Piezoelectricity in the
Globular Protein Lysozyme

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requirements for the degree of Doctor of Philosophy
by
Aimee Stapleton, B.Sc.

Supervisors:
Prof Tofail Syed
Prof Tewfik Soulimane
Dr Christophe Silien

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Declaration

I, Aimee Stapleton, declare that this submission is my own work and contains no material that has been submitted for any academic award or other qualification to any University or other institution. Where use has been made of the work of other people, it has been fully acknowledged and referenced accordingly.

Signed: ___________________________ Date: __/__/____

Aimee Stapleton
Abstract

Piezoelectricity is a particular type of electromechanical coupling in which a mechanical stress induces an electrical polarisation in materials that have a non-centrosymmetric crystal structure. A converse piezoelectric effect also exists in which an electrical field induces a mechanical strain. Although originally discovered in quartz, a solid-state material, the piezoelectric effect extends to include many biological materials such as wood, bone, viruses, amino acids and proteins. The classical theory of piezoelectricity describes the effect in solid-state materials fully; however, a comprehensive understanding of how piezoelectricity manifests in biological materials is lacking. Without this understanding, the physiological significance and potential applications of biological piezoelectricity cannot be realised. This thesis studied the electromechanical properties of lysozyme, a globular protein found abundantly in hen-egg whites. Crystals of the protein lysozyme were probed for piezoelectricity in order to understand whether the classical description of piezoelectricity applies to them. Films of lysozyme crystals were structurally characterised using synchrotron diffraction. The direct piezoelectric effect was quantified at the macroscale using static and quasi-static approaches based on the Berlincourt Method. The converse piezoelectric effect was also measured in crystals of lysozyme using Piezoresponse Force Microscopy. Furthermore, the related properties of ferroelectricity (spontaneous switchable polarisation) and pyroelectricity (changes of polarisation induced by a changing temperature) were investigated. Ferroelectricity was probed using Switching Spectroscopy Piezoresponse Force Microscopy and pyroelectricity was measured using a modified Byer-Roundy Method. A major finding of this thesis is that electromechanical coupling exists in crystals of lysozyme and can be described by the piezoelectric effect. This is the first example of piezoelectricity in a non-fibrous protein. Quantitative measurements show that the piezoelectric coefficients of monoclinic and tetragonal crystals of lysozyme are 1 pC N$^{-1}$ and 6 pC N$^{-1}$, respectively. This is significant; the piezoelectric coefficient of quartz is 2 pC N$^{-1}$. Unexpectedly, both longitudinal piezoelectricity and ferroelectricity was observed in tetragonal crystals of lysozyme whose symmetry should preclude these effects indicating that the crystals may be of lower symmetry. Observations of pyroelectricity and piezoelectricity in monoclinic lysozyme are consistent with classical theories. The findings contribute to our understanding of protein piezoelectricity and imply that the classical theory of piezoelectricity is applicable to biological materials.
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<th>Definition</th>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>BaTiO₃</td>
<td>Barium Titanate</td>
</tr>
<tr>
<td>IDE</td>
<td>Inter-Digitated Electrode</td>
</tr>
<tr>
<td>IOS</td>
<td>Inverse Optical Sensitivity</td>
</tr>
<tr>
<td>ITO</td>
<td>Indium Tin Oxide</td>
</tr>
<tr>
<td>LPFM</td>
<td>Lateral Piezoresponse Force Microscopy</td>
</tr>
<tr>
<td>NAG</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>NAM</td>
<td>N-acetylmuramic</td>
</tr>
<tr>
<td>NLC</td>
<td>Non-Linear Capacitance</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>OHC</td>
<td>Outer Hair Cell</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PFM</td>
<td>Piezoresponse Force Microscopy</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>PZT</td>
<td>Lead Zirconate Titanate</td>
</tr>
<tr>
<td>RCSB</td>
<td>Royal Collaboratory Structural Bioinformatics</td>
</tr>
<tr>
<td>SmA</td>
<td>Smectic A (liquid crystal)</td>
</tr>
<tr>
<td>SmC</td>
<td>Smectic C (liquid crystal)</td>
</tr>
<tr>
<td>SPM</td>
<td>Scanning Probe Microscopy</td>
</tr>
<tr>
<td>SS-PFM</td>
<td>Switching-Spectroscopy Piezoresponse Force Microscopy</td>
</tr>
<tr>
<td>STM</td>
<td>Scanning Tunnelling Microscopy</td>
</tr>
<tr>
<td>TSC</td>
<td>Thermally Stimulated Current</td>
</tr>
<tr>
<td>VPFM</td>
<td>Vertical Piezoresponse Force Microscopy</td>
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<tr>
<td>XRD</td>
<td>X-Ray Diffraction</td>
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# Nomenclature

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Name</th>
<th>Unit</th>
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<tbody>
<tr>
<td>a, b, c</td>
<td>Length of unit cell axes</td>
<td>m</td>
</tr>
<tr>
<td>A, Ae, Ai</td>
<td>Area, Area of electrode, Area of image</td>
<td>m²</td>
</tr>
<tr>
<td>C</td>
<td>Capacitance</td>
<td>F</td>
</tr>
<tr>
<td></td>
<td>Base-centred lattice</td>
<td>-</td>
</tr>
<tr>
<td>d</td>
<td>Piezoelectric coefficient</td>
<td>C m⁻¹, m V⁻¹</td>
</tr>
<tr>
<td>D</td>
<td>Electric Displacement</td>
<td>C m⁻²</td>
</tr>
<tr>
<td>dhkl</td>
<td>Inter-planar spacing</td>
<td>m</td>
</tr>
<tr>
<td>dhkl*</td>
<td>Inter-planar spacing of reciprocal lattice</td>
<td>m⁻¹</td>
</tr>
<tr>
<td>E</td>
<td>Electric Field</td>
<td>V m⁻¹</td>
</tr>
<tr>
<td>F</td>
<td>Force</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>Face-centred lattice</td>
<td>-</td>
</tr>
<tr>
<td>Fhkl</td>
<td>Structure factor</td>
<td>-</td>
</tr>
<tr>
<td>fn</td>
<td>Scattering amplitude</td>
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</tr>
<tr>
<td>g</td>
<td>Piezoelectric voltage constant</td>
<td>V m N⁻¹</td>
</tr>
<tr>
<td></td>
<td>Glide line/plane</td>
<td>-</td>
</tr>
<tr>
<td>h</td>
<td>Height of cantilever</td>
<td>m</td>
</tr>
<tr>
<td>h, k, l</td>
<td>Miller indices</td>
<td>-</td>
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<td>I</td>
<td>Current</td>
<td>A</td>
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<tr>
<td></td>
<td>Body-centred lattice</td>
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</tr>
<tr>
<td>k</td>
<td>Spring constant</td>
<td>N m⁻¹</td>
</tr>
<tr>
<td>L</td>
<td>length of cantilever</td>
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<td></td>
<td>Laevorotation</td>
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<tr>
<td>m</td>
<td>Mirror line/plane</td>
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</tr>
<tr>
<td>n</td>
<td>Axis of rotation</td>
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</tr>
<tr>
<td>N, Nb</td>
<td>Number of crystals, number of crystals in</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>counting box</td>
<td></td>
</tr>
<tr>
<td>Nm</td>
<td>Screw axis</td>
<td>-</td>
</tr>
<tr>
<td>P, Ps</td>
<td>Polarisation, spontaneous polarisation</td>
<td>C m⁻²</td>
</tr>
<tr>
<td>Symbol</td>
<td>Name</td>
<td>Unit</td>
</tr>
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<td>--------</td>
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<tr>
<td>$P$</td>
<td>Primitive lattice</td>
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<tr>
<td>$P_b, P_h$</td>
<td>Total pressure, Applied pressure</td>
<td>$N \ m^{-2}, Pa$</td>
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<tr>
<td>$p$</td>
<td>Pyroelectric coefficient</td>
<td>$C \ m^{-2} K^{-1}$</td>
</tr>
<tr>
<td>$Q$</td>
<td>Charge</td>
<td>$C$</td>
</tr>
<tr>
<td>$R$</td>
<td>Lateral sensitivity</td>
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<tr>
<td></td>
<td>Rhombohedral-centred lattice</td>
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<td>$S$</td>
<td>Entropy</td>
<td>$J \ K^{-1}$</td>
</tr>
<tr>
<td>$s$</td>
<td>stiffness</td>
<td>$N \ m^{-1}$</td>
</tr>
<tr>
<td>$T$</td>
<td>Temperature</td>
<td>$^\circ C, K$</td>
</tr>
<tr>
<td>$t$</td>
<td>time</td>
<td>$s$</td>
</tr>
<tr>
<td>$t_h$</td>
<td>thickness</td>
<td>$m$</td>
</tr>
<tr>
<td>$U$</td>
<td>Internal energy</td>
<td>$J$</td>
</tr>
<tr>
<td>$u,v,w$</td>
<td>Fraction of the unit cell length at which atom is located</td>
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</tr>
<tr>
<td>$V$</td>
<td>Voltage</td>
<td>$V$</td>
</tr>
<tr>
<td>$V_m$</td>
<td>Matthew’s coefficient</td>
<td>$\ Å^3 \ Da^{-1}$</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>Stress</td>
<td>$N \ m^{-2}, Pa$</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>Surface charge density</td>
<td>$C \ m^{-2}$</td>
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<tr>
<td>$\alpha$</td>
<td>Strain</td>
<td>-</td>
</tr>
<tr>
<td>$\alpha, \beta, \gamma$</td>
<td>Thermal expansion / Piezocaloric coefficient</td>
<td>$^\circ C^{-1}, K^{-1}$</td>
</tr>
<tr>
<td>$\Delta z$</td>
<td>Deflection</td>
<td>$m$</td>
</tr>
<tr>
<td>$\theta$</td>
<td>Angle, phase</td>
<td>$^\circ$</td>
</tr>
<tr>
<td>$\kappa, \kappa_o$</td>
<td>Permittivity, permittivity of free space</td>
<td>$F \ m^{-1}$</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Wavelength</td>
<td>$m$</td>
</tr>
<tr>
<td>$\Pi$</td>
<td>Electrostriction</td>
<td>$C^{-2}m^4$</td>
</tr>
<tr>
<td>$\rho_o, \rho_1$</td>
<td>Density of bulk water, hydration water</td>
<td>$kg \ m^{-3}$</td>
</tr>
<tr>
<td>$\omega$</td>
<td>Angular frequency</td>
<td>$rad \ s^{-1}$</td>
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Chapter 1  Introduction

The functionality of many devices including ferroelectric capacitors, piezoelectric energy-harvesters and actuators are controlled by the electromechanical properties of their composite materials. Similarly, many physiological processes rely on electromechanical coupling. Galvani’s historic experiment showed that an electrical stimulus applied to the legs of a frog would induce muscular contractions (1). Another example of the physiological significance of electromechanical coupling are voltage-gated ion channels, which provide a means of cell-cell communication (2). Recently, the outer hair cells (OHCs) of the mammalian cochlea were shown to respond to changes in the cell membrane potential by contracting or expanding their cell length. This process, known as electromotility, is responsible for the exquisite frequency selectivity and amplification necessary for hearing (3).

The focus of this thesis is a specific type of electromechanical coupling called piezoelectricity. Piezoelectricity is a property of materials where electrical polarization takes place at the surface due to a mechanical stress. Conversely, an applied electrical field causes a mechanical strain in these materials. This thesis is concerned with the manifestation of piezoelectricity and its associated properties of ferroelectricity (reversible spontaneous polarisation) and pyroelectricity (temperature-induced changes in polarisation) in proteins. The following section summarises the history of solid-state piezoelectricity. It then extends to include a brief summary of the existing work on biological piezoelectricity.

1.1  Background

Jacques and Pierre Curie discovered the direct piezoelectric effect in 1880 (4). Their finding was an extension of Pierre Curie’s work on pyroelectric materials. An awareness of pyroelectric materials has existed since ancient times when it was known that tourmaline would attract metals when heated or cooled (5, 6). Pierre Curie’s contribution to the field of pyroelectricity was in studying the relationship between pyroelectricity and crystal symmetry. This appreciation of the effect of symmetry on the physical properties of crystals led him and his brother to investigate the effect of
pressure on certain crystal classes and thus discover the direct piezoelectric effect. However, they had no inclination that a reciprocal piezoelectric effect would also exist. This was the contribution of Gabriel Lippman, who in 1881 predicted the converse piezoelectric effect from thermodynamic considerations (7). Later in the same year, the Curie brothers validated Lippmann’s predictions experimentally (8). Furthermore, they showed that the direct and converse piezoelectric coefficients of a material are equal.

It was during the First World War that the early applications of piezoelectricity became widely apparent with Langevin’s invention of the ultrasonic submarine detector (9). After the Second World War, the applications of piezoelectric materials were greatly expanded with the development of the first piezoceramics. Piezoceramics are agglomerations of small piezoelectric crystals whose overall piezoelectric effect is zero as the random orientation of crystals cancel one another out. Attributed to Jaffe (10), a process of poling (the application of an electric field usually at an elevated temperature) aligns the polar axis of the individual crystals within the ceramic. Thus, after poling the ceramic exhibits the piezoelectric effect at the macroscale. With their high dielectric constants and large piezoelectric co-efficients, piezoceramics such as barium titanate (BaTiO₃) and lead zirconate titanate (PZT) are widely employed in applications as diverse as medical imaging and car accelerometers (9). The third major advancement to the applications of piezoelectrics came with the discovery of piezoelectricity in the polymer polyvinylidene fluoride (PVDF) (11, 12). PVDF has good thermal stability, high chemical resistance and is mechanically flexible. Combined with relatively high piezoelectric and pyroelectric co-efficients, the properties of PVDF extend the range of actuating and sensing applications of piezoelectrics (13, 14). Biological piezoelectric materials, being inherently biocompatible, may extend the technical applications of piezoelectrics even further. In the next section, we give a brief background of piezoelectricity in biology to illustrate where this thesis sits within the existing literature.
1.2 Biological Piezoelectricity

In their seminal work, Fukada and Yasuda showed that piezoelectricity extends to biological materials with the observation of piezoelectricity in wood and bone (15-17). Soon afterwards, piezoelectricity was observed in tendon and collagen (18). The discovery of piezoelectricity in bone has been linked with Wolff’s which states that bone is adaptive, capable of regenerating in response to mechanical stress (19). The potential contribution of piezoelectricity to bone growth and healing has captured much attention and motivated research that has uncovered many other examples of biological-piezoelectricity. From the smallest biological building blocks, amino acids (20-24), to more complex structures including DNA (25), collagen (18, 26-28), peptide nanotubes (29) and viruses (30), it is clear that piezoelectricity is prevalent at all levels of biology.

The occurrence of piezoelectricity in biological samples is so prevalent that some speculate that it may be a universal feature of biology (31-33). Lemanov proposed a systematic method of investigating this claim. His approach was simple; to begin with the study of piezoelectricity in amino acids, the smallest biological unit, and then to progress towards the study of more complex systems such as peptides and proteins (33). Since then, piezoelectricity has been studied at various hierarchical levels of biology, however a complete understanding of the mechanisms underpinning biological piezoelectricity is still lacking. In particular, it is not fully apparent whether biological piezoelectricity is governed by the classical theory of piezoelectricity as it applies to solid-state materials.

In relation to the role of crystal symmetry in biological piezoelectricity, the body of work describing piezoelectricity in collagen and hydroxyapatite (the two main constituents of bone) offers an interesting insight. For a long time, piezoelectricity in bone was attributed solely to the collagen (34). Collagen has hexagonal symmetry and belongs a piezoelectric crystal class (point group 6) (28). In contrast, hydroxyapatite was originally assigned to a non-piezoelectric crystal class (point group 6/m), dispelling any possibility of it being piezoelectric. It was not until 2005, that computational studies of hydroxyapatite reassigned it to a polar crystal class (point group 2 or 6) (35, 36). In these classes, hydroxyapatite could exhibit the piezoelectric
effect as described by the classical theory of piezoelectrics. Soon afterwards, piezoelectricity was observed experimentally (37, 38), as was pyroelectricity and ferroelectricity (39, 40). These studies are of importance as they highlight that an understanding of physical properties begins with a clear understanding of the material’s crystal structure.

1.3 Objectives of Thesis

The objective of this thesis is to investigate whether or not proteins conform to the same definitions of piezoelectricity as classical solid-state materials. On the hierarchical scale of biological building blocks, proteins are intermediate structures that bridge between amino acid and more complex structures. However, knowledge of piezoelectricity in proteins is severely lacking. Stemming from the work on bone, piezoelectricity has been studied extensively in collagen (18, 27, 28, 41), and other fibrous proteins including keratin (42), elastin (43), myosin and actin (44). Aside from fibrous proteins, there have been no reports of piezoelectricity in other types of proteins. Unlike fibrous proteins, globular proteins can be crystallised. Proteins crystals are naturally non-centrosymmetric, resulting from the chirality of the amino acids from which they are composed. Thus, the study of protein crystals provides an exciting framework to investigate the nature of biological piezoelectricity.

1.4 Research Questions

This study aims to answer the following research questions.

**Research question 1:** Should lysozyme exhibit the piezoelectric effect?

**Research question 2:** Can the direct and converse piezoelectric effect be quantitatively measured in lysozyme using conventional methods?

**Research question 3:** Should lysozyme demonstrate ferroelectricity and pyroelectricity and can these properties be measured?
1.5 Hypothesis

The research questions outlined above were investigated based on the following hypotheses.

**Hypothesis 1:** Non-centrosymmetric crystals of lysozyme should be piezoelectric

**Hypothesis 2:** The tensor of piezoelectric coefficients for lysozyme are determined by its crystal symmetry

**Hypothesis 3:** Crystals of lysozyme with polar symmetry should be pyroelectric and can be measured by conventional methods

1.6 Methodological Approach

The main objective of this thesis was to study classical piezoelectricity in lysozyme. As piezoelectricity is found in non-centrosymmetric crystals, we hypothesised above that lysozyme in its crystallised form should be piezoelectric. Thus, the methodological approach taken in this thesis involved three interlinked aspects: protein crystallisation, structural characterisation and electromechanical measurements.

The approach used to crystallise lysozyme was a modified vapour diffusion technique. The modification was necessary so that the resulting lysozyme crystals could be electroded for later measurements. Modifying the vapour diffusion technique allowed films of lysozyme to be grown on a conductive substrate that acted as an electrode.

The methodology used to characterise the structure of lysozyme crystals within films took two directions. Firstly, x-ray diffraction analysis was employed to determine the structure of the crystals of lysozyme via synchrotron diffraction. Secondly, symmetry-dependent physical properties (piezoelectricity, pyroelectricity and ferroelectricity) were measured in order to ascertain the structure of the lysozyme crystals within the film.

The methodology used to characterise the electromechanical properties of lysozyme
was to employ conventional measurement techniques used in the study of solid-state materials. These approaches are summarised in Chapter 3 along with a justification of the techniques that are most applicable to the study of lysozyme crystals.

1.7 Contribution to the Field: The Gap in the Literature that this Study Fulfils

The work presented in this thesis is multidisciplinary; piezoelectricity in biological samples was studied from a classical physics perspective. While work on biological piezoelectricity began in the 1950s (16), the existing body of research has not extended to include proteins of the non-fibrous type until now. Perhaps this is due in part to the numerous challenges of working with crystallised globular proteins. Protein crystals are relatively small and fragile. Additionally, protein crystals require an aqueous environment to maintain their structure. In this thesis, methods of electroding and performing electrical and electro-mechanical measurements on protein crystals were realised. Overcoming the challenges of electroding and handling protein crystals is a contribution that should allow further studies of electrical properties of protein crystals to be realised.

Lysozyme is perhaps the most extensively studied protein. Within the literature, the mechanical properties of lysozyme have been well studied, contributing to our knowledge of its elastic properties and Young’s modulus (45-48), hardness (49, 50), as well as its crystal growth mechanisms and surface topography (51-56). Similarly, much is known about the dielectric properties of lysozyme (57-60). However, very little research has bridged the gap between the electrical and mechanical properties of lysozyme until now.

The literature survey conducted by the author revealed only one study dealing with electromechanical coupling in lysozyme. This study was conducted by Danielewicz-Ferchmin et al. (61) and is based on an observation by Ortore et al. (62) in which the density of the hydration water surrounding lysozyme was reported to be greater than the density of bulk water under the same conditions. Danielewicz-Ferchmin et al. propose that the changes in hydration water density observed at different applied pressures is the result of an electromechanical effect induced by charges on the surface of the lysozyme molecules. The surface charge of lysozyme is proposed to be the result of the combined effect of electrostriction and the direct piezoelectric effect under an
applied pressure. There are several inconsistencies in linking this observation to true piezoelectricity. Firstly, the so-called piezoelectric effect is observed indirectly, through observations of changes in the hydration water density at different pressures – which may or may not be related to a piezoelectric effect in lysozyme. Secondly, the observations were made for lysozyme in solution - thus the classical description of piezoelectricity based on crystal symmetry cannot be applied directly. Thirdly, the calculation used to determine an effective piezoelectric coefficient for lysozyme is incorrect (Appendix A). Finally, the claim that the result is invariant with the direction of the electric field is inconsistent with piezoelectricity. The approach taken in this thesis is to measure the piezoelectric effect in lysozyme directly using conventional methods.

As far as the author is aware, there have been no attempts to use conventional techniques to measure piezoelectricity in lysozyme or any other non-fibrous protein until now. In related studies, patch-clamping has been employed to measure electromechanical coupling (electromotility) in prestin, a transmembrane protein located in the OHCs of the cochlea that facilitates hearing (3). Patch-clamping is an electrophysiological technique that allows the current in patches of the cell membrane to be recorded with high resolution (63). The non-linear capacitance (NLC) measured by patch-clamping has been taken as a substitute measurement of electromotility (3, 64). Although the observations of electromotility in OHCs via patch-clamping are analogous to piezoelectricity, caution should be taken as they may not necessarily be equivalent. Recently, it has been shown that NLC measurements obtained through patch-clamping are not identical to direct motility measurement under normal physiological conditions (65). In addition, the coupling between electrical and mechanical properties in OHCs does not show the hallmark linearity of piezoelectricity (66, 67). Furthermore, electromotility of prestin have all been performed with the protein in the cell. The lipid bilayer of the cell is made up of phospholipids which are themselves piezoelectric (68) and may contribute to the measured electromotility.

The fact that piezoelectric measurements were performed directly on lysozyme using conventional techniques is one of the strengths of this study; it allows the observed phenomena to be distinguished from other electro-mechanical coupling effects.
Furthermore, piezoelectric measurements were performed at both the macroscale and the nanoscale. At the macroscale, the Berlincourt method measured the direct piezoelectric effect in lysozyme. Nanoscale measurements of piezoelectricity in lysozyme were achieved using piezoresponse force microscopy (PFM, discussed in Chapter 3). This technique, based on the converse piezoelectric effect, monitors deformations of the sample surface that arise in response to a voltage applied between a nanoscale tip and the sample. The limitations of PFM measurements are well documented; these include parasitic electrostatic contributions (69), crosstalk between the piezoresponse signal and topography (70), and artefacts arising from the background signal (71, 72). Thus, results obtained for lysozyme using PFM are regarded in light of these limitations.

While the focus of this thesis is to investigate the piezoelectric nature of lysozyme, the associated symmetry-dependent properties of pyroelectricity and ferroelectricity were also investigated. These properties have been studied in biological materials to a much lesser extent than piezoelectricity. To the author’s knowledge, neither pyroelectricity nor ferroelectricity has been reported in lysozyme or any other globular protein until now. Perhaps, this is due in part to the challenges of handling and electroding proteins described above. Accurate measurements of both pyroelectricity and ferroelectricity present significant challenges, even when conducted on solid-state materials. Pyroelectric measurements are subject to contributions from thermally stimulated currents if not heated linearly (73). Switching Spectroscopy PFM (SS-PFM, discussed in Chapter 3) is becoming a routine technique for probing ferroelectricity at the nanoscale. However, interpretation of the characteristic ferroelectric hysteresis loops must be done with caution, as increasingly false interpretations of ferroelectricity are being highlighted in the literature (74-76). Thus, the observations of pyroelectricity and ferroelectricity of lysozyme are presented tentatively within this thesis.

The study of piezoelectricity in lysozyme in this work contributes to the field of biological-piezoelectricity. We studied lysozyme as tetragonal and monoclinic crystals. As piezoelectricity is a symmetry-dependent property, measuring lysozyme in its crystallised form offers a unique perspective. This perspective of protein-piezoelectricity cannot be realised with fibrous proteins, as they do not crystallize...
readily. Thus, the research carried out in this thesis offers a new approach towards understanding if biological piezoelectricity is symmetry-dependent in the same way as solid-state piezoelectricity is.

1.8 Scope of Thesis

This thesis consists of seven chapters, the contents of which are organised as follows.

Chapter 1 introduced the background and main motivations of this study. Here, the objectives and research questions directing the research were outlined. The hypothesis on which the thesis is based were also stated. We then described the methodological approach taken and highlighted the gap in the literature that this thesis fills.

Chapter 2 reviews the most significant literature relevant to this research. It gives an account of how the symmetry of a crystal relates to the physical properties of piezoelectricity, ferroelectricity and pyroelectricity. Then it reviews underlying mechanisms that support these properties in biological materials. Finally, the chapter introduces lysozyme, the globular protein under study in this thesis. Relevant studies in the literature relating the structure and dielectric properties of lysozyme are discussed.

In Chapter 3, the experimental techniques used in this thesis are presented. Firstly, the principles of protein crystallisation and crystallography are discussed. Then methods used for investigating piezoelectricity, pyroelectricity and ferroelectricity are reviewed with the objective of selecting the methods most appropriate for the study of lysozyme in this work.

In Chapter 4, a suitable way to prepare films of lysozyme crystals for later measurements is determined. The films of lysozyme crystals are characterised by synchrotron diffraction to determine their structure.

Chapter 5 presents results of the direct piezoelectric effect in films of lysozyme crystals. Piezoelectricity in films of lysozyme will be measured quantitatively using
the Berlincourt Method and verified by two independent systems.

**Chapter 6** confirms the piezoelectric effect in lysozyme by presenting measurements of the converse piezoelectric effect via PFM. Measurements of pyroelectricity in films of lysozyme will also be presented. Furthermore, evidence of ferroelectricity via Switching Spectroscopy PFM will also be presented.

Finally, **Chapter 7** will summarise the main conclusions of this thesis and make recommendations for future work.
Chapter 2  Literature Review

Crystals are defined by their long-range, regular, repeating arrangement of atoms, ions or molecules in three dimensions. The symmetry of crystals determines their physical properties such as piezoelectricity, pyroelectricity and ferroelectricity. This chapter begins with an overview of the symmetry of crystals in 2D and 3D. Building on this knowledge, we will then discuss the symmetry conditions required for a material to demonstrate piezoelectricity, pyroelectricity and ferroelectricity. To understand how this thesis fits in to the state-of-the-art, biological examples of these three properties will be reviewed. The focus of each review will be on understanding the underlying mechanisms that support each phenomenon. Finally, background information will be provided on lysozyme, the protein studied in this work, with an emphasis on its structural and dielectric properties.

2.1  Symmetry of Crystals

2.1.1  The Lattice and the Unit Cell
An important concept in crystallography is that of a lattice. A lattice contains a periodic array of points (lattice points), where each point is indistinguishable from any other point and has identical surroundings. A 1-dimensional lattice is the easiest to understand. Imagine a line stretching from $-x$ to $+x$ with a number of points dotted along the line at regular intervals. The view from any of these points would look identical to the view from any other point along the line. It is important to note that the points of a lattice are just points – they are not atoms, molecules or any other physical object. However, physical objects such as atoms, ions or molecules are free to vibrate at these lattice points. Figure 2.1 extends the idea of a lattice to two-dimensions.
Figure 2.1: The 2D unit cell. A unit lattice combined with a motif creates a unit cell. Repeating the unit cell in two directions creates a 2D crystal. Adapted from (77)

The repeating entity, in this case a protein molecule, is called the motif. The combination of a lattice and a motif is called the unit cell. The unit cell is the smallest repeating unit that will fill all of space. The concepts of the lattice and unit cell are further extended to 3D in Figure 2.2.

Figure 2.2 The 3D unit cell. (a) A unit lattice combined with a motif creates a unit cell. Repeating the unit cell in three directions creates a 3D crystal. (b) Conventional notation used to identify the axis and angles of a unit cell. Adapted from (77)
Conventionally, $a$, $b$ and $c$ describe the distances between lattice points and $\alpha$, $\beta$ and $\gamma$ describe the angles between them. These six parameters define the unit cell. In many cases, there is more than one unit cell that could be chosen. By convention, the unit cell should be selected so that is (i) it has the same symmetry as the entire lattice, (ii) it has the greatest possible amount of right angles or equal angles and (iii) it has the greatest possible number of edges of equal length. For the 3-dimensional case, the Bravais lattices are the set of 14 possible lattice types that meet these requirements and will be discussed later. Lattices differ from one another in terms of their symmetry. The following section will describe the elements of symmetry.

2.1.2 Elements of Symmetry

In three dimensions, the elements of symmetry are axis of rotation, mirror planes, centres of symmetry, inversion axis, glide planes and screw axis. Three of these symmetry elements (axis of rotation, mirror planes and glide planes) have corresponding 2D analogues and will discussed from the 2-D perspective for simplicity. An asymmetric motif, the letter ‘R’, will be used to illustrate the effect of each element of symmetry.

An axis of rotation, denoted by $n$, is the symmetry element associated with the operation of rotation. Objects with a $n$-fold axis of symmetry look indistinguishable from their original after being rotated around the axis by $\frac{360^\circ}{n}$. Figure 2.3 shows the result of applying 2-fold (diad), 3-fold (triad), 4-fold (tetrad) and 6-fold (hexad) rotation to the asymmetric unit ‘R’.

![Figure 2.3 n-fold axis of rotation applied to the asymmetric motif ‘R’. Adapted from (78)](image-url)
A **mirror line** (or **mirror plane** in 3D) is a symmetry element of reflection and is denoted by the symbol ‘*m*’. Figure 2.4(a) shows the effect of a mirror line on the asymmetric unit ‘R’. Nature provides many examples of reflection symmetry; a butterfly is a simple example. In this case, the mirror line is along the long axis of the butterfly’s body. With its wings spread out on both sides, it is clear that reflecting any point on the left wing through the mirror line on to the right wing would not change its appearance in any way. This is the property of a mirror line.

![Figure 2.4 The effect of applying a (a) mirror line and (b) glide line symmetry operation to the asymmetric motif ‘R’. Adapted from (78)](image)

A **glide line** (or **glide plane** in 3D) performs a reflection across a plane and then performs a translation parallel to the plane. Glide lines and planes are denoted by the letter ‘*g*’. Figure 2.4(b) illustrates the effect of a glide line on the asymmetric unit ‘R’. The footprints left behind a person walking in the sand or snow in a straight line is often given as a real-world example of glide lines.

While axis of rotation, mirror lines (planes) and glide lines (planes) are symmetry elements in both 2D and 3D cases, there are three more symmetry elements that are unique to 3D crystals. These elements are centres of symmetry, axis of inversion and screw axis.

An **axis of inversion** is denoted as *n*. An inversion axis consists of two symmetry elements, a rotation followed by an inversion. Thus, a 4-*n*-inversion axis would consist of rotating the object by 90 ° and then inverting it through a centre of inversion. A
**centre of symmetry** is the special case of a $\bar{1}$-inversion axis. A centre of symmetry is the midway point along an imaginary line through an object that connects any object with its image. Figure 2.5 illustrates the concept by inverting an L-alanine molecule through a centre of symmetry. The result is a D-alanine molecule. This change of handedness is not permitted in chiral molecules and hence such molecules do not have axis of inversion.

![Figure 2.5](image)

**Figure 2.5** The effect of performing an $\bar{1}$ inversion (equivalent to a centre of symmetry) on an L-alanine molecule is to produce a D-alanine molecule. (77)

A **screw axis**, denoted by $N_m$, performs an $N$-fold rotation and then performs a $m/N$ translation along the axis. The result is a helical pattern. A $6_3$-screw axis is illustrated in Figure 2.6. The combination of a 6-fold rotation plus $3/6$ translation rotates the motif by 60° followed by a translation $\frac{1}{2}$ of the lattice repeat distance.
Figure 2.6 Performing a $6_3$-screw axis operation creates a helical translation in which the unit is rotated by $60^\circ$ and translated a distance of $\frac{3}{6}$ times the length of the repeat lattice distance. (78)

2.1.3 Bravais Lattices
There are 14 different ways the lattice can be arranged taking the above symmetry elements into account; they are known as the 14 Bravais lattices. Figure 2.7 shows the unit cell of each of the 14 Bravais lattices.
There are four types of elementary cell – primitive ($P$), face-centred ($F$), body-centred ($I$) and base-centred ($C$). The primitive cell has one lattice point per unit cell, located at one of the corners of the cell. The face-centred unit cell has a lattice point at the centre of each cell in addition to the one at its corner lattice point. The body centred unit cell is denoted $I$ from the German word *Innenzentrierte* meaning ‘body-centred’. It has a lattice point at the centre of its unit cell in addition to it the one at its corner lattice. The base-centred unit cell has a lattice point on its top and bottom faces, centred through the $c$-axis.
2.1.4 32 Point Group Symmetries

Each of the 14 Bravais lattice has a number of point symmetry elements associated with it. The group of symmetry elements associated with a lattice are called the point group symmetries. Combined together, there are 32 point group symmetries that describe all possible external arrangements of a crystal. Crystals with the same point group symmetry belong to the same point group or crystal class. The nomenclature used to differentiate point groups is a type of short-hand notation that lists the essential (but not all) symmetry elements of that point group. The full list of symmetry elements is generally not included in the nomenclature as it is understood that some symmetry elements produce other symmetry elements automatically. An example of this is an inversion monad ($\bar{1}$) which is equivalent to a centre of symmetry, thus in point group notation only one of these symmetry elements would be listed. The order of notation within the nomenclature for point group symmetries is different for each of the 7 crystal systems (described below) and is described in full in the International Tables for Crystallography (80).

2.1.5 Crystal Systems

Point group symmetry is independent of whether the unit cells are centred or not. Thus, based on point group symmetry each of the 14 Bravais lattices can be categorised in to one of seven basic systems. These are known as the seven crystal systems. In order of increasing symmetry, the crystal systems are: triclinic, monoclinic, orthorhombic, tetragonal, trigonal, hexagonal and cubic. The criteria defining each of the crystal systems are described in Table 2.1.
Table 2.1 The seven crystal systems and their corresponding Bravais lattice, axial lengths and angles

<table>
<thead>
<tr>
<th>Crystal system</th>
<th>Bravais lattices</th>
<th>Axial lengths</th>
<th>Axial angles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cubic</td>
<td>P    I   F</td>
<td>$a = b = c$</td>
<td>$\alpha = \beta = \gamma = 90^\circ$</td>
</tr>
<tr>
<td>Tetragonal</td>
<td>P    I</td>
<td>$a = b \neq c$</td>
<td>$\alpha = \beta = \gamma = 90^\circ$</td>
</tr>
<tr>
<td>Orthorhombic</td>
<td>P    I   C   F</td>
<td>$a \neq b \neq c$</td>
<td>$\alpha = \beta = \gamma = 90^\circ$</td>
</tr>
<tr>
<td>Trigonal</td>
<td>P    R</td>
<td>$a = b = c$</td>
<td>$\alpha = \beta = \gamma \neq 90^\circ$</td>
</tr>
<tr>
<td>Hexagonal</td>
<td>P    I</td>
<td>$a = b \neq c$</td>
<td>$\alpha = \beta = 90^\circ; \gamma = 120^\circ$</td>
</tr>
<tr>
<td>Monoclinic</td>
<td>P    C</td>
<td>$a = b \neq c$</td>
<td>$\alpha = \gamma = 90^\circ \neq \beta \geq 90^\circ$</td>
</tr>
<tr>
<td>Triclinic</td>
<td>P    I</td>
<td>$a \neq b \neq c$</td>
<td>$\alpha \neq \beta \neq \gamma = 90^\circ$</td>
</tr>
</tbody>
</table>

2.1.6 Space Groups

The 32 point group symmetries describe the different arrangements of point symmetry elements. Taking account of the effect of translational symmetry elements (i.e. glide planes and screw axis) on the lattice, leads to 230 possible internal 3D arrangements of a crystal. These are known as the 230 space groups and are fully described in the International Table for Crystallography Volume A (80), which numbers the space groups from 1 to 230. There are two dominant notation systems to describe the space group of a crystal: the Schoenflies system and the Hermann-Mauguin notation. The latter will be used throughout this thesis. As with the notation used to describe point group symmetries, space group symmetry notation only denotes essential symmetry elements. The first part of the notation is a letter – either P, F, I, C, B, A – denoting the type of elementary cell. Note that B and A describe crystals that are base-centred though the b-axis or a-axis, respectively. The remainder of the notation describes the essential symmetry elements of that crystal. As an example, space group P4_3212 belongs to the tetragonal crystal system, has a primitive Bravais lattice and has point group symmetry 422.

Protein Space Groups

Although 230 space groups are possible, in reality many of these do not have real world examples. Of those space groups that do have real world examples, a striking portion of crystals belong to just a few space groups. The number of space groups for protein crystals is less than that of inorganic crystals because the chirality of proteins
imposes some restrictions. Hands are a perfect example of a chiral object, so much so that \textit{handed-ness} is a synonym for chirality. A left-hand observed in a mirror appears as a right-hand. The left-hand and the image of the left-hand in the mirror have different chirality. Similarly, proteins are chiral and natural proteins all have left-handedness. Their chiral nature means that proteins cannot have mirror planes or inversion centres (81). The latter restriction is depicted in Figure 2.8, which shows that a mirror operation applied to a protein molecule changes its handedness.

![Image](image.png)

Figure 2.8 Protein molecules are chiral. Performing a mirror plane operation on a protein molecule is not permitted as it would change its handedness. (77)

In a similar way, an inversion operation performed on a left-handed chiral entity would result in right-handed structure, which cannot be. This was illustrated in Figure 2.5 with the example of alanine. Chirality, therefore, imposes restrictions that reduce the number of crystal point groups of proteins to 11 and the number of space groups to 65. The 11 chiral point groups and their 65 associated chiral space groups are listed in Table 2.2.
Interestingly, some of these 65 space groups describe protein crystal symmetry much more frequently than the rest. For example, space group P2₁2₁2₁ occurs three times more frequently than the other space groups (82).
2.2 Crystal Structure and Properties

Symmetry governs many physical properties including optical activity, piezoelectricity, pyroelectricity and ferroelectricity. Neumann’s principle relates the symmetry of a crystal with the symmetry of its physical properties. The principle states that the symmetry elements of any physical property of a crystal must include the symmetry elements of the point group of the crystal (83). Figure 2.9 creates subsets of the 32 crystal point groups, dividing them in those that are centrosymmetric or non-centrosymmetric, polar or non-polar, chiral or non-chiral.

![32 Crystal Point Groups Diagram](image)

Figure 2.9: The 32 point groups categorised by their physical properties that are symmetry dependent

The way to determine if a physical property has a particular symmetry element (for example, a 2-fold rotation) is to measure the physical property relative to some given axis, perform the 2-fold rotation and re-measure the physical property. If the measurements are the same, then the property contains this symmetry element. It is important to realise that the physical property may have a higher symmetry than the symmetry of the point group; Neumann’s principle allows this as long as all of the symmetry elements of the point group are present in the symmetry of the physical property.
2.2.1 Piezoelectricity and Crystal Structure

Piezoelectric materials are those that become electrically polarized when an elastic stress is applied to them. There also exist a converse effect; application of an electric field to a piezoelectric material causes an elastic strain to develop (84). Jacques and Pierre Curie discovered direct piezoelectricity first in 1880 while studying crystalline materials. Crystals of quartz, tourmaline, and sodium potassium tartrate exhibit piezoelectricity. Piezoelectricity is a linear, reciprocal effect. The converse effect, was predicted through Lippmann’s thermodynamic models and quickly verified by the Curie brothers in 1881 (85). Piezoelectric materials have useful applications forming the basis of actuators, sensors, motors and generators (86). They are also employed in energy harvesting devices (87).

From the Curie’s initial discovery, it was evident that the crystalline nature of the material was a deciding factor of piezoelectricity (85). Twenty-one of the 32 crystal classes are non-centrosymmetric. This asymmetry creates the anisotropic conditions that lie at the crux of piezoelectricity. With the exception of crystal class 432, all other non-centrosymmetric classes are piezoelectric (83). Applying stress to these materials alters the separation between positive and negative charges within these materials. Ultimately, this generates a net polarization at the crystal’s surface as shown in Figure 2.10. Conversely, materials with a centre of symmetry will always have a net polarisation of zero under an applied stress, even if it displays individual dipole moments. This is simply because the charges cancel one another out.

![Figure 2.10: The direct piezoelectric effect in (a) a non-centrosymmetric crystal under (b) tension and (c) compression](image)

Figure 2.10: The direct piezoelectric effect in (a) a non-centrosymmetric crystal under (b) tension and (c) compression
As illustrated in Figure 2.11(a), a force (or electric field) applied to a piezoelectric material can be longitudinal, transverse or shear. The conventional system of axis notation is illustrated in Figure 2.11(b). Normal axes 1, 2 and 3 denote the direction of the applied electric fields or mechanical stresses. Shear planes are denoted as 4, 5, and 6 and are perpendicular to the 1, 2, and 3 axes. Traditionally, the poling direction is defined to be always along the 3-axis.

Figure 2.11: Conventional nomenclature used in describing the piezoelectric effect. (a) The force or electric field can be applied in the longitudinal (blue), transverse (green) or shear (red) directions. (b) Conventional definition of the normal (1, 2 and 3) axis and shear planes (4, 5 and 6) in a piezoelectric material. (c) The piezoelectric tensor, \( d \), with its longitudinal (blue), transverse (green) and shear (red) components indicated.

Constitutive equations describe the piezoelectric effect mathematically. A stress \( \sigma \) applied to a piezoelectric material induces a polarisation per unit area \( P \) that is related to the applied stress by \( d \), the piezoelectric modulus.

\[
P_i = d_{ijk} \sigma_{jk}
\]

Polarisation is a vector with three components, stress is second rank tensor with nine components and the piezoelectric modulus is a 3\(^{rd}\) rank tensor with 27 components.
The piezoelectric tensor \( d_{ijk} \) is symmetrical in \( j \) and \( k \), i.e.

\[
d_{ijk} = d_{ikj}
\]  

2.2

Thus, the piezoelectric number of independent \( d_{ijk} \) components is reduced from 27 to just 18. This reduction also allows a matrix system of notation to be applied. Table 2.3 illustrates the rules for converting the second and third suffices of the \( d \)-coefficient from tensor to matrix notation.

Table 2.3 Conversion from tensor notation to matrix notation

<table>
<thead>
<tr>
<th>Tensor notation</th>
<th>11</th>
<th>22</th>
<th>33</th>
<th>23, 32</th>
<th>31, 13</th>
<th>12, 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrix notation</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

Thus, Equation 2.1 is simplified to

\[
P_i = d_{ij}\sigma_j
\]  

2.3

where \( i=1, 2, 3 \) and \( j=1, 2,..6 \).

Similarly, a constitutive equation for the converse piezoelectric effect can be described. An electric field with an electric field intensity \( E_i \) produces a strain \( \varepsilon_{jk} \) that is related to the applied field by the piezoelectric coefficient \( d_{ijk} \) as in Equation 2.4.

\[
\varepsilon_{jk} = d_{ijk}E_i
\]  

2.4

Applying the matrix notation described above for the direct effect, the constitutive equation for the converse piezoelectric effect can be simplified as

\[
\varepsilon_j = d_{ij}E_i
\]  

2.5

where \( i=1, 2, 3 \) and \( j=1, 2,..6 \).

The tensor of piezoelectric coefficients, \( d \), is shown above in Figure 2.11(c) using matrix notation. Each of the 18 components has two subscripts. The first subscript refers to the direction of the applied force or electrical field. The second subscript refers to the direction of the induced electrical charge or mechanical strain. The effect of crystal symmetry on the number of independent moduli of the piezoelectric tensor is discussed in Appendix B. The piezoelectric tensor associate with each point group is listed in Appendix C.
2.2.2 Piezoelectricity in Biological Materials

Many biological materials demonstrate piezoelectricity. Martin was the first to report on piezoelectricity in bundles of wool and hair (88). This was followed shortly by Fukada’s finding of piezoelectricity in wood (15, 17), bone (16) and tendon (89). Since then the list of biological materials that show piezoelectricity is growing; it includes amino acids (20-23, 90), DNA (25, 91), fibrous proteins (collagen (18, 27), elastin (92), keratin (42, 93)) and phospholipids (68, 94). In fact, the prevalence of piezoelectricity in living materials suggests that it may contribute to physiological functions. In this section, specific examples of biological-piezoelectricity will be reviewed with the goal of understanding how these materials support piezoelectricity.

Piezoelectricity in Bone

Bone piezoelectricity is of particular interest - not solely because it was the one of the first materials to be studied. Much of the enthusiasm surrounding the study of piezoelectricity in bone is because it is thought to contribute to bone healing and bone remodeling (19). Wolff’s law describes how bone reacts dynamically to mechanical stress. Anecdotal evidence seems to support the law. For example, astronauts returning from an extended period in a low-gravity environment present with reduced bone density because their bones were not sufficiently loaded. Conversely, athletes who regularly load their bones during training present with increased bone density. When piezoelectricity was discovered in bone it was enthusiastically promoted as the mechanism through which bone regenerates itself. In theory, bone remodeling cells, osteoblasts and osteoblasts, would be stimulated in to action by the electric potential induced by an applied stress via the direct piezoelectric effect (95).

The simplicity of Wolff’s law is attractive; however, more rigorous models have questioned its validity (96). Some concerns include the fact that while dry bone exhibits piezoelectricity, its presence in wet bone (living bone is wet) is severely diminished (26, 97). Also, the viscosity dependent nature of the induced potential as well as the duration of its relaxation times are inconsistent with piezoelectricity (19). Yet, studies that show greater bone formation on piezoelectric PVDF compared to non-
piezoelectric PVDF, make it difficult to completely dismiss the idea of bone piezoelectricity contributing in some way to bone healing (98). A dual mechanism which proposes that the combined contribution of piezoelectricity and streaming potentials may be the answer (19).

**Piezoelectricity in Collagen**

Fibrous proteins perform essential roles in cells and tissues, providing mechanical stability and support, stiffness and elasticity. In addition to their mechanical properties, some fibrous proteins have also exhibited electromechanical properties. Stemming from the seminal work on bone piezoelectricity, collagen unsurprisingly was the first protein to show piezoelectricity (18). Since then, piezoelectricity has been observed in keratin (42), fibroin (99) and elastin (43). Here, piezoelectricity in collagen is examined as a representative case for other fibrous proteins.

In Figure 2.12 cross-sections are cut through collagen at different hierarchical levels to illustrate the complex structure of the protein. Several collagen fibrils are packed together to form the overall collagen fibre. Within the fibril, collagen molecules arrange themselves in a quasi-hexagonally packing fashion. Each collagen molecules consist of three polypeptide chains that wind together to form a right-handed triple-helix. Each molecule lies parallel to its neighbour in a staggered manner such that each molecule sits approximately 64 to 67 nm above or below its neighbouring molecule in the fibril. This shift gives the fibrils their characteristic repeating D-band structure (100).
The complexity of collagen fibers makes it difficult to establish the origin of its piezoelectricity. The fibrils are highly oriented and have a crystalline arrangement (16) – attributes well associated with piezoelectricity. The molecules are packed in a quasi-hexagonal fashion which can be described by point group 6 (C6 in Schoenflies notation) (18). As discussed in Section 2.2, materials belonging to this symmetry group demonstrate shear piezoelectricity (102). The origin of piezoelectricity in collagen therefore may be the induced polarization or displacement of hydrogen bonds in the collagen polypeptide chains resulting from a shear stress or electric field, respectively (18). The early work on collagen piezoelectricity quantified its piezoelectric activity; a piezoelectric $d_{14}$ coefficient of $8 \times 10^{-8}$ cgs.esu (~2.64 pC N$^{-1}$) was reported (18). PFM has been used more recently to probe collagen at its different hierarchical levels. At the nanoscale, individual collagen fibrils demonstrate shear piezoelectricity. The piezoelectric tensor of collagen measured via PFM reports higher piezoelectric coefficients than those obtained via macroscopic measurements (28, 41, 103-105). The piezoelectric effect in collagen is sufficient to construct a successful collagen-based gramophone (18) illustrating its potential as an electromechanical transducer.

Piezoelectricity in Amino Acids
The prevalence of biological-piezoelectricity extends right down to the most basic biological units – amino acids. Amino acids are chains of simple compounds connected by peptide bonds. They are chiral (with the exception of α-glycine, which is achiral) and so belong to enantiomorphous symmetry groups. X-ray diffraction (XRD) or nuclear magnetic resonance (NMR) can determine the crystal structure of amino acids in their crystalline form. Based on their symmetry, 19 of the 20 protein amino acid crystals may demonstrate piezoelectric behaviour, as they are non-centrosymmetric.

Vasilescu et al. (90) reported the first piezoelectric resonance in powders of amino acids in 1970. Later work by Lemanov focused on crystals of amino acids and their compounds (20-23). While many of the reports were qualitative, one study calculated the piezoelectric $d_{33}$-coefficient of L-arginine phosphate as 8.6 pC N$^{-1}$ (21). These studies indicate that the classical symmetry requirements of piezoelectricity hold for amino acids too.

However, the observation of piezoelectricity in α-glycine and racemic amino acids and the absence of piezoelectricity in non-centrosymmetric amino acids contest the theory. In the case of α-glycine, its achiral nature prohibits piezoelectricity. This contradictory observation is accounted for as the sample was contaminated by its ambient form, γ-glycine (21). Similarly, while racemic amino acids should be achiral, classical piezoelectricity is still permissible in some cases – as long as they belong to non-centrosymmetric groups. Such is the case for DL-alanine which although a racemic variety, belongs to the piezoelectric point group mm2 (22). The explanation for the absence of piezoelectricity in some amino acids whose symmetry supports piezoelectricity is simple; the experimental setup was not sensitive enough to overcome the effects of damping within the amino acid crystals (23).

**Piezoelectricity in Phospholipids**

Phospholipids make up the flexible barrier between the cell and its environment. Like amino acids, they are chiral and demonstrate piezoelectricity. Interestingly, it was by comparing phospholipids with liquid crystals that an understanding of how piezoelectricity is supported in phospholipids arose (68). Chiral phospholipids and
Chiral liquid crystals are similar in that they both have the potential to demonstrate piezoelectricity, yet neither take on the traditional appearance of bulk, solid-state piezoelectric materials. Jakli, a liquid crystallographer, noted these strong parallels and hypothesized that chiral phospholipids would demonstrate piezoelectricity while racemic phospholipids would not (106).

There are two broad categories of liquid crystals - nematics and smectics, as illustrated in Figure 2.13.

![Figure 2.13 Types of liquid crystals. (a) Nematic liquid crystals have only orientation ordering, aligning themselves along a common director axis. (b-d) Smectic liquid crystals have both orientational and positional ordering and may orient (b) normal to the layer axis (SmA) or (c) at an angle to the layer axis (SmC). (d) Chiral liquid crystals (SmC*) form a helix as the orientation of molecules varies from layer to layer. The net spontaneous polarisation is zero. Adapted from (107)](image)

The molecules of nematic liquid crystals do not have any positional ordering – however, they maintain an alignment parallel to a common director axis, Figure 2.12(a). Smectic liquid crystals are more ordered. Each layer of molecules is ordered with a well-established interlayer spacing (108). Smectic A (SmA) and Smectic C (SmC) are illustrated in Figure 2.13(b) and Figure 2.13(c), respectively. The molecules in a SmA liquid crystal have their long axis pointing in the direction normal to the layer axis. The molecules in a SmC liquid crystal have their long axis tilted at an angle from the layer axis (108). If a liquid crystal is chiral it is denoted with an asterisks. A chiral smectic C liquid crystal (SmC*) is shown in Figure 2.13(d). The molecules long
axis points in a different direction from one layer to the next. Each molecule has a dipole moment normal to the tilt plane. The spontaneous polarisation \( P_s \) associated with each dipole precesses around the direction normal to the layer forming a helix. Meyer hypothesised that chiral liquid crystals would demonstrate piezoelectricity because of their low symmetry (109) and this has since been confirmed experimentally (110).

By drawing parallels with liquid crystals, the crystal symmetry of a phospholipid bilayer can be described. Figure 2.14(a) shows a schematic of a SmC* phospholipid bilayer that is described by point group 2. Each phospholipid has an associated dipole moment \( \vec{p} \) which is aligned normal to the direction of the tilt in each smectic layer.

![Diagram of liquid crystalline properties](image)

Figure 2.14: The liquid crystalline properties of (a) a phospholipid bilayer allow piezoelectricity to manifest when the bilayer is (b) subjected to a shear compressive force. Adapted from (101)

When the phospholipid is undisturbed, the net polarisation of the phospholipid is zero as illustrated by the top view schematic in Figure 2.14(c). Subjecting the phospholipid to a shear stress distorts the helix, inducing a net polarisation as depicted in Figure 2.14(d). The direct piezoelectric effect has been observed experimentally in phospholipids; a 5° tilt produces a net polarisation of 300 nC cm\(^{-2}\) (106).
2.2.3 Pyroelectricity and Crystal Structure

Pyroelectricity is “the manifestation of the temperature dependence of the spontaneous polarization of certain anisotropic solids” (84). Tourmaline crystals and poled barium titanate ceramics are examples of pyroelectric materials. Three conditions must be satisfied in order to be pyroelectric. Firstly, the molecular structure of the material must have a non-zero dipole moment. This means that on a molecular scale, the units or building blocks of pyroelectric materials possess a dipole moment. The way in which these building blocks are stacked together must be in a manner that does not result in individual dipole moments cancelling out one another. Secondly, the material must have no centre of symmetry. Finally, the material must either possess no axis of rotational symmetry or else have a single axis of rotational symmetry that is not included in an inversion axis (84). Ten of the 32 crystal groups, called polar groups, satisfy these criteria.

The spontaneous polarisation, $P_s$, is the dipole moment per unit volume. It defines the amount of charge, $Q$, that develops around a material of area, $A$.

$$|P_s| = \frac{Q}{A} \quad 2.6$$

A spontaneous polarization is one that is never zero; it is present even in the absence of an electric field. Lang proposed a simple thought experiment that allows the nature of pyroelectricity to be understood. If the material were free to float in air, charges at its surfaces would attract and bind to free charges in the atmosphere. The net effect would be a neutral surface charge, Figure 2.15(a). When the temperature is constant, the spontaneous polarisation remains unaltered and an ammeter connected in series with the sample would read zero current, Figure 2.15(b). However, if the material is pyroelectric, a change in temperature affects its dipoles, either shifting their atomic positions or affecting their interatomic bonding. The result is a change of spontaneous polarisation. In response, the free charges within the material must redistribute themselves to compensate for the change in bound charges. The net result is a flow of current, $I$, measured by the ammeter, Figure 2.15(c).
If heating causes a positive current to flow in a pyroelectric material, the cooling must cause a negative current to flow, and vice-versa. Equation 2.7 describes the pyroelectric current $I$ in terms of the pyroelectric coefficient ($p$) and the rate of change of temperature ($\frac{dT}{dt}$).

$$ I = pA \frac{dT}{dt} $$

From Equation 2.7, it is clear that the pyroelectric effect is the result of a linear change of temperature. Heating and cooling should be linear to avoid inducing false-pyroelectric currents. Chapter 3 outlines precautions to distinguish between primary, secondary and tertiary pyroelectric effects.

2.2.4 Pyroelectricity in Biological Materials
In 1966, Lang reported his observations of pyroelectricity in animal bone and tendon (89). This was the first report of pyroelectricity in biological materials. Other early works in the field found pyroelectricity in plant leaves (111) and the integument of cockroach insects (112).

The number of studies investigating pyroelectricity in biological materials is far less than the number of studies investigating their piezoelectric properties. One reason for this may be that because pyroelectricity has no physiological significance in the way that bone-piezoelectricity does, there has been less interest. Secondly, the difficulties of measuring pyroelectricity accurately, especially in biological materials, may be a barrier that has prevented researchers from uncovering pyroelectricity in other biological materials. Biological materials may degrade or denature when exposed to heating. Also, there size, shape and environment may pose challenges for creating electrode contacts. The high content of water in biological materials can also affect the reproducibility of data (113).

Despite these challenges, there is some evidence that pyroelectricity persists down to the smallest biological building blocks. While Lemanov’s primary interest was in studying the piezoelectric properties of amino acids, he was aware that triglycine sulfate, a derivative of the amino acid glycine, exhibits strong pyroelectricity. He also recognised that the polar symmetry of many amino acids would support pyroelectricity. Hence, while studying the piezoelectric effect in amino acids and their compounds, he reported that a number of them also showed pyroelectricity. Unfortunately, he did not publish details of the measurement approach, only commenting that the pyroelectric coefficient was not very high. On the contrary, the pyroelectric coefficient of $\gamma$-glycine reported was $13 \mu\text{C/m}^2\text{K}$ (21), more than three times the pyroelectric co-efficient of tourmaline.

In the same way that the symmetry assigned to hydroxyapatite erroneously dismissed it as non-piezoelectric, so too was the pyroelectric properties of hydroxyapatite kept hidden until recently. Originally, pyroelectricity in bone was attributed its polar collagen component. It was believed that hydroxyapatite played no role in bone pyroelectricity. After the reassigned of hydroxyapatite to point group 2/m (36), Tofail
et al. reported pyroelectricity in poled hydroxyapatite ceramics (114). Soon after, Lang et al. reported pyroelectricity in un-poled thin films of hydroxyapatite (39). These studies are important not only because they include hydroxyapatite as a contributor to bone pyroelectricity, but because they highlight the importance of Neumann’s principle in correlating material properties with their symmetry.

2.2.5 Ferroelectricity and Crystal Structure

All pyroelectric materials have a dipole; however, whether or not that dipole re-orientates in response to an electric field is the factor that distinguishes pyroelectrics from ferroelectrics. In ferroelectric materials, a sufficiently high electric field reverses the direction of the electric dipoles, re-orientating the direction of its spontaneous polarisation (84). Typically, ferroelectric materials are characterised by high dielectric constants and a Curie point, above which the material adopts a non-polar state.

The Sawyer-Tower circuit is the classical method of measuring ferroelectricity. Ferroelectric samples measured in this way demonstrate characteristic hysteresis loops, Figure 2.16(a). This hysteresis loop determines the spontaneous polarisation, remnant polarisation (polarisation at zero electric field) and coercive field (field required to reverse the spontaneous polarisation) of the sample. Also for samples that are ferroelectric, measurements of the strain induced due to the applied field reveal a characteristic butterfly loop, Figure 2.16(b).
While symmetry conditions govern ferroelectricity, a process of poling may alter polycrystalline materials in such a way that they become ferroelectric. The poling process involves heating the material above the Curie temperature. Applying a high DC voltage causes the dipoles to align in the direction of the applied field. The result is an overall net polarization. During cooling, the DC voltage remains in place, preventing the majority of dipoles from returning to their original state. After cooling and removing the DC voltage, a remnant polarization persists. Poling thus, induces an artificial anisotropic condition necessary to induce piezoelectricity.

Joseph Valasek formally reported observations of ferroelectricity in samples of Rochelle Salt in 1921 (116). Today’s ferroelectrics predominantly occupy roles in the data-storage, energy-harvesting, electro-optics and actuator industries. The majority of ferroelectrics are solid, bulk materials, often containing lead components. The discovery of piezoelectricity and ferroelectricity in PVDF extended ferroelectrics to applications that require flexibility and biocompatibility (117).

2.2.6 Ferroelectricity in Biological Materials

In the 1950’s and 1960’s, the discoveries of piezoelectricity and pyroelectricity in biological materials, naturally led to the idea that these materials may also be ferroelectric. The earliest reports of biological ferroelectricity were in samples of RNA (118) and DNA (119). However, since then further evidence has emerged that suggests that the observations made by Stanford and Polonsky were the result of nonlinear
conductivity rather than true ferroelectricity (120).

PFM has emerged as a tool capable of probing the ferroelectric properties of materials. Li et al. used PFM to demonstrate ferroelectric loops from green abalone shell (121). Liu et al. also used PFM to report evidence of ferroelectricity and weak piezoelectricity (1 pm V⁻¹) in the aortic wall (122), which they have attributed to its constituent material, the fibrous protein elastin (92). Ferroelectric switching in elastin is suppressed by glucose, an observation that may have physiological significance (43).

Heredia et al. used the same technique to measure relatively large piezoelectricity (10 pm V⁻¹) and ferroelectric switching in the amino acid, γ-glycine (123). Similarly, Lang et al. used Switching Spectroscopy PFM (SS-PFM) to demonstrate ferroelectricity in nanocrystalline hydroxyapatite (40). These examples fulfil the long held promise that biological materials might show ferroelectric behaviour. Studies such as these serve to motivate further research in to the area of biological-ferroelectrics. Eventually perhaps, a true physiological significance of ferroelectricity in biological materials will be uncovered.

2.3 Lysozyme: A Globular Protein

Amino acids form the basis of more complex biological units. A connected series of amino acids forms a peptide. A longer chain of amino acids arranged in biologically functional manner is called a protein. Proteins can be grouped in to three categories, fibrous, globular and integral membrane proteins. Lysozyme is a globular protein.

Sir Alexander Fleming is famously associated with the discovery of penicillin in 1928. However, before that in 1921, he made another significant discovery – that of lysozyme. By accident, a drop of nasal mucus fell on to a plate of bacteria and he noticed that it caused lysis of the bacteria (124). Lysozyme is effective in protecting us against infection and is used as a preservative in the food industry (125); however, it is not suitable as an anti-biotic. Although, it has not contributed to medicine in the way that penicillin has, the discovery of lysozyme has left a remarkable imprint. Ninety-five years on from its initial discovery, lysozyme has become the protein of choice in structural biochemistry, owing to its small size and stability.
Lysozyme is a globular protein, found in excretions such as tears and saliva. It is also found abundantly in hen egg whites. As Fleming discovered, its function is to protect against infection by breaking down the cell wall of gram-positive bacteria. Alternating N-acetylg glucosamine (NAG) and N-acetylmuramic acid (NAM) residues are linked together to form the peptidoglycan layer of the bacterial cell wall. Lysozyme lysis through these NAM–NAG links via hydrolysis as indicated in Figure 2.17. The weakened bacterial cell wall then collapses under its own internal pressure (126).

Like all proteins, the primary structure of lysozyme is a chain of amino acids connected by peptide bonds. Lysozyme is small in size – 14.7 kDa. Its 129 amino acids link together, folding into a unique conformation that is made up of α-helices and β-sheets (126). The ribbon diagram in Figure 2.18 illustrates lysozyme’s tertiary structure. The ribbon diagram begins at the red C-terminus and follows the colour of the spectrum until it reaches the blue N-terminus. Red coils represent α-helices and blue arrows represent β-sheets. Lysozyme has two domains linked by an α-helix. The N-terminus domain contains an anti-parallel β-sheet and some helices. The C-terminus domain contains α-helices. The hydrophobic groups are buried within and the hydrophilic groups are on the outside giving lysozyme its hydrophobic exterior and hydrophilic interior. The crevice between the two domains is the active site of lysozyme; this is

![Diagram of lysozyme's structure showing the point of hydrolysis and the N-acetylg glucosamine (NAG) and N-acetylmuramic acid (NAM) residues linked together.](image-url)
the part that binds to the carbohydrate chain of the bacterial cell before lysing it (126).

Figure 2.18 Ribbon diagram of the tertiary structure of lysozyme created using Chimera and I1EE. The sequence begins at the C-terminus (red) and follows the colour spectrum until the N-terminus (blue). Coils represent α-helices and arrows represent β-sheets.

2.3.1 The Crystal Structure of Lysozyme

X-ray diffraction (127), synchrotron diffraction and nuclear magnetic resonance (NMR) studies, reveal the structure of protein crystals. Compared to other protein types, globular proteins crystallise easily. Blake et al. solved the structure of the tetragonal lysozyme structure first in 1965 using XRD (128). Uniquely, lysozyme crystals can grow in more than one form. The Protein Data Bank (129), a depository of thousands of protein structures, reports findings of tetragonal, monoclinic, orthorhombic, triclinic and hexagonal crystals of lysozyme (130). Optical microscopy images of lysozyme crystal polymorphs are shown in Figure 2.19.
Figure 2.19 Optical microscopy images of lysozyme crystallised in different forms: (a) triclinic (51), (b) orthorhombic (131), (c) monoclinic (81), (d) tetragonal (132) and (e) hexagonal (130).

Diffraction studies reveal a lot of information about lysozyme. In the simplest sense, diffraction experiments determine the unit cell parameters of the protein crystal. In addition, these measurements add to our understanding of protein molecular packing arrangements, folding mechanisms and hydration shell properties. Diffraction data also reveals similarities and disparities between lysozymes from different sources. Structurally, lysozyme from hen egg whites, human, bacteriophage and bacterial sources are similar. However, lysozyme from the fungus species Chalarosis is different, having no sequence homology with avian, mammalian or phage lysozymes. It does share some sequence similarities with the bacterial form of lysozyme (133). Diffraction studies can also aid our understanding of how lysozyme binds to N-acetylglucosamine residues in bacterial cells (134).

While diffraction has emerged as a powerful tool in protein structural biochemistry, it may not always determine the best symmetry fit for a given protein. For protein crystals especially, artefacts such as twinning and pseudo-symmetry operations are common. This occurs when more than one protein molecule sit in each asymmetric unit. As diffraction systems and software are become increasingly sophisticated with automatic fitting programs, the risk of a protein being incorrectly assigned to an incorrect space group also increases (135). Crystals are assigned mistakenly when the non-crystallographic symmetry is very similar to the true crystallographic symmetry.
of the crystal. In this scenario, the non-crystallographic symmetry axis in the software program is biased towards the crystallographic axis (136). The extent to which misassignment of space group symmetry occurs may be significant; Zhart et al. estimate that 6% of the protein structures documented in the Protein Data Bank are pseudosymmetric and may be incorrectly assigned to space groups (135).

2.3.2 Atomic Force Microscopy Studies of Lysozyme

Many studies have investigated the ways in which nucleation and crystallisation occurs within lysozyme. Nucleation can be controlled by seeding, or by the application of a localized voltage (137) or magnetic field (138). The extension of Atomic Force Microscopy (AFM, described in Section 3.3.3) capabilities to include liquid environments offers another approach to investigate crystal growth mechanisms. AFM has been used to study the growth mechanisms of lysozyme in its triclinic (51), tetragonal (52), monoclinic (53) and orthorhombic (54) forms. In typical experiments, protein crystals are grown either directly on glass petri dishes or transferred from sitting drop wells to a petri dish. The protein crystal sits in a droplet of the mother liquor to prevent it from drying out. After some time, the protein crystals settle and adhere to the base of the dish. This allows the AFM tip to scan across their surface without the sample protein moving. AFM has revealed growth spirals, rounded steps, impurity pinning and 3D nucleation on the surface of lysozyme crystals.

Figure 2.20 shows the surface of a triclinic crystal of lysozyme obtained by liquid-AFM scanning in-situ (52), revealing concentric growth spirals on the crystal surface. At low supersaturation levels, screw dislocations are the growth mechanism that result in spirals at the crystal surface (139) shown in Figure 2.20(a-d) (52). In the latter stages of the liquid-AFM study, two-dimensional nucleation becomes the dominant growth mechanism. Further nucleation creates islands that merge with the spiral steps forming bulges on the spirals until eventually no evidence of the spiral step is left, Figure 2.20(e, f) (52).
Figure 2.20 The surface of a triclinic crystal of lysozyme imaged using AFM in liquid at (a) 0 min, (b) 20 minutes, (c) 30 minutes, (d) 40 minutes, (e) 87 minutes and (f) 99 minutes. The scale bar in (f) is 5 µm. At low supersaturation levels, a-d, spiral dislocations are the dominant growth mechanism which produce growth spirals. Later 2D-nucleation dominates, e-f, creating islands that merge together forming bulges. (52)

Other studies observed the adsorption of lysozyme on to mica substrates via AFM (55, 56). During adsorption, lysozyme changes its conformational state. Depending on the orientation of the molecule, the conformational change can result in a change of height that is detectable by AFM. Height images of lysozyme adsorbed on to mica demonstrated spikes that may correspond to induced changes in protein conformation. These spikes were not present in images of proteins with inhibited enzyme activity (55).

There have been very few attempts to extend PFM to the realm of liquid environments. The major difficulty is that most liquids are highly conductive and decompose even at low voltages (140). One approach is to use insulating probes that have a conductive apex. In this case, the voltage is localised, reducing stray currents and electromechanical artefacts (141). Other studies have investigated the feasibility of liquid PFM in contact (142) and intermittent contact modes (143) for samples of PZT and periodically poled lithium niobate. Surprisingly, Rodriguez et al. reported that
performing scans in distilled water improved the resolution compared to scans performed in ambient condition, probably due to reduced contributions from electrostatic forces. Replacing distilled water with NaCl solutions notably reduced the PFM contrast (142). This observation is one that will limit the usefulness of the technique for scanning biological materials in liquid environments.

Rodriguez et al. also used liquid PFM to probe the piezoelectric properties of dentin, a fibrous protein. The quality of the images reduced when collected in a liquid environment compared to images collected in an ambient environment, perhaps because of increased damping effects. Yet, the contrast between piezoelectric (intertubular) and non-piezoelectric (peritubular) regions was still evident (143). As the physiological environment of many biological materials, including proteins, is a liquid one, further work in this area could realise high-resolution PFM in biological samples under native conditions.

2.3.3 Dielectric Properties of Lysozyme

While, there are extensive studies dealing with the crystal structure and crystal growth mechanisms of lysozyme, relatively few studies have investigated its dielectric properties. Protein crystals may contain up to 80% water; to understand the nature of the biological water contained within the protein crystals, Rashkovich et al. investigated the dielectric properties of monoclinic lysozyme crystals. They reported that two-thirds of this water is structurally the same as ordinary water and the remaining one-third (bound water) is only slightly different (57). In the context of this work, Rashkovich’s study is useful in that it reports on the frequency dependence of the impedance and loss tangent of monoclinic lysozyme crystals. In the range of the frequency sweep, there was no indication of any impedance resonance that would point towards piezoelectricity.

Determining the dielectric constant of a protein is not a trivial task. The presence of an aqueous environment complicates both experimental and theoretical studies. In the literature, values for the dielectric constant of lysozyme range between 2 and 80. The range is so wide because some of the reports correspond to dry lysozyme (dielectric constant ~2) (58), while others investigated it in its reconstituted state. The dipole
moment of lysozyme is also temperature and pH dependent (144). Smith used long molecular dynamics simulations to show that an intermediate value for the dielectric constant of 30 may be most appropriate (59). More recently, Li et al. confirmed this, using a Gaussian approach to show that the dielectric constant inside the protein is relative low (dielectric constant \( \sim 6-7 \)), while at the surface it is larger (dielectric constant \( \sim 20-30 \)) (60).

In terms of measuring electro-mechanical properties of lysozyme, the literature survey only uncovered one study. Danielewicz-Ferchmin et al. conducted a computational study based on data in the existing to literature to study the hydration water density of in lysozyme (61). A publication from Ortore et al., which showed that the hydration water density of lysozyme under high pressure is greater than that at atmospheric pressures, prompted the investigation (62). Even at ambient pressures, this difference persists. At ambient pressure, protein surface charges create an electric field that increases the hydration water density via electrostriction. Interestingly, as the pressure is increased the hydration water density also increases in a manner that is analogous to the direct piezoelectric effect. A plot generated from literature data of the mean surface charge density as a function of the hydrostatic pressure is linear, but with a large y-intercept. Although incorrect units are derived, they calculate a strikingly high value for the effective piezoelectric coefficient, reporting a value of 250 pC N\(^{-1}\).

2.3.4 Crystal Properties of Lysozyme

As lysozyme crystal exist in more than one crystal form, we hypothesis that there will be more than one associated tensor of piezoelectric coefficients to describe it. We further hypothesis that the same symmetry restrictions that govern classical crystalline materials govern crystals of lysozyme also. Table 2.4 presents the predicted piezoelectric tensor for lysozyme in all its available forms, tetragonal, monoclinic, orthorhombic and triclinic.
Table 2.4: Piezoelectric tensor corresponding to various crystal point groups of lysozyme. Adapted from (102)

<table>
<thead>
<tr>
<th>Crystal</th>
<th>Point Group</th>
<th>Piezoelectric coefficient tensor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetragonal</td>
<td>422</td>
<td>( d = \begin{bmatrix} 0 &amp; 0 &amp; 0 &amp; d_{14} &amp; 0 &amp; 0 \ 0 &amp; 0 &amp; 0 &amp; 0 &amp; -d_{14} &amp; 0 \ 0 &amp; 0 &amp; 0 &amp; 0 &amp; 0 &amp; 0 \end{bmatrix} )</td>
</tr>
<tr>
<td>Tetragonal</td>
<td>4</td>
<td>( d = \begin{bmatrix} 0 &amp; 0 &amp; 0 &amp; d_{14} &amp; 0 &amp; 0 \ 0 &amp; 0 &amp; 0 &amp; d_{15} &amp; 0 &amp; 0 \ 0 &amp; 0 &amp; 0 &amp; -d_{14} &amp; 0 &amp; 0 \end{bmatrix} )</td>
</tr>
<tr>
<td>Orthorhombic</td>
<td>222</td>
<td>( d = \begin{bmatrix} 0 &amp; 0 &amp; 0 &amp; d_{14} &amp; 0 &amp; 0 \ 0 &amp; 0 &amp; 0 &amp; 0 &amp; 0 &amp; d_{36} \ 0 &amp; 0 &amp; 0 &amp; 0 &amp; 0 &amp; 0 \end{bmatrix} )</td>
</tr>
<tr>
<td>Hexagonal</td>
<td>622</td>
<td>( d = \begin{bmatrix} 0 &amp; 0 &amp; 0 &amp; d_{14} &amp; 0 &amp; 0 \ 0 &amp; 0 &amp; 0 &amp; 0 &amp; 0 &amp; -d_{14} \ 0 &amp; 0 &amp; 0 &amp; 0 &amp; 0 &amp; 0 \end{bmatrix} )</td>
</tr>
<tr>
<td>Monoclinic</td>
<td>2</td>
<td>( d = \begin{bmatrix} 0 &amp; 0 &amp; 0 &amp; d_{14} &amp; 0 &amp; d_{16} \ d_{21} &amp; d_{22} &amp; d_{23} &amp; 0 &amp; 0 &amp; 0 \ 0 &amp; 0 &amp; 0 &amp; 0 &amp; 0 &amp; d_{36} \end{bmatrix} )</td>
</tr>
<tr>
<td>Triclinic</td>
<td>1</td>
<td>( d = \begin{bmatrix} d_{11} &amp; d_{12} &amp; d_{13} &amp; d_{14} &amp; d_{15} &amp; d_{16} \ d_{21} &amp; d_{22} &amp; d_{23} &amp; d_{24} &amp; d_{25} &amp; d_{26} \ d_{31} &amp; d_{32} &amp; d_{33} &amp; d_{34} &amp; d_{35} &amp; d_{36} \end{bmatrix} )</td>
</tr>
</tbody>
</table>

Having the lowest symmetry possible, triclinic crystals of lysozyme have the potential to demonstrate all piezoelectric coefficients. As the symmetry increase from triclinic through to tetragonal, the corresponding piezoelectric tensor becomes more constricted. We predict that monoclinic crystals of lysozyme will demonstrate longitudinal \( (d_{22}) \), transverse \( (d_{21} \text{ and } d_{23}) \), and shear \( (d_{14}, d_{16}, d_{25}, d_{34} \text{ and } d_{36}) \) piezoelectric coefficients. Hexagonal crystals of lysozyme have the potential to demonstrate only the shear piezoelectric coefficients \( d_{14} \) and \( d_{25} \), which should be of equal magnitude and of opposite sign. Orthorhombic crystals of lysozyme may potentially demonstrate shear piezoelectric coefficients \( d_{14}, d_{25} \text{ and } d_{36} \). In its most common form (point group 422) tetragonal crystals of lysozyme may demonstrate only shear piezoelectric coefficients \( d_{14} \) and \( d_{25} \), which should be of equal magnitude and of opposite sign. If in the uncommon form, the symmetry of tetragonal point group 4
allows longitudinal ($d_{33}$), transverse ($d_{31}$, $d_{32}$) and shear ($d_{14}$, $d_{15}$, $d_{24}$, $d_{25}$ and $d_{34}$) piezoelectric coefficients.

2.4 Conclusions

While the dielectric properties of biological materials have been studied for decades now, there are still many gaps that need answers. Observed in the bulk and at the nanoscale, the properties of piezoelectricity, pyroelectricity and ferroelectricity, seem to persist at all hierarchical levels. The exact mechanisms behind each are not always clear. Lysozyme is an ideal case-study material for several reasons. Like all natural proteins, it is chiral and non-centrosymmetric. Additionally, crystallising lysozyme in its various forms allows one to investigate how symmetry affects its physical properties. The following chapter will look at the methods of measuring dielectric properties in biological materials, highlighting the challenges involved.
This chapter describes the techniques used to grow and characterise crystalline aggregate films of lysozymes. Firstly, we introduce the principles of protein crystal growth. Then, the structural characterisation of protein crystals will be discussed with a particular focus on synchrotron diffraction. Next, we review the state-of-the-art methods of piezoelectric measurement and justify the selection of two methods (the Berlincourt Method and Piezoresponse Force Microscopy) for investigating piezoelectricity in proteins. The principles underlying both the Berlincourt method and Piezoresponse Force Microscopy (PFM) are then detailed. This chapter also discusses Switching-Spectroscopy PFM for investigations of ferroelectricity at the nanoscale. Finally, techniques for measuring pyroelectricity are reviewed in order to select a suitable method of measuring pyroelectricity in lysozyme crystals.

3.1 Protein Crystallisation
Successful protein crystallisation begins with high protein purity. In addition, the choice of salt, pH, ionic strength, protein concentration and any additives is critical to the realization of protein crystals. Unfortunately, the set of parameters required is unique to each protein, making protein crystallisation a challenging endeavour. With so many parameters at play, screening methods identify suitable parameters that are optimised later.

The simple process of salt crystallisation is a good place to begin to understand how proteins crystallise. In both cases the laws of thermodynamics apply and supersaturation proceeds crystal nucleation. To induce supersaturation in a salt solution, the solution is heated to increase its solubility. As the solution cools, its solubility decreases allowing it to become supersaturated. The solution now contains more salt molecules than it would under normal conditions. It is no longer in equilibrium and seeks to find a more favourable thermodynamic state. The free-energy of the system is minimised when the salt begins to crystallise.

Although more complex, the process of protein crystallisation is similar, requiring a
slow reduction of protein solubility or alternatively, a slow increase in protein concentration. Reaching this supersaturated state triggers crystal nucleation. Although temperature can be used to vary the solubility of salts, using temperature to vary the solubility of proteins is unwise, as it may cause denaturation. Instead, precipitants are added which bind to water molecules in the solute – reducing the number of water molecules available to the protein. In effect, this reduces the protein solubility or thought about in another way, increases the effective protein concentration.

Figure 3.1 illustrates the phase transition diagram of a typical protein-precipitant mixture. Protein crystallisation is a two-stage process. Nucleation occurs during the first stage and crystal growth occurs in the second stage. Both stages require supersaturation conditions; however, nucleation needs a higher degree of saturation. Therefore, the protein must first be pushed into the highly supersaturated labile zone where rapid nucleation happens. The protein must not stay in this zone for too long as a surplus of crystal nuclei impedes the growth of large crystals. Instead, the protein is pushed to the lower-supersaturated metastable zone. In this zone, nucleation ceases and the existing crystals grow in size. There are various crystallisation techniques to control these processes so that good quality, large crystals can be grown for further studies.
Figure 3.1: Schematic protein crystallisation phase diagram showing the change of protein concentration with precipitant concentration. The solubility curves separate the undersaturated state from the supersaturated state. Nucleation occurs in the supersaturated state with nucleation being initialised in the labile zone and slow crystal growth occurring in the metastable zone.

3.1.1 Crystallisation Techniques
Vapour diffusion is perhaps the most common method of growing protein crystals. There are several variations of vapour diffusion techniques including the so-called ‘hanging-drop’ and ‘sitting-drop’ methods shown in Figure 3.2. In the hanging-drop method, the reservoir contains a relatively large volume (~100 µL) of precipitant—often called the mother liquor. The reservoir is sealed with a coverslip that has a drop (~1 µL to 2 µL) containing the protein and precipitant. Initially, the concentration of precipitant in the reservoir is higher than that in the drop – setting up a concentration gradient. Water from the drop evaporates, falling in to the reservoir below. Therefore, the volume of the drop decreases, and consequently the protein concentration within the drop increases. Eventually, the protein will supersaturate, initialising crystallisation.
Figure 3.2: Schematic of two variants of the vapour diffusion method of growing protein crystals, (a) the hanging drop method and (b) the sitting drop method

The sitting-drop process is essentially the same, except that the protein sits within a well that is surrounded by a reservoir. Both methods are relatively simple but the sitting drop has a slight disadvantage in that crystals may become stuck to the bottom of the well.

3.2 Synchrotron Diffraction
Discovered by Röntgen in 1895, X-rays are now employed in a wealth of applications most prominently in the fields of medicine and dentistry. However, the realisation made by Laue, Friedrich and Knipping in Munich in 1912 – that crystals diffract X-rays – would greatly extend the applications of X-rays and facilitate our understanding of the atomic structure of crystals. The father and son team, W.H. Bragg and W.L. Bragg, developed the technique of X-ray diffraction, earning a Nobel Prize for their efforts in 1915. Another important contributor at the time was P.P. Ewald who devised a means of interpreting the geometry of the diffraction pattern using a construct called the reciprocal lattice described below. In 1934, J.D. Bernal and D. Crawfoot-Hodgkin discovered that protein crystals would diffract X-rays (145). It took until 1960 to decipher a method of solving the protein structure from the diffraction pattern; myoglobin was the first protein to have its structure solved (146). Since then, the structures of thousands of proteins have been solved and deposited to the Royal Collaboratory for Structural Bioinformatics’ (RCSB) Protein Data Bank (PDB).
3.2.1 Production of X-rays

X-ray production in laboratory settings is accomplished by producing electrons via thermionic emission and using a high voltage to accelerate them towards the anode as depicted in Figure 3.3. The anode also acts as the target. X-rays are produced when high-speed electrons strike the target material (often made of chromium, copper or molybdenum). Only a small portion of electrons are converted to X-rays during the collision. The remaining energy is dissipated as heat.

Figure 3.3 Production of X-rays. Electrons produced by thermionic emission are accelerated by a high voltage. X-rays are produced when these high-speed electrons strike the target material. Adapted from (77).

A synchrotron is a powerful source of X-rays. A schematic of a synchrotron is shown in Figure 3.4. Electrons produced by an electron gun are pre-accelerated in the booster ring before entering the storage ring. The electrons are made to bend around the ring by strong magnets. Electrons circling the ring experience centripetal force and are accelerated to relativistic speeds producing X-rays.
Figure 3.4 Schematics of a synchrotron. (a) Electrons are produced by an injector-gun, are pre-accelerated by the booster ring and then fed in to the storage ring. Radio frequency generators compensate for energy losses and magnets direct the beam. (b) For experiments, the X-rays are directed out of the circular path, through the monochromater and into diffraction hutchess. (77)

Synchrotrons are advantageous in X-ray production because they can produce monochromatic X-rays at different wavelengths. Additionally, X-ray beams from synchrotron sources are of high intensity, allowing diffraction experiments to be performed on small crystals in a reasonable timeframe before degradation occurs.

3.2.2 Diffraction of X-rays

For diffraction to occur in crystals, the wavelength of the radiation must be comparable to the distance between the atoms in the crystal. The shortest distance between atoms in a crystal is the length of a covalent bond, i.e. approximately 0.1 nm. Thus, radiation in the visible spectrum (approximately 400 nm to 700 nm) will not diffract passing through a crystal. A higher energy radiation source is required. X-rays fulfil this criterion as their energy is in the range $10^2$ to $10^5$ eV. The corresponding wavelength range for X-rays is 10 nm to 0.1 nm; this wavelength is short enough that X-ray radiation will diffract as it passes through a crystal.

Laue’s interpretation of X-ray diffraction considered a crystal like a three-dimensional diffraction grating. While this interpretation is not incorrect, it is cumbersome requiring six angles, three lattice spacings and three integers to determine the direction of the diffracted beam (78). Bragg’s interpretation is simpler and thus, of more
practical significance. In the Bragg interpretation, atoms within the crystal are imagined to form layers or planes as in Figure 3.5.

![Diagram](image)

Figure 3.5 Bragg’s law: X-rays diffract when they hit a plane of atoms within a crystal. The diffracted X-rays interfere constructively when the path length difference (AB+BC) is equal to an integer number of wavelengths.

Incident X-rays of wavelength $\lambda$, strike atoms within the plane and diffract with the angle of incident equal to the angle of diffraction ($\theta$). The distance between planes is known as the inter-planar spacing and is denoted by $d_{hkl}$, where $hkl$ are miller indices. Diffracted X-rays interfere constructively when the path length difference ($AB + BC$) between diffracted X-rays equals an integer number of wavelengths. From Figure 3.5, the path length difference may be written in terms of the inter-planar spacings. Thus, Equation 3.1 describes Bragg’s law.

$$n\lambda = 2d_{hkl} \sin \theta$$  \hspace{1cm} 3.1

When X-rays diffract from a crystal, they produce a series of spots called a diffraction pattern, which is collected on a screen (typically a charge-coupled-device or CCD detector). Two abstract constructs that facilitate the interpretation of the diffraction pattern are shown in Figure 3.6; these are (i) the reciprocal lattice and (ii) the Ewald sphere.
Figure 3.6 Schematic illustrating the concept of the Ewald sphere and the reciprocal lattice. Incident X-rays $s_0$ of wavelength $\lambda$ strike a crystal and result in diffracted X-rays $s_1$. The Ewald sphere of radius $\frac{1}{\lambda}$ is drawn around the crystal. The Bragg equation is satisfied when the reciprocal lattice points intersect the Ewald sphere. Adapted from (77)

The diffraction pattern is a reciprocal lattice; it represents the Fourier transform of the Bravais lattice of the crystal. The inter-planar spacing of the reciprocal lattice $d_{hkl}$ is equivalent to the inverse of the real inter-planar spacing $d_{hkl}$. The Ewald sphere is a geometrical formulation of Bragg’s law. As shown in Figure 3.6, the Ewald sphere of radius $\frac{1}{\lambda}$ has the crystal at its centre. If the angle of incidence satisfies the Bragg equation (Equation 3.1), then the reciprocal lattice point will intersect the Ewald sphere. Conversely, if the Bragg equation is not met, the lattice point will not intersect the sphere.

Structural determination of the crystal involves two steps. In the first step, the lattice parameters of the unit cell are calculated from the geometry of the diffraction pattern. In the second step, the distribution of atoms within the structure from the relative intensities of the diffractions spots is determined. The intensity of the diffraction spots is directly proportional to the square of the structure factor, $F_{hkl}$. The structure factor
term is required as diffraction is a scattering process. While the Bragg equation considers a single atom to reside at each lattice point, it does not specify the scattering power of the each atom. To a large extend it is the electrons within the atom that contribute to scattering. Thus, the structure factor is the ratio of the sum of the atomic scattering amplitudes $f_n$ to the amplitude scattered by a single atom. The structure factor contains information about the amplitude and phase of the scattered waves (78).

$$F_{hkl} = \sum_{n=0}^{n=N} f_n \exp(2\pi i(hu_n + kv_n + lw_n))$$

Where $n$ is the number of atoms, $h$, $k$ and $l$ are the miller indices associated with the scattering planes and $u$, $v$ and $w$ are fractions of the unit cell length at which the atoms are located. Equation 3.2 is useful because it can be applied in reverse. That is, if the intensity of the diffraction pattern is known, the positions of the atoms within the unit cell can be determined.

As mentioned above, the intensity of the diffraction spot is proportional to the square of $F_{hkl}$. The value of $F_{hkl}^2$ is always a real number, i.e. information about the phase is lost. In crystallography, this is known as the phase problem. The phase problem can be overcome if the origin is placed at the crystal’s centre of symmetry. However, if the crystal does not have a centre of symmetry, as is the case of protein crystals, this solution is not valid. Other methods of solving the phase problem include molecular replacement (which makes use of the atomic co-ordinates of similar protein structure), isomorphic replacement (which uses heavy-atom substitution) and anomalous dispersion (which uses the anomalous scattering that occurs at the absorption edge) (147). These methods allow the phase information to be determined from some $a priori$ knowledge of the molecular structure.

3.3 Piezoelectric Measurement Techniques

3.3.1 Selection of Measurement Method

Methods of measuring piezoelectricity fall into three main categories, those based on the direct piezoelectric effect, those based on the converse piezoelectric effect and those based on dynamic methods. The Berlincourt Method is a direct method, laser-interferometry and piezoresponse force microscopy are converse methods and
resonance analysis is a dynamic method. Each method has advantages and limitations. This section reviews methods of measuring piezoelectricity, and justifies the choice of methods used in this work.

The **resonance method** is a dynamic method of piezoelectric measurement. It measures the frequencies at which the material demonstrates a mechanical piezoelectric response to an applied AC voltage. Piezoelectric materials each have a characteristic frequency at which it will vibrate freely, called the resonance frequency, and another frequency at which they resist vibration most effectively, called the anti-resonance frequency. Both the resonance and anti-resonance frequencies are characteristic of the sample, from which the piezoelectric coefficients of the sample are accurately calculated. With careful consideration of sample geometry, electroding, shielding and the clamping arrangement, the resonance method measures piezoelectricity with great accuracy and repeatability (148).

However, the resonance method is not ideally suited to thin-film measurement. In thin-films, the relationship between the piezoelectric and elastic properties and their resonant frequencies is not well established (149). Additionally, piezoelectric resonance in thin-films occurs in the gigahertz range, outside the scope of most commercial impedance analysers. The technique works well for bulk samples, establishing a complete piezoelectric tensor. However, achieving this requires the sample to be prepared in different geometries: as a disc, a plate and a cylinder (150). In many cases, including in this work, satisfying this requirement is unfeasible.

**Laser-interferometry** can measure displacements as low as $10^{-4}$ Å (151) with good accuracy. This resolution is sufficient to measure the small voltage-induced strains in piezoelectric materials. By knowing the voltage applied, one can calculate the piezoelectric coefficient of the sample (152). This method has successfully measured the piezoelectric coefficient of bulk (153), thin-film (154, 155) and biological samples (26).

While laser-interferometry is suitable for measuring piezoelectricity in thin-films, this type of measurement brings its own challenges. A single-beam interferometer only monitors the displacement from the front surface of the sample. The substrate may
bend away from its support substrate if it is not tightly bonded. In this case, the bending artificially enlarges the deformation measured by the interferometer (154). Simply strengthening the bond between the sample and substrate may not always be appropriate as it creates a clamping effect in the transverse direction (156). Double-beam interferometry provides a convenient solution, monitoring both the front and back surfaces of the sample simultaneously (154, 156, 157).

Using laser interferometry to detect protein piezoelectricity would be challenging as the method works best with flat, well-polished samples. However, laser interferometry has been used to study conformational changes (158) and crystal growth processes (159) in proteins. In terms of piezoelectric measurements, the effect of water on the piezoelectric properties of collagen were revealed by laser-interferometry (26). In this project, the cost of purchasing or custom building a laser-interferometry system was not feasible.

Owing to the limitations and constraints of both the resonance method and laser-interferometry, these were not pursued further in this research project. As such, the Berlincourt method and PFM, emerged as two viable techniques for investigating protein piezoelectricity. The next section discusses the basic principle of each, as well as their suitability and limitations in greater depth.

3.3.2 The Berlincourt Method
The Berlincourt method measures piezoelectricity in materials using the direct piezoelectric effect. The original design was static; a known weight placed on top of the sample caused a charge to develop due to the piezoelectric effect. Static measurements, however, suffer from thermal drift. For this reason, a quasi-static version of the method soon became the norm. The schematic in Figure 3.7 illustrates the basic principle of the quasi-static Berlincourt method.
Figure 3.7: Schematic of the Berlincourt Method for measuring the direct piezoelectric effect in materials quasi-statically

An oscillatory force, typically between 10 Hz and 1 kHz, induces a charge across the sample that is detected and amplified. The piezoelectric coefficient, \( d \), is calculated by employing the second constitutive equation from Appendix D (Equation D.20) with subscripts removed for simplicity.

\[
D = d\sigma + \kappa E
\]

The measurement is performed under zero electric field and so the piezoelectric coefficient is given by

\[
d = \left[ \frac{\delta D}{\delta \sigma} \right]_E = \frac{Q}{A} \frac{F}{A} = \frac{Q}{F}
\]

In general, the area of the electrode is the same as the area of the applied pressure. This results in a simple equation that forms the basis of the Berlincourt method.

The simplicity of the Berlincourt method is its main advantage. Taking measurements is quick and requires little sample preparation. Unlike the resonance method, only one type of sample geometry is required (150). However, the direct method has its limitations. The geometry of the sample, the magnitude of the preload force, the magnitude and the frequency of the oscillating force, the geometry of the electrodes as well as the temperature and humidity of the environment, can all affect the accuracy and consistency of the measurement (160, 161). The effects of non-uniform loading,
spurious contributions from the substrate and the choice of clamping arrangement can also influence the measurement. Thin-film samples are particularly challenging and often require modification of the method. The Berlincourt method relies on the comparison between the test sample and a reference material. Therefore, the measurement is inherently dependent on the accuracy by which the piezoelectric coefficient of the reference material was established (161).

Modifying the classical Berlincourt method overcomes many of these challenges. In cases where non-uniform loading or point loading is a concern, a pneumatic loading method is useful (162, 163). In cases where the substrate heavily influences the measurement (often true for thin films), the contribution of the substrate to the measured piezoelectric co-efficient can be altered by varying the geometry of the support substrate. In this way, by using several different substrate geometries, the contribution of substrate effects to the piezoelectric coefficient of the sample can be determined mathematically (149). Alternatively, finite element analysis can compensate for substrate effects by determine a calibration factor that relates the charge-force ratio to the true $d_{33}$-coefficient of the sample (164).

In this work, a commercial piezometer based on the Berlincourt method was available for the piezoelectric study. While many factors may affect the overall measurement, careful consideration of these limitations as outlined in the IEEE standard (165) and the National Physics Laboratory guide (166) allows for accurate and reliable measurements.

3.3.3 Piezoresponse Force Microscopy

Invented by Bennig and Rohrer in 1981, Scanning Tunnelling Microscopy (STM) measures the quantum-tunnelling current between an atomically sharp tip and a conductive surface to create images of the sample surface with atomic resolution. This advancement earned its creators a Nobel Prize in 1986. Scanning Probe microscopy (SPM) is an adaptation of STM that measures the interaction forces between the sample and a sharp probe (167). Today, SPM is an umbrella term capturing a range of techniques that measure forces (van der Waals, electrostatic, magnetic) and electrical properties at the nanoscale. In this project, the most notable of these techniques are
Atomic Force Microscopy (AFM) and its derivative, Piezoresponse Force Microscopy (PFM).

AFM generates topographical images with atomic-level resolution by rastering an ultra-sharp tip across the surface of a sample. Because of its high resolution, AFM has extensive applications in the semiconductor industry, as well as in material, polymer and biological sciences. Figure 3.8 illustrates the principle of operation of a typical AFM. The sharp tip, attached to a piezoelectric actuator, scans the sample surface. The tip deflects as it brought in to contact with the sample. Since the cantilever behaves as a spring with its own spring constant $k$, Hooke’s Law applies ($F = -k\Delta z$). A feedback system keeps the force ($F$) between the tip and the sample constant throughout. A photodiode monitors the deflection ($\Delta z$) of the cantilever by capturing the light reflected off the tip apex during scanning. The vertical deflection is proportional to the photodiode signal $(A + B) - (C + D)$ and lateral deflection is proportional to the photodiode signal $(B + D) - (A + C)$. The vertical deflection represents the topography of the sample and the lateral deflection represents the frictional forces involved.

![Figure 3.8: Schematic of the main components of an atomic force microscope. A laser focused on the apex of the cantilever tip is reflected on to a photodiode to track the deflection of the cantilever as it scans the sample surface. (168)](image-url)
There are three main modes of AFM scanning, contact, semi-contact (tapping) and hybrid mode. In contact mode, the tip is kept in constant contact with the sample and the deflection of the cantilever is monitored as it scans, generating an image of the surface topography. Contact mode AFM is fast and offers high resolution. However, continuous scanning in contact mode may damage the sample or degrade the tip. Scanning in semi-contact mode is gentler; by vibrating the tip at its resonant frequency (typically 10 - 200 kHz), it makes contact with the sample intermittently. Recently introduced, hybrid mode (also known as jumping mode) also maintains intermittent contact between the tip and the surface. However, it differs from semi-contact mode in that the tip vibrates at a frequency much lower than its resonance frequency. Each time the tip makes contact with the surface, it plots the force-distance curve at that point, providing information about the materials local mechanical properties, including adhesion, stiffness and Young’s modulus. As the mechanical properties are collected at each point along the scan, hybrid mode provides a means of mechanical mapping.

**Modification for PFM**

PFM is an extension of AFM that allows studies of electro-mechanical coupling at the nanoscale. It uses a conductive scanning probe. Typical commercial probes for PFM have platinum, gold or titanium nitride coatings, which unfortunately increase the tip’s radius of curvature and thereby reduce the resolution. PFM applies a voltage \( V_{\text{tip}} \) containing an AC and DC component between the sample and the conductive tip:

\[
V_{\text{tip}} = V_{dc} + V_{ac} \cos(\omega t)
\]  

The AC voltage, applied at a frequency \( \omega \), induces a deformation in piezoelectric materials. The deformation is ‘felt’ by the tip and detected by the photodiode, Figure 3.9.
Figure 3.9: Schematic of PFM operation. A sample deforms in response to an applied voltage, which causes the cantilever to deflect. (a) When the polarisation is parallel to the applied voltage, the sample will generate an out-of-plane piezoresponse, measured by the tip-photodiode system as a vertical deflection. (b) When the polarisation is perpendicular to the applied voltage, the sample will generate an in-plane piezoresponse that is measured as a lateral deflection by the tip-photodiode system.

The amplitude of the piezoresponse (deformation resulting from an applied bias) may be very small. For a sample with a sizeable piezoelectric co-efficient of 100 pC N$^{-1}$ (equivalent to 100 pm V$^{-1}$), applying 1 V generates a deformation of just 100 pm. For many samples, especially biological ones, the piezoelectric co-efficient is smaller still (typically less than 10 pC N$^{-1}$). While increasing the applied voltage stimulates a greater deformation response, this strategy cannot be employed indefinitely.

To overcome this challenge, piezoresponse force microscopes use a lock-in amplifier to amplify the piezosignal. A lock-in amplifier multiplies the piezosignal [$V(t) = V_0 \sin(\omega t + \phi)$] with a reference signal [$V_r(t) = \sin(\Omega t)$]. The product of the piezosignal and the reference signals is

$$V(t)V_r(t) = \frac{V_0}{2}[\cos((\omega - \Omega)t + \phi) - \cos((\omega + \Omega)t + \phi)]$$

When the frequency of the piezosignal and the frequency of the reference signal are unequal ($\omega \neq \Omega$), the product oscillates in time with an average value of zero. When the frequency of the input signal and frequency of the reference signal are equal ($\omega = \Omega$), then the product becomes
\[ V(t)V_r(t) = \frac{V_o}{2} [\cos(\theta) - \cos(2\omega t + \vartheta)] \]

The product contains a DC component and an AC component. By adjusting \( \vartheta \) to zero, and extracting the DC component using a low-pass filter, the product \( V(t)V_r(t) \) becomes directly proportional to \( V_o \). Two signals are outputted, \( X \) and \( Y \); the former is the demodulated signal and the latter is the signal demodulated by a second lock-in amplifier with a 90 degree phase shift.

\[ X = \frac{V_o}{2} \cos(\theta) \]

\[ Y = \frac{V_o}{2} \cos(\theta + \frac{\pi}{2}) = \frac{V_o}{2} \sin(\theta) \]

The magnitude and phase of the piezosignal are then given by:

\[ \text{Magnitude} = \sqrt{X^2 + Y^2} \]

\[ \text{Phase} = \tan^{-1}\frac{Y}{X} \]

The PFM magnitude signal reports on the magnitude of the deformation induced by the applied bias. The phase difference between the applied AC voltage and the resultant piezoelectric deformation indicates the direction of polarity of the sample.

**Quantitative PFM**

AFM is primarily a qualitative tool but quantitative analysis is possible. In the case of PFM, there are various methods of calibrating the vertical and lateral piezo-signals so that the piezoelectric co-efficient of the sample can be determined. Starting with the more straightforward case, the vertical piezo-signal is often calibrated by comparing it to an \( \alpha \)-quartz reference sample or by determining the so-called inverse-optical sensitivity (IOS) co-efficient (169).

The IOS co-efficient is essentially a conversion factor, relating the unit of deformation recorded by the photodiode (volts), to the unit needed for quantification (meters). The IOS depends on the alignment of the laser on the cantilever tip. It is determined from a force-distance curve performed on a hard substrate. Figure 3.10 shows a force-distance curve displaying both the approach and the retract traces as the tip is brought in to contact with the sample. Typically, the PFM system does not measure force, rather...
the magnitude of deflection, which is proportional to the force being applied. Therefore, the IOS coefficient can be calculated as the slope of the magnitude versus distance curve (Δmagnitude/Δdistance).

![Figure 3.10 Schematic of a typical force-distance curve for an AFM tip as it approaches and retracts from the sample surface. Initially the tip is far from the sample surface and does not exert any force on the sample (A). As the tip moves closer to the sample, attractive van der Waals forces pull the tip towards the sample (B). Once in contact with the surface, the tip exerts a force on the sample surface that causes the cantilever to deflect (C). On the retract cycle, the force exerted on the sample by the tip decreases (D). At the surface-air interface, the tip experiences adhesive forces (E) until finally the tip is fully withdrawn from the surface (F) (170).]

With the IOS conversion factor established the magnitude of the piezoelectric deformation (Equation 3.12) and hence the piezoelectric $d_{33}$ coefficient (Equation 3.13) can be calculated.

\[
\text{Displacement (nm)} = \frac{\text{Magnitude (nA)}}{\text{Gain} \times \text{Input} \times \text{IOS} \left(\frac{\text{nA}}{\text{nm}}\right)} \tag{3.12}
\]

\[
d_{33} \left(\frac{m}{V}\right) = \frac{\text{Displacement (m)}}{\text{Voltage applied (V)}} \tag{3.13}
\]
However, there are drawbacks to this approach. The frequency used during PFM imaging is much higher than the frequency at which the forces curves are performed, affecting the accuracy of calibration (171).

Calibrating the lateral piezoresponse signal is more challenging. In Lateral Force Microscopy (measures frictional forces between the tip and the sample), many approaches have been proposed to calibrate the in-plane motion of the tip. One approach involves attaching different masses to the cantilever tip and measuring the resonance frequency to determine the spring constant of the tip (172, 173). In another approach called the wedge-method, the lateral force is varied by using sample substrates with different slopes (called wedges) (174, 175). The complexity of each approach means that they are not transferable to lateral PFM quantification.

As such, new methods were required that are specific to PFM. One approach is to use a piezoresistive force sensor to calibrate the probes (176). Choi et al. (177) base their approach on the fact that increasing the scan speed increases the lateral force acting on the cantilever. Thus, a lateral inverse sensitivity co-efficient can be determined. Although, the $d_{31}$ coefficient of a BaTiO$_3$ single crystal determined by this approach was within the agreed literature values, the error associated with it was large ($d_{31} = 81.62 \pm 40.22 \text{ pm V}^{-1}$) (177). A third approach relies on a simple geometrical relationship between the length of the cantilever, $L$, the height of the tip, $h$, and the ratio of the vertical sensitivity to the lateral sensitivity, $R$.

$$R = \frac{4L}{3h}$$  \hspace{1cm} (3.14)

The sensitivity of the lateral response is generally greater than that of the vertical response. Calibrating the vertical sensitivity as described above, allows the lateral resolution to be estimated (178).

The simplest approach for lateral PFM quantification uses the fact that the in-plane piezo-signal for a y-cut lithium niobate crystal should be proportional to its $d_{15}$ co-efficient when the crystal is oriented in the correct way. By comparing the measured value with the standards true value, a calibration factor can be established.

**Contact Resonance**

Even with good amplifiers and noise reduction techniques, it can be difficult to detect
the small deformations from weakly piezoelectric materials. One way to enhance or amplify the piezo-signal is by performing PFM 'on-resonance'. The so-called contact-frequency is the frequency at which the entire tip-sample system resonates. Performing PFM at this frequency amplifies the voltage-induced deformation, magnifying the piezoresponse. This is demonstrated with an example in Appendix E. This approach to enhancing the piezoresponse signal is not always applicable. The frequency of contact-resonance may vary considerable from point to point within the scan area if the sample itself is not homogenous. In addition, as the enhancement at resonance cannot be easily quantified, quantitative PFM measurements are not possible in this regime (169).

Summary of PFM

Overall, PFM is an advantageous tool for probing electromechanical coupling at the nanoscale. Since its initial development, it has been employed extensively to measure these properties in solid samples such as doped PZT, bismuth ferrite films and crystals of triglycine sulphate (179). In recent years, PFM has been employed to investigate piezoelectric behaviour of biological samples. For example, PFM has extended our knowledge of piezoelectricity in single collagen fibrils (41), the M13 bacteriophage virus (30), and the fibrous protein chitin (180).

However, PFM is not without its limitations. Electrostriction and electrostatic interactions between the tip and the electric field are parasitic effects that contribute to tip motion (69). When PFM is used to investigate biological-piezoelectricity further precautions must be considered. Many biological materials require a liquid environment to sustain them. Recalling from Chapter 2, the contrast of PFM images is reduced when performed in liquid, especially if that liquid contains salt (142, 143). As mentioned above, the piezoresponse signal of a sample can be enhanced by performing the scan at the contact resonance frequency. However, for biological samples whose surface is often not homogenous, the contact resonance frequency is different at each point on the sample surface. Thus, enhancing the signal in this manner may not be feasible. Typically, the piezoresponse from biological materials is low (<10 pm V⁻¹). Quantitative measurements of weakly piezoelectric biological materials via PFM may be challenging.
3.4 Measurement of Ferroelectricity via Switching Spectroscopy-PFM

In addition to imaging piezoelectric domains, a sub-technique of PFM, called Switching Spectroscopy PFM (SS-PFM), can be used to study the switching characteristics of ferroelectric materials. In SS-PFM, the electric field applied between the tip and the bottom electrode contains a direct ($V_{DC}$) and alternating voltage ($V_{AC}$). As illustrated by Figure 3.11, $V_{DC}$ is applied in a series of pulses; the magnitude of each square pulse increases incrementally from $-V_{DC}$ to $+V_{DC}$. The alternating voltage is superimposed on to the DC voltage signal. $V_{AC}$ remains on throughout the measurement, while $V_{DC}$ steps back to 0 V between each pulse. The piezoresponse is measured. The piezoresponse induced by $V_{AC}$ is measured each time $V_{DC}$ goes to 0 V. Measuring the piezoresponse when $V_{DC}$ is off minimises contributions from electrostatic interactions.

SS-PFM is performed at a point on the sample surface, allowing us to study locally generated hysteresis loops. If a sample is ferroelectric and the applied voltage exceeds the coercive field of that sample, the voltage pulses cause the spontaneous polarization of the domain underneath the tip to switch. The amplitude and phase of the piezoresponse reflect the domain switching. Figure 3.12 shows typical butterfly-shaped amplitude (a) and switching phase (b) loops that are characteristic of ferroelectric materials. From these loops, the remnant polarization and coercive field of the materials can be determined. SS-PFM allows us to study the switching and ferroelectric properties of materials with high spatial resolution, thus it is well suited
to the study of ferroelectric domain wall structures.

Figure 3.12: Characteristic butterfly-shaped amplitude (black) and switching phase (red) loops for a true ferroelectric material, PZT. (182)

Unfortunately, measurements on non-ferroelectric materials, at the macro- and nanoscale, can yield false loops. The consequence of this has been a number of misinterpretation and false reports of ferroelectricity in non-ferroelectric materials. To highlight the difficulty in distinguishing between true and false ferroelectric hysteresis loops, Scott compares the closed loops obtained from a true ferroelectric material, Ba₂NaNb₅O₁₅, and a non-ferroelectric banana skin (74). Artefacts and misinterpretation of loops are often reported for leaky conductor samples (75). For these types of samples, a Schottky-contact best describes the contact between the sample and the electrode. Pintilie et al. have shown that a non-ferroelectric material with two back-to-back Schottky contacts yields misleading hysteresis loops that resemble true ferroelectric loops (76). Nanoscale measurements via SS-PFM are subject to artefacts caused by charge injection and electrostatic forces, which can induce ferroelectric-like loops.
3.5 Measurement of Pyroelectricity

Typical methods of measuring pyroelectricity involve heating and/or cooling the sample while measure the current generated. At this point, it is important to consider that the total pyroelectric effect is made up of the combined contributions of the primary pyroelectric effect and the secondary pyroelectric effect. The primary pyroelectric effect occurs when the sample is rigidly clamped, i.e. it is under constant strain conditions. A change in temperature induces a change in electric displacement that is measured as a flow of charge (current). The primary effect is illustrated in Figure 3.13 as the solid bold line connecting temperature and electrical displacement.

![Figure 3.13 Schematic illustrating the primary and secondary pyroelectric effect. A pyroelectric crystal at constant strain exhibits primary pyroelectricity (bold line). A pyroelectric crystal that is free to deform may exhibit the secondary pyroelectric effect (dashed line). Adapted from (6)](image)
All materials will exhibit thermal expansion effects. The secondary pyroelectric effect arises because all pyroelectric materials are inherently piezoelectric too. Secondary pyroelectricity involves two steps. Firstly, an unclamped pyroelectric material is free to expand when subjected to a change in temperature and causes strain within the material. This first step is indicated by the dashed line connecting temperature and strain in Figure 3.13. In the second step, the strain induces the converse piezoelectric effect within the material, indicated by the second dashed line connecting strain and displacement. These two combined steps define the secondary pyroelectric effect. Secondary pyroelectricity is not possible in materials that are piezoelectric but non-polar such as \( \alpha \)-quartz. While a thermal expansion may produce strains in these materials, they do not result in piezoelectricity (and hence pyroelectricity) because the net electrical displacement is zero.

Both primary and secondary pyroelectric effects are considered real pyroelectric effects in which a linear change in temperature produces a linear change in current. However, non-uniform heating can result in a different effect called tertiary pyroelectricity, which can be observed in non-pyroelectric materials. If a material is heated non-linearly, it induces a strain gradient. The piezoelectric charges that develop are not compensated for and result in a net charge. Tertiary pyroelectricity is false pyroelectric and in this study will be minimised by experimental design.

3.5.1 Pyroelectric Measurement Methods
Pyroelectric measurement methods can be broken down in to three main categories: static, indirect and dynamic. Static pyroelectric measurements involve heating the sample incrementally and measuring the charge displacement at discrete temperatures. Static methods are unreliable and time consuming. Indirect pyroelectric measurements involve measuring the pyroelectric coefficient of a sample indirectly through measurements of polarisation. Thus, indirect methods are limited to materials whose polarisation can be switched, i.e. ferroelectric materials. Dynamic pyroelectric measurements are most widely used; it involves continuous heating or cooling of the sample while recording the pyroelectric current generated. Several variations of the dynamic method exist. Three of the most frequently used dynamic methods are the Byer-Roundy method, the Laser Intensity Modulation Method and the Temperature Oscillation Method. Here we will briefly review each of
these dynamic methods, discussing their advantages and disadvantages. This information will be used to select and justify the method to investigate pyroelectricity in lysozyme later in this study.

In the **Byer-Roundy Method** (183), the electroded sample is heated or cooled at a constant rate, while measuring the current generated with an electrometer. Using Equation 2.7, the pyroelectric coefficient for the material can be determined. In Figure 3.14, a sample of PVDF is subjected to heating and cooling ramps, producing a square pyroelectric current signal. The pyroelectric current switches polarity when the temperature cycle changes from heating to cooling.

![Figure 3.14: Measurement of pyroelectricity of PVDF via quasi-static Byer-Roundy Method showing the ramping temperature cycle applied to the sample (upper) and the pyroelectric current generated (lower).](184)

The method is simple but precautions must be taken to ensure its accuracy. The most important factor is that the rate of change of temperature with time is linear. As discussed above, non-uniform heating-rates induce tertiary effects that are difficult to distinguish using this method. For some samples, achieving uniform heating and
cooling may be challenging. To ensure uniform heating, the rate of sample heating must be significantly smaller than the thermal diffusion time of the sample. Another difficulty is that trapped charges may be released as the sample is heated and contribute to the measured current. This is particularly troublesome for samples that have been poled. Subjecting the sample to repeated temperature-ramping cycles releases these trapped charges so that only the true pyroelectric current remains. Another challenge is accurately measuring high temperature rates (73). A precaution to address this is to place the thermocouple as close to the sample as possible so that the temperature of the sample itself is recorded.

In the **Temperature Oscillation Method**, the sample is subjected to an oscillating heat supply and the pyroelectric current generated measured with a lock-in amplifier. If the frequency of the oscillating heat supply is low, the current generated will be 90° out of phase with the thermal signal as shown in Figure 3.15. Thus, irreversible thermally stimulated currents can be distinguished from true pyroelectric currents.

![Figure 3.15](image)

Figure 3.15 Pyroelectric current (purple) generated in response to a sinusoidal heat supply using the Temperature Oscillation Method. Adapted from (185)

For this method to be successful, the frequency of the oscillation must be low enough to ensure that the sample is heated uniformly.

In the **Laser Intensity Modulation Method**, the sample is heated by a pulsed laser radiation source and the current generated is measured with an electrometer. The method was originally proposed by Chynoweth (186) and is often referred to as the Chynoweth method. As an example, the pyroelectric current generated in a sample of hydroxyapatite when subjected to a pulse radiation source is shown in Figure 3.16.
When the laser is on, the hydroxyapatite sample produced a negative pyroelectric current that relaxed towards zero. When the laser is off, the hydroxyapatite sample produced a positive pyroelectric current that again relaxed towards zero.

Figure 3.16 Pyroelectric current measured in thin films of hydroxyapatite via the Laser Intensity Modulation Method. (39)

This method is advantageous in that the measurement resembles operating conditions for samples used in technical applications. Very high heating and cooling rates can be achieved generating large pyroelectric currents over a small temperature range (73). Also, reversible and irreversible pyroelectric effects can be distinguished. This method also allows the frequency dependence of the sample to be investigated (187). As the overall temperature of the sample does not vary substantially throughout the measurement, the effect of thermally release trapped charges is greatly minimised (73). One challenge of using this technique is ensuring that the whole sample is heated uniformly. Non-uniform heating of the sample produces temperature gradients within the sample that produce irreversible pyroelectric effects (187). The other main limitation of this technique is in determining the temperature variation caused by the laser radiation. This is dependent on the heat capacity of the sample and emissivity of the front electrode (185).

3.5.2 Selection of a Pyroelectric Measurement Method to Study Lysozyme

In the literature, studies that accurately compare the pyroelectric effect using the different methods are scarce. In selecting a suitable pyroelectric measurement method to study crystals of lysozyme, we considered the advantages and limitations of each method as described above as well as the availability of equipment. The Byer-Roundy
method was selected for this study. It is a simple method to implement and when proper precautions are considered produces accurate pyroelectric measurements. The temperature range over which the sample of lysozyme may be heated is limited by the fact that proteins denature at elevated temperatures. The denaturation temperature for lysozyme (prepared with a pH of 4.5) is 78.8 °C. Thus, pyroelectric measurements were conducted well below this temperature.

3.6 Conclusions
This chapter reviewed the methods of growing and charactering protein crystals. Conventional methods of measuring piezoelectricity, ferroelectricity and pyroelectricity in materials were reviewed in respect of their applicability to biological materials. The Berlincourt Method and PFM were identified as two conventional techniques that may be appropriate to investigate piezoelectricity in lysozyme. The advantageous and limitation of using SS-PFM for ferroelectric measurements were described. Lastly, the Byer-Roundy method was justified as a pyroelectric measurement technique suitable for the study of lysozyme.
Chapter 4  Growth and Structural Characterisation of Lysozyme Crystals

Electromechanical properties of globular proteins are so far unstudied in the literature. Thus, investigating the most appropriate way of preparing protein samples for electromechanical measurement is a prerequisite step. Firstly, this chapter presents a brief rational explaining the suitability of studying lysozyme in its crystalline form. Then, we devise a method of preparing films of lysozyme crystals and optimise the films for this research work. Suitable means of electroding the protein crystals are also discussed. Finally, the structure of films of lysozyme will be characterised by synchrotron diffraction.

4.1 Suitability of Studying Lysozyme in its Crystalline Form

Lysozyme protein can exist in different forms - as a dry powder, as a reconstituted solution, or as crystals. The literature shows that powder samples of amino acids exhibit piezoelectricity (21). In the early stages of this research, we investigated if powder samples of lysozyme would exhibit piezoelectricity too. Pellets prepared by compressing lysozyme powder did not show any piezoelectric behaviour using a commercial piezometer. It is likely that the random orientation of powder particles results in a cancellation effect. If the piezoelectric effect exists in lysozyme powder, it may be impossible to detect for this reason. In ceramic materials, a poling process can overcome this problem of self-cancellation. Poling involves heating the material above its Curie point and applying a high electric field. The electric field orientates the dipoles in one direction. The electric field remains in place as the sample cools. If poling is successful, the majority of the dipoles will be orientated in the same direction and the piezoelectric effect may be observed. However, as of yet, there is no known method of poling proteins as they denature at relatively low temperatures.

Although piezoelectricity is mostly reported in solid materials, the relatively recent
discovery of piezoelectricity in liquid crystals (110) extends this phenomenon to the liquid phase. It is favourable to study lysozyme under physiological conditions. Therefore, in the initial stages of this research, we attempted to detect piezoelectric resonances in a lysozyme solution but were unsuccessful. Although liquid crystals support piezoelectricity in one dimension, they behave as traditional liquids that are unable to sustain or apply mechanical stress in the other two dimensions (188). In an attempt to restrict the flowability of the liquid, we prepared lysozyme in a lipidic cubic phase (a type of single lipid bilayer). However, we did not see piezoelectricity in lysozyme in this form either. It is worth noting also, that this latter approach was least favourable as the lipids themselves may be piezoelectric (68, 106). Thus, continuing with that approach would make ascertaining the true origin of any piezoelectric effect difficult.

As the classical theory of piezoelectricity has the crystal structure of the material at its crux, the most promising form to detect and examine piezoelectricity in lysozyme is its crystalline form. As discussed in Chapters 1 and 2, all protein crystals have the prerequisite non-centrosymmetric structure of piezoelectric materials. However, working with protein crystals creates additional instrumentational challenges, as we shall discuss below.

4.2 Growth, Handling and Electroding of Lysozyme Crystals

Protein crystals are soft and brittle (189) owing to their high water content. The crystal volume per unit of protein molecular weight (Matthews coefficient, $V_m$) is related to the solvent content of the crystal. Ruppweb’s online tool (190-192) calculates the Matthews co-efficient of a crystal based on the volume of the unit cell, the number of asymmetric units in the cell and the molecular weight. Table 4.1 shows that the solvent content of lysozyme ranges from 17.4% to 47.2% when crystallised in different forms. To maintain their crystalline structure and prevent cracking and degradation, protein crystals must stay hydrated at all times. This requirement presents a unique challenge to this research project. As the traditional solid piezoelectric materials are typically large, mechanically strong and stable within an air environment, experiments are generally straightforward. This ease-of-handling does not readily extend to fragile protein crystals.
Table 4.1: Solvent content of lysozyme when crystallised in different crystal groups

<table>
<thead>
<tr>
<th>Crystal System</th>
<th>PDB file</th>
<th>Molecular Weight (Da)</th>
<th>Unit Cell Volume ($\AA^3$)</th>
<th>$V_m$ ($\AA^3$/Da)</th>
<th>Solvent Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetragonal</td>
<td>1IEE</td>
<td>14531.49</td>
<td>221021.4</td>
<td>1.90</td>
<td>35.31%</td>
</tr>
<tr>
<td>Orthorhombic</td>
<td>1AKI</td>
<td>14331.24</td>
<td>123381.9</td>
<td>2.15</td>
<td>42.85%</td>
</tr>
<tr>
<td>Hexagonal</td>
<td>2FBB</td>
<td>15438.27</td>
<td>431465.0</td>
<td>2.33</td>
<td>47.19%</td>
</tr>
<tr>
<td>Monoclinic</td>
<td>2Z12</td>
<td>14496.04</td>
<td>43154.2</td>
<td>1.49</td>
<td>17.37%</td>
</tr>
<tr>
<td>Triclinic</td>
<td>2F2N</td>
<td>14765.27</td>
<td>25949.4</td>
<td>1.76</td>
<td>30.01%</td>
</tr>
</tbody>
</table>

It is not just in this research project that the fragility of protein crystals is problematic. In diffraction studies, it is necessary to find a way of handling the sample. Operating at cryogenic temperatures (typically below 120 K (193)) greatly reduces the amount of radiation damage during protein diffraction experiments. The addition of a cryoprotectant protects the protein crystal when exposed to such low temperatures, preventing it from cracking. Cryo-protectants commonly used include glycerol, sucrose and ethylene glycol. Another method of strengthening fragile protein crystals is to cross-link crystals with glutaraldehyde (194).

4.2.1 Means of Electroding

Designing electrodes for protein crystals is challenge worth addressing at this stage. Making good Ohmic contact between the electrode and the protein crystal is critical; however, this can be difficult since the protein crystal is small and fragile. The second major difficulty is that protein crystals do not survive well when extracted from solution; once extracted from the mother liquor they degrade quickly. Making electrical connections in liquid, especially those containing salts as is the case here, is
The solution to this challenge was inspired by the food industry where films of lysozyme are used in food packaging. By preparing lysozyme as a film on a conductive substrate, we could easily address the issue of making Ohmic contact with the protein crystals – assuming that crystals would indeed grow in the film. If successful, the film should be able to sustain the water content of the lysozyme crystals and at the same time provide a dry surface for electrode contacts.

In the food industry, glycerol is added to prepare protein films (195). For this work, glycerol is an appropriate additive because

1. is inherently non-piezoelectric  
   (We note that glycerol does not crystallise at room temperature (196))
2. prevents the film of crystals from drying out and cracking
3. allows the film to dry fully
4. does not alter the crystal structure of the protein (197)

For now, it is sufficient to say that electroding was accomplished by growing crystalline aggregate films of lysozyme. We note that throughout this thesis, “crystalline aggregate films” are often simply referred to as “films” of lysozyme. The films were grown on glass slides coated with indium-tin-oxide (ITO). The resistivity of the ITO glass (Delta Technologies) was 50 Ohms. Placing a second piece of ITO glass on top of the dry film created a parallel-plate type electrode arrangement. In other experiments, growing films on inter-digitated electrodes facilitated electroding. The next section describes in more detail the preparation of crystalline aggregate films of lysozyme.

4.2.2 Optimisation of Polycrystalline Films of Tetragonal Lysozyme

Tetragonal lysozyme crystals were prepared as aggregate films by modifying a protocol outlined by Hampton Research. A 100 mg/mL solution of lysozyme was prepared by reconstituting lysozyme powder (Sigma Aldrich, Catalogue number 62971-50G-F, used without further purification) in a 50 mM sodium acetate buffer. A small amount of glycerol was added to the lysozyme solution. Typically, between 50 and 100 µL of the protein solution was drop-cast on to a clean substrate. The films
were left to dry overnight in a temperature-regulated room at 20° C.

Film growth was heavily dependent on three factors: the pH of the buffer, the protein concentration and the amount of glycerol incorporated. These three factors were varied systematically to determine the optimum conditions for crystal film growth. The objective was to grow a film with a high density of good quality crystals. The film itself should dry completely to allow for electrical contacts. Films were assessed by taking optical microscopy images of the films with a Zeiss Microscope (Axio Imager .A1m) in bright-field mode with either 5x or 10x magnification.

The effect of the pH of the sodium acetate buffer was investigated by growing films from 100 mg/ml of lysozyme in a sodium acetate buffer with different pH levels. To prevent the films from cracking 40 uL of 100% glycerol was added to 1 mL of protein solution. Figure 4.1 shows optical microscopy images (magnification 10×) of crystals that grew in films prepared with (a) pH 4.2, (b) pH 4.4, (c) pH 4.6 and (d) pH 4.8 sodium acetate buffer.
Figure 4.1 Effect of buffer pH on the growth of crystalline aggregate films of tetragonal lysozyme. Films were prepared from a 100 mg/ml solution of lysozyme in a sodium acetate buffer at (a) pH 4.2, (b) pH 4.4, (c) pH 4.6 and (d) pH 4.8.

The crystals that grew in films prepared with a pH 4.2 buffer were relatively small (< 50 µm) and sparsely populated, Figure 4.1(a). Increasing the pH of the buffer 4.4 did not make any significant difference to the growth of crystals within the film, Figure 4.1(b). Crystals grown from this buffer were approximately 50 µm in size with a similar population density as those grown from the buffer with pH 4.2. When the pH of the buffer was increased to pH 4.6, the crystals grew much larger (approximately 100-150 µm) with a slightly lower population density, Figure 4.1(c). Finally, increasing the pH of the buffer to pH 4.8 resulted in small crystals (< 25 µm) which were sparsely populated within the film, Figure 4.1 (d). The crystals that grew from the pH 4.6 buffer were most suited to this work and so this pH was used in all the remaining work.
Next, we investigated the effect of protein concentration on the growth of crystalline aggregate films. Four protein concentrations were studied: 10 mg/ml, 20 mg/ml, 50 mg/ml and 100 mg/ml of lysozyme reconstituted in a 50 mM sodium acetate buffer at pH 4.6. Again, glycerol was added to the protein solution. To make the glycerol easier to pipette, it was diluted to 50% in DI water; for this study 1 µL of 50% glycerol was added to each 100 µL solution of lysozyme. Figure 4.2 shows optical microscopy images of tetragonal lysozyme crystals grown in films prepared with different protein concentrations. Note that images in Figure 4.2(a-c) were collected with 5× magnification while the image in Figure 4.2(d) was collected with 10× magnification. This is reflected in the scale bar of each image.

Figure 4.2: Effect of protein concentration on the growth of tetragonal lysozyme films. Films prepared from a 100 µL dropcast solution of (a) 10 mg/ml (b) 20 mg/ml (c) 50 mg/ml and (d) 100 mg/ml lysozyme in 50 mM sodium acetate buffer pH 4.6 with 1 µL of 50% glycerol added

Crystals grew in all four films but the size and quality of crystals in each film differed significantly. The crystals that grew from the protein solution with the lowest concentration (10 mg/ml, Figure 4.2(a)) were approximately 50 µm in size with poorly
formed crystal habits. Increasing the protein concentration to 20 mg/ml resulted in precipitates, Figure 4.2(b). Increasing the protein concentration to 50 mg/ml also produced precipitates surrounded by a few small individual protein crystals, Figure 4.2(c). Increasing the protein concentration to 100 mg/ml resulted in the growth of well-defined tetragonal lysozyme crystals, Figure 4.2(d). The crystals had well-formed habits and defined crystal faces and edges. The average size of the crystals was 50-75 µm. The films had a high density of such crystals. Typically, films with low protein concentration did not dry completely and thus, were unsuitable for electroding later. The films of crystals that grew from the highest protein concentration dried fully and were most suitable for the next stage of measurement.

Finally, we optimized the amount of glycerol added to the protein solution. As before, the protein solution was 100 mg/ml of lysozyme reconstituted in a 50 mM sodium acetate buffer pH 4.6. The amount of 50% glycerol added ranged from 1 µL to 6 µL per 100 µL of protein solution. Figure 4.3 shows microscopy images of films prepared with (a) 1 µL, (b) 2 µL, (c) 4 µL and (d) 6 µL of 50% glycerol. We note that the magnification for Figure 4.3(a) was 10× while for Figure 4.3(b-d) the magnification was 5×.
Figure 4.3: Effect of glycerol content on growth of tetragonal lysozyme films. Films prepared from 100 µL dropcast solution of 100 mg/ml lysozyme in 50 mM sodium acetate buffer pH 4.6 with (a) 1 µL (b) 2 µL, (c) 4 µL and (c) 6 µL of 50% glycerol added

Relatively large, well-defined crystals grew when the amount of glycerol was low (1 µL of 50% glycerol, Figure 4.3 (a)). Increasing the glycerol content to 2 µL of 50% glycerol did not have any significant effect on the growth of crystals within that film, Figure 4.3(b). Increasing the glycerol content to 4 µL of 50% glycerol produced similar sized crystals. However, the density of crystals within the film was much lower, Figure 4.3(c). Increasing the glycerol content to 6 µL of 50% glycerol reduced the density of crystals further. The crystals that grew in these films were also smaller, Figure 4.3(d). Additionally, when the glycerol content was low (1 µL to 4 µL of 50 % glycerol) the films dried fully. However, increasing the glycerol content above this resulted in films that never dried fully. From this study, we can see that incorporating glycerol allows lysozyme crystals to grow within a film. The amount of glycerol can be varied without affecting the crystal growth so long as the glycerol content remains less than 4 µL of 50% glycerol per 100 µL of protein solution. Increasing the glycerol content above
this suppresses the growth of crystals within the film. Thus, films for
electromechanical measurements were grown from protein solutions with low glycerol
content.

We note that the films could be prepared with good repeatability. While the exact
number and size of crystals within films that had been prepared in the same manner
was never identical, careful film preparation resulted in films with similar number and
size of crystals. However, variations in room temperature did affect the crystal growth
within the film. For this reason, lysozyme films were prepared in a 20 °C protein
crystallisation room to improve repeatability.

4.2.3 Optimisation of Polycrystalline Films of Monoclinic Lysozyme
Monoclinic lysozyme crystals were prepared in a similar manner to the tetragonal
lysozyme crystals, with the following exceptions. The lysozyme powder was
reconstituted in a 50 mM sodium acetate buffer (pH 4.6). The concentration of the
protein solution ranged from 5 mg/mL to 50 mg/mL. The lysozyme solution was then
combined with a 4% sodium nitrate buffer in the ratio 1:1. Again, adding glycerol
prevented the monoclinic crystals from degrading during drying. Drop-casted films
were left to dry at 20° C overnight, during which time a film containing monoclinic
lysozyme formed.

Figure 4.4 shows optical microscopy images of monoclinic crystals of lysozyme
prepared in the manner described above with varying amounts of glycerol added.
Figure 4.4: Effect of glycerol content on the growth of monoclinic lysozyme crystals within a film. Microscopy images with 10× magnification show crystals grown in a film with an initial protein concentration of 50 mg/ml and (a) 0.5 µL, (b) 1.0 µL, (c) 2.0 µL and (d) 3.0 µL per 100 µL of dropcast protein solution.

The microscopy image in Figure 4.4 shows a film of monoclinic crystals grown using an initial protein concentration of 50 mg/ml (diluted to 25 mg/ml during the addition of sodium nitrate in 1:1 ratio). When the glycerol content is low (0.5 µL, Figure 4.4(a)) the protein crystallises as spherulites. Increasing the glycerol content to 1.0 µL (Figure 4.4(b)) or 2.0 µL (Figure 4.4(c)), reduced the amount of crystals that collect together as spherulites but many still clump together sticking together forming agglomerates. Increasing the glycerol content further to 3.0 µL, Figure 4.4(d), produces crystals with better-defined faces, however the crystals still have a tendency to grow out of one another.

Reducing the initial protein concentration improves the size and quality of the crystals. The initial protein concentration used to prepare crystalline aggregate films in Figure 85.
4.5(a) and (b) was 50 mg/mL and 5 mg/mL, respectively. The crystals that grew from the 50 mg/mL solution were large (> 100 µm) but poorly faceted and stuck to one another. Crystals grown from the solution whose initial protein concentration was 5 mg/mL produced large, well-defined crystals. The size of these crystals ranged from 150 µm to >800 µm. In all films, the orientation of the crystals is random.

![Image](image_url)

Figure 4.5: Effect of initial protein concentration on the growth of monoclinic lysozyme crystals within a film. Microscopy images show crystals grown in a film with an initial protein concentration of (a) 20 mg/mL and (b) 5.0 mg/mL.

Although the crystals produced in films prepared from solutions with a low initial protein concentration and high glycerol produced the best crystals, these films were not always the most appropriate for further measurement. When the protein concentration was low or the glycerol content high, the films did not dry fully. Electroding these types of films is challenging and shorting between electrodes is a problem. Thus, a compromise between crystal quality and film quality is necessary.

### 4.3 Characterisation of Lysozyme Crystals

#### 4.3.1 Visual Characterisation using Optical Microscopy

Figure 4.6(a) shows the symmetry requirements of tetragonal crystals. The basic unit cell is an elongated cube; two sides of the cube are equal in length and the angles between sides are equal. Convention defines that the the c-axis is along the elongated
side as shown in Figure 4.6(d). Lysozyme is so extensively studied that we can predict which crystal system will develop based on the recipe used. Tetragonal crystals typically grow from solutions containing sodium acetate and monoclinic crystals grow from solutions containing a percentage of sodium nitrate. However, optical microscopy images alone cannot tell us everything about the symmetry of the lysozyme crystals. The crystal may be a tetragonal dipyramid, having one 4-fold axis (Figure 4.6(b)) and belong to point group 4. Alternatively, the crystal may be a tetragonal trapezohedron, having one 4-fold and two 2-fold axes, belonging to point group 422. Therefore, the space group and hence, the point group must be determined in another way, i.e. by diffraction.

\[ a = b \neq c \]
\[ \alpha = \beta = \gamma = 90^\circ \]

Figure 4.6: Tetragonal crystals: Schematic of a (a) tetragonal unit cell, (b) tetragonal dipyramid (point group 4) and (c) tetragonal trapezohedron (point group 422) (198). (d) Optical image of an individual tetragonal crystal of lysozyme with the c-axis denoted.

Determining the point group symmetry of monoclinic crystals of lysozyme is more straightforward. Figure 4.7(a) depicts the unit cell of all monoclinic crystals; the three sides of the cell are unequal in length, and two of the angles are equal to 90° but unequal to the third angle.
There are three point groups in the monoclinic system – point group 2, point group m and point group 2/m. The ‘m’ symbol denotes a mirror plane. We recall from Chapter 2, that mirror planes are not symmetry elements of protein crystals. Therefore, lysozyme can only adopt the symmetry of point group 2 when crystallised in its monoclinic form. In this form, lysozyme has one 2-fold axis, Figure 4.6(b). Convention defines that the b-axis is the unique axis (i.e. the 2-fold axis) direction and that the angle that is not restricted to 90° is the angle β.

4.3.2 Investigation of Crystal Structure via Synchrotron Diffraction

To understand if protein crystals conform to the classical rules that define piezoelectricity, knowing with some certainty which point group the crystals belong to is a prerequisite. According to the RCSB PDB (129), the most likely point groups for lysozyme crystals are 422 (tetragonal), 2 (monoclinic), 222 (orthorhombic) and 1 (triclinic). However, the studies accumulated in the PDB are of single protein crystals. The majority of the work in this thesis is on crystalline aggregate films of lysozyme prepared in the manner described earlier. The fact that the substrate may constrain the protein during growth may alter the structure of the crystal. It would be beneficial to determine the crystal structure of these crystalline protein films first before investigating their potential piezoelectric behaviour.

Samples of tetragonal lysozyme were sent to the synchrotron diffraction facility at
Diamond Light Source, Oxfordshire, UK for structural analysis. Dr Mohamed Noor collected the data whose contribution is graciously acknowledged. Films were prepared by drop-casting onto small pieces of coverslips made from borosilicate glass. We note that changing the substrate from ITO-coated glass to borosilicate glass is not ideal as the substrate can have a significant effect on the growth of protein crystals. For example, defects on the substrate surface can reduce the free energy barrier for nucleation and thereby increase the rate of crystal nucleation (199) while other substrates (e.g. mica) can induce epitaxial crystal growth (200). However, as ITO-coated glass would shatter if exposed to the cryo-stream, borosilicate was selected as an alternative.

Figure 4.8(a) shows a photograph of the film mounted on a standard SPINE pin. A magnet holds the SPINE pin in place in the goniometer during measurements. Figure 4.8(b) shows a microscopy image of the tetragonal film collected with 10× magnification.

![Figure 4.8 Film of lysozyme crystal prepared for synchrotron diffraction analysis. (a) Photograph of a protein film mounted on a standard SPINE pin. (b) Microscopy image at 10× magnification of the tetragonal film](image)

The diffraction experiments were performed with an X-ray wavelength of 0.968 Å. The diffraction pattern was collected with a Pilatus3 6M detector located 252.7 mm from the crystal. Initial experiments were performed with a cryo-stream. Despite the
fact that the films contained glycerol, the initial data sets showed ice formation that adversely affects the diffraction pattern. Therefore, the remaining experiments were conducted at room temperature. The crystals of lysozyme were sufficiently robust to withstand the radiation damage for the duration of the experiment. The crystal was rotated through an angle of 180° and the oscillation increment was 0.1°.

Figure 4.9 shows the diffraction pattern obtained for the film of lysozyme, produced with CPP4 (201) and MOSFLM (202). The diffraction pattern is visible to a resolution of 1.9 Å and beyond.

![Figure 4.9 Synchrotron diffraction pattern of lysozyme film showing visible diffraction spots extending to 1.9 Å and beyond](image)

The data from the diffraction pattern was processed with xia2 (203), using XDS to perform auto-indexing, cell-refinement and integration. During auto-indexing, the
lattice parameters and orientation is determined from the position of the diffraction spots. From this information alone, it is not possible to determine the space group of the crystal. In the next stage of data processing, the cell parameters are refined to provide accurate cell parameters. Then, the intensity of the diffraction spots are found by integration. After integration, a program called POINTLESS (204) was used to determine the space group. The result is shown in Table 4.2.

Table 4.2 Diffraction data statistics for a film of lysozyme crystals collected at Diamond Light Source UK

<table>
<thead>
<tr>
<th>Parameters</th>
<th>X-ray data collection statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ray detector</td>
<td>Pilatus3 6M</td>
</tr>
<tr>
<td>Wavelength</td>
<td>0.968620 Å</td>
</tr>
<tr>
<td>Space group</td>
<td>P422</td>
</tr>
<tr>
<td>Unit cell parameters</td>
<td>a = b = 82.95 Å; c = 39.59 Å</td>
</tr>
<tr>
<td></td>
<td>α = β = γ = 90°</td>
</tr>
<tr>
<td>Matthews coefficient</td>
<td>2.35 Å³ Da⁻¹</td>
</tr>
<tr>
<td>Solvent content</td>
<td>47.6 %</td>
</tr>
<tr>
<td>Molecules in asymmetric unit</td>
<td>1</td>
</tr>
<tr>
<td>Resolution range</td>
<td>34.25 - 1.14 Å</td>
</tr>
</tbody>
</table>

The film of lysozyme fits to a primitive tetragonal system. The unit cell parameters are $a = b = 82.95$ Å, $c = 39.59$ Å and $α = β = γ = 90°$. The resolution range was 34.25 Å to 1.14 Å. The lattice can accommodate 1 molecule per asymmetric unit. The Matthews co-efficient is $2.35$ Å³ Da⁻¹ with a corresponding solvent content of 47.6%. The tetragonal film was assigned to space group P422, which belongs to point group 422.
4.4 Conclusions

In this chapter, we determined a method of growing crystals of lysozyme within a film so that electrical measurements could be later performed. Two types of films were realised; one was a film of tetragonal lysozyme crystals and the second was a film of monoclinic lysozyme crystals. The films were grown on ITO-coated substrates, which will act as electrodes in the forthcoming experiments in Chapters 5 and 6. The crystals that grew were characterised visually by optical microscopy and structurally by synchrotron diffraction. Crystals of monoclinic lysozyme can only belong to point group 2. Crystals of tetragonal lysozyme were assigned to point group 422 by synchrotron diffraction experiments.
Chapter 5  Measuring the Direct Piezoelectric Effect in Lysozyme

Recalling from Chapter 3 that the Berlincourt Method may be an appropriate method of measuring piezoelectricity in protein films, this chapter will present measurements of the direct piezoelectric effect in crystalline films of lysozyme using this method. Firstly, we briefly describe the commercial piezometer used and its calibration. Next, we present and discuss the results obtained for lysozyme using the commercial piezometer. We then designed, developed and calibrated a custom-built rig based on the Berlincourt Method to verify these results. Finally, this chapter discusses the observations in relation to the classical theory of piezoelectricity and poses questions to direct the research described in the next chapter.

5.1  Direct Piezoelectricity Measurements of Lysozyme using a Commercial Piezometer

5.1.1  Description and Calibration of Commercial Piezometer

Piezometers are commercially available instruments based on the Berlincourt method. In this work, a piezometer (Model PM300, Piezotest, UK) quantitatively determined the piezoelectric effect of films of lysozyme protein. Figure 5.1(a) and (b) show a schematic representation and photograph of the piezometer, respectively. Two clamps hold the sample in place with a preload force of 10 N. The clamps also serve as electrodes to measure the charge generated from a piezoelectric sample. The piezometer applies an oscillatory force that can be varied from 0.05 N to 0.5 N. The frequency of oscillatory force can be varied from 30 Hz to 300 Hz. The accuracy of the piezometer is 0.01 pC N⁻¹.
The piezometer measures the charge that accumulates on the electrodes by measuring the voltage across a large capacitor (typically 0.1 \( \mu \)F) placed in parallel with the sample. The piezometer compensates for any variations in \( d_{33} \) due to changes of temperature by comparing with its internal reference sample - a well-aged sample of PZT (205).

The calibration procedure for the piezometer is straightforward. The manufacturer recommends checking the calibration routinely with standard positive and negative control samples. The manufacturer provides two standard control samples: Standard A has a piezoelectric coefficient of 360 pC N\(^{-1}\) (error 2\%) and Standard B has a piezoelectric coefficient of 389 pC N\(^{-1}\) (error 2\%). The piezoelectric coefficients of these standards were determined by the manufacturer using the resonance method. A third piezoelectric sample (APC850, APC International, USA) was also available for calibration which has a piezoelectric coefficient of 400 pC N\(^{-1}\) (error 20\%). Glass, or another non-piezoelectric material, is a suitable negative control sample. Table 5.1 shows the result of a routine calibration procedure. Each sample was measured three times in the upright configuration and three times in the inverted configuration. The average \( d_{33} \) coefficient of each sample is reported.
Table 5.1: Result of routine piezometer calibration measurements

<table>
<thead>
<tr>
<th>Sample</th>
<th>$d_{33}$ (pC N$^{-1}$)</th>
<th>$d_{33}$ (pC N$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Upright configuration</td>
<td>Inverted configuration</td>
</tr>
<tr>
<td>Standard A (PZT, Piezotest, UK)</td>
<td>355.5 ± 1.6</td>
<td>−351.7 ± 3.9</td>
</tr>
<tr>
<td>Standard B (PZT, Piezotest, UK)</td>
<td>381.5 ± 2.1</td>
<td>−382.5 ± 1.4</td>
</tr>
<tr>
<td>APC850, (PZT, APC International, USA)</td>
<td>408.7 ± 4.6</td>
<td>−400.8 ± 5.5</td>
</tr>
<tr>
<td>Glass (negative control)</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
</tbody>
</table>

For an ideal sample, the magnitude of the piezoelectric coefficient should be equal in the upright and inverted positions. For real samples, some slight inequality in the magnitude of $d_{33}$ is acceptable. The polarity of the piezoelectric coefficient must change sign upon inverting the sample from the 0° orientation to the 180° orientation; if this does not happen the sample is not piezoelectric.
Figure 5.2: Connecting the ITO electrodes to the electrode/clamps of the piezometer. Effectively, the switch system inverts the sample from the upright configuration (red) to an inverted configuration (black).

Rather than inverting the sample manually, we added a simple switch to invert the electrode configuration, Figure 5.2. In this way, we tested the exact same location of the lysozyme film in both upright and inverted states without disturbing the measurement.

5.1.2 Direct Piezoelectricity in Films of Lysozyme

Piezoelectricity was investigated in films of monoclinic and tetragonal lysozyme. Details of film preparation of crystalline films were presented in Chapter 3. For each measurement, the sample, sandwiched between two pieces of ITO-coated glass and placed in the jaws of the commercial piezometer. The ITO electrodes were connected to the electrodes of the piezometer with a switch (Figure 5.2). The sample was subjected to an oscillating force of 0.25 N at 111 Hz. The oscillation frequency was chosen such that it was not operating at any multiple of the mains frequency.

We measured the direct piezoelectric effect in both monoclinic and tetragonal
lysozyme films. The sign of the piezoelectric coefficient always changed when the sample was inverted - a confirmation of piezoelectric behaviour.

**Direct Piezoelectric Effect in Monoclinic Films of Lysozyme**

The piezoelectric $d_{33}$ coefficients of five monoclinic films prepared in an identical manner are reported in Table 5.2. For each sample, the piezoelectric coefficient was measured in the upright and inverted configuration five times; the average piezoelectric coefficient and standard deviation are shown for each sample. For each sample, the sign of the piezoelectric coefficient changed when the sample was inverted – this is confirmation of piezoelectricity.

Table 5.2: Piezoelectric $d_{33}$-coefficients of monoclinic lysozyme films all prepared by drop-casting a 100 µL solution consisting of 49.5 µL of 50 mg/ml lysozyme in 50 mM sodium acetate buffer pH 4.6, 49.5 µL of 4% sodium nitride and 1 µL of 50% glycerol. (206)

<table>
<thead>
<tr>
<th>Sample</th>
<th>$d_{33}$ (pC N$^{-1}$)</th>
<th>$d_{33}$ (pC N$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Upright configuration</td>
<td>Inverted configuration</td>
</tr>
<tr>
<td>Monoclinic_Lsz_A</td>
<td>0.93 ± 0.01</td>
<td>−0.72 ± 0.03</td>
</tr>
<tr>
<td>Monoclinic_Lsz_B</td>
<td>1.34 ± 0.01</td>
<td>−1.13 ± 0.02</td>
</tr>
<tr>
<td>Monoclinic_Lsz_C</td>
<td>0.67 ± 0.02</td>
<td>−0.51 ± 0.01</td>
</tr>
<tr>
<td>Monoclinic_Lsz_D</td>
<td>1.25 ± 0.04</td>
<td>−0.90 ± 0.03</td>
</tr>
<tr>
<td>Monoclinic_Lsz_E</td>
<td>1.17 ± 0.05</td>
<td>−0.78 ± 0.01</td>
</tr>
<tr>
<td>Average</td>
<td>1.07 ± 0.27</td>
<td>−0.81 ± 0.23</td>
</tr>
</tbody>
</table>
The piezoelectric coefficient measured in samples of monoclinic lysozyme ranged from $0.67 \, \text{pC} \, \text{N}^{-1}$ to $1.34 \, \text{pC} \, \text{N}^{-1}$. The magnitude of the $d_{33}$-coefficient for monoclinic lysozyme films is not trivial; it is of the order of longitudinal piezoelectricity in dry bone (97). The piezometer measures the piezoelectric coefficient of the aggregate film of lysozyme between the electrode clamps. The induced piezoelectric charge is only measured from those crystals that are in direct mechanical contact with the electrodes. As only a fraction of the crystals within the film contribute to the measured piezoelectric coefficient, the piezoelectric coefficients for the lysozyme films listed in Table 5.2 are likely to be underestimated.

The standard deviation for each sample (five measurements taken in upright and inverted) was calculated. The standard deviation was low, ranging from 0.75% to 4.17%. The average $d_{33}$ coefficient across all five samples is $1.07 \pm 0.27 \, \text{pC} \, \text{N}^{-1}$ in the upright configuration. The average $d_{33}$ coefficient across all five samples in the inverted configuration is $-0.81 \pm 0.23 \, \text{pC} \, \text{N}^{-1}$. The standard deviation between samples is high; 25.2% in the upright configuration and 28.4% in the inverted configuration. Although the samples were prepared in the same manner, the large standard deviation reflects the fact that the piezoelectric coefficient between samples is not constant.
Direct Piezoelectric Effect in Tetragonal Films of Lysozyme

The piezoelectric $d_{33}$ coefficient of five tetragonal films prepared in an identical manner are reported in

Table 5.3. The piezoelectric coefficients ranged from approximately 1 pC N$^{-1}$ to approximately 6.4 pC N$^{-1}$. As with the monoclinic aggregate film, the piezoelectric coefficient of tetragonal aggregate films reported in Table 5.3 may be underestimated, as only crystals that are in direct mechanical contact with the electrodes contribute to the measurement. For each sample, the standard deviation from five measurements was calculated.

Table 5.3 Piezoelectric $d_{33}$-coefficients of tetragonal lysozyme films all prepared by drop-casting 100 µL of 100 mg/ml lysozyme in 50 mM sodium acetate buffer pH 4.6 with 6 µL of 50% glycerol. (206)

<table>
<thead>
<tr>
<th>Sample</th>
<th>$d_{33}$ (pC N$^{-1}$)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Upright configuration</td>
<td>Inverted</td>
<td></td>
</tr>
<tr>
<td>Tetragonal_Lsz_A</td>
<td>1.04 ± 0.01</td>
<td>−0.89 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Tetragonal_Lsz_B</td>
<td>4.11 ± 0.03</td>
<td>−3.97 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Tetragonal_Lsz_C</td>
<td>2.93 ± 0.04</td>
<td>−2.76 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Tetragonal_Lsz_D</td>
<td>2.14 ± 0.03</td>
<td>−1.78 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Tetragonal_Lsz_E</td>
<td>6.44 ± 0.41</td>
<td>−6.50 ± 0.51</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>3.13 ± 2.07</td>
<td>−3.18 ± 2.18</td>
<td></td>
</tr>
</tbody>
</table>
The standard deviation was low, ranging from 0.73% for sample Tetragonal_Lsz_B to 6.37% for sample TetraLsz_E. The reason why the standard deviation of sample TetraLsz_E is greater than the standard deviation of the other samples is because measurements on this sample were conducted over several days. Thus, the exact part of the film under measurement would have been slightly different on each day. For the remaining samples, the five measurements were taken together. The switch system ensured that the same part of the film was measured in this case. Thus, the standard deviation of the TetraLsz A-D is low, ranging from 0.73% to 1.37%.

The average $d_{33}$ coefficient across all five samples is $3.13 \pm 2.07\ pC\ N^{-1}$ in the upright configuration. The average $d_{33}$ coefficient in the inverted configuration is $−3.18 \pm 2.18\ pC\ N^{-1}$. Again, the large standard deviation reflects the fact that the piezoelectric coefficient between samples is not constant despite the fact that samples were prepared in the same manner. There could be any number of reasons why this is so; differences in number and size of crystals within each films may affect the measured $d_{33}$ coefficient. We decided to investigate if the number of crystals within a given film had any correlation with its measured $d_{33}$ coefficient.

**Relationship between the number of crystals in a film and the $d_{33}$-coefficient**

Four samples, each containing a different number of tetragonal lysozyme crystals were compared. The films could be grown to contain different numbers of crystals by altering the pH of the crystallisation buffer used. Using buffers with different pH values results in films containing different number and size of crystals. There was not overall correlation between the number and size of crystals that grew and the pH of the crystallisation buffer.

A method of counting the number of crystals contributing to the piezoelectric effect was established. Crystals were counted from optical microscopy images with $10\times$ magnification. Only crystals that were in focus were included in the count. Five counting boxes were identified and the number of crystals in each counting box was manually counted. We then determined the average number of crystals per counting box. The area of the lysozyme film was larger than the area of the piezometer electrodes. Only the proportion of crystals inside the electrode area contributed to the overall piezoelectric coefficient measured. Equation 5.1 determines the number of
crystals contributing to the piezoelectric effect, N.

\[ N = N_b \times \frac{A_i}{A_b} \times \frac{A_e}{A_i} \]  

5.1

Where \( N_b \) is the average number of crystals in five counting boxes, \( A_i \) is the area of the microscopy image, \( A_b \) is the area of the counting box, and \( A_e \) is the area of the electrode. The area of the images was 565.9 nm² and the area of each counting box was 40 nm².

Figure 5.4 shows that the magnitude of the piezoelectric \( d_{33} \)-coefficient increases with the number of crystals present. The increase is non-linear. The magnitude of the piezoelectricity increased by an order of one when the number of contributing crystals increased by an order of two.

Figure 5.3: The piezoelectric \( d_{33} \) coefficient increases for films with a greater number of protein crystals. Optical images show the images of each film under 10 x magnification
The non-linear relationship between crystal count and piezoelectric is not unexpected. The crystals within the film are random in nature; the crystals are not textured nor are the distribution of crystals homogeneous. Also, the size of the crystals was not uniform across all films. If it were possible to texture crystals of uniform size along one direction, it may be possible to see an overall linear relationship.

5.2 Verification of the Direct Piezoelectric Effect in Lysozyme using a Custom-Built Rig

A commercial piezometer is a useful instrument to determine the piezoelectric coefficient of a material quickly. The PM300 piezometer can also report the capacitance and loss tangent of the sample. However, the piezometer only reports the calculated values of these coefficients. To analyze the electro-mechanical behavior of our samples more thoroughly, it is desirable to extract the raw information from the meter, i.e. to observe the applied force and charge generated signals in real-time. As the piezometer can be controlled remotely, it is possible to extract the raw data directly from the piezometer by connecting it to a PC via an RS232 cable and running a terminal emulator program. Typically, the frequency of force oscillations applied by the piezometer was set to 111 Hz. However, the number of readings the piezometer can report remotely is limited to one data point every four seconds. This rate of data recording is insufficient to capture the applied force and output charge signals in a meaningful way. Therefore, we decided to custom build a piezo-rig to monitor the applied force and induced voltage in real-time.

5.2.1 Design, Development and Calibration of a Custom-Built Rig

We made the piezo-rig by modifying an existing apparatus. The apparatus had been used previously by van Hout et al. (207) to analyze the stress-strain relationship of elastomers. The basic apparatus, Figure 5.4, consisted of a piezoelectric actuator (Pst 150/7/20, Piezomechanik) and a 10 lbs. load cell (LPM530, Cooper Instruments). The bottom plate of the stainless steel frame fixes the load cell in place. A platform attached to the top of the load cell holds the sample. A piezo-actuator, fixed to the top plate, applies stress to the sample in a controlled manner. The actuator can move a maximum
distance of 20 μm in increments of 0.01 μm. In its original form, the actuator and load cell system provided a sensible manner of applying a known force to our sample.

![Figure 5.4: Schematic (a) and photograph (b) of the basic apparatus of the custom-built rig](image)

To complete the piezo-rig we added the capability of measuring the induced voltage due to the piezoelectric effect by connecting the electrodes to an electrometer (Keithley 6514) as in Figure 5.5.

![Figure 5.5: Schematic illustrating the control connections of the custom rig](image)

When the sample impedance is high and the measured voltage is low, it is important to consider the electroding arrangement carefully. The Keithley electrometer connects to the sample with a special triax cable that has three inputs, high, low and ground. Leakage resistance and capacitance can exist between the inputs and adversely affect the measurement. Guarding is an effective way to eliminate these effects. In guard mode, the electrometer drives a conductive sheet surrounding the sample to have the
same potential as the high input terminal. With both ends at the same potential, current leakage is not possible. The electrometer used here can operate in guarded mode or un-guarded mode, as appropriate.

Finally, we automated the system using LabVIEW from National Instruments. The data-logging program controlled and monitored the actuator, load cell and electrometer.

Determining the force applied to the sample accurately is an essential part of direct piezoelectric measurement. As described above, a load cell measured the amount of force applied to the sample by the piezo-actuator. We note that the maximum capacity of the load cell is 10 lbs or approximately 4.54 kg. Of course, the load cell does not give a direct reading in units of newtons, instead it responds to an applied force by generating a proportional voltage. We calibrated the load cell by placing a series of weights with known mass on to the load cell and measuring the load cell voltage.

![Figure 5.6: Calibration of (a) load cell and (b) actuator used in the custom-built rig](image)

The graph in Figure 5.6(a) plots the load cell voltage as weights were added, in increments of 0.1 kg, to a maximum load of 1 kg. The slope of this graph (2.12 mV kg\(^{-1}\)) is the load cell calibration constant. Converting mass to weight, the load cell calibration constant becomes 0.218 mV N\(^{-1}\). Similarly, the actuator was calibrated by applying voltages from 0 V to 2.5 V in increments of 0.5 V to the actuator while recording its displacement as in Figure 5.6(b). The slope of this graph (3.85 µm V\(^{-1}\)) is the actuator calibration constant. We incorporated both of these calibration constants in to the VI program. The readout then displayed the amount of force applied to the sample by the actuator in units of newton.
To verify the custom-built rig, we used it to measure the piezoelectric \( d_{33} \) coefficient of a sample of lead zirconate titanate (APC850, APC International Ltd, USA). A preload force of 10 N held the sample in place. The piezo-rig applied a series of six force pulses to the sample. The electrometer measured the corresponding voltage generated due to the direct piezoelectric effect. In Figure 5.7, the magnitude of the force pulses is \( 2.49 \pm 0.01 \) N. When the sample was upright, positive voltage peaks were generated as seen in Figure 5.7(a). Negative voltage peaks were generated when the sample was inverted, Figure 5.7(b). The average magnitude of the voltage generated was \( 1.250 \pm 0.001 \) V.

![Figure 5.7: Force pulses applied by the piezo-rig induce positive voltage peaks when the sample is upright (a) and negative voltage peaks when the sample is inverted (b)](image)

To investigate if the relationship between the applied force and the voltage generated was linear, six cycles of force pulses were performed. Each cycle applied a series of six force pulses as in Figure 5.7. The magnitude of the applied force in each cycle increased incrementally from 0.81 N to 4.94 N. Figure 5.8 shows that the piezoelectric voltage increases linearly with the magnitude of the applied force. The piezoelectric voltage coefficient \((g)\) is a measure of the electric field generated due to piezoelectric conversion of stress and can be determined using the slope of the graph in Figure 5.8 and Equation 5.2.

\[
g = \frac{E}{\sigma} = \frac{\Delta V/t_h}{\Delta F/A} \tag{5.2}
\]

Where \( E \) is the electric field generated, \( \sigma \) is the applied mechanical stress, \( V \) is the
voltage generated, \( A \) is the electroded area and \( t_h \) is the thickness of the film.

The area and thickness of the PZT sample was 19.24 \( \mu \text{m}^2 \) and 0.35 mm, respectively, yielding a \( g_{33} \)-coefficient of 0.0287 V m N\(^{-1}\). The piezoelectric charge coefficient \( (d) \) can be calculated as \( d = g\kappa_s\kappa_o \), where \( \kappa_s \) is the permittivity of the sample and \( \kappa_o \) is the permittivity of free space. The permittivity of the sample was determined from its capacitance \( (C) \) as in Equation 5.3:

\[
\kappa_s = \frac{C \ell}{A \kappa_o} \quad 5.3
\]

The piezometer, which has the functionality to measure capacitance also, determined the capacitance of the samples to be approximately 798 pF. Therefore, the permittivity of the sample was 1641.4. Finally, the corresponding \( d \)-coefficient measured with the custom-built rig is 417.1 pC N\(^{-1}\). The \( g\) - and \( d\)-coefficients determined for the sample of APC850 using the custom built rig compare well with those values reported in the manufacturer’s datasheet (208) as shown in Table 5.4.

Figure 5.8: Linear piezoelectric response from APC850 sample verifies the custom-built set-up
Table 5.4: Comparison of experimentally determined values of permittivity, g and d-coefficients for APC850 sample with those values reported in the commercial datasheet

<table>
<thead>
<tr>
<th></th>
<th>Datasheet</th>
<th>Piezometer</th>
<th>Piezo-rig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permittivity</td>
<td>1900</td>
<td>1641.4</td>
<td>1641.4</td>
</tr>
<tr>
<td>g-coefficient (V m N(^{-1}))</td>
<td>0.0248</td>
<td>-</td>
<td>0.0287</td>
</tr>
<tr>
<td>d-coefficient (pC N(^{-1}))</td>
<td>400</td>
<td>408.7</td>
<td>417.1</td>
</tr>
</tbody>
</table>

The final step in calibrating the custom-built rig was to test it with a non-piezoelectric sample. A small piece of glass cut from a coverslip slide acted as the negative control. The glass sample had an area of 25 µm\(^2\) and a thickness of 1.0 mm. As with the positive control, the glass sample was subjected to a series of six force pulses while the voltage across the surface of the sample was recorded continuously.

Figure 5.9 shows the result of this test when 2.6 N was applied to the glass sample.
As expected, glass, being non-piezoelectric, does not generate any voltage in response to the force pulses. The voltage does creep upwards from approximately -80 mV to -20 mV over the course of the measurement. However, this does not relate to any piezoelectric effect. We observe the same type of voltage creep even while running the measurement without applying any force pulses.

Having confirmed the custom-built rig is suitable for measuring piezoelectric materials, we next use it to investigate the lysozyme films in more detail.

5.2.2 Verification of Direct Piezoelectricity in Monoclinic Lysozyme

We placed an electroded film of monoclinic lysozyme on the platform above the load cell. To hold the sample in place, the piezo-actuator applied a preload force to the sample. Typically, the preload force was 10 N. A flat rectangular disc (8 mm$^2$) placed between the actuator and the test sample ensured even loading. After preloading the sample, we connected the electrodes to the electrometer in the guarded configuration.
to minimize leakage effects. Then, the piezo-actuator applied a series of dynamic force pulses to the electroded film of lysozyme and the VI recorded the voltage induced due to the direct piezoelectric effect.

The monoclinic film of lysozyme produces a voltage in response to the force pulses, as shown in Figure 5.10. The rig applied six force pulses to the sample, each with the same magnitude (approximately 2.9 N). At the instant that the force is impacted, a voltage develops across the sample, which diminishes immediately after the impact. The insets in Figure 5.10 indicate the orientation, either upright (0°) or inverted (180°) of the sample during measurement. The voltage peaks are positive in the upright orientation and negative in the inverted configuration. The average voltage generated (measured peak-to-peak) in this case was 0.43 ± 0.08 mV.

![Figure 5.10: Piezoelectric voltage generated from a monoclinic film of lysozyme in response to dynamic force pulses when the sample is upright (0°) and inverted (180°).](206)

Linearity is a characteristic of piezoelectricity that distinguishes it from other electromechanical coupling phenomenon such as electrostriction (strain generated that is proportional to the square of the applied field) or flexoelectricity (electrical polarization induced by a strain gradient). Therefore, the next part of the study
investigated the relationship between force applied and voltage generated from the monoclinic lysozyme films. The rig applied seven cycles of force pulses. Each cycle applied six force pulses so that an average could be calculated. The magnitude of the forces applied were 1.8 N, 3.2 N, 3.7 N, 4.5 N, 5.3 N, 6.1 N and 6.8 N. For this particular sample, the corresponding voltage generated increased from 1.1 mV to 6.1 mV. Figure 5.11 plots the induced-voltage against the force applied. Although, the voltage increases with increasing applied force, the relationship is not fully linear. The R-squared value of the trend line (dashed line) fitted through the origin is relatively low ($R^2 = 0.8968$).

![Figure 5.11 Linear piezoelectric response from a monoclinic film of lysozyme. (206)](image)

From the error bars in Figure 5.11, it is clear that the magnitude of the voltage peaks vary considerably. The source of this large variation is most likely experimental. Unlike films of tetragonal lysozyme, the surface of the monoclinic lysozyme films was not smooth. Typically, larger monoclinic crystals of lysozyme sat above smaller crystals in the film creating an uneven surface. Consequently, the ITO-glass serving as electrodes may not have made perfect contact with the film. Poor electrode contact therefore, may be the causes of the large variation of voltage peaks. The amount of
voltage deviation from the average tends to increase with increasing force. This is not surprising, as one would expect that applying greater force would exacerbate the adverse effects of poor electrode contact.

Nonetheless, to give us a quantitative indication of the piezoelectric effect in monoclinic lysozyme films, we use the slope of the best-fit line in Figure 5.11 to estimate its $g$- and $d$-coefficients. The thickness of the sample is approximately 30 µm and the area exposed to force pulses is 64 µm$^2$. The capacitance of the sample was approximately 1000 pF. We note that this is the capacitance of the entire film, i.e. the capacitance of the lysozyme crystals plus the capacitance of the space between crystals in the film. The permittivity of the film was determined to be 43.2. The measured permittivity value lay in the range of values reported in the literature for lysozyme ($2 < \kappa_s < 80$) (58-60, 144). From Equation 2, the piezoelectric voltage co-efficient $g$ is $\sim$1340 µV m N$^{-1}$. Hence, the piezoelectric $d_{33}$-coefficient of this monoclinic lysozyme film is approximately 0.5 pC N$^{-1}$. This is in good agreement with the $d_{33}$ value determined with the commercial piezometer of 0.7 pC N$^{-1}$.

5.2.3 Verification of Direct Piezoelectricity in Tetragonal Lysozyme

As with the films of monoclinic lysozyme crystals, the custom-built rig applied force pulses to the film of tetragonal lysozyme crystals, while measuring the stress-generated-voltage at the film surface. As depicted in Figure 5.12, during each measurement, five force pulses were applied and the output voltage measured. In Figure 5.12, the average force applied was 3.05 N and average voltage generated (measured peak-to-peak) was 1.42 mV. Importantly, the polarity of the voltage generated changed sign when the sample was inverted between the electrodes – a distinguishing hallmark of piezoelectricity.
Next, we ran five cycles of force pulses to investigate if the voltage generated by the films was proportional to the applied force. The force applied ranged in magnitude from 0.7 V to 4.1 V. The electrometer measured the output voltage during each cycle. The output voltage generated by the tetragonal aggregate film increased \textit{linearly} with increasing applied force (Figure 5.13).
From the graph in Figure 5.13, we can calculate the piezoelectric voltage coefficient, $g$, using Equation 2. For this sample, the electrode area was 64 µm$^2$, and the thickness was approximately 30 µm, yielding a $g_{33}$-coefficient of $\sim$1300 µV m N$^{-1}$. The permittivity of lysozyme was determined from capacitance measurements using the commercial piezometer (C = $\sim$1000 pF, $\varepsilon$ = 43.2). Again, we note that this measure of capacitance represents the capacitance of the entire lysozyme film containing lysozyme crystals and amorphous lysozyme. Likewise, the permittivity value represents the permittivity of the entire lysozyme film. Finally, for this film, the corresponding $d$ coefficient measured with the custom-built rig is $\sim$0.5 pC N$^{-1}$. This same sample showed a $d_{33}$ value of 2 pC N$^{-1}$ in the piezometer.

**Stability of Lysozyme $d_{33}$ coefficient over time**

As a final study, the stability of the crystalline lysozyme films was monitored over time. The sample investigated was a film of tetragonal crystals. We recorded its piezoelectric $d_{33}$ coefficient over the course of 14 days. Figure 5.14 shows the results of the stability study.

Figure 5.13: Linear piezoelectric response from tetragonal crystalline films of lysozyme (206)
Figure 5.14: Stability of piezoelectric $d_{33}$-coefficient of crystalline lysozyme films over time

The $d_{33}$ value did indeed fluctuate over the course of the study. The lowest value of $d_{33}$ was recorded on Day 1 measuring $2.80 \pm 0.02 \text{ pC N}^{-1}$. The highest $d_{33}$ was recorded on Day 7 measuring $4.15 \pm 0.06 \text{ pC N}^{-1}$. The average $d_{33}$-coefficient measured over the 14-day period was $3.75 \text{ pC N}^{-1}$, indicated by the dashed line. The standard deviation between measurements over 14 days was $0.50 \text{ pC N}^{-1}$. The cause of these fluctuations may be environmental or experimental. Protein crystallisation and growth are sensitive to slight variations in atmosphere conditions namely, humidity and temperature. However, the magnitude of the effect on fully-grown crystals within a film may be less. Nonetheless, the fact that humidity places a large role in biological piezoelectricity is well accepted. The piezoelectric effect in dry bone is much greater than that of wet bone (26, 97, 211). As the crystals of lysozyme were grown within a film to maintain their moisture content, changing humidity levels in the environment are not expected to affect these experiments significantly. From an experiment design perspective, since the area of the film was greater than the area of the piezometer electrodes, the portion of film measured would have been slightly different on each day of measurement. Overall, the stability of the piezoelectric response from crystals of lysozyme is good.
5.3 Discussion: Interpretation of Results with regards to the Classical Theory of Piezoelectricity

5.3.1 Monoclinic Films of Lysozyme

The observation of direct piezoelectricity in films of monoclinic lysozyme crystals matches the classical theory of piezoelectricity. By definition, all protein crystals are non-centrosymmetric, satisfying the most fundamental requirement of piezoelectricity. The monoclinic lysozyme crystals here belong to point group 2. As discussed in Chapter 2, the symmetry of this crystal point group limits its piezoelectric tensor; crystals in point group 2 should only demonstrate the piezoelectric coefficients listed in Table 2.4. The fact that the longitudinal coefficient $d_{22}$ is permitted is important. It supports our experimental observation of longitudinal piezoelectricity in films of monoclinic lysozyme crystals. This observation was confirmed twice; using the commercial piezometer and the custom-built rig.

In general, monoclinic crystals in point group 2 are expected to demonstrate the piezoelectric $d_{22}$ coefficient. That is to say, the application of force along its 2-axis, generates a charge perpendicular to the 2-axis.

The piezometer measures the so-called $d_{33}$-coefficient. The term longitudinal coefficient describes this measurand more completely. A material has a longitudinal coefficient if the direction of its piezoelectric response is the same direction as its stimulus. Thus, the longitudinal coefficient measured by a piezometer may be $d_{11}$, $d_{22}$ or $d_{33}$. The difference between these three coefficients is merely the choice of coordinate axis assigned to the laboratory space. Rotating the laboratory coordinate system such that the b-axis now lies along the vertical laboratory direction, the piezometer becomes a “$d_{22}$-meter”. The same logic applies to the custom-built rig.

One final point, the situation described here considers only the response from crystals orientated with their 2-fold axis (b-axis) perpendicular to the piezometer electrodes. The optical images shown in Chapter 4 clearly illustrate the random orientation of crystals within the film. Those crystals whose b-axis lies perpendicular to the electrodes contribute directly to the measured $d_{22}$-coefficient. Additionally,
components of the crystals transverse and shear coefficients that lie parallel to the laboratory 2-axis, may contribute indirectly to the measured $d_{22}$-coefficient.

5.3.2 Tetragonal Films of Lysozyme

Matching the observation of direct piezoelectricity in films of tetragonal lysozyme crystals to the classical theory of piezoelectricity is more complicated. Of course, tetragonal crystals of lysozyme are also non-centrosymmetric, and should demonstrate piezoelectricity. However, the point group most commonly associated with tetragonal lysozyme crystals is point group 422. Recall from Section 2.3.4 that the piezoelectric tensor associated with point group 422 is limited to just two shear piezoelectric coefficients, $d_{14}$ and $-d_{14}$. Point group 422 does not permit longitudinal piezoelectric coefficients. However, we measured longitudinal piezoelectricity with a commercial piezometer and confirmed the observation with the custom-built rig. These experiments demonstrate that the origin of the piezoelectric voltage may be longitudinal piezoelectricity.

Our observation of longitudinal piezoelectricity is in disagreement with the classical theory of piezoelectricity. Below, two arguments to resolve the discrepancy between experiment and theory are proposed. Following that, we discuss the practical experiments to test both arguments.

**Proposed Argument 1: Averaging**

We have discussed in Section 5.3.1 that the random orientation of monoclinic crystals within a film can result in a longitudinal measurement containing contributions from non-longitudinal components. In much the same way, optical images of tetragonal lysozyme crystals show that they are arbitrary distributed and orientated. Thus, this randomness may cause shear piezoelectricity to appear as an apparent longitudinal piezoelectric response.

One way to test the first argument is to eliminate the effects of averaging. Orientating all crystals within the film in exactly the same way would accomplish this. We attempted to align and orient the crystals using several methods. One method tried to introduce directionality by coating the substrate with a layer of PVA. This method of alignment is common in studies of liquid crystals (212). By rubbing the layer of PVA
uni-directionally with a cloth, we tried to develop micro-grooves in the PVA along one
direction. These micro-grooves would then influence the crystals to grow in an
oriented manner also. In a second method, inspired by the work of Hammadi (137),
we tried to influence the orientation of protein crystals during growth by growing them
under an electrical field. However, all attempts to align the crystals were unsuccessful.

An alternative way to eliminate the effects of averaging is to measure just one
individual crystal at a time. As discussed in Chapter 4, growing and handling just one
isolated protein crystal is a challenge. Instead, piezoresponse force microscopy could
probe individual lysozyme crystals embedded within a film for piezoelectricity. This
will be studied in the next chapter.

**Proposed Argument 2: Symmetry lowering**

The observation of longitudinal, tensile piezoelectricity using direct piezoelectric
measurement methods indicates that the symmetry of the crystals may be lower than
that generally ascribed to tetragonal crystals of lysozyme. The classical theory of
piezoelectricity would support shear, transverse and in particular, the longitudinal
piezoelectric coefficient $d_{33}$ if the films of tetragonal lysozyme belong to point group
4 rather than 422.

This type of symmetry lowering is not unprecedented. Originally, bone was thought to
belong to point group 622 as only shear piezoelectric coefficients were measured
experimentally (16). Later, experimental evidence of non-shear piezoelectric
components (18) and pyroelectricity (89) emerged which did not fit with the original
symmetry description because point group 622 is non-polar. Instead, bone was
reassigned to the polar point group 6, which allows for longitudinal, transverse and
shear piezoelectricity as well as pyroelectricity (102). Similarly, the point group used
to describe wood original was point group 2 (15). However, recognising the
experimental observations of non-shear piezoelectric coefficients, the lower symmetry
point group 622 describes it more thoroughly (15, 213).

At this stage, we can only speculate what mechanisms may cause symmetry lowering
in tetragonal crystals. Crystal growth depends on several factors including temperature
(214), supersaturation (215), pressure (48, 62) as well as the pH and salt concentration
of the buffer. It is widely known that twinning, where two or more crystals interlink in a symmetrical way during growth, adds apparent symmetry to a crystal. Perhaps then, other mechanisms exist that lower the apparent symmetry of the crystal. Interestingly, Yamada et al. have reported that increasing the pressure to 950 MPa causes lysozyme to undergo a phase transition from P4\textsubscript{3}2\textsubscript{1}2 (point group 4222) to the lower symmetry P4\textsubscript{3} (point group 4) (48). While the pressure used in this study is not in this range, the study by Yamada et al. does show that symmetry lowering is possible. In our case, the substrate (ITO-coated glass slide) may strongly influence the lysozyme crystals during growth (200, 216). The substrate may restrict 3D growth and alter the overall symmetry of the crystals.

Testing the second argument can take two approaches. The first is a direct approach that uses either diffraction or nuclear techniques to determine the symmetry of the sample. In Chapter 4, the synchrotron diffraction fitted lysozyme to point 422 as expected. However, as discussed in the Literature Review, by Neumann’s principle apparent symmetry can be added mistakenly. An alternative approach to test the argument of symmetry lowering is to determine the point group indirectly by experimental observation of the physical properties of the sample.

Recall from Chapter 2 that the physical properties of a crystal (chirality, piezoelectricity, pyroelectricity and ferroelectricity) are inherent of its underlying symmetry. Crystals belonging to both point groups are optically active so there is no way to distinguish them optically. However, a close examination of Figure 2.9 in Chapter 2 establishes a set of criteria to distinguish between point group 422 and point group 4. Crystals in point group 422 should show only shear piezoelectricity, while crystals in point group 4 may show longitudinal, transverse or shear piezoelectric components. Additionally, crystals in point group 422 are non-polar and may not demonstrate either pyroelectricity or ferroelectricity. In contrast, crystals in point group 4 are polar, implying that there is a permanent polarization in the structure. This would allow the crystals to demonstrate pyroelectricity, and potentially ferroelectricity, if that polarization were switchable.

Therefore, to support the argument of symmetry lowering in tetragonal films of lysozyme from point group 422 to point group 4, one must observe experimentally: (i) longitudinal converse piezoelectricity, (ii) pyroelectricity and potentially (iii)
ferroelectricity. In the next chapter, we will investigate if tetragonal films of lysozyme demonstrate these properties.

5.4 Conclusions

The primary outcome of this chapter is the observation of the direct piezoelectric effect in crystalline films of lysozyme. This is the first observation of piezoelectricity in a non-fibrous protein. The magnitude of the piezoelectric effect (as large as 6 pC N\(^{-1}\)) is significant. It is larger than that of quartz, a material heavily exploited in for its piezoelectric effect. Thus, this points towards potential technically applications for lysozyme – perhaps in piezoelectric energy harvesting for medical applications were biocompatibility is a concern. At this point, a physiological underpinning for the observation of piezoelectricity in lysozyme in not known, but may motivate further research.

The second part of this chapter outlined the design, development and calibration of a custom-rig that verified our findings. Positive and negative controls calibrated the rig before it was used to measure piezoelectricity in films of lysozyme. The rig qualitatively and quantitatively validated the observation of the direct piezoelectric effect in lysozyme.

The third part of this chapter discussed the observations of piezoelectricity in lysozyme in relation to the classical theory of piezoelectricity. The unexpected observation of longitudinal piezoelectricity in tetragonal crystals of lysozyme poses fundamental questions. We proposed two arguments (averaging and symmetry lowering) to reconcile our experimental observations with classical theory.

Testing these two arguments will direct the remainder of the work in this project. In Chapter 6, we will investigate if longitudinal converse piezoelectricity, pyroelectricity and ferroelectricity are properties of lysozyme.
Chapter 6  Observation of Converse Piezoelectricity, Ferroelectricity and Pyroelectricity in Lysozyme

6.1  Introduction
Piezoresponse force microscopy (PFM) has emerged as a powerful tool for conducting piezoelectric measurements on samples at the nanoscale. Based on the converse piezoelectric effect, it applies of a bias through a conductive AFM cantilever to induce deformations in piezoelectric samples. The tip detects bias-induced deformations as it scans the sample surface. Recent studies on collagen (28, 41), amino acids (24, 123, 217) and viruses (30) show the versatility of PFM for studying biological materials. In this chapter, we use PFM to confirm that crystals of monoclinic and tetragonal lysozyme are piezoelectric by investigating the reciprocal converse piezoelectric effect. Quantitative measurements of the converse piezoelectric coefficient are determined for both types of lysozyme crystals. PFM is also used to investigate if a permanent switchable polarization exists in crystals of lysozyme. Monoclinic crystals of lysozyme are polar and thus may be ferroelectric. Tetragonal crystals of lysozyme described by point group 422 are non-polar and therefore, should not demonstrate switchable polarization. If the argument outlined in Chapter 5 (that the tetragonal films of lysozyme are of lower symmetry group and are described by point group 4) is correct, then a switchable polarization may exist in these crystals. Ferroelectricity will be investigated by PFM using two approaches, by performing polarizing scans and by using SS-PFM. Finally, pyroelectric measurements on polar, monoclinic crystals of lysozyme will be presented.

6.2  Interpretation of PFM Data
To illustrate how the PFM data is to be interpreted, first we study two well-studied piezoelectric materials, periodically poled lithium niobate (PPLN) and barium titanate (BaTiO₃) as examples. Figure 6.1 shows a schematic of the PPLN sample. The sample is ideal for PFM measurements because it is flat (roughness < 10 nm). The lithium
niobate has been poled so that the polarisation of neighbouring domains are in opposite directions; the polarisation either points upwards or downwards as indicated by the arrows labelled $P_s$ in Figure 6.1. Therefore, the $d$-coefficient of neighbouring domains will also be of opposite sign. The length ($L$) of the poled domains is 100 µm and the period of two oppositely polarised domains ($D$) is 7 µm. The thickness of the film ($t_h$) is 500 µm. The sample was grounded by fixing it to a grounded metal substrate with silver paste.

![Figure 6.1 Schematic of periodically poled lithium niobate (PPLN) used as a standard PFM sample. The direction of the polarization ($P_s$) is indicated by arrows and has opposite direction in neighbouring domains. The thickness of the sample ($t_h$) is 500 µm, the length of the domain ($L$) is 100 µm and the period ($D$) is 7 µm. Adapted from (218)]](image)

During PFM scanning, 20 V at 150 kHz was applied across the sample via a conductive tip (NSG03/Pt, NT-MDT, Russia) in contact mode. Both the topography and the vertical piezoresponse signals were collected during the measurement and the result is shown in Figure 6.2. The topography images show little or no surface features because the sample is flat (average roughness 6.1 nm). There are distinctive areas of contrast in the piezoresponse images. The contrast in the PFM magnitude images indicates the magnitude or strength of the piezoresponse in different domains. The contrast in the PFM phase images indicates the direction of the polarization.
Figure 6.2 AFM and PFM images of PPLN performed using 20 V at 150 kHz. The topography [(a) height, (b) deflection] shows a flat surface. The VPFM images [(c) magnitude, (d) phase] show the magnitude and direction of the piezoresponse in neighbouring domains. The white arrows indicate the direction of the piezoresponse. The scale bar in (a) is 4 µm.

If the poling voltage used to polarize the upward domain was exactly equal in magnitude to the poling voltage used to polarize the downward domain, then no contrast would be observed in the PFM magnitude image. Only the domain wall would be evident. This is because the magnitude of the $d$-coefficient would be equal in both domains; only the sign of the $d$-coefficient would be different. Because a clear contrast is observed in the magnitude image (Figure 6.2(c)) we can determine that the magnitude of piezoresponse in neighbouring domains is not exactly equal and is probably the result of imperfect poling. The contrast in the PFM phase image (Figure 6.2(d)) indicates that the direction of polarization in neighbouring domains differ. There is a $180^\circ$ phase difference between the domains, i.e. the polarization in...
neighbouring domains are in opposite directions, as illustrated by the white arrows.

The second sample, BaTiO$_3$, presents a more difficult case to interpret because of the presence of topography features. The lock-in amplifier serves to separate the deformation of the cantilever due to surface roughness and the deformation induced by the piezoelectric effect (219). However, if the surface of the sample is very rough, this separation may not be completely successful and an artificial response will result in the magnitude image. In this section, the manner in which a true piezoresponse is distinguished from an artificial response caused by mechanical crosstalk is laid out.

An industrial supplier provided commercial samples of BaTiO$_3$, which had been conventionally sintered. The samples were polished to a 4000-grit finish using a BUEHLER phoenix 4000 polishing machine. The film of BaTiO$_3$ was grounded in the manner described above for the sample of PPLN. Experiments were performed in contact mode using a platinum-coated cantilever (NSG03/Pt, NT-MDT) that has a spring constant of between 0.35 and 6.06 $N m^{-1}$ and a measured resonant frequency of 115 kHz. PFM was performed over a 10 $\mu m \times 10$ $\mu m$ area with an applied voltage of 10 V at 100 kHz. Figure 6.3 shows the topography, vertical piezoresponse and lateral piezoresponse. The height and deflection images in Figure 6.3(a) and (b), respectively, show the presence of large irregularly shaped grains. Although the samples had been polished, the surface is not completely flat. Within each grain, a number of parallel lines are evident. While interpreting the PFM images, it will be necessary to consider whether these topographical features contribute to the measured piezoresponse.

Figure 6.3(b) and (c), show the magnitude and phase of the vertical piezoresponse of BaTiO$_3$, respectively. Many striped domains are evident in both the magnitude and phase image. The magnitude image reveals piezoelectric domains of various strength and the phase image indicates the direction of the polarisation within each domain. This out-of-plane response corresponds to its c+ and c- domains (220). Similarly, the a-domains of tetragonal barium titanate are revealed by lateral PFM (220). Figure 6.2 (e) and (f), respectively, reports on the magnitude and phase (direction) of the piezoresponse in lateral domains.
Figure 6.3 AFM and PFM images of BaTiO$_3$ performed with 10 V bias at 100 kHz. Topography [(a) height, (b) deflection], vertical piezoresponse [(c) magnitude, (d) phase] and lateral piezoresponse [(e) magnitude, (f) phase] are shown. The scale bar in (a) is 2 µm.
Next, we must consider whether or not the topographical features of the BaTiO$_3$ sample contribute artificially to the observed piezoresponse images. Two areas of Figure 6.3 are considered and redisplayed in Figure 6.4 for clarity. The first area (labelled 1) has no obvious topographical features. In areas such as this, mechanical crosstalk is not a concern and the piezoresponse in this area can be considered genuine. The second area (labelled 2) has pronounced surface features. In areas such as this, the potential mechanical crosstalk between the topography and magnitude signal must be considered. This is done by taking a cross section of the height and deflection images and comparing them with cross-sections of the magnitude and phase images. Cross-sections of the height, deflection, VPFM magnitude and VPFM phase are shown in Figure 6.4 (b), (d), (f) and (h), respectively. Cross-sections were taken along the white line indicated in Area 2.
Figure 6.4 The (a) height, (c) deflection, (e) VPFM magnitude and (g) VPFM phase images presented in Figure 6.3 are redisplayed to highlight two areas of interest. Area 1 is flat and Area 2 has some striped surface features. To the right of each image is the corresponding cross-section (b, d, f, h). The cross-sections were taken along the line indicated in white in Area 2. The scale bar in (a) is 4 µm.

The cross-section of the height image (Figure 6.4 (b)) is the most important in determining if mechanical cross-talk is likely to have occurred. In this case, the height
varies less than 3 nm – well within the range in which the lock-in amplifier can separate the topography from the piezoresponse. If the sample is very rough (>> 20 nm), then the system may not fully separate the two signals. There is no general agreement over the maximum roughness that PFM can manage and operate normally. The engineers we consulted with (NT-MDT, Russia) recommended that the surface be as flat as possible and that care be taken for samples with a roughness greater than 100 nm.

6.3 Sample Preparation and Scan Parameters for PFM Measurements

PFM and SS-PFM measurements presented here were carried out on a research trip to Professor Kholkin’s lab in the University of Aveiro, Aveiro, Portugal. Measurements were performed under the supervision of Dr Ivanov and Prof Kholkin, who are graciously acknowledged here. An NT-MDT Integra Aura AFM microscope with a Px controller characterized the topographical and piezoelectric properties of proteins. Typically, during contact mode PFM, an alternating voltage, $V_{AC}$, with a frequency of 20 kHz and amplitude between 1 and 12 V was applied to the sample via a conductive probe. The laser and photodiode system monitored the piezoelectrically induced deformation. This method simultaneously measures the vertical piezoresponse signal (VPFM) and the lateral piezoresponse signal (LPFM) which correspond to an out-of-plane and in-plane response, respectively.

The sample was in the form of a crystalline aggregate film in which PFM could probe individual constituent single crystals for piezoelectricity. The films of lysozyme were prepared on ITO-coated glass by dropcasting as described in Chapter 4. By electrically grounding the ITO-coated glass, it also acted as the bottom electrode. The scan parameters were optimised to prevent damage to the delicate lysozyme crystals. If the scan parameters are inappropriate for the sample (hard cantilever, high set point, high scan rate) then the movement of the tip can damage the surface of the crystal as seen in Figure 6.5.
Damage to the crystals during scanning was minimized by using a soft, platinum-coated tip (CSG30/Pt, NT-MDT, Russia) which has a force constant of 0.6 N/m and a resonant frequency of 48 kHz.

The AFM instrument is fitted with an optical microscope. This made it possible to observe individual crystals within the film and position the tip so that it landed on the surface of the crystal during the approach, Figure 6.6.
6.4 Converse Piezoelectricity in Tetragonal Lysozyme Crystals

PFM was performed on a tetragonal lysozyme crystal over a 15 µm × 15 µm area. Initially, a relatively low bias of 1 V at 20 kHz was applied. The resulting topographical (Figure 6.7 a, b), vertical piezoresponse (Figure 6.7 c, d) and lateral piezoresponse (Figure 6.7 e, f) are shown below.

The height and deflection images show that the topography of this area of the crystal surface is granular in appearance. The topography images obtained here using ambient-AFM are similar to that reported by Durbin et al. (Figure 2.20(e, f) in Chapter 2) using in-situ liquid-AFM. Durbin et al. discussed two different growth mechanisms, spiral dislocations which produce concentric growth spirals and 2-D nucleation which produce growth islands and bulges (52). In our study using ambient AFM, growth mechanisms such as spiral steps were not discernible. Although preparing the crystals as a film provided sufficient moisture to maintain the structure of the crystal, it is likely that the crystals do not continue to grow once the film is dry. The rounded shapes in the topographical images collect here with ambient-AFM are likely to be bugs resulting from 2-D nucleation. Also, we performed AFM on crystals that were close to the surface of the film. The thin layer of film-material that covers these crystals may have hidden the subtle surface features which liquid-AFM can reveal.

At this bias, no vertical piezoresponse is evident in either the magnitude or the phase images. The lateral piezoresponse, however, does have some areas of weak contrast. The average roughness over this 15 µm × 15 µm area is 112.9 nm. With this level of roughness, the fact that areas of apparent piezoresponse coincide with the topography of the sample is a concern. Therefore, the approach described in section 6.2 is taken to discern if the piezoresponse is real.
Figure 6.7: PFM images performed with 1 V bias at 20 kHz on tetragonal lysozyme showing images of topography [(a) height, (b) deflection], vertical piezoresponse [(c) magnitude, (d) phase] and lateral piezoresponse [(e) magnitude, (f) phase]. The scale bar in (a) is 3 µm.
In Figure 6.8(a and b), the lateral piezoresponse images shown in Figure 6.7(e and f) are reprinted for further analysis. Three areas of interest are labelled as region 1, region 2 and region 3. Cross-sections of the height signal are taken along the white lines in each region and plotted in Figure 6.8(c).

Figure 6.8 Analysis of mechanical cross-talk in Figure 6.7. Lateral PFM (a) magnitude and (b) phase images taken from Figure 6.7(e and f) are re-displayed with three regions of interest highlight by dashed shapes. (c) Cross-sections of the height signal along white line in Region1, Region 2 and Region 3.
The areas of lateral piezoresponse contrast in Region 1 and Region 2 closely resemble that of the topography in Figure 6.7 (b), and may be artificial. The cross-sections of the height signal in these regions do indeed show severe surface roughness. The change of height across the line-segments in Region 1 and Region 2 are approximately 150 nm and 300 nm, respectively. Thus, these topographical features are likely to have contributed artificially to the lateral PFM contrast in Region 1 and Region 2.

However, the cross-section of the height signal in Region 3 shows that it is much flatter. The change of height across the line segment in this region is approximately 20 nm. In this relatively flat area, the PFM system is capable of effectively separating mechanical cross-talk. Therefore, the lateral piezoresponse in region 3 is not influenced by topography and is the result of a voltage-induced piezoelectric effect. The contrast in the piezoresponse of Region 3 is weak, however. The bias applied (1 V) may have been insufficient to induce a strong piezoresponse.

To confirm the nature of the piezoresponse, a second area (10 µm × 10 µm) adjacent to the first was investigated with a higher bias, 10 V at 20 kHz. Figure 6.9 shows the result of that scan. Horizontal scratches were removed from the height and deflection images with the Nova Image Analysis software installed on the AFM system. The height image was 1-D flattened post scan. Again, the topographical images (Figure 6.9 (a, b)) show that the surface of the crystal has a granular appearance. The average roughness over the 10 µm × 10 µm area is 79.0 nm. With this bias applied, the tetragonal crystal responded in both vertical and lateral directions. Figure 6.9 (c, d) shows areas of contrast in the magnitude and phase of the vertical piezoresponse signals, respectively. The areas of contrast in the vertical magnitude signal do not correspond to any topographical features and therefore mechanical cross-talk is not a concern. Additionally, the areas of contrast in the vertical phase image indicate that the polarisation in these domains are approximately 150° apart.
Figure 6.9: PFM images performed with 10 V bias at 20 kHz on tetragonal lysozyme showing images of topography [(a) height, (b) deflection], vertical piezoresponse [(c) magnitude, (d) phase] and lateral piezoresponse [(e) magnitude, (f) phase]. The scale bar is 2 µm.
Interpretation of the lateral piezoresponse (Figure 6.9 (e, f)) is more complicated. There are areas of contrast in both the magnitude and phase signals, however, these areas coincide strongly with the topography of the sample. As the sample is rough, it is difficult to discern whether the piezoresponse is genuine, an artefact resulting from rough topography or a combination of both. A second observation is that the areas that showed a vertical piezoresponse are also apparent in the lateral piezoresponse images, almost appearing to overlap the main signal.

The vertical and lateral piezoresponse signals correspond to the longitudinal and shear piezoelectric components, respectively. The lateral piezoresponse observed is as expected, conforming to point group symmetry 422. However, the out-of-plane piezoresponse is not predicted by this point group symmetry.

It is important to take into account the orientation of the crystal/tip system. The lateral piezoresponse is sensitive to surface displacements perpendicular to the cantilever axis of the tip. Therefore, the lateral piezoresponse may demonstrate some orientation dependence. The vertical piezoresponse signal consists of contributions from the longitudinal, transverse and shear components of piezoelectricity. If the tip approach is off vertical, a sample that should not have a longitudinal component may demonstrate a non-zero out-of-plane piezoresponse. Buckling vibrations, caused by in-plane surface displacements may be transmitted through frictional forces and contribute to the out-of-plane piezoresponse (103). Although, buckling vibrations may contribute to the vertical piezoresponse, the fact that we see longitudinal piezoelectricity in tetragonal lysozyme via PFM is credible considering that it was also measured via the Berlincourt Method in the previous chapter, which is devoid of these issues.

6.4.1 Polarisability of Tetragonal Lysozyme

To determine if the tetragonal lysozyme crystals are also polarizable, a series of three PFM scans were performed consecutively to an area of an individual tetragonal crystal. This type of scanning to investigate a material’s Polarisability is done routinely at room temperature, typically below the material’s Curie temperature (221, 222). As the
sample thickness is thin, the electric field across it is large enough to induce polarization. The first scan ($10 \times 10 \, \mu m^2$) applied 10 V of DC bias, the second ($40 \times 40 \, \mu m^2$) applied 5 V DC bias around the first scan area, and the final scan ($60 \times 60 \, \mu m^2$) applied a few millivolts of bias around the previous two scan areas. White dashed boxes in Figure 6.10(a) illustrate the areas biased. If the crystal is polarizable, the effect of the applied bias in the first two PFM scans will be evident in the third scan.

Figure 6.10 shows the result of the third scan in the series. The topography images, Figure 6.10 (a, b), show that the surface of the sample was effected during the first and second biasing scans.
Figure 6.10: PFM response after biasing a tetragonal lysozyme crystal with 10 V in the first scan and 5 V in the second scan. White dashed lines in (a) indicate the region over which the biases were applied. Images of topography [(a) height, (b) deflection], vertical piezoresponse [(c) magnitude, (d) phase] and lateral piezoresponse [(e) magnitude, (f) phase] are shown. The scale bar in (a) is 10 µm.
To investigate this, cross-sections were taken along the three sections of the height image which are indicated by white lines in Figure 6.11(a). The cross-section of the height image along line 1 shows that the change of height is greater than 400 nm in some parts. The cross-section along line 2 shows that this area is smoother with an average change in height of approximately 90 nm. This is similar to the cross-section of line 3 which has an average change of height of approximately 50 nm. We can conclude that the high bias used in the first scan appears to have caused significant surface damage. The lower voltage used in the second and third scans were less destructive and did not appear to influence the topographical features substantially.

Figure 6.11 Cross-sectional analysis of the height image in Figure 6.10(a). Cross-sections are along the white lines labelled 1, 2 and 3 in (a) are displayed in (b), (c) and (d), respectively. The scale bar in (a) is 10 µm.
Returning to the interpretation of the piezoresponse in Figure 6.10, it is clear that the polarizing effect of the bias field applied during the first two scans is evident in the piezoresponse of the third scan (Figure 6.10 (c-f)). The vertical and lateral piezoresponse in the centre $10 \times 10 \, \mu\text{m}^2$ may or may not be the result of the biasing effect. As discussed above, surface damage appears to dominate this area. In the region outside of this, however, surface damage is minimal. The piezoresponse in the $40 \times 40 \, \mu\text{m}^2$ area indicates that the protein can be polarized with the application of an external electric field. The polarization effect is evident in both the vertical and lateral piezoresponse.

An alternative explanation for the contrast observed in Figure 6.10 may be that the voltage applied to the sample induces local anodic oxidation which chemically modifies the surface of the sample. This effect is used in Local Anodic Nanolithography to pattern surfaces. Local Anodic Nanolithography makes use of the meniscus that forms on the surface of a sample due to ambient humidity. When a bias is applied across the sample via a conductive AFM tip, the water in the meniscus separates into $\text{H}^+$ and $\text{OH}^-$ ions. The $\text{OH}^-$ ions oxidize on the sample surface (223).

It is unlikely that local anodic oxidation occurs in the case of lysozyme as the water layer is likely to be much thicker than the thin meniscus required for local anodic nanolithography. Nonetheless, if the surface of films of lysozyme were chemically modified in this way it would impact the piezoelectric properties of the films. Therefore, future studies may use x-ray photoelectron spectroscopy to investigate this further.
6.5 Converse Piezoelectricity in Monoclinic Lysozyme Crystals

6.5.1 Polarisability of Monoclinic Lysozyme

From a classical viewpoint, monoclinic crystals of lysozyme belong to a polar symmetry class that can potentially demonstrate ferroelectricity. To investigate this, three successive scans were performed. The first two were polarisation scans; the first scan applied a 10 V bias to a 5 μm × 5 μm area and the second applied a 5 V bias to a 10 μm × 10 μm area. The third scan measured the piezoresponse of the 15 μm × 15 μm surrounding the previous two biasing scans using a voltage of 5 V at 20 kHz. The result of the third scan is depicted in Figure 6.12. The height image (Figure 6.12(a)) was 1D-flattened and horizontal scratches were removed post scan. The surface of monoclinic lysozyme is more rough than the surface of tetragonal lysozyme. The average roughness across the surface of monoclinic lysozyme is approximately 185 nm, therefore, mechanical cross-talk is possible.

The polarisation biases during the two proceeding scans has an effect on both the vertical (Figure 6.5 (c, d)) and lateral (Figure 6.12 (e, f)) piezoresponse images. For this particular crystal, the effect of polarising the crystal had a greater effect on the vertical signal compared to the lateral signal.
Figure 6.12: PFM response after biasing a monoclinic lysozyme crystal. White dashed lines indicate the region over which the biases were applied. The inner square was biased with +10 V and the larger square was biased with +5 V. Images of topography [(a) height, (b) deflection], vertical piezoresponse [(c) magnitude, (d) phase] and lateral piezoresponse [(e) magnitude, (f) phase] are shown. The scale bar in (a) is 3 µm. The scan was performed at 5 V and 20 kHz (adapted from (101))
In discerning the effect of topography on the contrast in the piezoresponse images of Figure 6.12, we notice three things. Firstly, the series of scans did not affect the topography of the crystal significantly. The outline of the second scan (10 µm × 10 µm) is just apparent in the deflection signal, Figure 6.12(b). Secondly, the areas of contrast in both the vertical and lateral piezoresponse do not resemble the topography of the surface. The areas of vertical and lateral PFM contrast do in fact correspond much more closely with the regions that were biased. Thirdly, both the vertical and lateral phase images (Figure 6.12 (e and f), respectively) show domains that are 180° apart. This is a sign that the direction of polarisation has been altered by the biasing scans.

In summary, these results imply that both monoclinic and tetragonal crystals of lysozyme demonstrate not just piezoelectricity but also appear to be polarizable, i.e. ferroelectric. From a classical viewpoint, monoclinic crystals of lysozyme belong to a polar symmetry class that can potentially demonstrate ferroelectricity. Even still, these observations are interesting as reports of ferroelectricity in biological materials are rare. What is even more intriguing is that tetragonal lysozyme also appears to be polarizable. As discussed in previous chapters, tetragonal crystals of lysozyme (point group 422) are non-polar and thus, ferroelectricity is not permitted. The polarizability observed here supports our earlier argument that the crystals may be of lower symmetry and belong to point group 4.

In section 6.7, ferroelectricity in lysozyme will be investigated further via SS-PFM. First, quantitative piezoelectric measurements of lysozyme via PFM will be discussed below.

6.6 Quantitative PFM Measurements

6.6.1 Verification with Poled PVDF

In this section, the quantitative PFM approach outlined in Chapter 3 will be applied to quantify the piezoresponse observed in monoclinic and tetragonal lysozyme. First, a piece of poled PVDF (Piezotech SAS, France) is measured to verify the quantitative approach. The poled PVDF should have a piezoelectric \( d_{33} \)-coefficient of +20.7 pC N\(^{-1}\).
according to the manufacturer’s datasheet. The error range associated with the $d_{33}$ coefficient of the poled PVDF is not reported in the datasheet nor is the measurement method.

The first step in the calibration is to determine the inverse-optical-sensitivity coefficient (IOS) for the particular tip/laser alignment by generating a force-distance curve on a reference material (in this case, an aluminium disk), as described in Chapter 3. We recall that the IOS coefficient relates the cantilever deflection (nA) and the tip-sample distance (nm). To calculate the IOS coefficient, force-distance curves are always taken on a hard substrate rather than the sample under investigation. A force distance curve performed on a soft sample (such as lysozyme) would produce a different slope and therefore an incorrect IOS coefficient would be determined. The tip used in this part of the study was conductive, coated with platinum (HA_C/Pt, Spectrum Instruments, Russia). Figure 6.13 shows a force-distance curve displaying both the approach and the retract traces as the tip is brought in to contact with the sample. The sharp dip is indicative of attractive forces, pulling the tip towards the sample. The IOS is calculated from the average of the forward and backward slopes of the force-distance plot. For this configuration of the tip and laser, the IOS is $0.0121$ nA nm$^{-1}$. In an ideal case, the forward and backward traces should be identical. The capillary force of the water monolayers on the surface of the film may affect the approach and retract traces differently resulting in the slight disparity between the forward and reverse slopes in Figure 6.13.
Determining how much the sample deforms in response to an applied voltage can be done in two ways. The first way involves extracting the magnitude of the piezoresponse manually from a single line within a regular PFM scan. The second way is to perform PFM at a single point. The latter is more convenient and was selected here. Figure 6.14 shows the magnitude of the piezoresponse at a point as the voltage is swept from 0 V to 50 V (forward) and then from 50 V to 0 V (backward). The magnitude of the piezoresponse increases with increasing applied voltage. For applied voltages greater than 30 V, the piezoresponse becomes non-linear.
Figure 6.14: Quantitative PFM performed on poled PVDF showing the magnitude of the piezoresponse to an applied voltage. The piezoelectric coefficient is determined from the slope of the linear part of the plot.

The slope of this graph (0.015 nA V\(^{-1}\)) is proportional to the piezoelectric coefficient of the sample. Recall that the magnitude of the piezoresponse in units of nano-ampere can be converted to displacement in units of nanometre using Equation 3.12 in Chapter 3. A piezoresponse magnitude of 0.015 nA is equivalent to a displacement of 0.0124 nm. This is the amount of displacement induced per applied voltage. Thus, the piezoelectric \(d_{33}\) coefficient for poled PVDF is 12.4 pm V\(^{-1}\). Because the direct and converse piezoelectric co-efficient are equal, 1 pC N\(^{-1}\) is equivalent to 1 pm V\(^{-1}\). Therefore, the \(d_{33}\) coefficient of poled PVDF quantified by PFM is less than that stated in the manufacturer’s datasheet (20.7 pC N\(^{-1}\)). In the literature, under-estimations of a samples piezoelectric coefficient via PFM have been attributed to the presence of an adsorbate layer on the sample surface (224). The adsorbate layer acts as voltage divider so that the applied voltage is overestimated and the corresponding piezoresponse is underestimated. Using a stiffer probe reduces this issue by providing better point contact, but this is not always feasible (225).
6.6.2 Quantitative PFM Measurements of Lysozyme

A soft tip (CSG30/Pt, National Instruments) was used for quantitative measurements of lysozyme crystals. The IOS coefficient for this tip/laser arrangement was determined from the slope of the force-distance curved performed on hard substrate. The IOS coefficient calculated in Figure 6.15(a) was 0.03 nA nm\(^{-1}\). PFM was performed at 100 points across the surface of the crystal of lysozyme. Figure 6.15(b) depicts a representation of the 10 x 10 grid of points at which single-point PFM was performed.

![Figure 6.15 (a) Force-Distance curve performed on a hard substrate to determine the inverse optical sensitivity (IOS) coefficient of the CSG30/Pt tip used to quantify the piezoresponse of lysozyme crystals. (b) A representative 10 x 10 grid illustrates the points on the sample surface at which PFM was performed.](image)

In the case of both tetragonal and monoclinic lysozyme, a voltage was swept from 0 V to 10 V at each point on the grid while monitoring the magnitude of its piezoresponse.

Figure 6.16 shows the average magnitude of the piezoresponse of tetragonal lysozyme obtained over six PFM points. The plot does not go through the origin, indicating that there is some contribution from electrostatic effects. Electrostatic interactions are also likely to be the cause of the non-linear piezoresponse. Electrostatic interactions are parasitic contributions that affect all types of samples, not just piezoelectric ones. The effect of electrostatic interactions can be corrected for by subtracting the background signal from the PFM signal (71). The background signal can be estimated in two ways. The first method involves performing a PFM scan across the positive and negative
domains of a sample of PPLN. If there is no electrostatic contribution, the PFM signal in the positive domain should be equal in magnitude but of opposite signal to the PFM signal obtained in the negative domain. However, if electrostatic interactions interfere with the measurement, the signals will not be of equal magnitude – the difference between them is the background signal. The second method estimates the background signal by performing PFM on a non-piezoelectric sample such as glass (226).

Unfortunately, measurements to estimate the background signal was not taken during this study in the University of Aveiro and therefore, the magnitude of the electrostatic contribution cannot be estimated and subtracted from the PFM measurements on lysozyme.

The piezoelectric coefficient is determined from the linear part of the plot indicated (i.e. from 2 V to 7.2 V). The slope in this region is 0.058 nA V$^{-1}$. Using the IOS coefficient to convert the units of the magnitude signal from nanoamperes to nanometers, this corresponds to a slope of 0.0193 nm V$^{-1}$. Thus the piezoelectric coefficient of tetragonal lysozyme as measured via PFM is 19.3 pm V$^{-1}$. 
Figure 6.16 Quantitative PFM performed on tetragonal lysozyme showing the average magnitude of the piezoresponse to an applied voltage. The piezoelectric coefficient is determined from the slope of the linear part of the plot.

Figure 6.17 shows the average magnitude of the piezoresponse of monoclinic lysozyme obtained over six PFM points. Again, a background signal prevents the plot from intersecting the origin. The piezoresponse is linear over the full voltage range, indicating that monoclinic lysozyme was less effected by electrostatic effects than the tetragonal lysozyme sample. We found that monoclinic lysozyme crystals were slightly more robust allowing better tip-sample contact which may have minimized electrostatic effects (227). The dashed line represents the line of best fit; the slope of which was 0.0083 nA V\(^{-1}\). This is converted to units of nanometer per volt using the IOS coefficient and Equation 3.12. Thus, the piezoelectric coefficient of monoclinic lysozyme as measured via PFM is 2.8 pm V\(^{-1}\).
The magnitude of the converse piezoelectric coefficient of lysozyme measured via PFM is larger than the magnitude of the direct piezoelectric coefficients measured using the Berlincourt method in Chapter 5. The converse piezoelectric coefficient of tetragonal lysozyme (19.3 pm V$^{-1}$) is three times greater than the largest $d_{33}$ value measured using the piezometer ($6.44 \pm 0.41$ pC N$^{-1}$). Similarly, the converse piezoelectric coefficient of monoclinic lysozyme (2.8 pm V$^{-1}$) is more than double the largest $d_{33}$ value measured using the piezometer ($1.34 \pm 0.01$ pC N$^{-1}$). Observing a larger piezoelectric coefficient via PFM compared to macroscopic methods is not unprecedented. The piezoelectric tensor of coefficients for collagen measured at the nanoscale via PFM (105) are four to ten times greater than those measured at the macroscale (18). In the case of lysozyme, the lower piezoelectric response obtained using macroscopic methods may reflect an overall averaging effect within the film. PFM measurements were performed on individual lysozyme crystals and would not have been effected in this way.
6.7 Investigating Ferroelectric Switching in Lysozyme with SS-PFM

To investigate the ferroelectric nature of lysozyme crystals further, switching-spectroscopy PFM (SS-PFM) was performed. As discussed in Chapter 3, SS-PFM applies voltage pulses to a specific area of the sample via the conductive AFM probe. If a sample is ferroelectric, the voltage pulses cause the spontaneous polarization of the sample underneath the tip to switch. Voltage pulses were applied from -10 V to +10 V (forward) and then from +10 V to -10 V (backward). The result of SS-PFM measurements performed on a monoclinic and a tetragonal crystal of lysozyme are shown in Figure 6.18 and Figure 6.19, respectively.

The shape of the loops obtained for monoclinic lysozyme in Figure 6.18 resemble ferroelectric loops, however, they are not. Both the magnitude and phase loop are of a closed nature. This type of behaviour is characteristic of a piezoelectric but non-ferroelectric material.
In contrast, the loops generated by performing SS-PFM on tetragonal lysozyme are characteristic of ferroelectric materials. The magnitude signal has a classic butterfly shape and the phase loop is open and switches polarity, Figure 6.19.
Figure 6.19: Characteristic ferroelectric (a) magnitude and (b) phase behaviour in a tetragonal single crystal of lysozyme in the forward and backward directions

The loops obtained for tetragonal lysozyme appear more noisy than those from monoclinic lysozyme; this may in part be because the number of data points recorded for the tetragonal lysozyme was half that recorded for monoclinic lysozyme. The loops are shifted slightly along the voltage axis. The asymmetric nature of the loops is
indicative of some internal bias within the sample. For ferroelectric materials, this internal bias is called an imprint field \((228)\). The mechanisms that underlie the phenomenon of imprint fields are not fully understood. Proposed mechanisms include asymmetrically distributed trapped space charges \((229)\), or the existence of a non-switching layer between the sample and the electrode caused by stress-induced lattice mismatch \((230)\). These mechanisms of imprint may also apply to lysozyme. As an alternative hypothesis, the internal bias observed here in lysozyme may arise from the bound water forming the so-called hydration layer. The hydration layer is polar and may be more difficult to switch in one direction than the other direction.

The experimental evidence here of ferroelectricity (i.e. lysozyme is polarisable and demonstrates ferroelectric loops) supports the hypothesis that tetragonal lysozyme may belong to a polar symmetry group, i.e. point group 4 rather than point group 422. As discussed in the methodology chapter, PFM and SS-PFM measurements are not without their limitations so we treat these observations tentatively.

### 6.8 Pyroelectricity in Films of Monoclinic Lysozyme

Pyroelectricity – the ability of certain materials to generate a current in response to a changing temperature – is a property of materials with polar symmetry. Monoclinic crystals of lysozyme belong to point group 2, which is polar. Thus, we hypothesize that films of monoclinic lysozyme will demonstrate pyroelectricity. This section first describes the development of a pyroelectric measurement system based on the Byer-Roundy method. Next, the pyroelectricity measurement system was tested and verified with a commercial PVDF standard. The system was used to investigate pyroelectricity in films of monoclinic lysozyme grown on ITO and on inter-digitated electrodes. The results of these measurements are presented.
6.8.1 Design, Development and Calibration of a Pyroelectricity Measurement System

The pyroelectric measurement system developed here is based on the Byer-Roundy Method described in Chapter 3. It is based on the principle that the pyroelectric current is proportional to rate of change of temperature. By measuring the pyroelectric current as a function of the rate of heating or cooling, the pyroelectric coefficient of the material can be established.

A schematic of the pyroelectric measurement system built for this study is shown in Figure 6.20. A Peltier heating stage (Linkam Scientific Instruments Ltd, UK) was used as the heating/cooling source. A Peltier controller (Linkam T95) controlled the set-point temperature and the rate of heating/cooling. The system has a temperature range of -20 °C to 120 °C. The rate of heating/cooling can be varied within the range 0.1 °C per minute to 20 °C per minute. The temperature controller uses an in-built thermocouple to control the temperature of the Peltier stage.

![Schematic of the pyroelectric measurement system.](image)

Figure 6.20 Schematic of the pyroelectric measurement system. The Peltier Stage is controlled by the Peltier controller and monitored with an external thermocouple. An electrometer measures the pyroelectric current generated. The system is automated with LabVIEW.

As discussed in Chapter 3, ensuring uniform heating and cooling is an essential criterion for Byer-Roundy type measurements. Therefore, a second external thermocouple was placed directly on the sample surface so that accurate measurements of sample temperature could be recorded throughout the measurement. An electrometer (Keithley 6514) measured the current through the sample. A custom LabVIEW program operated the Peltier controller, thermocouple and electrometer.
To verify that the pyroelectric measurement system worked correctly, the pyroelectric effect a commercial sample of poled PVDF (Piezotech SAS, France) was tested. The sample of poled PVDF was placed between two pieces of ITO-coated glass, which acted as electrodes. The area of the sample between the electrodes was 25 µm². The electroded PVDF sample was heated from 20 °C to 40 °C at a rate of 2 °C min⁻¹. Then the PVDF was cooled from 40 °C to 20 °C at the same rate. Measurements of current and temperature are recorded at 1 minute intervals.

Figure 6.21 shows the pyroelectric current generated from the sample of poled PVDF during the heating-cooling cycle. At the instant that the measurement begins, the current through the sample is positive. As the heating ramp is initiated, the sample of PVDF generates a negative pyroelectric current (approximately minus 20 pA). When the temperature ramp changes from heating to cooling, the pyroelectric current immediately switches polarity.

The average pyroelectric current generated during cooling is approximately +22 pA.
The pyroelectric coefficient calculated for this sample of PVDF is approximately 28 µC m⁻² K⁻¹. This compares well with literature values of the pyroelectric coefficient of PVDF \((p = 30 \mu\text{C m}^{-2}\text{K}^{-1})\) poled under similar conditions (184).

The measurement system was also tested with a negative control. The negative control was a piece of plain glass whose area was approximately 30 µm². As before, the sample was sandwiched between two pieces of ITO electrodes. The sample was cooled from room temperature to 11.4 °C at a rate of 4 °C per minute. Then it was heated from 11.4 °C to 36.3 °C with a heating rate of 4 °C per minute. This temperature cycle was repeated three times while recording the current and temperature. Figure 6.22 shows the current through the sample of glass the three heat cycles.
Figure 6.22 Testing the measurement system with negative control. (a) The current through a piece of glass is recorded over three heat cycles. (b) Replot of the area enclosed by the dashed box in (a).

The average current is just 0.45 pA. The current is predominantly positive, however, at higher temperatures it fluctuates between positive and negative. The region of the graph enclosed by the dashed box in Figure 6.22(a) is reprinted in Figure 6.22(b) to
observe the switching of current polarity in more detail.

The current is positive during heating and turns negative briefly when the temperature exceeds 30 °C. During the cooling ramp, the current turns positive again when the temperature goes below 33 °C. It remains positive (ignoring noise fluctuations) for the remainder of the cooling cycle. The change in current polarity does not coincide with the point in time when the temperature cycle changes from heating to cooling, as it would if it were a true pyroelectric current. The change in current polarity does not seem to be random. A similar brief change in current polarity is seen during the second and third temperature cycles also. We speculated that at temperatures above 30 °C thermally stimulated currents (TSCs) are activated. In this electrode configuration, the TSC adds negatively to the measured current.

6.8.2 Electrodng Samples of Monoclinic Lysozyme for Pyroelectricity Measurements

Films of monoclinic lysozyme were electroded in two different ways for pyroelectric measurements. The first electrode arrangement was essentially the same as that used for piezoelectric measurement of lysozyme films via the Berlincourt method outlined in the previous chapter. In this case, a film of lysozyme prepared by dropcasting was sandwiched between two pieces of ITO-coated glass. Electrical connection legs (Ossila, Sheffield, UK) were fitted to the pieces of ITO-coated glass allowing easy connection to the electrometer with crocodile clips.

The second electrode arrangement used inter-digitated electrodes (IDEs). Silver IDE’s were screen-printed using a commercial screen-printer (Fino Essemtec, Switzerland). Silver paint (9912-K, ESL, Europe) was pushed through a mesh screen (MCI Precision Screens Ltd, UK) on to an alumina substrate to create the desired IDE features. Figure 6.23 shows two different IDE templates (denoted wide IDE and fine IDE) that were used to design the mesh screen for screen-printing.
The wide IDE template produces an IDE with ten fingers of width 0.5 mm and with an inter-digit spacing of 0.5 mm. The fine IDE template produces an IDE with twenty fingers of width 0.2 mm and with an inter-digit spacing of 0.2 mm. The screen-printed IDE’s were fired in a furnace at 150 °C for 10 minutes and later at 850 °C for 30 minutes. The thickness of the IDEs post firing was 20 µm. Electrical wires were soldered to the IDE bond pads. The electrical wires were connected to the electrometer with crocodile clips. We note that because the IDEs were annealed in an ambient atmosphere, it is likely that oxidation occurred. The resulting IDEs are therefore silver-oxide IDE (although no chemical analysis was undertaken). As silver-oxide is less conductive than pure silver, the pyroelectric current measured in the following studies may be undervalued. Continuity between the silver-oxide IDEs and the electrometer inputs was verified with a multimeter before measurements.
6.8.3 Pyroelectricity Measurements of Lysozyme using ITO Electrodes

The first measurements of monoclinic lysozyme were performed on films sandwiched between ITO-coated glass. To improve the resolution, the number of data points recorded was increased from 1 data point per minute to 1 data point per second. The temperature range was selected so that it was well below the temperature at which lysozyme denatures and well above its freezing temperature. The temperature cycle comprised of three stages. During the first stage, the film of monoclinic lysozyme was cooled from 18.1 °C to 5.1 °C. Pyroelectric measurements at these low temperatures may be problematic because it coincides with the dew point. No obvious condensation was observed on the lysozyme films throughout the measurement. Furthermore, because the crystals were kept hydrated within the film, the effect of surface moisture may not adversely affect the measurement as significantly as it would with dry samples. The rate of cooling was set to 0.5 °C per minute. The actual temperature of the sample throughout the experiment was measured by the external thermocouple. The actual rate of cooling determined from the slope of the negative line in Figure 6.24(a) was 0.496 °C min⁻¹. The cooling ramp was linear with an R-squared value of 0.9998. The second stage of the heat cycle kept the temperature fixed at 5.1 °C for 25 minutes. The purpose of the ‘hold’ interval was to allow the sample time to stabilise at the minimum temperature value before initiating the heating ramp. During the third stage, the film was heated from 5.1 °C to 17.9 °C. The rate of heating was set at 0.5 °C min⁻¹. The actual rate achieved was 0.74 °C min⁻¹ as determined from the slope of the heating ramp. This is because the sample tried to return to ambient temperature at a rate that the Peltier stage could not fully compensate for. The heating ramp was not perfectly linear with a few small discontinuities where the Peltier tried to readjust the temperature. Overall, the linearity of the heating cycle is good with an R-squared value of 0.9967.
Figure 6.24 Pyroelectric effect in a film of monoclinic lysozyme sandwiched between two pieces of ITO-coated glass. (a) The current generated switches polarity becoming negative during the cooling cycle and positive during the heating cycle. (b) Temperature and current generated during the interval indicated by dashed box in (a) - the current becomes negative at approximately 9 °C.
The current through the sample at each stage of the temperature cycle is shown in Figure 6.24(a). For clarity, the section of the plot indicated by the dashed box in Figure 6.24(a) is rescaled and replotted in Figure 6.24(b). For the first 3.5 minutes of the measurement, the current through the sample fluctuates from positive to negative. After this, as the temperature decreases the current is approximately constant with an average positive current of 1.65 pA. At a temperature of 8.6 °C, the current switches polarity and becomes negative. It remains negative for the rest of the cooling ramp.

During the hold interval, the current stays negative. The magnitude of the current varies throughout this interval reaching a maximum value of 870 pA. When the heating ramp is started, the current decreases rapidly towards zero, changing to a positive current at approximately 9 °C. The current remains positive until the temperature exceeds approximately 15 °C where it becomes negative again.

The current measured through the monoclinic film of lysozyme in the temperature interval 5 °C to 9 °C is negative during cooling and positive during heating. The fluctuations in current outside of this temperature range are not consistent with pyroelectricity and may be the result of parasitic TSCs. The observed change of current polarity when the temperature ramp changed from heating to cooling during certain parts of the cycle warranted further investigation.

The choice of electrode arrangement used in this study may not be most appropriate. We recall that the pyroelectric current develops parallel to the polar axis of the crystal. For monoclinic crystals in point group 2, the polar axis is parallel to the diad axis (83), i.e. the long axis (b-axis) of the crystal. The monoclinic lysozyme crystals within the film are randomly orientated. Only crystals within the film whose polar axis is parallel to the electrodes would have contributed significantly to the measured current.

6.8.4 Pyroelectricity Measurements of Lysozyme using Wide IDE Electrodes

In the next experiment, the electrode used was the wide IDE described above. The wide IDE measures the current through crystals that touch two adjacent IDE fingers. In general, only crystals orientated with their b-axis perpendicular to the IDE fingers where long enough to touch two adjacent IDE fingers. Those crystals orientated with
their b-axis parallel to the IDE fingers presumably did not contribute to the measurement.

Before either the heating or cooling cycles were initiated, the sample was held at room temperature for 6.8 minutes under short-circuit conditions. As the pyroelectric current is proportional to the rate of heating/cooling, a higher temperature rate was selected in order to induce a larger current. The result of this measurement is shown in Figure 6.25. During the heating ramp, the film of monoclinic lysozyme was heated from 21.5 °C to 43.5 °C at a rate of 5 °C min⁻¹ (actual rate achieved was 4.30 °C min⁻¹). During the cooling ramp, the film cooled from 43.5 °C to 4.5 °C at a rate of 4 °C min⁻¹ (actual rate achieved was 4.43 °C min⁻¹). The rate of heating and cooling were linear (R-squared value of 0.9978 and 0.9887, respectively).

Figure 6.25 Pyroelectric effect in a film of monoclinic lysozyme dropcast on a wide IDE showing the reversal of current polarity when the temperature ramp is switched from heating to cooling.
Figure 6.25 shows the current generated from the film of monoclinic lysozyme on the wide IDE throughout the temperature cycle. During the hold interval, the current signal is affected by noise and oscillates between positive and negative. When the heating ramp is started, the current generated becomes negative. It reaches a maximum negative value of approximately -11nA at 26 °C. Above this temperature, the current tends towards 0 nA, becoming positive briefly in the temperature range between 29.5 °C and 33.3 °C. After this point, the current turns negative again and remains so for the remainder of the heating ramp.

When the temperature ramp changes from heating to cooling, the current does not switch polarity immediately. At approximately 35 °C, the current switches polarity and becomes positive. The current remains positive for the remainder of the cooling cycle, reaching a maximum positive value at 18.4 °C. The delay in switching polarity upon changing from heating to cooling indicates that TSC may dominate in this temperature region.

The reversal of current polarity (positive during heating and negative during cooling) is strongly indicative of the pyroelectric effect.

6.8.5 Pyroelectricity Measurements of Lysozyme using Thin IDE Electrodes

In the third and final study of pyroelectricity, the fine IDE arrangement was used. The pyroelectric current is proportional to the area of the sample electroded. By using the fine IDE, the number of monoclinic lysozyme crystals that are sufficiently large to touch two adjacent fingers is increased. Thus, the area of sample electroded is increased. The IDE spacing is still sufficient that crystals orientated with their polar axis parallel to the IDE electrodes do not contribute to the measurement.

A second modification was made for this study – the number of heat cycles was increased to five. By subjecting the sample to several heat cycles, trapped charges that contribute to TSCs are released so that subsequent cycles are less effected.

The film of monoclinic lysozyme was first cooled from 23.9 °C to 8.6 °C. The rate of
cooling was set to 5 °C min\(^{-1}\) (actual cooling rate was 4.17 °C min\(^{-1}\)). Then the film was heated at the same rate (actual heating rate was 5.11 °C min\(^{-1}\)) from 8.6 °C to the maximum temperature of 35.5 °C. This temperature cycle was repeated five times while measuring the current generated.

Figure 6.26 shows the temperature cycles applied to monoclinic films of lysozyme and the current measured using the fine IDEs. Although the signal contains noise, there is a clear change in polarity of the current in each measurement cycle when the temperature ramp changes from heating to cooling and \textit{vice versa}. The red annotated line in Figure 6.26 highlights this trend.

If we examine the current during the heat cycle in more detail, we see that just before the cooling cycle is initiated, the current is positive. When the cooling ramp begins,
the current immediately becomes negative. It stays negative throughout the cooling cycle. The current only changes polarity to positive when the heating ramp changes from cooling to heating. The current reaches its maximum positive value (taken from the red curve) at 25 °C. As the temperature continues to increase beyond this temperature, the current decreases towards zero but remains positive throughout the heating cycle. The current signal is clearly affected by noise. The noise affects the current during the heating cycle to a greater extent than during the cooling cycle.

Throughout the five temperature cycles, the monoclinic film of lysozyme responds in a similar manner to the cycle just described. Apart from the first temperature cycle, the current switches polarity immediately each time the cooling cycle is initiated. In contrast, when the heating cycle is initiated there is a slight delay before the current switches polarity in all but one of the temperature cycles. This indicates that during heating, TSCs contribute the pyroelectric current, delaying the polarity switch. The contribution of TSC to the pyroelectric current persists over the five temperature cycles. TSCs are irreversible thermoelectric effects, thus, they do not switch polarity if the heating cycle changes from heating to cooling. Thus, the reversible current observed here is pyroelectric in nature.

6.9 Conclusions
This chapter presented evidence of converse piezoelectricity in lysozyme. This is the first report of converse piezoelectricity in a non-fibrous type of protein. The piezoelectric coefficient of monoclinic and tetragonal lysozyme quantified by PFM is 2.8 pm V\(^{-1}\) and 19.3 pm V\(^{-1}\), respectively. Polarisation scans indicated that both monoclinic and tetragonal lysozyme may have switchable polarisation states. However, this was only confirmed via SS-PFM in the case of tetragonal lysozyme.

Observing converse piezoelectricity in lysozyme addresses the averaging argument laid out at the beginning of the chapter. Because we see converse piezoelectricity from an individual crystal, we can conclude that the longitudinal piezoelectricity observed at the macroscale via the Berlincourt Method was not solely the result of averaging.

The second argument under test in this chapter was whether or not the tetragonal
lysozyme crystals studied here belong to a lower symmetry group than typically ascribed to it. Three pieces of data reported in this chapter support that argument. Firstly, longitudinal piezoelectricity was measured in tetragonal lysozyme via PFM. A longitudinal piezoelectric response is only supported by crystals in point group 4, and not by crystals in point group 422. Secondly, tetragonal lysozyme was polarisable – as evidenced by polarisation scans. This indicates that tetragonal lysozyme may have a switchable polarisation state characteristic of ferroelectric materials. Thirdly, SS-PFM loops on tetragonal lysozyme were characteristic of ferroelectric materials. As ferroelectricity is not permitted in point group 422, these data support the hypothesis of symmetry lowering.

Finally, pyroelectricity was observed in monoclinic crystals of lysozyme using an adapted Byer-Roundy method. Monoclinic lysozyme is described by polar point group symmetry and thus, this observation agrees with the hypothesis made in Chapter 1.
Chapter 7  Conclusions and Future Work

7.1 Summary of the Key Findings

The objective of this thesis was to investigate whether the protein lysozyme conforms to the same definitions of piezoelectricity, pyroelectricity and ferroelectricity as classical solid-state materials.

The main findings of this study are:

- Crystals of lysozyme can be grown as films on conductive substrates to facilitate electrical and electromechanical measurements of lysozyme. Films of monoclinic and tetragonal lysozyme were grown using a vapour diffusion method modified to incorporate a small volume of glycerol to prevent the crystals from drying out.

- The structure of the crystals within the films was determined. The structure of monoclinic films of lysozyme was point group 2. The structure of tetragonal films of lysozyme was determined by synchrotron diffraction as point group 422.

- Electromechanical measurements performed on films of lysozyme showed that both monoclinic and tetragonal films of lysozyme demonstrate electromechanical coupling consistent with piezoelectricity.

- The direct piezoelectric effect was measured in tetragonal films of lysozyme using a quasi-static methods based on the Berlincourt method. The average $d_{33}$ co-efficient for tetragonal lysozyme films was $3.13 \pm 2.07 \text{ pC N}^{-1}$. The largest $d_{33}$ coefficient measured for tetragonal films of lysozyme was $6.44 \pm 0.41 \text{ pC N}^{-1}$.

- The direct piezoelectric effect was measured in monoclinic films of lysozyme using a quasi-static methods based on the Berlincourt method. The average $d_{33}$
co-efficient for monoclinic lysozyme films was $1.07 \pm 0.27 \text{ pC } N^{-1}$ and the largest $d_{33}$ coefficient measured was $1.34 \pm 0.01 \text{ pC } N^{-1}$.

- The direct piezoelectric effect was confirmed in both tetragonal and monoclinic films of lysozyme using a static method based on the Berlincourt method. The fact that the voltage generated from films of lysozyme in response to applied force was linear verified the direct piezoelectric effect in films of lysozyme.

- The converse piezoelectric effect was measured and quantified in individual crystals of tetragonal and monoclinic lysozyme using PFM. The converse piezoelectric coefficient for tetragonal and monoclinic lysozyme were measured as $19.3 \text{ pm } V^{-1}$ and $2.8 \text{ pm } V^{-1}$, respectively. These values are larger than that obtained by macroscopic methods and may be because PFM measurements were not subject to averaging effects.

- DC bias scans applied to tetragonal and monoclinic films using PFM indicated that both types of crystals may be polarizable

- SS-PFM loops obtained on monoclinic lysozyme indicated that it is piezoelectric but not ferroelectric

- Measurements on tetragonal lysozyme indicate that it may be ferroelectric as evidenced by the characteristic butterfly and switching phase loops obtained using SS-PFM. The asymmetric nature of the loops obtained may reflect an internal bias produced by the bound water of the protein hydration layer.

- A modified Byer-Roundy method applied to films of monoclinic lysozyme indicated that polar crystals of lysozyme are pyroelectric.
7.2 Answers to the Research Questions

The results and discussion presented in this thesis allow us to address the research questions proposed in Chapter 1.

**Research question 1: Should lysozyme exhibit the piezoelectric effect?**

Films of both monoclinic and tetragonal lysozyme exhibited the piezoelectric effect. Both crystal forms are non-centrosymmetric fulfilling the most fundamental requirement of classical piezoelectricity.

**Research question 2: Can the direct and converse piezoelectric effect be quantitatively measured in lysozyme using conventional methods?**

Conventional methods for measuring the piezoelectric effect can be applied to crystals of lysozyme. Lysozyme crystals can be electroded by growing them within a film on an electrode substrate. The Berlincourt Method is an appropriate method of quantifying the direct piezoelectric effect in films of lysozyme. Two variations of the Berlincourt method, static and quasi-static, verified the direct effect in lysozyme. PFM can be used to investigate the converse piezoelectric effect in individual crystals of lysozyme. However, quantitative measurement using this technique may underestimate the converse piezoelectric coefficient.

**Research question 3: Should lysozyme demonstrate ferroelectricity and pyroelectricity and can these properties be measured?**

Polar crystals of lysozyme should show ferroelectricity and pyroelectricity. Ferroelectricity in lysozyme crystals was investigated by polarising PFM scans and by SS-PFM measurements. Both methods indicated that tetragonal lysozyme may be ferroelectric. This observation challenges the structural characterisation study which assigned tetragonal lysozyme to a non-polar symmetry group. Ferroelectricity was indicated in monoclinic lysozyme by polarisation scans but was not verified by SS-PFM. Pyroelectricity was measured in monoclinic lysozyme using an adapted Byer-Roundy method.
7.3 Testing the Research Hypothesis

Based on the results presented in this thesis, the research hypothesis can be tested and evaluated as follows:

**Hypothesis 1:** Non-centrosymmetric crystals of lysozyme should be piezoelectric

This hypothesis has been proven correct. Two types of non-centrosymmetric crystals were tested for piezoelectricity and both demonstrated a significant piezoelectric effect using conventional methods. Furthermore, both the direct and converse piezoelectric effect were observed in crystals of lysozyme.

**Hypothesis 2:** The tensor of piezoelectric coefficients for lysozyme are determined by its crystal symmetry

The proof of this hypothesis is not straightforward. In the case of monoclinic lysozyme, the crystal structure did determine piezoelectric tensor of coefficients. Both longitudinal and shear piezoelectric coefficients were measured and correlate with the tensor of piezoelectric coefficients for monoclinic lysozyme described by point group 2.

However, for the case of tetragonal lysozyme proof of this hypothesis is complicated by two contradictory results. Synchrotron diffraction studies determined the structure of tetragonal lysozyme as belonging to point group 422. Based on this assignment, only shear piezoelectric coefficients are permissible. However, longitudinal piezoelectricity was observed by three methods, quasi-static and static Bellincourt methods and PFM. An argument to reconcile the observed piezoelectric tensor was proposed in which they point group describing tetragonal lysozyme was lowered to point group 4. SS-PFM measurements indicated that the crystals may be polar, supporting this argument.
Hypothesis 3: Crystals of lysozyme with polar symmetry should be pyroelectric and can be measured by conventional methods

This hypothesis has been proven. Pyroelectricity was qualitatively measured in monoclinic films of lysozyme which have polar symmetry. A conventional Byer-Roundy method was used successfully to detect the pyroelectric effect. Of the three electrode arrangements tested, an inter-digitated electrode with narrow spacings was found to be the most appropriate.

7.4 Limitations and Future Directions

This work presented in this thesis represents the first attempt at measuring and understanding piezoelectricity in lysozyme and indeed in any non-fibrous protein. Thus, the potential for future studies in this area is enormous. In this last section, suggested future works to address the limitations of the current work and to extend it further are proposed:

- The study of protein piezoelectricity was limited in this study to lysozyme. As all protein crystals are non-centrosymmetric, an obvious future study would be to investigate piezoelectricity in other proteins. Furthermore, future studies may be broadened to include the third category of protein, i.e. integral membrane proteins. As discussed in Chapter 1, the transmembrane protein prestin confer OHCs with electromotility (67). The methods used and approaches taken in this thesis should guide and facilitate future work on protein piezoelectricity and allow technical applications to be realised in the future.

- In this work, films of protein crystals were prepared by drop-casting. Future works may focus on improving density of crystals within the film and the uniformity of crystal orientation within the film. Epitaxial growth may be a suitable methods to improve film quality (200, 216).

- Protein piezoelectricity was probed using two conventional methods in this
thesis. Another avenue for further research would be to employ other conventional methods of piezoelectric measurement to the study of protein piezoelectricity. Laser-interferometry and resonance analysis were not pursued in this thesis for the technical reasons given in Chapter 3 as well as for practical reasons of accessibility. However, these techniques may offer a new approach from which to study protein piezoelectricity and in the case of resonance analysis might be realised by using a probe-station to make electrical contact with the protein crystals.

- The observations of ferroelectricity in tetragonal lysozyme were made possible by SS-PFM, a technique with many limitations. To complement the measurements presented in this thesis, conventional ferroelectric measurements performed at the macro-scale (i.e. Tower-Sawyer measurements) would supplement the findings of this thesis.

- This thesis took a solely experimental approach towards understanding protein piezoelectricity, pyroelectricity and ferroelectricity. Studies using computational simulations would complement the experimental work presented here.

- The properties of piezoelectricity, ferroelectricity and pyroelectricity were conducted on lysozyme in its crystallised form. While this offered a unique perspective from which to investigate protein piezoelectricity, future studies focused on non-crystallised lysozyme (or other proteins) might investigate if the inherent chirality of proteins is sufficient to sustain piezoelectricity, pyroelectricity and/or ferroelectricity.
References

21. Lemanov V. Piezoelectric and pyroelectric properties of protein amino acids
60. Li L, Li C, Zhang Z, Alexov E. On the Dielectric “Constant” of Proteins:


94. Harden JE. Electro-Mechanical Couplings in Liquid Crystals: Kent State University; 2009.


218. NTMDT. PFM03 Test Pattern for Piezoresponse Force Microscopy. p. 63-4.

Appendices

Appendix A: Comments on Paper by Danielewicz-Ferchmin et al. (2011)

The paper published by Danielewicz-Ferchmin et al. (61) deals with electromechanical coupling in lysozyme solution, based on observations made by Ortore et al. (62). The observation was that the density of hydration water at the surface of lysozyme is greater than in the bulk water under the same applied pressure.

The basic premise of the argument put forward by Danielewicz-Ferchmin et al. is that the difference in water density at different applied pressures is due to a piezoelectric effect in lysozyme which responds to the applied pressure by developing a charge at its surface. The surface charge of lysozyme is proposed to produce an electric field that compresses the water close to the surface via electrostriction.

In Chapter 1 of this thesis, we noted that the piezoelectric coefficient calculated by Danielewicz-Ferchmin et al. was incorrect. Here, we outline how we came to that conclusion.

Figure A. 1 shows the original plot of the relative mass density of the hydration water ($\rho_1$) to the mass density of bulk water ($\rho_o$) as the pressure is increased on which the observations are based.
The data points shown in Figure A.1 were extracted and used to calculate the total pressure ($P_b$), the electrostriction pressure ($\Pi$) and the surface charge density ($\sigma$). These parameters are shown in the table below, Figure A.2(a). Then to show the so-called piezoelectric effect in lysozyme, the calculated surface charge density was plotted as a function of the applied pressure, Figure A.2(b).
Table showing the relative mass density ($\rho_l/\rho_o$) of the hydration layer of lysozyme measured under pressure $P_h$ and the calculated total pressure ($P^b$), electrostriction pressure ($\Pi$) and mean surface charge density ($\sigma$) (61).

(b) Plot of the mean surface charge density of lysozyme as a function of applied pressure. The line of best-fit (solid line) does not intercept the origin. Adapted from (61)

The line of best-fit (solid line) does not intercept the origin. The relationship between the charge generated and the mechanical stress applied should be linear with no offset. In Figure A.3 the data is replotted by us and fitted through the origin (dashed line) for comparison. The line fitted through the origin clearly does not fit the data well ($R^2 = 0.669$).
Calculation of Piezoelectric Coefficient

Danielewicz-Ferchmin et al. used the slope of the graph in Figure A.2(b) to calculate the effective piezoelectric coefficient of lysozyme as follows:

\[
d = \frac{\Delta \sigma}{\Delta P^h} = 2.5 \times 10^{-10} \text{ C m}^{-2}
\]  \hspace{1cm} \text{A.1}

However, this calculation is incorrect. Ignoring the fact that the line does not intercept the origin, the slope of the best-fit line in Figure A.2(b) should be:

\[
\frac{\Delta \sigma}{\Delta P^h} = 2.52 \times 10^{-4} \frac{\text{C m}^{-2}}{\text{MPa}} = 2.52 \times 10^{-10} \frac{\text{C m}^{-2}}{\text{N m}^{-2}} = 252 \text{ pC N}^{-1}
\]  \hspace{1cm} \text{A.2}

This value is strikingly large; if it were to represent the piezoelectric co-efficient of lysozyme it would be approximately 100 times that of quartz.
Appendix B: Effect of Crystal Symmetry on the Number of Independent Moduli in the Piezoelectric Tensor

The full piezoelectric tensor is given by:

\[
\begin{pmatrix}
  d_{111} & d_{112} & d_{113} & d_{211} & d_{212} & d_{213} & d_{311} & d_{312} & d_{313} \\
  d_{121} & d_{122} & d_{123} & d_{221} & d_{222} & d_{223} & d_{321} & d_{322} & d_{323} \\
  d_{131} & d_{132} & d_{133} & d_{231} & d_{232} & d_{233} & d_{331} & d_{332} & d_{333}
\end{pmatrix}
\]  

B.1

As discussed in Section 2.1.2, the piezoelectric tensor $d_{ijk}$ is symmetrical in $j$ and $k$, so that $d_{ijk} = d_{ikj}$, reducing the number of independent components from 27 to 18 as given by Equation B.2.

\[
\begin{pmatrix}
  d_{111} & d_{112} & d_{113} & d_{211} & d_{212} & d_{213} & d_{311} & d_{312} & d_{313} \\
  0 & d_{122} & d_{123} & 0 & d_{222} & d_{223} & 0 & d_{322} & d_{323} \\
  0 & 0 & d_{133} & 0 & 0 & d_{233} & 0 & 0 & d_{333}
\end{pmatrix}
\]  

B.2

Crystal symmetry operations reduce the piezoelectric tensor further. By way of example, the reduction in independent moduli for a crystal with a centre of symmetry is discussed. A stress applied to the crystal induces a polarisation. Applying the centre of symmetry operation to the whole system (crystal, stress and resulting polarisation) has no effect on the crystal or the stress – they remain unchanged, as they are centrosymmetric themselves. However, the direction of the polarisation is reversed. This is only possible if the polarisation is zero; thus, centrosymmetric crystals cannot demonstrate a net polarisation under stress and are not piezoelectric. The same symmetry argument can be applied to determine whether individual moduli should be zero or non-zero in different crystal classes (83).

Another approach is to use an analytical direct inspection method. The method is best described with an example. We consider the effect of a 2-fold axis of rotation parallel to the $x_3$-axis on the number of independent piezoelectric moduli allowed. The effect of the 2-fold axis on the axis co-ordinates $x_1$, $x_2$ and $x_3$ is as follows

$x_1 \rightarrow -x_1, \quad x_2 \rightarrow -x_2, \quad x_3 \rightarrow x_3$  

B.3
The components of a tensor transform like the corresponding products of their coordinates, e.g. $d_{112}$ transforms in the same manner as $x_1^2 x_2$. Therefore,

$$d_{112} \rightarrow -d_{112} \quad \text{B.4}$$

However, because the crystal has a 2-fold rotation axis

$$d_{112} \rightarrow +d_{112} \quad \text{B.5}$$

The only way Equations B.4 and B.5 may be reconciled is if $d_{112}$ is zero. In the same way, the other moduli can be inspected. The overall effect of a 2-fold axis of rotation on the piezoelectric tensor is given by Equation B.6.

$$
\begin{pmatrix}
0 & 0 & d_{113} & 0 & 0 & d_{213} & d_{311} & d_{312} & 0 \\
0 & 0 & d_{123} & 0 & 0 & d_{223} & 0 & d_{322} & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & d_{333}
\end{pmatrix}
\quad \text{B.6}
$$

Employing Table 2.3 to transform the components from three subscripts to just two, Equation B.6 can be rewritten in the more familiar form as

$$
\begin{pmatrix}
0 & 0 & 0 & d_{14} & d_{15} & 0 \\
0 & 0 & 0 & d_{24} & d_{25} & 0 \\
d_{31} & d_{32} & d_{33} & 0 & 0 & d_{36}
\end{pmatrix}
\quad \text{B.7}
$$

Thus, the effect of a 2-fold axis of rotation on the piezoelectric matrix is to reduce it to just eight independent components.
Table C.1 The components of the piezoelectric matrix for each of the non-centrosymmetric crystal point groups. Adapted from (9, 83, 102)

<table>
<thead>
<tr>
<th>Point Group</th>
<th>Piezoelectric Matrix</th>
<th>Point Group</th>
<th>Piezoelectric Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$d_{11} \ d_{12} \ d_{13} \ d_{14} \ d_{15} \ d_{16}$</td>
<td>mm2</td>
<td>$0 \ 0 \ 0 \ 0 \ d_{15} \ 0$</td>
</tr>
<tr>
<td></td>
<td>$d_{21} \ d_{22} \ d_{23} \ d_{24} \ d_{25} \ d_{26}$</td>
<td></td>
<td>$0 \ 0 \ 0 \ 0 \ 0 \ 0$</td>
</tr>
<tr>
<td></td>
<td>$d_{31} \ d_{32} \ d_{33} \ d_{34} \ d_{35} \ d_{36}$</td>
<td></td>
<td>$d_{31} \ d_{32} \ d_{33} \ 0 \ 0 \ 0$</td>
</tr>
<tr>
<td>2</td>
<td>$0 \ 0 \ 0 \ d_{14} \ 0 \ d_{16}$</td>
<td>42m</td>
<td>$0 \ 0 \ 0 \ 0 \ 0 \ d_{14}$</td>
</tr>
<tr>
<td></td>
<td>$d_{21} \ d_{22} \ d_{23} \ 0 \ d_{25} \ 0$</td>
<td></td>
<td>$0 \ 0 \ 0 \ 0 \ 0 \ 0$</td>
</tr>
<tr>
<td></td>
<td>$0 \ 0 \ 0 \ 0 \ d_{33} \ 0 \ d_{36}$</td>
<td></td>
<td>$0 \ 0 \ 0 \ 0 \ 0 \ 0$</td>
</tr>
<tr>
<td>m</td>
<td>$d_{11} \ d_{12} \ d_{13} \ 0 \ d_{15} \ 0$</td>
<td>3</td>
<td>$d_{11} \ -d_{11} \ 0 \ d_{14} \ d_{15} \ -2d_{22}$</td>
</tr>
<tr>
<td></td>
<td>$0 \ 0 \ 0 \ 0 \ d_{24} \ 0 \ d_{26}$</td>
<td></td>
<td>$-d_{22} \ d_{22} \ 0 \ d_{15} \ -d_{14} \ -2d_{11}$</td>
</tr>
<tr>
<td></td>
<td>$d_{31} \ d_{32} \ d_{33} \ 0 \ d_{36}$</td>
<td></td>
<td>$d_{31} \ d_{31} \ d_{33} \ 0 \ 0 \ 0$</td>
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<tr>
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<td>$0 \ 0 \ 0 \ d_{14} \ 0 \ 0$</td>
<td>3m</td>
<td>$0 \ 0 \ 0 \ 0 \ d_{14} \ d_{15} \ -2d_{22}$</td>
</tr>
<tr>
<td></td>
<td>$0 \ 0 \ 0 \ 0 \ d_{25} \ 0$</td>
<td></td>
<td>$-d_{22} \ d_{22} \ 0 \ d_{15} \ -d_{14} \ 0$</td>
</tr>
<tr>
<td></td>
<td>$0 \ 0 \ 0 \ 0 \ 0 \ d_{36}$</td>
<td></td>
<td>$d_{31} \ -d_{31} \ 0 \ 0 \ 0 \ 0$</td>
</tr>
<tr>
<td>4</td>
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<td>4</td>
<td>$0 \ 0 \ 0 \ 0 \ d_{14} \ d_{15} \ 0$</td>
</tr>
<tr>
<td></td>
<td>$0 \ 0 \ 0 \ d_{15} \ -d_{14} \ 0$</td>
<td></td>
<td>$0 \ 0 \ 0 \ 0 \ -d_{15} \ d_{14} \ 0$</td>
</tr>
<tr>
<td>6</td>
<td>$d_{31} \ d_{31} \ d_{33} \ 0 \ 0 \ 0$</td>
<td>422</td>
<td>$0 \ 0 \ 0 \ 0 \ d_{14} \ 0 \ 0$</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>$0 \ 0 \ 0 \ 0 \ -d_{14} \ 0$</td>
</tr>
<tr>
<td>4mm</td>
<td>$0 \ 0 \ 0 \ 0 \ d_{15} \ 0$</td>
<td>622</td>
<td>$0 \ 0 \ 0 \ 0 \ 0 \ 0$</td>
</tr>
<tr>
<td>6mm</td>
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<td></td>
<td>$0 \ 0 \ 0 \ 0 \ -d_{14} \ 0$</td>
</tr>
<tr>
<td></td>
<td>$d_{31} \ d_{31} \ d_{33} \ 0 \ 0 \ 0$</td>
<td></td>
<td>$0 \ 0 \ 0 \ 0 \ 0 \ 0$</td>
</tr>
<tr>
<td>6</td>
<td>$d_{11} \ -d_{11} \ 0 \ 0 \ 0 \ -2d_{22}$</td>
<td>32</td>
<td>$0 \ 0 \ 0 \ 0 \ d_{14} \ 0 \ -2d_{22}$</td>
</tr>
<tr>
<td></td>
<td>$-d_{22} \ d_{22} \ 0 \ 0 \ 0 \ -2d_{11}$</td>
<td></td>
<td>$-d_{22} \ d_{22} \ 0 \ 0 \ -d_{14} \ 0$</td>
</tr>
<tr>
<td></td>
<td>$0 \ 0 \ 0 \ 0 \ 0 \ 0$</td>
<td></td>
<td>$0 \ 0 \ 0 \ 0 \ 0 \ 0$</td>
</tr>
<tr>
<td>23</td>
<td>$0 \ 0 \ 0 \ d_{14} \ 0 \ 0$</td>
<td>6m2</td>
<td>$d_{11} \ -d_{11} \ 0 \ 0 \ 0 \ -2d_{11}$</td>
</tr>
<tr>
<td></td>
<td>$0 \ 0 \ 0 \ 0 \ d_{14} \ 0$</td>
<td></td>
<td>$0 \ 0 \ 0 \ 0 \ 0 \ -2d_{11}$</td>
</tr>
<tr>
<td></td>
<td>$0 \ 0 \ 0 \ 0 \ 0 \ d_{14}$</td>
<td></td>
<td>$0 \ 0 \ 0 \ 0 \ 0 \ 0$</td>
</tr>
</tbody>
</table>

Table C.1

The piezoelectric tensors of the remaining point groups ($\bar{1}$, 2/m, mmm, 4/m, 4/mmm, $\bar{3}$, 3/m, 6/mmm, 6/m, 3m, m3m and 432) are zero.
Appendix D: Derivation of the Constitutive Piezoelectric Equations from Thermodynamics

The thermal, electrical and mechanical properties of a crystal are related, as illustrated in Figure D.1.

Figure D. 1 Illustration of the relationship between thermal, electrical and mechanical properties of a crystal. Adapted from (83)

In the corner of the outer triangle lie the independent variable, stress ($\sigma$), electric field ($E$) and temperature ($T$). The dependent parameters, strain ($\varepsilon$), electric displacement ($D$) and entropy ($S$) lie at the corners of the inner triangle. The bold lines connecting points on the outer triangle and inner triangle represent the direct effects: elasticity, heat capacity and permittivity. The other lines represent coupled effects and are labelled in the figure.
The independent variables are $\sigma_{ij}$, $E_i$ and $T$. The dependent variables are $\varepsilon_{ij}, D_i$ and $S$. The physical properties are determined by taking the differentials of $\varepsilon_{ij}, D_i$ and $S$ with respect to the independent variables.

\[
d\varepsilon_{ij} = \left( \frac{\partial \varepsilon_{ij}}{\partial \sigma_{kl}} \right)_{E_i,T} d\sigma_{kl} + \left( \frac{\partial \varepsilon_{ij}}{\partial E_i} \right)_{\sigma_{ij},T} dE_i + \left( \frac{\partial \varepsilon_{ij}}{\partial T} \right)_{\sigma_{ij},E_i} dT \tag{D.1}
\]

\[
dD_i = \left( \frac{\partial D_i}{\partial \sigma_{ij}} \right)_{E_i,T} d\sigma_{ij} + \left( \frac{\partial D_i}{\partial E_i} \right)_{\sigma_{ij},T} dE_i + \left( \frac{\partial D_i}{\partial T} \right)_{\sigma_{ij},E_i} dT \tag{D.2}
\]

\[
dS = \left( \frac{\partial S}{\partial \sigma_{ij}} \right)_{E_i,T} d\sigma_{ij} + \left( \frac{\partial S}{\partial E_i} \right)_{\sigma_{ij},T} dE_i + \left( \frac{\partial S}{\partial T} \right)_{\sigma_{ij},E_i} dT \tag{D.3}
\]

All of these properties are reversible. Therefore, the first and second laws of thermodynamics apply. We define a function $\varphi$ such that

\[
\varphi = U - \sigma_{ij} \varepsilon_{ij} - E_i D_i - TS \tag{D.4}
\]

Where $U$ is the internal energy of the system and $dU$ is given by

\[
dU = \sigma_{ij} d\varepsilon_{ij} + E_i dD_i + TdS \tag{D.5}
\]

Therefore, $d\varphi$ is given by

\[
d\varphi = dU - \sigma_{ij} d\varepsilon_{ij} - \varepsilon_{ij} d\sigma_{ij} - E_i dD_i - D_i dE_i - TdS - SdT \tag{D.6}
\]

\[
d\varphi = -\varepsilon_{ij} d\sigma_{ij} - D_i dE_i - SdT \tag{D.7}
\]

Since the terms on the right-hand-side of Equation D.7 are functions of $(\sigma_{ij}, E_i, T)$ it must follow that $\varphi$ is also a function of $(\sigma_{ij}, E_i, T)$. Next, $\varphi$ is differentiated with respect to these variables.

\[
d\varphi = \left( \frac{\partial \varphi}{\partial \sigma_{ij}} \right)_{E_i,T} d\sigma_{ij} + \left( \frac{\partial \varphi}{\partial E_i} \right)_{\sigma_{ij},T} dE_i + \left( \frac{\partial \varphi}{\partial T} \right)_{\sigma_{ij},E_i} dT \tag{D.8}
\]
Through a comparison of Equation D.7 and Equation D.8, we see that

\[
\left( \frac{\partial \varphi}{\partial \sigma_{ij}} \right)_{E,T} = -\varepsilon_{ij} \tag{D.9}
\]

\[
\left( \frac{\partial \varphi}{\partial E_i} \right)_{\sigma,T} = -D_i \tag{D.10}
\]

\[
\left( \frac{\partial \varphi}{\partial T} \right)_{\sigma,T} = -S \tag{D.11}
\]

Differentiating the Equation D.9 with respect to \( E_k \) and then with respect to \( \sigma_{ij} \) gives

\[
-\left( \frac{\partial^2 \varphi}{\partial \sigma_{ij} \partial E_k} \right)_T = \left( \frac{\partial \varepsilon_{ij}}{\partial E_k} \right)_{\sigma,T} = \delta_{ijkl}^T \tag{D.12}
\]

\[
-\left( \frac{\partial^2 \varphi}{\partial \sigma_{ij} \partial E_k} \right)_T = \left( \frac{\partial D_{ik}}{\partial \sigma_{ij}} \right)_{E,T} = \delta_{ijkl}^T \tag{D.13}
\]

where \( d \) is the piezoelectric co-efficient. Equations D.12 and D.13 show that the direct and converse piezoelectric effect are equal.

In the same way Equations D.10 and D.11 can be twice differentiate yielding Equations D.14 and D.15, respectively.

\[
-\left( \frac{\partial^2 \varphi}{\partial \sigma_{ij} \partial T} \right)_E = \left( \frac{\partial \varepsilon_{ij}}{\partial T} \right)_{\sigma,E} = \left( \frac{\partial S_{ij}}{\partial \sigma_{ij}} \right)_{E,T} = \alpha_{ij}^E \tag{D.14}
\]

\[
-\left( \frac{\partial^2 \varphi}{\partial \sigma_{ij} \partial T} \right)_E = \left( \frac{\partial D_{ik}}{\partial T} \right)_{\sigma,E} = \left( \frac{\partial S_{ij}}{\partial E_i} \right)_{\sigma,T} = p_{ij}^\sigma \tag{D.15}
\]

where \( \alpha \) is the coefficient of both the thermal expansion and the piezocaloric effect, and \( p \) is the coefficient of both the pyroelectric effect and the electrocaloric effect.

Equation D.14 shows that the coefficient of thermal expansion is equal to the coefficient of piezocaloric effect. Equation D.15 shows that the coefficient of the pyroelectric effect is equal to the coefficient of the electrocaloric effect.

From this information, Equations D1 to D3 can be rewritten in their integrated form as:
\[ \varepsilon_{ij} = s_{ijkl}^E \sigma_{kl} + d_{ijk}^T E_k + \alpha_{ij}^E \Delta T \] \hspace{1cm} \text{D.16}

\[ D_i = d_{ijk}^T \sigma_{kl} + \kappa_{ij}^\sigma E_j + p_i^\sigma \Delta T \] \hspace{1cm} \text{D.17}

\[ S = \alpha_{ij}^E \sigma_{ij} + p_i^\sigma E_i + \frac{\sigma_{ij}^E}{T} \Delta T \] \hspace{1cm} \text{D.18}

where \( s \) is the stiffness, \( \kappa \) is permittivity and \( T \) is the absolute temperature.

Experiments on piezoelectric materials are usually performed at constant temperature. Therefore, Equation D.19 and D.20 describe the direct piezoelectric effect and the converse piezoelectric effects, respectively.

\[ \varepsilon_{ij} = s_{ijkl}^E \sigma_{kl} + d_{ijk}^T E_k \] \hspace{1cm} \text{D.19}

\[ D_i = d_{ijk}^T \sigma_{kl} + \kappa_{ij}^\sigma E_j \] \hspace{1cm} \text{D.20}
Appendix E: Contact Resonance PFM

Contact resonance offers a means of amplifying the piezosignal detected by operating at the contact resonance of the tip-sample system. By way of example, a frequency sweep is performed on aluminium nitride while the tip is in contact with the sample, Figure E.1. The tip (HA_C/Pt, NT-MDT) has a normal free-resonance frequency of 19 kHz. A number of contact resonance peaks are evident with the first-order contact resonance at approximately 83 kHz. This resonance is mechanical and the sample does not need to be piezoelectric to see this effect. However, notice that the intensity of the magnitude and phase signals in Figure E.1 increases when the applied voltage increases. This is because poled PVDF is piezoelectric and so its deformation is directly proportional to the magnitude of the applied voltage. In the general case, we can induce resonance in the system by setting the frequency of the applied voltage to be the same as the frequency of the contact resonance. In this way, a weak piezoresponse signal may be amplified sufficiently so that it is detectable.
Figure E. 1 Contact resonance spectra for aluminium nitride showing (a) the magnitude and (b) the phase response at two different applied voltages. The resonance peaks are larger when the applied voltage is high because it induces a greater mechanical response in piezoelectric materials.

Figure E. 2 illustrates the enhancement effect by comparing the magnitude and phase images of the vertical piezoresponse signal obtained for a sample of aluminium nitride at three different frequencies. The image contrast of both the VPFM magnitude and phase images are lowest when the scan was performed at 50 kHz, far from the contact resonance frequency, Figure E. 2(a, b). Repeating the scan at a 100 kHz, i.e. at a frequency near the resonance frequency improves the scan contrast, Figure E. 2(b, c). In Figure E. 2(d, e) both the VPFM magnitude and phase images are enhanced because they were collected at 83 kHz, the resonance frequency for the system.
Figure E.2 Effect of frequency on the VPFM magnitude and phase images when the PFM scan is performed on a sample of aluminium nitride (a) off-resonance, 50 kHz, (b) near-resonance, 100 kHz and (c) on-resonance, 83 kHz.

This approach to enhancing the piezoresponse signal is not always applicable. The frequency of contact-resonance may vary considerably from point to point within the scan area if the sample itself is not homogenous. In addition, as the enhancement at resonance cannot be easily quantified, quantitative PFM measurements are not possible in this regime (169).