Degradation of bacterial DNA by a natural antimicrobial agent with the help of biomimetic membrane system

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The antimicrobial efficacy of methylglyoxal (MG) against several gram-negative bacteria including *Escherichia coli* has been reported. To determine the mechanism of action of MG, molecular interactions between lipid and MG within the liposomal membrane were also investigated. Multilamellar and unilamellar vesicles were prepared from 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). The effect of MG on DPPC liposomal membrane was studied by fluorescence spectroscopy and differential scanning calorimetry. The results indicate that MG interacts mainly with the DPPC head group that produces a significant increase in the fluidity of liposomal vesicles, which could be the cause of a fusion/aggregation effect in microbial cells. The agarose gel electrophoresis study with the genomic DNA extracted from *E. coli* ATCC 25922 revealed that addition of MG could completely degrade this DNA within 1 h, pointing out to their distinctly high degree of sensitivity towards MG.

Further, the drug was able to cross the cell membranes, penetrating into the interior of the cell and interacting with DNA for demonstrating antibacterial activity of MG.

**Keywords:** Agarose gel electrophoresis, Antibacterial activity, Fluorescence anisotropy, Liposomal membrane, van’t Hoff enthalpy change

Methylglyoxal (MG), an ingredient of New Zealand’s Manuka honey is a dicarbonyl compound containing two carbonyl groups. It is both an aldehyde and a ketone1. In 1967 Szent-Gyorgi et al.2 first observed the anticancer activity of MG, while the distinct curative property of this agent in mice, experimentally infected with cancer was reported soon after1. Subsequent studies have shown tumoricidal action of MG in malignant cells and further augmentation of this action in presence of ascorbic acid and creatinine4. Potentiality of MG as an antibacterial compound has been reported. MG was found to be a competent inhibitor of polyamine biosynthetic pathway5 and could induce lanthanum sensitive Ca++ transients for bacterial growth inhibition6, while the acute bactericidal activity of this agent resulted in death of viable multiplying bacteria within an hour7.

The severe and prompt action of MG is likely to be associated with smooth, secure and safe entry of this compound into the cell through the lipid membrane of bacteria followed by complete degradation of genomic DNA.

Since MG is hydrophilic in nature it may preferentially partition with the aqueous phase of bacterial membrane8. The present study describes the change in physical properties of phospholipids in bilayer that could be obtained by incorporating MG for dispersion of liposomes along with its effects on total disruption of bacterial DNA in a very short span of time.

**Materials and Methods**

Reagents—Spectral grade solvents (chloroform, acetone, methanol, ethanol and N,N'-dimethylformamide), sucrose, KCl, NaOH, NaCl, Na2HPO4, KH2PO4 were purchased from Merck (India) while 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was obtained from Avanti Polar Lipids (Alabaster, AL). The phospholipid

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yielded a single spot by thin-layer chromatography on silica-gel plate (E. Merck) using chloroform/methanol/7 M ammonia (230:90:15, by volume). Stock solution of DPPC was stored at -20 °C. 1,6-diphenyl-1,3,5-hexatriene (DPH), ethidium bromide and agarose were purchased from Sigma Chemical (St. Louis, MO). Stock solution of DPH was prepared in N, N′-dimethylformamide and stored in the dark at 4 °C. Tryptone soy broth and yeast extract were procured from Difco, USA and DNAzol reagent was purchased from Invitrogen, USA; a 40% MG was purchased from M.P. Biochemicals, USA. All the reagents and chemicals were used without further purification. Deoxygenated Milli Q water was used throughout the experimentation.

Preparation of drug incorporated DPPC vesicle—Liposomes with and without MG were obtained according to the following protocol. The phospholipid was dissolved in 3:1 chloroform:methanol solution. Afterwards a thin lipid film was obtained by evaporating the solvent in the atmosphere of dry nitrogen. Residues of solvents were removed in vacuum (the vacuum was maintained for several hours to remove trace amounts of any solvent). Liposomes were obtained by adding 1X phosphate buffer saline (pH = 7.4) to the film and then sonicated using a bath sonicator (Imeco Ultrasonicator UP 250F) for 1 h at a temperature above that of the phase transition temperature of DPPC. The samples were stored at 4 °C for about 16-18 h to assure complete hydration. MG solution was added to the dry DPPC film prior to sonication. Liposome suspensions were filtered repeatedly five times through 0.45 µM pore polycarbonate filter (Millipore, Ireland). Particle size of the filtered suspension was then analyzed using the method of dynamic light scattering with Zeta-sizer-5000 (Malvern Instruments, UK). The mean values of lipid size were ~88 nm for Small Unilamellar Vesicle (SUV) and ~250 nm for Multilamellar Vesicle (MLV). The suspension was stored at 2-4 °C and was taken for investigation within 24 h.

Determination of phospholipid concentration—The phospholipid concentration was measured as inorganic phosphate by a standard protocol. At least ten phosphate determinations were made on each sample with a resulting standard deviation of ± 1 % for small vesicle and ± 2.5% for large vesicle dispersions. Phosphatidylcholine concentrations in dispersions were examined spectrophotometrically at ~15 mM for calorimetric estimation and at ~1 mM for fluorometric estimation.

Relative lipophilicity determination—The relative lipophilicity (Rm) of the drug under investigation was measured by reversed phase high performance thin layer chromatography. Briefly, Whatman KC 18F plate was used as the non-polar stationary phase. The plate was dried at 105 °C for 1 hr before use. The mobile phase was a 2:1 (v/v) mixture of acetone and water. The drug was dissolved in absolute ethanol (50 mg/mL), and 2 µL of solution was applied to the plate. The experiment was repeated four times. The retardation factor (Rf) was expressed as the means of the four determinations. The Rm value was calculated from the experimental Rf value according to the formula Rm= log [(1/Rf) -1]. Lower Rm value indicated hydrophilicity of the drug.

Steady-state fluorescence anisotropy measurement—Liposome solutions with the addition of MG at 10-30 mol% ratios to DPPC were analyzed and compared with liposomes without MG. When the dispersion was prepared for fluorescence studies, 50 mM KCI in 15% (w/v) sucrose was used as the aqueous phase. This was done to prevent settling of the liposomes in the fluorescent cuvette during the course of an experiment. Labeling of the vesicles was achieved by adding a measured amount of DPH to the lipid solution, in a molar ratio yielding the desired final lipid/probe ratio of 1:10, such that DPH is directly incorporated into the membrane. The samples were kept at room temperature. Fluorescence anisotropy was determined as a function of temperature, the sample in the cuvette was heated up to 50 °C. Fluorescence intensity and temperature measurement were made continuously during heating. Perkin-Elmer luminescence spectrometer (LS 50B) was used to measure the steady-state fluorescence anisotropy,

\[ \gamma = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} \]  

where I_\parallel and I_\perp are the vertical and horizontal components of 428 nm emission band of DPH in the liposomes, while the sample was being excited at 360 nm.

Differential scanning calorimetric (DSC) study—The thermotropic phase behaviour of the lipid/MG samples was studied using a high-resolution differential scanning calorimeter (Microcal,
Northampton, MA, USA). A nominal heating rate of 30 °C/h was used for all samples and, for each sample, the reference cell of the calorimeter was filled with buffer solution containing the same MG concentration as that of the lipid/MG sample. In general each lipid/MG sample was run successively four times over a temperature range of 30–50 °C using the up scan mode. In this mode DSC measured continuously the excess heat capacity as a function of ascending temperature. The four DSC heating curves recorded for each lipid/MG sample were analyzed using the software provided by Microcal. Specifically the main phase transition temperature (Tm) exhibited by the lipid/MG sample was taken as the temperature at which the excess heat capacity reached a maximum in the DSC heating curve. In these DSC experiments, the values of Tm determined from the DSC heating curves were virtually identical, with a difference among them being well within ±0.05 °C. The Tm value determined from the second DSC heating scan was thus taken uniformly in this communication as the experimental Tm value.

Calculation of van’t Hoff enthalpy change—To calculate the van’t Hoff enthalpy change associated with gel to liquid crystalline phase transition in the liposomal membrane of DPPC and to study the effect of MG on the enthalpy change the method of Marky and Breslauer was employed. Let α be the fraction of lipids in the liquid crystalline phase. Since a fractional change in α denotes the extent of phase change, any anisotropy temperature curve can be converted to the corresponding α-temperature curve. By drawing an upper and a lower baseline to the anisotropy-temperature curve (Fig. 1) α is evaluated as the ratio of the distance between the curve and upper baseline to the distance between upper and lower baselines. The phase transition temperature (Tm) corresponded to the temperature for which the value of α = 0.5 (for drug free liposomal membrane of DPPC). In a similar way, α could be calculated for all liposomal systems incorporated with drugs (different mol %). The van’t Hoff enthalpy change (ΔH) was obtained from the relation:

\[ \Delta H = RT_m^2 \left[ \frac{d\ln K}{dT} \right]_{T=T_m} \]  

where ‘R’ is the gas constant and ‘K’ is the equilibrium constant.

For complete evaluation of ΔH, K must be expressed in terms of α. Assuming that each lipid molecule can exist either in the gel state or in liquid crystalline state, the transition would proceed in a two-state manner. The value of the equilibrium constant depends on the molecularity of the transition, n, which in this case was equal to unity. In terms of α, ΔH at the phase transition temperature, Tm, can be written as:

\[ \Delta H_{T_m} = RT_m^2 \left( 2 + 2n \right) \left[ \frac{d\alpha}{dT} \right]_{T=T_m} \]  

For n=1, this equation is reduced to

\[ \Delta H_{T_m} = 4RT_m^2 \left[ \frac{d\alpha}{dT} \right]_{T=T_m} \]  

With reference to Fig.1, the van’t Hoff transition enthalpy was calculated in all the liposomal systems (Table 1).

Preparation of genomic DNA and method of agarose gel electrophoresis—Escherichia coli ATCC 25922 was grown in liquid medium containing 1% tryptone soy broth (Difco), 0.5% yeast extract (Difco), 0.5% analar NaCl, pH 7.2-7.4 for 18 h. The growth was centrifuged and lysed with the help of 1 mL of DNAzol solution. Genomic DNA was precipitated from the lysate by adding 75% ethanol. The precipitate was washed twice in ethanol; DNA was solubilized in 8 mM NaOH, stained with 1 µL of ethidium bromide (10 mg/mL) for visualizing the DNA bands. This solution was subsequently electrophoresed in BIO-RAD documentation system for 2 h using 0.8% agarose gel.

Results and Discussion

It is known that when lipid bilayers prepared from pure one-component phospholipids are subjected to heating under the ambient pressure condition, these lipid bilayers often undergo multiple thermotropic phase transitions. These transitions may be detected by a wide variety of physical techniques such as differential scanning calorimetry (DSC), X-ray

<table>
<thead>
<tr>
<th>Drug in liposomal membrane (mol %)</th>
<th>Phase transition temperature (Tm in °C)</th>
<th>van’t Hoff enthalpy change (kcal mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>40.5</td>
<td>137.66</td>
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<tr>
<td>10</td>
<td>39.8</td>
<td>128.68</td>
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<tr>
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<td>113.35</td>
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diffraction, neutron diffraction, NMR and fluorescence spectroscopies. Although each of the physical techniques can provide specific information, much of the information concerning the thermodynamic changes accompanying the lipid phase transition in this study were taken from fluorescence and DSC techniques.

The rod like molecule 1,6-diphenyl-1,3,5-hexatriene (DPH) is one of the most popular fluorescence anisotropy probes for bilayer membranes and is located deep within the hydrophobic core region of the phospholipid bilayer\textsuperscript{13,18,19}. Thus any modification in the movement of the chains or packing is sensitively reflected in the fluorescence anisotropy of DPH. Fluorescence anisotropy of DPH was found to exhibit a potential response to the phase transition in phospholipid vesicles. The anisotropy produced a sudden drop in temperature at ~41 °C, which was the phase transition temperature of DPPC. Hence, it was chosen to monitor the effect of MG on DPPC vesicles.

The figure 1 exemplifies the character of changes induced by addition of cholesterol and then MG to DPPC. Here the anisotropy versus temperature curve of DPH-probed DPPC liposomes could be plotted and monitored to determine the change due to the incorporation of MG in the liposomal membrane. As it is known that cholesterol molecules enter into the hydrophobic core of the organized lipid assembly and disrupt the interactions between the hydrocarbon chains indicating an intermediate fluid condition, i.e., no detectable sharp transition. However, it is evident from Fig.1 that the drug could induce a decrease in phase transition temperature ($T_m$). Moreover, with the addition of the drug, the value of anisotropy decreased throughout the temperature range indicating an overall fluidizing effect of the drug which further increased with the increase in drug concentration. This $T_m$ value was found to decreases as the MG concentration (in mol %), in the aqueous dispersion was increased. Therefore, the drug could successfully adopt a well-defined position in the bilayer membrane. In particular, the drug was located near the water-lipid interface (due to its hydrophilicity as revealed in RP-HPTLC), interacting with the dipoles of the head group region according to a nonspecific, electrostatic mechanism and inducing a reorientation of the phosphocholine dipoles towards the water phase.

The scanned figure shows that the addition of MG caused gradual decrease in the phase transition temperature from about 41.0 °C to below 40 °C with the increase in MG concentration from 0-30 mol % relative to DPPC. The gel-to-liquid crystalline phase transition temperature ($T_m$) was determined from the endothermic peaks (Fig. 2). The highest value of these scans corresponded to the $L_{\beta}-L_{\alpha}$ phase transition temperature of DPPC in lipid bilayers. The phase

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**Fig.1**—Temperature dependent fluorescence anisotropy values (data are the average of 3 separate measurements) for liposomal systems of dipalmitoyl phosphatidylcholine (DPPC) and the drug MG. Inset: Variation of $\alpha$ with temperature in dispersions: control, with 10, 20 and 30 mol % MG

**Fig.2**—The effect of methylglyoxal (MG) concentration on phase transition behaviour of aqueous dispersion of DPPC. The representative DSC curves are the second DSC heating curves, and MG and $T_m$ values are indicated on the right side of each corresponding DSC curve.
transition in this case was related to a cooperative increase in the trans-gauche isomerization of acyl chains of lipid molecules. This resulted in a lipid membrane fluidization. Lowering of phase transition temperature, as observed in Figs 1 and 2, was possibly due to incorporation of MG in the polar region of lipid bilayer. Due to this property, it can incorporate into the lipid membrane hitching with its polar sides at the lipid polar heads, disturbing their acyl chains interactions and, consequently, decreasing the phase transition temperature and van’t Hoff enthalpy change of model membrane (Table 1).

The genomic DNA of E.coli ATCC 25922 was electrophoresed in agarose gel for 2 h followed by treatment with ethidium bromide and observed in BIO-RAD documentation system. The DNA procured from the culture that was not treated with MG or any other agent produced distinct fluorescent band. The intensity of fluorescence was much less in DNA that was extracted from the culture treated with MG for 30 min. Treatment of the culture with MG for 60 min failed to show presence of any distinct band. On the contrary there was a definite haziness in this lane that was visible at a position a little higher than the position of other bands (Fig. 3).

The results of fluorescence anisotropy and DSC measurements suggest a real transfer of MG through lipid bilayers; thus, the drug might be able to permeate through cellular membrane and interact with intracellular components. These observations may help to explain strong antimicrobial activity of MG.

Thus the initial observation in this study on degradation of bacterial DNA by MG indirectly indicated the presence of an unexplored activity of this compound on cell membrane. The search for a possible explanation to this action of MG led to speculations that the rapid action of MG on the DNA may be attributed to perturbation of the cell membrane. MG being a known metabolic intermediate of glycolysis pathway may have a significant action on membrane fluidity. Such a property can allow MG to enter the cell and potentiate its action as a very powerful bactericidal agent by destroying the machinery of DNA synthesis leading to accelerated cell death. The present study indicates that the action of MG on cell membrane is the key to its effectivity on DNA degradation in bacterial and even cancer cells.

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**References**


