INTERACTION OF METHOTREXATE
WITH BLM
8. Interaction of Methotrexate with BLM

Methotrexate influences both D.C and A.C electrical characteristics of the BLM.

8.1 Effect of methotrexate on D.C parameters

8.1.1 Effect of methotrexate on membrane current

Methotrexate increased the membrane current when added in micromolar quantities to aqueous NaCl bath media at a constant applied D.C potential. The increase becomes steeper above drug concentrations of 400 μM until the membrane ruptures. The lytic concentration varied from 900 – 1400 μM depending on the concentration of the bath medium. Table 8.1 summarizes the lytic concentrations of methotrexate in different concentrations of NaCl bath media. Similar increases in membrane current were observed in NaBr and MgCl₂ bath media also. Fig 8.1 shows the current-drug concentration profile of the BLM in 0.01 M NaCl bath medium at 40 mV potential.

<table>
<thead>
<tr>
<th>[NaCl]₀ M</th>
<th>Lytic dose of methotrexate (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>1400</td>
</tr>
<tr>
<td>0.10</td>
<td>1200</td>
</tr>
<tr>
<td>1.00</td>
<td>900</td>
</tr>
</tbody>
</table>

Applied D.C voltage : 40 mV.

Table 8.1: Lytic doses of methotrexate in different concentrations of NaCl bath medium.
Membrane Current (pA)
300
0 200 400 600 800 1000 1200 1400
Methotrexate Concentration (micromolar)

Bath medium : 0.01 M NaCl
Applied D.C voltage : 40 mV
* The membrane ruptured at a methotrexate dose of 1400 μM.

Fig.8.1: Dose dependent change in membrane current caused by methotrexate.
The increase in membrane current is obviously due to the passage of some charged species from one compartment to the other through the bilayer. These charged species are more likely to be the ions present in the medium as the charge density of methotrexate is too small to account for the 10-20 fold increase in membrane current. Also, the bulky size of methotrexate restricts its movement across the bilayer. However, entry of methotrexate into the bilayer cannot be completely ruled out. Methotrexate is added as the anion methotrexate sodium to the bath medium. Being bulky and lipophobic, it is unable to cross the highly structured and compact hydrocarbon barrier of the BLM. Therefore, it gets accumulated at the membrane surface. Here, it competes with water for binding to the membrane surface. It is now well known that the lipid-water interaction at the membrane surface is essential for maintaining the structural integrity of the membrane bilayer\textsuperscript{10}. The accumulation of methotrexate at the membrane surface breaks these hydrogen bonds and releases the electrostricted water. As a result, the rigidity of the bilayer structure is reduced and the fatty acid chains become more fluid. This fluidity induces small defects in the compact bilayer architecture. These defects act as conducting pathways between the two aqueous compartments resulting in the observed increase in the membrane current. As more methotrexate was added, more defects are created randomly on the BLM, resulting in further increase in the ion flux. This results in a local disordering effect in the hydrocarbon chains known as ‘fluidization’. As the membrane progressively gets fluidized, it tries to retain its structural integrity by allowing the passage of more number of ions through the defects in its structure. This manifests in the steep increase in the membrane current. However, the increased ionic pressure caused by the ion flux coupled with the perturbations induced by methotrexate ultimately leads to
the lysis of the membrane. Such ion conducting defects have been proposed by Deamer et al., to explain the increase in membrane current in the presence of alcohols. Ueda et al., have also reported a similar interaction of interfacial anesthetics on the hydrocarbon core that is responsible for the onset of anesthesia.

Similar ion-conducting defects have been postulated to explain the marginal increase in conductance observed when cisplatin was added to the bath medium. It is observed that like cisplatin, methotrexate did not produce an enormous increase in membrane current at doses below 100 μM. This shows that the permeability of methotrexate across the lipid bilayer at low doses is restricted. This is expected because methotrexate has a low lipophilicity.

The dose dependent changes in the membrane current caused by methotrexate depend upon a number of experimental factors.

8.1.1.a Effect of bath medium concentration on methotrexate-BLM interactions

The ionic strength of the medium influences the percent increase in membrane current for identical drug loads. The current-drug concentration plots obtained in different concentrations of NaBr are shown in Fig. 8.2.

It is seen from the Fig. 8.2 that though the trends are similar irrespective of the bath medium concentration, the relative percent increase in the membrane current is dependent on the bath medium concentration as shown in Table 8.2.
Table 8.2: Effect of bath concentration on the methotrexate-induced increase in membrane current

<table>
<thead>
<tr>
<th>Methotrexate doses (µM)</th>
<th>Conductance per area (nmho/cm^2)</th>
<th>0.01 M NaBr</th>
<th>0.1 M NaBr</th>
<th>1.0 M NaBr</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>2.6198</td>
<td>9.7386</td>
<td>17.671</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>6.4490</td>
<td>19.194</td>
<td>39.389</td>
</tr>
<tr>
<td>200</td>
<td></td>
<td>11.983</td>
<td>40.509</td>
<td>107.90</td>
</tr>
<tr>
<td>300</td>
<td></td>
<td>16.613</td>
<td>72.102</td>
<td>210.32</td>
</tr>
<tr>
<td>400</td>
<td></td>
<td>20.030</td>
<td>131.29</td>
<td>370.11</td>
</tr>
<tr>
<td>500</td>
<td></td>
<td>36.561</td>
<td>197.88</td>
<td>794.40</td>
</tr>
</tbody>
</table>

It is observed that the relative percent increase of membrane current in 0.01 M, 0.1 M and 1 M NaBr bath media for identical drug loads of 500 µM at 40 mV is in the ratio $1:2:4$.  

Applied D.C voltage : 40 mV
Fig. 8.2: Effect of NaBr bath medium concentration on the dose-dependent changes caused by methotrexate on membrane current.

- **Applied D.C voltage**: 40 mV
- **Crosses**: 1.0 M NaBr bath medium
- **Circles**: 0.1 M NaBr bath medium
- **Triangles**: 0.01 M NaBr bath medium
- **Maximum drug load**: 500 μM.
The increase in membrane current is caused by the creation of defects in the membrane matrix by methotrexate. Concentrated solutions have greater number of ions, which synergistically act with the drug molecules in building up the ionic pressure on the membrane. This could result in the release of electrostricted water from the membrane surface thereby increasing the vibratory motions of the polar head groups and hydrocarbon chains of the bilayer. As a result, more kinks are created in the bilayer resulting in passage of ions. Greater the ionic pressure on the bilayer, greater is the number of defects produced. Hence the changes in membrane current produced by the addition of methotrexate are maximal in 10 M solutions when compared with 0.01 M solutions. This also explains the requirement of lower lytic concentrations of methotrexate in concentrated bath media.

8.1.1.b Effect of ionic nature on methotrexate-BLM interaction

Fig. 8.3 compares the methotrexate-induced change in the membrane current in different ionic media. From Fig. 8.3, it is seen that when the applied voltage, molarity of the bath medium and the drug loads are maintained constant, the maximum increase in membrane current is observed in NaBr medium while MgCl₂ medium exhibits the least increase. Table 8.3 gives the values of membrane current for increasing doses of methotrexate in different ionic media of same concentration.
Fig. 8.3: Effect of ionic nature on the methotrexate-induced increase in membrane current.
From Fig. 8.3, it is observed that the trend of increase in membrane current remains the same irrespective of the nature of the ions present in the medium. Though the initial values of the membrane current differ, it is seen from Table 8.3 that the relative percent increase of membrane current remains almost the same in all the three media. This implies that the mode of action of methotrexate on the membrane bilayer is the same in all these media and the nature of the ions only influences the initial value of the membrane current. Methotrexate progressively fluidizes the membrane resulting in the creation of many defects in the matrix. These defects serve as ion conducting pathways causing the increase in membrane current. However, due to differences in the charge densities of the ions in the medium, the current flowing through these defects in the BLM varies.

Table 8.3: Effect of ionic nature on the methotrexate-induced increase in membrane current

| Methotrexate doses (µM) | Conductance per area (nmho/cm²) | | | |
|-------------------------|-------------------------------|----------------|----------------|
| 0                       | 14.311                        | 10.315         | 5.8683         |
| 100                     | 23.766                        | 14.704         | 11.042         |
| 200                     | 40.509                        | 23.734         | 16.007         |
| 300                     | 67.530                        | 37.546         | 24.186         |
| 400                     | 138.15                        | 76.971         | 51.550         |
| 500                     | 195.59                        | 124.86         | 80.560         |

Applied D.C voltage : 40 mV
Bath concentration : 0.1 M
It is well known that the hydrophobic interior of the BLM will offer lesser resistance to the passage of bromide and sodium ions that have lesser charge densities than chloride and magnesium ions respectively (Chapter 5). This is on expected lines and neutral molecules and ions have been reported to possess better permeability through the BLM\textsuperscript{11-13}. The experimental findings that the ions with lesser charge densities have greater permeability through the hydrophobic interior of the BLM also support this view. Mg\textsuperscript{2+} ions face stiffer resistance from the hydrophobic core of BLM to pass through than Na\textsuperscript{+} ions and hence its contribution to the total current is reduced. Similarly: the contribution of chloride ions to the total membrane current will be slightly lesser than that of bromide or iodide ions. Hence NaBr exhibits maximum current than MgCl\textsubscript{2} both in the presence as well as in the absence of methotrexate.

\textbf{8.1.1.c Effect of the mode of drug addition on the methotrexate-BLM interaction}

Fig. 8.4 shows the trends followed by current - drug concentration profiles of the BLM at a constant voltage when the drug is added to both sides (symmetric addition) and when it is added to only one side (asymmetric addition). From the plot, it is apparent that the trend of increase in both cases is similar. This suggests that the same mechanism namely creation of ion defects on the membrane matrix as discussed for cisplatin, operates during both modes of addition.
Fig. 8.4: Effect of the modes of drug addition on the increase in membrane current caused by methotrexate.

- D.C applied voltage: 40 mV
- Bath medium: 0.1 M NaCl
- Maximum drug load: 500 μM
- Crosses: Symmetric addition
- Circles: Asymmetric addition.
The number of drug molecules present on either side of the bath medium during asymmetric addition is only half that present during symmetric addition. This means that the number of drug-induced defects in the membrane matrix will also be lesser. This explains the slightly lower current observed during asymmetric addition.

The observed increase in membrane current during asymmetric addition is nearly 85% of that observed during symmetric addition. This implies that the methotrexate molecule has the ability to penetrate the bilayer thus creating defects on either side of the BLM. This passage must be diffusion controlled since the experiments are carried out in the absence of any protein receptor or charge carrier. The creation of defects on the membrane matrix results in the nearly similar increase in membrane current irrespective of the mode of drug addition.

At the outset, it appears that the difference between the symmetric and asymmetric modes of drug addition is more pronounced in the case of cisplatin than methotrexate. But it should be noted that the maximum drug dose added in cisplatin was 100 μM whereas it is 500 μM in methotrexate. Still, on closer examination of the values at 100 μM it is seen that the difference between symmetric and asymmetric additions is slightly higher in the case of cisplatin. This implies that the defects caused by cisplatin on the membrane matrix is slightly lesser than those caused by methotrexate. This is to be expected because structurally methotrexate is bulkier than cisplatin. It is also imperative to note that the difference between the symmetric and asymmetric modes of drug addition becomes progressively lesser as the methotrexate load in the medium is increased. This observation points out that the perturbation of the bilayer by methotrexate gains significance only at higher doses.
8.1.2 Effect of methotrexate on the dielectric strength of BLM

The current-voltage relationship of the BLM under both control and drug-infested conditions is shown in Fig. 8.5.

The membrane current follows a non-ohmic pattern as the breakdown voltage is neared due to creation of breaches in the membrane matrix by the ions at the interface. This fact holds good for both the control and drug-infested membranes. However, the deviation from the ohmic behavior is more pronounced for the drug-infested membrane. This observation indicates that methotrexate enhances ion flux through the bilayer by creating defects in the membrane.
Bath medium : 0.01 M NaCl
Drug load : 600 μM.

Fig. 8.5: Current-voltage plot of BLM under control (Lower curve) and drug-infested (upper curve) conditions.
The current-voltage study of the control and drug-infested BLMs gives the breakdown voltage of the BLM. The breakdown voltage of BLM is found to decrease with increasing loads of methotrexate. The dielectric strength of the BLM is calculated from the breakdown voltage using the following relationship.

\[
\text{Dielectric strength} = \frac{\text{Breakdown voltage}}{\text{Membrane thickness}}
\]

The membrane thickness was assumed to be 70 Å from literature and the calculated dielectric strength of the BLM under various drug loads in 0.01 M NaCl bath medium are tabulated in Table 8.4.

<table>
<thead>
<tr>
<th>Methotrexate concentration (µM)</th>
<th>Average breakdown voltage (mV)</th>
<th>Dielectric strength (V/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>180</td>
<td>2.571 x 10^7</td>
</tr>
<tr>
<td>200</td>
<td>160</td>
<td>2.229 x 10^7</td>
</tr>
<tr>
<td>400</td>
<td>140</td>
<td>2.000 x 10^7</td>
</tr>
<tr>
<td>600</td>
<td>110</td>
<td>1.571 x 10^7</td>
</tr>
<tr>
<td>800</td>
<td>90</td>
<td>1.286 x 10^7</td>
</tr>
<tr>
<td>1000</td>
<td>70</td>
<td>1.000 x 10^7</td>
</tr>
</tbody>
</table>

Bath medium : 0.01 M NaCl

Table 8.4: Dielectric strength of BLM under various drug loads.

From the Table 8.4, it is observed that the dielectric strength also proportionately decreases with the breakdown voltage. The progressive reduction in dielectric strength of the BLM highlights the fluidizing nature of methotrexate. From the earlier experiments, it is clear that methotrexate creates defects on the bilayer matrix. These ion-conducting defects tend to weaken the compact bilayer architecture as the close packing of the lipid
chains are disrupted. The continuous passage of ions through these defects leads to an enhanced ionic pressure on the bilayer. The accumulation of the bulky methotrexate molecules at the interface and the release of bound water further intensify the stress on the membrane. These effects together contribute to the observed reduction in the dielectric strength.

The possibility of the bulky methotrexate anion penetrating the bilayer also could not be ruled out. When the drug concentration at the membrane-solution interface becomes high, the stress on the bilayer increases and correspondingly the number of defects on the membrane also increases. The close packing that existed between the acyl chains of the bilayer is disrupted by the ion-conducting defects and the bilayer is now in a fluidized state. This enables the penetration of the drug molecules into the already weakened bilayer. This assumption is supported by the observation that the relative percent increase in membrane current during asymmetric mode of drug addition does not differ much from that observed during symmetric addition. Earlier studies on amphipathic drugs$^{24}$ and insecticides$^{57,62}$ have established that the length of the lipophilic part of a molecule determines the extent of its penetration into the bilayer. The lipophilic part will have greater affinity towards the hydrocarbon interior of the BLM. The longer the lipophilic part of a molecule, the greater will be the penetration of the molecule into the bilayer. This is true for long chained surfactants and detergents$^{114}$. The structure of methotrexate shows that it has a non-polar part and hence could penetrate the BLM. However, the presence of polar groups at either end will restrict its penetration. But the accommodation of the bulky methotrexate molecule within the bilayer even for a small distance will result in the destruction of the van der Waals' lipid-lipid interactions and
consequently, the volume of the bilayer will increase. Thus the intercalation of methotrexate between the fatty acid chains will perturb the compact bilayer assembly considerably. This perturbation would fluidize the hydrocarbon chains of the BLM causing an increased ion flux. Finally, disintegration of the entire bilayer assembly occurs when it becomes impossible to withstand the ionic stress.

As more methotrexate molecules are added to the bath medium, the fluidizing effect on the BLM becomes more profound. This will prompt disintegration of the bilayer at a lesser voltage. Hence, the breakdown voltage and consequently, the dielectric strength of the BLM are reduced at higher doses of methotrexate. Similar studies on the dielectric strength of membranes were carried out by Zimmermann et al., to ascertain the extent of interaction of benzyl alcohol on lipid bilayers.

Comparison of the dielectric strength of the BLM in the presence of identical loads of cisplatin and methotrexate (100 μM) shows that the decrease in the dielectric strength is more in the case of methotrexate. This supports the fact that methotrexate is capable of inducing more defects on the bilayer than cisplatin.

8.1.3 Effect of methotrexate on the D.C capacitance of BLM

The D.C capacitance of the BLM increases with increasing doses of methotrexate in the bath medium. The conductance of the BLM also progressively increases. These facts are manifested in the charge-discharge profile of the BLM. It is seen that the maximum voltage to which the BLM is charged decreases with increasing drug loads. Table 8.5 tabulates the charging time and acquired voltage of the control and drug-infested BLMs in 0.01 M NaCl bath medium.
<table>
<thead>
<tr>
<th>Methotrexate dose (μM)</th>
<th>0.01 M NaCl</th>
<th>0.1 M NaCl</th>
<th>1.0 M NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$ (mV)</td>
<td>$T$ (sec)</td>
<td>$C_m$/area (μF/cm²)</td>
</tr>
<tr>
<td>0</td>
<td>31.34</td>
<td>32.0</td>
<td>0.1126</td>
</tr>
<tr>
<td>100</td>
<td>27.24</td>
<td>18.0</td>
<td>0.1211</td>
</tr>
<tr>
<td>200</td>
<td>24.80</td>
<td>17.0</td>
<td>0.1243</td>
</tr>
<tr>
<td>300</td>
<td>23.98</td>
<td>16.0</td>
<td>0.1295</td>
</tr>
<tr>
<td>400</td>
<td>22.20</td>
<td>12.0</td>
<td>0.1324</td>
</tr>
<tr>
<td>500</td>
<td>21.03</td>
<td>10.5</td>
<td>0.1285</td>
</tr>
</tbody>
</table>

- $V_{\text{max}}$ is the maximum voltage to which the BLM is charged
- $T$ is the charging time
- $C_m$ is the membrane capacitance

Charging voltage: 40 mV

### Table 8.5: Effect of methotrexate doses on the charge-discharge characteristics of BLM in different bath concentrations

The discharge of the accumulated charge becomes faster with increasing drug doses. This confirms the existence of conducting pathways in the membrane matrix under drug-infested conditions. Fig. 8.6 depicts a typical charge-discharge profile of the BLM under control and drug-infested conditions.
Fig. 8.6: Charge-discharge profiles of control and methotrexate-infested BLMs.

* Solid lines denote control membranes and dotted lines indicate methotrexate-infested BLMs.

Charging voltage: 40 mV
Circles: 0.01 M NaCl
Dots: 0.1 M NaCl
Crosses: 1 M NaCl
The increase in membrane capacitance shows that the initial site of interaction of the drug is the membrane surface. The methotrexate molecule interacts with the polar head groups of the BLM and contributes to the total capacitance of the BLM by forming a small capacitor at the membrane-solution interface. This type of interaction is similar to that observed with cisplatin.

However, it is seen from Table 8.5 that at higher methotrexate doses (above 400 μM), the membrane capacitance shows a slight decrease. This implies that the methotrexate molecules at the membrane-solution interface start penetrating into the interior of the bilayer through the defects in the membrane matrix. This results in disruption of the 'mini' capacitors at the interface and hence the reduction in membrane capacitance. A similar pattern is also followed in the A.C measurements discussed in the forthcoming section.

The bath medium concentration affects the absolute values of membrane capacitance and conductance, but the pattern remains the same in all media. The difference in the absolute values of membrane capacitance is due to the presence of larger number of ions at the membrane-solution interface in concentrated solutions and hence the greater increase in membrane capacitance. The relative percent increase in membrane capacitance in 0.01 M, 0.1 M and 1.0 M NaCl bath media for identical drug loads of 400 μM is in the ratio 1 : 5 : 10.
8.2 Effect of methotrexate on A.C parameters

8.2.1 Effect of methotrexate on membrane capacitance

The A.C capacitance of the BLM at a constant voltage and frequency increases on addition of methotrexate and decreases slightly above doses of 400 μM similar to the observations made using the D.C method. The values are tabulated in Table 8.6.

<table>
<thead>
<tr>
<th>Methotrexate dose (μM)</th>
<th>A.C capacitance per area (μF/cm²)</th>
<th>Phase difference (°)</th>
<th>Dissipation factor (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.1242</td>
<td>-88.08</td>
<td>0.0335</td>
</tr>
<tr>
<td>100</td>
<td>0.1332</td>
<td>-88.05</td>
<td>0.0340</td>
</tr>
<tr>
<td>200</td>
<td>0.1425</td>
<td>-88.03</td>
<td>0.0344</td>
</tr>
<tr>
<td>300</td>
<td>0.1471</td>
<td>-88.02</td>
<td>0.0346</td>
</tr>
<tr>
<td>400</td>
<td>0.1461</td>
<td>-87.95</td>
<td>0.0358</td>
</tr>
<tr>
<td>500</td>
<td>0.1415</td>
<td>-87.84</td>
<td>0.0377</td>
</tr>
</tbody>
</table>

Applied R.M.S voltage = 40 mV  
Frequency = 40 Hz  
Bath medium = 0.01 M NaCl

Table 8.6: Effect of methotrexate on A.C capacitance of BLM

The variation of membrane capacitance with methotrexate doses is shown in Fig. 8.7. The increase in capacitance did not show much difference due to the mode of drug addition. This is consistent with the earlier observations on drug-induced changes in membrane current.

The polar groups at either end of methotrexate interact electrostatically with the zwitterionic polar head groups of the amphipathic phospholipids constituting the BLM and the non-polar middle part of methotrexate acts as a dielectric. This forms a ‘mini’ capacitor and contributes to the observed increase in the capacitance.

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The disengagement of the electrostricted water from the interface due to the influence of the added drug will increase the fluidity of the membrane. The creation of ion defects and fluidization of the BLM by methotrexate results in the formation of a leaky capacitor with an increased area. This is manifested in the A.C measurements by a slight reduction in the phase difference and a corresponding increase in the dissipation factor as shown in Table 8.6.
Fig. 8.7: Variation of membrane capacitance with increasing methotrexate doses.

Applied R.M.S voltage: 40 mV
Frequency: 40 Hz
Bath medium: 0.01 M NaCl
From the D.C studies, it is already seen that the perturbation of the bilayer by methotrexate becomes more pronounced at higher doses. After the initial interaction with the active sites at the membrane surface, the methotrexate molecules start perturbing the BLM, disrupting the electrical double layer at the membrane-solution interface. This perturbation is more pronounced at higher doses, which results in the slight dip in capacitance. As the drug molecules are capable of penetrating the bilayer, this type of interaction rules out any difference in the capacitance values due to symmetric or asymmetric modes of methotrexate addition.

Though a surface interaction with the polar head groups at the membrane surface is postulated for methotrexate, the extent of interaction when compared with that of cisplatin is small. This is manifested in the smaller increment in membrane capacitance (about 30%) even when 500 μM of methotrexate is added to the bath medium. This aspect distinguishes methotrexate from cisplatin. The dip in capacitance at higher doses indicates that the membrane perturbing action of methotrexate is greater than cisplatin. This dip in capacitance is not observed in cisplatin, which is indicative of its continued surface interaction.

8.2.2 Effect of methotrexate on the frequency dependent dispersion of BLM

Fig. 8.8 depicts the frequency dependent dispersion curves of the control and drug-infested membranes at constant R.M.S voltage in 0.01 M NaCl bath medium and Fig. 8.9 shows the same in 0.1 M NaCl bath medium.
Log Capacitance (pF)

1.5 2.5 3.5 4.5 5.5

Log Frequency (Hz)

Applied R.M.S voltage : 40 mV
Bath medium : 0.01 M NaCl
Drug load : 500 pM.

Fig.8.8: Frequency dependent dispersion of BLM under control and drug infested conditions.
Fig. 8.9: Frequency dependent dispersion of BLM under control and drug infested conditions.
All the curves show a Maxwell-Wagner dispersion pattern indicating that the same dielectric loss mechanisms dominate the dispersion behavior of BLM under both control and drug-infested conditions. This dispersion of behavior is similar to that of cisplatin discussed in Chapter 7.

From Fig.8.8, it is apparent that the initial differences in capacitance between the control and drug-infested membranes gradually taper and converge above 120 KHz in 0.01 M NaCl. The convergence of the capacitance curves implies the absence of any charged species at the membrane surface under both control and drug-infested conditions. It also indicates the relieving of all surface barriers at higher frequencies. At higher frequencies, the ions and drug molecules are nearly immobilized at the membrane surface. Therefore, the capacitance values of the drug-infested membrane become identical to that of the control membrane. In the case of cisplatin, the convergence of the curves for control and drug-infested BLMs was not observed in 0.01 M NaCl (Fig. 7.3), which implies surface interaction by the charged form of cisplatin even up to 200 KHz.

In 0.1 M NaCl bath medium, the curves for the control and drug-infested membranes converge above 60 KHz similar to that observed with cisplatin.

8.2.3 Effect of methotrexate on BLM impedance

The impedance of the BLM at a constant applied R.M.S voltage and frequency decreases progressively with increasing doses of methotrexate in the medium. Consequently the admittance of the BLM increases. The same trend is maintained in all bath media irrespective of the bath medium concentration. The results are tabulated in Table 8.7.
<table>
<thead>
<tr>
<th>Methotrexate concentration (µM)</th>
<th>BLM impedance (MΩ)</th>
<th>BLM admittance (µΩ⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.395</td>
<td>0.4175</td>
</tr>
<tr>
<td>100</td>
<td>2.247</td>
<td>0.4450</td>
</tr>
<tr>
<td>200</td>
<td>2.002</td>
<td>0.4995</td>
</tr>
<tr>
<td>300</td>
<td>1.9225</td>
<td>0.5202</td>
</tr>
<tr>
<td>400</td>
<td>1.8628</td>
<td>0.5368</td>
</tr>
<tr>
<td>500</td>
<td>1.8032</td>
<td>0.5546</td>
</tr>
</tbody>
</table>

Bath medium: 0.01 M NaCl
Applied R.M.S voltage: 40 mV
Frequency: 40 Hz.

Table 8.7: Effect of methotrexate on BLM impedance.

The results indicate that the ion flux through the bilayer increases with increase in the methotrexate dose. It substantiates the earlier observations that methotrexate induces defects in the membrane architecture resulting in the increase in membrane admittance and a corresponding decrease in the BLM impedance.

The frequency dependence of BLM impedance under control and drug infested conditions is shown in Fig.8.10. The figure shows that the distance between the control and drug-infested membranes broadens with increasing frequency and reaches a constant value above 10 KHz. The lower impedance of the drug-infested membrane suggests an increased BLM conductance under the influence of methotrexate-induced creation of defects in the BLM. However, at higher frequencies, the drug molecules and ions are immobilized near the BLM surface. This prevents any further damage to the membrane architecture and hence the constant impedance above 10 KHz.
Fig.8.10: Frequency dependent dispersion of membrane impedance under control and drug-infested conditions.

Applied R.M.S voltage : 40 mV
Bath medium : 0.01 M NaCl
8.3 Effect of pH on methotrexate

When methotrexate was added to an acidic buffer, a precipitate was formed which redissolved in the bath medium. The membrane current started increasing gradually at the start followed by a rapid increase leading to the membrane rupture. The membrane ruptures at methotrexate doses at 50 μM whereas the lytic dose in neutral pH is 900 μM and above depending on the bath concentration. This clearly indicated that methotrexate underwent some structural change and degradation in the acidic medium. These degradation products induce instability to the membrane resulting in its breakdown.

The maximum reported stability of methotrexate is between the pH range 6.6 – 8.0. Lower pH is reported to accelerate a complex degradation of methotrexate. The degradation products of methotrexate have not been completely identified. The possible degradation products of methotrexate in acid pH could include glycine, benzoic acid, p-amino benzoic acid, pteridine derivatives, glutamic acid and their derivatives.

Above pH 8.0, methotrexate degrades to 10-methyl folic acid. The presence of acetate, phosphate, borate or bicarbonate buffers is reported to catalyze the faster degradation of methotrexate. Since most of the biological buffers contain any of these components, the degradation of methotrexate occurs in them.

As the focus of this work is to study the interaction of methotrexate with the BLM and not on its degradation products, the experiments were conducted in unbuffered bath solutions. Moreover, buffered solutions resulted in faster lysis of the membrane. As the progressive addition of methotrexate to the bath medium did not change the pH beyond
8.0. The experiments were conducted in unbuffered electrolytes as they provided stable conditions for methotrexate.

**8.4 Mechanism of action of methotrexate on BLM**

The present experiments confirm that the anticancer drug methotrexate readily interacts with the unmodified BLM. The experimental findings support the following mode of interaction of methotrexate with the bilayer. Fig. 8.11 gives the pictorial representation of the step-wise action of methotrexate on the BLM.

The drug first interacts with the polar head groups at the membrane surface. It breaks the hydrogen bonds between the lipid and the interfacial water and consequently the fluidity of the hydrocarbon chains is increased. This increases the membrane capacitance due to the associated capacitors formed at the surface and a slight increase in the area of the BLM due to the release of electrostricted water. However, the extent of surface interaction is not as pronounced as in the case of cisplatin. Methotrexate then alters the conductance of the BLM by creating ion-conducting defects. The rate of formation of these defects is dependent on the methotrexate dose. As the drug dose is increased from 100 μM, methotrexate progressively accommodates itself within the defects in the bilayer. This breaks the van der Waals' attractive forces between the lipid chains and results in more fluidization of the membrane. Ultimately, the fluidized membrane ruptures unable to withstand the ion flux and the passage of the bulky drug molecules.
Fig. 8.11: Step-wise interaction of methotrexate with BLM. Stage (a) depicts the electrical double layer formed by the ions in the medium and the polar head groups of the BLM in the absence of methotrexate. Stage (b) depicts the situation when methotrexate is added to the medium. Ion conducting defects are induced by methotrexate allowing the passage of ions. Stage (c) depicts the next step when the methotrexate molecules start penetrating the fluidized membrane. Stage (d) depicts the disintegration of the membrane caused by the combination of ionic stress and penetration of methotrexate.
8.5 Implications of methotrexate-BLM interactions on its neurotoxicity

Methotrexate has been reported to cross the blood-brain barrier poorly\textsuperscript{1,75} because of its low lipophilicity. The present experiments conducted on a purely lipid model without any specific transport systems, confirm that methotrexate could not cross the lipid bilayer at low doses, but it is capable of crossing the bilayer at sufficiently high doses. This finding is substantiated by the clinical reports which have confirmed the presence of significant quantities of methotrexate in the cerebrospinal fluid after high dose and prolonged methotrexate therapy\textsuperscript{75,80}. This implies that a reasonable quantity of methotrexate could pass through the lipid bilayer under biological conditions also.

The increase in the membrane current at low doses of methotrexate is not very high. This means that the amount of methotrexate crossing the bilayer at low doses is less. Hence, the fluidizing effect of methotrexate on the lipid bilayer will not be manifested at low doses. This explanation is supported by clinical reports of mild neurotoxicity at low doses of methotrexate\textsuperscript{131-132}.

The present study shows that methotrexate progressively penetrates the bilayer at higher doses and fluidizes it. Such perturbations could lead to disruption of normal cellular activities apart from disintegrating the bilayer assembly. Since the drug is able to completely destroy the membrane architecture, the damage caused by the drug to the nerve membrane also is expected to be permanent. This hypothesis is supported by reports on the irreversible nature of methotrexate neurotoxicity in most cases\textsuperscript{78}. A case report showing the atrophy of the central nervous system due to prolonged high dose methotrexate therapy adds credence to the present findings that methotrexate causes irrevocable damage to the nerve membranes\textsuperscript{78,133}. 

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Clinical reports of enhanced neurotoxicity in patients treated with methotrexate in combination with cranial radiotherapy\textsuperscript{131} can be explained based on the above theory. It is a well-established fact that radiation weakens the cell membrane. Methotrexate probably causes neurotoxicity by fluidizing the bilayer. The combination of methotrexate chemotherapy with radiotherapy would enable methotrexate to easily fluidize the membrane bilayer already weakened by the radiation. This could result in the enhanced neurotoxicity.

Many recent \textit{in vitro} studies have tried to relate the observed methotrexate neurotoxicity to drug-induced defects in enzyme release\textsuperscript{82,335}. Such possibilities cannot be ruled out. It is well-established that the nerve membrane has a higher lipid content compared with other biological membranes\textsuperscript{5,6,42,13,129}. Hence, the probability of interaction of methotrexate with the lipid bilayer in the nerve membrane is greater than with the proteins and receptors. The present experiments confirm that methotrexate interacts with the lipid bilayer. But damage to the bilayer assembly takes place only at significantly high doses of the drug. Since, the neurotoxic side effects of methotrexate are reported to manifest during high dose chemotherapy, it could be believed that the non-specific interactions of methotrexate on the lipid bilayer architecture are responsible for the same. The methotrexate-bilayer interactions could modify the conformation of membrane-associated protein (enzyme / receptor) leading to activation or deactivation of the enzymes associated with the membrane. Thus, the direct (disintegration of bilayer) or indirect (alteration in the membrane associated proteins) involvement of methotrexate-bilayer interactions could be an important cause for the neuronal impairment during methotrexate therapy.