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Shunt Concept for Specific Active Transport Using Enzymatic Membranes: Review and Prospects

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Artifical enzymatic membranes (AEMs) were conceived with the aim of replicating the active transport found in vivo. The shunt concept has recently emerged from the use of two enzymes catalysing two opposite reactions occurring on both parts of a porous charged membrane (+ or −) and able to specifically add/remove (or the contrary) a charged group (+ or −) on the selected molecule to be transported. Historically, the Phosphatase (P)/Kinase (K) couple (or its inverse), frequently found in nature, was selected for creating these shunts. Modelling of these transports was realized using the Nernst-Planck equation. In parallel, experimental studies were conducted proving that these shunt topologies, involving enzymatic membranes, lead to specific and active solute transports at physiological temperature and pressure. Issuing from this concept, recent technological prospects, such as the specific separation and concentration of (L) substrate from the (D/L) racemic mixture and the selective transport of neutral molecules by electrophoresis, are presented. This review presents the main results obtained using polymeric membranes linked to enzymes with the aim of replicating the active transport found in vivo.

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KEYWORDS Enzymatic membranes, Specific active transports, Separation and purification technology, Shunt concept, Specific concentration technology

INTRODUCTION

Porous polymeric membranes still provide considerable insight into separation processes. However, these unspecific processes often need high pressure conditions. Therefore, advances in membrane technology focus now on the association of these passive membrane properties with the selective recognition of different biomolecules (1). These biofunctional membranes involving enzymes (or cells) immobilized onto polymeric porous matrices are now more and more studied. These investigations concern principally recent studies such as enzyme bioreactors (2,3), affinity membranes (4) and biosensors (5). As in biological membranes these biofunctional membranes take advantage of molecular recognition, i.e., a high selectivity into aqueous medium and under mild conditions of temperature and pressure. We present a short review dealing with a shunt concept aimed at specifically concentrate small molecules against their concentration gradients under physiological conditions of temperature and pressure only.

This shunt concept has emerged from the use of two enzymes catalysing two opposite reactions occurring on both parts of a porous charged synthetic polymeric membrane (+ or −) and able to specifically add/remove (or the contrary) a charged group (+ or −) on the selected molecule to be transported. Historically, the Phosphatase (P)/Kinase (K) couple (or its inverse), frequently found in nature, was selected for creating these shunts (6–10). Depending on the reaction sequence of the enzymes (P/K or K/P) as well as the membrane charges (+ or −), the membrane will permit either the transport of a charged (phosphorylated) molecule (7, 12–15) or the transport of a neutral (unphosphorylated) molecule against its electrochemical gradient, as illustrated in the overall topologies presented in Figure 1 (11).

The shunt functions as follows: in Figure 1(a), a phosphorylated substrate (SP^{2-}), present in the donor compartment (C_I) is dephosphorylated by the alkaline phosphatase immobilized on the side adjacent to (C_I). The neutral (dephosphorylated) substrate S^0, generated inside the unstirred layer (USL), always present (17), and adjacent to C_I (Δ_I) by this first reaction, crosses the membrane barrier by passive diffusion to be rephosphorylated by a specific kinase acting inside Δ_{II} the USL adjacent to receiver compartment (C_{II}). Negative charges, carried by the permeable membrane, by repelling the SP^{2-} regenerated by the kinase in C_{II}, cause it to accumulate. It is to be noted that this SP^{2-} active transport requires hydrolysis of the ATP as an energy source. In Figure 1(b), the shunt permitting the specific active transport of neutral substrate (S^0) functions in the same manner, the main

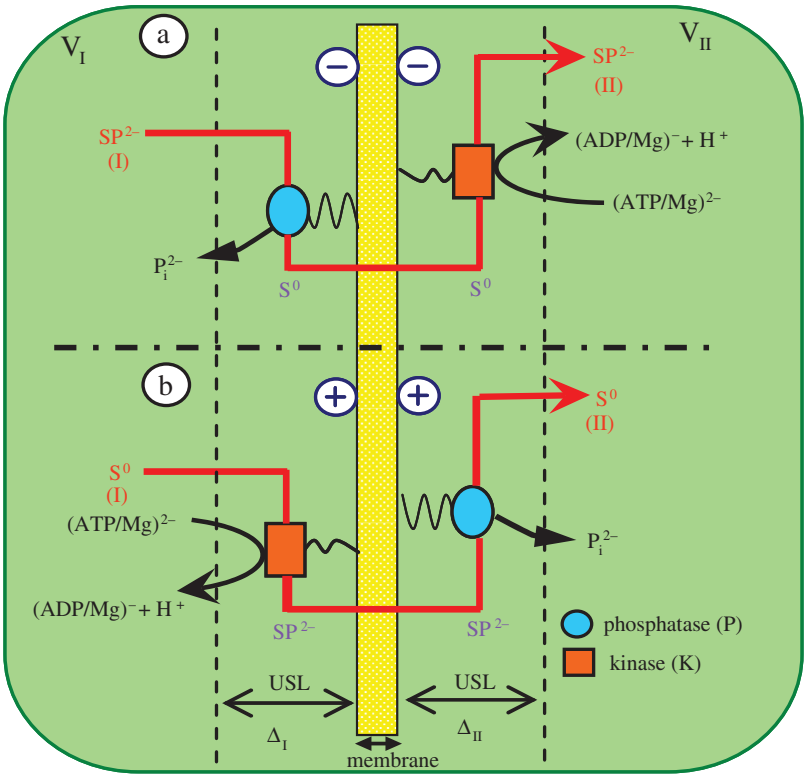


FIGURE 1 Overall topologies of shunts permitting (a) the specific active transport of a phosphorylated substrate (SP^{2-}) and (b) a neutral substrate (S^0). In (a) the membrane, with an overall thickness X is composed of a porous, negatively charged membrane inserted between a membrane carrying a phosphatase (●) and a membrane carrying a specific kinase (■). This composite membrane separates a donor compartment C_I from a receiver compartment C_{II} with a volume of V_I and V_{II}, respectively. The enzymes act in the unstirred layers of thickness Δ_I and Δ_{II} situated in compartments C_I and C_{II}, respectively. S^0 represents the substrate in its dephosphorylated form (e.g., glycerol) and SP^{2-} represents the substrate in its phosphorylated form (e.g., $Gro3P^{2-}$). (color figure available online.)

differences concern the enzyme positions, the membrane charges (+) and the ATP location in C_I.

Experimental studies suggest a coupling between: (i) diffusion phenomena taking place in diffusion layers adjacent to membrane surfaces (USLs); (ii) the two enzymatic reactions opposing one another; (iii) electrostatic interactions produced between membrane surface charges and at least one charged metabolite (intermediate substrate or product). Modelling of these transports was realized using the Nernst-Planck equation (8,9,16,18–21).

All the experimental studies presented proves that these enzymatic membranes lead to specific and active solute transports under physiological temperature and pressure conditions only (7, 13–15). Issuing from this

concept, we present the main results obtained as well as recent technological prospects, such as the specific separation/concentration of (L) glycerol-phosphate from a (D/L) racemic mixture (22) and the selective active transport of neutral molecules by electrophoresis (23).

It should be stressed that all the experimental results presented (early and recent) assume: (i) a detailed knowledge of the properties of the support membrane; and (ii) mastery of the techniques for immobilising biomolecules on different membrane supports. These fundamental technical aspects will not be covered in this review but can be found in the literature (1, 23).

The aim of this review paper is to present results and prospects on specific active transport using enzymatic membranes. The paper provides a literature section, and a discussion of both experimental and theoretical results obtained in this field. It will be emphasized throughout the paper that this purification method beside its high specificity offers several advantages, the major one being its mild conditions in temperature and pressure and the absence of solvent. In addition, this field of research is closely related to others well recognized areas such as enzymatic membrane bioreactors and biological membranes (6–10). Results presented in this paper could be found useful in these fields in terms of enzyme immobilization, applications and mathematical modelling.

RECALL AND REVIEW OF APPROACHES INVOLVING ENZYMATIC MEMBRANES IN COMPARTMENTALIZED SYSTEMS

Binding an enzyme to an insoluble membrane support creates heterogeneity as a result of several phenomena. Immobilising the enzymes on the support can differ for each molecule and, as a result, access to the catalytic sites can vary. In addition, binding is likely to provoke a change in enzyme conformation, leading to a possible modification in the enzyme's kinetic parameters. The support can also be the source of electrostatic and hydrophobic interactions with solutes involved in the reaction. Finally, substrate diffusion towards (or in) the support to reach the enzyme's catalytic site can be a limiting factor. It should be noted that there will always be a fluid zone close to an immersed solid support that is not subject to the turbulence of the surrounding medium. This fact recognized for a long time is now well established (17). This zone has been named the "Unstirred Layer" (USL). In this quiet zone, molecules migrate only by diffusion in relation to their electrochemical gradient.

Different methods can be considered for studying heterogeneous catalysis involving a single membrane as support. The membrane can be immersed in a solution or used to separate two compartments of a diffusion cell. Only

the latter approach permits the study of the vectorization and distribution of reagents on both sides of the membrane. Different groups have worked on compartmentalized systems. Historically, it was the Katchalski group who, in 1968, established the fundamental basis of heterogeneous enzymatic catalysis.

Katchalski's group was able to model the amplitude and direction of substrate and product fluxes, as well as their profiles in different types of membrane (symmetrical and asymmetrical) then predicted the local pH profile in the membrane when the enzymatic reaction produces acids or bases (24). They also showed that the effects of external diffusional limitations are more marked when the membrane enzyme is very active with a weak Km and were able to estimate the thickness of the USLs surrounding the membrane (25).

From 1970 to 1973, Thomas's group carried out several important studies aimed at achieving experimentally the active transport of a neutral molecule such as glucose. Using the hexokinase/phosphatase enzyme pair bound in a permeable membrane separating two compartments, these authors were able to obtain a concentration difference between the two compartments of 0.22 g/L, starting with identical initial concentrations of 0.5 g/L. This concentration difference then dropped rapidly over time (26, 27). This series of pioneering experiments that showed the possibility of obtaining active transport by using an artificial membrane integrating two enzymes catalysing two opposite reactions has not been followed up.

During this period, Caplan's group showed that the pairing coefficients linking material and reaction fluxes were non-existent in symmetrical membranes. This led to the enzyme's kinetic parameters (Vm/Km) (28, 29).

The experimental results obtained by these different groups led to a certain number of conclusions. Vectorization of the reaction product catalyzed by enzymes immobilized on a membrane can only take place if the system is anisotropic. This anisotropy can be achieved when the enzymatic reaction occurs in an unstirred layer, when the enzymatic membrane is structurally asymmetrical and depending on the origin of the substrate. An excellent summary of the results obtained during this period has been published (30).

Few groups in the world continued to use enzymatic artificial membranes with a view to making progress in the understanding of cellular compartmentalization (in its broadest sense).

In the case of asymmetric membranes, Caplan's group showed that the reaction product was always more concentrated in the compartment adjacent to the enzymatic face (31). This was due to the fact that these authors were using a diffusion cell containing two compartments of identical volume.

With the aim of optimizing the performance of enzymatic electrodes (which incorporate enzymatic membranes in their structures), Coulet's group focussed particularly on vectorization of the reaction product generated

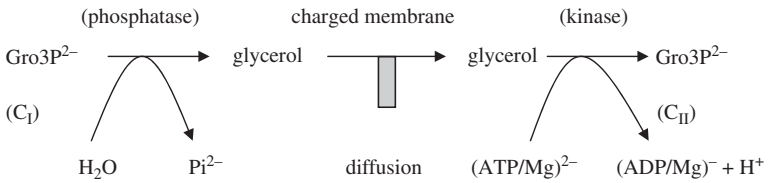
by an enzyme immobilized on a porous membrane separating two compartments of differing volumes, after taking into account the parameters described earlier (32–34). The results obtained showed that the asymmetry of the system was amplified by the difference in volume existing on either side of the membrane and by the presence of a diffusion layer adjacent to the enzymatic surface. This topography permitted a hyper-concentration of the reaction product to be obtained in the small compartment in contrast to the large compartment where the enzymatic reaction took place. These results were new and contradicted those obtained by *Bunow* and *Caplan* (31). In an asymmetrical compartmentalized system, the diffusion layer would appear to have a major role in enzyme kinetics and on product distribution on both sides of the membrane. Other experiments, using a membrane carrying a hexokinase (catalysing the reaction: $\text{Glucose} + (\text{ATP/Mg})^{2-} \rightarrow \text{Glucose-6-Phosphate}^{2-} + (\text{ADP/Mg})^{-} + \text{H}^{+}$) permitted a hyper-concentration of Glucose-6-Phosphate to be obtained in the compartment opposite to where the reaction took place (34).

Hervagault's group, concentrating on theoretical models suggested by Ricard's group (35, 36), have been able to show the major role of electrostatic membrane/solute interactions on solute vectorization (37, 38).

By the end of these two periods, no experiment had yet achieved the active transport of solute with the help of enzymatic artificial membranes. Theoretical models proposed by the different groups cited, to explain their results, only took into account a limited number, but never all the parameters identified by all these groups. These models were therefore incomplete. One experimental study in particular did make a significant advance in the AEM approach. This study, done with the magnetic resonance of ^{31}P, showed that the active transport of phosphocholine in higher plants occurred via dephosphorylation at the negatively charged cell wall, followed by re-phosphorylation of choline by a specific kinase situated inside the cell (39).

Thus, in vivo, active transport of a phosphorylated molecule was possible via a dephosphorylation/phosphorylation process. This assumed that choline, when uncharged, could diffuse through the cell wall. This negatively charged barrier, by trapping phosphocholine, also negatively charged, and regenerated by the intracellular kinase, caused an accumulation in the cell. From that time onwards one could imagine an AEM permitting the active transport of a phosphorylated molecule by coupling diffusion phenomena with two opposite reactions (phosphatase/kinase) and electrostatic interactions between a charged membrane and at least one charged solute involved in the reaction process.

From 1996, the structure of an AEM, allowing the active transport of a small phosphorylated molecule such as glycerol-3-phosphate, was presented in the form of a patent deposition (6), then published (7). The sequence of events giving this transport is as follows:



Glycerol-3-phosphate ($Gro3P^{2-}$), present in the donor compartment (C_I) is dephosphorylated by the alkaline phosphatase immobilized on the side adjacent to (C_I). The glycerol generated by this first reaction crosses the membrane barrier by passive diffusion to be rephosphorylated by the glycerokinase immobilized on the side of the membrane adjacent to receiver compartment (C_{II}). This last reaction requires hydrolysis of the ATP. Negative charges, carried by the permeable membrane, by repelling the $Gro3P^{2-}$ regenerated by the glycerokinase in $C_{II,}$ cause it to accumulate.

Following these first results, a number of experimental and theoretical studies were conducted.

MAIN EXPERIMENTAL RESULTS

The main experimental results obtained under a Phosphatase/Kinase (P/K) topology orientated in the C_I/C_{II} direction reported in Figure 2 concern the active transport of glycerol-3-phosphate ($Gro3P^{2-}$) performed using two different diffusion cells (7, 12) and an industrial reactor (13). In Figure 2(A), curves (1), (2) and (4) have been experimentally obtained by using a structured functional Phosphatase-GlyceroKinase membrane, a passive structured Bovine-Serum-Albumin membrane and a structured functional phosphatase membrane, respectively. Curve (3) corresponds to the differences between curve (1) and curve (4). These experiments confirm that the rate of the active transport of a phosphorylated substrate (i.e., $Gro3P^{2-}$) asymptotically reached a maximum value and also that without enzymes no transport occurs (7).

In Figure 2(B), these studies were performed with the same phosphatase and glycerokinase membranes associated with two different nonenzymatic intermediary membranes. Curve (1) was obtained using a PALL PES prototype membrane, while curve (2) was obtained using a more negatively charged membrane (PALL NAZ membrane negatively charged by glycine coupling). These experiments confirm the key role of membrane charges (12).

In Figure 2(C), $Gro3P^{2-}$, initially present in the same concentrations (0.6 mM) in both compartments, concentrated with time in C_I to reach a concentration 6 times greater than in C_{II} after 10 hours. Curve (1) shows change

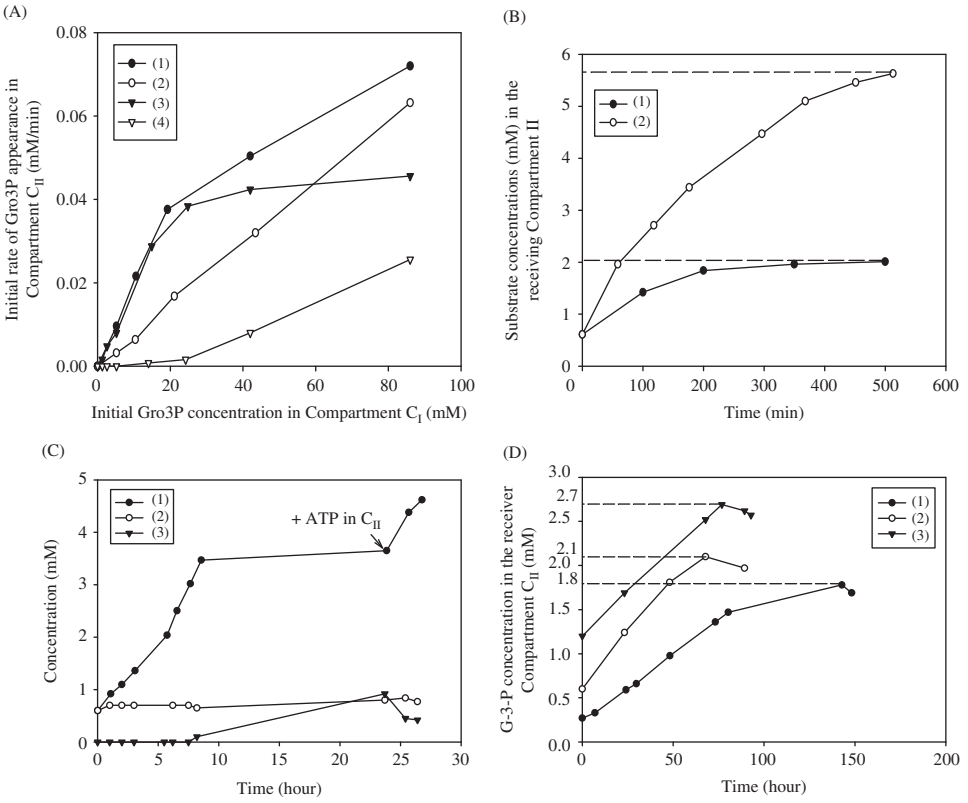


FIGURE 2 Active transport of $Gro3P^{2-}$ under a P/K topology involving a passive intermediate negatively charged membrane. (A**)** Initial rates of $Gro3P^{2-}$ appearance in the receiver compartment of a diffusion cell versus initial $Gro3P^{2-}$ concentrations in the emitter compartment. (B) Comparative studies of the active transport of $Gro3P^{2-}$ performed using two differently charged biomimetic membranes are presented. (C) Change in concentration of $Gro3P^{2-}$ in the receiver compartment of an industrial reactor. (D) Change in $Gro3P^{2-}$ concentration in the receiver compartment of a diffusion cell.

in concentration of $Gro3P^{2-}$ in the receiving compartment (C_I). The concentration of $Gro3P^{2-}$ in the emitter compartment (curve (2)) was remained constant with the aid of a drip perfusion. Contamination of the receiver compartment by glycerol (curve (3)) appears negligible up to 7 hours. Thereafter, because of a lack of $(ATP/Mg)^{2-}$, this contamination increases. Transport is restored by the addition of $(ATP/Mg)^{2-}$ in the receiver compartment; these experiments confirm the indispensable role of ATP (as fuel) in such transport (13).

In Figure 2(D), the three curves were obtained with the same initial concentration of 1.5 mM ATP in the receiver compartment. Initial $Gro3P^{2-}$ concentrations in the two compartments of 0.3 mM (1), 0.6 mM (2) and 1.2 mM (3) were used. For these three cases a concentration increase of 1.5 mM corresponds to the total consumption of the ATP and therefore to a stoichiometry of one molecule of $Gro3P^{2-}$ transported for one molecule

of $(ATP/Mg)^{2-}$ used and that a mole of ATP was hydrolyzed per mole of $Gro3P^{2-}$ transported (14). The four experiments reported in Figure 2 demonstrate that the specific active transport of small phosphorylated molecules can be achieved in vitro with the help of enzymatic membranes involving a P/K topology.

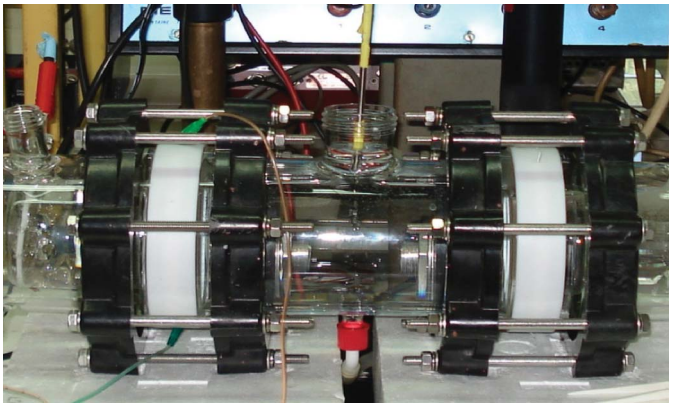
The slowness of the process must not overshadow the fact that these membranes allow an active transport which uses only ATP as its energy source (13). Surely, the transport time could be improved significantly, especially by using thinner membranes. As a matter of fact, the composite AEMs used in these studies were $4.5x10^4$ times thicker than a biological membrane. Simulations conducted on a biological scale have shown high substrate accumulations in a few minutes (20).

The active and selective transport of glucose and glycerol was carried out using electrophoresis and artificial enzymatic membranes. These positively charged composite membranes carry, on the face adjacent to the donor compartment of an electrophoresis module, a specific kinase (hexokinase or glycerokinase) and, on the opposite face, an alkaline phosphatase. Phosphorylation of the neutral substrate (glucose or glycerol) on the donor side by the kinase generates a negatively charged phosphorylated substrate, whose transmembrane migration is promoted by an electric field and by the membrane's positive charge. Dephosphorylation of the phosphorylated substrate by alkaline phosphatase on the opposite face regenerates the neutral substrate, which accumulates in the receiver compartment of the electrophoresis module (Fig. 3). Using an electrophoresis module specifically designed for this study, our experiments were carried out enabling glucose and glycerol to be concentrated approximately 8-fold and 12-fold, respectively, in 8 h (Fig. 4) (23).

As reported in Figure 5, we also demonstrate that a P/K topology, involving glycerokinase, leads not only to the separation of a racemic mixture of (D/L) glycerophosphate, but also to the concentration of the (L) form alone (22). One of the major advantages of this approach is that this specific separation-concentration is achieved in one step.

MATHEMATICAL ANALYSIS FOR P/K AND K/P TOPOLOGIES

In parallel to the experimental studies, exploratory theoretical studies were conducted from mathematical modelling based on mass balance, to understand the performance of the AEM and the role of different key parameters such as: the membrane porosity and thickness, the electrical resistance, the enzymatic reactions, the unstirred layers (USLs) thickness, the position of the enzyme in the USLs, etc. Two kind of topologies have been mentioned according to the position of the enzymes on the membrane surfaces: the P/K or phosphatase/kinase topology used for the active transport of a phosphorylated substrate denoted SP^{2-}, and the K/P or kinase/phosphatase topology



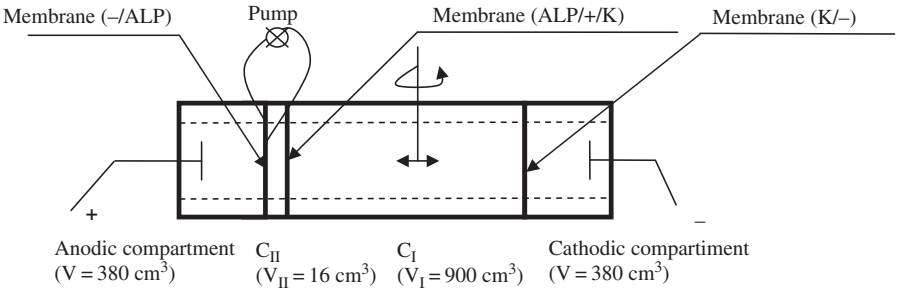


FIGURE 3 Photography and diagram of the electrophoresis module (not to scale). Three types of enzymatic membranes (active surfaces of 41.9 cm^2) separate the module's different compartments. From left to right, (i) a negatively charged (−) membrane connected to a membrane carrying alkaline phosphatase (ALP), referred to as membrane (−/ALP), separates the anodic compartment from the receiver compartment (C_{II}), the ALP face adjacent to C_{II}; (ii) a positively charged (+) membrane inserted between an ALP-carrying membrane and a kinase-carrying membrane (HK or GK), referred to as membrane (ALP/+/K), separates C_{II} from C_I, the ALP face adjacent to C_{II}; (iii) a kinase-carrying membrane (HK or GK) connected to a negatively charged (−) membrane, referred to as membrane (K/−) separates C_I from the cathodic compartment, the K face adjacent to C_I. (color figure available online.)

used to concentrate a neutral substrate denoted S against its concentration gradient (Fig. 1).

Under each topology, it was assumed that the two following reverse enzymatic reactions sequence were occurred inside the unstirred layers adjacent to the charged membrane surfaces:

$$SP^{2-} \xrightarrow[\text{Membrane-bound phosphatase } \mathbf{P}]{(H_2O)} S + P^{2-} \qquad (a)$$

$$S + (ATP/Mg)^{2-} \xrightarrow[\text{Membrane-bound kinase } \mathbf{K}]{} SP^{2-} + (ADP/Mg)^{-} + H^{+} \qquad (b)$$

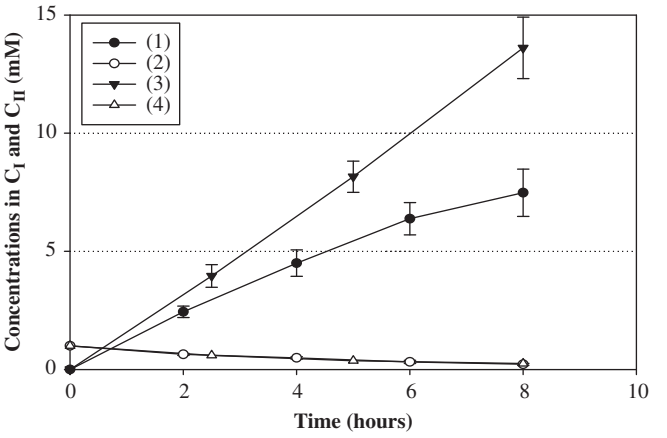


FIGURE 4 Typical changes in glucose and glycerol concentration over time in the two compartments of an electrophoresis module. Curves (1) and (3) show the typical changes in glucose and glycerol concentration (mM) over time in C_{II}, respectively, (average over 3 runs for each substrate). Curves (2) and (4) show the glucose and glycerol concentration evolution over time in C_I, respectively. Controls carried out under the same physico-chemical conditions, but with non-enzymatic composite membranes, show that weak passive diffusion of glucose and glycerol from C_I to C_{II} occurred (about 0.1 mM for both neutral substrate detected in C_{II} after 8 hours). These results were obtained at 30°C under a constant current of 200 mA (generating a tension of 40 Volts).

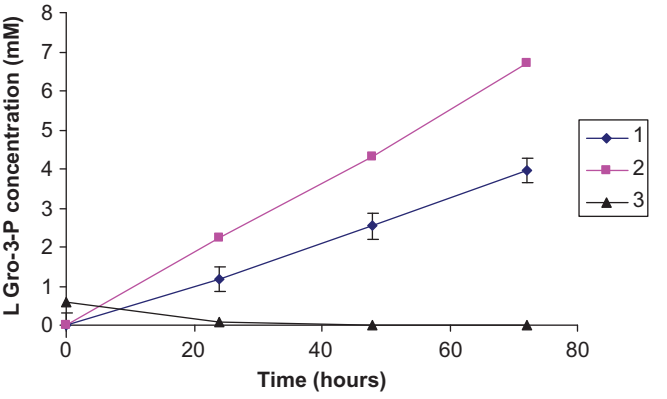


FIGURE 5 Changes in (L) Gro3P concentrations in the receiver compartment (C_{II}) of a diffusion cell, starting from a racemic mixture of (D/L) Gro3P (1.2 mM) initially present in the donor compartment (C_I). Curves (1) and (2) describe the changes in (L) Gro3P concentration in C_{II} obtained in the presence of initial $(ATP/Mg)^{2-}$ concentrations of 6 mM (Curve(1)) and 12 mM (curve(2)). Curve (1) is the average over 5 runs. Curve (3) describes changes in (L) Gro3P concentrations in C_I. (color figure available online.)

For this last reaction, the energy supply results from the degradation of the adenosine triphosphate $(ATP/Mg)^{2-}$ in the adequate compartment (emitter or receiver) according to the topology considered. During this reaction, $(ATP/Mg)^{2-}$ was consumed and thus, both reaction sequences appear

to behave as an ATPase. In the microenvironment of the membrane, it was assumed that each USL is composed of a passive diffusion layer of thickness δ and an ionic double layer of thickness DL in which an constant electric field exists (Fig. 6).

The model was derived from the mass balance by coupling mass transfer with enzymatic activities. The mass transfer flux of a charged compound, results from the combination of a diffusion flux described by Fick's law and a migration flux resulting from the potential gradient. Regarding the enzymatic reactions, the unphosphorylated substrate S of the kinase, but also the product the phosphatase, is phosphorylated using $(ATP/Mg)^{2-}$ as a phosphate group donor which therefore is the second substrate of the kinase. The

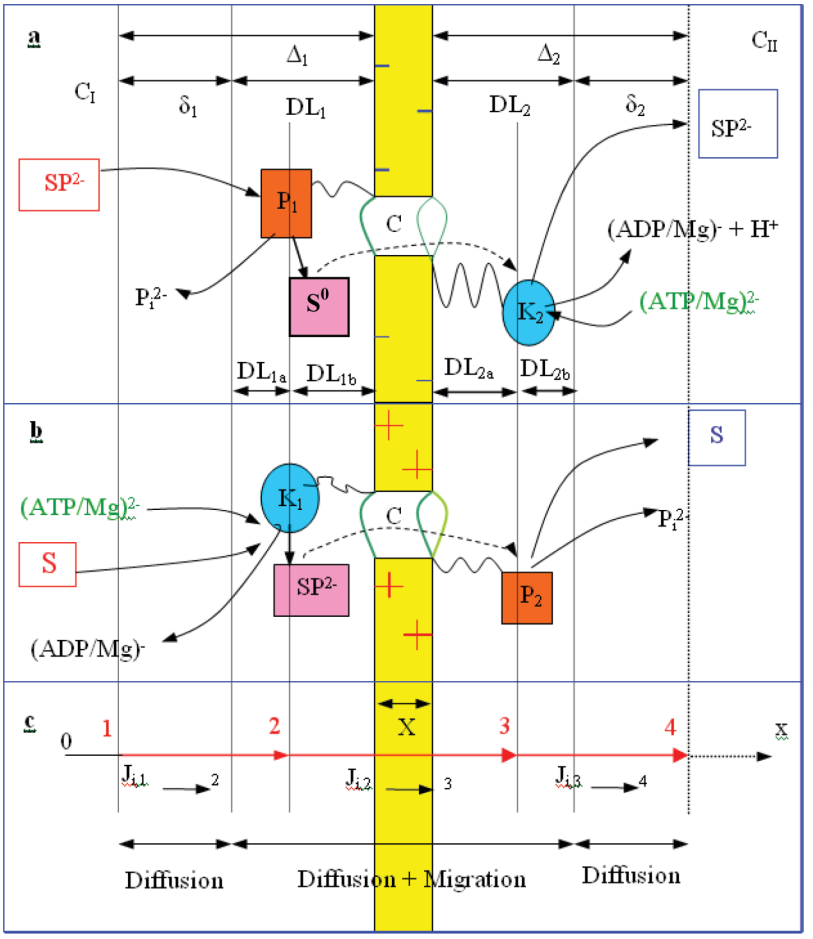


FIGURE 6 (a): Biomimetic membrane topology for the active transport of a phosphorylated substrate SP^{2-} (e.g., $Gro3P^{2-}$). (b): Biomimetic membrane topology for the active transport of an unphosphorylated substrate S^0, i.e., in most cases a neutral solute (e.g., glycerol). (color figure available online.)

kinetic enzymatic rate used in this case is described by the ping-pong expression (40, 41). SP^{2-} is the phosphorylated substrate of the membrane-bound phosphatase. An alkaline phosphatase belongs to the class of hydrolases, that is, it uses H_2O as second substrate to hydrolyze the phosphorylated substrate. Water concentrations are normally assumed not to be rate limiting, therefore, the rates of these enzymatic reactions can be expressed under Michaelis-Menten formulation.

All the derived equations with the boundaries conditions lead to an algebraic and differential equations which were solved by numerical methods. The simulations were performed with the glycerol-3-phosphate ($Gro3P^{2-}$) as an example of the active transport of SP^{2-}, and the glycerol as an example of the active transport of S.

MAIN SIMULATION RESULTS

First, analysis of this model with credible numerical values has permitted to observe that the relative position of the enzymes in relation to the membrane surface (more or less separated) play a key role in the transport process (18). For a given membrane surface potential, this relative position, within or outside the ionic double layers, can lead to different properties of the enzymatic membrane. Depending on these different topologies, the membrane can be either impermeable or capable of active transport. Therefore, on the technological level, depending on the technique used to immobilize the enzymes, involving linkers of different lengths, the performance of the enzymatic membrane (for fixed membrane surface potentials and under constant ionic strength in the medium) could be severely affected (19).

Second, a broad analysis of the role played by the main parameters taken into account in the model was conducted to precizely define the physicochemical conditions and the membrane topology needed for the highest active transports within the shortest time (20–21).

The main simulation results are the following: on the one hand, concerning the active transport of a charged molecule (P/K topology), anisotropic membranes (i.e., charged +/− or −/+), whatever the enzyme position (i.e., inside or outside the ionic double layer adjacent to the membrane) permit the active transport of a charged molecule. It is to be noted that isotropic membranes (charged −/−) also permit such active transport but only when enzymes are acting outside the ionic double layers (20). On the other hand, concerning the active transport of an uncharged molecule (K/P topology), only an isotropic positive-charged membrane (+/+) allows the active transport of a neutral molecule, whatever the enzyme positions (i.e., inside or outside the ionic double layer adjacent to the membrane) (21).

CONCLUSIONS AND PROSPECTS

Artificial enzymatic membranes were conceived with the aim to imitate the behavior of biological membranes in terms of selective active transport properties. All the studies carried out with the help of AEMs show that the primary active transport of small hydrophilic molecules (neutral and phosphorylated), can be achieved in vitro by using artificial permeable charged membranes involving the phosphatase/kinase enzyme pair (or the reverse). This specific ATP-dependent transport, created by these shunts, shows saturated kinetics comparable to that obtained with biological carriers (7). In relation to membrane charges (+ or −) : (i) the Phosphatase/Kinase (P/K) topology allows the active transport of a phosphorylated molecule (SP^{2-}); while (ii) the K/P topology allows the active transport of a neutral non-phosphorylated molecule S.

In all cases, for this transport to occur, the following conditions must be met simultaneously: (i) the membrane must be charged; (ii) two enzymes, catalyzing two opposite reactions (e.g. the phosphatase/kinase pair, or the reverse) act on each side of the membrane and in the USLs; (iii) at least one solute involved in the reaction sequence must be charged. It must be underlined that in absence of one of these conditions, no transport occurs.

These conditions, quite possible on a biological level, have led to the proposal of a new mechanism, which could explain certain types of biological transports (18). This proposal was supported by increasing evidence that channels play an important role in organic solute transport in a wide variety of cell types and organisms (42). However, it must be noted carefully that in the proposed mechanism, the diameters of the molecules were supposed to be smaller than the channel diameters.

Besides the high selectivity of enzymes, one major advantage of this technique of concentration (or purification) is its ambient temperature and pressure conditions. Such mild conditions are particularly attractive for labile molecules. Many active pharmaceutical ingredients and high value natural molecules are susceptible to thermal and pressure degradation, thus physiologic conditions during the preparation processes can minimize loss of activity and/or nutritive. Two applications of biomimetic enzymatic membranes were reported demonstrating that it was possible to simultaneously separate and concentrate (L) glycerol-phosphate from the (D/L) racemic mixture (22), as well as to specifically concentrate neutral solutes by electrophoresis (23). Hopefully, new application as the active enantioselective transport separation of (S)-ibuprofen from a mixture of (R/S)-ibuprofen (43) and others will emerge in the next future.

These experiments and theoretical analyses show that these enzymatic shunts allow active and specific transports of small molecules, without major conformational changes and under physiological conditions. The possibility of being able to either specifically concentrate or eliminate small organic

molecules with the use of AEMs opens up real technological prospects. Last but not least, the shunt concept presented by our research group and involving enzymatic membranes may be generalized using others couple of (bio)-catalysts grafted on both parts of a charged membrane and able to specifically add/remove (or the contrary) a charged group on the selected molecule to be concentrated or eliminated.

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