ON THE HYPERICIN INTERACTION WITH THE ARTIFICIAL LIPID MEMBRANE IN THE PRESENCE OF LYOTROPIC ANIONS

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Abstract. The modifications induced by hypericin on the electric properties of the artificial lipid bilayers suggest that it has apparent stabilizing effects on the membrane against strong chaotropic anions. We imply that this is due to the interference of hypericin's insertion into the bilayers with the mechanisms by which the lyotropic anions alter the electrical surroundings of the bilayer and/or the intrinsic membrane dipole potential.

Key words: hypericin, lyotropic anions, BLM, SSM.

1. INTRODUCTION

Hypericin (*Hyp*) is a naturally occurring polyphenol from *Hypericum* species. The group of polyphenols, containing from simple phenolic molecules to highly polymerized compounds with molecular weights of greater than 30,000 Da, constitutes one of the most numerous and widely spread group of plant metabolites, being an integral part of both human and animal diets. Among polyphenols characteristics, major ones consist in their free radical – scavenging capacity protecting the fatty membranes of cells, proteins and DNA, and in their ability to interact with proteins. The assumption that polyphenols exert their antioxidant, cell-protecting effects by free radical scavenging might be a rather simplified view on their mechanisms of action [2, 25]. More likely, cells respond to polyphenols mainly through direct interactions with receptors or enzymes involved in signal transduction and this may result in modification of the redox status of the cell and may trigger a series of redox-dependent reactions [27]. As antioxidants, polyphenols may improve cell survival. There is experimental evidence – *in vitro* studies at concentrations much higher than those found *in vivo* (20 μM) – that polyphenols may also present prooxidant activity leading to inhibition of cell

growth and transformation, enhanced apoptosis, reduced invasive behavior, and slowed angiogenesis [22, 34]. Polyphenols may exert their beneficial effects by altering stress signaling and neuronal communication, suggesting protection against age-related deficits in cognitive and motor function [20].

Belonging to the polyphenol group, *Hyp* also presents various properties: antidepressant, potent virucidal agent, anti-inflammatory activity, therapeutic agent in treatment/detection of cancer [1]. *Hyp* is capable of forming salts with sodium and potassium, it is hydrophobic and insoluble in water, oil, methylene chloride and most other nonpolar solvents. It is soluble in alkaline aqueous solutions, organic bases and polar organic substances (acetone, ethanol, methanol, ethyl acetate, ethyl methyl ketone) and in biological media, leading to complex formation with biological macromolecules such as DNA, human and bovine serum albumin and other plasma proteins, membrane fragments and cellular compounds [26]. *Hyp* interacts with artificial lipid membranes modifying their electric conductance [28].

In spite of all acquired data and of *Hyp* becoming the subject of intensive biochemical research during the last four decades, the mechanisms of its various activities remains largely unexplained.

Here we are interested to see whether this polyphenol may demonstrate a stabilizing effect on lipid membranes against some anions belonging to the Hofmeister series. These anions, commonly present in nutritional supplies as natural components or ingredients, can affect the kinetics of chemical reactions occurring in foods [8].

The Hofmeister series, or the lyotropic series, consists of two sequences in which anions and cations are ordered according to the magnitude of their effects, usually given in terms of the ability of the ions to stabilize the structure of proteins [16]. The order in which the effects of some anions seem to increase is the following one: $SO_4^{2-} < F^- < Cl^- < Br^- < NO_3^- < I^- < SCN^- < ClO_4^-$ [9]. Salt solutions have been used to stabilize and destabilize hydrophobic surfaces causing molecules with sufficient non-polar character to become more soluble (phenomena which is known as *salting-in*) or less (known as *salting-out*) soluble. It appears that anions are more likely to promote the *salting-out* of amphiphiles [9]. In order to explain these effects several theories have been promoted over the years and one that has been largely accepted suggests that ions modify the structure and properties of water. The character of water as a solvent for biomolecules would thus change in specific ways in the presence of electrolytes. The ions on the left of Cl^- in the Hofmeister series are called *kosmotropes* (exhibiting strong interactions with water molecules) or "structure makers", while ions on the right of Cl^- are called *chaotropes* (exhibiting weaker interactions with water than water itself) or "structure breakers" [7, 10, 33]. Small ions are strongly hydrated (they are situated on the right side of Cl^- in Hofmeister series) and they considerably increase the

difference between the hydrogen bond donating and accepting capacity of the linked water molecules resulting in the breakdown of the tetrahedral network, and this image is consistent with their chaotropic character.

The precise origin of action of the ions in the series has not been clarified yet and no generally accepted explanation exists at the molecular level. There are data that indicate that ions interact and destroy the natural hydrogen bonded network of water, with effects similar to increased temperature or pressure [23], while other experiments indicate that ions cause negligible changes to hydrogen – bond structure in liquid water [29, 35]. These different points of view may arise from different ionic concentrations that have been used, from the sensitivity of the methods regarding the scale of potential structural changes in the bulk water, from the difficulty in separating coexisting but opposite kosmotropic and chaotropic effects.

We chose to study the effects of the Hofmeister anions on the electric properties of artificial bilayers in the presence of *Hyp*, having in mind two major considerations. The first one is based on the fact that lyotropic anions have a relatively high incidence in human daily diet (nitrate, acetate) and they are also encountered in household products (for instance perchlorate). The second consideration is based on the physical fact that anions hydrate more strongly than cations for the same ionic radius and because of that they are energetically favored to cross a hydrophobic area (like the core of the lipid bilayer) and also anions are more polarizable than cations [15]. At the same time, the simplest anions are larger than most simple cations, therefore giving rise to smaller electrostatic potential.

In this paper, we intend to find an explanation for the changes in the artificial membrane electrical properties in the presence of Hofmeister anions and of *Hyp*, and to propose a mechanism based on the intrinsic effects that anions induce in the electrostatic environment in the vicinity of the lipid membrane and/or in membrane dipole potential.

2. MATERIALS AND METHODS

2.1. Chemicals

Hypericin, 7,14-dione - 1,3,4,6,8,13 – hexahydroxy 10, 11 - dimethyl - phenanthrol [1, 10 , 9, 8 - opgra] perylene (Fig. [1]) was purchased from Santa Cruz Biotechnology and it was prepared as 2 mM stock solution in 10% ethanol and 90% distilled water, subsequent dilutions being obtained in distilled water. For the BLM experiments, the desired concentrations were prepared by dilution with NaCl 100mM + HEPES 20mM. Sodium salts of Hofmeister anions (NaCH$_3$COO, NaCl, NaClO$_4$, NaNO$_3$, Na$_2$SO$_4$, NaSCN) and HEPES (N – 2 – hydroxyethylpiperazine - N' - ethanesulfonic acid) were purchased from Sigma-

Aldrich (purity > 98%) and were prepared as 4M stock salts solutions in distilled water and 1 M HEPES stock solution in distilled water. The black lipid membrane (BLM) forming solution contained 1.5% (w/v) diphytanoyl-phosphatidylcholine (Avanti Lipids) and 0.025% (w/v) octadecylamine in n-decane (Fluka, >98%). To facilitate the membrane formation by impregnating with lipid the margins of the hole, a small amount of 0.5% (w/v) diphytanoyl-phosphatidylcholine in hexane (Fluka, >98%) was used.
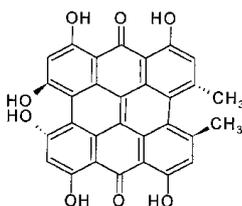
Fig. 1. Chemical structure of Hypericin.

All the solutions were applied in the BLM cuvette either on one side, which is referred to as CIS, (on the side of the cuvette were the lipids were added in order to form the artificial bilayer) or on the both sides of the cuvette, referred to as bilateral.

The solid supported membrane (SSM) had been prepared by linking an alkanethiol (octadecyl mercaptane – ODT purchased from Sigma-Aldrich – 1mM solution in ethanol) monolayer to a gold electrode deposited on a glass support and covering it with a lipid monolayer of diphytanoyl phosphatidylcholine (PC) (prepared as solution in decane, as described above for the BLM technique).

2.2. Methods and types of measurements

In order to investigate the influence of *Hyp* on electrical conductance and capacitance of the artificial lipid bilayers in the presence of lyotropic anions, we used two different electric methods. The first technique is the Black Lipid Method (**BLM**, for details see [3]) which allows the control of the solutions on both sides of the membrane. Complementary results were obtained by the Solid Supported Membrane (**SSM**) method, which is a relatively novel technique that combines the high sensitivity of the BLM with the mechanical stability required by a rapid mixing of the reactants [30 – 32].

In the BLM technique, the artificial lipid membrane is formed in the 1 mm diameter hole drilled in the thin wall that separates two compartments of 2 ml each. Typical values for the electric capacitance and conductance of the black lipid membrane are 400 nF/cm^2 and 7 nS/cm^2, respectively. The 1.5 ml electrolyte solutions from the both compartments of the BLM Teflon cuvette contained 100 mM sodium salt and 20 mM HEPES at pH = 7 (adjusted with NaOH). After the black lipid membrane formation, verified by its color and by the stabilized

values of the electrical capacitance and conductance (measured repeatedly, at 5 minutes intervals), *Hyp* was added either CIS or bilaterally into the cuvette, in increasing concentrations (with a 2 μM increment). After each addition, the electrolyte solutions were magnetically stirred and the electric capacitance and conductance of the membrane were measured three times in a 15 minutes interval. The final value was the average of the three recorded values, normalized to the values of the capacitance/conductance of the stabilized membrane before *Hyp* was added.

In the SSM technique, the planar artificial lipid membrane has an area of 1–2 mm^2 and is mounted in a flow-through cuvette with an inner volume of ca. 17 μl. The substrate containing solution is driven through the SSM cuvette by applying a pressure of about 0.6 bar to the solution containers connected to the cuvette. This way, the solid supported membrane acts as a capacitive electrode and allows the time resolved investigation of charge translocation after the concentration jumps of the substrates. After the formation of the SSM, its capacitance and conductance were determined until they became constant after a waiting time of ~90 minutes. Typical values were 300-500 nF/cm^2 for the capacitance and 50-100 nS/cm^2, for the conductance. A typical solution exchange protocol consists of three phases with duration of 0.5 or 1s each: 1) reference solution, 2) test solution and 3) reference solution. Electrical signals are observed at the concentration jumps taking place at the beginning (on-signal) and at the end of phase 2 (off-signal). Only the on-signal will be used throughout our analysis. The system is checked for artifacts by sending the buffer solutions under pressure in the SSM-containing cuvette.

All the experiments were carried out at room temperature (22^0- 24^0C). The Hofmeister anions solutions (Cl^-, SO_4^{2-}, NO_3^-, CH_3COO^-, SCN^- and ClO_4^-) were prepared as 50 mM Na^+ salts in 20 mM HEPES. We measured the capacitive currents resulted from rapidly applying Hofmeister solutions (active solutions) against NaCl at the same concentration (inactive solution). The control experiments were performed without *Hyp*; afterwards, the membrane was incubated for 15 minutes with 40 μl of *Hyp* at the desired concentration (5 μM, 20 μM and 50 μM), allowing *Hyp* attachment to the membrane. Following incubation, the membrane was rinsed with inactive solution, and a complete series of Hofmeister anions application was performed, following the same protocol as for the control experiments.

For each Hofmeister anion, the peak current values obtained for different *Hyp* concentrations were normalized by reference to the values elicited without *Hyp*. Each application was repeated 3 times on each membrane, and the normalized results were averaged for three different SSMs. Since the amplitude of the capacitive currents is highly dependent on the surface of the solid supported membrane and therefore cannot be accurately estimated, the comparison between different membranes was only possible considering normalized data. Statistical analysis of the results was made using Origin 7.5 software (OriginLab).

3. RESULTS AND DISCUSSIONS

3.1. BLM Experiments

Our previous experiments revealed that the interaction of *Hyp* with artificial lipid membranes depends on the type and concentration of the anion already found in the electrolytes in which the membranes are formed [13, 19].

Our present results show that the effects on the electric properties of the artificial lipid membrane depend on the site of *Hyp* application – bilateral or CIS – the applying of *Hyp* on both sides of the black lipid membrane or only on one side of it, when the buffer solution consisted only in 20 mM HEPES at pH = 7. The conductance of the lipid bilayer increases in both cases almost similarly (Fig. [2]. b)), exhibiting a stepwise augmentation, due to possible pore formation.

As for the bilayer capacitance (Fig. [2]. a)) we noticed that the augmentation in the bilateral application of *Hyp* is more accentuated than in the CIS *Hyp* application, for high *Hyp* concentrations (higher than 35 μM). At Hyp concentrations larger than 30 μM, the bilayer capacitance increases more significantly when *Hyp* is applied bilaterally. In addition, when *Hyp* is applied bilaterally the capacitance does not show a saturating behavior, while in the case of CIS application of *Hyp* one can notice the slight saturating tendency of the capacitance.

Fitting with the Hill equation the normalized capacitance *versus Hyp* concentration, we obtained for CIS application a binding constant of 430 μM and the Hill coefficient is 3, while for the bilateral application, the binding constant is 37 μM and the Hill coefficient has a value of 9. These findings suggest that there are different mechanisms involved in the *Hyp* – membrane interactions when *Hyp* is applied CIS, compared to bilaterally. The apparent increased cooperativity of the process, in bilateral application, can be due to the influence of the different polarization of the electrical field in the TRANS cuvette.

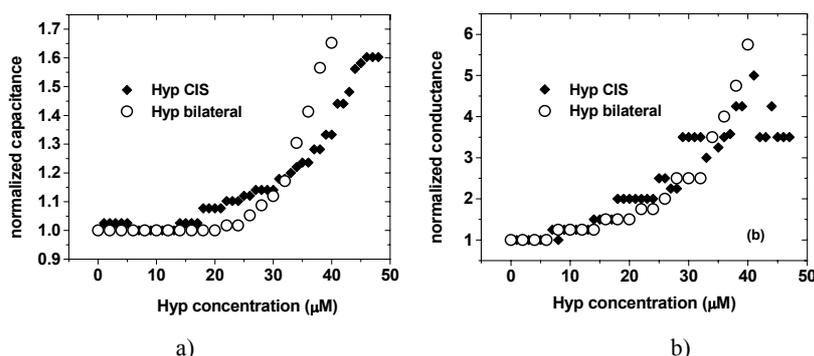a)   b)

Fig. 2 – The effect of CIS and bilateral application of *Hyp* on the electrical properties of the artificial lipid membrane: a) capacitance, and b) conductance. The buffer solution contained 20 mM HEPES, pH = 7.

The differences between the capacitance and conductance profiles suggest that *Hyp* effects on capacitance and on conductance probably take place through partially different mechanisms. A possible explanation could be that the increase of capacitance is due to insertion of *Hyp* on the surface of the membrane that results in the modification of the bilayer dielectric constant, in the bilateral application of *Hyp* a larger amount of the substance being inserted.

Let us consider the artificial lipid bilayer being made of three layers with different electric properties: a hydrophobic core layer and two interfacial layers. Each of these three layers has a specific capacitance given by the formula for a plane capacitor:

$$C = \frac{\varepsilon S}{d} \, , \tag{1}$$

where S represents the membrane area, d is the thickness of the membrane, ε is the dielectric constant.

Therefore, the equivalent electric capacitance of the bilayers, $C_{equivalent-bilateral}$, may be calculated as it follows:

$$\frac{1}{C_{equivalent-bilateral}} = \frac{1}{C_{core}} + \frac{2}{C_{interface1}} \, , \tag{2}$$

$$C_{equivalent-bilateral} = \frac{C_{core} \cdot C_{interface1}}{C_{interface1} + 2 \cdot C_{core}} = \frac{C_{core} \cdot C_{interface1}}{C_{interface1} + C_{core} + C_{core}} \, , \tag{3}$$

admitting that the two interfaces, having the electric capacitance $C_{interface1}$, behave identically, which is the case of bilateral application of *Hyp*.

When *Hyp* is applied only CIS, the formula becomes:

$$\frac{1}{C_{equivalent-CIS}} = \frac{1}{C_{core}} + \frac{1}{C_{interface1}} + \frac{1}{C_{interface2}} \, , \tag{4}$$

$$C_{equivalent-CIS} = \frac{C_{core} \cdot C_{interface1} \cdot C_{interface2}}{C_{core} \cdot C_{interface1} + C_{core} \cdot C_{interface2} + C_{interface1} \cdot C_{interface2}} \, , \tag{5}$$

where index "2" stands for the side with no *Hyp*, while index "1" denotes the *Hyp* side.

Equation (5) may be rewritten in the following form:

$$C_{equivalent-CIS} = \frac{C_{core} \cdot C_{interface1}}{C_{interface1} + C_{core} + C_{core} \cdot \dfrac{C_{interface1}}{C_{interface2}}} \, . \tag{6}$$

Because of its unique molecular structure, *Hyp* has both a hydrophobic side (the one containing the methyl groups) as well a hydrophilic side. Therefore, it is possible that the molecule will bond to the outer face of the membrane, and its hydrophobic side would insert into the lipid bilayer, modifying the dielectric constant of the interfacial layer and/or its thickness.

From Fig. 2b, we notice that there are three different *Hyp* concentration intervals in which the capacitance behavior for the two configurations differs:

a) In the interval of 0–18 μM *Hyp* concentration, the capacitance of the artificial lipid bilayer in the two different situations is almost the same. Comparing equations (3) and (6), and considering $C_{equivalent-CIS} = C_{equivalent-bilateral}$ we get $C_{interface1} = C_{interface2}$, probably the *Hyp* concentration is to low.

b) In the interval of 19–34 μM *Hyp* concentration, $C_{equivalent-CIS}$ is greater than $C_{equivalent-bilateral}$. Comparing again equations (3) and (6), one obtains $C_{interface2} > C_{interface1}$, that meaning the interfacial layer exposed to *Hyp* presents either a smaller dielectric constant, than the one not exposed to the polyphenol, because of the insertion of the *Hyp*, or rather a higher thickness (see equation (1)).

c) Finally, for a *Hyp* concentration higher than 35 μM, $C_{equivalent-CIS}$ is less than $C_{equivalent-bilateral}$, and that gives $C_{interface2} < C_{interface1}$ that is in agreement with the hypothesis that the dielectric constant of the layer covered with Hyp molecules increases due to the adsorbtion of *Hyp* on the surface. *Hyp* concentration becomes high enough, and this way the increase in the dielectric constant of the bilayer should overtake the increase in the bilayer thickness.

Because this opponent behavior of the bilayer's capacitance function on the *Hyp* concentration and given the fact that the conductance of the lipid membrane increases in both configurations (Fig. 1b) in the same way, we conclude that not only the amount of *Hyp* inserted into the bilayer is responsible for the modification of these electric parameters, but also another mechanism is implied.

Our previous experiments [18] on the variation of the electric parameters of the artificial lipid membrane in the presence of anions belonging to the lyotropic series showed that the electric capacitance of the bilayer does not depend on the type or on the concentration of the anion, while its conductance is increasing with the augmentation of the anions concentration. We suggested that there is a relationship between the conductance increase rate and the position of the anion in the Hofmeister series.

In this paper, we studied the behavior of the electric parameters of the lipid bilayer in the presence of the following lyotropic anions: CH_3COO^-, Cl^-, NO_3^-, ClO_4^- and in the simultaneous presence of *Hyp,* which were all added bilaterally into the BLM cuvette. All the anions were dissolved in 100 mM Na salt solutions in 20 mM HEPES at pH = 7. The black lipid membrane was

formed in 20 mM HEPES solution, then in each compartment of the cuvette the appropriate amount of Na salt was added in order to obtain a final anion concentration of 100 mM, and afterwards *Hyp* was added in increasing concentration, with a 2 μM increment.

For all the anions that have been tested, the conductance increased almost similarly, as the concentration of *Hyp* went higher (Fig. 3b), although the augmentations in the membrane conductance in the presence of only 100 mM Hofmeister anions solutions (no *Hyp*) were concentration and anion type dependent [18]. Probably the effects of *Hyp* on the artificial lipid membrane are significantly stronger than the ones induced by the Hofmeister series anions. This suggests that the preponderant mechanism influencing the membrane conductance is *Hyp* insertion and, moreover, that *Hyp* insertion prevents the interaction between Hofmeister anions and the membrane, possibly due to membrane stabilization.

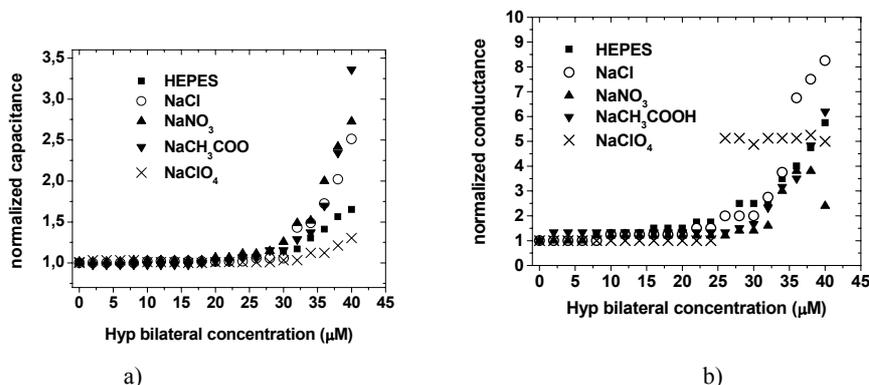a)   b)

Fig. 3 – Compared behavior of the artificial lipid membrane capacitance (a) and conductance
(b) in the presence of 100 mM lyotropic anions in 20 mM HEPES, pH = 7, and *Hyp*.

As the *Hyp* concentration increases, differences in the lipid bilayer capacitance augmentation profiles appear (Fig. 3a). The presence of 100 mM acetate induces the most accentuated capacitance augmentation at high *Hyp* concentration. In order, acetate, the nitrate and chloride also increase the bilayer capacitance.

Let us consider the membrane being constituted from the same three layer system as before, equivalent with three plane capacitors connected in series. In the following $C_{Hyp\text{-}interface}$ will denote the capacitance of the interface capacitors, while $C_{Hyp\text{-}core}$ will denote the capacitance of the inner hydrophobic layer. Thus, we shall have, for the equivalent capacitance $C_{Hyp\text{-}equivalent}$ of the lipid membrane, considering that the two faces of the membrane are in the same conditions (*Hyp* and anions were added bilaterally to the cuvette):

$$\frac{1}{C_{Hyp-equivalent}} = \frac{2}{C_{Hyp-interface}} + \frac{1}{C_{Hyp-core}}, \tag{7}$$

$$C_{Hyp-equivalent} = \frac{C_{Hyp-core} \cdot C_{Hyp-interface}}{2 \cdot C_{Hyp-core} + C_{Hyp-interface}} = \frac{C_{Hyp-interface}}{2 + \dfrac{C_{Hyp-interface}}{C_{Hyp-core}}}. \tag{8}$$

The capacitance of the artificial lipid bilayer does not vary with the anion type [18]. That might be explained by the fact that the dielectric constant of the membrane and its thickness, as well, do not vary in the presence of different anions. However, this is not the case anymore, when *Hyp* is present in a concentration higher than 30 μM (Fig. 3a), when obvious differences between the capacitance values of the membrane appear, in the simultaneous presence of *Hyp* and different Hofmeister anions.

For any *Hyp* concentration higher than 30 μM, it can be seen in Fig. 3a, that the capacitance of the artificial lipid membrane in the presence of 100 mM neutral chloride, chaotropic nitrate and kosmotropic acetate, in this particular order, is greater than the capacitance of the bilayer in the presence of the buffer. Applying eq. (8) in this case, one gets for acetate, for instance, the following:

$$C_{Hyp-equivalent-acetate} > C_{Hyp-equivalent-buffer}, \tag{9}$$

$$\frac{C_{Hyp-interface-acetate}}{2 + \dfrac{C_{Hyp-interface-acetate}}{C_{Hyp-core-acetate}}} > \frac{C_{Hyp-interface-buffer}}{2 + \dfrac{C_{Hyp-interface-buffer}}{C_{Hyp-core-buffer}}}, \tag{10}$$

$$2 \cdot C_{Hyp-interface-acetate} + \frac{C_{Hyp-interface-buffer}}{C_{Hyp-core-buffer}} \cdot C_{Hyp-interface-acetate} >$$
$$> 2 \cdot C_{Hyp-interface-buffer} + \frac{C_{Hyp-interface-acetate}}{C_{Hyp-core-acetate}} \cdot C_{Hyp-interface-buffer}. \tag{11}$$

Considering that the hydrophobic core capacitance of the bilayer is not affected by the presence of the anions ($C_{Hyp-core-acetate} = C_{Hyp-core-buffer}$), eq. (11) becomes:

$$C_{Hyp-interface-acetate} > C_{Hyp-interface-buffer} \tag{12}$$

and generalizing for all the anions sequence that we had used in our experiments:

$$C_{Hyp-interface-acetate} > C_{Hyp-interface-nitrate} > C_{Hyp-interface-chloride} > C_{Hyp-interface-buffer} \tag{13}$$

This sequence is correlated rather with the ions radii (the larger the radius, the larger the capacitance) than with their position in the Hofmeister series. Let us consider the two capacitors situated at the interface in the simultaneous presence of *Hyp* and of a certain anion as being plane having the thickness proportional with the hydrated anion diameter. Equation (13) can now be written in terms of dielectric permittivity, surface and thickness:

$$\frac{\varepsilon_{Hyp-acetate}S}{R_{Hyp-acetate}} > \frac{\varepsilon_{Hyp-nitrate}S}{R_{Hyp-nitrate}} > \frac{\varepsilon_{Hyp-chloride}S}{R_{Hyp-chloride}} > \frac{\varepsilon_{Hyp-buffer}S}{R_{Hyp-buffer}} \qquad (14)$$

where R stands for the anion radius.

Because $1 < \dfrac{R_{Hyp-acetate}}{R_{Hyp-nitrate}}$, it results that the ratio $\dfrac{\varepsilon_{Hyp-acetate}}{\varepsilon_{Hyp-nitrate}}$ is also greater than 1. That means $\varepsilon_{Hyp-acetate} > \varepsilon_{Hyp-nitrate}$. This shows the increase in the dielectric permittivity of the interface layer as the anion radius increases, the large anions facilitating probably *Hyp* insertion.

But, at the same time, perchlorate, which is strongly chaotropic, behaves differently, and the small increase of the capacitance in the presence of *Hyp* suggests a possible protective effect of the polyphenol on the lipid membrane against this chaotropic anion influence.

As for the conductance behavior, its augmentation is rather small at a 100 mM anion concentration, being 1.4 for acetate, 1.15 for nitrate and close to one for chloride, in the absence of *Hyp* as it shows in Fig. 3b. From Fig. 3b, one can see that for a *Hyp* concentration higher than 26 μM, the increase of the electric conductance is accentuated, the augmentation profile being dominated by the presence of the polyphenol, rather than by the anions type.

SSM Experiments. In the SSM experiments we measured the capacitive signals elicited by concentration jumps of the sodium salts of several anions belonging to the Hofmeister series in the presence of hypericin at various concentrations.

In the solution exchange process, two solutions of different composition were applied to the surface, the test solution and the reference solution, both of which were buffered with 20 mM Hepes at pH 7.0. The test solution contained the salt of interest and the reference solution contained NaCl, which was used as an internal reference. NaCl is a popular reference since Hofmeister effects often show a sign inversion at Na^+ and Cl^- [10]. We have chosen for the test solutions two kosmotropic (SO_4^{2-}, CH_3COO^-) and three chaotropic (NO_3^-, SCN^-, ClO_4^-) anions of the Hofmeister series. Concentration jumps of 50 mM sodium salts of these anions were performed in the absence (control) and in the presence of *Hyp* (applied directly on the lipid bilayer) at various concentrations. Fig. 4 presents the signals obtained when the solutions containing Hofmeister anions were applied in the absence of polyphenols.
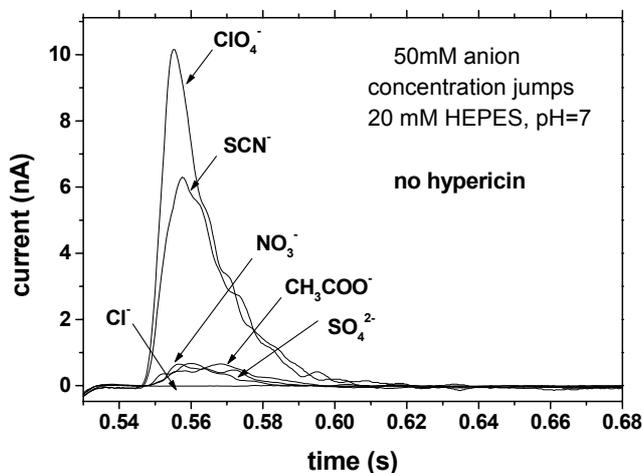
Fig. 4 – Signals elicited by concentration jumps of sodium salts of Hofmeister anions
in the absence of *Hyp*.

The results in this control experiment are similar to those of Garcia-Celma *et al.* [14].

The evolution of the peak currents as a function of *Hyp* concentration shows again a dependence of the position of the anion in the Hofmeister series (Fig. 5).

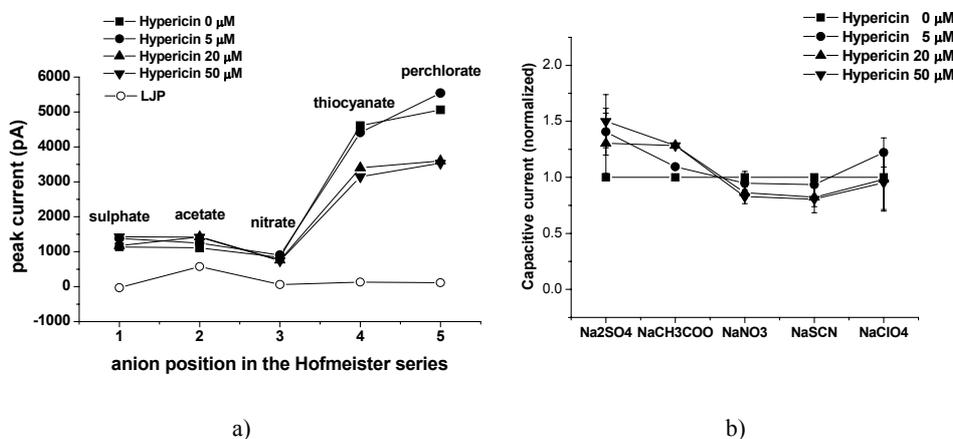a)   b)

Fig. 5 – a) The evolution of the peak current after 50 mM concentration jumps of the anion solutions at various hypericin concentrations, and the calculated value of the liquid junction potentials (LJP) for the solution exchange. b) Dependency of the normalized peak current values on the hypericin concentration, for the same tested anions (*n*=2).

While, as a general tendency, the amplitude of the capacitive currents shows a slight increase for the kosmotropic anions as compared to the control, the peak

current for the signals elicited by chaotropic anions decreases as *Hyp* concentration increases and this effect appears to be more pronounced as the chaotropic character is higher, i.e for perchlorate. The normalized results indicate, however, that *Hyp* influences more the amplitude of kosmotropic ions induced currents, while the effect on chaotropic anions is milder. The mirror-image profile (increase signals with increased *Hyp* concentrations for kosmotropes and opposite dependency for chaotrops) is also evident when looking at the normalized data.

A legitimate question concerning the origin of the SSM measured currents is how much of the electrical signals is due to liquid junction potentials (LJP). In order to test this, we computed the LJP corresponding to our precise experimental conditions and, based on this, the expected amplitudes of the capacitive currents due exclusively to LJP (Fig. 5a). In order to do this, we considered the following equation [17]:

$$E_J = \left(\frac{RT}{F}\right)\left(\frac{\sum u_i \frac{|z_i|}{z_i}(c_{i,2}-c_{i,1})}{\sum u_i |z_i|(c_{i,2}-c_{i,1})}\right)\ln\left(\frac{\sum u_i |z_i| c_{i,1}}{\sum u_i |z_i| c_{i,2}}\right), \tag{15}$$

where z_i is the ionic charge, u_i is the mobility of the ions, $c_{i,1}$ the concentration of the first solution, $c_{i,2}$ the concentration of the second solution, R the universal gas constant, T the absolute temperature and F the Faraday constant. The amplitude of the capacitive current I was computed as $I = CU/t$; we considered an average capacitance for an SSM C = 2.5 nF, tension U equal to the computed value of the LJP and t = 70 ms the average duration of an experimentally measured signal. We can observe in Fig. 5a that the values of the LJP currents are much smaller (a different order of magnitude) by comparison to the measured signals, which proves that other kinds of charge translocations occur at the membrane-solution interface when Hofmeister anions solutions are applied to the membrane. We can also notice an increase in the gap between the experimental data and the computed LJP-currents as me move towards the chaotropic edge of the Hofmeister series.

In a recent paper [14], by means of SSM experiments, the authors have analyzed the charge translocation after the rapid exchange of various cations and anions, belonging to the Hofmeister series, on a lipid surface of different composition and interpreted this in terms of binding of the ions in the lipid headgroup region. In an SSM measurement after concentration jumps of solutions with different ionic composition a charge displacement can be registered which can provide information about the relative distribution of cations and anions within or close to the lipid headgroup region [14]. This effect was called "binding" with the specification that it could be anything between a diffuse accumulation close to the

lipid surface up to specific binding at a well defined binding site within the lipid headgroup [24]. It was also shown that the chaotropic anions bind best to or in a lipid surface and the more chaotropic the ion, the stronger its interaction with the lipid will be. Our results are in agreement with these observations.

The dependency of the capacitive current amplitude on the free energy of hydration of the tested anion indicate a striking similarity of our dependency curve (not shown) to the results reported by Clarke and Lüpfert [5, 6], who noticed a change in the fluorescence intensity ratio of unilamellar DMPC vesicles which depends on the free hydration energy of the Hofmeister anions present in the environment, as a result of changes of the dipole potential of the lipid membranes. Clarke and Lüpfert explained the reduction of the dipole potential by the binding of the chaotropic anion at the positive side of the dipole deep in the lipid headgroups. Changes in the dipole potential of an SSM, due to Hofmeister interactions, can be another mechanism responsible for our experimental results. Furthermore, it can be inferred that the attractive potential at lipid interface is much stronger for chaotropic than for kosmotropic anions, an idea also supported by the findings of Garcia-Celma *et al.* [14].

We took a further step by studying the modulatory effect of *Hyp* on the interaction of several lyotropic anions with the lipid membranes. Our experiments show an opposite effect of *Hyp* on the interactions between kosmotropic or chaotropic anions and the lipid membrane (Fig. 5). While the signals elicited by applications of kosmotropic anions increase at higher concentrations of *Hyp*, suggesting more important charge translocations, the interaction with the membrane of the chaotropic anions is hindered. Such behaviour is consistent with a protective effect of *Hyp* on the membrane, against the chaotropic-induced destabilization. In order to try to explain the intimate processes behind these effects, we have to take into account previously reported data that point towards a very complex mechanism. One of them is the above-mentioned theory of Clarke and Lüpfert [6] about the effect of Hofmeister anions on the dipole potential. Recently, it was shown that dispersion forces have to be taken into account at physiological ion concentrations where electrostatic interactions are screened [4, 21]. Collins [11] proposed a "principle of matching water affinities" according to which the chaotropic ions bind to chaotropic surface groups and kosmotropic ions to kosmotropic surface groups. The geometry of the water molecules around the ions and headgroups could, therefore, also play an important role in Hofmeister effects. The polyphenols bind at the surface of the lipid membrane possibly hindering the action of chaotropic anions which have the tendency to enter the hydrophobic core of the bilayer. They could screen the electrostatic interactions due to the anions and, thus, the effects on intrinsic dipole potential of the membrane. They also might disturb the geometry of water in the vicinity of the

lipid bilayer with consequences on the lyotropic anions-bilayer interaction. The precise mechanisms are still to be investigated and further progress could be probably also achieved by means of molecular simulation studies.

Among the interactions of lyotropic anions with lipid bilayers, one can mention the interaction with polar groups from the bilayer surface implying an effect on intrinsic membrane dipole potential and/or the dehydration of the region neighboring the lipid polar heads, a possible effect in the case of the anions with high hydration energy.

Our results show that the lyotropic anions effects on lipid membrane are modulated by the presence of *Hyp*, and this modulation appears to be mutual. *Hyp* insertion into the lipid bilayer seems to be ruled by two different mechanisms concentration dependent. The *Hyp* presence results in protection of the membrane against the electrical and structural perturbances effects that Hofmeister series anions have on artificial lipid membranes. This effect might be due to *Hyp* incorporation mainly on the outer surface of the membrane and interference in the alterations that lyotropic anions bring to the electrical properties at the interface solution/lipid bilayer and/or to membrane dipole potential.

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