RSC Advances

PAPER

Cite this: *RSC Adv.*, 2016, **6**, 62594

Determining the substrate permeability through the bilayer of large unilamellar vesicles of DOPC. A kinetic study

Maria Alejandra Luna, Juana J. Silber, Leonides Sereno, N. Mariano Correa* and Fernando Moyano*

In this work we determine the permeability of 1,2-dioleoyl-*sn*-*glycero*-3-phosphatidylcholine (DOPC) vesicles in the presence of different cholesterol (Cho) contents, by using the enzymatic hydrolysis of *N*-benzoyl-L-tyrosine *p*-nitroanilide (Bz-Try-*p*NA) catalyzed by α-chymotrypsin (α-CT). The reaction was first studied in homogeneous media in a 4% p/p ethanol–water mixture and then in DOPC vesicles at different Cho content. In homogenous media, ethanol helps to solubilize the substrate, which is almost insoluble in water and therefore increases the effective concentrations. In DOPC vesicles, ethanol does not destroy the bilayer. In both cases, the enzymatic hydrolysis can be followed by UV-visible spectroscopy. The hydrolysis of Bz-Try-*p*NA catalyzed by α-CT follows the Michaelis–Menten mechanism and the kinetic parameters: k_{cat}, K_M and k_{cat}/K_M were evaluated in both systems at the same solvent mixture compositions. To obtain the kinetic parameters and the permeability of the reactant in DOPC : Cho vesicles, we use a simple mathematical model and dynamic light scattering (DLS) measurements. The results show that the hydrolysis reaction takes place in the water entrapped in the interior of the DOPC vesicles and, that the enzyme encapsulated inside the vesicles, despite the significant differences in the permeability values of Bz-Try-*p*NA, has similar catalytic effects independently on the Cho composition used.

Received 17th May 2016
Accepted 18th June 2016

DOI: 10.1039/c6ra12847e

www.rsc.org/advances

Introduction

Vesicles or liposomes can be considered as large cooperative units with very different characteristics from the individual structural units which constitute them.[1] They are spherical aggregates formed by some amphiphilic compounds called surfactants. There are a wide kind of surfactants (anionic, cationic, nonionic and zwitterionic) that form vesicles and, probably the most frequently used are the phospholipids, especially the phosphatidylcholine[2] since they are the fundamental matrix of natural membranes and, represent the environment in which many substrates, like proteins and enzymes, can go through the membranes displaying their activity.[1–5] This is why one of the many physicochemical studies[6–9] on vesicles is about the permeability of different substrates through their bilayers structures. The capacity of the bilayer to permeate different types of solutes has been of great interest to biophysicists, especially for phospholipid-based membranes. One of the reasons for such an interest is that vesicles are regarded as drug delivery systems, *e.g.*, vectors for gene transfer,[10,11] in the diagnostic imaging of tumors,[12] or as cosmetic agents for the delivery of moisturizers and anti-inflammatory agents to the skin.[13] There are a variety of methods to measure permeability in vesicles, such as: radioactivity counting using radioactive tracers,[14] spectrophotometry[15–17] and photochemical[18] using enzymes, the surface selective technique based on second harmonic generation,[19,20] and the measures of electrical resistance or electrical capacitance of ion.[21]

In relation to different components that can contain the phospholipid-based membranes, we can name to sterols. Cholesterol (Cho) is one of many sterols present in membranes and, some effects on its properties are known.[22,23] For example, Cho will abolish the gel phase in saturated phospholipid bilayers (decrease acyl chain order or increase fluidity) but, in unsaturated phospholipid bilayers, the effect of Cho is just the opposite: the acyl chain order increases and decreases the fluidity of the bilayer.[24] However, it is complicated to determine the value of the permeability in the presence of Cho on membranes due to specific and nonspecific interactions.[22–26]

The aim of this study is to determine the permeability coefficient across different bilayers varying the Cho content, in 1,2-dioleoyl-*sn*-*glycero*-3-phosphatidylcholine (DOPC) large unilamellar vesicles (LUVs), of the substrate *N*-benzoyl-L-tyrosine *p*-nitroanilide, (Bz-Try-*p*NA) by using an enzymatic reaction. The experimental data allows us to propose a mathematical model to obtain the kinetic parameters and the permeability coefficient. The model was suggested by Sato *et al.*[18] which calculated the

Departamento de Química, Universidad Nacional de Río Cuarto, Agencia Postal # 3, C.P. X5804BYA Río Cuarto, Argentina. E-mail: fmoyano@exa.unrc.edu.ar

permeability (P) of a solute through a single bilayer of LUVs, by using the Fick's first law, and the Michaelis–Menten mechanism. This model is very attractive, among others, since it determines, jointly, substrate permeability across the bilayer and the kinetics parameters. The P values obtained with our model have a very good agreement with other values obtained in bilayers by using different techniques. Furthermore, we demonstrate that the Cho content considerably modifies the permeability value of the substrate without changing the kinetic parameters, which are comparable with the values obtained in pure homogenous media.

Methods

General

α-Chymotrypsin, (α-CT), MW 24800, from bovine pancreas (Sigma), *N*-benzoyl-L-tyrosine *p*-nitroanilide, (Bz-Try-*p*NA) (Sigma) and cholesterol (Cho) (Sigma), were used as received. Absolute ethanol (Merck HPLC quality) was used as received and, ultrapure water was obtained from a Labonco equipment model 90901-01. The pH of the bulk water solution has been maintained at 8.7 by using a 20 mM phosphate buffer (Na_2HPO_4/NaH_2PO_4).

The absorption spectra were measured by using a Hewlett-Packard, UV-visible 8453 spectrophotometer equipment at 25 ± 0.1 °C with a thermostated cell (3 mL of volume and 1 cm path length). To subtract the background contribution of the absorption spectra, samples of the same concentrations of phospholipids were prepared.

The diameters of the LUVs were determined by dynamic light scattering (DLS, Malvern 4700 with a goniometer and 7132 correlators) with an argon-ion laser operating at 488 nm. Multiple samples of each size were made, and thirty independent size measurements were made for each individual sample at the scattering angle of 90°. The instrument was calibrated before and during the course of experiments by using several different size standards. Thus, we are confident that the magnitudes obtained by DLS measurements can be statistically significant for all the systems investigated. The DLS experiments show that the polydispersity of the LUV size is less than 5%.

OriginPro 8.0 program was used for edit equations, analysis, and calculations of data.

Reactions in homogeneous media

Scheme 1 shows the hydrolysis reaction of Bz-Try-*p*NA catalyzed by α-CT in ethanol–buffer at 4% p/p. The reactions were followed by measuring the increases at the maximum absorption band of the product *p*-nitroaniline, (*p*NA) at $\lambda = 386$ nm ($\varepsilon = 12\,500\ M^{-1}\ cm^{-1}$) and 25.0 ± 0.1 °C. The UV-visible spectroscopic analysis shows that the hydrolysis reaction of Bz-Try-*p*NA catalyzed by α-CT in ethanol–buffer at 4% p/p (Scheme 1), produces *p*NA in quantitative yields.

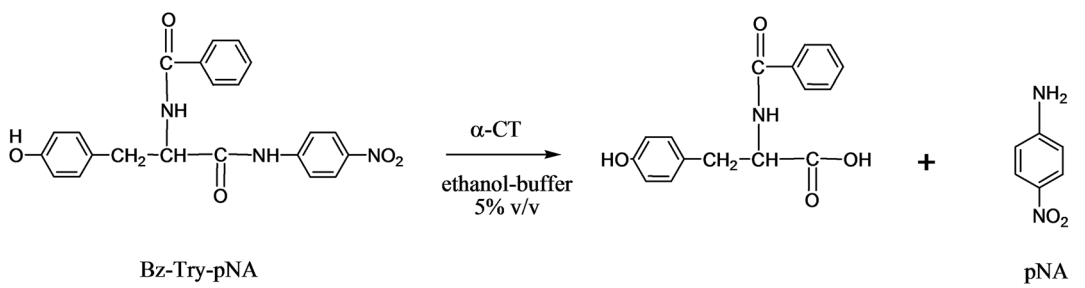
The experimental reaction was conducted as follows: a thermostated stock solution of Bz-Try-*p*NA 0.01 M in ethanol was prepared and it was set at 25.0 ± 0.1 °C. To start a kinetic run in homogeneous media, 3 mL of the thermostated α-CT in ethanol–buffer solution at 4% p/p (20 mM phosphate buffer at pH 8.7 and [α-CT] = 1×10^{-6} M) was introduced in the thermostated cell at 25.0 °C. Then, the enzymatic reaction was initiated by addition of different μL of the stock ethanol solution of Bz-Try-*p*NA in order to have 3 mL of solution with the desired [Bz-Try-*p*NA]. For example, for [Bz-Try-*p*NA] = 1×10^{-5} M, 3 μL of the ethanol stock solution was added to the cell. The concentrations of Bz-Try-*p*NA were varied between 10^{-5} M and 10^{-4} M.

Reactions in DOPC : Cho vesicles

The LUVs were formed, at 25 ± 0.1 °C by using the phospholipid 1,2-dioleoyl-*sn-glycero*-3-phosphatidylcholine (DOPC T_c = −17.3 °C (ref. 27)). The stock solution of DOPC in chloroform was obtained from Avanti Polar Lipids, Inc. (Alabaster Al, USA).

The vesicles with Cho were typically obtained as follows: solutions were prepared by mixing the appropriate amount of Cho (by weight) and DOPC in chloroform (Sintorgan HPLC) to obtain the desired mole fraction ($X_{DOPC} = 1$, $X_{DOPC} = 0.90$, and $X_{DOPC} = 0.80$); [DOPC] = 1.27×10^{-3} M. After the solvent was evaporated and the film was dried under reduced pressure. Large multilamellar vesicles (MLV) were obtained by hydrating the dry lipid film with an ethanol–buffer solution at 4% p/p that contains α-CT (20 mM phosphate buffer at pH 8.7 and [α-CT] = 1×10^{-6} M), through mixing (Vortex – 2-Genie) for about 5 minutes at room temperature. The resulting solutions of MLVs have the desired DOPC : Cho concentrations with the enzyme encapsulated. To prepare LUVs, the MLVs solutions were extruded ten times (Extruder, Lipex biomembranes) through two stacked polycarbonate filters of pore size 200 nm under nitrogen pressure up to 3.4 atm. The LUVs with enzyme incorporated were separated from the free enzyme by gel chromatography as it is described below.

To prepare the gel filtration column, we added 2 or 3 g of Sephadex G-50 (Sigma, Aldrich) to 30 mL of ethanol–buffer (4%



Scheme 1 Bz-Try-*p*NA hydrolysis reaction catalyzed by α-CT.

p/p) solution at 70 °C and we allowed it to swell for 20 minutes. The well-mixed gel suspension was poured into the column (1.0 × 30 cm) and this was calibrated over its entire length using vesicles previous characterized.[28] The ethanol–buffer mixture at 4% p/p with the presence of 100 μM of bovine pancreatic typsin inhibitor (BPTI, from FLUKA) was used as the mobile phase. This was made in order to be confident that, any enzyme that is not trapped by the LUV media is not reactive. The amount of LUVs that were seed was 2 mL. Then, the fractions that contained LUVs with the enzyme entrapped were determined by DLS and absorption spectrophotometry. It is necessary to clarify that the enzyme is hydrolytic and it does not cross the hydrophobic bilayer. All samples were used immediately after preparation.

Finally, the enzymatic reaction started with the addition of different μL of the stock ethanol solution of Bz-Try-*p*NA to the samples, in order to have a final volume of 3 mL with the desired [Bz-Try-*p*NA] as described above. The concentrations of Bz-Try-*p*NA was around 10^{-5} M.

Kinetic equations and model used

The hydrolysis of Bz-Try-*p*NA catalyzed by α-CT, in the ethanol–water mixture follows the Michaelis–Menten mechanism depicted by:

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E-S} \xrightarrow{k_{\mathrm{cat}}} \mathrm{E} + \mathrm{P} \tag{1}$$

Applying the steady state approximation to (E − S), the rate law was given as:

$$v_0 = \frac{k_{\mathrm{cat}}[\mathrm{E}][\mathrm{S}]}{(K_{\mathrm{M}} + [\mathrm{S}])} \tag{2}$$

where v_0 is the initial reaction rate (M s^{-1}), [E] and [S] are the analytical enzyme and substrate concentration, respectively; k_{cat} is the catalytic rate constant and, K_{M} is the Michaelis–Menten constant defined by:

$$K_{\mathrm{M}} = (k_{-1} + k_{\mathrm{cat}})/k_1 \tag{3}$$

Then, k_{cat} and K_{M} in homogenous media were determined by fitting the experimental data according to the mechanism using OriginPro 8.0 and, the catalytic efficiency ($k_{\mathrm{cat}}/K_{\mathrm{M}}$) was calculated. The pooled standard deviation of the kinetic data, using different samples, was less than 5%.

When the kinetics is performed in the vesicle media, the enzyme is entrapped inside the LUV media and, the substrate should pass through the vesicles bilayer in order to start the chemical reaction. Scheme 2 depicts a schematic representation of the DOPC vesicle, its radius (R_v), thickness (δ), α-CT entrapped, the processes of the substrate (S) permeability and, the evolution of the reaction.

Eqn (4) shows the permeability (P) through one single bilayer of DOPC vesicles considering that the flux of substrate through bilayer obeys the Fick's first law.[29] At time t different of zero, the substrate is present inside and outside of the bilayer named as $S_{\mathrm{in}(t)}$ and $S_{\mathrm{out}(t)}$, which represent the molar concentration of substrate inside and outside the vesicle system, respectively; where V_{out} represents the volume of the outer phase. The value of A_v denotes the sum of the outer and inner surface area of the vesicles.

$$V_{\mathrm{out}}\delta S_{\mathrm{out}(t)} = \frac{A_v P}{2}\left[S_{\mathrm{in}(t)} - S_{\mathrm{out}(t)}\right]\delta t, \tag{4}$$

The substrate distribution in time (t) is obtained by taking into account the mass balance, (eqn (5)) and taken into consideration that V_{in} is much smaller than V_{out} of medium eqn (6) is obtained

$$V_{\mathrm{total}}S_{\mathrm{total}} = V_{\mathrm{out}}S_{\mathrm{out}(t)} + V_{\mathrm{in}}S_{\mathrm{in}(t)} \tag{5}$$

$$S_{\mathrm{in}(t)} = \frac{V_{\mathrm{out}}}{V_{\mathrm{in}}}\left[S_{\mathrm{total}} - S_{\mathrm{out}(t)}\right] \tag{6}$$

Then, combining eqn (4), (6) after rearranged the differential equation is obtained

$$\frac{\mathrm{d}\left(\frac{S_{\mathrm{out}(t)}}{S_{\mathrm{total}}}\right)}{\mathrm{d}t} = \frac{A_v P}{2V_{\mathrm{in}}}\left[1 - \frac{S_{\mathrm{out}(t)}}{S_{\mathrm{total}}}\right] \tag{7}$$

The solution of this differential equation is a single exponential relaxation:

$$S_{\mathrm{out}(t)} = S_{\mathrm{total}} + [S_{\mathrm{out}(0)} - S_{\mathrm{total}}]\mathrm{e}^{-t/\tau} \tag{8}$$

where τ is the characteristic time of the substrate to pass through the vesicle given by

$$\tau = \frac{2V_{\mathrm{in}}}{A_v P} \tag{9}$$

In the case of a spherical vesicle of outer-radius R_v and wall-thickness δ, the geometrical factor (sphere) in eqn (9) is given by the following equation

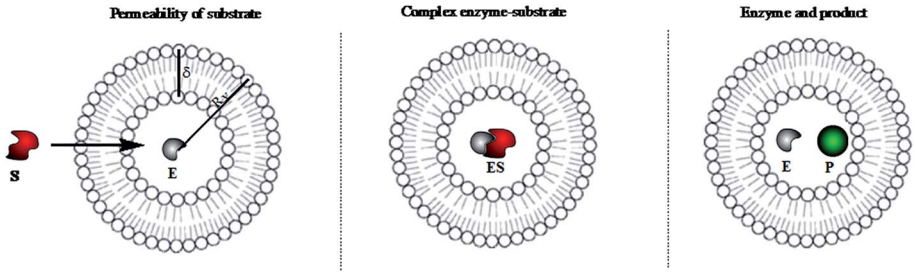
$$\frac{V_{\mathrm{in}}}{A_v} = \frac{1}{3}\,\frac{[R_v - \delta]^3}{R_v^2 + [R_v - \delta]^2} \tag{10}$$

which leads, in the limit where $\delta \ll R_v$, to a characteristic time of leakage expressed for unilamellar vesicles as

$$\tau = \frac{R}{3P} \tag{11}$$

By using the Michaelis–Menten equation (eqn (2)), the solution of the differential equation (eqn (8)) and considering that $[\mathrm{S}]_{\mathrm{out}(t)} \ll K_{\mathrm{M}}$, the following eqn (12) is obtained. This consideration is possible to do when K_{M} value is, at least 10 times greater than the substrate concentrations.[36]

$$v_{(t)} = \frac{k_{\mathrm{cat}}[\mathrm{E}]_t}{K_{\mathrm{M}}}\left[S_{\mathrm{total}} + \left[S_{\mathrm{out}(0)} - S_{\mathrm{total}}\right]\mathrm{e}^{-t/\tau}\right] \tag{12}$$



Scheme 2 Representation of the processes occurring in the enzymatic hydrolysis in DOPC vesicles.

Results

Reactions in homogeneous media

In previous work,[30] we studied the hydrolysis reaction of Bz-Try-*p*NA catalyzed by α-CT in dimethyl-sulfoxide (DMSO)–water mixtures by UV-visible spectroscopic. DMSO was necessary because the substrate solubility in water is very low but, unfortunately, herein we could not use this solvent because it destroys the vesicles. Thus, we choose to perform the reaction in a mixed ethanol–buffer solution. The choice of this mixture is because it does not destroy the vesicle structure as the DLS experiments show (results not shown) and, the substrate solubility is enhanced in comparison with pure water. Similar behavior was observed for DOPC vesicles in glycerol–water mixtures.[31]

Fig. 1A shows typical absorption spectra for the Bz-Try-*p*NA hydrolysis, at different reaction times, in the ethanol–buffer mixture at 4% p/p. We observed that the reaction was completed in 450 seconds and, the absorption spectra follows the same profile that in the DMSO–water mixture.[30] The absorption spectra taken at different times of reaction shows an increase in the absorbance at $\lambda_{max} = 386$ nm which indicates the formation of the product (*p*NA). Furthermore, the clear isosbestic point observed at $\lambda = 345$ nm evidences the lack of intermediates and/or product decomposition.[32,33]

The best mechanism found that explains the experimental data obtained at different Bz-Try-*p*NA concentrations, is the Michaelis–Menten discussed above. Fig. 1B shows typical results obtained by treating the data according to with the Michaelis–Menten mechanism (eqn (2)), which are consistent with the Michaelis–Menten mechanism.

The k_{cat} and K_M values were determined by fitting the experimental data to eqn (2) and, the catalytic efficiency (k_{cat}/K_M) was calculated. The values obtained are well-matched with those obtained by other authors[34,35] and make us confident that, in our experimental conditions the reaction follows the same mechanism.

Reactions in DOPC vesicles

A model for solutes permeability through one single bilayer of LUVs has been proposed by Sato *et al.*,[18] which was applied successfully to different solutes.[36–38] In the Experimental section, we described the principal concepts and equations that we will use in this work for the analysis of the data. In addition, we know that it is necessary to determine the vesicles sizes in order to calculate the permeability of the substrate used (eqn (11)). The size of DOPC vesicles, in presence and absence of the enzyme and Cho, were determined by using DLS technique to evaluate if the encapsulated enzyme and Cho would alter and/or modify the size of the vesicles (see Table 1).

We observe that the size of the vesicles decreases when the Cho content increases, results that we will discuss later. Herein,

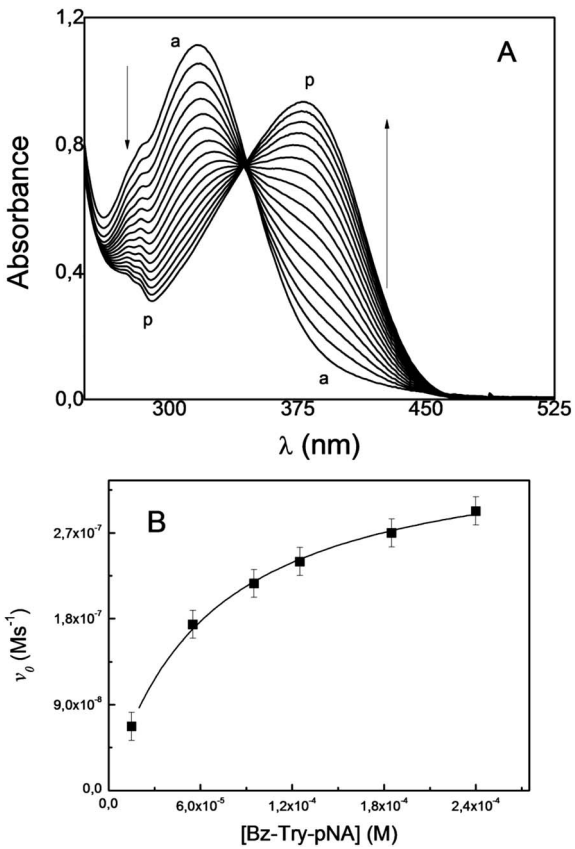


Fig. 1 (A) Representative absorbance spectra at different times for the hydrolysis of Bz-Try-*p*NA catalyzed by [α-CT] in ethanol–water at 4% p/p. Time (s): (a) 0, (b) 30, (c) 60, (d) 90, (e) 120, (f) 150, (g) 180, (h) 210, (i) 240, (j) 270, (k) 300, (l) 330, (m) 360, (n) 390, (o) 420, (p) 450. [α-CT] $= 1 \times 10^{-6}$ M. [Bz-Try-*p*NA] $= 9 \times 10^{-5}$ M; pH = 8.7. (B) Effect of Bz-Try-*p*NA concentration on the Michaelis–Menten plot for the α-CT catalyzed hydrolysis of Bz-Try-*p*NA in ethanol–water. [α-CT] $= 1 \times 10^{-6}$ M. pH = 8.7. The experimental data were fitted according to eqn (2), (–) solid lines.

we will focus on the results found for vesicles without Cho content.

The independence of R_v values on the presence of α-CT has to be considered only as an indirect proof that the enzyme probably does not alter the bilayer properties.

However, there are two regions where the enzyme, α-CT, could be located: (a) in the water entrapped core of vesicles, far from the bilayer, environment which similar properties to pure water[39] or (b) near to the interface were the specific interactions could modify the activity of the enzyme.[32,40]

In systems where the size of the droplets are around 5–15 nm, such as reverse micelles (RMs), the specific interactions and the confinement of α-CT play a fundamental role in the catalytic efficiency (k_{cat}/K_M) value of the enzyme. We demonstrated[30,32] that the catalytic efficiency values obtained in RMs systems, are higher than the value reported in water. Even more, there is a remarkable increase in the α-CT efficiency in the cationic RMs in comparison with the anionic RMs believable due to the unique water properties found inside these confined media.[32] The cationic RM forms a net of hydrogen bonds around the enzyme reduce the conformational mobility, which leads to an increase of the enzyme stability and activity.[32] However, the vesicles sizes (Table 1) are bigger than those for RMs and the hydrolytic enzyme is far away from the bilayer interface.

To elucidate which could be the regions of the DOPC vesicles where the enzyme can be located, we perform a spectroscopic study. Fig. 2A shows typical absorption spectra for the Bz-Try-*p*NA hydrolysis at different reaction times in DOPC vesicles, at the same conditions studied in homogeneous media. As described above, we detect spectrophotometrically the product of the reaction at $\lambda_{max} = 386$ nm and, we observe that the absorption spectra of *p*NA shows similar profile than in homogeneous media, however, there are important differences in the reaction times. In DOPC vesicles, the reaction needs longer times to complete. It is necessary to remark that the increase in the time of reaction is related to the permeability value of Bz-Try-*p*NA through the bilayer because the substrate has to be in contact with the enzyme in so as to the hydrolysis reaction occurs.

Fig. 2B shows typical results obtained treating the data according to the model described before and fitted to eqn (12). The results show that the model proposed is valid for α-CT in all the concentrations of Bz-Try-*p*NA studied (1.5×10^{-5} M to 2.4×10^{-4} M). Moreover, the kinetic parameters (k_{cat} and K_M) and the characteristic time of the substrate that pass through of bilayer

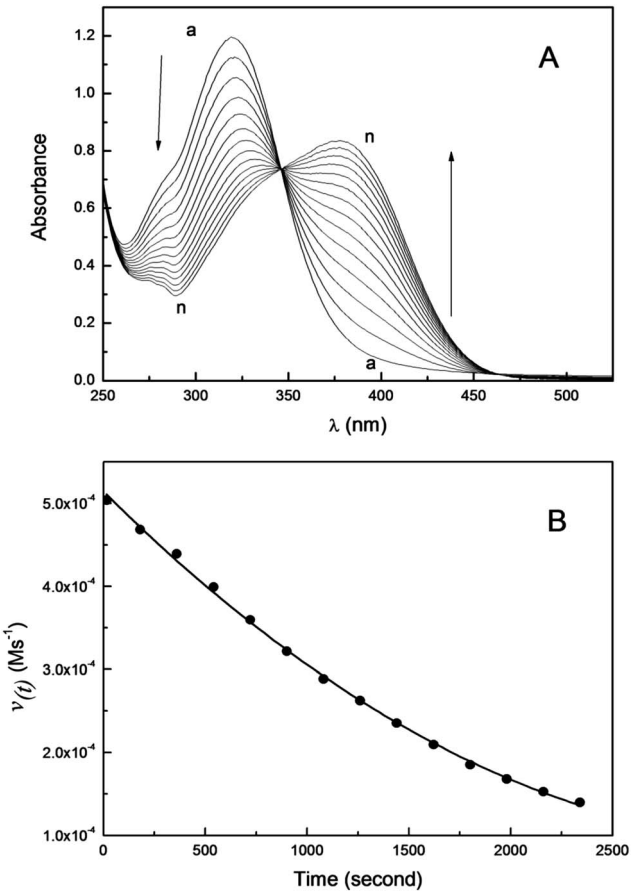


Fig. 2 (A) Representative absorbance spectra at different times for the hydrolysis of Bz-Try-*p*NA catalyzed by [α-CT] in DOPC vesicles in ethanol–water at 4% p/p. Time (s): (a) 15, (b) 180, (c) 360, (d) 540, (e) 720, (f) 900, (g) 1080, (h) 1260, (i) 1440, (j) 1620, (k) 1800, (l) 1980, (m) 2160, (n) 2340. [α-CT] = 1×10^{-6} M. [DOPC] = 1.27×10^{-3} M. [Bz-Try-*p*NA] = 1×10^{-4} M; pH = 8.7. (B) The time rate of hydrolysis of Bz-Try-*p*NA catalyzed by α-CT hydrolysis inside of DOPC vesicles in ethanol–water 4% p/p. [α-CT] = 1×10^{-6} M. [Bz-Try-*p*NA] = 1×10^{-4} M. [DOPC] = 1.27×10^{-3} M. pH = 8.7. The experimental data were fitted according to eqn (12), (–) solid lines.

Table 1 Shows the hydrodynamic radius (R_v) of DOPC : Cho vesicles in presence and absence of the enzyme at different mole fraction studies. [DOPC] = 1.27×10^{-3} M

DOPC : Cho vesicles X_{DOPC}	R_v in presence of α-CT (nm)	R_v without α-CT (nm)	Polydispersity
1	101.3	101.0	0.11
0.90	95.0	95.1	0.10
0.80	86.5	86.2	0.11

(τ) were obtained and gathered in Table 2. Furthermore, by using the vesicles radius (R_v) determined with DLS (Table 1) and eqn (11), we calculate the permeability (P) values which are shown also in Table 2.

It is interesting to comment that Walde *et al.*[39] determine the permeability coefficient for the same substrate (Bz-Try-*p*NA) in 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phosphocholine (POPC) vesicles using another model and simulation dynamics. They obtained a P value of 2.45×10^{-7} cm s^{-1} for a bilayer of a thickness of 3.7 nm and a mean POPC head group area of 0.72 nm^2. The value that we obtained for the DOPC vesicle (Table 2) is of the same order but lower than for POPC (Table 2). This fact might indicate a different interaction of the substrate with saturated and unsaturated carbon chains. These confirm that the model is validated and, it can be used in order to determine the influence of the addition of Cho in the DOPC bilayer lipid.

Table 2 Summary of experimental kinetics parameters of the enzymatic reactions in homogeneous media and in DOPC vesicles with and without cholesterol

Parameters	Water	DOPC vesicles $X_{DOPC} = 1$	DOPC : Cho vesicles $X_{DOPC} = 0.90$	DOPC : Cho vesicles $X_{DOPC} = 0.80$
k_{cat} (s^{-1}) $\times 10^{-2}$	36	33	40	37
(K_M) (M) $\times 10^{-4}$	1	1	1.2	1.1
(k_{cat}/K_M) (M^{-1} s^{-1})	3600	3300	3332	3363
τ (s^{-1})	—	13.5	4.5×10^2	3.7×10^2
P (cm s^{-1})	—	1.7×10^{-7}	7.1×10^{-9}	7.8×10^{-9}

Mechanism of the hydrolysis of Bz-Try-*p*NA catalyzed by α-CT in DOPC : Cho vesicles

We present the study of the membrane by using the model proposed above to investigate the effect that the Cho has on the permeability values of the substrate in the DOPC vesicles.

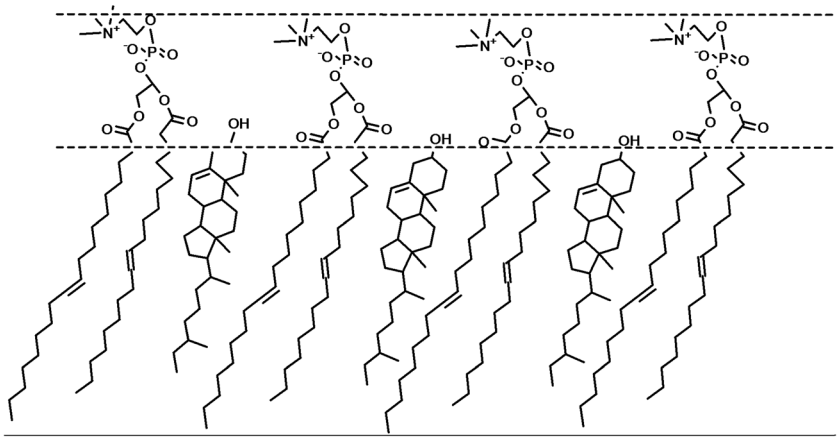
Previously, the sizes of the DOPC : Cho vesicles, in the presence and absence of the enzyme, were studied using DLS (see Table 1). The data obtained show that the sizes of the vesicles decrease when the Cho content increases. One possible explanation is that the decrease in the sizes of the vesicles are due to the fact that the Cho molecules are oriented in a way that its hydroxyl group points outward of the bilayer, while its hydrophobic ring system is within the bilayer, with the fatty acid tails of phospholipid, as shown in Scheme 3. Furthermore, the fatty acid heads of the phospholipids can form hydrogen bonds with the hydroxyl group of Cho and, this interaction reduces the mobility of the hydrocarbon tails.

In other words, Cho helps the phospholipids to pack in the membranes,[41] decreases the sizes of the vesicles and hence could decrease the fluidity across the bilayer.

Fig. 3A shows typical absorption spectra for the Bz-Try-*p*NA hydrolysis at different reaction time in DOPC : Cho vesicles with $X_{DOPC} = 0.80$. We observed the clear isosbestic point at $\lambda = 345$ nm evidencing that the lack of intermediates and/or product decomposition. Fig. 3B shows typical results obtained treating the data according to with eqn (12). The data was treated as described before and, the values of k_{cat}, K_M, τ and, the permeability obtained are gathered in Table 2. An interesting interpretation can be obtained.

There are not significant differences in the k_{cat} and K_M values found in DOPC vesicles (with and without Cho). For example, the catalytic efficiency (k_{cat}/K_M) in the DOPC vesicles is 3300 M^{-1} s^{-1} and, 3363 M^{-1} s^{-1} for DOPC : Cho vesicles with $X_{DOPC} = 0.80$; in comparison with the value found in homogeneous media: 3600 M^{-1} s^{-1}. The similar values found in different systems confirms that the α-CT is localized in the water core of the vesicles. It seems that the presence the Cho does not affect the kinetic parameters values.

On the other hand, it is very interesting the fact that the DOPC bilayer permeability change by two the order of magnitude even at 10% of presence of Cho. The value of the permeability in the DOPC vesicles without Cho is 1.7×10^{-7} cm s^{-1} and, 7.1×10^{-9} cm s^{-1} and 7.8×10^{-9} cm s^{-1} for DOPC : Cho vesicles with $X_{DOPC} = 0.90$ and $X_{DOPC} = 0.80$, respectively. As discussed before, the data of permeability obtained is of the same order of magnitude than that found by Walde *et al.*[39] in different vesicles validate the model used. Even more, the values found are in agreement with results obtained for another substrate in different bilayers of vesicles. For example, Faure *et al.*[36] investigated the glucose permeability in phosphatidylcholine vesicles by an enzymatic reaction and the method proposed by Sato *et al.*[18] The value found was 8.3×10^{-9} cm s^{-1} and comparable to a value obtained by Lidgard *et al.*[37] (7.5×10^{-8} cm s^{-1}) and Wood *et al.*[38] (5×10^{-8} cm s^{-1}) when bilayer was prepared from egg phosphatidylcholine–cholesterol



Scheme 3 Schematic representation of the region occupied by cholesterol in DOPC vesicles.

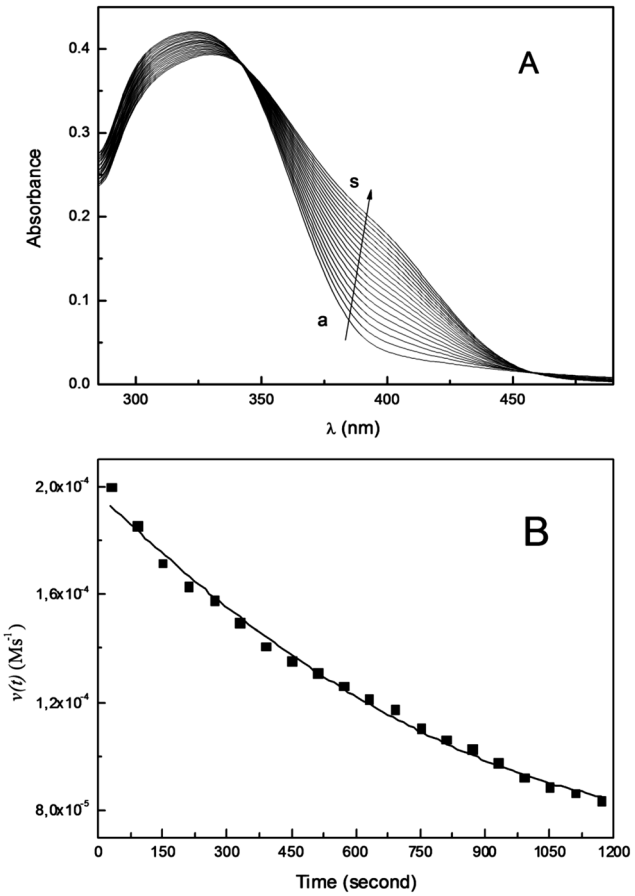


Fig. 3 (A) Representative absorbance spectra at different time for the hydrolysis of Bz-Try-*p*NA catalyzed by [α-CT] in DOPC : Cho vesicles with $X_{DOPC} = 0.80$. The mixer ethanol–water at 4% p/p was used as dispersant media. Time (s): (a) 30, (b) 90, (c) 150, (d) 210, (e) 270, (f) 330, (g) 450, (h) 510, (i) 570, (j) 630, (k) 690, (l) 750, (m) 810, (n) 870, (o) 930, (p) 990, (q) 1050, (r) 1110, (s) 1170. [α-CT] $= 1 \times 10^{-6}$ M. [DOPC] $= 1.27 \times 10^{-3}$ M. [Bz-Try-*p*NA] $= 5 \times 10^{-5}$ M; pH = 8.7. (B) The time rate of hydrolysis of Bz-Try-*p*NA catalyzed by α-CT hydrolysis inside of DOPC : Cho vesicles with $X_{DOPC} = 0.80$. The mixer ethanol–water at 4% p/p was used as dispersant media. [α-CT] $= 1 \times 10^{-6}$ M. [Bz-Try-*p*NA] $= 5 \times 10^{-5}$ M. [DOPC] $= 1.27 \times 10^{-3}$ M. pH = 8.7. The experimental data were fitted according to eqn (12), (–) solid lines.

mixtures and from phospholipids extracts of human red cells, respectively. The differences found on the values of the permeability were assigned to the different capacity of the surfactants to alter the structural organization of the lipids, which is directly related to the bilayer fluidity and permeability.[36] Thus, we attribute that the presence of Cho decreases the fluidity across to bilayer dramatically to a different lipid organization in the presence of the sterol, which makes more propitious the interaction of Bz-Try-*p*Na with the carbon chain.

Conclusion

The results show that reactions hydrolysis of Bz-Try-*p*NA catalyzed by α-CT follows the Michaelis–Menten mechanism in both system studied, homogenous media and DOPC : Cho vesicles. The value of the catalytic efficiency is around 3300 M^{-1} s^{-1} in all systems and, these indicate that the cholesterol is not modifying the kinetic parameters of the reaction, changing dramatically the permeability values of the substrate.

Here, we demonstrated that, using a simple mathematical model, it is possible to determine the values of the permeability of Bz-Try-*p*NA through the vesicle bilayer. The permeability value found for DOPC vesicles was 1.7×10^{-7} cm s^{-1} while, in the presence of Cho the value found was around 7.0×10^{-9} cm s^{-1}. Thus, there is a notable decrease in the permeability values even at low Cho content. This was attributed to a different lipid organization in the presence of the sterol, which makes more propitious the interaction of Bz-Try-*p*Na with the carbon chain.

We highlight that using an enzymatic hydrolysis reaction inside DOPC vesicles and, a simple mathematical model, it is possible to determine jointly the kinetics parameters and the permeability of the bilayer to the Bz-Try-*p*Na.

Acknowledgements

Financial support from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP CONICET 112-201101-00204), Universidad Nacional de Río Cuarto, Agencia Nacional de Promoción Científica y Técnica (PICT 2012-0232), (PICT 2016-2151), and Ministerio de Ciencia y Tecnología, gobierno de la provincia de Córdoba (PID-2012) is gratefully acknowledged. J. J. S., N. M. C., and F. M. hold a research position at CONICET. M. A. L. thanks from CONICET for a research post-doctoral fellowship.

References

1 A. Chattopadhyay and S. Mukherjee, *Biochemistry*, 1983, **32**, 3804.
2 O. G. Mouritsen and L. A. Bagatolli, *Life – As A Matter Of Fat: Lipids In A Membrane Biophysics Perspective*, Springer, 2nd edn, 2016.
3 M. Kępczyński, K. Nawalany, B. Jachimska, M. Romek and M. Nowakowska, *Colloids Surf., B*, 2006, **49**, 22.
4 R. N. A. H. Lewis and R. N. McElhaney, in *The Structure of Biological membranes*, ed. P. Yeagle, CRC Press, Boca Ratton, FL, 1992.
5 M. Poznik and B. König, *RSC Adv.*, 2016, **6**, 44456.
6 M. Poznik, U. Faltermeier, B. Dick and B. König, *RSC Adv.*, 2016, **6**, 41947.
7 S. Chaves, L. M. Pera, C. L. Avila, C. M. Romero, M. Baigori, F. E. Morán Vieyra, C. D. Borsarelli and R. N. Chehin, *RSC Adv.*, 2016, **6**, 8528.
8 T. Zheng, M. Bulacu, G. Daudey, F. Versluis, J. Voskuhl, G. Martelli, J. Raap, G. J. Agur Sevink, A. Kros and A. L. Boyle, *RSC Adv.*, 2016, **6**, 7990.
9 A. A. Yaroslavov, O. V. Zaborova, A. V. Sybachin, I. V. Kalashnikova, E. Kesselman, J. Schmidt, Y. Talmon, A. R. Rodriguez and T. J. Deming, *RSC Adv.*, 2015, **5**, 98687.
10 P. C. Ross, M. L. Hensen, R. Supabphol and S. V. Hui, *J. Liposome Res.*, 1998, **8**, 499.

11 N. Mignet, A. Bup, C. Degert, B. Delord, D. Roux, C. Hélène, J. C. Francxois and R. Laversanne, *Nucleic Acids Res.*, 2000, **28**, 3134.

12 V. P. Torchilin, V. S. Trubetskoy, A. M. Milshteyn, J. Canillo, G. L. Wolf, M. I. Papisov, A. A. Bogdanov, J. Narula, B. An Khaw and V. G. Omlyanenko, *J. Controlled Release*, 1994, **28**, 45.

13 G. Vanlerberghe, *Liposomes in cosmetics: how and why? In Non-Medical Applications of Liposomes*, ed. D. D. Lasic and Y. Barenholz, CRC Press, Boca Raton, FL, 1996, vol. IV.

14 S. M. Johnson and A. D. Bangham, *Biochim. Biophys. Acta*, 1969, **193**, 82.

15 T. Kitagawa, K. Inoue and S. Nojima, *J. Biochem.*, 1976, **79**, 1135.

16 R. A. Demel, S. C. Kinsky, C. B. Kinsky and L. M. Van Deenen, *Biochim. Biophys. Acta*, 1968, **150**, 655.

17 J. Davidsen, K. Jorgensen, T. L. Andresen and O. G. Mouritsen, *Biochim. Biophys. Acta*, 2003, **1609**, 95.

18 T. Sato, M. Kijima, Y. Shiga and Y. Yonezawa, *Langmuir*, 1991, **7**, 2330.

19 A. Srivastava and K. B. Eisenthal, *Chem. Phys. Lett.*, 1998, **292**, 345.

20 E. C. Y. Yan and K. B. Eisenthal, *Biophys. J.*, 2000, **79**, 898.

21 R. T. Hamiltonand and E. W. Kaler, *J. Colloid Interface Sci.*, 1987, **116**, 248.

22 P. L. Yeagle, *Biochimie*, 1991, **73**, 1303.

23 T. P. McMullen, R. N. Lewis and R. N. McElhaney, *Biochemistry*, 1993, **32**, 516.

24 R. R. C. New, *Liposomes. A practical approach*, Oxford University Press Inc., New York, 1997.

25 V. De Los Rios, J. M. Mancheno, P. A. Martinez, C. Alfonso, G. Rivas, M. Onaderra and J. G. Gavilanes, *FEBS Lett.*, 1999, **455**, 27.

26 V. De Los Rios, J. M. Mancheno, M. E. Lanio, M. Onaderra and J. G. Gavilanes, *Eur. J. Biochem.*, 1998, **252**, 284.

27 D. Marsh, *Handbook of Lipid Bilayers*, CRC Press, Boca Raton, 2nd edn, 2013.

28 T. C. Lauren and J. Killander, *J. Chromatogr. A*, 1964, **14**, 317.

29 G. Cerv, Solute Transport Across Bilayer, in *Phospholipid handbook*, ed. G. Cerv, Marcel Dekker, Inc., New York, 1993.

30 F. Moyano, E. Setien, J. J. Silber and N. M. Correa, *Langmuir*, 2013, **29**, 8245.

31 M. F. Barroso, M. A. Luna, J. S. Flores Tabares, C. Delerue-Matos, N. M. Correa, F. Moyano and P. G. Molina, *Beilstein J. Nanotechnol.*, 2016, **7**, 655.

32 F. Moyano, R. D. Falcone, J. C. Mejuto, J. J. Silber and N. M. Correa, *Chem.–Eur. J.*, 2010, **16**, 8887.

33 R. D. Falcone, M. A. Biasutti, N. M. Correa, J. J. Silber, E. Lissi and E. Abuin, *Langmuir*, 2004, **20**, 5732.

34 E. Abuin, E. Lissi and C. Calderón, *J. Colloid Interface Sci.*, 2007, **308**, 573.

35 A. Kumar and P. Venkatesu, *Chem. Rev.*, 2012, **112**, 4283.

36 C. Faure, F. Nallet, D. Roux, S. T. Milner, F. Gauffre, D. Olea and O. Lambert, *Biophys. J.*, 2006, **91**, 4340.

37 G. P. Lidgard and M. N. Jones, *J. Membr. Biol.*, 1975, **21**, 1.

38 R. E. Wood, F. P. Wirth and H. E. Morgan, *Biochim. Biophys. Acta*, 1968, **163**, 171.

39 M. Blocher, P. Walde and I. J. Dunn, *Biotechnol. Bioeng.*, 1999, **62**, 36.

40 D. M. Davis, D. Mcloskey, D. J. S. Birch, P. R. Gellert, R. S. Kittlety and R. M. Swart, *Biophys. Chem.*, 1996, **60**, 63.

41 T. M. Konyakhina, J. Wu, J. D. Mastroianni, F. A. Heberle and G. W. Feigenson, *Biochim. Biophys. Acta, Biomembr.*, 1828, **9**, 2204.