JOANNA SIERPOWSKA

Electrical and Dielectric Characterization of Trabecular Bone Quality

Doctoral dissertation

To be presented by permission of the Faculty of Natural and Environmental Sciences of the University of Kuopio for public examination in Auditorium L3, Canthia building, University of Kuopio, on Friday 24th August 2007, at 12 noon

Department of Physics
University of Kuopio
ABSTRACT

Electrical stimulation has been used for decades to stimulate bone growth. However, the relationships between bone structure, mechanical properties, organic composition and electrical properties are still not fully understood. Better understanding of these relationships could enable more accurate estimation of electrical fields in bone during electrical stimulation. Furthermore, measurements of electrical properties may provide diagnostically valuable information about bone quality.

In this thesis, bovine and human trabecular bone were investigated using electrical impedance spectroscopy (EIS) with a parallel-plate capacitance cell in a wide range of frequencies (20 Hz - 5 MHz) (data published as original articles I-IV). These results were supplemented with high frequency measurements (300 kHz - 3 GHz) which were published in this thesis but were not included in the studies I-IV. The additional experiments were conducted with an open-ended probe. The sample preparation procedure and the measurement protocol were designed to preserve the physiological state of the tissue throughout the investigation. As a reference, mechanical properties, microstructure, density and organic composition of trabecular bone were determined.

Strong significant frequency-dependent relations between the electrical characteristics and the density, composition, structural and mechanical properties of trabecular bone were found. The dissipation factor and especially the relative permittivity at frequencies between 100 kHz and 5 MHz were found to be sensitive to variations in human bone quality. Further, these parameters were able to detect mechanically induced damage in trabecular architecture. The surface-specific parameters of trabecular structure were found to be the main determinant of relative permittivity, while the shape and number of trabeculae related primarily to other electrical measures. It was demonstrated that fat and water content can be estimated by a simple measurement of relative permittivity and conductivity at two separate frequency bands. Additionally, bone tissue possessing a healthy dense trabecular structure showed lower conductivity and higher relative permittivity at frequencies between 100 kHz and 5 MHz than tissue with sparse structure.

To conclude, the EIS showed potential as a tool to determine bone characteristics. The relationships presented in this study enable the evaluation of bone electrical properties for the purpose of current and field distribution calculations during stimulated osteogenesis. The method may be feasible during some special cases of open surgery or used in vitro for the quantitative evaluation of bone grafts. It could provide a basis for future improvement of existing electrical impedance tomography techniques towards clinical in vivo bone diagnosis. However, further theoretical, experimental, clinical and technical developments of the method are needed to confirm the present findings and to fully reveal its potential, especially at radiofrequencies.
ACKNOWLEDGMENTS

This study was carried out during 2000-2006 in the Department of Physics, University of Kuopio.

I owe my deepest gratitude to my head supervisor, Professor Reijo Lap-palainen, for the opportunity of working in his research group and guidance. His support and encouragement during this work are greatly appreciated.

I wish to thank my supervisor Docent Juha Töyräs, for his extensive collaboration and for the practical supervision, especially for his constructive suggestions and critical reading of the manuscripts.

I am grateful to my supervisor Professor Jukka Jurvelin, for fruitful discussions and professional guidance.

I express my gratitude to the official pre-examiners Professor Stig Ollmar, and Professor Timo Jämsä, for their constructive criticism, which helped to improve this thesis. I am also grateful to Vivian Michael Paganuzzi for revising the language of the thesis.

I owe my thanks to the Delphin Technologies Ltd company for providing the equipment for high frequency measurements. I am especially grateful to Jouni Nuutinen, for valuable discussions.

I would like to thank all my friends in the Biomaterial research group: Arto Koistinen, Hannu Korhonen, Jari Leskinen, Mikko Selenius, Laura Tomppo, Markku Tiitta, Sami Myllymaa, Siru Turunen, Hanna Matikka, and Esa Miettinen. I am grateful to Juhani Hakala for his technical assistance in constructing the measurement setups. I give special thanks to Ritva Sormunen for the assistance in sample preparation and invaluable companionship. The atmosphere in our group has been inspirational and supportive through these years. I would also like to acknowledge my co-workers in the Biophysics of Bone and Cartilage (BBC) group, especially Mikko Hakulinen for extensive cooperation.

I am indebted to the secretaries of the Physics Departments, especially to Tarja Toivanen for administrative support. Additionally, I would like to thank Heikki Viisänen for the assistance with computer troubles and keeping up my spirits.

I owe my gratitude to Kati Niinimäki and Anna Ruuskanen for companionship and morale support.

I wish to thank Atria Lihakunta Oyj, Kuopio, and their personnel for providing material for the part of the study involving bovine bones.

I am grateful to the Centre for International Mobility (CIMO) and the Graduate School of Musculoskeletal Diseases and Biomaterials Graduate School for their financial support.

Finally, I wish to thank my parents, Bożena and Zenon, friends and relatives for their encouragement. I owe my warmest thanks to my loving Risto for his understanding and faith during this work.

Kuopio, August 2007

Joanna Sierpowska
NOTATION

\begin{itemize}
\item \( A \) \quad \text{area}
\item \([A]\) \quad \text{tensor}
\item \( C \) \quad \text{capacitance}
\item \( D \) \quad \text{surface charge density}
\item \( d \) \quad \text{distance}
\item \( E \) \quad \text{Young’s modulus}
\item \( F \) \quad \text{force}
\item \( f \) \quad \text{frequency}
\item \( G \) \quad \text{conductance}
\item \( j \) \quad \text{imaginary unit}
\item \( K^* \) \quad \text{complex cell factor}
\item \( m \) \quad \text{unit vector}
\item \( n \) \quad \text{number of specimens}
\item \( L \) \quad \text{length}
\item \( P_L \) \quad \text{number of intersections}
\item \( P_p \) \quad \text{areal density}
\item \( p \) \quad \text{statistical significance}
\item \( p \) \quad \text{structure point}
\item \( R \) \quad \text{resistance}
\item \( r \) \quad \text{correlation coefficient}
\item \( S \) \quad \text{cross-section area}
\item \( t \) \quad \text{time}
\item \( \tan \delta \) \quad \text{dissipation factor}
\item \( U \) \quad \text{resilience}
\item \( u \) \quad \text{amplitude of voltage}
\item \( X \) \quad \text{reactance}
\item \( x \) \quad \text{variable of integration}
\item \( x_c \) \quad \text{center of sphere}
\item \( \bar{Y}^* \) \quad \text{complex admittance}
\item \( \bar{y} \) \quad \text{mean}
\item \( Z \) \quad \text{impedance}
\item \( Z_{sp} \) \quad \text{specific impedance}
\item \( Z_0 \) \quad \text{characteristic impedance}
\item \( Z^* \) \quad \text{complex impedance}
\item \( \alpha \) \quad \text{distribution parameter of Cole-Cole model}
\item \( \beta \) \quad \text{attenuation coefficient}
\item \( \Gamma^* \) \quad \text{complex reflection coefficient}
\item \( \gamma^* \) \quad \text{complex propagation constant}
\item \( \delta_0 \) \quad \text{number of bone particles}
\item \( \delta_1 \) \quad \text{connectivity}
\item \( \delta_2 \) \quad \text{number of cavities}
\item \( \epsilon \) \quad \text{strain}
\item \( \epsilon^* \) \quad \text{complex permittivity}
\item \( \epsilon, \epsilon' \) \quad \text{permittivity (real part)}
\item \( \epsilon'' \) \quad \text{loss factor (imaginary part)}
\item \( \epsilon_r \) \quad \text{relative permittivity}
\end{itemize}
\(\varepsilon_0\)  permittivity of vacuum
\(\eta\)  trabecular thickness
\(\theta\)  phase angle
\(\kappa\)  stress
\(\lambda\)  wavelength
\(\sigma, \sigma'\)  conductivity (real part)
\(\tau\)  relaxation time
\(\nu\)  standard deviation
\(\varphi\)  angle
\(\chi\)  Euler number
\(\omega\)  angular frequency
\(\Omega\)  structure volume

**ABBREVIATIONS**

2D  two dimensional
3D  three dimensional
AC  alternating current
BMD  bone mineral density
BS  bone surface
BS/BV  bone surface-to-volume ratio
BV  bone volume
BV/TV  trabecular bone volume fraction
CV  coefficient of variation
CT  computed tomography
DA  degree of anisotropy
DC  direct current
DXA  dual energy x-ray absorptiometry
DNA  deoxyribonucleic acid
EDTA  ethylenediaminetetraacetic acid
EIS  electrical impedance spectroscopy
FC  femoral head
FG  femoral groove
FLC  femoral lateral condyle
FMC  femoral medial condyle
FTM  femoral greater trochanter
GAG  glycosaminoglycans
\(\text{max}\)  maximum
\(\text{min}\)  minimum
MIL  mean intercept length
NMR  nuclear magnetic resonance
PC  principal component
PCA  principal component analysis
PBS  phosphate buffered saline
Res  resilience
ROI  region of interest
\(s\text{CV}\)  standardized coefficient of variation
SMI  structure model index
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tb.Th.</td>
<td>trabecular thickness</td>
</tr>
<tr>
<td>Tb.Sp.</td>
<td>trabecular spacing</td>
</tr>
<tr>
<td>Tb.N.</td>
<td>trabecular number</td>
</tr>
<tr>
<td>TMP</td>
<td>tibial medial plateau</td>
</tr>
<tr>
<td>TV</td>
<td>tissue volume</td>
</tr>
<tr>
<td>US</td>
<td>ultimate strength</td>
</tr>
</tbody>
</table>
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred in the text by their Roman numerals (I-IV):


The original articles have been reproduced with permission of the above copyright holders.
1 Introduction

2 Structure and composition of trabecular bone
  2.1 Trabecular bone microstructure .............................................. 20
  2.2 Trabecular bone composition .................................................. 22

3 Mechanical properties of trabecular bone
  3.1 Mechanical testing of bone .................................................... 25
  3.2 Determinants of trabecular bone mechanical properties ................. 26
  3.3 Damage of trabecular structure .............................................. 27

4 Computed tomography of bone structure ........................................ 29

5 Electrical impedance spectroscopy (EIS) of bone
  5.1 Introduction to the relaxation theory ...................................... 33
  5.2 Microwave frequency measurements ........................................ 38
  5.3 Limiting Factors of EIS ....................................................... 40
  5.4 Electrical characteristics of trabecular bone .......................... 41

6 Aims of the present study .................................................................. 45

7 Materials and methods
  7.1 Sample preparation ................................................................. 47
  7.2 EIS methods
    7.2.1 Low frequency impedance spectroscopy ............................... 49
    7.2.2 Microwave frequency impedance spectroscopy (supplementary data) 51
  7.3 Bone mineral density measurements ......................................... 53
  7.4 Mechanical testing ................................................................. 53
  7.5 Microstructural analysis ........................................................ 54
  7.6 Compositional analysis ............................................................ 54
  7.7 Statistical analysis ................................................................. 54

8 Results
  8.1 Variation and frequency dependence of electrical properties .......... 58
  8.2 Relations between electrical and mechanical properties ............... 59
  8.3 Relations between electrical and structural properties ................. 61
  8.4 Relations between electrical and compositional properties ............ 65

9 Discussion ..................................................................................... 67

10 Summary and conclusions .................................................................. 75

References ......................................................................................... 77

Erratum ............................................................................................. 91

Appendix: Original publications
Bone is a connective tissue which extracellular components are calcified yielding a hard and rigid substance. Its constituents are organized on all hierarchical levels to provide elasticity and high tensile and compressive strength together with minimal weight and use of material [1]. As such, bone provides mechanical support and protection for the vital organs of the body, including the brain, spinal cord and structures within the thoracic cavity. Furthermore, bone enables locomotion by serving as levers for the muscles and tendons. In addition to its mechanical function, bone acts as a store of inorganic salts that can be easily released to maintain balanced concentration of certain ions in body fluids. In particular, the skeleton stores about 99% of the body calcium [2]. Moreover, bone houses marrow tissue, which is a hemopoietic organ.

Wolff’s law [3] states that “under the direction of functional pressure the given form of bone changes by placing or displacing its elements” [4] and gives the theoretical basis of bone remodeling. The adaptation of a bone to external forces requires a strict control system [5]. The discovery of the piezoelectric properties of bone by Fukada and Yasuda [6] led to speculation that remodeling could be governed by endogenous electrical potentials. “Yasuda’s hypothesis” suggests that the cellular response leading to remodeling is caused by electrical potentials generated in the tissue as a consequence of applied stress. Further, the hypothesis suggests that osteogenesis is stimulated by negative potentials while positive potentials result in bone resorption. This theory led to three main branches of research.

The first branch concentrated on investigating whether cellular responses leading to osteogenesis can be stimulated by externally applied electrical potentials without simultaneous application of mechanical stress. This research resulted in a number of clinical applications of the theory, including treating nonunions [7, 8], promoting spinal fusions [9, 10] and distraction osteogenesis [11, 12]. Investigations of the mechanisms underlying electrical and electromagnetic stimulations are currently underway [13, 14], especially at the cellular level [15, 16, 17, 18].

The second branch focused on the validity of Yasuda’s hypothesis. Some investigators (e.g., [19]) argue that endogenous electrical currents are not involved in bone remodeling but this process is directly modulated by mechanical strains. Further, it has been proposed that the effect of exogenous electromagnetic fields on bone remodeling is secondary. It might modulate the activity of
other factors, including mechanical strains. However, to the present date, the physiological significance of endogenous electrical signals is not fully understood [19]. Proof of the hypothesis would require a difficult separation of electrical and mechanical stimuli and should demonstrate that cellular response is caused by electrical potentials experienced by cells during mechanical stress but is not caused by the stress itself [20].

The third branch is the most fundamental, uniting the other two. It focuses on determining and understanding the electrical properties of bone. By construction of an advanced theoretical model of bioelectric phenomena in bone that could enable objective investigation of the "Yasuda's hypothesis," the electrical environment of bone cells must be understood and values of electrical and magnetic fields and current densities in the vicinity of cells must be known. This knowledge could also clarify the uncertainties in the electrical characteristics of the bone-electrode interfaces and in the current paths between electrodes during stimulated osteogenesis [21, 22].

The fast development of techniques that expose humans to electromagnetic radiation has led to the foundation of electromagnetic dosimetry. This new branch of biomedical science deals with the calculation of internal fields within exposed tissues. A three-dimensional anatomical model of a human body has been created to calculate specific absorption rate [23], electrical fields at the extremely low frequencies [24, 25] and to assess the effect of electrostatic discharges on the human body [26]. As the permittivity and conductivity of a tissue play a dominant role in the overall consideration of interactions between field and matter [23], electromagnetic dosimetry requires knowledge of the exact values of the electrical properties of the various tissues at all frequencies to which a human is exposed [27, 23]. The electrical properties of bone have been of particular interest in investigations of the possible interrelation between leukemia and exposure to strong electrical fields [26].

Similarly, during the image reconstruction of electrical impedance tomography, a non-invasive and inexpensive imaging method [28], all tissues must be accurately characterized electrically in order to obtain the maximum sensitivity. Electrical properties of bone are of major importance in brain imaging, as the electrodes are placed on the skull (see e.g., [29]). However, a strong variability or the lack of appropriate data along with the systematic errors associated with the measurement technique make both the dosimetry model and accurate image reconstruction difficult to realize.

Measuring electrical properties could provide valuable diagnostic information about bone quality, too. Impedance measurements have been shown to be efficient in certain cases of open surgery, where traditional techniques are not feasible [30, 31]. For example, during the limb lengthening with the Ilizarov technique both the frequency and rate of distraction have to be controlled in order to avoid premature fusion or insufficient bone formation [32, 33]. The rate of new bone formation cannot be controlled by plain radiography as it is not sensitive enough to detect the small changes in the newly formed bone. In contrast, electrical impedance measurements have been shown to be sensitive to changes occurring during the bone healing process [31]. By investigating the interrelations between the electrical properties and mechanical or structural characteristics of bone, it might be possible to further develop this technique towards more comprehensive monitoring of the tissue quality and clinical applications.
In this thesis, the electrical and dielectric properties of trabecular bone were measured using a wide frequency range (20 Hz - 3 GHz) and compared with systematically determined mechanical, microstructural and compositional parameters. The main aim was to clarify the effect of the mechanical, structural and compositional characteristics of bone tissue on its electrical and dielectric properties. We aimed at gathering the relevant data in order to contribute to the understanding of Yasuda’s hypothesis, to provide the information needed for modeling and to test the sensitivity of the impedance technique in determining bone characteristics.
1. Introduction
Structure and composition of trabecular bone

Long bones consist of a shaft, i.e., diaphysis, located between two heads, i.e., epiphysis [34]. The epiphysis is covered by hyaline cartilage forming an articular surface. The middle portion of the shaft, called the medullary cavity, is filled with bone marrow. Short bones (e.g., the carpal bones of the wrist) are approximately cube-shaped with nearly equal vertical and horizontal dimensions. Flat bones are plate-like, flat and thin, e.g., the bones forming the skull cap. Bones with shapes that do not fit into any of above mentioned groups, such as bones that contain air spaces, e.g., ethmoid bones, are called irregular. Sesamoid bones (e.g., patella), embedded within certain tendons, reduce the ability of tears to propagate across the tendon and improve the mechanical performance of the muscle across a joint.

Two different types of bone tissue can be distinguished. The spongy-like meshwork of the epiphysis and the inner part of the medullary cavity and short and flat bones is called trabecular bone (Figure 2.1). The dense structure forming a cortex around the trabecular tissue and the diaphysis of long bones is called compact bone. The distinction between the two tissues is made on the basis of the porosity of the structure. Compact bone has a porosity of 5% - 10%, while the porosity of a trabecular bone is 75% - 95% [35]. However, as bone is a dynamic porous structure, its porosity may change. A change in porosity can result from a pathological situation or from a normal adaptive response to a mechanical or physiological stimulus [1].

All bone surfaces are covered by a thin layer of connective tissue called periosteum, except at places where muscle and tendons insert the bone [2] or where they articulate with another bone. The periosteum consists of outer layer of collagen fibers and fibroblasts and the inner cellular layer [36]. When the bone is actively growing, the inner layer consists of osteoprogenitor cells, whereas when there is no active bone formation, the inner layer is not well developed. It consists of few cells capable of dividing and differentiating into osteoblasts [34]. The periosteum is attached to the bone by the bundles of periosteal collagen fibers, called Sharpey’s fibers, that penetrate the bone matrix. The endosteum, composed of a small amount of connective tissue and a single layer of flattened osteoprogenitor cells, covers the internal cavities of bone, including the marrow spaces within trabecular bone. Under an appropriate stimulus, the cells of endosteum can differentiate into osteoblasts [34]. The main function of both
Figure 2.1: Bone is a connective tissue with calcified extracellular components. The shaft is formed by a dense compact bone. A spongy-like meshwork of trabecular tissue, arranged in the form of plates or struts called trabeculae, occupies the epiphysis. Trabeculae consist of parallel layers of an anisotropic matrix called lamellae. Lamellae, in turn, contain uniformly spaced cavities that encompass bone cells, or osteocytes. Bone marrow can be found in the medullary cavity and in the spaces between trabeculae.

membranes is to nourish the bone tissue and provide it with osteoblasts, cells necessary for the repair and growth [36].

Bone is a dynamic structure. It is removed and formed by means of specialized cells, osteoclasts and osteoblasts, throughout life. This process of constant modification of tissue architecture to meet physical stress, to repair microscopic damage and prevent the accumulation of fatigue damage is called remodeling [37, 1]. It has been estimated that the total remodeling of bone takes around 4 months, with resorption lasting about 3 weeks and formation about 3 months [1]. Osteoclasts and osteoblasts function on the tissue surface, as they are the size of trabeculae and thus cannot tunnel within trabeculae without damage [1]. Trabecular bone turnover, or the rate of remodeling, is about 25 % a year. It varies, however, with skeletal site and the person’s age.

2.1 Trabecular bone microstructure

In trabecular bone, the calcified tissue is arranged in the form of plates or struts called trabeculae, around 200 µm thick [1], creating numerous interconnected cavities. Those cavities are filled with bone marrow.

Trabecular bone matrix

Trabeculae are composed of a mosaic of lamellar bone pieces [38], which is a highly organized material. It consists of parallel layers of an anisotropic matrix composed of mineral crystals and collagen fibers, called lamellae. Lamellae
2.1 Trabecular bone microstructure

contain uniformly spaced cavities, lacunae, that encompass osteocytes. Those cavities are spread in all directions, penetrate the lamellae and connect with neighbouring lacunae. Calcified bone matrix is impermeable and osteocytes cannot be nourished or can metabolites be removed through diffusion. These processes occur through thin cylindrical spaces, called canaliculi, that perforate the matrix and extend to the endosteal surface of the trabeculae. All nutrients are supplied from the marrow cavity. However, a sufficiently thick trabeculae can contain osteons [34]. An osteon is a structural unit consisting of concentric lamellae of bone matrix surrounding the central osteonal canal, which contains nerves and blood vessels.

Cells of bone tissue

There are four types of bone cells: osteoprogenitor cells, osteoblasts, osteoclasts, and osteocytes.

Osteoprogenitor cells are located in the periosteum, endosteum and osteonal canals. In mature bone with no active remodeling processes, osteoprogenitor cells become flattened and spread closely on the bone surface. However, in growing bone or during periods of high tissue turnover these cells become much larger and more numerous. Osteoprogenitor cells are derived from embryonic mesenchyme [2] and, as stem cells, are capable of differentiating into specialized bone-forming cells, i.e., osteoblasts [37]. Moreover, it has been suggested [34] that under certain stimuli these cells can modify their morphological characteristics and function to differentiate to adipose cells, chondroblasts or fibroblasts. Osteoprogenitor cells take part in the healing processes of fractured bones and are involved in creating cartilage and connective tissues in the callus [34].

Osteoblasts are differentiated bone-forming cells that constitute a closely packed sheet on the surface of the bone. Active osteoblasts have cuboidal or columnar shapes. As their activity declines, they become flat and resemble osteoprogenitor cells. Bone remodeling and changes in the growth of the tissue are activated by osteoblasts as a response to mechanical stimuli [34]. The cells secrete collagen and the ground substance that constitute the unmineralized bone, or osteoid. Initially, osteoid forms a layer that separates osteoblast from calcified bone, but as the secreted matrix accumulates, osteoblasts become buried within and transform into osteocytes. This process results in the formation of a lacuna, a cavity occupied by an osteocyte, its extensions and a small amount of noncalcified matrix. The calcification process, or the deposition of calcium salts into osteoid, completes the bone formation. The apposition rate, or the speed of osteoid formation, is about 1 µm/day [1].

Osteocytes are derived from osteoblasts and are situated in lacunae within calcified matrix. The cavity of lacunae conforms to the shape of the cell [34]. Osteocytes communicate with neighbouring cells by cytoplasmic processes housed in canaliculi. Cells receive nutrients through the extracellular substance located between osteocytes, cytoplasmic processes and the bone matrix. The main function of osteocytes is to maintain the bone matrix. They also help to maintain homeostasis of blood calcium by synthesizing and resorbing bone matrix. The resorption of the bone by osteocytes that release calcium ions is called osteocytic osteolysis. Because of the small size of osteocytes, lacunae and canaliculi, they occupy only about 1% of bone volume. However, there are about 15000 lacunae per cubic millimeter of bone and the surface area of canaliculi in an adult male
2. Structure and composition of trabecular bone

is about 1200 m$^2$ (trabecular bone surface area is about 9 m$^2$) [1].

Osteoclasts are large cells, 150 $\mu$m in diameter [2], with multiple nuclei. They are responsible for bone resorption, which occurs at the rate of tens $\mu$m per day [1]. The cells first demineralize adjacent bone matrix with acids and subsequently dissolve collagen with enzymes [1]. Osteoclasts can be found in enzymatically etched depressions called resorption bays. The resorption of the bone occurs through finger-like dynamic processes called ruffled borders. They are connected to the portion of the cell which creates a microenvironment for bone resorption called a clear zone [36]. Ruffled borders reflect the activity of the osteoclasts and thus bone resorption rate. The lack of ruffled borders can lead to osteopetrosis, a disease characterized by dense bone [36].

2.2 Trabecular bone composition

As an organ of the skeletal system, bone consists of calcified bone tissue, blood vessels, hemopoietic, fat and nerve tissues. In the synovial joints, articular cartilage covers the heads of bones.

Osteoid, *i.e.*, the bone extracellular matrix, may be lamellar, *i.e.*, deposited in regular parallel sheets, or woven, *i.e.*, deposited haphazardly. Bone formed from lamellar osteoid is strong and healthy. In contrast, tissue synthesized on the basis of woven osteoid is comparatively weak and pathological [37]. Osteoid is composed of organic and inorganic components. These two components comprise 35% and 65% of bone dry weight, respectively [38, 2]. Collagen embedded in a supporting gel containing specific proteins constitutes the organic phase. Deposition of the inorganic phase in the form of mineral salts into this gel provides bone with rigidity and functional strength [37].

Collagen type I constitutes 90% of the organic phase of bone matrix. It is highly cross-linked and organized into strong fibres typically 50 – 70 nm in diameter [38, 2]. It also provides substrate for bone mineral crystals nucleation [38]. The gel, or ground substance, wherein the collagen is embedded, contains proteoglycan aggregates and several specific glycoproteins. Glycoproteins are covalently bound to short proteoglycan cores and are responsible for promoting bone matrix calcification [34, 37, 36].

Calcium and phosphorous, along with other components such as magnesium, sodium and potassium, constitute the inorganic phase of bone matrix. Calcium and phosphorous form hydroxyapatite crystals $Ca_{10}(PO_4)_6(OH)_2$ in a rod-like form with a length 40 nm, width of 25 nm and thickness of 1.5 – 3 nm. The crystals reside mainly in gaps roughly 40 nm long and 25 nm wide formed by collagen fibers [38]. Additionally to crystal hydroxyapatite, calcium phosphate is also present in amorphous form. In adults, the mineral phase constitutes about 65% of the fat-free dry weight. However, in individuals suffering from *e.g.*, osteomalacia, which is characterized by poorly calcified pathological bone, the mineral content may be as low as 35% [38].

About 60% of the water in the calcified bone matrix is free and about 40% is bonded to molecules [1]. The surface ions of hydroxyapatite are hydrated, facilitating the exchange of ions between the crystals and the body fluids [36].

Nonmineralized spaces within trabecular bone contain marrow, which is highly cellular soft tissue consisting of the precursors of blood cells, macrophages and adipose tissue [38]. The main function of marrow is to generate blood cells [1]. In infants, all bones contain red hemopoietically active marrow. At the age
of 4-5 years the number of blood-forming cells decrease with a simultaneous increase in adipose cells. As the amount of adipose tissue grows, the colour of the marrow changes from red to yellow [38]. Under an appropriate stimulus, such as extreme blood loss, yellow marrow can be converted into red [34]. In adults, red marrow can be found in the proximal ends of the humerus and femur, and in the vertebrae, ribs, sternum and ilia of the pelvis [38].
2. Structure and composition of trabecular bone
3.1 Mechanical testing of bone

In three dimensions, stress $\kappa_{ik}$ on the plane $i$ along the direction $k$ can be defined as follows:

$$dF_i = \sum_{k=1}^{N} \kappa_{ik} dA_k,$$  \hspace{1cm} (3.1)

where $dF_i$ is the force acting on a small element represented by a vector $dA_k$ which has a length equal to the area of the element, is perpendicular to it and faces outwards. Stress can be categorized, based on the manner how the force is applied, into compressive, tensile or shear. Compressive stresses are developed as material is compressed along its length. A deformation of an object in such a way that one region is shifted parallel with respect to the adjacent one will generate a shear stress.

Strain $\epsilon$ is defined as a relative change in the sample dimension. When bone is deformed in length, its width will normally change: for instance, when compressively loaded, the tissue will bulge. The ratio of transverse to axial strain is called Poisson’s ratio.

A compressive test is a common way to determine the mechanical properties of a material. In this test a sample is compressively loaded, and the strain is recorded as a function of the stress applied, $\kappa(\epsilon)$ (Figure 3.1). The stress-strain curve can be divided into two parts: the elastic and plastic deformation region. Within the elastic, or linear, region, the deformation increases linearly with increasing load, and when the load is released the sample retains its original shape. However, due to the presence of the fluids in the bone matrix, some of the elastic energy is lost, causing viscous effects during the deformation [39].

The slope of the linear response is known as Young's modulus. The area under the linear region of the stress-strain curve is called resilience, $U$:

$$U = \int_{\epsilon_{\text{yield}}}^{\epsilon_{\text{yield}}} \kappa(\epsilon) d\epsilon.$$  \hspace{1cm} (3.2)

Resilience is a measure of the capacity of a material to store energy while elastically deformed and can be interpreted as the resistance of an elastic material to deform under an applied force. At yield point the elastic deformation ends
and a material starts to deform plastically. Strains within the plastic strain region are called postyield strains. They represent permanent deformations of bone structure induced by trabecular microfracture, crack growth, slip at cement lines or a combination of these [39, 40]. Ductility represents the amount of postyield strain that a material is able to sustain before fracture. In contrast to a ductile material, a brittle material can support no or only minor post-yield strain and, thus, is liable to fracture.

If the load continues to increase, the maximum stress a material can withstand, or ultimate strength, is reached. As such, strength is an intrinsic property of bone, i.e., its values do not depend on the size and shape of a sample [39]. The ultimate strength of trabecular bone on the tissue level varies from 1 MPa to 20 MPa and is strongly dependent on apparent density and trabecular orientation [41, 39]. As the load continues to increase, bone does not fracture into pieces and the stress does not fall to zero, but is followed by a plateau. Bone marrow spaces collapse and trabeculae are compacted against one another. When all void spaces are filled with fractured bony matrix, the stress rises again.

3.2 Determinants of trabecular bone mechanical properties

The mechanical properties of trabecular bone are determined by the material properties of the tissue in the individual trabeculae, tissue apparent density and the anisotropy of the trabecular structure [42, 1].

Figure 3.1: Stress-strain ($\kappa - \epsilon$) behavior of trabecular bone. The curve is divided by a yield point into elastic and plastic strain regions. Yield stress, $\kappa_y$, and yield strain, $\epsilon_y$, are recorded at the yield point. Young’s modulus, $E$, is defined as the slope of the linear part of the stress-strain curve. Resilience represents the amount of energy stored during elastic deformation. Ultimate strength, $\kappa_{max}$, marks the point where the fracture of bone occurs. The $\epsilon_0$ denote the zero strain.
The material properties of the tissue on the trabecular level, i.e., the degree of mineralization of the extracellular matrix and the variations in canalicular porosity, influence the mechanical performance of whole bone [43]. Even though trabecular and compact bone are considered to consist of the same tissue [3, 44], there are some differences in the elastic modulus. The largest value of Young’s modulus, 14.8 GPa, reported in the literature for individual trabeculae is smaller than the frequently quoted modulus for dense cortical bone, $E = 17.1$ GPa [43, 45].

Young’s modulus and strength depend strongly on the apparent density [46] and thus on the bone volume fraction. The dependence between Young’s modulus and density has been reported to be cubic [44] or squared [47] for multiple anatomical sites analyzed together, or linear for a single site when a sample is loaded along the main trabecular orientation [48, 49, 50]. These differences may be a consequence of the imperfections introduced during mechanical testing or of the neglect of the importance of anisotropy. However, bone volume fraction alone cannot completely explain the variation in elastic properties. Both Young’s modulus [51, 52] and the strength [50, 51] of trabecular bone have been found to depend on the orientation, spacing, number and size of trabeculae [53, 46] as well as on the anisotropy of these variables [1]. Consistent with Wolff’s law [54] anisotropy is a form of adaptive response to functional loading. Trabecular bone has been reported to display orthotropic symmetry\(^1\) [55, 56], or in some cases transverse isotropy\(^2\) [57]. For instance, the mean value of Young’s modulus of human vertebral bone in the superior-inferior direction is 3.4 times higher than that in the transverse directions [51, 46]. Remarkably, bone volume fraction together with trabecular orientation and anisotropy is able to explain about 90% of the variation in mechanical properties [58].

In addition, organic composition was found to be imbalanced in certain diseases such as osteoporosis [59, 60, 61]. Thus, it has a significant impact on tissue mechanical properties. In particular, collagen has been shown to be a significant determinant of bone toughness [62]. The orientation of the collagen fibers relates to the tissue strength in such a way that longitudinal fibers promote strength in tension, while transverse fibers promote strength in compression [1, 63, 64].

### 3.3 Damage of trabecular structure

During the course of daily activities skeletal tissue is subjected to repetitive cyclic loading. A fatigue failure may occur if this loading is of sufficient magnitude or duration. Compressive stress fractures in the proximal femur, calcaneus, and proximal tibia are the most common fatigue fracture sites observed clinically within trabecular bone [65]. The physical damage that occurs with overloading will not start with a fracture of entire trabeculae, but with subtle damage within them [46, 66, 67]. Cracks extending only over a part of trabeculae are called microcracks [65] and can be as small as 10 µm in length [68]. They can be repaired by microcallus formation. On the other hand, fatigue microfractures, or complete fractures of individual trabeculae, are confined to elements

---

\(^1\)An orthotropic material is an anisotropic material that possesses different properties in each of three perpendicular directions [39].

\(^2\)Transversely isotropic material is an orthotropic material whose properties are the same in two of the three directions, i.e. in a plane [39].
oriented transversely to the loading direction. They originate from age-related femoral neck fractures, aseptic necrosis of the femoral head or degenerative joint diseases. They might trigger trabecular bone remodeling.

Fractured trabeculae are likely to be completely resorbed by osteoclasts [4,5] and may not be replaced during tissue repair [4,6,7]. Hence a microfracture weakens the tissue by decreasing the load-carrying capacity of the trabecular structure [69] and has a permanent detrimental effect on bone volume fraction, microarchitecture, and its mechanical properties [70]. The resistance of trabeculae to fracture is determined by their ductility which, in turn, is governed by collagen cross-linking [71, 70].
Modern high resolution computed tomography (microCT) methods enable the gathering of three-dimensional images of trabecular bone with micrometer resolution. Based on the cross-sections of an object obtained by means of X-ray radiation, a virtual 3D model of the specimen is reconstructed nondestructively. In the following, a microCT technique and computational methods for determining quantitative structural parameters are introduced.

The analysis of the 3D images begins with separating hard tissue from soft tissues. For this purpose, a segmentation of the original gray-scale data set is introduced and a single CT number is selected with a histogram technique [72]. Voxels with a CT value higher than the predefined threshold are interpreted as bone whereas the other voxels are defined as soft tissues. The morphological parameters can be determined from such a segmented data set using various algorithms [72, 73, 74, 75, 76]. For instance, a "marching cubes" method has been suggested for modeling the bone surface using continuous triangles (surface triangulation) [77, 78]. As the segmentation method affects significantly the calculated values of structural parameters [72, 74, 79], it has been suggested that using local threshold values instead of global ones may improve the segmentation [80].

The tissue volume (TV) is the volume of the entire sample [81]. It can be calculated by multiplying the total number of voxels within a sample, both solid and void, by voxel volume. Bone volume (BV) is calculated similarly to TV, but only voxels of the solid phase are counted. BV can be normalized with TV to obtain the bone volume fraction (BV/TV).

Bone surface (BS) is defined as the total area of the triangles. In order to enable the comparison of surfaces among samples with different sizes, BS can be normalized with BV to obtain a surface-to-volume ratio, BS/BV. This parameter characterizes the complexity of bone structures.

The degree of anisotropy, DA, reflects the orientation of trabeculae, their main direction and the degree of dispersion around it [82]. It is a dimensionless quantity defined as a ratio of eigenvalues of primary and tertiary direction [82, 58].

The degree of anisotropy can be determined using mean intercept length (MIL) analysis [83]. A circular region of interest (ROI) is defined on each 2D microCT slice. The areal density, \( P_r \), of the ROI is calculated as the ratio of
the number of bone pixels to the total number of pixels in the analysis region [58]. A reference direction is defined. A grid of parallel test lines is sent through the ROI at an angle \( \phi \) with respect to a reference direction. For each test line the number of intersections between the hard and soft tissues is counted. Subsequently, the number of intersections per unit length of test line, \( P_L(\phi) \), is calculated by dividing the total number of intersections for all test lines by the total lines length. Thus the MIL for trabecular bone is defined as follow:

\[
MIL(\phi) = \frac{2P_0}{P_L(\phi)} \quad [58]
\]

As the grid of test lines is rotated of \( \Delta \phi \), the MIL can be determined as a function of the angle MIL(\( \phi \)), where \( 0^\circ \leq \phi < 180^\circ \). A polar plot of MIL(\( \phi \)) versus the angle \( \phi \) can be fitted to an ellipse from which the number of trabeculae in a certain direction can be found.

For 3D analysis, a spherical ROI is defined and a grid of lines is superimposed over this volume in a large number of planes with different angles. The MIL for each plane is calculated and a polar plot of MIL versus angle is constructed. The uneven length of the MIL will result in an elongated, not spherical, line distribution. Using a statistical fit, an equation for the ellipsoid can be determined:

\[
\frac{1}{MIL^2(m)} = m^T[A]m,
\]

where \( m \) is the unit vector in the direction of interest, and \([A]\) is the orthogonal second rank symmetric anisotropy tensor formed from the coefficients of the ellipsoid equation (Figure 4.1). The principal structural directions and magnitudes can be determined by solving the eigenvalue problem for the tensor \([A]\).

The degree of anisotropy is defined as the maximum (MIL\(_{max}\)) to minimum (MIL\(_{min}\)) MIL ratio:

\[
DA = \frac{MIL_{max}}{MIL_{min}}
\]

The mean trabecular thickness, \( \bar{\eta} \), can be determined as an average of the local thickness of the individual trabeculae [84, 73]:

\[
\bar{\eta} = \frac{1}{V(\Omega)} \int \int \int_\Omega \eta(\vec{z})d^3\vec{z},
\]

where

\[
V(\Omega) = \int \int \int_\Omega d^3\vec{z}
\]

and \( \Omega \) is the set of points in the trabecular structure under investigation. The local thickness for a point in solid is defined as the diameter of the largest sphere which contains the point (\( \vec{p} \)) and fits completely inside the trabeculae [73] (Figure 4.2). Trabecular bone volume can also be characterized as a distribution of thicknesses. For instance, for a specimen showing a large variation of trabecular thickness, \( i.e., \) having thick plates interconnected with thin rods, the thickness distribution will be widespread. In contrast, a homogenous structure with plates and rods of almost constant thickness will yield a narrow distribution [73].
Figure 4.1: Using a statistical fit, an ellipsoid can be fitted to the 3D distribution of mean intercept length.

Figure 4.2: Local thickness for a point $p$ in solid structure $\Omega$ is defined as the diameter $d$ of the largest sphere with the center in $x$, which contains the point $p$ and fits completely inside the trabeculae.

The trabecular separation is the thickness of the marrow spaces between trabecular structures. It can be calculated by applying the same technique as used for the trabecular thickness calculation to the non-bone parts of the 3D image [84].

The trabecular number, $Tb.N.$, is defined as a number of intersections with solid per unit length of a linear path through the trabecular structure. It can be calculated from the following equation:

$$Tb.N. = \frac{BV/TV}{Tb.Th}$$

(4.6)
Connectivity reflects the degree to which a structure is interconnected [85]. It is an important parameter, reflecting tissue integrity. For instance, a decrease in connectivity in osteoporotic bone may reflect a structure for which the restoration of original architecture will be difficult [86]. Trabecular bone can be represented as a node-and-branch network, where more than one path exists between any two nodes (unlike in the case of a tree). A number of branches may be cut in this structure without separating the network. In contrast, in the case of a tree, cutting one branch will split the unit into two parts [76]. The maximum number of trabecular connections that can be ruptured without dividing the structure is defined as connectivity [87]. It can be interpreted as the number of trabeculae minus one [76].

The Euler number $\chi$ is a determinant of connectivity:

$$\chi = \delta_0 - \delta_1 + \delta_2,$$

(4.7)

where $\delta_0$ is the number of disconnected bone pieces, $\delta_1$ is the connectivity, and $\delta_2$ is the number of marrow cavities enclosed in bone matrix [87]. Equation (4.7) can be simplified, assuming that the trabecular bone matrix is one interconnected structure ($\delta_0 = 1$), and that the marrow is placed in one main marrow space, and thus no marrow cavities are enclosed within the bone ($\delta_2 = 0$) [76]:

$$\chi = 1 - \delta_1.$$

(4.8)

Equation (4.8) holds for all cases except bone during formation and healing, where isolated tissue parts may exist [85].

The structure model index (SMI) reflects shapes in the trabecular structure, i.e., plate-like, rod-like or a combination of the two. An ideal plate structure displays the SMI value of zero, while for an ideal rod structure the value is three. The intermediate values depend on the rods-to-plates volume ratio [88]. Osteoporosis of trabecular bone is characterized by a transition of trabeculae from plate-like to rod-like shapes [75]. Determination of SMI requires an artificial addition of one voxel of thickness to all binarised object surfaces, or dilation. It can be calculated as follows:

$$SMI = 6 \left( \frac{BS' \cdot BV}{BS^2} \right),$$

(4.9)

where $BS$ is the trabecular surface area before dilatation, $BS'$ is the change in the surface area after dilatation and $BV$ stands for the undilated volume of the trabeculae [89].
Electrical impedance spectroscopy (EIS) of bone

The bulk electrical properties of tissues determine the pathways of current flow through the body [90]. They are essential in research on the interaction mechanisms of electromagnetic waves with biological systems [90], especially in calculating the specific absorption rate and its distribution in animal and human models [91]. They enable the measurement of physiological parameters using impedance techniques. Further, the theoretical description of electrocardiography, muscle contraction, nerve transmission and electrosurgery would not be feasible without detailed knowledge of the electrical parameters [90].

5.1 Introduction to the relaxation theory

If a material is placed in the parallel-plate capacitor of a plate area \( A \) and separation \( d \), then an electrical field between the plates will induce a charge density. The passive electrical properties are characterized by capacitance \( C \) and conductance \( G \) that can be calculated as follows:

\[
G = \frac{A\sigma}{d} \tag{5.1}
\]

and

\[
C = \frac{A\varepsilon}{d} = \frac{A\varepsilon_r\varepsilon_0}{d}. \tag{5.2}
\]

Conductivity \( \sigma \) describes the readiness of charged particles to move through a material under the influence of an electrical field. The factor \( \varepsilon_0 \) is the permittivity of free space \( (8.854 \cdot 10^{-12} \text{ F m}^{-1}) \), \( \varepsilon \) is the permittivity of a material and \( \varepsilon_r \) is the permittivity of a material relative to the permittivity of free space. Permittivity indicates the extent to which charge distribution can be polarized when an electrical field is applied. In biological materials the charges are associated with either polar molecules or electrical double layers near membrane surfaces or solvated macromolecules [92].

Each polar or polarisable particle in a material exhibits characteristic responses to the applied electrical field. The field disturbs the system. After the excitation has ceased, the system relaxes to a new equilibrium. Relaxation in the time domain can be described by a relaxation time \( \tau \). This parameter depends on the polarization mechanisms in a material. The shortest \( \tau \) within times of
a picosecond is connected to electronic polarization. On the other hand, the polarization of large organic molecules such as proteins can take up to seconds.

The Debye relaxation model describes a simple case. If a material excited by a step voltage at time $t = 0$ possesses only one relaxation process with a single characteristic time constant $\tau$, then the surface charge density on a capacitor plates $D$ is given by:

$$D = D_\infty + (D_0 - D_\infty)(1 - e^{-t/\tau}). \quad (5.3)$$

The instantaneous response of a system is included in $D_\infty$. This parameter is a surface charge density just after the step voltage has been applied ($t = 0^+$) and arises from electronic polarization. In contrast, $D_0$ is the charge density long after the application of the step, when a system has obtained a new equilibrium. The response of this system in a frequency domain can be obtained by a Laplace transform:

$$\varepsilon^* = \varepsilon' - j\varepsilon'' = \varepsilon_\infty + \frac{\varepsilon_s - \varepsilon_\infty}{1 + j\omega\tau}. \quad (5.4)$$

The factor $\varepsilon_\infty$ denotes permittivity measured at a high frequency where the polar or polarisable particles are not able to respond to the applied electrical field. Static permittivity $\varepsilon_s$ is the limiting low-frequency permittivity where the polarization is fully manifested (Figure 5.1A). It must be emphasized that parameters $\varepsilon_\infty$ and $\varepsilon_s$ are used at frequency extremes, and thus are real: $\varepsilon_\infty = \varepsilon'_\infty$ and $\varepsilon_s = \varepsilon'_s$ [93]. The factor $\omega$ is the angular frequency of the electrical field.

**Figure 5.1**: Dielectric dispersion of pure water at 20°C [92].
5.1 Introduction to the relaxation theory

The real part of the complex permittivity $\varepsilon'$, corresponding to the permittivity in equation (5.2), can be expressed in the form:

$$\varepsilon' = \varepsilon_{\infty} + \frac{\varepsilon_s - \varepsilon_{\infty}}{1 + (\omega\tau)^2}. \quad (5.5)$$

Dielectric loss, or the imaginary part of the complex permittivity $\varepsilon''$, reflects the losses associated with the movement of charges in phase with the electric field. It is related to the in-phase (lossy) component of the conductivity:

$$\varepsilon'' = \frac{\sigma'}{\omega\varepsilon_0} = \frac{(\varepsilon_s - \varepsilon_{\infty})\omega\tau}{1 + (\omega\tau)^2}. \quad (5.6)$$

The characteristic relaxation frequency $f_c$ corresponds to the maximum value of the dielectric loss factor $\varepsilon''$ ($\omega\tau = 1$) and is given by:

$$f_c = \frac{1}{2\pi\tau}. \quad (5.7)$$

Using an appropriate model for the Debye single relaxation model, the complex conductivity can be determined. In particular, the real (lossy) part is given by:

$$\sigma' = \frac{(\varepsilon_s - \varepsilon_{\infty})\omega^2\tau\varepsilon_0}{1 + (\omega\tau)^2} = \frac{(\sigma_{\infty} - \sigma_s)(\omega\tau)^2}{1 + (\omega\tau)^2}. \quad (5.8)$$

The above equation follows from the fact that the limit values $\varepsilon_s$, $\varepsilon_{\infty}$, $\sigma_s$ and $\sigma_{\infty}$ (Figure 5.1B) are interrelated as follows:

$$\sigma_{\infty} - \sigma_s = \frac{(\varepsilon_s - \varepsilon_{\infty})\varepsilon_0}{\tau} = 2\pi f_c (\varepsilon_s - \varepsilon_{\infty}). \quad (5.9)$$

Equation (5.9) is a special case of the Kramers-Kronig relation that interrelates permittivity and losses in the system. It states that the permittivity and conductivity of a material cannot vary independently; any decrease in permittivity with increasing frequency yields an increase in conductivity. This relation can be explained by the fact that for a given voltage the total energy of the electric field is constant. It is either stored or dissipated by the material the field is interacting with. The real part of a complex permittivity ($\varepsilon'$) expresses storing of the energy, while the imaginary part ($\varepsilon''$) reflects the losses. Equation (5.9) holds only for a system with a single relaxation time. However, any material with a relatively small spread of relaxation times can be approximated by this simple form of the Kramers-Kronig relation.

The ionic dc conductivity $\sigma_{dc}$ is often added to the equation (5.8) even though it does not represent a relaxation mechanism [93]:

$$\sigma' = \sigma_{dc} + \frac{(\sigma_{\infty} - \sigma_s)(\omega\tau)^2}{1 + (\omega\tau)^2}. \quad (5.10)$$

A Cole-Cole plot or a plot of $\varepsilon''$ against $\varepsilon'$ with frequency as a parameter yields a semicircle with a center on the $\varepsilon'$ axes (Figure 5.2). It intersects the $\varepsilon'$ axes at points $\varepsilon_{\infty}$ and $\varepsilon_s$. A similar plot can be obtained for conductivity. The maximum values of $\varepsilon''$ and $\sigma''$ correspond to the characteristic frequency $f_c$.

In contrast to the simple Debye model of a material with a single relaxation time, biological systems are characterized by a multiple relaxation processes...
(corresponding to charge densities $D_1$, $D_2$, ...), usually each with different relaxation times ($\tau_1$, $\tau_2$, ...):

$$D = D_\infty + D_1(1 - e^{-t/\tau_1}) + D_2(1 - e^{-t/\tau_2}) + ...$$  \hspace{1cm} (5.11)

Also, the center of the Cole-Cole semicircle is shifted below the $\varepsilon'$ axes (Figure 5.2). To meet this problem, a simple modification in Debye’s equation has been made by Cole and Cole [94]:

$$\varepsilon^* = \varepsilon_\infty + \frac{\varepsilon_s - \varepsilon_\infty}{1 + (j\omega \tau)^{1-\alpha}}.$$  \hspace{1cm} (5.12)

The $\alpha$-parameter in the above equation is purely experimental and thus lacks a proper physical or molecular basis [92]. For most biological materials the $\alpha$-parameter falls in the range between 0.3 and 0.5 [90]. For compact bone this parameter was found to be 0.6 [95].

![Figure 5.2](image-url)  

**Figure 5.2:** The complex permittivity plot of the Cole-Cole equation (5.12) [90, 93]. The dotted line corresponds to the single Debye relaxation.

The general form of a Kramers-Kronig relation for a material with multiple relaxation times is given by:

$$\varepsilon'(f) - \varepsilon_\infty = \frac{2}{\pi} \int_0^\infty \frac{x \varepsilon''(x)}{x^2 - f^2} dx$$
$$\varepsilon''(f) = \frac{\sigma_s}{\omega \varepsilon_0} = -\frac{2f}{\pi} \int_0^\infty \varepsilon'(x) - \varepsilon_\infty \frac{1}{x^2 - f^2} dx,$$  \hspace{1cm} (5.13)

where $x$ is the variable of integration. This relation holds for dielectric materials with any distribution of relaxation times, even if the system displays a resonance behavior [90].
5.1 Introduction to the relaxation theory

The electrical relaxation in tissues can be described in terms of only three parameters: the dielectric increment $\varepsilon_s - \varepsilon_\infty$, the relaxation time $\tau$ (alternatively characteristic frequency $f_c$) and the $\alpha$-parameter [92]. Dispersion is the corresponding concept to relaxation in the frequency domain [93]. Electrical permittivity in biological tissues decreases with increasing frequency. It is a consequence of different electrical charges not being able to follow the changes in the applied electrical field. Three major drops in the permittivity value, or dispersions, are observed, named consecutively the alpha, beta and gamma dispersions (Figure 5.3). In addition, a tissue may exhibit a small dispersion between 0.1 and 3 GHz called a delta dispersion [90]. Clearly separated single dispersions are present typically in cell suspensions. In contrast, tissues display much broader and overlapping dispersions, occasionally in the form of a continuous fall almost without plateaux and over a wide range of increasing frequencies [93].

![Figure 5.3: The value of permittivity decreases with increasing frequency. Three major drops are distinguished: the alpha ($\alpha$), beta ($\beta$) and gamma ($\gamma$) dispersions.](image)

In the alpha dispersion region, relative permittivity attains values as large as $10^6 - 10^7$ [90, 93]. This can be explained by e.g., counterion effects or charging of intracellular organelles [90] and does not mean that the capacitive properties of living tissue are dominant [93]. On the other hand, the alpha dispersion is hardly noticeable in the conductivity. Tissue conductivity is small compared with background conductivity. Theoretically the increase in conductivity at 100 Hz associated with the alpha dispersion would be about 0.005 Sm$^{-1}$, provided that the value of permittivity is around $10^6$. However, the ionic conductivity of typical tissues is about 200 times higher. It follows that at low frequencies, despite the enormous permittivity values, the impedance of most tissues is resistive [90]. For beta and gamma dispersions the associated increase in conductivity is more pronounced (Table 5.1).

It is worth emphasizing that dispersion is related to the electrical and physical behavior of molecules, as opposed to such analytical techniques as nuclear
magnetic resonance (NMR), which examine molecular structure [93]. These analytical methods are based on resonance phenomena. In contrast, dielectric spectroscopy below 1 GHz deals with relaxation, routinely presented as a non-resonance event. Of course, if the frequency is sufficiently high, a resonance might be observed. For instance, the resonance frequency for deoxyribonucleic acid (DNA) molecules is in the lower gigahertz region [93].

5.2 Microwave frequency measurements

A high inductance of electrode leads makes the use of a parallel-plate measurement cell at frequencies above 10 MHz problematic. Although a method allowing an accuracy of the order of 1% up to frequencies of 100 MHz has been designed [96], it is accepted that the classical techniques should not be used for high radiofrequencies and microfrequencies [97].

Measurements at above 10 MHz are based on the transmission-line theory. A transmission line is characterized by its characteristic impedance \( Z_0 \). It is defined as a ratio of the longitudinal current to the transverse voltage in the line. The voltage and current waves move along the line in opposite directions with a complex propagation constant \( \gamma^* \):

\[
\gamma^* = \beta + j\frac{2\pi}{\lambda},
\]

where \( \beta \) is the attenuation coefficient and \( \lambda \) is the wavelength. When the transmission line is terminated with a load that has impedance different from \( Z_0 \) then the waves will be reflected at the point of the discontinuity. A complex reflection coefficient \( (\Gamma^*) \) can be defined as the ratio of the amplitude of the incident voltage \( (u_e) \) to the the amplitude of the reflected voltage \( (u'_e) \) at the reflection point:

---

Table 5.1: Dielectric dispersions [90, 93].

<table>
<thead>
<tr>
<th>Type</th>
<th>Characteristic frequency</th>
<th>Mechanism</th>
<th>Increase in conductivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )</td>
<td>( MHz - kHz )</td>
<td>Counter ions effects, Ionic diffusions, Active membrane effects, Charging of intercellular structures</td>
<td>( \sim 0.005 \text{ Sm}^{-1} )</td>
</tr>
<tr>
<td>( \beta )</td>
<td>( 0.1 - 100 MHz )</td>
<td>Capacitive charging of cellular membranes, Dipolar orientation of tissue proteins</td>
<td>( \sim 0.4 \text{ Sm}^{-1} )</td>
</tr>
<tr>
<td>( \gamma )</td>
<td>( 0.1 - 100 GHz )</td>
<td>Dipolar mechanisms in polar media such as water salts and proteins</td>
<td>( \sim 70 \text{ Sm}^{-1} )</td>
</tr>
<tr>
<td>( \delta )</td>
<td>( 0.1 - 3 GHz )</td>
<td>Dipolar relaxation of water, Rotational relaxation of polar side chains, Counterions diffusion along small regions of charged surfaces</td>
<td>( \sim 0.5 \text{ Sm}^{-1} )</td>
</tr>
</tbody>
</table>
5.2 Microwave frequency measurements

\[ \Gamma^* = \frac{u_e}{u'_e}. \]  

(5.15)

The impedance of the load, \( Z_e \), can be expressed in terms of the characteristic impedance of the line and the reflection coefficient:

\[ Z_e = \frac{U_e}{I_e} = \frac{u_e + u'_e}{Z_0 - \frac{u_e}{Z_0}} = \frac{Z_0(1 + \Gamma^*)}{1 - \Gamma^*}, \]

(5.16)

where \( U_e \) and \( I_e \) are the voltage and current through the load, respectively. It follows from equation (5.16) that it is possible to determine the impedance of the load from the measurement of the reflection coefficient, \( \Gamma^* \).

**Figure 5.4**: The microwave frequency measurements are conducted with a coaxial probe consisting of one layer of insulating material between two conductors. The probe is connected to the network analyzer through a coaxial cable (A). A coaxial probe can be represented electrically as a parallel connection of two capacitors (B).

The function of an open-ended coaxial line at frequencies where its inner and outer diameters are small compared with the wavelength of the electromagnetic field, can be represented as a parallel connection of two capacitors (Figure 5.4):

\[ C_T = C_f + \varepsilon^*C_0. \]  

(5.17)

In the above equation, \( C_T \) is the total capacitance, which is independent from the electrical properties of the load. The capacitance \( C_f \) is called the fringe capacitance and represents the energy of the field inside the line. The terminal capacitance, \( \varepsilon^*C_0 \), represents the energy inside the load, or the material under investigation. The term \( C_0 \) is the terminal capacitance of the line in the air and \( \varepsilon^* \) is the complex permittivity of the dielectric material. On the basis of the model depicted in Figure 5.4 and on equation (5.16), \( \varepsilon^* \) can be determined.

The complex admittance can be written:

\[ Y_e^* = \frac{1}{Z_e} = j\omega(C_f + \varepsilon^*C_0). \]  

(5.18)

Hence, when the reflection coefficient is measured and \( C_0 \) and \( C_f \) are determined, \( e.g. \), from the calibration measurements, the complex permittivity of a material can be calculated from:
When a lossy material, e.g., trabecular bone, terminates the coaxial line, then the term $\varepsilon^* C_0$ also contains a conductance representing the dielectric losses according to the equation (5.6):

$$\varepsilon^* = \varepsilon' - j\varepsilon'' = \varepsilon' - \frac{j\sigma'}{\omega\varepsilon_0}.$$  
(5.20)

Thus, the conductivity can be obtained from:

$$\sigma' = \varepsilon'' \varepsilon_0 \omega.$$  
(5.21)

The measurements can be performed in the frequency or time domain. In the former case, the excitation is produced by a radiofrequency generator. In the latter case a pulse generator with a very short rise time is used for excitation. The reflection coefficient is measured at the input of the line by a network analyzer.

### 5.3 Limiting Factors of EIS

The major limiting factors of impedance spectrometry include the polarization impedance of the measurement electrodes, the geometry of the measuring cell and the material used for the electrode.

**Polarization impedance.** During the measurement, the acquisition of the potential and injection of the current into the sample occurs through the electrodes. However, due to the finite impedance of the electrode material, complex electrochemical reactions can occur at the interface between the tissue and electrode, leading to polarization phenomena. As these phenomena may introduce errors in the measurements, the polarization impedance and low frequency limit above which the measurements can be carried out with sufficient signal-to-noise ratio shall be determined [97]. High polarization impedance is especially pronounced in a system where the same set of electrodes is used to inject current and measure the voltage.

When a metal is placed into an electrolyte, two current transport mechanisms occur [93]. First, when the charges are moved across the electrode-tissue interface, a faradic current will result. Second, a non-faradic, or capacitive, current will originate from the electrical double layer that acts as a capacitance. Due to the molecular scale distance between layers, the capacitance per unit area is large. The current across the electrode interface will charge this capacitance and results in its polarization. The combination of the faradic and non-faradic mechanisms acts as a high-pass filter [97]. For signals with relatively small amplitude and frequencies approximately above 100 Hz, the polarization impedance $Z_p$ can be represented as a resistance $R$ and reactance $X$ in series:

$$Z_p = R - jX = R + \frac{1}{j\omega C}.$$  
(5.22)

The polarization impedance decreases with increasing frequency. Its value depends on the electrode material and the nature of the electrolyte. The series
resistance in equation (5.22) decreases as the frequency increases. The magnitude of the capacitance depends on the surface condition of the electrode and is lowest for polished surfaces and highest for roughened ones [98].

**Cell factor.** The use of simple mathematical tools for analyzing the measurement system, such as Ohm’s law, is possible when the recorded signals are determined solely by the input stimulation, the electrical parameters of the investigated material and the geometry of the electrode system. However, this assumption of linearity is fulfilled only when the electrical field is uniform and undisturbed by objects placed in the measurement cell [97]. The errors introduced by the measurement cell can be determined by investigated a phantom material as a function of frequency and the geometry of the cell. The complex cell factor $K^*$ can be represented as

$$K^* = K' + jK'' = \frac{Z_{pha}}{Z_{th}},$$

(5.23)

where $Z_{th}$ is the theoretical impedance of the phantom and $Z_{pha}$ is the impedance of the phantom measured in the cell of cross-section $S$ with electrodes separated by $d$:

$$Z_{pha} = \frac{1}{\sigma_{pha}} \frac{d}{S}.$$  

(5.24)

The tissue impedance $Z_{tissue}^*$ can be estimated from the measured impedance spectrum $Z^*$ corrected by the cell factor:

$$Z_{tissue}^* = Z^*/K^*.$$  

(5.25)

**Electrode material.** The polarization impedance $Z_p$ is strongly affected by the electrode material [97]. For a given electrolyte, enlarging the electrode surface area decreases the cut-off frequency of the high-pass filter equivalent to the polarization impedance. Additionally, the risk of infections or allergies due to the toxic or catalytic effects of the electrode surface must be taken into account.

Silver-silver chloride electrodes are the most common in electrophysiological measurements. They exhibit a stable potential and are easy to manufacture in any desired shape [97]. However, the potential may change with exposure to light, and the chloride coating can be easily removed by abrasion.

Stainless steel electrodes are cheap and easy to use. However, they exhibit a high polarization impedance at low frequency. The frequency characteristic can be improved by increasing the roughness of the electrode, e.g., by grinding with sand paper [99, 100]. The roughness of the electrode can disappear with time, and after a few measurements the polarization impedance approaches that of polished stainless steal.

The polarization impedance of platinum electrodes is lower than that of other materials such as stainless steel or silver [101, 102]. If metal electrodes cannot be used, then porous carbon fiber electrodes have been suggested as an alternative [103].

### 5.4 Electrical characteristics of trabecular bone

The frequency dependence of bone electrical properties [104, 105] is comparable to that of other tissues with low cellular content, e.g., ligaments [104]. The
relatively low water content of these tissues might be the main reason for the differences in permittivity values between tissues with low and high cellular content [104].

A significant contribution to relative permittivity is expected from the polarization of ions near charged surfaces in the tissue. A large number of interfaces, e.g., between lamellae, fibrils and in many small capillaries that penetrate the tissue, affect electrical properties. The polarization of membranes within the tissue and fluid-solid interfaces has been hypothesized to have a lesser effect [90].

The electrical properties of compact bone have been widely studied [106, 107, 108, 109, 110, 111, 112, 95, 104, 113, and others]. The tissue has been investigated in a dehydrated [114] state as well as in a range of moisture content [115]. To obtain results resembling those of in vivo conditions, bone tissue has been measured wet [116]. Also, there have been some attempts to conduct measurements in vivo [30, 117, 118, 119]. Recently, Skinner et al. [31] reported that the healing process during corticotomy and distraction in the Ilizarov technique can be followed by impedance spectroscopy.

In contrast to the extensive data existing on compact bone, few reports have been published on trabecular bone (e.g., [120, 104, 121]). Moreover, many studies have just hypothesized the interpretation of results leaving certain phenomena unexplained. Despite the fact that compact and trabecular bone are quite different in physiological state (due to e.g., differences in porosity and the inclusion of marrow in trabecular tissue), some findings obtained in investigations of compact bone are applicable to trabecular bone. The conclusions drawn from studies of dried tissue (e.g., [114]) serve as a good example. The calcified matrixes of compact and trabecular bone are very similar (see Chapter 2). However, the findings should be carefully interpreted when describing fluid-saturated matrix. The electrical behavior of hydrated matrix containing water bound to its molecules differs from the behavior of a dry network. Further, the results obtained in experiments on decalcified compact tissue [122] or as a function of immersion fluid [112] are useful in analyzing trabecular bone, remembering that it is the interaction between different tissue components and their internal microstructure that govern the electrical properties.

The electrical properties of different bone components, such as bone marrow [123, 21], collagen [124], hydroxyapatite [125] and water [126] have been studied. The combination of these components is responsible for the overall electrical properties of trabecular tissue, and thus the data should be used with care and interactions between the phases should not be omitted. Typical values of relative permittivity and conductivity for bone and marrow in physiological state are presented in Table 5.2 and Table 5.3. Due to the large variability in low-water-content tissues, no single value of permittivity and conductivity can be presented [123]. A range of values or an approximate number is given, as the exact composition of the tissue is not known.

Trabecular bone was found to be electrically transversely isotropic. Relations between the electrical properties in different directions have been studied [104]. In particular, resistivity in longitudinal $R_L$ direction was related to resistivity in anterior-posterior direction $R_{AP}$ as follows:

$$R_{AP} = -1.70 + 1.23R_L.$$ (5.26)
5.4 Electrical characteristics of trabecular bone

Table 5.2: Typical values of relative permittivity for bone and marrow in physiological state.†

<table>
<thead>
<tr>
<th>Type of tissue</th>
<th>Species</th>
<th>10 kHz</th>
<th>1 MHz</th>
<th>10 MHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trabecular bone</td>
<td>Human distal tibia [121, 104]</td>
<td>600 - 800</td>
<td>~600</td>
<td>~100</td>
</tr>
<tr>
<td></td>
<td>Bovine femur [120]</td>
<td>~6000</td>
<td>~500</td>
<td>~100</td>
</tr>
<tr>
<td></td>
<td>Ovine skull [127]</td>
<td>-</td>
<td>~800</td>
<td>~100</td>
</tr>
<tr>
<td>Compact bone</td>
<td>Human distal tibia [104]</td>
<td>~1050</td>
<td>~500</td>
<td>~80</td>
</tr>
<tr>
<td></td>
<td>Bovine femur [120, 111, 128, 27]</td>
<td>600 - 1000</td>
<td>60 - 110</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Ovine skull [127]</td>
<td>-</td>
<td>~500</td>
<td>~60</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Calf femur and tibia [123]</td>
<td>~1000</td>
<td>~100</td>
<td>~50</td>
</tr>
</tbody>
</table>

† Values estimated from graphs.

Table 5.3: Typical values of conductivity (Sm$^{-1}$) for bone and marrow in physiological state.†

<table>
<thead>
<tr>
<th>Type of tissue</th>
<th>Species</th>
<th>10 kHz</th>
<th>1 MHz</th>
<th>10 MHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trabecular bone</td>
<td>Human distal tibia [121, 104]</td>
<td>0.2 - 0.5</td>
<td>0.2 - 0.5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bovine femur [120]</td>
<td>~0.07</td>
<td>~0.07</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Ovine skull [127]</td>
<td>-</td>
<td>~0.09</td>
<td>~0.2</td>
</tr>
<tr>
<td>Compact bone</td>
<td>Human distal tibia [104]</td>
<td>~0.05</td>
<td>~0.06</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Bovine femur [120, 111, 128, 27]</td>
<td>0.05 - 0.5</td>
<td>0.05 - 0.6</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Ovine skull [127]</td>
<td>-</td>
<td>~0.3</td>
<td>~0.8</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Calf femur and tibia [123]</td>
<td>~0.2</td>
<td>~0.2</td>
<td>~0.3</td>
</tr>
</tbody>
</table>

† Values estimated from graphs.

Some attempts have been made to explain these variations in terms of trabecular orientation. It has been hypothesized that an increase in the density of a healthy bone results in an increase in the area of trabeculae in all directions. However, in specimens obtained from diseased persons, such as osteoporotic bone, horizontal trabeculae are selectively resorbed. Thus, a pathological condition of trabecular bone may be reflected by the altered directional relations in electrical properties [104].

In the frequency range between 100 kHz and 1 MHz specific capacitance has been shown to correlate highly and significantly with wet density [121]. De Mercato and Garcia-Sanchez [120] have suggested that trabecular bone conductivity is a consequence of the tissue microstructure and liquid content. The permittivity of trabecular bone is additionally affected by bone marrow, which exhibits its own dielectric behavior. Moreover, the relative contribution of bone matrix to the total impedance has been found to be more significant at higher frequencies (i.e., above 100 kHz) [121, 120].

The electrical properties of bone are affected by various factors, e.g., moisture content, temperature, immersion fluid, and the age and disease state of the person from whom the specimen was obtained, in the following ways.

Moisture content. It has been suggested that the electrical behavior of a material is connected to the rotational mobility of water molecules [105].
An increase in conductivity of almost an order of magnitude per percent of moisture content has been found [129]. Possibly at 98% relative humidity, only the small pores of bone are filled with fluid, leaving the large pores empty [130]. Thus, if the tissue is allowed to dry, only solid phase and perhaps a thin layer of water absorbed on it is measured. Already a 5-minute exposure of bone tissue to air already affects significantly the measured electrical values. For instance, resistance may increase by 92% [105, 131]. On the other hand, in most measurement geometries the surface conductivity changes due to free liquid can increase the apparent conductivity of the sample and lead to erroneous results.

**Temperature.** The electrical properties of bone have been found to be temperature dependent [104, 92, 132]. In particular, conductivity increases with increasing temperature [133].

**Immersion fluid.** The electrical properties of fluid-saturated wet bone are influenced by the properties of the fluid in the pores [105]. For instance, a deviation in pH by ±2 from the neutral solution (pH = 7.0) can change the measured value of resistance by 70% [131, 105]. Moreover, the permittivity of compact bone has been found to exhibit a dispersion with a relaxation frequency proportional to the conductivity of the immersion fluid [112].

**Age.** Little information is available on age-related changes in the electrical properties of bone [104]. However, it is believed that the chemical changes that occur in bone with increasing age may affect its electrical properties [104, 108, 134]. Peyman et al. [135] reported a decrease in the values of the dielectric properties of rat tissue in the microwave frequency range due to changes in the water content and organic composition of tissue.

**Species and bone status.** The electrical response is different in human and in bovine bone. Bovine bone is generally more compact, dense and brittle than human bone [105]. Various bone diseases affect bone microstructure and chemical composition, e.g., osteoporosis [136, 137] or osteogenesis imperfecta [138]. However, at present little information is available on the quantitative effects of these factors.
The electrical and dielectric properties of bone, especially human trabecular bone, and their relations with structure, composition and mechanical properties are poorly known. It has been suggested that electrical stimulation increases bone growth and repair after fracture. Better knowledge of the determinants of trabecular bone electrical properties would make it possible to calculate the electrical field and currents during stimulation. Furthermore, knowledge of these relations might make it possible to use electrical measurement in the evaluation of bone composition, structure and mechanical properties in situ.

To clarify these issues, the present study aimed to:

1. Determine the optimal electrical and dielectric parameters for the assessment of bone mechanical, structural and compositional properties and mineral density.

2. Reveal the most sensitive frequency range for the prediction of the mechanical, structural and compositional properties of human trabecular bone.

3. Investigate the interrelationships between electrical quantities and trabecular bone mechanical properties including evaluation of the EIS potential to detect bone microdamage.

4. Understand the role of the composition and microstructure of bone in creating its electrical properties as well as describing a number of correlations between the impedance parameters of bone and a variety of parameters related to bone structure and composition.

5. Provide a basis for the development of a possible method of bone evaluation in vitro or in situ, as well as for the improvement of the existing EIT technique towards clinical in vivo bone diagnostics.
6. Aims of the present study
Four studies (I-IV), supplemented with the data obtained from the measurements at frequencies above 5 MHz (not included in the original articles I-IV), constitute the present thesis work. The study design is summarized in Table 7.1.

### 7.1 Sample preparation

Specimens for Study I were obtained from bovine proximal and distal femur within few hours post mortem. After slaughter at the local abattoir (Atria Lihakunta Oyj, Kuopio, Finland), an entire femur was excised. Soft tissues were removed and the epiphysis was cut out. Forty cylindrical trabecular bone samples (diameter: 25.4 mm, height: 14.2 mm) were prepared from four anatomical locations: femoral lateral condyle (FLC; \( n = 9 \)), femoral medial condyle (FMC; \( n = 11 \)), femoral head (FC; \( n = 10 \)) and femoral greater trochanter (FTM; \( n = 10 \)) (Figure 7.1). The plugs were drilled in the medio-lateral direction using a hollow drill bit. The faces of the samples were cut parallel in the sagittal plane using an Isomet low-speed diamond saw (Buehler, Lake Bluff, IL, USA). After preparation, the samples, immersed in PBS, were sealed in plastic bags, frozen \((-20^\circ C)\) and thawed prior to measurement.

For Studies II-IV, knee joints from thirteen human cadavers (age = 28 – 58 years, 1 female, 12 males) were obtained from Jyväskylä Central Hospital with the permission of the Finnish National Authority for Medico/legal Affairs (TEO, 1781/32/200/01). After the knees were excised, the soft tissues were removed and the distal femur and proximal tibia were wrapped in a bandage moistened with saline, sealed in plastic bags and stored in a freezer \((-20^\circ C)\) for about 12 months. The joints were thawed before sample preparation. Twenty six samples (diameter: 16 mm, height: 8 mm) were drilled from trabecular tissue of the femoral medial condyle (FMC, \( n = 10 \)), tibial medial plateau (TMP, \( n = 10 \)) and the femoral groove (FG, \( n = 6 \)) in a direction perpendicular to the articular surface (Figure 7.1), i.e., along the preferred loading direction. The end-faces of the resulting cylindrical plugs were cut parallel with a micro-grinding system (Macro Exakt 310 CP, Exakt, Hamburg, Germany). The samples were subsequently immersed in phosphate buffered saline (PBS) in a plastic tube, frozen \((-20^\circ C)\) and thawed prior to measurement.

For the bovine and human samples, an increase in temperature and drying were prevented during the cutting process by spraying the sample with PBS.
Table 7.1: Summary of materials and methods.

<table>
<thead>
<tr>
<th>Study</th>
<th>Material</th>
<th>n</th>
<th>Electrical testing</th>
<th>Reference method and parameters</th>
<th>Other methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Bovine proximal and distal femur</td>
<td>40</td>
<td>20 Hz - 5 MHz $\varepsilon'$, $\varepsilon''$, $\theta$, $\sigma'$</td>
<td>Mechanical testing $E$, $\sigma_y$, $\sigma_{max}$, $Res$</td>
<td>BMD$_{vol}$</td>
</tr>
<tr>
<td>II</td>
<td>Human distal femur and proximal tibia</td>
<td>26</td>
<td>50 Hz - 5 MHz $\varepsilon'$, $\varepsilon''$, $\theta$, $\sigma'$, $Z_{sp}$, tan $\delta$</td>
<td>Mechanical testing $E$, $\sigma_y$, $\varepsilon_y$, $\sigma_{max}$, $Res$</td>
<td>BMD$_{vol}$</td>
</tr>
<tr>
<td>III</td>
<td>Human distal femur and proximal tibia</td>
<td>26</td>
<td>50 Hz - 5 MHz $\varepsilon'$, $\varepsilon''$, $\theta$, $\sigma'$, $Z_{sp}$, tan $\delta$</td>
<td>Structure analysis WV/TV, SMI, Tb.N., Tb.Sp., BS/BV, Tb.Th., Connectivity, Anisotropy</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>Human distal femur and proximal tibia</td>
<td>26</td>
<td>50 Hz - 5 MHz $\varepsilon'$, $\varepsilon''$, $\theta$, $\sigma'$, $Z_{sp}$, tan $\delta$</td>
<td>Composition analysis Wet and dry density; Fat, water, GAG and collagen content</td>
<td>-</td>
</tr>
<tr>
<td>I*</td>
<td>Human distal femur and proximal tibia</td>
<td>26</td>
<td>300 kHz - 3 GHz $\varepsilon'$, $\sigma'$</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Unpublished data

Explanation of the measurement parameters:

* $BMD_{vol}$: Volumetric bone mineral density
* $n$: number of specimens
* $\varepsilon'$: Relative permittivity
* $\varepsilon''$: Loss factor
* $\theta$: Phase angle
* $\sigma'$: Conductivity
* $Z_{sp}$: Specific impedance
* tan $\delta$: Dissipation factor
* $E$: Young’s modulus
* $\sigma_y$: Yield stress
* $\varepsilon_y$: Yield strain

No fixation agent was used because electrical properties are sensitive to fixation treatments. In order to improve the comparison between the samples and minimize the artifacts originating from preparation, the procedure was carefully standardized. Samples were harvested from different anatomical sites to obtain specimens with a variety of structural, composition and mechanical properties.

7.2 EIS methods

Electrical and dielectric properties were measured in a wide frequency range: 20 Hz - 5 MHz for bovine trabecular bone and 50 Hz - 5 MHz for human trabecular bone. A parallel-plate capacitance cell was used in the experiments. Additionally, the results published in the four original articles (I-IV) have been supplemented with measurements at frequencies 300 kHz - 3 GHz conducted only on human trabecular bone. A method using an open-ended coaxial trans-
Figure 7.1: The bovine bone samples were prepared from four anatomical positions: femoral lateral condyle (FLC), femoral medial condyle (FMC), femoral head (FC) and femoral greater trochanter (FTM). The samples from human tissue were obtained from distal femur (femoral medial condyle (FMC) and femoral groove (FG)) and proximal tibia (tibial medial plateau (TMP)).

mission line was used in this study.

### 7.2.1 Low frequency impedance spectroscopy

Measurements were made with an LCR meter (HIOKI 3531 Z HiTester, Koizumi, Japan). The parallel-plate cell consisted of two round stainless steel electrodes (AISI 316L) mounted in insulating supports that allowed adjustment of the distance between the electrodes (Figure 7.2). Measurements of empty and short-circuited cell allowed corrections for the stray capacitance, the series lead inductance and resistance and shunt conductance arising within the instrument. Repeated measurements with a sample permitted checking of the reproducibility of the set-up. For the experiments, the samples were removed from the PBS and the excess liquid was removed with a paper towel, and the sample was placed in the measurement cell. To avoid drying of the sample, which is known to significantly affect electrical properties, the time between the removal of a sample from the PBS and the end of the measurements was minimized and standardized. The modulus of impedance, parallel resistance, parallel capacitance and phase angle were measured as a function of frequency. From these measurements relative permittivity, conductivity, loss factor, specific impedance and dissipation factor were calculated according to equations shown in the Table 7.2.

In Study I electrodes with a 30 mm diameter were used. In order to provide good contact between the electrodes and tissue, a thin layer of conducting gel (Spectra 360, Parker Laboratories, New Jersey, USA) was applied to the sample surface. To assess the effect of gel, a phantom material as well as five samples of five different lengths (20 mm, 14 mm, 10 mm, 8 mm and 2 mm) were measured.
Figure 7.2: Schematic representation of the low-frequency measurement setup. A sample was placed in the holder between two stainless steel electrodes and closed in a plastic box containing PBS at the bottom. A small weight of 300 g was applied to the holder to ensure good contact between the specimen and electrodes. The data were collected over a frequency range of 50 Hz - 5 MHz with an LCR meter and further processed with MatLab 6.5.1 (The Mathworks Inc., Natick, MA, USA).

with and without gel. To minimize measurement artifacts, the electrical examination was conducted in a Faraday cage. The current was directed through samples in a direction perpendicular to the in-vivo loading axis.

In Studies II-IV, a current was applied through electrodes 16 mm in diameter. Good contact between sample and electrodes was assured by placing a weight (300 g) on top of the parallel-plate cell. The electrical parameters measured were found to be practically independent of the amount of weight above a certain threshold. The current was directed through all samples along the long axis of the cylindrical sample, i.e., along the in vivo loading axis. A phantom material (resistor of a known value) was measured before each measurement in order to ensure the reproducibility and validity of the data. During measurement, the specimens and electrodes were enclosed in a cylindrical plastic insulating cage of a slightly larger diameter than the samples. PBS was placed at the bottom of the cage, but not in contact with samples. This measurement geometry prevented the samples from drying.

Additionally, in Study II, the samples were also measured after mechanical
7.2 EIS methods

Table 7.2: Basic equations for low frequency EIS [93, 139, 121].

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex admittance</td>
<td>( Y^* = \frac{1}{Z^*} = \frac{1}{R_p} + j\omega C_p )</td>
</tr>
<tr>
<td>Complex relative permittivity</td>
<td>( \varepsilon_r^* = \varepsilon_r' + j\varepsilon_r'' = \frac{Y^*}{j\omega C_e} )</td>
</tr>
<tr>
<td>Relative permittivity</td>
<td>( \varepsilon_r' = \frac{C_p}{C_e} )</td>
</tr>
<tr>
<td>Loss factor</td>
<td>( \varepsilon_r'' = \frac{j\omega C_e}{\varepsilon_0} )</td>
</tr>
<tr>
<td>Conductivity</td>
<td>( \sigma' = \frac{R_p}{R_p C_e} )</td>
</tr>
<tr>
<td>Specific impedance</td>
<td>( Z_{sp} = \frac{R_{sp}}{\sqrt{(2\pi f R_{sp} C_{sp})^2 + 1}} )</td>
</tr>
<tr>
<td>Dissipation factor</td>
<td>( \tan \delta = \frac{\varepsilon_r''}{\varepsilon_r'} )</td>
</tr>
</tbody>
</table>

Explanation of the parameters:

- \( Z^* \): Complex impedance
- \( \varepsilon_0 \): Permittivity of free space
- \( R_p \): Parallel resistance
- \( R_{sp} \): Specific resistivity
- \( \omega \): Angular frequency
- \( C_{sp} \): Specific capacitance
- \( f \): Frequency
- \( C_e \): Capacitance of an empty measuring cell
- \( C_p \): Parallel capacitance

Destructive testing. The time between electrical measurements was 10 months. To minimize artifacts and provide straightforward comparison between values in the parameters before and after micro-damage, the data were normalized. The values after destructive testing were normalized with values measured before destructive testing. Normalization was also conducted for control group by dividing the values from the latter measurement by the values obtained from the first measurement.

The polarization impedance of the parallel plate measurement cell was studied. Six samples (diameter = 16 mm) of different lengths (L = 55 mm, 35 mm, 24 mm, 20 mm, 16 mm, 14 mm, 10 mm, 8 mm and 2 mm) were investigated at frequencies 20 Hz - 5 MHz. The measurement protocol was identical to that used in Studies II-IV.

7.2.2 Microwave frequency impedance spectroscopy (supplementary data)

Human trabecular bone specimens were also investigated (unpublished data) with radio-frequencies (300 kHz - 3 GHz) using the reflection principle. Samples were always measured first at low frequencies, followed by high-frequency investigation. The electromagnetic waves were generated by an HP 8753C Network Analyzer (Agilent Technologies, Palo Alto, CA, USA) and applied through a coaxial probe to the tissue (Figure 7.3). A probe with an intrinsic impedance of 50 Ω with an electrical field penetration depth of about 3 mm\(^1\) were used. The coaxial open-ended sensor consisted of two brass conductors with gold-coated

\(^1\)Penetration depth was confirmed in personal communication with Dr E. Alanen.
surfaces with diameters of 4 mm (center pin diameter) and 10 mm (inner diameter of the outer electrode) (see Figure 5.4). The space between the conductors was filled with insulating Teflon. Errors arising from reflections from the probe insulator and other instrumental artifacts were corrected by the standard calibration procedure employing short, open and 50 Ω terminations (matched load). Additionally, measurements of a phantom material of known properties (water) were conducted to assure the validity of the data. As in the low-frequency EIS protocol, all measurements were conducted at room temperature (22°C). A sample was removed from the PBS and the excess liquid was removed with a paper towel. The time between the removal of a sample from the PBS and the end of the measurements was minimized. The end-face of the specimen cylinder was placed against the probe and the real and imaginary parts of the reflection coefficient were measured with reference to the plane of the sample-line interface. A specially designed holder ensured good contact between the coaxial probe and the trabecular bone sample. An open container with PBS at the bottom was mounted on the sample holder just below the tissue. The liquid could evaporate during the measurements, which prevented sample drying. As the measurement of both end-faces were found to yield similar results, only the end-face of the cylinder closer to the cartilage surface was investigated. Relative permittivity and conductivity were calculated from equations (5.19) and (5.21), respectively.

Figure 7.3: Schematic representation of the microwave measurement setup. The sample was placed in the holder and in contact with the coaxial probe. A small weight of 300 g was applied to the holder to ensure good contact between the specimen and the probe. An open plastic box with PBS on the bottom was placed below the sample. The data were collected over a frequency range of 300 kHz - 3 GHz with a network analyzer and further processed with MatLab 6.5.1 (The Mathworks Inc., Natick, MA, USA).
7.3 Bone mineral density measurements

The bone mineral density (BMD) of the bovine and human specimens was determined using a dual energy x-ray absorptiometry (DXA) technique in a direction perpendicular to the parallel ends of the cylindrical samples. Volumetric bone mineral density ($BMD_{vol}$) was calculated by normalizing the measured BMD with the sample thickness determined with a micrometer (Mitutoyo, Kawasaki, Japan).

In Study I, the investigations were performed with a clinical Lunar Expert densitometer (Lunar Co. Madison, WI, USA). In order to optimize the measurement accuracy, the soft tissues were simulated by placing samples in a PBS bath of a constant depth.

In Study II, the BMD of human trabecular bone was measured with a Lunar Prodigy instrument (GE Medical, Wessling, Germany). The AP spine measurement protocol was used. Samples were immersed in a water bath of a constant depth simulating soft tissues to improve the accuracy of the density measurements.

7.4 Mechanical testing

A destructive mechanical test was performed on all the bovine and human trabecular bone samples, and Young’s modulus, ultimate strength, yield stress, yield strain and resilience were determined.

In Study I, mechanical testing was performed with a 12.5 kN servohydraulic material testing device (Matertest Oy, Espoo, Finland). To stay within the capacity of the device, plugs of diameter 19 mm were drilled from the original samples prior to the examination. In order to prevent drying of the tissue, the samples were immersed in PBS bath during the test. A small 0.13 MPa prestress was applied to a specimen for 2 min followed by five non-destructive cycles up to 0.7% strain. This ensured good contact between the loading platens and sample. After preconditioning, a destructive strain of 4.5% was applied to the tissue with a strain rate of $3.5 \cdot 10^{-3} \text{s}^{-1}$.

A 200 kN material testing device (Zwick 1484, Zwick GmbH & Co. KG, Ulm, Germany) equipped with a 10 kN force transducer was used in Study II. Frictionless contact between platens and the tissue was ensured by placing a Teflon foil on each end-face of the sample. Prior to the destructive testing, a sample was subjected to a small 25 kPa prestress and subsequently preconditioned with five consecutive cycles up to 0.5% strain. Finally, samples were destructively compressed to a destructive strain of 5% at a strain rate of $4.5 \cdot 10^{-3} \text{s}^{-1}$. The samples were moistened during the mechanical testing to avoid drying.

Young’s modulus was calculated as a slope of linear fit to a stress-strain curve between 40% and 65% and between 45% and 60% of the maximum stress for bovine and human bone, respectively. Yield stress was determined with the offset method [39]. In this technique, a line parallel to the linear portion of the stress-strain curve but offset by 0.2% is constructed. Yield stress is defined as an intersect between this line and the stress-strain curve. A tangent to the stress-strain curve at the point 15% of the yield stress was constructed. The zero-strain point was defined as an intersect of this tangent and the strain axes. Yield strain was defined as the difference between the strain corresponding to yield stress and that at the zero-strain point. The maximum stress detected
during the experiment represented ultimate strength. Resilience was calculated by integrating the stress-strain curve from zero strain to the yield strain.

7.5 Microstructural analysis

In Study III, the microstructure of human trabecular bone was investigated using a high-resolution micro-computed tomography (microCT) system (SkyScan 1072, Aartselaar, Belgium). Each cubic voxel measured 18 x 18 x 18 µm³. Accurate 3-D data sets were obtained by segmenting each image with a local threshold method [80]. Several structural parameters were calculated based on true and unbiased methods. Firstly, trabecular bone volume fraction (BV/TV) and the bone surface-to-volume ratio (BS/BV) were quantified. Subsequently, trabecular thickness (Tb.Th.), spacing (Tb.Sp.) and number (Tb.N.) were calculated using a direct 3-D analysis without any model assumptions of trabecular structure. The degree of anisotropy (DA) was defined as the ratio between the maximal and minimal eigenvalues of the fabric tensor of the architecture [76]. Connectivity was determined according to Odgaard and Gundersen [85], whereas the structural model index (SMI) was determined according to Hildebrand and Rüegsegger [75].

7.6 Compositional analysis

In Study IV, biochemical composition analysis was conducted on human trabecular bone tissue. Wet samples were weighed and their volume determined using the Archimedes principle. Subsequently, the specimens were freeze-dried and weighed in order to determine dry weight and water mass. Fat was removed from the specimens with acetone, and the mass of fat was determined.

Hydroxyproline and uronic acid assays were performed on fat-free bone powder. Approximately 20 mg of each sample was portioned for acid hydrolysis in 5 M HCl at 108°C for 16 hours. A microplate assay was used to analyze the hydroxyproline content in the hydrolysate [140]. The estimates for the total collagen content can be obtained by multiplying the analyzed hydroxyproline content by a factor of 7, since the hydroxyproline content of collagen is approximately 14% of the collagen mass [141]. Proteoglycans were extracted from a 20 mg sample of the fat-free bone powder using 4 M GuHCl including 0.2 M EDTA in 50 mM sodium acetate buffer, pH 6.0, for 70 hours. Uronic acid content was determined with a spectrophotometric assay [142]. The GAG content was obtained by multiplying the uronic acid content by a factor of 3.2. This follows from the fact that 31% of chondroitin sulfate weight comes from uronic acid.

Wet and dry densities were calculated by normalizing wet and dry weight, respectively, with sample volume. Water, fat, collagen and GAG contents were computed by normalizing individual weights with corresponding wet masses of the samples.

7.7 Statistical analysis

In study I, the significance of the variation in the measured parameters between different anatomical sites was tested with the Kruskall-Wallis H-test. To reveal the relation between electrical and mechanical parameters, linear Pearson’s
correlation analysis was used. The reproducibility of the electrical impedance measurement setup was expressed in terms of standardized coefficient of variation (sCV). This parameter can be calculated from the coefficient of variation (CV), which can be expressed as a ratio of standard deviation \( \sigma_y \) to the mean \( \bar{y} \) of repeated measurements of parameter \( y \). For \( n \) samples, the CV becomes a root mean square averaged value \( CV_{RMS} \):

\[
CV_{RMS} = \sqrt{\frac{1}{n} \sum_{i=1}^{n} \left( \frac{v_{yi} - \bar{y}_i}{\bar{y}_i} \right)^2}.
\]

(7.1)

The CV provides only a short-term precision and does not take into account the biological variation of the parameters measured \[144\]. The true diagnostic sensitivity of the measurements can be obtained with the standardized coefficient of variation, determined as follow:

\[
sCV = \frac{\bar{\sigma}}{4\sigma_{\mu}} CV_{RMS},
\]

(7.2)

where \( \bar{\sigma} \) is the average of standard deviation within the population and \( \sigma_{\mu} \) is the population standard deviation. A smaller sCV value indicates better reproducibility, with an extreme case of 0% meaning a perfect reproducibility \[144\].

In study II, Pearson’s correlation analysis was used to investigate the relationships between electrical and mechanical parameters. The significance of the anatomical site-dependent variations of parameters measured was tested with the Wilcoxon signed rank test. The effect of bone microdamage was studied using the Wilcoxon signed rank test for two related samples by comparing the electrical parameters of samples obtained before and after destructive mechanical testing. In order to eliminate possible confounding factors in this comparison, the results were normalized and further tested for variations solely due to microdamage. The differences in the normalized electrical parameters between the control and the mechanically tested groups were compared using the Mann-Whitney U-test. The electrical properties of human and bovine trabecular bone were investigated with the Mann-Whitney test.

In Study III, the Friedman test for several dependent samples was used to assess the site-dependent variations in electrical and structural parameters. Pearson’s correlation analysis was used to determine the linear correlation coefficients between parameters tested. Principal component analysis (PCA) was used to classify and reduce the number of electrical and structural variables (Table 7.3). Two PCA approaches were used for electrical parameters. The frequencies (50 Hz - 5 MHz) used for the measurement of electrical properties were grouped into the main principal components. Extreme multicollinearity was avoided by excluding specific frequencies from the analysis. In addition, PCA was conducted on relative permittivity, conductivity, phase angle, dissipation factor and specific impedance at 1.2 MHz. Loss factor was not included in the analysis, as it correlates strongly with conductivity. Varimax rotation, i.e. rotation of the axes to a position in which the sum of the variances of the loadings reaches the maximum values, was used to improve the interpretability of the components. The eigenvectors remained orthogonal during the rotation. Multiple stepwise linear regression was used to reveal whether a combination
7. Materials and methods

Table 7.3: Principal components extracted from electrical and structural parameters.

<table>
<thead>
<tr>
<th>PC</th>
<th>Name</th>
<th>Explained variation</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Electrical</strong></td>
<td><strong>principal components</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field propagation component</td>
<td>68.2%</td>
<td>$\theta$, $\sigma'$, $Z_{sp}$, $\tan\delta$</td>
<td></td>
</tr>
<tr>
<td>Energy component</td>
<td>27.0%</td>
<td>$\tan\delta$, $\varepsilon'$</td>
<td></td>
</tr>
<tr>
<td>Low frequency permittivity</td>
<td>26.3%</td>
<td>Permittivity at 50 Hz - 1.2 kHz</td>
<td></td>
</tr>
<tr>
<td>Middle frequency permittivity</td>
<td>46.3%</td>
<td>Permittivity at 300 Hz - 500 kHz</td>
<td></td>
</tr>
<tr>
<td>High frequency permittivity</td>
<td>25.3%</td>
<td>Permittivity at 300 kHz - 5 MHz</td>
<td></td>
</tr>
<tr>
<td>Low frequency dissipation factor</td>
<td>56.6%</td>
<td>Dissipation factor at 50 Hz - 120 kHz</td>
<td></td>
</tr>
<tr>
<td>High frequency dissipation factor</td>
<td>39.5%</td>
<td>Dissipation factor at 50 kHz - 5 MHz</td>
<td></td>
</tr>
<tr>
<td><strong>Structural</strong></td>
<td><strong>principal components</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trabecular structure component</td>
<td>49.1%</td>
<td>SMI, Connectivity, Tb.Sp., BV/TV, Tb.N.</td>
<td></td>
</tr>
<tr>
<td>Surface component</td>
<td>32.5%</td>
<td>BS/BV, Tb.Th.</td>
<td></td>
</tr>
<tr>
<td>Anisotropy component</td>
<td>14.8%</td>
<td>Anisotropy</td>
<td></td>
</tr>
</tbody>
</table>

Explanation of the parameters:

- PC: Principal component
- $\varepsilon'$: Relative permittivity
- $\theta$: Phase angle
- $\sigma'$: Conductivity
- $Z_{sp}$: Specific impedance
- $\tan\delta$: Dissipation factor

of electrical parameters or principal components could predict bone structure. Moreover, a combination of structural parameters and their PCs were applied to linear regression analysis in order to investigate the effect of bone microarchitecture on electrical parameters.

In study IV, the same statistical procedures as in study III were used to reveal relations between the electrical properties of bone and its composition.

For the EIS at microwave frequencies, Pearson’s correlation analysis was used to relate relative permittivity and conductivity with bone structure. The significance of site-dependent variation in the electrical parameters measured was studied with the Friedman test for several dependent samples.
The measurements of the electrical and dielectric properties of bone were found to be reproducible over a wide frequency range. The standardized coefficient of variation (sCV) was below 1% for all parameters investigated at a frequency range 1 kHz - 5 MHz. Below 1 kHz, the sCV of conductivity and loss factor remained at the level of 1%, while the sCV of relative permittivity and phase angle increased up to 4.5% and 8.5% at 50 Hz, respectively.

Polarization impedance of the parallel plate cell was investigated (Figure 8.1 A). It was found to decrease with frequency from 211 Ω at 100 Hz to 44 Ω at 1 MHz (Figure 8.1 B). The effect of contact impedance for 8 mm samples decreased with increasing frequency from 19% at 100 Hz through 10% at 1 kHz to 4.5% at 1 MHz. No significant difference in impedance values was observed between the samples with and without gel applied to the surface ($p > 0.05$). Additionally, the gel was found to have no or only minor effect (< 1%) on the impedance values of the phantom material investigated.

![Figure 8.1](image-url)  
Figure 8.1: The mean modulus of impedance of six bone samples was measured at nine different lengths. Polarization impedance was determined from these measurements by a linear fit and was found to be e.g., 211 Ω at 100 Hz (a). Polarization impedance decreased with increasing frequency (b). The error bars indicate standard deviation.
8.1 Variation and frequency dependence of electrical properties

The electrical and dielectric properties of bovine and human trabecular bone were dependent on frequency. The observed dispersions were broad. For instance, relative permittivity dropped steadily over all frequency ranges from $5 \cdot 10^6$ at 50 Hz to 10 at 3 GHz (Figure 8.2 A). Conductivity was almost frequency independent at low frequencies but increased from 0.125 Sm$^{-1}$ to 0.582 Sm$^{-1}$ over the frequency range 700 MHz - 3 GHz (Figure 8.2 B). A small shift in the values of the parameters measured with parallel plate cell and open-ended cell configurations over the same frequency range (300 kHz - 5 MHz) was observed (Figure 8.2).

![Figure 8.2: The spectrum of relative permittivity (a) and conductivity (b) of human trabecular bone at frequencies 50 Hz - 3 GHz obtained by parallel-plate (50 Hz - 5 MHz) and reflection methods (300 kHz - 3 GHz). The error bars indicate standard deviation.](image)

Interestingly, at low frequencies, a strong relation was found between conductivity and relative permittivity. This association decreased with increasing frequency. For instance, at 100 Hz the correlation was 0.79 ($p < 0.01$) (Figure 8.3 A), while at 1.2 MHz it was only $-0.18$ ($p > 0.05$) (Figure 8.3 B).

In addition, the electrical parameters of bovine trabecular bone showed significant site-dependent variation (Figure 8.4) ($p < 0.05$). Relative permittivity at 1.2 MHz reached the highest value in femoral medial condyle (FMC) (79.9 ± 10.8) and the lowest in femoral trochanter major (FTM) (34.0 ± 7.3). However, the site-dependent variation in human trabecular bone samples was found to be insignificant ($p > 0.05$, Figure 8.4).

Statistically significant ($p < 0.01$) differences in electrical parameters between bovine and human trabecular bone were observed. Relative permittivity at frequencies of 50 Hz - 30 kHz was higher in human bone, while at frequencies of 50 kHz - 5 MHz it was higher in bovine bone (Figure 8.5 A). The conductivity of human samples was higher than that of bovine specimens at all frequencies (e.g., 0.08 ± 0.03 Sm$^{-1}$ for human and 0.03 ± 0.01 Sm$^{-1}$ for bovine at 1.2 MHz) (Figure 8.5 B). The peak value of dissipation factor was seen at a higher frequency in human bone (120 kHz) than in bovine bone (1 kHz, Figure 8.5 C).
8.2 Relations between electrical and mechanical properties

The electrical properties of human trabecular bone samples were measured twice. The samples were frozen for about 10 months between measurements. Among the parameters measured with a parallel-plate cell, significant differences were observed in the values of permittivity at frequencies of 80 Hz - 2.5 MHz and in the values of dissipation factor at frequencies of 50 Hz - 50 kHz (p < 0.05, n = 6). The difference observed in conductivity and loss factor was statistically insignificant (Figure 8.6).

At all frequencies (300 kHz - 3 GHz), relative permittivity and conductivity measured with the open-ended coaxial probe showed significant differences in values before and after long-term-freezing (p < 0.05, n = 6) (Figure 8.7).

8.2 Relations between electrical and mechanical properties

Significant but frequency-dependent relationships were found between the electrical and mechanical characteristics of bovine and human trabecular tissue. Relative permittivity displayed strong linear correlations with ultimate strength (bovine: r = 0.62, p < 0.01, n = 40, f = 50 kHz; human: r = 0.72, p < 0.01, n = 20, f = 1.2 MHz) and Young’s modulus (bovine: r = 0.55, p < 0.01, n = 40, f = 50 kHz; human: r = 0.71, p < 0.01, n = 20, f = 1.2 MHz) (Table 8.1).

In bovine bone, correlations between mechanical properties and relative permittivity or phase angle were strongly frequency dependent, being significant above 6 kHz (Figures 5 and 6 in Study I). Conductivity and loss factor of bovine bone did not correlate significantly with any of mechanical properties at any frequency (p > 0.05). Phase angle correlated only moderately with all mechanical properties (r ≤ 0.45).

In human bone, correlations between mechanical properties and relative permittivity or dissipation factor were strongly dependent on frequency, changing

Figure 8.3: At frequencies below 300 kHz, about 62% of the variation in relative permittivity can be explained by conductivity (a). The association between relative permittivity and conductivity gets weaker with increasing frequency (b).
the sign from negative to positive for relative permittivity and from positive to negative for dissipation factor with increasing frequency. Correlations with other parameters were almost frequency independent, being either negative or positive. Conductivity, phase angle and loss factor displayed only weak or moderate relations with mechanical characteristics \( (r \leq |0.45|) \). Specific impedance showed significant correlation with yield strain \( (r = 0.65, p < 0.01, n = 20, f = 1.2 \text{ MHz}) \).

In human samples, electrical principal components correlated significantly with mechanical properties \( (Table 8.2) \). Relatively strong correlations \( (r \geq |0.57|, p < 0.01) \) were found between ultimate strength, Young’s modulus, yield stress or resilience and high frequency permittivity, dissipation factor as well as field propagation component. Low frequency permittivity correlated moderately with resilience \( (r = -0.45, p < 0.05) \). Yield strain correlated only with the energy component \( (r = -0.46, p < 0.05) \). No significant correlations were found between mechanical properties and relative permittivity or conductivity.
8.3 Relations between electrical and structural properties

The strength and sign of linear correlations between human trabecular microstructure and relative permittivity as well as dissipation factor were strongly frequency dependent. Relative permittivity correlated significantly with all of human samples measured with the open-ended coaxial probe.

Relative permittivity and dissipation factor at the frequency range 300 Hz - 1.5 MHz displayed significant \( p < 0.05 \) differences in their values before and after destructive mechanical testing as compared with the control group (Figure 8.8 A). The differences in the other parameters measured with a parallel-plate cell were insignificant. Normalized conductivity and relative permittivity determined with an open-ended coaxial line showed small, but statistically insignificant variations between destructively tested and control groups (Figure 8.8 B).

### Figure 8.5

Statistically significant \( p < 0.01 \) differences in electrical parameters between bovine and human trabecular bone were observed: relative permittivity (a), conductivity (b) and dissipation factor (c). Samples were obtained from the same anatomical site (femoral medial condyle (FMC)) but in different direction (bovine: medio-lateral; human: perpendicular to the articular surface). The error bars indicate standard deviation.
structural parameters, except for degree of anisotropy (Figure 4 in Study III and Table 8.1). On the other hand, dissipation factor did not depend on trabecular thickness nor on degree of anisotropy. The significant correlations were found at low and high frequencies, while at the range 5 kHz - 50 kHz the relations were insignificant.

The linear correlations between microstructure and loss factor, conductivity, phase angle as well as specific impedance parameters were almost frequency independent and exhibited either positive or negative sign. The values of these correlations were significant with all structural parameters excluding BS/BV, Tb.Th. and degree of anisotropy.

Importantly, the dense trabecular structure with high BMD_{col}, BV/TV and collagen content, but low SMI, fat and water contents showed higher values of relative permittivity at high frequencies (e.g., 1.2 MHz) but lower at low frequencies (e.g., 100 Hz), than did more sparse low density trabecular bone (Figure 8.9). Interestingly, conductivity displayed the opposite behavior, being
8.3 Relations between electrical and structural properties

Figure 8.7: Relative permittivity (a) and conductivity (b) measured with an open-ended coaxial probe showed significant differences in values before and after long-term-freezing. The error bars indicate standard deviation.

Figure 8.8: Only relative permittivity and dissipation factor at the frequency range of 300 Hz - 1.5 MHz were able to distinguish between intact and mechanically damaged trabecular structure. Normalized electrical parameters at 1.2 MHz (a) and 30 MHz (b) for destructively tested and control groups (** p < 0.01). The error bars indicate standard deviation.

Structural parameters showed significant relations with all electrical principal components, except for middle frequency relative permittivity and low frequency dissipation factor. Bone surface-to-volume ratio correlated highly with high frequency permittivity and field propagation component (r > |0.71|, p < 0.01). High frequency dissipation factor was strongly related to BV/TV and SMI (r > |0.72|, p < 0.01).

In contrast, anisotropy component correlated with no electrical parameter measured at 1.2 MHz, and the surface component was strongly related only with relative permittivity (r = 0.64 p < 0.01). Trabecular structure component

low for dense bone and high for low density bone.
Table 8.1: Linear correlation coefficient for selected electrical (measured at 1.2 MHz for human and at 50 kHz for bovine tissue) and biomechanical, structural and compositional parameters in human and bovine bone.

<table>
<thead>
<tr>
<th>Type of test</th>
<th>Relation</th>
<th>Human bone</th>
<th>Bovine bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical testing</td>
<td>$\varepsilon'$ vs. US</td>
<td>0.72**</td>
<td>0.62**</td>
</tr>
<tr>
<td></td>
<td>$\varepsilon'$ vs. $E$</td>
<td>0.71**</td>
<td>0.55**</td>
</tr>
<tr>
<td></td>
<td>$\sigma'$ vs. US</td>
<td>-0.47</td>
<td>-0.19</td>
</tr>
<tr>
<td></td>
<td>$\sigma'$ vs. $E$</td>
<td>-0.36</td>
<td>-0.15</td>
</tr>
<tr>
<td></td>
<td>tan$\delta$ vs. US</td>
<td>-0.61**</td>
<td>-0.50**</td>
</tr>
<tr>
<td></td>
<td>tan$\delta$ vs. $E$</td>
<td>-0.57**</td>
<td>-0.44**</td>
</tr>
<tr>
<td>Structural testing</td>
<td>$\varepsilon'$ vs. Tb.N.</td>
<td>0.45</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$\varepsilon'$ vs. SMI</td>
<td>-0.59**</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$\sigma'$ vs. Tb.N.</td>
<td>-0.64**</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$\sigma'$ vs. SMI</td>
<td>0.61**</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>tan$\delta$ vs. Tb.N.</td>
<td>-0.63**</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>tan$\delta$ vs. SMI</td>
<td>0.71**</td>
<td>-</td>
</tr>
<tr>
<td>Compositional testing</td>
<td>$\varepsilon'$ vs. fat$^1$</td>
<td>-0.85**</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$\varepsilon'$ vs. collagen$^1$</td>
<td>0.64**</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$\sigma'$ vs. fat$^1$</td>
<td>0.10</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$\sigma'$ vs. collagen$^1$</td>
<td>-0.43**</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>tan$\delta$ vs. fat$^1$</td>
<td>0.42</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>tan$\delta$ vs. collagen$^1$</td>
<td>-0.55**</td>
<td>-</td>
</tr>
<tr>
<td>DXA testing</td>
<td>$\varepsilon'$ vs. $\text{BMD}_{\text{vol}}$</td>
<td>0.67**</td>
<td>0.87**</td>
</tr>
<tr>
<td></td>
<td>$\sigma'$ vs. $\text{BMD}_{\text{vol}}$</td>
<td>-0.50**</td>
<td>-0.48**</td>
</tr>
<tr>
<td></td>
<td>tan$\delta$ vs. $\text{BMD}_{\text{vol}}$</td>
<td>-0.61**</td>
<td>-0.65**</td>
</tr>
</tbody>
</table>

$^1$ content, $^* p < 0.05, ^{**} p < 0.01$

Explanation of the parameters:

$\varepsilon'$ Relative permittivity
$E$ Young's modulus
$\sigma'$ Conductivity
Tb.N. Trabecular number
$\tan \delta$ Dissipation factor
SMI Structure model index
US Ultimate strength
$\text{BMD}_{\text{vol}}$ Volumetric bone mineral density
DXA Dual energy x-ray absorptiometry

Table 8.2: Linear correlation coefficients between the electrical principal components and mechanical properties of trabecular human bone samples ($n = 20$).
8.4 Relations between electrical and compositional properties

Strong or moderate correlations were found between the electrical parameters and densities. Collagen content was strongly related to relative permittivity at 1.2 MHz ($r = 0.64$), but only moderately to other parameters (Table 8.1). Trabecular bone GAG content showed no or only minor impact on electrical properties at all frequencies. The sign and strength of correlations between relative permittivity and composition parameters, water and fat content in particular, was dependent on frequency (Figure 8.10).

Fat content was significantly related to dissipation factor and relative permittivity only at some specific frequency bands (>100 MHz), but showed no correlations with other electrical parameters at any frequency. Water content strongly affected all electrical parameters, conductivity in particular ($r = 0.79$ at 1.2 MHz, $p < 0.01$, $n = 26$) (Figure 8.11). The linear combinations of electrical principal components accounted for more than 50% of the variation in
organic composition or densities. The high frequency permittivity was a strong predictor of fat content ($r^2 = 0.72$). In contrast, a good predictor of variation in water content was the linear combination of low and middle frequency permittivity ($r^2 = 0.66$).

Figure 8.11: Conductivity correlated highly significantly with water content.
In present study the interrelations between trabecular bone composition, structure, mechanical and electrical properties were investigated. Initially, bovine trabecular bone samples were measured to verify the reproducibility and reliability of the system as well as to reveal the potential of the EIS technique to diagnose mechanical properties of the tissue. Later, electrical measurements of human trabecular bone samples from distal femur and proximal tibia were conducted over a wide range of frequencies using a parallel-plate capacitance cell and an open-ended coaxial probe. The sample preparation procedure and the measurement protocol were designed to preserve the physiological state of the tissue. The bone samples were never allowed to dry and no fixation agent was used. Bone mineral density was measured with a clinical DXA technique followed by high resolution microCT investigations and biochemical analysis. This study protocol provided a unique insight into the electrical properties of bone. The findings indicate that, when further developed, the EIS may prove to be a useful technique for the rapid assessment of bone status in vivo or during certain special cases of open surgery.

The results of the present study would suggest that the interstitial liquid of trabecular bone determines its conductivity. In particular, the dc conductivity was found dominating, possibly due to the shunt pathways provided by the structural organization of trabecular tissue. At frequencies below 100 kHz, only free, extracellular water, originating from both hard and soft tissues, is available to current flow. The mineralized bony matrix contains about 25% water, of which 40% is free. Yellow bone marrow contains mostly lipids, the molecules of non-polar and insoluble in water triglycerides. As such, they are shielded from water, leaving the tissue with a substantial amount of unbound liquid. Additionally, the increase in conductivity associated with the $\beta$-dispersion is masked by a high ionic-, frequency-independent dc conductivity. The mineralized bony matrix contains an insufficient quantity of cells to significantly increase the amount of current paths, while the lipids of bone marrow are known to be poor conductors at all frequencies. The increase in conductivity values at frequencies above 10 MHz is associated with $\delta$ dispersion.

1 Supplementary data not included in studies I-IV
2 Cells are known to possess capacitive properties. If the frequency is sufficiently high (e.g., above 100 kHz), the cell membranes will be short-circuited, increasing the amount of current paths and, thus, conductivity.
It can be interpreted as a significant contribution of bound water to the current carriers [90].

A previous study [146] investigated bone conductivity as a function of bathing solution and found a limiting value of 0.1 mS m$^{-1}$ as the conductivity of the medium fell to zero. It has also been reported that the measured values of conductivity of dry bone are higher than those predicted theoretically [115]. Both of the previous investigations attributed those results to the conductivity of the matrix, which is strongly controlled by bounded water. This might explain the strong correlation observed in Study IV between conductivity and dry density ($r < -0.7$ for 1 kHz - 5 MHz).

The observed dispersion curve of trabecular bone is broad and appears featureless. This may be due to the superposition of several processes with different relaxation times [114], originating from both mineralized matrix and marrow. At frequencies below 300 kHz, about 62% of the variation in relative permittivity can be explained by conductivity, the movement of free water ions in particular. The unaccounted variation may originate from the diffusion of counterions near the charged interfaces and the spatial distribution of water within the tissue [112, 111]. This possibility is further supported by the strong correlation between relative permittivity and the densities or microstructure found in the present study. The association between relative permittivity and conductivity diminishes with increasing frequency, possibly due to the polarization processes in collagen protein [114, 95, 122] and in bone marrow [123]. The collagen bundles in particular appear to be an important source for permittivity, as they are rich in interfaces. Further, in an aqueous environment, the charged groups of proteins as well as the charged membrane surfaces electrostatically interact with water molecules. The non-polar hydrophobic groups of proteins are surrounded by water molecules, forming hydrogen bonds. This results in the formation of one or two layers of water molecules near protein or membrane surfaces. This bound water is characterized by different physical properties than bulk, free water [92]. The rotation of the bound water in both mineralized and soft tissues determines the $\delta$ dispersion.

The frequency spectrum of the dissipation factor is consistent with the results presented by Saha and Williams for human trabecular bone [104]. The rather high value is probably due to the large amount of water present in trabecular tissue [116] and might serve as further evidence for the significance of the dc conductivity contribution to the overall dielectric response of the tissue [147, 148]. The peak in the dissipation factor spectra indicates the Debye-like relaxation processes [149, 148]. However, the width of the peak suggests the existence of processes with different relaxation times [148, 150].

The difference in spectra between bovine and human bone can be attributed to the differences in the tissue structure, composition and the organization of one phase relative to the other. Bovine bone is known to be denser [151, 152, 153], and hence contains less bone marrow. The higher amount of liquid in human bone is directly reflected in its higher conductivity. Water, the universal solvent, is particularly effective in increasing ionic concentrations and the mobility of such particles as surface ions of minerals [115]. The association of water with ionizable constituents within, on the surface of, or on the interface of dielectrics results in higher values of the peak dissipation factor and relative permittivity at low frequencies in human bone. Additionally, a certain amount of energy is lost by particles vibrating in the ac field. The amount of such energy is determined
by the number and size of the particles participating in the motion (see e.g., [154]) that can be different for bovine and human bones. At frequencies above 100 kHz, the substantial amount of water in human bone prevents polarization of the tissue by increasing the current flow. At the same time, the higher amount of collagen in dense, or bovine, bone may significantly increase the values of relative permittivity.

The electrical properties varied to some extent between different anatomical sites. Those variations may be attributable to the topographical differences in the amount and orientation of collagen fibers [114], the microstructure and the composition between samples. In human bone the variation in collagen content was statistically insignificant, possibly also reflected by the lack of significant fluctuations in electrical parameters. In contrast, significant variations in density and mechanical parameters and hence electrical characteristics were found in bovine bone. It is worth emphasizing that the largest difference in bovine bone was found between the femoral greater trochanter (FTM) and femoral groove (FG). However, human bone samples from these locations were not investigated, and this might explain the insignificant differences.

The effect of long-term freezing on human trabecular bone has been investigated. The values of electrical parameters except dissipation factor have been found to decrease after freezing. However, in previous studies conductivity was reported to increase after the removal of a tissue from the body, over a period of time ranging from 50 h up to one month [130, 95, 155]. This clear contradiction may be explained in two ways. First, the significant changes in tissue occur within a few hours post mortem [156, 157, 90]. For instance, Saha and Williams [155] observed that the resistivity of bone samples remained similar after undergoing a certain number of freezing-thawing cycles, while the most rapid changes occurred during the first days of the experiment. In the present study, due to the prolonged procedure of harvesting human bones, freezing and thawing of the specimens prior to measurements was unavoidable. It is possible that already during those first cycles cell membranes may have been destroyed, and the washout of the cellular components and changes in the ionic content occurred, leading to the increase in conductivity. With time and during further cycles, the value of conductivity could reach equilibrium. Thus, the changes observed in this study might be different in nature and cannot be attributed to the same phenomena as reported earlier. Second, it has been shown that freezing may change the physical state of lipids and the structure of proteins [158] in such a way that the electrical double layer formations [159] could be impaired. Further, extended storage of a sample in a freezer affects the properties of cells, and possibly also the whole tissue, in different ways than does a short time freezing [160]. Thus, it can be hypothesized that long-term freezing reduces the possible current paths and decreases conductivity, although the precise mechanism remains unclear. The alternations in the protein structure might explain the observed changes in permittivity. Long-term freezing of the samples for electrical measurements should therefore be avoided when-ever possible.

Relative permittivity and dissipation factor showed strong associations with mechanical properties in both human and bovine trabecular bone. Additionally, in human bone all mechanical properties showed significant correlations with electrical characteristics at certain frequencies, but in bovine bone conductivity and loss factor did not correlate significantly with any mechanical parameter. This may be due to the differences in structure and composition of human and
animal tissues, as discussed above. Dissipation factor and permittivity in the $\beta$ relaxation frequency range was demonstrated to be sensitive to variation in bone strength and, in particular, to its elastic behavior, with the exception of yield strain. The latter parameter related to specific impedance at all frequencies.

In this study, the significant linear correlations between mechanical characteristics and electrical parameters of bovine trabecular bone, particularly relative permittivity, appeared to be stronger for low rather than for high density tissue. For instance, a significant difference was found in BMD between femoral caput (FC) (0.586 g cm$^{-3}$) and femoral greater trochanter (FTM) (0.198 g cm$^{-3}$). However, the significant correlations between certain mechanical parameters and relative permittivity at specific frequencies persisted only for FTM. This might explain the stronger associations between mechanical properties and relative permittivity in human bone, as a low density tissue, when compared with bovine bone. This hypothesis should be further verified by experiments with a large number of samples. Some of the correlations in the present study may appear insignificant due to the small number of specimen within anatomical sites.

Fractures of trabecular tissue can occur even without a traumatic event and for this reason they cannot be detected by simple measurements of the amount of bone [86], such as determining BMD. At frequencies below 5 MHz, relative permittivity and dissipation factor, i.e., parameters strongly associated with the postyield behavior of bone, distinguished microfractured and intact trabecular structure. The microCT investigations revealed the sensitivity of relative permittivity to the processes occurring on the surface of mineralized bony matrix. This might explain the sensitivity of EIS in detecting the microcracks accompanying microdamage. In contrast, the parameters measured with an open-ended coaxial probe did not change as the structure fractured, possibly because of the significance of free and bound water abundant in superficial surface layer. This water is the main determinant of the gamma and delta dispersions.

In human trabecular bone, strong relations were found between EIS measures and microstructure. However, as structural parameters are strongly interrelated [77, 161], it is difficult to evaluate the effect of individual structural parameters on electrical properties. Nevertheless, the PCA showed that the trabecular structure component, reflecting in particular the degree to which the mineralized matrix is multiply interconnected, the number of trabeculae and space between them, correlated strongly with the electrical parameters, except for relative permittivity. Interestingly, after adjustment for these structural parameters in the regression analysis, the degree of anisotropy provided additional information on the dissipation factor at 1.2 MHz. On the other hand, the variation in trabecular properties related to surface characteristics, such as the amount of surface, the thickness and shape of trabeculae were the main determinants of the relative permittivity. Additionally, trabecular orientation has been reported to be strongly associated with the mechanical behavior of bone [162], as well as to be the main reason for the existing relationships between permittivity in different directions [104]. In the present study, despite the wide variation (2.0° - 22.6°) in mean trabecular orientation relative to the axes of the cylindrical samples, no correlation between the electrical parameters and trabecular orientation was found. This lack of correlation emphasizes the importance of other differences between the samples. The specimens with different trabecular orientation were not obtained from adjacent sites of the same bone.
The difference in the data obtained with parallel-plate and open-ended probes (Figure 8.2) may be due to the heterogeneity and anisotropy of the tissue investigated and to the different volume of the sample probed by each technique [123]. The parallel-plate cell measured the tissue in the direction parallel to the main loading axis of the bone. The electric field fills the volume of the whole sample, approximately 1.6 cm³. In contrast, the field from the open-ended probe penetrated a specimen in the medio-lateral direction, or perpendicular to its long axis. The volume occupied is much smaller, approximately 0.5 cm³. Moreover, the superficial surface layer contains more damage and liquid than the bulk sample. This may lead to higher values of permittivity and conductivity for the open-ended probe measurements.

The size of the sample was optimized based on the feasibility of all experiments conducted. A compromise was made between, e.g., the capacity of the servo-hydraulic material testing device and the size of the impedance electrodes. The preferred dimensions of specimens limited the possible electrode configurations. For instance, there were certain practical limitations when introducing four electrodes big enough for errorless experiment. The feasibility of a measurement setup described by Gandhi et. al. [163] was investigated. The impedance probe consisted of four stainless-steel electrodes coated with gold placed in one plane. Contact between the probe and specimen was found to be poor, because of the small size of the electrodes (2 - 3 mm in diameter). This led to too high impedance values and not reproducible results. Poor contact may be due to roughnesses on the surface of the specimen. Flat surfaces can be obtained by polishing, but this may affect the microarchitecture of the sample. Additionally, due to the small inter-electrode distance (1 - 2 mm) and the presence of a certain amount of liquid on the surface of a sample, the electrodes easily become short-circuited, leading to an unreliable measurement protocol. Similar shortcomings were found at microwave frequency range. The results were found to be erroneous when a probe with dimensions below a certain threshold was used. Further, the size of the sample was also restricted by biological factors. Due to the limited volume of trabecular bone in the human skeleton, only relatively small specimens will be homogeneous. Measuring inhomogeneous samples would result in averaging the mechanical, structural and compositional properties, impeding the investigation of EIS sensitivity.

Consequently, two relatively large electrodes, ensuring good contact without short-circuiting, were chosen for the measurements. They probe a sufficiently big volume of the sample so that the surface condition does not introduce a significant error, but the sample remains homogenous. As discussed in the 5.3 Section, a two-point method is prone to electrode polarization. However, the latest research shows that even a four-electrode setup, which is believed to be suitable for the measurements of biological materials and to be a remedy for the shortcomings of the two-point technique [98, 164], is not free from inaccuracies due to polarization impedance, and may be even more susceptible to errors than a two-point system [165]. In the present study, the error due to the polarization impedance was found to be 20% - 4.5% in the frequency range of 20 Hz to 5 MHz and below 8% in the most relevant frequency range of this study. This is of the same order of magnitude as in a previous investigation on bone [123], so the present results are in line with those of previous studies [121, 104, 27]. Electrical and dielectric properties are sensitive to factors such as the technique of the sample preparation, quality of the surface, method of preservation or
Even if all these factors are kept constant, the electrical and dielectric properties may vary from specimen to specimen by as much as 20%. Consequently, the average error introduced by the polarization impedance does not significantly increase the uncertainty of the results. Nevertheless, the values of the electrical and dielectric properties obtained in this study should be used with caution. The main aim of the present study was to conduct a comparison analysis. For instance, the values of the electrical and dielectric properties before and after mechanical testing were compared with each other. Alternatively, the effect of variations in bone composition on the change in electrical and dielectric properties was investigated. A shift in absolute values will not affect the correlations obtained and the conclusions drawn remain valid.

Electrode gel was used in Study I to improve the contact between the electrode and the sample, especially at low frequencies (< 1 kHz). However, the gel may contain long jelly molecules that might interact with the electric field and might influence the outcome of the investigations. For this reason, the use of gel has not been recommended in impedance measurements [166]. The results obtained in Study I were analogous to those of previous studies without gel [27]. Interestingly, previous studies report no significant differences in bone tissue impedance due to the use of gel (see e.g., [131]). In order to determine the possible effect of gel on the results, a phantom material was tested with and without gel rendering indistinguishable results. Moreover, no significant difference was observed in bone samples tested with and without gel. However, the difference may appear insignificant due to the small number of specimens and relatively high standard deviation of the bone sample set. The largest variation between the results with and those without gel among the five tested samples was 10%. As discussed above, this is less than the variation in electrical and dielectric properties due to specific biological factors, such as composition or microstructure, which are often not accounted for in the literature. Further, only a thin layer of gel was applied to the surface of the sample, just prior to the experiment. During the short measurement time, it is unlikely that the gel penetrated the sample and changed its properties significantly.

The results of the present study suggest that a low density trabecular bone structure can be distinguished from a high density structure by measurements of permittivity and conductivity at low (< 100 Hz) or high (> 100 kHz) frequency bands. A sparse structure possesses higher relative permittivity below 100 kHz and higher conductivity at all the frequencies considered than does tissue with more dense architecture. Possibly, high water and fat content as well as low BMD and BV/TV value for sparse structure result in a high DC conductivity that masks the AC conductivity and increases relative permittivity at low frequencies. On the other hand, the high values of SMI and BMD for high density bone are associated with high amount of mineralized matrix and collagen content, increasing the β dispersion for this structure. Interestingly, the differences in electrical parameters between human and bovine bone showed similar behavior to the sparse-dense structure relationship. This could possibly result high collagen content and low water content found in bovine trabecular bone. It could also suggest that the basic structural and compositional

---

3It has been hypothesized in study IV that the interstitial bone marrow water significantly contributes to the overall trabecular bone conductivity.
properties are the main determinants of electrical characteristics, and that the other differences between human and animal bone tissue are consequently less significant.

The findings of the supplementary data suggest that EIS may be feasible and useful also at microwave frequencies. Measurements at this high frequency band may provide information on e.g., microstructural anisotropy not obtainable from low-frequency measurements. However, further research is needed to confirm the findings. Contact between electrode and sample must be optimized, by for instance improving the flatness of the sample surface or employing Micro-Electro-Mechanical System (MEMS) technology when preparing the probe. Additionally, the small number of samples could be the reason for the insignificance of certain correlations obtained in this study.

Even though electrical properties, relative permittivity in particular, were good predictors of trabecular bone BMD as well as other structural, mechanical and compositional quantities, the applications of EIS techniques in clinical bone diagnosis are limited. The method may be feasible during some special cases of open surgery. Further, it may be used for quantitative evaluation of bone grafts in vitro. Additionally, it might be useful in laboratory practise, providing fast and cheap estimation of bone mechanical, structural and compositional characteristics. The results presented here might be useful in the development of electrical impedance tomography methods, where the quality of bone could be assessed based on the electrical parameters measured. On the other hand, the detailed electrical characterization of bone is necessary for determining the relationship between the current and the electrical field, as well as for the current paths during electrical stimulation of bone fractures [130]. For instance, conductivity should be known if we are to choose the correct magnitude of the voltage [119]. Thus, if the mineral density of bone is known, the relationships presented in this study provide a tool for the estimation of electrical or dielectric properties.
9. Discussion
In this study electrical and dielectric properties of trabecular bone have been characterized. The wide range of frequencies applied improves the significance and potential application of the results. The quality of bone samples, understood as the architecture of trabecular elements, their connectivity, the amount of minerals, the mechanical strength, the presence or absence of microdamage and the chemical composition of the matrix, was evaluated and correlated with EIS results. The most important results can be summarized as follows:

1. Dissipation factor and especially relative permittivity were sensitive parameters to changes in mechanical, structural or compositional characteristics of trabecular bone. Additionally, conductivity showed a strong dependence on water content as well as on the shape and amount of trabeculae. Yield strain in trabecular tissue was only predicted by specific impedance.

2. Due to the frequency dependence of linear correlations between certain electrical and dielectric parameters and other investigated quantities, frequency bands with the highest potential to estimate bone quality were determined. In bovine trabecular bone, parameters obtained at frequencies above 50 kHz were sensitive to variation in mechanical characteristics. In human trabecular bone, however, electrical spectra obtained at frequencies between 100 kHz and 5 MHz contained the most valuable information. Additionally, some parameters, such as water content, dry density or connectivity, could be characterized more efficiently by measurements at frequencies between 100 Hz and 1 kHz.

3. In human bone, electrical and dielectric parameters were more sensitive to variation in mechanical properties than in bovine bone. Especially tissue strength was reflected by the EIS parameters. Relative permittivity and dissipation factor were found to be capable of detecting microdamage in trabecular structure.

4. Microstructural parameters related to the surface of trabecular structure were found to be the main determinants of relative permittivity, while the shape and amount of trabeculae were primarily related to other electrical...
and dielectric parameters. This suggests that the variation in different microstructural elements may be detected by various electrical parameters.

Water content was strongly related to all electrical and dielectric parameters, conductivity in particular. It is possible that that interstitial bone marrow water has a major impact on overall trabecular bone conductivity. On the other hand, fat content correlated only with the relative permittivity at frequencies higher than 100 kHz. Additionally, collagen content and the polarization effects on its surface associated with the hydration layer, were the main determinant of $\beta$ dispersion.

5. Bone with healthy, dense trabecular structure was found to have lower conductivity and higher relative permittivity at frequencies between 100 kHz and 5 MHz than tissue with a sparse structure. Moreover, tissue water and fat contents could be determined by a measurement of relative permittivity and conductivity at two separate frequency bands. This, along with the ability of EIS to detect microdamage of trabecular structure, suggests the potential of EIS as a tool to assess bone quality. However, further theoretical, experimental, and technical developments of the method are needed to confirm the present findings and to fully reveal the potential of the method, especially at radiofrequencies. The geometry of the measurement cell at frequencies below 1 MHz could consist of two electrodes in one plane in order to make the measurements fast and simple.


ERRATUM

In the study II the Figure 3C, as well as the values for phase angle at 1.2 MHz, has been misprinted. The correct figure (Figure E.1) and the values of the parameter (Table E.1) are presented below.

![Figure E.1](image)

**Figure E.1**: Phase angle as a function of frequency (mean ± υ) for the femur and tibia.

**Table E.1**: Mean values (± υ) for the phase angle at 1.2 MHz of human trabecular bone in femur and tibia.

<table>
<thead>
<tr>
<th></th>
<th>Femur (n = 10)</th>
<th>Tibia (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase angle (°)</td>
<td>−2.0 ± 0.8</td>
<td>−1.3 ± 0.5</td>
</tr>
</tbody>
</table>
Additionally, in the study III, the name of the 2nd and 3rd column in the Table 1 has been exchanged. The correct table is presented below (Table E.2).

**Table E.2:** The variances and coefficients of the principal components for electrical parameters of trabecular bone samples \( n = 26 \) measured at 1.2 MHz.

<table>
<thead>
<tr>
<th>Field propagation component</th>
<th>Energy component</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of explained variation</td>
<td></td>
</tr>
<tr>
<td>68.2%</td>
<td>27.0%</td>
</tr>
<tr>
<td>Phase angle</td>
<td>0.985</td>
</tr>
<tr>
<td>Conductivity</td>
<td>0.982</td>
</tr>
<tr>
<td>Specific impedance</td>
<td>-0.939</td>
</tr>
<tr>
<td>Dissipation factor</td>
<td>0.770</td>
</tr>
<tr>
<td>Relative permittivity</td>
<td>-0.042</td>
</tr>
</tbody>
</table>

92
Kuopio University Publications C. Natural and Environmental Sciences


