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# Monitoring of Cytochrome c Adsorption at Supported Lipid Membranes Using Multiharmonic QCM Method †

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**Abstract:** Cell apoptosis is initiated by release of cytochrome c (cyt c). This protein can be used as biomarker to evaluate chemotherapy efficacy. Therefore, detection and study of cyt c interaction with lipid membranes is a challenging task. Recent studies suggest innovative development in cyt c detection by binding it to specific DNA aptamers. To achieve this goal, it is necessary to provide surface with bound cyt c close to the real biomembranes. Our research was focused on preparation of supported lipid membranes on the gold surface of the quartz crystal. Lipid membranes were prepared by liposome fusion at the surface of 1-dodecanethiol chemisorbed at gold surface of the crystal. The liposomes were composed of a mixture of L-$\alpha$-Phosphatidylcholine (lecithin) and Dimyristoyl phosphatidylglycerol (DMPG), which were used in different ratios. Subsequent formation of the lipid membrane was monitored by multiharmonic quartz crystal microbalance (QCM). Cyt c was applied on lipid layer and we observed changes in fundamental and several higher harmonic frequencies. Energy dissipation changes were recorded as well. The data on frequency and dissipation changes were used for the analysis of the viscoelastic properties of adsorbed layers.

**Keywords:** cytochrome c; quartz crystal microbalance; lipid layers; viscoelasticity

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## 1. Introduction

Cytochrome c (cyt c) is a small protein that serves as an initiator of several critical processes in the living cells. It plays a key role in cellular metabolism, energy, and protein regulation. Because if this role it became the aim of many publications. Cyt c is positively charged at physiological conditions. It proved to be electrochemically active compound. Cyt c is released from mitochondria during the process of programmed death—apoptosis. It is a useful tool to evaluate chemotherapy effect on cancer cells. Therefore, it is crucial to develop the methods of detection of cyt c as well as to study the interaction mechanisms of these proteins with lipid membranes. The detection methods can be based on application of antibodies or DNA aptamers that specifically bind cyt c [1]. First step in the study these interactions and to model biomembranes is to select suitable model membranes that can bind cyt c. Because cyt c is positively charged it can be adsorbed electrostatically at the surface of negatively charged lipid membranes [2]. The supported lipid films provide sufficient stability for such a study and can serve as a model of mitochondria membranes.

Several studies were focused on the analysis of the interaction of the cyt c with the lipid membranes. Because the lipid membrane is composed of neutral and negatively charged lipids, the positively charged cyt c can interact electrostatically with the lipids and can also change the membrane structure. These structures usually include neutral

phosphatidylcholine or cardiolipin to simulate interaction with the membrane of mitochondria. Many articles also reported the interaction of cyt c with certain lipid mixtures, for example DMPC and DMPG [3].

We applied of multiharmonic quartz crystal microbalance (QCM) to study the viscoelastic properties of the supported lipid films composed of a mixture of DMPC and DMPG during interaction with cyt c.

## 2. Materials and Methods

### *2.1. Chemicals*

For liposomes preparation and QCM measurements, the following chemicals were used: PBS (phosphate buffered saline composed of 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 137 mM NaCl and 2.7 mM KCl diluted in MiliQ water, pH 7.4), HEPES (50 mM HEPES, 10 mM $MgCl_2$, pH 7.4), MiliQ water has been prepared by Purelab Classic UV (Elga, High Wycombe, UK).). The standard chemicals such as ethanol, NaCl, $NH_3$ and $H_2O_2$ were purchased from Slavus (Bratislava, Slovakia). Liposome solution was prepared from 1,2-dimyristoyl-sn-glycero-3-phosphocholine—DMPC (Avanti Polar Lipids Inc., Birmingham, AL, USA) and 1,2-Dimyristoyl-sn-glycero-3-phospho-rac-(1-glycerol) sodium salt—DMPG (Sigma Aldrich, Darmstadt, Germany). 1-dodecanthiol (Sigma Aldrich, Darmstadt, Germany) was used to modify crystal surface.

### *2.2. Piezocrystal Cleaning and Preparation of Lipid Film*

Quartz crystal (Total Frequency Control, Storrington, UK, fundamental frequency 8 MHz, working area 0.2 $cm^2$ consisted of a thin gold layer) were cleaned before each measurement. They were submerged to basic Piranha solution (29% $NH_3$, 30% $H_2O$ and $H_2O_2$ with volumetric ratio 1:5:1, respectively) for 25 min. After this treatment, the crystal was washed three times with deionized water and stored in ethanol. The crystal was then submerged to 2 mM solution of 1-dodecanthiol (DDT) in ethanol and stored at room temperature for 16 *h*. After washing by ethanol and drying in a flow of nitrogen the crystal was placed in an acryl flow cell (JKU Linz, Austria) connected to the syringe pump (Genie Plus, Kent Scientific, Torrington, CT, USA).

The lipid monolayer has been prepared by liposome fusion. For this purpose, the small unilamellar liposomes were prepared by sonication method. Briefly, 8 mg of the phospholipid mixture (DMPC:DMPG in molar ratio 10:1) was dissolved in a small quantity of chloroform and dried under nitrogen in order to deposit a layer on the walls of the flask. A 4-mL aliquot of PBS was then added, and after a 30-min incubation, the mixture was ultrasonicated for 20 min with a sonicator (Bandelin Sonorex RK31, Berlin, Germany) in a water bath at room temperature [4]. The liposomes in a concentration of 0.5 mg/mL were then added in a flow to a surface of piezo crystal modified by DDT.

### *2.3. The Principles of QCM and Evaluation of Viscoelastic Properties of Lipid Layers with Cytochrome c*

The principle of the QCM consists in measurements of the changes in the resonant frequency that are related to the changes of the mass at the crystal surface. According to Sauerbrey [5], the changes in the resonant frequency, $\Delta f$, of the quartz crystal in vacuum are related to the changes of mass, $\Delta m$, by equation:

$$\Delta f = \frac{-2nf_0^2\Delta m}{A\sqrt{\mu_q\rho_q}} \quad (1)$$

where $n$ is harmonic number, $f_0$ is the fundamental resonance frequency, $A$ is effective crystal area, $\mu_q = 2.947 \times 10^{11}$ g.cm$^{-1}$.s$^{-2}$ is the shear modulus of elasticity and $\rho_q = 2.648$ g.cm$^{-3}$ is the crystal density.

In a water environment, the frequency can also be affected by viscous forces, therefore additional term should be added to the Sauerbrey equation:

$$\Delta f = 2f_0^{\frac{3}{2}}\sqrt{\frac{\eta_L\rho_L}{\pi\mu_q\rho_q}} \quad (2)$$

where $\eta_L$ is the viscosity and $\rho_L$ the density of the liquid, respectively [6].

The acoustic waves in QCM transducer are generated by applying a high-frequency voltage to the electrodes sputtered at both sides of the crystal [7]. As the oscillation is modelled by Butterworth-Van Dyke equivalent electric circuit, this attenuation can be estimated by motional resistance $R_m$. Corresponding decay of acoustic wave is characterized by penetration depth (decay length) $\Gamma$, that can be expressed as:

$$\Gamma = \sqrt{\frac{2\eta_L}{\omega\rho_L}} \quad (3)$$

where $\eta_L$ is liquid viscosity, $\omega$ is circular frequency of the oscillations and $\rho_L$ is density of the liquid. Another factor related to penetration depth is dissipation factor, $D$, expressed as:

$$D = \frac{2\Gamma}{f_0} \quad (4)$$

The QCM experiments were performed using the computer-controlled Sark 110 vector analyzer (Seeed, Shenzhen, China). The device allowed measurement of fundamental and higher harmonic frequencies. The frequency changes increased linearly with the harmonic number, $n$ (see Equation (3)). All measurements were performed in a flow mode at ambient temperature at around 20 ± 0.5 °C.

By measurement of the frequency and dissipation it is possible to estimate the viscoelastic properties of the surface layer at the piezocrystal. It is, however, useful to verify whether the analysis properly reflects the viscoelastic properties. For this purpose, the analysis of the normalized frequencies for several overtones $f_n/n$ and their relative changes $\Delta f_n/n$ are helpful. As more these values differ from each other, the higher the viscoelastic component of the sample is.

For a study of the viscoelastic properties of the sample, it is useful to analyze the thickness of the hydrated layer, since water molecules are an integral part of this system. It is possible to calculate the thickness $h_0$ of the crystal using the resonant frequency, $f_0$, at the beginning of the measurements according to the equation:

$$f_0 = \frac{u}{2h_0} \rightarrow h_0 = \frac{u}{2f_0} \quad (5)$$

where $u$ is the velocity of the acoustic wave in the crystal ($u$ = 3336 m/s). Once the thickness of the crystal has been calculated and the frequency shift obtained, the thickness $h_1$ of the adlayer can be calculated, using the equation:

$$\Delta f = f_1 - f_0 = \frac{u_f}{2(h_0 + h_1)} - \frac{u}{2h_0} \quad (6)$$

Using this equation, it is also possible to calculate the thickness of subsequent layers at the piezocrystal surface. Considering that the contribution of the layer in the variation of the acoustic wave along the crystal is practically negligible, then $u_f = u$ [8].

By means of the viscoelastic analysis it is possible to obtain information about the characteristics of sensing layer, such as stiffness, viscosity, elasticity and loss moduli. Furthermore, the formation of crosslinking bonds or the possibility of freedom of movement of the molecules constituting the adlayer can be analyzed. The Kelvin-Voigt viscoelastic model has been developed for this purpose. Using this model, it is possible to calculate the viscoelastic properties of the adlayer considering the differences in frequency and dissipation at the different harmonics. This model was described mathematically by Voinova

et al., [9], according to which the viscoelastic properties of the film are related to the variations in frequency and dissipation by the equations:

$$\Delta f \approx -\frac{1}{(2\pi\rho_0 h_0)}\left[\left(\frac{\eta_3}{\Gamma_3}\right) + h_1\rho_1\omega - 2h_1\left(\frac{\eta_3}{\Gamma_3}\right)^2\left(\frac{\eta_1\omega^2}{\mu_1^2} + \omega^2\eta_1^2\right)\right] \quad (7)$$

$$D \approx \frac{1}{(\pi f_n \rho_0 h_0)}\left[\left(\frac{\eta_3}{\Gamma_3}\right) + 2h_1\left(\frac{\eta_3}{\Gamma_3}\right)^2\left(\frac{\eta_1\omega}{\mu_1^2} + \omega^2\eta_1^2\right)\right] \quad (8)$$

where $\Gamma_3$ is the decay length of the shear wave in the liquid; $\rho_3$ and $\eta_3$ are the density and viscosity of the liquid (water or very dilute saline solutions have approximately the same density, 0.9982 g/cm$^3$, and dynamic viscosity, 1.0016 mPa.s, at 20 °C), $\rho_0$ and $h_0$ are the density and thickness of the quartz crystal, respectively 2.648 g·cm$^{-3}$ and 0.208 mm for the crystal with fundamental frequency of $f_0$ = 8 MHz (but the thickness can be precisely calculated using Equation (5)), $h_1$, $\mu_1$, $\eta_1$ and $\rho_1$ are the thickness, the elastic shear modulus, viscosity and density of the adsorbed film; $\omega$ is the angular frequency of the oscillation.

## 3. Results and Discussion

At the first series of the experiments we studied the changes in the fundamental resonant frequency and their overtones (3rd, 5th, 7th and 9th harmonics) following additions of liposomes prepared from DMPC:DMPG mixture at molar ratio 10:1 to a surface of piezo crystal modified by DDT. After 35-min of liposome application in a flow mode (flow rate 50 μL/min), the crystal was rinsed with buffer and 1 μM cyt c in PBS was applied on the surface. The kinetics of the changes of fundamental frequency and their overtones following application of liposomes and cyt c are presented on Figure 1.

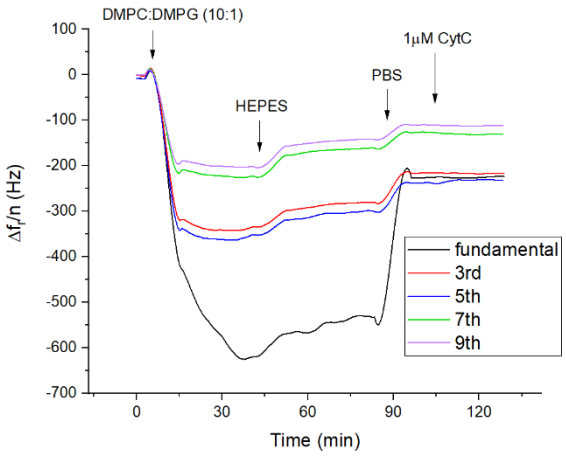


**Figure 1.** The kinetics of the changes of fundamental frequency, 3rd, 5th, 7th and 9th harmonic frequencies vs. time following addition of DMPC:DMPG (10:1) liposomes and 1 μM cyt c on 1-dodecanthiol layer. The moments of addition of liposomes, cyt c and HEPES/PBS wash are shown by arrows.

Because the changes in the frequency following addition of cyt c were not significant, we used liposomes with higher content of negatively charged DMPG (DMPC:DMPG = 1:1 mol/mol). As it can be seen from Figure 2a, a significant decrease in the frequency has been observed following addition of cyt c. This has been accompanied by decrease of dissipation (Figure 2b).

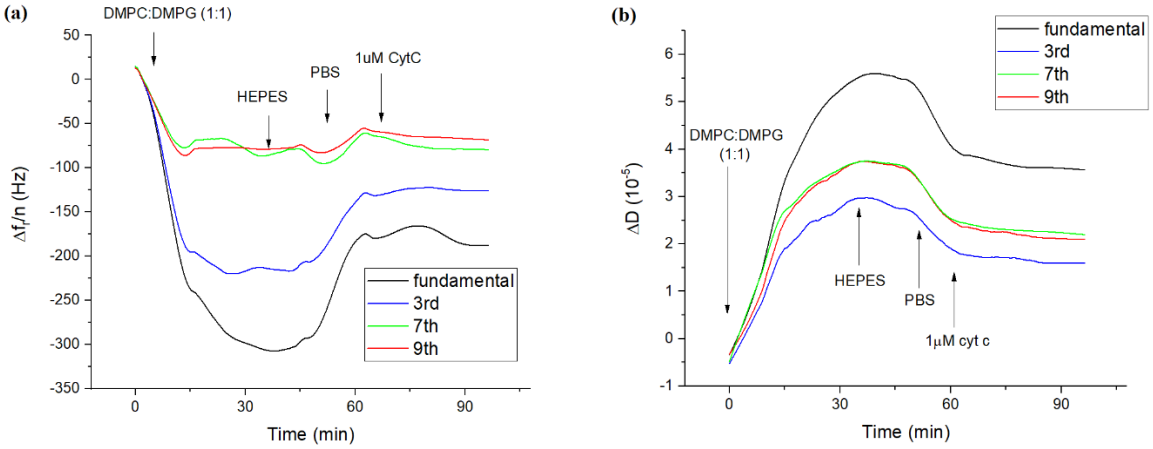


**Figure 2.** The kinetics of the changes of (**a**) fundamental frequency, 3rd, 7th and 9th harmonic frequencies and (**b**) energy dissipation vs. time following addition of DMPC:DMPG (1:1) liposomes and 1 μM cyt c on 1-dodecanthiol layer. The moments of addition of liposomes, cyt c and HEPES/PBS wash are shown by arrows.

To obtain additional information about the properties of the lipid layers we applied Kelvin-Voigt-Voinova model for analysis of viscoelastic properties. For this purpose, the fundamental frequency and their overtones 3rd and 7th were used. Other overtones were excluded due to irregularities and anomalies appeared during their recording. Using the testimated lipid layer thickness from literature, we approximated this thickness as $h = 4.76 \pm 0.1$ nm and layer thickness after the cyt c adsorption as $h = 6.26$ nm ± 0.1 nm [10,11]. We determined also the viscosity coefficient, η, and shear elasticity modulus, μ. The kinetics of the changes of these values at various modification of QCM transducer are presented on Figure 3.

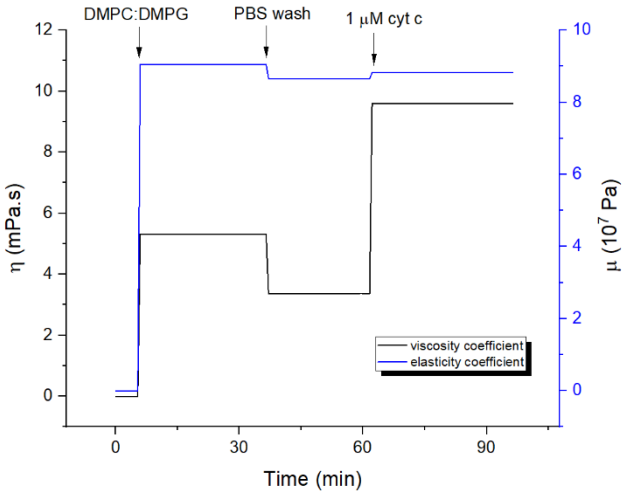


**Figure 3.** Kinetics of the changes of viscosity coefficient ($\eta$, black, left axis) and shearing modulus of elasticity ($\mu$, blue, right axis) during formation of the lipid layer and interaction with 1 μM cyt c. Addition of various compounds and washing of the surface by buffer is shown by arrows.

It can be seen that while formation of lipid layer caused prominent increase in both viscoelastic parameters characteristics ($\Delta\eta$=5.313 mPa.s and $\Delta\mu$=9.05 × $10^7$ Pa), washing the surface by buffer and addition of cyt c resulted only in small changes in elasticity coefficient ($\Delta\mu = 0.164 \times 10^7$ Pa). However, addition of the cyt c caused prominent change in viscosity coefficient ($\Delta\eta = 6.234$ mPa.s). This can be contributed to its loading on the lipid layer surface and cyt c molecules being affected by the flow. However, in order to obtain

further inside on the mechanisms of interaction of cyt c with the lipid layer additional methods can be applied such as laser ellipsometry. Also, the effect on the viscoelastic properties of the variation in cyt c concetration must be performed. These experiments are in progress.

## 4. Conclusions

Several measurement using multiharmonic acoustic method was performed to study formation of mixed lipid layer. Liposomes formed by different DMPC:DMPG molar ratio were applied on quartz crystal surface that was modified by 1-dodecanthiol. Equimolar ratio of DMPC:DMPG proved to be optimal for study the interaction of cyt c with supported lipid film. Kinetics of fundamental frequency and several overtones together with dissipation were used to estimate changes in viscosity and elasticity coefficients using Kelvin-Voight-Voinova model. We have shown that addition of cyt c resulted in increase of the shear modulus, $\mu$, and viscosity coefficient, $\eta$. In the latter case the changes were more remarkable. This is evidence on the interaction of cyt c with the lipid layer surface. Further experiments are, however, required to confirm this finding, together with the selection of alternative methods for accurate film thickness determination. However, the experiments and analysis of viscoelastic properties performed are evidence of advantage of the multiharmonic detection of the interaction of cyt c with the surface of supported lipid films.

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