BIOPHYSICS AT THE INTERSECTION OF HEALTH SCIENCE AND NUCLEAR TECHNOLOGY

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Abstract

We're all on a quest for improved heart health, but what do we really know about it? A daily regimen of aspirin can help some people with heart disease. We need to lower our cholesterol, and increase our intake of omega fatty acids. There is simply no health benefit to taking extra vitamin E, and it's not known why. Apart from cardiac tests with radiopharmaceuticals, what role does nuclear technology play in this story? It turns out that cold and thermal neutrons are important tools for the biophysicists studying these topics. We will review some recently published studies that are advancing our understanding of how cholesterol, vitamin E, and aspirin all work at the molecular level, inside the membrane of our cells. These insights could not have been learned without access to research reactor neutron beams such as those at the Canadian Neutron Beam Centre, and how this new knowledge has really engaged the broader health science community into new ways of thinking about these molecules.

1. Introduction

The molecular mechanism by which drugs and nutrients interact with the membranes of our cells has become a central issue in pharmacological sciences. Cellular membranes are complex assemblies that are much more than simple permeable barriers or passive substrates for proteins. Rather, they play an active role in many cellular functions, and they have a rich metabolism of their own. Many of these functions rely on a diverse array of lipids, vitamins, sterols, proteins and carbohydrates.

One area of particular interest for the health of Canadians is cardiovascular diseases, which are the leading cause of death in adult Canadians. Of the six types of cardiovascular diseases highlighted by Health Canada, ischemic heart disease is the leading cause of death, accounting for 54% of all cardiovascular deaths [1, 2]. Ischemic heart disease occurs when the blood supply to the heart muscle (myocardium) is cut off. Commonly, ischemia is a result from the accumulation of cholesterol-rich plaques in the coronary arteries (atherosclerosis).

The blockage of blood flow is not the only life threatening condition, which arises from ischemia. When treated, the restoration of the blood supply (reperfusion) can cause further damage to the myocardium, through oxidative stress, specifically free radical damage. The damage done during blood restoration is known as ischemia–reperfusion injury and also

occurs during surgery when blood vessels are cross-clamped [3]. Ischemia-reperfusion injury has been extensively studied, but the underlying molecular mechanisms of the pathology and treatments remain a mystery [4].

Below we discuss the role neutrons have played in the understanding of three small molecules with significant implications in the cause (cholesterol), preventative measure (aspirin) and recovery (vitamin E) of myocardium ischemia and reperfusion injury. Most interestingly, the availability of neutron beams is crucial to obtain molecular level information in these systems.

2. The Need for Neutrons

Compared to other biophysical techniques, neutron scattering offers many advantages for the study of biological systems at the atomic level. Firstly, it does not rely on bulky fluorophore or spin label probes, which can drastically alter the physical properties of model membrane systems. Instead, neutrons scatter from even light elements (e.g., H, C, N, O, etc.) commonly found in biological systems, and are able to distinguish between isotopes of the same element, with the substitution of hydrogen for deuterium being commonly used to systematically manipulate contrast, as shown in Figures 1 and 2 [5]. Scattering from individual components of the system (i.e. lipid, solvent or protein) can be suppressed through contrast matching, allowing for robust determination of bilayer organization, as shown in Figure 2.

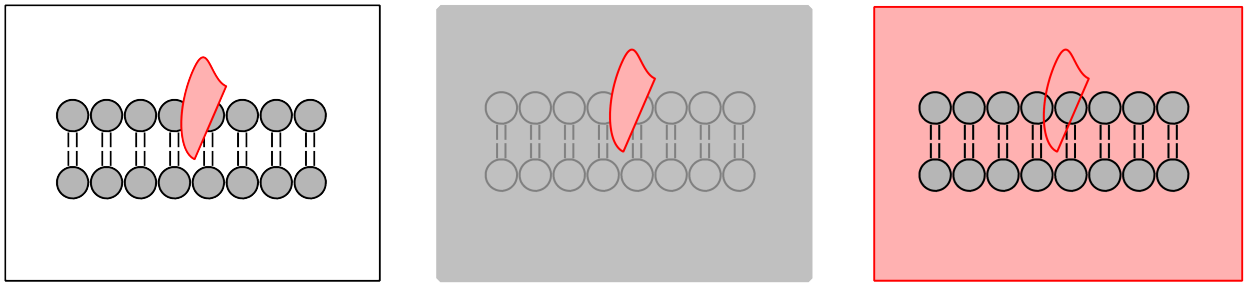

Figure 1: Schematic of possible neutron contrast variation experiments for a membrane (gray) with a protein inserted (pink). The left diagram represents the system with no contrast matching. The protein is highlighted in the centre diagram when the solvent (water) is contrast matched to the lipid bilayer. Membrane properties can be studied the diagram on the right when the solvent (ie. water) is contrast matched to the protein.

3. Cholesterol

In mammalian cells, as much as 90% of all cholesterol can be found in the plasma membrane [14]. Cholesterol has been well established as a mediator of cell membrane fluidity. By interacting with lipid tails, cholesterol causes the membrane tails to be constrained thereby reducing membrane fluidity.

The action of cholesterol's membrane mediation is observed through the formation of highly ordered domains (patches) of membrane enriched with cholesterol, as depicted in Figure 3. Interestingly these patches are a unique lipid phase which requires the presence of cholesterol and is named the liquid ordered phase (L_o). The cholesterol poor counterpart to L_o is the

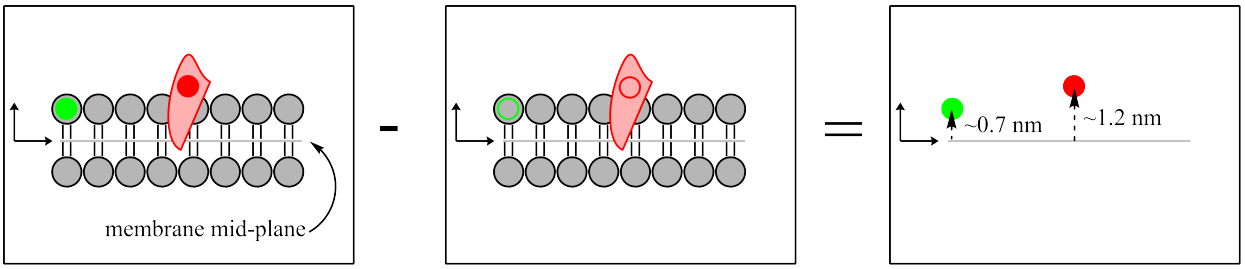

Figure 2: Biological systems have an intrinsic ability to be labeled due to the abundance of hydrogen (^{1}H) atoms that can be replaced (labeled) by deuterium (^{2}H). The substitution of deuterium atoms for hydrogen, at selective locations, provides contrast between the "labeled" sample (left) and the "unlabeled" sample (middle). The difference in scattering length density between the labeled and unlabeled sample yields the precise location and distribution of the ^{2}H label (red circle, right).

thinner and more disordered liquid disorder (L_d) phase. At high concentrations of cholesterol, immiscible cholesterol bilayers may form [15, 16]. These cholesterol 'plaques' often occur in people with elevated cholesterol, and play a role in diseases such as atherosclerosis [17].

An study of nanosized domain formation in free-floating bilayers was conducted by Heberle *et al.* by **small angle neutron scattering**. In order to mimic a complex biological membrane, this pioneering study examined four-component model systems containing a saturated phospholipid, varying ratios of mono- and di-unsaturated phospholipid and a constant cholesterol concentration for the presence of domains [18]. Domain sizes were found to increase with unsaturation (di-unsaturation : mono-unsaturation ratio) but more interestingly there is a direct correlation between the domain size and the bilayer thickness mismatch of L_d and L_o. These results were one of the first probe-free to observe these cholesterol rich nanodomains, as well as the first to demonstrate how functional domains in cells may be regulated through changes in phospholipid composition.

Armstrong *et al.* has observed the existence of cholesterol induced highly ordered lipid domains within the L_o phase of a binary phospholipid:cholesterol system [19]. Using **coherence length dependent neutron diffraction**, the authors were able to, unambiguously and for the first time, resolve signals of L_o domains from L_d regions. In single phospholipid systems L_o was believed to be a homogeneous phase. In addition to the presence of these ordered domains existing Armstrong determined, for the first time, dynamic properties cholesterol imposes on the L_o and did so before the formal observation of these domains [20, 21]. The nanoscale dynamics L_o were observed using an **in-elastic neutron scattering technique**, which does not rely on the use of bulky and perturbing probes. The domains in the cholesterol induced L_o phase appeared softer than the L_d phase, with a reduced membrane viscosity, but were more ordered than the gel phase. It is believed that cholesterol's "property amplifying" ability is one of the the driving forces for the formation of the hypothetical lipid rafts.

These studies, for the first time, give a detailed molecular picture of the fluid structure of lipid membranes. Cholesterol leads to the formation of ordered patches, which are enriched with cholesterol, and drastically different properties as compared to their surroundings. This change in membrane homeostasis has been shown to lead to reduced health in individuals

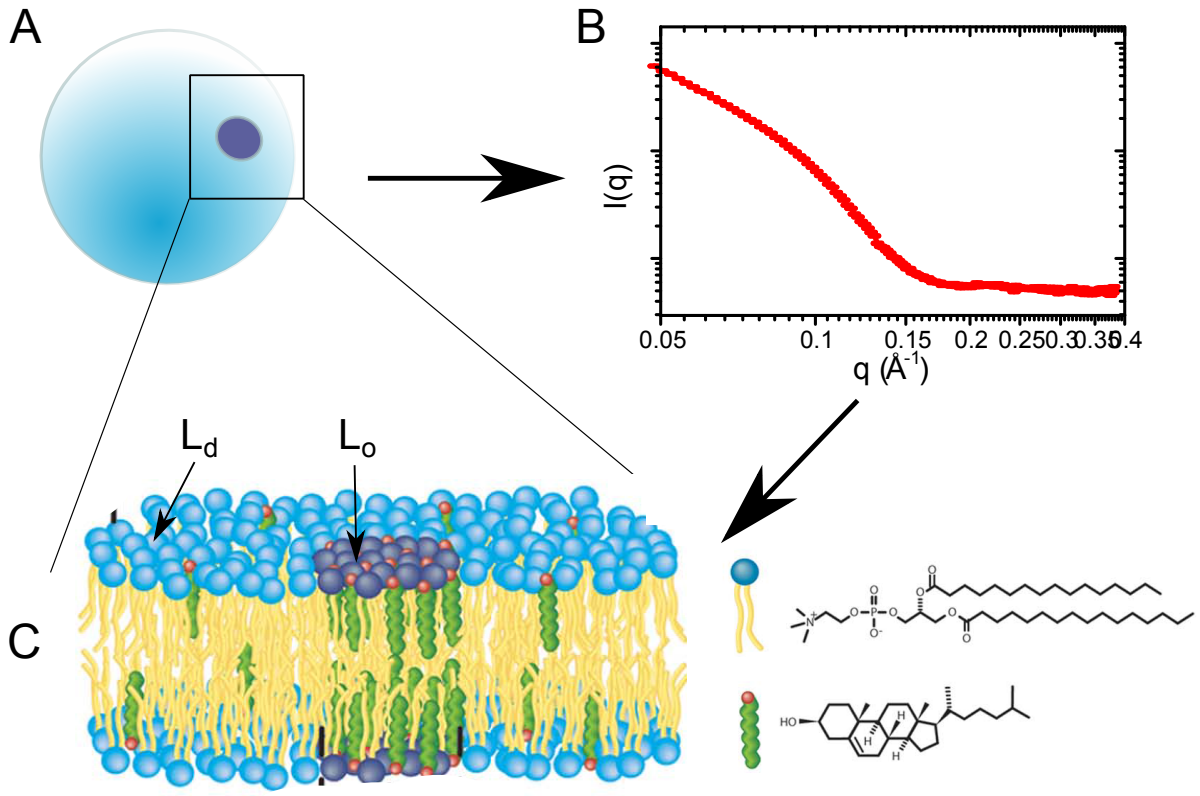

Figure 3: A) Lipid vesicle containing containing L_o domains. B) Small angle neutron scattering (SANS) curve which contains structural information about the bilayer as well as bilayer organization. C) Schematic of the membrane information contained within the SANS curve.

with high cholesterol. Some of the reduced health effects include high blood pressure and hypertension, which increases the risk for ischemic heart disease.

4. Aspirin

A common treatment for the prevention of ischemia related events, in individuals with increased cholesterol levels, is a daily low-dose of acetylsalicylic acid (aspirin) [22, 23].

Unlike α-tocopherol, aspirin has long been associated with specific interactions when introduced into the body. Aspirin interacts with the cycloxygenase (COX) pathway, inhibiting platelet aggregation [24]. In patients with high cholesterol, a reduction in platelet aggregation can decrease the incidence of blocked arteries and reduce the chance of myocardial events [25]. This was long believed to explain the low-dose aspirin therapy. Recently, the role of the COX pathway in the low-dose aspirin therapy has been called into question, given the growing awareness of so-called "aspirin resistance" [26]. Platelets from aspirin resistant patients often appear unaffected by the drug, likely through COX independent mechanisms [27]. The confusion surrounding aspirin has been recently discussed in the media [28].

At the same time, there is an increasing evidence for a role of the lipid membrane structure and composition in platelet function [29]. Aspirin has recently been shown to strongly interact with membranes, both real and synthetic, residing in the lipid headgroup region [30, 31]. In particular, when introduced in model membranes, aspirin has been shown to dissolve harmful cholesterol plaques leading to a more fluid, healthy bilayer [32]. In addition, aspirin is believed to interact with the membranes of red blood cells, making them more fluid and compressible, which could allow them to flow past barriers with greater ease [33].

We have recently performed **neutron diffraction** experiments on model membranes containing cholesterol and aspirin. The data suggests aspirin locally alters the lipid environment when introduced into membranes. By interacting with lipid headgroups, aspirin is able to increase lipid fluidity and compressibility, opposing the effect of cholesterol. By working against the effects of cholesterol, aspirin is able to frustrate the formation of lipid domains, fundamentally changing the membrane's structure and organization. Using the **coherence length dependent neutron diffraction** technique, we were able to well resolve the nanoscale changes in lipid structure induced by aspirin. Neutron diffraction gives unprecedented details of the molecular organization in membranes and enables us to develop molecular models, as shown in Figure 4.

5. Vitamin E

There is simply no clear evidence for the health benefits of supplementing our diets with additional vitamin E (α-tocopherol), except of course, for specific deficiency syndromes [6]. This is true whether for general heart-health, or as part of conventional treatments of conditions such as ischemia-reperfusion injury. This despite in the case of myocardial ischemia reperfusion injury, where maintaining redox homeostasis is pivotal in the survival of victims [7].

Tocopherol pretreatment is often used to prevent myocardial ischemia reperfusion injury in the case of bypass surgery patients [3]. However, different studies examining the benefits of tocopherol pretreatment yield contradictory results [3, 8]. What is missing is a clear molecular mechanism of vitamin E antioxidant action in a cellular membrane, or if such antioxidant action exists *in vivo* at all. This is especially true when considering the conflicting data in the literature. For example, some argue that it functions as an antioxidant, while others argue from the same evidence that it has some other, not yet identified task. For example, Traber and Atkinson write: "...all of the observations concerning the in vivo mechanism of action of α-tocopherol result from its role as a potent lipid-soluble antioxidant" [9]. However in the same journal issue, Azzi takes the counter argument that "... α-tocopherol is not able, at physiological concentrations, to protect against oxidant-induced damage..." [10].

Recently we have shown evidence of an antioxidant mechanism for α-tocopherol, which correlates strongly with its physical location in a model lipid bilayer [11]. The data addressed the overlooked problem of the physical distance between the vitamins reducing hydrogen and lipid acyl chain radicals. Our combined data from **neutron diffraction**, nuclear magnetic resonance (NMR) spectroscopy, and ultraviolet (UV) spectroscopy studies all suggest that

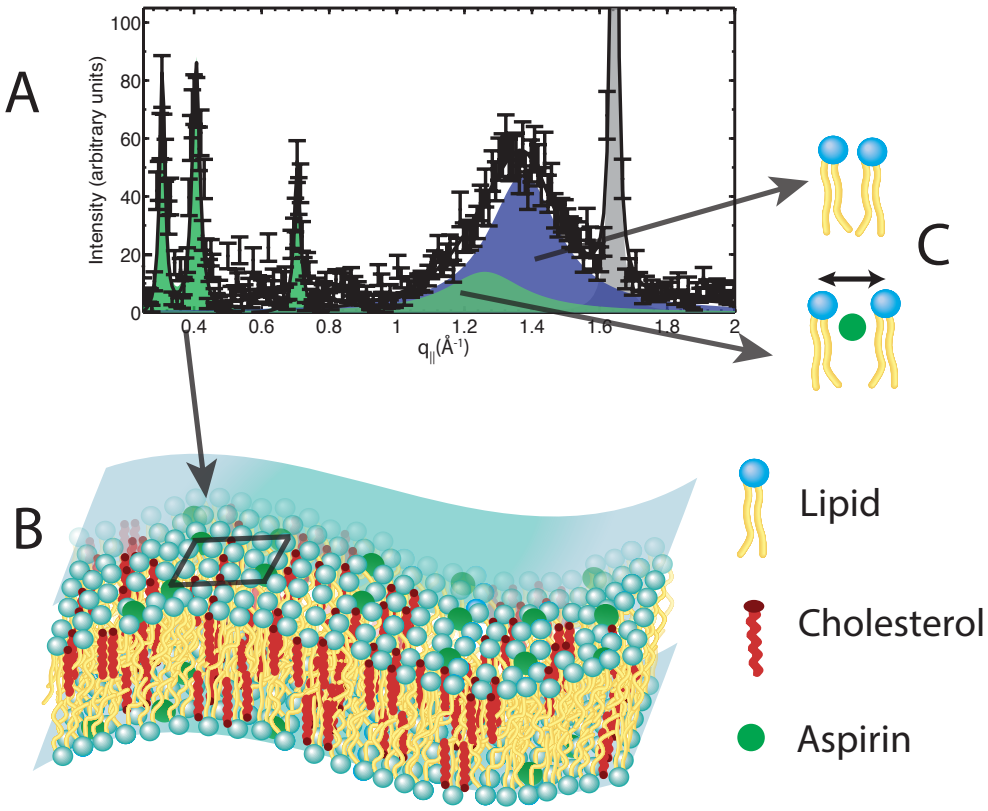

Figure 4: A) Neutron diffraction data obtained from model membranes containing cholesterol and aspirin. B) A 3D cartoon of a membrane containing cholesterol and aspirin, as determined by the neutron data. The cartoon highlights the regular distribution of aspirin(dark square on the membrane), leading to the frustration of lipid raft structures C) Cartoons highlighting the altered lipid environments introduced by aspirin. Aspirin interacts with the lipid headgroups leading to an increase in lipid tail separation, and an increase in lipid fluidity

reduction of reactive oxygen species and lipid radicals occurs specifically at the membrane's hydrophobic-hydrophilic interface, as shown in Figure 5. Such a conclusion has eluded scientists for decades because no one had yet determined the location α-tocopherol with precision until we applied neutron diffraction with **deuterium labeling**

A follow up study determined, by means of **small angle neutron diffraction**, that not only is α-tocophero's hydroxyl group located high in the membrane, but its tail also resides far from the center of bilayers of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) [12]. In addition, Marquardt *et al.* located the hydroxyl group of α-tocopherol above the lipid backbone in 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) and sphingomyelin, suggesting that α-tocopherol's location near the lipid-water interface may be a universal property of the vitamin [12].

Another important result which has originated from thermal neutron scattering was determining the location of vitamin E in the prototypical lipid dimyristoyl–phosphatidylcholine (DMPC). Without exception, the data point to α-tocopherol's active chromanol moiety re-

siding deep in the hydrophobic core of DMPC bilayers, a location that is in stark contrast to α-tocopherol's location in other lipids. The discovery of α-tocopherol's residence in the centre of a DMPC bilayer explains some of the conflicting and inexplicable data found in the literature regarding α-tocopherols behaviour in DMPC bilayers versus other phospholipid bilayers [13].

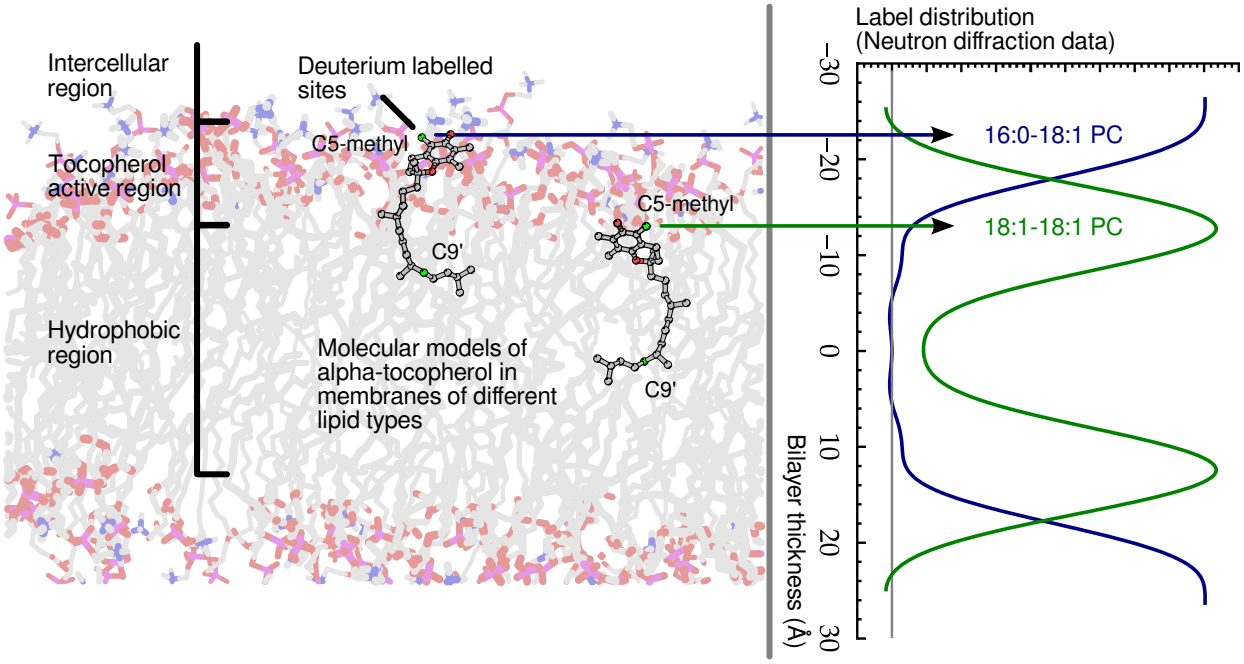

Figure 5: Schematic of α-tocopherol in a model lipid membrane as determined by neutron diffraction. The zone of α-tocopherol antioxidant action is confined to the region of the glycerol ester and above, extending practically to the aqueous phase. Although α-tocopherol can either terminate a lipid radical or intercept diffusing reactive oxygen species, its different locations within bilayers correlate well with its primary activity.

6. Concluding Remarks

In this paper, we hope to have shown that neutron beams are an indispensable tool for cutting–edge research in molecular biology and pharmaceutical sciences.

Biological themed research remains a small and slowly growing component of the science conducted at neutron beam facilities. Annual report data from the Institut Laue–Langevin (Grenoble, France) tracks growth in experimental proposals classified as "biology" from 6% in 2002 to 10% in 2013. However, many experiments classified as "soft condensed matter" often have applications in biochemistry and molecular biology, and including these, as many as 1 in 8 instrument–days at the ILL is devoted to science involving some biologically related material.

One reason for the slow growth of using neutrons for biological research is the difficulty of new knowledge breaching the wall separating biology from neutron physics. Translating the

results described above to clinical use is a daunting challenge, as these results are guided by methods and techniques drawn more from physics, and are far removed from petri dishes and cages of biochemical and animal research.

Most of these experiments are guided by physics–trained biophysicists, working in collaboration with colleagues from biochemistry and biology departments. Ultimately, it will be up to these biologists to flesh-out the theories necessary to reach clinical application.

However, we continue to recruit biochemists and biologists to consider conducting neutron beam experiments. Insights such as these shown above afford physiologists a molecular picture otherwise unattainable without the use of neutrons. One important way to make entry into this field easier for biologists lay outside the research reactor.

Deuterium plays an important role in neutron scattering for biology, and to that end many neutron beam laboratories have established their own ancillary laboratories dedicated to the incorporation of deuterium into biological molecules and systems.

For example, the Center for Structural Molecular Biology at the Oak Ridge National Laboratories (utilizing the High Flux Isotope Reactor) established the Bio-Deuteration Lab for this express purpose. The European Photon and Neutron Campus (EPN-campus), home of the Institut Laue–Langevin reactor, now shares its grounds with the Institut Biologie Structurale, and through the Partnership for Structural Biology, has established the Deuteration Laboratory platform (D–LAB).

It is thought that with a better knowledge foundation of how to incorporate deuterium into biological materials, more biologists will feel free to design more interesting neutron experiments. This also has the additional benefit that this knowledge will also help those researchers using nuclear magnetic resonance techniques.

References

[1] Mortality, summary list of causes 2008, 2011. Catalogue no. 84F0209X.

[2] C.D. Mathers and D. Loncar, "Projections of global mortality and burden of disease from 2002 to 2030", *PLoS Med*, Vol. 3, Iss. 11,2006, pp.e442.

[3] V. Braunersreuther and V. Jaquet, "Reactive Oxygen Species in Myocardial Reperfusion Injury: From Physiopathology to Therapeutic Approaches", *Current Pharmaceutical Biotechnology*, Vol. 18, Iss. 1, 2012, pp. 97–114.

[4] P. Venditti, P. Masullo, S. Di Meo, and C. Agnisola, "Protection against ischamia-reperfusion induced oxidative stress by vitamin e treatment", *Archives Of Physiology And Biochemistry*, Vol. 107, Iss. 1, 1999, pp. 27–34.

[5] T.A. Harroun, J. Katsaras, and S.R. Wassall, "Cholesterol Hydrozyl Group Is Found To Reside in the Center of a Polyunsaturated Lipid Membrane", *Biochemistry*, Vol. 45, Iss. 4, 2006, pp. 1227–1233.

[6] M.G. Traber and J.F. Stevens, "Vitamins c and e: Beneficial effects from a mechanistic perspective", *Free Radical Biology & Medicine*, Vol. 51, 2011, pp. 1000–1013.

[7] W. Dröge, "Free radicals in the physiological control of cell function", *Physiological Reviews*, Vol. 82, Iss. 1, 2002, pp. 47–95.

[8] M.W. Clarke, J.R. Burnett, and K.D. Croft, "Vitamin E in human health and disease", *Critical Reviews in Clinical Laboratory Sciences*, Vol. 45, Iss. 5, 2008, pp. 417–450.

[9] M.G. Traber and J. Atkinson. "Vitamin E, antioxidant and nothing more", *Free Radical Biology and Medicine*, Vol. 43, Iss. 1, 2007, pp. 4–15.

[10] A. Azzi, "Molecular mechanism of α-tocopherol action", *Free Radical Biology and Medicine*, Vol. 43, Iss. 1, 2007, pp. 16–21.

[11] D. Marquardt, J.A. Williams, N. Kučerka, J. Atkinson, S.R. Wassall, J. Katsaras, and T.A. Harroun, "Tocopherol activity correlates with its location in a membrane: A new perspective on the antioxidant vitamin e", *Journal of the American Chemical Society*, Vol. 135, Iss. 20, 2013, pp. 7523–7533.

[12] D. Marquardt, N. Kučerka, J. Katsaras, and T.A. Harroun, "α-tocopherol's location in membranes is not affected by their composition", *Langmuir*, 2014, Submitted.

[13] D. Marquardt, J.A. Williams, J.J. Kinnun, N. Kučerka, J. Atkinson, S.R. Wassall, J. Katsaras, and T.A. Harroun, "Dimyristoyl phosphatidylcholine: A remarkable exception to α-tocopherol's membrane presence", *Journal of the American Chemical Society*, Vol. 136, Iss. 1, 2014, pp. 203–210.

[14] Y. Lange and B.V. Ramos. "Analysis of the Distribution of Cholesterol in the Intact Cell", *Journal of Biological Chemistry*, Vol. 258, Iss. 24, 1983, pp. 5130–5134.

[15] M.A. Barrett, S. Zheng, L.A. Toppozini, R.J. Alsop, H. Dies, A. Wang, N. Jago, M. Moore, and M.C. Rheinstädter. Solubility of cholesterol in lipid membranes and the formation of immiscible cholesterol plaques at High cholesterol concentrations *Soft Matter*, Vol 9, 2013, pp. 9342 - 9351.

[16] R. Ziblat, I. Fargion, L. Leiserowitz, L. Addadi Spontaneous Formation of Two-Dimensional and Three-Dimensional Cholesterol Crystals in Single Hydrated Lipid Bilayers *Biophysical Journal*, Vol 103, 2012, pp. 255-264.

[17] R.P Mason, T.N. Tulenko, R.F. Jacob. Direct evidence for cholesterol crystalline domains in biological membranes: role in human pathobiology *Biochimica et Biophysica acta*, Vol 1610, 2003, 198-207.

[18] F.A. Heberle, R.S. Petruzielo, J. Pan, P. Drazba, N. Kučerka, R.F. Standaert, G.W. Feigenson, and J. Katsaras, "Bilayer thickness mismatch controls domain size in model membranes" *Journal of the American Chemical Society*, Vol. 135, Iss. 18, 2013, pp. 6853–6859.

[19] C.L. Armstrong, D. Marquardt, H. Dies, N. Kučerka, Z. Yamani, T.A. Harroun, J. Katsaras, A.-C. Shi, and M.C. Rheinstädter, "The observation of highly ordered domains in membranes with cholesterol", *PLoS ONE*, Vol. 8, Iss. 6 2013, pp. e66162.

[20] C.L. Armstrong, M.A. Barrett, A. Hiess, T. Salditt, J. Katsaras, A.-C. Shi, and M.C. Rheinstädter. "Effect of cholesterol on the lateral nanoscale dynamics of fluid membranes", *European Biophysics Journal With Biophysics Letters*, Vol. 41, Iss. 10 2012, pp. 901–913.

[21] C.L. Armstrong, T. Seydel, W. H aussler, J. Katsaras, M.C. Rheinstädter. Nanosecond Lipid Dynamics in Membranes with Cholesterol *Soft Matter*, Vol 10, Iss 15, 2014, pp. 2600-2611.

[22] L. Hansson, A. Zanchetti, S.G. Carruthers, B. Dahl of, D.Elmfeldt, S. Julius, J. Ménard, K.H. Rahn, H. Wedel, and S. Westerling, "Effects of intensive blood-pressure lowering and low-dose aspirin in patients with hypertension: principal results of the hypertension optimal treatment (HOT) randomised trial." HOT study group, 1998", *Lancet*, Vol. 13, Iss. 351, 1998, pp. 1755-62.

[23] S.M. Weisman and D.Y. Graham, "Evaluation of the benefits and risks of low-dose aspirin in the secondary prevention of cardiovascular and cerebrovascular events", *Archives of Internal Medicine*, Vol. 162, Iss. 19, 2002, pp. 2197–2202.

[24] G.J. Roth, N. Stanford, P.W. Majerus, Acetylation of prostaglandin synthase by aspirin. *Proc. Natl. Acad. Sci.*, vol 72, 1975, pp. 3073-3076.

[25] C. Patrono, L.A. Garcia Rodriguez, R. Landolfi, and C. Baigent. Low-dose aspirin for the prevention of atherothrombosis. *New England Journal of Medicine*, Vol 353, pp. 2005, 2373-2383.

[26] C.N. Floyd and A. Ferro. Mechanisms of aspirin resistance. *Pharmacology and Therapeutics*, Vol 141, Iss 1, 2014, pp. 69-78.

[27] A. Assadian, J. Lax, U. Meixner-Loicht, G.W. Hagmüller, P.M. Bayer, and Wolfgang Hübl. Aspirin resistance among long-term aspirin users after carotid endarterectomy and controls: flow cytometric measurement of aspirin-induced platelet inhibition. *Journal of Cardiovascular Surgery*. Vol 45, 2007, pp. 1142-1147.

[28] A. Picard Self-medicators, beware the Aspirin myth. *The Globe and Mail*, May 27, 2014.

[29] V.B O'Donnell, R.C. Murphy, S.P. Watson. Platelet Lipidomics: Modern day perspective on lipid discovery and characterization in platelets *Circulation Research*, Vol 114, 2014, pp. 1185-1203.

[30] L.M. Lichtenberger, Y. Zhou, J. V. Jayaraman, J.R. Doyen, R.G. O'Neil, E.J. Dial, D.E. Volk, D.G. Gorenstein, M.B. Boggara, R. Krishnamoorti. Insight into NSAID-induced membrane alterations, pathogenesis and therapeutics: characterization of interaction of

NSAIDs with phosphatidylcholine. *BBA - Molecular and Cell Biology of Lipids*, Vol 1821, 2012, pp. 994-1002.

[31] M.A. Barrett, S. Zheng, G. Roshankar, R.J. Alsop, R. K. R. Belanger, C. Huynh, N. Kuerka, and M.C. Rheinstädter, Interaction of aspirin (acetylsalicylic acid) with lipid membranes. *PLoS ONE*, Vol. 7, Iss. 4, 2012, pp. e34357.

[32] R.J. Alsop, M.A. Barrett, S. Zheng, H. Dies, and M.C. Rheinst adter, "Acetylsalicylic acid (asa) increases the solubility of cholesterol when incorporated in lipid membranes", *Soft Matter*, Vol. 10, 2014, pp. 4275–4286.

[33] J.N. Frydman, A.S. Adenilson, C. Vanessa, M.O. Benarroz, G.S. Rocha, M.O Pereira, M.J Pereira, A.C Medeiros, and M. Bernardo-Filho Acetylsalicylic acid and morphology of red blood cells. *Brazilian Archives of Biology and Technology*, Vol 53, 2010, pp. 575-582.