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Noufissa Zanati
Cleveland State University

Michael Ellen Mathews
Buckeye Pharmaceuticals

Perera N. Indika
Cleveland State University

John J. Moran
Cleveland State University

Jean A. Boutros
Cleveland State University

See next page for additional authors

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Authors

Noufissa Zanati, Michael Ellen Mathews, Perera N. Indika, John J. Moran, Jean A. Boutros, Alan T. Riga, and Mekki Bayachou

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Noufissa Zanati Michael Ellen Mathews
Indika N. Perera John J. Moran Jean A. Boutros
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Abstract The long-term goal of this investigation is to study the effects of increased cholesterol levels on the molecular activity of membrane-bound enzymes such as nitric oxide synthase, that are critical in the functioning of the cardiovascular system. In this particular investigation, we used differential scanning calorimetry (DSC) and dielectric thermal analysis (DETA) to study the effect of added cholesterol on melting/recrystallization and dielectric behavior, respectively, of phosphatidylcholine (PC) bilayered thin films. We also used electrochemical methods to investigate the effect of added cholesterol on the redox behavior of the oxygenase domain of nitric oxide synthase as a probe embedded in the PC films. The results show that added cholesterol in the PC films seems to depress the molecular dynamics as indicated by lowered current responses in the presence of cholesterol as well as a slight increase of the transition temperature in the overall two-phase regime behavior observed in PC cholesterol films. These results are rationalized in the context of the general DSC and DETA behaviors of the PC chol films.

Keywords Cholesterol · Calorimetry · Dielectric · Nitric oxide synthase · Phosphatidylcholine · Redox

Introduction

Although high cholesterol levels as a risk factor for cardiovascular disease is well established [1, 2], the direct underlying mechanism of this implication is not well understood. Clinical findings show that high cholesterol levels are unequivocally linked to dysfunction of vascular dilation, which results in impaired regulation of systemic blood pressure and vascular tone. Despite numerous clinical observations of this sort, the causal relationship, in the form of a molecular link between hypercholesterolemia and systemic blood pressure is lacking. Increased levels of cholesterol are known to contribute to increased rigidity of cell biomembranes [2–9]. Whether the increased rigidity would affect the dynamics and the molecular function of membrane-bound enzymes such as endothelial nitric oxide synthase is not known.

In these preliminary studies, we electrochemically characterized the fluidity of phospholipid films as a function of cholesterol content, and measured resulting effects on the transport of NOS oxygenase as a redox active probe. In this regard, we monitored the effect of added cholesterol on the kinetics and thermodynamics of electron transfer to NOS oxygenase in our membrane-like films. We also used differential scanning calorimetry (DSC) and dielectric thermal analysis (DETA) to study the thermal and dielectric behaviors of our phosphatidylcholine (PC) bilayered films and how these are affected by added cholesterol.

Experimental, materials and methods

Mixtures of PC aqueous solutions with the required level of cholesterol content are cast onto graphite electrodes and allowed to form bilayered films prior to electrochemical

analysis. For electrodes with embedded NOS oxygenase as a redox probe, 10 μL suspensions of PC with or without the required levels of cholesterol (0 or 40 mol%) are mixed with 10 mL of NOS oxygenase (inducible NOS oxygenase, $\sim 65 \mu\text{M}$) and then cast on the electrode surface. The mixture is allowed to equilibrate under a closed vessel overnight and then allowed to dry the following day before analysis. Cyclic voltammetry is used to measure changes in the transport properties of NOS oxygenase in the thin film. Square wave voltammetry is used to measure reduction current of NOS oxygenase embedded in PC films with or without cholesterol as a function of temperature. The cholesterol phosphatidylcholine preparations are lyophilized and re-suspended in Tris buffer and kept overnight at about 2°C before use.

A TA Instruments (TAI) 2970 DEA was used to determine the electrical conductivity profile of pure PC and 40/60 mol% cholesterol/PC and dried overnight in a microscopic slide. A sample of typically 10 μL was placed on a microscopic slide and then on a surface of ceramic plate with interdigitated gold electrodes in an isolated atmosphere under dry nitrogen. The temperature was scanned at a rate of $10^\circ\text{C min}^{-1}$ from below room temperature (-40°C) to about 60°C . The $10^\circ\text{C min}^{-1}$ rate is not too fast to observe typical transitions of PC films since at higher rates (20 – 40°C), previous studies have shown that complete gel-to-liquid transition of PC-based membranes require no more than 1 s [10]. Conductivity measurements were recorded at controlled interval frequencies ranging from 0.10 to 10,000 Hz for all temperatures.

A TAI 2920 DSC was used to measure the heat flow transmitted (as W g^{-1}) from our PC films with various levels of cholesterol at constant pressure in consecutive heating cooling cycles. Mixtures of PC and cholesterol were prepared and stored as described above.

Between 7 and 14 mg samples in aluminum pans holders were subjected to a cooling heating cycles between -50 and 150°C at a rate of $10^\circ\text{C min}^{-1}$ in an isolated nitrogen atmosphere.

Results and discussion

Differential scanning calorimetry (DSC) analysis of films of pure PC shows peaks of thermal transitions around 0°C and then at ca. 30°C during the first heating cycle (first ramp in Fig. 1). The endothermic process at exhibits a corresponding heat of fusion of about 1 J g^{-1} and is possibly due free water present within the bilayers of the PC film. It is worth mentioning that strongly bound water .e. nonfreezing is known to exhibit very different melting enthalpy and temperature, and the related DSC transition is not typically seen around 0°C [11–13]. The second major

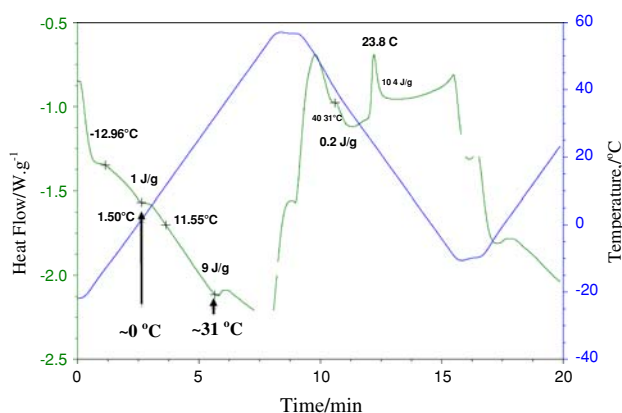


Fig. 1 Differential scanning calorimetry (DSC) on phosphatidylcholine only films. Arrows in the first heating cycle point to major transitions observed

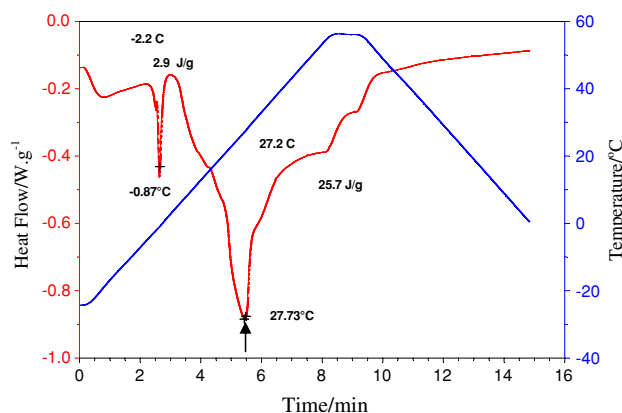


Fig. 2 Differential scanning calorimetry (DSC) on PC/cholesterol films (40 chol/60 PC mol%). Arrow in the first heating cycle point to a sharp phase transition occurring around $\sim 28^\circ\text{C}$ as a result of the presence of cholesterol in the film

transition occurring around $\sim 30^\circ\text{C}$ shows a corresponding heat of 11 J g^{-1} , and represents a bulk phase transition typical of the gel-to-liquid crystalline-like behavior, also known as the $L\beta$ -to- $L\alpha$ phase transition [14, 15]. The exothermic peaks (at ~ 40 and 24°C) recorded during the subsequent cooling cycle probably indicate the existence of metastable microstructures as the film phase reorganizes during cooling. While this overall behavior does not change in the presence of added cholesterol, a major change in the shape of the transitions is noted in the presence of 40% (mol%) cholesterol in PC films; the endothermic gel-to-liquid crystal phase transition becomes very sharp and is slightly shifted toward lower temperatures compared to PC-only films, Fig. 2. The sharp transition in the presence of 40 mol% cholesterol in the PC membrane is indicative of increased order induced by the presence of cholesterol. In fact, it is known that cholesterol

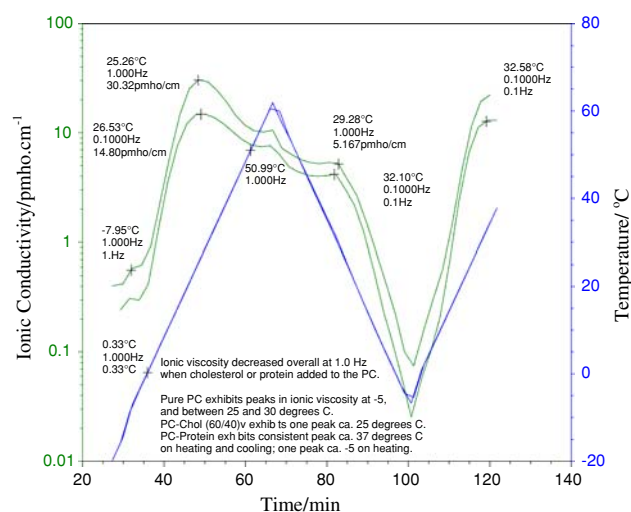


Fig. 3 Dielectric thermal analysis (DETA) of pure PC films

helps shift the main phase of PCs into a liquid-ordered (Lo) state in which the orientational order of the bilayer is increased and the rate of motion of hydrocarbon chains is decreased. This general DSC behavior of the PC film allowed us to define the temperature window in which the DETA experiments are carried out.

In DETA experiments, we represent the results in terms of overall observed “conductivity” within the film as a function of temperature. In this context, we are using qualitatively the property of bulk “conductivity” to follow the major phase changes that affect viscosity of the medium and thus the ease movement or transport within the film. Figure 3 shows the DETA results of PC-only. This figure shows that the overall observed conductivity within PC exhibits two peaks, the first just below 0 °C (probably the melting of frozen unbound or free water), and the second around 25 °C. This supports the DSC results in terms of the temperature where the major phase transition occurs, i.e. around 25 °C, Fig. 3. DETA of 40/60 (mol%) cholesterol PC films shows that the addition of cholesterol causes the major DETA peak to shift in a reproducible manner to 31 °C, Fig. 4. This reproducible shift in dielectric properties as a result of addition of cholesterol to PC films is in line with the DSC findings in terms of the changes in the gel-to-liquid crystalline-like phase transition. The dielectric changes observed in PC films in the presence of 40 mol% cholesterol represent variations in dipole orientation not mainly due to ionic transport. This interpretation is based on the quantitative observation in cyclic voltammetry (*vide infra*), which indicates that the measured current is at a minimum or viscosity at a maximum at the temperature cited. In fact, the DETA observed “conductivity” is at maximum, which is counter to the minimum electrochemical current observed in cyclic voltammetry. On the other hand, the “ionic conductivity” in

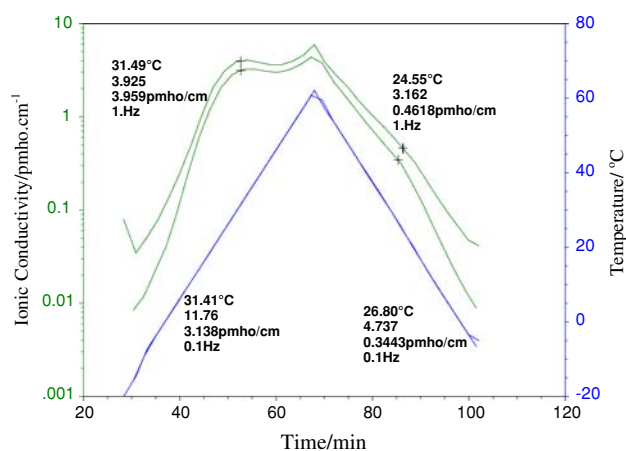


Fig. 4 Dielectric thermal analysis (DETA) of 40/60 mol% cholesterol PC film

DETA corresponds to both the energy to align dipoles as well as to move ions; the observed transition is therefore due primarily to dipole variations not ionic mobility.

The electrochemical current response of inducible nitric oxide synthase oxygenase as a redox protein in PC films at different cholesterol levels was used to monitor the changes in film rigidity as a function of temperature. We compared the behavior of 60/40 PC/cholesterol films to PC-only films as controls. In these electrochemical measurements, we used square wave voltammetry and focused on the FeIII/FeII redox couple of the embedded protein as a current reporter [16, 17]. In general, we note that the presence of cholesterol in the film is accompanied by a significant decrease in the overall redox current measured. The current temperature curves show two distinct regimes separated by a region with minimum current in the temperature range of 20–30 °C, Fig. 5. This temperature transition represents a major phase transition in the bilayered film. The regime transition detected by our electrochemical method is consistent with the findings of DSC and DETA of this study, and is in line with the known phase transition of PC lipids in terms of the temperatures at which a major transition between the gel-like phase to the liquid-crystalline lamellar phase occurs [18].

The first regime where the redox current decreases as a function of temperature is rationalized in terms of the gradual temperature-driven disruption of organized structures within the film that support electron transfer. After the minimum, the second regime where the redox current increases as a function of temperature is the result of both increased electron transfer kinetics and structural changes within the film due to increased fluidity in the initially liquid-crystalline film medium. While the two-regime behavior with the current minimum is seen for both PC-only and PC/Cholesterol films, the overall current response of embedded NOS oxygenase in the 60/40 mol% PC/

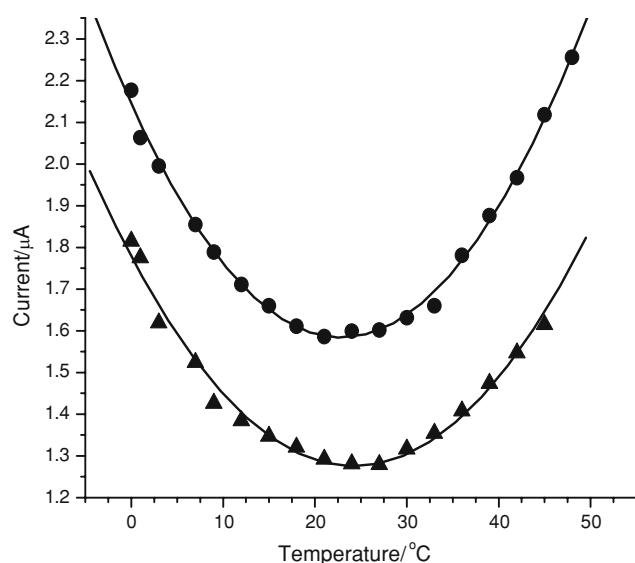


Fig. 5 Current response as a function of temperature for PC only (filled circle) and for the 60/40 mol% PC/cholesterol film (filled triangle); solid curves are second order polynomials fitting curves modeling the two regime behavior of the current response of the redox probe in the film

cholesterol film, as we noted earlier, is ca. 20% lower than in PC-only films. In addition, the observed current minimum in the cholesterol-containing film is slightly shifted towards higher temperatures in the presence of added cholesterol as determined with nonlinear second order fitting of the two-regime current response. Another aspect that is worth monitoring in terms of the second-order modeling procedure of the current behavior is the relative broadness of the parabolic curves; while this is still preliminary, we do observe a general trend that added cholesterol results in the broadening of the parabolic current temperature curve representing the two-phase regime change. The overall decreased redox current, the slight shift of the minimum current towards higher temperatures, as well as the current temperature curve broadening observed with added cholesterol, are all consistent with findings of thermal methods and further support the hypothesis that added cholesterol contribute to the structural rigidity of the film and depresses the molecular dynamics in this microenvironment.

In conclusion, three independent analytical techniques, namely DSC, DETA, and voltammetry, used to study the effect of added cholesterol to PC films yield consistent results in terms of detection of distinct regime behaviors and ranges of observed temperature transitions. Particularly, PC films exhibit an overall two-regime behavior with a characteristic temperature transition in the 20–25 °C range. Added cholesterol in the PC films seem to depress the molecular dynamics as indicated by lowered current responses in the presence of cholesterol as well as a slight increase of the transition temperature within the film.

Future investigation of the PC/cholesterol system will further explore structural details about the effect of cholesterol in PC films on the molecular dynamics of embedded the oxygenase domain of nitric oxide synthase. We will also further explore the effect on the kinetics of electron transfer to this protein serving as a redox probe in the bilayered PC/cholesterol microenvironment.

References

1. American Heart Association. Heart disease and stroke statistics 2008 update (2008).
2. Tabas I. Consequences of cellular cholesterol accumulation: basic concepts and physiological implications. *J Clin Invest.* 2002;110:905–11.
3. Chauhan NB. Membrane dynamics, cholesterol homeostasis, and Alzheimer's disease. *J Lipid Res.* 2003;44:2019–29.
4. de Lange MJL, Bonn M, Müller M. Direct measurement of phase coexistence in DPPC/cholesterol vesicles using Raman spectroscopy. *Chem Phys Lipids* 2007;146:76–84.
5. Kakorin S, Brinkmann U, Neumann E. Cholesterol reduces membrane electroporation and electric deformation of small bilayer vesicles. *Biophys Chem.* 2005;117:155–71.
6. Nasseri B. Effect of cholesterol and temperature on the elastic properties of niosomal membranes. *Int J Pharm.* 2005;300:95–101.
7. Pande AH, Qin S, Tatulian SA. Membrane fluidity is a key modulator of membrane binding, insertion, and activity of 5 lipooxygenase. *Biophys J.* 2005;88:4084–94.
8. Song J, Waugh RE. Bending rigidity of SOPC membranes containing cholesterol. *Biophys J.* 1993;64:1967–70.
9. Gondre Lewis MC, Petrache HI, Wassif CA, Harries D, Parsegian A, Porter FD, et al. Abnormal sterols in cholesterol deficiency diseases cause secretory granule malformation and decreased membrane curvature. *J Cell Sci.* 2006;119:1876–85.
10. van Osdol WW, Johnson ML, Ye Q, Biltonen RL. Relaxation dynamics of the gel to liquid crystalline transition of phosphatidylcholine bilayers. Effects of chainlength and vesicle size. *Biophys J.* 1991;59:775–85.
11. Hino T, Ishimoto H, Shimabayashi S. Thermal gelation of aqueous curdlan suspension: preparation of curdlan jelly. *J Pharm Pharmacol.* 2003;55:435–41.
12. Sakai Y, Kuroki S, Satoh M. Water properties in the super salt resistive gel probed by NMR and DSC. *Langmuir.* 2008;24:6981–7.
13. Takei T, Kurosaki K, Nishimoto Y, Sugitani Y. Behavior of bound water in polyethylene oxide studied by DSC and high frequency spectroscopy. *Anal Sci.* 2002;18:681–4.
14. Halling KK, Slotte JP. Membrane properties of plant sterols in phospholipid bilayers as determined by differential scanning calorimetry, resonance energy transfer and detergent induced solubilization. *Biochim Biophys Acta.* 2004;1664:161–71.
15. Pentak D, Sułkowski WW, Sułkowska A. Calorimetric and EPR studies of the thermotropic phase behavior of phospholipid membranes. *J Therm Anal Calorim.* 2008;93:471–7.
16. Bard AJ, Faulkner LR. *Electrochemical methods: fundamentals and applications*, 2nd ed. New York: Wiley; 2001. p. 293.
17. Bayachou M, Boutros JA. Direct electron transfer to the oxygenase domain of neuronal nitric oxide synthase (NOS): exploring unique redox properties of NOS enzymes. *J Am Chem Soc.* 2004;126:12722–3.
18. Small DM. The physical chemistry of lipids. In: Hanahan DJ, editors. *Handbook of lipid research*, vol. 4. New York: Plenum Press; 1986.