Synchrotron and Ion Beam Studies of the Bone-Cartilage Interface

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Abstract

The investigations reported herein concern the bone and overlying cartilage in thin sections of articular joints. The focus is on essential and trace elemental distribution and the structural organization of the component collagen fibres. Use is made of several nuclear analytical techniques (NAT’s), including micro-proton induced x-ray emission ($\mu$-PIXE), Rutherford back scattering technique (RBS), micro proton-induced gamma emission ($\mu$-PIGE), synchrotron radiation micro x-ray fluorescence (SR-$\mu$XRF) and synchrotron radiation small angle x-ray scattering (SR-SAXS).

Sections of healthy and diseased human femoral heads were examined by a combination of $\mu$-PIXE, RBS and PIGE to investigate the accumulation of some essential and trace elements such as zinc (Zn), calcium (Ca), phosphorus (P), sulfur (S), sodium (Na) and potassium (K) and their concentrations in these sections. Enhanced accumulations of these elements were noted at the bone-cartilage interface in both normal and diseased sections, and suggest an increased activity of bone/cartilage formation and degrading enzymes whose function rely on the presence of Zn, Ca, K and P as co-enzymes. Their high concentrations in the calcified zone of cartilage affected by OA may reflect an enhanced metabolic activity of chondrocytes that are associated with their reparative response to osteoarthritis and also as an indicator of bone growth.

SR-$\mu$XRF has also been employed in mapping the distribution of Ca, P, K, S, Na and Cl in equine metacarpophalangeal joint, the samples comprising healthy and diseased tissues at the bone-cartilage interface. Of interest was that the $\mu$-SXRF technique showed for the first time the change in shape of the tidemark and cement line in accord with the expectation of an abraded cartilage surface.

To investigate the molecular and structural features of bone and cartilage on the nanometre scale, a Pilatus 2M detector was used for the first time in investigation of collagen fibres arrangements in diseased calcified human femoral head sections. Well defined intensity and orientation maps were obtained.

The findings of this study have shown that the use of these physical techniques in qualitative analysis and quantification of the elemental content and collagen orientation in bone and cartilage tissues offer the ability to make an assessment of the initiation and progress of OA at the micro and nanometre scale.
Acknowledgments

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Last and not least I am grateful to the secretariat of Education and Scientific Research in Libya for the financial support of my studies abroad.
Declaration

This thesis and the work to which it refers are the results of my own efforts. Any ideas, data, images or text resulting from the work of others (whether published or unpublished) are fully identified as such within the work and attributed to their originator in the text, bibliography or in footnotes. This thesis has not been submitted in whole or in part for any other academic degree or professional qualification. I agree that the University has the right to submit my work to the plagiarism detection service Turnitin UK for originality checks. Whether or not drafts have been so-assessed, the University reserves the right to require an electronic version of the final document (as submitted) for assessment as above.

Wejdan Kaabar
# Acronyms and Abbreviations

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<table>
<thead>
<tr>
<th>Acronym</th>
<th>Term</th>
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<tbody>
<tr>
<td>2D</td>
<td>Two Dimension</td>
</tr>
<tr>
<td>AB</td>
<td>Atomic Bremsstrahlung</td>
</tr>
<tr>
<td>AC</td>
<td>Articular Cartilage</td>
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<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AR</td>
<td>Atomic radiation</td>
</tr>
<tr>
<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra Cellular Matrix</td>
</tr>
<tr>
<td>ED</td>
<td>energy dispersive</td>
</tr>
<tr>
<td>E DXRF</td>
<td>Energy dispersive x-ray fluorescence</td>
</tr>
<tr>
<td>ERD</td>
<td>Elastic Recoil Detection</td>
</tr>
<tr>
<td>FCD</td>
<td>fixed charge density</td>
</tr>
<tr>
<td>hrs</td>
<td>Hours</td>
</tr>
<tr>
<td>IBA</td>
<td>Ion Beam Analysis</td>
</tr>
<tr>
<td>IBC</td>
<td>Ion Beam Centre</td>
</tr>
<tr>
<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>keV</td>
<td>Kilo electron volt</td>
</tr>
<tr>
<td>Si(Li)</td>
<td>lithium drifted silicon detectors</td>
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<tr>
<td>L MF</td>
<td>List Mode Files</td>
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<tr>
<td>LS</td>
<td>Light scattering</td>
</tr>
<tr>
<td>MeV</td>
<td>Mega electron volt</td>
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<tr>
<td>mins</td>
<td>Minutes</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>NAT's</td>
<td>Nuclear analytical techniques</td>
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<tr>
<td>NB</td>
<td>Nuclear Bremsstrahlung</td>
</tr>
<tr>
<td>NRA</td>
<td>Nuclear Reaction Analysis</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OMDAQ</td>
<td>Oxford Micobeams Data Acquisition System</td>
</tr>
<tr>
<td>P</td>
<td>Phosphors</td>
</tr>
<tr>
<td>Pb glass</td>
<td>Lead glass</td>
</tr>
<tr>
<td>PIGE</td>
<td>Proton Induced Gamma-ray Emission</td>
</tr>
<tr>
<td>PIXE</td>
<td>Proton Induced X-ray Emission</td>
</tr>
<tr>
<td>ppm</td>
<td>Part per million</td>
</tr>
<tr>
<td>QFEB</td>
<td>quasi-free electron bremsstrahlung</td>
</tr>
<tr>
<td>RBS</td>
<td>Rutherford Back Scattering</td>
</tr>
<tr>
<td>S</td>
<td>Sulphur</td>
</tr>
<tr>
<td>SANS</td>
<td>Small angle neutron scattering</td>
</tr>
<tr>
<td>SAS</td>
<td>Small angle scattering</td>
</tr>
<tr>
<td>SDD</td>
<td>silicon drift diode</td>
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<tr>
<td>SR</td>
<td>Synchrotron Radiation</td>
</tr>
<tr>
<td>SR-SAXS</td>
<td>Synchrotron Radiation Small Angle X-ray Scattering</td>
</tr>
<tr>
<td>SR-XRF</td>
<td>Synchrotron Radiation X-ray fluorescence</td>
</tr>
<tr>
<td>TXRF</td>
<td>Total XRF</td>
</tr>
<tr>
<td>WAXS</td>
<td>Wide Angle X-ray Scattering</td>
</tr>
<tr>
<td>WDXRF</td>
<td>Wavelength dispersive x-ray fluorescence</td>
</tr>
<tr>
<td>Zn</td>
<td>Zinc</td>
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Analysis of bone
Scan area
A point on the trabecular/spongy bone
- Indicates below lower limit of detection.
Scan area
Spot size ~ 3 \( \mu \)m x 5 \( \mu \)m and beam current ~ 70 pA

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1 Introduction

The advent of Synchrotron Radiation (SR) and Ion Beam Analysis (IBA) has added a new dimension to the use of Nuclear Analytical Techniques (NAT's). These include the proton induced X-ray emission technique (PIXE), the Rutherford Backscattered technique (RBS), synchrotron radiation X-ray fluorescence (SR-XRF) and synchrotron radiation small angle X-ray scattering (SR-SAXS) [1-6]. These techniques have continuously evolved over the last few decades, attracting for instance the attention of scientists and researchers in areas of medical physics and various clinical applications. The high sensitivity and non-destructive nature of NAT’s have made them extremely employable in various applications for the detection and distribution of trace elements such as zinc (Zn), calcium (Ca), phosphorus (P), sulfur (S) and potassium (K) in healthy and diseased biological tissues of bone, cartilage of articular joints as well as brain and mammary tissues [6-11]. These elements are essential for biological systems and metabolic processes in which their presence play an important role as co-factors of the different enzymes, catalyzing the various reactions in living cells [12, 13].

Another application of SR is the utilization of SAXS in investigating the nano-structural alteration of collagen fibre organisation in bone and cartilage at the articular joints [14, 15]. The current study is an attempt to exploit various NAT’s to enhance our understanding of the presence and distribution of some essential and trace elements in bone and cartilage. In particular PIXE, RBS, PIGE, XRF and SAXS have been employed with respect to trace element accumulation and collagen fibre packing in healthy and diseased bone and cartilage sections.

Trace element deficiency in humans has been found to be among several factors that may contribute to many diseases, including cancer and degenerative joint diseases. The greatest emphasis has been placed on the elucidation of the accumulation or deficiency of specific elements in various organs which may result in dysfunction or disease in the human and animals [16-20]. These elements are usually classified into three categories: major elements Ca, P, S, H, C, O, Na, N, Mg and Cl; minor and trace elements: F, Si, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se, Mo, Sn and I; and toxic elements: Be, Hg, Pb, U and transuranic elements. The first two of the three groups are essential to human health and, in particular in regard to the trace elements, any surplus or deficiency in the availability of these elements, in the body may lead to a multitude of diseases or organ dysfunction. Acknowledging the inherent limitations of conventional imaging systems such as X-rays tubes, which suffer from insufficient spatial resolution, contrast and problems of quantitative scaling, synchrotron radiation (SR) and ion beam analysis (IBA) have been employed in this study to minimise such problems.
Qualitative and quantitative investigations of some essential and trace elements and their distribution in healthy and diseased bone and cartilage tissues by the simultaneous use of SR-XRF, PIXE and RBS can potentially be exploited as diagnostic indicators of clinical conditions and may enhance better understanding of the pathogenesis of the underlying mechanisms involved in various degenerative joint diseases such as rheumatoid arthritis (RA), osteoporosis (OP) and osteoarthritis (OA).

OA in particular is listed among the major global health concerns as a result of an increasingly aging population [21]. OA affects large numbers of people of all ages (including those involved in high activity situations), and various animals including equine species. OA manifests as alterations of compositional, organizational and physico-chemical make up in the articular cartilage, subchondral bone and other joint tissues [22-24].

This thesis starts with an introduction that shows the development of SR and Ion Beam Analyses (IBA) and their applications as NAT’s in PIXE, PIGE, RBS, SR-XRF and SAXS techniques in areas of medical physics. It also presents the role of some trace and essential elements in healthy and diseased bone sections affected by OA. The potential of some NAT’s are also discussed.

Chapter two is a general description of NAT’s giving the general concepts and theory of PIXE, RBS and PIGE and also SR sources, detailing their advantages with brief discussion of the basic components of SR facilities and SR for XRF and SAXS. An underpinning general review of bone and cartilage biology and the applications of NAT’s in biology and medicine is given at the end of this chapter.

Chapter three details the materials and methods, starting with the preparation of human and equine cartilage-bone sections and then proceeds to methods. In the methods section, a brief description of the University of Surrey Ion Beam Centre is given, in particular the microbeam line that was employed in present PIXE, RBS and PIGE. A summary explanation of the Swiss Light Source (SLS) at the Paul Scherrer Institute (PSI) has been presented, more emphasis being placed on the c-SAXS beam line and LUCIA beam line that have been utilized in this study and their relevant experimental conditions.

Chapter four is dedicated to bone-cartilage interface studies at the University of Surrey Ion Beam Centre. It includes three experiments; in experiment 1 the elemental distribution of some trace and essential elements in two diseased sections of human femoral heads were examined by using μ-PIXE and RBS. The first sample was probed with a beam spot size ~ 4 μm x 5 μm at beam current ~40 pA while the second sample was probed with a beam spot size ~ 3 μm x 5 μm at beam current ~100 pA. In the second experiment a normal and diseased human femoral head section were examined. In the third experiment simultaneous measurements of PIXE and PIGE were carried out on healthy and diseased bone
sections to determine the distribution of moderate and light elements. A proton beam (3 MeV) was focused to \( \sim 60\mu m \times 60\mu m \) spot size at a beam current of \( \sim 20\mu A \). The results from these experiments are reported.

Chapter five is dedicated to bone-cartilage interface studies at the SLS. The first section of the chapter shows the distribution of Ca, P, K, S, Na and Cl in three sites in which there were no visible lesions and one area in which a lesion was visible on an equine metacarpophalangeal joint, use being made of SR-\( \mu \)XRF at the LUCIA beam line. The second part of the chapter displays measurements of spatial alterations of collagen fibres and their molecular features investigated by employing SAXS on two diseased decalcified human femoral head sections.

Finally, chapter six gives a general discussion and conclusions of the study.
2 Review of the Literature

2.1 Nuclear Analytical Techniques

Nuclear analytical techniques (NAT's) are a group of analytical techniques dealing with nuclear excitations, nuclear reactions, electron inner shell excitations and/or radioactive decay [25]. NAT's, including Ion Beam Analysis (IBA), Synchrotron Radiation (SR), neutron activation analysis and other techniques have been very well developed. Their non-destructive nature and high sensitivity make the applications of these techniques plausible in the analysis of various materials. In the following sections of this literature review, the main emphasis is dedicated to IBA and SR techniques.

2.1.1 Ion Beam Analysis

Ion Beam Analysis (IBA) is based on interaction, at both the atomic and nuclear level, between accelerated charged particles and the target material [26]. IBA comprises Rutherford Backscattering (RBS), Elastic Recoil Detection (ERD), Proton/Particle Induced X-ray Emission (PIXE), Proton/Particle Induced Gamma-ray Emission (PIGE) and other forms of Nuclear Reaction Analysis (NRA) [27, 28]. The burgeoning and extensive employment of RBS and NRA since 1957 has been followed by other forms of IBA in the early 1960's, hastened by developments in accelerators and resulting in the advent and commissioning of several IBA facilities in many places around the world [29]. These technological advances made it possible to carry out investigations not only on inanimate but also on biological tissues such as bone and cartilage [4, 6-9].

2.1.1.1 Proton Induced X-ray Emission (PIXE).

Concept and Theory

Particle / Proton Induced X-ray Emission (PIXE) [1] is a powerful multi-elemental analytical technique that has been widely used in scientific investigations for detection of trace elements in various materials, with samples of small mass (~μg to g) and typically taking a few minutes to acquire an x-ray spectrum. In PIXE analysis, the samples are bombarded with particles (typically 1-4 MeV and usually protons) produced by an ion accelerator; interactions between sample atoms and beam particles causing bound electrons (predominantly from the K and L shells) to be ejected, ionizing the atoms in the sample, ejecting in particular the most tightly bound electrons. External/ higher shell electrons drop down to replace inner shell vacancies. As a result, either x-rays of a characteristic energy of the element or Auger electrons are emitted (Fig.2-1). Not only are the energies of the X-rays
that are produced characteristic of the emitting atoms, but also, from the relative intensities, the composition of the sample can be determined in examination of the X-ray energy spectra acquired during an appropriate beam bombardment. Use of a standard can lead to an estimation of the absolute concentrations of particular atoms. Normally semiconductor detectors such as lithium drifted silicon detectors Si (Li), with a good response over the range of photon energies 1-20 keV are used to record and measure the X-rays; the intensities are then converted to elemental concentrations with the aid of standards as previously mentioned [30].

In line with Moseley's law, targets with low atomic number Z results in lower energy x-ray emission while high Z targets produce higher energy x-ray emissions. When an electron drops from an L shell to K shell the X-ray line is called a Kα X-ray, and from an M shell to K shell it is called a Kβ X-ray. Similarly, L X-rays are produced when electrons drop from higher shells to the L shell and so on as shown in (Fig 2-2).

Figure 2-1: Simplified illustration of ionization of the K-shell by an incident X-ray particle.

Figure 2-2: Electronic transitions producing various x-ray lines [31].
An often cited advantage of PIXE over x-ray fluorescence (XRF) is the largely absent problem of photon interactions (photoelectric effect and scattering in particular) that lead to a significant source of background in XRF techniques. Having outlined the basic principles of PIXE, it is also worth remembering that with the advent of the Si (Li) high resolution detectors in the early 1970's, PIXE was established to be a highly powerful and sensitive tool for elemental trace analysis.

Johansson et al. [32] reported the first analytical use of PIXE in the examination of air particulate contaminants on carbon foils. Since that work, PIXE analysis has been developed to encompass various applications in biology, geology, medicine, archaeology and forensic sciences. This proven spectroscopic technique has now been extensively used for more than three decades. In addition to its main advantages such as high sensitivity and simultaneous multi-elemental analysis of elements of atomic number $Z \geq 11$ at all concentrations, one can also mention the non destructive nature of the technique and minimal sample preparation, and the short time required for data acquisition (typically a few seconds/few minutes) and the demand for only small sample areas allowing good elemental concentration details to be examined. Another major feature of this technique is, as previously agreed, that the background radiation is lower in intensity than that encountered by more conventional techniques such as x-ray excitation or excitation by electromagnetic radiation from X-ray tubes or radioisotopes, collectively referred to as x-ray fluorescence (XRF). The latter can be inconvenient for routine every day applications since data acquisition time is relatively long due to the relatively poor signal to noise ratio.

A relatively recent research development is PIXE using a tightly focused beam (~1 μm) to give additional potential for micro analysis, making it possible to determine the distribution of trace elements in a variety of smaller-scale samples [33, 34].

**Ionization cross section, $\sigma_z(E)$**

The probability that an inner shell electron of an atom will undergo ionization thus leaving a vacancy is known as the ionization cross-section $\sigma_z(E)$.

$\sigma_z(E)$ depends on the energy of the bombarding protons and the atomic number ($Z$) of the element. Usually $\sigma_z$ increases with energy of the incident protons, reaching a maximum when the proton velocity matches that of the removed electron. Several theoretical approaches such as the Binary Encounter Approach (BEA) [35] have superseded the Plane Wave Born Approximation (PWBA) [36] and the Semi-Classical Approximation (SCA) in determining $\sigma_z$. 
The combined theoretical BEA and experimental proton cross section were used by Johansson and Johansson [35] in the calculation of ionization cross-section \( \sigma_z \), given by:

\[
\ln(\sigma_z u^2) = \sum_{n=0}^{s} b_i, n \left[ \ln \left( \frac{E_p}{\lambda u_i} \right) \right]^n
\]

Where: \( \sigma_z \) concerns K and L shell ionization cross sections \( (10^{-24} \text{ cm}^2) \)

\( u_i \) is the ionization energy (eV)

\( b_i, n \) are values of coefficients that are determined by fitting a fifth-order polynomial to the available experimental proton ionization data.

\( E_p \) is the proton energy (eV).

\( \lambda \) is the ratio of proton to electron mass \( (\lambda = 1836.1514) \).

Although this equation does not provide accurate values for heavy elements with atomic number \( |Z| > 50 \), its simplicity has made it convenient for use for moderate \( Z \) values.

The need for more accurate \( \sigma_z \) values, led to development of new models. The ECSSSR model, a modified version of the PWBA [36], takes into account energy loss (E) during the collision, Coulombic deflection (C), Perturbed Stationary States (PSS), and relativistic effects (R) [37]. Values obtained in this model have been found to conform with experimental values [38].

**Branching ratio, b**

Elements yield characteristic x-rays with different energies depending on which transition takes place. These can be additionally divided according to which sub shells are operative although the energy differences will be small. For instance, if the K-shell is ionized an electron from the L-shell will fill the vacancy producing a K\( \alpha \) peak in the spectrum. In this, the branching ratio can be defined as the relative probability of the transition occurring.

**Fluorescence Yield, \( \omega \)**

The fluorescent yield is defined as the ratio of characteristic x-rays to the number of primary vacancies created in the shell [39]. It is well known that when an electron from a higher shell descends to fill a vacancy created in an inner shell, an X-ray photon is emitted from the atom. Occasionally, the emitted x-ray on its way out may be absorbed by the electrons in outer shells, which may result in the emission of an outer shell electron known as a low-energy Auger electron which can also create soft x-rays through attenuation.
In addition, the $\omega$ can also be defined depending on the shell of the ejected electron, for instance, $\omega_k$ is the fluorescent yield of characteristic x-rays formed from the removal of electrons from the K-shell. Higher shell ionisation is complicated further by several sub-shells; as mentioned earlier, the limited resolution of Si (Li) detectors makes it difficult to distinguish between x-rays released from various sub-shells.

The quasi-empirical formula by Bambynek [40] is generally applied to calculate the fluorescent yield:

$$\left( \frac{\omega_z}{1-\omega_z} \right)^{1/4} = \sum_{i=0}^{3} B_i Z_i$$

where $\omega_z$ is the shell fluorescent yield

$B_i$ are values of coefficients, calculated by fitting the formula to the experimental data.

The formula (2.2) is applied for elements $Z > 20$ and every element is described by four coefficients. However, the theoretical data by Scofield is still used for elements $Z \leq 20$ [41].

**X-ray production cross section, $\sigma(E)$**

The branching ratio $b$, fluorescent yield and ionization cross-section can be combined to give the X-ray production cross-section which can be calculated from equation 2.3:

$$\sigma(E) = b \omega_z \sigma_z(E)$$

The data by Reis and Jesus [42] is used to calculate the x-ray production cross-section while the energies of these x-rays are taken from GeoPIXE [43].

**X-ray attenuation**

X-ray attenuation occurs when traversing atoms in the sample matrix. Consequently, x-ray yields need to be corrected. Normally, lower energy x-rays are more attenuated than higher energy x-rays and heavier elements attenuate more than lighter elements.

The attenuation of x-rays by materials offers an extensive variety of information about the basic properties of matter in atomic, molecular and the solid state including electron density and Archimedean (mass) density.

The mass attenuation coefficient in the matrix can be used to describe the total reduction in intensity of x-rays at a detector due to both energy absorption and scattering and can be calculated by using the following equation:
\[ \mu = \sum_{i=1}^{n} \mu_i \omega_i \]  

Where \( \omega_i \) is the weight fraction of element \( i \).

\( \mu_i \) is the mass attenuation coefficient of element \( i \).

To calculate the mass attenuation coefficients of each element for x-ray energies above 1 keV the theoretical data published by Berger and Hubbell are typically used [44].

PIXE spectrum analysis and background radiation

The resulting spectrum obtained by PIXE analysis consists of two basic parts (Fig.2.3). The first is the continuous background of electromagnetic radiation and the other is the characteristic x-ray peaks superimposed upon the background radiation. From the x-ray spectrum, it is essential to resolve the characteristic x-ray peaks in order to distinguish between the K lines of an element, for instance between \( K_a \) and \( K_b \). Consequently, reference materials are available to provide for discrimination between the peaks.

![Figure 2-3: A typical PIXE spectrum of a sample of femoral head bone irradiated by a 2.5 MeV proton beam.](image)

The amount of the background limits the detection threshold; the lower the background, the better the detection limits of the technique. The background radiation is produced from a collection of effects such as Compton scattering, \( \gamma \)-radiation from nuclear reactions, environmental sources and charge build up. However, the main contributing factor to the background radiation is bremsstrahlung / braking radiation, being the electromagnetic radiation produced by charged particles passing through matter. There are two forms of bremsstrahlung, primary and secondary. The primary is produced when the incident proton beam passes through the Coulomb field of the nuclei of the matrix matter. As a result, a continuous background radiation is produced which is the main source of background at high values of energy. The secondary bremsstrahlung is produced by the deceleration of secondary
electrons in the target, which is the main source of background at low energies. The bremsstrahlung emitted by deceleration of particles is proportional to the square of the acceleration.

\[ \text{Intensity} \propto \left(\frac{\text{acceleration}}{m}\right)^2 \]

Then

\[ \text{Acceleration} = \frac{F}{m} \]

\[ \text{Intensity} \propto \left(\frac{F}{m}\right)^2 \]

where \( F \) is the electrostatic force between the particles and \( m \) is the mass of the decelerating particle.

The lower bremsstrahlung background of the proton beam revealed in formula (2-5) makes PIXE more sensitive than XRF which uses photon beams.

It is understood that the continuous background contains:

- quasi-free electron bremsstrahlung (QFEB),
- atomic bremsstrahlung (AB),
- nuclear bremsstrahlung (NB),
- the Compton tail of \( \gamma \)-rays produced by nuclear reactions and the tail of the response function of the detector.

QFEB occurs in the following circumstance: if the proton velocity is much higher that the velocity of orbital electrons then the electrons can be considered as free and stable and therefore can be scattered by the proton's Coulomb field.

Atomic radiation (AR) occurs in the circumstance that a bound electron is excited to a non-bound continuum state, after that being slowed down to a bound state, consequently releasing radiation. This radiation is called atomic bremsstrahlung. AB is usually very small compared with QFEB however; its contribution from heavy elements in the sample is noteworthy.

The bremsstrahlung is angular dependent [45], becoming high at 90° and a minimum at 180° relative to the beam. Therefore to reduce the background the detector is usually placed at a convenient backward angle of 135° relative to the beam.

Filters

Most PIXE work is performed with filters/absorbing foils placed between the sample and the x-ray detector; this offers better absorption of low energy bremsstrahlung background, thus increasing the throughput of higher-energy characteristic x-rays that carry information on the trace elements of interest. Filters are also essential in preventing recoiling protons from entering the detector. Various types of filters can be used; in particular Mylar and aluminium are the most widely used absorbers. Materials with low Z numbers are sometimes used to
filter low energy x-rays as they have low binding energy, often below 1keV. For more localised detection, a combination of K-edge filters can be employed so that only elements in the energy range required are detected [46]. In the present study a Be filter of 130 µm thickness was used.

Detection

A wide variety of x-ray detectors are used to detect x-rays. While the number and types of these detectors has increased dramatically, an appropriate detector of high efficiency in the energy region of interest, a high count rate capability and the use of positron sensitive facilities on occasions is what is required. For example, the detector most often used for PIXE is the Si (Li) detector. The detector must be operated at the temperature of liquid nitrogen in order to reduce the leakage current (and hence noise). This requires a large insulated container to hold the liquid nitrogen and a ‘cold finger’ to mount the crystal. The first transistor of the signal amplifier is also mounted on the cold stage. The use of Si (Li) detectors of high efficiency is now widespread for the detecting of photons in the 1-20 keV range, allowing detection of various elements simultaneously.

Charge build-up

When a sample is bombarded with positively charged protons with consequent release of electrons, charge build-up in the sample may happen and this phenomenon commonly occurs with thick samples. High voltages on the surface of the sample will accelerate the free electrons resulting in high background in the entire region of the x-ray spectrum and this may mask the presence of small peaks on the spectrum and prevent their detection. To decrease this effect, different methods have been suggested including:

- Lower beam current (although this will increase the analysing time).
- An electron gun which may be used to neutralise positive charge.
- For thick-target homogenous samples a conductor may be mixed into it (however contaminations can present a problem).
- A conducting coat such as carbon is usually painted between the sample and its holder. However, as before, this may cause contamination.
- Increase in the pressure of the chamber, therefore reducing charge build-up.
2.1.1.2 Rutherford Back Scattering (RBS) technique

Rutherford Back Scattering (RBS) is an analytical technique based on measuring the number and energy of protons in a beam which back scatter after colliding with atoms in the near surface region of a sample at which the beam has been targeted [47]. A schematic diagram is shown in Fig. 2.4.

The collision does not actually involve direct reactions between the projectile proton and target atom. Energy exchange occurs due to coloumbic forces between nuclei in close proximity to each other. The interaction between the proton and the nuclei can be modelled accurately as an elastic collision using classical physics.

The energy produced is measured by detecting protons back scattered at a given angle and the associated loss of energy depends upon two factors: firstly, loss in energy while they pass through the sample, and secondly, the energy lost depends on the masses of the protons and the target atom. The ratio of the energy of the protons before and after collision is named the Kinematic factor (K). The number of backscattered protons that arise as a result of colliding with a specific element in a sample depends on two main factors: the concentration of the element and the scattering cross-section for the nucleus. Here it should be noted that the probability that a material will cause a collision is called its scattering cross-section.
Kinematic factor

The elemental concentration with depth can be determined using the K factor, equation 2-6, which determines the amount of energy transferred from the protons to the target atoms.

\[ K = \frac{E_s}{E_0} = \left[ \frac{\left(1 - \left(\frac{M_1 \sin \theta}{M_2}\right)^2\right)^{\frac{1}{2}} + \frac{M_1 \cos \theta}{M_2}}{1 + \frac{M_1}{M_2}} \right]^2 \]

Where \( E_s \) and \( E_0 \) are the energies of the incident particle after and before scattering, \( M_1 \) and \( M_2 \) the mass of the incident particle and target atom and \( \theta \) the scattering angle.

From the formula (2.6), it is interesting to note that, despite the cross-section being less, more energy is transferred from protons to nuclei that are closest in mass to the protons such as the lighter elements C, N and O.

The sum of the transferred energy and the energy loss due to the stopping power of the sample equals the total amount of energy difference between the energy measured by the RBS detector and the initial proton energy. The stopping power of the sample is dependent on the depth of the atom in the sample. The scattering cross-section shows the probability of backscattering in a given solid angle.

2.1.1.3 Proton/particle Induced Gamma-ray Emission (PIGE)

Light elements detection is often difficult to determine by PIXE which is mainly employed to detect element of atomic number \( Z > 11 \). Therefore PIGE is an ideal complementary technique to PIXE and PIGE is usually utilized in the analysis of light elements such as Li, B, and F as well as being highly efficient for perhaps dominating elements like Al, Mg, Si and Na (Fig.2-5) [48, 49]. Of interest is that However, these techniques (PIXE and PIGE) can be simultaneously performed [50, 51].
Proton/ Particle Induced Gamma-ray Emission (PIGE) analysis is a rapid, non-destructive technique that, in theory, may be employed in the analysis for any element. In PIGE analysis, a charged particle (typically protons) approaches the nucleus of a target atom; the Coulomb force usually prevents it in engaging in a collision. However, when the incident particle has energy greater than that required to overcome the repulsive Coulomb force, the charged particle can then penetrate through the electrostatic barrier into the nucleus, resulting in interactions with nuclear forces. Depending on the energy of the incident particle and the type of target nucleus a number of interactions occur during that process (Fig.2-6). In general, a nuclear reaction will follow, resulting in the emission of high energy γ-rays (emitted from the nucleus) and other nuclear particles. The energy of the gamma ray is indicative of the element present and therefore its value is used to fingerprint elemental composition while yields are used to quantify elemental concentrations. Because the technique is based upon specific nuclear reactions, the sensitivity of PIGE varies greatly from one element to another. In addition, the sample matrix will also influence the detection limit of an element because of variations in the background of the gamma ray spectrum.
The emitted gamma rays are generally collected with a high-purity germanium detector (Ge) when good energy resolution is required, or with sodium iodide / bismuth germinate crystals when good efficiency for high energy gamma-rays is needed.

While here the physical principles of IBA have been presented, it is also worthwhile to recall the main technical advantages and limitations of these consolidated analytical techniques[53]:

**Advantages**
- Multi-elemental capability including for light elements;
- Non-destructive for most materials, excluding organic compounds which might be sensitive to heat or radiation damage.
- Highly quantitative and sensitive, in particular PIXE.
- Minimal sample preparation.
- Complementary and the techniques (PIXE and PIGE) can be employed simultaneously.
- Provides spatial information via depth profiling and elemental-mapping.

**Limitations**
- Requires access to particle accelerator.
- Cost ineffective in some analyses.
- Lacks information on the chemical state of elements (speciation not possible conversely XANES analysis through use of SR provides for such analysis).
- Unsuitable for the analysis of insulating materials because of electrical charge build up.
2.1.2 Synchrotron Radiation for Microscopic X-ray fluorescence and Small Angle X-ray Scattering analysis

Synchrotron radiation

Synchrotron Radiation (SR) is the electromagnetic radiation [54] emitted by charged particles like electrons and positrons accelerated to high energies (up to several GeV) and then injected into a storage ring, and forced to change direction under the action of magnetic fields. They lose their energies by emitting photons of light that can be guided through beam tubes towards experimental facilities [55, 56].

SR was observed for the first time at the General Electric company in the USA in 1947 in a newly developed type of accelerator, an electron synchrotron [57]. At the beginning SR was considered a nuisance as it caused the particles to lose energy, however subsequently in the 1960s it was recognised to be a light source with brilliant properties and pioneering studies were made employing the so-called first-generation SR sources. In the period between the 1970s and 1980s, second-generation SR sources were specially made at different locations around the world. These second-generation radiation sources were generally taken out from the bending magnet of a dedicated storage ring. Second–generation synchrotrons have represented a tool that is well adapted to the study of material properties.

The great benefits accrued from second-generation SR sources led to further developments of such x-ray technology, and consequently to the development of third-generation synchrotrons during the 1990s. In the third-generation of SR the facilities provide low emittance of the electron beam and extensive utilization of insertion devices like wigglers and undulators [58, 59]. The result is x-ray beams of extremely high spectral brightness which can be utilized in a number of ways. At the same time, the development of fourth-generation synchrotrons, represented by the free electron laser (FEL) source is in progress [60].

Among the main features of SR is the ability to generate high spatial resolution images with high signal- to-noise ratio [55, 58], high intensity, coherence and polarization, wide spectral coverage, and a known time structure. These features make SR a powerful tool in a wide range of research fields, including chemistry, medicine, biology, material science, earth science, engineering and recently nanoscience [55, 59]. In addition SR is an extremely intense and brilliant source over a wide range of wavelengths, from infrared, through to the ultraviolet and x-ray regions, making it possible to examine the specific composition of various materials. SR sources have numerous advantages which are briefly described below.
**High intensity and high collimation**

As SR is emitted in an extremely small angular range around the tangential direction of the electron direction and the photon flux (intensity) is extremely high over the small beam sizes achievable (<1 μm), this makes SR suitable for experiments involving smaller samples [61]. The highly collimated SR is greatly suited for micro analysis and this feature enables analysis of trace metallic elements contained in a biological sample, even down to the single cell level [55, 62].

**Polarization**

SR is linearly polarized (in the plane of the electron beam) either from a bending magnetic or a plane undulator. In contrast, the x-rays from an x-ray tube are randomly polarized. Linear polarization is used to good effect in reducing the background radiation during x-ray fluorescence analysis [61].

**Pulsed time structure**

Photons radiated from bunched electrons running periodically in the storage ring are pulsed at controlled intervals. This characteristic makes it possible to perform time-resolved measurements [61].

**Broad spectral range and energy tunability**

The radiation spectrum from an electron that travels in a curved path ranges from the infrared through to highly-energetic x-rays. Predominantly the photon energies used range from the vacuum ultraviolet to hard x-rays. By using a suitable monochromator, it is possible to extract monochromatic beams from this continuous spectrum and to tune the radiation energy to given values [55]. The radiation from a series of magnets in an undulator immediately gives quasi-monochromatic beams. The peak energy can be changed by changing the magnetic gap.
Basic components of an SR Facility

SR facilities consist of an injection system, a storage ring and beam lines. A schematic diagram of the main components of a SR facility is provided in Fig.2.7.

![Schematic diagram of a synchrotron](image)

Figure 2-7: A schematic diagram of a synchrotron [63].

with:

1) the electron gun 2) linear accelerator 3) booster synchrotron | Injection system
4) storage ring
5) beam lines 6) experiment station | Beam lines

Electrons or positrons are usually generated in the injection system and pre-accelerated. Sometimes a second accelerator further accelerates the electrons/positrons up to ~1 GeV before insertion into the storage ring. The storage ring consists of radio-frequency cavities, bending magnets, other magnets, insertion device and control systems. Inside the storage ring, bunches of electrons periodically circulate at relativistic speed, typically for many hours or sometimes for many weeks under continuous top-up when electrons are added to the ring at regular small intervals. The beam lines combine x-ray optics, detectors, computer interface electronics and computer software. Beam lines deliver the SR from the storage ring and prepare the beam for experiments and protect the users against radiation. The main components of the storage ring and beam lines are briefly described below:

Storage Ring

Radio-frequency cavities

When the electrons lose energy due to the emission of SR the radio-frequency cavity system restores the energy and steadies the bunch of electrons.

Bending magnets

The bending magnets turn the path of electrons and force them to circulate in an orbit. The magnitude of the relativistic angular width of the bending magnet radiation (\(\Delta\Psi\)) is calculated from the formula:

\[\Delta\Psi = \frac{1}{\gamma}\]
where \( \gamma \) is the relativistic factor for the experiment, while the critical wavelength is given by:

\[
\lambda(c) = \frac{18.64}{(B \times E^2)}
\]

where: \( B \) is the magnetic field in Tesla and \( E \) the beam energy in GeV.

**Insertion devices**

Initially the source was radiation produced by the electrons as they travelled through the bending magnets. More recently, in third-generation synchrotrons, in order to obtain enhanced radiation from the storage ring, a series of short-pitch dipole magnets with alternating polarity are used to cause the path of the electrons to oscillate (wiggle). These magnetic arrays are placed in the straight sections (between the bending magnetic) of the storage ring and are named insertion devices (Fig 2.8). Insertion devices fall into two main categories, undulators and wigglers [64]. The undulator is a device that causes the electron beam to follow a gentle, periodic, undulating path with the result that the radiation wave fronts emitted overlap with each other and so interference effects occur. At some wavelengths the interference is beneficial and significant enhancement in the intensity is observed, in other cases the interference is destructive and consequently there are dark regions in the spectrum. The wiggler has a stronger effect upon the electron beam path, causing it to wiggle from side to side in greater amounts. This prevents the wave fronts from overlapping and so no interference effects occur. The advantage of this type of insertion device over a bending magnet source is that each wiggle produces the same number of photons as a bending magnet and so if tens of wiggles are produced in a straight section then the observer will see an intensity enhancement of the same order.

![Figure 2-8: A schematic diagram of Insertion Devices [65, 66]](image)
Beam lines
Monochromator
To pick an extremely narrow energy band of the spectrum a monochromator is used while a focusing system such as Kirkpatrick-Baez mirror system is used to obtain sub micron beam diameter [67].

End station
The end station consists of different tools depending on the nature of the experiment (diffraction, scattering, absorption and others). Some of these tools are sample holders used for introducing samples to the beam, detectors for recording the original and fluorescent radiations and other equipment are used to control the end station and measuring the response of the detectors [68].

2.1.2.1 X-Ray Fluorescence Spectrometry (XRF)

X-ray fluorescence spectrometry (XRF) is counted among the various analytical techniques existing for elemental analysis. In this particular technique, the sample is irradiated by energetic photons causing the emission of fluorescent x-rays from the sample. The produced x-rays are collected and displayed in a spectrum with either wavelength dispersive or an energy dispersive detector. There are two main XRF methodologies: the energy dispersive (EDXRF) and wavelength dispersive (WDXRF) techniques. The elements in the sample are identified in terms of characteristic wavelengths of x-ray emission while the concentrations of the elements are determined by the intensity of those x-rays. EDXRF has two main disadvantages: it is count-rate limited and generally offers lower energy resolution which limits its accuracy and precision when compared with WDXRF. On the other hand, WDXRF is rarely used due to the slow measurement process and the relatively low resolution. In association with the particular instrument configuration and set up, the range of detectable elements will be variable. Usually EDXRF covers all elements from sodium (Na) to uranium (U), while WDXRF can detect down to beryllium (Be) but clearly only for in-vacuo conditions. The limits of detection depend upon the specific element and the sample matrix; however, it is apparent that heavier elements generally have better detection limits.

The need for a fast and non destructive analytical technique suitable for a variety of materials led to the development of XRF instruments capable of high spatial resolution analysis by micro-XRF/μ-XRF (generally based on use of synchrotron sources) which is based on localized excitation and analysis of a very small area on the surface of a larger sample. This offers information on the lateral distribution of the elements in the test sample [69]. A basic μ-XRF assembly is illustrated in Fig.2.9. In this technique, the micro x-ray beam irradiates the sample to induce the emission of fluorescence x-rays at a microspot.
These fluorescent x-rays are usually detected by an optimum suitable detector. The test sample might be moved manually or by computer control which makes possible the obtaining of 2-D image collection or line profile analysis. In the past, before the advent of third-generation SR, the production of sufficiently intense x-ray beams for sensitive microanalysis had been a major obstacle.

![Figure 2-9: Basic assembly of \( \mu \)-XRF](image)

**2.1.2.1.1 Bench-Top -\( \mu \)-XRF**

The primary means of producing a \( \mu \)-XRF source in the laboratory is from a broad cone of radiation from a conventional x-ray tube collimated using an aperture or cross-slit system.

While the idea was first employed in the 1960s, the very low count rates observed for small-area samples made it unacceptable. In the 1980s, with the advent of energy dispersive (ED) detectors, the use of \( \mu \)-XRF was again considered for x-ray imaging applications. As the solid angle of acceptance of Si (Li) detectors can be much larger than that of crystal spectrometers, fluorescent signals of much lower intensity could be exploited. To produce a microbeam with a smaller diameter (10-100\( \mu \)m), Nicholas and Ryon (1986) equipped a rotating anode x-ray generator with a customized microdiffractometer system. The use of the diffractometer allowed manual translation of the sample through the beam and included a microscope for sample observation. The aim was to construct a relatively economical system from off-the-shelf components that would offer a deeper depth analysis through the sample than near surface analysis by using electron optics instrumentation. More recently still, rapid developments of Synchrotron Radiation (SR) in the 1980s and 1990s has now made it possible to employ this form of source as an efficient source of \( \mu \)-XRF.
2.1.2.1.2 Synchrotron Radiation for Micro- X-ray Fluorescence analysis

As mentioned earlier, the SR x-ray source offers a great improvement in x-ray production technology and has opened up modern applications such as trace elements analysis, surface and micro analysis. SR-XRF analysis is a unique method to analyze trace elements. Compared to conventional X-ray tube excitation, synchrotron radiation has the advantages of brightness, energy tunability, collimation and polarization.

In summary, the arrangement of a micro SR-XRF set-up is entirely different to that of bench-top XRF systems and can be arranged in different modes using components like mechanical collimators, monochromators selecting a specific energy from the white beam by for example Bragg reflection and flat mirrors to deflect the beam and to remove high energy photons. To focus the beam, curved mirrors are used. To move the sample with respect to the beam a motor-driven stage is used. A typical SR-μXRF assembly is shown in the schematic of Fig.2.10.

![Figure 2-10: A schematic illustration of SR-μXRF.](image)

The microbeams are usually generated at the storage rings. To achieve submicron spots different optical systems such as circular or linear Fresnel zone plates are employed. For example, in the Hasylab storage ring, Germany, a pinhole or conical capillary are utilized at the μ-XRF beam lines.

**Detectors**

For detecting fluorescent x-rays, different detectors are employed, however the most commonly used are proportional counters and solid-state ionization detectors, Si(Li) detectors and charge coupled devices. These detectors detect the characteristic x-rays, whose intensities are relative to the concentration of elements in the sample. Due to their high detection efficiency, the most exclusive and commonly used are Si(Li) detectors. They are placed normal to the beam in the horizontal plane, benefiting from the linear polarization in reducing
the background. However, in some experimental conditions it is difficult to place the detector normal to the beam. In these detectors, ionization by the x-rays in the essential region creates electron-hole pairs that are swept by the reverse bias electric field to give a current pulse. The number of pairs produced is proportional to the incident x-ray energy. The charge composed at the anode is converted to a voltage by an amplifier. Afterwards these voltages are converted into voltage pulses by a preamplifier, and then amplified and shaped by a linear amplifier to optimize the signal-to-noise ratio. By using a multi-channel pulse height analyzer the signals are fed to be stored into an energy spectrum. Conversely, a crystal spectrometer is generally used for laboratory XRF analysis for bulk samples. In continuum excitation SR-XRF assemblies these detectors are still employed due to their lower detection efficiency.

2.1.2.2 X-Ray Scattering techniques

Basic concepts

Around the turn of the 20th century, Roentgen discovered radiation of unusually small wavelengths, naming it x-rays. In 1912 Max Von Laue discovered x-ray diffraction, its applications being developed by W.H and W.L. Bragg to the study of crystalline structures. An example of these applications is Small Angle X-ray Scattering (SAXS), established by Andre Guinier in 1930's. In the 1950's Andre Guinier and Otto Kratky demonstrated that SAXS provides insights into the internal structures of materials in addition to their size and morphology. Most of the earlier theoretical work to SAXS was established by Gunther Porod [71].

The advancement of high flux, third generation synchrotron sources and developments in detectors for data acquisition analysis have made SAXS a unique technique in terms of angular and time resolution and for smaller sample volumes in investigations of a range of biological applications [72-74]. Recently, the small angle x-ray scattering initiative for Europe (SAXIER) [75] has developed the use of the SAXS technique as one complementary to other analytical techniques such as polarized light microscopy (PLM), Fourier-transform infrared microspectroscopy (FT-IRM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) [49-51], also developing a new generation of SAXS sources for present and future light sources. This project has also aimed at investigating the potential of measuring scattering from biomolecules in the gas phase while the molecules are confined in an ion-trap mass spectrometer.

Overall, Small Angle Scattering (SAS) is formed of a broad family of techniques that encompasses x-ray scattering techniques such as (SAXS) and Wide Angle X-ray Scattering (WAXS), Small Angle Neutron Scattering (SANS), and Light Scattering (SALS/LS). X-ray scattering analysis are usually described in terms of the scattering angle 2θ [76] where the
Bragg angle $\theta$, is the angle formed between the incident beam and the plane containing the scattering centres of interest while $2\theta$ is the angle through which the incident beam is turned to form the scattered beam. When this scattering angle decreases, the resultant pattern of scattering is indicative of larger structures. Within this scheme it is understood that SAXS provides information on nanostructures (1-100 nm), while WAXS offers information on the sub-nanometre scale structure and is more typically employed on amorphous structures and crystal packing. SANS also provide information on nanostructure (1-100 nm) [77]. Since the wavelengths of visible light is much greater than that of either neutrons or x-rays, light scattering (LS) is applied in investigating even larger structures. Nevertheless, the physical principles of these techniques are identical.

2.1.2.2.1 Small angle x-ray scattering (SAXS)

SAXS is a well established technique that has become a powerful probe in the determination of nanostructures of condensed and soft materials offering insight on their size, morphology and internal features [78, 79]. Investigations of amorphous materials such as biological polymers, in particular proteins, make use of SAXS as the technique of choice [77, 80].

When compared to complicated conventional x-ray tube techniques, which are cumbersome, the advent of SR as a source of SAXS offers extensive monochromatic x-ray beams that render examination of weak scattering materials achievable and data acquisition attainable within period of a few minutes, being referred to as SR-SAXS [59, 60].

Regarding the advantages of SAXS, these include its non-destructive nature, its applicability to a multitude of systems (solutions, powders, thin films and biological molecules) [61-63] and the minimal sample preparation requirements. Furthermore, SAXS also provides determination of internal features and surface-to-volume ratios in materials. For routine analysis, SAXS is not a cost-effective tool being almost entirely a research tool.

The basic principle of SAXS is to scatter a highly collimated and monochromatic x-ray beam of wavelength $\lambda$ elastically and to record the scattering intensity as a function of the scattering angle ($2\theta$). Figure 2.11 shows a schematic of a typical SAXS measurement. The recorded scattering profile provides information about the large-scale structure and conformation of the studied molecules.
Figure 2-11: Schematic diagram of a SAXS setup representing the incident, scattering and transmitted x-ray beams, beamstop, the 2-D detector, and the definition of the scattering vector (q).

Note that \( \theta_s \), the scattering angle is twice the Bragg angle \( \theta \).

Usually a beam stop is placed before the detector to absorb the transmitted primary beam and the whole path before and after the sample is kept under vacuum to prevent absorption and scattering by gases. To enhance spread of the SAXS signal over a large area to allow detection and splitting of the signal, the detector and beam stop are typically placed at a distance one meter or more from the sample [81]. On such basis, the incident \( \vec{S}_0 \) and scattered \( \vec{S}_s \) wave vectors are equal, assuming that the refractive index is close to unity. The relationship between the absolute value of the scattering vector, \( |\vec{q}| \), and the scattering angle \( 2\theta \) is given by the formula:

\[
q = |\vec{q}| = \frac{4\pi}{\lambda} \sin(\theta_s/2)
\]

where \( \lambda \) is the wave length and \( q \), the momentum transfer parameter, the latter referring to the length scales probed by the underlying scattering trial and expressed in units of reciprocal length.

\[
I(\vec{q}) = \left| \sum b_n e^{i\vec{g_n} \cdot \vec{r}_n} \right|^2
\]

where \( b_n \) is the scattering length and \( \vec{r}_n \) position vectors of scattering centres.

Two limiting factors must be satisfied for SAXS: (i) the measured length scales are much larger than the atomic length scale, and; (ii) a multi-phase model is linearly separable; as an instance if the examined material is comprised of two components (\( \alpha \) and \( \beta \)), then these can be considered in terms of these separate electron densities (\( \rho_\alpha \) and \( \rho_\beta \)), separated by a sharp boundary.
If these two constraints are satisfied, the previous equation could be rewritten as:

\[ I(\bar{q}) = I_0 \cdot (\rho_\alpha - \rho_\beta)^2 \left| \int \sigma(r) e^{i\bar{q} \cdot r} dr \right|^2 \]

where \( V \) is the total irradiated volume and \( \rho \) is a step function, equal to one for the phase \( \alpha \) and zero for the phase \( \beta \). In the case of bones which consist of minerals and collagen, the scattered intensity of bone is enabled by the electron density contrast between the minerals and collagenous components of bone [82].

2.1.2.2 Synchrotron Radiation for Small Angle X-ray Scattering

In early days, SAXS investigations were achieved by using x-ray tubes and slits collimation were extensively employed to balance the low intensity of the source from x-ray tubes. Recently, a sufficient intensity to perform SAXS can easily be gained from SR sources. The advent of powerful SR offered more enhanced photon flux which allows researchers to examine different materials and in particular small scattering volumes. Figure 2-14 depicts the schematic layout of SAXS using a SR source.

![Figure 2-12: Schematic layout of SAXS arrangements at a synchrotron source.](image)

2.2 Applications of Nuclear Analytical Techniques to the Bone-Cartilage interface

Before describing some applications of NATs, it is essential to present the general features of bones and cartilage, in particular in regard to the compositional and structural features of articular joints. This section of the literature also includes a general review concerning osteoarthritis (OA) and its relevance to the mineral and cartilage metalloproteinases.
2.2.1 An underpinning general review of bone and cartilage biology

Bones

Bone as a part of the skeletal system is a dynamic structure of vascularity and living tissue that transforms throughout life. It is a form of connective tissue, consisting of cells embedded in collagenous matrix and minerals [83, 84]; its structure and function concern elements completely interacting with each other. In addition to its strong mechanical properties which are protective of soft organs such as brain, heart, liver and eyes, it also provides structural support and movement and maintenance of mineral homeostasis.

As a dynamic material, bone is also a connective tissue / osseous material, having a central marrow cavity containing the bone marrow that produces blood cells. The osseous tissue is made of water, organic materials such as protein, inorganic minerals (predominantly calcium, phosphorus and magnesium) and trace amounts of iron, sodium, potassium, chlorine and fluorine). The organic part in the bone is approximately 95 per cent by weight collagen fibers, and the remaining fraction is cementing substances in the form of gels surrounding the collagen. The organic part represents approximately a third of bone weight, whereas water constitutes about a quarter of the bone weight [85]. The proportions of these components vary with age, health, diet, and tissue location [86]. The organic components provide bone with resilience and flexibility, whereas the inorganic components give the bone its hardness [84].

As for the structural and the functional properties of bone, there are three vital mechanisms: bone cells, bone mineralization and bone matrix of bone substance that are essential. Bone cells are important for both bone modelling and remodelling, providing storage of extracellular calcium and maximizing bone strength. Bone cells also provide the extracellular calcium and phosphate for bone mineralization required for the skeletal matrix, highly essential for bone muscles [87].

Bone matrix

Bone is a dynamic connective tissue composed of cells and matrix [88], the latter is the major constituent of bone, surrounding the cells. Bone matrix is the intercellular substance of bone tissue, consisting of collagen fibres and ground substance. The collagen fibres are mainly type I collagen forming the main shape of bone, and small amounts of non-collagen proteins. The ground substance on the other hand, is an amorphous material rich in proteoglycans with chondroitin sulphate side-chains [88, 89]. Bone matrix has organic and inorganic parts.
Organic components

The organic components of bone matrix are nearly 90 per cent by weight collagen fibers that are almost all type I collagen and 5 per cent by weight cement substance [90]. Collagen fibrils are huge collections similar to that of cotton or silk. The fibers are cross-linked which gives rise to their high tensile strength required for bone flexibility [84] and low solubility [85]. The cementing substances exist in the form of gels. The organic matrix of bone also contains sulphated glycosaminogycans, known as chodroitin sulphate and keratin sulphate forming small proteoglycan molecules [86].

Inorganic components

The inorganic components of the bone matrix are minerals, mainly calcium, hydroxytions and phosphate, but which might also contain small amounts of cationic strontium and magnesium and bicarbonate and fluoride. Calcium exists in the form of small needle-shaped crystals (hydroxyapatite \(\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2\)), parallel to the collagen fibres. In bone apatite calcium might be replaced by other divalent ions such as strontium, barium and lead and the monovalent hydroxyls may also be replaced by fluoride, even though in some states the ionic distance may slightly change. Thus said, the apatite character remains unchanged [84, 85].

The amorphous constituents in the inorganic part are calcium phosphate, citrates, magnesium, bicarbonate, potassium and sodium. The hydroxyapatite crystals are arranged along the organic collagen fibres (type I), and sited in the spaces of the collagen. The dimensions of hydroxyapatite crystal is about 40 nm in length by 25 nm in width and between 1.5 to 3 nm in thickness, parallel to the long axis, and its outer surface is surrounded by an amorphous ground layer similar to crystals of common salt. As bone contains both amorphous and crystalline calcium phosphates, through aging the proportion of the crystalline hydroxyapatite increases whereas the amorphous calcium phosphate decreases [91].

Various solvents are used in the removal of the inorganic components of bones that consequently make them flexible.

Bone cells

Bone is composed of different types of cells constituting the bone [87]:

**Osteoclasts** are multinucleated cells present only in bone [92, 93], arising from the fusion of cells called monocytes in the hemopoietic portion of the bone marrow. These are primarily responsible for the degradation of mineralized bone during bone development, homeostasis and repair [94-97].
Osteoblasts are mononuclear cells that produce osteoids, the organic portion of the bone matrix; located near / at the bone surface covering roughly one per cent of it, these cells are responsible for the synthesis of organic components of the bone matrix. When active, they show high alkaline phosphatase activity [98, 99]. For skeletal health, balance between the number of osteoclast and osteoblast cells is required.

Lining cells when the activity of osteoblasts declines, they flatten in shape and become elongated cells that cover the bone surfaces [100]. These cells are named bone lining cells. They play a major role in the coupling of bone formation and resorption, as they prepare the bone surface by digesting normalized collagen.

Osteocytes are the most abundant bone cells, embedded deep within the mineralized bone matrix in lacuna. They are mature and non-dividing bone cells derived from osteoblasts [101], although they are smaller and less active than osteoblasts. They are essential to the maintenance and routine turnover of the matrix and represent the final stage of maturation of the bone cell lineage [88, 102]. Osteocytes may control mineral exchange between bone and plasma [101].

Bone structure

Histologically, at the macroscopic level the mature human skeleton appears as two distinctive structures. The first type, being very dense on the outside surface, is known as "compact" or "cortical" bone. This cortical bone comprises 80 per cent of the skeleton and is largely to be found in the shafts of long bones [103]. The second type is the "trabecular" bone, also known as cancellous or spongy bone. The spongy bone is the main inner component of bones in the axial coating of the marrow cavity (Fig.2-13) [104].

Figure 2-13: Schematic drawing of the cortical and trabecular bone[105].
Cortical and trabecular bones have the same matrix composition and structure, but differ in density and porosity; cortical bone is 10 per cent less porous than trabecular bone but is more dense [106]. Moreover, trabecular bone has much higher surface area than cortical bone and therefore it is metabolically more active and responsive to pathological effects [103], while the cortical bone functions as a mechanical support [102].

At the microscopic level, bone consists of two types viz lamellar and woven bone. It is well known that woven bone is to be found in fetal and in newborns of less than a month; after that the lamellar bone replaces it.

According to their shape, bones vary from long to wide. Long bones are those that are longer than they are wide. Long bones include the femurs, tibias, and fibulas of the legs, the humeri, radii, and ulnas of the arms, and the phalanges of the fingers and toes. A long bone has a shaft and two ends. The long bone (Fig.2-14) consists of the diaphysis, epiphysis, metaphysis and the articular cartilage. The diaphysis is the shaft of the long bone; it is a hard hollow tube made of hard compact bone, but lightweight enough to allow for easy movement while the epiphysis are the ends of the long bone covered with the cartilage, consisting of a thin layer of compact bone surrounding spongy bone. The epiphyseal plates separate the diaphysis from each epiphysis and allow for extra growth in the bone. The metaphysis is the region that joins diaphysis with epiphysis; it is an area of transition between the epiphyseal plate and the diaphysis; columns of spongy bones are usually located there [107, 108].

Bone is a highly vascular structure, supplied by several blood vessels. Long bones for example receive blood from nutrient, metaphyseal, periosteal and epiphyseal arteries [88].

Figure 2-14: Diagram of a Long Bone (Left Femur) [109].
Bone marrow

Bone marrow is a soft fatty tissue located in the centre of the bone, containing a combination of hematopoietic, vascular, stromal and mesenchymal cells competent to produce skeletal repair and regeneration [110]. Bone marrow cells are discriminated into osteoclasts and osteoblasts and they are involved in degradation of mineralized bone during bone growth and fracture healing [97, 110, 111].

Bone marrow is classified into red marrow and yellow marrow depending on the predominance of vascular (red) or fatty (yellow marrow)[112]. Red marrow in human is found in ribs, heads of long bones, pelvic bones, sternum, and cranium. The production of red blood cells, platelets and most of white blood cells take place in red marrow.

Yellow marrow takes its name from the huge number of fat cells inside it. The yellow marrow is found in the empty core of long bones. Most of red marrow is replaced by yellow marrow with aging [112]. In critical cases of blood loss, the body exchanges the yellow marrow to red marrow to increase the production of blood cells.

Bone modelling and remodelling

Bone is a dynamic tissue that constantly undergoes modelling and remodelling processes [113]. They are involved in the morphogenesis of biological tissues. Modelling/growth in bone simply implies changes in relaxed lengths, while bone remodelling /turnover implies changes in mechanical properties [114].

Bone is usually undergoing constant rebuilding; approximately 30 per cent of bone mass is annually remodelled and this normal process occurs to ensure that healthy bone is growing and maintaining normal bone structure [90, 115, 116].

Bone remodelling / bone replacement is a complex cyclical coupled process. It starts with removal of the old bone (resorption) by osteoclasts followed by the replacement with newly formed ones (Fig.2-15). This process starts with recruitment of osteoclasts in which an ill-defined margin expands under the osteoclasts covering the cavities under the cell. Simultaneously, the osteoclasts produce hydrogen ions, proteolytic enzymes and lactate, that cause degradation to the bone matrix and allow calcium and bone mineral components to be lost to the system. After the osteoclasts have excavated a lacuna (in other words a resorption cavity), osteoblasts or bone formation cells differentiate from connective tissue precursors and start to fill osteoid (protein matrix) into the lacuna. The osteoid is subsequently mineralized to form new bone. The new bone is formed in situ because the load-bearing requirement is unchanged; this process normally occurs because the old bone cannot carry out its function (mechanical in cortical bone and support in trabecular bone) [90].
The process of bone remodelling usually starts with a positive balance between the osteoblast and osteoclast cells during maturation and a negative balance with aging [117].

A balance between the amounts of osteoclasts and osteoblasts is highly essential in the process of bone remodelling because the uncoupling (formation vs. resorption) requires a balanced remodelling to avoid excessive bone losses/gains during this process [119].

2.2.2 Joints

A joint is formed where two or more bones meet. They are simply known as the junctions between bones. They are constructed to allow movement and provide mechanical support. Based on their structure in which how they connect to each other, they can be classified as [120]:

- Fibrous joint - joined by fibrous connective tissue.
- Cartilaginous joint - joined by cartilage.
- Synovial joint - not directly joined.

Joints can be also classified functionally; i.e. by the degree of mobility they allow. There is three functional classifications of joints:

- Synarthrosis - permits little or no mobility such as the skull. Most synarthrosis joints are fibrous joints.
- Amphiarthrosis- allows slight mobility like vertebrae. The majority of amphiarthrosis joints are cartilaginous joints.
- Diarthrosis- permits a variety of movements for example, shoulder, hip and knee. All diarthrosis joints are synovial joints [121].
Synovial joint

The synovial joint or joint capsule is one of the most permanent joints in the skeleton (Fig. 2-16). In health, this highly specialized structure provides frictionless recurring, painfree movement [22]. The synovial joints are lubricated by variable amounts of a colourless/yellow pale viscous fluid named synovial fluid, allowing the hyaline glassy cartilage (otherwise known as articular cartilage) covering the articulating bones within the joint capsule to move freely. The assembly is responsible for the exclusive biomechanical properties of the joint. Bones are normally held in position by soft tissue structures that cover synovial fluid [121, 122]. Surrounding the joints is the articular capsule, ligaments, tendons and muscles, and these protect and stabilize the joint [22].

The synovial joint consists of two layers, a fibrous protein layer and the synovial membrane (i.e. the synovial lining cell layer) [22, 121, 123]. The fibrous layer plays a part in the mechanical stability of the joint and determines the flexibility of the possible movement while the synovial membrane provides major functions as follows: connects the bones and lines the interal cavity of the joint as one with the articular cartilage, regeneration of protein and hyaluronate of synovial fluid, regeneration of joint capsule, phagocytosis and removes wastes and filters nutrients out of and into the joint cavity [22, 121].

The cells in the synovial membrane are type A synoviocytes (or M cells), the majority consisting of macrophages (active phagocytic); the second type is type B synoviocytes (or F cells) that resemble fibroblasts and secrete hyaluronic acid [121, 124, 125]. These molecules offer great viscosity to the synovial fluid. Synovial fluid is a viscous clear amber coloured liquid that contains protein and nutrients derived from blood plasma and lymph produced in
small amounts into the joints by synovial membrane. It has two main functions: the participation in the nutrition of cartilage and lubrication of the synovial cavity [126].

Three types of sensory nerve innervations are found in the synovial membrane, bone marrow, muscles and ligaments. These nerves are connected with movements and pain; the pain may occur when abnormal stress is applied on the joint capsule; the responsible component for this pain is C fibres which are only found in the synovial membrane and nearby blood vessels [127]. The synovial membrane with its metabolically highly active surface cells (synoviocytes) plays a major role in removing metabolites from the synovial space, thus the metabolic activity of the synovial membrane is low, and it may increase in pathologic situations because of an increase of lining cells [22].

If the synovial membrane is affected by damage /disease the consequence is: inflammation with the motivation of the C fibres, the type A cells, plasma, leukocytes and lymph arising moving to the joint cavity, resulting in joint pain and stiffness because of the amount of synovial fluid having increased and the pressure inside the joint cavity also having increased [127]. Besides the inflammation, the enzymes also speed up the erosion of the articular cartilage. In addition, other factors may change the synovial content such as blood plasma, leukocytes and lymph when they enter to the joint during cartilage injury.

**Cartilage**

Cartilage is an elastic, flexible, semi transparent connective tissue surface of the bones composed of a highly charged part known as the extracellular matrix (ECM) [128] and cells named chondrocytes embedded into the ECM, and collagenous fibers and/or elastic fibers [121]. Cartilage has different functions in its covering of the joints, allowing bones to slip over each other, reducing the friction at joints and acting as a shock absorber [129]. Cartilage can be hyaline cartilage, elastic cartilage and fibro cartilage, the most common type being hyaline (glassy-like) cartilage [130-132].

**Articular cartilage**

The articular cartilage is the thin layer of smooth hyaline cartilage. It is a uniquely designed biomaterial [22] and is to be found covering the ends of the bones in the diarthrodial joints, cartilaginous parts of trachea (windpipe), bronchi and nose [122, 133]. Articular cartilage is also a connective tissue, its specific structure being composed of chondrocytes and ECM [123, 134]. By volume chondrocytes represent about 20 per cent of the total cartilage volume in the human adult [129]; situated in small cavities named lacunae, their function are to produce and maintain the ECM [109, 123]. The ECM is composed of tissue fluid and macromolecules; tissue fluid is composed of water (~80 per cent) , gases, dissolved ions, proteins, and metabolites, while the macromolecules consist of collagen, proteoglycans, and a
very heterogeneous group of non-collagenous non-proteoglycaneous proteins [22]. Extracellular matrix provides smoothness to the subcondral bone which allows movement of the joint without friction, while the collagens and proteoglycans provide stability and structure to the cartilage.

Different types of collagen fibrils are present in the articular cartilage, mainly collagen type II, type VI, type X, type XI and type IX [135]. Collagen type II is the most dominant form, along with the other major cartilage collagens forming a meshwork of high tensile-strength fibrils. It consists of a triple helix of hydrophilic chains. Besides the cartilage, it is also found in the vitreous humour of the eye, inner ear and annulus fibrosus of the intervertebral disks; fibrils are three-dimensional lattice networks and contain non-collagenous protein components besides the collagen [135, 136]. Collagen type XI is located in the centre of the collagen type II fibrils and is thought to be associated with fibril initiation and in limiting fibril diameter [136]. Collagen type IX is situated cyclically in an antiparallel direction along the surface of collagen type II and has been suggested to be responsible for the cross linking in the collagen network with itself [137]. While both types XI and IX collagens are present in small amounts they are nevertheless essential for cartilage stability [137]. In addition, collagen type X is expressed in the calcified zone by chondrocytes. While other types of collagens such as collagens III, XII, and XVI have also been found in articular cartilage, they are not typically classified as cartilage as they even appear in the noncartilaginous tissue [138]. These various types of collagen create a network that provides essential physical properties to the cartilage matrix, especially tensile strength [22].

The other major component of cartilage is proteoglycan and the main composition of this is the macromolecule aggrecan (providing for water to be bound to the proteoglycan). Aggrecan is composed of a protein core to which many chondroitin and keratin sulphate chains are joined. As inferred, aggrecan is a highly hydrophilic structure, its resultant low viscosity offering the compressive stiffness of the cartilage [137, 139]. In aging, the number of chondrocytes progressively decrease and consequently the production of the extracellular matrix decreases [22].

**Zones differentiation of articular cartilage**

Articular cartilage has four distinctive zones based on the organization of the chondrocytes, orientation of the collagen fibres (Fig.2-17) and distribution of proteoglycan. These zones have been characterised by using electron microscopy, polarised light microscopy, differential interference contrast light microscopy and small angle x-ray diffraction [140-144]. The zones (from superficial to deep) are as follows:
a. **Superficial Tangential Zone**

In this zone the collagen fibrils are oriented parallel to the surface of articular cartilage giving it the ability to resist shear stresses [145]. The chondrocytes are relatively few in number and are in the form of flattened discoids. This zone is about 50 μm thick and has a high water content and a low concentration of proteoglycan [146].

b. **Intermediate/Transitional Zone**

This zone is the transition layer representing a buffer between the shearing forces experienced in the superficial zone and the downward forces experienced in the deeper zone. It is also metabolically active. In this zone, the chondrocytes are larger than in the superficial zone, the proteoglycan richer in amount and the collagen fibrils thicker, with random orientation [120, 147].

c. **Deep / Radial Zone**

The collagen fibrils are increased in number, being also oriented, this time vertically to the surface and the tidemark (see below). This zone is one of high proteoglycan concentration and the cells are organized in groups as clusters. The high concentration of proteoglycan allows it to resist compressive loads [148].

d. **Calcified Zone**

The deepest zone in the cartilage, the collagen fibrils are radially oriented with little presence of proteoglycan. At the osteochondral junction, this zone is separated from the uncalcified zone by a distinct line, known as the tidemark that is a narrow seam of gradually increasing mineral content between the two zones. This calcified zone acts as yet another buffer, this time between the more complicit superficial layers and the rigid bone, avoiding a large pressure change at a boundary between cartilage and bones [120, 147].

![Figure 2-17: Structure of articular cartilage. A, schematic diagram of the cellular organization in the zones of articular cartilage; B, diagram of the collagen fiber architecture [149].](image-url)
The mechanical behaviour of AC bears resemblance to that of sponge. Throughout rest, for instance sitting or lying down, the osmotic swelling pressure produced by the proteoglycan aggregates fills the tissue with water equal to its maximum capacity. The swelling pressure is entirely controlled by the flexible collagen meshwork. Under weight, for example walking or standing up, the weight of the body compresses the cartilage, squeezing water out, the osmotic pressure producing a force equal to the compressive force to the whole body weight. As the weight is taken off the cartilage returns to its full amount of liquid [150].

**The bone-cartilage interface**

In summary, and as already mentioned, the cartilage structure consists of four zones, from the superficial zone to the calcified/deepest zone. The calcified zone is separated from the uncalcified zone by a distinct line viz "tidemark", and is attached to the subchondral bone by a "cement line" this area between the tidemark and cement line being known as the bone cartilage interface. While the structure and composition of this interface has yet to be fully described, it is nevertheless understood to undertake an important role as the mechanical buffer between cartilage and bone [151]. It is a zone of active tissue remodelling and is believed to be a key site in the development of joint disease such as OA [16].

**Subchondral bone**

In synovial joints, the subchondral bone is a layer of dense bone underlying the articular cartilage that separates trabecular (cancellous) bone from cartilage and supports the joint surface. It forms the major supporting media for the cartilage and transmits loads from the cartilage into the underlying cancellous bone [152]. Subchondral bone plays an important role in osteochondral diseases, the precise details of which still requires elucidation. In OA the sclerosis of the subchondral bone is a clinical feature of OA [153].

**Joint disease**

Joint diseases such as osteoarthritis, osteoporosis and rheumatoid arthritis affect huge numbers of patients around the world, causing pain and disability. The increasing life expectancy of people will greatly affect both individuals and society. Even though extensive study on disease etiology and development has been made to-date there remains no really effective medical treatment for these diseases. There are instead many other treatment modalities presented for managing of joint diseases including psychotherapy, education, analgesics, weight reduction, physical therapy, occupational therapy, intra-articular corticosteroid injections and others.
Osteoarthritis

Osteoarthritis (OA) is the most prevalent joint disease in humans, also known as degenerative joint disease and otherwise as "wear and tear", increasingly becoming one of the major health problems worldwide. It is a particularly debilitating disease, and more so among the elderly, affecting approximately 70% of 70 year olds and a total of 8.5 million people in the UK [128]. OA is not only a common problem among the elderly population, also becoming more widespread among younger people in the third or fourth decades of life, being most often caused by genetic predisposition, traumatic injury to the joint, subsequent to an infection of the joint or as a result of aging and the wear and tear associated with the stresses of daily life [154]. OA is characterized by the breakdown of the joint cartilage with the consequence that the articulating bones will perhaps come into contact, resulting in pain, stiffness and loss of movement [155]. In OA, progressive degradation of the articular cartilage is accompanied by abnormal subchondral trabecular bone remodelling and secondary inflammation of the synovial membranes [156-158].

OA usually affects the feet, hands, spine and those joints bearing the greatest weight, as in for instance, the knees and hips. However, theoretically every joint in the body can be affected [156].

Clinical features, diagnosis and classification of OA

Osteoarthritis is a complex disease for which different symptoms and signs may occur. Clinically it has been found that there is no swelling in the first phase of the disease, but as the disease develops limited and painful inflammation occurs [121], together with damage and deformity, with throbbing and swelling to the joints [159]. Thus diagnosis of osteoarthritis can usually be made clinically and then confirmed by radiography. Among the most prominent features of OA are included joint pain, limitation of movement, softness, crepitus, and variable degrees of intra-articular inflammation without systemic effect [156, 160]. Clinical diagnosis by radiography and other imaging techniques can provide good evidence of the progression of the disease; in particular, those changes that occur in the joints, such as joint space narrowing, subchondral bone sclerosis and subchondral cyst formation, these being the main features of the disease. However, in early OA, X-rays may not be able to detect the disease [79, 118, 140, 141]. In addition changes of subchondral bone are often seen in radiographs of patients with recognized OA and increasingly these are being viewed as being a vital cause of OA rather than the sequelae of cartilage damage [161].
**Etiopathogenesis of osteoarthritis**

Although the most common joint disease, OA is not easy to be diagnosed specifically, in large part because of its slow development and progression [84]. OA is no longer regarded as a simple effect of aging and cartilage degradation [162], however, the pathogenesis of OA includes different factors such as mechanical, biochemical, molecular and genetic factors that have an important role in the imbalance among cartilage matrix degradation and synthesis [84, 160]. These factors cause changes of both cells and the cartilage matrix. In addition, the risk factors include a person’s age, ethnicity, sex and bone density [162, 163]. Clinical features are to be found in the pathophysiology, varying from joint to joint. As such, OA might not be a single disorder but a group of overlapping separate diseases [137, 164].

Osteoarthritis is usually seen as a disease of articular cartilage even though the changes that occur in the subchondral bone are very significant. In fact, in idiopathic OA, it remains unknown as to whether the first abnormality takes place in subchondral bone or articular cartilage [137].

What is known is that the chondrocytes are the main cells found in cartilage, and they are involved in maintaining the extracellular matrix. In OA the pathophysiology and joint damage are correlated with a breakdown of cartilage and a high degree of chondrocyte apoptosis. In particular, during the loss of extracellular matrix in OA, the chondrocytes become susceptible to apoptosis. In normal mature cartilage, the chondrocytes do not regenerate and the differentiation of articular chondrocytes is arrested before terminal differentiation. The apoptosis is a result of creation of apoptosis bodies usually taken away by phagocyte cells, therefore avoiding any reaction of an inflammatory nature that may occur. However, articular cartilage is non vascular tissue. It does not have any mononuclear phagocytes that clear the products of chondrocyte apoptosis [165]. Recently, enzymatic degradation has been recognized to be an essential feature of OA. The main sources of the enzymes that are responsible for cartilage breakdown are chondrocytes and synoviocytes [166, 167].

Cartilage matrix turnover is a balanced process of synthesis and degradation, and any failure in this balance, accepted as being involved in OA, is either because of increased catabolism or reduced formation. As an instance, matrix metalloproteinases, enzymes that catalyse collagen and proteoglycan degradation as well as gelatinase, collagenase, and stromelysin, are usually found at elevated concentrations in OA, and their synthesis by chondrocytes can be motivated by interleukin 1 (IL-1) [168]. Additionally, reduction in the severity of OA lesions can be achieved, at least in animal models, by obstructing these enzymes by chemically modified tetracyclines or doxycycline. Synthesis of cartilage components depends on a number of growth factors, including insulin-like growth-factor
and the transforming growth-factor β (TGF β). The first factor has been shown to decrease the development of osteoarthritis in animal models; however, in human beings the role of this factor is conflicting, increased, decreased, and normal concentrations having been described in patients with OA [137].

During OA pathology, synovial membrane undergoes a moderate inflammation, that might be generally closest to the OA lesion, indicating a relation between cartilage synovium inflammation and cartilage lesion [169]. Although it is not clear whether it proceeds or occurs subsequently to cartilage damage, subchondral bone remodelling is a great feature in the pathophysiology of OA. In the early stage of the disease there will be increase in the thickness of the subchondral bone plate as well as underlying trabeculae. Therefore, new bone formation is found at several foci in early to middle stage patients [170]. With subchondral bone thickness increase, increase is also found in shear stress while the shock absorbing capacity will decrease in the articular cartilage leading to cartilage fibrillations [171]. These fibrillations are the first observation in the degradation at the articular cartilage [172]. The fibrillations occur when collagen type II damage is restricted to pericellular and superficial sites [173]. The superficial fibrillations involve cracks roughly parallel to the articular surface and associated with the losing of collagen type II from the cartilage fibrils [172]. Besides the fibrillation there is a limited loss of aggrecan [174] and small proteoglycans, including decorin and biglycan [175]. As a result of the increase in water content of the articular surface, clonal formation of chondrocytes is often observed [176]. In advanced OA, the bone formation process is increased and characterized by periarticular osteophyte formation and sclerosis of subcondral bone. Cleavage of the collagen network results in progressive loss of cartilage, causing deep fibrillation and vertical clefts.

**Bone minerals**

Bone as a dynamic tissue carries out chemical, biological, and mechanical functions. It also acts as a metabolic reservoir for important minerals such as calcium (Ca), phosphorus (P), zinc (Zn), strontium (Sr), copper (Cu) and others. Bone consists of minerals, mainly hydroxyapatite \(\text{Ca}_{10} (\text{PO}_4)_6 (\text{OH}_2)\) deposited in an organic matrix. Bone minerals are deposited in great numbers of small crystals, providing large surface areas for bone. For instance one gram of bone mineral is approximated to provide for an area of 100 m²; this large surface area permits for fast exchange of certain ions, such as Ca and P, between interstitial fluid and bone in order to maintain circulating levels. Seventy per cent of bone tissue consists of minerals that give the bone tissue rigidity and the great amount of it is in the form of Ca and P, accounting for some 99 per cent of the minerals [102]; some 99 per cent of Ca and 85 per cent of P in the body are contained in the bones and teeth. For example, in hydroxyapatite
the proportion of Ca and P is 2.15:1 g/g [101]. In spite of this, the crystallization of bone salt takes place in multiple steps, and in every step the ratio between Ca: P is lower than the hydroxyapatite, usually the ratio in young bone being in the range 1.7:1 – 2.14:1 g/g. Moreover the hydroxyapatite contains cations such as Mg and Zn and anions such as carbonate and citrate. Both Mg and Zn are present in higher concentrations in the skeleton than in other tissues in the body.

In general, elements in the body can be classified into four categories: major elements: such as S, H, C, O, N, and Mg; minor elements: like Ca, Cl, P, K and Na; trace elements: such as F, Si, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Se, Mo, Sn and I; and toxic elements: like Be, Hg, Pb, U and transuranic elements [177]. In regard to elements essential to human health, particular attention has been placed upon measuring the trace elements. In bones, trace elements are understood to be essential for the growth and development of the skeletal system. While they are minor structural components of bone, they nevertheless play a vital role in bone metabolism and bone turnover.

In the following, a brief description is provided concerning some of the important minor and trace elements which play an essential role in human health in general:

**Calcium**

Calcium (Ca) is the fifth most prevalent, essential mineral in the human body, ideally comprising some 1.5 -2 per cent of an adult’s total body weight (depending on gender, race, size of the body). More than 99 per cent of Ca is found in the skeleton, and deposited as the hydroxyapatite [101]. Ca is an important element as it is responsible for a great many body functions, every cell and organ in the body depending on it for proper functioning. In bones, for example, it represents some 20 per cent of the total mass but it is alone not sufficient for proper bone strength and health. In addition to contributing to skeletal health, Ca has a role in digestion, blood clotting, and wound healing, Ca being an activator or stabilizer of enzymes. The amounts that are needed to achieve these functions are small and usually do not lead to signs of deficiency, there being significant reserves in bones.

**Phosphorus**

Phosphorus (P) is an inorganic element and the second most prevalent minor element in the human body next to calcium, making up about 1 percent of our total body weight. About 85 per cent of P in the body is contained in the bones as hydroxyapatite while the remaining amount is distributed in soft tissues and blood [178]. In the blood, P is a fraction of the monobasic-dibasic buffer system. P has an essential role in filtering out waste in the kidneys. It also aids in reducing muscle pain. In addition, it has another vital role in the
growth, maintenance, and repair of all tissues and cells, and for the production of the genetic building blocks, DNA and RNA. Furthermore, phosphoproteins are contained in many enzyme systems [178].

Deficiencies in P in the body can be caused by an excess intake of aluminium in such as certain antacids, aluminium binding to P. The main signs of P deficiency are bone pain, loss of appetite, bone weakness, stiffness in joints, and irregular breathing. In children P deficiency can be responsible for poor bone and tooth development and decreased growth. Conversely, an excess of P can be even more problematic and indeed is more common. In adults for example, having a low calcium-to-P ratio is most likely to generate problems, and bone resorption is often brought on by a low calcium intake. In addition to this problem there are other problems including skin disease, tooth decay, and arthritis [178].

**Zinc**

Zinc (Zn) is an abundant element present in all parts of the body located in organs, fluids, tissues, bones and cells, it is an essential element required by the body in small amounts. It has a structural and fundamental role in a large number of macromolecules and is essential for over 300 enzymatic reactions. Zn ions contribute in all features of intermediary metabolism, transmission, and regulation of the expression of genetic information, synthesis, storage, and action of peptide hormones and structural maintenance of chromatin and biomembranes [179]. Body tissues high in Zn include bone, liver, pancreas, prostate, and kidney [180]. Bones are also relatively rich in Zn, containing approximately 30 per cent of the total body Zn which is between 2 and 3 grams [181, 182], the Zn remaining for relatively long periods of time in bone. Using different investigative techniques, the mean Zn content in bone has been quantified to be 110-300 µg/kg.

Zn as an essential trace element, has been verified to have a large variety of roles in humans and animal systems, this element being also required by enzymes that have specific functions in bone metabolism [183, 184]. As examples, alkaline phosphatase, which is essential for bone calcification, and collagenase, which is essential for bone resorption and remodelling, are Zn metallo-enzymes [185]. There is much evidence from human and animal studies that a decrease in tissue Zn concentrations may lead to a reduction in the activity of a large series of enzymes that are dependent on this element for regular function and may lead to different problems such as bone growth retardation and improper bone formation and bone fractures [164-166]. Zn can excite the protein synthesis in osteoblastic cells and can also inhibit the formation of osteoblastic cells from bone marrow cells [186]. Thus Zn may play a vital role in the protection of bone, by motivating bone formation and slowing down bone resorption [185]. In addition to its role in this process, Zn has a number of other functions; it is a required mineral for production of steroid hormones essential for growth, maturation,
development, reproduction and tissue repair and it is also an anti-aging and degenerative disease nutrient that stabilizes cell membranes, defending them against oxidation [181]. Moreover, Zn protects against ultraviolet radiation and is suggested to decrease the relative risk of cancer and cardiovascular disease and enhances wound healing [187].

In bones, it is well known that Zn plays an extremely important physiological role in bone growth by exciting protein synthesis [188]. In animals studies it is reported that deficiency of zinc possibly will lead to damage in bone tissue, abnormalities in bone growth, bone formation, and mineralization [189]. In human studies, Zn intake has been reported to be coupled with low bone mass in women [190].

**Metalloproteinases**

Matrix metalloproteinases (MMPs) are zinc-dependent endoproteinases that are involved in many physiological processes such as bone development, vascular remodeling, and tissue homeostasis [191]. In articular cartilage, a group of enzymes including alkaline phosphatase play a pivotal role in the growth and degeneration of the collagenous bone-cartilage interface of articulating joints. However, their roles rely on the presence of some elements such as Ca, P, and Zn, known as co-factors.

**Alkaline phosphatase**

Alkaline phosphatase is a substrate non-specific phosphomonoesterase and zinc-bearing metalloenzyme. It is to be found in almost all living forms, from bacteria to plants to humans; almost all cells have alkaline phosphatase in their plasma membranes. Its activity can be reversibly reduced and restored by the taking away and adding of zinc ions at the active site [192].

This enzyme is well known in medicine in many diagnostic applications, but the physiological role of this enzyme has not been fully clarified [192]. It is associated with calcification, through cells that calcify their extracellular matrix, including tooth forming cells (odontoblasts) and cartilage cells at the end of long bones (growth plate chondrocytes), each likely to have higher levels of this enzyme [193]. In addition, the activity of this enzyme increases when osteoblasts produce the extracellular matrix. In such a case this enzyme could be used as an early marker of osteoblast differentiation as the activity is greatest just before mineralization begins, alkaline phosphatase causing increase in the local level of inorganic phosphate, one of the components of apatite, the mineral part of bone. More evident is that levels of free P ions are important and not just in calcification as such. Many investigators have incorrectly supposed that a positive reaction shows the presence of actively mineralizing osteoblasts, although they may really be observing evidence of high levels of phosphatase activity caused by alkaline phosphates [193].
Joint lubricant

In human joints, most of the applied load is supported by synovial fluid, pressurized owing to soft lubrication while a mixed lubrication region also inevitably exists. Various types of complementary lubrication such as phospholipids also take an active part in the lubrication process and protect the outer boundary of the articular cartilage [194]. As has been demonstrated [195] the synovial fluid provides limited lubricating capacity to artificial materials, therefore, there is an apparent need for complementary process as in phospholipids control of friction and wear. This boundary lubrication has been shown to be important in maintaining low friction between opposing cartilage surfaces when contact occurs [196, 197]. In view of the fact that joint loss lubricant is one of the main feature of OA, a study by Hills et al. [198] showed a deficiency in phospholipids at the outer boundary of articular cartilage affected by OA. In the final stage of this work we have concentrated on the outer edge of the diseased articular cartilage seeking to use PIGE to detect the accumulation of some elements such as Na and F present in phospholipids. These could be exploited as markers in the detection of OA.

2.2.2 Applications of NAT’s in biology and medicine

NAT’s are increasingly finding a wide range of applications in the biological and medical fields. Their main applications are the determination of some essential elements in many clinical and pathological dysfunctions arising from an imbalance of essential element such as Ca, P, K, S and Zn. In this respect the various techniques are also expected to provide information on trace element distributions in bone and cartilage which could enhance our understanding of the nature and role of these elements in these biological systems.

This section intends to review the applications of the various NAT’s in biology and medicine. It is not the purpose to give an extensive overview of all published material but rather to make clear the potential of NAT’s in this area of research and to indicate developments in these applications. Specifically, more emphasis is given to the application of SR-XRF, SR-SAXS, PIXE, RBS and PIGE in human biology and medicine. In particular, in the present study it has been demonstrated that the combined use of all of these techniques in the imaging and analysis of the cartilage structure and elemental composition of bone and cartilage can be of great importance when compared with other conventional analytical techniques. μ-PIXE and SR-μXRF are generally acknowledged to be amongst the most powerful analytical techniques available for quantifying elements at the subcellular level. As an instance, the use of PIXE in conjunction with RBS and PIGE has been reported by several authors to have contributed to the identification of pathological features of OA and Alzheimer’s disease [179-181].
Various healthy and diseased tissues have now been popularly examined in studies by NAT's. As an instance extensive analyses of bone, breast, kidney and brain tissues have been investigated by SR and IBA facilities. Investigations by Geraki et al. [182-184] on breast tissues by SR have revealed increased concentrations of K, Fe and Zn in tumour breast tissue, being also higher in healthy tissues close to tumours as compared to normal tissues. Their results verify that higher levels of trace elements are valuable indicators in the identification of disorders in breast tissues. Other investigations [199, 200] have also reported enhanced concentrations of trace element in diseased breast tissues as compared to benign and normal tissues.

Another biological sample that has been in the subject of such studies is lung tissue, investigated through a combination PIXE and Total XRF (TXRF) analysis. Thus for instance, Kubala-Kukus et al.[199, 201] detected increased levels of Ti, Cr, Mn in cancerous lungs. SR-XRF has been also employed to detect the distribution of some trace elements in brain tissues affected by Alzheimer's disease (AD); for example a study by Miller et al. [202] reported that there were high accumulations of Cu, Zn and Fe in AD brain tissue. Other groups observed high concentration of Pb in calcified AD tissues [189, 190]. Danscher et al. [203] employed PIXE on 16 AD patients and 15 control subjects, revealing high accumulation of Zn in some tissues while other showed low accumulations; they were able to explain some of this range to be simply a result of inter subject variations.

Extensive analyses of bone samples have been carried out by Zoeger et al. [9]. Earlier this group examined the accumulation of Pb in the tidemark of normal human articular cartilage from patients with no history of work related exposure to Pb. While their work is novel the mechanisms of the accumulation remains unknown. In another study by this group [11], the combined use was made of confocal μXRF and backscattered electron (BE) for elemental imaging on diseased femoral heads at the bone-cartilage interface; they found enhanced concentrations of some trace elements in that region, also a double tidemark (which we suggest to be a reflection of fibrillation) and a thickening of sunchondral bone which is believed to be a sign of increased turnover in the affected joints. More recently, they have employed SR-μXRF to determine the distribution of Pb, Ca, Zn and Sr at the bone-cartilage interface in human femoral heads and patellae [204].

In another study by Gomez et al. [3], slices of normal stained bovine bones were examined employing μ-PIXE to identify Zn and Ca. Both the PIXE and histochemical analysis showed Zn to be deposited at the surfaces of various structures in the bone tissue and in compact mineralized zones

Zizak and his coworkers [205] have employed SR-SAXS and back scattered electron imaging to study the nanostructure of human bones of different age, at the interface between bone and mineralized cartilage and also measured the mineralized density with polarized
light. They observed higher concentration of Ca for all samples at the cartilage interface and the highest of this at the growth cartilage. In addition for tissue apparently unaffected by OA, they established the orientation of collagen to be perpendicular to the interface at the bone-cartilage interface and parallel at the trabecular bone.

Studies by Zaichick and Tzaphlidou [194-196] concerned determination of Ca, P and the Ca/P ratio; they investigated healthy human neck, femoral heads and rib bones. They also established range of values of the Ca/P ratio in diseased human ribs affected by osteoporosis [206].

Results reported by Bradley et al. [16], concerning Zn deposition at the bone-cartilage interface in equine articular cartilage, showed enhanced concentration of Zn at the bone-cartilage interface, the study also recommending employing most of the same techniques used by others in the micro-mapping detection and distribution of essential elements in human samples. It is in respect of micro-mapping that such study may reveal more information on OA in both human and equine samples than that found in existing literature. μSR-XRF analysis of bone-cartilage interface has been carried out by this same group [19]. It was found that the Ca and Zn each display a peak in elemental presence at the cartilage surface and also at the tidemark; conversely within the lesion there is an absence of surface elemental enhancement.

SAS has for long time been used for the characterization of inhomogeneous materials and in particular collagen fibres on the nanometre scale [72, 82, 207, 208]. In a study by Cedola et al. concerning orientation of mineral crystals by collagen fibres during in vivo bone engineering, they employed SR-SAXS to study the local structure of the interface between the deposited bone and the biomaterial. The group proposed an original methodology of semi-quantitative analysis to achieve microscopic images showing the spatial variation of different structural feature [208].

Studies by Fratzl and his co-workers [207, 209, 210] have concerned determination of collagen fibres orientation and arrangements in human bone using SAXS and Fourier-transform Infrared Microscopy (FT-IRM). They reported detailed information on human bone sections regarding their structure and orientation of collagen fibres in the trabecular bone.

Recently, the nanostructure of the neurocentral growth plate of pig bone have been shown using SAXS, atomic force microscopy (TFM) and scanning electron microscopy (SEM) by Bunger et al. [82]; they have reported gradual changes from cartilage to bone.
3 Materials and Methods

3.1 Materials

Human femoral heads and equine metacarpophalangeal joint sections were investigated in this study.

3.1.1 Bone Sections Preparation

3.1.1.1 Human femoral heads

Human femoral heads were obtained from subjects who had undergone total hip replacement procedures in response to degenerative joint disease. Fig.3-1 shows a schematic diagram of a human femoral head, illustrating the portion of bone that is removed during total hip replacement.

![Figure 3-1: Schematic diagram of human femoral head (the part within the dotted line is that excised during total hip replacement).](image)

The femoral heads were maintained at a licenced tissue bank at City University (London) in the Laboratory of Professor Farquharson and stored at -81°C. Using a water-cooled diamond saw (Isomet 1000 Precision saw) [211] various sections (0.8-1.5 mm thick) were cut perpendicular to the articular surface from the superior (weight bearing region) of the femoral head, as shown in (Fig.3-2). The diameter of these sections ranged between ~15-25 mm and their height was ~15-20mm. These sections were subsequently soaked in distilled water to remove any bone marrow and loose particulate matter.
In the preparation of thin sections (~200μm), the prepared sections were ground and polished using a pair of wet silicon carbide grit paper. One was placed under the section of the bone as shown in Fig.3-3 while the other paper was used to smooth the surface and remove saw cut marks. Both surfaces of the femoral heads were ground and polished using 800/1200 grit paper, taking some 15 minutes, followed by used of 2000 grade grit paper for 10 minutes, to obtain a useful section thickness (~200μm). The grinding procedure was carried out under continuous flow of water.

To keep these sections moist, they were placed in Petri dishes containing distilled water and stored at +5°C pending SRµ-XRF, µ-PIXE, RBS, PIGE and SAXS examinations.
3.1.1.2 Equine Metacarpophalangeal joint

Preparation of the equine sections was performed at the equine hospital located at Langford Farm, Exeter, by Dr Donald P Attenburrow.

Thin sections (~1mm thick) of equine articular cartilage were prepared by cutting perpendicularly to the articular surface of the metacarpus using a band saw as shown in Fig.3-4a, maintaining the bone cartilage area moist during the cutting process. To prepare thinner sections, the thicker sections were lapped gently on a rotating lapping machine (Fig.3-4b) equipped with fine silicon carbide paper under running water. A smooth slice (~0.125 μm) is shown in Fig. 3-4c.

![Figure 3-4: a) Slicing of equine metacarpus by band saw  b) Lapping of sections under running water  c) Equine metacarophalangel joint section [212].](image)

The sections were kept moist in Petri dishes containing low formalin concentration, sealed with moisture-resistant laboratory film and stored at +5°C pending μ-XRF examinations.
3.1.1.3 Decalcification

Decalcification of five sections (four human femoral heads and one equine articular cartilage) for structural investigation of the collagen fibres was carried out by soaking in 8.0 per cent formic acid /24 hrs, and their wet weights recorded immediately. The weights of the thin sections were recorded daily. When variations in weights of the thin sections were noticed, formic acid was replaced and the sections were left for another 24 hrs. To confirm the completion of the decalcification, the procedure was repeated daily until a stable weight of sections was achieved. To minimize undesirable experimental artefacts (structural/histochemical alterations) no stains or chemicals other than formalin were used during preparation of samples.

3.2 Methods

3.2.1 University of Surrey Ion Beam Centre Facilities

The Ion Beam Centre (IBC) at the University of Surrey [213] is a National Facility funded by the Engineering and Physical Sciences Research Council (EPSRC). It employs a 2 MeV Tandetron accelerator which was installed in 2002 to encourage research by varying interest communities in the UK in the applications of IBA [214]. The centre comprises several beamlines (millbeam, microbeam and nanobeam, in-vacuo and in-air [215, 216]) providing a variety of facilities for a range of analytical applications and materials [217]. A schematic diagram of the Surrey beam lines is shown in Fig.3-5 (all located in the Stephens Laboratory).

3.2.1.1 Microbeam line

In the current study, the in-vacuo microbeam line was employed in the combined PIXE and RBS experiments while the in-air microbeam was used for the combined PIGE and PIXE experiment.

The microbeam line is equipped with a magnetic quadrupole triplet lens and magnetic scanner unit from Oxford Micro beams Ltd. It is also able to produce a micron-scale beam spot size with sufficient current for realistic IBA. In addition the microbeam is able to scan over an area of 2 mm². The accelerator is a 2 MV tandetron, accelerator from High Voltage Engineering Europe (HVEE). It is capable of running H⁺ ions up to 4 MeV. A large beam of protons is produced by the accelerator and is subsequently focused into the beam line by a beam steerer while the shape of the beam is defined via object slits. Collimator slits are used to gain a straight beam and the scanning coils scan the beam over the sample and the magnetic lenses focus the beam onto the sample. A Si (Li) detector is usually used for PIXE
analysis while detection of the RBS signal is achieved by a thick silicon surface barrier detector and PIGE products are detected by a high pure germanium detector.

The collected signals are displayed using OMDAQ (Oxford Micobeams Data Acquisition System) software produced by Oxford Microbeams Ltd [218]. The events are recorded in binary form into List Mode Files (LMF). This software also enables computerization of analyses of scanning modes (scanning area, line scan and point analysis) and programmed control of the sample stage. It also allows rapid and economic quantitative analysis of large batches of complex samples.
Figure 3-5: A schematic diagram of the Stephens Laboratory [219].
Experimental conditions

μ- PIXE and RBS

Elemental mapping and quantification of element content of two diseased human femoral head sections was carried out at the in-vacuo microbeam line with incident proton energy 2.5 MeV. Two beam spot sizes (~ 4 μm x 5 μm and ~ 3 μm x 5μm) and two beam currents (~40 pA and ~70 pA) were applied. The experiment was carried out on two consecutive days (27-28/06/2007).

The PIXE detector was an e2v Si(Li) 80 mm² crystal 2.7 mm thick with an energy resolution of 130 eV and a solid angle of approximately 32 msr when mounted at a scattering angle of 45° to the beam. The detector has a 6 μm Be window and a 130 μm Be filter to reduce the intensity of low energy x-rays from major components of the sample. The particular crystal has a 5μm Si dead layer. Fig.3-6 shows the Si(Li) PIXE detector placed at 45° to the beam line.

The RBS detector was set at a scattering angle of 155° (above the beam, at 25° to the beam) producing 167 msr solid angle and an energy resolution of 17 keV.

Figure 3-6: In-vacuo Surrey micro beam line set-up.
μ-PIXE and RBS

The μ-PIXE and RBS experiment was carried out at the University of Surrey Ion Beam Centre, at the in-vacuo microbeam line on one diseased human femoral head section and another one normal section. The experiment was also carried out on two consecutive days (09-10/06/2008).

The beam spot size and beam current on the first day of investigation was ~ 4μm x 6μm and ~150pA respectively, while a beam spot size of ~ 5μm x 6μm and beam current ~200pA were obtained on the second day of the investigation.

μ-PIXE and PIGE

The μ-PIXE and PIGE experiment was carried out at the University of Surrey Ion Beam Centre, at the in-air microbeam line on two consecutive days (20-21/07/2009). The 3 MeV proton beam was focused to ~ 60 μm x 60μm spot size and a beam current of ~ 20 nA was obtained.

For the particular elements of interest, namely F and Na, the following reactions apply (together with the characteristic γ emission energies):

\[ ^{19}F(p,p'\gamma)^{19}F; \gamma \text{-} \text{rays of 110 and 197 keV [220].} \]
\[ ^{23}Na(p,p'\gamma)^{23}Na, ^{23}Na(p,\alpha\gamma)^{23}Na \text{ and } ^{23}Na(p,\alpha\gamma)^{20}\text{Ne; }\gamma \text{-} \text{rays of 440, 1634 and 1636 keV respectively [221].} \]
\[ ^{35}Cl(p,p'\gamma)^{35}Cl \text{ and } ^{37}Cl(p,\gamma)^{38}\text{Ar; }\gamma \text{-} \text{rays of 1220 and 1640 keV [222].} \]

The peaks for these typically are the result of nuclear excitation by inelastic scattering, with transitions to the ground state for the 1634 keV peak of Na which feeds transitions to the ground state. Since the 1636 keV line for Na overlaps with the 1640 keV line for Cl, therefore use was not made of this line. γ-rays were detected by a HPGe detector fixed at 90° relative to the incident beam. Fig.3-7 shows the apparatus set-up.
3.2.2 Swiss Light Source (SLS)

The Swiss Light Source (SLS) is a third-generation synchrotron facility located at the Paul Scherrer Institute (PSI) in Switzerland and was launched in October 2001, producing electromagnetic radiation of high brightness. It comprises of a 100 MeV Linac full energy booster synchrotron and a storage ring of 288 m circumference offering a beam emittance of 5 nm rad at 2.4 GeV [223]. The SLS synchrotron has been improved since it was first opened, with the addition of further beamlines and beam stations; in June 2009 the SLS had eighteen experimental stations (undulators and bending magnets) and seventeen operational beamlines, Fig.3-8 shows a schematic diagram of the SLS beam lines. Two undulator beamlines that have been used in this work provide a microfocus for Synchrotron X-ray Absorption Spectroscopy (SXAS) and fluorescence analysis in the energy ranges 0.2-5 keV and 5-18 keV. A full description of the SLS synchrotron is described by Boge and Joho et al. [223, 224]. For the coherent Small Angle X-ray Scattering (cSAXS) and Line for Ultimate Characterization by Imaging and Absorption (LUCIA) facilities, the SLS web site provides a detailed description for both beamlines [225-227].
3.2.2.1 c-SAXS

The c-SAXS, beamline produced first monochromatic light in May 2007. Between August to December 2007 it was utilized for preliminarily investigations until January 2008, subsequently being made available for normal user operation.

The primary detector at the c-SAXS beamline is the PILATUS 2M (the M indicating ‘millions’ of pixels). The PILATUS detectors (PIxel apparATUs for the SLS) [229], including the Pilatus 2M, Pilatus 6M and Pilatus 100 K, are a new category of x-ray detectors that have been developed at the PSI for SLS. They operate in the single-photon counting mode and are based on the new hybrid pixel technology. A PILATUS detector is generally composed of a silicon sensor that is a 2D collection of pn-diodes processed in high-resistivity silicon, connected to an array of readout channels [230]. The latter is also connected to its equivalent element by a microscopic indium ball. This form of assembly was developed at the PSI and is referred to as microbump-bonding.
The main advantage of this assembly is based on the small size of the pixel (172\(\times\)172 \(\mu\)m) and the main features include: no readout noise, superior signal-to-noise ratio, read-out time of 5 ms, a dynamic range of 20 bit, high detector quantum efficiency and the possibility to suppress fluorescence by an energy threshold that is set individually for each pixel. The collected signals are displayed using MATLAB software. Alternative analysis software, such as fit2d, IDL, or PRIMUS, are also available for data analysis [227].

3.2.2.2 LUCIA beamline

The beamline LUCIA at the SLS is a powerful and sensitive (0.8-8 keV) x-ray microprobe offering elemental mapping of a wide range of elements by \(\mu\)-XRF and chemical speciation by \(\mu\)-XAS [225]. This beam line offers combination investigations using these two techniques, \(\mu\)-XRF offering the elemental maps and \(\mu\)XAS offering chemical analysis, quantitative and local geometric structure around the absorbing atom being obtained. A schematic diagram of the beamline is shown in Fig.3-9.

A mono-element energy dispersive silicon drift diode (SDD) for \(\mu\)-XRF analysis is usually used, on which is mounted a special very thin window so that the fluorescence of elements down to carbon can be detected. The collected signals are displayed using MATLAB software.
3.2.2.3 Experimental conditions

**μ-XRF**

Mapping of the elemental content of the thin sections was carried out at the LUCIA beamline, using an incident x-ray energy of 4.06 keV. The cross-section of the beam was approximately $3 \times 3 \, \mu m^2$ at the position of the section. Each of the scanned areas was $1.5 \, mm (H) \times 0.5 \, mm (V)$. The X-ray beam was monochromated using a Si (111) crystal pair in a water-cooled fixed-exit double-crystal monochromator.

**c-SAXS**

Small-angle X-ray scattering measurements were carried out at the c-SAXS beamline at the SLS. A monochromatic photon energy of 12.4 keV was used as the photon energy of the incident beam (giving a wavelength of $1 \, \text{Å}$), with the beam having rectangular dimensions of $20 \, \mu m (H) \times 5 \, \mu m (V)$ and a sample to detector distance of 2.1 m. The sample section was mounted on a motorised sample-stage to allow the alignment and orientation of the sample to the beam. Subsequently raster scanning was performed in the horizontal and vertical directions, covering areas of up to several square mm to encompass the cartilage surface and underlying bone, the scans being made for a range of momentum transfer values to include the relevant order-spacings of the predominating types of collagen and bone. The two-dimensional X-ray scattering patterns were acquired employing a Pilatus 2M detector [229] with a dwell time of 1 s per point, individual scans taking periods of between a few hours up to 8 hours. Before scanning, camera length calibration of the set-up was performed using a silver behenate powder standard. Figs.3-10, 11 and 12 show the SAXS apparatus set up, Pilatus detector and camera length calibration with 2-D SAXS scattering of the silver behenate standard and the sample in its motorised stage in front the beam respectively.
Figure 3-10: SAXS apparatus set up at PSI indicating that a source to detector-distance of up to 7.0 m can be obtained to provide for very large scale structure analysis.

Figure 3-11: a) camera length calibration of the set-up using a silver behenate powder standard. b) SAXS diffraction patterns of the standard.
Figure 3-12: The examined sample located just in front of the beam exit position at the PSI c-SAXS facility.
4 Bone-Cartilage Interface Studies at the University of Surrey Ion Beam Centre

4.1 Experiment 1 (on diseased bone sections only)

The elemental distribution of some trace and essential elements in two diseased sections of human femoral heads were examined by using μ-PIXE and RBS.

**Spot size ~ 4 μm x 5 μm and beam current ~40 pA**

In the first day of a two-day long experiment, and for a sample of diseased human femoral head designated bone2, two scanned areas and a point on the trabecular (spongy) bone (Fig.4-1) were probed with a beam spot size and current of ~ 4 μm x 5 μm and ~40 pA respectively.

![Figure 4-1: A thin section of human femoral head (bone2) showing the areas investigated. It is clear from the photograph of the section that this is a highly diseased bone.](image)

The calibration of the RBS detector was carried out by a fitted RBS spectrum (Fig. 4-2) of the calibration sample (lead glass). The RBS spectrum has been fitted using GUPIX software [218]. The heights of the RBS spectrum were used to determine the charge/protons delivered to the sample.
Fig. 4-3 shows the fitted PIXE spectrum of the Pb glass standard, the GUPIX software being used for the construction of the PIXE spectrum of the Pb glass. Since the composition of the standard was known and (from the RBS spectrum) the charge received by the sample was known, thus the PIXE spectrum was used to determine the solid angle of the PIXE detector for this analysis. Knowledge of both the solid angle and charge delivered during an analysis are essential in defining the solid angle of the detector, subsequently allowing determination of the concentration of elements in the sample.

![Energy spectrum](image)

**Figure 4-2:** The fitted RBS spectrum of the standard (lead glass).

**Figure 4-3:** The fitted PIXE spectrum for the calibration sample (lead glass).
Analysis of bone2

Scan area1

Fig.4-4 shows a $\mu$-PIXE spectrum for scan area1, revealing the presence of a range of essential elements, including Si, P, S, K, Ca, Fe and Zn. The 5.7 keV peak represents the P and Ca pileup whereas the double peak at 7.4 keV represents the poorly resolved Ca $K_a$ and Ca $K_\beta$ lines.

![Energy (keV)](image)

**Figure 4-4:** $\mu$-PIXE spectrum for scan area1. The counting time was ~24 mins.

Fig. 4-5 shows 2-D $\mu$-PIXE maps of Si, P, S, K and Ca obtained in a counting time of ~24 mins; these maps show the structural features of bone2. The air interface, most clearly seen in the map for sulphur, is shown as the dark region to the extreme right edge of the maps, below which is a layer of ligament, cartilage and then bone.
Figure 4-5: μ-PIXE maps for Si, P, S K and Ca revealing the elemental makeup of bone2.

Figure 4-6: μ-PIXE maps for S, Ca and Zn 2 mm to the left into bone2 with respect to the previous scan (Fig. 4-5), revealing in particular the trabeculation most clearly depicted by the Ca scan. The colour gradation is a 'colour-wash' scale, with red indicating the greatest presence of an element and blue the least.

Fig.4-6 shows μ-PIXE maps for S, Ca and Zn 2 mm to the left into bone2 of scan area1 with respect to the previous scan (Fig. 4-5). The counting statistics for Zn are very low, in line with the low elemental presence indicating the need for extended counting times.

Fig.4-7 shows μ-PIXE maps for S, Ca, P and Zn a further 2 mm to the left into the sample with respect to the previous scan (Fig. 4-6).
Figure 4-8: μ-PIXE maps showing the elemental makeup of bone2 at the air-bone cartilage interface and the overlying ligament. An extended counting time has improved the mapping of Zn, showing correlations with the localisation of Ca.
From the μ-PIXE maps (Fig. 4-8), obtained for a counting time of ~ 40 mins, the concentration of P and Ca are clearly highest in the bone as expected, while S is highest in the ligament and cartilage overlying the bone (resulting from the presence of chondroitin sulphate). Zn is present at elevated concentration closest to the bone interface (as will be demonstrated in the associated line profiles of Fig. 4-11).

Fig. 4-9 displays zoom maps of the bone-cartilage interface. Ca and P are as expected higher in concentration in the bone while S is higher in the cartilage and Zn appears to have its highest levels in the region of the bone-cartilage interface.

![Figure 4-9: Zoom maps of the bone-cartilage interface.](image)

A line profile was taken from left to right (normal to the bone-cartilage interface) using the maps above. The μ-PIXE spectrum for this line scan is shown in Fig. 4-10.

![Figure 4-10: μ-PIXE spectrum for the line scan from bone to cartilage.](image)
Fig. 4-11 depicts the line profiles obtained for Ca, P, K, S and Zn, being the essential elements of interest detected in the air to bone cartilage interfaces. Apparent is that there is a lesser prevalence of the Ca, P, K and Zn within the cartilage region whereas there is an enhanced presence in the bone cartilage interface. Conversely, S is localised within the cartilage, again being in line with the expected locations of chondroitin sulphate.

Figure 4-11: Line profiles of Ca, P, K, S and Zn along the bone-cartilage interface.
A point on the trabecular/spongy bone

To quantify the concentrations of some trace and essential elements present in the bone, a fitted RBS spectrum (Fig.4-12) has been employed at a particular location in the spongy bone.

**Figure 4-12:** The fitted RBS spectrum for a point taken on the surface of spongy bone.

Fig.4-13 displays the fitted μ-PIXE spectrum for the same point. The PIXE spectrum was fitted using GUPIX software.

**Figure 4-13:** The fitted μ-PIXE spectrum for a point taken on the trabecular bone.
Table 4-1 lists the concentrations of some essential and trace elements on the surface of diseased spongy bone (measured at point P Fig.4-1). The concentrations are expressed as ppm (ug/g). It is evident in Table 4-1 that concentrations of Ca and P are as expected high compared to the other detected elements, these two elements being the main elemental components of the mineral part of bones. Si, Cu and Fe are below the limits of detection for the particular counting time (which was ~ 24 min).

<table>
<thead>
<tr>
<th>Element</th>
<th>Detection limit (ppm)</th>
<th>Concentration (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Si</td>
<td>700</td>
<td>-</td>
</tr>
<tr>
<td>P</td>
<td>690</td>
<td>45100</td>
</tr>
<tr>
<td>S</td>
<td>170</td>
<td>918</td>
</tr>
<tr>
<td>K</td>
<td>90</td>
<td>244</td>
</tr>
<tr>
<td>Ca</td>
<td>140</td>
<td>235000</td>
</tr>
<tr>
<td>Fe</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Cu</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>Zn</td>
<td>15</td>
<td>136</td>
</tr>
</tbody>
</table>

- Indicates below lower limit of detection.

**Scan area2**

For scan area2, the same procedures as scan area1 were repeated. A 2 x 2 mm² area scan was made for a counting time of ~ 13 min. The μ-PIXE maps for P, S, Ca and Zn is shown in Fig.4-14.

**Figure 4-14**: μ-PIXE maps show the structure of the bone2 scan area2 for representative elemental components.
A line scan was taken on the μ-PIXE map (Fig. 4-14) for a location on the lower left portion of the map. Fig. 4-15 displays the μ-PIXE spectrum for this line scan. As before, the double peak around 6 keV represents Ca pile up whereas the double peak around 7.5 keV represents the Ca Kα, Ca Kβ, P Kα and P Kβ.

![Figure 4-15: μ-PIXE spectrum for the line scan (counting time ~ 8 mins).](image)

Fig. 4-16 depicts line profiles of Ca, P, K, S and Zn detected at the air–bone interface, producing results similar to that for scan area1. The Ca and P line profiles show low concentrations in cartilage, increasing at the region between the tidemark and cement line (bone-cartilage interface) to reach peak values in the surface bone. A reverse trend for S accumulation is noted, the lowest concentration being present in bone and the highest within the cartilage zone. The Zn line profile shows a fluctuating distribution in both the bone and cartilage regions (expected by the limitation imposed by the counting time available) but again enhanced accumulation at the bone-cartilage interface region. It is also apparent that the double Zn (and Ca) peaking in cartilage suggestive of cartilage fibrillation (and associated enzymatic activity) and also elevation of Zn at the bone-cartilage interface (again strongly suggestive of enzymatic activity).
Figure 4-16: line profiles of Ca, P, K, S and Zn along the bone-cartilage interface (100 pixels~0.6mm).
In the second day of the experiment, the sample designated bone1 was examined; the scanned areas are shown in Fig. 4-17. Repeat calibration procedures similar to that bone2 were carried out.

Spot size ~ 3 μm x 5 μm and beam current ~ 70 pA

Scan area2

Fig. 4-17: A thin section of diseased human femoral head (bone1).

Scan area2

Fig. 4-18 shows a μ-PIXE spectrum for scan area2, a range of elements including Si, P, S, K, Ca, Cu and Zn being detected in a counting time of ~ 13 mins.

Figure 4-18: μ- PIXE spectrum for bone1 scan2.
Fig. 4-19 shows the μ-PIXE map for P; in this area the scan was positioned only on the trabecular to cortical bone region.

![Figure 4-19: μ-PIXE map of P shows the structure of bone at scan area2.](image)

**A point on the trabecular bone**

For quantitative analysis a selected area on the trabecular bone was chosen within scan area2 to carry out in situ examination of elements in a point. Fig.4-20 shows the μ-PIXE map for P within the selected area.

![Figure 4-20: μ-PIXE map of P for a selected area chosen for quantitative analysis.](image)

Table 4-2 lists concentrations of some essential and trace elements for bone1 scan2 for the selected area shown in Fig.4-20.

<table>
<thead>
<tr>
<th>Element</th>
<th>Concentration (ug/g)</th>
<th>Errors (fit and statistics)%</th>
<th>Limit of Detection (ug/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>50750</td>
<td>1.4</td>
<td>1020</td>
</tr>
<tr>
<td>S</td>
<td>1140</td>
<td>11.8</td>
<td>270</td>
</tr>
<tr>
<td>Cl</td>
<td>340</td>
<td>13.5</td>
<td>86</td>
</tr>
<tr>
<td>Ca</td>
<td>343000</td>
<td>0.17</td>
<td>250</td>
</tr>
<tr>
<td>Fe</td>
<td>19</td>
<td>37</td>
<td>9.7</td>
</tr>
<tr>
<td>Ni</td>
<td>220</td>
<td>11</td>
<td>90</td>
</tr>
<tr>
<td>Sr</td>
<td>120</td>
<td>55</td>
<td>92</td>
</tr>
<tr>
<td>Zn</td>
<td>131</td>
<td>13.2</td>
<td>12</td>
</tr>
</tbody>
</table>
From Table 4-2 the concentration of Zn was 131 ppm being in accord with that for point P in bone2. In this area the Ca escape peak overlaps the P K\alpha peak. This is expected to have an effect of up to 2% on the Ca:P ratio.

Another selected area was taken at scan area2 for quantitative analysis. Fig.4-21 shows the μ-PIXE map for P for the selected area, the dark blue, light blue, yellow and red areas representing the ligament, cartilage, bone-cartilage interface and bone respectively.

![Figure 4-21: μ-PIXE map of P shows the cartilage, bone-cartilage interface and bone region.](image)

Table 4-3 lists concentrations of essential and trace elements for bone1 scan2 for the selected area across the bone-cartilage interface shown in Fig.4-21.

<table>
<thead>
<tr>
<th>Element</th>
<th>Concentration (ug/g)</th>
<th>Errors (fit and statistics)%</th>
<th>Limit of Detection (ug/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>17200</td>
<td>1.5</td>
<td>360</td>
</tr>
<tr>
<td>S</td>
<td>6540</td>
<td>1.2</td>
<td>58</td>
</tr>
<tr>
<td>Cl</td>
<td>76</td>
<td>20</td>
<td>26</td>
</tr>
<tr>
<td>K</td>
<td>1107</td>
<td>1.35</td>
<td>27</td>
</tr>
<tr>
<td>Ca</td>
<td>80000</td>
<td>0.18</td>
<td>46</td>
</tr>
<tr>
<td>Zn</td>
<td>100</td>
<td>8.7</td>
<td>8.9</td>
</tr>
</tbody>
</table>

Table 4-3 reveals concentrations that are significantly different from these given in Table 4-1 and 2 as are the ratios (as an example from Table 4-2 Ca/P is 6.76 while in Table 4-3 it is 4.65). Clearly, the present selected area represents both subchondral bone and cartilage tissue, the mixture giving a lower Ca/P ratio. A further discussion of this will be provided later (see section 4.1.2).
Scan area1

Fig. 4-22 displays μ-PIXE maps of Ca, P and S showing the trabecular structure of bone1.

Figure 4-22: μ-PIXE maps showing the structure of bone1.

To probe the bone-cartilage interface, the scan area was moved 1 mm deeper into bone1 with respect to the previous scan (Fig.4-22). Fig.4-23 shows μ-PIXE maps of Ca, P, S, and Al for the new area scan. Taking the Ca map as an example; the red, yellow, light blue and dark blue areas represent bone, bone-cartilage interface, cartilage and air respectively. Also apparent is a low presence of Al.

Figure 4-23: μ-PIXE maps show the bone-cartilage interface.
Elemental quantification was carried out in an interesting point on the previous μ-PIXE maps (Fig.4-23) where Al was higher. The concentrations of some essential and trace elements in this region are listed in Table 4.4. The presence of Al is possibly as a result of the subject being on long term dialysis [233].

Table 4-4: concentrations of some essential elements (ug/g).

<table>
<thead>
<tr>
<th>Element</th>
<th>Concentration (ug/g)</th>
<th>Errors (fit and statistics)%</th>
<th>Limit of Detection (ug/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>4470</td>
<td>18</td>
<td>980</td>
</tr>
<tr>
<td>Si</td>
<td>1940</td>
<td>11</td>
<td>270</td>
</tr>
<tr>
<td>P</td>
<td>2650</td>
<td>5.7</td>
<td>160</td>
</tr>
<tr>
<td>S</td>
<td>1100</td>
<td>6.4</td>
<td>90</td>
</tr>
<tr>
<td>K</td>
<td>360</td>
<td>8.1</td>
<td>44</td>
</tr>
<tr>
<td>Cl</td>
<td>430</td>
<td>10</td>
<td>69</td>
</tr>
<tr>
<td>K</td>
<td>360</td>
<td>8.1</td>
<td>44</td>
</tr>
<tr>
<td>Ca</td>
<td>2206</td>
<td>2.6</td>
<td>21</td>
</tr>
<tr>
<td>Fe</td>
<td>104</td>
<td>18</td>
<td>24</td>
</tr>
</tbody>
</table>

4.2 Experiment 2 (on one diseased bone section and one normal bone section)

In this experiment two sections of human femoral head were examined by μ-PIXE and RBS. The diseased and normal sections were designated Fem3 and norm respectively. The aim of this experiment was to investigate trace element distribution and their intensities in these sections.

4.2.1 Results

Spot size ~ 4 μm x 6 μm and beam current ~150 pA

Under the experimental conditions defined in section 3.2.1.2 the experiment was conducted over a period of two days (09-10/06/2008). Fig.4-24 shows the two area scans for elemental maps and a point on the trabecular bone chosen for elemental quantitation. The first area scan was 6 x 2 mm² and subdivided into three separate scanning areas (each of area 2 x 2 mm², this being the maximum scan area allowed by the PIXE facility), and designated as scan area1, scan area2 and scan area3.
Figure 4-24: A thin section of human femoral head showing the areas investigated and a point in the trabecular region.

The lead glass standard was again used to calibrate the RBS detector and the GUPIX software was again used to fit both the RBS and PIXE spectra as previously shown in section 4.1 in experiment 1.

Analysis of Fem 3 (first scan area)

Scan area1

A series of μ-PIXE spectra for scan area1 were captured, the location of peaks along the energy scale indicating the various elements identified, including Si, P, S, K, Ca, Fe and Zn. A counting time of ~6 mins was used.
Fig. 4-25 shows the two dimensional maps obtained for the elements Ca, P, K, Zn and S; these maps showing the structure of Fem3. The right edge of the maps is the air interface.

Figure 4-25: μ-PIXE maps for Ca, Zn, S, K and P revealing the elemental makeup of the sample.
As for scan area 1, \( \mu \)-PIXE spectra for scan area 2 for a range of elements were obtained, including Si, P, S, K, Ca, Fe and Zn. A counting time of 6 mins was used.

Fig. 4-26 shows the associated \( \mu \)-PIXE maps obtained for Ca, K, S, P and Zn for scan area 2 a further 2 mm deeper into the sample with respect to the previous scan (Fig. 4-25). While the bone-cartilage interface presents as a smooth transition between cartilage and bone, closest inspection shows a disrupted surface (see Ca map) indicative of OA.

![Figure 4-26: \( \mu \)-PIXE maps for Ca, K, S, P and Zn corresponding to scan area 2.](image-url)
Scan area3

Fig. 4-27 illustrates μ-PIXE maps for Ca, K, S, P and Zn corresponding to scan area3, again with the effect of OA apparent in the disrupted cartilage to bone interface.

![Image of μ-PIXE maps for S, Ca, P and Zn on scan area3.](image-url)

From the μ-PIXE maps shown in Figs (4-25, 26 and 27) it is apparent that the concentration of Ca and P dominate within the bone as expected, while S is highest in the cartilage overlying the bone. Zn is present at elevated concentration closest to the bone interface, again indicative of enzymatic activity.

A line profile was taken from left to right (normal to the bone-cartilage interface) on the elemental maps of scan area2. Line profiles for Ca, P, K, S and Zn are shown in Fig.4-28. The line profiles for Ca, P and K indicate some evidence for elemental elevation within the cartilage region, gradually increasing at the bone-cartilage interface to reach maximum values within the bone region. Ca variation in the subchondral bone is possibly indicative of microfibrillations associated with OA. An elevated presence for S is found in the non-calcified cartilage zone, slightly decreasing within the bone-cartilage interface, reaching its lowest value in the subchondral bone region.
Figure 4-28: line profiles of Ca, P, K, S and Zn along the bone-cartilage interface (100 pixel = 0.6 mm).
Spot size ~ 5 µm x 6 µm and beam current ~200 pA

Analysis of Fem 3 (Second scan area)

As before μ-PIXE spectra were obtained for the second area scan showing the expected range of elements, including Si, P, S, K, Ca, Fe and Zn.

![Image](image.png)

Figure 4-29: μ-PIXE maps for Ca, P, S, K and Zn, the trabeculation of bone being shown with a considerable degree of clarity.

The μ-PIXE maps (Fig.4-29) display the structure of Fem3 at the second area scan. The Ca and P maps show high relative concentrations within the bone region, slightly decreasing at the bone-cartilage interface to reach their lowest relative counts in the cartilage region. K and Zn maps show similar patterns to P and Ca but with lesser elemental concentration. Conversely, the S map shows the highest concentration at the cartilage region and the lowest within the bone.

Another scan was taken on the area of trabeculation to examine the structural composition there. The μ-PIXE elemental maps are shown in Fig.4-30.
A point on the trabecular bone

To quantify the concentrations of essential and trace elements in bone, a fitted RBS spectrum was employed (see section 4.1).

Table 4-5 lists the concentrations of some essential and trace elements on the surface of the trabecular bone. The concentrations are expressed in ppm (ug/g). The concentration of Zn is 125 ppm which is again in accord with that represented in Table 4-2. The value for Ca is ~ 30 per cent of that shown in Table 4-2 for compact bone, whereas P has a value of the order of that in Table 4-2. The significant presence of Al attracts interest, a strong possibility being that it is due to dialysis sustained over a number of years [233].
Table 4-5: Concentrations of some essential and trace elements in the trabecular bone (ug/g).

<table>
<thead>
<tr>
<th>Element by weight</th>
<th>Concentration (ppm)</th>
<th>%Errors</th>
<th>Detection limit (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>7718</td>
<td>18</td>
<td>2707</td>
</tr>
<tr>
<td>P</td>
<td>54255</td>
<td>0.4</td>
<td>283</td>
</tr>
<tr>
<td>S</td>
<td>23367</td>
<td>0.6</td>
<td>111.4</td>
</tr>
<tr>
<td>Cl</td>
<td>169</td>
<td>26</td>
<td>62</td>
</tr>
<tr>
<td>K</td>
<td>-</td>
<td>-</td>
<td>103</td>
</tr>
<tr>
<td>Ca</td>
<td>120489</td>
<td>0.1</td>
<td>32</td>
</tr>
<tr>
<td>Cu</td>
<td>21</td>
<td>24</td>
<td>7</td>
</tr>
<tr>
<td>Zn</td>
<td>125</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Sr</td>
<td>30</td>
<td>47</td>
<td>25</td>
</tr>
</tbody>
</table>

- : not detected.

Analysis of Norm

A normal sample was obtained from the cadaver of a young male road accident victim (referred to as ‘Norm’). The scanned areas “first scan” and “second scan” and a point P on the trabecular bone, are shown in Fig.4-31. Before examining the Norm sample the calibration procedures were repeated, as in previous cases.

Figure 4-31: The scan areas and the selected point (P) for the elemental quantitation.
First scan

Fig.4-32 displays μ-PIXE maps of Ca, P, K, S and Zn respectively. The Ca and P maps indicate the retention of high concentrations in the bone region and low concentrations in the cartilage region while the S map reveals relatively high concentration in the cartilage region. The Zn map demonstrates enhanced concentrations at the bone-cartilage interface.

Figure 4-32: μ-PIXE maps show the structure of the sample in the scan area.

On the above maps a line scan was taken from bone to air, the duration of the measurement being ~ 1 hour.
Fig. 4-33 depicts line profiles of Ca, P, K, S and Zn.

![Line profiles for Ca, P, K, S and Zn along the bone-cartilage interface (100 pixels ~ = 0.6 mm). The underlying trabeculation is most apparent in the Ca map.](image)

The line profiles represent the variation in concentrations of Ca, P, S, Zn and K from cartilage to bone. The S line profile demonstrates a relatively high concentration within the cartilage, gradually decreasing at the bone-cartilage interface to reach its minimum values in bone. The Ca line profile shows low concentration in the cartilage, enhanced concentration at the bone-cartilage interface, increasing at the bone surface region. The sharp, highly delineated variations thereafter are indicators of the porous nature of the trabecular bone. The Zn line profile shows its highest concentration at the bone-cartilage interface.
Second scan

As for first area scan μ- PIXE spectra for second area scan for a range of elements were obtained, including Si, P, S, K, Ca, Fe and Zn. A counting time of 6 mins was used.

Fig. 4-34 displays μ- PIXE maps for Ca, K, S, P and Zn. Ca, P, K and Zn maps, again demonstrating elemental enhancement at the bone-cartilage interface while the S map illustrates the reverse trend.

Figure 4-34: μ- PIXE maps for Ca, P, S, K and Zn
The corresponding line profiles for P, K, Ca, S and Zn are shown in Fig.4-35.

**Figure 4-35:** line profiles of Ca, P, K, S and Zn along the bone-cartilage interface (100 pixels $\sim 0.6$ mm).

The Ca, P, K and Zn line profiles demonstrate that there is minor enhancement in elemental concentration at the bone-cartilage interface for the first three elements while for Zn the enhancement is pronounced. The S line profile shows the reverse trend, with elevation in the cartilage.
A point on the trabecular bone

Table 4-6 lists the concentrations of some trace and essential elements at a point P on the trabecular bone; K, Fe and Cl were below the limits of detection. The highest concentration was for Ca at 54455 ppm, whereas for Zn it was 119 ppm, the latter being in the range of normal mean content for bone that has been quantified as 110-300 ppm by others using different analytical techniques [3, 234, 235].

Table 4-6: Concentrations of some essential and trace elements at a point on the trabecular bone.

<table>
<thead>
<tr>
<th>Element by weight</th>
<th>Concentration (ppm)</th>
<th>Errors (fit and statistics)%</th>
<th>Detection limit (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>1469</td>
<td>93</td>
<td>2898</td>
</tr>
<tr>
<td>Si</td>
<td>197</td>
<td>264</td>
<td>801</td>
</tr>
<tr>
<td>P</td>
<td>13294</td>
<td>2.4</td>
<td>461</td>
</tr>
<tr>
<td>S</td>
<td>19049</td>
<td>0.8</td>
<td>109</td>
</tr>
<tr>
<td>Cl</td>
<td>-</td>
<td>-</td>
<td>171</td>
</tr>
<tr>
<td>K</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Ca</td>
<td>54455</td>
<td>0.3</td>
<td>56</td>
</tr>
<tr>
<td>Fe</td>
<td>-</td>
<td>-</td>
<td>21</td>
</tr>
<tr>
<td>Cu</td>
<td>21</td>
<td>65</td>
<td>24</td>
</tr>
<tr>
<td>Zn</td>
<td>119</td>
<td>21</td>
<td>35</td>
</tr>
</tbody>
</table>

- : not detected

4.3 Experiment 3 (on one diseased bone section and one normal bone section)

In this experiment two sections of human femoral heads were examined by employing μ-PIXE and PIGE techniques. The diseased and normal sections were simply designated as diseased and normal sections. This experiment aimed at investigating differential distribution of Ca, P, K, S, Zn, Na and F and their intensities in these sections. The experiment also focussed on the outer boundary of the articular cartilage to explore the possible presence of lipid layers (phospholipids) that take an active part in the lubrication process, in particular quantifying the presence of Na using the PIGE technique. Here it is appreciated that the ion Na$^{2+}$ is attracted to the phospholipids. Through this, the presence or depletion of phospholipids may also be used as an indication of the development of OA.
4.3.1 Results

Fig.4-36 shows a background spectrum for the HPGe detector without Pb shielding. 5 cm lead blocks were used to shield the Ge detector and measurements of background radiation were again taken with no beam on target. The background spectrum (Fig.4-37) reveals an absence of any significant peaks at 1635 keV or 440 keV, which are the energies of gamma emission that are characteristic of Na.

Figure 4-36: Background spectrum for the Ge detector without Pb shielding, obtained in a counting time of 30 mins.

Figure 4-37: Background spectrum for the Ge detector with Pb shielding, obtained in a counting time of 59 hrs.
Spot size ~ 60 μm x 60 μm and beam current ~20 nA

Analysis of diseased sample

Fig.4-38 shows a thick section of diseased human femoral head and the areas in which scans were made.

Figure 4-38: A thick section of diseased human femoral head showing the areas investigated.

A standard Pb glass was used for calibration and the GUPIX software was used for the construction PIGE and PIXE spectrum of the Pb glass.

Fig.4-39 shows the fitted PIGE spectrum for the Pb glass calibration sample. The peaks at 440 keV and 1635 keV are due to Na, the peak at 511 keV is the $e^+/e^-$ annihilation and the peaks at 429 keV and 478 keV are due to Li. The peaks at 1779 keV and 1273 keV are due to Si.

Figure 4-39: The fitted PIGE spectrum of the calibration sample (lead glass).
Fig. 4-40 displays the fitted PIXE spectrum for the Pb glass standard.

Figure 4-40: The fitted μ-PIXE spectrum of the standard (lead glass).

To enable quantitative analysis of element concentrations between bone and cartilage, a point on bone and cartilage were taken and the concentrations of Na and F in bone and cartilage were evaluated by simulating the PIGE spectrum using the GUPIX code. To confirm the peaks for Na and F were from the sample and not due to the background; another point was taken without the presence of the sample.

Fig. 4-41 shows a PIGE spectrum without the presence of the sample (air). The Si γ-rays are due to the Si exit window. The other peak in the spectrum is the 511keV e⁺/e⁻ peak.

Figure 4-41: PIGE spectrum for a point taken in air.
Fig.4-42 shows the PIGE spectrum for a point taken on cartilage (green) and a point taken on bone (black). These spectra demonstrate the presence of Na peaks are from the bone section.

![PIGE spectrum](image)

**Figure 4-42:** PIGE spectrum for a point taken on cartilage (green) and a point taken on bone (black).

Table 4-7 lists the concentration of Na at a point on the cartilage and another point on bone at 440 keV. The Na content of bone is almost 28-fold higher than in cartilage.

<table>
<thead>
<tr>
<th>Location</th>
<th>440 keV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartilage</td>
<td>0.04 %</td>
</tr>
<tr>
<td>Bone</td>
<td>1.10 %</td>
</tr>
</tbody>
</table>

**Table 4-7:** Concentration of Na at 440 keV for a diseased sample.

Scan area1

An area scan of 1.5 x 1.5 mm² was taken on the sample. Figs.4-43 and 44 show PIGE and PIXE spectra for this area scan respectively.
Figure 4-43: PIGE spectrum for scan area 1.

Figure 4-44: μ-PIXE spectrum for scan area 1.

The μ-PIXE spectrum illustrates the presence of essential trace elements, including Ca, P, S and Zn, while the PIGE spectrum reveals the presence of Na and F.
The $\mu$-PIXE elemental maps of S, Zn, P and Ca respectively for scan area1 are displayed in Fig.4-45. Fig.4-46 represents the Na and F PIGE maps for scan area1. The counting time for the PIXE and PIGE maps were 30 mins.

![Figure 4-45: $\mu$-PIXE maps of S, Zn, P and Ca for scan area1.](image)

![Figure 4-46: PIGE maps of Na and F for scan area1.](image)

The $\mu$-PIXE elemental distributions of Ca, P, S and Zn show similar patterns with presence both in cartilage and subcondral bone. Also apparent is fibrillation, the Ca is particular showing multiple (double) fibrillation to have occurred.

The Na and F PIGE maps show high localisation within the surface of subchondral bone. A line profile was taken on the $\mu$-PIXE and PIGE maps perpendicularly from the bone-air interface. Fig.4-47 shows this on the $\mu$-PIXE map of Ca as an example.

![Figure 4-47: $\mu$-PIXE map of Ca showing the location of the line profile produced on the elemental maps.](image)
The corresponding line profiles from the μ-PIXE and PIGE maps are displayed in Fig. 4-48.

![Line profiles for S, Zn, P, Ca, Na and F at scan area 1, the cartilage being to the left and the bone to the right side of the profile.](image)

Figure 4-48: Line profiles for S, Zn, P, Ca, Na and F at scan area 1, the cartilage being to the left and the bone to the right side of the profile.

The line profile of S shows enhanced concentration at the bone-cartilage interface while P and Ca line profiles reveal low concentrations in the non-calcified cartilage regions; the presence increases within the bone-cartilage interface to achieve their peak values in the bone surface, thereafter decreasing within the subchondral bone. The Zn line profile shows a fluctuating pattern, the low statistics pointing only to enhanced concentration in the subchondral bone.
PIGE line profiles for both Na and F show enhanced concentrations within the calcified cartilage-subchondral bone interface, achieving a maximum at the border of bone and then decreasing beyond this.

**Scan area2**

Fig.4-49 displays μ-PIXE maps for S, Zn, P and Ca. The Ca and P maps demonstrate high concentration within the bone region while the S map illustrated the reverse trend, high concentration of S with the cartilage.

![Figure 4-49: μ-PIXE maps for S, Zn, P and Ca at scan area2.](image)

Fig.4-50 displays PIGE maps of Na and F. These maps clearly demonstrate that the intensities of Na and F are increased in the calcified zone of the cartilage-bone interface.

![Figure 4-50: PIGE maps of Na and F at scan area2.](image)

A line profile was taken on the elemental maps from bone-cartilage as shown on the elemental maps (Figs.4-49 and 50). Fig.4-51 displays the associated elemental line profiles at scan area 2.
The S profile shows the reverse trend to the Ca and P, the presence of S being indicative of relatively healthy cartilage. The Na and F line profiles show relatively low elemental presence at the cartilage and enhanced concentrations at the bone-cartilage interface. Zn shows elevation in the calcified cartilage, supportive of the presence of Zn matrix metalloproteinase and osteoblastic/osteoclastic activity.

Figure 4-51: Line profiles of S, Zn, P, Ca, Na and F at scan area2, with the cartilage on the left and the bone to the right side of the profile.
Analysis of the Normal section

Figure 4-52: A thick slice of normal human femoral head showing the area scans.

Scan area 1

Fig.4-53 presents the μ-PIXE maps for S, Zn, P and Ca for scan area1. The S map again reveals localisation within the cartilage region.

Figure 4-53: μ-PIXE maps of S, Zn, P and Ca at scan area1.

Fig.4-54 presents PIGE maps of Na and F at scan area1.

Figure 4-54: PIGE maps of Na and F at scan area1.
Given the relatively low counts obtained in scan area 1, a selected small area scan was located within scan area 1 and counting was made over an extended period of time. Fig.4-55 shows an associated PIGE spectrum, in particular the Na signal in the sample at 440keV.

Figure 4-55: PIGE spectrum of selected area on scan area1.

Figs.4-56 and 57 show the μ-PIXE maps for S, Zn, P and Ca and PIGE maps of Na and F for the selected area scan on scan area1.

Figure 4-56: μ-PIXE maps of Ca, P, Zn and S at the selected area scan on scan area1.
The Ca map shows clearly the three interesting regions of this normal section, the red, yellow and light blue areas representing bone, bone-cartilage interface and cartilage respectively. It is apparent here that there is a strong presence of S in cartilage, indicative of tissue minimally affected by OA and that the Na and F are localised in the subchondral bone. While there are emergent features within such analyses, the lack of an entirely consistent picture is almost certainly related to the underlying make up of the tissue, varying with location around the surface of such joints in response to the demands placed upon it.

On the selected area scan, two points for elemental quantification were taken, on the cartilage and the other on bone region (Fig.4-58).

Figure 4-57: PIGE maps for Na and F for the selected area scan within scan area1.

Figure 4-58: μ-PIXE map of S showing the two points for elemental quantification.
For elemental quantification two points one on the cartilage and another on the bone were taken. In the cartilage region, Na was not observed as it was below the limit of detection. Table 4-8 provides the concentration of Na at a point on bone at 440 keV.

Table 4-8: Concentration of Na at 440 keV, for healthy tissue.

<table>
<thead>
<tr>
<th>Location</th>
<th>440 keV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartilage</td>
<td>-</td>
</tr>
<tr>
<td>Bone</td>
<td>0.29 %</td>
</tr>
</tbody>
</table>

- : not detected.

Scan area2

Fig.4-59 shows PIXE maps of S, Zn, P and Ca. The top right dark blue of the map is the air interface below the cartilage region and then the bone. P and Ca maps show high concentrations within the bone and cartilage region. The S map is localised in concordance with the Ca and P maps (see caption of Fig.4-61).

Figure 4-59: µ- PIXE maps of S, Zn, P and Ca at scan area2. The maps show the scan to have included both bone-cartilage interface1 and bone-cartilage interface2 (see Fig.4-52).

Figure 4-60: PIGE maps of Na and F at scan area2.
Line profiles from the PIXE and PIGE maps at scan area2 displayed in Fig.4-61.

Figure 4-61: Line profiles of F, Ca, P, Na, Zn and Ca respectively at scan area2. The S is in this case is not differentially localised from that of Ca, possibly being an artefact of a relatively thick sample with bone and cartilage overlapping.
Analysis of diseased section

Scan area3

Figure 4-62: A thick section of diseased human femoral head showing scan area3 and a point on the bone and another point on the cartilage for elemental quantification.

Fig. 4-63 displays μ-PIXE maps of S, Zn, P and Ca showing the structure of a diseased sample at scan area3. From the Ca map it is apparent that the scan was made on the bone-cartilage interface to bone region. The pores within the trabecular bone are clearly visible in all maps.

Figure 4-63: μ-PIXE maps of S, Zn, P and Ca at scan area3.
Fig.4-64 show PIGE maps of Na and F at scan area3. Again PIGE maps illustrate the structure of diseased section at scan area3.

Figure 4-64: PIGE maps of Na and F at scan area3.

4.1.2 Discussion

The application of μ-PIXE, PIGE and RBS micro probes as effective means for producing cartilage and bone elemental maps and quantification in this study have demonstrated the major advantages of these techniques, mainly the high sensitivity, non-destructive nature and multi-elemental analysis capability. Presently these techniques are being applied to investigations of degenerative joint diseases in particular (OA), and the monitoring of the progression and development of the disease. μ-PIXE for instance, has been found to be sensitive in the detection of low atomic number (13 < Z < 30) trace elements in cartilage and bone. Compared with μ-XRF, μ-PIXE is more time efficient in detecting and mapping elements taking only of the order of a few minutes for similar areal extent (~1 mm²).

In present investigations, made at the University of Surrey Ion Beam Centre, the focus was on the bone-cartilage interface, being of particular interest as an active site of both bone growth and remodelling. Among its subcomponents, a class of enzymes known as metalloproteinases play a fundamental role in the growth, degeneration and inflammatory processes of OA. However, the role of these enzymes relies on the presence of divalent ions such as Ca, P, K and Zn. The scientific explanation of the elemental composition and distribution of some essential elements in this region are still lacking, implying a need for further investigations to address the mechanisms occurring at the bone-cartilage interface.

In experiment one, two and three included herein, the objective was to detect the distributions (accumulations) of some essential trace elements, in particular Ca, P, K, S and Zn and their quantification at the bone-cartilage interface. The final part of this study also focussed on the outer boundary of the articular cartilage to explore the presence of lipid layers (phospholipids) that take an active part in lubrication process by mapping the associated Na⁺.
ion using the PIGE technique. The presence or depletion of phospholipids may be used as an indication of the development of OA.

µ-PIXE elemental maps and line profiles of diseased human femoral heads sections

The results obtained herein using a beam spot size ~ 4 \( \mu \text{m} \times 5\mu \text{m} \) indicate an accumulation of Ca, P and Zn at the bone-cartilage interface. The µ-PIXE maps of Ca, P and Zn shown in Fig. 4-8 reveal the presence of these elements to be slightly increased at the border of the calcified region (t纪念馆ark), with a more pronounced increase at the cement line.

Taking the cement line as a benchmark of measurements, line profiles (Fig.4-11) taken on the µ-PIXE maps (Fig.4-8) more clearly reveal the enhancement of these elements at the bone-cartilage interface, of the order of 300, 928 and 314 per cent for Ca, P and Zn respectively. These elements are known to be associated with high activity of cartilage degradation enzymes [9, 16]. The results are in accordance with previous results by others [4, 236].

Similar patterns of elemental enhancement at the bone-cartilage interface were observed in the µ-PIXE maps displayed in Fig.4-14. Line profiles indicated enhancements of Ca, P, and Zn at the bone-cartilage interface, of the order of 1421, 1214 and 350 per cent respectively. The accumulation of these elements in active sites and in particular Zn might be due to new bone formation as Zn is an essential element in normal growth of the skeleton [237, 238]. These results are in accordance with previous findings by several other authors who have suggested the accumulation of these elements to be due to enzymatic activation [4] and also due to new bone formation [3].

Results using a beam spot size ~ 3\( \mu \text{m} \times 5\mu \text{m} \) showed similar results to those obtained for a beam spot size ~ 4\( \mu \text{m} \times 5\mu \text{m} \), i.e. accumulation of trace elements at the bone-cartilage interface (Fig.4-23). Similarly, the µ-PIXE maps achieved by using a beam spot size ~ 4 \( \mu \text{m} \times 6\mu \text{m} \) displayed in Figs. (4-25, 26, 27 and 29) and the line profiles in Fig.4-28 have again revealed increases of Ca, P, K and Zn levels at the calcified zone of cartilage, the elemental intensities starting to increase at the tidemark to reach their maximum levels at the cement line.

Accumulation of essential elements are suggestive of remodelling and growth [239], specifically at the bone-cartilage interface; in diseased sections this may be suggestive of increased activity of cartilage degrading enzymes whose function rely on the presence of Zn, Ca, K and P as co-enzymes [3, 16, 19, 240]. Elevated concentrations of these elements in the calcified zone of cartilage affected by OA may further reflect an enhanced metabolic activity of chondrocytes that are associated with their reparative reaction to osteoarthritis [240]. These results are in accord with previous results by others [4, 16, 19, 236]. The µ-PIXE elemental
maps obtained using a beam spot size ~ 60µm x 60µm showed similar patterns to those of previous elemental maps obtained under other conditions. Again, the results are in a good agreement with results reported by others [11, 16, 17, 204].

**μ-PIXE elemental maps and line profiles of normal human femoral heads sections**

The elemental maps for healthy bone and cartilage sections scanned using a beam spot of 4µm x 6µm are presented in Figs.4-32 and 34, the associated line profiles shown in Fig.4-33 denoting elevated metal accumulation in the calcified zone of the cartilage. This suggests evidence of a relationship between the enzymatic activity and metal accumulation, these elements (Ca, P, K and Zn) being essential in bone calcification [241-243]. At a beam spot size 60µm x 60 µm, the PIXE maps displayed in Fig.4-53 and 58 showed similar results, namely elevated elemental concentrations at the bone-cartilage interface.

Pertaining to P and Ca maps, the expected large presence in the bone tissue was obtained but in addition there is a notable presence in cartilage, it being well known that, these elements play an important role in bone structure and growth, forming hydroxyapatite [104, 241, 244]. Elevated concentrations of P in cartilage result from P acting as a precursor in phospholipids biosynthesis, forming a lubricant material and energy source [245, 246]. Ca in cartilage results from its presence as the Ca$^{2+}$ ion.

Relating to K intensity maps in Figs.4-32 and 4-34 and their associated line profiles (Figs.4-33 and 35 respectively), these reveal an elevation of K within the cartilage region and this is an indication of low degradation of proteoglycans which keeps the level high [247].

With regard to S, it was observed to be highly localised within the cartilage regions, Figs.4-5, 23, 25, 26, 27 and 45 showing maps of S present within the diseased bone and cartilage tissues. Figs.4-32, 34 and 53 present the maps of S for normal cartilage and bone tissues. Both S diseased maps and normal maps reveal that the distribution of S to be non-uniformly distributed in the cartilage, the superficial zone having the lowest concentration and the intermediate zones having the maximum concentrations, followed by a decline in the calcified zone. S is a well known essential component of the proteoglycans and forms sulphate SO$^{3-}$ that accounts for the highly negative charged side-chains of proteoglycans [245] binding large amounts of water. Thus, the high levels of S in normal sections indicates a low level of degradation of the proteoglycans, keeping hydration high [245]. It is to be noted that the higher concentration of S in the normal section is caused by increased production by the hypertrophic cells [245].
RBS elemental quantifications in bone, cartilage and ligament

To quantify the presence of some essential elements in healthy and diseased bone, points were taken on the trabecular bone or selected uniform areas comprising bone and cartilage or ligament. We have summarised the results of Tables 4-1, -2, -3, -4 and -6 in comparison tables. Thus Table 4-10 lists measured elemental concentrations for trabecular bone, obtained from present investigations obtained herein, comparing these with other reported values, specifically those reported by Zhang et al. [248]. For the diseased tissues it is clear that there are significant differences between the two sets of data, almost certainly reflecting the stage of disease and the likelihood that the samples were affected not only by OA but also by osteoporosis [248] and possibly by rheumatic arthritis.

Table 4-9: elemental concentration in diseased spongy bone tissue (current results versus Zhang [248]).

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Source of data</th>
<th>Spongy bone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ca</td>
</tr>
<tr>
<td>Diseased</td>
<td>A</td>
<td>0.235</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.098</td>
</tr>
</tbody>
</table>

A: current study, B: Zhang [248].

Table 4-11 records presently measured concentrations for Ca, P and Zn in selected diseased areas comprising trabecular and cortical bone, comparing these with data again reported by Zhang [248] but now for cortical and spongy bone.
Table 4-10: elemental concentration in various diseased tissues (current results versus Zhang [248]).

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Source of data</th>
<th>Dry weight elemental concentration in tissue type (in g of element /g tissue, except where otherwise noted).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>An area comprising cortical and spongy bone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ca</td>
</tr>
<tr>
<td>Diseased</td>
<td>A</td>
<td>0.343</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.217</td>
</tr>
</tbody>
</table>

A: current study, B: Zhang [248].

It can be seen that present findings for P concentration agree with those reported by Zhang, while significant differences are noted for concentrations of Ca between the present results and literature values. In regard to Zn, elevated concentrations are found in present results for both of the data sets, tables 4-10 and -11, compared with the available literature values.

Table 4-12 lists concentrations of Ca, P and Zn in a selected diseased area comprising bone, bone-cartilage interface and bone, comparing these against concentrations in cartilage, cortical and spongy bone as reported by Zhang [248]. Again elevated differences may be attributed to the osteoarthritic condition.

Table 4-11: elemental concentration in various diseased tissues (current results versus Zhang [248]).

<table>
<thead>
<tr>
<th>Tissue State</th>
<th>Source of data</th>
<th>Dry weight elemental concentration in tissue type (in g of element /g tissue, except where otherwise noted).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>An area comprising cartilage, bone-cartilage interface and spongy bone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ca</td>
</tr>
<tr>
<td>Diseased</td>
<td>A</td>
<td>0.080</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.217</td>
</tr>
</tbody>
</table>

A: present results, B: Zhang [248].
It is apparent that the same degree of variation in concentration for Ca, P and Zn occurring between diseased tissues also prevails in the trabecular bone of diseased and normal bone sections, as shown in Table 4-13. While the table shows relatively small differences in concentration of Zn between our results and those of Zhang in normal and diseased bone sections, noticeable differences are shown for Ca and P concentrations in normal and diseased bone sections. In normal tissue, the literature values are ~2.42-fold and 4.60-fold higher for Ca and P respectively than present values; this may also reflect the age of bone section and gender or might also be a sign of osteoporosis, the Ca in bone migrating to other tissues [248, 249].

Table 4-12: elemental concentration in various healthy and diseased tissues (current results versus Zhang [248]).

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Source of data</th>
<th>Spongy bone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ca</td>
</tr>
<tr>
<td>Healthy</td>
<td>A</td>
<td>0.054</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.132</td>
</tr>
<tr>
<td>Diseased</td>
<td>A</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0.098</td>
</tr>
</tbody>
</table>

A: present results, B: Zhang et al. [248].

It is worth remembering that the diseased and normal sections examined in this study were not chemically treated i.e. no chemicals or solvents were used to remove any lipids or bone marrow from the sections, thus some of the variation in the elemental concentrations may be due to the presence of bone fat, lipids or marrow [250, 251].

In Tables 4-1, 2, 3, 4 and 5 the Ca/P ratios for diseased sections were 5.21, 6.75, 4.65, 0.83 and 2.2 respectively and in Table 4-6 the Ca/P ratio for the normal section was 4.09 whereas the stoichiometric value for hydroxyapatite is 2.16. Comparing the findings of this study and the stoichiometric value for hydroxyapatite, it is evident that the Ca/P ratio in diseased sections represented ~2.41, 3.12, 2.15 fold increase and also a ~2.60 decrease in one section while in the normal section the value was ~1.89-fold that of the stoichiometric ratio. In a study by Tzaphlidou and Zaichick [206], the Ca/P for healthy human rib bones for women and men of different ages were 2.33 and 2.35 respectively. The same study showed that differences between the stoichiometric value and the observed values were due to the
presence of bone fat, lipids or marrow which in rib bone accounts for a 5-10\% increase in the Ca/P ratio. However, in other bones this influence may be even higher [250, 251]. It is well known that the increase of Ca in intracellular and extracellular spaces is a result of toxicity produced by metals like Cd, Fe, Cu, Pb and or possibly to increase of pro-oxidants in the diseased condition [252] which are associated with mechanisms leading to cellular death [253]. Another explanation of the higher Ca/P ratios in diseased sections might be the intense calcification of Ca with other anions such as (oxalate and hydroxide). Conversely the lower Ca/P ratio might be attributed to an extra inorganic phosphate [254]. In Table 4-4 a Ca/P ratio of 0.83 was recorded; the lower Ca/P ratio here may indicate an additional P component and this may be due to the presence of phospholipids [254]. It has been shown previously that the Ca/P ratio may not be distributed evenly in osteoporotic bone and a possible cause for the change in bone quality could result in a degree of non-uniformity in the high Ca/P regions [255].

In conclusion, the present results illustrate that \( \mu \)-PIXE, \( \mu \)-PIGE and RBS are suitable micro-analytical techniques for elemental distribution mapping and their quantification in bone and cartilage sections. \( \mu \)-PIXE and RBS offer simultaneous mapping and quantification of Ca, P, K, S and Zn which may be used as markers of bone growth, remodelling and disease. Light elements like Na and F can be mapped and their concentrations quantified by the PIGE technique.

In this study, the analysis of the bone-cartilage interface has illustrated that the latter is an active site for cartilage changes and degradation enzymes such as alkaline phosphatase whose function relies on the present of metallic co-factors such as Ca, P, K and Zn. In addition to the Ca, P, K and Zn maps, the S maps may be used as an indicator of cartilage viability.

Another major advantage of the RBS microprobe lies in its ability to provide quantitative analyses of some trace and essential elements in trabecular bone; this is important in determining the composition of bone. Thus said, it is apparent that the large inter-variations observed between elemental concentrations for diseased tissues and between diseased and normal tissues renders such measurements of doubtful utility as diagnostic indicators. In future such studies, the sources of inter-variation (for example, stage of disease, subject age and gender) would need to be more rigorously controlled in an effort to determine the utility of quantitative analysis.

To sum-up, \( \mu \)-PIXE, PIGE and RBS are appropriate microprobes for elemental distribution in bone and cartilage, their high sensitivity, non-destructive nature, multi-elemental capability data and rapid acquisition of data making them valuable techniques in the field of biomedical research.
5 Bone-Cartilage Interface Studies at the Swiss Light Source

5.1 SR-μ XRF

In this experiment a thin slice (~170μm thick) of an equine metacarpophalangeal joint was examined. The aim was to use the high sensitivity synchrotron μ-XRF LUCIA beam line at PSI in an imaging mode to examine the distribution of Ca, P, K, S, Cl and Na for better understanding of whether there exists correlation between articular enzymes (e.g. alkaline phosphatase) and Ca, P, K and S distributions in the interfacial region between cartilage and subchondral bone. As these elements are co-factors of articular enzymes, an attempt has been made to elucidate the basic mechanisms underlying the causes of OA and the role of some trace elements in the onset and development of OA.

5.1.1 Results

This experiment has been carried out under the same experimental conditions mentioned in section (3.3.2.1). The scanned locations (Fig.5-1) were two areas in which there were no visible lesions (P1 and P3) and one area in which a lesion was visible (P2).

![Figure 5-1: A thin section of an equine metacarpophalangeal joint. The finest scale division in this scale illustrated is 1mm.](image)

Fig. 5-2 shows 2-D intensity maps of Ca, P, K, S, Cl and Na for location P1. Gross structural features may be easily recognized via the associated Ca, P, K and S intensities; the dark blue region on the top of the maps refers to the air interface, below which is the cartilage and then bone. Ca, P and K were found to have a relatively low presence throughout the whole region of cartilage, the maps showing increased intensities in the calcified region of cartilage at the inner border of the calcified cartilage (tidemark), the intensities then rapidly increasing to maximal values at the border of subchondral bone (i.e. at the cement line) and
high values of concentration of these elements in the region of subchondral bone. The S intensity map shows the reverse pattern, with high concentrations of S in the cartilage region, in particular in the uncalcified regions, and subsequently decreasing in the calcified region and remaining relatively constant at a low level across the bone region. Na intensity map shows presence of Na on the top surface of cartilage, the intensity gradually increasing beyond that. Conversely, the Cl intensity map shows relatively high concentration within the cartilage region and low concentration in the bone region. In contrast to the more or less homogeneous distribution of Ca, P, K and S in single tissue components (e.g. cartilage) the distribution across the various tissue components is inhomogeneously distributed.

Most apparent is that at location P1 the bone-cartilage interface presents as a smooth and regular surface, with no evidence of OA to be seen.

Figure 5-2: 2-D intensity maps of Ca, P, K, S, Cl and Na at location P1. To the right of each map is a colour-wash scale that represents the relative concentration of each element and the scan size 1.5 (h) x 0.5 (v) mm.
Figure 5-3 shows line profiles which have been extracted from the elemental maps (taken along lines normal to the bone-cartilage interface) at different positions across the surface on the maps (three in all) for each element, to represent the reproducibility.
The line profiles for Ca, P, K and Na illustrate there are slight increases intensity in the tidemark, with dramatic increases to the cement line, reaching the highest intensity above this. The S and Cl line profiles show the reverse trend, the region of non-calcified cartilage revealing the concentration of S to be the highest, decreasing to reach its lowest values in the bone region. The results are entirely expected, there being a rich content of chondroitin sulphate in cartilage to ensure a high level of water content (as functionally demanded of healthy cartilage).

Comparing the elemental intensities to that in subchondral bone (at the border between the cement line and bone) (table5-1), the mean values show a ~ 2.78, 3.75 and 2.74 fold increase in intensities of Ca, P and K respectively in the transitional zone between the non-calcified and calcified articular cartilage (from tidemark to cement line). Conversely a ~2.37 -fold decrease in the same region is observed for S.
Table 5-1: Elemental distribution at location P1 (no visible lesion) at the tidemark and cement line taken from line profiles obtained at different positions along lines down normal to the bone-cartilage interface.

<table>
<thead>
<tr>
<th>Element</th>
<th>Transitional Zone first line profile</th>
<th>Transitional Zone second line profile</th>
<th>Transitional Zone third line profile</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TM</td>
<td>CL</td>
<td>Ratio</td>
<td>TM</td>
</tr>
<tr>
<td>Ca</td>
<td>$2 \times 10^4$</td>
<td>$5.5 \times 10^4$</td>
<td>2.75</td>
<td>$2 \times 10^4$</td>
</tr>
<tr>
<td>P</td>
<td>300</td>
<td>1300</td>
<td>4.33</td>
<td>300</td>
</tr>
<tr>
<td>K</td>
<td>320</td>
<td>880</td>
<td>2.75</td>
<td>320</td>
</tr>
<tr>
<td>S</td>
<td>980</td>
<td>400</td>
<td>2.45</td>
<td>980</td>
</tr>
</tbody>
</table>

TM: tidemark  
CL: cement line  
Ratio: CL/TM

In order to study the accumulation of these same essential elements in the bone-cartilage interface in regions of articular lesions, a second area scan (location P2) was examined where the lesion is clearly seen. Results in terms of the 2-D intensity maps of the element of interest are displayed in Fig.5-4.
The elemental maps for Ca, P and K disclose similar intensity distributions with the previous scan (P1). The intensity maps for Ca, P and K reveal low relative elemental presence in cartilage, increasing in the transitional zone, then remaining relatively constant within the bone. The intensity map of S again shows similar presence in cartilage and bone and a decreased level in the bone-cartilage interface. Additionally, a visibly abraded outer surface of the cartilage and change in the shape of the bone-cartilage interface are observed.
Figure 5-5: line profiles for Ca, P, K, S, Cl and Na at location P2, (1 pixel =40 μm).
Line profiles were also extracted from the elemental maps shown in Fig 5-4 along the bone cartilage interface at different positions, as shown in Fig. 5-5. These line profiles also indicate increase in the concentration of Ca, P and K at the bone-cartilage interface and a decrease of S concentration at the bone-cartilage interface. In Table 5-2 a comparison is made of the elemental intensities to that in subchondral bone, the mean values showing ~4.7, 6 and 4.55 - fold increase in intensities for Ca, P and K respectively in the transition zone. A ~2-fold decrease in the same region is noted for S.

Table 5-2: Elemental distribution at location P2 (visible lesion) at the tidemark and cement line taken from line profiles obtained at different positions along lines down normal to the bone-cartilage interface.

<table>
<thead>
<tr>
<th>Element</th>
<th>Transitional Zone</th>
<th>Transitional Zone</th>
<th>Transitional Zone</th>
<th>Mean</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>first line profile</td>
<td>Second line profile</td>
<td>Third line profile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TM</td>
<td>CL</td>
<td>Ratio</td>
<td>TM</td>
</tr>
<tr>
<td>Ca</td>
<td>1x10^4</td>
<td>4.5x10^4</td>
<td>4.5</td>
<td>0.9x10^4</td>
</tr>
<tr>
<td>P</td>
<td>140</td>
<td>850</td>
<td>6</td>
<td>140</td>
</tr>
<tr>
<td>K</td>
<td>150</td>
<td>650</td>
<td>4.33</td>
<td>150</td>
</tr>
<tr>
<td>S</td>
<td>400</td>
<td>220</td>
<td>0.55</td>
<td>400</td>
</tr>
</tbody>
</table>

Comparing the elemental profiles in Fig 5-3 with those in Fig. 5-5, it is evident that there exists a decrease (~ 51.7 % at the tidemark) and (~ 18.5 % at the cement line) in the concentration of Ca at the bone cartilage interface in P2. There is also a similar decrease in the concentration of P at the same region of bone-cartilage interface (~ 52.27 % and ~ 28.79 % receptively) with regard to the concentration of P in Fig. 5-5. There is also a reduction (~ 56.18 % and ~ 27.81 % respectively) in the concentration of K at the same region compared with its concentration at P1. From the line profile for S at P1 and P2 shown in Figs 5-3 and 5-5 respectively, there is an obvious change in the trend of the distribution of S, the fluctuation in S concentrations being more pronounced in Fig. 5-5, and also a significant reduction in the concentration of these elements at P2.
Fig. 5-7 displays 2-D intensity maps for Ca, P, K and S at location P3.

From the elemental maps for location P3, the Ca intensity shows a slight increase in intensity on the top surface of the cartilage but otherwise remains equally distributed over the whole region of cartilage with no noticeable intensity increase in the bone region. In general, compared to sites P1 and P2 the concentration of Ca shows a significant decrease in both cartilage and bone, while P shows a relatively constant value over the whole region of the cartilage with a dramatic intensity increase in the bone region compared with Ca. The presence of K is low and almost of the same value across the entire scanned for the three scanned positions. A significant elevation of S intensity is detected on the surface of cartilage, the intensity dropping off in the non calcified cartilage region with a marked intensity decrease in bone.
More quantitative conclusions can be drawn from line profiles (Fig. 5-7) which have been extracted from the previous relative elemental concentration maps (Fig. 5-6).
From the line profiles, comparing the elemental intensities to subchondral bone baseline intensities (Table 5-3) the mean ratios were found to indicate a ~2.74, 11 and 6.69 fold increase of Ca, P and K with respect to those in the transition zone. More markedly, a ~20.6-fold decrease in the same region is observed for S.
Table 5-3: Elemental distribution at location P3 (no visible lesion) at the tidemark and cement line undertaken from line profiles obtained at different positions along lines down normal to the bone-cartilage interface.

<table>
<thead>
<tr>
<th>Element</th>
<th>Transitional Zone</th>
<th>Transitional Zone</th>
<th>Transitional Zone</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>first line profile</td>
<td>Second line profile</td>
<td>Third line profile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TM</td>
<td>CL</td>
<td>Ratio</td>
<td>TM</td>
</tr>
<tr>
<td>Ca</td>
<td>360</td>
<td>1000</td>
<td>2.77</td>
<td>400</td>
</tr>
<tr>
<td>P</td>
<td>500</td>
<td>5000</td>
<td>10</td>
<td>500</td>
</tr>
<tr>
<td>K</td>
<td>200</td>
<td>1100</td>
<td>5.5</td>
<td>140</td>
</tr>
<tr>
<td>S</td>
<td>10000</td>
<td>5000</td>
<td>0.50</td>
<td>11000</td>
</tr>
</tbody>
</table>

TM: tidemark, CL: cement line, Ratio; CL/TM

In making comparison between elemental concentrations obtained in the previously scanned areas (P1 and P2) and the present scanned area (P3), it can be concluded that, firstly, the concentration of Ca between P1 and P2 shows a decrease (for example at the tide mark and cement line respectively ~ 52 and ~ 19 per cent respectively, but an even more dramatic decrease at P3 ~ 99 and 98 per cent respectively. Similarly, the concentration of P between P1 and P2 decreased ~ 52 and 29 per cent respectively but dramatically increased ~ 71 and 400 per cent respectively at P3. Thirdly, the concentration of K between (P1 and P2) and (P2 and P3) shows decrease levels ~ 56 and ~ 28 per cent respectively and ~ 55 % and 6 per cent respectively, and finally, the concentration of S between P1 and P2 again shows decreased levels ~ 61 and ~ 68 per cent but dramatically increased ~ 954 per cent and ~ 900 per cent respectively at P3.

To sum up, at location P3 there was a far greater presence of both P and S. There were also rapid reductions observed in both Ca and K at increasing depths in bone, away from the bone-cartilage interface, a feature not observed at the other two locations.
5.1.2 Discussion

SR-XRF has been employed in several measuring modes (transmission and reflection), providing a powerful technique in identifying simultaneously the spatial distribution of a range of trace elements, including Ca, P, K, Sr and Zn in biological tissues including bones [9, 10, 16, 17]. Certain trace elements are well known to be co-factors of articular cartilage metalloproteinases, a family of enzymes playing a pivotal role in the growth and degeneration of bone and cartilage [16]. While the bone-cartilage interface is an active site for such enzymes, mechanisms such as bone degradation and remodeling in this region are not fully understood and in particular the distribution of trace elements as co-factors of these enzymes has not yet been quantified [151]. For this purpose SR-XRF was employed to determine the distribution of some of trace elements in an equine metacarps joint section comprising of both healthy and diseased tissue, as an attempt to enhance our understanding of this and also to improve the potential for prognosis and diagnosis of OA and its monitoring.

As one of several nuclear analytical techniques, SR-μXRF is a highly sensitive, non-destructive procedure, capable of mapping micro-areas such as at the bone-cartilage interface, the multi-elemental detection capability enabling the detection of various elements of interest simultaneously [17, 204].

Intensity elemental maps shown in Fig. 5-2 reveal high intensities of Ca, P and K in bone relative to that in cartilage as expected, bone tissue being primarily composed of Ca and P structurally organized as hydroxyapatite [Ca_{10} (PO_{4})_{6} (OH)_{2}]. Conversely, the intensity maps for S show high levels of S in healthy cartilage compared to that in bone and this high concentration of S reflects the strong presence of chondroitin sulphate in cartilage, an important mucopolysaccharide whose highly electronegative fixed charge density (FCD) structure provides greater capacity to attract/retain water molecules, resulting in the cartilage being elastic but yet highly resistant to friction and impact forces [256].

The intensity elemental maps (Fig.5-2) and the extracted profiles (Fig.5-3) for Ca, P and K reveal enhanced concentrations of these elements at the bone-cartilage interface in which there is no visible lesion, possibly indicating high enzymatic activity in this region which may be due to the growing of bone [3, 204, 205].

The presence of the three elements Ca, P and K at the site of the lesion (Fig.5-4) showed the same trend as that in a locality with no visible lesion at the bone-cartilage interface but with a marked decrease in the elemental concentrations. One explanation of this finding is that it may reflect the increased activity of degrading enzymes and consequently higher concentration of these co-factor elements [10, 11, 16, 17, 19]. From the elemental maps it is clear that there was a dramatic loss of AC as a result of degradation of the collagen fibril network. The decrease of these elements in bone, cartilage and at the bone-cartilage
interface might be due to some metabolic abnormality of bone leading to reduction in mineral content. The apparent reduction of S (by a factor of 2 over that away from the lesion, at location P1) in the abraded cartilage has also been noticed. This is almost certainly due to the loss of chondroitin sulphate (C₁₂H₂₁O₁₄NS). S is a good indicator for aggrecan (aggregating proteoglycan of (C₁₂H₂₁O₁₄NS), keratin sulphates (C₁₆H₃₉O₁₃NS) and hyaluronic acid (C₁₄H₂₀O₁₁N) [257]. The findings mentioned above are also noted to be in agreement with earlier reports [4, 258].

The line profile of Cl map at location P2 showed enhancement of Cl at the bone-cartilage interface. Cl is well known to be an important electrolyte for physiological processes in biological systems, therefore higher Cl accumulation at the bone-cartilage interface may be an indication of cartilage degradation, hypochloric acid being demonstrated to be involved in cartilage degradation [257].

Compared to locations P1 and P2, increased intensities of P and S were noted at P3 where remarkable reductions in both Ca and K were found with increasing depths in bone away from the bone-cartilage interface, a feature not observed at the other two locations (P1 and P2); both elements are well known as co-factors of cartilage enzymes and it is therefore also of interest to note that both displayed a high accumulation at the bone-cartilage interface when compared to their elemental presence at greater depths. The remarkable reduction in Ca and K in bone is perhaps unsurprising given that osteoarthritic bone is noticeably less dense and has a reduced mineral content [259]. Another possibility is that bone turnover is reduced at the time the secondary ossification centre forms. The associated reduced osteoclastic bone resorption may lower the Ca²⁺ concentration in the bone and hence the Ca gradients close to the bone-cartilage interface [260].

In conclusion, this investigation has demonstrated that SR-μXRF is a powerful technique that provides sensitive, high spatial resolution, non-destructive mapping, simultaneously detecting the distribution of a number of key trace elements such as Ca, P, K and S in an equine metacarpophalangeal joint section comprising healthy and diseased tissue. It can be concluded that Ca, P and K were observed to accumulate around the calcified cartilage in tissue with visible lesion and also through the lesion. The observation of accumulation of these elements between the tidemark and cement line may lead to further understanding and research on the effects of some trace elements on cartilage, bone-cartilage interface and bone biology. Since the bone-cartilage interface plays a vital role in the onset and development of OA, the enhancement of Ca, P and K may find use as indicators of the progress of the osteoarthritic lesion.
5.2 SR-SAXS

Generally, many diseases find their initiation in molecular changes/alterations in the tissue. As an example, in osteoarthritis (OA), damage to the collagen network of the articular cartilage is known to be involved in the initiation, development and progress of the disease [261, 262]. Appraisal of compositional and structural changes in AC affected by OA can be carried out through a number of instrumental detection techniques and SAXS is generally considered one of the most powerful of these, providing an insight into structural deformations on the nanometric scale [263].

Herein, regarding OA, direct measurements of spatial alterations of collagen fibres and their molecular features were investigated by employing SAXS on two diseased decalcified human femoral head sections. In this investigation, the 2D SAXS patterns are shown to be a potentially rapid and sensitive diagnostic tool.

5.2.1 Results

For anisotropic (direction-dependent) scattering two decalcified diseased bone sections ~200 µm thick, referred to as sample1 and sample2, were examined in exploring the morphological and internal structure, the relationship between the scattering vector (q), intensity and orientation of collagen fibrils being shown.

Fig.5-8 shows sample1 and the area in which a SAXS was taken.

Figure 5-8: A thin section of decalcified human femoral head (sample1) showing the area of the SAXS scan. In the picture, the decalcified diseased section is shown in its aluminium holder, sandwiched between Mylar windows and immersed in a thin layer of water to maintain hydration.
Fig. 5-9 displays a 2-D SAXS pattern at a point within the scan area shown in Fig. 5-8. It should be noted here that this sample (sample 1) displays signs of the presence of osteoarthritic lesions.

**Figure 5-9:** 2-D SAXS pattern at a point within the cartilage-bone section of sample 1 (shown in Fig. 5-8), revealing evidence of cartilage structure organization and preferential alignment.

It is apparent that one can analyse such data with respect to several variables: intensities, orientation and d-spacing. The SAXS intensity maps (4.5 (H) x 2.25 (V) mm$^2$) obtained for the identical area scan on sample 1 are shown in Fig. 5-11 for different length scales. Fig. 5-10 extracted from Fig. 5-11, depicts the various regions of the area scans on sample 1, the immediate upper dark region referring to the air interface following by a layer of ligament (bright red), below which is cartilage and then subchondral bone.

In the uppermost high intensity map (96.2-83.3 nm) (Fig. 5-10), a layer of ligament (orange colour) is attached to a highly defined cartilage region. The deepest zone of cartilage (the calcified region) is clearly seen, located between the tidemark and cement line. The latter follows an irregular contour and is attached to the bone, while the former can be recognized as a dark fine line after the light brightly coloured region on the cartilage. In subsequent intensity maps (rotated by 90° to allow efficient display) covering various length scales (13.9-11.8 nm; 11.5-10.6 nm; 9.8-9.1 nm; 9.0-7.7 nm; 7.6-7.1 nm; 6.8-6.4 nm), it is apparent that the lower relative intensities render it difficult to distinguish between any microscopic features. Conversely, the intensity map (96.2-83.3 nm) provides the highest relative intensity of the set and here detailed internal structures of bone/cartilage are clearly observed. Thus it is clear that in these intensity maps, intensity changes are significantly dependent on length scale.
Figure 5-10: Intensity map shows the structure of sample 1.
Figure 5-11: SAXS intensity maps for sample1, covering length scales from 2.5nm to 96.2 nm.
In regard to orientation maps for sample1 (Fig.5-13), the predominant collagen fibres orientations have been extracted from the SAXS patterns of the area scan shown in Fig.5-11. These maps represent the degree of orientation of sub-components, from 0° to 90° at various length scales. In the present coding, 0° of orientation is shown as a dark blue area, the collagen fibres being oriented parallel to the surface. On the other hand, dark red areas represent orientation of 90°, the fibres being perpendicularly oriented to the surface. Variations in orientation indicate different alignment directions of the collagen fibres, again depending upon length scale.

It can be seen from the SAXS orientation map (96.2-83.3 nm) (Fig.5-12 extracted from Fig.5-13) that in cartilage regions, the collagen fibres are perpendicularly oriented to the surface of the bone in the calcified zone (dark red, 90°). In other words, at this length scale, the SAXS orientation map reveals that the collagen fibres are preferentially vertically oriented to the direction of the trabecular bone, while in the region of the bone; the collagen fibres are randomly oriented. Similar patterns of orientation can also be observed in the orientation maps (46.3-25.0 nm; 19.5-16.9 nm and 6.2-2.5 nm). Again, it is difficult to recognize any orientation in lower intensity maps such as (9.8-9.1 nm; 9.0-7.7 nm; 7.6-7.1 nm and 6.8-6.4 nm).

In the orientation maps (46.3-25.0 nm; 19.5-16.9 nm and 96.2-83.3nm) offering relatively high intensity, the collagen fibres are vertically oriented to the articular surface (~90°) in the cartilage region. In this region orientation can be quite easily resolved, so that on the surface of the cartilage (the superficial zone) collagen fibre orientation is between (~ 50°-52°), followed by a degree of orientation of the order of between (60° -70°), followed by nearly 90° orientation in the radial and calcified zones. In bone, it is clearly seen that values of orientation range from 0° to 90°, being present over significant areas of the area scan. Conversely, other SAXS orientation maps of relatively high intensities (78.1-52.1 and 78.1-2.5nm) show high order orientations that are often non-congruent with that shown in Fig. 5-12. It is apparent that the collagen fibres are presenting in a mesh work.
Figure 5-12: SAXS orientation map shows the degree of orientation structural components in sample 1.
Before presenting the relationship between momentum transfers (q) versus intensity (I), it is important to first explain the calibration employed in this investigation and also the standard against which results have been compared.
Calibrations

Camera length calibration of the set-up was performed using a silver behenate powder standard, being highly suitable for use as an angle-calibration for small-angle diffraction, displaying as it does a number of well defined diffraction peaks. However, since it also suffers from line broadening, care must be taken if it is to be used as a peak-profile calibration standard. Silver behenate peaks are uniform, with d-spacing of \( d = 5.83 \) nm and is often used as an absolute standard \[264\]. Collagen on the other hand is often used as a spatial calibration sample as its SAXS pattern displays equidistant peaks with a primary spacing of nearly 67.0 nm. It should also be noted that the value of d-spacing for such media will vary depending on humidity. Consequently, wet rat tail collagen is typically only employed to determine the position of the direct beam (zero order). Dry rat tail has a shorter period ranging approximately between 65.0-66.0 nm and the diffraction pattern is thus slightly different.

Fig.5-14 shows plots of intensity versus \( q \)-values for the experimental standards wet rat tail (left) and silver behenate (right).

![Figure 5-14: Plots of intensity versus \( q \)-values for the experimental standards wet rat tail collagen (left) and silver behenate (right) \[265\].](image)

In order to obtain the relationship between \( I (q) \) vs. \( q \) and their relevance to the packing of collagen fibres, various locations on sample1 were scanned (Fig. 5-15).
Figure 5-15: 2-D SAXS patterns at four locations of the sample within the cartilage of bone section of sample 1.

The central panel indicates the locations of the four areas that were scanned, S1 to S4. The exploded views indicate the typical feature types that are being explored as a function of depth; evident are the crystalline features of the hydroxyapatite of bone and the shorter x-ray orders of the collagenous component more prevalent in cartilage.

SAXS profiles (intensity versus momentum transfer/scattering vector q) for the location S1 on sample 1 are shown for cartilage and bone respectively in Fig 5-16.
In the cartilage region (Fig. 5-16 a) the second-, third, fifth, sixth, seventh, and ninth-order collagen peaks of collagen type II can be clearly distinguished in the momentum transfer range ($q = 0.10 - 1.0 \text{ nm}^{-1}$). The 4th and 8th orders are less evident being of insignificant intensity). In bone; three peaks are clearly visible; the 1st and the 3rd order of collagen type I ($q \approx 0.05-0.17 \text{ nm}^{-1}$) and in addition a distant peak ($q \approx 1.5 \text{ nm}^{-1}$) is clearly observed. Comparing the two SAXS profiles in Fig.5-16, the cartilage tissue presents relatively sharper peaks, in particular the 1st order peak (64.1 nm). The range of d-spacing is 13.3 nm to 65.8 nm while in the bone profile fewer peaks are observed and d-spacing ranges from 12.9 nm to 63.5 nm. The d-spacing 4.2 nm is obtained from the peak at $q= 1.5 \text{ nm}^{-1}$ (the origin of which is discussed later). In regard to the SAXS intensity profiles for location S2 (Fig. 5-17), for both the cartilage and bone regions, the first-, third and fifth-order collagen peaks are clearly distinguished. The momentum transfers $q$ for cartilage orders range from $q = 0.125$ to $0.468 \text{ nm}^{-1}$ whereas in bone the range is from $0.106 - 0.5 \text{ nm}^{-1}$. 

Figure 5-16: SAXS profile of the intensity versus momentum transfer/ scattering vector $q$, for S1.
Fig. 5-18 depicts the SAXS intensity profile for the third location S3. At this location, similar order peaks to S2 are also observed. However, in cartilage the third and the fifth order peaks are less prominent with d-spacing range (13.0-62.8 nm) and q (0.108-0.428 nm\(^{-1}\)). In bone, a peak with associated d-spacing =4.2 nm and q \(\sim\) 1.5 nm\(^{-1}\) is again observed.

![Image](image_url)

**Figure 5-17:** SAXS profile of the intensity versus momentum transfer/scattering vector q, for S2.
At location S4 (Fig.5-19) for the outer cartilage only, one peak (the 1\textsuperscript{st} order of collagen II) is recorded at \((q \approx 0.14 \text{ nm}^{-1})\) and d-spacing of 44.6 nm, previously unobserved and due to the presence of a phospholipid outer layer forming the protein lubricin that provides the cartilage with its smooth outer surface. In bone the first-, and third-order collagen peaks are present at \((q \approx 0.109-0.50 \text{ nm}^{-1})\). The 1\textsuperscript{st} order peak in cartilage is relatively broad compared with other peaks of bone. In the four scan locations (S1, S2, S3 and S4) the peaks for bone are not as uniform as that of the silver behenate standard.
Figure 5-19: SAXS profile of the intensity versus momentum transfer/scattering vector $q$, for S4.
Sample 2

Regarding SAXS intensity maps and orientation, the same procedures were followed as for sample 1. Fig. 5-20 shows sample 2 and the area in which the scan was taken.

Figure 5-20: A thin section of decalcified human femoral head showing the areas investigated. In this picture the section is in an aluminium holder, sandwiched between Mylar windows and immersed in a thin layer of water to maintain hydration.

The SAXS intensity maps (4.50 x 2.25 mm²) of sample 2 are shown in Fig.5-21. The air interface, the dark region seen to the extreme right edge of the maps, precedes a layer of ligament, then cartilage and then bone. In higher relative intensity maps the microscopic structure features are more highly pronounced. For instance, in the intensity map associated with the scale range (46.3-25.0 nm) the cement line is clearly apparent. Intensity maps (78.1-52.1 nm; 19.5-16.9 nm; 78.1-2.5 nm and 65.8-56.8 nm) partially reveal the molecular features of the bone/cartilage region. In lower intensity maps (9.0-7.7 nm; 6.8-6.4 nm and 6.2-2.5 nm) it is difficult to distinguish any such features in both of the regions, cartilage and bone.
Figure 5-21: SAXS intensity maps of sample 2.
scan_0110: no. 1: 7.8 - 52.1 nm, orientation

scan_0110: no. 2: 46.3 - 25.0 nm, orientation

scan_0110: no. 3: 24.0 - 20.2 nm, orientation

scan_0110: no. 4: 19.5 - 16.9 nm, orientation

scan_0110: no. 5: 11.5 - 10.6 nm, orientation

scan_0110: no. 6: 9.8 - 9.1 nm, orientation

scan_0110: no. 7: 9.0 - 7.7 nm, orientation

scan_0110: no. 8: 6.0 - 7.7 nm, orientation
Figure 5-22: SAXS orientation maps of sample 2.

In order to provide more detailed profiles in regard to locations on sample 2 affected by OA, the orientation of collagen fibres has been investigated and the SAXS orientation maps are shown in Fig. 5-22. The degree of orientation from 0° - 90° is shown as dark blue and dark red respectively. In SAXS orientation maps with low intensities (11.5-10.6 nm; 9.8-9.1 nm; 9.0-7.7 nm; 7.6-7.1 nm; 6.8-6.4 nm and 6.2-2.5 nm) it is difficult to distinguish any orientational features in the bone and cartilage region. Conversely, SAXS orientation maps with high intensities (46.3-25.0 nm; 78.1-2.5 nm and 96.2-83.3 nm) show characteristic non-uniform orientation of the collagen fibres. However, in the deep region of bone the main orientation is approximately perpendicular to the articular surface.
5.2.2 Discussion

The SAXS technique makes it possible to explore nanoscale hierarchical structures in media such as bone with a lateral resolution determined by the SR-x-ray beam size. SAXS also provides direct measurements of morphology, internal structure and orientation of collagen fibres on the nano scale [73, 205, 266]. This makes it the technique of choice in investigating the integrity of various biological tissues such as bone.

Discussion of SAXS results obtained in this investigation will be covered under the following headings:

SAXS intensity maps;
SAXS orientation maps; and
SAXS profiles; (I(q) vs. q) and their relevance to the packing of collagen bundles.

SAXS intensity maps

As is well known, bone is a composite material, consisting of a mineral phase surrounded by an organic fraction. Thus, any contrast in its intensity maps is due to variations in the electron density between these two parts [267]. In the un-mineralized regions, intensity contrasts stem from the difference in the electron densities between organic molecules and air. The 2D intensity maps of sample1 and sample2 (Figs.5-11 and 5-21) show large variations in relative intensity across the length scale. For example, in fig.5-11 the intensity map (96.2-83.3 nm) provides relatively high intensity contrast in both the bone and cartilage regions, while other maps show much less contrast.

Higher SAXS contrast indicates that the tissue is likely to contain an unmineralized part and consequently there are wider variations observed in the electron density, related to structures on the nanometre length scale. In the bone region, for the intensity maps (96.2-83.3 nm) shown in Figs.5-11 and 5-21, the relatively higher intensity of SAXS signals is a good indication of the presence of mineral nanocrystals in the organic matrix. On the same intensity maps (96.2-83.3 nm), noticeable contrasts were observed in both regions of bone and cartilage, these contrasts are also arise from differences in the electron densities between organic molecules and air [267] and, this is consistent with earlier work reported elsewhere [267]. In the cartilage region, the relatively higher intensity of SAXS signals can also be attributed to the presence of minerals and fibrous tissue within the same sample volume [72, 73].

It is interesting to note that for sample1 and sample2 internal features varied across the entire set of 14 maps (Figs.5-11 and 5-21). One plausible explanation for this might be due to their different physical origin; the apex peripheral region of the articular joint is for instance slightly thinner compared with the sides. It is well known that wear and tear primarily initiates at the apex of the joints. As such, the articular cartilage features vary from
one region to another along the cartilage joint [74]. However, variations in the internal structure, could also be due to a more porous nature of the diseased samples and not directly related to their particle size [82]. An earlier explanation to this variation in the internal feature of articular cartilage has been proposed by Bunger and coworkers [82] who indicated that collagen fibres bundles tighten up whereby the internal distances between collagen fibres are reduced. This in turn may affect the internal feature rather than the outer morphology. These changes may however, serve other purposes like the mechanical alignment between areas of different stiffnesses as proposed by Zizak et al.[205] and this may serve as an adaptive response mechanism to OA.

**SAXS orientation maps**

Collagen is a common name for the most abundant form of protein in the human body, being found in bone and connective tissues such as skin and cartilage. The articular joint contains different types of collagen are mainly types I and II. The structural integrity and orientation of collagen fibrils in articulating joints are essential for many physicochemical properties that may affect the function of the articular joint. Thus, any damage/reorientation of collagen fibres may indicate initiation of OA [207]. In OA, it is known that the arrangement of collagen fibrils is altered as a result of collagen network breakdown and consequently collagen becomes more disorganized with the progression of the disease [268]. The use of SAXS techniques in investigating the structure of collagen fibrils [269] has been reported for more than six decades, the collagen structure, organization/ disorganization being highly complex and not completely understood. Herein, the SR-SAXS technique combined with the newly developed highly pixilated detector Pilatus 2M has been used in an attempt to provide more detailed insight into collagen fibre arrangements.

The variations in the direction of predominant orientation and the degree of alignment of the collagen fibres within the examined samples 1 and 2 are shown in Figs.5-13 and 5-22. The dark blue areas refer to zero degree of orientation, indicating that collagen fibres are oriented parallel to the articular surface, while dark red areas correspond to an angle of 90° indicating that these fibres are perpendicularly oriented to the articular surface. Variations in orientation indicate different alignment directions of the collagen fibres at different length scales. For example, in Fig.5-13, the orientation maps (46.3-25.0 nm; 19.5-16.9 nm; 6.2-2.5 nm and 96.2-83.3 nm) show that for the particular sample the orientation of collagen fibres on the surface of the cartilage is approximately 50° gradually increasing to achieve ~ 90° in the middle of the cartilage and then decreasing to angles between ~ 60°-80°. These findings are not in complete agreement with earlier reports for healthy cartilage [143, 270, 271] where a model for collagen fibres arrangement in articular cartilage has been shown (chapter 2, Fig. 2-17). In this model, the orientation of collagen fibres are parallel to the surface in the...
superficial zone, thicker and randomly orientated in the transitional zone and vertically orientated in deeper and calcified zone. The disagreement with this model is in particular in regard to the orientation of the superficial zone which in healthy tissue is expected to be oriented parallel to the articular surface to resist shear stresses. The disorganization in the investigated samples is almost certainly a result of OA as cartilage normally manifests itself as damage to the collagen fibres around the superficial chondrocytes and proceeds deeper into the tissue by means of progressive damage [261]. Further, while the model shows that the calcified zone should be perpendicular oriented to the bone surface. Present findings indicate the collagen fibres to be oriented in this region with angles of 60° to 80°. Other orientation maps (78.1-52.1 nm, 24.0-20.2 nm, 78.1-2.5 nm and 65.8-56.8 nm) of Fig.5-11 show other orientation patterns from those previously referred to (46.3-25.0nm; 19.5-16.9 nm; 6.2-2.5 nm and 96.2-83.3 nm). The orientation in these maps was about 25° -30° in the superficial region of the cartilage, gradually decreasing in degree of orientation, achieving ~ 0° in the middle area of the cartilage. Damage to the collagen network in articular joints has been proposed as an initiating event in the development of OA [272, 273]; as noted, the examined samples (sample1 and sample2) showed the alignment of collagen fibres to be altered, indicating break down of the collagen network. Changes in the zonal orientation of collagen fibrils may reflect the extent of disease severity, the collagen fibres becoming more disorganized with progression of OA [274]. In the bone region, random orientation of collagen fibres is also related to disease stage, this morphological changes usually only being noticed at advanced stages of the disease [275]. Earlier studies by Fratzl et al. [72, 73] demonstrated that the collagen fibres in bone are oriented along the long axis and follow the direction of the trabeculae [210]. The current observations of this study are inconsistent with this established picture [73, 205] and might be explained in light of the fact that samples 1 and 2 investigated in this study were affected by OA.

**SAXS profiles (I(q) vs. q) and their relevance to the packing of the collagen bundles.**

The SAXS technique can also be employed in the investigation of alterations of collagen structures on the nanoscale by determining the d-spacing. Here, the latter can be understood to be the molecular spacing within the tissue and can be defined as the distance (d) between fibre layers [276]. This can be determined from the relation:

\[ n\lambda = 2d \sin \theta \]  

where n is an integer, \( \lambda \) is the wavelength of the X-ray, \( \theta \) is the angle between the incident and reflected rays and d is the unknown distance between layers in the organized medium. In this study, \( \lambda = 12.4 \text{ nm} \), and \( \theta = 5^\circ \).
Collagen fibrils (Type I) are a highly ordered axial arrangement of molecules where neighbouring bundles are staggered by 67 nm [277]. Thus, any alteration in the tissue will change this fundamental periodicity. The results of this study are explained in the light of the published data for wet rat tail collagen (Table 5-4), identifying any alterations in the packing of collagen fibres affected by OA (sample1).

**Table 5-4**: d-spacing values found in sample1 at four locations around the periphery of the joint.

<table>
<thead>
<tr>
<th>Order No.</th>
<th>Collagen type I (Wet rat tail* )</th>
<th>Location (S1)</th>
<th>Location (S2)</th>
<th>Location (S3)</th>
<th>Location (S4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cartilage centre</td>
<td>In the bone</td>
<td>Outer cartilage</td>
<td>In the bone</td>
<td>Outer cartilage</td>
</tr>
<tr>
<td>1</td>
<td>67.0</td>
<td>64.1</td>
<td>62.8</td>
<td>65.8</td>
<td>65.3</td>
</tr>
<tr>
<td>2</td>
<td>33.5</td>
<td>32.1</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>3</td>
<td>22.3</td>
<td>21.4</td>
<td>21.3</td>
<td>22.0</td>
<td>21.5</td>
</tr>
<tr>
<td>4</td>
<td>16.75</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>5</td>
<td>13.4</td>
<td>13.0</td>
<td>x</td>
<td>13.3</td>
<td>12.9</td>
</tr>
<tr>
<td>6</td>
<td>11.6</td>
<td>10.7</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>7</td>
<td>9.6</td>
<td>9.3</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>8</td>
<td>8.4</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>9</td>
<td>7.4</td>
<td>7.3</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

* Taken as standard for comparison in this study, since the examined sample was wet. The symbol x indicating undetected.

By and large, the figures in table 5-4 for (sample1) showed a slight decrease in the axial periodicity between normal collagen type I and that in diseased tissue in the various sites probed (S1, S2, S3 and S4). The figures reported in this study are in contrast with earlier work reported by Fernandez et al. [278] who observed an increase in the axial period of damaged collagen, a matter for which at this time there is no resolution. Thus said it is apparent that variations in d-spacing between diseased and normal tissue (standard) are available for use as a marker for demarcation between normal and diseased tissue.
The relationship between momentum transfer (q) and intensity (I(q)) for SAXS profiles can be employed in the measurement of the arrangement of collagen fibres. As an example of its utilisation, Fig.5-23 shows published SAXS profiles for normal, benign and malignant breast tissues, presenting the collagen peaks and the relationship between q and their relative intensities. This profile will be used as a reference to our present findings. Comparing the SAXS profiles (Figs.5-16, 5-17, 5-18 and 5-19) with SAXS normal profile of collagen Type I taken from healthy breast tissue, the scattering data of diseased tissues showed lower intensities, exponentially decreasing as compared with the healthy curve. The exponential decay behaviour related to the spread scattering component, which is caused by disordered collagen fibres as a result of degeneration of collagen fibres and other component of the extracellular matrix [279]. Further, the scattering intensities of the diseased tissue are relatively higher than the normal; one possible explanation of this is that the diseased tissue is repairing itself [280]. However, an increase of the specific surface area of the scatters in disease has been demonstrated by Fernandez et al. [278] and he attributed that to the higher intensity of damaged collagen, a condition which could not be found in healthy tissue. On the other hand, SAXS profiles reported in this study showed lesser diffraction orders compared to the normal SAXS profiles and higher q(nm⁻¹) values (Table5-5), and this may be due to the lower degree of collagen orientation in damaged tissue [278]. It should be taken into consideration that the examined samples are not adipose-free and this may explain the presence of an anomalous peak (q~ 1.5 nm) which has been reported in the literature to represent the triacylglycerol molecules in adipose tissue [279].

![Figure 5-23: Example of SAXS profiles of normal, benign and malignant breast tissues [281].](image-url)
Table 5-5: momentum transfer q (nm⁻¹) values found in sample1 at four locations around the periphery of the joint.

<table>
<thead>
<tr>
<th>Order</th>
<th>Wet rat tail*</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cartilage</td>
<td>bone</td>
<td>Cartilage</td>
<td>Bone</td>
</tr>
<tr>
<td>1</td>
<td>0.09</td>
<td>0.104</td>
<td>0.104</td>
<td>0.125</td>
<td>0.125</td>
</tr>
<tr>
<td>2</td>
<td>0.18</td>
<td>0.208</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>3</td>
<td>0.28</td>
<td>0.291</td>
<td>0.291</td>
<td>0.291</td>
<td>0.291</td>
</tr>
<tr>
<td>4</td>
<td>0.38</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>5</td>
<td>0.47</td>
<td>0.5</td>
<td>x</td>
<td>0.479</td>
<td>0.479</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>0.587</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>7</td>
<td>0.65</td>
<td>0.687</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>8</td>
<td>0.75</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>9</td>
<td>0.8</td>
<td>0.875</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

* Taken as standard for comparison in this study, since the examined sample was wet. The symbol x indicating not detected.

It is worth while to mention that the current findings are different from other studies concerning the degree of orientation of collagen fibrils. Their values of momentum transfer (q) might be due to methodology and in particular investigated cartilage-bone sections. These sections were washed with deionised water, which may introduce artefacts. Washing in deionised water could affect minerals in contact with the surface of the sample.

In conclusion, with the bourgeoning interest in nano-technology and nano-biology, the need to image smaller scale structures has arisen. SAXS studies of the type herein concern investigation of collagen fibres arrangements. Also for other applications, improvements in spatial resolution are of large importance. For biomedical applications, it is now possible to study damage to bone and cartilage and changes of this on the nanostructural scale as a result of injury or disease.

In this study, it has been possible using the c-SAXS beamline at SLS PSI to use a high-resolution technique in investigating collagen fibres in bone and cartilage.
6 Discussion and Conclusions

The advent of NAT's such as p-PIXE, RBS, PIGE, SR-XRF and SAXS as analytical techniques for various biomedical tissues has lead to them becoming an indispensable means of research at the micro and nanometre scale levels [17, 20, 74, 204, 207, 282-285]. These developments now provide for mapping of relatively large areas from (~ mm²) in such tissues.

The main objectives of this study were an attempt to elucidate some basic features involved in the development and progress of OA in human and animal tissues. The study comprises two main parts. The first involved the determination of distribution of some essential and trace elements at the bone-cartilage interface in healthy and diseased human femoral head sections and a section of equine metacarpophalangeal joint. Interest is in monitoring for any signs that could be used as valuable indicators of the onset and progress of OA, examining cartilage degradation and the relationship between elemental distribution and enzymatic cartilage degradation and quantification of some of the elements present. To achieve this goal µ-PIXE, PIGE and SR-XRF techniques were employed. The quantification of these elements was obtained through use of RBS. The second major part of the work reported herein was the characterization of collagen fibres arrangements and any alterations that might take place during the progress of OA in bone and cartilage by employing SR-SAXS.

In this study special emphasis has been placed on the bone-cartilage interface, it being reported that cations such as Ca, P and Zn play an important role in the normal growth and remodelling of articular cartilage and subchondral bone as well as in the degenerative and inflammatory processes associated with the disease [3, 10, 16, 19, 286]; these cations act as co-factors of a class of articular enzymes known as metalloproteinases, including alkaline phosphatase, and are believed to be active during the initiation, progress and remodelling processes associated with osteoarthritis [9, 16, 204]. Preliminary trials were performed on diseased human femoral head sections to establish the viable use of the in-vacuo scanning µ-PIXE and RBS techniques in the detection of Ca, P, K, S and Zn and beyond that to determine their concentrations in spongy bone. This allowed comparisons to be made between diseased sections and healthy tissues. The elemental maps showed the distribution of elements in both intact and affected areas. Where present findings have been found to be inconsistent with previous studies [9, 11, 16, 204], efforts have been made to define these in terms of sample preparation, technique or disease.

Further investigations on healthy and diseased human femoral heads sections were carried out using PIXE, RBS and PIGE. Enhancements of Ca, P, K and Zn have been observed at the bone-cartilage interface. The findings of these experiments are in accordance
with previous findings by several other authors who have suggested the accumulation of Ca, P, K and Zn to be due to enzymatic activity at the bone-cartilage interface during synthesis and cartilage degradation [4, 10, 19, 204, 236] and also due to new bone formation [3].

The concentrations of some trace and essential elements in spongy bone underlying the subchondral surface have been measured by RBS in selected small areas, including the bone-cartilage interface. Ca/P ratios for healthy and diseased tissues differed to a considerable extra from the stoichiometric value for hydroxyapatite (2.16), the higher Ca/P ratios in this study from diseased sections being suggested to be due to the calcification of Ca with other anions such as (oxalate and hydroxide) while the lower Ca/P ratio could be attributed to additional inorganic phosphate [254] or otherwise an indication of additional phosphate due to the presence of phospholipids [254].

The SR-XRF technique has shown for the first time the changes occurring in collagen fibre organization during the development of OA and in particular at the site of a visible lesion (Fig.5-4). The shape of the surface and calcified zone of articular cartilage has been observed to change and in particular the tidemark and cement line transform from being straight and smooth in surface locations of the sample that precede and follow the lesion to an abraded outer surface at the lesion. Conventional radiography is incapable of allowing visualization of these transformational changes of cartilage on the micrometer scale. It was the advent of high flux techniques such as SR-XRF which have made it possible to obtain high definition imaging of cartilage and bone. In a study by Zoeger et al. [8], they used five human samples with thickness of ~5 mm. They employed backscattered electron (BE) imaging to map the distribution of Ca, Zn, Pb and Sr at the cartilage region and also employed SR μ-XRF to study the trace element distribution at the bone-cartilage interface. Their results regarding the trace elemental distribution at the bone-cartilage interface are in a good agreement with the present study, the presence of elements being enhanced at the bone cartilage interface. The group also obtained spatial information on the elemental distribution. However, their elemental maps were not as clearly defined as those obtained herein (see section 5.1.1), almost certainly due to their use of thick samples, the XRF photons originating from the inner part of the samples being heavily absorbed by the sample. Therefore, the thickness of the tissue is of great importance when using NAT's, an aspect which should be dealt with in detail during preparation of bone and cartilage sections.

The advances in scanning x-ray scattering technologies and in particular the use of the pixelated detectors e.g. the Pilatus family developed at PSI, [229, 230], allow for rapid data acquisition and high resolution imaging making it possible to investigate the molecular and structural features of biological tissues such as bone and cartilage on the nanometre scale. In this study the Pilatus 2M detector was used for the first time in the investigation of collagen fibre arrangements in diseased calcified human femoral head sections. Papers describing the
development of Pilatus detectors have cited the SAXS patterns obtained in our current study and it is evident that the use of this detector has provided for versatile interpretation of a massive amount of data regarding SAXS patterns and their integrated intensity. In particular, the nanoscale disarrangements of articular joint collagen fibre arrangements, investigated using the novel Pilatus 2M detector combined with high brilliance synchrotron x-ray scattering, have lead to final understanding of OA progression.

For detection of the arrangement of collagen fibres using different detection tools, comparison can also be made between present results and that of other authors. As an example, in a study by Camacho et al. [210], using a gas-filled area detector for data acquisition, this concerned the predominant orientation and degree of orientation of collagen fibres in vertebral human bone. It was evident that their SAXS orientation maps gave less detailed features as compared to the present findings (see section 5.2.1). In another study by Bunger et al. [82], despite the improvement in the quality of their SAXS intensity maps, the intensity maps provided less detail to the extent that even the microvascularity within the bone was clearly visible.

With the emergence of interest in the application of nano-scale investigations of biological tissues, it is apparent that SAXS imaging techniques proves to be a promising approach and it is clear that it can be applied in assessing any damage or injury to bone and cartilage fibres affected by disease or injury.

It is also worth mentioning here that the present work required no special treatment of the bone and cartilage sections during sample preparation and storage. In this regard, these sections were left to dry at ambient temperatures in present work while freeze-drying and staining manipulations reported in previous studies in preparation of bone and cartilage tissues might have induced pre-experimental artefacts.

In regard to sources of error during samples preparation is keeping the biological tissues in distilled water for several hours. This may also lead to the leaching out of ions, thus samples must be maintained in isotonic physiological medium. Since our sections were washed and kept in distilled water this might have resulted in the leaching out of some of the ion of interest.

Another aspect of the experimental design was the limited number of bone and cartilage sections used in the study and the medical history and this may not be enough to infer statistically significant results. Thus, in future studies larger number of samples of well-recorded provenance must be examined.

To sum-up, OA is an increasingly important issue in an aging population. Recently good progress has been made in our understanding of OA using advances in the imaging techniques applied herein. The results illustrate that micro-PIXE, RBS and PIGE are powerful analytical techniques for the quantification of elements in bone and cartilage tissues, also
allowing simultaneous mapping of heavy and light elements which may constitute an indicator of bone growth and bone pathology. In this study we have also demonstrated that SR-μXRF is a highly sensitive, non-destructive procedure, capable of mapping micro-areas such as at the bone-cartilage interface, the multi-elemental detection capability enabling the detection of various elements simultaneously. It was also possible to make use of the c-SAXS beamline at SLS to obtain high-resolution orientation mapping of collagen fibres in bone and cartilage.

In summary, through use of Ion beam and synchrotron facilities, elemental and organisational features at the bone-cartilage interface have been investigated at an unprecedented level of detail. It is apparent that both could be sensitive methods in understanding the progression of osteoarthritis. Present work has also established the methodology for such studies, also indicating a number of pitfalls that need to be avoided in ensuring that data are meaningful. Clearly it would be desirable to engage in further such studies, moving to investigations of a greater number of samples, from specimens with which are associated a more complete medical history.
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