Interference of coumarin with the insertion of lyotropic anions and cadmium in artificial lipid bilayers

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Abstract
The interference of the natural polyphenol coumarin derivative, coumarin-3-carboxylic acid, with the insertion of lyotropic anions and cadmium into artificial lipid bilayers was investigated using the solid supported membrane (SSM) method. This electrophysiological method allows the study of artificial lipid membranes by successively applying substrate solutions of different concentrations (“concentration jumps”), which results in measurable capacitive currents. Heavy metals, like cadmium, and lyotropic or Hofmeister anions are among pollutants that can have noxious effects on living organisms. Therefore, it is of interest to try to find remedies to counteract or diminish their negative effects. An important number of studies have evidenced the beneficial role of polyphenols in the treatment and prevention of various human pathologies, due to both their antioxidant as well as prooxidant properties. In the present work, concentrations jumps of lyotropic anions and cadmium have been applied to artificial lipid membranes and the resulting electrical capacitive currents have been measured in the presence of several concentrations of the polyphenol coumarin. It has been found that in the presence of coumarin the electrical signals elicited by chaotropic anions and cadmium decreased significantly, suggesting a protective role of the polyphenol at the lipid bilayer level.

Keywords: coumarin; lipid membranes; solid supported membranes; Hofmeister anions; cadmium

Introduction
In the last decades the interest in food polyphenols has been steadily increasing due to their antioxidant capacity and, therefore, of their implications in treatment and prevention of different human pathologies such as cancer and cardiovascular diseases. It is, nowadays, widely accepted that the oxidative stress is a major phenomenon that can lead to chronic diseases. The quantification of the oxidative stress in a population appears to be a possible indicator for the magnitude of risk factors. Diet plays a major role in the control of oxidative stress. A diet rich in fruit, vegetables and plant-derived beverages decreases oxidative stress, mainly due to their high content of polyphenol antioxidants (F. LEIGHTON & al. [1]), playing thus an important role in the prevention of diseases like cancer, atherosclerosis, osteoporosis, cardiovascular and neurodegenerative diseases, diabetes mellitus etc. (J.M.C. GUTTERIDGE & al. [2] and references therein); therefore diet appears to be a potential tool for the control of such affections.

Polyphenols have some major properties that recommend them as beneficial molecules for human health. Among them, their free radicals scavenging capacity stands out, as being a mechanism of protection against oxidative stress damage at the lipid membrane level, protein
and DNA level. Another property consists in their direct interactions with receptors or enzymes involved in signal transduction pathways, resulting in the modification of the redox status of the cell (A. AZZI & al. [3], B. HALLIWEL & al. [4], J.O. MOSKAUG & al. [5]). On the other hand, polyphenols can act not only as antioxidants, but also as prooxidants. While as antioxidants they contribute to improve the cell survival, as prooxidants they may induce apoptosis and prevent tumor growth (J.D. LAMBERT & al. [6], A. SCALBERT & al. [7]). In this respect, more than a decade ago, our group has started to study the effects of various polyphenols, both at the level of model lipid membranes and at the level of cancer cell lines as well, evidencing their ability to act either as antioxidants or as prooxidants (IONESCU & al. [8, 9], IFTIME & al. [10], D. IONESCU & C. GANE A [11], A. POPESCU & al. [12], A. POPESCU [13], I. BARAN & al. [14 - 16], M. M. MOCANU & al. [17 - 18], D. MARGINA & al. [19]). We have found that polyphenols interact with model lipid membranes modulating their electrical and thermodynamical properties (IONESCU & al. [8, 9], IFTIME & al. [10], D. IONESCU & C. GANE A [11], A. POPESCU & al. [12]). They also can have protective effects against various pollutants or noxious agents interfering with membrane structures. We have shown, for example, that hypericin has a protective effect against Hofmeister anions (D. IONESCU & al. [9]) and the flavonoid quercetin protects the lipid bilayer against heavy metals (A. POPESCU & al. [12], A. POPESCU [13]). On the other hand, we have found that quercetin and epigallocatechin gallate (EGCG), due to their ability of acting as prooxidants, can induce apoptosis in cancer cell lines (I. BARAN & al. [14 - 16], M.M. MOCANU & al. [17 - 18]).

One of the polyphenols that can be used for the prevention and treatment of various diseases is coumarin. As an example, Melilotus officinalis, a plant that contains coumarinic compounds, is used for the treatment of respiratory, stomach, liver, renal, genital, cardiovascular, eye, oral, skin, nervous and rheumatic diseases (K.N. VENUGOPALA & al. [20], M. IRANSHAHI & al. [21]). As coumarin can be synthesized, some of its synthetic derivatives, like warfarin, are widely used as anticoagulants in the secondary prophylaxis of thrombotic events (deep veins thrombosis, stroke etc)(M. GREAVES & al. [22]).

A lot of potentially noxious agents, including certain anions of the Hofmeister series (F. HOFMEISTER [23]), are found in foods or pharmaceutical products, as a result of preservation and manufacturing techniques or as an effect of pollution. The Hofmeister series consists of two sequences in which anions and cations are ordered based on the magnitude of their effects, usually given in terms of the ability of the ions to stabilize the structure of proteins. COLLINS & WASHABAUGH [24] have shown that the Hofmeister effects of anions are much stronger than those of cations and they have classified the ions in kosmotropes, also called "structure makers", (exhibiting strong interactions with water molecules) and chaotropes (which exhibit weaker interactions with water than water itself) called "structure breakers". The effects of anions, corresponding to their ability to stabilize the structure of proteins (A.L. HODGKIN & P. HOROWICZ [25]) increase in the following order: \( \text{SO}_4^{2-} < \text{F}^- < \text{Cl}^- < \text{Br}^- < \text{NO}_3^- < \Gamma < \text{SCN}^- < \text{ClO}_4^- \) (E.J. COHN & J.T. EDSALL [26]). A number of studies were dedicated to Hofmeister effects of anions and cations on membrane proteins, pumps, channels, enzymes etc. (A.L. HODGKIN & P. HOROWICZ [25], A. DÉR & J.J. RAMSDEN [27], G.Y. RYCHKOV & al. [28], C. GANE A & al. and ref therein. [29], A. NEAGU & al. [30], and on lipid membrane or vesicles (P.W. SANDERSON & al. [31], R.J. CLARKE [32], R.J. CLARKE & C. LÜPFERT [33], J. GARCIA-CELMA & al. [34], A. IFTIME & al. [35], M. DRAGUSIN & al. [36], D. IONESCU & al. [37]). In particular, it was shown by one of us that the Hofmeister anions, by inducing changes in the lipid environment, could influence the function of membrane proteins.
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(C. GANEA & al. [29]). A number of studies have shown that, in the case of lipid membranes and vesicles, the Hofmeister ions can induce modifications of the lipid phase behavior, of the surface potential or of the membrane dipole potential. (P.W. SANDERSON & al. [31], M. G. CACACE & al. [38], J. CLARKE [32], R.J. CLARKE & C. LÜPFERT [33], J. GARCIA CELMA & al. [34]). At the lipid membrane interface the chaotropes have the tendency to insert themselves in the hydrophobic core (salting-in effect), while the kosmotropes distribute themselves in the surrounding water (salting-out effect). The interaction of Hofmeister anions with artificial lipid membranes is electrogenic and can be evidenced through capacitive currents by means of electrophysiological techniques like that of SSM. SSM (Solid Supported Membrane)-based electrophysiology is a technique which combines a high sensitivity with the mechanical stability required by a rapid mixing of the reagents. (A. BAZZONE & al. [39], PINTSCHOVIUS & al. [40], GANEA & al. [41]). This technique is very useful in the study of membrane proteins or the lipid environment in which they are embedded (for recent reviews see F. TADINI-BUONINSEGNI & al. [42], C. GANEA & K. FENDLER [43]).

One of the aims of the present study is to find out if the polyphenol coumarin, by means of one of its derivatives (coumarin-3-carboxylic acid), has a possible protective effect against the chaotropic anions of the series. A similar effect has already been proven for hypericin (D. IONESCU & al. [9]). The experiments that we have performed using the SSM method show that the interaction of coumarin with artificial lipid membranes depends on the type and concentration of the anion found in the electrolytes in which the membranes are formed, as previously shown in the case of hypericin (D. IONESCU & al. [9]).

Another aim of our study refers to the interference of coumarin with the insertion of a heavy metal, cadmium, into the artificial lipid bilayer. It is known that heavy metals are major pollutants of the environment and it is important to find possible remedies against their negative effects. The toxicity of the heavy metals affects mainly the kidney due to their ability to be reabsorbed and to accumulate in the organism (D. CUCU & al. [44]). At cellular level the heavy metals have toxic effects on the metabolic activity of enzymes and can generate free radicals, thus initiating the peroxidation of the lipid bilayer. Therefore, finding compounds that have the ability to counteract the interference of heavy metals with physiological processes is a matter of major importance. In a previous work we have shown that the flavonoid quercetin induces a decrease in the membrane affinity for cadmium and zinc, therefore exerting a protective effect (A. POPESCU & al. [12], A. POPESCU [13]). In the present study, we investigated the possible protective effects of coumarin against cadmium interference with lipid bilayers, by measuring the changes in the electrical properties of artificial lipid membranes in the presence of both coumarin and cadmium.

Our present results show that the changes occurring in the electrical properties of the artificial lipid bilayers are ion type and concentration dependent, coumarin having possible protective and/or stabilizing effects on the membrane against strong chaotropic anions, such as perchlorate, and against the heavy metal cadmium. We conclude that by inserting itself into the lipid bilayer coumarin interferes with the mechanisms by which lyotropic anions affect the electrical surroundings of the lipid bilayer and/or the intrinsic membrane dipole potential.

**Materials and Method**

**SSM method**

The solid supported membrane (SSM) was prepared as previously described (A. BAZZONE & al. [39], PINTSCHOVIUS & al. [40], GANEA & al. [41]). In short, an alkanethiol (octadecyl mercaptane - ODT) monolayer was spread on top of a gold electrode.
deposited on a glass support. This first monolayer was covered with a lipid monolayer of diphytanoyl phosphatidylcholine (PC). The planar membrane formed had an area of 1-2 mm². The SSM was mounted in a flow-through cuvette with an inner volume of ca. 17 μL. The gold electrode was connected to an amplifier and as reference electrode we used an Ag/AgCl electrode separated from the solution by an agar salt bridge. The solution containing the reagents to be investigated (in our case the Hofmeister anions or cadmium salts) (the so called activating or test solution) is driven through the cuvette by applying a pressure of about 0.6 bar to its container and the same is applied for a so called nonactivating or reference solution (i.e. NaCl) The solid supported membrane thus constructed acts as a capacitive electrode allowing the time resolved investigation of charge translocation after concentration jumps of the substrates. Further details can be found in (A. BAZZONE & al. [39], PINTSCHÖVIUS & al. [40], A. IFTIME & al. [35]).

Chemicals
The coumarin derivative (coumarin-3-carboxylic acid) (Fig. 1) was prepared as 100 mM stock solution in DMSO and was diluted correspondingly in NaCl 100mM + HEPES 20mM to reach the desired concentration and then applied directly to the SSM. Activating and nonactivating solutions were prepared in 0.1 M KPi (a mixture of KH2PO4 and K2HPO4 at pH 7) and 0.1 mM dithiothreitol (DTT, 99.5% Roth, Karlsruhe, Germany). Sodium salts (NaCH3COO, NaCl, NaClO4, NaNO3, Na2SO4, NaSCN) and HEPES (N-2-hydroxyethylpiperazine - N’-ethanesulfonic acid) were purchased from Sigma-Aldrich (purity > 98%) and were prepared as 4M stock salt solutions in distilled water and 1 mM HEPES stock solution in distilled water. The membrane forming solution contained 1.5% (w/v) diphytanoyl-phosphatidylcholine (Avanti Lipids) and 0.025% (w/v) octadecylamine in n-decane (Fluka, >98). Cadmium chloride solutions (CdCl2) (Sigma-Aldrich) were prepared in several concentrations between 1 M - 1 mM in HEPES 20 mM. All the experiments have been carried out at pH 7.0.

Measuring procedure
The experiments were carried out at room temperature (22°C). In order to allow the formation of an SSM, i.e. of a bilayer, a flow of KPi buffer was sent to the cuvette and, after a waiting time of 90 minutes, necessary for the electrical parameters to become constant, the capacitance and conductance of the lipid membrane have been measured. Typical values were 300-500 nF/cm² for the capacitance and 50-100 nS/cm², for the conductance. A typical solution exchange protocol consisted of three phases with a duration of 0.5 or 1s each: 1) reference (nonactivating) solution, 2) test (activating) solution and 3) reference solution. The data recording and the solution exchange were controlled via computer as previously described (J. GARCIA CELMA & al. [34], C. GANE & al. [41]). 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 was used throughout our analysis. The system is

![Figure 1. Chemical structure of coumarin-3-carboxylic acid](image-url)
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Results and discussion

Effect of coumarin on Hofmeister anions insertion in the lipid bilayer

Two solutions of different composition were applied to the surface, the test (activating) solution and the reference (nonactivating) 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 was taken as reference due to the fact that Hofmeister effects usually show a sign inversion at Na+ and Cl-. (K.D. COLLINS & M.W. WASHABAUGH [24]). As test solutions we have chosen two kosmotropic (SO\textsubscript{4}\textsuperscript{2-}, CH\textsubscript{3}COO\textsuperscript{-}) and three chaotropic (NO\textsubscript{3}\textsuperscript{-}, SCN\textsuperscript{-}, ClO\textsubscript{4}\textsuperscript{-}) 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 coumarin (applied directly on the lipid bilayer) at various concentrations. In a previous work (D. IONESCU & al. [9]) as well as in the present study, we have found results similar to those of J. GARCIA CELMA & al. [34], i.e. that the maximal amplitude of the capacitive signal (I\textsubscript{p}) can be observed for the perchlorate anion (ClO\textsubscript{4}\textsuperscript{-}), followed by thiocyanate (SCN\textsuperscript{-}). No significant differences could be noticed between the signals due to concentration jumps of NO\textsubscript{3}\textsuperscript{-}, SO\textsubscript{4}\textsubscript{2-} and CH\textsubscript{3}COO\textsuperscript{-}, their peak currents (I\textsubscript{p}) being one order of magnitude lower than in the case of the extreme chaotrope ClO\textsubscript{4}\textsuperscript{-}.

![Figure 2](image-url). The evolution of the capacitive signals measured in the presence of several coumarin concentrations (5, 10, 20 µM) following concentration jumps of the Hofmeister anions solutions. The ionic salts were at 50mM concentration in 20 mM HEPES at pH 7.

Fig. 2 shows the capacitive signals obtained in the presence of several coumarin concentrations. It can be noticed that in the presence of coumarin the amplitude of the corresponding capacitive signals (peak currents) decreases as the coumarin concentration increases (Fig. 2) in the case of chaotropic anions. In the case of kosmotropic ions the signal...
Changes are of much lower amplitude. In order to interpret the data obtained in SSM experiments one has to take into account the so called "liquid junction potentials (LJP)" arising as a consequence of different mobilities of the ions employed in our measurements. In our experiment such LJP could appear during the exchange between the activating and nonactivating solutions. Their values are given by the equation (E.O. Holmes & H.P. Candy [45]):

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

where $E_j$ is the LJP, $z_i$ 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. By using the formula $I = CU/t$ we could calculate the amplitude of the capacitive current $I$; the average capacitance for the SSM was $C = 2.5$ nF and the average duration of the experimentally measured signal was $t = 70$ ms. The mobilities of the anions was taken from tables. (D. Ionescu & al. [9], present work). In a previous study (D. Ionescu & al. [9], as well as in the present one, we have calculated the corresponding LJP's and the associated peak currents in the case of activating and nonactivating solutions of 50 mM salt.

From Fig. 3, representing the peak currents elicited by concentration jumps of anions as a function of coumarin concentration, it can be seen that the values of the LJP currents are much smaller in comparison to the measured signals due to Hofmeister anions, proving thus that the role of LJP is negligible in our measurements. It can also be noticed that the magnitude of the signals elicited by concentration jumps of kosmotropic anions does not differ significantly for increasing coumarin concentrations and, moreover, its value is very close to that of the theoretical value of the corresponding LJP (Figs. 3, 4). These data suggest that the kosmotropic anions do not interfere significantly with the artificial lipid membranes. On the other hand, the peak currents elicited by chaotrope anions concentration jumps decrease as the coumarin concentration increases (Figs. 3, 4), without reaching the corresponding LJP values in the coumarin concentration range studied. These results might suggest a protective effect of coumarin against the modifications induced by chaotropic anions in the lipid membrane.

The lipids present in the cell membranes have polar groups comprising a negatively charged phosphate (PO$_4$) group and a positively charged trimethylammonium (N(CH$_3$)$_3^+$) group.
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The lipid used in our SSM experiments is diphytanoyl phosphatidylcholine (PC) and, if we take into consideration that among the lipids of the cell membrane phosphatidylcholine is present in about 50%, one can say that PC can constitute an appropriate model of study (J. GARCIA CELMA & al. [34]). The study of J. GARCIA CELMA & al. [34] provided information about the relative distribution of cations and anions within or close to the lipid headgroup region by means of the SSM method. According to their results, 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 concerning the effects of chaotropic anions of the Hofmeister series in the absence of coumarin are in agreement with those of (J. GARCIA CELMA & al. [34])(see Fig. 3) and also with those of another type of experiments concerning the influence of anions on the dipole potential of PC liposomes (R.J. CLARKE & C. LÜPFERT [33]). R.J. CLARKE & C. LÜPFERT [33] 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 headgroup. It can be noticed that kosmotropic anions have a much more reduced effect than the chaotropic ones. From SSM experiments it could be inferred that the attractive potential at lipid interface is much stronger for chaotropic than for kosmotropic anions (J. GARCIA CELMA & al. [34]).

**Figure 4.** The peak currents elicited by chaotropic anion concentration jumps decrease as the coumarin concentration increases. The points represent the experimental data and the lines are drawn in order to guide the eye.

In a previous work (D. IONESCU & al. [9]) we have studied the modulatory effect of another polyphenol, hypericin, on the interaction of several lyotropic anions with the lipid membranes. In this study we have obtained similar results by using the polyphenol coumarin. Our experiments show that coumarin has a pronounced effect on the binding of chaotropic anions to the lipid bilayer, while in the case of kosmotropic anions their effect is much more reduced (Figs. 2, 3, 4). Thus, it seems that in the presence of coumarin the “binding” of chaotropic anions is hindered. We have tried to understand the reason of this apparent protective effect against the chaotropic anions. We took into account several hypotheses concerning the Hofmeister effect of anions. Beside the theory of R.J. CLARKE & C. LÜPFERT [33] about the effect on the dipole potential, an important part seems to be played by the so-called “principle of matching water affinities” according to which the chaotropic ions bind to chaotropic surface groups and kosmotropic ions to kosmotropic surface groups (K.D. COLLINS [46]). Coumarin, similarly to hypericin (D. IONESCU & al. [9]), might 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. It could screen the electrostatic interactions due to the anions and, thus, the effects on intrinsic dipole potential of the membrane. It 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 to be further investigated.

The interaction between coumarin and cadmium at the level of the artificial lipid bilayer

Through the SSM method we have also studied the interplay between coumarin and a heavy metal — cadmium at the level of the artificial lipid bilayer. For this study we increased the cadmium concentration stepwise (in reference with a nonactivating solution of sodium, having the same concentration; both ionic metals where in the form of chloride salts), in the absence of coumarin (control) and in the presence of different coumarin concentrations (Fig. 5).

![Figure 5. Capacitive signals measured from increasing cadmium concentration jumps in the absence (A) or presence of coumarin 10 µM (B)](image)

The amplitudes of the electrical signals are proportional with cadmium concentration and decrease at the application of coumarin (see Fig. 5), which suggests a protective-stabilizing effect in the presence of coumarin on the lipid bilayer membrane. The dependency between the maximum value of the measured current and the concentration of cadmium is hyperbolic, which suggests that cadmium binds to the membrane with a first-order Michaelis-Menten kinetics. We computed the cadmium biding constant by fitting the experimental data (see Fig. 6). The values of the measured signals were normalized as ratios to the value of the signal measured in presence of 1 µM cadmium concentration. The binding constant varies in relationship with the coumarin concentration having a greater value in the presence of 50mM coumarin than in its absence. These changes could be explained by a direct effect of coumarin on the membranes and also by the formation of a cadmium-coumarin complex with a different affinity towards the membranes than free cadmium ions.

The values obtained for the cadmium-membrane binding constants through this method are 168.373 µM in the absence of coumarin and 221.98 µM in the presence of 50 µM coumarin. The presence of coumarin at a concentration of 50 µM decreases the affinity of the membrane for cadmium, which can be interpreted as a protective effect for the membrane. The mechanism for this decrease of membrane-cadmium affinity could involve both the binding of coumarin to the membrane and its subsequent electrical stabilization (as it is implied by the first series of experimental measurements in the presence of Hofmeister anions), and the formation of a coumarin-cadmium complex with a different kinetics of
interaction (insertion / surface attachment) with the lipid bilayer than the kinetics of the separate components.

**Figure 6.** The capacitive signals to cadmium concentration jumps dependency on the cadmium concentration, in the absence and the presence of coumarin 50 µM. The points correspond to the measured experimental data, the lines to the theoretical data fitting with a hyperbolic function. The values of the measured signals were normalized as ratios to the value of the signal measured in presence of 1 µM cadmium concentration.

**CONCLUSIONS**

The coumarin derivative, coumarin-3-carboxylic acid, interferes with the insertion in the membrane of the extremely chaotropic anions, such as perchlorate, probably affecting in an opposite manner the transmembrane dipole moment than perchlorate does, those two actions being mutually balanced. The coumarin presence results in protection of the membrane against the electrical and structural destabilizing effects that Hofmeister chaotropic anions have on artificial lipid membranes, probably due to its 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. The stabilizing effect of coumarin on lipid membranes was also proven in the presence of cadmium. The apparent affinity of cadmium to the lipid bilayer decreased as the coumarin concentration increased.

In conclusion, our results show that cadmium and chaotropic anions effects on lipid membrane are reduced when the coumarin derivative is added to the artificial lipid membrane, recommending it as a possible protective agent against their insertion in the artificial lipid bilayer.

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