EFFECTS OF EPIGALLOCATECHIN GALLATE ON ARTIFICIAL LIPID MEMBRANES

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Abstract. Epigallocatechin-3-gallate (EGCG), a natural antioxidant belonging to the family of catechins, revealed itself as a potent drug having multiple medical applications. In order to better understand the mechanisms underlying its action at the cellular level, we have studied the modifications induced by EGCG on the electric properties, i.e. capacitance and conductance, of artificial lipid membranes, by means of the electrophysiological method Black Lipid Membrane (BLM). While the electrical conductance of the lipid bilayer has not been affected by EGCG in the concentration range studied, its capacitance evolved in a concentration dependent manner. Our results suggested a complex mechanism of insertion of EGCG in the lipid membrane.

Key words: EGCG, BLM, capacitance, conductance.

INTRODUCTION

Epigallocatechin-3-gallate (EGCG) (Fig. 1), a catechin included in the group of flavanols, is encountered in significant amounts in tea leaves, especially in those of green tea (*Camelia sinensis*). Various effects of EGCG concerning not only its antioxidant properties, but also its interference with the intra- and intercellular signaling pathways are already well documented [3, 11, 12, 13, 18, 22].

Kobayashi *et al*. [8] have shown that the green tea polyphenols (−)-epigallochatechin gallate (EGCG) and (−)-epichatechin gallate (ECG) also inhibit glucose transport recommending thus the catechins in the prevention and treatment of *diabetes mellitus*. Another important direction, less investigated so far, concerns the effects of EGCG as a neuroprotectant [10] or at the level of neurodegenerative diseases. It was shown that EGCG has favorable effects in multiple sclerosis, either by a non-specific protective mechanism, or by immunomodulation [4, 15]. Studies on mice indicate a reduction of beta-amyloid correlated with Alzheimer disease [13].

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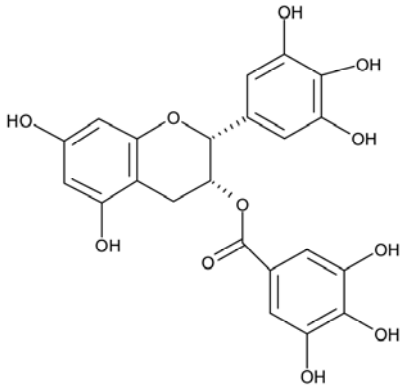

Fig. 1. (−)-Epigallocatechin gallate structure.

An important aspect that cannot be dissociated from the *in vivo* action of EGCG concerns its interaction with lipid membranes. Caturla *et al*. [2] have shown, by means of differential scanning calorimetry and spectroscopic studies, that ECG and EGCG partitioned efficiently into biological membranes being located deep in the phospholipids bilayer intercalating with the hydrocarbon chains and affected the extent of hydration of the phospholipids water-interface perturbing the membrane structure. On the basis of NMR spectroscopy studies it was proposed that EGCG interacts with the surface of lipid membranes via choline groups at their surface [21]. Recently, by means of molecular dynamic simulations it was shown that several tea catechins have a strong affinity for the lipid bilayer via hydrogen bonding to the bilayer surface and among them EGCG showed the strongest interaction due to the great number of hydrogen bonds formed with lipid headgroups [17]. It is well known the fact that EGCG attaches itself predominantly to the *lipid rafts* and this attachment is more important than for other catechins [5,9]. Kajiya *et al*. [7] identified some of the factors that influence the affinity of EGCG for lipid bilayers. Thus, the affinity seems to depend on EGCG concentration, on the ionic composition of the extracellular moiety and on the electrical charge of the membrane. More recent studies [19] on single giant unilamellar vesicles (GUVs) of egg phosphatidylcholine (egg PC) showed that low concentrations of EGCG $\geq$ 30 μM induced rapid leakage of internal contents from GUVs due to a possible permeabilization of the lipid membrane. Moreover, from the analysis of the EGCG-induced shape changes the authors conclude that the binding of EGCG to the external monolayer of the GUV increases its membrane area, inducing an increase in its surface pressure. Nevertheless, in spite of the increasing interest in the effects of EGCG at various levels of the living structures, coherent data about EGCG interaction with lipid membranes are not yet available.

Our study aims to obtaining new data concerning the mechanisms of EGCG insertion in artificial lipid bilayers. By means of Black Lipid Membrane (BLM) method, we have monitored the evolution of the electrical parameters of lipid bilayers (the electrical capacitance and conductance) as a function of EGCG concentration.

MATERIALS AND METHOD

THE BLM METHOD

The black lipid membranes (BLM), having an area of ca. 10^{-2} cm^2, are formed in a Teflon cuvette, consisting of two compartments, each with a volume of 1.5 mL. The compartments are filled with an appropriate electrolyte solution and, via agar bridges and Ag/AgCl electrodes, each compartment is connected to an external electric circuit that contains a current amplifier, an oscilloscope, a function generator, an electric filter, and a computer for data acquisition. The whole set-up is placed inside a compact Faraday cage, with the possibility of optional light inside. The stirring of the solutions inside the cuvette was performed by a magnetic stirrer operated by a small magnet placed under the cuvette, which is rotated with variable velocity. Using an electrical function generator it was possible to measure the electric capacitance of the lipid bilayer (by applying 10 mV triangular pulses) and its conductance (by applying continuous 100 mV voltage jumps). Typical values for those electric characteristics of the black lipid membrane are ca. 400 nF/cm^2 for the specific capacitance and ca. 7 nS/cm^2 for the specific conductance (for further details see [1]).

CHEMICALS AND PROTOCOLS

The lipid film forming solution contained 1.5% (w/v) diphytanoyl-phosphatidylcholine (Avanti Polar Lipids, USA) and 0.025% (w/v) octadecylamine in n-decane (Fluka, Sigma-Aldrich Germany, purity >98%). Before painting the lipid film the cuvette hole was impregnated with a solution of 0.5% (w/v) corresponding lipid in hexane (Scharlau, Germany, purity >96%). The artificial lipid membranes were formed in the presence of 20 mM HEPES (purity >99.5%, Sigma) and 100 mM NaCl (Merck, purity 99%) in both compartments of the cuvette. All the experiments were carried out at pH = 7 and at room temperature (22 – 24 ºC). The pH was adjusted with KOH (LobaChemie, purity >97%) and before use the solution was filtered through a cellulose-acetate filter 0.2 mm (Sartorius). EGCG extracted from green tea (purity >95%, Sigma) was prepared as 0.5 mM stock solution in ultrapure water (Millipore). The solution was kept and manipulated in opaque recipients as EGCG is photosensitive. The statistical data processing was performed by using the OriginPro 7.0 software.

RESULTS

We present here the results concerning the modifications of the electric properties of the artificial lipid membrane in the presence of various concentrations of EGCG, obtained by means of the BLM technique.

TIME DEPENDENCE OF ELECTRICAL PARAMETERS AT CONSTANT EGCG CONCENTRATION

The first category of experiments was designed to study the insertion kinetics of EGCG in the artificial lipid bilayers. For this purpose we have performed several measurements at a constant concentration of EGCG (5 μM, 20 μM and 50 μM) in the BLM cuvette, applied bilaterally (i.e. on both sides of the lipid bilayer). The solutions were prepared as described in Materials and Method. The instant of introducing EGCG in the cuvette was taken as the starting point of the measurement (t = 0). We measured then the electrical properties of the lipid bilayer (capacitance, conductance) every 10 minutes, until these properties reached a stable state (plateau). For each membrane the experimental data values were normalized to the initial values measured at t = 0, and the results obtained from all studied membranes were averaged. The control experiment was performed at 0 μM EGCG, and it has confirmed the stability of the artificial lipid bilayer parameters for the time interval we used to perform the experiments in the absence of EGCG.

The electrical conductance of the membrane remained constant throughout all the measurements, regardless of EGCG concentration. This observation suggests that the insertion of EGCG in the lipid bilayer does not appear to produce pores in the membrane – a phenomenon observed for other flavonoids, i.e. quercetin [6, 14]. The normalized values of electrical conductance vs. time for different EGCG concentrations are shown in Fig. 2.

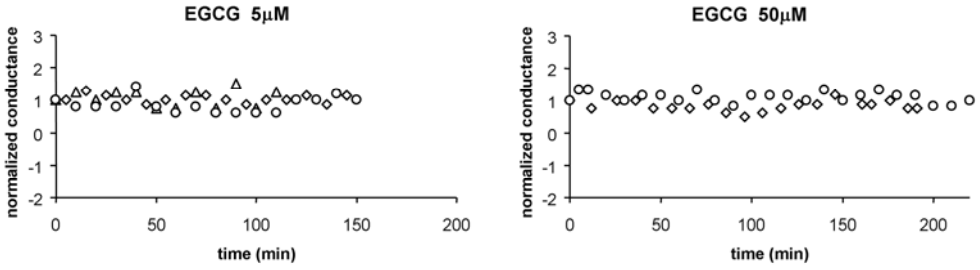

Fig. 2. Membrane electrical conductance appears to remain constant during the experiment, regardless of EGCG concentration. A similar situation happens for 20 μM EGCG (not shown).

The profile of electrical capacitance time dependence suggests an insertion of EGCG in the lipid bilayer in a dose-dependent manner (Fig. 3).

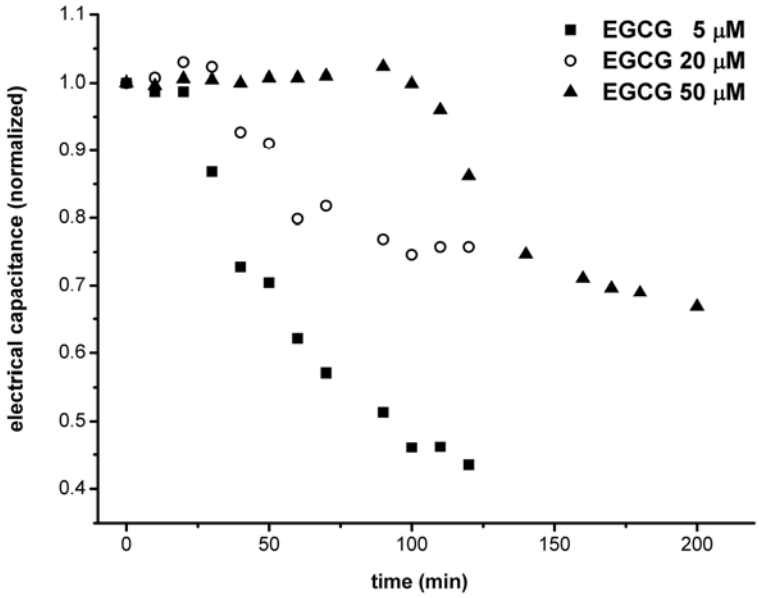

Fig. 3. The variation of membrane electrical capacitance (normalized) in time at different EGCG concentrations.

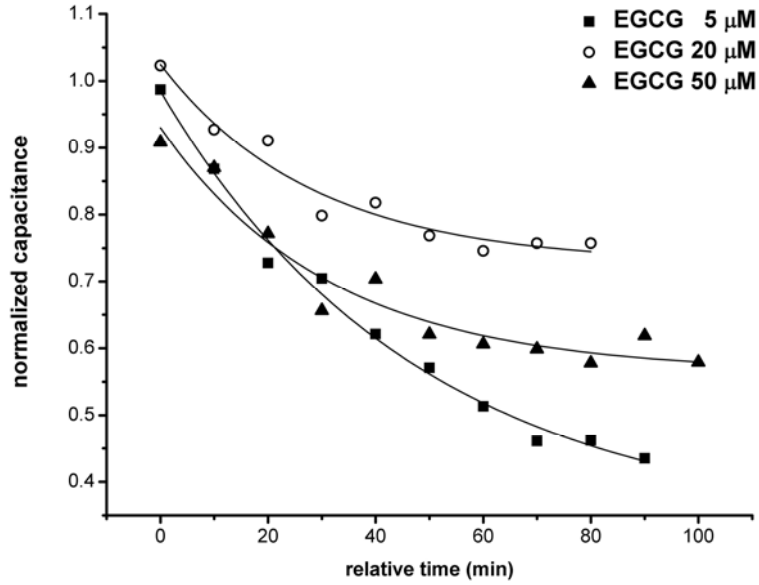

Fig. 4. The descending slopes of membrane capacitance variations. The normalized capacitance is plotted against the relative time from the beginning of descending phase of EGCG at different concentrations. The fitting equation is presented below (Equation 1) and the fitting parameters are given in Table 1.

EGCG concentration appears to influence both the duration of the plateau phase (the time interval after which the capacitance starts to decrease) and the descending slope of the capacitance variation (the time constant of the fitting exponential function) (Fig. 4 and Table 1).

$$y = A_1 \cdot e^{-\frac{x}{t_1}} + y_0 \quad (1)$$

Table 1

The parameters for the descending slopes of membrane capacitance variations presented in Fig. 4 and Eq. (1)

Parameter	EGCG 5 μM	EGCG 20 μM	EGCG 50 μM
y_0	0.33084±0.04205	0.72704±0.02813	0.56402±0.02905
A_1	0.65378±0.03773	0.29741±0.02945	0.36601±0.03324
t_1	47.98233±1.82343	28.4894±7.83527	31.60846±1.01361

A statistical analysis of the three data sets corresponding to the descending slopes of membrane capacitance variations shows no statistically significant difference between the slopes corresponding to 20 μM and 50 μM EGCG concentration in the cuvette, while the difference between the 5 μM and 20 μM, respectively between 5 μM and 50 μM is statistically significant. This information suggests a saturation-like insertion kinetics of EGCG in the lipid bilayer. Additional studies are required to elucidate how the saturation is influenced by the complex insertion kinetics (see the section below about the titration experiments) and by the available amount of lipids (the volume of the membrane which is directly dependent on the aperture diameter of the BLM cuvette).

TITRATION EXPERIMENTS

The second category of experiments was designed for observation of the variation of electrical parameters of the lipid bilayer in relation with the varying concentration of EGCG in the BLM cuvette. In these titration experiments the EGCG concentration was progressively increased. Two subgroups of measurements have been performed: in the first subgroup the concentration variation step was 1 μM, while in the second subgroup this step was 5 μM. The measurements of the electrical conductance of the membrane have shown stable values (within normal statistical fluctuations) regardless of EGCG concentration in the BLM cuvette; this observation is consistent with the results from the first category of experiments (presented above).

The measured variation of membrane capacitance (Fig. 5) hints at a complex, biphasic insertion kinetics for EGCG. The results show that the data dispersion is greater in the descending phase than in the plateau phase, suggesting thus a greater dependence on the membrane geometry (the measurements were performed with several different BLM cuvettes, each one with a different aperture) in the first phase. The plateau phase seems to be less influenced by the geometrical parameters, the differences in the results obtained in measurements performed with different cuvettes being almost zero.

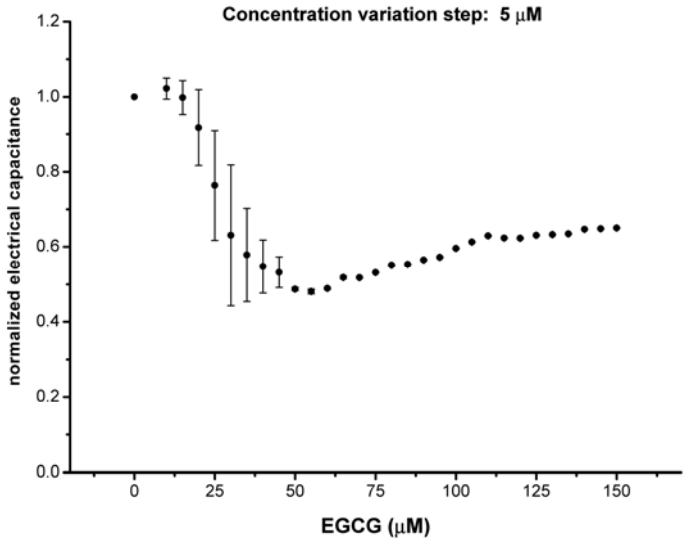

Fig. 5. Variation of membrane capacitance in an EGCG titration experiment. A greater data dispersion is present in the descending phase.

The obtained experimental data can be modeled with a biphasic dose-response equation:

$$y = A_{\min} + \frac{A_{\max 1} - A_{\min}}{1+10^{(x-x_{0\_1})h_1}} + \frac{A_{\max 2} - A_{\min}}{1+10^{(x-x_{0\_2})h_2}} \tag{2}$$

The fitting parameters and theoretical functions are presented in Fig. 6 and Table 2. The results suggest that EGCG behaves differently in lower concentrations than in higher concentrations. Membrane insertion of EGCG (and consequently its protective effects due to membrane attachment) is significant at concentrations up to 50 μM. A hypothesis that needs further research is that two different mechanisms might be responsible for the observed changes in the electrical capacitance; these mechanisms are working with different efficiencies for different concentrations.

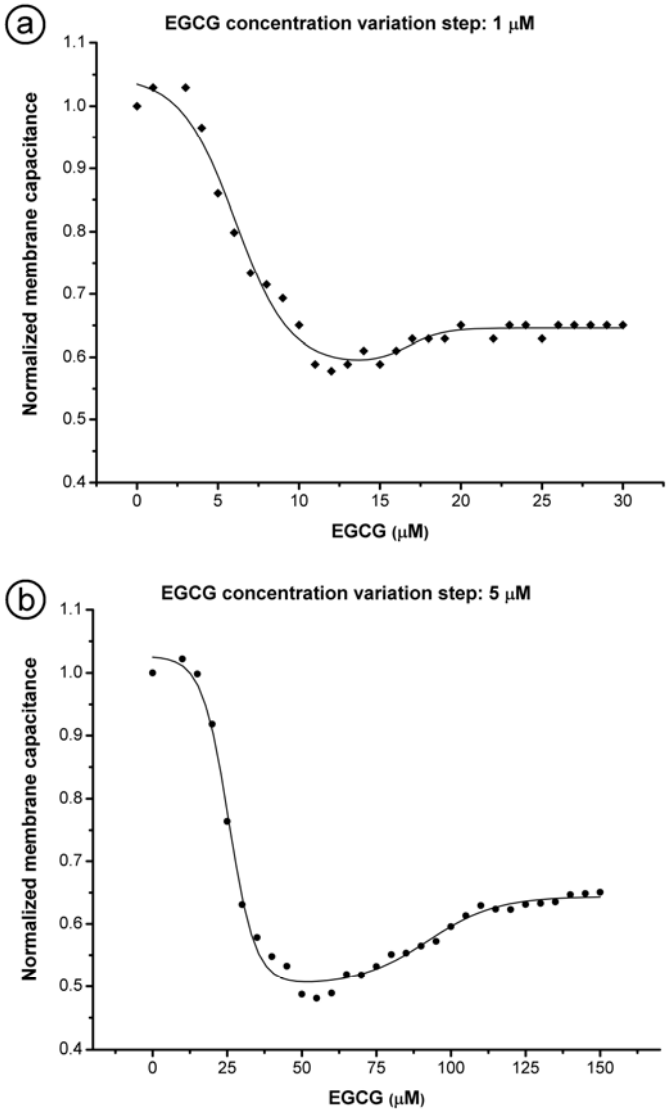

Fig. 6. The fitting functions for normalized membrane capacitance variation in EGCG titration experiments. a) concentration increment step: 1 μM. b) concentration increment step: 5 μM. The fitting parameters are given in Table 2.

Thus, at lower concentrations of EGCG the predominant mechanism would be the insertion of EGCG, which modifies the thickness of the membrane and leads to the decrease of capacitance in a concentration-dependent manner. For higher concentrations of EGCG the predominant mechanism appears to be a modification of the lipid bilayer electrical permittivity (possibly because of the appearance of

new complexes between EGCG and phospholipids), which results in an increase in electrical capacitance. According to Tarahovsky [20] the polyphenols rich with gallate moieties may attach to the cell surface and change the physical properties of lipids. The stabilization of the capacitance values in a plateau phase (with a different absolute value than in the beginning of the experiment, when the EGCG was introduced) is an argument against a possible disruptive effect of the EGCG on the membranes. If this would have been the case (i.e. disruptive effect) we reason that the observed phenomena would not have been stabilization in a plateau phase after the increase in capacitance, and also there would have been an observed increase in electrical conductance, which is contradicted by our experimental data.

Table 2

The fitting parameters for functions presented in Fig. 6

Parameter	a) EGCG titration step 1 μM	b) EGCG titration step 5 μM
A_{min}	0.58722±0.02033	0.50183±0.0103
A_{max1}	1.047±0.02215	1.02645±0.01197
A_{max2}	0.64581±0.00719	0.64384±0.00803
x_{0_1}	6.06459±0.27851	25.5015±0.47334
x_{0_2}	16.7336±1.4004	92.2462±3.19912
h_1	0.25826±0.04379	0.09797±0.00927
h_2	0.39428±0.41536	0.0379±0.01026

CONCLUSIONS

Our experimental data concerning the EGCG insertion in the artificial lipid membranes suggest the following observations:

The kinetics of EGCG insertion in artificial lipid bilayers has two phases: a plateau phase (its length in time is non-linearly dependent on EGCG concentration) and an exponential – descending phase. The electrical parameter of the artificial membrane which optimally reflects the insertion phenomena is the capacitance.

The values of electrical conductance of the artificial membrane were constant during the experiments, regardless of EGCG concentration and of the experiment category (constant EGCG concentration or titration, i.e. a stepwise increase of EGCG concentration). The slope of exponential-descending phase of insertion varies also with EGCG concentration, but for concentrations above 20 μM a saturation phenomenon has been observed – there is no statistically significant difference between the slopes obtained for 20 μM and for 50 μM EGCG. The

saturation phenomenon depends probably on the EGCG insertion kinetics, but also on the volume of the membrane (itself dependent on the amount of lipids used – kept constant across the experiments and on the diameter of BLM cuvette aperture, i.e. the place where the membrane resides).

The membrane electrical response to the progressive increase of EGCG concentration is biphasic: after an initial decrease in capacitance (for lower concentration of EGCG), a second phase of slight increase followed by a plateau (which differs from the initial values, recorded in absence of EGCG) can be observed. The much smaller dispersion of measured data values when the EGCG concentration is higher suggests a weaker dependence on the geometrical properties of the membrane during this phase.

The insertion kinetics is dependent on the concentration up-step used in the titration experiments. For smaller steps (1 μM), the observed phenomenon happens faster (i.e. the observed decrease in electrical capacitance of the membrane when the EGCG concentration is lower), and also the plateau phase is reached for lower EGCG concentration, but the absolute values of the plateau are higher than in the case of up-step concentration variation of 5 μM.

The measured values for the membrane electrical capacitance during the second phase of EGCG insertion probably reflects the equilibrium between two opposite phenomena influencing the capacitance: the increase in membrane thickness (due to EGCG insertion) and the decrease of dielectric constant of the membrane. The results concerning EGCG insertion confirm the data previously published [2, 7, 17].

Our data do not suggest an effect on the lipid membrane area. This difference between our results and the ones reported by Tamba *et al*. (2007) [19], which used vesicular lipid mono-layers and not planar lipid membranes, suggests how important is the spatial arrangement of lipid molecules in their interactions with EGCG. As the cellular membranes are in fact lipid bilayers, our experimental data obtained through BLM method could offer valuable clues regarding *in vivo* actions of EGCG.

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