Location of Chlorhexidine in DMPC Lipid Model Membranes

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Chlorhexidine (CHX, Figure 1) is a common and effective biocide, used widely in antiseptic products, especially in hand washing and oral products such as mouthwash.

CHX effectiveness as a bactericidal agent was discovered over 40 years ago by Hugo and Longworth [1], who noted that the uptake of the drug by bacteria was very quick, within seconds, and a maximal effect of the drug occurs in as little as 20 seconds.

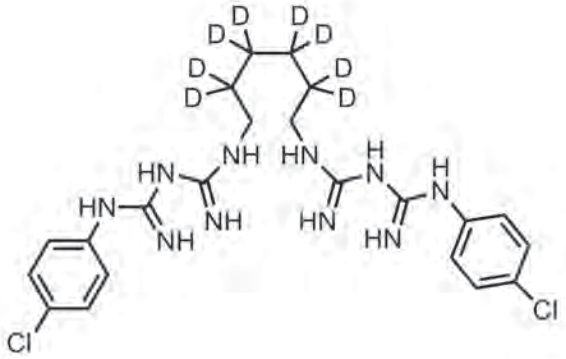

Fig 1. *Chlorhexidine Hydrochloride, deuterium-labeled.*

CHX presents an interesting biophysical modelling challenge. Structurally, CHX is of the family of N1, N5-substituted biguanides, in a bis configuration with a hexamethylene connector, and chlorophenol rings at the ends. The hexamethylene give the molecule a hydrophobic affinity, and the cationic nature of the bigaunide adds hydrophilic and ionic components. In the literature, both characteristics have been separately advanced as the modes of CHX action against membranes, though in actuality, both must play important roles. Furthermore, the aromatic rings are lipophilic, since they are now accepted to be lipid/water interface anchoring motifs, especially in proteins.

The molecular structure in various environments has, to the best of our knowledge, not been highly investigated. We have begun a series of biophysical experiments to hopefully provide some insight into the molecular basis of CHX's mode of action. In all of our experiments, we use the simple, neutral lipid 1,2-dimyristoyl-*sn*-glycero-3- phosphatidylcholine (14:0-14:0 PC, DMPC). The thickness of the DMPC bilayer is comparable to the extended length of CHX, and contrary to some assertions in the literature, we find that CHX has a high affinity for neutral lipids. The central experiment uses neutron diffraction and isotopic/isomoprhic substitution by deuterium labelling, to locate the depth of chlorhexidine's central hexamethylene chain in a DMPC bilayer with nanometre resolution.

Polarized light microscopy (Figure 2) and langmuir monolayer (Fig. 3) experiments were performed at the labs of the CNBC. The only easily identifiable liquid-crystalline phases are multilamellar vesicles (MLV) by the white circles with dark crosses, which are more numerous and larger in the 10:1 sample than the the 3:1 sample. The 3:1 samples, on the other hand, are almost entirely characterized by non-lamellar/non-ordered aggregate blobs, which are reminiscent of the damage caused to bacterial plasma membranes. There was no change in gross morphology with respect to temperature in the range of room temperature to 80°C. CHX thus has a concentration, but not temperature, dependant membrane disruption effect.

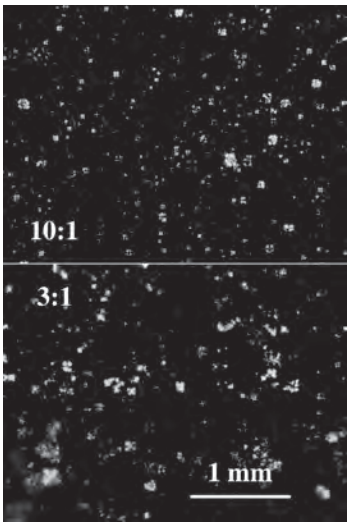

Fig 2. *Polarized light micrographs of DMPC:CHX at 10:1 (top) and 3:1 (bottom).*

Langmuir data shows the slow but dramatic increase in surface area of a DMPC film after injection of CHX digluconate into the subphase. After injection of CHX, the surface area immediately begins to increase, representing ~ 0.8 Å^2 or ~ 1.5% increase in area per lipid.

This is contrary to previous monolayer results at constant area, but agree with the general conclusions that CHX and neutral lipids have a strong binding affinity.

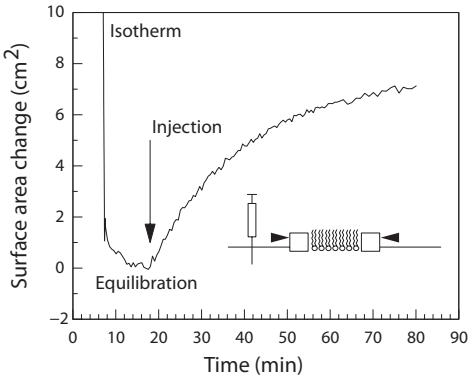

Fig 3. *The time course of the surface area of a DMPC Langmuir monolayer, adjusted to show the change in area from that at a surface pressure of 30 mN/m.*

Neutron diffraction data was recorded at the CNBC D3 beamline, using 2.37 Å wavelength neutrons from a pyrolytic graphite (PG) monochromator. A PG filter was used to eliminate higher-order reflections. During data collection, samples were kept at 36 °C, and hydrated at fixed humidity using saturated salt solutions of KNO3 (90.79 ± 0.83% relative humidity (RH)), KCl (82.95 ± 0.25% RH), $NaNO_3$ (72 ± 0.32% RH), and K_2SO_4 (96.71 ± 0.38% RH), with 70, 16, 8 and 0 mol % D_2O. Note that at 8 mol % D_2O, the contribution to the total scattering intensity from the inter-bilayer water is nullified, which means the only contribution to the scattering arises from the lipid bilayer and CHX.

Reconstruction of the bilayer profile followed the method outlined in [2]. The Fourier reconstruction method takes the integrated area of the Bragg peak intensities for each order, and corrects for neutron absorption, geometry of beam and sample widths, and the Lorentz factor. The scattering length density (SLD) profile ρ(z) is calculated from the Fourier transform of the structure factors, and placing the data on a SLD per mole basis, rather than the more common per lipid basis, is more straightforward.

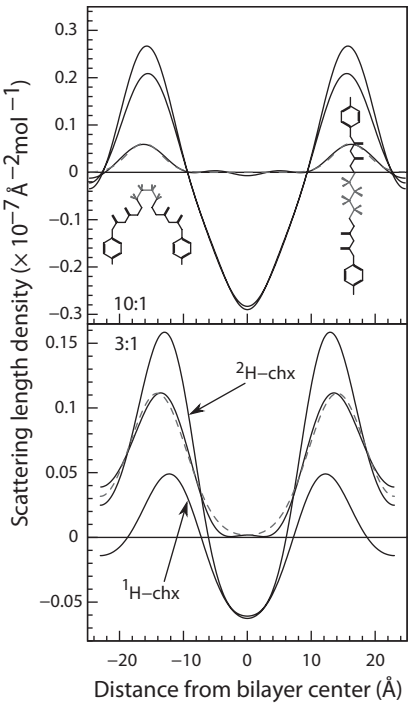

Fig 4. *SLD difference profiles between CHX labelled and unlabelled samples of (upper frame) 10 mol % and (lower frame) 3 mol % chlorhexidine in 14:0-14:0 PC. In this case the thick line is the time and sample averaged mass distribution of the deuterium of CHX shown in Fig. 1.*

The difference between labelled (L) and unlabelled (U) samples can also be calculated using the difference in the structure factors $F_h = F^L_h - F^U_h$, as long as the structure factors for the labelled and unlabelled experiments can be placed on the same relative scale. If they can, then the difference SLD profile is simply the center of mass of the isotopic/isomorphic substitution label, with all other molecular components subtracted away.

Figure 4 shows the SLD profiles of the DMPC containing (upper frame) 10 mol % and (lower frame) 3 mol % unlabelled chlorhexidine or labelled chlorhexidine. The bold curve is the difference in SLD between the two samples, while the dashed curve is a fit of a single Gaussian function to this peak. The fact that the label areas independently work out to the correct values (the total scattering length density calculated based on the chemical composition) indicates that the scaling was done correctly.

In both samples, the distribution of the center of mass of the deuterium label in chlorhexidine indicates an affinity between the drug and the glycerol backbone of the lipid. In the 10:1 sample, the center of mass of the deuterium is found to reside 16 Å from the center of the bilayer, and in the 3:1 sample, the center of mass of the deuterium is found to reside 14 Å from the center of the bilayer. In DMPC, this location is between the headgroup and the glycerol backbone, based on SANS data, where the thickness of a DMPC bilayer is 38.3 Å. Note the bilayer is proportionally more thin in the 3:1 sample, so the hexamethylene is at nearly the same location in this sample as in the 10:1 sample.

With the center of mass of the deuterated chlorhexidine lying at the junction between lipid headgroup and glycerol backbone, there are a few different structural arrangements that can be imagined. We propose that the molecule is bent on the center chain. This

would mean the molecule sits as a cleavage point in the lipid bilayer. Here, the chlorhexidine molecule bends on a central axis, providing the chance for the hydrocarbon chain to be near the lipid chain, while allowing the urea and aromatics to be interacting with the headgroup, and sticking out into the water outside of the cell. This would seem to be a structurally stable configuration of the drug, and the cleaving is a likely scenario to explain the biocidic properties of the drug.

References

[1] W.B. Hugo and A.R. Longworth. Some aspects of the mode of action of chlorhexidine. J. Pharm. Pharmacol., 16:655–662, 1964.

[2] .A. Harroun, J. Katsaras, and S.R. Wassall. Cholesterol is found to reside in the center of a polyunsaturated lipid membrane. Bio chemistry, 47:7090–7096, 2008.