Rate of solute incorporation to liposomes evaluated from encapsulated enzymes activities

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Abstract There are numerous studies on systems comprising an enzyme encapsulated in unilamellar liposomes and its substrate initially present in the external aqueous media. Most of these studies are focused on enzyme stability and activity in a restricted media. However, the rate of the process is also determined by the capacity of the substrate to permeate towards the liposome inner pool. In spite of this, there are few studies aimed at a quantitative evaluation of the substrate permeation rate and its lifetime inside the liposome pool. In the present work, we describe, in terms of a very simple mechanism, the permeation of glucose and hydrogen peroxide in DPPC unilamellar liposomes. To this aim, we evaluated the rate of the process employing encapsulated glucose oxidase and catalase in the kinetic diffusion controlled limit. Under this condition, the rate of the process becomes zero order in the enzyme and allows a direct evaluation of the rate constant for the permeation process and the lifetime of a substrate molecule incorporated into the liposome inner pool.

Keywords Liposomes · Diffusion · Enzyme kinetics

Introduction

In biological systems, most of the chemical reactions that support their life occur in compartmentalized environments, either in the cellular media or inside intracellular organelles. An important part of compartmentalization in biological reaction media is given by the presence of lipidic membranes

which play an important role by conditioning the flow of molecules essential for successful life maintenance.

The passage of solutes across lipidic membranes can take place by different mechanisms, depending on conditions such as temperature, salinity, and membrane composition, the presence of macromolecular solutes, and the characteristic of the permeating solute (Naoi Naoi et al. 1977). In spite of the large number of factors that condition the rate of the process, three main mechanisms can usually be recognized:

- i) Passive transport across the lipid bilayer. The rate of this process increases with the mobility of the membrane chains and the hydrophobicity of the solute and decreases with its size. Frequently, this transport has its maximum rate at the phase transition temperature and is mediated by transient pore formation (Paula et al. 1996; Mouritzen et al. 1995)
- ii) Passive transport throughout channels that go across the membrane. This mainly depends upon the size of the channel section and the size of the solute (Graff et al. 2001; Kobayashi et al. 1982).
- iii) Active transport throughout protein carriers. This process is generally very selective and can take place increasing the free energy of the system by its coupling to a spontaneous process (Hediger et al. 2004).

In order to evaluate the extent of the effect of compartmentalization and the mechanisms of solute transport across lipidic membranes in biological reactions, extensive use has been made of liposomes composed of a wide variety of phospholipids (Hauser 1993), in which one (or multiple) lipidic bilayer(s) surrounds a given volume of water (usually referred to as the aqueous pool). Liposomes have become an easy-to-work cell model for studies aimed at assessing the importance of the presence of lipid membranes in different types of reactions at the cell level. There are several characteristics that made

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liposomes so useful in this kind of studies. Liposomes show high stability in a wide range of environmental conditions, their size can be easily controlled by using a wide array of preparation techniques (sonication, freezing/thawing, dry lipid film, etc.) (Gregoriadis 1993), and molecules such as proteins, nucleic acids, and sugars can be easily encapsulated. Furthermore, the influence of membrane properties (viscosity, rigidity, charge, lipid distribution, and organization) that are relevant to solute permeation can be assessed by changing the lipidic composition of the membranes (Naai et al. 1977), or by factors such as temperature, pH, and low frequency radiation (Ramundo-Orlando et al. 2000a, b).

Enzymatic reactions have been by far the most widely used type of reaction in studies related to compartmentalization, essentially due to the ubiquity and diversity of enzymatic reactions in living organisms. A number of studies have focused on developing techniques for incorporating enzymes into liposomes and the study of the behavior of entrapped macromolecules. The influence of encapsulation on enzymatic activity in general has its origin in a number of variables, such as the stabilization of the structure of the enzyme due to the interaction with the lipid bilayer constituents (Madeira 1977), modification of enzyme activity due to intra-pool changes in the concentration of the substrate (Annesini et al. 1992), and control of enzyme activity due to the selective passage of molecules (substrates) through the lipid membrane (Nasseau et al. 2001).

In recent decades, several studies have been published dedicated to the study of the activity of liposome-entrapped enzymes in conditions such that the intake of solute molecules plays a pivotal role. Hydrolysis of two p-nitroanilide substrates catalyzed by α -chymotrypsin entrapped in POPC liposomes (Blocher et al. 1999) reveals that substrate permeation across the membranes can be the rate-limiting step. RNA polymerase encapsulated in DMPC liposomes (Chakrabarti et al. 1994) shows a five- to sevenfold lower activity (compared to that observed in bulk solution) when the substrate adenosine diphosphate was present in the external solvent. Substrate flux was maximized by performing the reaction at the phase transition temperature of the lipid. Kinetic analysis on the hydrolysis of starch catalyzed by aminoglucosidase (Li et al. 2007) showed a decreased activity of the liposome-entrapped enzyme due to a combination of limited access of the substrate and a less relevant modification of the intrinsic enzymatic activity. This last effect was explained in terms of the interaction between the entrapped enzyme and the phospholipid heads of the inner face of the membrane. Organophosphorous antidotal treatment by encapsulation of OPA anhydrolase within sterically stabilized liposomes composed of POPC/cholesterol/PEG-phosphatidyl ethanolamine lipids (Petrikovics et al. 2000) revealed that the rate of hydrolysis was first order in substrate and was proportional to the amount of liposomes with encapsulated enzymes. Horseradish peroxidase

incorporated into PC/biotinylatedphosphoetanalamine liposomes (Piwonski et al. 2012) shows noncompetitive enzymatic inhibition by the charged product resorufin which accumulates inside the vesicles.

The passage of solutes has been modulated by incorporating pore-forming membrane proteins and additives to the liposome membrane. Diffusion of cephalosporin (Kobayashi et al. 1982) was demonstrated to be highly dependent on the number and type of OmpFporins, revealed by analysis of the activity of liposome-entrapped cephalosporinase. On a similar topic, it has been demonstrated (Graff et al. 2001) that OmpF acts as a selective filter, which allows the passage of molecules with a molecular weight below 400 g/mol. This was demonstrated by studying the hydrolysis of ampicillin catalyzed by β -lactamase encapsulated in POPC liposomes. The incorporation of gramicidin, a pore-forming polypeptide, to liposomal preparations showed an increase in the water flux across the bilayer due to the contribution of passive channels generated by the polypeptide organization (Soto-Arriaza et al. 2012). Regarding the presence of additives, sodium cholate solubilized into the membrane of POPC molecules enhances the uptake of nucleotides (Treyer et al. 2002). The activity of β -galactosidase (Annesini et al. 1997) entrapped in PC liposomes remains latent and is recovered after surfactant-mediated disruption of the vesicles while sublytic surfactant concentration enhances liposomal enzyme activity due to increased permeability of the substrate.

In spite of the relevance of the diffusion of substrates across lipidic membranes, most of the studies comprising encapsulated enzymes were focused on the efficiency and stability of the incorporated enzyme and the mechanism of the intraliposomal catalysis, without a quantitative evaluation of the rate of solute incorporation into the liposomes and its mean life inside their inner pool. In this paper, we present the main advantages and drawbacks of studies comprising an entrapped enzyme and externally added solute, with emphasis on the experimental conditions that leads to a quantitative evaluation of the rate of solute permeation across the bilayer and discuss some of the results obtained by these procedures.

Experimental

Glucose oxidase (GOx; from *Aspergillus niger*); 1, 2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), and Sepharose-CL4B from Sigma and glucose from Merck were employed as received. Experiments were carried out in Hepes buffer (10 mM, pH 7.4). Water employed to prepare the solutions was obtained from a Modulab Type II equipment. The rate of the process was followed by registering the consumption of oxygen as a function of time on an Oxygraph System (Hansatech Instruments). Liposomes were prepared by adding, into a round-bottom tube, a known DPPC amount

dissolved in chloroform. The solvent was removed by slow evaporation at 40 °C in a water bath. Re-suspension of the film was carried out with an appropriate volume of the GOX containing buffer to ensure the desired working concentration of lipids. The suspensions were subjected to ten freeze–thaw cycles, transferred into a homemade extruder, and extruded 10 times back and forth through polycarbonate filters (400 nm nominal pore size, Nucleopore; Corning Costar) at 52 °C. This procedure maximizes the formation of unilamellar liposomes (Walde and Ichikawa 2001; Hwang et al. 2012). External GOX was removed by eluting the solution through a 30-cm column containing Sepharose-CL4B. This procedure did not affect the activity of the entrapped enzyme (data not shown). The diameter (number average) of the liposome ensemble was 225 ± 39 nm, as determined by dynamic light scattering in a Zetasizer Nano S-90 (Malvern Instrument, USA). Details on catalase (CAT; from bovine liver) incorporation are given in Abuin et al. 2012.

Results and discussion

Simplified modeling of the solute permeation towards the liposomal inner pool

Figure 1 presents a naive description of a simple system comprising an encapsulated enzyme, externally added substrate, and an ensemble of large unilamellar vesicles. In this system, the enzyme is only present in the internal water pool and/or associated to the internal liposomal surface. If the enzyme substrate (S) is added to this system, the mechanism of the reaction taking place inside the water pools can be represented by Eqs. 1 to 3

Fig. 1 Scheme of reaction for the influx of solute to the liposomal inner pool

where TLS is the total area of the external surface of the liposomal ensemble, and S_{encap} and S_{bulk} are the analytical concentrations of encapsulated and bulk solute, respectively. The rates of the corresponding steps can be expressed as:

$$R_1 = k_1 \text{TLS}[S_{\text{bulk}}] \quad (4)$$

$$R_2 = k_2 [S_{\text{encap}}] \quad (5)$$

$$R_3 = k_3 [\text{Enz}] [S_{\text{encap}}] \quad (6)$$

Applying the steady state approximation to (S_{encap}),

$$R_1 = R_2 + R_3 \quad (7)$$

is obtained and, hence,

$$[S_{\text{encap}}] = k_1 \text{TLS}[S_{\text{bulk}}] / (k_2 + k_3 [\text{Enz}]) \quad (8)$$

The rate of reaction (R_3) is then given by

$$R_3 = k_3 [\text{Enz}] k_1 \text{TLS}[S_{\text{bulk}}] / (k_2 + k_3 [\text{Enz}]) \quad (9)$$

which, under conditions such that $R_2 \ll R_3$ (irreversible capture), reduces to

$$R_3 = k_1 \text{TLS}[S_{\text{bulk}}] \quad (10)$$

And evaluation of R_3 allows an evaluation of R_1 and, if TLS is known (or estimated), can afford the value of k_1 , which is the rate constant of solute permeation into the liposomes.

In the absence of enzyme,

$$R_1 = R_2 \quad (11)$$

and, if R_2 is expressed as

$$R_2 = k_2 [S_{\text{encap}}] \quad (12)$$

, it follows that

$$\Gamma = 1/k_2 = (S_{\text{encap}}/R_1) \quad (13)$$

where Γ measures the average residence time of glucose molecules incorporated into a liposomal pool.

System preparation

A quantitative evaluation of k_1 , based on Fig. 1, requires unilamellar liposomes with a sharp distribution of sizes. There are many liposome preparation methods but only a few of them can be used for encapsulation of enzymes since an inadequate treatment of the lipid/enzyme system can lead to low encapsulation efficiency, high dispersion of liposomes size and lamellarity, reduced enzyme activity, or denaturation (Walde and Ichikawa 2001).

Among most frequently used methods for preparing liposomes are lipid film hydration, sonication, freezing–thawing, extrusion, reverse-phase evaporation, and detergent depletion. Some of these procedures (reverse-phase evaporation and detergent depletion) are not suitable for protein encapsulation since organic solvents and/or detergents can lead to their denaturation (Hwang et al. 2012). Similarly, sonication-based methods may lead to inactivation of the encapsulated enzyme (Walde and Ichikawa 2001). The lipid film hydration method has the disadvantage of wide dispersion of size and lamellarity (Lichtenberg and Markello 1984). Freezing–thawing methods can render high encapsulation efficiencies but produces multilamellar liposomes (Pick 1981; Anzai et al. 1990). Extrusion-based methods provides a better control over the size and lamellarity of liposomes, and best results have been obtained coupling the extrusion technique to other methods, like freezing–thawing (Walde and Ichikawa 2001; Liang et al. 2005). An interesting result is that, frequently, the encapsulation efficiency of the enzyme increases with the number of cycles (Colletier et al. 2002; Chaize et al. 2004) achieving enzyme concentrations much higher than those expected from a water pool/external solvent equilibration.

Another crucial point is remotion of “all” the enzyme outside the liposomes. Independent of the method used for the determination of transport across the lipid bilayer, a low percentage of active enzymes outside the liposomes can lead to an overestimation of the rate of solute transport. The external enzyme is generally removed by size-exclusion chro-

matography, centrifugation, dialysis, or by a combination of these procedures (Dipali et al. 1996; McNamara and Rosenzweig 1998; Walde and Ichikawa 2001; Grabielle-Madelmont et al. 2003). In size exclusion chromatography, an “ideal” result is a total separation between the fractions containing free proteins and the liposomes. The amount of protein in the liposome-containing fractions is then a measure of the efficiency of enzyme encapsulation (Abuin et al. 2012).

How to measure the reaction rate of enzymatic processes taking place in the liposome internal pool. Application to hydrogen peroxide decomposition and glucose oxidation

An ideal experimental procedure must involve the continuous evaluation of a reactant and/or product by spectroscopic or electrochemical analysis. The use of an oxigraph to measure oxidations or reductions is an example of this type of approach. Examples of data obtained in this type of systems are shown in Table 1

The decomposition of hydrogen peroxide catalyzed by catalase has also been measured in DMPC and POPC liposomes following the decrease in hydrogen peroxide absorbance at 240 nm (Mathai and Sitaramam 1994; Yoshimoto et al. 2007).

Glucose oxidation catalyzed by glucose oxidase can also be followed with an oxygen electrode by oxygen uptake (Hertz and Barenholz 1973; Solomon and Miller 1976; Taylor and Jones 1995; Antunes and Cadenas 2000). Another method employed is the spectrophotometric quantification of H_2O_2 based on the HRP-catalyzed oxidation of o-dianisidine. In this system, GOx catalyzes the oxidation of glucose to gluconic acid and H_2O_2 (Yoshimoto et al. 2010; Jo et al. 2009; Hill et al. 1997; Bankar et al. 2009). In both cases, the initial rate is closely related to the rate of glucose influx.

Figure 2 shows a typical profile of oxygen uptake in glucose oxidation catalyzed by glucose oxidase, both in the absence and presence of liposomes. The large difference in rates results from the smaller analytical enzyme concentration

Table 1 Systems studied by changes in oxygen concentration

Substrate	Enzyme	Lipids	Study	Reference
HOOH	Catalase	DPPC	HOOH permeation	Abuin et al. 2012
Glucose	GOX	DPPC	Glucose permeation	This work
Aminoacids	D-amino acid oxidase	Egg PPC	Amino acids permeation	Naoi et al. 1977
Ascorbate	Ascorbate oxidase	DPPC+Cholesterol	Interaction of vesicles with enzyme	Mossa et al. 1989
5,6-Dihydroxyindole	tyrosinase	Egg PPC	Regulation of the Raper-Mason pathway	Miranda et al. 1988
HOOH	Glutathione peroxidase	“Jurkat Cells”	HOOH permeation	Antunes and Cadenas 2000
HOOH	Catalase	“Saccharomyces cerevisiae”	HOOH permeation	Folmer et al. 2008

Fig. 2 Oxygen consumption in the glucose oxidase catalyzed oxidation of glucose. *Black line* data obtained in homogeneous solution. Glucose 10 mM; GOx 6.25 μ M. *Red line* data obtained in the catalysis by GOx entrapped enzyme in DPPC (1.36 mM) liposomes

inside the liposomes and the restriction in glucose incorporation inside the liposomes generated by the bilayer.

Evaluation of the Total Lipid Surface (TLS)

The starting point is to assume that all phospholipids are included in near-spherical liposomes, and that nearly half of them are present in the outer leaflet. This leads to:

$$\text{TLS} = 0.5 S_{\text{ph}}[\text{PL}]N_{\text{A}} \quad (14)$$

where S_{ph} is the surface cover by one phospholipid polar head, $[\text{PL}]$ is the molar concentration of phospholipids and

Fig. 3 Rate of O_2 evolution plotted as a function of entrapped catalase analytical concentration. *Black squares* data obtained at 20 $^{\circ}\text{C}$; *red circles* data obtained at 42 $^{\circ}\text{C}$. Data taken from Abuin et al. 2012

Table 2 Values of k_1 and residence times obtained in DPPC LUVs at 25 $^{\circ}\text{C}$

Enzyme	k_1 ($\text{m}^{-2} \text{s}^{-1}$)	Residence time of substrate (s)	Reference
Catalase	1.06×10^{-3}	25	Abuin et al. 2012
Catalase plus nonanol	1.47×10^{-3}	18	Abuin et al. 2012
Glucose oxidase	6.11×10^{-6}	4,300	This work

N_{A} is the Avogadro number. This formulae assumes that:

1. The system is made up only of unilamellar liposomes. This supposition is validated by the method employed (extrusion and freezing–thawing cycles).
2. The external surface of liposomes is nearly equal to the internal. This is taken into account by introducing a factor of 0.5 in the calculation of the TLS.

Taking for DPPC a value of 60 $\text{\AA}^2$ for lipid heads (Nagle and Tristram-Nagle 2000), a TLS of 180.7 m^2 per mol of lipids incorporated into the LUVs ensemble is obtained.

When k_2 is negligible and how to realise it

From Eq. 7, the value of k_1 can be obtained

$$k_1 = R_3 / \text{TLS}[\text{S}_{\text{bulk}}] \quad (15)$$

Fig. 4 Rate of O_2 consumption plotted as a function of temperature. Data obtained for the initial rate of externally added glucose to DPPC (1.36 mM) liposomes containing GOx (1 mg/mL)

The only assumption in this calculation is that the process is diffusion-controlled, i.e., that $R_2 \ll R_3$. In particular, the procedure does not require knowing whether the substrate is free in the water pool and/or adsorbed into the bilayer. Regarding the enzyme, is not required to know the incorporated amount if it is partially inactivated or hyperactive or if it is present in the pool or adsorbed in the internal leaflet with its active site oriented towards the liposome pool. The only condition required is enough active enzyme inside the pool to avoid a significant back liberation of the solute. In this sense, extended incorporation of enzyme inside the pool, an effect very common and difficult to control, is beneficial and not an extra difficulty. The crucial point is then to know if the condition $R_2 \ll R_1$ is fulfilled. A necessary (and sufficient) condition to support this is that the rate of the process must be independent of the bulk enzyme concentration. In Fig. 3, is shown data obtained for the liberation of oxygen in the catalase-promoted decomposition of HOOH. It can be observed that the rate of the process is not dependent on catalase concentration, indicating that this system can be treated according to the above proposed simplified mechanism (Abuin et al. 2012). Similar conclusions can be obtained for the oxidation of glucose catalyzed by glucose oxidase (data not shown). The values of k_1 derived in these systems at room temperature are collected in Table 2.

Table 2 includes values estimated for the residence time of the substrate inside a liposomal water pool. The relatively large times, particularly for glucose, are compatible with the assumption that $R_2 \ll R_3$.

What factors determine k_1 ?

The fluidity of the bilayer is an important factor affecting the rate of incorporation of a given solute to the liposome inner pool. This is in agreement with the increase in k_1 reported in Table 2 elicited by nonanol addition (Abuin et al. 2012). Similarly, it can be expected that k_1 increases when the temperature increases. However, the data obtained for hydrogen peroxide and glucose show a more complex behavior. In fact, in both systems, there is an increase in k_1 with temperature below the lipid phase transition (42 °C) and a sharp peak at this temperature, as can see in Fig. 4 for glucose. This is a common feature of solute diffusion across lipidic bilayers that have been explained in terms of enhanced fluctuations at this critical temperature at which the bilayer comprises co-existing liquid crystalline and gel-like rafts (Walde and Ichikawa 2001). It is interesting to note that this peak in diffusion is not observed in the fluorescence anisotropy of probes incorporated into the liposomes, where there is a steady increase over all the temperature range considered (Abuin et al. 2012).

Is an intravesicular product accumulation relevant?

Glucose oxidation catalyzed by glucose oxidase can be represented by the following scheme

It can be determined that D-glucono-1,5-lactone accumulation favors the reverse reaction diminishing the oxygen uptake. However, the increase of the rate of irreversible gluconic acid formation due to the relatively high pH (7.0) employed argues against this possibility. Furthermore, the reverse process from the lactone requires H_2O_2 . Under our experimental conditions, H_2O_2 accumulation is not relevant due to its short residence time (Table 2) and the fact that we measure initial rates after external glucose addition.

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Conflict of interest Manuel Ahumada, Cristian Calderón, Luis León, Eduardo Lissi declare that they do not have any conflict of interest regarding the present manuscript.

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