

Methodology article

## Protein encapsulation in liposomes: efficiency depends on interactions between protein and phospholipid bilayer.

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### Abstract

**Background:** We investigated the encapsulation mechanism of enzymes into liposomes. The existing protocols to achieve high encapsulation efficiencies are basically optimized for chemically stable molecules. Enzymes, however, are fragile and encapsulation requires in addition the preservation of their functionality. Using acetylcholinesterase as a model, we found that most protocols lead to a rapid denaturation of the enzyme with loss in the functionality and therefore inappropriate for such an application. The most appropriate method is based on lipid film hydration but had a very low efficiency.

**Results:** To improve it and to propose a standard procedure for enzyme encapsulation, we separate each step and we studied the effect of each parameter on encapsulation: lipid and buffer composition and effect of the different physical treatment as freeze-thaw cycle or liposomes extrusion. We found that by increasing the lipid concentration, increasing the number of freeze-thaw cycles and enhancing the interactions of the enzyme with the liposome lipid surface more than 40% of the initial total activity can be encapsulated.

**Conclusion:** We propose here an optimized procedure to encapsulate fragile enzymes into liposomes. Optimal encapsulation is achieved by induction of a specific interaction between the enzyme and the lipid surface.

### Background

Genomics studies and molecular biology evolutions in mutagenesis have brought to us a large quantity of different proteins, and notably enzymes, that would be useful to biotechnology. Different applications may be considered such as their use in biosensors, protein chips or as biocatalysts. However, most enzymes are fragile and any of their conformational changes may induce a loss of ac-

tivity. Therefore their stabilization is required. One possibility to is to encapsulate the protein into nanometer sized vesicles. This method protects the enzyme from self denaturation due to dilution effects and furthermore protect the enzyme from the aggression by external agents such as proteases [1]. Beyond its stabilizing effect, enzymes encapsulation put forward other assets that biotechnological applications may benefit from. For example, encapsula-

tion allows to control indirectly the enzyme specificity; by entrapping different porines in the liposomes membranes, it is possible to regulate substrates entrance or products exit from the vesicle [1,2]. Another application of encapsulation is the delivering of actives molecules for treatment of malignancy [3,4].

Nanometer sized capsules can be readily prepared upon rehydrating lipid films. The size calibration is usually performed by ultrasonic irradiation, extrusion through a filter of calibrated pore size, centrifugation or molecular sieve chromatography. However, liposomes are unstable and are therefore not suited for many applications. Liposomes can be stabilised by adding hydrophobic monomers of a polymer. The monomer will participate into the hydrophobic core of the lipid. Crosslinking can be induced by UV light or temperature [5]. Another possibility to create long term stable vesicles is to formulate them using diblock or triblock copolymers leading to mechanical and chemical stable nanocapsules while preserving the membrane fluidity [6]. Both techniques lead to stable nanometer sized vesicles.

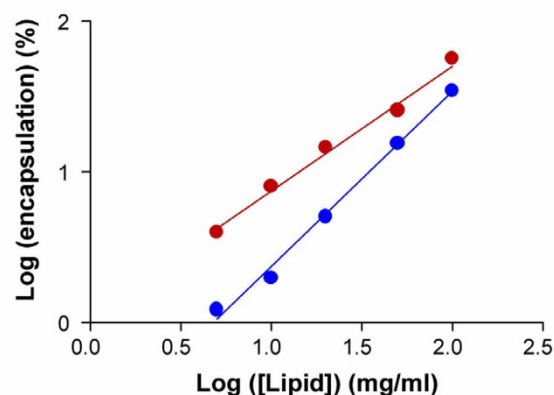
To encapsulate molecules in vesicles, several methods can be used. In a first method, lipids are dissolved in a suitable organic solvent, mixed with an aqueous medium followed by removal of the solvent [7]. In a second method, mixed lipid-detergent micelles are prepared and detergent is eliminated by dialysis below its critical micellar concentration [8]. We tested both approaches but the protein was denatured either by organic solvents or by detergent. A third popular method is the lipid film's hydration: a phospholipid solution in chloroform is dried under vacuum to obtain a lipid film; this lipid film is subsequently hydrated in a solution containing the protein [9,10]. This method allows to encapsulate proteins in its functional form but efficiency of encapsulation was weak.

Our purpose was to improve the film hydration method for loading liposomes with proteins while preserving their functionality. We chose to encapsulate *Drosophila melanogaster* acetylcholinesterase (AChE) as a reporter enzyme because of the availability of an easy and accurate functional assay. Moreover, this type of enzyme is highly interesting as a biosensor to detect insecticide residues and many groups are trying to develop an adequate detection systems [11,12]. Here, we show following our suggestion that 40% of the initial active enzyme can be encapsulated without any denaturation.

## Results

### Effect of lipid concentration

In a first series of experiments, we investigated the influence of the lipid concentration on the encapsulation. Various amounts of egg PC and POPS (from 0.5 to 10 mg in



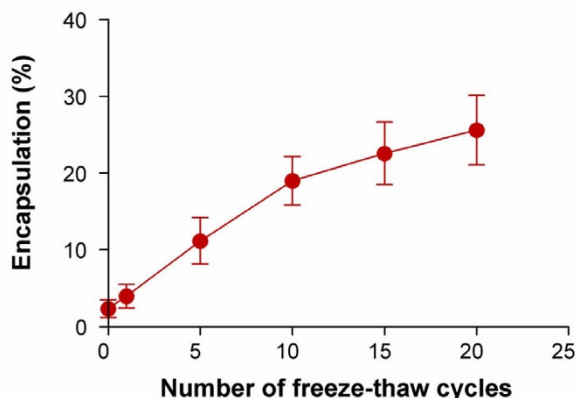
**Figure 1**

Effect of lipid concentration on AChE encapsulation. Two lipids compositions were used: egg POPC (red) and POPS (blue).

0.1 to 1 ml buffer) were used and the encapsulation efficiency of AChE was recorded. Figure 1 clearly shows a linear relationship between the lipid concentration and the encapsulation efficiency. The liposome surface is proportional to the lipid concentration, in contrast the encapsulated inner volume is proportional to the lipid concentration to the power 3/2. Thus a double logarithmic plot of the encapsulated activity versus lipid concentration allows to discriminate between the relevant parameter. For eggPC and POPC lipids, values of the slope ( $0.86 \pm 0.01$  and  $1.16 \pm 0.04$ , respectively) were close to 1 suggesting that encapsulation is proportional to the number of lipids and thus proportional to the surface; and is significantly far from 1.5 relevant for encapsulation via internal volume.

### Effect of freeze-thaw number

Hydration of lipid films results in inhomogeneous multilamellar vesicles. Application of a series of freeze-thaw cycles breaks the multilamellar vesicles into unilamellar vesicles. As each freeze-thaw cycle usually causes denaturation of proteins, we investigated the effect number of freeze-thaw on encapsulation. About 5 mg eggPC lipid films were solubilized with 1 ml of a 25 mM MOPS pH 7 solution containing AChE. The tubes then underwent different numbers of freeze-thaw cycles from 0 to 20. The liposome solutions were then extruded. The encapsulation efficiencies were compared and are shown in figure 2. We observed an increase of encapsulation efficiency according to the number of freeze-thaw cycles without significant denaturation of the enzyme. This suggests that in the lipid hydration method, the encapsulation mainly occurs during the freeze thaw step. In the following experiments we fix the freeze-thaw cycles to 10.

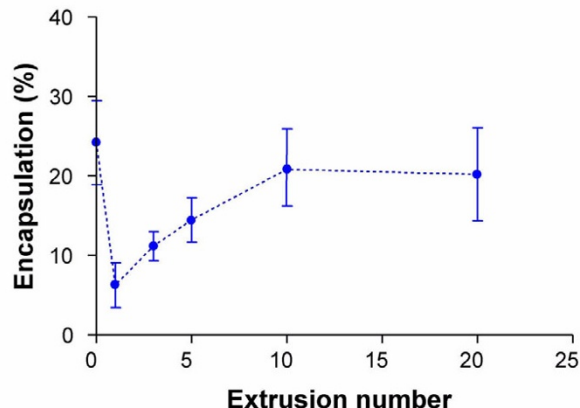


**Figure 2**  
Effect of freeze-thaw number on AChE encapsulation under similar conditions as in figure 1.

#### Effect of extrusion

Vesicles formed by spontaneous swelling as described do have a rather polydisperse size distribution [13]. Narrow size distribution can be obtained by extruding the suspension through a filter of defined size. About 5 mg eggPC lipid films were solubilized with 1 ml of a 25 mM MOPS pH7 solution containing AChE. Then the tubes underwent ten freeze-thaw cycles and the liposome solutions were then subjected to different numbers of extrusions (from 0 to 20) through a disposable 200 nm cellulose acetate pore filter. Surprisingly we observed a strong decrease of encapsulation efficiency with the first extrusion and then a progressive recovery (Fig. 3). However, if the solution is passed through a new filter, no recovery was observed. This suggests the retention of much material inside the filter. Increasing the extrusion number did not result in an increase of encapsulation efficiency beyond the encapsulation observed following the freeze-thaw step.

As several types of filters are available, we investigated the effect on the composition of the filter on encapsulation efficiency (Fig. 4). AChE was encapsulated using the above conditions with ten freeze-thaw cycles followed by ten extrusions through a polycarbonate filter usually sold for the liposome extruder (Fig. 4, line 1). After extrusion we compared the final encapsulates enzymatic activity with the initial total one. This gives the encapsulation efficiency. In addition, we compared also the final total (encapsulated and free enzyme) activity after extrusion with the initial total one. This gives the enzyme recovery. This allows an estimate of the amount of enzyme lost, either adsorbed inside the filter or denatured. We observed 20% encapsulation without any significant loss of enzyme activity with the polycarbonate filter. The same result was

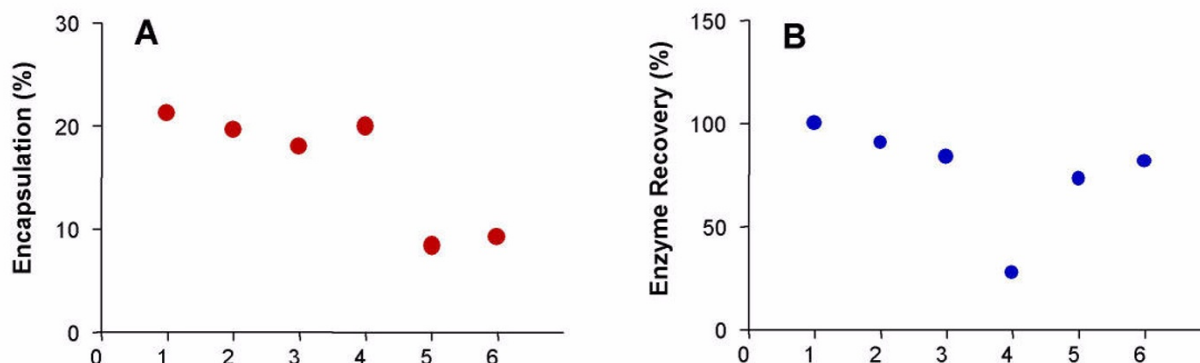


**Figure 3**  
Effect of extrusion number on AChE encapsulation. The filter was made of polycarbonate under similar conditions as in figure 1.

obtained using disposable filter in cellulose acetate or in regenerated cellulose (Fig. 4, line 2 and 3). With nylon filter (Fig. 4, line 4), the encapsulation is identical to encapsulation observed with cellulose acetate but recovery was weak. Encapsulation and recovery were identical suggesting that non-encapsulated AChE was retained on the membrane while encapsulated AChE passes through the filter. This result is consistent with the absence of effect of extrusion on encapsulation which took place before, during the freeze-thaw cycles. It is interesting to note that the use of nylon filter shows an unexpected advantage: it permits to remove the non-encapsulated enzymes without any detrimental effect on encapsulation. Extrusions performed on membranes made of PTFE (polytetrafluoroethylene) and Cellulose Acetate with Glass Fiber premembrane (Fig. 4, line 5 and 6) gave much lower encapsulation efficiencies. As these filters are hydrophobic, we may hypothesize that retention of some vesicles or lipids occurred on these filters. Dynamic light scattering experiments were done concurrently and in agreement with published literature [13], a narrow size distributions were obtained for all extruded liposomes solutions.

#### Effect of solution composition

In the following series of experiments, we investigated the effect of the buffer composition on the encapsulation efficiency. Four buffer compositions were tested: 25 mM Tris HCl, 25 mM MOPS, 25 mM MES and 25 mM sodium phosphate, all buffers were at pH 7. AChE was encapsulated under standard conditions as described above, and encapsulation efficiencies were compared. No significant difference in the encapsulation efficiency was observed. Several pH values were tested for the encapsulation of AChE in 25 mM sodium phosphate buffer from 6.0 to 8.5,

**Figure 4**

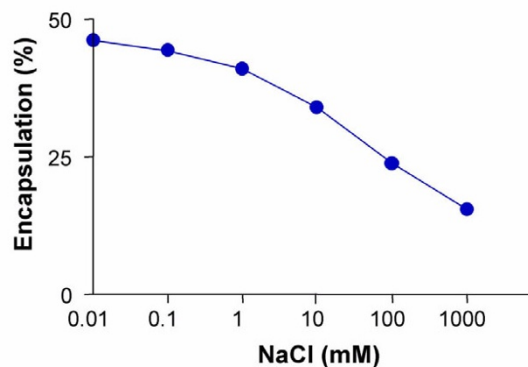
4A. Effect of filter composition on AChE encapsulation percentage: encapsulated enzymatic activity/ total activity. 4B. Enzyme recovery percentage: Total enzymatic activity after extrusion/total enzymatic activity. The filter used were: Polycarbonate filter specially designed for liposome extruder (line 1), Cellulose Acetate (line 2), Regenerated Cellulose (line 3), Nylon (line 4), PTFE (polytetrafluoroethylene) (line 5) and Cellulose Acetate with a Glass Fiber premembrane (line 6).

with an increment of 0.5 and no significant difference in the pH range we tested could be observed.

In the next step we tested the effect of the ionic strength. Different concentrations of NaCl were used for the encapsulation of AChE from 10  $\mu$ M to 1 M. It appeared that increasing concentration of NaCl lowers encapsulation (Fig. 5). According to the Debye-Hückel theory, addition of ions screens charged groups and leads to a reduction of electrostatic interactions between the enzyme and the lipid film. The observed decrease of the encapsulation efficiency while increasing the salts concentration suggests that encapsulation is related to an interaction between phospholipids and protein.

#### Effect of co-encapsulation of stabilizers

Most of proteins are denatured during freeze-thaw cycles. Since this step of the encapsulation process is a key step for the encapsulation efficiency, it cannot be skipped. One solution to avoid denaturation of proteins sensible to thermal fluctuations would be the coencapsulation of stabilizers. Indeed, addition of stabilizing additives in protein formulations is the most common tool to increasing the shelf life of the product. These compounds are often chosen on an empirical basis since the protective effect of solutes is variable, depending on protein characteristics. We therefore tried to coencapsulate AChE with different molecules (sugars, polymer or protein) usually employed to stabilize enzymes and we checked if these molecules affect encapsulation efficiencies. Here our enzyme was stable to resist the freeze-thaw cycle. We therefore

**Figure 5**

Effect of NaCl concentration on AChE encapsulation under similar conditions as in figure 1.

investigated if the coencapsulation reduces the activity of the encapsulated enzyme. Mannose, sucrose and trehalose were coencapsulated at 100 mM with AChE as described in materials and methods. It appeared that those stabilizers did not affect encapsulation (data not shown), suggesting that proteins that would be stabilized by those sugars could be coencapsulated without any loss of efficiency. PEG (polyethylene glycol) and BSA (bovine serum albumin) protect the AChE from denaturation [14]. Enzyme buffered solutions containing various concentrations of PEG 3350 or BSA were used for encapsulation and

efficiencies were compared. It turned out that PEG 3350 and BSA strongly disfavor encapsulation (Fig. 6) suggesting that PEG and BSA immobilize water molecules and compete with AChE for interaction with phospholipids.

#### Effect of lipid composition

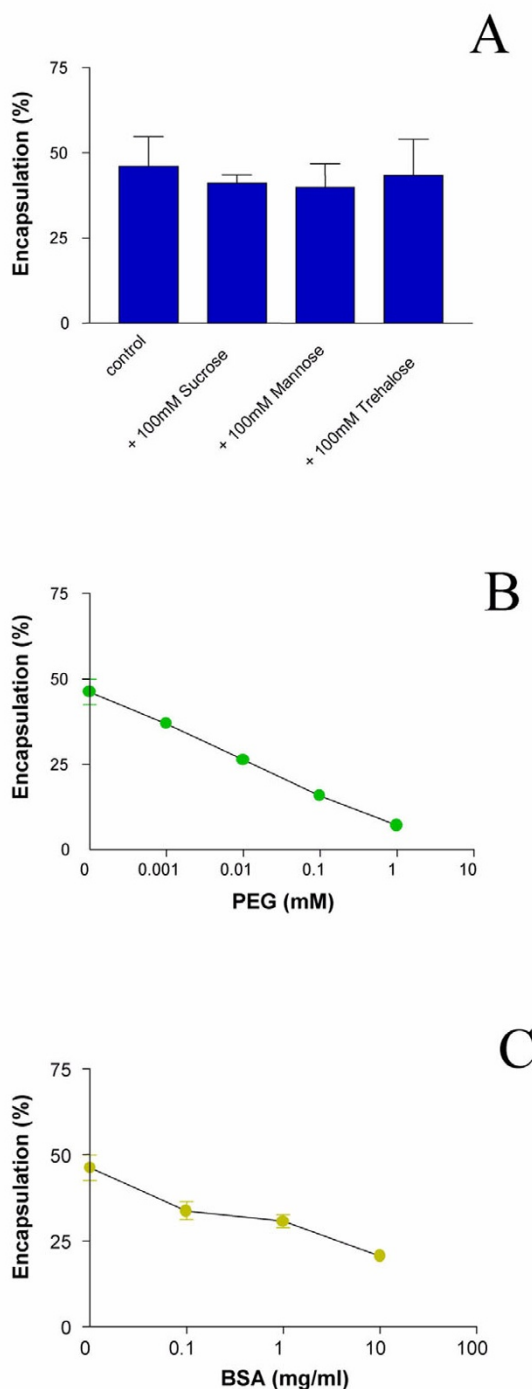
Different phospholipid compositions were used for the formulation of liposomes loaded with AChE, and encapsulation efficiencies were compared. Liposomes with the same polar head groups (eggPC and POPC) but with hydrocarbon chains of different length and saturation degrees had nearly the same encapsulation efficiencies (Fig. 7) suggesting that encapsulation efficiency does not depend on the hydrophobic component of the phospholipid. Encapsulation efficiency was better with POPC than with POPS (Fig. 1 and 7). Encapsulation was performed at pH 8.5, the enzyme peripheral surface was globally negatively charged. Surface of lipid membrane made with POPC was neutral and surface of lipid membrane with POPS was negatively charged. These data suggest that encapsulation depends on electrostatic interactions between the enzyme peripheral surface and the polar head group of phospholipids. With 5% of POPE, no significant encapsulation efficiency differences were observed.

#### Effect of lipids with a functional group to bind proteins on encapsulation efficiency

The previous experiments suggest that electrostatic interactions between the enzyme and the lipidic surface favor encapsulation. However, increasing the unspecific electrostatic interactions with the lipid surface is rather limited as the ionic strength and the surface charge density of the protein can only be varied within a small range. In order to provide "universal method" for high efficient encapsulation we then test the possibility to use a specific interaction using the affinity of histidine tag for Nickel. The AChE we used has three histidine tag in a peptidic loop lying at the surface of the protein. This enzyme binds to NTA-Ni column chromatography confirming the functionality of the histidine tag. 5 mg of eggPC mixed with 0.12 mg DOGS-NTA-Ni lipids were used to formulate 1 ml lipid suspension. In order to observe the enhancement due to the specific interaction we minimize the electrostatic interaction by addition of 500 mM NaCl to the solution. It appeared (Fig. 8) that the addition of DOGS-NTA-Ni lipids in the composition of the liposomes enhanced the encapsulation efficiency of AChE.

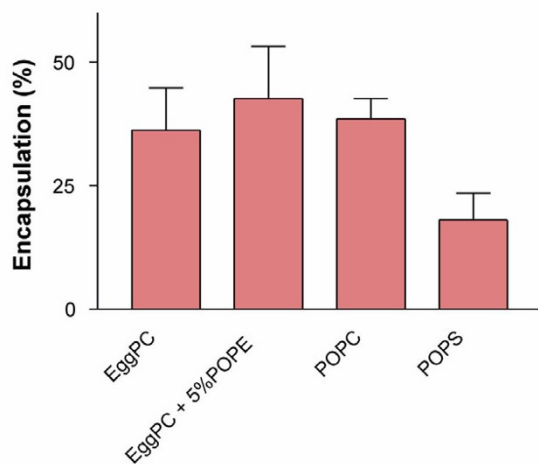
#### Discussion

Several methods have been described to encapsulate macromolecules. First assays using the dissolution of lipids in a suitable organic solvent failed because the protein was always denatured. Methods using mixed micelles of detergents (n-octyl- $\beta$ -D-glucopyranoside or CHAPS) according to Mok et al. [15] also resulted to a denaturation of the en-

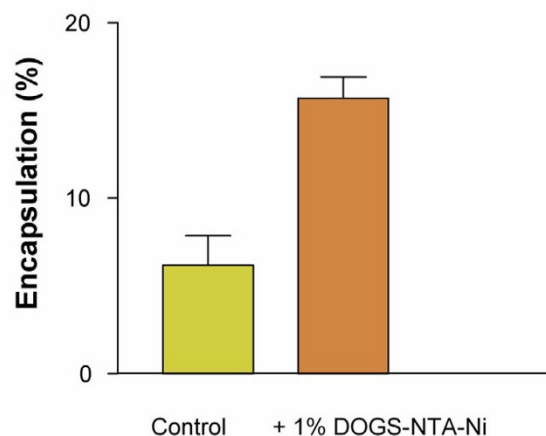


**Figure 6**

Effect of coencapsulation of stabilizers on AChE encapsulation. The measurements were done under similar conditions as in figure 1. (A) Encapsulation efficiency in absence (control) or with sugars (sucrose, mannose or trehalose). (B) Encapsulation efficiency as function of Polyethylene Glycol (PEG) concentration. (C) Encapsulation efficiency as function of Bovine Serum Albumin (BSA) concentration.

**Figure 7**

Effect of lipid composition on AChE encapsulation. Effect of eggPC, eggPC+5%POPE, POPC, POPS under similar conditions as in figure 1.

**Figure 8**

Effect of addition of 1% DOGS-NTA-Ni to egg-PC lipids on AChE encapsulation efficiency in presence of 0.5 M NaCl.

zyme during the dialysis steps necessary to eliminate the detergent molecules. Thus we turned to the film hydration method and we performed various experiments to increase the efficiency of active enzyme encapsulation.

In the film hydration method, several evidences suggest that efficient encapsulation is obtained by favoring interactions of the protein with the lipidic film during the freeze-thaw cycles.

- AChE encapsulation efficiencies in PC liposomes was better than that obtained with POPS liposomes. As the enzyme is negatively charged as well as POPS, we can hypothesize that the difference in encapsulation is due to an electrostatic interaction between the enzyme and the surface of the lipidic wall.

- During the successive extrusions, the enzyme stayed inside the liposome suggesting that interaction between lipid membranes and the protein did not allow free displacement of the protein.

- Encapsulation decreases with increase of salt concentration. This result leads to hypothesize that ionic strength decreases electrostatic interactions between liposomes wall and amino-acids present at the surface of the protein resulting in a decreased encapsulation.

- With an His-tagged enzyme, the addition of DOGS-NTA-Ni lipids in the liposome formulation induces a specific

interaction of the lipid with the enzyme, and thus allow its better encapsulation.

A drawback of freeze-thaw cycles is the denaturation of proteins. Stabilization of the enzyme by addition of protective compounds can be used. Utilization of a specific interaction such as the Nickel-histidine interaction permits to use stabilizers without any competition for the interaction with the membrane. Neglecting the unexpected adsorption of the material in the filter which can be recovered after several repeated extrusion steps no reduction nor an enhancement of the encapsulation efficiency could be observed. Another interest of extrusion appeared when we tested different filter materials: using filter surfaces with a strong affinity for protein allows to eliminate non-encapsulated protein from a solution.

## Conclusions

We showed that the encapsulation efficiency of protein depends on interaction between the protein and the lipid bilayer. The enzyme entrapment can be increased by manipulation of the liposomal lipid composition, or by increasing the lipid concentration, in order to favor electrostatic interactions. Because of the important role of those interactions during encapsulation, one has to take care of the ionic strength of the enzyme solution, as ions could hide electrostatic interactions, preventing a good encapsulation efficiency. It is also possible to induce a specific interactions such as an interaction between an His-tagged enzyme and the lipid surface of the liposomes by addition of DOGS-NTA-Ni lipids in their formulation.

## Material and methods

### Materials

#### Lipids

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), 1,2-Dioleoyl-*sn*-Glycero-3- $\{[N(5\text{-Amino-1-Carboxypentyl})\text{iminodiAcetic Acid}]\text{Succinyl}\}$  (Nickel salt) (DOGS-NTA-Ni), were from Avanti Polar Lipids, Inc (Alabaster, AL) and eggPC extract (98% pure) was from Lipoid, Ludwigshafen.

#### Proteins

Pronase was purchased from Sigma (St. Louis, MO). Recombinant acetylcholinesterase was produced in baculovirus infected cells and purified by affinity chromatography as previously described [14]. Drosophila AChE is a dimer which is linked to the membrane via a glycolipid anchor. This form can be converted to an hydrophilic dimer by expression of a truncated cDNA [16]. This hydrophilic form was used. Furthermore, each monomer of the dimer was tagged with three histidines by site directed mutagenesis using the QuickChange site-directed mutagenesis kit from Stratagene.

#### Preparation of liposomes

Liposomes were prepared using the film hydration method. Under standard conditions, 5 mg of lipids in solution in chloroform were dried at the bottom of a 8 ml tube for 15 min. under nitrogen gas stream and desiccated for 6 hr. under vacuum. Multilamellar vesicles were obtained by hydration of the film with 0.1 ml of 25 mM MOPS buffer pH 8.5, containing 0.3 nmole of enzyme. The tube was vortexed until the lipid film was peeled off from the tube surface. To break the multilamellar vesicles into monolamellar, ten cycles of freezing (liquid nitrogen) and thawing (30°C water bath) were applied. The sample was then diluted to 1 ml in 25 mM MOPS buffer pH 8.5. Size of liposome was homogenized by extrusion by passing the sample 10 fold through a 200 nm pore polycarbonate filters unless otherwise indicated. Modifications of this method are indicated for each experiment.

#### Quantification of the encapsulation efficiency

AChE activity is measured according Ellman [17]. AChE catalyses the hydrolysis of acetylthiocholine, producing acetate and thiocholine; thiocholine is then able to react with di-thionitrobenzoic acid, producing a thiocholine coupled with a thio-nitro-benzoic acid by a disulfide bond and a thio-nitro-benzoate. This molecule is detectable by O.D. measurements at 412 nm with an  $\epsilon = 13600 \text{ M}^{-1}\text{cm}^{-1}$ . AChE activity is equal to the absorbance variation plotted versus time.

To determine the fraction of encapsulated AChE, the solution was incubated in a solution of the proteolytic enzyme pronase. Pronase does not pass through the liposomal bilayer and therefore the encapsulated AChE is protected from enzymatic digestion. Free enzyme and enzyme bound to the external surface of the liposome are inactivated according to a first order kinetics. Variation of proportion of remaining active enzyme amount ( $[E]/[E_0]$ ) with time ( $t$ ) follows equation 1:

$$\frac{[E]}{E_0} = (1 - A) \cdot e^{-k \cdot t} + A \quad (1)$$

where A is the proportion of encapsulated enzyme and  $k$  the rate constant of enzyme inactivation. To estimate  $[E]/[E_0]$ , the enzyme was incubated with 1 mg ml<sup>-1</sup> pronase at 25°C in 25 mM phosphate buffer pH 7. The variation of the remaining free enzyme  $[E]/[E_0]$  with time was estimated by sampling aliquots at various times and recording the remaining activity with 1 mM acetylthiocholine in presence of 0.1% Triton X-100. The proportion of encapsulated enzyme (A) was estimated by non-linear regression with  $t$  as variable. As far as mixed lipid-detergent micelles were found to be reversible inhibitors of AChE, inhibition kinetics of AChE by POPC, POPS and eggPC were recorded and the results shown in this paper take those inhibitions in account.

#### Authors' contributions

Author 1 (J-P C) carried out experiments illustrated in figures 1, 3 and 4. Author 2 (B C) carried out experiments illustrated in figure 8. Authors 1 and 2 carried out together experiments illustrated in figures 2, 5, 6 and 7; Author 3 (M W) and 4 (D F) conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

#### Abbreviations

AChE acetylcholinesterase

BSA bovine serum albumine

DOGS-NTA-Ni 1,2-Dioleoyl-*sn*-Glycero-3- $\{[N(5\text{-Amino-1-Carboxypentyl})\text{iminodiAcetic Acid}]\text{Succinyl}\}$  (Nickel salt)

EggPC egg extracted lipids with a Phospho-L-Choline polar head

MOPS 3-[N-morpholino]propanesulfonic acid

PEG polyethylene glycol

POPC 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

POPE 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine

POPS 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine

PTFE polytetrafluoroethylene

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