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PROBING THE MICROSTRUCTURE OF BIOMATERIALS WITH POSITRONS

Doctoral Dissertation

Petri Sane

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Faculty of Information and Natural Sciences
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Abstract

Voids in biological membranes are vital to the diffusion characteristics of the membranes and as such information gained from e.g. void sizes elucidates the understanding of the functionality of the membrane. In this thesis the focus is on lipid bilayers, a type of membrane that exists in almost all organisms as a diffusion barrier surrounding the cells. The lipid bilayer consists of hydrophilic and hydrophobic parts that can form the bilayer structure in aqueous solution and the structural properties of the inner core of the bilayer (aka. hydrocarbon tail area) have a strong effect on the diffusion of particles through the bilayer. One of the key elements in the bilayer structure is the distribution and size of the free volume pockets, voids. The size of the voids depend both on the lipid type, but as well as the structural parameters of the bilayer, so called phase behaviour of the bilayer structure.

Positron annihilation lifetime spectroscopy is a widely used tool to characterize atomic-scale structural properties of both solid state and soft matter and as such the correlation with positron (or to be specific positronium, the bound state of positron and electron) lifetime and the size of voids in e.g. polymers has been observed. In this thesis positron annihilation lifetime spectroscopy has been applied to probe the voids in lipid bilayers in several manners: Firstly as a proof-of-concept-type of experiment reveals the feasibility of positron annihilation lifetime spectroscopy as a tool to detect subtle differences in the void sizes depending on the lipid concentration in aqueous solution, secondly it is found possible to characterize the phase transition temperature by observing the change of void sizes below and above the phase transition temperature. In addition, the effect of dopants, namely cholesterol, on the bilayer structure is studied as a function of cholesterol concentration.
The feasibility of positron annihilation lifetime spectroscopy to study intact tissues in-situ is also presented in a study where the temperature-induced structural transition of mammalian lens is observed and traced down to the lipids the lens strongly consists of.

Keywords positron lifetime, lipid bilayer, positronium, voids

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Tiivistelmä

Tutkimustyössä on kokeellisen ja laskennallisen tutkimustyön avulla osoitettu positroniannihilaatiospektroskopian soveltuvan erinomaisesti biologisten materiaalien tutkimiseen. Menetelmän toimivuutta testaavan tutkimuksen lisäksi on kyetty tuottamaan uutta tietoa kokonaisten biologisten kudosten atomitasolla tapahtuvista rakenteellisista ominaisuuksista. Menetelmän taustalla on positronin ja tutkittavan aineen elektronin muodostaman positronium-atomin soveltuvuus materiaalien atomitasolla olevien tyhjien tilavuuksien tutkimiseen.

Tutkituista materiaaleista tärkeimpiä olivat lipidikaksoiskalvot, jotka koostuvat polaarisista lipidimolekyyleistä. Lipidimolekyylin hydrofobinen hiilivetyhäntä ja hydrofiilinen pääryhmä muodostavat vesiseoksessa itseisesti erilaisia rakenteita, mistä kaksoiskalvo-rakenne on erityisen mielenkiinnon kohteena. Kaksoiskalvorakenteessa on kaksi kerrosta lipidimolekyylejä missä hydrofobiset hiilivetyhännät osoittavat toisiaan kohti rakenteen keskellä muodostaen suhteellisesti tyhjän vedestä vapaan ”ydin-alueen” ja hydrofiiliset pääryhmät toimivat rakenteen ulkoreunoina. Yksi kaksoiskalvorakenteen tärkeistä rakenneominaisuuksista on vapaan tilavuuden määrä kalvon sisällä, mikä vaikuttaa merkittävästi mm. kalvon läpi tapahtuvaan diffuusioon. Lipidityyppi vaikuttaa olennaisesti vapaan tilavuuden määrään, mutta lisäksi lipidissä tapahtuvat rakenteelliset faasitransitio muuttavat rakenneparametrejä merkittävästi.
Tämän tutkimuksen yhtenä päämäärä on ollut pystyä tutkimaan faasitransitiossa tapahtuvaa vapaan tilavuuden muutosta käyttäen positronium-atomia nk. ”probe”-hiukkasena. Tulosten valossa on selvää että Positronium-atomi reagoi voimakkaasti rakennemuutoksiin ja saatavasta mittausdatasta voidaan tehdä päätelmiä vapaan tilavuuden määrästä. Menetelmän yleisen soveltuvuuden toteennäyttämisen lisäksi tutkimustyössä on kyetty havaitsemaan erilaisten seosaineitten vaikutus vapaan tilavuuden määrään. Lisäksi tutkimuksissa havaittiin lämpötilariippuvaisia pieniä rakenteen uudelleenorganisoitumisia tutkituissa kokonaisissa mykiöissä. Jatkotutkimuksissa kyettiin jäljittämään havaitut muutokset tapahtumaan pääasiassa lipidirakenteissa, jotka edustavat merkittävää osaa mykiön materiaalikoostumuksesta.

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Preface

This thesis has been prepared in the positron group in the Department of Applied Physics at the Aalto University during the years 2006-2010. Major supervision the work has been performed by Dr. Filip Tuomisto to whom I am grateful for being able to work in the positron group. Regarding the technical details on digital lifetime spectrometers, I have also received valuable support from Dr. Klaus Rytsölä. However this work would have been severely harder without the existing studies and testing of the digital lifetime setup by present colleague Simo Kilpeläinen and the major work on the digital lifetime spectroscopy by Dr. Reino Aavikko whose work has made my work so much simpler.

As well as personnel in positron group, also the insight on physiology by Dr. Juha Holopainen from the University of Helsinki and discussions with our collaborators Prof. Ilpo Vattulainen and Dr. Emppu Salonen have helped me to gain additional information on this project which spans over many branches of science. Working on joint research collaboration with scientist from different fields has been enlightening and rewarding, hopefully for each of us.

Naturally I must thank my parents for providing me a well-supplied and stimulating environment during the childhood years. Furthermore, discussions and activities on on- and off-work related matters with my brothers have kept the motivation level high during the studies.

And of course, without my greatest idol and favorite TV-character, MacGyver, I might have never been interested in physics and science in general.

Espoo, September 2010

Petri Sane

Contents

List of Publications

This thesis consists of an overview and of the following publications which are referred to in the text by their Roman numerals.

I P. Sane, S. Kilpeläinen and F. Tuomisto, *4-channel digital positron lifetime spectrometer for studying biological samples,* Materials Science Forum **607** pp.254-256 (2009). www.scientific.net/MSF.607.254

II P.Sane, E. Salonen, E. Falck, J. Repakova, F. Tuomisto, J.M. Holopainen, and I. Vattulainen, *Probing biomembranes with positrons*, Journal of Physical Chemistry B **113** 1810-1812 (2009). http://dx.doi.org/10.1021/jp809308j

III P.Sane, F. Tuomisto, S.K. Wiedmer, T. Nyman, I.Vattulainen and J.M. Holopainen, *Temperature-induced structural transition in-situ in porcine lens – Changes observed in void size distribution*, Biochimica et Biophysica Acta - Biomembranes **1798** 958-965 (2010) http://dx.doi.org/10.1016/j.bbamem.2010.01.011

IV P.Sane, F.Tuomisto and J.M. Holopainen, *Void volume variations in contact lens polymers,* Contact Lens and Anterior Eye, *in press*. http://dx.doi.org/10.1016/j.clae.2010.06.008

The author has had an active role in all the phases of the research reported in this thesis. He has had an active role in the planning and performing of the positron lifetime experiments, analysis and the interpretation of the data. The author has been a main author in publications I, III and IV and has written the experimental parts in publication II.

Chapter 1 – Introduction

Positron annihilation spectroscopy is an experimental method for characterizing different material properties of condensed and soft matter on an atomic level. Two major branches of positron annihilation spectroscopy typically employed are positron annihilation lifetime spectroscopy and Doppler broadening spectroscopy. This work concentrates on positron annihilation lifetime spectroscopy, which is a spectroscopic method for measuring the lifetime of positrons in the sample material. This lifetime is observable via the time difference of the creation of a positron in radioactive decay of the parent-isotope and the annihilation of the positron with electron of the sample material. The method is widely used to study for example defects in semiconductors, pore size distribution in polymers etc. and has been employed for material studies for over half a century worldwide. At the former Helsinki University of Technology the positron studies began in late 1950's, involving among others the future Academy of Finland academician Prof. Pekka Jauho.

Every cell in every living being is strongly dependent on the transport of particles and substances through the cell membranes. A cell membrane can be easily characterized as a skin for the cell, which protects the interior of the cell and prevents unwanted particles entering the cell, while at the same time allowing the transport of vital particles in and out of the cell. The cell membrane contains a wide variety of biological materials, molecules and complexes, mainly consisting of lipids and proteins. The lipids in the cell membrane are primarily phospholipids arranged in a bilayer structure surrounding the cell as the transport layer for all cell trafficking. The lipid molecule itself is an amphiphilic molecule, consisting of hydrophilic and hydrophobic components, thus arranging spontaneously to a bilayer structure in aqueous solution. This bilayer is the key element for particle diffusion and therefore under intensive studies regarding the characteristics and functioning of the bilayer system. The major focus on bilayer characteristics in this work is the free volume properties inside the bilayer. Free volume changes in different phases have been thoroughly studied with different methods, both

experimental and theoretical (namely molecular dynamics simulations in recent years). In this work the viability of positron annihilation lifetime spectroscopy for such studies is presented as an alternative to traditional experimental methods (differential scanning calorimeter, nuclear magnetic resonance etc.) providing more detailed information on the micro-structural level of the membranes. For cell membranes, it is generally believed that free volume pockets inside a membrane have a prominent role in a variety of dynamic processes. Such processes include for example permeability of small molecules across membranes and the diffusion of, e.g., lipids, drugs and electron carriers in the plane of the membrane, thus based on results on the free volume parameters, the diffusion parameters can be interpreted from the data.

Different solutes implanted to the bilayer can alter the structural characteristics of the bilayer and open a wide range of possibilities for controlling the functionality of the bilayer for, e.g., medical purposes. As an example changes in free volume distribution due to anaesthetics partitioning into a membrane are also believed to perturb the lateral pressure profile, thus inducing a change in the distribution of ion channel state, which controls the majority of the diffusion through the membrane. As for clinical applications, there are drugs that need to penetrate tissues, such as in the topical application of eye drops and in transdermal drug delivery, implying that understanding molecular diffusion through membrane structures is crucial for the development of improved drug delivery carriers without a need for invasive intraocular injections.

The sensitivity of positron annihilation lifetime spectroscopy opens also additional possibilities of materials research as by employing positrons as probe particles, it is possible to gauge the structure of different materials without perturbing the systems significantly or destroying the material in the process. Probing the microstructure of intact tissues makes it possible to gain novel information about processes occurring inside the tissues in-situ. This information may be used to solve medical problems related to the malfunctioning of the tissues, e.g., formation of glaucoma inside the mammalian eye. Combining the understanding of the function of lipid bilayers and biological tissues, it might be possible to control the diffusion of solutes to and from

cells by doping the bilayers with fixed concentrations of dopants in order to prevent hazardous processes occurring in the tissues.

From an instrumentation point of view, the digitalization of the positron lifetime measurement equipment has been an ongoing process in several laboratories worldwide for the past 10 years. Modern fast digitizers have opened up a possibility to perform the data analysis digitally, removing the need for large racks of analogue instruments for the same purpose. This development has lead to a situation where the construction and use of digital lifetime setup has become relatively cheap and reliable as the degradation of digital components doesn't affect the results of the lifetime measurements. Malfunctioning components are easy to replace due to the large supply of digital electronic components, whereas year by year it is harder to find spare parts for older analogue electronics components. Also, the use of a digital measurement setup also enables the tuning of the setup for specific purposes and all necessary calibrations can be automated. This has lead to a situation where the measurements of biomaterials in this thesis have been performed solely on a new digital lifetime setup as the requirements for measurements on biomaterials compared to semiconductor measurements differ and existing analogue lifetime setups are not well suited for such measurements. Besides using a standard type of a digital lifetime setup, a novel multi-purpose detector system has been developed during the recent years and construction was executed during the last year. This setup allows researchers to combine simultaneously measured data from different detector pairs to gain more detailed information from the sample materials, more specifically, to solve the relevant problem of efficiency vs. resolution.

This thesis organises as follows; a general overview on biophysics and lipid bilayers is presented in chapter 2, after which an introduction to positron annihilation lifetime is given in chapter 3 and in chapters 4 and 5 proof of concept study and several applications are presented based on the Publ. II-IV.

Chapter 2 – Physics of soft and biological matter

Quoting the **Encyclopædia Britannica** [1]:

"*Biophysics is a discipline concerned with the application of the principles and methods of physics and the other physical sciences to the solution of biological problems*."

Biophysics is a cross disciplinary field, combining the efforts of physicists, chemists, biologists and specialists in medicine. When the understanding of these fields is combined, one is able to perform physical experiments (or create models) on biological materials and most importantly, interpret the data in a way that provides meaningful answers to the problems occurring in biology. One of the most famous examples of biophysical research is the unraveling of the DNA's molecular structure by Watson and Crick in 1953, biologist and physicist, respectively.

An other excellent example of interdisciplinary work in biophysics is the research on biological membranes, namely lipid bilayers, on which most of this thesis concentrates and which are more thoroughly presented in following sections.

2.1 – Lipid bilayers

A lipid bilayer is a structure of two layers of lipid molecules, which in aqueous solution will form spontaneously a bilayer due to the amphiphilic nature of the lipid molecule. In the framework of this thesis the focus is on phospholipids as they are the dominant type of lipids in organisms. Lipid bilayers have been under intense research for the past 40 years: for a more thorough introduction on the topic see for example following Refs. [2], [3], [4], [5]. The lipid molecule consists of a denser molecule compound, so called head group, (phosphate group in phospholipids) and a long, typically more than ten CH_2-molecules long, lighter hydrocarbon chain or as typically called, hydrocarbon tail. In most lipids the hydrocarbon tail is paired, but the length and composition of the individual tail vary [6]. The charge density in the head group is not evenly distributed, resulting in the polar nature of the molecular compound. The phosphate group in the head group is typically charged negatively (anionic state) and thus it feels strong attraction to water molecules due to the capability of hydrogen bonding, whereas the neutrally charged tail is non-polar and unable to form hydrogen bonds. The lack of hydrogen bonding (making it preferable for water to bond with other water molecules or hydrophilic molecules) causes a pseudo-repulsion between hydrophobic molecules and the water molecules. The chemical composition of dipalmitoylphosphatidylcholine-lipid molecule (DPPC) is illustrated in Fig. 1 to give an example of the lipid structure.

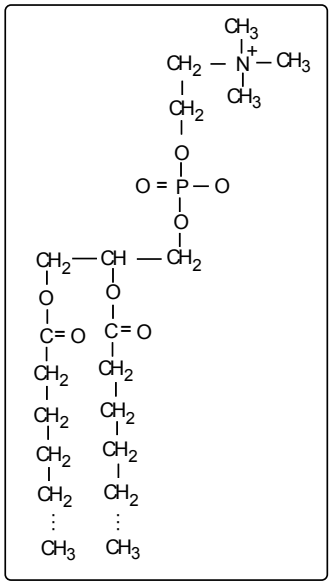

Figure 1. *Chemical composition of a DPPC-lipid molecule. The hydrocarbon tail is shortened for visual purposes, composition based on [6]. Typical length of the hydrocarbon tail varies within 12-18 CH_2-molecules depending on the lipid type.*

When inserted in aqueous environment with sufficient water concentration, the lipid molecules will auto-organize themselves forming a bilayer structure. The hydrophilic head groups will form a densely packed layer adjacent to the water, and the hydrophobic tails will point away from the water. However it must be noted that not all lipids form a bilayer in aqueous solution, some prefer other structures e.g. micelles or inverse micelles depending on the lipid type and concentration. A lipid bilayer can exist at a given temperature in either a liquid (fluid) or a gel (solid) phase, which are mainly defined by the relative mobility of the individual lipid molecules. This relative mobility is also known as fluidity and the response of the fluidity to the temperature changes is known as the phase behavior of the bilayer. All lipids have a lipid specific critical temperature at which they transition from the gel to the liquid phase (a transition that is in most lipids reversible), though when certain dopants are induced to the bilayer the transition can occur to the less ordered liquid-order phase which is a state containing characteristics of both phases [7]. The formation of the liquid order phase prevents the normal phase transition to occur in specific critical temperature. In the gel phase the

lipid molecules are, in practice as the mobility is close to zero, prevented from flip-flopping across the bilayer, but in the fluid phase a given lipid molecule will exchange locations with its neighbors at a frequency of $>10^6$/s. In some cases the frequency is significantly lower, occurring few times/hour. This random walk exchange allows a lipid to diffuse within the membrane [8]. The specific phase also affects the hydrocarbon tail by changing the thickness of the chains and reducing the ordering. This structure of a DPPC bilayer is visualized in Fig 2. [Publ. II].

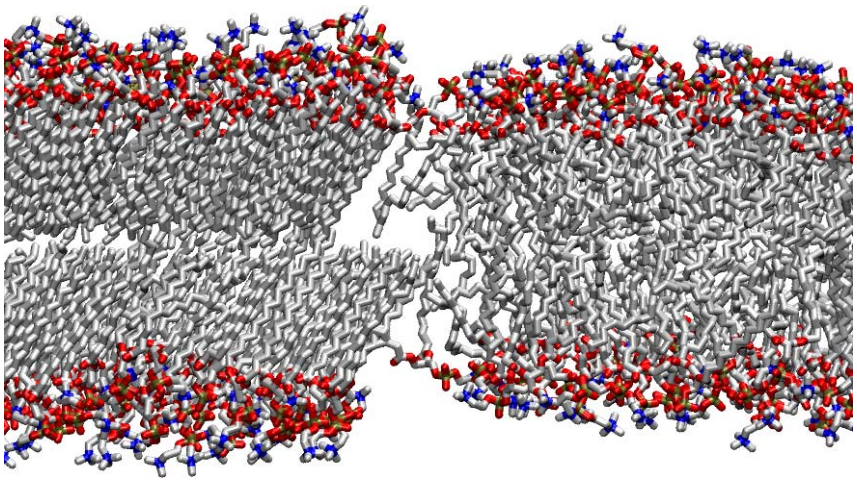

Figure 2. *Illustration (molecular dynamics simulation snapshot) of a DPPC bilayer structure on two different phases, left gel, right fluid. Carbon-atoms are colored gray, blue denotes for N-, red O- and brown P-atoms (barely visible).*

The thickness of the whole bilayer structure varies from 35 to 60Å depending on the lipid type, its chemical composition and the water concentration in the mixture [2]. As seen in Fig. 2, most of the space is occupied by the tails, typically named as the core region, taking up to 70-80% of the whole thickness of the bilayer. The mass density in the head group layer is significantly higher and the core is very much empty space in terms of material density. The electron density of the head group layer is of the same magnitude as in the normal condensed matter (~$4x10^{23}$ electrons/cm^3 in, e.g. DPPC-lipid vs. ~$7x10^{23}$ electrons/cm^3 in silicon). Together with the polar nature of the head groups, the bilayer is almost impenetrable to polar molecules/atoms, though naturally

enabling osmotic permeation of water through the membrane. At the same time of acting as a barrier between the outside world and the cell, the lipid membrane is a complex heterogeneous structure consisting of hundreds different lipids, whose collective dynamics cause the formation of nanoscale functional areas, called as domains. These domains have a strong role in the different cellular functions (e.g. intracellular trafficking) and it has been suggested that ordered domains, known as lipid rafts govern most of the functionality [3],[9].

The critical parameter characterizing the lipid membrane is the free volume within the core region, as the voids in the core region have a crucial role for a variety of dynamic processes in the lipid membrane. The diffusion in the membrane plane is facilitated by the voids and they have a relevant role in the permeation of small solutes. The free volume could be characterized as concept of volume that is not occupied by any molecule at a given moment, though the concept is not as clearly defined as e.g. a vacancy in an atomic lattice. Its distribution and the amount of the free volume vary across the membrane and as a function of time.

2.2 – Computational modeling of lipid membranes

The structure of a bilayer can be modeled using computational methods such as molecular dynamics simulations [6], [10]. It has also been shown that the distribution of free volume affects the packing and the ordering of the molecules in the membrane [5]. The basis for computational studies on biomaterials was first developed by several groups in the 70's and 80's and more recently laid out by Schulten et al in 1996 [11]. A good overview to the methodology can be found in Ref. [12]. During the last decade computational biomolecular modelling has developed into an invaluable method to study the detailed atomic structure and dynamics of complex biological systems.

Because molecular systems generally consist of a vast number of particles, it is practically impossible to find the properties of such complex systems analytically, unless the study is performed on a statistical level with the means of statistical physics. To resolve this problem, numerical methods must be used at this point of computational development. The most common technique is applying the aforementioned molecular dynamics simulations, where atoms and molecules are allowed to interact for some fixed period of time by using approximations of known physical interactions (e.g. not taking into account the quantum mechanical effects in the calculations, yet the fundamental parameters of the system are derived from quantum mechanics). Essentially the MD simulation consists of numerical step-by-step solution of the classical equations of motion, i.e., '$\Sigma F=ma$'. This type of simulation gives a snapshot type of view into the motion of the particles. The method is based on statistical mechanics and as such well-defined. In some cases (excited states, chemical interactions) the standard MD functions are not sufficiently accurate to reproduce the dynamics of molecular systems. For such more demanding systems "ab initio"-method based on the quantum mechanics (Density functional theory, DFT, overview on the method see, e.g., [13]) could be applied to provide more accurate modeling. Even though the development of new computational facilities around the world and the increase in computing power, a typical MD simulation still consists of only 10^4-10^5 atoms; thus it is always only a small snap shot of the whole system and limited as such. The MD data used in this thesis is based on well-known and validated methods [14],[15]. These methods have been shown to give reliable results on the structure and dynamics of DPPC membranes (and as such it were found to be feasible for these studies) and it was successfully used in earlier studies of the collaboration [5]). The simulations were carried out with the GROMACS package [16].

2.3 – Experimental techniques for biophysical research

Several different experimental techniques can be used for biophysical research and are still under development, originating from fundamental physics and chemistry. Modern biophysics studies interactions occurring at the molecular or cellular level, thus optical microscopy has not been a viable tool for several decades. Luckily the development of electron microscopy techniques and atomic force microscopy has opened up possibilities to see inside a cell. However, as many of the cellular functions take place at the molecular level (in the scale of 1-10nm) and in the normal room temperature which creates substantial thermal noise; even the aforementioned sensitive techniques are not sufficiently accurate or not suited for studies of biological samples, though several transmission electron microscopy facilities are used in studies of biological material in extremely low temperatures, e.g. BIO-TEM at Aalto University. On the other hand in many applications of electron microscopy samples need to be studied in vacuum, which prevents studies of living cells or materials that would need to studied, e.g., in hydrated state (however some techniques enable such studies to some extent, e.g., environmental scanning electron microscopy). Many of the techniques need the sample to be conductive to prevent charge accumulation. These requirements limit the usability of microscopy techniques to mainly non-living samples, or to samples that are taken away from their natural surroundings.

Still, the structural characteristics can be studied in the atomic level by other experimental methods of which three are the most common in the field of biophysics: X-ray scattering techniques, differential scanning calorimeter and nuclear magnetic resonance.

X-ray scattering techniques, Refs. [17], [18],[19] , where the scattering of X-rays reveal information about the electron density in the sample are commonly used for different soft matter studies and even to study hydrated lipids [20]. The most significant drawbacks of this method are the limited resolution in the low electron density areas (in the case of lipid bilayers the lipid core region) and the destructivity of the method; the

intensity of the X-ray flux is so high that sample material typically vaporizes during the measurements. Besides X-ray diffraction, also neutron diffraction techniques have been employed to study material characteristics on biomaterials [21] with greater resolution, down to the Ångström-level. It should be noted that with neutrons, one is only able to probe the density of the nuclei.

Diffrential scanning calorimetry (DSC) is a thermodynamical tool to characterize the changes in the latent heat imported in to the sample system. The method was developed by Watson and O'Neill in 1960's [22] and ready-to-use systems are commercially available from several vendors. The measurements are performed with a reference, to which the heat flux to the sample is compared. The purpose of the method is to find out how much heat is needed to gain fixed temperature change in the sample. One major application of DSC is to study structural transitions, e.g., gel/fluid transitions in lipids [23], [24] or glass transitions in polymers. The working principle is that when the sample undergoes a structural transition, more or less heat will be needed to maintain same temperature as in the reference. Regardless of the type of the transition (exo- or endothermic process), the difference in heat flux occurring at a specific temperature provides information about the structural change. DSC is simple to use and reasonably fast, however as the method is based on measuring the changes in the temperature in the system, the sensitivity is limited at the microscopic scale and sensitivity is in essence also affected by the thermal characteristics of the studied subject.

Nuclear magnetic resonance (NMR) spectroscopy is a technique involving the magnetic properties of certain radiotracer nuclei (e.g. ^{3}H or ^{13}C) inserted in the sample material [25]. NMR is a property that a magnetic nucleus has in a magnetic field when electromagnetic pulses are applied to the nucleus causing the nucleus to absorb energy from the electromagnetic pulse. This absorption occurs at a specific resonance frequency that depends on the strength of the magnetic field and other factors e.g. the magnetic shielding effect of surrounding electrons. Measuring the absorption allows the observation of specific magnetic properties (e.g. angular momentum) of the nuclei, which are affected by the chemical surroundings inside the sample material. Depending

on the local chemical environment, different protons in a molecule resonate at slightly different frequencies that can be measured (in NMR spectroscopy this measurable quantity is known as chemical shift). This information can be used to gain information about the molecular structure of the sample. One of the goals in NMR is the possibility to gain 3D structural information of, for example proteins, or characterizing the diffusion parameters trough membranes. The method requires implantation of an artificial radiotracer-nucleus into the sample and the effect of this perturbation to the sample is ensured to be negligible. And as it is vital to get the radiotracer inside of the studied structure, some membrane types cannot be studied with NMR. The diffusion of the solutes occur only (or if at all) trough specified ion channels and the diffusion in the membrane plane is limited, thus information is gathered only from the vicinity of the diffusion path which limits the usability of the method.

Chapter 3 – Positron Annihilation Spectroscopy

In this chapter the basic principles of positron annihilation spectroscopy are introduced as well as the basis for the measurements in biomaterials. Studies on crystalline material have been performed routinely i.e. in the positron group at the Aalto University (former Helsinki University of Technology) and the understanding of positron processes on crystalline or semi-crystalline materials are well known. However when applying positron annihilation spectroscopy to soft matter research, i.e., disordered materials, new reactions and novel concepts arise. Whereas the positron annihilation process in the crystalline solids, e.g., silicon, is a well understood quantum mechanical process and produces easily interpretable discrete results both from computational and experimental point of view, the case for the disordered materials is quite different and vastly more complex, bringing up not only new physical phenomena such as significantly larger fraction of positronium formation but as well chemical interactions such as positronium oxidation [26].

Though it is relatively easy to study theoretically/computationally the voids in lipid bilayers, direct experimental information on the subject has been harder to achieve. Based on the extensive studies of positron annihilation lifetime spectroscopy on polymers (e.g. [27] or thorough introduction to different applications on Refs. [26], [28]) where free volume pockets of the order of 0.1 nm^3 in polymers are routinely studied, it can be assumed that positron lifetime spectroscopy can also be applied to probe the free volume pockets inside bilayers as well where the void sizes are in 0.1-10 nm^3 range. Studies performed by Jean et al. in early 1980's showed that positron lifetime spectroscopy can indeed be used to characterize the changes in free volume occurring in a phase transition of lipid bilayer, but the interpretations were constrained by the limited understanding of the bilayer structure prior to the modern MD techniques [29, 30]. During the last 30 years positron annihilation spectroscopy has been applied to several different materials that resemble biological membrane. More over, several studies on artificial membranes are performed that reveal similar phase behavior as in

biological membranes [31]. For a reason unknown to the author, the research on biological membranes stopped in mid 80's and further development on positron studies or theory on biological membranes has not occurred until know. In addition, several studies are published in related materials, ranging from studies on micelles [32] to porous chemical structures such as cyclodextrines [33]. Different approaches and examples over the years can also be found in the proceedings of both International Conference on Positron Annihilation (ICPA) and Positron and Positronium Chemistry (PPC)-conference series.

3.1 – Positron Annihilation Lifetime Spectroscopy

In positron annihilation lifetime spectroscopy the focus is on the accurate measurements of the time difference between the decay of the positron source and the detection of the annihilation gamma quanta. When using ^{22}Na-atoms as a positron source, the decay and the almost simultaneous birth of a positron can be detected via a 1.27 MeV gamma quantum released in the process. When a positron annihilates, depending on the annihilation process, it will in most cases (>99% in normal material research) produce two identical annihilation gamma quanta with energies of approx. 511 keV each. Due to the conservation of the momentum, as the positron and electron have been almost still during the annihilation process, the resulting gamma quanta will have almost the opposite momenta thus travelling opposite directions. The minor difference in momentums compared to fully parallel trajectory can be used to study the chemical surroundings of the positron annihilating with the electron in Doppler broadening studies see, e.g., Ref [34]. Positron annihilation occurs from different states. The probability for a single positron being "alive" at a moment **t** is a sum of probabilities of annihilations from different states **i**:

$$n(t) = n_0 \sum_i I_i e^{-\lambda_i t} \quad , \tag{1}$$

where I_i is relative intensity of the respective decay constant λ_i and n_0 is the total number of observed annihilation incidents. The measured lifetime spectrum is the time derivate of Eq. (1), i.e., the probability for annihilation occurring at the moment **t**. The term positron lifetime is defined as the inverse of the decay constant $\tau_i=\lambda_i^{-1}$ (not for the lifetime of a single positron).

Positron lifetime data can then be gathered by using the detection of decay gamma quantum as a start signal and then the detection of either of the annihilation gamma quanta as a stop signal. This time difference is known as the positron lifetime as the time delays for the detection of decay and annihilation quanta signals are similar in both

cases. For the detection, scintillation detectors are used due to their simplicity and cost efficiency. In the studies presented in this thesis, plastic scintillation heads are used. Scintillation detectors are coupled with photomultiplier tubes (PMT) to convert the scintillation light signal to electrical signal [35]. A more thorough description on the equipment and methodology can be found in Section 3.4.

Depending on the setup type (analogue or digital) the electrical pulses from PMT's are then analyzed for the pulse height to determine START/STOP-signals. From this data the positron annihilation spectrum is collected in the form of histogram to produce the positron lifetime spectrum presented in Figure3.

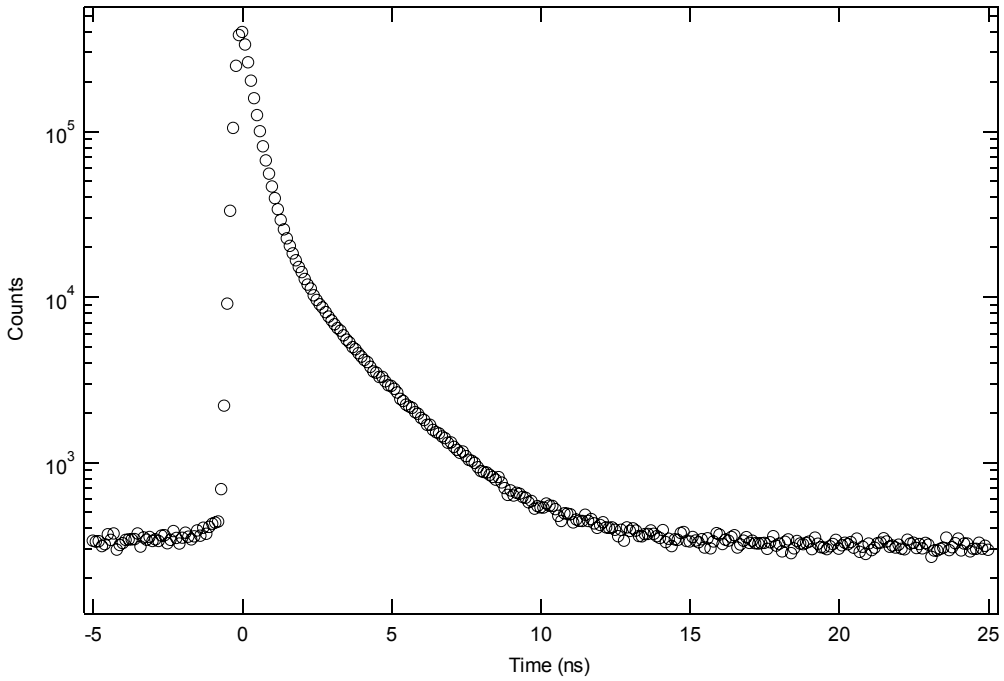

Figure 3. Raw *annihilation histogram gathered from a SM-lipid sample showing the spectrum of measured time differences in the sample.*

3.2 – Positronium

When a positron emitted from a radioactive isotope (typically ^{22}Na in lifetime studies) interacts with the sample material, it will travel a short distance in the material, losing kinetic energy in collisions with the sample material atoms. This process is known as stopping and thermalization and will take few picoseconds for a positron in condensed matter from a ^{22}Na-atom with mean energy of ~180 keV [26] and can be as long as 100 ps in soft matter. This results in a range of approximately hundred micrometers (0.1mm) in condensed matter such as silicon or a range of ~0.3 mm in water. These ranges can be calculated based on the empirical particle range model by Katz and Penfold [36]. After thermalization, the positron can annihilate as a free positron[1] together with an electron after diffusing in the material for some hundreds of picoseconds. Or in special cases positron can form a meta-stable state Positronium with the electron, annihilating later on. The Positronium-state can be viewed as a hydrogen-like atom, where the proton is replaced by the positron.

Typically the electron density in condensed matter is so high that almost every positron annihilates as free positrons and only a small fraction (due to e.g. shadowing effects) of positrons form positronium, typically near large pockets of free volume (e.g. voids) or surfaces. A thermalized positron can not form positronium with one of the bound electrons in a material due to the low binding energy (6.8eV) of an electron in positronium compared to ordinary molecules where it can be 9-12eV. Thus positronium formation occurs mostly only with free electrons.

In this thesis the formation of positronium (Ps) is interpreted to occur by the Spur model introduced by O.E. Mogensen in 1974 [37]. In the spur model, while stopping and thermalizing, the positron will form spurs of residual ionized atoms and free electrons due to the release of the kinetic energy in the collisions with individual atoms. This process lasts until the positron does not have energy for additional ionizations and the

[1] here ’free’ means free from being bound to an electron

rest of the thermalization occurs just by the in-elastic collisions. This state is called the terminal spur, where the positron is now thermalized and surrounded by free electrons and ions (Fig 4.).

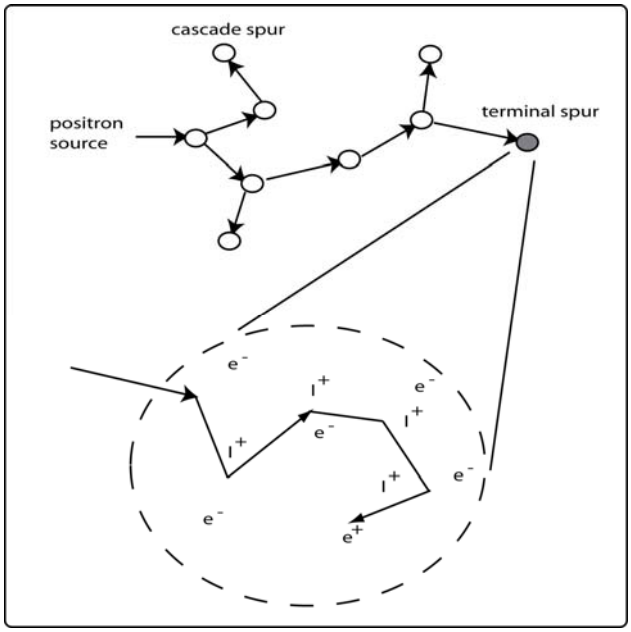

Figure 4. *Illustration of the trajectory of positron in soft matter. During the thermalization positron will create free electrons and ions, which can have their own trajectories and produce cascade spurs. When the positron energy is not sufficient for additional ionizations, it will stop in the terminal spur for final thermalization.*

In the terminal spur the positron has two possibilities; either it goes through the normal annihilation or it forms positronium. The meta-stable bound state has two states, para- and ortho-state which depend on the spins of the electron and positron. 25% Ps forming is in the para-positronium state (singlet), with the spins of the two particles antiparallel (total spin=0), resulting to a quick self-annihilation producing two 511 keV annihilation gamma quanta. Rest of the formed positronium-atoms are in ortho-state (triplet), where the spins are parallel (total spin=1). This state results to a longer lifetime (due to the electromagnetic theory [28]) with a three-gamma annihilation with continuous distribution of energies to each gamma quantum due to the spin conversion. The

lifetime for para-positronium (p-Ps) and ortho-positronium (o-Ps) in vacuum are approx. 125 ps and 142 ns, respectively. In a medium, the o-Ps prefers to undergo the so called pick-off annihilation with an electron of opposite spin during collision with molecules in the material in which they are localized. The pick-off process reduces the lifetime of o-Ps from 142 ns down to a few ns and produces easily detectable gamma quanta. In material studies, the pick-off annihilation lifetime of o-Ps is the one which provides the most relevant information about the material properties. In the self annihilation of p-Ps a narrow Doppler broadening spectrum is produced, i.e., the energy distribution of the 511 keV annihilation gamma spectrum is tight. In pick-off process the o-Ps picks the electron from the molecular matter, thus having a different momentum distribution resulting in a wide energy spectrum.

In molecular materials the Coulombic repulsion and the exchange repulsion (due to the exclusion principle of electrons) of the polar Ps-atom can create a bubble-like empty space surrounding it. If the local material density is low enough creating a temporary cavity in the material. The typical size of this o-Ps bubble is in the range of 6-8 Å in diameter in solutes. In denser molecular matter the bubble can be smaller [28], but the lowest limit to the size is the actual size of the o-Ps atom, ~1,1Å in diameter [38].

The pick-off annihilation lifetime of o-Ps is inversely proportional to the overlap of the surrounding materials electron wave function and the positron component of Ps wave function and thus it has a correlation to the mean size of the void where the electron density is low. In polymers and in biomaterials this site can also be called as a free volume pocket or a void and the size of such space (spherical shape assumed) can be estimated from the o-Ps lifetime (τ_i) using the so called Tao-Eldrup model for the positronium trapping [39], [40]. The pick-off lifetime is assumed to be inversely proportional to the overlap of the wave function of electron in the surrounding material and to the positron wave function component of o-Ps wavefunction. Thus the lifetime can be correlated to the size of the low electron density site where the annihilation takes place:

$$\tau_i(ns) = \frac{1}{2}\left[1 - \frac{R}{R+C} + \frac{1}{2\pi}\sin\left(\frac{2\pi R}{R+C}\right)\right]^{-1}, \quad (2)$$

where R is the mean radius of the free volume pocket, C a semi-empirical constant for which a value of 1.66Å has been found valid for polymers [41]. The o-Ps in a free volume site is approximated by a particle in spherical potential well of radius R+C. Different extensions for large pockets [42] and cylindrical voids [43] exists. Further research is carried out for finding similar correlations between the o-Ps lifetimes and size of the voids in biomaterials. Typically several different processes, both chemical (e.g. oxidation reactions) and physical (e.g. inhibition) affect the o-Ps lifetime and contrary to the discrete lifetimes of free positrons in solids, o-Ps lifetimes are rarely discrete but exhibit more or less wide distributions. For the sake of clarity, typically the median value of the distribution is used as the respective o-Ps lifetime component in analysis and interpretations.

3.3 – PALS on Lipid Bilayers

In this study, the analysis of annihilation events is performed digitally using the Agilent (formerly Aqciris) DP240-Digitizer to convert the pulses gained from the PMTs to a digital format for pulse height analysis. The basic principles for the digital lifetime setups can be found in the following references; [44], [45] and [46]. In the simplest form the digital lifetime spectrometer consists of a scintillation detector pair and a logical circuit (gate-module), which detects coincidences in the detector data and gives triggering signal to a digitizer card to save pulse data for further analysis on the measurement computer. For the purposes of efficient biomaterial studies a novel digital lifetime setup was developed and explained in greater detail in Section 3.4 and [Publ. I].

Evaluation of the lifetime spectra in the samples studied in this thesis has been performed by using semi-commercial and well-documented analysis programs such as PALSfit [47] for the characterization of the resolution function of the measurement setup and MELT [48] for resolving the lifetime component distributions in the spectra. In a typical spectrum 3-4 lifetime components (τ_i) can be resolved. In lipid samples τ_1 originates from the annihilation of free positrons and p-Ps, τ_2 is a component related to o-Ps annihilations in the sample material, τ_3 correspond to a o-Ps lifetime in either water, which is always present in the biological samples, or depending on the time resolution of the setup, τ_3 can be characterized as an intensity weighted sum of the lifetime in water and lifetime inside the lipid bilayer (τ_4). For the sake of clarity, the intensity weighted sum lifetime in measurements with poor time resolution, is denoted as $\Sigma\tau_3$. In table 1, the lifetime distribution values obtained using MELT from a (typical) lipid bilayer/water-mixture are presented.

Table 1. *Lifetime components extracted from a DPPC lipid bilayer sample. Lifetime values correspond to a mean value of the distribution, which is assumed to be Gaussian and the FWHM is presented in the table. From the FWHM it is clearly seen that the free positron and p-Ps lifetimes (τ_1) are fairly discrete compared to the o-Ps lifetimes (τ_2 and $\Sigma\tau_3$). The literature value for o-Ps in water is approx. 1.8 ns [*28*], and thus it is easily seen that the $\Sigma\tau_3$ is a sum of that and of a much longer component τ_4.*

Lifetime component	*Lifetime (ns)*	*Relative Intensity%*	Distribution FWHM (ps)
τ_1	0.366	71.4	42
τ_2	1.036	9.7	217
$\Sigma\tau_3$	2.321	18.9	502

When performing positron lifetime studies with ^{22}Na positron source injected in to the sample as NaCl-solution the Na^+ ions do not penetrate the bilayer, due to the strong potential barrier the head groups pose to the charged Na-ions. Because of the high kinetic energy of the emitted positrons, they can however penetrate some number of bilayers during the thermalization, causing minor radiation damage to the system.

There is no pre-existing theory or model on o-Ps interactions or formation in lipid bilayers, however based on an existing understanding of similar reactions in polymers following hypothesis has been developed by the author and as such it is still more or less just a working theory:

After the positron has lost most of its energy, it will interact with the bilayer structure in a similar fashion to any soft matter. A fraction of positrons is almost completely

thermalized after penetrating the head groups of the bilayer for the last time. However some of them are still able to ionize some atoms in the structure. This interaction will finalize thermalization of a specific positron, leaving the positron in to the terminal spur inside of the bilayer core as illustrated in Fig. 5. In the spur, positrons interact with the surroundings via different processes, e.g., positron-positive ion (aka. positron scavenger)-interaction and only a fraction of the positrons will form Ps-atoms. Based on the experiments, a typical Ps-yield in biomaterials is of the order of 30-40%, the same order of magnitude as Ps-yields in polymers.

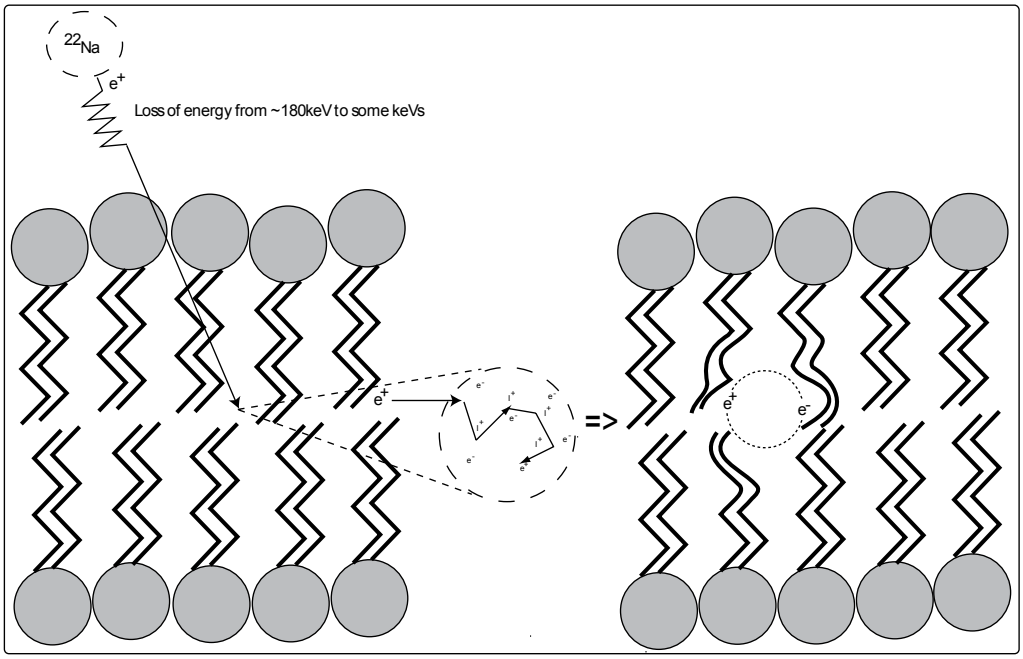

Figure 5. *Ps-formation in lipid bilayer structure. First the positron will thermalize to almost zero kinetic energy in several collisions with the sample material. During the final stages of thermalization the positron has no more kinetic energy to escape out of the bilayer when penetrating the head group layer (marked in gray). In the terminal spur, some fraction of the positrons will eventually form o-Ps atoms, possibly forming a bubble and skewing the structure slightly.*

After the Ps-formation, the o-Ps atom might create a bubble due to the short range repulsion with hydrocarbon tails. This phenomenon could to some extent skew the free volume data based on Eq. (2), but as the effect can be assumed to be similar for every o-Ps atom inside the bilayer, it can be interpreted as a systematic error, inseparable from other error sources in the measurement. The tail group, which consists of strongly bounded molecule chain, is definitely different compared to pure liquids (weaker molecular bonds between each molecule) from which the bubble-model originates. This could mean that the weak repulsive surface tension of an o-Ps molecule can not "push aside" the tail molecules and as such bubble formation is inhibited, or at least the bubble size would be significantly smaller than in liquids (~6-8 Å in diameter). The effect, or more importantly the magnitude of it, i.e., whether bubble formation occurs at all among tail groups would need the simulation of o-Ps-atom inside the lipid bilayer using computational methods but such work is still in progress and at this moment such interpretation is not possible.

In addition, depending on the phase and dynamics of the core region, the interaction frequency with the tails dictates the pick-off lifetime. In the gel phase the tails are highly ordered and densely packed, thus opposing the bubble formation even more. In addition, the smaller number and size of the voids in the core increase the local electron density surrounding the o-Ps atom, increasing the pick-off probability. In the fluid phase, the voids are bigger and the tails move more freely, thus decreasing the pick-off probability because the local electron density around the o-Ps atom decreases. As mentioned earlier, our hypothesis is that as the electron density and packing ratio of the head group layer is high and the layer acts as a barrier for the Ps, preventing escape from the bilayer by any Ps-atom forming inside the bilayer. On the other side near the head group layer, the pick-off probability increases significantly resulting in a small lifetime. The o-Ps atom trapped inside the bilayer yields information about the structure of the bilayer, say whether the bilayer is in the gel or fluid state, or whether some dopants have affected the free volume parameters inside the bilayer.

Lifetime experiments were performed by preparing samples of DPPC-lipid in water with increasing molar concentrations to be certain that the lifetime results really provide information on the bilayer structure. It was assumed and later experimentally verified that the o-Ps lifetime in water is relatively stable (~1.8ns±0.05ns) and the free volume distribution in water is uniform, as there are no relevant free volume pockets in pure water. Then again, the lipid bilayer has pockets of free volume and these voids would be seen in the data as a new lifetime component (τ_4). The sample materials were prepared inside plastic test tubes; identical test tubes have been used on all of the experiments to keep the results comparable, as a minor fraction of the o-Ps atoms will annihilate inside the test tube walls and contribute slightly to the o-Ps lifetime distribution. The fraction of the o-Ps annihilating in the walls is estimated to be less than 1% of the overall o-Ps yield.

The results of the preliminary experiments are shown in Fig. 6. They show that the Ps-spectroscopy is sensitive to lipid content and that it is possible to measure lipid bilayer samples and gain relevant information on them. When the lipid concentration in the mixture increases and closes to 80% concentration, the lipid packing ratio changes in head group affecting the tail groups as well which creates excessive and/or slightly larger free volume pockets that can be seen on the prolongation of τ_4. When calculating the intensity weighted lifetime average, it is clear that the lifetime increases at higher lipid concentrations, as the amount of free volume in the system increases.

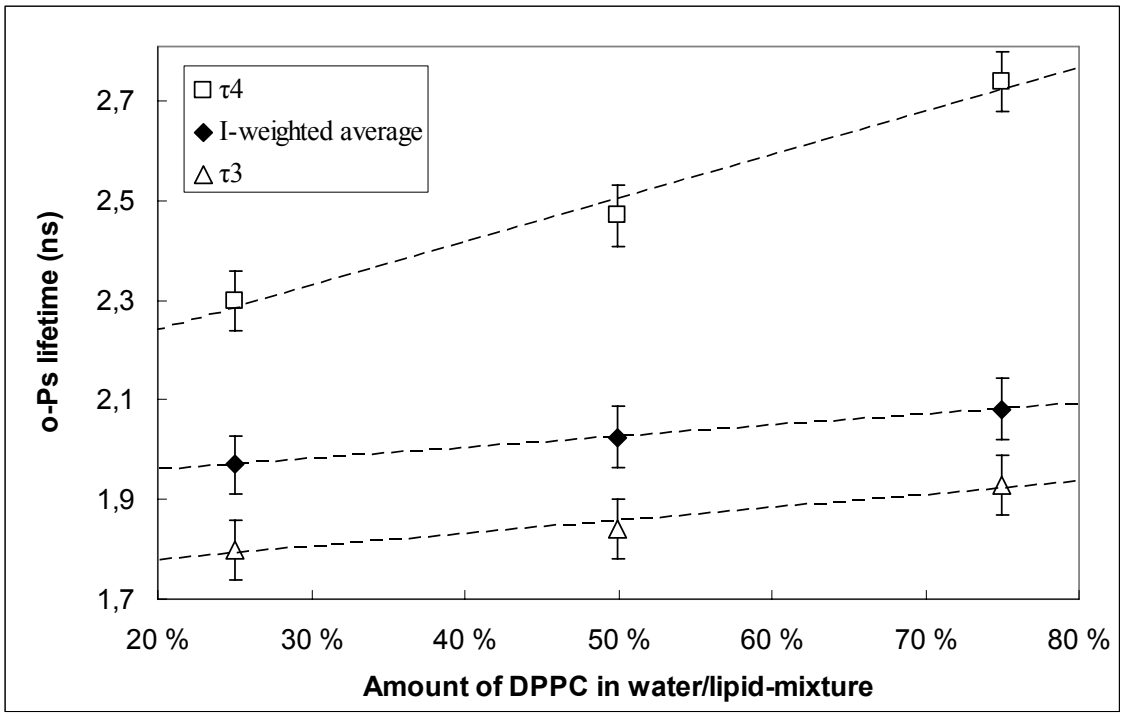

Figure 6. *The intensity weighted average of τ_3 and τ_4 shows that the free volume in the system is increasing generally when increasing the lipid concentration. Lines between markers are just a guide to the eye.*

The above experiments show that positron annihilation lifetime spectroscopy can be used to probe the free volume parameters inside the bilayers. One of the relevant questions is, whether the o-Ps atom will be localized in the premises of its formation or will it diffuse inside the bilayer during its lifetime. In late 1990's a debate arose regarding this behavior in polymers, where Yu et al [49] argued that Ps-atoms would tunnel into many different holes thus skewing the relation of lifetime and free volume completely. However Jean and Baughner, [50] and [51] respectively, later showed that the premises for Yu's calculations were false. Moreover based on the experimental diffusion coefficients, o-Ps atom would generally diffuse only a distance of 20 Å during its lifetime, thus trapping itself quite definitely into a single void. Baughner calculated the tunneling rate for o-Ps atom between potential wells of different sizes and it was found out that only in the case of nearly identical voids the tunneling rate is relevant.

Thus, tunneling between voids of different sizes does not occur. In a lipid bilayer, where the voids are continuously changing and mostly not spherical (that is the structural distribution of the voids is not uniform), these findings can also be interpreted in a similar fashion, i.e., the tunneling rate of o-Ps between the voids in lipid bilayer is extremely low and the overall concept of tunneling is negligible. Ps-formation may not always occur within the voids but due to the Coulombic forces it is likely that the Ps-bubble would be pushed into the closest void as the material density in such places is lower.

Hence, based on the current understanding of the phenomena, it seems to be possible to characterize changes occurring in the bilayer structure with positrons. Being able to extract specific free volume parameters from the lifetimes (τ_4 or $\Sigma\tau_3$) is however still out of the scope of this work and current level of research.

In regards of the radiation damage, most of the possible radiation damage to the lipid bilayer is caused by the thermalizing and stopping positrons. The interaction rate of gamma quanta is insignificant in such low density material. The typical activity of the source injection was approximately 1-2 MBq (with a ^{22}Na half-life of ~2.5a), causing roughly 5 Sv equivalent radiation dose to the samples during the average ~24 hour measurement cycle. The dose is reasonably high, tenfold the recommended dose for, e.g., the human skin [52], or 30 times more than allowed for the human lens. However, the mean positron energy of 180 keV is not high enough for severe microstructural damage that would skew the results significantly during the measurement period. During the measurements o-Ps lifetimes indicate no effect of radiation damage in lipid membranes within the 24 hour measurement period. The inevitable contamination of the sample during the positron studies is perhaps the largest drawback as samples can not be used in additional studies with alternative experimental techniques after positron studies.

3.4 – Digital 4-Detector setup for PALS studies

Development of fast digitizers and the increase in computational resources in personal computers has made it possible to perform spectroscopic measurements digitally. Before positron lifetime measurements, digital techniques have been applied in the field of Nuclear science for some pulse-height (for example [53]) and time-interval spectroscopies (for example [54]). In the field of positron spectroscopy the development towards digital spectrometers begun in early 2000's both at the Helsinki University of Technology [44] and the University of Tokyo [55], independently of each other. After these pioneering studies, further development has been carried out on, e.g., ultra-fast digitizers at Charles University in Czech Republic [56]. Currently, two types of digital lifetime setup are dominantly used, one based on the use of digitizer card inside the PC and the other using a digital oscilloscope from which the digitized data is transferred to the PC. From the hardware point of view, the use of the digitizer is simpler, however as the state of the art digitizer-cards typically have a smaller sampling rates (frequency of samples taken from the pulse) than digital oscilloscopes the digitizing resolution is a bit lower. By the development of the digitizers in recent years the difference in resolution is negligible as meanwhile the accuracy of the digitizing has increased from 8 to 12 bits in digitizer cards. The main components in time resolution are both the photomultiplier tubes as well as the structure of the measurement setup, i.e., how well the random coincidences are filtered by either the means of the analysis software, by using more than one scintillation detector to detect parallel annihilation quanta or by adjusting the detector geometry to minimize the unnecessary background. Further development has also been performed to improve the time resolution by, using the Cherenkov radiation created by the thermalizing positron (a highly energetic positron from a ^{68}Ge-isotope) as a START-signal and then using several scintillation detectors to detect the annihilation quanta [57]. However, improving the time resolution to infinity is not relevant in many cases. Typical time resolutions of ~0.250ns FWHM of the resolution function of the setup are sufficient for most of the studies. The decrease (=improvement) of time resolution comes usually with the price that the measurement efficiency decreases dramatically. In a typical lifetime spectrum at Aalto University, some 10^6 lifetime

events are recorded with a normal counting rate of 100-800 lifetime events/s, the highest values being enabled by latest development on the digitizing software. These parameters will provide reasonable timing accuracy within a reasonable measurement time, making the actual materials research more feasible.

A normal positron annihilation spectrum measured with large scintillation heads (SHs, resolution function FWHM ~0.5 ns, physical volume ~55 cm^3) in a biomaterial (lipid bilayer in this case) consists of three lifetime components as shown in Table 1. With smaller heads (FWHM~0.270 ns, V=24 cm^3) it is possible to find one component more, τ_4, which corresponds to the o-Ps annihilation occurring inside of the bilayer. As the o-Ps lifetime in water stays reasonably constant during, e.g., temperature changes the general effect of an annihilation rate change in the biomaterial to $\Sigma\tau_3$ is smaller than it should be in terms of the actual lifetime prolongation due to, e.g., increasing free volume. With smaller scintillation heads, it is typically easy to separate the biomaterial specific lifetime component τ_4 independently thus giving more precise information on the changes in free volume parameters. On the other hand as biomaterials often degrade rapidly over the time, doing separate measurements with good statistics and resolution is out of the question. The use of a digital positron spectrometer with two separate detector pairs and a dual channel digitizer card (8 bit Agilent DP240) make it possible to measure both types of spectra (high and low resolution from different detector pairs) simultaneously with minimal hardware requirements. Constructing the system in the analogue mode (using conventional timing electronics) would require substantially more electronics that are both expensive and furthermore, in the year 2010, quite hard to acquire. At the present status the system consists of two detector pairs, two coincidence modules (gate-module) and a digitizer card in a measurement computer. The design on the setup is based on earlier setups [45], with necessary modification to the original design [Publ. I].

When either of the two START-detectors (say pair 1) detects a 1.27 MeV quantum, a pulse is formed and the gate-module time window opens. If the STOP-detector of the same pair detects an annihilation quantum within the fixed time window (100ns in this

case), the gate-module will pass a triggering signal to the digitizer at the same moment it detects the pulse from the STOP-detector. The pulse combiner simultaneously combines the START and STOP-pulses with a fixed cable delay for the prevention of overlapping of the pulses which makes the pulses impossible to digitize separately.

When triggered, the digitizer sweeps inputs for a fixed time period (300ns in this case) and saves the input data from both inputs, which consists of both the START and STOP-pulses. The data from the inputs is first digitized and then further analysed with a self-made application Digilife [46], [45], to discriminate the false lifetime events. The discrimination is made in several steps including the pulse height comparison and energy window settings for energy discrimination and the timing is performed with the constant fraction smoothing spline-method [45] to gain actual START-STOP-pulse time differences, i.e., lifetime events. The lifetime events gained from input 1 (see Fig 7.) are saved to the lifetime spectrum corresponding to the detector pair 1. The connection diagram for the 4-detector setup is presented in Fig 7. For additional filtering, the angles between the detectors are fixed to specific angles differing from 90° to reduce the possibility of detecting “false” annihilation quanta. The angles (75°, 85°, 115° and 85° counting counter clockwise from STOP 2, see Fig. 8) are chosen so that no two detectors are parallel.

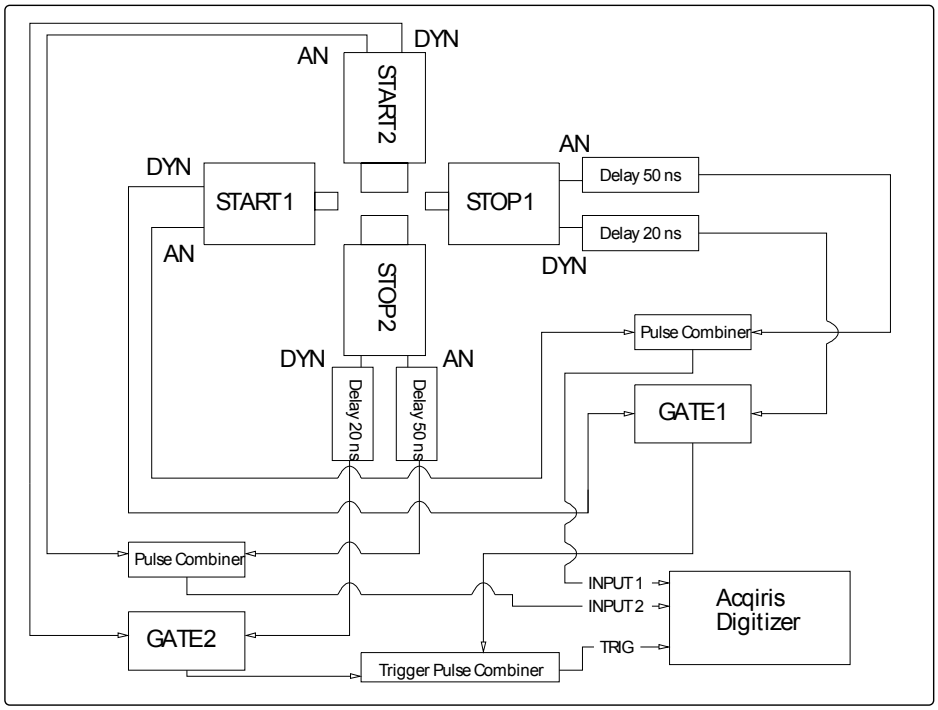

Figure 7. *Operation principle of the 4-detector setup. The anode signals from the PMTs are used for the analyzed data (going to inputs) and the dynode signals are used for the triggering with gate module. The signal cables are 50 Ω coaxial cables and pulse combination is performed with impedance fitted power splitters. Drawing by J. Heikinheimo.*

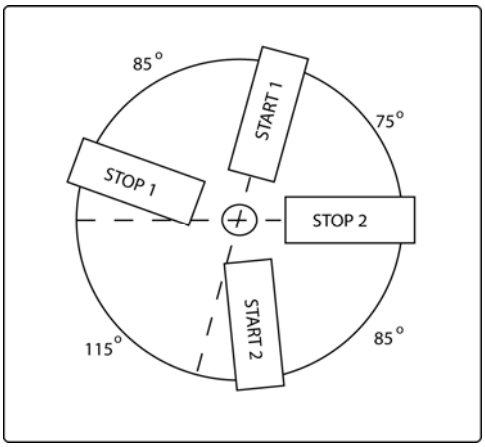

Figure 8. *Presentation of the detector geometry in the setup.*

As shown in Table 1 the o-Ps lifetimes of lipid bilayers and water can be mixed if the time resolution is not adequate. The results from a lipid/water-mixture measured with the 4-detector setup are presented in Table 2 and it is clearly visible that the enhancement in resolution makes it possible to separate the material specific lifetime components from $\Sigma\tau_3$. However, as seen in the count rate, the detection efficiency is significantly lower in the detector pair with smaller SH's, however recent development of the digitizing software has increased the count rates so significantly that even medium sized SHs provide reasonable measurement efficiency with much higher resolution than with very large SHs, which are used in the studies in [Publ. II and III]..

Table 2. *Measured lifetimes of Sphingomyelin-lipid/water mixture (very low source activity) with 4-detector setup using medium sized scintillation heads and small scintillation heads. The measurements were performed simultaneously in the geometry presented in Fig. 8 with detector distance to the sample being defined by the temperature shielding of the sample (~5cm detector to detector).*

SH size	Ø45x35mm	*Ø35x25mm*
FWHM	~0.370 ns	~0.270 ns
τ_3	2.19 ns	1.89 ns
I_3	20,4 %	17,8 %
τ_4	-	3.14 ns
I_4	-	4,4 %
$\mathbf{\Sigma(\tau_3 I_3+\tau_4 I_4)}$	2.19 ns	2.14 ns
Count Rate	68 cts/s	18 cts/s

Chapter 4 – Probing biomembranes with positrons – proof of concept

In this chapter some examples of applying positron lifetime spectroscopy to study biomaterials are presented based on the work in Publ. II. The focus of this work is to present positron spectroscopy as a viable tool for biomaterial characterization and present results obtained by performing positron lifetime measurements on different lipids. In the framework of the research collaboration in this thesis, molecular dynamics simulations are used to provide additional theoretical insight to the interpretation of the results obtained with positron measurements. By coupling atomistic simulations to positron experiments, one is provided with a means to characterize the void sizes in a quantitative manner.

Most importantly, as the studies presented in this work are more or less the proof of concept of the method, further work on the theory of Ps-interactions in biomaterials is under way. The aim of the theoretical work by Dr. A. Zubiaga at the Department of Applied physics in Aalto University is to validate the hypotheses of Ps-behaviour presented in this thesis as well as to create new theoretical models for quantitative free volume information based on the experimental o-Ps lifetimes. This theory project has just been launched during the final stages of this thesis and thus at this point no major interpretations can be made.

4.1 – Phase transition in lipid bilayers

Considering the phenomena of phase transition in lipid bilayers and applications for manipulating the free volume parameters, it is of exceptional interest to understand how the total amount and size distribution of the free volume pockets depend on thermodynamic conditions and molecular composition. However, it is exceedingly difficult to gauge the free volume pockets in membranes through experiments in a non-perturbative manner. The techniques commonly used are largely based on probes such as fluorescent markers, which inevitably cause major perturbations in the vicinity of the probe. Alternatively, neutron and X-ray scattering techniques yield information of mass and electron density distributions respectively that may provide some insight into average free volume distribution in a sample. However, such methods do not directly characterize local free volume distribution, which would be decisive to understand diffusion phenomena

The positron lifetime measurements were performed by injecting ^{22}NaCl (radioactive positron source) in an aqueous solution into a test tube containing multilamellar vesicles in purified water with lipid to water ratios varying from 1:3 to 3:1. The concentration of NaCl (<0.01 μM) was low enough not to have a significant effect on the properties of the lipid membranes. The temperature of the samples was controlled by placing the test tube in a heat bath and the temperature of the bath was controlled with commercial Lakeshore TC331 Temperature controller. As the temperature inside the sealed test tube cannot be measured during the measurements to prevent contamination, temperature inside the sample test tube was tested prior measurements by calibration measurements. In the calibration measurements the test tube was filled with purified water and a thermocouple sensor was placed in to the water via a hole drilled on the cap of the test tube, the hole itself was sealed with elastic plastic. The temperature of the heat bath was fixed to several different temperatures and the corresponding temperature inside the test tube was measured at the respective temperatures. From this linear correlation the sample temperature inside the test tube can be approximated with an accuracy of 0.1°. However it must be pointed out that the actual temperatures are not really relevant to

such accuracy, the more important is that the changes in the test tube temperature were directly comparable to changes in the heat bath with an error of less than 0.05°. Based on these calibrations, it can be concluded that the temperature inside the sealed test tube is well controlled and known with a reasonable accuracy and the changes of the temperature can be controlled with a greater accuracy. Similar calibrations have been performed in all of the latter measurements, where the sample materials have been measured inside sealed test tubes. As mentioned earlier, the type of the test tube has been the same in all of the measurements and several studies, e.g., temperature scans with ^{22}Na-active water injected in to the test tube were performed to obtain information about the presence of the o-Ps lifetime component originating from the formation of o-Ps within the test tube walls. All of these studies showed no observable effect (either decrease or increase) in the only found o-Ps lifetime component, which corresponded well to the literature value of water.

Dipalmitoylphosphatidylcholine (DPPC) was purchased from Sigma Chemical (St Louis, MO, USA) and the lipids were used without further purification. All sample processing prior measurements were performed by J. Holopainen from the University of Helsinki. The main phase transition temperature of the single-component lipid bilayer is ~ 41.5 °C for DPPC. It has for long been assumed that the total amount and size distribution of free volume pockets is different in the two phases, since the packing is likely increased in the fluid-to-gel transition. This idea is supported by a decreasing area per lipid [58] and an increasing tilt [2] as the fluid membrane transforms to a gel one. However, the bottom line is that there have not been experimental methods available to test this assumption directly. Multilamellar vesicles (MLVs) were prepared from chloroform solutions (20 mM) of DPPC, POPC[2] and cholesterol. Appropriate amounts of the lipid stock solutions in chloroform were mixed to obtain the desired compositions. The resulting mixture was evaporated to dryness under a stream of nitrogen, and traces of solvent were removed by evacuation under reduced pressure overnight. The lipid residues were hydrated in purified water at 65°C to yield MLVs with indicated lipid concentrations, and the MLVs were maintained at this temperature

[2] Palmitoyloleoylphosphatidylcholine

for 60 min with subsequent shaking. During hydration, the vesicle-containing solution was vortexed four times.

For the temperature-dependent measurements, large scintillation heads (51xØ51mm) were used for maximal detection efficiency at the cost of resolution. As the sample material was un-sterilized biomaterial, the deterioration of the material in normal room temperatures raised concern and the measurement duration was to be minimized. This resulted in the 550-570ps FWHM of the time resolution. Lipid/water ratio in samples was fixed to 40% DPPC/ 60% distilled water, the ratio in each sample changing slightly when injecting the e+-source (NaCl) in to the test tube, explaining the small differences in the measured absolute lifetimes. Three identical samples were measured with ^{22}Na positron-source activity of 0.75 ±0.1 MBq. The first sample was measured at temperatures from 22 to 51 °C, far below and above the main phase transition temperature to test whether any change might occur in the o-Ps lifetime. To illustrate the difference in the spectra in gel and fluid phases, spectra from temperatures of 22 °C and 51 °C respectively are shown in Fig 9. In the figure the lifetime spectrum is divided to three time regions which are characteristics of different type of positron or positronium annihilation. Of the regions in the spectrum the region III specifies the area where o-Ps pick-off annihilation occurs inside the lipid bilayer.

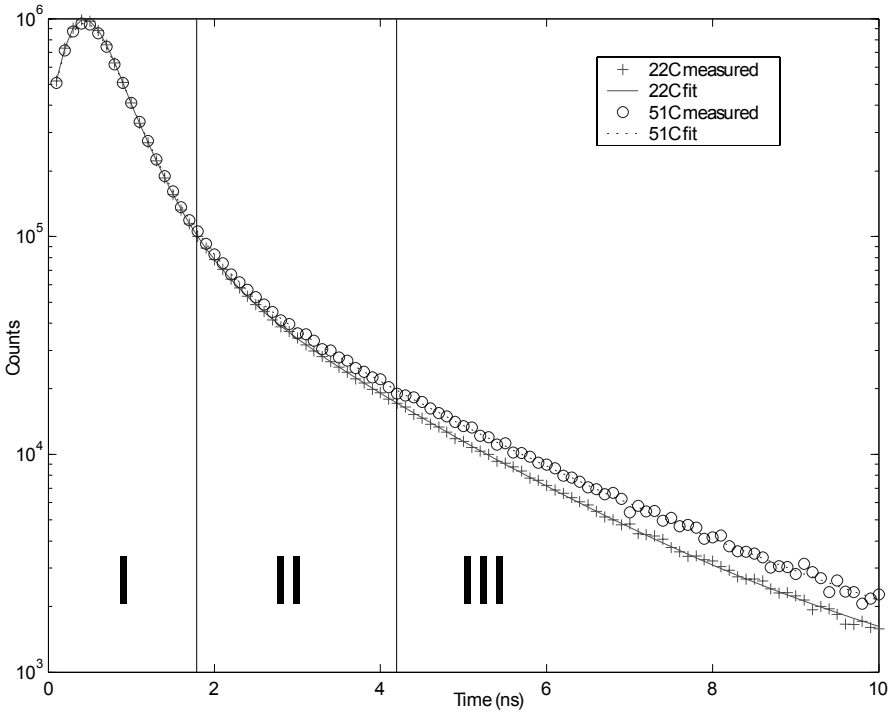

Figure 9. *Measured positron lifetime spectrum at 22°C and 51°C. Region (III) indicates the o-Ps lifetime distribution ($\Sigma\tau_3$), region II being additional o-Ps lifetime and region I being lifetimes of p-Ps and free positrons. The difference in the slope is clearly seen in region III indicating a difference in the lifetimes as seen in Fig. 10.*

The analysis revealed three different lifetime components, the longest of them ($\tau 3$) being the o-Ps annihilation lifetime (see Table 1 for a detailed composition). The temperature dependence of the lifetime component $\Sigma\tau_3$ for samples No. 2 and No. 3 can be seen in Fig. 10 and the molecular dynamics simulation data for the same lipid structure is shown in Fig 11. The lifetime increases slowly with increasing temperature, starting from the room temperature, and at the main phase transition the slope rises rapidly indicating a drastic change in free volume properties in the sample caused by the transition from the gel to the fluid phase.

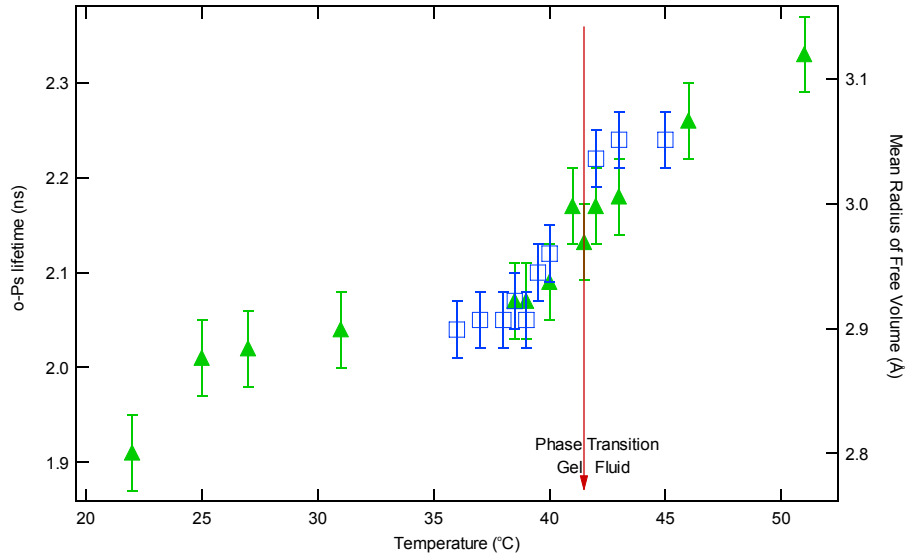

Figure 10. *Temperature dependence of o-Ps lifetime in two DPPC-samples (green, sample No. 2; blue, No. 3). Values for sample No. 2 have been normalized to correspond to the amount of added water in sample No. 3. The position of the main phase transition temperature has been marked with an arrow. Approximations for the mean radius of the void based on Eq. (2) are plotted to the right y-axis.*

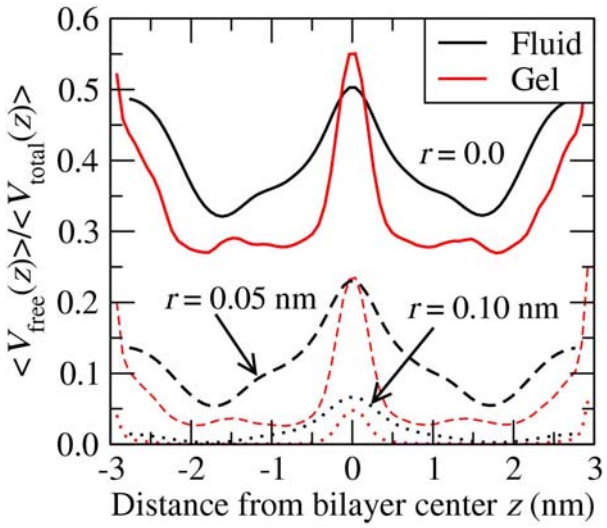

Figure 11. *Molecular Dynamics free volume profile (r=simulated probe particle radius) of DPPC-lipid in gel and fluid phases. The edge of the bilayer is at approx. distance of 1.5-2 nm from the center.*

In Fig. 11 the free volume parameters are calculated along the membrane axis from head groups to head groups showing that as the free volume in the gel phase is mostly confined in the region between the tails whereas in fluid phase the free volume distribution has spread towards the whole core region. Curves in the bottom of the figure show the density of the test particles (sized 0.5 and 1 Å) and it is easily seen that in the fluid phase the amount of larger voids have increased in the bilayer center as well as the smaller voids are more thoroughly present in the core region in generally.

The difference in o-Ps lifetime below and above the primary phase transition temperature, as well as for the sub-transition (structural rearrangement of the gel structure) at 23 °C, confirms that the positron annihilation lifetime spectroscopy is a viable method to distinguish the change in free volume in these lipid bilayer samples. The wide temperature range (40 – 43 °C) of the phase transition in the lifetime data is similar to that observed in polymer glass transition studies with PALS [26],[50]. The difference of the slopes in Fig. 8 in the third region III of the spectra clearly shows that the difference in analyzed lifetimes is not only a matter of fitting and analysis, but a physical phenomenon. The experiments clearly show (Fig. 10) that the lifetime of o-Ps in an aqueous solution of multilamellar DPPC vesicles increases with increasing temperature. At the main phase transition temperature of T_m ~ 41.5 °C, where the membrane undergoes a gel-fluid transformation, the slope rises rapidly indicating a clear change in free volume properties. Similar behaviour has been found earlier by Jean and Hancock [29].

As for void sizes, Eq. (2) gives an average void radius of ~2.9 Å and ~3.0 Å in the gel and fluid phases, respectively (see Table 3). While there is a reason to take these numbers with some caution due to the approximations of the model, the 7% increase in the void size in the gel-to-fluid transition (39 °C → 42 °C) is consistent with the 5% increase predicted by atomistic simulations [Publ. II]. The results presented here are in full agreement with the results from MD simulations, however as the simulations have not been performed by the author these results are not presented here, but can easily be found in Publ. II.

Table 3. Estimates for the corresponding free volume void sizes in gel (plain) and fluid (bold) phases. The volume estimations are based on the Tao-Eldrup model (Eq. 2) for polymers using the Nakanishi's semi-empirical constant of 1.66 Å.

Temperature (°C)	*o-Ps lifetime (ns)*	*Mean Void radius (Å)*	**Mean Volume ($Å^3$)**
39	2,07±0,02	2,91±0,02	104±1
40	2,09±0,02	2,93±0,02	106±1
42	**2,17±0,02**	**3,00±0,02**	**113±1**
43	**2,18±0,02**	**3,05±0,02**	**115±1**

To confirm that the results gained by measuring DPPC-lipid were not just a coincidence, additional measurements on egg-yolk Sphingomyelin(SM) were performed. Since SM-lipids are known to exhibit a main phase transition at ~40°C [59] it seemed reasonable to assume that a similar effect as observed for DPPC in Fig. 10 should be seen with SM.

Measurements were carried out in the same order, mixing the SM-lipid with water in approx. 50/50 mol% concentration and after injecting positron active saline to the sample, lifetime spectra were gathered over a wide range of temperatures below and above the main phase transition temperature. The results are presented in Fig. 12.

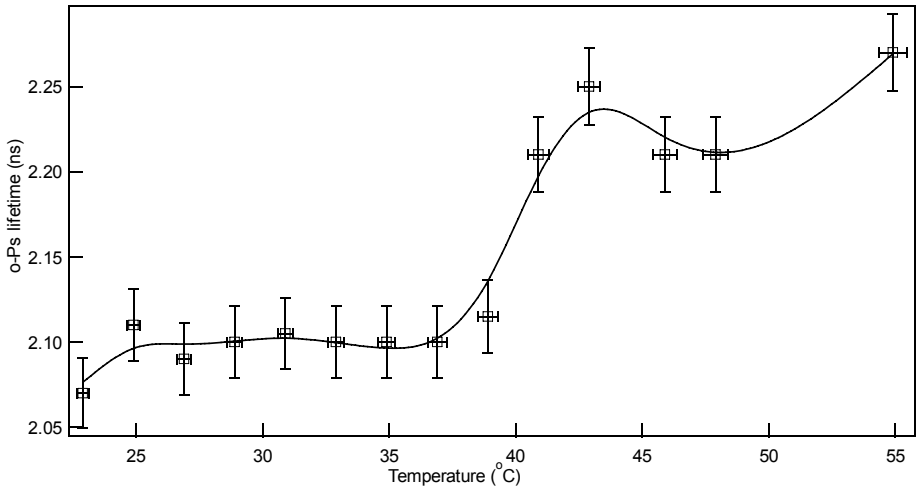

Figure 12. *O-Ps lifetimes ($\Sigma\tau_3$) measured over the main phase transition temperature of ~40°C in Egg-SM-lipid. The measurements performed up/down over T_c and the lifetime results are averaged over both runs, no relevant hysteresis behavior was observed in the data.*

Based on the measurements of phase transitions on lipid bilayer structures with PALS, it is safe to say that positron annihilation lifetime spectroscopy is a viable tool to characterize such transitions. However, due to the time that is needed (~24h) for a temperature scan presented in Figs. 10 and 12 the benefits of the method lie within the information of the void size distribution that could be extracted from the lifetime data (given time to further develop the theory of Ps-interactions in lipid membranes), not from the observation of the transition itself, which can easily detected in couple of minutes with DSC. Interestingly, simultaneously and independently of us Dong et al. at CSIRO, Australia have performed similar positron annihilation lifetime studies on lipid bilayer structures [60] and their results (complemented by results obtained from X-ray studies) show as well the suitability of the positron lifetime spectroscopy to characterizing the microstructure of the bilayers.

4.2 – Effect of Cholesterol in the bilayer

Based on the computational studies it is predicted that some dopants alter the free volume parameters in the bilayer and thus such changes should be observable with positrons. Of main interest for the present work are the previous simulation results for membranes including cholesterol [10]. It was shown that increasing the cholesterol concentration in a DPPC-cholesterol mixture reduces the total amount of free volume in a lipid bilayer, decreases the sizes of voids, and changes void orientations to lie along the membrane normal direction. The cholesterol acts by stabilizing the membrane and the effect of free volume decrease is most prominent in those parts of the bilayer, where cholesterol's steroid ring structures and the hydrocarbon tails are located, thus in the same region where significant fraction of the o-Ps formation is supposed to be occur.

The lipid type studied was chosen to be POPC and samples with different cholesterol concentrations were prepared. PALS measurements were performed in a static room temperature above the main phase transition temperature (T_m) for POPC/Chol-mixture. The results (Fig. 13) clearly depict that the o-Ps lifetime and consequently the average free volume size decrease substantially when the cholesterol content is increased above 30 mol%. Interestingly, these major changes in o-Ps lifetime coincide with the phase transition boundary: at ~30 mol% of cholesterol there is a transition from the coexistence region (between the liquid-disordered and liquid-ordered phases) to the liquid-ordered phase dominated by cholesterol [61]. Further, at the largest cholesterol concentration of 50 mol%, one finds a minor increase in lifetime, reflecting the formation of cholesterol crystals that takes place in this regime. These findings highlight the sensitivity of PALS for detecting changes in the phase behaviour, as well as the structural differences in void distributions for fluid and raft-like membrane domains.

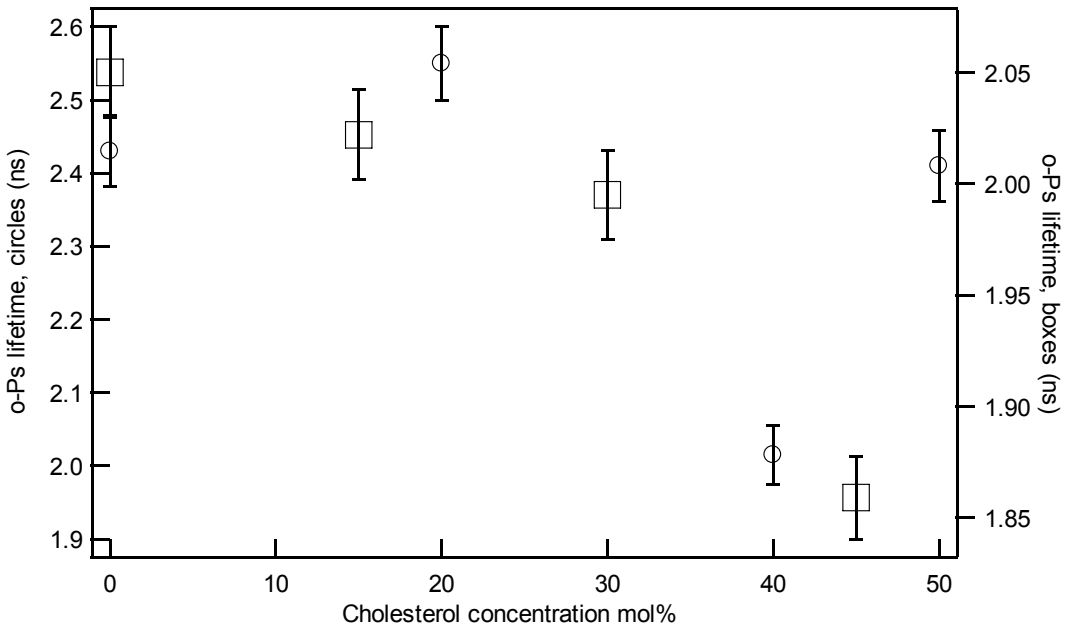

Figure 13. *$\Sigma\tau_3$ – lifetimes in POPC/Cholesterol mixture ratios show that the increase in cholesterol concentrations reduces the Ps-lifetimes due to the reduction to the free volume distribution. The figure consists of data from two samples with different water concentrations affecting the scale of the change. The crystallization of the cholesterol molecules at ~45 mol% concentrations then again increases the free volume as seen in the figure.*

As with the earlier measurements, the positron annihilation lifetime measurements are backed up with MD simulation data shown in Fig 14. [Publ. II]. As for the role of cholesterol, simulation studies, as well as our PALS measurements for POPC/cholesterol membranes highlight the prominent role of cholesterol as a membrane stabilizer, reducing void sizes and densities

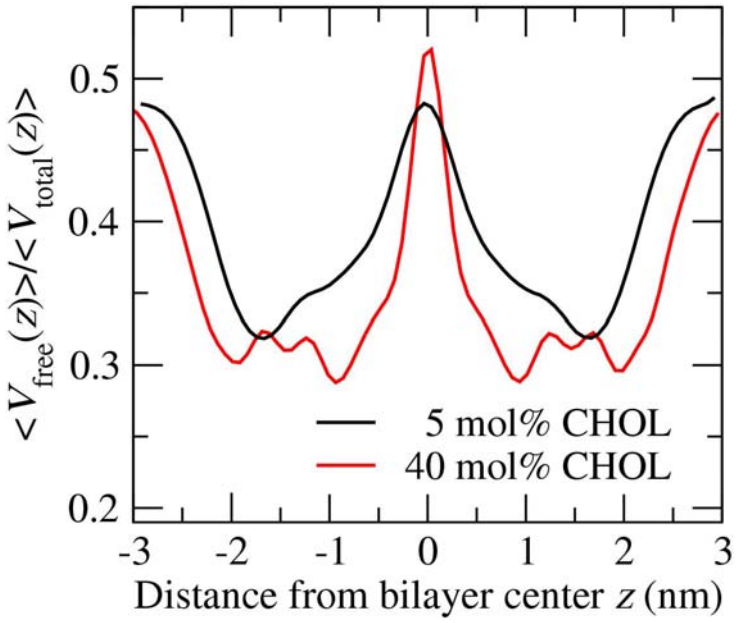

Figure 14. *MD free volume profiles on the effect of cholesterol in lipid bilayer. When the cholesterol concentration is increased from 5% to 40% the free volume in the middle of the bilayer decreases significantly. The edge of the bilayer is at approx. 1.5-2nm range from the bilayer center.*

Chapter 5 – Application to natural and artificial lenses

In the previous chapter the feasibility of positron annihilation lifetime spectroscopy in the biomaterial research was shown and based on the data, it is clear that the positron annihilation lifetime spectroscopy is capable of sensing subtle changes in the properties of biological membranes.

In this chapter some applications are presented to show that the positron lifetime spectroscopy opens up a new level of sensitivity enabling the observation of phenomena in biological materials that are not directly visible by other means. This type of novel information opens possibilities to create new scientific information as well as validating results gained with other methods. Not only studying natural materials such as the lenses of the mammalian eye, in this chapter positron annihilation lifetime spectroscopy is also applied on studies on void properties in contact lens polymers. Yet, even if the functionality of the free volume properties in the materials differs, the positron lifetime spectroscopy is able to provide valuable information in both cases.

5.1 – Studies on intact mammalian lens tissue in-situ

Many tissues in the human body consist mostly of lipids and the lipid characteristics dominate the functionality of the tissue itself. For such an example, mammalian lens tissue was studied to find out the microstructural characteristics of the lens tissue near the normal body temperature (see Fig. 15 for illustration of the human eye). The function of the mammalian ocular lens is to provide a sharp image on the retina, for such a function the lens must be transparent and minimize the scattering of light.

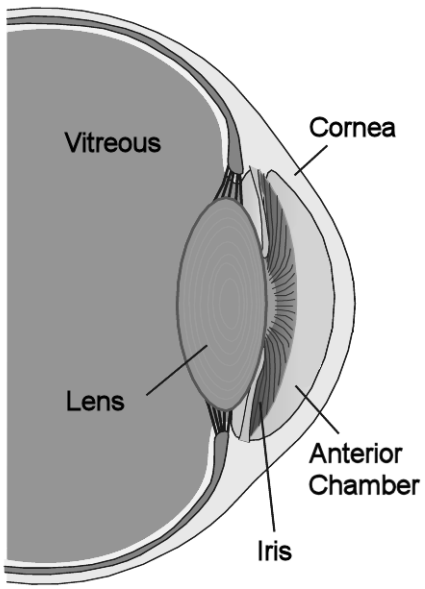

Figure 15. *Cross-section of human eye to visualize the role of lens. In principle, all mammalian eyes are closely similar to each other and as such experiments could be performed with bovine eyes [*62*].*

For maintaining an optimal optical property the lens fiber cells reorganizes its composition, e.g., arranging the fiber cell membranes. Because the fiber cells are metabolically inactive, the plasma membrane becomes the only cellular organelle and consequently, the phase behavior of these membranes determines the physiological state of the lens. In order to preserve transparency and minimize light scattering, these membranes need to be highly ordered. The plasma membrane is unique in that it contains high concentrations of certain proteins, lacks polyunsaturated phospholipids, and has very high concentrations of Sphingomyelin-lipid (SM), which is a similar long-tailed lipid as the DPPC illustrated in Figure 2, and cholesterol [63]. Previous studies have shown lipid membranes isolated from the cortical and nuclear fiber cell plasma membranes display a temperature dependent phase transition close to the body temperature and close to the main phase transition temperature of SM [64],[65]. Still, intact lens tissues have not been studied previously on the atomic/molecular level and as the lens tissue is highly consisted of lipid membranes, positron annihilation lifetime spectroscopy should be able to provide novel information about the microstuctural parameters of the tissues itself; at least it seems feasible that with positrons the phase transition in membranes should be observable. Furthermore, the physiological function of this phase transition remains an open issue, and equally unclear is whether such a phase transition could play a role in the formation of cataract, a physiological symptom where the transparency of the lens is diminishing resulting eventually to blindness.

Altogether six individual porcine lenses were measured inside the same type of test tubes as in previous positron lifetime studies.^{22}Na positron source material was injected as aqueous solution (saline) directly inside the lens with a 30 gauge needle, causing minimal damage to the lens. After the source injection the test tube was sealed and inserted into the temperature controlling setup between the detector pair. Based on the results of preliminary positron lifetime experiments which showed anomalous behavior at ~35 °C, the temperature range from 33 to 36 °C was studied more carefully. For the first two lenses, a temperature step of 0.5 °C was used, while for lenses #3-6 the temperature dependence was studied more carefully in terms of a temperature step of 0.3 °C. The annihilation spectrum revealed three lifetime components, the longest of

them again originating from the o-Ps annihilation in the material. The conditions for conducting measurements were not ideal since the nominal sample temperature fluctuated to some extent within each sample, and the amount of saline inside the test tube may have varied. These uncertainties create minor differences in the o-Ps lifetimes between different lenses at specific nominal temperatures. Consequently, since the lifetime data for lenses No. 1- No. 5 were in agreement, all behaving in a similar manner, the averaged data from these samples are shown in Fig. 16. For the lens No. 6, no temperature dependence was found. While the reason for this remains unclear, it is likely that this is related to air bubbles in the sample which formed while injecting the active saline inside to the sample. Contrary to the previously presented lipid phase transitions the effect observed here was not reversible.

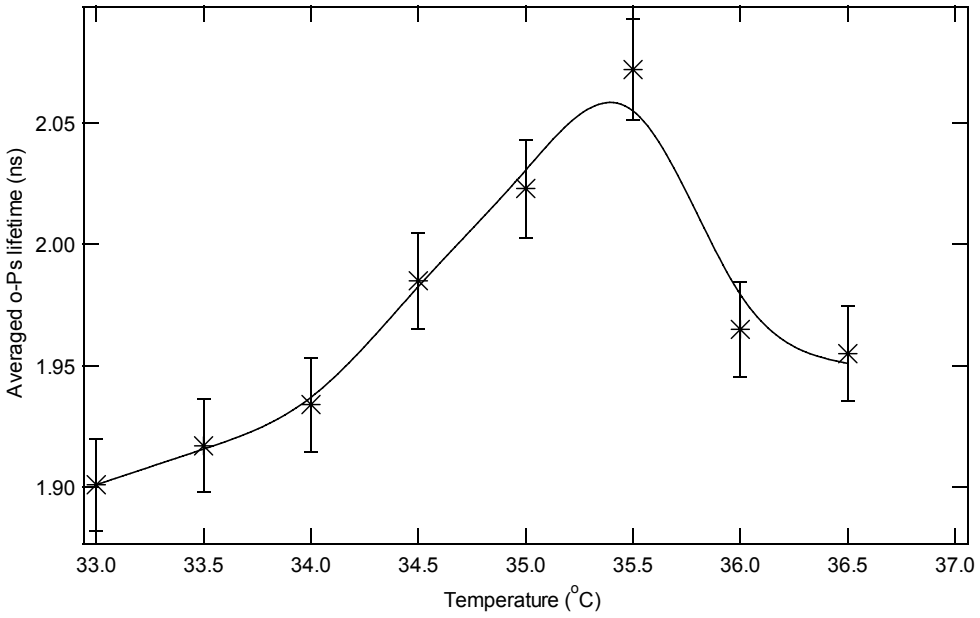

Figure 16. *o-Ps lifetimes (τ_3) averaged over data for lens #1-5. A temperature induced anomalous transition is observed between 34.5 and 35.5 °C. Averaging was performed by calculating the average of measured lifetimes for all the data points within 0.5° steps, e.g., the marker at 34.5 °C is an average of results from temperatures at 34.3, 34.5 and 34.6 °C.*

To gain better understanding of the origin of the anomalous behavior of intact lenses, two additional lipid samples were examined by PALS: lipids separated from lenses and SM-cholesterol mixtures at different cholesterol concentrations to have a controlled measurement as the lens lipids are mostly consisted of SM and cholesterol. The yield of separated lipids was low, resulting to a larger impact of annihilations in water compared to lipids, thus the measured average lifetime was significantly lower than in the more lipid- rich samples.

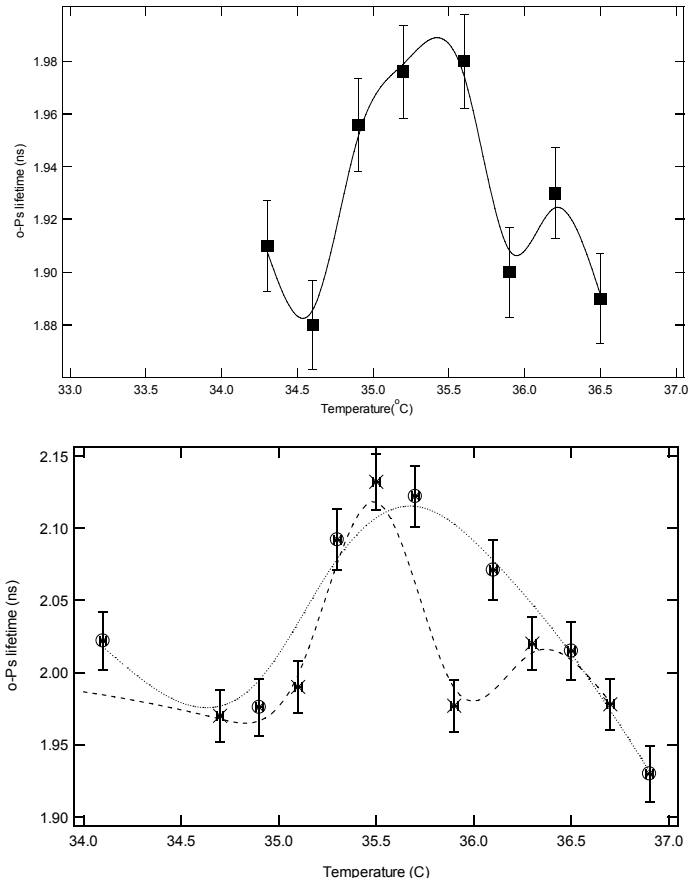

Figure 17. *o-Ps lifetimes measured in lipids separated from lenses (graph above) and control measurements of Sphingomyelin+cholesterol mixtures (volume ratio of 85/15, crosses; 70/30, circles, graph below). It is clear that the same temporal transition is visible as observed in Fig 14. The effect of cholesterol on the free volume can be seen on the SM+cholesterol figures where the increase of cholesterol has an effect on the lifetimes in the lipid, i.e., by widening the temperature range of the effect.*

Figure 17 shows the temperature dependence of o-Ps lifetime as a function of temperature. It essentially shows that both lipid mixtures display a peak-like increase in the o-Ps lifetime at ~35-36°C. Furthermore, at 30 mol% of cholesterol the peak in the o-Ps lifetime is wider than that in the 15 mol% cholesterol sample, in agreement with the lens lipid sample where the cholesterol concentration is assumed to be significantly high. In essence the same phenomena as observed in intact lenses can be seen in these control studies.

The data essentially shows that at approximately 35.5 °C an anomalous behavior in the o-Ps lifetime τ_3 is observed in-situ in intact lenses. Based on purified lens lipid extract and pure lipid measurements, we suggest that the observed effect is caused by the lipids in the lens itself. This finding is in a very good agreement with those from Borchman et al. [65] who showed that the cortical lipids displayed a main phase transition at 36-39 °C. Based on the results, it seems reasonable to assume that the structural transition observed in positron lifetime experiments in-situ can be derived to the SM-lipids in these lenses. Even if the exact means of o-Ps interactions with the structures and molecules of the biomolecular materials remain partly unknown, it is clear that the changes in the o-Ps lifetime are real rather than due to random fluctuations. The averaged data in Fig. 16 contains tens of millions of lifetime events and provide a reliable statistics for the analysis. As the increase in o-Ps lifetimes is strongly temperature related, it is most likely caused by a small change in the dynamics and free volume characteristics of the lens material. The change could be small and insignificant on a macrostructural level, but the nanometer-sized microstructure definitely undergoes a structural transition, which the o-Ps probe is able to gauge. Whether the enhanced free volume and the transition have physiological effects on the function of the lens remains to be clarified. Intriguingly, a similar phenomenon was also observed and shown in Fig 17 in artificial lipid membranes composed of SM and cholesterol which also showed a peak-like increase in the lifetime of o-Ps at approximately the same temperature as in lens lipid samples. This behavior in o-Ps lifetime can be explained by melting of a SM-rich gel-like phase in an otherwise cholesterol-rich liquid-ordered phase, which concurs with recent results [66] obtained with X-ray scattering techniques.

Still, based on the results on actual intact tissues, it is clear that positron annihilation lifetime spectroscopy is a suitable tool to characterize even whole tissues as the positrons (and resulting Ps formation) can be directed to specific location by macroscopical manipulation, i.e., direct injections of the source material. When combined with atomistic simulations for detailed considerations of free volume pockets in biomolecular simulations, positron annihilation lifetime spectroscopy can provide significant added value to better understand both structural and dynamical features in biological matter.

5.2 – Voids in contact lens polymers

Oxygen diffusion is one of the most important factors in contact lens (CL) properties. Typically the permeation of oxygen through the CL is much less efficient in soft disposable CLs compared to harder CLs designed for daily long term use. In an earlier study by Singh et al. [67] where PALS and measurements of gas diffusion rates were compared, it was shown that oxygen diffusion through CLs was strongly dependent on the void sizes. This effect was explained by the fact that gas permeability is the product of solubility and diffusion coefficient, causing the permeability to change with free volume. The oxygen diffusion and o-Ps-lifetimes in different CL polymers were observed to exhibit a clear linear correlation. O_2-permeability values (unit described in detail in footnote[3]) in the study at 35 °C were measured from 32.8, corresponding to the o-Ps lifetime of 2.36 ns, to 109.0 for the lifetime of 2.80 ns. This information can be used to compare the changes in oxygen diffusion as a function of the void size, even if actual measurements of oxygen diffusion are not performed. Furthermore, the contamination of CL by proteins, ions, and impurities present in the tear fluid will eventually reduce the diffusion properties of the CL by filling the voids within the polymer. The studied lenses were: Bausch&Lomb Daily Disposable (DD, 59 % water), B&L Soflens® (SL, 38 %), and B&L Purevision® (PV, 36 %). The base materials in these CLs are polymer-based Hilafilcon B (DD), Polymacon (SL) and a silicone-hydrogel based Balafilcon A (PV).

The positron measurements were performed using a sandwich-geometry of the sample material and the positron source. A layer consisting of at least five contact lenses was placed on both sides of the small (2x2x0.003 mm) positron source. This geometry enables complete surrounding of the small source and thus the vast majority of positrons annihilated in the lenses based on the estimate of positron range in the CL polymers.

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[3] Gas Permeability **P** [$\frac{cm^3(O_2(STP)}{cm^3\,polymer \cdot mmHg}\frac{cm^2}{s}$] x 10^{-11}

It was observed that different types of CLs differ also on their microstructural level, CLs designed for longer use periods (i.e. enhanced oxygen diffusion) had vastly larger voids than those in disposable lenses (Ø0.59 nm, V~0.11 nm^3 vs. Ø0.78nm, V~0.25 nm^3), the detailed data can be found on Publ. IV.

In order to study the possible filling of the voids during CL use, Purevision CLs were submerged into albumin (Bovine serum albumin, BSA) solution (12.5 mg/ml in water). The lenses (5+5 lenses in each case) were removed from the solution with tweezers and measured after 2-5 minutes, 1, 2, 3, and 4 weeks of submersion in the protein solution. The results from these measurements are shown in Fig. 18. It was found that the albumin in the solution either fills the voids or causes decrease in the void size in the CL over a time period of weeks. Based on the present data, it is not possible to state whether the voids are actually filled with denatured proteins or is the observed effect due to a change in the polymer's structural dynamics (the protein chains could change the packing ratios of the polymer chains and cause an increase of the material density of the structure when albumin permeates the polymer structure, reducing the void size). In any case, the conclusion remains: the average size of the free volume pockets in the polymer structure decreases when subjected to usage-mimicking conditions. This is likely to result in a lower oxygen diffusion rate through the lens.

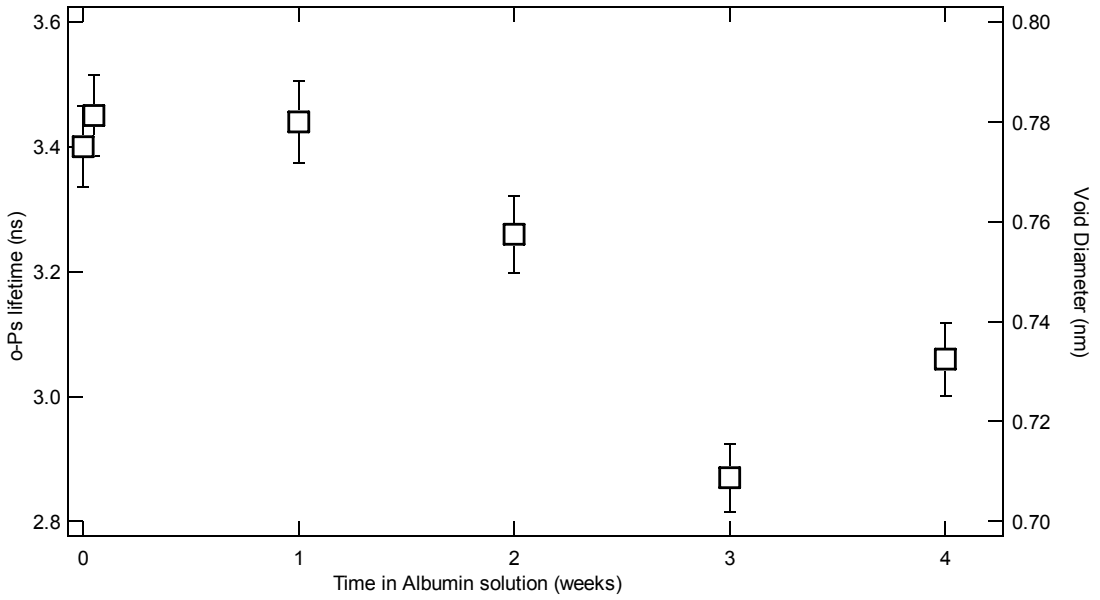

Figure 18. *Measured lifetimes and corresponding void diameters (Eq. (2)) in Purevision CLs submerged into 12.5 mg/ml aqueous albumin solution. The intensity of the o-Ps lifetime component was in the 10-15 % range in all measurements. The first data (without albumin immersion and after 2-5 minute incubation in albumin solution) coincide, they are plotted with a small horizontal shift for visual purposes. Overall void volume change between, i.e., zero incubation time and 3 weeks of submersion is ~ 25 %.*

More interestingly, when comparing the data from disposable CLs and monthly CLs, it is clear that even after a 4 weeks of submersion in albumin solution the void properties in Purevision CLs are still superior to disposable lenses, indicating that disposable CLs are inferior regarding to oxygen diffusion properties and proper monthly lenses would seem to provide extensive oxygen diffusion throughout the usage period. This is an important notion due to the large difference in price between disposable and continuous use lenses. Daily disposable and Purevision CL users might be tempted to over-extend the use of these lenses or instead of monthly lenses use cheaper disposable or semi-disposable CLs as monthly CLs.

Chapter 6 – Summary

In this work the feasibility of positron annihilation lifetime spectroscopy as a tool for biophysical research is studied and found to be a good method to gain novel information on structural properties of biomaterials. Firstly it was demonstrated that positrons can be employed as a probe of free volume pockets in lipid bilayer structures and that the resulting positron (Positronium to be precise) lifetimes correlate well with structural transitions in the bilayers. Comparing the lifetime data with results from computational simulations reveals that the results are in good agreement with each other. Secondly it was shown that the method is also suited for characterizing more subtle changes in the bilayer structure, namely the effect of dopants on the bilayer free volume.

Positron annihilation lifetime spectroscopy was also applied to study intact tissues, namely mammalian lenses (consisting mostly of lipids) and hint of minor structural transition was observed in the data. When further studies were performed on the separated lens lipids and manufactured lipid samples they were found to match with the results on intact tissues. This further confirms the observation in intact tissues and emphasizes the sensitivity and suitability of positron annihilation lifetime spectroscopy to studies on biomaterials. With the positron lifetime method it is possible to study the tissues in situ, gaining additional information on the processes occurring inside the tissues. When broadening out the research focus to artificial medical polymers, studies on commercial contact lens polymers revealed significant differences in the void sizes in different types of contact lens polymers. Additional studies on the contact lens deterioration during the emulated use period were performed and void sizes were found to decrease dramatically (to 1/3 of original value) during the incubation period in protein-solution, emulating a typical protein concentration of tear liquid. The void sizes are directly correlated to the vital oxygen diffusion through the contact lens and as such the changes in void properties provide important information on the safety of the contact lenses in prolonged use periods.

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