Electromechanical Properties of Bone at the Nanometre and Micrometre Scale

by

Yuqi Zhang
B.Eng Materials, M.Sc Biomed
Department of Physics and Energy
Materials and Surface Science Institute

Thesis submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy

Supervisors: Dr. Syed A. M. Tofail (University of Limerick)
Dr. Brian Rodriguez (University College Dublin)
Prof. Hugh J. Byrne (Dublin Institute of Technology)

Submitted to the University of Limerick
October 2012
Declaration

“I, Yuqi Zhang, hereby declare that this thesis is entirely my own work and has not been submitted to any other university or higher education institute, or for any academic award in this university. Where use has been made of the work of other people, it has been fully acknowledged or referenced.”

___________________________
28th September 2012
Acknowledgements

I would like first and foremost to thank my supervisor Dr. Tofail Syed, for all his support, encouragement and advice during my research. He has brought me into a new scientific world and has inspired me with his philosophy and beliefs of life. He was a true source of inspiration and confidence when I had become lost in my study.

I sincerely appreciate the generous research contributions from my co-supervisors – Dr. Brian Rodriguez and Prof. Hugh Byrne. Their patient guidance and excellent advice have driven my scientific achievements to a higher level.

I would like to express my gratitude to Dr. Abbasi Gandhi for his academic and personal support throughout my studies. Special thanks are due to Dr. Maros Gregor, Dr. Jacek Zeglinski, Dr. Calum Dickinson, Dr. Fathima Laffir, Dr. Anna Piterina, Dr. Serguei Belochapkin, Dr. James Butler and Dr. Wynette Redington for their assistance in completion of my experiments.

Our manager John Mulcahy, my colleagues Karina McNamara, Patrick Cronin, Eoghan Twohigs, Drahomir Chovan and all my friends in University of Limerick deserve credit for helping solve problems, sharing information in the past.

Denise Denning and Liam Collin from University College, Dublin for their help with PFM experiments.

I am also very grateful to the Joyce and Keely families, who have taken me in and treated me as a part of their family.

Finally and never enough thanks to my mum and dad, my wonderful mother in-law Eileen, my families in China and Ireland, for all their constant love, kindness and encouragement. Especially to my husband Ivan, for his love and support. I love you all!
Dedication

For Grandmother Josephine Joyce, very sadly missed,
(1921-2010)
who said to me ‘Never give up on your dreams!’
Abstract

The mechanical and electromechanical behaviour of bone such as elasticity and piezoelectricity have long been considered to be a consequence of its hierarchical architecture, the basic building block of which, at the nanostructural level, is a finely interleaved composite of collagen fibrils and apatite, a substituted calcium orthophosphate. Also, stress generated surface charge in bone in the form of piezoelectricity and streaming potential is believed to be the driving force behind bone remodelling. However, very little is known about the basic mechanism for dissipating stress and surface charge at the local level of organisation between the composites. In this study, the relationship between electromechanical properties of bone and its molecular foundation is investigated. To achieve this, the organic and inorganic constituents of a bovine bone were separated from each other using chemical extraction methods. Microscopic techniques were then employed to analyse the morphology of the unextracted (raw) bone and the results were compared with that of the extracted bone. Chemical characterisation techniques were used to determine the purity of the extracted constituents of bone. The electromechanical properties of bone were studied using both vertical and lateral Piezoresponse Force Microscopy (PFM). To obtain a common framework for comparison of quantitative values obtained for piezoelectricity measured in both nano and microscopic scales, the standard equivalent single crystal structures of bone was resorted. For this, a transformation of reference axes was necessary to take into consideration the PFM probe/sample orientation as well as the mode of scanning. Piezoelectric coefficients measured in lateral PFM (represented as \(d_{34}\) constants) showed a trend of increasing value when the angle of the sample was varied between 0°, 45° and 90° with respect to the bone’s macroscopic axis. The shear piezoelectricity measured by PFM in micro and nanoscopic scale, 3.48±0.08 pC/N and 4.06±0.30 pC/N respectively, are comparable to collagen’s macroscopic piezoelectric constant (1.4 pC/N) and its single crystal equivalent standards (2.89 pC/N). Finally, the work revisited the original investigation of the orientation dependence of macroscopically measured piezoelectricity in light of the PFM technique and suggested that there was a variation in PFM response for bone and collagen if one switches from a transverse lateral measurement to a longitudinal lateral measurement. While the subject matter of this article is bone and collagen, this developed methodology can be useful in quantitative analysis of nano and microscopic piezoresponse measured on any piezoelectric composite or biopolymer possessing uniaxial texture.

Keywords: Bone, Piezoelectricity, Orientation dependent, Piezoresponse Force Microscopy
# Table of Contents

Declaration ........................................................................................................................................... I  

Acknowledgements ......................................................................................................................... II  

Dedication .......................................................................................................................................... III  

Abstract ............................................................................................................................................ IV  

Table of Contents ............................................................................................................................ V  

List of Tables ..................................................................................................................................... IX  

List of Figures .................................................................................................................................... X  

List of Abbreviations ........................................................................................................................ XIV  

Chapter 1 Introduction ..................................................................................................................... 1  
  1.1. Introduction .............................................................................................................................. 1  
  1.2. Research Questions and Hypotheses ....................................................................................... 2  
  1.3. Methodological Approach: Why Nanoscale Characterisation is Important ......................... 2  
  1.4. Importance of the Study: The Gap in Literature that it Fulfils .................................................. 4  
  1.5. Scope of the Thesis: Chapter Overviews ................................................................................ 8  

Chapter 2 Literature Review ............................................................................................................. 9  
  2.1. Introduction .............................................................................................................................. 9  
  2.2. Anatomy of Bone ..................................................................................................................... 10  
    2.2.1. Skeletal System ................................................................................................................... 10  
    2.2.2. Structure of Bone .............................................................................................................. 12  
    2.2.3. Major Types of Bone ....................................................................................................... 14  
    2.2.4. Chemical Composition of Bone ....................................................................................... 19  
    2.2.5. Bone as a Composite Material ......................................................................................... 28
2.2.6. Bone Growth and Substitutes ......................................................... 28

2.3. Bone Remodelling and Modelling ......................................................... 31

2.4. Mechanical Properties of Bone and its Constituents ............................. 35
   2.4.1. Ultimate Strength ................................................................. 36
   2.4.2. Ultimate strain ................................................................. 39
   2.4.3. Hierarchical Organisation and Anisotropic Mechanical Properties of Bone 40
   2.4.4. Elastic Properties of Collagen and its Role in Bone ....................... 43
   2.4.5. Elastic Properties of Hydroxyapatite .............................................. 45
   2.4.6. Mechanical Properties of Bone at the Nanoscale ....................... 49

2.5. Electromechanical Properties of Bone ................................................... 50
   2.5.1. Dielectric Properties and Crystal Physics ................................... 50
   2.5.2. Piezoelectricity, Pyroelectricity and Ferroelectricity of Bone .......... 59
   2.5.3. Piezoelectricity of Bone at Nanometric scale ................................. 65
   2.5.4. Physiological Significance of Piezoelectricity ............................... 70

2.6. Piezoelectric and Micromechanical Modelling of Bone ........................ 72

2.7. Conclusions ......................................................................................... 77

Chapter 3 Methodology: Characterisation Techniques ........................................ 79

3.1 Introduction ............................................................................................ 79

3.2 Chemical Extractions............................................................................. 79

3.3 X-Ray Diffraction (XRD) ...................................................................... 82

3.4 X-Ray Photoelectron Spectroscopy (XPS) ............................................ 85

3.5 Fourier Transform Infrared (FT-IR) Spectroscopy ................................ 89

3.6 Microscopy ............................................................................................ 92
   3.6.1 Optical Microscopy ................................................................... 92
   3.6.2 Scanning Electron Microscopy ................................................... 94
   3.6.3 Transmission Electron Microscopy ............................................. 96
3.6.4 Confocal Laser Scanning Microscopy (CLSM) ..................... 98

3.7 Scanning Probe Microscopy ................................................ 100
  3.7.1 Atomic Force Microscopy .............................................. 101
  3.7.2 Piezoresponse Force Microscopy .................................... 105
  3.7.3 Force Distance Curve .................................................. 107
  3.7.4 Comparison of AFM Instrumentations ......................... 108

3.8 Conclusion .......................................................................... 115

Chapter 4 Structural Characterisation of Bone and its Constituents .... 117
  4.1 Introduction .................................................................... 117
  4.2 Morphology of Raw Bone and Extracted Bone ................. 117
  4.3 Purity of Extractions ....................................................... 128
  4.4 XPS Analysis .................................................................. 135
  4.5 X-Ray Diffraction and Rietveld Refinement ...................... 137
  4.6 Conclusion .................................................................... 141

Chapter 5 Piezoresponse of Bone and its Constituents .................. 143
  5.1 Introduction .................................................................... 143
  5.2 Morphology Analysis of Bone at Nanoscale ..................... 143
    5.2.1 Investigation of Piezoresponse in Bone and its Constituents ... 147
    5.2.2 Comparison of Piezoresponse in Extracted Bone ............. 148
    5.2.3 Further Investigation of Piezoresponse in Etched Bone ...... 151
    5.2.4 Adhesion Mapping for Bone Elasticity ......................... 156
    5.2.5 Secondary PFM Testing of Bone Apatite ...................... 159
  5.3 Orientation Dependence in Bone ...................................... 162
    5.3.1 Orientation Dependence of Microscopic Piezoresponse of Bone 162
    5.3.2 A Comparison of Orientation Dependence of Piezoelectricity of Bone at Nanoscopic, Microscopic and Macroscopic Scales .... 166
5.3.3. Significance of the Investigation of Orientation Dependence of Macroscopic Piezoelectricity for PFM ....................................................... 170

5.4. Conclusions .......................................................................................... 172

Chapter 6 Conclusions and Future Work ...................................................... 175

6.1 Summary of Findings ........................................................................... 175

6.2 Solutions to the Research Questions and Testing of the Research Hypotheses ...................................................................................... 177

6.3 Future Work ......................................................................................... 179

References .......................................................................................................... 180

Bibliography ....................................................................................................... 200

Appendices ......................................................................................................... 203

Appendix I Cypher Demonstration .................................................................. 203

Appendix II Unit Conversion ............................................................................. 206
List of Tables

Table 2.1 Levels and structures of bone ................................................................. 19
Table 2.2 Terms and definitions of grafts .............................................................. 31
Table 2.3 Tensile Strength of Bone ..................................................................... 37
Table 2.4 Effects of Strain Rate on Bone properties .......................................... 40
Table 2.5 Stiffness matrix for HAP, bone and tendon in Gpa ............................. 47
Table 2.6 A comparison of materials tensors between limiting group $\infty 2$ and lattice groups $P2_1$ and $P6_3$ ................................................................................................. 49
Table 2.7 Macroscopic, microscopic and nanoscopic shear piezoelectric constants of poly- and single- crystal bone and collagen................................. 63
Table 3.1 Comparison of PFM$s$ ......................................................................... 115
Table 4.1 FT-IR data of Bone, Collagen and Apatite .......................................... 134
Table 4.2 XPS analysis of raw bone, extracted bone and apatite....................... 136
Table 4.3 R-factors and lattice parameters of HA crystal phases in bone refined over the range 25-55° 2$\theta$ by Rietveld method ......................................................... 141
Table 5.1 Rationalisation of macroscopic, microscopic and nanoscopic shear piezoelectric constants of bone and collagen ..................................................... 169
List of Figures

Figure 2.1 Human Skeletal System .............................................................. 11

Figure 2.2 Macroscopic structure of a long bone (femur) .......................... 13

Figure 2.3 Cross-Section of Bone Showing Compact and Trabecular Structures ........................................................................................................ 14

Figure 2.4 Lamellar structure of compact bone ....................................... 15

Figure 2.5 Microscopic structure of compact and spongy bone ................. 17

Figure 2.6 The hierarchical levels of structure found in secondary osteonal bone ........................................................................................................ 18

Figure 2.7 Hierarchical structure of collagen protein materials ............... 22

Figure 2.8 AFM images of different types of collagen fibrils ..................... 24

Figure 2.9 TEM photographs of HAP crystals synthesised hydrothermally at 200°C under 2 MPa for 5 hours ..................................................................... 27

Figure 2.10 Broad steps in bone growth, development and remodelling .... 32

Figure 2.11 BMU with possible estrogen action. Bone remodelling on the surface of trabecular bones is illustrated................................................................. 34

Figure 2.12 Schematic models of a cross section of bovine femoral compact bone ........................................................................................................ 41

Figure 2.13 Schematic stress-strain profiles of bone samples taken from a given bone but at different orientation to the long axis............................................. 43

Figure 2.14 Stress-strain curves of a collagen molecule (filled circles), a collagen fibril (open circles) and tendon (open squares) .............................. 45

Figure 2.15 Classification of Conductivity and Dielectric Properties of Solids.. 52

Figure 2.16 Classification of crystal symmetry point groups ........................ 54

Figure 2.17 Ferroelectric Hysteresis .............................................................. 59

Figure 2.18 Converse effect of piezoelectric measurement of bone............. 60

Figure 2.19 PFM images of tibia ................................................................. 66

Figure 2.20 Piezoresponse curve and PFM images of collagen fibril ............ 67

Figure 2.21 High-resolution shear piezoresponse in compact bone ............ 68

Figure 2.22 PR of an isolated collagen fibre at three cantilever orientations with respect to the sample ................................................................. 69
Figure 2.23 Feedback Control System in Bone owing to mechanical Stimuli mechanism .......................................................................................................................... 73
Figure 2.24 Representation of the double-layer .......................................................... 74
Figure 2.25 Simulation of the gradual straightening of a femur that is broken and heals with a malposition .......................................................................................... 75
Figure 2.26 Directions of stress and the piezoelectric vector ..................................... 77
Figure 3.1 Procedure of obtaining bovine bone matrix ............................................ 80
Figure 3.2 Chemical extraction procedures of bovine bone .................................... 82
Figure 3.3 Bragg’s law .......................................................................................... 84
Figure 3.4 Schematic of the photoemission process .............................................. 87
Figure 3.5 Schematic of the Auger process .............................................................. 88
Figure 3.6 Universal curve showing the attenuation of an element’s path in a solid as a function of its KE .......................................................................................... 89
Figure 3.7 Potential energy of a diatomic molecule as a function of the atomic displacement during a vibration for a harmonic oscillator (dashed line) and an anharmonic oscillator (solid line) ................................................................................. 91
Figure 3.8 Principle of Optical Microscopy ............................................................. 93
Figure 3.9 Schematic drawing of SEM .................................................................... 95
Figure 3.10 Objective/intermediate lens system of TEM ......................................... 97
Figure 3.11 Rejection of light not incident from the focal plane ................................ 99
Figure 3.12 Schematic diagram of AFM setup .......................................................... 103
Figure 3.13 Repulsion and attraction force .............................................................. 104
Figure 3.14 Schematic image of how the sample surface reacts to the applied electric field in PFM .......................................................................................... 106
Figure 3.15 Four brands of AFMs .......................................................................... 109
Figure 3.16 Agilent 5500 SPM ........................................................................... 111
Figure 4.1 Extracted collagen vs. Extracted apatite ............................................... 117
Figure 4.2 Optical microscopic images .................................................................... 118
Figure 4.3 SEM images of early extracted bone matrix ........................................... 120
Figure 4.4 SEM image of unpolished raw bone ..................................................... 121
Figure 4.5 SEM images of extracted bone apatite .................................................. 121
Figure 4.6 SEM images of extracted bone collagen ............................................... 122
Figure 4.7 EDX result of Raw bone ....................................................................... 123
Figure 4.8 EDX result of Extracted bone apatite ............................................... 124
Figure 4.9 EDX result of Extracted bone collagen ............................................ 124
Figure 4.10 Confocal microscopic images of bone and the bone matrix .......... 126
Figure 4.11 Confocal microscopic images - 3D optimal deflection of the controlled samples (Bio-Oss®) ................................................................. 126
Figure 4.12 TEM images of bone apatite ......................................................... 127
Figure 4.13 Fourier Transform Infrared Spectra of bovine femur: Raw bone, Pure HAP, Extracted collagen and Extracted apatite ........................................ 129
Figure 4.14 FT-IR spectra of bovine bone heated from room temperature (RT) to 1200°C ............................................................................................................... 132
Figure 4.15 Infrared spectra of bone, HAP, CaHPO4 and CaCO3, before and after deproteination ................................................................. 133
Figure 4.16 Survey spectra of extracted bone sample (demineralised and deproteinated bone) .............................................................................. 136
Figure 4.17 High resolution C 1s spectra of (a) raw bone, (b) demineralised bone, (c) deproteinated bone and (d) apatite .................................................... 137
Figure 4.18 Molecular modelling of HAP. Coloured balls represent atoms: green – Calcium; red – Oxygen; and white – hydrogen ........................................ 139
Figure 4.19 X-ray diffraction pattern of bone (top) and apatite extracted from bone (bottom). The figures on the right panels show high resolution spectra within the region of 35-40°. The traces of thermally diffusive, hexagonally forbidden, weak monoclinic reflections are indicated by arrows ................. 140
Figure 5.1 Tapping mode AFM of polished bovine femur bone ...................... 144
Figure 5.2 Contact mode AFM of polished bovine femur bone ...................... 145
Figure 5.3 Contact mode AFM of HA film, HA pellet and Bio-Oss HA ............ 146
Figure 5.4 Lateral PFM of Raw Bone (a, b, c), Extracted collagen (d, e, f), Scanning size: 5µm ............................................................................................. 148
Figure 5.5 Vertical PFM of (a) Raw bone, (b) Extracted collagen and (c) Extracted apatite from early extractions, Scanning size: 2µm ......................... 150
Figure 5.6 (a) VPFM and (b) LPFM images and phase histograms of raw bone before and after etching. Scanning size: 5µm; Applied bias: 60V (peak to peak) ......................................................................................................................... 153
Figure 5.7 (a) Tapping mode AFM and (b) VPFM of etched extracted bone apatite ..................................................................................................................................... 154

Figure 5.8 Spot analysis of extracted bone apatite on bright and dark regions. Applied bias: 0-120V peak to peak ............................................................................................................ 155

Figure 5.9 Tapping mode topography images of raw bone ................................................................................................................................. 157

Figure 5.10 Topography (left) and the adhesion map (right) ................................................................................................................................. 157

Figure 5.11 Adhesion map of dry bovine bone at the size of 2µm ......................................................................................................................... 158

Figure 5.12 Force distance curve map of hydrated bovine bone ................................................................................................................................. 159

Figure 5.13 High resolution tapping mode AFM topography of bone apatite. Scanning size: 1µm, Resolution: 1024x. (L) 2D topography (R) 3D topography ..................................................................................................................................... 159

Figure 5.14 PFM of bone apatite with AC bias 6V and DC bias 3V ................................................................................................................................. 160

Figure 5.15 Line profile of PFM amplitude and phase ................................................................................................................................. 161

Figure 5.16 PFM phase and histogram ..................................................................................................................................... 161

Figure 5.17 Schematic model of collagen fibrils alignments in the bone with macroscopic orientations ..................................................................................................................................... 162

Figure 5.18 AFM tapping mode amplitude images of bovine femur bone (5x5 µm2) with macroscopic orientations ..................................................................................................................................... 164

Figure 5.19 PFM images of collagen fibrils near a lacuna ..................................................................................................................................... 165

Figure 5.20 Schematic representation of PFM measurements of collagen fibrils orientated in bone in 0°, 45° and 90° samples and the piezoelectricity coefficients that can be measured Vertical PFM and Lateral PFM ..................................................................................................................................... 167

Figure 5.21 Transformation of coordinates in determining ..................................................................................................................................... 171
## List of Abbreviations

### Chemical Formula

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BaTiO$_3$/BTO</td>
<td>Barium Titanate</td>
</tr>
<tr>
<td>C</td>
<td>Carbone</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>Ca$_3$(PO$_4$)$_2$</td>
<td>$\beta$-whitlockite</td>
</tr>
<tr>
<td>EDA</td>
<td>Ethylenediamine</td>
</tr>
<tr>
<td>H</td>
<td>Hydrogen</td>
</tr>
<tr>
<td>HAP Ca$_{10}$(PO$_4$)$_6$(OH)$_2$</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>He</td>
<td>Hellum</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>Potassium hydrogen phosphate</td>
</tr>
<tr>
<td>Li</td>
<td>Lithium</td>
</tr>
<tr>
<td>Mg</td>
<td>Magnesium</td>
</tr>
<tr>
<td>N</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>O</td>
<td>Oxygen</td>
</tr>
<tr>
<td>P</td>
<td>Phosphate</td>
</tr>
<tr>
<td>PbZrO$_3$</td>
<td>Lead Zirconate</td>
</tr>
<tr>
<td>S</td>
<td>Sulfur</td>
</tr>
</tbody>
</table>

### Instrumentations and Techniques

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFAM</td>
<td>Atomic Force Acoustic Microscopy</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscope</td>
</tr>
<tr>
<td>EBSD</td>
<td>Backscattered electron detectors</td>
</tr>
<tr>
<td>EDS</td>
<td>Energy Dispersive Spectroscopy</td>
</tr>
<tr>
<td>FLIM</td>
<td>Fluorescence Lifetime Imaging</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier Transform InfraRed</td>
</tr>
<tr>
<td>LPFM</td>
<td>Lateral Piezoresponse Force Microscopy</td>
</tr>
<tr>
<td>PFM</td>
<td>Piezoresponse Force Microscopy</td>
</tr>
<tr>
<td>SAXS</td>
<td>Small angle X-ray scattering</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SPM</td>
<td>Scanning Probe Microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>UPS</td>
<td>Ultraviolet Photoelectron Spectroscopy</td>
</tr>
<tr>
<td>VPFM</td>
<td>Vertical Piezoresponse Force Microscopy</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray Photoelectron Spectroscopy</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray Diffraction</td>
</tr>
</tbody>
</table>

### Other Terms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D</td>
<td>Three-Dimensional</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone Mineral Density</td>
</tr>
<tr>
<td>BMUs</td>
<td>Basic Multicultural Units</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>BSE</td>
<td>Backscattered Electrons</td>
</tr>
<tr>
<td>BSUs</td>
<td>Bone Structural Units</td>
</tr>
<tr>
<td>CAO</td>
<td>Computer-Aided Optimisation</td>
</tr>
<tr>
<td>DIT</td>
<td>Dublin Institute of Technology</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EMPA</td>
<td>Electron Microprobe Analysis</td>
</tr>
<tr>
<td>FLS</td>
<td>Fibrous Long Spacing</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>MEMS</td>
<td>Micro-Electro-Mechanical Systems</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
</tr>
<tr>
<td>PGs</td>
<td>Proteoglycans</td>
</tr>
<tr>
<td>PVDF</td>
<td>Poly vinylidene Fluoride</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SFA</td>
<td>Surface Force Apparatus</td>
</tr>
<tr>
<td>SGP</td>
<td>Stress Generated Potential</td>
</tr>
<tr>
<td>SLRPs</td>
<td>Small Leucin-rich Proteoglycans</td>
</tr>
<tr>
<td>UCD</td>
<td>University Collage Dublin</td>
</tr>
<tr>
<td>UL</td>
<td>University of Limerick</td>
</tr>
</tbody>
</table>
Chapter 1 Introduction

1.1. Introduction
The quality of life and the survival rate is often greatly affected by factors such as medical complications caused by diseased, damaged, or aged tissues or organs. Bone is an important organ of the human body. It provides support and protection to other important organs in the body. A better understanding of the fundamental properties of bone is important for medicine, medical device technology, industry and especially for everyday life of patients suffering from bone related diseases.

Bone is a highly orientated tissue and is electrically active. The research on piezoelectricity in bone, a property that enables bone to generate electricity when stressed, can be traced back to 1957, when Eiichi Fukada and Iwao Yasuda [1] linked the empirical evidence of stress generated electrical potential in bone to linear piezoelectricity. This discovery, together with Yasuda’s earlier observation of a link between an electrical stimulus and bone growth [2], led to a number of interesting studies in the past few decades. It has been suggested that electrical potentials might be the basic link in the clinically observed adaptive response of bone to mechanical stress [3] and physiological functions controlling bone remodelling [4]. There is a consensus that bone piezoelectricity originates from collagen fibres, the main organic constituent of bone [5]. Collagen has also been found to be piezoelectric macroscopically, and very recently, microscopically [6].

The objectives of this work are to provide the peer understanding of the origin of piezoelectricity of bone through high lateral resolution characterisation techniques. For this, the study has employed chemical extractions and high resolution electromechanical techniques and investigations of influences of the hierarchical nature of bone on its piezoelectric response.
1.2. Research Questions and Hypotheses

Based upon background discussed in the preceding section this study deals with the following research questions (RQ):

- **RQ 1**: What is the local orientation of bone apatite nanocrystals?
- **RQ 2**: Is bone apatite piezoelectric?
- **RQ 3**: How does the local orientation of collagen fibrils in bone affect bone piezoelectricity?
- **RQ 4**: What are the instrumentation factors that affect quantitative values of bone piezoelectricity obtained by Piezoresponse Force Microscopy?

The above research questions were investigated on the basis of the following research hypotheses (RH):

- **RH 1**: Bone apatite contributes to the piezoelectricity in bone.
- **RH 2**: The local orientation in bone has direct effect to the piezoelectricity in bone at nanometre scale.

1.3. Methodological Approach: Why Nanoscale Characterisation is Important

To study the origin of the piezoelectricity in bone, the first understanding is the formation and the organisation of bone. The formation of bone is a complex process and it initiates from nanometre scale. In the beginning, apatite nucleates in collagen (a substituted calcium orthophosphate) channels or gaps, which have been created with periodic (~67 nm) holes and overlap zones. These nucleated crystals grow longitudinally along their crystallographic c-axes, and also laterally in width through the channels or gap spaces in the direction of collagen fibrils.

As collagen macromolecules grow to form nanofibrils of ~20 nm in diameter, crystals fuse into larger and thicker plates in which their periodic deposition (50–70 nm) occurs. Crystal plates then grow larger in all dimensions at the level of collagen fibres (~80 nm and greater in diameter). Fibres associate to create a
series of parallel plate aggregates that may vary in length, width, and thickness [7].

Mineral deposition proceeds in the tissue, and aggregates grow to thicknesses of ~130 nm. Edges of plate aggregates still maintain a periodicity of between 50 and 70 nm, and indicate the role of a basic collagen skeleton structure underlying the mineral formation. Finally the plate aggregates become lamellar in shape and constitute a portion of bone or mineralised tendon [7].

Although piezoelectricity in bone has long been studied at macro- and microscopic levels and contemporary investigations have revealed the bioelectric potential as the possible stimulus for bone formation, very little is known at the local level. Since there is a lack of basic information on bone organisation, the mechanism for orientation dependent piezoelectricity of bone remains uncertain.

Nanotechnology has opened the door for researchers to reveal more mysteries in a smaller world. With advanced characterisation techniques, electron microscopy has been widely used in investigation of material properties at nanometre scale. Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) are powerful tools for studying the morphology of biomaterials. In this study, SEM was used to analyse the surface of polished bone and to check the nanocrystal residues remaining on the extracted bone samples. TEM was used to observe the shape, size and the local orientation of nanocrystal apatite at nano and at atomic level. Piezoresponse Force Microscopy (PFM) allows the electromechanical analysis of biological systems at nano scale [6, 8-11]. PFM is so far the most suitable tool to investigate the nanoscopic electromechanical properties of materials. Therefore, PFM was the major characterisation technique in this study for understanding the local orientation dependent piezoelectricity of bone.
1.4. Importance of the Study: The Gap in Literature that it Fulfils

Fundamental physical properties of bone such as elasticity and electromechanical properties originate from the composite and hierarchical nature of bone. The dynamic process of bone formation and destruction accounts for its growth during the different development stages of the body and enables its regeneration in the case of fracture. Bone is known as a composite material which contains an inorganic matrix of hydroxyapatite (HAP) nanocrystals (~65% in mass) and an organic matrix of type I collagen fibrils (~35%) [12]. Collagen fibres and nanocrystals of carbonated HAP make up the hierarchical structure of bone.

Piezoelectricity of bone was considered as the primary mechanism of potential measured in bone resulting from an applied stress (stress generated potential - SGP) in dry bone, but for wet bone the role was less clear. Recently, Ahn and Grodzinsky [13] have brought in a new perspective to the physiological role of wet bone by ascribing piezoelectricity of collagen in wet bone to work in combination with the streaming potential, which has been strongly correlated with bone growth. It has been suggested that apatite nanocrystals perform a shielding mechanism that protects collagen from being wet by the body fluid, so that collagen fibrils remain mainly dry.

Piezoelectricity has been experimentally observed in bone both in dry and wet state [14-16]. The methods used include *inter alia* static, quasi static and low frequency dynamic methods using direct piezoelectric effect. A converse piezoelectric effect was also observed [1]. Samples from different origins such as human, bovine and equine have been tested. Most of these samples were obtained from femur, perhaps due to the compactness of the samples that can be obtained and convenience in handling, see Section 2.4.1 for details.

The proposition that bone piezoelectricity originates from collagen fibre has been primarily taken from the experiments reported by Marino and Becker [5], who measured piezoelectricity in demineralised bone, but could not measure piezoelectricity in de-collagenated bone. The accepted crystal structure for bone...
Chapter 1 Introduction

[17] at the time when such measurements were carried out was similar to that for $\infty m$ – a centrosymmetric structure devoid of any piezoelectricity. Naturally, the conclusion was that piezoelectricity in bone originated from its collagenous nature. Piezoelectricity could still be found in bones that have been boiled for 2 hours, boiled and dried at 120 °C (collagen denatures at ~60 °C) for 5 hours, freshly excised bones and bones that have been just dried. These treatments should affect the structural and mechanical properties of collagen and consequently, piezoelectricity should have been substantially compromised if collagen were solely responsible for bone piezoelectricity, as denatured collagen is not piezoelectric. Interestingly, even after using a similar method of de-collagenation as used by Marino and Becker [5], bone (i.e. bone apatite) was found to be piezoelectric in dynamic measurements [18, 19]. Notably, partially extracted bones towards a complete demineralised and decollagenated bone also showed piezoelectric resonance [15].

The mechanical and electromechanical behaviour of bone have thus been considered for a long time to be influenced by its hierarchical architecture, and its composite nature between collagen fibrils and apatite. Also, stress generated surface charge in bone in the form of piezoelectricity and streaming potential is believed as the driving force behind bone remodelling. However, very little is known about the basic mechanism for dissipating stress and surface charge at the local level of organisation between the composites – an important issue that the current study addresses. For this, the organic and inorganic constituents of bone have been separated using a chemical extraction method. Unlike previous studies, such separation has been meticulously characterised using chemical characterisation techniques to establish the purity of the extracted constituents of bone.

Lang [20] has shown that bone and tendon (predominantly made of collagen fibres with no apatite) are also pyroelectric, a subset of piezoelectric materials. A pyroelectric material has at least one direction along which a spontaneous polarisation exists. As a result of this spontaneous polarisation, surface charge exists in a pyroelectric material. In the case of bone, the direction of the
spontaneous macroscopic polarisation is along the bone fibre axis whereas in the case of tendon, it is the long fibrillar axis of collagen. Piezoelectricity is fundamentally related to crystal structure, order, orientation, and to polarisation that occurs as a result of mechanical stress. Besides, a material without a macroscopic anisotropy may not exhibit piezoelectricity in macroscopic measurements even though its individual building blocks have been piezoelectric due to the cancellation of the piezoelectricity in isolated materials. To date, the crystal structural of bone apatite has not been reconciled with the perspective of the newly discovered piezoelectric symmetries of HAP [21].

There is now experimental evidence at the macroscopic scale that stoichiometric HAP can be both piezoelectric and pyroelectric [22, 23]. Nanocrystalline HAP films have recently been found piezo ($d_{33} - 16pC/N$) and pyroelectric ($12 \mu C m^{-2} K^{-1}$) of the order of the conventional ferroelectric polymer - poly vinyledene fluoride (PVDF) [24]. Apatite crystals in bone closely resemble HAP although they contain carbonates and other important impurities, which may have important contributions to bone apatite’s piezoelectricity. It is therefore important to reconcile why macroscopic measurements could not measure piezoelectricity in bone apatite at a macroscopic scale.

On the other hand, high resolution experimental techniques such as Piezoresponse Force Microscopy (PFM), allows nanoscale characterisation of converse piezo effects with high sensitivity and spatial resolution [25]. PFM can detect local electromechanical mechanisms through an electrical bias-introduced surface deformation and has been used for biological systems at high resolution such as proteins, dentin, tendon and calcified tissues [8, 9, 26]. The piezoelectric coefficient in the Haversian canals in human bone is reported to be ~8pm/V [10], the value of shear piezoelectric coefficient is ~0.3pm/V for bone [6, 11], ~0.1-2 pm/V for individual collagen fibrils [6, 8, 27], ~0.15-0.25pm/V for dentin and enamel in human tooth [28] and ~1pm/V for chitin rods in butterfly wing [29].
The present study deals with both marco- and nanoscopic piezoelectric coefficient values and we have developed a common framework for quantitative analysis of piezoelectricity for bone. For measurements made in nano, micro and macroscopic scales, the standard equivalent single crystal description of bone was resorted following the schema proposed by Gundjian et al. 1974 [30] after modifying it for the case of PFM using Euler angles in laboratory coordinate system as proposed by Harnagea [27].

The study also investigated, for the first time, the orientation dependence of piezoelectricity at the nano and microscopic scale and compared these data with those measured macroscopically by Fukada and Yasuda [1]. The study finds significant dependence of PFM response of bone on the orientation of bone collagen. It also reconciles the measured piezoresponse of bone collagen with respect to that measured for tendon and the common quantitative framework obtained from the standard equivalent single crystal structures of bone. In conclusion, the characterisation method of nano and microscopic piezoresponse in this study has provided a detailed quantitative analysis for any piezoelectric composites in future.
1.5. **Scope of the Thesis: Chapter Overviews**

This thesis is organised in five Chapters and four Appendices:

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Overview</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>Discusses the historical development of the physiology of bone and describes the aim of the present study along with the research questions, research hypothesis and methodological approach</td>
</tr>
<tr>
<td>Two</td>
<td>Reviews the fundamental knowledge of bone with a major emphasis on the mechanical and electromechanical properties of bone at the nanometre scale</td>
</tr>
<tr>
<td>Three</td>
<td>Explains the chemical and the electrical characterisation techniques and the importance of a nano approach</td>
</tr>
<tr>
<td>Four</td>
<td>Presents the experimental observations of chemical extraction of bovine bone</td>
</tr>
<tr>
<td>Five</td>
<td>Presents our micro- and nanoscopic studies that led us to discover an orientation dependence in bone piezoelectricity</td>
</tr>
<tr>
<td>Six</td>
<td>Presents our conclusions based on the experimental findings and proposes some possible future works.</td>
</tr>
</tbody>
</table>
Chapter 2 Literature Review

2.1. Introduction

Bone is a living system and is never static. It responds and adapts to the external mechanical environment due to its capacity of remodelling. In vivo, bone cells act continuously to maintain the remodelling [31]. Thus, living bone is in a constant state of dynamic equilibrium, both in terms of its composition and structure, and responds to external mechanical forces by adopting changes in its normal architecture. This amazing organ possesses various physical, solid-state and electro-mechanical properties. These properties are characteristics of bone and are modified under the action of external stimulus and changes of Calcium (Ca) and Phosphate (P) metabolism. However, its mechanism and biological significance are not completely understood. Also, the origin of functional significance of bone renewal is still a matter of speculation.

The solid structure of bone has been a subject of continuing investigation because of its physical properties, e.g. elasticity, pyroelectricity, piezoelectricity, etc. [32]. The prime reason seems to be that it retains and reveals several of its physical properties, even after extraction from the body. Intrinsically, it is subjected to stresses and consequent generation of charges and currents that contribute to bone growth and development. In Section 2.4 the details of mechanical and electromechanical properties of bone, particularly compact bone, from macroscopic to nanoscopic metre scale, are explained in details.

In Section 2.5.2, the cause of bone fracture and bone related diseases, such as osteoporosis are described and the significance of studying the electromechanical properties of bone and some suggested research of electric field-induced bone healing are highlighted.
2.2. Anatomy of Bone

2.2.1. Skeletal System

Bone tissue is a vital organ that makes up about 18% of the weight of the human body [33]. The unique structure of human body makes it possible for us to survive. The skeletal system serves as a scaffold for the human body and consists of both fused bone (only in children) and individual bones (in both children and adults). It provides support and protection for various organs, e.g. heart, brain, and also is responsible for the strength for movements, e.g. grabbing, walking, etc. It is also responsible for storing minerals, i.e. mineral homeostasis, especially Calcium and Phosphate. In an adult human skeleton system, 206 bones are named and most of them exist in pairs. In infants and children, more than 206 bones are found, with additional structures in areas such as the pelvis and cranium, which fuse into mature bones along the longitudinal axis in their later lives [33]. Differences of skeletal structure between male and female has also been reported. The weight of the adult skeleton accounts 30-40% of the total body weight and the half of this weight is water [34].

There are two primary divisions of adult skeleton – the axial skeleton (80 bones) and the appendicular skeleton (126 bones). The axial skeleton is the major skeletal structure that defines the longitudinal axis of the body to transmit the weight from the upper extremities to the lower extremities, and protects the soft organs, e.g. vertebral column, rib cage and skull, etc. The appendicular skeleton (126 bones) is attached to the axial skeleton to enable the majority movements, e.g. the upper limbs, the lower limbs, pelvic girdle, etc. [35]. Figure 2.1 demonstrates the human skeletal system from front view to rear view.
Figure 2.1 Human Skeletal System [36]
2.2.2. Structure of Bone

In gross anatomy of human body, bones are the hardest organs in a human body and are classified into five main categories based on their shape – long, short, flat, irregular and sesamoid bones. Bone tissue makes up ~18% of the body weight and is described as a type of dense connective tissue that frameworks the entire body with cells, fibres and extracellular matrix (ECM) [37].

Macroscopically, the bone structure is illustrated using a long bone that has a most detailed structure, for example, the femur, as shown in Figure 2.2. The structure of a long bone consists of diaphysis, epiphyses, metaphyses, articular cartilage, perosteum, endosteum and medullary cavity. The spongy bone tissue of the epiphyses and metaphysis also contains red bone marrow, whereas the medullary cavity of the diaphysis contains yellow bone marrow in the case of adults. A typical long bone has greater length than width and is slightly curved, consisting of a hollow shaft and a numerous of extremities. This special design provides strength and the ability of absorbing the stress of the body weight at different points, so that the stress can be evenly distributed, as described in Wolff’s law (Section 2.3). The design also minimises any shocks to the body and the risks of bone fracture due to sudden loading [33].
In the histology of connective tissues, bones or osseous tissues are made of living cells that are surrounded by a great amount of non-living materials - abundant ECMs. The living cells are embedded in ECMs and the matrixes are secreted by the cells. The living cells include osteoblasts (bone-forming cells), osteoclasts (bone-destroying cells), osteocytes (mature bone cells) and osteogenic cells (unspecialised stem cells). Bone ECM is composed of a complex mixture of water (~25%), collagen fibres (~25%) and crystallised mineral deposits (50%), as determined by Tortora et al [33]. The matrix, which is primarily responsible for the mechanical strength of the bone tissue, is formed by an organic and an inorganic phase.

Figure 2.2 Macroscopic structure of a long bone (femur) [38]
2.2.3. Major Types of Bone

Two major forms of bone tissue can be distinguished from their macroscopic appearance, the compact or cortical bone, and the cancellous or trabecular bone (sponge bone) [39]. Figure 2.3 shows the compact and trabecular structures at a cross section of a long bone. Compact bone is dense and uniform in composition and has a low level of porosity (5%-10%). In contrast, trabecular bone has a high level of porosity (50%-95%) and low density [40, 41].

![Figure 2.3 Cross-Section of Bone Showing Compact and Trabecular Structures](image)

(Reproduced from Sampson et al. 2002 [42])

Compact bone is the strongest form of bone tissue. It can be found beneath the periosteum and makes up the bulk of the diaphysis of long bones. Compact bone provides protection and support. It also resists to the stresses produced by weight and environment. Compact bone can respond in two ways to a change in loading environment:

(i) By adapting its local mechanical properties by changing the porosity of compact bone tissue, which is called internal remodelling;

(ii) By adapting its shape by deposition or resorption of bone materials in surface, which is called surface remodelling [43].
Compact bone bulk and surface are dense materials with a density of $\sim 2\text{gm/cm}^3$. The external surface of bone is smooth and is called the periosteal surface, where the interior surface is called the endosteal surface [44], as seen in Figure 2.2.

Compact bone consists almost entirely of extracellular material. Osteoblasts deposit in the matrix in the form of thin sheets which are called lamellae. Lamellae are microscopic structures [45]. Collagen fibres within each lamella are parallel to each other. In adjacent lamellae they are at oblique angle to each other. Fibre density is lower at the border between adjacent lamellae, which gives rise to the lamellar appearance of the tissue. This type of bone reveals such lamella organisation when viewed under the microscope, so that it is also called lamellar bone. Figure 2.4 shows the microscopic lamellar structure of compact bone and the unstained histological image of an osteon (diameter: $\sim 200\ \mu\text{m}$), including the Haversian systems.

Figure 2.4 Lamellar structure of compact bone

(a) Lamellar structure of osteons in compact bone, optical microscopic image, Magnification: $\times 200\mu\text{m}$ ; (b) Details of a section of lamella within an osteon, ground image [46]

In the process of the deposition of bone matrix, osteoblasts become encased in small hollows within the matrix, known as lacunae. Canaliculi are the microscopic canals between lacunae, eventually, with larger, vessel-containing
canals within the bone [33]. The function of canaliculi is to provide the means for the osteocytes to communicate with each other and to exchange substances by diffusion.

In mature compact bone, most of the individual lamellae form concentric rings around large longitudinal canals (~ 50 \( \mu \text{m} \) in diameter) within the bone tissue [45]. These canals are the Haversian canals. Haversian canals typically grow parallel to the surface and along the long axis of compact bone. The canals and the surrounding lamellae (8-15) constitute a Haversian system or an osteon. Generally, a Haversian canal also contains one or two capillaries and nerve fibres.

Irregular areas of interstitial lamellae that apparently do not belong to any Haversian system, have been found in between Haversian systems. Some lamellae are found immediately beneath the periosteum and endosteum, which run parallel to the inner and outer surfaces of the compact bone. These are the circumferential lamellae and endosteal lamellae [47].

A second system of canals are the Volkmann's canals, which penetrate the bone more or less perpendicular to its surface [33]. The function of these canals is to connect the Haversian canals with the inner and outer surfaces of the bone. The vessels in Volkmann’s canals are connected with the vessels in the Haversian canals on the one hand and with the vessels in the endosteum on the other. A few other communications also exist with the vessels in the periosteum [48], shown in Figure 2.5.

In contrast, trabecular bone exists only in the metaphaseal region of long bones and within the confines of the compact bone covering the smaller flat and short bones. Trabecular bone is also considered as a unit, which is called trabeculae. The connected trabeculae give cancellous bone a spongy appearance, therefore, cancellous bone is also called spongy bone [43]. No blood vessels or osteons are found within the trabeculae, but there are vessels immediately adjacent to the tissue and they weave in and out of the large spaces between the individual trabeculae. A trabecular bone has a vast surface area as suggested by its spongy
Chapter 2 Literature Review

appearance [49]. **Figure 2.5** illustrates the difference of microscopic structure between compact and spongy bone.

**Compact Bone & Spongy (Cancellous Bone)**

![Diagram of Compact Bone & Spongy (Cancellous Bone)]

**Figure 2.5** Microscopic structure of compact and spongy bone [50]

The macroscopic spaces between the trabeculae can help making bones lighter and are sometimes filled with red bone marrow. Within each trabecula, there are lacunae that contain osteocytes. There are also canaliculi that radiate outward from the lacunae. The osteocytes of spongy bone are located on the superficial surfaces of trabeculae, so that they receive nourishment directly from the blood circulating through the medullary cavities [33]. At a microscopic level, trabecular tissue consists of a matrix of collagen fibres impregnated with bone mineral. The collagen fibres are generally oriented parallel to a main direction and they contribute the toughness of the tissue structure. The bone mineral is brittle in character but is critical to provide strength to the bone structure. Collagen fibres and bone mineral are arranged into geometric entities – Bone Structural Units (BSUs) which are also referred to as ‘trabecular packets’ [48]. The BSUs are shallow crescents in shape, and have a radius of 600 µm, a thickness of 50 µm and a length of 1 mm on average. BSUs are combined by cement lines which are the layers of mineralised tissue deficient in collagen fibres [39].
Figure 2.6 The hierarchical levels of structure found in secondary osteonal bone

(Reproduced from: Weiner and Wagner, 1998 [51])
Chapter 2 Literature Review

It is clear that bone has a complex hierarchical structure, as shown in Figure 2.6, which, despite much investigation, is still not well understood [52, 53]. This hierarchically organised structure has an irregular, yet optimised, arrangement and orientation of the components, making the material of bone heterogeneous and anisotropic. Levels and structures of bone are listed in Table 2.1:

Table 2.1 Levels and structures of bone [52]

<table>
<thead>
<tr>
<th>Levels</th>
<th>Scale</th>
<th>Structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrostructure</td>
<td>from mm to m</td>
<td>cancellous and compact bone</td>
</tr>
<tr>
<td>Microstructure</td>
<td>from 10 to 500 mm</td>
<td>Haversian systems, osteons, single trabeculae</td>
</tr>
<tr>
<td>Sub-microstructure</td>
<td>from 1 to 10 mm</td>
<td>lamellae</td>
</tr>
<tr>
<td>Nanostructure</td>
<td>from a few hundred nanometres to 1 mm</td>
<td>fibrillar collagen and embedded mineral</td>
</tr>
<tr>
<td>Sub-nanostructure</td>
<td>below a few hundred nanometres</td>
<td>molecular structure of constituent elements, such as mineral, collagen, and non-collagenous organic proteins</td>
</tr>
</tbody>
</table>

2.2.4. Chemical Composition of Bone

Bone consists of mineral and organic material and water. Minerals account for about 45–70 % of total weight and are formed mostly of calcium phosphate (bone apatite), the remaining weight is essentially accounted for by organic materials consisting principally type I collagen with smaller amounts of non-collagenous proteins (NCP) and lipids, and for about 10wt% by water [54]. The mineral in bone is located primarily within the collagen fibril. During mineralisation, the fibril is formed first and the water within the fibril is replaced with minerals [55]. While bone is a chemical reservoir for phosphorous, which is a life essential element, bone must possess the physical properties required for the functionality of the tissue, such as structural support [56]. Bone strength and fracture risk are
Chapter 2 Literature Review

generally assessed by measuring bone mineral density (BMD), however; the mechanical properties of bone are determined not only by bone mass, but also by the bone architecture and geometry, and by the bone materials intrinsic properties. It is considered that bone strength is determined by 70% bone density and 30% bone quality. Bone quality is defined by at least four factors:

1. The rate of bone turnover;
2. Properties of the collagen-mineral matrix;
3. Microdamage accumulations;
4. Architecture geometry of cancellous and compact bone [57].

The relative distribution of these components may lead to changes in the mechanical properties, and alterations in the transmission and the distribution of stress in the joint [58].

The weight of dry bone is accounted for 65-70% by inorganic crystals of the calcium phosphate mineral, apatite, and for 30-35% by organic matrix of which collagen makes up the major fraction (95-99%) [59, 60]. Collagen is one of the long, fibrous structural proteins whose functions are quite different forms those of globular proteins such as enzymes. Collagen fibres are a major component of the extracellular matrix that supports most tissues and gives cells a support structure outside [61]. From an X-ray diffraction point of view, bone collagen is practically similar to the collagen obtained from other sources. Also no marked differences have been reported by electron diffraction studies [62]. Collagen in bone structure is often considered similar to glass fibres impregnated in a resin matrix within a fibre-glass reinforced composite [63, 64].

Collagen is the most abundant protein in vertebrates and accounts for 66% of all proteins in humans [65]. Up to now, 25 types of collagen have been identified [66]. Collagen can be found in both fibril and non-fibril forming structures. The fibril-forming collagens include type I, II, III, V and XI, which provide the structural framework and the mechanical strength of tissues [67]. In general, collagen type I is a major component of connective tissue, such as skin, bone, cartilage, and tendons [68]. Collagen fibres form through the parallel
arrangement of a large quantity of collagen fibrils. The fibrils within the fibres are tilted, resulting in a macroscopic crimped structure visible under optical microscope [69]. Furthermore, small proteoglycans (PGs), especially the small leucin-rich proteoglycans (SLRPs) play an important role in the assembly of adjacent fibrils although the exact molecular interactions involved in the binding are not yet understood [70, 71].

As mentioned in Section 2.2.3, bone is a highly organised material from the molecular level to the gross macroscopic level. At the macroscopic level, bone has an overall linear arrangement with collagen fibres lying parallel to each other and effectively in line with the original long axis of bone. At the molecular level, the apatite crystals (about 50nm axis dimensions) surround the surface of the collagen fibrils. Collagen is a macromolecule and has a hierarchical structure, as shown in Figure 2.7. The basic collagen unit is defined as tropocollagen, which consists of polypeptides organised in the form of a triple helix [60]. These three left-handed helices are twisted together into a right-handed coil, a triple helix or "super helix", a cooperative quaternary structure stabilised by hydrogen bonds, ~300nm long, ~1.5 nm in diameter, which has a molecular weight of 300,000Da [72].
Figure 2.7 Hierarchical structure of collagen protein materials

Each collagen molecule is made of three peptide chains that form the \( \approx 300 \text{ nm} \) long triple helical collagen molecule. Collections of collagen molecules aggregate both in lateral and longitudinal directions to form fibrils. Fibrils in cornea are normally thin (\( \approx 30 \text{ nm} \)) and uniform in diameter, while tissues such as tendon contain a wide-ranging distribution of diameters (100-500 nm). Fibrils include tiny hydroxyapatite crystals in bone tissue, which provide stiffness and compressive load resistance. In tendons and ligaments, multiple fibrils make up collagen fibre, formed with the aid of proteoglycans

(Adapted from Gautieri et al. 2011 [73])

The periodicity of the collagen band structure is 64-67nm, is also called ‘D-band structure’, arises through an overlap of the tropocollagen molecule with respect to the nearest neighbours by about one quarter of its length. This has been revealed for example by Atomic Force Microscope (AFM), see Figure 2.8. In type I collagen and possibly all fibrillar collagens if not all collagens, each triple-helix associates into a right-handed super-super-coil that is referred to as the collagen microfibril. Each microfibril is interdigitated with its neighboring microfibrils to a degree that might suggest that they are individually unstable although within collagen fibrils they are so well ordered as to be crystalline [74, 75].
Because the helix in collagen is stabilised by hydrogen bonds formed between amino groups of polypeptide chains and carboxyl groups of adjoining polypeptide chains, the peptide groups forming the intramolecular hydrogen bonds have a permanent electric dipole moment, which may primarily contribute to the piezoelectric polarisation of collagen fibrils [76].

A number of spectroscopic studies [77-79] have focused on the structural aspects of collagen to elucidate the relationship between its molecular structure and functions. These studies have showed the elastic modulus of the tropocollagen molecule and an estimate of hydrogen bonding, the values of which compare well with the data obtained for other solids on the basis of neutron inelastic scattering. It suggests that the classical central force constant model can be applied in such cases [77]. A low frequency Raman study of collagen in the region 200-600 cm\(^{-1}\) was reported by Diem [78]. Also Berney have reported results of an elastic neutron scattering study of collagen at ambient and low temperatures [79]. These studies provide evidence of the possibility of significant structural changes accompanying the low temperature cooling.
**Figure 2.8** AFM images of different types of collagen fibrils

(a) a native collagen type I fibril; (b) native and Fibrous Long Spacing (FLS) collagen type I fibrils; (c) an intermediate FLS-fibril, assembled with increased a1-acid glycoprotein concentration; (d) native collagen type II fibrils  
(Adapted from Strasser *et al.* 2006 [80])

HAP is an inorganic crystal that is present as the main constituent of bones. In biomineralisation, the functions of organic macromolecules are important in the regulation of growth of HAP [81]. This concerns molecular recognition between functional groups on the surface of organic macromolecules and ions on the surface of a crystal nucleus [60]. X-ray diffraction studies showed that most basic calcium phosphates belonged to the apatite group. There is a marked meridional orientation of the 00.2 reflection from apatite if the apatite crystals are elongated in the c direction and they run parallel to the fibre axis of the collagen
fibrils [72]. Some other scientists interpreted the broadening of wide-angle X-ray patterns of bovine bone mineral in terms of nanocrystalline structure. Their nanocrystalline dimensions are \( \sim 22 \) nm and \( \sim 7 \) nm for the basal and prism planes, which are similar to the crystallite size obtained in earlier studies [60].

Apatite crystals are the most important component of bone. In general, three aspects of recognition at inorganic-organic interfaces leading to specificity in the nucleation of biominerals are considered effective:

1. Electrostatic accumulation of cations;
2. Structural correspondence; and
3. Stereochemical requirements [82].

Depending on the Ca/P ratio, presence of water, impurities, and temperature, calcium phosphate can be crystallised into the salts of mono-, di-, tri-, and tetra-calcium phosphate, HAP, and \( \beta \)-whitlockite \( (\text{Ca}_3(\text{PO}_4)_2) \) [83]. The most important element is HAP, due to its presence in natural bone and teeth. In a wet environment and at lower temperature \( (<900^\circ\text{C}) \), it is more likely that the hydroxyl or hydroxyapatite will form, while in a dry atmosphere and at higher temperature, \( \beta \)-whitlockite will be produced. Both forms are very tissue compatible and are used for bone substitute in granular form or as a solid block [84]. The mineral part of bone and teeth is made of a crystalline form of calcium phosphate similar to HAP \( [\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2] \). The apatite family of minerals, \( \text{A}_{10}(\text{BO}_4)_6\text{X}_2 \), crystallises into hexagonal rhombic prisms and has unit cell dimensions \( a = 0.9432 \) nm and \( c = 0.6881 \) nm. Six of the ten calcium ions in the unit cell are associated with the hydroxyls in these columns, resulting in strong interactions. The ideal Ca/P ratio of HAP is 1.667 and the calculated density is \( 3.16 \) g/cm\(^3\). **Figure 2.9** displays Transmission Electron Microscopy (TEM) images of synthesised HAP nanocrystals. It is interesting to note that the substitution of OH with F will give greater chemical stability due to the closer coordination of F (symmetric shape) as compared to the hydroxyl (non-symmetric, two atoms) by the nearest calcium [84].

![Figure 2.9](image_url)
Mineral crystals are responsible for hardness, rigidity, and the great compressive strength of bone, but similar to other crystalline materials, bone mineral has low tensile strength. On the contrary, the collagen fibrils of bone possess high elasticity, low compressive strength, and considerable intrinsic tensile strength. The tensile strength of bone depends, however, not only on collagen but also on the intimate association of mineral with collagen, which confers to bone many of the general properties exhibited by two-phase composite materials such as fibre glass and bamboo. In such a combination, the dispersion of a rigid but brittle material in a matrix of quite different elasticity prevents the propagation of stress failure through the brittle material and therefore allows a closer approach to the theoretical limiting strength of single crystals [59]. Therefore, studying the mechanism and the orientation of HAP nanocrystals at such scale is vital to understand the origin of bone piezoelectricity.
Figure 2.9 TEM photographs of HAP crystals synthesised hydrothermally at 200°C under 2 MPa for 5 hours

(a) without additives, (b) KOH (10 wt%) added, (c) K3PO4 (10 wt%) added, and (d) EDTA (5 wt%) added. (Adapted from Yashimura et al. 1994 [85])
2.2.5. Bone as a Composite Material

The strongest and the most efficient materials created by nature are composite materials, e.g. wood, teeth and bone. They are made of different substances with contrasting properties of strength and elasticity. It has been observed that increasing amounts of calcium minerals in single osteons cause an increase in their modulus of elasticity [58] [72].

As mentioned in Section 2.2.4, bone tissue is considered as a composite material consisting of an organic phase, mainly collagen fibres and an inorganic phase, predominantly apatite crystals. Crystallites of HAP are distributed throughout the collagen matrix. In the simplest model the HAP from McConnell et al. 1965 is uniformly dispersed without order, but to account for the observed anisotropy a more complex model has been suggested which includes the orderly distribution of the microcrystallites in the collagen fibrils of bone and dentine, or in the prisms of dental enamel [60]. Some studies show that calcified tissues should be considered as two phase composite systems of HAP and collagen. Bone becomes stiffer as the hard filler (HAP) is added until a maximum elastic modulus is reached. It is postulated that increased density of cross-linking in collagen is associated with increased HAP content and HAP crystallites provide rigid bases for shortened links to stiffen the composite by reversible enzyme-directed processes [86-88].

2.2.6. Bone Growth and Substitutes

Bone growth, maintenance, destruction, modelling and remodelling is governed by the behaviour of three related cell types: the osteoblasts, the osteoclasts and the osteocytes [89]. Long branching structures are due to osteocytes that are contained in bone, and are encased in calcified cavities - lacunae. These are connected together to the capillaries in Haversian canals by the narrow channels - canaliculi permeating in a solid bone matrix. Bone matrix consists of bundles of collagenous fibres in an amorphous ground substance (cement) impregnated with calcium phosphate complexes deposited in the osteoid [90]. The osteoblasts formed within the matrix are converted into osteocytes. Osteocytes then
surrounded by nearly solid bone receive nourishment and excrete waste products through diffusion and fluid transport. The above physiological processes are dependent upon the existence of an adequate blood supply.

Bone is produced and resorbed along orientation patterns corresponding to the functional type of stress [91]. Mechanical influences upon bone are the sum total of a variety of intrinsic and extrinsic effects: such as ballistics of cardio-vascular action, gravity muscle tones, voluntary muscle activities and the impact between skeletal systems and the external environment [49]. Old bone tissue is first resorbed by osteoclasts and new un-mineralised tissue is then deposited by osteoblasts. The new tissue is then gradually mineralised to synthesised new bone. In adults, the bone remodelling process is usually in a state of dynamic equilibrium, with the amount of new bone removed being balance by the newly deposited tissue. Disturbances of this balance can be triggered by several types of intrinsic stimuli, e.g. genetic, hormonal and metabolic [49].

Although bone growth induced by mechanical stress is of considerable physiological importance the mechanism at work still remains a matter of conjecture. It appears that both osteoblastic and osteoclastic activities are regulated by several hormones, such as parathyroid, growth factor interleukin 6 (IL-6) [92]. Since bone responds to mechanical stress by differential growth so as to overcome the effect of the former, it satisfies the requirements of a stimulus-response adaptive system [93]. Its behaviours have thus been analysed by the control system theory of cybernetics. Mechanically induced bone remodelling can be thought of as being regulated by a closed loop negative feedback system, an approach in which the response feeds back to the original signal and tends to cancel it out. Behari et al. concluded that in the language of a system approach this can be written in a sequence:

1. an external mechanical stimulus applied to the bone;
2. biotransduction to convert it into a biological response;
3. the response triggers osteoblastic and/or osteoclastic activity, and finally
4. the bone structural changes take place appropriately to resist the stress [94].
In general, the simplest control system is a closed loop feedback in which the response feeds back to the original signal and tends to annul it. This also satisfies Wolff’s law, which states ‘bone is a dynamic organ adaptive to its mechanical environment’ [13]. Examples of such hypothetical mechanisms of bone are:

1. relative motion between the lamella causing impingement on osteocyte processes,
2. stress-induced fluid motion resulting in improved nutrition of osteocytes,
3. stimulation of bone cells by piezoelectrically or stress generated potentials [95].

Bone growth and bone healing are cell-mediated phenomenon although the factors that activate or stimulate such activity are expected to influence the healing process. Healing of a primary bone wound involves haematoma formation, Mesenchymal stem cell (MSC) differentiation, platelet aggregation and growth factors release [96].

Bone grafts are used to replace the missing bones with material from the patient’s own body, an artificial, synthetic, or natural substitute [97]. The need for bone grafting procedures to correct skeletal defects or augment reconstruction has become more prevalent because of enhanced opportunities to salvage major bone loss [97]. Many bone graft options are available nowadays, including compact and cancellous autografts and allografts, each of which has specific biologic and mechanical properties [98]. Other terminologies, such as xenografts, alloplastic grafts, demineralised bone matrix, synthetic variants, growth factors are applied to clinical trials [99-101]. Knowledge of these biologic events is important in understanding the larger processes that influence graft incorporation into the host skeleton. Some terms are explained in Table 2.2.
Table 2.2 Terms and definitions of grafts

<table>
<thead>
<tr>
<th>Terms</th>
<th>Definitions [98]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autograft</td>
<td>A tissue or organ that is taken from the patient and transplanted to a new location of the patient</td>
</tr>
<tr>
<td>Allograft</td>
<td>A tissue or organ that is taken from a donor from the same species and transplanted to a recipient</td>
</tr>
<tr>
<td>Xenograft</td>
<td>A tissue or organ that is taken from a donor from different species and transplanted to a recipient</td>
</tr>
<tr>
<td>Alloplastic graft</td>
<td>The graft that includes non-biologic materials, such as metal, ceramic, plastic, etc.</td>
</tr>
</tbody>
</table>

2.3. Bone Remodelling and Modelling

Bone growth and development to a stage of maturity is controlled by the combination of intrinsic and extrinsic forces. The mass and structure of bones are governed by adaptive mechanisms to some extent, which are sensitive to external mechanical environment.

As early as 1892, Wolff suggested that when bone is bent under a mechanical load it modifies its structure so as to resist external pressure by bony apposition in the concavity and by resorption in the convexity [102]. It has been experimentally proved that mechanical stresses affect the form of bone [31, 103, 104]. The skeletal unweighting associated with spaceflight is the result of reduction in bone formation mineral content, and bone matrix protein production [105-107]. Conversely, it has shown that increasing skeletal loading through exercise can increase bone mass and retard bone loss caused by postmenopausal osteoporosis [108, 109].

Figure 2.10 shows a sequence of bone mechanical stimuli leading to subsequent bone remodelling, according to the modified Wolff’s law. It states: ‘The form of a bone being given, the bone elements place or displace themselves in the direction of functional forces and increase or decrease their mass to reflect the amount of functional forces.’ [102]
The effects of mechanical loading from moderate exercise are complex in the process of bone [111]. This is because bone is not homogenous, either morphologically and mechanically, but interestingly its homogeneities do not disturb the functional adaptation [112]. On the other hand, homogeneities contribute towards bone remodelling. Other considerations in most experiments and theory on bone adaptation are the size and the location of the bone. Remodelling occurs in cancellous bone, in which osteoclasts and osteoblasts work together in coordinated sequence to replace bone. However, the secondary osteons, i.e. Haversian systems remain unaltered.
Bone remodelling can conserve and reduce bone strength and mass but does not change at the same rate. The remodelling process arranges a given amount of calcified material to support the naturally occurring loads with the largest possible safety factor. Remodelling process achieves three goals [113]:

1) It creates a system for the body to alter the balance of essential minerals by increasing or decreasing the concentration of these in serum;
2) It provides a mechanism for the skeleton to adapt to the external mechanical environment;
3) It improves the metabolism that repairs damage within bone by repetitive cycles of mechanical loading.

The mechanical properties of bone are a result of a compromise between the need for stiffness and the need for ductility to absorb impacts from external environment changes. The mechanical properties of whole bones depend both on their material properties and on the bone architecture. During bone growth, the material properties of bone can change in synergy with its architecture as the function of the bone. Therefore, the material properties are determined over a long and evolutionary time, while the architecture of the bones may also respond to the applied strains in the bone over a short period [114]. It is well studied that bone remodelling is the mechanism of bone replacement in the vertebrate skeleton. One of the primary reasons for this replacement is the functional capacity of bone, which in some way is compromised if bone cells are allowed to become too old [115].

In bone remodelling, the metabolic activity of cancellous bone is tenfold greater than at the compact sites. According to Wolff’s law, living cells are constantly subjected to mechanical stimulations arising from the external environment and internal physiological conditions. Bone cells can respond in various ways depending on the magnitude, direction and distribution of these mechanical stimuli. Mechanical signalling for bone remodelling that is translated into biological and chemical responses in a cell is still under investigation [114]. Also, induced within or outside the body many chemicals can affect the
mechanical properties of living cells. Other studies show that mechanical loads exerted at the tissue level can be transmitted to individual cells and can influence their physiological functions [116, 117].

Bone remodelling process is performed by groups of cells functioning as organised unit, which are known as Basic Multicultural Units (BMUs) [118]. The BMUs operate on bone periosteum and endosteum, trabecular and compact bone, replacing old tissue with new tissue in discrete packets. Figure 2.11 demonstrates the cycle of remodelling stages, which can be described as: Activation → Resorption → Reversal → Formation → Quiescence.

*Figure 2.11* BMU with possible estrogen action. Bone remodelling on the surface of trabecular bones is illustrated.

(Adapted from Raisz, 2005 [119])

Adult bone change over time, becoming damaged by microfractures as a result of loading or reduction in quality as it ages. The best evidence for bone modelling is that bone grows during childhood and slows down on attainment of adulthood. Bone modelling has the characteristics of:
1) Bone structure changes on the basis of the existing structure and
2) Bone structure alerts by independent action of osteoblasts and osteoclasts, which causes bone resorption and formation on different surfaces [114].

Bone modelling can increase bone “mass” and hence its strength. This occurs when bone strains equal or exceed a threshold range. Bone formation and resorption use osteoblasts and osteoclasts, respectively, to modify the cross sectional/longitudinal shape and size of compact and cancellous bones and to increase their strength and mass [120]. If strain is greater than a particular threshold the modelling takes place, *vice versa*. It can be expressed as below:

\[
\text{Strain} < \text{Threshold} < \text{off} \\
\text{Strain} > \text{Threshold} > \text{on}
\]

In conclusion, bone remodelling is an important mechanism adopted by the body to replace and repair bone. Modelling is a different process to remodelling, whereby bone formation and resorption processes are coupled and isolated to the same location of bone. Remodelling occurs to replace the Haversian systems upon their demise and to respond to altered stress and strains placed on the bone.

### 2.4. Mechanical Properties of Bone and its Constituents

Bone is a dynamic tissue that, when properly organised and distributed in a whole bone, acts as both a mechanically capable skeletal structure and a physiological unit. Bone is also a very rigid anisotropic material. The functions of bone are manifold and at times too complex to understand by any simplistic model. One of its apparent roles is to provide support to the body, which is carried out mainly by compact bone throughout the skeleton and by peripheral cancellous bone [110].

As the hardest organ, bone has the following main functions: (1) supporting soft tissues and providing attachment points for the tendons of most skeletal muscles; (2) protecting internal organs from injuries; (3) assisting skeletal muscles to produce movements; (4) maintaining mineral homeostasis; (5) producing blood
cells by red bone marrow; (6) reserving potential chemical energy and storing triglyceride by yellow bone marrow [33, 121].

### 2.4.1. Ultimate Strength

Bone strength depends on its material properties and hierarchical structure. Genetic information in osteoblasts mainly determines the material properties of bone, including stiffness, ultimate strength, true density, proportional limit and yield point [93]. However, these properties change with age, disease, gender and species. The architectural features that affect a whole bone’s strength also include the amount of bone in a cross section.

The bulk of the literature describing the mechanical properties of bone concerns itself with the determination of intrinsic strength. Usually the strength is defined as the maximum stress sustained by the bone specimen without fracture in some loading configuration, usually tensile, compressive, bending, torsion, or direct shear. How the maximum stress is to be measured dictates quite often the shape of the specimen used, which can lead to different results [122].

Several types of mechanical testing on bone have become common tests for bone tissues. Tensile tests conventionally are performed on the specimen fabricated with a gauge section reduced in size (so as to lessen the influence of the device used to grip the specimen) and are subjected to a uniaxial tensile load, so that the ultimate stress can be found by dividing the highest load sustained by the original cross-sectional area [123]. Compressive tests are a little more difficult to perform compared to tensile tests, due to the edge effect from the testing machine and the axial inaccuracies are harder to eliminate. The ultimate compressive stress (or strength) is calculated using the same method as tensile tests and the same assumption regarding area changes than those used for the tensile tests [124]. Bending tests are often used to determine tensile strength of bone, but the results must be interpreted from an elastic-plastic viewpoint if they are to be used to calculate absolute numeral values, not simply for comparison. In other words, if the elastic beam formulation:
Chapter 2 Literature Review

\[ \sigma = \frac{M c}{I} \]

Equation 2.1

(where \( \sigma \) = stress in extreme fibre; \( M \) = bending moment supported by the beam; \( c \) = distance from the neutral axis to extreme fibre; and \( I \) = moment of inertia of the cross section) is used, the calculation of the tensile stress in the extreme fibre of bone becomes less accurate as the beam material displays more plastic deformation [93]. In 1974, Reily’s group calculated the value of ultimate strength for bovine bone [125]. The results showed that, with a material which displays the amount of plastic deformation found in bone, using a bending test, and assuming bone to be elastic, gave values which overestimate the stress in the extreme fibre by 50 to 100 per cent [125]. The values for ultimate strength reported in the literature have been concluded in Table 2.3.

<table>
<thead>
<tr>
<th>Bone Species/Type</th>
<th>Ultimate Stress (x10^4 N/m^2)</th>
<th>Load Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femur, tibia and humerus [126]</td>
<td>78.8</td>
<td>Longitudinal</td>
</tr>
<tr>
<td>Femur</td>
<td>122±1.1</td>
<td>Longitudinal</td>
</tr>
<tr>
<td>Tibia</td>
<td>140±1.2</td>
<td>Longitudinal</td>
</tr>
<tr>
<td>Fibula [127]</td>
<td>146±1.5</td>
<td>Longitudinal</td>
</tr>
<tr>
<td>Tibia [128]</td>
<td>95.3±27.0</td>
<td>Longitudinal</td>
</tr>
<tr>
<td></td>
<td>9.9±2.9</td>
<td>Transverse</td>
</tr>
<tr>
<td>Tibia [129]</td>
<td>138</td>
<td>Longitudinal</td>
</tr>
<tr>
<td></td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>Femur [130]</td>
<td>86.5</td>
<td>Longitudinal</td>
</tr>
<tr>
<td>Femur [131]</td>
<td>151±18</td>
<td>Longitudinal</td>
</tr>
<tr>
<td><strong>Bovine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femur [132]</td>
<td>112</td>
<td>Longitudinal</td>
</tr>
<tr>
<td>Femur [133]</td>
<td>129</td>
<td>Longitudinal</td>
</tr>
<tr>
<td>Femur [134]</td>
<td>172±22.0</td>
<td>Longitudinal</td>
</tr>
<tr>
<td></td>
<td>52±8.0</td>
<td>Transverse</td>
</tr>
</tbody>
</table>

Wall et al. [135] studied many of the variables and suggested how they should be standardised. One of the considerations is the bone and site selected for testing. Differences have been shown in the strength of bone tissue from different bones. Testing embalmed wet bone, Evans et al. [136] found that specimens from the
human femur are 14 per cent weaker in tension than specimens from the tibia and fibula, the specimens from the tibia and fibula having the same strength. Koh et al. [127] showed differences in the tensile strength of specimens from different bones, the sequence from strongest to weakest being: radius, fibula, tibia, humerus and femur. According to them, the tensile strength of the tibia was approximately 17 per cent higher than that of the femur. Almost the reverse sequence for compression strength was reported by Yokoo et al. [137] that the femur being strongest, followed by the tibia (the strength of the tibia being 98\% that of the femur), the fibula, humerus, radius, and ulna (ulnar strength being 77\% of that of the femur). Therefore, magnitude and significance of the strength differences vary not only with the type of test and loading conditions, but also with the bone specimen from which it was obtained, and any discussions of the strength of bone tissue should be restricted to the same bone.

The effect of the histological type of fresh bovine bone on the ultimate tensile stress was investigated by Currey et al. [132]. They found a strong negative correlation between the number of Haversian systems and tensile strength, with the strength of totally Haversian bone being ~ 30 per cent less than that of laminar bone. They attributed this decrease in strength to the combined effect of decreased density of bone due to secondary osteons having a larger central canal, and to the lower degree of calcification of the secondary osteons. Evans [138] working with embalmed human bone tissue found similarly that a large number of osteons and osteonal fragments in the specimen reduced the ultimate tensile strength. Aoji et al. [139] reported that for bovine bone the ultimate compressive strength was inversely proportional to the amount of interstitial lamellae in the cortex.

The anisotropic strength and the differences in total strain to failure are considered as being due to the number of collagen fibres oriented in the direction of the load [140, 141]. A pronounced anisotropy of the proportions of 6:2:1 for longitudinal, transverse, and radial directions have been reported for bovine bone. Evans et al. and Vincentelli et al. [138, 142] also considered the "predominant" collagen-fibre orientation in relation to mechanical properties of embalmed
human bone. In other two independent investigations, the direction of the collagen fibres was determined by means of a polarising microscope, a method which has been challenged in Boyde et al. and Hobdell et al. [143, 144]. Using Scanning Electron Microscopy, they found that the collagen fibrils are in a "predominant" direction only in very small domains within the lamella, and that only a few micrometres away, but in another domain, the fibrils are in a different "predominant" direction. Other evidence regarding the relation between bone-tissue strength and collagen-fibre direction is still lacking.

### 2.4.2. Ultimate strain

From equine and canine studies applying surgically implanted strain gauges, Rubin et al. [145] concluded that physiological strain rates during walking and running are in the range of 0.005–0.08 s⁻¹ and that this is a realistic range also for humans. Lanyon et al. [146] measured strain rates of 0.013 s⁻¹ in humans during running and Burt et al. [147] measured maximum strain rates of 0.050 s⁻¹ during sprinting and downhill running. In a failure scenario, bone will often have been exposed to high strain rates.

Strain rates during traumatic events clearly depend on the particular circumstances and it is not possible to specify a single traumatic strain rate [134]. Hansen et al. [148] made an estimate of the strain rate of knee and suggested that strain rates on the order of 25 s⁻¹ provide an upper bound relevant to bone failures during traumatic events. There has been some previous work investigating the effect of strain rate on bone’s mechanical behaviour. Table 2.4 is a summary of these results showing that usually high strain rates result in increasing yield and preyield properties (σᵣ, εᵣ and E) and this appears to be a consistent finding among previous studies. Some more recent studies reported the occurrence of a more brittle behaviour of bone at higher strain rates [145, 149, 150], while the earlier ones did not [151-153]. Few of the above studies include strain rates relevant to failure during traumatic events and even fewer tested human bones. Furthermore, the results obtained from these studies are inconsistent with respect to postyield properties.
Table 2.4 Effects of Strain Rate on Bone properties [122]

<table>
<thead>
<tr>
<th>Bone Spice/Type</th>
<th>Test</th>
<th>Strain Rate (s(^{-1}))</th>
<th>E</th>
<th>(\sigma_r)</th>
<th>(\varepsilon_r)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femur [154]</td>
<td>Tension</td>
<td>133</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femur [155]</td>
<td>Compression</td>
<td>0.001-1500</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td><strong>Bovine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femur [156]</td>
<td>Tension</td>
<td>0.00013-0.16</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Femur [150]</td>
<td>Tension</td>
<td>0.00014-17</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Femur/Tibia [154]</td>
<td>Tension</td>
<td>0.0025</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tibia [157]</td>
<td>Tension</td>
<td>0.001-200</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Femur [158]</td>
<td>Tension</td>
<td>0.00053-237</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femur [159]</td>
<td>Compression</td>
<td>0.00013-0.16</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femur [155]</td>
<td>Compression</td>
<td>0.001-1500</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: ↑ increase; → little change with strain rate

2.4.3. Hierarchical Organisation and Anisotropic Mechanical Properties of Bone

An anisotropic material has mechanical properties that are strongly dependent on the orientation at which the measurements are made, e.g. wood, which is easier to cut along the grain than against the grain. This is in contrast to an isotropic material such as polycrystalline steel, which behaves in the same way regardless of the direction of measurements. Bone is also considered anisotropic because it responds differently if the external forces are applied in different directions. Bone can handle large forces applied in the longitudinal plane, in terms of high values of tension and compression; but it is weak in handling forces applied transversely to the bone axis. As mentioned in Section 2.2.3, compact bone is solid and dense, whereas cancellous bone is more porous and brittle. These differences between the properties of the compact and cancellous bone contribute to the anisotropy of the bone. Cancellous bone provides bending strength, and compact bone provides significant compressive strength [49].

Simplified structure of elements that assemble the major structure of bone is presented in Figure 2.12 that explains the local organisation of collagen fibrils in the entire femoral compact bone in a bottom-up method from the nanoscopic scale to the macroscopic scale. Collagen fibrils in the bundle of fibre are parallel to each other and are pointing to the same direction. There are also collagen
fibrils randomly orientated on the lateral plane of osteons and cross between collagen fibres. The lacunae that are at the edge between the lamellae (single lamella is \(\sim 3\text{-}7 \text{ \(\mu\text{m}\}} \text{ in thickness}) are surrounded by layers of collagen fibrils that are orientated radially. An osteon at the size of \(\sim 200 \text{ \(\mu\text{m}\}} \text{ is composed of layers of lamellae, which is the major unit of compact bone. Numerous osteons parallel to each other packed with pores construct the compact bone.

**Figure 2.12** Schematic models of a cross section of bovine femoral compact bone

(a) collagen fibrils orientated radially near a lacuna at [001] plane; (b) Single lamella contains lacunae surrounded by collagen fibrils and bundles of collagen fibre; (c) Osteons are formed with layers of lamellae and Haversian canals in the centre; (d) The macroscopic view of a compact bone and the location of osteon
Compact bone is an anisotropic material, whose mechanical properties are determined by its composition as well as microstructure. At the microstructural level, compact bone is composed primarily of osteonic lamellae and interstitial lamellae. A typical secondary osteon is a cylinder-like measuring approximately 200-250 μm in diameter and is surrounded by interstitial lamellae. Osteonic lamellae and interstitial lamellae exhibit significantly different mechanical properties [160-162]. These differences may be due to a combination of factors, such as collagen fibre orientation, degree of mineralisation, and arrangements of the lamellae [160, 163-165]. Even within an osteon, a tendency of decline has been observed in both elastic modulus and hardness from the centre of the osteon outward in mature secondary osteons [160]. The complexity of compact bone also arises from its hierarchical structural organisation. The character of anisotropy may be partly due to the highly anisotropic structure of mineralised collagen fibrils of compact bone. The fibrils are in the forms of bundles or aligned arrays, which can be arranged in a variety of different patterns, resulting in different mechanical properties in all three orthogonal directions. Although the patterns of lamellae are still a matter of dispute, many researchers have indicated that compact bone is also an orthotropic material [166-169]. Figure 2.13 shows the different stress-strain rate of bone from different orientation to the bone axis. A better understanding of bone anisotropy can help determine the basic mechanical and electromechanical functions of compact bone.
2.4.4. Elastic Properties of Collagen and its Role in Bone

The main function of type I collagens is to provide the structural framework and the biomechanical properties of tissues [67]. Hence, there is a fundamental understanding of these properties at different scales and levels of hierarchy to be discovered. For example, any anisotropy or inhomogeneity of collagen fibrils is likely to manifest itself in a corresponding anisotropy and inhomogeneity of its mechanical properties. The challenge is that conventional macroscopic technical tools based on direct manipulation and visual observation that determine the mechanical properties are not easily applicable to fibrils of nanoscale dimensions [171].

Tensile tests at different strain rates reveal that tendons and collagen fibres or fascicles from tendon have strain-rate dependent mechanical properties. Wu et al. [172] reported that the elastic moduli of fresh flexor tendons immersed in PBS buffer were $427 \pm 10$, $653 \pm 21$ and $837 \pm 11$ MPa at strain rates of $0.6 \, \%/s$, $1.2 \, \%/s$ and $5 \, \%/s$ respectively. Lynch et al. [173] investigated the strain rate
dependent properties of tendon in details and found that only the slope of the linear region (the modulus) was strain rate dependent. Yamamoto et al. [174] recorded the elastic modulus of collagen fascicles increases from 160 ± 49 MPa to 216 ± 68 MPa when increasing the strain rate from 0.01 %/s to 1 %/s. Robinson et al. [175] tested the collagen fascicles from rat tail tendon without small proteoglycans (decorin) and revealed a reduced strain rate dependency. Martin et al. [176] found that the longitudinal collagen fibre orientations are correlated with the modulus and strength but not the fatigue life of bone. The results also indicated that the proteoglycans play a role in the viscoelastic behaviour of both bone and tendon.

Sasaki and Odajima et al. [177] studied the small angle X-ray scattering (SAXS) performed on the loaded Achilles tendon to measure the changes in the D-band structure as explained in Section 2.2.4, the strain at the fibril level, with changing the stress. Using the force applied to the bulk tendon they found an almost linear stress-strain relationship of the collagen fibrils as shown in Figure 2.14. A Young’s modulus of 400 MPa of the collagen fibrils in the hydrated state was estimated from these measurements. By analysing the intensity of the X-ray diffraction pattern, they also suggested that molecular elongation is the major contribution to fibril elongation.
2.4.5. Elastic Properties of Hydroxyapatite

Compared to collagen, the elastic properties of HAP is much lower. The elastic modulus is at the range of 40-117 GPa [83]. Polycrystalline HAP has a high elastic modulus. Hard tissues such as bone, dentin, and dental enamel are natural composites that contain HAP (or a similar mineral) as well as protein, other organic materials, and water, and their elastic properties usually follow the composite mixture rules. Enamel is considered as the stiffest hard tissue with an elastic modulus of 74 GPa, and it contains the most mineral, followed by dentin (E = 21 GPa) and compact bone (E = 12 ~ 18 GPa) containing comparatively less mineral. The Poisson's ratio for the mineral or synthetic HAP is about 0.27, which is close to that of bone (≈ 0.3) [178]. Moreover, dense HAP does not have the mechanical strength to enable use in long term load bearing applications.

Experimental measurements of the elastic constants of single crystals of HAP have not yet been reported mainly due to the difficulties in obtaining large enough single crystals of pure HAP in natural or synthetic form. Gilmore and Katz [179] were the first to circumvent this difficulty in part by measuring

**Figure 2.14** Stress-strain curves of a collagen molecule (filled circles), a collagen fibril (open circles) and tendon (open squares)

(Adapted from: Sasaki and Odajima, 1996 [177])

![Stress-strain curves of a collagen molecule (filled circles), a collagen fibril (open circles) and tendon (open squares)](image)
isotropic elastic behaviour in polycrystalline HAP. In this experiment, they used a purpose-built ultrasonic interferometer, which contained a solid media pressure apparatus. This technique enabled them to determine the technical elastic moduli as a function of pressure applied to obtain better compaction of the polycrystalline aggregate. The values of bulk, shear and Young’s moduli at atmospheric pressure were then determined by back-extrapolating the high-pressure measurements. A similar approach was used for the measurements of the technical moduli of fluor- and chlorapatite. A comparison between Gilmore and Katz’s work and that of Yoon and Newnham [180] on fluorapatite shows that the isotropic elastic moduli measured by Gilmore and Katz are roughly 10% larger than the values calculated from the work of Yoon and Newnham. Gilmore and Katz [179] explained this phenomenon in terms of the evolution of a compression texture as the polycrystalline apatite samples that they have used were subjected to uniaxial compression.

Katz and Ukrainick [181] have determined the anisotropic elastic constants of HAP by an indirect method. They employed the data from Yoon and Newnham’s work on single crystal fluorapatite and averaged it for polycrystalline fluorapatite. Anisotropic values for Young’s modulus (E), bulk modulus (K) and shear modulus (G) were also calculated as a function of the orientation angle to the hexad axis of polycrystalline fluorapatite. They also compared the moduli calculated this way with those of the experimentally measured isotropic moduli, which showed a good agreement [181]. Based on this result and the consideration of the similarity of crystal structures between fluorapatite and HAP, Katz and Ukrainick [181] suggested a scaling method on the assumption that the single crystal stiffness coefficients can be calculated for HAP. This method used a mere scaling of the isotropic moduli of HAP against the corresponding fluorapatite moduli. The assumption was that the ratios of measured isotropic moduli to their respective Voigt averages are the same for both HAP and fluorapatite crystals. To obtain five independent elastic stiffness coefficients required for hexagonal crystal systems of HAP or fluorapatite from the only available two isotropic moduli, there were three more assumptions [181]:

46
1) Certain linear combinations of stiffness coefficients have a constant ratio between fluorapatite and HAP;
2) The ratio of linear compressibility along the crystallographic c-axis to that perpendicular to the c-axis is the same for the two apatites;
3) The ratio of the two shear moduli \( c_{44} \) and \( c_{66} \) is also the same for both fluorapatite and HAP.

The results of this calculation for HAP are a set of elastic stiffness, which are listed in Table 2.5.

<table>
<thead>
<tr>
<th>Stiffness constant</th>
<th>From Fluorapatite</th>
<th>From First Principle</th>
<th>Calculated average for polycrystal</th>
<th>Ceramic HAP</th>
<th>Bone</th>
<th>Tendon</th>
</tr>
</thead>
<tbody>
<tr>
<td>( c_{11} )</td>
<td>137.0</td>
<td>138.0</td>
<td>159.6</td>
<td>137.2</td>
<td>23.4</td>
<td>2.93</td>
</tr>
<tr>
<td>( c_{12} )</td>
<td>42.5</td>
<td>45.9</td>
<td>64.9</td>
<td>53</td>
<td>9.1</td>
<td>1.71</td>
</tr>
<tr>
<td>( c_{13} )</td>
<td>54.9</td>
<td>69.1</td>
<td>57.5</td>
<td>55.1</td>
<td>9.1</td>
<td>3.00</td>
</tr>
<tr>
<td>( c_{33} )</td>
<td>172</td>
<td>172.8</td>
<td>138</td>
<td>123.2</td>
<td>32.5</td>
<td>3.90</td>
</tr>
<tr>
<td>( c_{44} )</td>
<td>39.6</td>
<td>51.4</td>
<td>48.7</td>
<td>42.2</td>
<td>8.7</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*Calculated from measured \( c_{66} \) value by using the relation \( c_{66} = \frac{1}{2} (c_{11} - c_{12}) \)

Recently, Haverty et al. [21] have found a significant difference in the crystal structure between fluorapatite and HAP. While fluorapatite belongs to \( P6_3/m \) symmetry, Haverty et al. argues that, due to the presence of a hydroxyl dipole in the place of a spherical fluorine ion, \( P6_3/m \) is not a valid symmetry description for pure HAP. From \textit{ab initio} calculations, they found that, despite the pseudo-hexagonal nature of HAP, its most stable phase at absolute zero belongs to \( P2_1/b \) symmetry. It is interesting to note that Rietveld refinement of X-ray diffraction pattern of synthetic polycrystalline HAP at room temperature revealed it to belong to a mixed phase between \( P2_1 \) and \( P2_1/b \), \( P2_1 \) being the dominant one [21]. On the basis of the newly derived crystal structures of HAP from \textit{ab initio} geometry optimisation, elastic, dielectric and piezoelectric properties for HAP were computed by using inter-atomic potential method [182]. The stiffness
matrix obtained this way is listed in Table 2.6 showing the formal coefficients of the various symmetry descriptions of HAP.

Although the values listed in Table 2.5 agrees well with those calculated by Katz and Ukrainick, it is important to find experimental values to compare with calculated values. The difficulty in obtaining large single crystals of pure HAP still prevails thus making measuring anisotropic elastic properties complex. As an alternative, quasi-isotropic polycrystalline ceramics of HAP has been used to study the anisotropic elastic constants. Sintered ceramics have recently been used to measure isotropic elastic properties [183].

Furthermore, in determining elastic properties of bone, ultrasound offered a non-destructive means. Lang et al. measured the elastic constants of bovine bone employing an ultrasonic technique at two different frequencies, a dilatational mode at 5 MHz and an equivoluminal mode at 2.25 MHz [184]. Yoon and Katz [185-187] used an ultrasonic pulse transmission method at a fixed frequency of 5 MHz to measure anisotropic velocities in dry bone and assessed the effect of ‘bone-symmetry’ on the anisotropy of wave propagation. Yoon and Katz [185] have also provided a review of the theory of ultrasound propagation in hexagonally isotropic media.
**2.4.6. Mechanical Properties of Bone at the Nanoscale**

The unique properties of bone are apparent contradictions: rigid, but flexible; lightweight, but solid enough to support tissue growth; mechanically strong, but porous. Bone can withstand body weight without breaking and its compressive strength is about twice that of its tensional strength. These outstanding properties of bone are the results of its complex hierarchical structure and composition of type I collagen fibrils reinforced with apatite nanocrystals (See Figure 2.6) [51]. Bone, thus, is an example of a natural nanocomposite of apatite and collagen.

Nanotechnology, therefore, provides tremendous opportunities to understand the nanoscopic organisation of bone. Electron microscopy has long been used to visually identify the structure and properties of the bone constituents. For example, Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) have been widely used to study the morphology of bone structure and HAP nanocrystals [51, 85, 188-190]. Micro-Raman now provides
the ability to assess the chemical bonding within collagen fibrils [191, 192]. Micro-X-ray diffraction, available at synchrotron light sources, provides an analysis of the crystal structure [192-195]. More advanced microscopes made it possible to understand the nano-mechanical properties of bone constituents. Piezoresponse Force Microscopy (PFM) can detect the local electromechanical properties of bone with an applied electric field [6, 8, 11, 25]. Nanoindentation is a new capability for assessing mechanical properties at the 1-10 micron level [161, 196-200].

2.5. Electromechanical Properties of Bone

2.5.1. Dielectric Properties and Crystal Physics

Depending on its electric properties, the atoms in a solid are fixed in a rigid structure. There are three general types of solids: crystalline (single crystal), polycrystalline and amorphous (non-crystalline). Each type is characterised by the size of its ordered region (spatial volume), where atoms or molecules have a regular geometric arrangement or periodicity [201, 202].

1) Crystalline

Crystalline solid is the solid form of a substance in which the atoms or molecules are arranged in a definite, repeating pattern in three dimensions that extends to a few hundred microns, to a millimetre range, or even larger. Single crystals, usually have a high degree of order, or regular geometric periodicity throughout the entire volume of the material. A single crystal also has one atomic structure which repeats periodically across the whole volume. Even at infinite length scales, each atom is related to every other equivalent atom in the structure by translational symmetry.

2) Polycrystalline

Polycrystals consist of an aggregate of many small single crystals, i.e. crystallites or grains. Polycrystals have a high degree of order over many atomic or molecular dimensions. The regions in a polycrystal are grains or domains and are separated from one another by grain boundaries. The size of the grains is usually
between 100 nm and 100 μm in diameter. Polycrystals with grains that are <100 nm in dimension are called nanocrystalline. Also, the atomic order within a polycrystal material can vary from one domain to the next [203].

3) **Amorphous**

An amorphous solid consists of randomly orientated atoms, ions, or molecules that do not form defined patterns or lattice structures. Amorphous materials have low degree order and the order is only within a few atomic or molecular dimensions. Therefore, amorphous solids do not have any long-range order, but they have varying degrees of short-range order [203].

Electrical conductivity of solids may arise through the motion of electrons and positive holes, i.e. electronic conductivity or through the motions of ions, i.e. ionic conductivity [201, 202]. The conduction through electrons is the $n$-type conduction and through positive holes is the $p$-type conduction. Electrical conductivity of metal is due to motion of electrons and it increases with the number of electrons activated to participate in the conduction process. Insulators are the pure ionic solids where conduction can take place only through motion of ions. The presence of defects in the crystal structure also increases their conductivity [202].
Figure 2.15 Classification of Conductivity and Dielectric Properties of Solids

On the basis of the Band theory, solids can be classified into three types: Insulators, semiconductors and conductors. Insulators have low conductivities i.e. the electric circuit is impossible to flow through them. The electrical conductivity is in the range of $10^{-10} \sim 10^{-20} \text{ohm}^{-1} \text{cm}^{-1}$. Semiconductors are solids with intermediate conductivities at room temperature, which allow a small electric current to flow through them. The conductivity is in the intermediate range of $10^{-6} \sim 10^{-4} \text{ohm}^{-1} \text{cm}^{-1}$. Conductors are usually referred to as metals, which allow the maximum applied electric current to flow through them. The electrical conductivity is of the order of $10^{6} \sim 10^{8} \text{ohm}^{-1} \text{cm}^{-1}$ [202]. Figure 2.15 summarises the classification of conductivity and dielectric properties of solids.

Among all solids, only insulators have dielectric character. These substances do not allow electricity to pass through them but when an electric field is applied, induced charges are produced on their faces. Within an insulator, the electrons are strongly held by the individual atoms. When an electric field is applied,
polarisation occurs because the nuclei are attracted to one side and the electron cloud to the other side. As a result of polarisation, dipoles are produced. Dielectric properties include piezoelectricity, and its sub category pyroelectricity, and its sub category ferroelectricity, ferrielectricity, anti-ferroelectricity and paraelectricity (see Figure 2.15). Dielectric displacement \( D \) occurs when an electric charge is acting on a dielectric material, so that the molecules in the material are stressed and polarised. The equation is as follow:

\[
D = xP
\]

where \( x = \) electric field strength, and \( P = \) permittivity [202].

The difference in dielectric properties is due to the symmetry of the crystal materials, which is a fundamental postulate of crystal physics, as stated in Newmann’s Principle [204]:

‘The symmetry elements of any physical property of a crystal must include the symmetry elements of the point group of the crystal.’

The point group of a crystal is the group of macroscopic symmetry elements that its structure possesses. According to the number of rotation axes and the reflection planes, it is divided into 32 point groups [205]. Of these 32 point groups, 11 point groups contain an inversion centre, which are centrosymmetric. The rest of 21 are non-centrosymmetric and may support piezoelectricity. However, 1 of this group is a cubic point group whose symmetry elements prevent charge separation. This leaves 20 point groups in total that are piezoelectric. In further division, only 10 point groups are polar point groups that support spontaneous polarisation of charge without any mechanical stress due to a non-vanishing dipole moment associate with the unit cell. These 10 polar point groups are pyroelectric. The subgroup of the polar point groups includes ferroelectric materials that have reversible polarisation. Figure 2.16 displays the classification of crystal symmetry point groups.
Piezoelectricity

Piezoelectricity is the electric phenomenon that in some crystals, electricity is produced when mechanical stressed is applied due to the displacement of ions [202]. Typical examples of piezoelectric materials are Quartz, Rochelle’s salt, bone and tendon. Piezoelectric crystals can act as mechanical-electric transducers. These crystals are used as pick-ups in record players where they produce electric signals by application of pressure [206].

In piezoelectric matrix, four piezoelectric constants, $d$, $e$, $g$ and $h$, are used to correlate the mechanical and electrical parameters to each other. The piezoelectric strain constant $d$ expresses the electric polarisation $P$ produced by unit of stress $T$ or the strain $S$ produced by unit of field $E$. Since $T$ has six components, i.e., the tensile stresses $T_1$, $T_2$, and $T_3$ in the $x$, $y$, and $z$ directions and
the shear stresses \( T_4, T_5 \) and \( T_6 \) in the \( yz, zx \) and \( xy \) planes, and \( P \) has three components \( P_1, P_2 \) and \( P_3 \) in the \( x, y \) and \( z \) directions, their coefficients \( d_{ij} \) may have 18 components. The strain \( S \) has six components, and the field \( E \) has three components [207]. Their coefficients are also given by 18 components of \( d_{ij} \). According to the symmetry of a polymer, some components of \( d_{ij} \) become zero [208].

Piezoelectric materials are intrinsically anisotropic. Piezoelectricity provides a coupling between elastic and dielectric phenomena. Guzelsu et al. [209] emphasised the importance of coupling between the mechanical and electrical fields in a quasi-static case. Piezoelectric matrix of an anisotropic material that contains oriented crystallites can be expressed as [110]:

\[
P = \sum_{j=1}^{6} d_{ij} T_j
\]

where \( d \) is the constant of proportionality between the applied mechanical stress \( T \) and the generated electric polarisation \( P \). The \( d \) matrix contains 18 piezoelectric coefficients relating to the piezoelectric polarisation in different directions with the applied tensile and shear stress in different directions; the index \( i = 1, 2, 3 \) corresponds to \( x, z \) coordinates. The macroscopic electric field \( E \) across the bone is zero. The six \( j \) indices correspond to three tensile stress and three shear stresses. In general, the \( d \) coefficients are complex quantities and are expressed as

\[
d^* = d' - d''
\]

where the real part \( d' \) is proportional to the charge generated in phase with the applied stress and the imaginary part \( d'' \) is proportional to the charge generated out of phase with the applied stress. When \( d'' \) is non-zero, a relaxation dispersion is present and measurements as a function of frequency become a necessity [110].

The piezoelectric relations in a crystal producing a single mode of motion can be expressed as [110]:

\[

\]
\[ S_2 = S_2^E T_2 + d_{21} E_1 \quad \text{Equation 2.5} \]

\[ D_1 = d_{31} T_2 + \varepsilon_1^T E_1 \quad \text{Equation 2.6} \]

where \( S_2 \) and \( T_2 \) are the extensional strain and stress; \( S_2^E = S_{11}^E \), the elastic compliance, i.e., inverse of Young’s modulus along the length of crystal measured at constant electric field. \( d_{21} \) is the piezoelectric constant relating to the strain with applied field \( E_1 \). \( D_1 \) is the electric displacement and \( \varepsilon_1^T \) is the dielectric constant measured at constant stress. These equations are used to determine the static and low frequency behaviour of piezoelectric crystals.

The constant strain dielectric constant \( \varepsilon_1^s \) is related to the constant stress dielectric constant \( \varepsilon_1^T \) by the relation [110]:

\[ \varepsilon_1^s = \varepsilon_1^T (1 - k^2) \quad \text{Equation 2.7} \]

where \( k \) is the electromechanical coupling factor.

The square of the coupling factor is written as:

\[ K^2 = \frac{d_{21}^2}{S_2^E \varepsilon_1^T} \quad \text{Equation 2.8} \]

By solving Equation 2.7, it is readily shown that the clamped dielectric constant \( \varepsilon_1^s \), obtained by setting \( S_2=0 \), and elastic compliance of constant displacement \( S_2^D \), obtained by setting \( D_1=0 \), are related to the constant stress dielectric constant \( \varepsilon_1^T \) and the elastic compliance at constant field \( S_2^E \) by the equation [110]:

\[ \frac{\varepsilon_1^s}{\varepsilon_1^T} = \frac{S_2^D}{S_2^E} = 1 - k^2 \quad \text{Equation 2.9} \]
For crystals and polar molecules, the simplest example of linear electromechanical coupling is piezoelectricity, in which the application of stress, \( X \), results in electrical polarisation

\[
P = dX \quad \text{Equation 2.10}
\]

whereas the application of electric field, \( E \), results in strain;

\[
x = dE. \quad \text{Equation 2.11}
\]

From the thermodynamic Maxwell relations, the piezoelectric constant, \( d \), for direct and converse effects is equal; thus, studies of electromechanical response provide insight into the polarisability of material, and vice versa [210].

For composite materials, the macroscopic piezoelectric constant is a function of the piezoelectric moduli, elastic moduli, dielectric constants and geometry of the different phases and connectivity [210]. It is generally believed that the protein collagen is responsible for the piezoelectric effects in bone. It is also apparent that the dielectric effects in bone (including the conductivity) have a strong influence on the piezoelectric effect, and the presence of a finite conductivity introduces a frequency (or time) dependence into the measured voltages. A simple system is represented by a dielectric (capacitor) in parallel with a resistance, where that ratio \( \varepsilon / \sigma \) is just the RC time constant. In this case \( \varepsilon (\omega) \) can be a complex quantity; the real part, \( \varepsilon' \), represents the dielectric constant, and the imaginary part, \( \varepsilon'' = \sigma / \omega \) represents the conductivity divided by the frequency \( \omega \). Then

\[
\varepsilon = \varepsilon' - i \cdot \sigma / \omega \quad \text{Equation 2.12}
\]

The resistance and the capacitance depend on the frequency so \( \varepsilon' \) is frequency-dependent [72].

**Pyroelectricity**

If a spontaneous polarisation already exists, a change of temperature alters it. This phenomenon is pyroelectricity [204]. If the temperature within the crystal stays constant at its new value, the pyroelectric voltage then gradually disappears
due to leakage current. The cause of the leakage is due to electrons moving through the crystal, ions moving through the air, current leaking through a voltmeter attached across the crystal [211]. The total pyroelectric coefficient measured at constant stress is the sum of the pyroelectric coefficients at constant strain (primary pyroelectric effect) and the piezoelectric contribution from thermal expansion (secondary pyroelectric effect). If there is a small temperature change $\Delta T$, uniform over the crystal, the change in the polarisation vector $\Delta P_i$ is given by

$$\Delta P_i = p_i \Delta T$$

Equation 2.13

where the $p_i$ are the three pyroelectric coefficients [212].

Pyroelectric crystals are a subset of piezoelectric materials. The pyroelectric effect has been observed in mineral crystals such as tourmaline since antiquity and in hundreds of natural and artificial crystals, ceramics, and polymers during the past century [20, 32, 208, 213, 214].

**Ferroelectricity**

Ferroelectricity concerns materials that show a spontaneous polarisation that can be reversed by a sufficiently large electric field [215]. The electric field can reverse the polarisation by causing a small relative shift of the atoms in the crystal, and so turning the crystal into its electric twin. If the atoms make half this shift, or if half the atoms make this shift, the crystal will be in a more symmetrical and non-polar state. This intermediate configuration can be caused by temperature change, hence most ferroelectrics have a transition temperature (Curie point) above which they are normal and non-polar [204]. Ferroelectricity shows hysteresis in a strong alternating field as shown in Figure 2.17. It is a plot of polarisation ($P$) versus electric field strength ($E$).
Examples of common ferroelectric materials are potassium hydrogen phosphate (KH$_2$PO$_4$) and barium titanate (BTO/ BaTiO$_3$). Furthermore, antiferroelectricity occurs when the dipoles in alternate polyhedra point up and down so that the crystals do not possess any net dipole moment, e.g. lead zirconate (PbZrO$_3$). Ferrielectricity describes the phenomenon in which, even though the spontaneous polarisation of the dipoles is not uniformed, a net polarisation is present. Paraelectricity occurs when ferroelectrics lose their intrinsic polarisation at the temperature above the transition temperature. The dipoles disappear in the paraelectric phase [202].

### 2.5.2. Piezoelectricity, Pyroelectricity and Ferroelectricity of Bone

Bone has electrical properties that depend upon external mechanical loading. Studies of the mechnano-electrical response of bone under a wide range of conditions have shown this response to be a feature of living, freshly excised and dry bone [1, 14, 217-219]. This is considered as a material property of bone. Analyses of the direct and induced current flow into the bone require accurate information on the electrical properties of bone. Such data is also important for understanding the mechanism of charge transport. Electrical conductivity of bone is found to be dependent upon 1. humidity, 2. temperature, 3. magnitude of
applied electric field, and 4. exposure to radiation [72]. There are more studies that focus on the piezoelectricity and pyroelectricity of bone and these are discussed below.

**Piezoelectricity of Bone**

Fukada and Yasuda first demonstrated that dry bone is piezoelectric in the classic sense, i.e. mechanical stress results in electric polarisation, the direct effect; and an applied electric field causes strain, the converse effect [1]. In a piezoelectric material such as bone, the application of an electric field causes a change in physical dimensions. The electrical charges bound within the lattice of the material contract with the frequency of the applied filed to produce a mechanical stress. This converse effect leads to a potential difference across the end faces of the transducer in response to an applied stress. Piezoelectricity marks thus a relationship between elasticity and dielectricity; dielectricity being the relation between electric field and polarisation [72, 220]. **Figure 2.18** explains the converse effect measurements of bone, where the bone axis, collagen fibril direction and applied bias are the same direction.

**Figure 2.18** Converse effect of piezoelectric measurement of bone
Bone has a microscopic polycrystalline structure with a degree of ordering that varies substantially from one point to the other. Gundjian and Chen [30] assumed that the microscopic crystallite units of bone consist of collagen and HAP crystals aligned with their c-axes parallel to each other. Therefore, a bone crystallite unit is a composite of these two substances, having an equivalent c-axes of infinite rotational symmetry [$T(\infty)$]. This unique structure of bone results in a physical behaviour corresponding to that of a single crystal of piezoelectric symmetry [17, 20]. In conformity to this model, an idealised parallel plate lamellae model has been suggested for a specimen in bending test, in which the collagen orientations in a local region can be approximately parallel to each other. Tensile stresses were applied to the crossed (90°) orientations of collagen parallel to the plane, which is the same orientation to the collagen axes.

This work reminds us that the task of streamlining quantitative data on bone piezoelectricity is for macroscopic piezoelectricity measurements as well. There exists a big debate with regard to the overall macroscopic symmetry of bone obtained from the piezoelectric tensors measurements, suggesting a much lower (triclinic) symmetry instead of the commonly accepted (polar hexagonal) symmetry [221]. The original measurements of piezoelectricity in bone involved both direct and converse piezoelectric effects of human and ox femur and indicated an orientation dependence of the measured converse piezoelectric response with respect to the angle between the pressure axis (in reality the electrode axis) and the bone axis (taken as the long axis of bone fibre direction) [1]. The relevance of this study for PFM will be discussed in Section 5.3.

Two important conclusions from this study were that the maximum value of piezoelectricity occurred at 45° with respect to the bone axis and that a non-zero piezoelectricity value was measured at the 0°. From these orientation dependences, it was deduced that the macroscopic symmetry was similar to that of a crystal possessing hexagonal symmetry, $D_6$ (equivalent to an infinity symmetry $\infty2$), which has a nonpolar unidirectional symmetry that allows only shear piezoelectricity.
Measurements made by Fukada and Yasuda [16] on bovine and horse Achilles tendons determined a similar orientation dependence of the piezoelectric coefficient with the maximum value occurring at $45^\circ$ with respect to the macroscopic tendon axis (long axis of tendon) but with a zero piezoelectricity value at $0^\circ$. As tendons are constituted mainly by collagen but no mineral apatite, these measurements have been taken as benchmark values for collagen piezoelectricity. The macroscopic symmetry was found to be similar to that of a crystal possessing hexagonal symmetry, $C_6$ (equivalent to an infinity symmetry $\infty$), which has a polar unidirectional symmetry possessing a polar axis along the direction of macroscopic tendon axis. The numerical value of piezoelectricity along the polar axis (the so called $d_{33}$ coefficient) was however found to be much weaker than the piezoelectricity measured at $45^\circ$ to the tendon axis. Importantly, they also measured a non-zero $d_{33}$ value of piezoelectricity in horse femurs, thus alluding to the fact that bone’s macroscopic symmetry should be considered as $C_6$ rather than the originally suggested $D_6$, which does not allow a polar axis and hence rules out pyroelectricity. This finding essentially corroborated the latter report of bovine bone pyroelectricity [20].

There are two independent studies by Martin [221] and Liboff and Furst [222] that have challenged this apparent consensus due to the measurements of piezoelectricity in orientations that are not allowed by either $D_6$ or $C_6$ symmetries for bone (Table 2.7). Gundjian and Chen [30] reconciled the apparent discrepancy with these symmetries by incorporating polycrystalline bone X-ray texture analysis into the distribution of macroscopically measured piezoelectric coefficients to obtain an axially invariant single crystal tensor description. By employing a mathematically rigorous approach, they were able to convert macroscopically measured polycrystal piezoelectric tensors $d_{ij}$ (non-zero unique coefficients of $d_{14} = -0.27$; $d_{15} = 0.044$; $d_{31} = 0.0035$ and $d_{33} = 0.0093$) into Standard Equivalent Single Crystal Structure (SESCS) for bone systems with the single-crystal piezoelectric tensor $d^e_{ij}$ (non-zero unique coefficients of $d^e_{14} = 0.966$; $d^e_{15} = 0.205$; $d^e_{31} = 0.0746$ and $d^e_{33} = -0.125$).
This study by Gundjian and Chen [30] has two other significances. Firstly, it demonstrated mathematically that bone piezoelectricity, is primarily derived from the piezoelectricity of bone collagen. This was accomplished in a simple manner by comparing the volume of collagen in tendon and in bone, which was a factor of 3. They calculated the SECS for tendon and multiplied the SECS of bone by a factor of 3 to reach comparable results. Secondly, they derived a piezoelectric tensor of much lower symmetry than $C_6$, and very similar to the tensors suggested by Martin [221] and Liboff and Furst [222], by simply assuming a 10° misorientation from what has been postulated during the SECS calculation for bone piezoelectricity. If anything, the above discussion highlights that local measurements are different than macroscopic measurements on hierarchical structures. PFM measurements of bone in this work will be benchmarked with respect to its SECS.

Table 2.7 Macroscopic, microscopic and nanoscopic shear piezoelectric constants of poly- and single- crystal bone and collagen

<table>
<thead>
<tr>
<th>Reference</th>
<th>Bone symmetry</th>
<th>Bone</th>
<th>Tendon symmetry</th>
<th>Tendon</th>
</tr>
</thead>
<tbody>
<tr>
<td>$d$ coefficients</td>
<td>pC/N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>1.904</td>
<td>0.2171</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>-1.904</td>
<td>-0.2172</td>
<td>0.64</td>
<td>-0.09</td>
</tr>
<tr>
<td>36</td>
<td>-</td>
<td>0</td>
<td>0.18</td>
<td>-0.01</td>
</tr>
<tr>
<td>32</td>
<td>-</td>
<td>0.00033</td>
<td>-0.09</td>
<td>-0.05</td>
</tr>
<tr>
<td>31</td>
<td>-</td>
<td>0.00033</td>
<td>0.08</td>
<td>0.05</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>0</td>
<td>0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>0</td>
<td>-0.08</td>
<td>-0.01</td>
</tr>
<tr>
<td>24</td>
<td>-</td>
<td>0.04342</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>0.04342</td>
<td>0.05</td>
<td>-0.03</td>
</tr>
<tr>
<td>21</td>
<td>-</td>
<td>0</td>
<td>0.04</td>
<td>-0.36</td>
</tr>
<tr>
<td>23</td>
<td>-</td>
<td>0</td>
<td>-0.03</td>
<td>0.36</td>
</tr>
<tr>
<td>33</td>
<td>-</td>
<td>0.00033</td>
<td>-0.02</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>-</td>
<td>0</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Pyroelectricity of Bone

Pyroelectricity has been found in many biological systems, such as, bone, tendon, teeth, etc, although its source remains uncertain. It is also the manifestation of the temperature dependence of the spontaneous polarisation of certain anisotropic solids [208]. The pyroelectricity of bone depends strongly on the moisture content and the polarisation in wet bone is nonuniform [223]. The polarisation of bone appears to be due to an internal field at the interfaces between the solid matrix and the fluid within the pores.

Lang was the first scientist to study the pyroelectricity in bone [20]. Since then, he and his colleague used the dynamic pyroelectric method to study both wet and dry turkey femur and found an extremely large pyroelectric effect in moist bone. These phenomena observed were apparently related to the high orientational mobility and to the viscous relaxation, characteristics of dipolar water molecules in moist bone. In dry bone, the pyroelectric coefficient was very small, spatially uniform, and independent of bias field [224, 225]. Lang also found that the strong dependence of the pyroelectric coefficient of moist bone on the bias electric field is caused by the orientational mobility of the water molecules with their large dipole moments. By contrast, the large polar collagen molecules cannot be easily reoriented, and the pyroelectric coefficient of dry bone is field-independent [223].

Ferroelectricity of Bone

Because of the composite nature of bone, many interests have arisen in investigating the origin of the mechano-electrical phenomena. However, its physiological consequences are still not fully understood. The strain-dependent potentials are a consequence of its ferroelectric nature and have an important role in normal stimulated bone growth and fracture treatment [110].

Although ferroelectricity of tibia was reported by Hastings et al. [226], the effect from the water content in moist bone are unreliable because of the presence of leakage currents.
2.5.3. Piezoelectricity of Bone at Nanometric scale

So far, the fundamental mechanical and electromechanical properties of bone have been discussed mainly from macroscopic point of view. As discussed in Section 2.2.2, bone has the characteristics of nanostructure and therefore nanomechanism. Early research was based on relatively large scale measurements of mechanical behaviours, which are macro or micrometre scale, whereas recent studies look more into the nanoscale behaviours. Predominantly, piezoelectric effect of bone at nanoscale has drawn a great attention from scientists since the advent of Scanning Probe Microscopy (SPM).

Piezoresponse Force Microscopy is a special type of SPM, which allows the electromechanical analysis of biological systems at nanometre scale. The technique and the applications will be discussed in details in the Methodology section in Chapter III. PFM experiments on bone and related tissues began in recent years. In 2004, Halperin and associates studied the piezoelectric effect of both wet and dry human humerus and tibia with PFM at the nanoscale [10]. They recorded the piezoelectric behaviour in a collagen matrix and successfully obtained the 3D piezoresponse images near the Haversian canal and combined theoretical calculations with the conductivity analysis. The average value of piezoelectricity in human bone was measured at ~8 pm/V. Figure 2.19 shows the results of PFM measurements near Haversian canal of human compact bone.
Figure 2.19 PFM images of tibia
(a) Transverse cut of tibia bone. Piezoelectric coefficient was measured in four marked points. Piezoresponse image was studied in the marked square region; PFM image of cross-section of a sample from mature human cortical bone (tibial shaft); (b) topography image; (c) piezoresponse image near the Haversian canal; (d) nanoscale resolution piezoresponse image in the vicinity of the Haversian canal  (Adapted from: Halperin et al. 2004 [10])

Minary-Jolandan and Yu (2009) took the type I collagen fibrils from both bovine Achilles tendon [8] and bone [6] for nanoscale characterisations. Studying of the isolated collagen fibrils in tendon with ~100 nm in diameter, they found that the collagen fibrils behave predominantly as shear piezoelectric materials with a piezoelectric coefficient on the order of 1 pm/V, and have unipolar axial polarisation throughout the entire fibril [8].
Minary-Jolanda and Yu further examined the shear piezoelectricity in bovine bone and its collagen fibrils. They revealed with high-resolution PFM that these fibrils consist of piezoelectrically heterogeneous gap and overlap regions, with the overlap regions being apparently piezoelectric and the gap regions showing little piezoelectricity [6]. Later on, they concluded the shear piezoresponse in animal compact bone at nanoscale, indicating the direct contribution of collagen fibrils to the shear piezoelectricity of bone [11]. The piezoelectric constant calculated from the ten random point of the bone sample is at the range of 0.1~0.3 pm/V. Figure 2.21 shows the high-resolution shear piezoresponse in compact bone and the curve of shear piezo deformation vs. the applied AC bias.
Figure 2.21 High-resolution shear piezoresponse in compact bone
(a) AFM deflection image, and (b) the simultaneously acquired lateral PFM; (c) AFM tapping mode amplitude image of a cortical bone surface, revealing individual collagen fibrils randomly oriented on the bone surface. (d) Representative shear piezoresponse vs input amplitude curve acquired at a sample location on the bone surface showing the linear piezoelectric coupling. The slope provides a measure of the effective shear piezoelectric coefficient $d \approx 0.3$ pm/V.
(Reproduced from: Minary-Jolandan and Yu, 2009 [6])

Haenagea’s recent work [27] explained the importance of the scanning direction of single collagen fibril in PFM on the measurement of the strength of the piezoresponse (PR) and revealed the shear piezoelectricity was parallel to the fibril axis. Another discovery was that individual fibrils within bundles in skeletal muscle fascia can have opposite polar orientations and are organised into domains, though groups of several fibres having the same polar orientation. Figure 2.22 demonstrates the isolated collagen fibril at different scanning
directions to the sample in PFM receiving different vertical piezoresponse (VPR) and lateral piezoresponse (LPR).

Figure 2.22 PR of an isolated collagen fibre at three cantilever orientations with respect to the sample: at an initial, arbitrary orientation (b and c); after a 90° clockwise rotation (e and f); and at 180° orientation (h and i). Images b, e, and h are constructed from the out-of plane deflection signal, and c, f, and I represent the LPR. The topography of the area is shown in a (10 mm scan) and d (2 mm zoom of a). The plot in g shows the change in VPR along the same line of the sample (b and h) before and after the sample has been rotated by 180°. The cantilever orientation and the detection direction are overlaid on the PFM images.

(Adapted from: Harnagea et al. 2010 [27])
2.5.4. Physiological Significance of Piezoelectricity

Biophysical parameters governing bone growth and development have been studied for the understanding of bone disease and treatment [15]. Osteoporosis is one of the most common metabolic disorders of bone and may be caused by drug induced, idiopathic and postmenopausal or oophorectomy, but is mostly a result of aging. As a person ages, bone becomes more brittle, fracturing more easily and healing more slowly. Many elderly people also are less active and deficient in protein. This is a multifactorial and polygenic disease [227]. Osteoporosis occurs when bone remodelling is slower than modelling, i.e. the rate of resorption exceeds that of new bone formation coupled with a defective osteoblast function [212, 228]. This causes an imbalance between bone resorption and bone formation. Bone fragility is caused by reduced bone size, reduced bone mineral density or architectural changes [229, 230]. There is evidence that the change of microstructure of trabecular bone, such as loss of connectivity or removal of trabeculae, can significantly decrease bone strength in osteoporosis [231, 232]. Women are found to be more affected by osteoporosis. It has been proposed that postmenopausal osteoporosis is the consequence of impaired bone formation due to estrogen deficiency [233]. In contrast to women, male osteoporosis is secondary bone disease in most cases [234]. The other type of osteoporosis is related to calcium deficiency and aging of the skeleton [235]. The multiple pathogenetic mechanisms converge to contribute to bone loss and microarchitectural deterioration of skeletal structure. The mechanism of osteoporosis at the cellular level remains to be fully understood. However, it is believed that osteoporosis is mainly caused by the uncoupling of osteoclastic–osteoblastic activity, where the osteoclastic activity dominates [228].

Fracture healing is a natural process that bone has the ability to reconstitute injured tissue and recover its original function and form, which is a complex process that demands the coordinated participation of immigration, differentiation and proliferation of inflammatory cells, and osteoblasts, etc. [110]. The fracture evolution depends on many factors, e.g. mechanical loads, type of fracture, gap size, hormones [110]. Several authors have suggested the experimental methods to initiate and control this transient osteogenic response [218, 236], which
provides a means of accelerating the rate and capacity of fracture healing. Bassett and Becker et al. [155, 237] and McElhaney et al. [155, 237] suggest that bone growth is related to physiologically generated electric fields in vivo. Therefore, electrical characteristics of bone during growth are potentially important in understanding bone remodelling dynamics and in controlling bone growth by the application of electric fields.

As mentioned in Section 2.4.2, bone adapts to external mechanical environment and responds to cyclic rather than static strain. Lanyon et al. [92] reported that in an isolated turkey ulna preparation a few cycles of load per day are enough to prevent disuse osteoporosis. The precise mechanism for the response remains uncertain, but it has been proposed that strain produces transient pressure changes in bone, leading to interstitial fluid flow, which produces shear forces on the osteocyte cell membrane [238]. This theory has been modified by You et al. [239] to provide a mechanism for amplification of the signal detected by the osteocytes. In bone, two dissimilar materials (collagen and HAP) are combined together by polysaccharides, whose role is very significant in explaining the mechanical properties of bone. So that bone is considered as a two-phase composite. Piezoelectricity of bone is closely related to mechanical, electrical, bone growth and the remodelling, and also for understanding life processes in general.

A good understanding of physical phenomena is of paramount importance in finding treatments of bone disease. Many researches aim to establish a link with clinical behaviour. While the treatment of fracture healing by conventional methods has long been in practice, the use of electrostimulation has been successfully reported, and its use is promising [240-247]. Other studies on osteoporosis recorded that in the light of exogenous biochemical supplement are also considered along with electrical stimulation [248-257]. This combination significantly decelerates the progress of osteoporosis. An emerging aspect related to nanoparticles to treat bone related disease has also been reported [258-264]. A greater depth in understanding the mechanism of such interactions in bone may go a long way in improving the quality of human life.
2.6. Piezoelectric and Micromechanical Modelling of Bone

Since Wolff’s law was born, investigators have sought to formulate and test mathematical rules to simulate the adaptation of bone to its various functions. Many investigations have focused upon mechanical functions of bone, but some also encompass “biological functions” - including mineral homeostasis, development and growth processes, age-related changes, and hormonal influences [93].

In 1957, Fukada and Yasuda first demonstrated that dry bone is piezoelectric in the classical sense [1]. Since that time, many other researchers have confirmed the capacity of bones to produce piezoelectric potentials [265-269]. It is well known that bone matrix is considered as an amphoteric ion exchanger with both positive and negative fixed charges, and is composed in large part by:

(a) Inorganic HAP crystals which are not asymmetrical and thus cannot contribute to any piezoelectric effect;
(b) Collagen which has an asymmetric structure and is believed to be capable of producing piezoelectricity for bone [270].

Theoretical analyses of bone piezoelectricity are relevant to the issue of bone remodelling. Some studies have explored electromechanical effects in wet and dry bone [14, 209], see Figure 2.23. It suggests that two different mechanisms are responsible for these effects: The first mechanism describes classical piezoelectricity mainly due to the molecular asymmetry of collagen in dry bone, and streaming potentials found in moist or living bone, and generated by the flow of a liquid across charged surfaces. The second mechanism has been argued by dielectric measurements, and has suggested that the electromechanical effect in wet (fluid saturated) bone is not resulted from a piezoelectric effect [271, 272]. Thus, fluid transport plays a significant role not only in various aspects of bone metabolism such as mineralisation, but also in the electromechanical properties of bone.
Double-layer and streaming potentials in the fluid

Hunter et al. mentioned that an important property inherent to most of the biological charged porous media is the negative charge of their pore surface due to the presence of some negative sites, such as hydroxyl complexes [273]. This negative charge is partially compensated by the adsorption of cations on the surface forming the inner compact layer which is commonly referred to as the immobile Stern layer. However, the majority of the excess of positively charged counter-ions is located in the electrolyte aqueous solution forming an outer diffuse layer composed of mobile charges, as shown in Figure 2.24. Together with the fixed charged groups on the solid matrix, these ions form the so-called electric double-layer [273].

When advected by the interstitial fluid, the mobile charge population of the double-layer generates the streaming currents that can be macroscopically observed. In parallel, to conserve charge, the movement of the net charge generates an electric potential, which is often referred to as streaming potential.
In bone tissue, the flow of interstitial electrolyte may be driven by, for instance, the deformation of the bone under external forces [274]. Thus, the streaming potentials can be attributed to strain-induced fluid flow [275] and can be obtained from the hydraulic profiles within bone [276-278].

![Double-layer diagram](image)

**Figure 2.24** Representation of the double-layer
(Adapted from: Lemaire et al. 2011 [279])

**Homogeneous Stresses at the Surface**
An optimisation approach has been reported by Mattheck *et al.* [280] in developing a computer-aided optimisation (CAO) hypothesis. They observed that a good mechanical design is characterised by a homogeneous stress distribution at its surface. As a result, a computerised implementation of CAO was developed and has been used to simulate shape change of bone as well as plants, as shown in **Figure 2.25**.

With this model, the implementation is possible with commercial finite-element codes that support swelling materials or thermal expansion. The finite element model can be constructed with a uniformly thick surface layer. This method is developed based on changing the volume of the surface layer of elements.
Adaptive Elasticity of compact bone

Beginning in 1976, Cowin et al. developed and published a series of papers on “Adaptive Elasticity” of bone [282, 283]. Adaptive elasticity is a comprehensive phenomenon that is based on linear elasticity supplemented by additional constitutive equations that allow for changes in the density, hence the stiffness of bone and also changes in the external shapes of bone. Based on customary loadings, material properties and boundary conditions, the mechanical condition of remodelling equilibrium is described by the remodelling equilibrium strain state $E^0$. A new strain state $E$ is produced by altered loading or implantation of bone prosthesis. Then it generates local strain differences ($E - E^0$). The differences are used to formulate first-order remodelling rate equations that dictate the change in the density and/or shape of bone [282, 283].

Piezoelectric model

Gjelsvik et al. [284] made an assumption that a relationship exists between formation and deformation of bone on one hand, and stress induced electrical polarisation on the other, so that surface bone deposition and resorption is governed at least in part by a piezoelectric signal. However, the actual mechanism responsible for this system is unknown. For any relationship between
polarisation and bone development, there are four distinct parts that must be determined:

1) If bone is to be deposited or removed;
2) The rate at which these two processes are taking place;
3) Where bone is being deposited, the material direction of the new bone; and
4) The stress state of the new bone [284].

Since formation and destruction of bone on surface are considered, it is assumed that this activity is governed by a surface-generated signal. The polarisation vector on the bone surface is the activity signal. The restriction to the surface signal is not a very serious one because most of the bones are fairly thin walled and have nearly constant stress, and therefore constant polarisation through the wall [284].

The direction of the material axis relative to the primary stress direction is critical for the generation of the piezoelectric signal in bone. To find the actual relative direction, both the stress direction and the material direction everywhere in the skeleton must be determined. Bone direction is easier to find since it can be determined for isolated parts of the skeleton or for parts of bone in vivo. Therefore, the primary stress direction for a normal bone is also the material direction, lie in the surface. See Figure 2.26.

Piezoelectric experiments have been done directly using a piezoelectrical technique at the few points in both human and ox femur by Fukada [1]. However, this is a concern that it may be limited to remodelling of the unloaded surfaces of the bones, since bones typically are loaded only at the joints and muscle attachments, and a large portion of the bone surface falls into this category.
2.7. Conclusions

This chapter reviewed the characteristics of bone from anatomic system, chemical composition to biophysical behaviours. Bone is a remarkable tissue, combining such features as rigidity, compliance and regeneration capacity. It is characterised physically by the fact that it is a tissue that is hard, rigid and strong, and microscopically by the presence of relatively few cells and much extracellular substance formed of collagen fibres and stiffening substances. Bone provides us with structural support, levers for locomotion and undergoes growth and remodelling throughout the life. It is a compositionally and structurally complex material capable of remodelling itself in response to mechanical loads. The understanding of processes which relates load of bearing and growth has
become an area of considerable interest. It has also been reported that bone also has many interesting mechanical electrical and acoustic properties.

The solid state behaviour of bone appears to be one of the most interesting observations. It has been reported that bone has wide variety of properties. Among these important properties, piezoelectricity is a bilinear interaction between electrical and mechanical variables, while pyroelectricity is the interaction between electrical and thermal variables.

The mechanical and electromechanical behaviour of bone such as elasticity and piezoelectricity have been considered to be a consequence of its hierarchical architecture, the basic building block of which, at the nanostructural level, is a finely interleaved composite of collagen fibrils and HAP, a substituted calcium orthophosphate. Despite that the earliest discovery of bone mechanism dates back to the times of Galileo Galilei in the early 1600’s, the molecular foundation of such mechanical and electromechanical behaviour of bone is still uncertain.

Stress generated surface charge in bone in the form of piezoelectricity and streaming potential has long been considered as the driving force behind bone remodelling. Very little is, however, known about the basic mechanism for dissipating stress and surface charge at the local level of organisation between HAP nanocrystals and collagen. Recent studies suggest that an understanding of this behaviour of HAP/collagen nanocomposite is important in explaining a number of *in vivo* processes, most important of which are the biomineralisation and bone remodelling processes.

Therefore, the study of bone requires an interdisciplinary approach to understand and quantify its various functions and adaptations. Continued progress will depend upon approaches that blend principles and experiments in mechanics, cell biology, and biochemistry, and also incorporate clinical problems and findings.
Chapter 3 Methodology: Characterisation Techniques

3.1 Introduction

The unique chemical characteristics of bone give it special electrical and mechanical properties. It has been suggested that the electrical potentials might mediate biological processes [218]. Studies of the mechano-electrical response of bone under a wide range of conditions have shown it to be a feature of living, freshly excised and dry bone. It is a bone material property and is possibly unrelated to the cellular or other in vivo factors.

This chapter introduces the chemical, electrical and mechanical methods for biomechanical evaluation of bone and its constituents. There are a variety of experimental techniques available for evaluation of bone structure, microstructure, and biomechanics, including XRD, FT-IR, mechanical testing and electrical microscopy. The details of the techniques chosen are described in this chapter as well as the reasons that the author decided to use them to answer the fundamental questions: Is bone apatite piezoelectric? The main nancharacteralisation technique is Atomic Force Microscopy (AFM), which has been established as a standard tool in nano- and biotechnology for the imaging of surfaces. With the help of this technique, many details of bone at the nanometre scale can be revealed.

3.2 Chemical Extractions

As there are two major phases (collagen and apatite crystals) in bone, chemical extractions were carried out to separate these two phases from each other in order to study the chemical-physical properties of bone. The femur is one of the strongest bones of the body, having high tensile strength as shown in Table 2.3 of Section 2.4.1. Bovine femur was obtained from local source. The animal age is at ~13 months. The tissue on the surface of the bone and bone marrow was removed. The femoral heads were also removed and only the compact bone was
kept and was mechanically sliced at room temperature using hand saw. The ring shape bone section was then further reduced to the size of 5 mm x 5 mm x 1 mm squares by hand sawing and polishing with a series of abrasive paper. The cutting direction towards the bone axis was always marked (see Figure 3.1). The thickness of the samples was kept at ~1 mm, so that it is easy for the chemicals to penetrate to the centre of the sample. An average weight of a bone square is 0.35 ± 0.05g and the total weight of 10 samples in each batch is 3.3 ± 0.16g. At this stage, the bone samples were ready for the chemical extractions.

These plates were polished with a series of abrasive papers. For comparison, the cut samples were divided into three groups (A, B, C):

Group A - Raw bone;
Group B - Deproteinated bone;
Group C - Demineralised bone.
Many studies in literature have used similar extraction methods to obtain the bone matrix. The details of the extraction procedure are as follows (Figure 3.2):

**Bone Deproteination** [285]:
Ten bone samples were first rinsed with deionised water. They were put in a Fisher Pyrex coarse-fritted extraction thimble that fitted into the Soxhlet flask. A round-bottom flask with 100 mL of aqueous 80 per cent ethylenediamine (EDA) solution was connected to the bottom of the Soxhlet flask and a water flow system was connected to the top of the Soxhlet flask. The procedure of refluxing took 20 – 30 cycles, ~50 hours at the temperature of 115 - 119°C in the oil bath, which is the boiling point of ethylenediamine. The extracted samples were then cooled to room temperature and were rinsed thoroughly with deionised water until the reddish-brown amine agent was decanted. Another three times re-rinsing with cold deionised water was required and a continuous flow of water rinsed the samples to ensure the impurities were washed off. Finally, the samples were dried in an oven at 80 °C overnight. Thus, the inorganic matrix of bone (apatite) was obtained (Figure 3.2a). The average of the weight of the bone apatite from each batch was 2.86 ± 0.07 g.

**Bone Demineralisation** [286]:
Another ten bone samples were first rinsed with deionised water and were then treated with prolonged agitation in 5% formic acid in an Erlenmeryer flask until the samples became transparent (usually 72 hours). This procedure was completed in the oil bath to help dissolve out the mineral component. After cooling to room temperature, the samples were re-rinsed three times with deionised water and were washed with continuous flow of water until the samples were clean. Finally, they were dried in an oven at 80 °C overnight. Thus, the organic matrix of bone (mainly collagen) was obtained (Figure 3.2b). The average of the weight of the bone collagen from each batch was 2.92± 0.06 g.

**Bone Matrix with Orientations**
For optimising the extractions, other solutions and procedures were also used in different batches of samples. It was found that the methods reported above were
the most suitable methods for this experiment. To study the orientation dependent properties of bone, bone samples were cut with angles, i.e. 0°, 45°, and 90° to the bone axis. After the extraction methods were optimised, the samples with orientation marks were extracted following the procedures above. The successful samples were verified with the analytical methods, which will be discussed in the next sections.

![Chemical extraction procedures of bovine bone](image)

**Figure 3.2** Chemical extraction procedures of bovine bone
(a) deproteination (b) demineralisation

### 3.3 X-Ray Diffraction (XRD)

X-ray was discovered by W. C. Röntgen in 1895 and since then this radiation has been widely used in diagnostic methods for medicine and industry. The ability of X-ray to detect, characterise and to identify crystal materials is based on the nature of crystal materials. Every crystalline substance has a unique diffraction pattern when interacting with X-ray, even in a mixture, each substance can produce its pattern independently from the others [287]. Therefore, the X-ray
diffraction pattern of a pure substance is like a fingerprint of the substance. The powder diffraction method is thus ideally suited for characterisation and identification of polycrystalline phases [288].

The term of ‘crystal’ is used to designate a class of solid exhibiting certain characteristic properties [289]. Among all solid materials, 95% can be described as crystalline. 50,000 inorganic and 25,000 organic single components, crystalline phases, and diffraction patterns have been collected and stored on magnetic or optical media as standards [290]. The main use of powder diffraction is to identify components in a sample by a search/match procedure. Furthermore, the areas under the peak are related to the amount of each phase present in the sample.

X-ray diffraction starts with an electron in an alternating electromagnetic field oscillate with the same frequency as the field. When an X-ray beam is projected and hits an atom, the electrons around the atom start to oscillate with the same frequency as the incoming beam. The destructive interference exists in almost all directions, because the combining waves are out of phase and there is no consequent energy leaving the solid sample [290]. However, the atoms in a crystal are arranged in a regular pattern so that constructive interference exists in a very few directions. The combining waves are then in phase and the well-defined X-ray beams are ready to leave the sample at various directions. Hence, a diffracted X-ray beam can be described as ‘a beam composed of a large number of scattered rays mutually reinforcing one another’ [291]. This complex model can be simply described as X-ray reflections from a series of parallel planes inside the crystal. The orientation of X-ray reflection and interplanar spacing of the parallel planes are defined by Miller indices [205].

W.L. Bragg was the first to give the mathematical explanation of the actual positions of the X-ray diffraction spots, which is well known as Bragg’s law. The diffraction is considered by a two-dimensional array of scatterers [291]. Figure 3.3 is shows X-rays being reflected from a crystal obeying Bragg’s law. A
number of sets of equi-spaced parallel lines are shown which pass through all the scatterers. Each plane acts like a mirror and reflects X-rays strongly at an angle of reflection that equals the angle of incidence. Bragg’s diagrams show the reflection from successive parallel planes. If the path difference between the beams from successive plane is a whole number of wavelengths, then there is constructive interference.

Figure 3.3 Bragg’s law

(a) Parallel rays reflected from different points of a plane are in phase after reflection; (b) Parallel rays reflects from points on neighbouring partially reflecting planes are in phase when Bragg’s law is obeyed

(Adapted from: Woolfson, 1997 [291])

In this case, an X-ray beam incident on a pair of parallel planes, separated by an interplanar spacing $d$. The two parallel incident planes make a permitted angle of reflection ($\theta$) with these planes. A reflected beam of maximum intensity will result if the waves represented by the two parallel planes are in phase. The
difference in path length between the first beam and the second beam must then be an integral number of wavelengths (\(\lambda\)). This relationship can be mathematically expressed in Bragg’s law:

\[ n\lambda = 2dsin\theta \]  
\textbf{Equation 3.1}

The process of reflection occurs where Bragg’s law is satisfied. It is described in terms of incident and reflected (or diffracted) rays, each making an angle \(\theta\) with a fixed crystal plane. Bragg’s law often refers to 2\(\theta\) theory, because reflections occur from planes set at angle \(\theta\) with respect to the incident beam and generates a reflected beam at an angle of 2\(\theta\) from the incident beam. The possible \(d\)-spacing is determined by the shape of the unit cell. Bragg’s law can be written as [205]:

\[ sin\theta = \frac{n\lambda}{2d} \]  
\textbf{Equation 3.2}

The possible 2\(\theta\) values of reflections are determined by the unit cell dimensions. However, the intensities of reflections are determined by the distribution of the electrons in the unit cell [289]. The highest electron density is found around atoms. Therefore, the intensities of electrons depend on the types of atoms and the location in the unit cell.

XRD Rietveld refinement is a well recognised method to be uniquely valuable for structural analyses of almost all classes of crystalline materials [292]. In this experiment, X-ray diffractometer - PANalytical X’Pert was used to anlyse the crystal structre of bovine bone and extracted bone apatite. Rietveld refinement of bone apatite and constructed HAP structure was also carried out to study the electromechanical properties of bone and its constituents. The results are discussed in Section 4.5.

### 3.4 X-Ray Photoelectron Spectroscopy (XPS)

Photoelectron spectroscopy is a chemical analysis technique that involves the measurement of kinetic energy of photoelectrons to determine the bonding energy, intensity and angular distributions of these electrons and also uses this
information to examine the electronic structure of molecules [293]. It differs from the conventional methods of spectroscopy in which it detects electrons rather than photons to study only the electronic structures of a material. This new technique can be divided according to the source of exciting radiation into Ultraviolet Photoelectron Spectroscopy (UPS) and X-ray Photoelectron Spectroscopy (XPS). The energy of ultraviolet rays (<41 eV) is sufficient to only eject electrons from valence orbitals, in comparison to the high energy that X-rays (1000-1500ev) uses can eject electrons from core orbitals.

XPS is a typical example of a surface-sensitive technique which is based on the principle that X-rays hitting atoms generate photoelectrons. An important strength of XPS is that it can provide both elemental and chemical information. Only electrons that are generated in the top few atomic layers can be detected. In this way, quantitative information can be obtained about the elemental composition of the surface of all kinds of solid material [293].

Back to 1905, Einstein was the first to enunciate the basic principle of the photoelectric effect that placing a sample in vacuum with X-rays gives rise to the emission of electrons. If monochromatic X-rays are used with a photon energy $h\nu$, the kinetic energy of the emitted electrons $K_e$ is given by the following [294]:

$$K_e = h\nu - B_e - \phi$$

Equation 3.3

where $B_e$ is the binding energy of the atomic orbital from which the electron originates and $\phi$ is the work function. The work function is the minimum amount of energy that an individual electron needs to escape from the surface. Each chemical element can produce a unique set of electrons with specific energies. In these techniques, the kinetic energy distribution of the emitted photoelectrons can be measured using any appropriate electron energy analyser, so that a photoelectron spectrum can be recorded. See Figure 3.4. All elements can be detected, except hydrogen (H) and Helium (He).

In addition to the photoelectric process, relaxation of the excited ion may occur by the emission of Auger electrons of fluorescent X-ray photons, as shown in
Chapter 3 Methodology: Characterisation Techniques

**Figure 3.5.** When an electron from a core level is emitted, another electron from a higher level falls to occupy the core level vacancy; this causes emission of a third electron known as the Auger electron to conserve energy released in the previous step. Auger emission is independent of the photon energy and therefore the X-ray source is dominant for elements with low atomic numbers ($Z < 30$) [294].

![Figure 3.4 Schematic of the photoemission process](image-url)
XPS is surface sensitive technique that can probe a depth of ~10nm from the top of the surface. Sampling depth is the depth from which 95% of all photoelectrons are scattered when they reach the surface ($3\lambda$), where $\lambda$ ranges from 1 ~ 3.5nm [294]. The path length of the X-ray photons is in the order of few micrometres, whereas the inelastic mean free path of electrons is in the order of tens of angstroms. Although electrons are generated from the entire X-ray probe volume, kinetic energy losses due to inelastic scattering and it allows only those electrons a few atomic layers deep beneath the surface to escape without energy loss to be detected, thus giving its useful property of surface sensitivity. Figure 3.6 shows the ‘Universal curve’ of attenuation length or inelastic mean free path ($\lambda$) as a function of electron kinetic energy (KE). Electrons generated in XPS correspond to ~10 monolayers.
Figure 3.6 Universal curve showing the attenuation of an element’s path in a solid as a function of its KE

(Adapted from: Wagner, 2010 [294])

X-ray photoelectron spectrometer (Kratos AXIS -165) based in University of Limerick was used in this experiment to verify the purity of the extracted bone substances. The results were discussed in Section 4.4.

3.5 Fourier Transform Infrared (FT-IR) Spectroscopy

Infrared (IR) radiation refers to a broad range of frequency, i.e. the electromagnetic spectrum between the visible and microwave regions. Infrared spectroscopy measures the wavelength and intensity of the absorption of mid-infrared light by a sample. Mid-infrared is energetic enough to excite molecular vibrations to higher energy levels [297]. The wavelength of infrared absorption bands is one of the characteristics of specific types of chemical bonds. Infrared spectroscopy provides greatest utility to identify organic and organometallic molecules. The high selectivity of the method enables the estimation of an analyte in a complex matrix. This technique involves examination of the twisting, bending, rotating and vibrational motions of atoms in a molecule.
In infrared spectroscopy, when IR radiation is subjected to a sample, some of the infrared radiation is absorbed by the sample and the rest is transmitted. The resulting spectrum represents the molecular absorption and transmission of IR radiation, creating a molecular fingerprint of the sample. Like a fingerprint, there is no two unique molecular structures produce the same infrared spectrum. This makes infrared spectroscopy unique and useful for several types of analysis.

Spectra in the mid-infrared region are usually measured at a resolution of \(~4\ \text{cm}^{-1}\). When such spectra between 4000 and 400 cm\(^{-1}\) are measured with a prism or grating monochromator, only one 4 cm\(^{-1}\) resolution element in the 3600 cm\(^{-1}\) wide spectral range of interest is measured at any instant and the remaining 899 cm\(^{-1}\) resolution elements are eliminated. Thus, the efficiency of this method is only \(~0.1\) [298].

Infrared spectra are the results from transitions between quantised vibrational energy states. Molecular vibrations have a wide range from the simple coupled motion of the two atoms of a diatomic molecule to the much more complex motion of each atom in a large polyfunctional molecule [297]. Molecules with \(N\) atoms can have \(3N\) degrees of freedom, three of which represent translational motion in mutually perpendicular directions (the \(x\), \(y\), and \(z\) axes) and two represent rotational motion. The remaining \(3N-6\) degrees of freedom allow the atoms in a nonlinear molecule to vibrate in a number of vibration modes. Each mode \((i)\) has harmonic displacements of the atoms from their equilibrium positions. For each mode, all the atoms vibrate at a certain characteristic frequency \((v_i)\). The potential energy \((V(r))\) of a harmonic oscillator is a function of the distance between the atoms \((r)\), shown by the dashed line in Figure 3.7. For any mode in which the atoms vibrate with simple harmonic motion, which obeys Hooke’s law. Therefore, the vibrational energy states \((V_{iv})\) is described as: [297]

\[
V_{iv} = hn_i(n_i + \frac{1}{2})
\]

\textbf{Equation 3.4}
where $h$ is Planck’s constant, $n_i$ the fundamental frequency of the particular mode, and $n_i$ the vibrational quantum number of the its mode ($n_i = 0, 1, 2, \ldots$). The frequency in units of Hertz is usually given the symbol $\nu$. Vibrational frequencies are often in the form of units of wavenumber, the number of waves per unit length. The unit of wavenumber is cm$^{-1}$ and is given the symbol $\delta$ by many chemists and $s$ by many physicists [297].

![Figure 3.7](image.png)

**Figure 3.7** Potential energy of a diatomic molecule as a function of the atomic displacement during a vibration for a harmonic oscillator (dashed line) and an anharmonic oscillator (solid line)

(Adapted from: Griffiths et al. 2007 [297])

Fourier Transform InfraRed (FT-IR) is a preferred method of infrared spectroscopy. In FT-IR spectrometry, all the resolution elements in the sample are measured at all times during the measurement. In comparison to IR spectrometry, more radiation can be passed between the source and the detector.
for each resolution element [297]. As a result, transmission, reflection, and even emission spectra can be measured significantly faster and with higher sensitivity than IR technique.

FT-IR spectrometer (PerkinElmer Spotlight™) in both University of Limerick and Dublin Institute of Technology (DIT) was used in this experiment to analyse the chemical structure of the bovine bone and extracted bone collagen and apatite, compared with the commercial pure HAP powder. The results were discussed in Section 4.3.

3.6 Microscopy

Electrons have very special properties that they are very surface sensitive at certain energy level. Electrons in this energy range carry enough momentum to explore the whole surface Brillouin zone of the targeted material and they are easy to generate and to control. Electron spectroscopy is a surface analytical technique to study the electronic structure and its dynamics in atoms and molecules, using electrons as a probe. There are many experimental applications of electron spectroscopy, such as high-resolution measurements on the intensity and angular distributions of emitted electrons on the total or partial ion yields. Since ejected electrons can escape only from a depth of approximately 3 nm or less, it makes electron spectroscopy one of the most useful techniques to analyse surfaces of solid materials. For detecting deep layers of the sample surface, depth profiling technique can be accomplished by combining an electron spectroscopy with a sputtering source that can remove surface layers.

3.6.1 Optical Microscopy

Optical microscopy belongs to a larger group of microscopic techniques, which magnifies the object by using visible light and a system of lenses. Optical microscopy is the oldest design of microscope, but it is an important analytical technique in modern scientific fields and has been widely used in research [299].
Chapter 3 Methodology: Characterisation Techniques

The main components of an optical spectroscopy are ocular lens, revolver, objective, focus wheel, frame, light source, diaphragm and condenser lens, and stage. The basic working principles are quite simple, though the modern design could be complex containing a number of objectives and a camera system [299]. The objective lens used is a high powered magnifying glass. The light from the sample to be examined comes to a focus when the objective lens is brought close to the sample. By adjusting the brightness of the light source, a highly enlarged image is obtained. The image is further magnified by the eyepiece lenses. Finally, this image can be viewed either by eyepiece or on the screen of a computer. The essential principle of an optical microscope is that the objective lenses have short focal length [299]. Figure 3.8 demonstrates the principle of optical microscopy.

![Figure 3.8 Principle of Optical Microscopy [300]](image)

The two major optical microscopy techniques are brightfield and darkfield microscopy. In this study, brightfield microscopy was used to observe the morphology of the bone samples. Brightfield microscopy is the simplest of all the optical microscopy illumination techniques and its simplicity makes it a popular technique [299]. Sample illumination is transmitted white light and the contrast in the sample by illuminating from below the sample and by observing from above it. This is caused by the absorbance of some of the transmitted and scattered light in dense areas of the sample. This technique is suitable for
unstained, stained samples or other types of samples that can naturally absorb and scatter visible light. The images of the sample produced appear dark or highly coloured against bright background that usually white or light grey.

The ability to distinguish two points apart from each other determines the resolving power of the microscope. The resolution of a microscope depends on a number of factors in its construction, such as the size of the lenses used, the distance between the lenses, etc. There is also an inherent theoretical limit to the resolution related to the wavelength of visible light, i.e. 400-600nm. This theoretical limit is the smallest distance observed between two points and is described as follow [299]:

\[ \text{Resolution} = \frac{0.61 \ l}{\text{N.A.}} \]  

Equation 3.5

where \( l \) represents the wavelength of light used and \( \text{N.A.} \) is the numerical aperture. The resolution of an optical microscope is dependent on the quality lenses and illumination systems.

Optical Microscope (Zeiss AX10) was used to analyse the morphology of the extracted bovine bone. The images and the discussion are in Section 4.2.

### 3.6.2 Scanning Electron Microscopy

Compared to optical microscope, scanning electron microscope uses electrons rather than light to form an image. SEM has many advantages over a light microscope. It has a large depth of field, which allows a number of samples to be in focus at the same time. The SEM also produces much higher resolution images, i.e. the closely spaced features can be examined at a high magnification. Sample preparation is not complicated since most SEMs only require the samples to be conductive. The combination of higher magnification, larger depth of focus, greater resolution, and easy sample preparation makes SEM one of the most widely used instruments in scientific research [301]. **Figure 3.9** illustrates the set-up of a typical SEM.
Imaging samples with an SEM, accelerated electrons carry significant amounts of kinetic energy. This energy gradually disappears due to various signals produced by electron-sample interactions when incident electrons are decelerated on the surface of the solid samples. These generated signals include [301]:

- Secondary electrons (that produce SEM images);
- Backscattered electrons (BSEs);
- Diffracted backscattered electrons (that determine crystal structures and orientations of minerals);
- Photons (characteristic X-rays that are used for elemental analysis and continuum X-rays);
- Visible light (cathodoluminescence - CL); and
- Other signals, e.g. heat.

Figure 3.9 Schematic drawing of SEM [302]

Among all produced signals, secondary electrons and backscattered electrons are commonly used for generating sample images. Secondary electrons produce the morphology and topography of the samples and backscattered electrons are responsible for showing contrasts in composition in multiphase samples, which is rapid phase discrimination [303]. X-ray generation is produced by inelastic collisions as the incident electrons hit the electrons in discrete orbitals of atoms in the sample. When the excited electrons return to lower energy states, X-rays are
yielded with a fixed wavelength. Hence, characteristic X-rays are generated for each element in a mineral sample that is excited by the electron beam. SEM technique is a non-destructive method because x-rays generated by electron interactions do not cause volume loss of the sample, and the same materials can be analysed repeatedly [303].

The SEM is also a routinely used analysis not only to generate high resolution images but also to show spatial variations in chemical compositions:
1) Acquiring elemental maps or spot chemical analyses using Energy Dispersive Spectroscopy (EDS);
2) Discrimination of phases based on mean atomic number using BSEs; and
3) Compositional maps based on differences in trace element activators using CL (that are useful for typical transition of metal and rare earth elements) [303].

Furthermore, the SEM is widely used to identify sample phases based on qualitative chemical analysis and crystalline structure. SEM also provides precise measurements of very small features and objects down to 50 nm in size. For other applications, e.g. SEMs equipped with diffracted backscattered electron detectors (EBSDs) can be used to examine micro fabric and crystallographic orientation in many materials.

Scanning Electron Microscope (HITACHI SU-70) was used to analyse the morphology and the chemical elements of the raw bone extracted bovine bone. The images and the discussions are in Section 4.2.

3.6.3 Transmission Electron Microscopy

Transmission electron microscopy is a valuable technique for the characterisation of materials by using the very small wavelengths of high-energy electrons as a probe to image solids at the atomic scale. The TEM can provide significantly higher resolution images and various information, such as local structure of the sample by imaging of defects e.g. misorientations, average structure by using diffraction to identify crystal class and lattice parameter, chemical composition
and electron phase shift [304]. When imaging with the TEM, the electrons are focused with electromagnetic lenses and the image is generated on a fluorescent screen, or recorded on film or digital camera. The electrons in the TEM are accelerated at several hundred kV, giving the wavelength of 0.025Å much smaller than that of light - 200kV. However, like other electron microscopes, TEM is limited by aberrations inherent in electromagnetic lenses, which is ~1-2 Å [304]. Figure 3.10 explains the basic objective/intermediate lens system of TEM. Using the TEM requires highly skilled operators, sample preparations, the interpretation of the information obtained as well as a good understanding of the structure of materials and the processes occurring in the microscope.

![Objective/intermediate lens system of TEM](image)

**Figure 3.10** Objective/intermediate lens system of TEM [302]

Even in very thin samples, individual atoms cannot be observed. In high resolution imaging mode of TEM, the crystal lattice of a material is used as an interference pattern between the transmitted and diffracted beams. Therefore, information such as planar and line defects, grain boundaries, interfaces, etc. is obtained with atomic scale resolution. Combined with electron diffraction and imaging in the brightfield /darkfield modes, the TEM gives information about the morphology, crystal phases, and defects in a material. Finally, equipped with a
special imaging lens, TEM allows to observe micromagnetic domain structures in a field-free environment [304].

The TEM can also form a very small focused electron probe (~ 20 Å), which can be positioned on very fine features in the sample for microdiffraction information or compositional information. The analysis of x-rays for compositional information is the same signal as that used for EMPA (Electron Microprobe Analysis) and SEM composition analysis, where the resolution is of 1 μm due to the electron beam spreading in the bulk sample. The spatial resolution for this compositional analysis is much higher that is near the probe size due to the TEM sample is so thin [305]. Using a high brightness field-emission gun in modern TEMs can improve the sensitivity and resolution of x-ray compositional analysis over traditional thermionic sources.

Transmission electron microscope (JEOL, 2100F) was used in this experiment to study the morphology and the local orientation of extracted bone apatite. The images and the discussions are in Section 4.2.

3.6.4 Confocal Laser Scanning Microscopy (CLSM)

Compared to conventional microscopes, confocal microscopes generate much sharper images of a sample by excluding most of the light from the sample that is not from the microscope’s focal plane [306]. Confocal images have better contrast that allows better observation of fine details and represent a thin cross-section of the sample. It is also capable to build 3D reconstructions of a sample by assembling the images of a volume of thin slices taken along the vertical axis. Confocal laser scanning microscopy is for obtaining high resolution images with depth sensitivity by controlling and limiting the depth of focus [306]. Confocal microscopes consist of the pinhole apertures and point-by-point illumination of the specimen, which are the key features from the first design by Minsky in 1955 [306]. Modern designs of confocal microscopes combine the advances of optics and electronics to improve scanning speed, image quality, and data storage. Two major methods to obtain confocal microscope images, i.e. by reflecting light off the sample or by stimulating fluorescence from dyes (fluorophores) applied to the
sample. The difference between the two techniques is small. The latter method - fluorescence confocal microscopy is most commonly used in biological applications. Other methods, for example, using transmission of light through the sample, are much less popular [306].

The basic of confocal microscopy can be explained as a pair of lenses that focuses light from the focal point of the first lens to the focal point of the second lens, as illustrated by the dark blue rays in Figure 3.11. The other rays in light blue colour are the light from another point in the sample that is not the same focal point of the first lens as the dark blue rays. So that, the image formed by the light blue point is not at the same location as the image from the dark blue point [306]. Obviously, the light blue point is out of focus at the location of the screen. The aim of this design is to see only the image of the dark blue point, as explained in Minsky, 1988 [305].

![Figure 3.11 Rejection of light not incident from the focal plane](image)

All light from the focal point that reaches the screen is allowed through. Light away from the focal point is mostly rejected [307]

The setup of confocal microscope greatly minimises the illumination of the sample by reducing the amount of light scattered from light blue points. In fluorescence microscopy, the entire view of the sample is completely illuminated, which makes the observed area fluoresce at the same time. Although the highest intensity of the excitation light is at the focal point of the lens, the other areas of the sample get fluoresce by some of this light. This indicates that the light at a dark blue point may also include light that has been scattered from other light
blue points, thereby reducing the effect of fluorescence. To eliminate the interference of the light from the light points, the design is to focus a point of light at the in-focus dark blue point by imaging a pinhole aperture placed in front of the light source [308]. Thus, the only regions that are illuminated are a cone of light above and below the dark blue focal point and the images obtained are much sharper.

Confocal Microscope (Zeiss LSM 710 META) was used in this study to compare the morphology of raw bone and the commercial extracted bone samples. The images and the discussions are in Section 4.2.

3.7 Scanning Probe Microscopy

Scanning probe microscopy (SPM) is an imaging technique that forms high resolution images of sample surface using a physical probe at atomic level. It has diverted into many branches with different application proposes. Atomic Force Microscopy is a basic type of SPM. Since 1986, the atomic force microscope has become a standard tool in surface physics and has been used for many biological systems [309]. In contact-mode AFM setups, a sharp AFM tip of a microstructured cantilever is approached to the sample surface in direct mechanical contact to create a force between tip and sample. Along with the tip scanning the sample surface, maps of constant tip–sample interaction force are generated, which are the topography of the sample. Simultaneously, the deflection of the cantilever constant was kept for recover the maps. This is achieved by connecting a feedback loop to the scanner, which continuously adjusts the z-position (the vertical axis) of the scanner and thus the tip during raster scanning the uneven sample surface. So that the output of the deflection sensor can remain unchanged at a pre-set value, i.e. the setpoint value [309].

Contact-mode AFM has been successfully used in many applications. However, the resolution was found to be limited in many cases by lateral forces interfering between tip and sample, in particular for soft samples. To avoid this effect, vibration can be applied to the cantilever in vertical direction near the sample
surface. AFM imaging with oscillating cantilever is also known as dynamic force microscopy (DFM) [309].

With SPM technique, samples can be studied at nanometre level rather than using light or electrons. It is also possible to observe the sample in three dimensions and to manipulate the sample in real time through an application of external force, e.g. an external electric field is applied in piezoresponse force microscopy to study the electromechanical properties of the sample.

### 3.7.1 Atomic Force Microscopy

Atomic force microscopy allows to view and to measure surface structure with unprecedented resolution and accuracy at nanometre scale. It is capable to image the arrangement of individual atoms in a sample or the structure of individual molecules [310]. AFM can also be carried out in physiological buffers at 37 °C to monitor biological reactions and even see them occur in real time. AFM can be used for image most samples, e.g. hard surface, such as a ceramic material, a texture with metallic nanoparticles, or soft surface, such as highly flexible polymers, animal cells, or individual molecules of DNA [310]. As well as an imaging tool, AFM has various spectroscopic modes that measure other properties of the sample at the nanometre scale. Because of all the advantages of AFM over other types of microscopy techniques, AFM has become popular to deliver quantitative high-resolution images in all fields of science, such as chemistry, biology, physics, materials science, nanotechnology, astronomy, medicine, etc.

Not like an optical or electron microscope that works by focusing light or electrons onto a surface, an AFM uses a sharp probe to physically ‘feels’ the sample’s surface and builds up a map of the height of its surface in three dimensions. This is very different from traditional imaging microscope, which can only measure a two-dimensional projection of a sample’s surface [310]. The data from the raster scan process is then treated by the computer software to generate expect images. This treatment is simple and flexible. With the collected AFM height data, images of the sample surface can be generated from any
conceivable angle. The height data is very useful to quickly analyse the morphology of the sample and to measure the height, length, width or volume of any feature in the image [310].

As mentioned earlier, the AFM scans the sample surface with a sharp probe, building up a map of the height/ topography of the surface as it goes along. **Figure 3.12** illustrates the basic setup of the AFM. To understand the operation of an AFM, three basic concepts are introduced:

1) Piezoelectric transducers (or piezoelectric scanners);
2) Force transducers (force sensors); and
3) Feedback control.

Basically, the piezoelectric transducer brings the AFM tip near the sample surface and moves it over the surface, the force transducer senses the force between the tip and the surface, and the feedback control receives the signal sent from the photodiode detector to generate images and also feeds the signal from the force transducer back in to the piezoelectric scanner to maintain a fixed force between the tip and the sample surface [310].
The AFM measures the forces between the tip and sample surface, therefore knowing these forces is important for image interpretation. The forces are not measured directly, but calculated by measuring the deflection of the cantilever, and the stiffness of the cantilever that has been calibrated. Hooke’s law is the basic principle of the AFM:

\[ F = -kz \]  \hspace{1cm} \text{Equation 3.6}

where \( F \) is the force, \( k \) is the stiffness of the lever, and \( z \) is the distance the lever is bent [310].

Usage of oscillating cantilever in the AFM was firstly suggested by Binnig [310]. In earlier experiments, the effect of lateral forces acting between the tip and sample was realised. Therefore, scanning with oscillated cantilever was practised in later works. This effect also demonstrates the influence of the force gradients on the cantilever frequency shift and possibility of non-contact scanning sample surface [310].
Figure 3.13 demonstrates the possibility of materials sensing under abrupt decreasing of cantilever oscillation amplitude. When scanning sample surface, not only attractive force exists but also repulsive forces play an important role. In most cases, these two tip-sample forces are responsible for the topographic contrast in AFM. Relatively, small shifts of oscillating frequency during sensing repulsive forces means that the contact of tip with surface under oscillation is not constant. Since the oscillation amplitudes of the oscillating cantilever are typically much higher than the interaction range of these forces, and only during small period of time, it makes nanoscale cantilever dynamics in AFMs inherently nonlinear [309]. Scanning in this manner is intermittent contact rather than absolute contact. Corresponding mode of Atomic Force Microscope operation is Intermittent Contact mode or Semi-contact mode. Sensing the contact repulsive forces leads to the additional phase shift of cantilever oscillations [312]. This phase shift is dependent on the characteristics of the sample. Recognition the
phase shift in Phase Contrast Imaging mode has been found very useful for nanostructured and heterogeneous materials [313].

### 3.7.2 Piezoresponse Force Microscopy

Piezoresponse Force Microscopy investigates the local effect of the piezoelectric sample surface caused by an applied electric field and analyses the resulting displacements of the sample surface [314]. The PFM technique is based on the converse piezoelectric effect, which is a linear coupling between the electrical and mechanical properties of a material. PFM is able to measure the surface deformations in the sub-picometre regime and to map the ferroelectric domains at nanometre scale. To detect the polarisation orientation, the AFM tip is used as a top electrode moving over the sample surface [315].

Operating in contact mode, PFM shows the reaction of out-of-plane and in-plane domains in the sample on the AC bias applied to the scanning tip as demonstrated in Figure 3.14. The electric field causes the domains with the polarisation parallel to the field to expand and the domains with opposite polarisation to contract. However, if the polarisation vector is perpendicular to the applied electric field, there is no deformation along the field direction, but a shear strain appears, which leads to displacements of the sample surface parallel to itself, along the polarisation direction [314].

Depending on the surface displacement, the movements of the AFM tip causes cantilever normal or torsion deflections. This is because of friction. The final direction of the deflection depends on the mutual orientations of the electric field as well as domain polarisation. Correspondingly in the case of the AC electric field phase lag between the electric field and cantilever deflections will depend on the their mutual orientations. By analysing the amplitudes and phases of the normal and torsion cantilever vibrations, the sample domain structure can be reconstructed [315].
In PFM, a voltage is applied to the conductive tip as \([313]\)

\[ V_{\text{tip}} = V_{DC} + V_{AC} \cos(\omega t) \]  \hspace{1cm} \text{Equation 3.7}  

where \(V_{DC}\) is the DC bias (switching bias), \(V_{AC}\) is the AC bias (probing bias) and \(\omega\) is the AC bias frequency (driving frequency). As the sample expands and contracts due to the converse piezoelectric effect, the tip deflection is monitored using a lock-in amplifier so that the tip oscillation \([313]\)

\[ A = A_0 + A_{1\omega} \cos(\omega t + \varphi) \]  \hspace{1cm} \text{Equation 3.8}  

where \(A_0\) is the static surface displacement, \(\varphi\) is the phase shift between the driving voltage \(V_{AC}\) and \(A_{1\omega}\) is the voltage induced deformation.

The PFM amplitude provides information on the magnitude of the local electromechanical coupling and the PFM phase image gives local polarisation orientation. Typically, the imaging resolution of PFM is less than ~10-30 nm as determined from half of the width of a domain wall in the mixed PFM signal, which is mostly used for the characterisation

\[ PR = A_{1\omega} \cos(\varphi) \]  \hspace{1cm} \text{Equation 3.9}  

here \(\varphi\) is ether close to 0° or to 180°. The resolution is limited by the tip-sample contact area, which is nominally determined by the radius of the tip apex, though additional mechanisms for broadening such as electrostatic interactions and the formation of a liquid neck in the tip-surface junction are possible \([313]\).
In this study, each bone sample was glued to a stainless steel metal plate with conductive silver paste and placed under the AFM tip. A commercial AFM (Asylum Research MFP-3D™AFM) was connected to an external lock-in amplifier (Stanford Research System, SR830), an external AC signal generator (Tektronix, AFG320) and an oscilloscope monitor (HITACHI, V-252). The high resolution probes (Nanosensors, ATEC) coated with Pt-Ir were chosen for the experiments with a tip radius of curvature < 20 nm, force constant of 0.2 N/m and a typical resonant frequency of 15 kHz. These unique probes, with the tip visible from the top of cantilever, effectively eliminate the electrostatic interference. Both vertical and lateral PFM modes were driven with the harmonic AC voltage signal of 0~120 V (peak to peak) applied to the AFM probe. The piezoresponse amplitude and phase signals of interested regions were monitored at the scanning rate of 0.5 ln/sec for both Vertical PFM (VPFM) and Lateral PFM (LPFM).

The out-of-plane deflection sensitivity was obtained by the standard analysis of the force curve from indenting a hard substrate - a glass slide (e.g., ~14 mV/nm ) [317]. The value of in-plane torsional twist sensitivity was proportional to the measured out-of-plane deflection sensitivity. According to the geometry of the AFM probe, the in-plane/out-of-plane sensitivity ratio \( R \) is expressed as [317]:

\[
R = \frac{4L}{3h}
\]

where \( h \) is the height of the tip (10 \( \mu \)m) and \( L \) is the length of the cantilever (450 \( \mu \)m). Therefore, \( R = 60 \) in this study, and the in-plane torsional twist sensitivity for this tip is \( \approx 840 \) mV/nm.

Four brands of PFM instruments have been used in this study, which are Agilent 5500 Scanning Probe Microscope, Asylum MFP 3D™, JPK NanoWizard 3 AFM and NT-MDT Ntegra system. Their differences are discussed in Section 3.7.4. The PFM results of bone and its constituents are discussed in Chapter 5.

### 3.7.3 Force Distance Curve

Atomic Force Microscopy force distance curves have been employed for the study of many materials’ properties and for the characterisation of surface forces.
Chapter 3 Methodology: Characterisation Techniques

Force in an AFM experiment is measured by collecting a force curve, which is a plot of cantilever deflection \((dc)\) as a function of sample position along the \(z\)-axis, i.e. towards or away from the probe tip [315]. Hooke’s Law is applied to force distance curve and it assumes a simple relationship between the force \(F\), and the cantilever deflection. There are other forces included in force distance curve, such as tip-sample interaction under \(dc\) approach or retracting motion [315].

The established force laws describe force as a function of the tip-sample separation distance \((D)\) rather than as a function of the \(z\)-piezo position [318]. The interpretation of AFM force curves entirely relies on these force laws, particularly those determined using the surface force apparatus (SFA). Therefore, the force curves must be transformed into the descriptions of force as a function of distance, \(F(D)\). Although current SFMs do not have an independent measure of \(D\), this transformation to \(D\) can be achieved by subtracting the cantilever deflection from the \(z\)-piezo movement [318]. For a very hard surface, zero separation is defined as the region in the force curve in which the cantilever deflection is coupled 1:1 with the sample movement. This appears in the force curve as a straight line of unit slope. The final plot after the corrections is the force-distance curve of the sample. Determining \(D\) by this approach requires that the tip is always in contact with the sample surface. A complete force curve includes two important stages: the probe approaches the sample and the probe is retracted to its starting position. Furthermore, in practice, there are two factors that can make determining the point of contact very difficult, i.e. long-range forces and sample elasticity [319].

3.7.4 Comparison of AFM Instrumentations

Piezo-response Force Microscopy was a crucial characterisation technique in this study. Four different brands of PFM have been used for investigation the electromechanical properties of bone, which are Agilent 5500 Scanning Probe Microscope, Asylum MFP 3D™, JPK NanoWizard 3 AFM and NT-MDT Ntegra system (Figure 3.15). Each instrument has their advantages and also disadvantages. The comparison of these instruments was made as a reference for future purchase and study.
Each instrument contains the basic components – a scanner, nose assembly, a photodiode detector, laser exciter, samples stages, video system, main controller, an anti-vibration system and a noise control isolation chamber. However, each instrument has their own unique design to tailor the needs for different purpose of research.

**Agilent 5500 Scanning Probe Microscope**

The Agilent 5500 SPM was used for the early stage of this study. Agilent’s multipurpose, top-down scanners come in a range of sizes, both open- and closed-loop, all offering good linearity, accuracy, versatility. The 5500 SPM has many technological features, including precision temperature control and environmental control. The Agilent 5500 SPM has been used in many research areas, such as materials science, polymers, nanolithography and general surface characterisation. Its unique features include top-down scanning and unrivalled environmental and temperature control, while providing maximum flexibility and modularity. The universal microscope base provides integration with an environmental chamber or an inverted optical microscope. Sample preparation is
made simple with its own sample plates, which have been designed for many different applications [311].

The Agilent 5500 SPM system includes the X/Y motion controls, scanner, high-resolution probe/tip, and detector. The control system for the microscope includes, at minimum, a high-speed computer, AFM controller and Head Electronics Box. Optional components include additional electronics, specialized scanners and probes for particular SPM techniques, and an environmental enclosure to control acoustic and vibration noise [322].

The advantages of the Agilent 5500 from the author’s experience are: (1) The design of the microscope is simple and light; (2) Most important components of the system are separated, e.g. scanner, photodiode detector, camera system, which is easy to repair if broken; (3) The system can work with both AFM and STM; (4) The automatic surface approaching function is easy to use especially for the beginners; (5) The anti-vibration system is simple but effective with a unique stone stage hooked with four elasticity robes (See Figure 3.16). (6) Agilent has its own image processing program under the main operation program.
However, there are also problems with this type of instrument: (1) The set-up procedure is relatively complex. The minor wires of each component are easy to be damaged; (2) The image resolution is not as high as other instruments; (3) All the cables connected to the microscope can be kept at one side of the chamber, so that the microscope itself cannot perfectly keep parallel to the horizontal level. For the samples with liquid, the surface may not be even; (4) Because the design of the isolation chamber left a window on the left side for the cables passing through, it may not be helpful if in a noisy environment; (5) The chamber is made of timber and foam, which is a not desirable shielding for any electromechanical measurements that are highly sensitive to the external electrical signal interferences; (6) So far, the system in UL does not have an external voltage supply connected to the controller. It is not possible to complete the experiments that require higher applied voltage than 10V.
Chapter 3 Methodology: Characterisation Techniques

Asylum MFP 3D™

The Asylum system was used most in this study. The MFP 3D™ provides the high sensitivity and measurements possible on an inverted optical platform [324]. The NPS™ closed loop nanopositioning sensors on all three axes ensure distortion-free images on samples as small as proteins and as large as cells - in both air and liquid. The MFP 3D™ measures the cantilever deflection to better than 20 pm (8 pm typical) without artifacts, which is ideal for force measurements, such as unfolding single molecules or probing cell mechanics [324].

The MFP 3D™ combines optical and atomic force microscopies in a single integrated tool. MFP 3D™ has been optimised with an inverted optical microscopes to improve the productivity from the combination of these useful techniques. It also accommodates high numerical aperture, water immersion, etc. The system has excellent performance for optical integration, high resolution imaging, quantitative force measurements, and environmental controls. It also supports many optical microscopy techniques, such as Brightfield, Phase Contrast and Confocal Laser Scanning [324].

The advantages of Asylum system from the author’s experience are: (1) It is a high resolution imaging system that allows to observe a very small area in nanometre scale; (2) The design of the scanner is unique with three height adjustable legs, which allows to scan the uneven samples with slope; (3) The operation software allows that one can observe the individual real-time profile line under each image window at the same time observing the scanning images; (4) The microscope is protected by an enclosed faraday cage, which greatly minimise the external static and non-static electrical signal interferences; (5) The microscope is placed on an isolated anti-vibration stage that is stable and safe; (6) Currently, the system is connected with an external high voltage supply which can provide up to 60V.

However, the problems of this model of Asylum AFM are: (1) The automatic approaching function is not an option; (2) The current model can only preform AFM imaging, but cannot multi-function; (3) Its operation software dose not
include image processing function. Therefore, a relevant software IGOR PRO has to be purchased to perform the best interpretation of the results.

During the Asylum workshop, Cypher™ AFM was demonstrated by the demonstrators. The biggest advantage over all types of AFM is that this system is the fastest and has highest resolution. It saves tremendous time for researchers from system set-up, tip mounting and surface approaching, not to mention the accuracy and the fast location of an interested area.

**JPK NanoWizard 3 AFM**

The JPK company had a demonstration in UL using NanoWizard 3 AFM by the demonstrators. The resulting NanoWizard 3 NanoScience system design provides high AFM performance in liquids and air, integrated with optical microscopy. It provides optimum imaging for single molecules, polymers and nanomaterials. The tip-scanning head equipped with a flexure scanner gives good flexibility for a large variety of different samples. In particular, large sample size imaging is possible [325]. The expanded flexibility and modularity of design coupled with the widest range of operation modes and accessories from electrochemistry to the tip-assisted optics module makes is suitable for multiple users and applications.

HyperDrive is a high resolution imaging technique, which is the core of the new system. It allows extremely low tip-sample interactions, and samples are kept from being damaged. The system is very stable to drift and has the ability to detect the smallest cantilever deflections enabling repeatable high resolution images [325].

This digital controller allows flexible operation. The design of NanoWizard 3 also allows for optimal use in liquid and comes with a vapour barrier, encapsulated piezos and a variety of dedicated liquid cells for applications ranging from single molecule experiments to corrosion in an electrochemical environment [325].
NT-MDT Ntegra system

NT-MDT Ntegra system was not operated by the author in this study. The samples were sent to the NT-MDT lab for test. From the description of the instrument, we studied that it is a multifunctional device for performing the most typical tasks in the field of Scanning Probe Microscopy.

The device is capable of performing various measuring methods, which allows analysing physical and chemical properties of the surface with high precision and resolution. Like the AFMs mentions above, Ntegra system is also possible to carry out experiments in air, as well as in liquids and in controlled environment. The new generation electronics provides operations in high-frequency (up to 5MHz) modes. This feature appears to be essential for the work with high-frequency AFM modes using high-frequency cantilevers [326].

There are several scanning types implemented in NTEGRA Prima: scanning by the sample, scanning by the probe and dual-scanning. On account of that, the system is ideal for investigating small samples with ultra-high resolution (atomic-molecular level) as well as for big samples and scanning range up to 100x100x10 µm. The unique DualScan TM mode allows investigating even bigger fields on the surface (200x200 µm for X, Y and 22 µm for Z) that can be useful, for example, for living cells and MEMS (Micro-electro-mechanical systems) components [326].

Built-in three axes closed loop control sensors trace the real displacement of the scanner and compensate unavoidable imperfections of piezoceramics as non-linearity, creep and hysteresis. The sensors, which are used by NT-MDT, have the very low noise level, thus allowing working with closed loop control on the very small fields (down to 10x10 nm). This is especially valuable for carrying out nanomanipulation and lithography modes. Furthermore, NTEGRA Prima has a built-in optical system with 1 µm resolution, which allows imaging the scanning process in real-time [326].
Chapter 3 Methodology: Characterisation Techniques

From the author’s imaging experience, the comparison of four brands of AFM was concluded in Table 3.1.

### Table 3.1 Comparison of PFMs

<table>
<thead>
<tr>
<th></th>
<th>Agilent</th>
<th>Asylum</th>
<th>JPK</th>
<th>NT-MDT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model</strong></td>
<td>5500 SPM</td>
<td>MFP 3D™</td>
<td>Cypher</td>
<td>NanoWizard 3 AFM</td>
</tr>
<tr>
<td><strong>Resolution</strong></td>
<td>Average</td>
<td>High</td>
<td>Highest</td>
<td>High</td>
</tr>
<tr>
<td><strong>Max. Applied voltage</strong></td>
<td>10V</td>
<td>60V</td>
<td>10V</td>
<td>10V</td>
</tr>
<tr>
<td><strong>Force distance curve</strong></td>
<td>No</td>
<td>Available</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Adhesion map</strong></td>
<td>No</td>
<td>No</td>
<td>Unknown</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Wet samples</strong></td>
<td>No</td>
<td>No</td>
<td>Unknown</td>
<td>Yes</td>
</tr>
</tbody>
</table>

#### 3.8 Conclusion

We have studied a large range of characterisation techniques that can be applied to evaluate the properties of bone. The details of each technique have been reviewed. The design of this experiment started with studying the chemical composition of bone by chemical extractions. Two major components of bone – collagen (organic) and apatite (inorganic) have been separated from each other. The nanostructure and morphology of raw bone and the extracted bone was revealed by a range of microscopy. To verify the purity of the extracted bone, chemical characterisations were completed with XRD, FT-IR. Further electromechanical characterisations on raw bone and pure extracted bone samples were carried out by AFM. This technique has become a fundamental tool in characterisation of biological samples at nanometre scale.

A functional AFM with an applied external electric field has been often used in investigation of physiological properties of biomaterial, that is PFM. The
characters and functions of four types of PFMs were also compared in this chapter, while each one has its own strengths for specific purpose. When choosing a PFM, one should overview at the applications, functions and performance of the instruments; also consider the individual research needs. We should never compromise in a research by choosing an ‘inexpensive, easy to use’ system with limited capabilities.
Chapter 4 Structural Characterisation of Bone and its Constituents

4.1 Introduction

As bone is a composite material consisting collagen fibrils and apatite nanocrystals, the design of this experiment is set to separate these two major components and to study their morphology, chemical composition, crystal structure and the comparison with HAP models, therefore the implication of their contribution to bone piezoelectricity. In the beginning of this chapter, the morphology of raw bone and the extracted bone were studied from microscopic to nanoscopic level using optical microscopy, SEM and TEM. The microscopic structure of a commercial bone sample was also compared with the raw bone. To proof the success of the chemical extraction, the physical state of bone was characterised by the measurements of various parameters using XPS, XRD, FT-IR and spectrochemical analysis. The experimental findings provided new insights of properties of bone and its constituents.

4.2 Morphology of Raw Bone and Extracted Bone

The primary focus of the extraction methods described in Section 3.2 were to cause minimal damage to the targeted constituent while removing the other completely. The freshly extracted samples show differences in properties ranging from appearance to mechanical properties. For example, the extracted collagen in wet state showed low rigidity, high compliance and high optical transparency, while apatite crystals in either wet or dry state showed fragility, more porosity, low compliance and low optical transparency, as seen in Figure 4.1.

Figure 4.1 Extracted collagen vs. Extracted apatite (Adapted from Zhang et al. 2012 [327])
Figure 4.2 Optical microscopic images

Raw bone (a, b), Extracted collagen (c, d) and Extracted apatite (e, f) before and after polishing. The Haversian Canals can be seen in the polished Raw Bone and unpolished Extracted Apatite at the same scale. (Adapted from Zhang et al. 2012 [327])

The extracted samples in general maintained the original plate-like shape of the starting raw bone sample. The hierarchical organisation within bone can be resolved by optical microscopy (Figure 4.2). In mature compact bone, the unique Haversian systems, which contribute to mineral deposition and
homeostasis, are present in the shape of ellipses (Figure 4.2b). Bundles of parallel collagen fibres can be seen in extracted collagens before polishing (Figure 4.2c), while after polishing, most of the surface became smooth, but some deep gaps between the bundles became larger and deeper (Figure 4.2d). The extracted apatite has largely retained the structure of the Haversian canals (Figure 4.2e), although the size and shape are much larger than the Haversian canals in the polished raw bone. Because of its nature of high fragility and high porosity, the polished apatite showed relatively smooth with stretching marks, but the centre of the Haversian canals still remained (Figure 4.2f).

The SEM morphology of bone and its extracted constituents revealed further details of the organisation. By studying the morphology of the extracted bone, the purity of the bone matrix can be predicted. Early extractions did not result in pure bone matrix. Some residues of bone apatite were found in the extracted bone collagen. The green arrows in Figure 4.3a show the apatite crystals are like larvae (~30nm) embedded in the valley of a cave of collagen matrix. But in the early extracted bone apatite matrix, it is difficult to observe any collagen residues. The surface of the extracted apatite appeared dense and rigid. The red arrows in Figure 4.3b points out the cracks on the apatite surface due to the drying process.

The later extractions were verified to be successful (see the Chemical analysis in Section 4.3). The morphology of pure bone matrix was compared to the untreated bone. The SEM images of bone were taken at different magnifications. Unpolished raw bone at low magnification reveals only the cutting marks from the hand saw (Figure 4.4a), but at high magnification, it exhibited rock-like and porous structure and more apatite crystals on the surface (Figure 4.4b, c). The extracted bone apatite was left with many holes on the surface after extraction (Figure 4.5a, b). Similar to the raw bone, but it was more porous in morphology. Apatite crystals are clustered and compact with an average crystallite size of ~30nm, confirming earlier observations made on bone apatite (Figure 4.5c, d). In contrast, the extracted bone collagen did not exhibit any apatite impurities but Haversian systems of approximately 100µm in diameter are clearly visible.
Bundles of collagen fibres were apparent on the surface at higher magnification (Figure 4.6c, d).

**Figure 4.3** SEM images of early extracted bone matrix

- a) extracted bone collagen, Magnification x1.10k
- c) extracted bone apatite, Magnification x500
Figure 4.4 SEM image of unpolished raw bone

a) Mag. x150; b) Mag. x10k; c) Mag. x100k; d) Mag. x110k

Figure 4.5 SEM images of extracted bone apatite

a) Mag. x150; b) Mag. x3k; c) Mag. x10k; d) Mag. x100k
Figure 4.6 SEM images of extracted bone collagen

a) Mag. x150; b) Mag. x400; c) Mag. x13k; d) Mag. x3k

For elemental analysis of bone surface, EDX was used to detect the chemical elements on the sample surface. The calculated results were the average value of five randomly selected areas on the sample surface. Both weight and atomic percentage of the elements are displayed in the figures below. Raw bone contains the major elements of Calcium (Ca), Carbon (C), Oxygen (O) and Phosphate (P), as well as the minor elements Nitrogen (N), Sodium (Na) and Magnesium (Mg) (Figure 4.7). Another element Sulfur (S) was also expected, but the percentage was too small to be detected. If the extractions were successful, the protein elements of bone should not be shown in bone apatite, and similar the inorganic elements should not be in bone collagen.

As expected, the element N that represents protein did not show on the surface of extracted bone apatite. The elements Ca and O are responsible for a larger portion of the total weight than in the raw bone, but C reduced to a quarter
Chapter 4 Structural Characterisation of Bone and Its Constituents (Figure 4.8). The element S was again too small to show. In contrast, the elements Ca and P that represent inorganic materials in bone were completely removed from the surface of extracted bone collagen, as well as Na and Mg. The only elements left were C, O and N, where the portion of N has dramatically increased compared to in the raw bone (Figure 4.9). The element C may be from the carbon tape used for sample attachment to the sample stage.

Figure 4.7 EDX result of Raw bone
Figure 4.8 EDX result of Extracted bone apatite

Figure 4.9 EDX result of Extracted bone collagen
Chapter 4 Structural Characterisation of Bone and Its Constituents

Under the confocal microscope, the microstructure of raw bone can be visualised and was compared with the extracted bone matrix. Obviously, the bone tissue is not homogenous and the difference of bone component was presented in colours (Figure 4.10b Excitation: 443nm; Emission: 486nm). Some substances of bone were autofluorescent and they can be visualised without staining, e.g bone apatite. However, bone cells were not autofluorescent and appeared as dark coloured circles. The extracted collagen in Figure 4.10c is somewhat distorted after the chemical and the heat treatments (Excitation: 485nm; Emission: 523nm). Some holes were left when the building blocks- bone apatite were washed away. The extracted bone apatite in Figure 4.10d showed a rigid inorganic matrix (Excitation: 425nm; Emission: 478nm). Compared to the organic matrix, the inorganic matrix presented high porosity. The holes left in the extracted apatite were where the collagen fibres existed. It is amazing how nature designs the complex structure of bone tissues. Even without a collagen template, the building blocks can still be held together without breaking down.

To compare the microstructure with the samples, the commercial samples - Bio-Oss® were also studies. Bio-Oss® is a medical bone supplement for bone implants, which is extracted from bovine bone [328]. There are two types of samples used as control samples- Bio-Oss® collagen and Bio-Oss® apatite. 3D optimal deflection images of the two control samples were taken with determined parameters using confocal microscopy. Bio-Oss® apatite containing 100% bone apatite showed that the apatite substances combine to form a rigid matrix as a whole (Figure 4.11a Excitation: 405nm; Emission: 425nm). Bio-Oss® collagen also contained some apatite residues showing how collagen fibres construct an organic matrix, where the apatite residues were not distinguishable (Figure 4.11b Excitation: 488nm, 535nm; Emission: 490 – 695nm).
Figure 4.10 Confocal microscopic images of bone and the bone matrix

(a) a piece of raw bone marked where the area was studied; (b) raw bone under confocal microscope (unstained); (c) the extracted collagen and (d) the extracted apatite, scale: 100 µm

Figure 4.11 Confocal microscopic images - 3D optimal deflection of the controlled samples (Bio-Oss®)

(a) 100% bone apatite; (b) bone collagen with apatite residues
A closer look at the nanocrystalline structure of bone apatite with TEM showed that the nanocrystals presented two shapes – rods and plates, where the former shape is predominate in the morphology. The size of these crystals were 7-10nm in diameter and 80-100nm in length (Figure 4.12a). The polycrystalline pattern of TEM was applied, which was co-responding to the apatite structure.

![TEM images of bone apatite](image)

**Figure 4.12** TEM images of bone apatite

(a) Polycrystalline of bone apatite; (b) extracted bone apatite showing nanocrystalline nature and an average orientation of apatite crystallites along the dotted line; (c, d) the defects of crystal lattices within individual nanocrystal of bone apatite, compared with straight lines (pink and yellow)

TEM also reveals the presence of local orientation of apatite crystals in bone, which is amazingly preserved and improved in extracted apatites despite the
aggressiveness of the extraction process employed. Even without the templated support from collagen fibres, the nano apatite crystals are pointing to a major direction and the parallel nature is still maintained (Figure 4.12b). The red dotted line shows the main orientation of the apatite crystals. Therefore, each individual nanocrystal rod was suspected to have polars. Finally, at much higher magnification, some defects in individual apatite rods can be found. Figure 4.12c, d discloses that the lattice pattern with an apatite rod does not always parallel to each line. There are misorientated lattice exist, which are considered to be the defects within crystal materials. The straight coloured lines in Figure 4.12c, d indicates the existing of the defect lattice.

### 4.3 Purity of Extractions

FT-IR provides the knowledge of the vibrational origins of the chemical bonds and as such can be used to determine the purity of the extracted components. FT-IR was used to distinguish the extracted phases by their vibrational signatures e.g. from the phosphate ions only in apatite and the amino groups only in collagen [329]. One distinct difference between the samples is that three main amide regions occur in both bone and collagen as the chemical markers of protein, which have been reported as amino I (~1600 cm\(^{-1}\)), amino II (~1550 cm\(^{-1}\)) and amino III (~1240 cm\(^{-1}\)) [54]. In this investigation, the FT-IR spectrum shows the amino regions of the raw bone and the extracted collagen showing at amino I (1624 cm\(^{-1}\)), amino II (1550 cm\(^{-1}\)) and amino III (1237 cm\(^{-1}\)). As expected, no amino peaks appear in the commercial sample of synthesised HAP (CAMCERAM HAP Sponge Powder). In the extracted apatite, there is a hint of spectral features in the region 1200-1650 cm\(^{-1}\) with low intensity, which is considered as a contribution from inorganic impurities in bone, such as carbon ions, and the organic chemical used for extraction. A significant evidence of the presence of the apatite phase is the phosphate group, which distinguishes apatite from collagen. The pure apatite used as a benchmark presents high intensity peaks of phosphate in the regions of PO\(_4\) \(v_1\) ~960 cm\(^{-1}\) and PO\(_4\) \(v_3\) ~1000 cm\(^{-1}\). These phosphate peaks were observed in both the extracted apatite and raw bone, but were removed from the extracted collagen, which confirms a high level of the
success of extraction. The presence of hydroxyl ions in these samples also appears in the form of OH\(^{-}\) stretching vibrations, in the region of ~3100-3500 cm\(^{-1}\) (Figure 4.13).

**Figure 4.13** Fourier Transform Infrared Spectra of bovine femur: Raw bone, Pure HAP, Extracted collagen and Extracted apatite

(Adapted from Zhang *et al.* 2012 [327])
FT-IR of Bone, Collagen and Apatite

IR spectra have been used extensively to assess mineral crystallinity in synthetic apatites and normal and diseased bones [330-332]. The introduction of computerised FT-IR spectrometers made calculation of spectra parameters easier, and there are now numerous papers that rely on FT-IR for characterisation of bone mineral [54, 333-335]. Coupling an IR spectrometer to a light microscope allowed FT-IR spectra to be recorded and mapped at anatomically distinct portions in bone [336-338]. The advantage of FT-IR microspectroscopy or imaging is that changes in mineral properties can be mapped at a spatial resolution of -20µm. Thus, the variation in mineral quality and quantity across an osteon can be documented [339], and changes across developing bones going from the periosteum to the endosteum or across the trabeculae can be noted [340].

In recent years, Boskey [341-343] and Morris [344, 345] have succeeded in determination of bone quality with microscopic FT-IR, FT-IR imaging and Raman spectroscopy without homogenisation or stain. The Morris group has characterized micro crack in bone mineral using a newly developed Raman system. In 1997 the Boskey group focused on the state of collagen cross-links in bone and characterized nonreducible = reducible collagen cross-link ratio in disease bone matrix with microscopic FT-IR and FT-IR imaging. They also studied the changes in mineral and matrix content and composition in replicate biopsies of nonosteoporotic human compact and trabecular bone and found that changes in osteonal bone in these same samples were reported previously. Spectral maps along and across the lamellae were obtained from iliac crest biopsies of two necropsy cases. Mineral: matrix ratios, calculated from the integrated areas of the phosphate ν1, ν3 band at 900–1200 cm$^{-1}$ and the amide I band at ~1585–1725 cm$^{-1}$, respectively, were relatively constant in both directions of analysis, i.e., along and across the lamellae. Analysis of the components of the ν1, ν3 phosphate band with a combination of second-derivative spectroscopy and curve fitting revealed the presence of 11 major underlying moieties. Of these, the ratio of the relative areas of the two underlying bands at ~1020 and ~1030 cm$^{-1}$ has been shown to be a sensitive index of variation in crystal perfection in both human osteonal bone and in
synthetic, poorly crystalline apatites. This ratio was calculated in both compact and trabecular bone from human iliac crest biopsies along and across the lamellae. The protein and mineral constituents produce intense, structure-sensitive IR bands. The protein Amide I (peptide bond C = O stretch) and Amide II (mixed C-N stretch and N-H in-plane bending) modes near 1650 and 1550 wave numbers (cm\(^{-1}\)) undergo wave number and intensity changes as a result of changes in protein secondary structure. The apatite phosphate \(\nu_3\) (P-O asymmetric stretch) and \(\nu_4\) (in-plane bending) modes have been used to monitor changes in mineral crystallinity [346, 347].

OoI et. al. [329] reported the properties of porous HAP bioceramic produced by heat treatment (annealing) of bovine bone over temperatures between 400°C and 1200°C and revealed the characteristics of a natural bone with the interconnecting pore network being retained in the structure. Figure 4.14 shows the FTIR spectra of bovine bone heated from room temperature (RT) to 1200°C.

Bertazzo et. al. [348] reviewed the effect of hydrazine deproteination on bone mineral phase. They found that several alterations in the IR spectra of bone after deproteination: all peaks are more narrow, peaks at 2920, 2860 and 1250 cm\(^{-1}\) are absent, and peaks at 870 and 961 cm\(^{-1}\) are more intense. The narrowing of peaks observed in the diffactograms of bones after deproteination can be connected to the elimination of organic matrix. The presence of organic matrix caused peaks to appear wider in the spectra obtained before deproteination. Peaks at 2920, 2860 and 1250 cm\(^{-1}\), present in the spectrum of bone before deproteination (Figure 4.15), correspond to bands of organic matrix that are eliminated after deproteination. The peak at 961 cm\(^{-1}\), seen in Figure 4.15, is attributed to group \(\text{HPO}_4^{2-}\) and the peak at 870 cm\(^{-1}\) to group \(\text{CO}_3^{2-}\), found in carbonated HAPs. The presence of such peaks in the spectra of bone both before and after deproteination shows that this process does not significantly eliminate those ions from bone mineral.
Figure 4.14 FT-IR spectra of bovine bone heated from room temperature (RT) to 1200 °C

(Adapted from Ooi et al. 2007 [329])
Figure 4.15 Infrared spectra of bone, HAP, CaHPO4 and CaCO3, before and after deproteination

(Adapted from Bertazzo and Bertran 2008 [348])

Table 4.1 summarises and compares the FT-IR spectral characteristics and assignments of bone from the literature and from this work.
<table>
<thead>
<tr>
<th></th>
<th>Bone</th>
<th>Collagen</th>
<th>Apatite</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat</td>
<td>Bovine</td>
<td>Human</td>
</tr>
<tr>
<td>OH liberation</td>
<td>630[329]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PO$_4$ v$_4$</td>
<td>560[329]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO$_3$ v$_4$</td>
<td>870[348]</td>
<td>873[54]</td>
<td></td>
</tr>
<tr>
<td>CO$_3$ v$_2$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PO$_4$ v$_1$</td>
<td>961[348]</td>
<td>962[329]</td>
<td>960</td>
</tr>
<tr>
<td>PO$_4$ v$_3$ or CO$_3$ v$_1$</td>
<td>900-1200 (v$_1$-v$_3$) [54]</td>
<td>1049-1090[329] 1000</td>
<td>1029 vs[351]</td>
</tr>
<tr>
<td></td>
<td>500-1100[349]</td>
<td>1000-1060(C-O stretching) [329]</td>
<td>1000</td>
</tr>
<tr>
<td>CO$_3$ v$_3$</td>
<td></td>
<td></td>
<td>1237</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1281[349]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OH stretching</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Code: vs; very strong, s; strong, m; medium, w; weak, vw; very weak, sh; shoulder, br; broad bands [351]; the bolded numbers were obtained from this experiment.

134
4.4 XPS Analysis

XPS provides quantitative information of chemical compositions of bone. The purity of the extracted samples was compared with raw bone and pure apatite. Nitrogen, a component of collagen and calcium, a component of apatite were used as markers to determine the purity of extracted collagen and apatite. The first two batches of extracted samples still contained residues giving comparable values for N/Ca ratios in both demineralised and deproteinated bone samples. Thus, the ratios suggested that the extraction was not complete.

Eventually, after purification process, successful extraction was achieved in the third batch. The results of XPS analysis is given in Table 4.2 where the compositions of the different samples are compared. The demineralised bone sample has high nitrogen and carbon content similar to raw bone and attributed to proteinaceous material. Absence of calcium in the sample shows high purity of the demineralised bone. The deproteinated bone also showed high purity with relatively high concentration of calcium and phosphorous and Ca/P ratios comparable with the pure apatite samples. A small amount of nitrogen was detected which may be a residue of the amine from the extraction solvent. The survey spectra of the extracted bone samples are shown in Figure 4.16 High resolution C 1s spectra of the samples are shown in Figure 4.17 in which the component peaks corresponding to C-N and O=C-N has relatively high intensity in raw bone and demineralised bone and are attributed to N-containing components of protein present in collagen.
Table 4.2 XPS analysis of raw bone, extracted bone and apatite

<table>
<thead>
<tr>
<th>Samples</th>
<th>O</th>
<th>N</th>
<th>C</th>
<th>Ca</th>
<th>P</th>
<th>Ca/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw Bone</td>
<td>24.2</td>
<td>15.2</td>
<td>58.5</td>
<td>1.1</td>
<td>1.0</td>
<td>1.19</td>
</tr>
<tr>
<td>Demineralised Bone</td>
<td>20.3</td>
<td>11.9</td>
<td>66.6</td>
<td>0.0</td>
<td>1.2</td>
<td>-</td>
</tr>
<tr>
<td>Deproteinated Bone</td>
<td>43.7</td>
<td>2.6</td>
<td>30.7</td>
<td>14.4</td>
<td>8.6</td>
<td>1.68</td>
</tr>
<tr>
<td>Bio-Oss® Bone Apatite</td>
<td>52.4</td>
<td>0.4</td>
<td>16.5</td>
<td>18.8</td>
<td>11.9</td>
<td>1.58</td>
</tr>
</tbody>
</table>

Figure 4.16 Survey spectra of extracted bone sample (demineralised and deproteinated bone)
Chapter 4 Structural Characterisation of Bone and Its Constituents

4.5 X-Ray Diffraction and Rietveld Refinement

Atomic modelling can help us have a better understanding of the molecular structure of bone. There is no certain model for bone apatite due to the complicity of its nature. However, it is known that bone apatite has similar structure to HAP (Ca_{10}(PO_{4})_{6}(OH)_{2}). Models of HAP were made using Material Studio software. There are four possible structures, which are Hexagonal polar P6_3, Hexagonal non-polar P_{63/m}, Monoclinic polar P_{21} and Monoclinic non-polar P_{21/b} (See Figure 4.18). From the structure, P6_3 has 6 tetrahedral phosphate groups, 2 hydroxyl groups and 10 Caesium ions. The 2 hydroxyl groups along the c axis in every corner of the unit cell repeat in the lattices. Similar but different to P6_3, P_{63/m} has 4 hydroxyl groups, where the 2 extra groups are the mirror reflects to the original 2 groups. The hydroxyl groups in this model are too close to each other and in
Chapter 4 Structural Characterisation of Bone and Its Constituents

reality when the repulsion exists, the angles between the atoms and the distance between the lattices may change. P2\(_1\) has a structure of 2 combined P6\(_3\) unit cells. The phosphate groups have slightly rotated from the original crystallographic orientation compared to P6\(_3\). The hydroxyl ions relax in certain places, which allow dipole moments. Because the P2\(_1\) symmetry is lower than P6\(_3\) and P2\(_{1/h}\) and its lattice is shorter in length, this model is most likely to the real structure of bone apatite. Compared to P2\(_1\), the neighbouring hydroxyl groups in P2\(_{1/h}\) orientate in the opposite direction. XRD patterns were also generated using the software according to the crystal structures. Most intensive peaks concentrate at 35 to 39 degree. Small shifts can be found in the less intensive peaks.

**Figure 4.19** shows the XRD patterns of bone and bone apatite taken at a high spectral resolution. Both bone and bone apatite had a small amount of amorphous content, which has decreased in bone apatite, as it can be seen from a lower background and slightly narrower peaks in bone apatite. There are traces of weak, thermally diffusive monoclinic reflections within the range of 35-40°, as indicated by arrows in **Figure 4.19**. The appearance of the (212) crystal reflection indicates e.g. alternate orientation of OH ions and therefore presence of the monoclinic P2\(_{1/b}\) crystal phase. A lowering of the symmetry from hexagonal to monoclinic symmetry is further seen from the presence of the hexagonally forbidden, monoclinic reflections (2 -7 -1) and (1 5 1) detected in the pattern [21]. Rietveld analysis is employed to find the best fit to bone apatite of the four contending symmetries [24] of HAP (i.e. hexagonal non-polar P6\(_3/m\) and polar P6\(_3\), and monoclinic non-polar P2\(_{1/b}\) and polar P2\(_1\)).
Figure 4.18 Molecular modelling of HAP. Coloured balls represent atoms: green – Calcium; red – Oxygen; and white – hydrogen.
Figure 4.19 X-ray diffraction pattern of bone (top) and apatite extracted from bone (bottom). The figures on the right panels show high resolution spectra within the region of 35-40°. The traces of thermally diffusive, hexagonally forbidden, weak monoclinic reflections are indicated by arrows.

Adapted from Zhang et al. 2012 [327]

Table 4.3 summarises the results of Rietveld refinements with the respective fitting parameters, $R_p$ and $R_{wp}$ and the resulting lattice parameters obtained from such fitting. A lower $R$-value indicates a better fit of a model structure to the experimental data and hence higher possibility of the existence of the particular crystal phase. As seen from Table 4.3, the experimental apatite pattern fits best to the polar $P6_3$ hexagonal crystal phase, showing the lowest $R_{wp}$ and $R_p$ values. It is noteworthy that the Rietveld fit to the $P2_1/b$ monoclinic structure is also comparable to this fit with a $P6_3$ model. It can be thus assumed that both these phases can co-exist in bone making-up the mineral hard tissue. Importantly, the presence of $P6_3$ symmetry in the pattern signifies that there are crystallites of bone apatite that belongs to the piezoelectric class and should exhibit piezoelectricity as an individual crystal. The local orientation of these crystals as
revealed in TEM (Figure 4.12 TEM images of bone apatite) also indicates that it may be possible to detect local piezoelectricity in bone apatite.

Table 4.3 R-factors and lattice parameters of HA crystal phases in bone refined over the range 25-55° 2θ by Rietveld method [327]

<table>
<thead>
<tr>
<th>Structure</th>
<th>( R_{wp} ) (%)</th>
<th>( R_p ) (%)</th>
<th>Refined lattice parameters ( (\text{Å/°}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>P6(3/m )</td>
<td>4.01</td>
<td>5.20</td>
<td>( a = b = 9.424, \ c = 6.879 )</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( a = \beta = 90, \ \gamma = 120 )</td>
</tr>
<tr>
<td>P6(3 )</td>
<td>3.77</td>
<td>4.90</td>
<td>( a = b = 9.424, \ c = 6.879 )</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( a = \beta = 90, \ \gamma = 120 )</td>
</tr>
<tr>
<td>P2(1/b )</td>
<td>3.89</td>
<td>5.08</td>
<td>( a = 9.407, \ b = 18.871, \ c = 6.882 )</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( a = \beta = 90.000, \ \gamma = 119.999 )</td>
</tr>
<tr>
<td>P2(1 )</td>
<td>4.57</td>
<td>6.02</td>
<td>( a = 9.436, \ b = 18.909, \ c = 6.842 )</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>( a = \beta = 90.000, \ \gamma = 119.965 )</td>
</tr>
</tbody>
</table>

4.6 Conclusion

The question of piezoelectricity in bone constituents from the perspective of recent findings of piezoelectricity in HAP has been revisited. While there are significant differences between synthetic HAP and the apatite in bone, Rietveld analysis of XRD data on apatite extracted from bone show that there is a strong possibility of the presence of apatite crystals that belong to a piezoelectric symmetry, P6\(3 \). The morphology of collagen fibrils in any form of bone, with or without the inorganic shielding can be analysed. TEM data has shown that apatite can have a local orientation. Based on the above results, it is assumed that microscopic measurements by PFM may determine whether bone apatite is piezoelectric.
Chapter 5 Piezoresponse of Bone and its Constituents

5.1. Introduction

While many papers have reported the piezoelectric coefficients in both animal and human bones, the debate on the roles of organic and inorganic components continues. The complex architecture and composite nature of bone is designed to provide unique physical properties. The results support that the contribution to piezoelectricity of the organic matrix of collagen fibrils in bone, tendon and tooth may be the major source due to the permanent electric dipole moment forms in collagen molecules. Years after the first discovery of piezoelectricity in bone and collagen at macroscopic level, the orientation dependence of piezoelectricity in bone and collagen is now studied at nanometre scale with the high resolution PFM.

This chapter discussed the morphology of raw bone, extracted bone and synthesised HAP samples using AFM. The results obtained from four different brands of PFMs were compared. It also revealed the orientation dependent piezoelectricity in bone and its constituents at nano scale, which were compared with the studies at macro- and microscopic scales. By imaging near a lacuna within a lamella, the hierarchical organisation of bones sub-microstructure was disclosed and compared with the simplistic models.

5.2. Morphology Analysis of Bone at Nanoscale

The investigation of electromechanical properties of bone and its constituents began with mastering the AFM technique and studying the morphology of bone with AFM at the nanometre scale. Different types/brands of Atomic Force Microscope have been used throughout the study, as described in Section 3.7.4. Agilent 5500 SPM is available the University of Limerick (UL). The instrument was used to obtain the best AFM images of bone in the tapping mode and contact mode. Figure 5.1 shows high resolution AFM images of polished bovine femur bone in tapping mode with a resolution of 512x and a scanning range of 10µm.
The Agilent system has its own imaging processing program that allows processing of the images within the same operation system. Three dimensional images can also be displayed, so that the surface roughness can be easily observed.

![Figure 5.1 Tapping mode AFM of polished bovine femur bone.](image)

Resolution: 512x, Scanning size: 10µm: (a) Topography; (b) Amplitude; (c) Phase; (d) 3D of Topography

**Figure 5.2** shows the AFM images of polished bovine femur bone in contact mode with a resolution of 256x and a scanning range of 1µm. Compared to tapping mode, contact mode gives more details of the sample surface. Even at smaller scanning area, the polishing mark was detected. In data analysis, the Agilent software can provide a distribution and segmentation map according to the roughness of the sample surface, where the regions with red colour have higher value of height than the regions with blue value. Because the bone sample
Chapter 5 Piezoresponse of Bone and Its Constituents

used for both contact and tapping mode was not chemically etched, bone apatite was the most abundant component on the surface and the periodic band structure of collagen fibrils could not be seen clearly. The extracted bone collagen and the extracted bone apatite samples were also tested with tapping and contact mode AFM. However, the extracted collagen sample had shrunk to half of the original size due to the drying process and the polished surface did not show any trace of collagen fibrils. Neither did the polished bone apatite show any interested features. The AFM images of both types of samples were similar to the raw bone sample (images not shown here).

![AFM Images](image)

Figure 5.2 Contact mode AFM of polished bovine femur bone

Resolution: 256x, Scanning size: 1µm: (a) Topography; (b) Deflection; (c) Friction; (d) Roughness distribution of Deflection

At the same time, some related samples were obtained in the lab from other researchers. The morphology of the samples was also observed using AFM.

145
Because their surface roughness is relatively small, the scanning mode was chosen as contact mode and the scanning size was 1μm. Synthesised HAP films and pellets were made in our lab for other research. They have similar components to bone apatite. The HAP film is in the form of a gel and is spray-coated onto a silicon wafer and dried. The surface of the HA film presented grains with the average size of 0.2μm. The HAP pellet is a transparent solid cylinder and is fine polished. The surface of the HA pellet is very smooth but the polishing marks can be seen. Bio-Oss apatite is the extracted bone apatite from bovine bone. The sample is solid but brittle. The surface is flat with no interesting patterns or features. These three types of related sample all exhibited higher roughness than the polished raw bone in morphology. See Figure 5.3.

Figure 5.3 Contact mode AFM of HA film, HA pellet and Bio-Oss HA
Resolution: 256x, Scanning size: 1μm: (a) Topography; (b) Deflection; (c) Friction.
5.2.1. Investigation of Piezoresponse in Bone and its Constituents

After studying the morphology of bone, the investigation of piezoelectricity of bone and its constituents at nanometre scale was carried out using PFM. The first PFM experiments of bone were completed with the Agilent 5500SPM instrument in the UL. The maximum applied bias was limited to 20V (peak to peak). To compare the properties of the three types of samples – raw bone, extracted collagen and extracted apatite, both vertical and lateral PFM were carried out. As the results of the vertical PFM (VPFM) were not desirable, lateral PFM (LPFM) were the only results kept. Figure 5.4 shows the topography, amplitude and phase data of lateral PFM of raw bone, extracted collagen from the later batch. While all three samples show a lateral piezoresponse, the response is much weaker in bone apatite, almost at the level of instrument noise (not shown here). Collagen fibres show a uniform alignment and an indication of its band structure with a period of ~67nm in raw bone can be identified. Significant differences of signal amplitude indicate the interaction between the sample and the bias-induced tip. Clearly, the piezoresponse from extracted collagen is the highest with a signal averaging at 8.5V. The response of raw bone and extracted apatite are lower with a signal averaging at 0.5 V and 0.025V respectively.

The phase shift of both raw bone and the extracted collagen is almost 180°. There is a hint of a phase shift in the PFM phase image of the extracted apatite; however it cannot be calculated due to the small value of phase change (PFM images were not shown here). While the piezoresponse in bone and extracted collagen is in line with expectations and previous piezoelectricity measurements at both macroscopic and microscopic scale, the piezoresponse of bone apatite is rather weak. This response may originate from the organic residue from the extraction process, as discussed in Section 2.5.2. Further work is required to resolve this. Therefore, an instrument with much higher applied bias and an electric signal shielding was needed to continue the study.
5.2.2. Comparison of Piezoresponse in Extracted Bone

Although the Agilent system provided good quality AFM and PFM images of bone, it is not yet equipped with high external voltage supply. Due to the limitations of the instrument in the University of Limerick, the samples were taken to University Collage Dublin (UCD) for further investigation using Asylum Research MFP-3D™ AFM system (Section 3.7.4). The instrument was placed in the centre of an enclosed electric shielding cage which kept the instrument away from external electrical signal interference. Also, an external AC signal generator (Tektronix) was connected to the main controller which can provide maximum 120V (peak to peak) bias. The high applied bias may provide the answer as to whether bone apatite is piezoelectric. Better understanding of PFM and better operational techniques were gained through the study in UCD.

Firstly, the same raw bone and the extracted bone samples from the first batch of extractions were tested with Asylum system. Figure 5.5 shows vertical PFM of...
raw bone, extracted collagen and extracted apatite from early extractions at a bias of 60V peak to peak. All three samples exhibited a piezoelectric response. As expected, the extracted collagen had a higher response than the extracted apatite. Because all sample surfaces showed high roughness and it was difficult to distinguish the samples from their surfaces, these samples needed further polishing and maybe etching. The force distance curve mapping on a 2\( \mu \)m area of the raw bone sample was also carried out on the same day. However, it was not accurate data due to the indentation function he system was not available.
Figure 5.5 Vertical PFM of (a) Raw bone, (b) Extracted collagen and (c) Extracted apatite from early extractions, Scanning size: 2μm
5.2.3. Further Investigation of Piezoresponse in Etched Bone

Considering the purity of the extractions, the extracted apatite may contain organic residues. Later, the best batch of extractions, which was verified to have high purity, was taken to UCD for testing. Raw bone sample was tested before and after etching by 5% acetic acid. Figure 5.6 compares the VPFM and LPFM images and phase histograms of raw bone before and after etching. In VPFM, the surface roughness of raw bone was high before etching and the piezoelectric response was low due to bone apatite exposed on the surface. After etching, the collagen band structure could be observed on the surface and the roughness became low. The phase histogram indicated two mixed phases – out-of-plane and in-plane. Similar observations made from the LPFM images. Therefore, one can conclude that bone collagen has a higher piezoelectric response than bone apatite.

With external high voltage stimulation, the investigation moved onto the electromechanical properties of extracted bone apatite. The sample has been verified to have high purity. The sample surface was etched by 5% acetic acid for different periods and the sample etched for 10 seconds showed the best results of the exhibition of collagen fibrils on the surface. Figure 5.7 is the AFM/PFM of etched extracted apatite. From tapping mode AFM, the grains on the surface could be observed at the size of ~100nm. In VPFM, the 120V (peak to peak) bias was applied to the sample; the piezoelectric response was low and was suspected to be cross-talk. Also, the phase histogram showed only one major phase, which cannot prove that bone apatite is piezoelectric.
Chapter 5 Piezoresponse of Bone and Its Constituents

(a) Raw Bone
Before Etching

After Etching
Figure 5.6 (a) VPFM and (b) LPFM images and phase histograms of raw bone before and after etching. Scanning size: 5µm; Applied bias: 60V (peak to peak)
Figure 5.7 (a) Tapping mode AFM and (b) VPFM of etched extracted bone apatite

For further investigation of bone apatite, spot analyses were carried out on the same sample. Bright and dark spots from the VPFM amplitude image were selected to see if they have any linear response to the increasing applied bias 0 to 120V (peak to peak). On the bright spots (1nm area), the response from the PFM amplitude image gradually was brightened with increasing bias (See Figure 5.8), whereas the changes of the response for the dark spots were not obvious. However, there was a tendency for the response from both bright and dark spots
to be linear from 20-60V. After 60V, the response was random which may be caused by the instrument itself or the external interferences. The reasons were still unknown.

Figure 5.8 Spot analysis of extracted bone apatite on bright and dark regions.
Applied bias: 0-120V peak to peak

So far, the piezoelectricity tests on pure extracted bone apatite were completed using PFM. The piezoelectric response and the linear response are inclusive.
Therefore, the conclusion that bone apatite is piezoelectric cannot be drawn. An instrument with higher voltage or another technique may give the answer.

A short Asylum Cypher PFM demonstration was given in UCD. The raw bone and the extracted bone apatite were tested with high resolution. Although the Cypher can perform automatic recordings of piezoresponse with a functional bias, this program in the demonstration instrument had not been calibrated. The linearity of the piezoresponse of bone apatite is uncertain from the obtained results. The PFM images and piezoresponse curves of raw bone and the extracted bone are shown in Appendix I Cypher Demonstration.

**5.2.4. Adhesion Mapping for Bone Elasticity**

During the JPK demonstration, both dry and hydrated raw bone were tested. The raw bone sample was polished and etched. **Figure 5.9** is a tapping mode topography image. In an area of 500nm, the collagen band structure can be seen clearly.
Figure 5.9 Tapping mode topography images of raw bone

The selected area with the collagen band structure was tested for adhesion. Figure 5.10 is topography and the adhesion map. These images were taken in air which means that the adhesion map is likely a map of hydrophobicity.

Figure 5.10 Topography (left) and the adhesion map (right)
In a 2µm area of the collagen, the adhesion of bovine bone was calculated. **Figure 5.11** the adhesion image on the right ranges from 8.22 nN to 24.77nN.

![Figure 5.11 Adhesion map of dry bovine bone at the size of 2µm](image)

After hydrating the raw bone sample the fast force mapping was performed again. In this condition the adhesion map shows no contrast (and is not presented in this report). In **Figure 5.12**, the left hand image is topography (with a z range of 340nm) and the right hand image is the Young's Modulus as determined by applying a Hertz indentation fit to each curve taken in the map. This analysis is performed with a batch process and automatically generates a new image based on the chosen parameter. The data range is 0 to 15 MPa. There are still some hints of the collagen banding structure, as indicated, for example, by the white box.
5.2.5. Secondary PFM Testing of Bone Apatite

Some pure extracted bone apatite samples were taken to the NT-MDT laboratory for testing. Although a high external bias could not be applied, the image resolution was good quality and the scientist in NT-MDT claimed that they have found the piezoelectric response in our bone apatite sample. Figure 5.13 is the high resolution tapping mode AFM topography of bone apatite in both 2D and 3D format.

Figure 5.12 Force distance curve map of hydrated bovine bone

Figure 5.13 High resolution tapping mode AFM topography of bone apatite. Scanning size: 1µm, Resolution: 1024x. (L) 2D topography (R) 3D topography
Chapter 5 Piezoresponse of Bone and Its Constituents

PFM measurements were completed on the surface of the bone apatite sample with applied AC bias at 6V and DC bias at 0V/3V (both same). A TiN coated cantilever was used with 30nm tip and ~1.5N force constant. The green and blue boxed areas in the entire scanning area indicated the phase changes (Figure 5.14). A line was drawn at the same position of PFM amplitude and phase (Figure 5.15). The cross line profile showed the out-of-plan and in-plane piezoresponse in amplitude correlated to the phase image. A PFM phase histogram can be derived and it indicates two mixed phases: one major out-of-plane phase and one small in-plane phase – in Figure 5.16.

Figure 5.14 PFM of bone apatite with AC bias 6V and DC bias 3V.

Scanning size: 700nm, Resolution: 512x
Figure 5.15 Line profile of PFM amplitude and phase

Figure 5.16 PFM phase and histogram
5.3. Orientation Dependence in Bone

5.3.1. Orientation Dependence of Microscopic Piezoresponse of Bone

The piezoelectricity of collagen is primarily responsible for piezoelectricity in bone. A simple approach to investigate the orientation dependence of the microscopic piezoresponse of bone can thus be to follow the orientation of collagen or collagenous superstructures (e.g., lamella or individual lamellae) in bone with respect to the bone axis. As it has been often assumed during macroscopic piezoelectricity measurements by earlier researchers [353], the orientation of collagen fibrils at the sub-microstructural level, as an initial approximation, can be considered to be coinciding with bone’s long axis taken as an arbitrarily defined $c$ axis (Figure 5.17a), which is perpendicular to the plane of the Figure 5.17e, lower left). If PFM samples are cut at angles of 0°, 45° and 90° with respect to this plane, perpendicular to the $c$-axis, three different orientations of collagen fibrils for PFM analysis result, as shown in Figure 5.17(a-c) schematically.

**Figure 5.17** Schematic model of collagen fibrils alignments in the bone with macroscopic orientations of (a) 0°, (b) 45° and (c) 90° to the plane perpendicular to the bone axis; (d) the top view and the size of the collagen fibrils appear on the sample surface with the orientations; (e) the location of AFM probe detecting the area of 5x5 µm2 (f) on the sample (5x5x1mm3) surface
If collagen fibrils are parallel to each other and perfectly aligned along the $c$ axis they should have presented, at the sub-microstructural level, all circular cross sections in a $0^\circ$ bone sample (Figure 5.17a), elliptical cross sections in a $45^\circ$ bone sample and rod like cross section in a $90^\circ$ bone sample. For such presentations, and with high resolution topographic images, one should then expect the circular cross sections of collagen fibrils to be of a diameter in the range of ~50 nm (in $0^\circ$ sample) [354], the calculated elliptical cross section of dimensions ~71 nm in the long axis and ~50 nm in the short axis (in $45^\circ$ sample), and the horizontal rod like cross sections to be full of oblique circular cylinders with the width of ~50 nm and exhibiting the ~67 nm [354], so called D-band structure characteristic of collagen (in $90^\circ$ sample).

This simplistic hypothesis is tested using both VPFM and LPFM, typical results of which are shown in Figure 5.18 along with their corresponding topography. The AFM topography show that there are more D-periodic collagen fibrils, as expected, in the $90^\circ$ samples than others, in contrast to the expectation that $0^\circ$ samples should show all circular cross sections (red), $45^\circ$ sample elliptical (yellow) and $90^\circ$ sample all rod like periodic features of collagen fibrils (green). Some selected areas where such expectations have been somewhat fulfilled. These areas were chosen from 20 regions over 5 different images and they have been used to obtain quantitative values of local nanoscopic piezoresponse. Importantly, the overlapping regions with large size and stronger signals were avoided to choose, because of the complication of mixed orientations. Collagen fibrils in $90^\circ$ samples could be identified with relative ease due to their periodic D-band structures, but the identification of $0^\circ$ and $45^\circ$ fibrils was difficult due to the image resolution used. For this reason, an average from over twenty regions was taken to reach a mean value for the nanoscopic values of each orientation, although the confidence level in these measurements remained low, as indicated in Table 2.7 in Section 2.5.2.
Figure 5.18 AFM tapping mode amplitude images of bovine femur bone (5x5 μm²) with macroscopic orientations of (a) 0°, (b) 45° and (c) 90° to the plane perpendicular to bone axis, showing the misorientations of collagen fibrils; Piezoresponse of bone with macroscopic 45° angle to the bone axis; also piezoresponse of the individual collagen fibrils with nanoscopic orientations of 0° (red), 45° (yellow), 90° (green); (d) High resolution contact mode deflection image; (e) VPFM response; and (f) LPFM response
Chapter 5 Piezoresponse of Bone and Its Constituents

The use of higher resolution PFM may improve this confidence level although complications from overlapping regions with large size and stronger signals, interference from apatitic regions, and, most importantly, a predominantly hierarchical organisation of collagen fibrils would still play a role in PFM quantification. Therefore, averaging the PFM response was resorted over the field of view of these images according to their cuts. This approach provided more robust microscopic measurements which it was preferred to the measurements taken at the nanoscopic level measurements on single fibrils.

That hierarchical organisation still plays a dominant role can be seen from the high resolution topography and corresponding LPFM in Figure 5.19 of a bovine bone sample that was cut so that the bone axis is perpendicular to the imaging plane (0° sample). The imaging was near a lacuna within a lamella. In complete contrast to the simplistic local model of proposed in Figure 5.17, D-band structure of collagen fibrils clearly dominated the topography. The remarkable correspondence of the LPFM to the concentric but radially outward topography of collagen fibrils is, however, expected from the hierarchical organisation of bones sub-microstructure as shown schematically in Figure 2.12 in Section 2.4.3.

![Figure 5.19 PFM images of collagen fibrils near a lacuna](image)

(a) topography in deflection mode (b) LPFM, showing collagen fibrils orientate radially at [001] plane in a bovine bone sample cut at a 0° with respect to the bone axis. The fibrils that are perpendicular to the scanning direction showed less response than those parallel to the scanning direction. Topographic roughness has also influenced the response especially in the peripheral region.
5.3.2. A Comparison of Orientation Dependence of Piezoelectricity of Bone at Nanoscopic, Microscopic and Macroscopic Scales

PFM contrast, however, does not readily discern orientation dependence, neither vertical or in plane, due to the complexities associated with hierarchical organisation of collagen in bone, topographic influence and PFM measurement itself. In addition to the texture of the sample to be analysed, the link between the sample and the laboratory coordinate systems must be clarified. These two coordinate systems can be transformed through the Euler angles \((\varphi, \theta, \psi)\) [355]. Following the schema set by Harnagea et al. 2010 for quantitative PFM analysis of collagen fibrils, the rotation angle \(\varphi\) is determined by the rotation of the longitudinal axis of the symmetry of collagen fibres and the piezoresponse is considered independent of \(\varphi\). Collagen fibre orientation then can be found using z-y-z convention as follows [27]:

\[
d_{zz} = d_{33}^{lab} = \cos \theta [(d_{33} + d_{15})(1 - \cos^2 \theta) + d_{33}\cos^2 \theta]
\]

Equation 5.1

\[
d_{xyz} = d_{34}^{lab} = \sin \theta [2(d_{33} - d_{31} - d_{15})\cos^2 \theta \sin \psi + d_{14}\cos \theta \cos \psi
\]

+ \(d_{15}\sin \psi\]

Equation 5.2

\[
d_{zxz} = d_{35}^{lab} = \sin \theta [-2(d_{33} - d_{31} - d_{15})\cos^2 \theta \sin \psi + d_{14}\cos \theta \cos \psi
\]

- \(d_{15}\sin \psi\]

Equation 5.3

In these expressions, the coefficients are not related to \(\varphi\), so that the twisting of collagen fibril does not affect the PFM signal [27]. However, as PFM measures converse piezoelectric effect where piezoelectric deformation due to an applied electric field is measured, the angle between the PFM probe and the textured axis (in this case collagen fibril axis) and the mode of detection of the deformation (vertical or lateral) are important to define the piezoelectric coefficients being measured. Figure 5.20 shows an idealistic view of vertical and lateral PFM
measurements with respect to the collagen fibril orientation in 0°, 45° and 90° samples respectively. In such cases, Equation 5.1 represents the VPFM, while Equations 5.2 and 5.3 represent longitudinal transverse LPFM. Vector PFM techniques can then measure the following piezoelectric coefficients with respect to bone’s own reference coordinate system: 0° sample (VPFM = d33; LPFM = d34 and d35), 45° (VPFM = d33'; LPFM = d34' and d35') and 90° (VPFM = d11 or d22, LPFM = d15 or d16 and d24 or d26). From the knowledge of piezoelectric coefficients of tendon (Table 2.7 in Section 2.5.2), the schema was expected (Figure 5.17 in Section 5.3.1) that VPFM would have the maximum and minimum response for 0° and 90° samples respectively with 45° sample showing some intermediate value. Similarly for LPFM, the maximum and minimum response can be expected for 90° and 0° samples respectively with 45° sample showing intermediate values.

Figure 5.20  Schematic representation of PFM measurements of collagen fibrils orientated in bone in 0°, 45° and 90° samples and the piezoelectricity coefficients that can be measured Vertical PFM and Lateral PFM
Table 5.1 lists both nanoscopic (discussed in the previous section) and microscopic measurements of 0°, 45° and 90° samples in VPFM ($d_{33}$) and LPFM ($d_{34}$). It was not possible to conduct $d_{35}$ measurements in our PFM system. It can be seen that there is very little change in the $d_{33}$ values, which are in general rather weak in both collagen and bone (Table 5.1). However, we measured reasonably strong VPFM due to the inherent nature of VPFM. Microscopic $d_{34}$ constants are, expectedly, strong for 0°, 45° and 90° samples and follow the trend of increasing value with increasing angle, as expected. The shear piezoelectricity, $d_{34}$, measured on 90° samples is comparable to the shear piezoelectricity reported for collagen using PFM.

In an attempt to benchmark this nanoscopic and microscopic data with those from macroscopic measurements, orientation dependent piezoelectric constants for both polycrystalline and SESCS values were converted into expected values to be measured by VPFM and LPFM. For this, Equations 5.1-5.3 were used. It is noted that microscopic and nanoscopic measurements from the present study lie, both in trend and in numerical values, between the axially invariant SESCS values for collagen and bone. The numerical values are in general are comparable to the SESCS of collagen. This is expected, as we have etched our samples to partially decalcify the surface to facilitate PFM measurements.
Table 5.1 Rationalisation of macroscopic, microscopic and nanoscopic shear piezoelectric constants of bone and collagen

<table>
<thead>
<tr>
<th></th>
<th>Collagen</th>
<th>Bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>d33 (pm/V)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0°</td>
<td>-0.37</td>
<td>0.009</td>
</tr>
<tr>
<td>45°</td>
<td>0.154</td>
<td>0.522</td>
</tr>
<tr>
<td>90°</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>d34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0°</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45°</td>
<td>2.5</td>
<td>-0.041</td>
</tr>
<tr>
<td>90°</td>
<td>2.89</td>
<td>1.4</td>
</tr>
<tr>
<td>d35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0°</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>45°</td>
<td>-0.217</td>
<td>-1.33</td>
</tr>
<tr>
<td>90°</td>
<td>0.615</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: * Despite the low level of confidence in these data, they follow the overall trend of the microscopic measurements.
#Nanoscopic shear piezoelectricity values reported in the literature for comparable measurements on collagen is ~2 pC/N [6] and ~1 pC/N [8]
* SD = Standard Deviation

169
5.3.3. Significance of the Investigation of Orientation

Dependence of Macroscopic Piezoelectricity for PFM

In Figure 5.21, orientation dependent piezoelectricity of bone as measured by PFM in the current study were compared and contrasted with those measured by Fukada and Yasuda on bone and collagen [1, 16]. Clearly, there is a discrepancy in the overall trend. For example, Fukada and Yasuda’s measurements indicate a maximum at 45° orientation while our measurements show the maximum in shear piezoelectricity occurs at 90° (as expected from Figure 5.17). This apparent discrepancy can be resolved by considering the fact that Fukada and Yasuda measured orientation dependence of piezoelectricity by a direct method, while in PFM we are restricted to study converse effects only. A proper understanding of the original experiments by Fukada and Yasuda is therefore needed.

In measuring the orientation dependence, Fukada and Yasuda cut samples in a manner similar to us, but during measurements they rotated the pressure axis with respect to the bone or tendon axis (z axis in Figure 5.21a). This rotation took place about the [100] direction (x-axis), which was the polarisation direction and remained unchanged with respect to the bone/tendon axis. When the pressure axis coincides with the z-axis, the situation is similar to the measurement of $d_{13}$ coefficient, which they measured as zero for collagen and near zero for bovine bone. When the pressure was applied at an angle of 90° to the z-axis, the situation is equivalent to the measurement of the $d_{12}$ coefficient, which they measured also as zero for both collagen and bone samples. When the pressure axis made a 45° angle to the z-axis, they measured a finite value which was also the maximum. The measurement was equivalent to the measurement of $d_{14}$ coefficient [23] and turned out to be the maximum in value.

In our case, PFM measured the converse piezoelectric by applying the electric field along the PFM probe, which has been taken as the z axis (Figure 5.21b). For the 0° sample this means that the electric field is applied along the bone’s axis but then the angle between the field axis (polarisation axis) and the bone axis changes to 45° and 90° respectively. This implies a completely different scenario.
than what Fukada and Yasuda employed, so a direct comparison with the latter will be inappropriate. To replicate Fukada’s work, one needs to take the 90° sample so that polarisation is always perpendicular to the bone axis, as in Figure 5.21a, and measure LPFM. Deformation measured transverse to the bone fibre direction will then represent the case of the 90° sample of Fukada and Yasuda, whereas that measured along the fibre direction will represent the case of 0° sample. In other words, the measurements would be equivalent to the $d_{34}$ and $d_{35}$ coefficients for our 90° sample. The SESCS indicates that transverse measurements would provide higher values than longitudinal measurements. All values were converted to the same unit ‘pm/V’ according to the conversion in Appendix II Unit Conversion. Due to the instrument limitations, we could not measure $d_{35}$ coefficients to verify this, but PFM measurements on a single collagen fibre loop by Harneaga et al. has indicated such a dependence of PFM values on PFM scanning directions.

**Figure 5.21** Transformation of coordinates in determining (a) Direct piezoelectric effect of bovine tendon collagen fibre using a galvanometer -Fukada 1964 (b) Converse piezoelectric effect of bovine bone collagen fibrils using PFM; Orientation dependent piezoelectric constants of bovine tendon fibres and bovine bone collagen fibrils
5.4. Conclusions

Piezoresponse Force Microscopy, a powerful characterisation technique, has been performed on biological system from macroscopic to nanoscopic scale over the past five decades. This investigation focused on the orientation dependence of piezoelectricity of bovine bone using both vertical and lateral PFM.

Working with Agilent system in UL, PFM data on raw bone and extracted collagen has shown piezoresponse as expected; the weak piezoresponse observed in bone apatite still requires further experiments to confirm to the presence of piezoelectricity. The application of a higher AC bias during PFM is being pursued to improve the signal to noise ratio so that piezoelectric nature of bone apatite can be determined conclusively.

With Asylum PFM in UCD, misorientations of collagen fibrils in bone were detected and defined using PFM for the first time. This further proved the existence of misorientations of collagen fibrils in bone play an important role of enhancing the strength and elasticity of bone and therefore update the understanding of bone growth. The findings confirm the shear stress is most effective on bone, and also the orientations of collagen fibrils in bone are crucial to bone piezoelectricity. Compared to Fukada’s results which showed macroscopically the piezo constant of 45° is the highest, our results showed micro- and nanoscopically 90° is the highest. One need to bear in mind that the systems and the characterisation techniques used is different in these two experiments. When bone is cut into smaller size, the water content in the tested samples is reduced. The piezoelectricity of bone in the living environment could be different again.

Although the PFM results from four different manufactories were concluded, the main question that ‘is bone apatite piezoelectric?’ still remains uncertain. It has been arguing the piezoresponse from the images of bone apatite may be affected by topography or a cross-talk phenomenon. Current technology cannot solve these problems.
Chapter 5 Piezoresponse of Bone and Its Constituents

A research gap has been filled by developing a framework to conclude the current studies for the piezoelectric coefficients of single and Polycrystal structure of bone and tendon in nano and microscopic measurements. In comparison to the original investigations of the orientation dependence of macroscopically measured piezoelectric, this study suggested that there is a significant variation in PFM response for bone and collagen between transverse and longitudinal lateral measurement.

Finally, the piezoresponse of bone in sub-microstructure was analysed by lateral PFM near a lacuna at [001] plane, which indicated that collagen fibrils are highly organised at local level. In conclusion, the characterisation method of nano and microscopic piezoresponse in this study has provided a detailed quantitative analysis for any piezoelectric composites in future.
Chapter 6 Conclusions and Future Work

6.1 Summary of Findings

This study was designed to understand the origin of piezoelectricity of bone through high lateral resolution characterisation techniques. For this, chemical extraction and high resolution electromechanical characterisation techniques have been employed to determine the influence of the hierarchical nature of bone on the bone piezoelectricity. A detailed literature review has been conducted to obtain necessary background information related to bone anatomy, chemical composition, hierarchical organisation and physico-mechanical properties of bone, with a particular emphasis on piezoelectricity and stress generated potential in bone. A comprehensive background study was also undertaken to understand the mechanism of the characterisation techniques used in this study, in particular the piezoresponse microscopy (PFM). A careful comparison was also made between the available instrumentation to carry out PFM.

The primary constituents of bone, namely bone apatite and collagen, have been separated from each other by chemical extractions. Unlike previous studies, the detailed chemical analyses were carried out using EDS, IR Spectroscopy and XPS in conjunction with high resolution optical microscopy, c-LSM, SEM, TEM, and AFM to establish the purity of such constituents derived from the extraction. The crystal structure of bone apatite was investigated by using XRD and Rietveld analysis. Finally, the electromechanical characterisations on raw bone and pure extracted bone samples were carried out using PFM.

Important findings from this study are listed below:

i. The atomic structure modelling of HAP indicated the P2\textsubscript{1} model with a lower symmetry is similar to the real structure of bone apatite, which is possibly piezoelectric

ii. Significant differences were found between synthetic HAP and the apatite in bone. Rietveld analysis of X-Ray diffraction data on
apatite extracted from bone show that there is a strong possibility of the presence of apatite crystals that belong to a piezoelectric symmetry, $P6_3$.

iii. In morphological studies, TEM data has shown that apatite can have a local orientation at atomic scale and there are misorientations within the lattice.

iv. PFM results on the bovine bone supported the proposal that the major contribution to piezoelectricity in bone is from collagen fibrils. The value of shear piezoelectricity of collagen fibrils is $\sim 4 \text{ pm/V}$, which matches the results from previous literature. However, the contribution from bone apatite remains uncertain due to its complex nature and the limitations of current technology.

v. Novel finding on the orientation dependence of piezoelectricity in bone showed that at nanometre scale the shear piezoresponse of bone greatly depended on how the original piezoelectric source – collagen fibrils orientated and the bone with a 90° cut to the bone axis had the maximum shear piezoelectricity at nano level. This discovery is different to the previous result of shear piezoelectricity in bone at macro- and microscopic level which was concluded by Fukada in 1957.

vi. Different to the ideal bone model showing how collagen fibrils align in bone, the AFM image revealed there were misorientations of collagen fibrils, which may affect the piezoelectricity in bone at nano level.

vii. There is no ‘best’ microscope in the world. The four tested models of PFM from different manufactories have their own strengths. The Asylum Cypher AFM may be more advanced than other
models on the market, in terms of performance. A good selection of a PFM instrument depends on the application and the outcome that one expected.

### 6.2 Solutions to the Research Questions and Testing of the Research Hypotheses

**RQ 1: What is the local orientation of bone apatite nanocrystals?**

The local orientation of apatite nanocrystals in bone were revealed by TEM. These nanocrystals have shown that they followed a major direction and that the parallel nature was still maintained even without templated support from collagen fibrils. Therefore, each individual nanocrystal rod was suspected to have polars. This phenomenon has not yet been reported. TEM also revealed that, at much higher magnification, some defects in individual apatite rods existed. The lattice pattern within an apatite rod does not always lie parallel to each line. Misorientated lattices exist, which are considered to be the defects within crystal materials.

**RQ 2: Is bone apatite piezoelectric?**

Although the XRD and Rietveld analysis indicated that there is a possible presence of bone apatite belonging to the piezoelectric class \((P2_1\text{ or } P6_3)\) within the extracted bone apatite matrix, the PFM did not conclusively show that bone apatite is piezoelectric. Three different PFM instrumentations were used to determine the PFM response of bone apatite. The hint of PFM obtained in one of these instruments is weak, thus suggesting that piezoelectricity of bone apatite may still remain as an open question that needs to be studied in future.

**RQ 3: How does the local orientation of collagen fibrils in bone affect bone piezoelectricity?**

Horizontal and lateral comparisons were made between the raw bone and the etched bone (mainly collagen fibrils) with 0°, 45° and 90° angle to the bone axis. As collagen fibrils are so far considered to be main contributors to bone piezoelectricity, macroscopically Fukada’s results have showed that the piezo
constant of 45° but the highest, whereas this work showed micro- and nanoscopically 90° is the highest.

Closer to the lacuna, the hierarchical organisation of collagen fibrils still plays a dominant role. The lateral piezoresponse of the concentric but radially outward collagen fibrils showed remarkable correspondence to the sub-microstructure near a lacuna at [001] plane, which was reported for the first time.

RQ 4: What are the instrumentation factors that affect quantitative values of bone piezoelectricity obtained by Piezoresponse Force Microscopy?
Four makes of PFMs were used for electromechanical characterisation of bone and their characters and functions of were compared according to the current performance in the experiments. While each instrument has its own strengths for specific purpose, there are a number of instrumentation factors should be taken into account when concluding obtained quantitative values of bone piezoelectricity in this study, which are the resolution, maximum applied voltage, anti-vibration ability, anti-electrical interference ability, multi-functions (e.g. force distance curve, adhesion mapping, wet stage, vacuum chamber, etc), instrument set-up. When choosing a PFM, we should overview at the applications, functions and performance of the instruments; also consider the individual research needs.

RH 1: Bone apatite has certain contributions to the piezoelectricity in bone.
From the chemical analysis, XRD and Rietveld analysis has positively shown the evidence of bone apatite to be piezoelectric; however, from the electromechanical characterisation, PFMs did not conclusively show such phenomenon in bone apatite and future studies are required. Another positive evidence is from the morphology analysis. TEM revealed the local orientation and the parallel nature of bone apatite nanocrystals, which indicated that the nanocrystals may have bipolar. Therefore, it can be concluded as bone apatite are formed into the inorganic matrix of bone that provides structure support and a waterproofed shielding for bone collagen fibrils, which are secondary contributions to the piezoelectricity in bone.
RH2: The local orientation in bone has direct effect to the piezoelectricity in bone at nanometre scale.

As predicted, the local orientation of collagen fibrils in bone has direct related to bone piezoelectricity and the fibrils of the etched bone with 90° angle to the bone axis showed the highest piezoresponse. At lamellar level, the collagen fibrils orient radially around a lacuna, the lateral piezoresponse was then the averaged in that area.

6.3 Future Work

There are still many properties of this mystical material, – bone, waiting for us to discover. Studying bone at nano level is still new and promising; however, future studies may go into the next levels – atomic or even sub-atomic level. On the basis of the present study, some future works are suggested as following:

i. Study of the elastic properties of bone at nano scale using Atomic Force Acoustic Microscopy (AFAM) or using an attachment, e.g. nano-indenter

ii. More advanced technology may be used to discover the electromechanical properties of bone apatite and to find out if it is piezoelectric

iii. Lateral study on bone from other species can be completed to compare with bovine bone

iv. Biological study on nanocomposite materials may be done to find an alternative material for bone apatite
References


W. W. Reynolds and W. J. Karlotski, "The Allometric Relationship of Skeleton Weight to Body Weight in Teleost Fishes: A Preliminary


[38] (02.05.2012). *SKELETAL SYSTEM*. Available: http://www.methuen.k12.ma.us/mmelan/skeletal%20system.htm


188


Bibliography

Conference Proceeding

Publications on Progress

Conference/Workshop Presentations


Appendices

Appendix I Cypher Demonstration

Figure A-1 PFM images of raw bone from Cypher demonstration, scanning size 4x4µm, (a) Topography; (b) Deflection; (c) PFM Amplitude; (d) PFM Phase

Figure A-1 and A-2 are the high resolution PFM images of raw bone obtained from Cypher demonstration, although the PFM tip used was not calibrated. The piezoresponse are clear and the mixed phases can be identified.
Figure A-2 PFM images of raw bone from Cypher demonstration, scanning size 1x1µm, (a) Topography; (b) Deflection; (c) PFM Amplitude; (d) PFM Phase
Figure A-3 PFM amplitude with functional bias: (a) Raw bone, applied bias 0-10V, showing linear from 0-7V; (b) Extracted bone apatite, applied bias 0-100V, trend to be linear from 40-100V, but curve is lower and digitised.

In Cypher demonstration, the PFM amplitude with functional bias was also tested with raw bone and the extracted bone apatite samples. The signal for raw bone is high and clear, while for apatite is much lower and digitised as shown in Figure A-3. Therefore, more confirmation and test will be needed.
Appendix II Unit Conversion

Unit Conversion: cgs.esu → pm/V

Cgs: centimetre-gram-second

1 dyne = 10⁻⁵ newtons

Esu: electrostatic units

1 Fr = 1 Statcoulomb = 1 esu charge

1 statC = 3.34x10⁻¹⁰ C

Therefore:

1 cgs.esu = 1statC /1dyne
= 3.34x10⁻¹⁰ C/ 10⁻⁵ N
= 3.34x10⁻⁵ C/N =3.34x10⁻⁵ pC/N
= 3.34x10⁻⁷ pm/V

The unit in Fukada’s calculation is converted to 10⁻⁹ x 3.34x10⁷ pm/V = 0.0334 pm/V