X-RAY SCATTERING STUDIES OF BIOLOGICAL AND BIOMIMETIC MATERIALS

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Science of the University of Helsinki, for public criticism in auditorium E204, Gustaf Hällströmin katu 2, on August 26th, 2016, at 12 o’clock noon.

Helsinki 2016
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Preface

Like all good things life, science should not be attempted alone. The research for this thesis was carried out at the Department of Physics of the University of Helsinki, and I am grateful to the head of the department, Prof. Hannu Koskinen, for the opportunity to work here. I also owe to Vilho, Yrjö and Kalle Väisälä’s foundation, and to the financially lesser but no less appreciated support of my doctoral schools ISB and, later, ILS.

I first came to the Laboratory of Electronic Structure to work under Doc. Mika Torkkeli and the late Prof. Ritva Serimaa, and I thank them for the faith they showed in me, the freedom they gave me to find my own way, and the patience they had when I found myself in a dead end. After things took an unexpected turn, Doc. Susanne Wiedmer took over the role of my supervisor, and working with her has been a privilege. With her around, things progress at an amazing pace. I am also grateful to Dr. Kirsi Svedström, who has guided me with patience and kindness through the marvels of X-ray scattering. Prof. Airi Palva and Dr. Ulla Hynönen have patiently answered my questions about biology and always offered a wonderful collaboration. I am profoundly in debt to my thesis committee, which in addition to Susanne, Airi, Ulla and Kirsi included Prof. Sarah Butcher, who was always on top of all things formal, asked the right questions and offered encouragement to make things trodgle along.

I am grateful for the company of my fellow students. I thank Dr. Paavo Penttilä for his help, his music and his cooking during the beamtime. I’ve been honoured to collaborate with Patrik Ahvenainen, who, in addition to always being up for a discussion of science, also has made the laboratory atmosphere his priority and organized countless floorball games and counted board game events (the current number standing at 99). I am thankful that Dr. Filip Duša and Suvi-Katriina Ruokonen have always had time for my chemistry-related questions. I thank Dr. Kirsi Mikkonen and Dr. Geza Szilvay for introducing me to new and exciting projects, all my collaborators for their efforts, and MAX IV Laboratory for giving me an opportunity to conduct research there.

But there is more to university than research. I am deeply honoured to have worked with such excellent educators as Prof. Hanna Vehkamäki, Dr. Szabolcs Galambosi and Ilkka Hendolin, who have made teaching and developing the courses at the department a real pleasure. I also want to thank everyone at the Laboratory of Electronic structure. Doc. Simo Huotari owes a mention for shouldering the burden of running a laboratory and taking on things — and persons — which were not really his responsibility. In the laboratory I was, in particular, appreciative of the company of Aki Kallonen. We have not done research together, but when the inanity of this profession became too much, I always knew he would be there to share my woes.

Of course, there is friendship outside the laboratory as well. I can only hope I have been able to repay the love and support of my friends equally, because I do not know what I would have done without them. In particular, I want to thank Jan Jansson, who has walked by my side since high school, and offered me advice in all things related and unrelated to chemistry and teaching.
I want to thank my parents and siblings for all the support they have showed me during the years. My parents have shown faith in me and given me the freedom to find my own way. I also want to extend the thanks to my other family for allowing me into their lives and welcoming me back time and time again. I am privileged to have been loved by so many, and the knowledge that there are people both near and far who care for me has been invaluable.

Above all, there is one person who has comforted me when I was anxious, shared my joy when things went well, and listened to me rant about things he had no interest in; a person who has stood by me during all this time, and for that I thank Mikko. For reciprocated love, for perseverance, for all the times he offered to end a board game prematurely to spare me the experience of total humiliation — and for all the times he did not.

13.6.2016 Helsinki

[Signature]
Abstract

Elastic X-ray scattering is a probe which provides information on the structure of matter in nanometer length scales. Structure in this size scale determines the mechanical and functional properties of materials, and in this thesis, small- and wide-angle X-ray scattering (SAXS and WAXS) have been used to study the structure of biological and biomimetic materials. WAXS gives information on the structures in atomistic scales, while SAXS provides information in the important range above atomistic but below microscale.

SAXS was used together with dynamic light scattering and zeta potential measurements to study protein and liposome structures. The S-layer protein of *Lactobacillus brevis* ATCC 8287 was shown to reassemble on cationic liposomes. The structure of the reassembled crystallite differed from that of the S-layer on native bacterial cell wall fragments, and the crystallite was more stable in the direction of the larger lattice constant than in the direction of the shorter.

Anionic liposomes were used as a biomembrane model to study the interactions of phosphonium-based ionic liquids with cell membrane mimics. All studied ionic liquids penetrated multilamellar vesicles and caused a thinning of the lamellar distance that was dependent on ionic liquid concentration. The ability of the ionic liquids to disrupt membranes was, however, dependent on the length of hydrocarbon chains in the cation. In most cases, ionic liquids with long hydrocarbon chains in the cation induced disorder in the system, but in one case also selective extraction of lipids and reassembly into lamellae was observed. The effects depended both on ionic liquid type, concentration, and lipid composition of the vesicle.

WAXS was used as a complementary technique to provide information on the structure-function relationship of a novel biomimicking material composed of a genetically engineered protein, chitin and calcium carbonate, and films composed of hydroxypropylated xylan. The presence of calcium carbonate and its polymorph (calcite) was determined from the biomimetic material. For the xylan films, crystallinity was assessed. In both cases, also the crystallite size was determined. These parameters influence the mechanical properties of the developed materials.

In all cases, X-ray scattering provided information on the nanostructure of biological or biomimetic materials. Over a hundred years after the principle behind X-ray scattering was first explained, it still provides information about the properties of matter which is not available by other means.
List of original publications


The papers I-IV are included as appendices in the printed version of this thesis. Paper I and III-IV are reprinted with kind permission from the publishers Elsevier (paper I and IV) and RSC Publishing (paper III).

Author’s contributions

In Paper I the author was involved in designing the experiment and prepared the samples. The author performed all of the SAXS, DLS and zeta-potential measurements, analyzed all data and wrote the paper. In Paper II the author was involved in designing the experiment, prepared a part of the samples and performed all of the SAXS experiments and a part of the DLS and zeta-potential measurements, analyzed all data and wrote the paper. In SAXS measurements of Papers I-II, the author was in charge of the synchrotron experiments.

In Paper III and IV the author performed the WAXS measurements, analyzed the WAXS data, wrote the experimental section related to the WAXS measurements and commented on the paper. Paper IV has previously been included in the doctoral thesis of Dr. Jani-Markus Malho.
1 Introduction

Biological materials are inherently complex. Many natural materials have properties which are hard to mimic — they self-assemble into complicated structures that are hard to replicate by technology, they display complex biological functions and can have superior mechanical properties to man-made materials [1]. Biological matter has evolved to what it is in billions of years, and this is reflected in its complexity.

In the 21st century, the importance of bio-based materials is again growing. Though surplanted by oil for nearly a century, natural polymers are returning to the field of materials science by offering an alternative to fossil materials [2]. Bio-based materials are interesting because of their availability and for the possibility of tuning their properties by designing self-assembling structures. By and large, biological matter is biodegradable.

The importance of materials of biological origin is manyfold. First, a distinction between different kinds of biological matter should be made. Biological materials are materials that are found in nature. Biomaterials, on the other hand, are usually synthetic materials which are used in biomedical applications. Biomimetics refer to materials and design which take their inspiration from nature [3].

Naturally, there is a large overlap between these segments. Many biomaterials are also biological materials that have been used in new biomedical applications. This includes e.g. the use of corals and collagen in medical implants [3, 4]. Biomimetic materials can either be materials that mimic the function of biological structures by synthetic means, or materials that mimic the molecular properties of the biological structures. A commonly used example of the first kind is the famous velcro, which is an adhesive structure that mimics the hooks in burrs. Burrs use their hooks to attach to animal fur. Velcro is made of synthetic polymers: its hooks attach to a counterpart that has synthetic loops, although it also attaches to wool and other fuzzy fabrics. It provides fast closure and opening of clothes, shoes, bags et cetera.

An example of biomimetic materials of the second kind are liposomes, which are spherical shells composed of phospholipids. Liposomes are self-assembling structures, and their biomimetic aspects originate in the molecular self-assembly which is also present in nature: the cell membrane of organisms is composed of a similar structure [5, 6]. The properties of liposomes can be tailored for different purposes by varying the kind(s) of lipid(s) that are used to control e.g. surface charge, size, transition temperature and mechanical properties of the lipid bilayer.

Liposomes can be used both as biomimetic membranes, to study the reactions of the cell membrane, or as delivery vehicles or platforms for molecular organization. They can be used as a complementary tool for evaluating the harmfulness of e.g. ionic liquids and other substances that are known to affect the cell membrane [7, 8]. Their use in drug delivery is propagated by them being biodegradable, however, unmodified liposomes are unstable in a biological environment [9]. They can be stabilized for example by coating them with polymers [10] or self-assembling proteins [11].

In general, the smaller structures biomimetic materials try to emulate, the
more important the molecular-level processing aspect is. The unique aspects of biological materials are related to their hierarchical structure: biological materials have structure in many different length-scales, and their superior properties result from optimization in all length-scales from the molecular to the macroscopic [3, 12, 13]. Many materials found in nature display remarkable mechanical properties, even though the interactions holding them together are weak [1].

For example, the properties of nacre are influenced by the organization of atoms and molecules at all length-scales. Nacre, found in mollusk shells, employs a complex structure that combines properties of hard and brittle and soft and flexible materials for a superior tenacity [14, 15, 16]. Composed of the polymer chitin, silk-like proteins, acidic proteins and stacked calcium carbonate platelets, nacre is a hardy material whose formation is not fully understood. At the atomic level, it is composed of both ordered and amorphous materials: calcium carbonate crystals are embedded in the polymer matrix.

The crystals themselves are hard and brittle, but the matrix they are embedded in gives nacre a work of fracture which exceeds pure ceramics by three orders of magnitude [15]. Nacre compares favorably to man-made composites both in terms of the elastic modulus, stress-intensity factor and the work of fracture [16]. In the build-up of nacre, the amorphous matrix is present first. The chitin matrix is highly aligned and porous, and the silk-like proteins and acidic proteins facilitate the crystallization of calcium carbonate, likely from a bound, amorphous layer. The porous matrix allows the calcium carbonate crystals to align with respect to each other [14].

The alignment of the crystals is a factor which is important in many biological materials. Natural materials are often polycrystalline, that is, composed of many crystals. Crystallites are typically mixed in an amorphous matrix — nacre, as mentioned above, is a case where mineral platelets are suspended in an organic matrix, but matter with the same chemical structure can also be organized in a variety of ways. A prime example of this hierarchical structure is wood.

Wood is composed of cellulose, lignin and hemicelluloses. Cellulose, the most abundant biopolymer with a mass fraction of 40–55% in wood, is a linear polymer consisting of \( \beta - (1 \rightarrow 4) \) linked D-glucose units. Lignins, cross-linked phenol polymers, come in at 15–35 wt-% and hemicelluloses, which are water-soluble heteropolysaccharides, at 25–40 wt-% [17]. The cellulose in wood comprises both crystalline and amorphous regions. This, along with the rest of the hierarchical structures of cellulose, including bundling into microfibrils that wrap around the wood cells in three distinct layers with certain preferred orientation distributions, in part give wood its mechanical properties.

However, the fraction of crystalline material (crystallinity) of a sample does not only affect its strength but also reactivity, solubility and other chemical properties. These factors become particularly important when looking at biological materials from the other direction: as a source of raw materials for refinement. From an ecological point of view, natural fibers are in many ways preferable to petroleum-based products, although the harmfulness of all chemicals used in processing need to be evaluated critically. Wood, for example, has been used as a source of energy and for construction since prehistoric times. Later, when it has partly been
replaced in these applications by, respectively, less combustible and more energy
dense materials, its importance grew with the advent of the paper and packaging
industry. However, with the evolving nature of these, more refined applications are
continuously developed. Where previously whole logs were used, modern materials
science strips wood into its constituents, and we are again faced with its nanoscale
structure: crystallinity, crystallite size, organization into fibrils and fibers. Some-
times, even these characteristics are eliminated: as amorphous cellulose is more
reactive, reducing the crystallinity of cellulose by solvents such as ionic liquids is a
hot topic in materials science. Functional groups are stripped from the backbone of
hemicelluloses such as xylans, yielding linear polymers. By controlled substitution
of new side chains, these polymers can be processed into films with tailored barrier
properties that depend on the molecular organization. The nanoscale matters.

Methods to study the organization of matter at nanoscale are many. Some,
like atomic force microscopy and electron microscopy, are direct. They are gaining
in popularity due to the maturing of the techniques, and because interpreting
visual information is often easier than indirect measurements. However, many
indirect methods give information from which the nanometer-scale structure can
be calculated, and often the obtained information is inaccessible by any other
means. One approach is to use electromagnetic radiation with a wavelength in the
same length scale as the size scale to be studied. X-ray scattering has been used
for materials research for over a hundred years, and with the advent of new and
more powerful sources, it is more current than ever.
2 Theory of X-ray scattering

Electromagnetic radiation with a wavelength in the Ångström ($10^{-10}$ m) region is called X-rays. Discovered by Wilhelm Röntgen in 1895, they were first used to probe the internal structure of macroscopic objects, such as humans.

X-rays interact with the electrons in the material they are passing through. Some processes, such as the photoelectric effect or (for high energies) pair production lead to the absorption of X-rays into the medium, but X-rays can also be scattered by electrons. X-ray scattering is divided in elastic and inelastic scattering, depending on whether the energy of the scattered X-ray is conserved. In elastic scattering, the photons preserve their energy and only change the direction of propagation, whereas in inelastic scattering, part of the X-ray energy is transferred to the electron. Elastic scattering has been one of the first ways to observe the atomic structure of materials. As early as in 1912, Max von Laue discovered diffraction of X-rays by crystals, and soon after, Lawrence Bragg presented an explanation for the phenomenon in the form of the famous Bragg’s law [18, Ch.1].

X-ray scattering has been used to study biomaterials from the very beginning: Nishikawa and Ono did diffraction experiments with lamellar and fibrous materials such as asbestos, talc, bamboo and hemp already in 1913 [19] and discovered that biopolymers and other fibrous materials are similar in structure.

2.1 Elastic scattering of X-rays

Elastic scattering can be explained classically through a wave description of photons as Thomson scattering of electromagnetic radiation from a free charged particle. The electric field of the incoming X-ray makes the electron oscillate, and this in turn generates a spherical wave with the same frequency as the incoming photon, but with a phase shift of $180^\circ$. The intensity of the scattered X-ray, $I$, is a fraction of that of the incoming one, $I_0$:

$$I = I_0 \frac{e^4}{r^2 m^2 c^4} \left( \frac{1 + \cos^2 2\theta}{2} \right). \quad (1)$$

In the above formula, $r$ is the distance from the scattering point to the point of observance and the term $\frac{e^4}{m^2 c^4}$ is the square of the classical electron radius that depends of the unit charge $e$, the mass of the electron $m$, and the speed of light $c$. $2\theta$ is the scattering angle in which the radiation is observed [20, Ch.4].

Thomson scattering is the low-energy limit of the more general case of Compton scattering, where part of the photon energy is transferred to the electron. Compton scattering is thus inelastic: both the energy and phase of the scattered photon are different from the incoming one. Effects of Compton scattering are often ignored when elastic scattering studies are conducted, and this is also the case for the works presented in this thesis.

As mentioned, in elastic scattering the energy of the incoming and outgoing X-ray are equal, but there is a transfer in momentum between the electron and X-ray, and we can define a scattering vector $\mathbf{q}$ with the help of the momenta of the photon before ($\hbar \mathbf{k}$) and after ($\hbar \mathbf{k}'$) the interaction:
2.1 Elastic scattering of X-rays

\[ \hbar q = \hbar k - \hbar k'. \]  

(2)

Here, \( k \) and \( k' \) are the wave vectors of the incident and scattered wave, respectively. The scattering vector is visualized in Figure 1.

When considering light elements, all electrons in the atom, or, on a larger scale, in the sample, can be regarded as free electrons. Thus the form factor \( f^0(q) \) for an atom can be obtained by integrating over its electron density \( \rho(r) \):

\[ f^0(q) = \int \rho(r)e^{iqr}dr. \]  

(3)

For a molecule, the form factor \( F_{\text{mol}}(q) \) is a sum of the atomic form factors:

\[ F_{\text{mol}}(q) = \sum_j f_j(q)e^{iqr_j}, \]  

(4)

where \( f_j \) are the form factors of the \( j \) atoms and \( r_j \) their respective positions in the molecule. The form factor is equivalent to the amplitude of the scattered beam. Thus, the amplitude of the scattered X-rays is essentially a Fourier transform of \( \rho(r) \), and the observed intensity \( I(q) \) is the square of the form factor \( F(q) \):

\[ I(q) = |F(q)|^2. \]  

(5)

The length of the scattering vector \( |q| \) can also be used to tie together the angle in which the X-rays are scattered, \( 2\theta \), and the wavelength of the X-rays, \( \lambda \):

\[ |q| = \frac{4\pi \sin \theta}{\lambda}. \]  

(6)

This provides a convenient way of comparing scattering intensities obtained by using different X-ray wavelengths, and as will later be shown, is a particularly simple way of transforming distances between the reciprocal and real space.

Figure 1: X-ray diffraction in the perpendicular transmission geometry. The incoming, transmitted and scattered beams and the orientation of vectors \( k, k', \) and \( q \).
2 THEORY OF X-RAY SCATTERING

2.2 Diffraction from crystalline matter

The following derivation follows the presentation of Als-Nielsen and McMorrow [21, Ch.4].

For an infinite lattice with point-like scatterers which are organized into a crystal, we can define a lattice vector $\mathbf{R}_n$:

$$\mathbf{R}_n = n_1 \mathbf{a} + n_2 \mathbf{b} + n_3 \mathbf{c},$$

where $\mathbf{a}$, $\mathbf{b}$ and $\mathbf{c}$ are the basis vectors for the lattice and $n_1$, $n_2$ and $n_3$ are integers. The scattering factor of a crystal can be factorized into the product of the form factors of a unit cell and the lattice:

$$F_{\text{crystal}}(\mathbf{q}) = \sum_j f_j(\mathbf{q}) e^{i\mathbf{q} \cdot \mathbf{r}_j} \sum_{\mathbf{R}_n} e^{i\mathbf{q} \cdot \mathbf{R}_n},$$

where the first sum is an analogue to Equation 4, with index $j$ denoting the atoms of the unit cell, and the second is a sum over the positions in the crystal lattice.

The amplitude of the scattered wave, Equation 3, will be negligible unless the scattering vector fulfills the condition

$$\mathbf{q} \cdot \mathbf{R}_n = 2\pi n,$$

where $n$ is an integer. A solution to this equation can be found with the help of a reciprocal lattice, in which the reciprocal lattice vectors $\mathbf{a}^*$, $\mathbf{b}^*$, and $\mathbf{c}^*$ are defined using $\mathbf{a}$, $\mathbf{b}$, and $\mathbf{c}$:

$$\mathbf{a}^* = 2\pi \frac{\mathbf{b} \times \mathbf{c}}{\mathbf{a} \cdot (\mathbf{b} \times \mathbf{c})}, \quad \mathbf{b}^* = 2\pi \frac{\mathbf{c} \times \mathbf{a}}{\mathbf{a} \cdot (\mathbf{b} \times \mathbf{c})}, \quad \mathbf{c}^* = 2\pi \frac{\mathbf{a} \times \mathbf{b}}{\mathbf{a} \cdot (\mathbf{b} \times \mathbf{c})}.$$ (10)

An arbitrary site in the reciprocal lattice, $\mathbf{G}_{hkl}$, is given by the reciprocal lattice vectors and a set of integers, $h$, $k$, and $l$:

$$\mathbf{G}_{hkl} = h\mathbf{a}^* + k\mathbf{b}^* + l\mathbf{c}^*,$$

and diffraction is observed when $\mathbf{G}_{hkl} = \mathbf{q}$. This is the Laue condition. A translation into real space gives that the distances between lattice planes in reciprocal space are related to those in real space, $d$, by

$$d = \frac{2\pi}{|\mathbf{q}|}.$$ (12)

This is equivalent to the well-known Bragg diffraction condition, which is usually expressed as a function of real-space distance of lattice planes $d$, the angle in which radiation is scattered $2\theta$ and the wavelength of the incident radiation $\lambda$:

$$n\lambda = 2d\sin\theta.$$ (13)

The positions of the diffraction peaks can be computed from Equation 11. For example, the hemicellulose xylan has a hexagonal structure [22]. In a hexagonal lattice, the length of two lattice vectors is equal, $|\mathbf{a}| = |\mathbf{b}| = a$, and the angle
between them is 120°. The third lattice vector, \( c \), of length \( c \), is perpendicular to the plane spanned by \( \mathbf{a} \) and \( \mathbf{b} \). According to Cullity [20, Appendix 1], positions for the diffraction peaks for the hexagonal structure can be calculated from

\[
q_{hkl} = 2\pi \sqrt{\frac{4}{3} \left( \frac{h^2 + hk + k^2}{a^2} \right) + \frac{l^2}{c^2}}. \tag{14}
\]

For two-dimensional crystals, only two lattice vectors (\( \mathbf{a}, \mathbf{b} \)) and consequently reciprocal lattice vectors (\( \mathbf{a}^*, \mathbf{b}^* \)) exist. The reciprocal lattice vectors are given by Equation 10, where the place of the third lattice vector is taken by a unit vector \( \mathbf{n} \) which is perpendicular to the plane defined by \( \mathbf{a} \) and \( \mathbf{b} \):

\[
\mathbf{a}^* = 2\pi \frac{\mathbf{b} \times \mathbf{n}}{\mathbf{a} \cdot (\mathbf{b} \times \mathbf{n})}, \quad \mathbf{b}^* = 2\pi \frac{\mathbf{n} \times \mathbf{a}}{\mathbf{a} \cdot (\mathbf{b} \times \mathbf{n})}. \tag{15}
\]

Thus, the magnitudes of the reciprocal lattice vectors, \( a^* \) and \( b^* \), are given by

\[
a^* = \frac{2\pi}{a \sin \gamma} \quad \text{and} \quad b^* = \frac{2\pi}{b \sin \gamma}, \tag{16}
\]

where \( \gamma \) is the angle between the two lattice vectors [23, Ch.2]. The positions of the diffraction peaks of any two-dimensional lattice are given by

\[
q_{hk} = \frac{2\pi}{\sin \gamma} \sqrt{(\frac{h}{a})^2 + (\frac{k}{b})^2 - 2(\frac{hk}{ab}) \cos \gamma}. \tag{16}
\]

Diffraction from a single crystal leads to diffraction maxima emerging only at distinct positions (spots), as specified by the Laue condition. However, single crystals are rare in biological materials. More commonly, these materials contain numerous crystallites that are at random orientations, or are somewhat but not perfectly oriented. A polychrystalline material like this may be formed from crystallites only, or be partly crystalline and partly amorphous [18, Ch.1].

Randomly oriented crystallites result in a diffraction pattern where the Laue condition is fulfilled over the whole azimuthal angle \( 2\pi \). Instead of spots, diffraction rings are observed. This is usually called powder diffraction, but the phenomenon is more general, and happens also when X-rays are scattered from any random particles in aqueous suspension.

### 2.3 Finite crystals

In scattering experiments from a perfect, infinite crystal, only sharp lines (theoretically delta peaks) would be observed [18, Ch.1]. An inherent broadening of diffraction peaks comes from the fact that many crystals, especially in biological materials, are not infinite or even practically infinite, when compared to the X-ray wavelength. The crystallite size, also known as the length of the scattering domain \( s \), can be calculated from the full width at half maximum (FWHM) of the diffraction peak, \( B \):

\[
s = \frac{K \lambda}{B \cos \theta_B}. \tag{17}
\]
s is thus the function of peak width, the wavelength $\lambda$, and half of the scattering angle $\theta_B$. The factor $K$ is a constant, which is usually given a value of 0.9–1.0 [20, Ch.3].

2.4 Scattering from amorphous matter

In amorphous matter, no lattice planes can be determined and hence no diffraction peaks are observed. However, atoms are not completely randomly situated in an amorphous structure: some distances are still more common than others. This leads to one or two wide scattering maxima in the scattering pattern [18, Ch.1].

If the system is statistically isotropic and there is no long range order, the factor $e^{i\mathbf{q}\cdot\mathbf{r}}$ in Equation 3 can be replaced by its average over all directions of $\mathbf{r}$. Hence, the whole equation can be reduced to

$$I(q) = \int 4\pi r^2 dr \cdot \tilde{\rho}(r) \frac{\sin qr}{qr},$$

(18)

where $\tilde{\rho}(r)$ is the autocorrelation of the electron density in different points in the studied material:

$$\tilde{\rho}(r) = \int \int dV_1 \rho(r_1) \rho(r_2).$$

(19)

Here, $r = r_1 - r_2$.

2.5 Crystallinity

Many materials contain a mix of several components, e.g. crystalline and amorphous components. X-ray diffraction can be used to determine the degree to which matter is crystalline, as the total elastic scattering from the atoms does not depend on whether they are in crystal. The crystallinity $w_c$, corresponding to the mass fraction of the crystalline component, can be calculated from the volume integrals of the scattering intensity:

$$w_c = \frac{\int_0^\infty q^2 I_c(q) dq}{\int_0^\infty q^2 I(q) dq},$$

(20)

$I_c$ and $I$ are the intensities associated with scattering from the crystalline part and the total scattering, respectively [24, 25, Ch.7].

2.6 Small and wide angle domains

In practice, elastic X-ray scattering is divided into two regimes by the magnitude of the scattering angle. As distances in real space are inversely related to the magnitude of the scattering vector (Equation 12), small angle X-ray scattering (SAXS) is employed when the studied system contains larger correlation distances, and wide angle X-ray scattering (WAXS) when the correlation distances are small. Commonly, the SAXS region is limited to scattering angles of a few degrees and
2.6 Small and wide angle domains

size scales of \(\sim 1–100\ \text{nm}\), and WAXS deals with scattering angles exceeding five degrees and atomistic scales.

The WAXS region, therefore, is useful in solving \(\text{e.g.}\) the atomic positions in crystal lattices, whereas in SAXS, the scattering originates from larger scale inhomogeneities in the electron density. While all previously derived results for elastic scattering hold also in the small-angle region, SAXS is also an art in itself. The dimensions of electron density inhomogeneities that give rise to scattering in the small angle region are much larger than the used wavelength, and the shape of the scattering curve will remain essentially unaffected by small changes in the position of electrons. Thus, particles that have relatively small internal electron density differences can be approximated as homogeneous [26]. In most cases, the system can be considered as a matrix with intrinsic inhomogeneities, whether it be particles in solution, pores in solid phase or a mixture of different solid components [27, Ch.2].

In this thesis, SAXS is used for looking at the structure of crystallites composed of proteins and the changes in the electron density of biomimetic liposomes, whereas WAXS is used to look at the crystallinity of the biopolymer xylan and the structure of calcium carbonate in a novel biohybrid material. In all cases, the sizes of the ordered domains have also been evaluated.

In the SAXS region, the form factor of nanometer-sized particles can be obtained analogously to the calculation of the atomic form factor in Equation 3, replacing the atomic form factor \(f^o(q)\) with a form factor for the particle, \(f(q)\), and integrating over the entire particle. For example, the scattering intensity of large planes which are randomly oriented is calculated simply from

\[
I_{\text{plane}}(q) = \frac{\left|\int \rho(z)e^{iqz}dz\right|^2}{q^2},
\]

where \(\rho(z)\) is the electron density on the axis perpendicular to plane surface. Complex structures such as proteins, fibers and membranes can be modeled as sums of Gaussian electron density differences or as composites of dummy atoms [28].
3 Materials

3.1 Liposomes

Molecules with both a hydrophilic and a hydrophobic group are called amphiphiles. Such molecules self-assemble in aqueous solutions to minimize the exposure of hydrophobic parts to water. For molecules that have a large hydrocarbon volume and a small head-group area, the most favorable conformation is in bilayers or vesicles that are closed surfaces formed by bilayers [29]. An important subset of such vesicles are liposomes, which is the name commonly given to spherical structures composed of phospholipid bilayers. Since their first description in the 1960s, various uses have emerged.

The importance of liposomes is two-fold. Firstly, their structure mimics that of the cell membrane: the basic building block common for all eucaryotes and prokaryotes. Secondly, they can be used to store and transport functional molecules. In pharmaceutical research, liposomes are used both as delivery vehicles for therapeutic molecules and as a model for cell membrane, to study the penetration of functional molecules into the cells [30]. The liposomes can be tailored for different purposes by use of lipids. One important aspect is the transition temperature, that is, the temperature at which the lipids become free to move in the lipid bilayer plane. At temperatures exceeding the transition temperature, the lipids are in the $L_\alpha$-phase.

For the biomimetic approach, it is important to remember that the phospholipid bilayer that separates the inside of a cell from its environment is naturally more complex than a simple bilayer. The cell membrane incorporates proteins and carbohydrates; transmembrane proteins that act as channels into the cell span the entire bilayer. Nevertheless, the bilayer is the basic structural block, and though its composition varies between domains, species and cell types, the common principle remains. As such, liposomes can be used as a membrane model.

On the other hand, liposomes can be used for storage and transport of functional molecules. Liposomes are nowadays commonly used in customer products. They can be used for controlled delivery of for example proteins and enzymes, vitamins, and flavors in foods [31], for cosmetics, and for drug delivery [32]. They also have applications outside biology, and can be used to facilitate dyeing of textiles [33].

3.1.1 Modeling of liposomes

Lipid bilayers have been studied with SAXS for a long time. For multilamellar samples composed of a single lipid, the lamellar distance (approximately 6 nm) gives rise to well-defined diffraction peaks that can be used to calculate the lamellar distance by assuming them as infinite planes. From good quality data, also the relative electron density in the lipid bilayer can be calculated by modeling the bilayer as a sum of three or more Gaussian functions, where the lipid headgroups are assumed to have a higher electron density than the surrounding water and the hydrophobic tails a lower one. From this, also the thickness of the interbilayer water layer can be calculated [34, 35].
3.1 Liposomes

No diffraction peaks are naturally observed in SAXS from large unilamellar vesicles (LUVs), as a well-ordered lamellar structure is absent. A similar situation is observed in the scattering intensity of multilamellar vesicles (MLVs) that are composed of a mixture of lipids: the thermal unbinding of the lipid layers results in a diffraction pattern with one large, wide maximum. Multilamellar vesicles in the L\textsubscript{α}-phase can be modeled as unilamellar vesicles [36].

A simple way to look at liposomes is to model them as large unilamellar planes with an electron density composed of three Gaussians. The relative electron density $\rho_R$ can be expressed with the electron density of the headgroups, $\rho_H$, the electron density of the hydrocarbon tails, $\rho_C$, and the ambient electron density $\rho_A$:

$$\rho_R = \frac{\rho_C - \rho_A}{\rho_H - \rho_A}. \quad (22)$$

Thus, the form factor of the membrane becomes

$$F(q) = \sqrt{2\pi}(2\sigma_H e^{-\sigma_H^2 q^2/2} \cos(qz_H) - \rho_R \sigma_C e^{-\sigma_C^2 q^2/2}), \quad (23)$$

where $\sigma_H$ and $\sigma_C$ are the widths of the Gaussian functions used to model the electron density and $z_H$ is the distance of the center of the maximum of the headgroup Gaussian to the center of the bilayer [34, 36]. The scattering intensity $I_{LUV}$ is

$$I_{LUV}(q) = \frac{|F(q)|^2}{q^2}. \quad (24)$$

By stacking $N$ of these bilayers together, the structure factor of the multilamellar structure is

$$S(q) = N + \sum_{k=1}^{N-1} (N - k) \cos(kqd), \quad (25)$$

where $d$ is the lamellar distance of the bilayers. The scattering intensity for a multilamellar structure, $I_{MLV}$ is then

$$I_{MLV}(q) = \frac{S(q)|F(q)|^2}{q^2}. \quad (26)$$

While more complicated models, such as those accounting for the presence of unilamellar liposomes adding to the diffuse scattering [36], models including effects of liposome size [10, 37] and models utilizing two Gaussian functions for the head group electron density [38] exist, many features of the scattering from lipid bilayers can be explained with simple models.

Scattering patterns calculated from Equations 24 and 26 are shown in Figure 2. The scattering pattern of LUVs has a single, wide maximum, whereas the scattering patterns of MLVs have diffraction peaks. The figure illustrates that the peaks are narrowed and the intensity is amplified when more lamellae are added. This observation corresponds to the premise set out by the Scherrer equation, Equation 17.
3 MATERIALS

Figure 2: The theoretical scattering patterns of multilamellar (main figure) and large unilamellar (inset) vesicles calculated from equations 26 and 24, respectively. The MLV patterns are calculated with ten (blue), twenty (black), or thirty (red) layers. The other parameters used in the calculation are shown in the text box.

3.2 S-layer coated liposomes

Surface (S-) layers are crystalline protein layers that cover the surface of many bacteria and archaea. S-layers are composed of identical protein subunits, which assemble into a regular lattice with evenly shaped and distributed pores. When present, they are the most abundant cellular proteins, but no a common function has emerged and there is wide variation in the protein encoding sequences [39, 40].

The functions of S-layers range from supporting, acting as sieves and protecting the organism against environmental factors, to bacterial adhesion and factors in virulence. Some bacterial species express different S-layer proteins in different environmental conditions [39, 40].

The interesting property that all S-layers share is self-assembly into 2D crystallites. S-layers form regular arrays on suitable surfaces (the air-water interface, on solid supports or lipid layers) or in solution. They have a tendency to neither stay as monomers in solution nor to crystallize into three-dimensional structures, although bilayers have been formed [41, 42].

S-layer proteins reassemble on liposomes [43] and stabilize the liposomes against e.g. temperature and pH changes, and exposure to bile salts and pancreatic extracts [11]. S-layer coatings also prevent leakage of functional molecules enclosed in the liposome and offer a template for the attachment of functionalized molecules on the surface of the particle. S-layer coated liposomes can also be further coated with silica to make cages with a surface texture [44].
Lactobacilli are rod-shaped lactic acid bacteria. Many lactobacilli have an S-layer, and their S-layer proteins share several common characteristics, such as a relatively low molecular weight (20-70 kDa) and a high predicted pI [45].

The functions of Lactobacillus S-layers are varied. Also their self-assembly properties vary: the S-layer protein of Lactobacillus acidophilus ATCC 4356 crystallizes on negatively charged phospholipid monolayers composed of various lipids, but forms only small patches on zwitterionic phosphocholine monolayers [46]. On the other hand, despite their positive total charge at neutral pH, S-layer proteins of Lactobacillus kefir JCM 5818 and Lactobacillus brevis ATCC 14869 reassemble on positively charged liposomes by modifying the lipid order [11, 47].

3.3 Liposomes and ionic liquids

Ionic liquids (ILs) are salts which are liquid at room temperature. They are more and more commonly used as solvents in industrial processes, as they have many desirable properties: they dissolve biomass and have a high chemical and thermal stability. Due to the possibilities of fine-tuning the properties of the ILs by choosing the anion and cation freely, ILs have been considered “designer solvents” [48]. ILs have a variety of uses ranging from cellulose regeneration [49] and nanoparticle synthesis [50] to use as electrolytes in fuel cells [51].

Phosphonium-based ILs are quaternary phosphonium salts that have recently started to become available in industrial scale. The higher thermal stability and a lower acidity than ILs with an imidazolium cation make them an attractive option for industry [52].

However, ILs are often toxic, and while their water-solubility is an advantage for their industrial use, it poses a threat to e.g. aquatic organisms [53, 54]. The harmfulness of ILs, both with and without phosphonium cations, has been linked to their lipophilicity and thus their effects on the cell membrane [7, 55, 56]. The length of hydrocarbon chains in the cation seems to play a significant role, although the size of the anion also matters, especially for large and bulky anions.

In particular, phosphonium-based ILs have generally shown more toxicity than other moieties [57]. Phosphonium-based ILs with long hydrocarbon chains (14 carbons) are significantly more harmful than those with moderately long hydrocarbon chains (4-8 carbons) [7]. The interactions between model phospholipid bilayers and various ILs have been studied by computational means [58, 59, 60] (for a review, see [61]) and by several experimental techniques, such as neutron reflectometry [62], confocal laser scanning microscopy [60] and SAXS [63]. The variety of methods used manifests the usability of liposomes and more generally phospholipid bilayers as model systems for the cell membrane.

3.4 Biohybrid material from ceramophilic chitin

Calcium carbonate (CaCO₃) is a common mineral which is present in nature both as a mineral from inorganic sources — it precipitates from an amorphous form — and in the load bearing structures of many biological structures, such as mollusk shells and egg shells. CaCO₃ comes in several polymorphs. Calcite is the most
stable form, but in biological materials, the less stable aragonite is often present. In nacre in mollusk shells, CaCO$_3$ is usually aragonite, although also species with calcite or both calcite and aragonite exist [14]. This iridescent inner layer of the shell is composed of an organic matrix composed of chitin, silk-like proteins and highly acidic proteins that contain a lot of aspartate. The formation of nacre is not fully understood, but a similar material has been made with a biomimetic layer-by-layer assembly, though without protein and synthetic polymers [64]. Also in nature, the chitin matrix is present first. The chitin strands are aligned and the matrix is porous, so when CaCO$_3$ starts to crystallize — likely from an amorphous layer present in the matrix — the crystal axes of the CaCO$_3$ platelets are aligned with each other [14].

3.5 Xylan films

Wood is composed primarily of cellulose, hemicelluloses and lignins. For example in birches (Betula), the hemicellulose component forms 25-30% of the wood [17, Ch.3 &10]. With the decrease of consumption of printing paper, a need has arisen to develop new materials from kraft pulp in the pulp industry. However, many applications, such as cellulose-based clothing fibers and other cellulose derivatives, have little use for hemicelluloses, and several industrial processes, such as the production of nanocrystalline cellulose, yield hemicelluloses as a by-product. With the rising need of advanced renewable materials, putting these by-products into use has several attractive sides.

The main hemicelluloses in hardwood are xylans. In birch, the hemicelluloses are mainly glucuronoxylans, where β-(1 $\rightarrow$ 4)-linked xylopyranosyls form a chain that carries (1 $\rightarrow$ 2)-linked 4-O-methyl-α-d-glucopyranosyl uronic acid residues and acetyl groups. In alkaline extraction of glucuronoxylans, these residues are lost and a linear polymer is obtained. Advantages to synthetic polymers are that no polymerization needs to be done and, like many other natural polymers, the obtained xylans are inherently biodegradable.

The properties of films can be tailored with plasticization. In external plasticization, chemicals such as polyols are added to the polysaccharide matrix, whereas in internal plasticization the polysaccharides are modified to reduce their tight packing. Plasticization can also be necessary for film formation. In xylans, introducing hydroxypropyl groups to the xylan backbone has been investigated as a way to tailor the properties of the material [65, 66]. Hydroxypropylation is also necessary for processing, as xylans obtained from alkaline extraction are not water soluble.

The plasticization of films affects their mechanical and barrier properties. In addition to mechanical strength, desirable properties for a packaging material include low permeability for gases (especially oxygen), water vapor, aromas and lipids [67]. Crystallites add strength to the material and are less permeable than amorphous matter [68], but can make the material hard and brittle, whereas amorphous materials add softness and tenacity. Here again, desirable properties for a material result from a combination of different components.
3.6 Aims of the studies

In this thesis, biological and biomimetic materials were studied with X-ray scattering. The aim was to characterize the nanostructure of the materials to either study their organization in a new environment, changes brought on by external factors, or examine the structure-function relationship.

In Paper I, the S-layer protein SlpA of *Lactobacillus brevis* ATCC 8287 was reassembled on liposomes. S-layer lattice constants have previously been determined by electron microscopy or atomic force microscopy, but primarily for native S-layers. On the other hand, many studies of S-layer reassemblies on liposomes focus on the effects of S-layer coatings in adding stability to the liposomes, using S-layers to keep functional molecules in or as templates for the attachment of other molecules on liposome surface. The aim here was to study whether SlpA attaches on liposome surfaces with different electrical properties, and whether SAXS can be used to assess its structure on liposomes, as this had not previously been done.

In Paper II, the effects of phosphonium-based ionic liquids on liposome model systems were studied with SAXS, zeta potential measurements and dynamic light scattering measurements. The purpose of this study was to assess the effects of the studied ionic liquids on liposomes that mimic the cell membrane, which in turn can be used to understand toxicity mechanisms of ionic liquids. Previous SAXS studies of ionic-liquid interactions with liposomes are limited to multilayer systems.

In Paper III, a new biomimicking material was developed and characterized. The mechanical properties of the material, composed of chitin and a genetically engineered hybrid protein, were characterized and its nanostructure was studied by electron microscopy and WAXS to reveal structure-function relations.

In Paper IV, the aim was to develop new xylan-based films for package material use. The mechanical and barrier properties of the package materials were characterized, and their nanostructure was studied with WAXS to yield crystallite size and crystallinity, as the permeability and mechanical properties of the films depend on the amount and distribution of crystallites in the sample.
4 Methdology

4.1 X-ray scattering experiments

4.1.1 X-ray sources

X-rays are emitted by accelerating charged particles. Traditionally, X-rays are produced by X-ray tubes, where electrons are accelerated to hit the anode, which leads to the production of a continuous spectrum of bremsstrahlung as well as the characteristic emission peaks of the anode material. More intense X-ray beams can be produced at synchrotrons, where electrons or, in some rare instances, positrons are kept circulating at a relativistic speed in a storage ring using magnetic fields [21, Ch.1]. The intensity obtained at synchrotrons makes it possible to perform experiments at a high time resolution, for example scattering experiments with millisecond resolution. More recently, X-ray sources with an even higher intensity have been built: the intensity of free-electron lasers surpasses that of synchrotrons by several orders of magnitude [69].

4.1.2 X-ray scattering set-ups

X-rays produced both by X-ray tubes and synchrotrons are polychromatic. Laboratory X-ray sources produce a highly divergent beam [20, Ch.1], whereas the radiation cone obtained from the bending magnets or insertion devices from a synchrotron is more compressed [21, Ch.2]. In small- and wide angle X-ray scattering, a monochromatic and focused beam is needed for experiments, and this is achieved by the use of monochromators and focusing optics.

In laboratory sources, the characteristic X-ray wavelength of the anode material is selected, due to its vastly higher intensity than other wavelengths. Depending on the optics used, either the K\(_{\alpha}\) doublet or the more intense K\(_{\alpha1}\) line is selected from the spectrum and either collimated or focused to the detector. A schematic of a SAXS set-up is shown in Figure 3. This set-up was used in the home-laboratory SAXS experiments in Paper I, but the basic principle of the set-ups is similar both at beamline I911-SAXS in MAX IV Laboratory, Lund, Sweden [70] (Papers I-II) and in the home-laboratory WAXS experiments (papers III-IV).

4.2 Crystallite size

In experiments, broadening of the diffraction peaks arises both due to the finite crystallite size, as described earlier, and instrumental effects. The intensity of a diffraction peak is always smeared due to non-ideal characteristics of the incoming beam (divergence, scattering) and the point-spread function of the detector, even when scattered from a perfect crystal.

Crystallite size is an important parameter to describe the properties of the sample. It affects, for example, the thermal degradation of natural polymers [71] and the degree of cellulose polymerization [72]. Crystallite size can be calculated from the width of the diffraction peak \(B\) (Equation 17).
4.3 Crystallinity determination

Figure 3: Schematic of the home laboratory X-ray scattering set-up used in Paper I. The mirror focuses the scattering in the vertical direction and the crystal in the horizontal direction.

When determining $B$, the width of the diffraction peak must be corrected for the instrumental broadening. At large scattering angles, this is done by measuring a calibration sample with crystallites of “infinite” size, that is, crystallites that would be large enough to result in diffraction peaks with a negligible width if the instrumental conditions would be ideal. These are typically minerals. In these cases, for Gaussian line profiles $B = \sqrt{\text{FWHM}_{\text{peak}}^2 - \text{FWHM}_{\text{inst}}^2}$, where $\text{FWHM}_{\text{peak}}$ is the FWHM of the peak in the sample, and $\text{FWHM}_{\text{inst}}$ is the width of a peak of a calibration standard, located close to the sample peak on the $2\theta$ scale, as the instrumental broadening depends on the scattering angle.

However, for small angles, no standards exist for which the crystallite scattering domain can be considered infinite. The calibration standards available for small-angle calibration, such as silver behenate, have a crystallite sizes around some hundreds of nanometers, and this means that calibration peaks are broadened both by the instrumental factors and by the inherent properties of the calibration sample itself.

4.3 Crystallinity determination

In theory, determining the crystallinity of a sample requires determination of total scattering of the sample from $q = 0$ to $q = \infty$ (see Equation 20). In practice, determining the whole diffraction pattern from zero to infinity is not possible, but a fair approximation is to use the whole measured WAXS range for samples which have correlation distances in the length-scale probed by WAXS.

Separating the scattering originating from the crystalline portions of the sample is difficult, particularly for samples with a small crystallite size and thus wide diffraction peaks. Instead, the approach taken in paper IV in this thesis is the amorphous fitting method, where an amorphous standard is measured. The amorphous standard is ideally chemically equivalent to the studied sample, though such
a sample is not always available. Fortunately, often the fits are not very sensitive to the choice of amorphous standard [73], and e.g. sulfate lignin can be substituted for amorphous cellulose [74] and glucomannan [75] or arabinoxylan [76] for xylan.

If the sample is assumed to be a two-component system, the contribution of the scattering intensity of the amorphous component, \( I_a \), is related to the total scattering intensity and crystalline scattering by \( I_a = I - I_c \). The height of the amorphous background is fixed by fitting a linear combination of it and Gaussian peaks situated at the positions of the diffraction peaks of the crystal into the measured scattering intensity. The peak positions are adjusted to the positions of the reflections of known lattice constants, and the width of the reflections is set to fit the diffraction pattern. The crystallinity index \( w_c \) is then calculated from

\[
    w_c = 1 - \frac{\sum I_a}{\sum T}.
\]

For cellulose, results from this method agree with crystallinity determinations by NMR [73, 77].

4.4 Dynamic light scattering

Light scattering from the visible wavelengths of electromagnetic radiation can be used to determine particle sizes. Dynamic light scattering (DLS) is based on the fact that particles in a dispersion undergo Brownian motion, which is related to their size: small particles move faster than large particles. The Stokes-Einstein equation,

\[
    a = \frac{k_B T}{6\pi \eta D},
\]

relates the hydrodynamic radius of the spherical particles, \( a \), with the diffusion coefficient \( D \), and the viscosity of the solvent \( \eta \) at thermal energy \( k_B T \).

In DLS, the incident laser light at frequency \( \omega_0 \) is scattered from particles, and it may exchange energy while scattered, corresponding to a change in its frequency. This change in frequency, \( \Delta \omega = \omega - \omega_0 \), is the Doppler shift and depends on particle velocity. The intensity distribution of the scattered radiation \( I(\omega) \) is a Lorentzian with a width \( Dq^2 \) [78] and the diffusion constant can be determined from the equation

\[
    I(\omega) \propto \frac{Dq^2}{(\omega - \omega_0)^2 + (Dq^2)^2}.
\]

The obtained \( D \) can then be used in Equation 28 to yield the hydrodynamic radius of the particles. In practice, the measurement system measures intensity fluctuations and calculates the correlation function for them. The correlation functions for small particles decay faster than for large particles, and the size distribution of the sample is derived from a combination of a number of size classes [79].
4.5 Zeta potential measurements

The electrokinetic behavior of a suspended, charged particle in solution depends on the electric potential at the surface of shear between the particle surface and the electrolyte solution. This potential is called the electrokinetic or zeta ($\zeta$) potential [80, Ch.7]. It is related to, but not equivalent with the surface potential of the particles: the charged particles accumulate counterions from the solute to their surfaces. These attached ions travel with the particle and are called the Stern layer (Figure 4). Outside of the Stern layer, in the diffuse layer, ions are less firmly attached. The shear surface is the outer limit of the stationary layer of fluid attached to the particle. Its thickness decreases with increasing ion concentration of the surrounding medium [81, Ch.3]. The Stern layer and the diffuse layer form the electrical double layer.

The zeta potential is related to the stability of the dispersion. The larger the zeta potential, the less likely the particles are to aggregate, as there is increased electrostatic repulsion between the charged particles. Generally, particles with $|\zeta| > 30$ mV are considered stable [79].

In zeta potential measurements, the electrophoretic mobility (the velocity per field strength) is derived by applying an electric field over the sample, which causes the charged particles to migrate. In a measurement called laser Doppler velocimetry, the scattered light is combined with a reference beam and the fluctuations of the combined intensity are measured. The rate of fluctuation is proportional to particle velocity and hence the electrophoretic mobility $u$.

The zeta potential $\zeta$ can then be calculated from the Henry equation:

$$u = \frac{2}{3} \frac{\varepsilon \zeta}{\eta} f(\kappa a). \quad (30)$$

In the above equation, $\varepsilon$ is the dielectric constant, $\eta$ the viscosity of the fluid and $f(\kappa a)$ Henry’s function [79, 81, Ch.3]. Henry’s function attains different values for particles of different size and depends also on the electrolyte concentration. A common approximation, also used in the works in this thesis, is the Smoluchowski approximation ($f(\kappa a) = 1.5$), which is valid for moderate electrolyte concentrations ($> 10^{-3}$ M), or when the particle diameter $a$ is much larger than the thickness of the electrical double layer $1/\kappa$: $\kappa a \gg 1$ [80, Ch.7].
Figure 4: Schematic of the surface and zeta potentials of a negatively charged particle with potential as a function of distance from particle surface $r$. Zeta potential $V_z$ is the potential at the shear surface located at $r_z$. Outside the shear surface, a diffuse layer of counter and co-ions is formed. The total width of the electrical double layer is $r_d$. 
5 Results and discussion

5.1 S-layer coated liposomes (Paper I)

Paper I concerns the reassembly of the S-layer protein SlpA of *Lactobacillus brevis* ATCC 8287 on positively charged liposomes composed of 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt) (DOTAP) (70:30 mol%). SlpA facilitates adhesion to intestinal epithelia [82], and the bacterial strain itself has been proposed as potential vaccine vector — like many other lactobacilli, it is a putative probiotic [83, 84].

An example of an SlpA coated liposome with a reference uncoated liposome is shown in Figure 5. The liposome in this figure differs from those studied in Paper I by composition of lipids, though not by surface charge, as it is composed of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and DOTAP in a 70:30 mol-% ratio.

The SlpA results were compared to results of native SlpA from bacterial cell wall fragments (CWF). The measurements of CWF were done in home laboratory, whereas the SAXS experiments from SlpA coated liposomes were conducted at MAX IV Laboratory in Lund, Sweden. The size distributions of the samples and their zeta potentials were measured with Zetasizer Nano-ZS (Malvern Instruments Ltd, Worcestershire, UK).

![Figure 5](image.png)

Figure 5: Cryo-electron micrograph of a large unilamellar vesicle composed of 70 mol-% of DPPC and 30 mol-% of DOTAP. a) Reference uncoated liposome and b) liposome coated with SlpA. The size difference between these two liposomes is due to the liposome size distribution produced by the extrusion method. The images were obtained with a FEI Tecnai F20 electron microscope (Philips Electron Optics, Holland, voltage 200 kV) and Gatan Ultrascan 4000 CCD-camera (Gatan Inc., USA) at a magnification of 50k and defocus of 4000 nm.
5.1.1 Native SlpA

The lattice constants $a$, $b$, and $\gamma$ were solved from the SAXS pattern of *L. brevis* ATCC 8287 cell wall fragments. The results agree well with the previous electron microscopy results [85], where the lattice constants of the S-layer have been determined from freeze-fractured whole *L. brevis* cells (Table 1). Comparing the results from CWF to those from the freeze-fractured whole cells, a 6% difference in the value of the lattice constant $b$ was observed. It is noteworthy that this larger change is in the lattice constant $b$, as, in light of the results from SlpA coated liposomes, this is the less stable direction for the S-layer crystallite. However, this difference is very close to the error margins of the electron microscopy measurement, which are 5% of the determined values, and those of the values derived from the SAXS measurement (3-5%). Thus, the observed difference may either reflect a true change due to the relaxation of the crystallite as the cell is fractured and emptied for the SAXS measurement, or just measurement uncertainty.

5.1.2 SlpA on liposomes

In studies of the S-layer coated liposomes, a clear difference between the behavior of charged and net neutral liposomes emerged. The SAXS pattern of anionic and cationic liposomes that had been hydrated with the soluble fraction of SlpA showed a clear difference from the SAXS pattern of reference liposomes in the low $q$-range, while the curves were congruent at higher $q$. The scattering patterns of the zwitterionic POPC liposomes showed no such feature: while a small difference between the scattering patterns was observed, this was related to the two scattering maxima that indicated that the sample was not fully unilamellar. Indeed, the difference between the samples was palpable during preparation: during the extrusion, the zwitterionic sample with SlpA was almost impossible to extrude because of the aggregates that had formed in solution, whereas the SlpA containing samples that had liposomes with a positive or negative surface charge were supple and easy to extrude.

However, diffraction peaks related to SlpA were only detected for the sample with cationic liposomes. If reassembly took place also on the anionic liposomes, the crystallites that formed were too small to give rise to discernible peaks, and more closely resembled amorphous matter. X-ray scattering only detects crystallites which are composed of several repeat units on equal distance from each other. Two or three repeat units do give rise to amplified scattering, but no discernible peaks, whereas ten repeat units result in clear diffraction peaks. Between these cases, the detection of diffraction peaks depends greatly on the crystallite properties: if broad diffraction peaks overlap, their detection and separation are difficult. Indeed, the scattering domain of the POPC sample is quite small, but the lamellar structure allows the observation of the well-separated diffraction peaks.

It is clear that of the studied liposomes, SlpA prefers to reassemble on cationic liposomes. No significant differences was found between the samples extruded through 50 nm and 100 nm membranes. However, the ratio of the diameter of the liposomes in the two samples was not 1:2, but smaller, so the difference in the curvature of the surface was not that significant.
5.2 Liposome and ionic liquid interactions (Paper II)

The lattice constant $a$ remained virtually unchanged in the reassembly. However, both $b$ and $\gamma$ grew markedly (Table 1). This means that the lattice spanned by the proteins opens up and its pores must become larger. The symmetry of the crystal is likely $p2$, as the protein is fairly small to begin with and does not fill the unit cell. The crystallite is clearly more stable in the direction of $a$, as this is the direction that gives rise to the most well-defined diffraction peak and also the spacing which remains unchanged in the reassembly of the proteins.

The crystallite size of SlpA on cationic liposomes was estimated from the full width at half maximum of the diffraction peak corresponding to the (1 0) lattice planes. As the peaks were wider than those of the calibration sample, a lower limit of the crystallite size was obtained by neglecting the instrumental broadening and the upper limit by setting it equal to the FWHM of silver behenate. Thus, a range for $s$ was obtained: $92 \text{ nm} < s < 114 \text{ nm}$.

Table 1: The lattice constants $a$, $b$ and $\gamma$ of SlpA on different surfaces. Lattice constants for SlpA on cationic liposomes and on cell wall fragments are determined by SAXS, lattice constants for SlpA on whole bacteria are determined by EM in ref. [85].

<table>
<thead>
<tr>
<th>Sample</th>
<th>$a$ (nm)</th>
<th>$b$ (nm)</th>
<th>$\gamma$ (deg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SlpA reassembled on cationic liposomes</td>
<td>9.29±0.33</td>
<td>8.03±0.33</td>
<td>84.9±2.5</td>
</tr>
<tr>
<td>Native SlpA on cell wall fragments</td>
<td>9.41±0.40</td>
<td>6.48±0.40</td>
<td>77.0±2.4</td>
</tr>
<tr>
<td>Native SlpA on whole bacteria</td>
<td>9.39</td>
<td>6.10</td>
<td>79.8</td>
</tr>
</tbody>
</table>

5.2 Liposome and ionic liquid interactions (Paper II)

Paper II is a study of the effects of phosphonium-based ILs on multilamellar MLVs composed of POPC and biomimicking LUVs composed of L-α-phosphatidylcholine (eggPC) and L-α-phosphatidylglycerol (eggPG) (80:20 mol%) or eggPC, eggPG and cholesterol (60:20:20 mol%). The results from tributylmethylphosphonium acetate ([P$_{4441}$][OAc]) were compared to those from trioctylmethylphosphonium acetate ([P$_{8881}$][OAc]) to see the effect of longer hydrocarbon side-chains in the cation, and to those from tributyl(tetradecyl)phosphonium acetate and tributyl(tetradecyl)phosphonium chloride ([P$_{14444}$][OAc] and [P$_{14444}$]Cl respectively), to see the effect of a cation with one very long, lipophilic hydrocarbon chain. Results were also compared to effects of 1-ethyl-3-methyl imidazolium acetate ([emim][OAc]), which is an imidazolium-based IL with a small, not very lipophilic cation. [emim][OAc] is much less harmful than the long-chained phosphonium-based ILs, and one of the commonly used ILs [7].

The effects of ILs on liposomes were studied with SAXS experiments at beamline I911-SAXS at MAX IV Laboratory, Lund, Sweden. Due to experimental difficulties, suppression of the background scattering could not be done reliably for these data. The lamellar distance of MLVs was examined quantitatively and the scattering patterns of LUVs were interpreted qualitatively.

In addition, size distribution and zeta potential measurements were conducted with Zetasizer Nano-ZS (Malvern Instruments Ltd, Worcestershire, UK).
5.2.1 Multilamellar vesicles

The effects of ILs on MLVs can be divided into two categories: effects on the lamellar distance and effects on the order in the sample. The short-chained ILs \([\text{P}_{4441}]\text{[OAc]}\) and \([\text{emim}]\text{[OAc]}\) influenced only the lamellar distance in the MLVs at all studied concentrations (3-50 mM). They had little effect on peak intensity: the intensity of the observed diffraction peaks remained the same or even grew slightly, and the shape and width of the peaks did not change markedly. This indicates that the multilamellar structure of the vesicles remains intact, but the ILs penetrate the multilamellar structure. The shift in the position of the diffraction peaks was dependent on IL concentration: higher concentrations lead to greater shrinking of the lamellar distance. The results are summarized in Table 2.

However, the ILs with longer hydrocarbon chains had more drastic effects on the MLVs. At low concentration (0.8 mM for \([\text{P}_{8881}]\text{[OAc]}\) and 0.4 mM for \([\text{P}_{14444}]\text{[OAc]}\), a similar shift of diffraction peak positions that with the short-chain ILs was found, but the intensity of the diffraction peaks decreased markedly, indicating a loss of order in the lamellae. At higher concentrations (8-17 mM for \([\text{P}_{8881}]\text{[OAc]}\) and 3 mM for \([\text{P}_{14444}]\text{[OAc]}\), the diffraction peaks were lost or found to be rapidly dissolving during measurement. For the MLVs mixed with 3 mM \([\text{P}_{14444}]\text{[OAc]}\) only a wide maximum remained, indicating that the multilayer structures had lost their order, but structures with electron density differences in this size scale still remained in the sample. As the position of the diffraction maximum was at a higher \(q\)-value than that of the characteristic maximum of unilamellar vesicles, it is possible that this new structure is a bilayer which has expanded to incorporate the large IL cation.

Effects of \([\text{P}_{8881}]\text{[OAc]}\) were even more drastic (Figure 6). At 8 mM concentration, all scattering features were lost, indicating a total loss of structure at the length scale probed by SAXS. At an even higher concentration, 17 mM, a new wide scattering maximum emerged at much lower angles, indicating that new aggregates

<table>
<thead>
<tr>
<th>Ionic liquid</th>
<th>(c) (mM)</th>
<th>(d) (nm)</th>
<th>(s) (nm)</th>
<th>MLV characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>-</td>
<td>6.45</td>
<td>210</td>
<td>MLV</td>
</tr>
<tr>
<td>([\text{emim}]\text{[OAc]})</td>
<td>5.0</td>
<td>6.33</td>
<td>180</td>
<td>MLV</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>5.95</td>
<td>200</td>
<td>MLV</td>
</tr>
<tr>
<td>([\text{P}_{4441}]\text{[OAc]})</td>
<td>2.0</td>
<td>6.41</td>
<td>200</td>
<td>MLV</td>
</tr>
<tr>
<td></td>
<td>30.0</td>
<td>6.04</td>
<td>190</td>
<td>MLV</td>
</tr>
<tr>
<td>([\text{P}_{8881}]\text{[OAc]})</td>
<td>0.8</td>
<td>6.39</td>
<td>200</td>
<td>dissolving MLV</td>
</tr>
<tr>
<td></td>
<td>8.4</td>
<td>-</td>
<td>-</td>
<td>no order</td>
</tr>
<tr>
<td></td>
<td>16.8</td>
<td>-</td>
<td>-</td>
<td>liquid crystal</td>
</tr>
<tr>
<td>([\text{P}_{14444}]\text{[OAc]})</td>
<td>0.4</td>
<td>6.43</td>
<td>190</td>
<td>MLV</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>6.39</td>
<td>-</td>
<td>dissolving MLV + liquid crystal</td>
</tr>
</tbody>
</table>

Table 2: Effects of ILs on POPC MLVs. Concentration \(c\), lamellar distance \(d\), and the lower limit of the length of the scattering region \(s\) calculated from the Scherrer equation.
with order in the corresponding length-scale appeared. The scattering pattern had the characteristics of a liquid crystal with order only in one direction, or a thicker lipid bilayer.

Previous studies show that the effects of ILs on the model systems are varied. For example, both computational methods, neutron reflectometry, and SAXS results show that the lamellar distance in both supported phospholipid bilayer stacks and multilamellar vesicles shrink when the system is exposed to ILs. However, both the magnitude of the change and whether it is dependent on IL concentration vary. Molecular simulations and neutron reflectometry show an even thinning of ∼0.1 nm independent of concentration [58, 62], whereas SAXS results show a thinning that is concentration-dependent and can exceed 1 nm [63]. Confocal laser scanning microscopy, on the other hand, shows conformational changes to a lipid monolayer: as a function of IL concentration, supported bilayers turn into multilayers, tubes, vesicles, or the membrane is completely disrupted. The lipid bilayer also shows swelling due to incorporation of the IL cations and due to bending [60].

These previous results are congruent with results in paper II: the difference between the thinning observed in computational and neutron reflectivity measurements and SAXS from MLVs may stem from the difference in dynamics of supported

![SAXS intensities of POPC MLVs mixed with [P$_{8881}$][OAc].](Figure6.png)

Figure 6: SAXS intensities of POPC MLVs mixed with [P$_{8881}$][OAc]. Reference MLVs (black) and MLVs mixed with 0.8 mM (blue), 8.4 mM (green), and 16.8 mM (red) [P$_{8881}$][OAc].
multilayers and MLVs, which has been previously observed with salts [86].

In summary, the results show that more harmful ILs destroy the lamellar structure of MLVs. This interaction can be followed in situ using synchrotron radiation, as for particular concentrations, the time-scale of the reaction are of the order of seconds to minutes. Less harmful ILs do not influence the lamellar order, but shrink the lamellar distance. Clearly, even the ILs with a short hydrocarbon chain are able to penetrate the entirety of the multilamellar structure (>30 bilayers), as peak shape and width are virtually unaffected by the addition of ILs, so the chemical environment must be the same throughout the multilayer.

5.2.2 Large unilamellar vesicles

**DLS and zeta potential results** The results from large unilamellar vesicles shed light on the interaction of ILs with the lipid bilayer. LUVs are better models for cell membranes, as the cell membranes are unilamellar. Here, the unilamellar vesicles are a better mimic for the cell membrane also due to the inclusion of cholesterol, which is a molecule that increases the rigidity of the cell membrane [87].

The ability of ILs to disrupt liposomes were, again, dependent on the length of the hydrocarbon chains in the IL cation. DLS and zeta potential measurements showed that the IL cations coat the anionic liposomes, reversing the surface charge. The concentrations at which this occurred were dependent on the chain length of the IL cations. If concentration was increased after the reversal of the surface charge, the liposomes were disrupted. The concentration in which this happened is close to the the critical micelle concentration of the ILs.

The size of the LUV was also affected by IL concentration. In all studied cases, the liposomes shrank slightly when exposed to ILs. Around or above the critical micelle concentration, particle aggregation is observed. It is likely that the ILs are incorporated into these novel structures.

**SAXS results** The SAXS data from LUV samples was analyzed by comparing the shape of the scattering pattern of LUVs mixed with ionic liquids to the SAXS patterns of reference samples. The reference patterns clearly showed the typical features of a lipid bilayer, with a diffuse characteristic maximum around \( q = 1.1 \, \text{nm}^{-1} \) (for LUVs composed of eggPC, eggPG, and cholesterol) and \( q = 1.2 \, \text{nm}^{-1} \) (for LUVs composed of eggPC and eggPG).

For the ILs with the longest hydrocarbon chain, \([P_{14444}][\text{OAc}]\) and \([P_{14444}][\text{Cl}]\), concentrations around the critical micelle concentration did not affect the scattering pattern of the liposomes. However, a higher concentration caused a shift of the SAXS maximum to lower \( q \)-values, indicating a change in the structure of the lipid bilayer.

The IL with medium length hydrocarbon chains, \([P_{8881}][\text{OAc}]\), showed markedly different behavior from other samples when mixed with eggPG/eggPG/cholesterol liposomes at a concentration of 0.8 mM. During the measurement, diffraction peaks that resembled those of multilamellar vesicles appeared. These diffraction peaks also grew in intensity and narrowed in width during the ten-minute measurement.
Higher concentrations of $[\text{P}_{8881}][\text{OAc}]$ wiped out all features related to the bilayer structure. In the eggPC/eggPG sample lacking cholesterol, a similar change did not occur: the characteristic maximum of the lipid bilayer is present in the sample mixed with 0.8 mM $[\text{P}_{8881}][\text{OAc}]$. However, based on the lamellar spacing calculated from the diffraction peaks, the most likely source of diffraction is not cholesterol but eggPC, displaced from the liposome and aggregated into well-ordered multilayers.

In this sample, the concentration of LUVs was 4 mM, and the fraction of cholesterol in the sample is 20 mol%, which brings the cholesterol concentration of the sample to 0.8 mM. The separation effect is likely due to matching the concentration of $[\text{P}_{8881}][\text{OAc}]$ to that of cholesterol and possibly also that of the anionic lipid species eggPG. Only matching the latter is clearly not enough, as similar separation did not happen in the sample lacking cholesterol, even though the concentration of eggPG was 0.8 mM also in that sample.

The effects of $[\text{P}_{8881}][\text{OAc}]$ are summarized in the schematic in Figure 7.

![Figure 7: Schematic of the effects of $[\text{P}_{8881}][\text{OAc}]$ on LUVs composed of eggPC, eggPG, and cholesterol (60:20:20 mol-%). SAXS curves of reference LUVs (black) and LUVs mixed with 0.8 mM (blue), 8.4 mM (green), and 16.8 mM (red) $[\text{P}_{8881}][\text{OAc}]$. Exposure to 0.8 mM $[\text{P}_{8881}][\text{OAc}]$ is shown after 20 min (solid line) and 30 min (dotted line) of exposure.](image)

### 5.3 Biohybrid material from ceramophilic chitin (Paper III)

Paper III is a study of a biohybrid material, which was composed of chitin and a genetically engineered hybrid protein (ChBD-aspein). ChBD-aspein consists of a chitin-binding domain and a highly acidic fragment of a shell-specific protein from the pearl oyster. The studied samples were films prepared by vacuum filtration of a solution of chitin nanofibers, with and without ChBD-aspein, CaCO$_3$, and CaCl$_2$. The samples were tested for their mechanical properties: stiffness, ultimate tensile strength, and maximum elongation, and studied by scanning electron microscopy (SEM), cryo-electron microscopy, and WAXS.
The presence of the ChBD-aspein protein in the films influenced their mechanical properties. Surprisingly, both samples prepared with CaCO$_3$ and samples prepared with CaCl$_2$ showed changes in the mechanical properties of the material: ultimate tensile strength of the films increased, as did the stiffness.

WAXS was used to study the nanostructure of the biohybrid material. The research question was to find out whether CaCO$_3$ was formed and if it was, which polymorph(s) were present. Additionally, the crystallite size and orientation of CaCO$_3$-crystallites were objects of interest, together with their correlation with the mechanical properties of the samples. The samples studied were films prepared with equal molar amounts of ChBD-aspein and Ca$^{2+}$ and CO$_3^{2-}$ ions, and ChBD-aspein with a tenfold excess of Ca$^{2+}$ and CO$_3^{2-}$ ions. As a reference, samples prepared without protein but with equivalent amount of CaCO$_3$ were measured.

5.3.1 WAXS results

WAXS studies revealed crystalline chitin in all studied samples, but only the sample containing ChBD-aspein and CaCO$_3$ in a ratio of 1:10 showed diffraction peaks from crystalline CaCO$_3$, which were identified as calcite. Samples prepared without the protein but with CaCO$_3$ or with protein and CaCO$_3$ in a 1:1 molar ratio showed no evidence of crystalline calcium carbonate. The crystallites were randomly oriented and so no preferred orientation was found. The scattering patterns of the samples prepared with the 10-fold excess of calcium and carbonate ions are shown in Figure 8.

The mean size of the crystallites for the [104] direction was calculated to be (50.2 ± 1.2) nm. Cryo-electron micrographs showed the crystallites to be well integrated into the chitin matrix, and tensile testing showed that the integrity of the material was not compromised by inclusion of crystallites into the polymer matrix. The ChBD-aspein protein was shown to provide nucleation sites for crystallization of calcium carbonate. The results provide a possibility of tailoring mechanical properties by producing proteins that combine specific functionalities, to produce functional biomaterials from a combination of natural functionalities and inorganic compounds.
5.4 Hydroxypropylated xylan (Paper IV)

The hydroxypropylated films of Paper IV were prepared from alkali-extracted xylan with varying degree of substitution (DS) of the hydroxypropyl groups, with and without an external plasticizer (sorbitol). The properties of the films were studied by differential scanning calorimetry, mechanical testing, oxygen and water vapor permeability, and their nanostructure was examined by WAXS.

The DS of the hydroxypropylated films influenced both the ability of the xylans to form films and their mechanical properties. A lower DS lead to brittle films, although addition of external plasticizers resulted in cohesive films even for the lower DS (0.3) xylans. The water and oxygen permeability increased with increasing DS, but an addition of the external plasticizer lowered both properties.

WAXS was used to study the crystallinity and crystallite size of xylan in the HPX films. The research hypothesis was that increasing DS leads to decreasing crystallinity, as the packing of the xylan chains becomes more difficult with an increasing amount of HP-residues surrounding the linear backbone of the polymer. Samples with a DS of 0.3, 0.6, and 1.1 were studied (samples HPX0.3/20s, HPX0.6/20s and HPX1.1/20s respectively). All samples studied by WAXS contained 20 wt-% of sorbitol.

From the WAXS data, the crystallinity was determined by the amorphous fitting method, where an amorphous background measured from rye arabinoxylan and Gaussian peaks at the positions of the crystalline peaks were fitted into the WAXS intensity. The positions of the diffraction peaks were based on the structure determined in [22] but with lattice constants determined from the positions of the first two diffraction peaks. The width of the diffraction peaks was determined from the scattering pattern.

Figure 8: The scattering patterns of the sample with 10x CaCO$_3$. a) No ChBD-aspein, b) with ChBD-aspein. The center area with the primary beam and beam-stop is masked away.
5.4.1 WAXS results

The high DS was associated with a lower crystallinity, but even at the highest studied DS (1.1) and with 20% sorbitol content, the films showed crystalline characteristics. The remaining crystallites were few, but they were surprisingly large (Table 3) compared to crystallites in native biopolymers, such as cellulose. The crystallinity could not be determined for sample HPX1.1/20s, but the crystallinities of the two other samples were determined. The result for sample HPX0.3/20s is shown in Figure 9.

With increasing DS, the amorphous background of the sample started to change, and in the HPX1.1/20s sample, a wide maximum was visible at low $q$ values. The maximum corresponds to a Bragg distance of 1.26 nm, and similar features have been observed in the scattering patterns of hydroxypropylated cellulose [88, 89], indicating possibly that this maximum arises from a characteristic separation length of hydroxypropylated polysaccharides. Other changes attributable to a change in the amorphous background were not observed, which strengthens the conclusion that this amorphous maximum is due to packing in one direction only.

The most promising film for packaging purposes was HPX0.3/20s, which had the best qualities in terms of permeability. The correlation between a higher crystallinity and lower permeability was expected, as the permeability of crystallites is much lower than that of amorphous matter. HPX0.2/20s also had the highest biocontent and is known to be biodegradable. All in all, xylans from forest sources were shown to be highly promising as barrier materials for ecologically friendly packaging materials.

![Figure 9: The crystallinity determination for sample HPX0.3/20s. WAXS intensity as a function of scattering angle $2\theta$. The position of the diffraction peaks from which the lattice constants were determined are marked with arrows.](image)
Table 3: The crystallinity and crystallite size of HPX samples (low = not determined, but crystallites were present).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Crystallinity (%)</th>
<th>$s_{100}$ (nm)</th>
<th>$s_{110}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPX0.3/20s</td>
<td>20.4±3.0</td>
<td>15.5±0.6</td>
<td>15.2±0.8</td>
</tr>
<tr>
<td>HPX0.6/20s</td>
<td>16.0±5.0</td>
<td>14.0±0.6</td>
<td>13.8±0.8</td>
</tr>
<tr>
<td>HPX1.1/20s</td>
<td>low</td>
<td>12.0±2.0</td>
<td>11.2±1.0</td>
</tr>
</tbody>
</table>
6 Conclusions and future prospects

X-ray scattering provides valuable information on the nanostructure of biological materials and biomimetics. Although the studied systems are complex, they can often be reduced into simple two- or three component systems, or parts of the structure can be evaluated. A definite advantage of X-ray scattering is its sensitivity to crystalline materials, which means that information on the crystalline part of a multi-component system can be extracted with relative ease.

Even though the use of direct methods, such as microscopy, has advanced in large steps in the near past, there is still a strong advantage in using indirect methods. X-ray scattering provides information from a relatively large area or volume compared to microscopy methods with a similar resolution, thus they are often used as complementary methods. The better statistical average from X-ray scattering, as well as the possibility to conduct measurements on fully hydrated systems at wide temperature ranges, offset the difficulty of interpreting indirect information.

As new and more complicated materials are constructed, also the need to understand the mechanisms behind the molecular organization is growing. The path to miniaturizing large structures is proving insufficient, and more and more novel materials are constructed by self-assembly of molecules. The bottom-up approach is taking over, hence the importance of understanding the resulting structures in detail. The nanostructure of materials, such as the crystal polymorphism, crystallinity, crystallite size and orientation, and the organization of amorphous matter influences greatly the mechanical and biological properties of materials.

In this thesis, the properties of a new biohybrid material was examined (Paper III). This material was synthesized from chitin and the protein ChBD-aspein in the presence of calcium carbonate precursors. WAXS provided valuable information on the crystallite polymorph, the crystallite size and the distribution of crystallites in the sample. Unexpectedly, no crystallites formed in samples with a 1:1 molar ratio of ChBD-aspein and CaCO$_3$, and it was shown that their formation was outcompeted by the protein binding the large Ca$^{2+}$ cation.

The mechanical properties of materials are related to their nanostructure. X-ray scattering naturally cannot determine the tensile strength or barrier properties of a material, however, results from X-scattering give information on the structure-function relationship. When hydroxypropylated xylan was studied (Paper IV), WAXS studies provided information on the crystallinity of xylan, which declined as expected with increasing substitution degree. However, even the sample with the largest DS showed evidence of few but relatively large crystallites. In addition, correlation distances in nanometer scale emerged in this sample, due to the ordering of the hydroxypropylated xylan. The combination of this large separation length at nanometer level and few but large crystallites in atomic scales yielded worse barrier properties than the higher crystallinity of samples with a lower degree of substitution.

All in all, the results show that films made from hydroxypropylated xylan are a good candidate for biodegradable packaging materials. Changes in their nanostructure correspond to changes in mechanical and barrier properties.
The barrier properties and stability of liposomes is important in drug delivery. Liposomes can be stabilized e.g. with coatings of S-layer proteins. Work included in this thesis showed that SlpA of *L. brevis* ATCC 8287 reassembles on cationic liposomes (Paper I). This strengthens the view that S-layer reassembly is not a process governed simply by the electrostatic interactions of the reassembling protein and the reassembly surface, as both the surface and the proteins had an overall positive charge in the reassembly conditions. We also showed, to our knowledge for the first time, that the structure of the S-layer crystallite can change in the reassembly. The crystallite of SlpA is more stable in the direction of the larger lattice constant $a$, as this is the lattice constant that does not change in the reassembly and also corresponds more well-defined diffraction peaks. Yet unpublished results indicate that the reassembly requires the lipid surface to be in the L$_\alpha$-phase, but these preliminary results need further verification.

Liposomes are an important model system also for the cell membrane, and in this thesis, they were also used as a membrane analogue to look at the effects of ionic liquids. Paper II shows that SAXS provides information on the effects of phosphonium-based ionic liquids, and these effects can be followed *in situ*, though with a low time resolution of minutes. This work shows that even ILs with small cations penetrate the entirety of MLV and cause shrinking of the lamellar distance throughout. This shrinkage is dependent on IL concentration and possibly independent of IL type. ILs with long hydrocarbon chains induce a similar shrinkage of the lipid bilayer at low concentrations, but induce also disorder in the lamellae, and destruct the lamellar structure at concentrations exceeding the critical micelle concentration. For LUV, results were similar and backed up by DLS and zeta potential data: ILs with small cations had little effect, but ILs with larger cations disrupted the vesicles and changed the structure of the bilayer, possibly by incorporating the IL cation in the lipid bilayer. In particular, [P$_8$881][OAc] at a concentration of 0.8 mM not only ruptured LUV composed of eggPC, eggPG, and cholesterol but separated one lipid species, likely eggPC, from the phospholipid matrix and induced the formation of well-ordered multilayers. This kind of lipid separation has not been otherwise observed and requires further study. It will be followed up by a time-resolved SAXS experiment at the European Synchrotron Radiation Facility in Grenoble to look at the dynamics of ionic liquid interactions with biomimicking liposomes on a millisecond time-resolution.

In conclusion, in the work presented in this thesis, SAXS and WAXS provided valuable information that was not accessible by other means. In all cases, the scattering experiments were either supplemented by other more direct methods to study the properties of biomaterials and biomimetic materials. The value of X-ray scattering lies in its inherent sensitivity to crystalline matter, and in the possibilities provided by measurements *in situ* in ambient temperature and in aqueous solutions, which make it an extremely relevant technique in studies of interesting bio-based systems.
References


REFERENCES


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