Neutron diffraction and Vitamin E

T. A. Harroun,[1] D. Marquardt,[2] J. Katsaras[3] and J. Atkinson[2]

[1]Department of Physics, Brock University, St. Catharines, Ontario, L2S 3A1 Canada
[2]Department of Chemistry, Brock University, St. Catharines, Ontario, L2S 3A1 Canada
[3]Canadian Neutron Beam Centre, Building 459, Station 18, Chalk River, Ontario K0J 1J0, Canada

E-mail: tharroun@brocku.ca

Abstract. It is generally accepted that neutron diffraction from model membrane systems is an effective biophysical technique for determining membrane structure. Here we describe an example of how deuterium labelling can elucidate the location of specific membrane soluble molecules, including a brief discussion of the technique itself. We show that deuterium labelled α-tocopherol sits upright in the bilayer, as might be expected, but at very different locations within the bilayer, depending on the degree of lipid chain unsaturation.

1. Introduction

Despite its discovery over 80 years ago, the biological role of vitamin E remains a subject of tremendous controversy and confusion, with proposed theories being put forward with diametrically opposed views of its molecular function *in vivo* [1, 2].

Vitamin E occurs in two family groups, the tocopherols and tocotrienols, and there are two well established observations acknowledged by all competing theories. First, that α-tocopherol is the only member of the vitamin E family that is preferentially retained in all mammalian tissues, regardless of the amount of the four tocopherol or tocotrienol types found in an animal's diet. The discovery of the ubiquitous α-tocopherol transfer protein (αTTP) shows *how* this is accomplished, but the evolutionary reasons as to *why* α-tocopherol is selected in preference to the other family members remains a mystery.

The second observation is that the antioxidant properties of α-tocopherol in the test-tube is very well established. In fact, the fat-soluble α-tocopherol combined with water soluble ascorbic acid is arguably among the best anti-oxidant additives that can be used in food and chemical preservation.

In contrast, however, to these two observations, *in vivo* experiments on α-tocopherol have produced many confusing and contradictory results. Notably, vitamin E has been implicated in the regulation of cellular processes seemingly far removed from any antioxidant activity, including apoptosis and gene regulation [1, 2]. A majority of the biophysical studies regarding α-tocopherol have been on saturated acyl-chain model membranes, hardly a relevant model for determining whether α-tocopherol is really a lipid anti-oxidant.

We have begun a series of biophysical experiments based on neutron diffraction and isotopic/isomorphic labelling hoping to see whether α-tocopherol *could* act as an antioxidant protector of unsaturated lipids. To this end we were fortunate to have several milligrams of

© 2010 IOP Publishing Ltd

deuterium labelled α-tocopherol, as shown in Fig. 1. These were labelled either by $^2H_3$ on the methyl of the 5 carbon on the chromanol ring, or $^2H_2$ on the 9' carbon along the acyl chain.

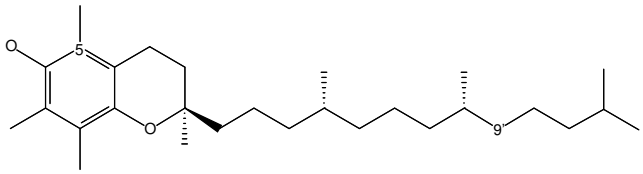

Figure 1. α-tocopherol, with deuterium label centres indicated by 5 and 9'.

Using aligned multi-lamellar stacks in hetero and homo-monounsaturated phosphocholine lipids, we have found the sample mass and time-averaged mass distribution of these labels within the membrane. From this data we hope to formulate whether, at these locations in the lipid bilayer, α-tocopherol is more capable of being a signalling molecule or a proper anti-oxidant.

2. Method of membrane diffraction and deuterium labelling

The entire preparation of aligned multilayer samples was carried out in a nitrogen-filled glove-box. A total of 12 mg of phospholipid with 5 mol % α-tocopherol were dissolved in chloroform-trifluoroethanol (3:1). The solution was deposited on a silicon crystal substrate, and the solvent evaporated while gently rocking the sample. This produces well aligned lamellar samples in a reproducible manner. The samples were then placed in a vacuum for $\sim$1 h to remove traces of the solvent. The samples were then sealed into sample holders and equilibrated in a humid nitrogen atmosphere at room temperature for several hours.

Samples were hydrated either; 1) at varying humidities using saturated salt solutions of KCl (84 % RH) and KNO_3 (94 % RH) or K_2SO_4 (97 % RH) with 8 mol % ^2H_2O, or 2) at a fixed humidity of 94 % RH with 70, 16, 8 and 0 mol % ^2H_2O.

Neutron diffraction data were taken on the N5 and D3 beam-lines at the Canadian Neutron Beam Centre (Chalk River, Ontario, Canada), using 2.37 wavelength neutrons selected by the (002) reflection of a pyrolytic graphite (PG) monochromator, while a PG filter was used to eliminate higher order (i.e., $\lambda/2$, etc.) reflections. Typically 5 to 6 pseudo-Bragg peaks were recorded, meaning that the reconstructed unit cell has a crystallographic resolution of 8-10 Å.

The bilayer profile was reconstructed by Fourier synthesis following the method outlined in [3]. The method records the integrated area of the Bragg peaks for each order I_h, and corrects for neutron absorption (C_{abs}), geometry of beam and sample width (C_{flux}), and the Lorentz factor (C_{Lor}), which are given by

$$C_{\text{abs}} = \frac{\alpha}{1-e^{-\alpha}}, \quad \alpha = \frac{2\mu t}{\sin\theta}, \tag{1}$$

$$C_{\text{flux}} = \frac{1}{\sin\theta}, \tag{2}$$

$$C_{\text{Lorentz}} = \sin(2\theta). \tag{3}$$

Here, μ is the absorption coefficient, and t is the sample thickness, calculated alongside the zeroth order Bragg peak F_0 (the scattering length density [SLD] of the entire unit cell, per mole of sample), using the total neutron cross section (rather than just the coherent scattering cross section), and some simple assumptions – the assumptions being the chemical composition (assuming 10% water by mass), and secondly, a mass density of 1 g/cm^3, which is likely correct to 10% with little effect on the final results.

Phasing of the structure factors was done either by 1) fitting a single continuous smooth form factor F_h versus scattering vector q to the swelling series of 8 mol % ^2H_2O data [4], or

2) by fitting straight lines to F_h as a function of the ^2H_2O percentage for each Bragg order h, expecting opposite signed slopes for even and odd values of h [5]. The first method has the advantage of being able to extrapolate and smooth out differences in the measured unit cell size, while the second method enables the difficult phasing of higher order peaks $h \geq 5$.

The SLD profile $\rho(z)$ is calculated from the Fourier transform of the structure factors according to,

$$\rho(z) = F_0 + 2 \sum_{h=1}^{h_{max}} F_h \cos(2\pi z h/d) \ , \tag{4}$$

where z is the distance along the bilayer normal, $z = 0$ is defined as the centre of the bilayer.

The SLD difference between labelled (L) and unlabelled (U) samples can also be calculated with Equation 4 using the difference between the structure factors $F_h = F_h^{\mathrm{L}} - F_h^{\mathrm{U}}$, with one important caveat: The structure factors for the labelled and unlabelled experiments must be placed on the same relative scale. If they can, then the difference SLD profile is simply the centre of mass of the isotopic/isomorphic substitution label, with all other molecular components subtracted away. Unfortunately, since these are two different samples, unknown experimental factors mean F_h^{L} and F_h^{U} are, in fact, on different scales.

Placing these data on a similar, if not an absolute, scale is the heart of the method of neutron diffraction with isotopic and isomorphic labelling. We have followed closely the discussion and methods of this topic from Han et al. [6] and our earlier work on deuterium labelled cholesterol [3]. In the end we have adopted a scaling method similar to Wiener and White [7, 8], but we achieve the same results by several scaling methods: 1. Equating $\rho^{\mathrm{L}}(z)$ and $\rho^{\mathrm{U}}(z)$ at one point z which results in a set of simultaneous equations in h with scaling factors and label parameters as unknowns. 2. Equating $\rho^{\mathrm{L}}(z)$ and $\rho^{\mathrm{U}}(z)$ at two points, results in an analytical result for scaling factors. 3. Scale inter-bilayer water profiles to be the same, which places $\rho^{\mathrm{L}}(z)$ and $\rho^{\mathrm{U}}(z)$ on the same relative scale.

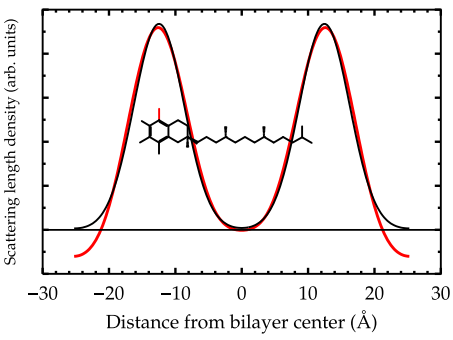

Figure 2. The deuterium mass distribution of labelled α-tocopherol at the C5a methyl (cf. Fig. 1), incorporated into 18:1-18:1 PC bilayers.

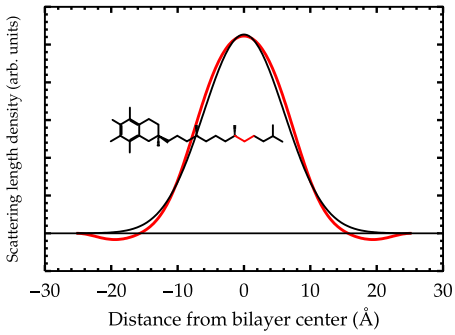

Figure 3. Similar to Fig 2, only for the C9' acyl chain label. The chemical diagram is to orient the viewer to the rough location of tocopherol.

3. Results

Figure 2 shows the the scattering length density (SLD) distribution of α-tocopherol labelled with three ^{2}H on the methyl attached to carbon 5 of the chromanol ring, as measured in a 18:1-18:1 phosphocholine membrane. The lighter curve is the label distribution, and the overlapping dark

curve is the fit using a single Gaussian function. The unit cell contains one bilayer, and the origin of the abscissa is at the centre. The inter-lamellar water layers are located at the edges of the unit cell.

The data places the reactive hydroxyl group at 13 Å from the bilayer centre. This is at, or perhaps just below, the hydrophobic/hydrophilic interface, nearly at the same depth as the phospholipid glycerol backbone ester.

The location of the distal end of the α-tocopherol is shown in Figure 3. In this case, the deuterium label resides on the 9' carbon of the tocopherols acyl chain. The distribution is also well fitted by a single Gaussian function fixed at the $z = 0$ Å, and with a standard deviation width of 8.6 Å. In fact, the difference can also be fit by a narrower Gaussian whose centre lies within $z \lesssim 4$ Å, since the mirror distribution appearing on the opposing bilayer leaflet overlaps this Gaussian distribution, creating a much broader single distribution. This is because of the unit cell's mirror symmetry, where atoms in one half of the unit cell are reflected across the centre (and edges) of the unit cell.

Figures 2 and 3 together show that α-tocopherol stands "upright" in the bilayer, the hydroxyl group just above the depth of the first carbon in the 18:1-18:1 PC acyl chain. The chain of the tocopherol is highly disordered, unlike the chemical schematic shown in the figures, since the C9' carbon, only 3/4 along the chain, is localized to the centre of the bilayer. If the chain were extended it would be interdigitated into the opposing bilayer leaflet, but the additional branched methyls make an all-*trans* configuration highly unlikely due to higher gauche probability at the *tert*-carbons.

The location of the C5a-methyl $^2H_3$ label was also measured in the hetero-unsaturated lipid of 16:0-18:1 PC. Shown in Figure 4, the carbon 5 methyl is surprisingly found to reside much higher in the bilayer, around the choline groups. The C9' label was also found to reside at the centre of the bilayer as in Fig. 3 (data not shown). Tocopherol's curled chain spends most of its time at the center of the bilayer.

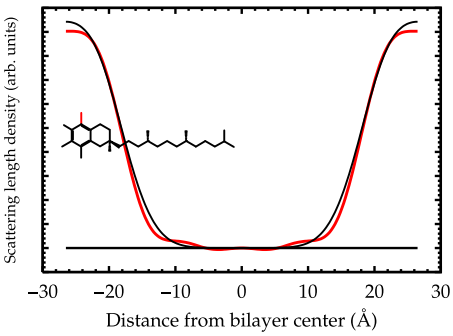

Figure 4. The deuterium mass distribution of labelled α-tocopherol at the C5a methyl (cf. Fig. 1), incorporated into 16:0-18:1 PC bilayers. The lighter line is the data, the darker line a Gaussian fit.

This unexpected result shows that the degree of lipid acyl chain unsaturation has a tremendous effect on the location of tocopherol in the bilayer. When all the lipid chains are unstaturated, the molecule sinks lower into the bilayer. This might indicate that tocopherol prefers to position itself near oxidizable bonds, which it would do to fulfil its anti-oxidant purpose.

References

[1] Traber M G and Atkinson J 2007 *Free Radical Biology and Medicine* **43** 4–15
[2] Azzi A 2007 *Free Radical Biology and Medicine* **43** 16–21
[3] Harroun T, Katsaras J and Wassall S 2008 *Biochemistry* **47** 7090–7096
[4] Darkes M J M and Bradshaw J P 2000 *Acta Cryst.* **D56** 48–54
[5] Worcester D L and Franks N P 1976 *J. Mol. Biol.* **100** 359–378
[6] Han X, Mihailascu M and Hristova K 2006 *Biophys. J.* **91** 3736–3747
[7] Wiener M C and White S H 1991 *Biophys. J.* **59** 162–173
[8] Wiener M C and White S H 1991 *Biophys. J.* **59** 174–185