

Biomechanical characterisation of the bone-implant interface

Manon Fraulob

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Biomechanical characterisation of the bone-implant interface

by

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supervised by Guillaume HAIAT

defended on October 14th 2020

in front of the following board of examiners :

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Acronyms and abbreviations

- AFM : Atomic Force Microscopy
- BIC : Bone-Implant Contact
- BII : Bone-Implant Interface
- DMA : Dynamic Mechanical Analyses
- FDTD : Finite-Difference Time-Domain
- FE : Finite Element
- FTIR : Fourier-Transform Infrared Spectroscopy
- FWHM : Full Width at Half Maximum
- μ CT : Micro-Computed Tomography
- PMMA : Polymethyl Methacrylate
- PTFE : Polytetrafluoroethylene
- QUS : Quantitative UltraSound
- RFA : Resonance Frequency Analysis
- ROI : Region Of Interest
- SAXS : Small Angle X-ray Scattering
- SEM : Scanning Electron Microscopy
- WAXS : Wide Angle X-ray Scattering
- 1D : One-Dimensional
- 2D : Two-Dimensional
- 3D : Three-Dimensional

Introduction

The present thesis aims at characterising periprosthetic bone tissue. In the current clinical context, with population ageing and the high number of accidents, orthopaedic and dental implant surgeries are common procedures. However, the rate of implant failures is still important, which triggers the need for research studies to understand and then prevent such surgical failures. Indeed, even if the reasons for the loss of an implant appear related to a lack of stability, they still remain unclear.

Implant stability corresponds to the quality of the implant fixation within the bone tissue just after the surgery as well as after a few weeks or months of healing. During the healing period, bone tissue develops around the inserted implant thanks to the osseointegration process forming a bone-implant interface (BII). The extent of such boneimplant contact and its multi-scale compositional, structural and mechanical properties determine the BII strength and are then of foremost importance to achieve an adapted implant stability. The properties of the BII are interdependent since bone tissue is a composite material composed of minerals and collagen and displays a complex hierarchical structure, from the nano- to the macroscale. Those properties are also in constant evolution during healing as bone adapts to its environment, and many environmental factors such as implant surface roughness may affect osseointegration. Consequently, evaluating the intricate variations of bone quantity and properties at the BII over healing time is essential to understand osseointegration phenomena and implant stability.

However, experimental characterisation of the BII may be tricky because it requires *in vivo* experimentations following ethical standards and with high interspecimen variability. The preparation of the samples may be challenging to access the BII without damaging it and, around clinical implants, newly formed and mature bone tissues are undifferentiated at the BII, which complicates the analysis of tissue formation. To extensively understand osseointegration and access all interdependent bone properties, research studies should associate various techniques, which may be incompatible with each other limiting sitematched analyses and the reuse of samples.

Using an *in vivo* standardised bone chamber implant model to clearly distinguish newly formed from mature cortical bone, this PhD work implements a multi-modal and multi-physics approach to experimentally characterise the BII throughout the bone scales (macro-, micro- and nanoscale) in site-matched regions of interest. The quantity and local properties of bone tissue around the implant surface are evaluated as a function of healing time to clarify the conditions leading to long-term implant stability and then to surgical success. First, the literature review in chapter 1 sets the context of the work, with an emphasis on the description of bone multi-scale organisation, osseointegration and implant stability. The investigated properties, bone-implant contact, bone quantity as well as quality, gathering bone composition, structure and mechanics, are defined in the context of the BII. This chapter also introduces the necessary methods for the following work: the *in vivo* standardised coin-shaped implant model considered in the thesis' experimental studies and an overview of some of the most important bone characterisation techniques to access various bone features (content, structure, composition or mechanics).

Chapter 2 explores the influence of healing time and of implant surface roughness on the amount of bone formed in direct contact with the implant surface. This study combines quantitative ultrasound (QUS) and histological analyses to evaluate the boneimplant contact at the macro- and microscale, respectively. The obtained experimental results are validated with a finite element (FE) model simulating the propagation of ultrasounds through the BII modelled as a sinusoid.

To pursue the BII characterisation by taking into account bone quality properties, the difference in tissue composition and nanostructure between newly formed tissue within the bone chamber and mature bone is investigated with Raman spectroscopy in chapter 3. The impact on the respective microscopic elastic properties is also assessed in site-matched locations using nanoindentation.

Then, combining nanoindentation with micro-Brillouin scattering and histology, chapter 4 deepens the characterisation of bone elastic properties, through a spatial investigation within the bone chamber and temporal evolution along healing time. This study considers two healing times and the bone chamber below the implant surface is divided into four regions of interest to investigate spatial variations of indentation elastic moduli within the newly formed bone chamber depending on healing duration. Furthermore, elastic properties are also assessed by wave velocities within the same regions using micro-Brillouin scattering and, in combination with elastic moduli, they inform about bone mass densities.

Based on the results of this PhD work, future research directions are presented in chapter 5 to pursue the bone characterisation at the BII, to expand the investigation of resulting biomechanical properties and to enlarge the study to the effects of mechanical loading and mechanotransduction. This research work aims at clinical applications to improve long-term implant stability by optimising implants' design and treatments in orthopaedic and dental surgery. New medical devices to assess implant stability can also emerge from the used research techniques.

Finally, a synthesis and conclusion of the whole thesis highlights the relations between all the conducted studies especially the interconnection between bone properties, in order to broaden our knowledge on osseointegration and orthopaedic and dental implant stability.

Chapter 1. Bone and osseointegration

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1.1 Bone tissue

Bones are basic units of the skeleton ensuring numerous functions such as protecting organs, supporting other biological tissues and enabling motion. Their composition also ensures mineral storage and they host the marrow. To achieve all those functions, bone is a complex material with an highly organised multi-scale composition and structure.

1.1.1 Bone components and hierarchical structure

Bone tissue is a composite material composed of around 70 % of an inorganic phase and 30 % of an organic phase. The inorganic phase is a mineral constituted of hydroxyapatite $Ca_5(PO_4)_3OH$ crystals. The organic phase is mainly composed of type I collagen, of noncollagenous proteins, proteoglycans (GAGs) and lipids [163, 172]. Both phases contain water as well. At the scale of tens of nanometres, the collagen molecule is a triple helix made of three polypeptides which are chains of amino acids [113] such as phenylalanine, proline, and hydroxyproline. At the scale of hundreds of nanometres, bone crystals, which are thin plate-shape crystals, gather into crosslinks between collagen molecules forming bundles, grouped into mineralised collagen fibrils (Fig. 1.1) [113, 209]. Surrounded by extrafibrillar crystals, fibrils finally gather into lamella at the scale of 2–9 µm forming lamellar bone microstructure [189].



Figure 1.1 – Schematic multi-scale hierarchical structure of bone tissue (adapted from [189])

Composed of the same nano- and microscale hierarchical structures previously described, lamellar bone tissue assembles into two distinctive structures at the mesoscale. Cortical bone, the outer layer of bone (Fig. 1.1 and Fig. 1.2), is a dense and stiff tissue made of osteons at the scale of 100 μ m. Under the cortical layer, trabecular bone (Fig. 1.1 and Fig. 1.2) is a more porous and softer tissue, made of plates and rods.



Figure 1.2 – Mesoscopic architecture of cortical and trabecular bone (adapted from [15])

1.1.2 Bone remodelling and healing

Bone is a dynamic, vascular, living tissue that changes throughout life [45] as its matrix is formed, repaired and restructured in a continuous process called bone remodelling. Every year, an adult remodels 25 % of his trabecular bone and 4 % of his cortical bone [59].

Several bone cells are involved in the bone remodelling process. First, osteoclasts remove the old bone tissue. After bone resorption, osteoblasts form new bone tissue by producing bone organic matrix and depositing hydroxyapatite enabling then bone mineralisation. Some osteoblasts are trapped during the bone formation process and become osteocytes. The precise role of those cells is still investigated, but osteocytes, in association with bone lining cells on the bone surface, appear to be part of the signalling pathway triggering the bone remodelling process in response to mechanical stress [107].

This basic multi-cellular unit ensures bone formation in four different situations: i) initial formation of bone in embryos and fetus ; ii) bone growth during youth until reaching an adult size ; iii) bone remodelling during all life ; and iv) bone healing. During adult life, in addition to the continuous bone remodelling, bone formation is triggered when bone is affected by microcracks, fracture or bone damages due to a disease or surgery. To reconstitute the tissue continuity, a healing process will then start creating first an irregular woven bone which will later undergo remodelling to form lamellar and structured bone [45]. The case of healing around an implant after surgery, studied in this thesis, is presented in the next section.

1.2 Bone-implant interface (BII)

1.2.1 Implants

Nowadays, trauma injuries provoked by road traffic, sports and work accidents often occur. Moreover with population ageing, bone disorders, like osteoporosis weakening bone, become more and more common. In these clinical situations, inserting implants characterised by their design and their biomaterials within bone tissue has become a common practice in orthopaedic and dental surgery [221]. Replacing a joint or a tooth, the main functions of implants are to provide mechanical and structural support, restore the functionality of the treated organ, integrate with the damaged tissue and promote healing [3].

Implant material is chosen to be biocompatible and to have mechanical properties adapted to the implantation site [221]. More than 70 % of implants (and up to 95 % in orthopaedics) are made of metal [79]. Metal implants are used for their mechanical support. They aim to strongly bind to bone, limiting movements between implant and host tissue, and providing physiological load bearing functionality to the implantation site [3]. With their biocompatibility, low elastic modulus and high resistance to corrosion [180], titanium alloys are often used to design orthopaedic and dental implants, especially Ti6Al4V (the material studied in this thesis) composed of 90 % of titanium (Ti), 6 % of aluminium (Al) and 4 % of vanadium (V).

1.2.2 Implant stability and osseointegration

Implant stability is essential as it determines the long-term surgical success. Immediately after surgery, primary stability mostly depends on the surgical procedure, the implant design and bone properties at the implantation site [76]. Primary stability of the implant is a necessary condition to obtain a long-term stability resulting from osseointegration phenomena.

During the weeks following surgery, peri-implant healing process takes place at the implantation site and bone tissue develops around the implant, and in particular in direct contact with the implant surface. Initially defined by Branemark et al. in 1977 [27] and by Albrektsson et al. in 1981 [7], osseointegration is described as a direct contact between the living bone and implant on the microscopic level [7]. It creates a bone-implant interface (BII), which allows the long-term implant stability defined as secondary stability. Secondary stability relies on the implant's design, material and surface properties, on the frictional properties between the implant and surrounding bone, as well as on the geometry and quality of bone tissue at the implantation site and its remodelling activity [76].

During osseointegration, osteoinduction occurs [6] as osteogenic cells differentiate into osteoblasts which will synthesise new bone through a woven collagenous matrix, later mineralised into bone and the whole process will spatially progress. Contact osteogenesis corresponds to *de novo* bone formation on the implant surface which progresses towards the host bone. In parallel, distance osteogenesis corresponds to bone formation from existing host bone following surgery and progresses towards the implant surface [45, 112]. When both contact and distance osteogenesis occur, bone tissues formed in the two directions merge at late osseointegration stage representing the end of the healing process with a tight BII [77].

1.2.3 Osseointegration performance

Despite their routine clinical use, failures of implant surgery still occur and improvement is needed [3]. One reason of osseointegration failure is the amplitude of micromotions at the BII. During bone healing, low-amplitude micromotions (lower than 40-70 μ m) stimulate bone remodelling, whereas fibrous tissues may develop instead of an osseointegrated interface in the case of excessive interfacial micromotions (above 150 μ m) following surgery [26]. The effect of micromotions explains why primary stability (subsection 1.2.2) should not be too low to avoid excessive interfacial micromotions [53, 197] causing implant failure. It should also not be too high since an excessive level of stresses may lead to bone necrosis [44, 54].

To improve osseointegration outcomes, various factors have been identified to affect the amplitude of micromotions and bone properties at the implantation site, such as the surgical procedure, as well as the implant geometry and material. Among implant properties, surface roughness influences osseointegration. Surface roughness can be modified with sandblasting and/or chemical treatments [121, 186]. It is usually evaluated using the S_a value, the average surface roughness, measured by mechanical contact profilometres, optical profiling devices or scanning probe microscopes [218]. Rough surfaces increase the friction coefficient at the BII, thus reducing micromotions and increasing primary stability. Moreover, rough implants present a higher specific surface area on which bone cells can interact, also stimulating bone tissue repair [67]. However, a compromise should be found with a high roughness to sufficiently stimulate bone remodelling without creating stress concentration and debris, which could damage bone tissue and thus hamper the osseointegration process. In particular, surface roughness between 3.6 and 3.9 μ m seem to be optimal for osseointegration [179].

1.2.4 Bone content and tissue quality at the BII

As explained in subsection 1.2.2, the BII formed during healing will condition longterm stability. Therefore, to evaluate osseointegration performances, the BII needs to be fully characterised to identify properties ensuring long-term implant stability. Resulting in the formation of the BII, osseointegration phenomena lead to an increase in the amount of bone tissue in direct contact with the implant surface. The obtained bone-implant contact (BIC) at the BII, defined as the first tens micrometre-thick layer of bone tissue at the implant surface, enables implant anchorage and is besides an important determinant of implant stability [76]. Another factor is the amount of bone tissue in the surroundings, further from the implant surface, which also develops during healing. This bone content beyond the direct contact layer around the implant surface is defined as bone quantity [76]. Within bone ingrowth, bone properties evolve during mineralisation as bone adapts to its mechanical environment at every scales from its composition to its nano-, microand mesostructure, inducing changes in its mechanical properties [67]. The combination of bone structural, compositional and mechanical properties define bone quality [50]. Therefore, bone-implant contact, bone quantity and quality within a region of interest located at a distance up to 200 μ m from the implant surface determine surgical success [76] and should then be investigated.

1.3 In vivo models of the BII

The study of osseointegration to understand implant stability requires to be able to replicate the conditions of surgical implantations. Different strategies can be adopted. In vitro studies consider bone-implant contact outside any living being using synthetic materials to mimic bone tissue [216]. Conversely, when the implant is inserted within a living being to monitor osseointegration evolution during healing, this is an *in vivo* study [212, 215]. In vivo studies in the human body are called clinical studies [111]. At the end of an animal *in vivo* study, samples can be harvested to conduct *ex vivo* analyses on the resulting osseointegrated implants [212]. However, due to the interdependency of all bone parameters and properties, bone experimental characterisation may not give clear explanations, justifying *in silico* approaches to characterise the BII at various scales using computer simulation [85, 134, 214].

1.3.1 Animal models

In vivo animal studies are interesting ways to produce osseointegrated samples for ex vivo analyses as in vitro studies cannot replicate many of bone features. Even if bone tissue has many common points throughout species, every animal model is specific. Common animal models include mice, rabbits, sheeps, pigs, dogs or primates [167, 205]. The choice of the animal model depends on the topic of the study (such as bone disease or characteristic bone structure) and the animal convenience (such as size, housing or treatment).

Rabbit (often New Zealand white rabbit) is a common animal model to study bone ingrowth and the BII around an implant [205], on the distal part of the femur or the bilateral tibiae [122]. Despite different size, shape and loading, rabbit and human bones have similar composition with comparable bone mineral density and fracture toughness of mid-diaphyseal bone [122, 167]. Rabbit bone microstructure is made of vascularised osteons parallel to the long axis of the bone [167]. Furthermore, rabbits quickly reach skeletal maturity at only 6 months and have a faster bone turnover than other species (primates or some rodents, for instance) [122, 143, 167, 205]. This small animal model is also often chosen for its availability and convenience in housing and treatment compared to other species such as sheeps or pigs [122, 167].

1.3.2 Implant models

Different implant models have been studied in the literature, from clinical implants to adapted models to isolate phenomena and consider standardised configurations aiming at a better understanding of the osseointegration process.

Clinical dental implants have often been inserted into *in vivo* animal models to evaluate bone composition and structure [89] as well as mechanical properties of the tissue developed around the implant [36] or within the thread [228], in order to understand implant stability [212]. To isolate the implant surface from its biological environment and study the dependency between contact and distance osteogenesis (subsection 1.2.2), the implantation setup was modified by inserting a titanium tube between the implant and neighbouring bone (Fig. 1.3A) [38].



Figure 1.3 – A) Dental implant with titanium tube [38]. B) Screw-shaped implant with bone chambers in regions b. The red dotted line represents the cut bone wall [19]. C) Hollow dental screw with a bone chamber inside [17]. D) Implant design with an inner hollow canal (adapted from [114]). E) Titanium pin inserted in rat tibia [46]. F) Cut view of the subperiosteal titanium plate. The bone chamber filled with new bone is surrounded by red [42]. G) Hydraulic bone chamber [109].

Around clinical implants, newly formed and mature bone tissues are intertwined, which does not allow precise characterisation of new bone tissue. To study more specifically the bone ingrowth resulting from healing, bone chamber compartments isolating newly formed bone were created within threads (Fig. 1.3B) by modifying the shape of cylindrical titanium implants [30] and of commercial titanium screw implants [19]. A bone chamber was also designed inside a hollow titanium dental screw (Fig. 1.3C), where new bone could develop thanks to ingrowth openings and bone graft could be introduced [17]. Other implant designs were used to place bone graft [49, 114] or bone substitute [49] in an inner canal to evaluate the impact on bone growth located in the bone chamber in the inner hollow canal and/or outside in the gap between the implant surface and cortical bone (Fig. 1.3D) [49, 100, 114].

Complex implant geometry generates multi-axial stress distribution at the BII, making the process inducing the observed bone properties difficult to understand. Thus, simplified implant geometry have been used such as non-weight bearing titanium rods (Fig. 1.3E) avoiding any mechanical effect of the threading to characterise more specifically bone microstructure at the BII [46, 124] and its strength [46]. Implant geometry has also been adapted to study a plane BII limiting complex stress distribution with, for example, a subperiosteal titanium plate inserted with a trough to create a bone chamber (Fig. 1.3F) [42]. Cylindrical coin-shaped implants also allow a plane BII and such implants were used with the hydraulic bone chamber (Fig. 1.3G) where cyclic compressive loading through an hydraulic pressure was applied to assess the effect on bone microstructure within a new bone chamber [109]. Similarly, based on the implant model described in [175] (Fig. 1.4A), a coin-shaped implant model presented in Fig. 1.4B has been designed our group [136, 139] and is used in this thesis. Its simple geometry with a planar interface allows reproducible and standardised biomechanical conditions. The coin-shaped Ti6Al4V implant is surrounded by a polytetrafluoroethylene (PTFE) cap in order to i) limit bone growth and attachment on its lateral sides and ii) create a bone chamber between the cortical bone surface and the implant. The bone chamber is initially empty and will be filled with new bone tissue during healing, allowing a clear distinction between newly formed and mature bone (see Fig. 1.4B).



Figure 1.4 – A) Schematic cross-sectional view of two coin-shaped titanium implants covered with PTFE caps and stabilised in the cortical bone with a titanium plate retained by two screws [175]. B) Schematic cross-sectional view of the coin-shaped Ti6Al4V implant model inspired from [175] with a bone chamber between the cortical bone surface and the implant.

1.4 Experimental characterisation methods

Multi-modal, multi-physics and multi-scale methods need to be implemented to investigate all aspects of osseointegration phenomena including bone-implant contact at the BII, bone quantity further from the implant surface and bone quality properties (structure, composition and mechanics). In this PhD work, an experimental approach has been implemented with multiple interdisciplinary *ex vivo* analyses.

This section presents the current state of the art related to the techniques used or discussed in the following chapters of this thesis. Some other available methods, which won't appear in the thesis, are also mentioned to give an overview of existing experimental methods to characterise bone tissue and the BII. All the presented techniques are summarised at the end of the chapter in Fig. 1.10. A more complete review of the literature was published during the PhD to deepen the techniques applied to the BII [67]: Gao X., Fraulob M., and Haïat G., "Biomechanical behaviours of the bone-implant inter-

face: a review", Journal of the Royal Society Interface, 16: 20190259, 2019.

1.4.1 Bone-implant contact, bone quantity and structure

To ensure implant stability, a sufficient implant anchorage within the surrounding tissues is important. It is evaluated by analysing bone content around an implant and depends on bone-implant contact, bone quantity and its micro- and nanostructure. Different techniques can be used to reach different hierarchical level of periprosthetic bone, from the nano- to the macroscale.

1.4.1.1 Quantitative ultrasound (QUS) technique

Based on the sensitivity of the ultrasound wave to bone elastic properties with an axial resolution of a few tens of micrometres [115], the non-destructive QUS technique can estimate the bone-implant contact (with a lateral resolution around 0.5 mm [138]) from the ultrasonic response of the BII. The principle is to use the intensity of the received echo, which depends on the type of tissue in contact with the implant surface: the lower the acoustical impedance gap is, the lower the intensity of the QUS response is. Indeed, tissues in contact with an osseointegrated implants allow the propagation of ultrasounds (represented in Fig. 1.5).



Figure 1.5 – US wave propagation at the BII in the case of A) an osseointegrated implant and B) a non-osseointegrated implant.

Currently developed in our group [135, 138, 212, 215, 216], a decrease in the echo amplitude at the BII has been evidenced experimentally as a function of healing time, which could be explained by the increase in the BIC and bone quality around the implant surface, leading to a decrease in the gap of the mechanical properties at the BII during healing.

1.4.1.2 Histology

Histology is considered as the gold standard to assess osseointegration [61], but it is a destructive method. The process of sample preparation (presented in Fig. 1.6), can be complicated in presence of metallic implants with possible damage of the BII requiring the embedding of the sample in a resin. Then, a slice is observed with a microscope to visualise bone spreading at the implant surface and calculate the BIC ratio. In addition, histological analysis can also inform about bone quantity in the surroundings of the implant. However, it is only a 2D measure allowing the quantification of bone only in some slices [20, 25, 69] and it does not quantify bone quality. The resolution of histology, of the order of the micrometre, depends on the microscope resolution.

Histology could describe bone repair in the case of fracture [123]. Around dental implants [139], histomorphometric results have been related to the primary stability of the implant [154] and histological analyses have also been used to evaluate osseointegration [193]. The BIC ratio evaluates the amount of bone formed in direct contact with the implant surface. For instance around dental implants inserted in rabbit femures, the BIC



Figure 1.6 – A) Sample preparation process before histological analysis (adapted from [157]). B) Resulting histological slice of a dental implant (bone tissue is coloured in red).

was found to vary from BIC values of 30 % after 2 weeks of healing to BIC values of 50 % after 11 weeks of healing [212].

1.4.1.3 Micro-computed tomography (μ CT)

Micro-computed tomography (μ CT) analysis is of interest in order to quantify bone by 3D imaging [159]. It gives a full volumetric information at a resolution of a few tens of micrometres, and is fast to set up with no sample preparation, but it irradiates the sample affecting the bone properties [94]. μ CT often analyses the attenuation of an X-ray beam by the sample [227], but it may also use a neutron beam. Indeed, in presence of metal components, X-rays induce artefacts limiting the analysis of the interface, which is not the case with a neutron beam [94].

Within bulk bone tissue, X-ray μ CT gives access to the microstructure [75, 119]. In the surroundings of metallic implants, neutron μ CT was used in combination with X-ray μ CT [94] and pull-out tests [116] to visualise in 3D the quantity and microstructure of bone tissue developed at the BII.

1.4.1.4 X-ray scattering techniques

To deepen the investigation and access bone ultrastructure, X-ray scattering techniques analyse X-ray beams diffracted by the sample with a high resolution of a few nanometres. However, these techniques are destructive as they necessitate similar sample preparation as histology (subsubsection 1.4.1.2) to work on thin slices and they are irradiating too. The mineral crystals diffract the X-rays and the diffraction patterns at wide angles, acquired by the so-called wide angle X-ray scattering (WAXS), are representative of mineral crystalline structure, while the small angles with the small angle X-ray scattering (SAXS) contain information about particles size and alignment [161].

The inorganic structure of bulk bone, such as the shape, thickness and orientation of hydroxyapatite crystals could be characterised with WAXS/SAXS analyses [201, 207]. Little work has been done to investigate the periprosthetic bone tissue, but mineral crystals close to the implant surface were found to be preferentially aligned with the implant surface [28].

1.4.1.5 Other techniques evaluating bone structure

Scanning electron microscopy (SEM) SEM is a high resolution (around 20 nm [50]), versatile and easy-to-use 2D imaging method [157, 189], but its sample preparation may

affect bone properties [50]. SEM scans the specimen surface with a focused electron beam to access structural information at the bone surface like the morphology, the degree of mineralisation as well as osteocytes' alignment and the lacunae and canaliculi network [50, 157].

Electron tomography Electron tomography is a promising technique to visualise 3D structure with a high resolution (around 5-20 nm), but limited angles of 2D images may create artefacts in the 3D scan reconstruction. This technique has been used around hydroxyapatite or metal implants [72, 158].

1.4.2 Bone composition

1.4.2.1 Raman spectroscopy

Raman spectroscopy is a chemical analysis technique based on the interaction of light with the chemical bonds within a material, which can be applied to bone to extract information from the mineral and organic phases. This technique has a spatial resolution approximately equal to 1 μ m [50], it does not require any specific sample preparation and can be applied for various types of samples. It represents an interesting technique to investigate local bone tissue composition through spectra representing peaks at characteristic wavelengths corresponding to vibration of each bone component from the mineral and the organic phases. Such Raman spectrum is a fingerprint of the material. Bone composition is then evaluated based on parameters obtained from the spectrum such as mineral-to-matrix ratio, carbonate-to-phosphate ratio and mineral crystallinity [50]. Note that Raman spectroscopy also provides information about nanoscale bone structural properties since mineral crystallinity represents crystal size [188].

Raman spectroscopy has been used on bulk bone to characterise effects of metabolic disorders on bone tissue [91, 106, 162] and to investigate the remodelling process of healing bone tissues [4, 188]. Raman spectroscopy has been used at the BII to quantify the quality of bone formed *in vivo* around titanium implant coated with hydroxyapatite [89] or with a film containing a drug against osteoporosis [101] and around 3D printed Ti6Al4V implants [187].

1.4.2.2 Other techniques evaluating bone composition

Fourier-transform infrared spectroscopy (FTIR) FTIR spectroscopy has a spatial resolution of approximately 6 μ m and works with the same principle as Raman spectroscopy retrieving similar parameters from FTIR spectra, but with a higher signal-tonoise ratio than Raman spectroscopy [50]. However, FTIR requires very thin slices (tens of microns) which is barely impossible to reach with a metal-to-bone interface without any support. In bulk bone, FTIR spectroscopy has shown that bone compositional properties change with age and disease [24] and they are correlated to nanomechanical properties of bone tissue [217].

Elemental analysis For the observation of bone components, elemental analysis derived from the SEM method (paragraph 1.4.1.5) with an analysis called energy dispersive X-ray [50, 189] provides mapping at the BII of calcium and phosporus, the most abundant elements in bone aside from carbon. Such elemental analysis can also be obtained from electron tomography (paragraph 1.4.1.5) which suggested the existence of a nanoscopic interfacial layer forming an intimate contact between bone and the implant surface [73].

1.4.3 Bone mechanical properties

Bone quality is determined by bone structure and composition related to bone mechanical properties. Therefore, probing mechanical properties at different hierarchical levels is crucial to evaluate bone tissue quality.

1.4.3.1 Nanoindentation

Nanoindentation is a reference technique to retrieve the mechanical properties of a medium at a resolution of 10 μ m [1, 48], but it is destructive [67]. A rigid indentation tip with known properties and geometry (such as a Berkovich diamond three-sided pyramid probe [8, 31]) is pressed into a material to retrieve its elastic modulus and hardness from the curves representing the applied force as a function of the displacement, using the Oliver and Pharr method [1].

Nanoindentation assessed locally that cortical bone tissue (approximately 14 GPa) is stiffer than trabecular bone (approximately 2 GPa) [31]. Moreover, the changes in bone tissue properties have also been evaluated especially the evolution of the elastic modulus with age [93, 165, 220] and during healing in the case of bone defects [10]. Typical values of Young's modulus and hardness obtained in rabbit bone with nanoindentation are respectively 6-12 GPa and 200-500 MPa in newly formed bone and 9-17 GPa and 300-700 MPa in mature bone [10, 22].

At the BII, nanoindentation has been applied to assess the changes in periprosthetic tissue properties with different configurations. Using dental implants, the technique has been used to assess the effect of implant material and surface treatment [8, 99, 104], of location around the implant [104, 228], of mechanical loading [9] and of bone maturation and healing [99, 105]. Lower indentation moduli were measured within newly formed bone tissue compared to mature bone using the *in vivo* titanium plate model described in subsection 1.3.2 (Fig. 1.3F) [42]. Nanoindentation was also conducted using the *in vivo* standardised implant model (presented in subsection 1.3.2 (Fig. 1.4B)) to compare the properties of mature and newly formed bone tissue and show the increase in the indentation parameters with healing time [210].

1.4.3.2 Micro-Brillouin scattering

Micro-Brillouin provides complementary information to nanoindentation since it measures the ultrasonic wave velocity at the microscopic scale, which is related to both the elastic modulus and mass density of bone tissue. From the interaction between light and bone tissue, Brillouin scattering creates acoustic phonons corresponding to elastic wave and inducing a frequency shift of the incident wave. Therefore, the measurement of the frequency shift (of the order of the GHz) evaluates the wave velocity assessing elastic properties of the medium with a resolution around 10 μ m [140]. Even if this technique is non-destructive and does not apply any contact, only limited data is available at the BII as it is time-consuming and requires a high precision setup. Micro-Brillouin scattering has been used to investigate bone wave velocities, in particular within the femoral head [32] or a bone defect [5], as well as to assess bone anisotropic properties [102], bone structure and alignment [204] and the effect of decalcification [65]. Using the coin-shaped implant model (Fig. 1.4B), micro-Brillouin scattering has been used to quantify the differences between elastic properties of periprosthetic (4.97.10³ m/s) and mature (5.31.10³ m/s) bone tissues after 7 weeks of healing time [136].

1.4.3.3 Macroscopic tests

Push-out and pull-out tests The BII strength has been evaluated using push-out (Fig. 1.7a) [35, 203] and pull-out (Fig. 1.7b) [155] tests, where an osseointegrated implant is subjected to mechanical loading. However, these tests are very sensitive to misalignment errors and cracks propagate at the BII in an unstable manner and shear stress is observed as well [35, 203]. During push-out and pull-out tests, the maximal force and stiffness are recorded to evaluate the strength of the BII. For example, the impacts of surface roughness [179], surface treatment [178] and healing time [177] have been evaluated on the pull-out force.



Figure 1.7 – Push-out (a), pull-out (b) and torque (c) test configurations (adapted from [67]).

Torque tests In torque macroscopic tests on osseointegrated implants [98], the bone sample is attached and the implant is rotated until complete debonding (Fig. 1.7c). Contrary to push-out and pull-out configurations, torque tests allow crack propagation in a steady-state manner at the BII by generating shear stress, which is likely to occur *in vivo*. However, the test is sensitive to misalignment errors too and a specific implant model with a planar interface is necessary [67]. The debonding of Ti6Al4V coin-shaped implants (Fig. 1.4B) inserted into rabbit bone described the debonding of the BII coupling friction and mode III crack propagation thanks to an analytical model [137]. Similar torque test configurations have also been used to estimate the removal torque of dental implants [47].

Friction tests At the BII, assessing the friction coefficient which provides initial mechanical fixation for the implant's primary stability allows the understanding and prevention of micromotion that can hamper osseointegration (subsection 1.2.3). Friction tests require a standardised geometry, generating a plane contact between bone and implant [67]. Some measurement configurations developed to measure the friction coefficient are presented in Fig. 1.8.



Figure 1.8 – Schematic description of different friction tests at the BII. (a) Applied normal pressure, (b) applied normal load using a roller, and (c) using a constant weight (adapted from [67]).

1.4.3.4 Other techniques evaluating bone mechanical properties

Atomic force microscopy (AFM) At the nanoscale, bone local elastic properties can be studied with AFM at a resolution of around 10 nm [149], by monitoring the deflection of a cantilever with a predetermined stiffness resulting in a force–displacement curve similarly to nanoindentation. However, the geometry of the AFM cantilever tip is not precisely known, inducing errors in the estimation of the tip displacements in all directions [67]. In rabbit femur, a gradient of elastic properties was evidenced radially to the implant surface with AFM measurements of the mechanical response at different distances from the titanium implant [43].

1.5 Numerical models

All the experimental characterisation techniques described above have the advantage of probing properties on *ex vivo* samples, but measurements remain localised and dependent on the biological environment. Furthermore, all determinants of implant stability, i.e. bone-implant contact, bone quantity and bone quality, with its compositional, structural and mechanical properties, are interdependent. All these simultaneous bone modifications make the occurring phenomena intricate. Numerical modelling, allowing the control of all parameters independently, represents a complementary tool to better understand each influence on osseointegration phenomena. Furthermore, numerical models are often validated by experimental tests or based on experimental data used as realistic biological inputs (density or elastic modulus within cortical and trabecular bone, for instance). Thus, although numerical models do not completely replace animal experimentations, they limit their number, which makes them an ethical approach.

1.5.1 Mechanical models

1.5.1.1 Modelling the BII

Modelling the BII and the osseointegration process is a challenging task but it is necessary as osseointegration strongly affects the stress and strain fields observed around the implant. Some finite element (FE) studies considered a fully bonded interface corresponding to a fully integrated BII with an acetabular cup implant [152] or a dental implant [129], even if the implant is overconstrained since such configuration does not occur in the clinic. Then, around a dental implant, trabecular bone was considered fully bonded at the BII, while the cortical layer was divided into four sublayers on the top of the trabecular bone surface to allow a partial integration of the cortical BII [78]. Friction between implant and bone tissue was enabled for non-osseointegrated parts of the BII. Around an orthodontic miniscrew [196], partial osseointegration was also simulated with a BIC value by randomly selecting a percentage of the soft tissue layer which surrounds the miniscrew and assigning bone properties to it.

During osseointegration, the BII evolves as it undergoes remodelling, which could be introduced in FE models by changing iteratively bone density [74] or with a thin BII layer described by Drucker-Prager plasticity model [126]. The BII was also modelled with a network of linear springs whose stiffness vary with time in response to a remodelling feedback [56]. This spring network model generates a dynamic mechanical system to transfer loads from the implant to the surrounding bone tissue. More recently, a non-linear spring model was developed in our group to model an osseointegrated BII with an effective contact stiffness [170], suggesting new methods to integrate evolving microstructure of the BII into large scale FE models.

1.5.1.2 Complementing the experimental characterisation methods

Using such BII models, ex vivo analyses can be validated with numerical models to further understand osseointegration. FE models are often obtained by segmentation of μ CT images (subsubsection 1.4.1.3) to work with realistic bone microstructure [168, 192]. To better understand bone failure in ex vivo push-out tests, shear stress distributions around cylindrical implants were visualised using such FE models [141] and a fracture mechanics approach was implemented leading to matching results with experiments [198]. Furthermore, after an implant was cyclically stimulated *in vivo*, mechanical macroscopic tests (subsubsection 1.4.3.3) were applied until rupture, and the μ CT image-based FE model was able to predict the obtained bone properties [168]. In the case of pull-out tests on screws (paragraph 1.4.3.3), a FE model was first validated by correlation with *in vitro* preliminary results and calibrated with ex vivo measurements after rats' sacrifice, before it enabled the monitoring of the adaptation of bone mechanical parameters over healing time, which could not be accessed *in vivo*. The results were validated by *in vivo* μ CT scanning throughout the whole healing process [192].

1.5.1.3 Preventing stress shielding

Stress shielding is a common cause of implant failure in orthopaedic surgery, occurring in the case of an important difference in terms of material properties between the implant and bone tissue [67]. The implant is stiffer than the surrounding bone, and therefore carries loads forming a "shield" against mechanical stimulation for the host bone and causing its resorption which may lead to aseptic loosening [97]. The stress shielding effect comes from stress and strain fields distributed around the osseointegrated implant. To better understand this phenomenon at the BII, a microscopic FE model investigated factors influencing shear stress triggering stress shielding [171]. High shear stress at the BII were associated with low BIC values corresponding to low implant stability and implant surface roughness, modelled with a sinusoid, was proven to have an impact on shear stress within a distance of the order of the interface wavelength away from the implant surface. To limit stress shielding effects, many other factors have been numerically investigated considering stress, strains and displacements at the BII. Since stress shielding results from the contrast in material properties between implant and bone tissue, titanium alloys [153] or surface coating using polyether ether ketone (PEEK) [11] have been studied to consider implants with Young's modulus closer to Young's modulus of bone. To approach bone properties more specifically, 3D printed implants mimicking bone microstructure were created allowing a reduction of bone loss [13]. Functionally graded materials, consisting of gradual patterns of composition and/or microstructure to better fit with bone tissue properties, appear to be a promising solution too [81]. Furthermore, different implant design such as threaded or non-threaded femoral stem [169] and stems with different lengths [97] have been tested to identify the optimal geometry reducing the stress shielding effect. For example, in spite of low mechanical stability, short stems seem to reduce stress shielding and stress concentration [97]. Surgical procedure may also play a role as a strong inclination of the femoral stem at the insertion in an uncemented total hip replacement arthroplasty can lead to severe stress shielding affecting long-term surgical outcomes [103].

1.5.2 Acoustical models

Experimentally validated with the QUS methodology described in subsubsection 1.4.1.1, complementary acoustical numerical models have been built by our group [134, 213, 214] to better understand the propagation of ultrasonic waves at the BII, and the impact of bone-implant contact and bone quality. Considering dental implants, osseointegration level can be modelled with a layer surrounding the implant, of variable thickness, depth and properties, to mimic newly formed bone, fibrous tissue or water (Fig. 1.9A,B). The ultrasonic response of the BII is sensitive to bone healing and it decreases when primary and secondary stability increase corresponding to an increase in bone-implant contact and in surrounding bone quality including for instance mass density and elastic constants. Such phenomenon was evidenced first around a cylindrical implant using a 2D finite-difference time-domain (FDTD) model [134] and a 3D axisymmetric FE model [213] (Fig. 1.9A), and then around the geometry of a clinical implant in a 3D axisymmetric FE model (Fig. 1.9B) [214].

At the microscale, the effects of the BIC and of the implant surface roughness on the ultrasonic propagation at the BII have been evaluated using FE models [83]. Idealised (Fig. 1.9C) [84] and realistic (Fig. 1.9D) [85] 2D roughness profiles were studied showing that the reflection coefficient of the BII at 15 MHz becomes lower when osseointegration progresses reducing the thickness of the soft tissue layer between bone and implant. The model with idealised roughness profile evidenced that the reflection coefficient is affected by material properties within the first 25 μ m away from the implant surface [84]. Furthermore, the model with the realistic roughness profile was proven to be equivalent to a 2D sinusoidal profile [85].



Figure 1.9 – 3D axisymmetric FE models of A) a cylindrical implant (adapted from [213]) and B) a clinical implant (adapted from [214]). Microscopic 2D models of the BII with C) a sinusoidal roughness profile and D) an original roughness profile (adapted from [85]).

1.6 Key points

- Bone is a complex composite material with a hierarchical multi-scale organisation.
- During healing, osseointegration corresponds to bone formation in direct contact with the implant surface. It creates a bone-implant interface (BII) and ensures implant's long-term stability.
- An *in vivo* standardised coin-shaped implant model has been developed to study osseointegration in standardised conditions and to clearly distinguish newly formed bone, within a bone chamber, from mature bone.

- Numerous multi-physics experimental methods, summarised in Fig. 1.10, can be used to characterise bone-implant contact, bone quantity and quality (structural, compositional and mechanical properties) at different scales (macro-, micro- and nanoscale).
- During the healing period, bone tissue at the BII has changing structure and composition affecting their biomechanical behaviour.
- As bone structure, composition and mechanics are interdependent, investigating the evolution of multiple properties in site-matched locations using a multi-scale, multi-modal and multi-physics approach can help the understanding of the osseoin-tegration phenomena.
- Complementing and validating experimental characterisation, numerical simulation, with mechanical and acoustical FE models for instance, enables further investigations about osseointegration by controlling independently each parameters and it limits animal experimentations.





Chapter 2. Bone-implant contact at the BII

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2.6	Key po	pints
2.1 Introduction

As explained in chapter 1, to better understand implant stability leading to surgical success, research studies need to be elaborated to retrieve information about osseointegration phenomena, the resulting bone growth and bone quality at the BII. A first parameter to be investigated in order to assess osseointegration is the BIC which quantifies bone content in direct contact with implant surface (chapter 1, subsection 1.2.4).

In this chapter, the BIC is investigated comparing a non-destructive QUS technique developed at MSME (chapter 1, subsubsection 1.4.1.1) with the gold standard histology (chapter 1, subsubsection 1.4.1.2) to quantify the contact conditions between new bone tissue and the whole implant surface, with a macro- and microscopic resolution. To ensure the visualisation of only newly formed bone tissue, this bi-modal characterisation approach has been implemented with the previously described *in vivo* bone chamber model (chapter 1, subsection 1.3.2). The study has been designed to evaluate the influence of healing time (7 and 13 weeks) and implant surface roughness (smooth and rough levels) on the BIC. Moreover, the experimental results from the QUS technique have been compared to numerical simulation, adapting a 2D FE model developed in our group (chapter 1, subsection 1.5.2).

To successfully conduct the study, the implantation phase was enabled by the collaboration with clinical surgeons from Hôpital Henri Mondor AP-HP (Créteil, France) and veterinary experts from École Nationale Vétérinaire d'Alfort ENVA (Maisons-Alfort, France). This *in vivo* study involved researchers from LAMIH (Laboratoire d'Automatique, de Mécanique et d'Informatique Industrielles et Humaines) UMR CNRS 8201 (Université Polytechnique Hauts de France, Valenciennes, France) for the surface analysis. This interdisciplinary work has resulted in the submitted paper [64]:

Fraulob M., Vayron R., Le Cann S., Lécuelle B., Hériveaux Y., Albini Lomami H., Flouzat-Lachaniette C. H., and Haïat G., "Quantitative ultrasound assessment of the influence of roughness and healing time on osseointegration", *Scientific Reports*, submitted.

2.2 Methods

2.2.1 Implants

Following the implant model described in subsection 1.3.2 (chapter 1), twenty-eight coin-shaped implants made of medical grade titanium Ti6Al4V alloy were prepared (diameter 5 ± 0.05 mm and thickness 3 ± 0.05 mm) (Fig. 2.1A,B). To evaluate the influence of implant surface roughness, the implant surfaces were first mirror polish and then sandblasted with two different aluminium oxide (Al_2O_3) powders (Cobra and Basic Quattro, Renfert, Hilzingen, Germany), leading to two series of samples with different surface roughness. The smooth (S) (respectively rough (R)) series was obtained after sandblasting the implants with 25 µm (respectively 250 µm) particles for 8 seconds (respectively 30 seconds) at 5 bar (respectively 6 bar). During sandblasting, the implant surface was maintained perpendicularly to the particle direction at a distance of 20 mm from the blasting nozzle.

All implants were cleaned with ethanol and put in an ultrasound bath first with

absolute ethanol for 20 min and then with demineralised water for 30 min. Before surgery, they were sterilised by autoclaving (1.5 atm at 121°C for 20 min).

All coin-shaped implants were surrounded by PTFE caps creating a 200 μ m-thick bone chamber (corresponding to the region of interest influencing osseointegration success mentioned in subsection 1.2.4 (chapter 1)) below the implant surface (Fig. 2.1A,B).



Figure 2.1 – A) Sandblasted coin-shaped implant with a PTFE cap. B) Schematic crosssectional view of the model with the 200 μ m-thick gap. C) Medial view of an implanted distal femur, after sacrifice.

2.2.2 Topographical analysis

The surface roughness properties affecting osseointegration subsection 1.2.3 (chapter 1) were quantified for both implants groups via topographical analysis, performed at LAMIH UMR CNRS 8201. The surface profiles of one implant from each surface roughness series were then analysed using the Alicona Infinite Focus device with a x10 objective and a resolution of 1.09 μ m. Surface properties were extracted from 10 regions of interest (ROIs) of 1x1 mm² spread over the implant surface. Each topographical analysis led to a 3D image of the ROI, analysed to extract five typical roughness parameters: the surface roughness S_a , the sum of the largest peak height and pit depth S_z , the mean dale area S_{da} , the mean dale volume S_{dv} and the auto-correlation length S_{al} following a method described in details in [66].

2.2.3 Surgical procedure

After the design and preparation of the *in vivo* study (Fig. 2.2A), the implantation on rabbits was performed by clinical surgeons from Hôpital Henri Mondor AP-HP (Créteil, France) at ENVA (Maisons-Alfort, France) (Fig. 2.2B). Seven New Zealand white male rabbits (average weight 3.9 kg) were implanted with four coin-shaped implants each. Two implants from the S (respectively R) series were inserted medially in the left (respectively right) posterior limb: one at the distal-medial femur and one at the proximal-medial tibia (Fig. 2.2B).

After a subcutaneous injection of 0.03 mg/kg buprenorphine (Bupaq[®], Virbac, Carros, France) 30 minutes before surgery, the animals were anaesthetised via intramuscular injection of 0.5 mg/kg diazepam (Valium[®], Roche, Basel, Switzerland), 0.25 mg/kg metedomidine hydrochloride (Domitor, Virbac, Carros, France) and 20 mg/kg ketamine hydrochloride (Ketamine1000[®], Virbac, Carros, France). They were intubated with a 3 mm endotracheal tube and ventilated during the whole procedure at controlled pressure



Figure 2.2 – Stages of the *in vivo* study: design and preparation of the study (implant model, sandblasting, topographical analysis...) (A), surgical implantation (B) with a picture of the operating room (top left), a picture of an inserted coin-shaped implant maintained by elastic strings (bottom left) and a schematic representation (front view) of the surgical implantation on rabbit posterior femures and tibiae (right), *ex vivo* analysis (C) on the samples harvested after healing.

with an air-oxygen mixture enriched with isoflurane (between 1.5 and 2 %) in particular EtO2 > 50 %. The animal monitoring consisted in controlling and following the O_2 saturation, the electrocardiogram and respiratory parameters.

After exposing the implantation site (medial knee), a flat bone surface of 5.6 mm diameter was levelled to i) create a cortical bone planar surface to receive the implant and ii) stimulate osseointegration phenomena after surgery. Four irrigation holes (diameter 0.9 mm) were drilled through the cortex to allow blood supply, and four holes (diameter 1.2 mm) were equally created around the implantation zone to stabilise the implant with osteosynthesis screws (diameter 1.6 mm, Easy Implant, Chavanod, France), attached in a cross-pattern with two elastic strings (see Fig. 2.1B,C).

After surgery, 25 μ g/h fentanyl was transdermally delivered regularly and continuously for 3 days through a patch which could be changed once if necessary and 100 mg/L enrofloxacine (Baytril[®] 10 %, Bayer Healthcare, Loos, France) was put in water for 5 days. The animals were housed in a metal hutch in an environment (ambient temperature 19°C and a humidity of 55 %) in accordance with the requirements of the European Guidelines for care and use of laboratory animals and the ethical committee of ENVA (Ecole Nationale Vétérinaire d'Alfort). Artificial lightening and air conditioning systems were used in the animal housing facility. The animals were fed with commercial food and water was provided ad libitum.

At the end of the defined healing period (Fig. 2.2C), three rabbits were euthanised after 7 weeks of implantation and the four others after 13 weeks, using an overdose of pentobarbital. The samples, consisting in the coin-shaped implants integrated in bone tissue, were carefully harvested.

Samples were classified based on their implant surface roughness and integration time. Four groups were created, labelled X-Y, where X represents the level of surface roughness and Y the number of weeks of healing time. For instance, R-7 corresponds to the group of samples having a rough surface profile and a healing duration of 7 weeks.

2.2.4 Quantitative ultrasonic (QUS) device

Shortly after sacrifice, QUS measurements were performed on 5 samples from group S-7, 6 samples from group S-13, 6 samples from group R-7 and 8 samples from group R-13. In addition, one control implant with each surface roughness, which had not been in contact with any bone tissue (denoted S- θ and R- θ), was also analysed using the QUS device described below.



Figure 2.3 – A) Photo of the quantitative ultrasonic (QUS) device developed in the MSME laboratory. B) Schematic representation of the QUS device. The green arrows indicate the translation of each component. C) Photo of the measurement setup including the ultrasonic transducer acting as an emitter-receiver and the sample. The top schematic view of the implant indicates the position of the water-implant and bone-implant interfaces.

The QUS device presented in Fig. 2.3A,B was developed in the MSME laboratory and used in previous studies [86, 138]. It comprised a broadband focused transducer (CMF-25; Sonaxis, Besançon, France) with a centre frequency of 15 MHz, a diameter of 6 mm and a focal length of 40 mm, which led to an approximate 0.5 mm beam width at focus. The transducer, immersed in a container filled with water at room temperature, worked

in echographic mode and its axis was aligned in the y-direction (see Fig. 2.3B,C). The supporting electronics included a pulse-receiver amplifier (5052A; Panametrics, Waltham, MA, USA) and an A/D conversion card of 100-MHz sampling frequency with 12-bit resolution (Spectrum, Grosshansdorf, Germany).

All bone samples were carefully degassed before each measurement to remove air bubbles. The samples were hung by a clamp exposing first the water-implant interface and then the bone-implant interface to the ultrasonic beam (Fig. 2.3C). Both interfaces were aligned so that i) the BII was in the xz plane, approximately at the focus of the transducer, and ii) the normal of the implant surface and the axis of the transducer coincided with the y-direction (see Fig. 2.3B). The parallelism between the implant and the transducer surfaces was adjusted by rotating the x- and z-axes, to reach a maximum orientation error of around 1° relatively to both axes.

The displacement of the sample in the xz plane was controlled using two translation stages (Physik Instruments, Pantin, France), as shown in Fig. 2.3B. A custom-made human machine interface was developed under LabVIEW (National Instruments, Austin, TX, USA) to synchronise the displacement of the sample and the ultrasonic acquisitions. The spatial displacement step of 0.3 mm was chosen to approximately correspond to half of the ultrasonic beam diameter. 2D measurements (10x10 mm², 256 points) were carried out for each sample (Fig. 2.4B) by spanning the entire coin-shaped implant surface and radiofrequency (rf) signals were collected.

2.2.5 Signal processing

For each rf signal (which corresponds to a given position of the sample relatively to the transducer) presented in Fig. 2.4A, two echoes could be distinguished. The first echo corresponds to the water-implant interface and the second one to the BII. The envelope of each rf signal (dashed lines in Fig. 2.4A) was determined by computing the modulus of its Hilbert's transform. The maximum amplitudes of both echoes were noted A_1 (water-implant interface) and A_2 (BII), and the ratio $r = A_2/A_1$ was computed. This normalisation allowed the reduction of the measurement variability due to i) possible orientation errors of the sample relatively to the axis of the ultrasonic beam and ii) noise related errors.

Typical spatial variations of A_1 , A_2 and r as a function of the sample position are shown in Fig. 2.4B. For each sample #i ($i \in 1, ..., 25$), a ROI was defined by considering the sample position for which $A_2 > 0.21$, to remove rf signals corresponding to the periphery of the implant where the PTFE cap may have disrupted the signal. The choice of the value of 0.21 for the threshold will be discussed in the discussion (subsection 2.4.7). This ROI corresponded approximately to a 2D circular ROI centred on the implant, with a diameter close to 2.5 mm (non-black pixels in the ratio map in Fig. 2.4B).

For each sample #i, the average value of r (noted $\overline{r_i}$) as well as its standard deviation (noted r_i^{sd}) were determined by considering only sample positions within the ROI. r_i^{sd} represents the intraspecimen variability, which corresponds to the spatial variation of r among the interface illustrating the heterogeneity of bone contact at the interface. Moreover, within each sample group, the average value \overline{r} and the standard deviation value \overline{r}^{sd} (representing the interspecimen variability) of all $\overline{r_i}$ in the considered group were calculated. The mean intraspecimen variability r^{sd} was also calculated by averaging all values r_i^{sd} of the considered sample group.



Figure 2.4 – A) Typical radiofrequency signals obtained for rough implants inserted for 7 (black line) and 13 weeks (grey line) and their corresponding envelopes in dashed lines. The lower script x in A_x corresponds to the interface: 1 for water-implant and 2 for bone-implant. The upper script y in A^y corresponds to the number of weeks of healing time. B) 2D maps corresponding to the spatial variations of A_1 , A_2 and r for a 13 week osseointegrated sample, ratios $r = A_2/A_1$ are shown only for positions for which $A_2 > 0.21$.

2.2.6 Histological analysis

After the QUS measurements, all samples were embedded in polymethyl methacrylate (PMMA) (Fig. 2.5) so that they could be cut without debonding the BII. The embedding procedure consisted in fixing the samples for 1 week in 10 % phosphate-buffered formalin, rinsing them with water, dehydrating in ethanol, clearing in two baths of xylene for 12h and finally embedding them in methyl methacrylate (MMA) [37, 191].



Figure 2.5 – Sample preparation procedure: PMMA embedding, cutting into slices, and polishing and staining for histological analysis.

The embedded samples were then cut (Fig. 2.5) in 400 μ m-thick slices with a low-speed cut-off machine (Minitom, Struers[®], Ballerup, Denmark) in the transverse plane, orthogonal to the implant surface. The closest slice from the centre of the implant was selected

and polished (LabPol-5, Struers[®], Ballerup, Denmark) with abrasive paper, SiC Foil with grit 1200 and 2000 using a sample holder (AccuStop, Struers[®], Ballerup, Denmark) to control flatness and limit material removal. The face to be histologically analysed was then polished with 9 μ m alumina powder and 0.3 μ m alumina suspension on polishing cloths, before being coloured with van Gieson picro-fuchsin for 1 min (Fig. 2.5) and then rinsed in absolute ethanol. After rinsing, the dye fixed on collagen fibers coloured bone tissues in red.

The stained slices, from the samples previously studied with QUS, were analysed by light microscopy (Stemi 305, Zeiss[®], Jena, Germany). The BIC ratio, corresponding to the ratio between the length of bone in contact with the implant surface and the total length of the implant surface, was calculated using ImageJ (NIH, Bethesda, USA) [184]. For each sample #i ($i \in 1, ..., 25$), the BIC ratio was determined and noted BIC_i . For each sample group, the mean BIC ratio of all considered BIC_i was determined.

2.2.7 Measurement errors and statistical analysis

The measurement error of QUS, E_{QUS} , corresponding to the error on the estimation of r, was determined for N=14 samples by repeating the measurement. To do so, a first measurement was realised, leading to an average value of $r = \overline{r_{i,1}}$ (over the ROI). Then, the sample was removed from the clamp and the measurement was repeated, leading to a second value of $r = \overline{r_{i,2}}$. E_{QUS} is an estimation of the reproducibility of r and was defined according to Eq. 2.1:

$$E_{QUS} = \frac{1}{N} \sum_{i=1}^{N} |\overline{r_{i,1}} - \overline{r_{i,2}}|$$
(2.1)

In order to assess the reproducibility of the BIC estimation using histological analysis, two consecutive slices were identically prepared and analysed for M=13 samples, leading to two values of BIC noted $BIC_{i,1}$ and $BIC_{i,2}$. The error corresponding to the BIC estimation E_{hist} was defined by Eq. 2.2:

$$E_{hist} = \frac{1}{M} \sum_{i=1}^{M} |BIC_{i,1} - BIC_{i,2}|$$
(2.2)

Non-parametric Mann-Whitney U-tests (Matlab R2017b, MathWorks[®], Natick, USA) were performed to investigate whether the values of $\overline{r_i}$ (average ratio over a sample surface), r_i^{sd} (intraspecimen variability or heterogeneity of the surface) and BIC_i were sensitive to healing time and to surface roughness. The Mann-Whitney U-test on the BIC results was performed considering all analysed slices.

2.3 Results

2.3.1 Implant surface characterisation

From the surface profile of two samples corresponding to the S and R series (Fig. 2.6), the topographical analysis confirms that the implant of the R series presents a rougher surface, with an average roughness S_a equal to 3.46 µm for the R series and to 0.492 µm for the S series, as indicated in Table 2.1. Except for S_{al} , which corresponds to a distance between peaks in the surface plane, all other roughness parameters (S_z, S_{da}, S_{dv}) are higher for the surface corresponding to the implant from the R series compared to the S series.



Figure 2.6 – Implant surfaces sandblasted with A) 25 μ m diameter particles (S series) and B) 250 μ m diameter particles (R series). C, D) Corresponding topographical results of 1x1 mm² maps, and distribution of the measured peaks heights (Gaussian distribution on the right-hand scale).

Table 2.1 – Roughness parameters (mean \pm standard deviation) obtained from the analysis of 10 ROIs (1x1 mm²) spread over the implant surface of samples from the S and R series.

Topographic parameters	$S_a \; (\mu \mathrm{m})$	$S_z \; (\mu \mathrm{m})$	$S_{da} \; (\mu \mathrm{m}^2)$	$S_{dv} \; (\mu \mathrm{m}^3)$	S_{al} (µm)
S series	0.492 ± 0.036	6.48 ± 1.35	1371 ± 247	88.3 ± 25.9	51.7 ± 25
R series	3.46 ± 0.25	39 ± 4.61	5518 ± 620	2783 ± 582	33.9 ± 2.63

2.3.2 QUS analysis

Considering non-osseointegrated implants from both series of samples, ultrasonic measurements are first carried out before surgery, when no bone tissue is attached to the implant surface. The mean value \bar{r} is equal to 0.448 ± 0.01 and 0.447 ± 0.01 for intact implants from the S and R series respectively. The corresponding results are shown with grey lines in Fig. 2.7A.



Figure 2.7 – Average value of the ratio $r(\bar{r})$ (A) and standard deviation r^{sd} (B), which corresponds to the heterogeneity of the distribution of r, for the samples belonging to each group. The dots represent the mean value, the grey lines the median value, the bottom (respectively top) edges of the box the 25^{th} (respectively 75^{th}) percentiles and the whiskers extend to the most extreme data points. The light grey lines correspond to the values for the intact implants ((A) average ratio and standard deviation, (B) standard deviation). The Mann-Whitney U-tests lead to: * = p-value < 0.05, ** = p-value < 0.01, NS (non-significant difference) = p-value > 0.05.

For a given roughness, the average QUS ratios \overline{r} are found to be significantly lower after 13 weeks of healing than after 7 weeks (Fig. 2.7A). No significant difference in \overline{r} is obtained when considering different roughness levels after 7 weeks of healing time. However, after 13 weeks of healing time, the values of \overline{r} are significantly lower for the rough implants compared to the smooth implants.

The average value of the reproducibility of the QUS measurements E_{QUS} is equal to 0.01.

All obtained intraspecimen variations r^{sd} shown in Fig. 2.7B are higher than the nonosseointegrated r^{sd} (0.01, shown with grey line in Fig. 2.7B). For each roughness level (R and S series), the value of r^{sd} is significantly higher after 7 weeks than after 13 weeks of healing time. For a given healing time, the results are similar for the S and R series.

2.3.3 Histological analysis

The BIC ratios are significantly higher after 13 weeks of healing time than after 7 weeks for both series of implants (Fig. 2.9), as can be visually observed in Fig. 2.8 with examples of slices from samples of the R series. Furthermore, for a given healing time, the average value of the BIC is significantly higher for implants of the R series compared to implants from the S series (Fig. 2.9).

The reproducibility of the BIC estimation given by histological analysis E_{hist} is equal to 0.1.



Figure 2.8 – Examples of stained histological slices from samples of the R series obtained after A) 7 weeks of healing and B) 13 weeks of healing. Bone tissues are represented in red and the implant in black.



Figure 2.9 – Average BIC ratios for the samples belonging to each group. The dots represent the mean value, the grey lines the median value, the bottom (respectively top) edges of the box the 25^{th} (respectively 75^{th}) percentiles and the whiskers extend to the most extreme data points. The Mann-Whitney U-tests lead to: * = p-value < 0.05, *** = p-value < 0.001.

2.3.4 Comparison of QUS and histological measurements

The present study compares the QUS and histological measurements qualitatively, presented in Fig. 2.10 through images obtained for a sample belonging to the S-13 group. The C-C' plane in Fig. 2.10 represents a site-matched measurement region of interest where the grey cross-hatched area corresponds to a region of interest where no bone is in contact with the implant (Fig. 2.10B) and where the value of r is the highest (Fig. 2.10A).



Figure 2.10 – A) Spatial variation of r obtained for a sample belonging to the group *S*-13 (same as Fig. 2.4). B) Corresponding histological slice (in the C-C' plane) with stained bone tissue in red. The white line corresponds to the locations where bone is in contact with the implant. The grey cross-hatched region (in A and B) corresponds to a region of interest where no bone is in contact with the implant, as observed in the histological slice (B) and confirmed with higher values of r (A).



Figure 2.11 – Variation of the ratio \overline{r} as a function of the BIC values for all samples. The markers (see legend) correspond to the experimental results and the dotted lines to their linear regressions (S series in grey and R in black).

2D maps of the bone contact over the whole implant surface resulting from QUS measurements allow the assessment of 2D BIC values through the ultrasonic \bar{r} ratios. QUS results are compared with gold standard histology on 2D slice, from which 1D BIC values are extracted. QUS and histological results are plotted together in Fig. 2.11. For each sample, the values of the histological BIC and of ultrasonic \bar{r} are noted by a marker, which depends on the group of the sample. Linear regression analyses (dotted lines) have been carried out for each roughness series to compare the variations of \bar{r} and of the BIC

obtained experimentally and lead to correlation coefficients equal to R=-0.62 and R=-0.64 for the S and R series respectively. The correlations show that the values of \bar{r} decrease when BIC values increase with a steeper slope for rough implants.

2.4 Discussion

The present study combines QUS and histological analyses in order to study osseointegration and in particular bone-implant contact at the BII. Thanks to the *in vivo* coinshaped implant model, we could ensure that bone measured at the implant surface came from the osseointegration process. This has allowed the investigation of the effects of differences in healing time and surface roughness on the BIC. Results show an increase in the BIC with healing time and with increased surface roughness. The QUS technique has the advantage of being non-destructive, keeping the samples intact for further measurements to better identify bone-implant contact. The comparison of the QUS and histological results reveals a correlation between the two techniques.

2.4.1 Bone-implant contact increases with healing time

The variation of the ultrasonic response of the BII has been explained through the evolution of the biomechanical properties of the BII and in particular of the tissue in contact with the implant surface [138, 212, 215]. The reflection coefficient of an interface (which is related to r in the present study) increases as a function of the gap of acoustical impedance between the two materials [2]. The gap in acoustical impedance between titanium and mineralised bone is smaller than between titanium and non-mineralised tissue. Therefore, the reflection coefficient at the BII, affected by both i) the amount of bone contact and ii) bone tissue properties in contact with the BII, is lower when mineralised tissue has formed at the implant surface (see in Fig. 1.5 (chapter 1)).

First, the BIC ratio increases in the present study during bone healing with averaged BIC values increasing between 7 and 13 weeks of healing for both surface roughness levels (from 0.35 ± 0.07 to 0.55 ± 0.25 for the S series and from 0.48 ± 0.16 to 0.71 ± 0.09 for the R series (Fig. 2.9)), in agreement with a previous study on the same coin-shaped implant model [138]. Such results correspond to larger amounts of bone in contact with the implant surface for longer healing times. Second, the elastic properties [210, 211] as well as mass density [211] of newly formed bone tissue around an implant surface are known to increase as a function of healing time, which also leads to a decrease in the gap of the acoustical impedance hence of the reflection coefficient.

The two aforementioned phenomena have cumulative effects and can explain the decrease in \bar{r} (from 0.432 ± 0.028 to 0.376 ± 0.016 between 7 and 13 weeks of healing for the S series and from 0.425 ± 0.051 to 0.351 ± 0.018 for the R series (Fig. 2.7A)) as a function of healing time, consistently with the previous study [138]. Such decrease as a function of healing time is due to both increasing bone-implant contact at the implant surface (Fig. 2.9) and increasing newly formed bone quality according to literature [210, 211]. Similar variations have been obtained using QUS method on dental implants [212, 215]. This cumulative effect also explains the high values of r obtained in the cross-hatched region of interest in Fig. 2.10, where no bone is in contact with the implant surface.

2.4.2 Heterogeneity of bone-implant contact during osseointegration

The heterogeneity of the ultrasonic response over the implant surface is assessed with the standard deviation r_i^{sd} for each sample, corresponding to the intraspecimen variability. While non-integrated samples present low standard deviations attesting an homogeneity of the interface, osseointegrated samples have higher standard deviations, what can be explained by the heterogeneity of bone contact at the BII (Fig. 2.7B). In particular, the average value r^{sd} obtained for the samples with 7 weeks of healing time is significantly higher than for the samples with 13 weeks of healing time, attesting a higher intraspecimen variability for lower healing time.

Such heterogeneous bone contact over the implant surface obtained with the 2D QUS scans explains the important differences obtained in the histological analysis according to the considered slice ($E_{hist}=0.1$). It indicates that isolated histological slices are not sufficient to describe the BIC [25].

2.4.3 Implant surface roughness influences osseointegration

Two levels of surface roughness (Table 2.1) have been considered within typical ranges corresponding to clinical situations [121, 179]. The R series presents S_a values around 3.46 µm, which is of the same order of magnitude as values shown to optimise osseointegration when using a comparable animal model (3.6-3.9 µm) [179]. For both healing times, the BIC ratio is higher for samples from the R series compared to implants from the S series (Fig. 2.9), which is consistent with the results obtained in [176]. In Ronold et al. [176], higher strength of the BII with rougher implants is also emphasised, suggesting a relationship between implant surface roughness and mechanical properties of the BII. Mechanical behaviour at the BII will be further considered in this thesis.

In parallel to the increase in BIC values with implant roughness, after 13 weeks of healing, a significantly lower value of \bar{r} is obtained for *R-13* than for *S-13* (Fig. 2.7A), in agreement with the explanation of subsection 2.4.1. However, no significant differences are observed between *S-7* and *R-7*, which can be explained by the numerical results presented in the next section.

2.4.4 Validation with the numerical microscale BII model

The results have been compared with numerical simulations based on a FE model developed in our group (see chapter 1, subsection 1.5.2) which models the interaction at the microscale between an ultrasonic wave and the BII modelled with a sinusoidal profile. As any original roughness profile may be approximated by a 2D sinusoidal profile [85], the model has been adapted to validate the present experimental study.

The original roughness profiles of coin-shaped implants from S ($S_a=0.492 \ \mu m$) and R ($S_a=3.46 \ \mu m$) series have been converted into sinusoidal roughness profile with a half-period $L_{eq}=60 \ \mu m$ and an amplitude h_{eq} determined with the polynomial regression (Eq. 2.3) obtained in [85]:

$$h_{eq} = 5.6R_a - 77000R_a^2 \tag{2.3}$$

Therefore, $h_{eq}=2.83 \ \mu\text{m}$ corresponds to $R_a=0.5 \ \mu\text{m}$ and $h_{eq}=19.5 \ \mu\text{m}$ to $R_a=3.46 \ \mu\text{m}$.

Assuming the interaction between a plane wave and a sinusoidal BII, the variation of the numerical reflection coefficient is evaluated as a function of the BIC value for the two considered roughness levels. These numerical results may be compared to the experimental QUS ratios measured in the present study. Both experimental and numerical results are combined in Fig. 2.12 where solid lines represent the numerical results added to the experimental linear regressions of Fig. 2.11.



Figure 2.12 – Variation of the ratio \overline{r} as a function of the BIC values for all samples. The dotted lines correspond to the experimental linear regressions (S series in grey and R in black). As a comparison, the solid lines correspond to the variation of the reflection coefficient of the BII as a function of the BIC obtained numerically [84, 85].

A good qualitative agreement is obtained between the experimental and numerical results. In particular, the slope of the decrease in \bar{r} as a function of the BIC obtained experimentally and numerically is higher in absolute value for implants of the R series (black lines) compared to the S series (grey lines), suggesting that the QUS response of the BII is more sensitive to the BIC value for higher roughness levels.

Besides, the numerical model has proven (Fig. 2.13a) that for a relatively low osseointegration level corresponding to high soft tissue thickness W, the roughness represented by the parameter h weakly influences the value of the reflection coefficient [84]. This is consistent with the non-significant differences in \bar{r} found between S-7 and R-7 in Fig. 2.7A and with the similar \bar{r} values between the S and R series for intact implants. The similar ultrasonic reflection found for all roughness levels at low osseointegration levels can be explained by the similar gap in acoustical impedance as almost only soft tissues are in contact with titanium implants.

Numerical simulation can also explain why some values of $\overline{r_i}$ after 7 weeks of healing time are slightly higher than those for non-osseointegrated implants (Fig. 2.7A), which seems inconsistent with the decrease in the reflection coefficient in presence of bone tissue. In Fig. 2.13a,b, a similar trend was numerically obtained, in particular, for an approximately 40 µm-thick soft tissue layer at the BII. The presence of constructive interferences could potentially lead to higher values of the reflection coefficient compared to a non-osseointegrated interface (maximal soft tissue thickness) [84, 85]. Note that such



Figure 2.13 – Variation of the reflection coefficient as function of the soft tissue thickness W (a) for different values of the roughness amplitude h at the microscopic scale (adapted from [84]) and (b) for six laser-modified surface roughness profiles characterised by their roughness level (adapted from [85]).

behaviour is more likely for low healing times corresponding to quite thick soft tissue layers, which explains why it has not been obtained for 13 weeks of healing time (Fig. 2.7A).

Therefore, the numerical study [84] and its adaptation to our experimental conditions display the same variations of \bar{r} observed experimentally, as a function of the BIC and roughness levels. The differences obtained in Fig. 2.12 between the experimental and numerical results may be explained by the fact that i) the reflection coefficient obtained numerically is not strictly equivalent to the ratio \bar{r} and ii) a focused ultrasonic beam was used experimentally whereas the wave field was considered to be plane in the finite element simulations.

2.4.5 A higher sensitivity of the QUS technique compared to histology

The advantage of the QUS technique is that it is a non-destructive method that allows a 2D analysis of the BII. The QUS method can be used *in vivo* [212, 215], while histological analyses are restricted to a 1D view and to *ex vivo* analyses.

The performances of this QUS technique can be compared to histological analysis by comparing their respective reproducibility to the interspecimen variability. While E_{QUS} is lower than the interspecimen variability \bar{r}^{sd} for all four studied groups, E_{hist} is lower than the BIC standard deviations only for groups S-13 and R-7, indicating that histology does not appear sensitive enough to assess differences in the two other groups S-7 and R-13.

Another simple way to compare the performances of the two techniques is to consider the ratio P between the reproducibility errors (i.e., E_{QUS} and E_{hist}) and the range of variation of the values obtained for the corresponding parameters (i.e., $\overline{r_i}$ and BIC_i), as described in details in [216]. A low (respectively high) value of P indicates that the considered technique is strongly (respectively weakly) sensitive to variations of the properties of the BII. The value of P obtained for the QUS technique (respectively histological analysis) is equal to 0.07 (respectively 0.17), suggesting that QUS is a more sensitive method compared to histology measurements.

2.4.6 Influence of bone quality

In this study, QUS and histological analyses have been used to investigate boneimplant contact at the BII. However, bone quality also affects the results. In histological images, mineralised tissues stained with van Gieson picro-fuchsin may present stronger colours [210] (which did not appear clearly in this study), so even if identifying the type of bone tissue can be difficult, histological analysis is qualitatively sensitive to the degree of mineralisation, and thus to bone mineral content. Concerning the QUS method, as mentioned in subsection 2.4.1, bone properties, in particular mechanical properties, play a role in the ultrasonic reflection at the BII by affecting the gap of acoustical impedance: mineralised tissue leads to a lower r value than non-mineralised tissue. These two characterisation techniques suggest an interdependence between bone properties which should be all taken into account to fully characterise osseointegration at the BII motivating the following studies of this thesis.

2.4.7 Limitations

This study presents limitations. First, because the ultrasonic wave is first reflected from the water-implant interface before interacting with the BII, any geometrical variation or imperfection of the implant (such as thickness or alignment) may affect the value of r. To limit this effect, the same implant geometry was considered throughout the study and the alignment of the QUS device was checked, leading to a low error in reproducibility. Second, the sample sandblasting was done manually, which may lead to possible surface roughness heterogeneity, which is shown in Table 2.1 to be around 7 % of variation for the S_a values. Third, a relatively low number of samples per group has been considered, which is however of the same order of magnitude than what was done in previous studies [177, 178, 179]. Further studies should be conducted on more samples to confirm the results. Fourth, the resolution of the QUS technique is limited since the beam diameter is around 500 μ m and the size of the ROI (diameter of around 2.5 mm) could not be increased because of the limited size of the animal model. Fifth, the ROI where the values of rwere averaged had been defined by comparing the value of A_2 with an arbitrary threshold equal to 0.21, which corresponds to a compromise between a sufficiently high value in order to reject positions corresponding to the PTFE cap and to a sufficiently low value to obtain enough measurements in the ROI. Moreover, changing the value of the threshold within acceptable values (e.g. 0.20 to 0.22) induces slight variation of the average QUS ratio (e.g. 0.414 to 0.417) approximately three times lower than the calculated error of the technique $(E_{QUS}=0.01)$.

2.5 Conclusion

Quantitative ultrasound as well as histological measurements could quantify the increase in the bone-implant contact due to longer healing time and rougher surfaces, in agreement with the numerical microscale BII model with a sinusoidal profile adapted to the present experimental conditions.

This QUS technique represents an interesting and sensitive echographic method to investigate osseointegration phenomena, providing a macroscopic 2D mapping of the bone in contact with an implant. Unlike 1D histological analyses, QUS techniques capture the heterogeneity of bone-implant contact over the implant surface, which reduces as bone invades the whole bone chamber during healing.

This chapter focuses on bone-implant contact, however, as explained in subsection 2.4.6, the quality of the newly-formed tissue may impact the present results and is important to characterise. Therefore, bone compositional and mechanical properties should be quantified to better understand the osseointegration around an implant.

2.6 Key points

- The *in vivo* coin-shaped implant model is an interesting experimental tool to characterise osseointegration since the bone chamber ensures that the quantified boneimplant contact comes from new bone formation only.
- Being sensitive to the difference in acoustical impedance, the QUS technique enables the detection of bone formation at the implant surface.
- The bone-implant contact assessed by the QUS technique has been correlated with the BIC ratio obtained with the gold standard histological technique.
- The bone-implant contact increases with longer healing times and in the presence of rougher surfaces.
- The adaptation of the numerical microscale BII model with a sinusoidal profile confirms the experimental results and could explain the observed trends.
- The non-destructive QUS technique provides a mapping of bone contact over the whole implant surface and is more sensitive than the reference histological analysis restricted to a specific 1D view of the interface.

Chapter 3. Influence of nanoscopic bone composition and structure on microscopic elastic properties at the BII

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3.1 Introduction

In chapter 2, an increase in the bone-implant contact has been evidenced during osseointegration. However, as introduced in chapter 1, not only is the presence of bone tissue at the interface important to ensure sufficient anchorage of the implant, but the quality of this tissue needs to be investigated too. Starting from the nanoscale, bone tissue composition and structure impact the overall bone quality. Bone tissue is a composite material divided into a mineral phase constituted of hydroxyapatite crystals and an organic phase, the collagen matrix (chapter 1, subsection 1.1.1). The proportion, size and arrangement of these components define the tissue local quality and play a role in the tissue's mechanical behaviour at the larger microscopic scale [21, 68, 165]. How compositional and structural properties differ in the newly formed bone tissue close to an implant during osseointegration is still unclear and needs to be investigated as well as their impact on the BII's mechanical behaviour.

The study presented in this chapter evaluates nanoscopic bone composition and crystal size with Raman spectroscopy (chapter 1, subsubsection 1.4.2.1). Their effect on the local microscopic elastic properties has been investigated by site-matched measurements using nanoindentation (chapter 1, subsubsection 1.4.3.1). The properties of newly formed bone have been compared to mature control bone using the *in vivo* coin-shaped model, thanks to the bone chamber allowing a clear distinction between those two tissues.

As in chapter 2, we collaborated with Hôpital Henri Mondor AP-HP (Créteil, France) and ENVA (Maisons-Alfort, France) during the *in vivo* phase. Moreover, collaborations with other research groups were established for Raman spectroscopy measurements with the Department of Mechanical Science and Engineering (University of Illinois at Urbana-Champaign, USA) and for nanoindentation measurements with Institut de Chimie et des Matériaux Paris-Est ICMPE (Thiais, France). Those multi-physics and international collaborations have given access to experimental techniques that are not available at MSME and led to the following paper [63], currently under revision:

Fraulob M., Pang S., Le Cann S., Vayron R., Laurent-Brocq M., Todatry S., Soares J. A.N.T., Jasiuk I., and Haïat G., "Multimodal characterization of the bone-implant interface using Raman spectroscopy and nanoindentation", *Medical Engineering and Physics*, submitted.

3.2 Methods

3.2.1 Sample preparation

A Ti6Al4V coin-shaped implant similar to the ones used in chapter 2 was used (see Fig. 2.1 (chapter 2)). In this study, the implant was blasted with titanium dioxide particles to reach an average surface roughness of R_a =1.9 µm.

Identically to subsection 2.2.3 (chapter 2), the implant was inserted into the distalmedial part of the right femur of a New Zealand white male rabbit. After 10 weeks of healing time, the rabbit was euthanised and the distal femur was carefully dissected for analyses. Described in subsection 2.2.6 (chapter 2), the sample was embedded in PMMA and cut transversally through the middle of the implant. For this study, the distal part of the sample was analysed (Fig. 3.1). The surface of interest was manually polished using abrasive paper, SiC foil with grit 1200 and polishing cloths with 9 μ m alumina powder and then 0.3 μ m alumina suspension. Next, Raman spectroscopy measurements were collected on the sample surface, as described in subsection 3.2.2. Non-decalcified histology was done to distinguish newly formed and mature bone tissue. Finally, nanoindentation measurements were carried out, as described in subsection 3.2.3.



Figure 3.1 – Picture of the analysed distal half of the sample.

3.2.2 Raman spectroscopy

The sample was sent to the Department of Mechanical Science and Engineering (University of Illinois at Urbana-Champaign, USA) for Raman spectroscopy measurements. Due to difference in molecule and ion vibrations, the scattered light reflects compositions of mineral and collagen phases of the scanned bone tissue (see chapter 1, subsubsection 1.4.2.1). From the observed bands in the obtained Raman spectrum, information can be retrieved about the tissue chemical components as described later in this section.



Figure 3.2 – Sample coloured using van Gieson picro-fuchsin to expose bone tissue (red). The white lines correspond to the 400 μ m-long lines where Raman measurements were carried out: (a, b, c) in newly formed bone tissue and (d, e, f) in mature cortical bone tissue. Nanoindentation was performed in site-matched regions of interest, see Fig. 3.4 for detailed locations.

A confocal Raman microscope (Nanophoton RAMAN-11, Osaka, Japan) was used with a 785 nm (infrared light) laser with 1 mW power for beam excitation. The sample was scanned through a 20x/0.45 objective with 100 s exposure time. Six lines were measured: three in newly formed bone tissue (labelled a, b, c in Fig. 3.2) and three in mature cortical bone tissue (d, e, f in Fig. 3.2). Each measured line extended over a length of 400 µm (pixel size 1 µm), leading to a total of 400 spectra per line.

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The spectra intensity was measured between 400 and 1800 cm⁻¹ [128] and the 400 spectra obtained for each line were averaged. All six averaged spectra were then corrected by removing the baseline coming from fluorescence background by cubic spline interpolation. Eleven bands could be identified (Fig. 3.3A), corresponding to bone tissue components from i) the mineral phase: phosphate bands ($\nu_1 PO_4^{3-}$ and $\nu_2 PO_4^{3-}$) and carbonate band ($\nu_1 CO_2^{2-}$), and ii) the organic phase: amino acid (proline (Pro 853 and Pro 920), hydroxyproline (Hyp), tyrosine (Tyr), phenylalanine (Phe)), collagen bands (amide I and amide III), and proteins bands with CH₂ deformation δ (CH₂).



Figure 3.3 – A) Average spectrum of line d (mature bone tissue) with characteristic bands of bone tissue components. The bands corresponding to the mineral (respectively organic) phase are indicated in grey (respectively black). B) Zoom between 900 and 1000 cm^{-1} presenting the maximum intensity, full width at half maximum (FWHM) and area under the phosphate band at 959 cm⁻¹.

For each averaged spectrum, bands of interest were analysed using Origin 2019b (OriginLab[©], Northampton, USA). For each band with the maximum at the Raman shift Y, the area under the band A_Y , the maximum intensity I_Y and full width at half maximum $FWHM_Y$ (see example for the phosphate band in Fig. 3.3B) were determined. From the measurements for each line, a total of 13 parameters were computed, as commonly reported in the literature to investigate bone tissue (Table 3.1) [128, 150]. These parameters can be divided into three categories: indicators investigating the mineral phase (n=2), the organic phase (n=3) and the ratio of the mineral and organic phases (n=8).

Table 3.1 – Rama	n parameters $(n=1)$	3) and their corre	esponding ratio,	calculated for
each average spectr	um. The subscript	represents the R	aman shift (cm ⁻	$^{-1}), A = area,$
$I = \max \min \min$	sity, $FWHM = $ full	width at half max	kimum.	

Mineral phase		Mineral phase/Organic phase		
Mineral crystallinity	$\frac{1}{FWHM_{959}}$	Mineral-to-matrix (Pro 853)	$rac{I_{959}}{I_{853}}$	
Carbonate-to-phosphate	$\frac{A_{1070}}{A_{959}}$	$\begin{array}{llllllllllllllllllllllllllllllllllll$	$\frac{I_{959}}{I_{853}+I_{872}}$	
Organic phase		$\begin{array}{c} \mbox{Mineral-to-matrix} & (\mbox{Pro} \ 853 \ + \\ \mbox{Hyp} \ + \ \mbox{Pro} \ 920) \end{array}$	$\frac{I_{959}}{I_{853}+I_{872}+I_{920}}$	
Phenylalanine-to-proline $\frac{A_{1003}}{A_{853}}$		Mineral-to-matrix $(\delta(CH_2))$	$rac{I_{959}}{I_{1450}}$	
Hydroxyproline-to-proline $\frac{I_{872}}{A_{853}}$		Mineral-to-matrix (Phe)	$\frac{I_{959}}{I_{1003}}$	
Amide I/Amide III $I_{1620-1700}$ I_{1250}		Mineral-to-matrix (Tyr)	$rac{I_{959}}{I_{1607}}$	
		Carbonate-to-matrix	$\frac{A_{1070}}{A_{1620-1700}}$	
		Calcium content	$\frac{A_{422}}{A_{1250}}$	

3.2.3 Nanoindentation measurements

After being analysed by Raman spectroscopy, the sample was brought to ICMPE (Thiais, France) where nanoindentation measurements were performed with a nanoindenter TI 950 TriboIndenter (Hysitron[®], Minneapolis, USA) equipped with a diamond Berkovich tip (Fig. 3.5A). The sample was indented in Raman site-matched ROIs, at room temperature and in dry conditions. Thirteen indents were done in newly formed bone tissue and nine in mature cortical bone tissue (Fig. 3.4).

The indentation was load-controlled at a rate of 20 mN/s until a maximum force of 100 mN was reached, corresponding to approximately 3 μ m of indentation depth (represented in Fig. 3.5B). Then, the tip was maintained for a 2 s holding time and unloaded at 20 mN/s. A minimal distance of 70 μ m between two indents was chosen to avoid interactions [225].

Prior to measurements, the nanoindenter was calibrated by performing measurements in fused silica and the device setup was verified by performing three measurements in PMMA, which led to a mean value of $E = 2.9 \pm 0.1$ GPa, in agreement with the literature [70].

For each measurement, the variation of the force as a function of the penetration depth was retrieved and the contact point identified on the loading curve. The Oliver and Pharr method [156] allowed the evaluation of the reduced modulus E_r from the maximal slope of



Figure 3.4 – Location of the nanoindentation measurement points (black triangles) in respect to the Raman measurements (white lines, see Fig. 3.2) in newly formed bone tissue (A, B) and mature cortical bone tissue (C).



Figure 3.5 – A) Nanoindentation device during measurement. B) Schematic representation of the Berkovich tip indenting a sample until a maximum depth of 3 μ m with a maximum force of 100 mN. C) Load-displacement curve obtained from nanoindentation measurement with the loading and unloading phases. The maximal slope of the unloading phase considered to evaluate the reduced modulus E_r is represented.

the load-displacement curve (Fig. 3.5C) at the beginning of the unloading phase assuming bone tissue as a homogeneous, linear elastic, and isotropic material using Eq. 3.1:

$$E_r = \frac{S\sqrt{\pi}}{2\beta\sqrt{A_p}} \tag{3.1}$$

where β is a geometric factor equal to 1.034 for a Berkovich tip, A_p is the projected contact area and S is the maximal slope of the unloading curve (represented in Fig. 3.5C).

From the reduced modulus, the bone Young's modulus E could then be determined following Eq. 3.2:

$$\frac{1}{E_r} = \frac{1 - \nu_b^2}{E} + \frac{1 - \nu_i^2}{E_i} \tag{3.2}$$

where $E_i=1141$ GPa and $\nu_i=0.07$ are the Young's modulus and the Poisson's ratio of the diamond indenter tip and $\nu_b=0.3$ is the Poisson's ratio of bone tissue, chosen based on literature [99, 104, 105, 228].

3.2.4 Statistical analysis

Raman spectroscopy parameters and Young's moduli were averaged over newly formed bone ROIs (i.e. lines a-c and corresponding 13 indents) and mature bone ROIs (i.e. lines d-f and corresponding 9 indents). Due to the relatively small sample size, nonparametric Mann-Whitney U-tests (Matlab R2017b, MathWorks[®], Natick, USA) were performed to assess a statistical significance between newly formed and mature bone tissue values. The significance level was defined at p=0.05.

3.3 Results

Among the two parameters representative of the bone tissue mineral phase (Table 3.2), the carbonate-to-phosphate ratio is significantly higher for newly formed bone tissue. Mineral crystallinity average values are similar between newly formed and mature bone tissue but its standard deviation is seven times higher for newly formed bone.

All three parameters representative of the organic phase (Table 3.2), i.e., phenylalanineto-proline, hydroxyproline-to-proline, and amide I-to-amide III ratios, are significantly lower in the newly formed bone tissue compared to mature bone tissue.

Concerning parameters representative of the amount of the bone mineral phase relatively to organic phase (Table 3.2), the mineral-to-matrix ratios defined as $\nu_1 PO_4^{3-}/Pro$ 853, $\nu_1 PO_4^{3-}/(Pro 853 + Hyp)$, $\nu_1 PO_4^{3-}/(Pro 853 + Hyp + Pro 920)$, $\nu_1 PO_4^{3-}/\delta(CH_2)$, $\nu_1 PO_4^{3-}/Phe$ and $\nu_1 PO_4^{3-}/Tyr$ are all significantly lower in the newly formed bone tissue compared to mature bone tissue. Moreover, the carbonate-to-matrix ratio is significantly higher, and its standard deviation is three times higher for the newly formed bone tissue. The calcium content $\nu_2 PO_4^{3-}/Amide$ III is higher in the mature than in the newly formed bone tissue without being significantly different.

The elastic modulus of the newly formed bone tissue measured by nanoindentation is significantly lower than that obtained for mature bone tissue (Table 3.2).

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Table 3.2 – Comparison between different parameters (mean \pm standard deviation over the different ROIs) derived from Raman spectroscopy and nanoindentation for newly formed and mature bone tissues. Mann-Whitney U-test: significant differences with * = p-values ≤ 0.05 , ** = p-values ≤ 0.01 ; NS = non-significant differences with p-value > 0.05.

Bone phase	Parameter		Newly formed bone		Mature bone
Mineral phase	Mineral crystallinity	NS	0.0590 ± 0.0007	≈</td <td>0.0595 ± 0.0001</td>	0.0595 ± 0.0001
	Carbonate-to-phosphate	*	0.391 ± 0.029	>	0.320 ± 0.020
	Phenylalanine-to-proline	*	1.100 ± 0.084	<	1.434 ± 0.136
Organic phase	Hydroxyproline-to-proline	*	0.377 ± 0.042	<	0.562 ± 0.030
-	Amide I/Amide III	*	0.373 ± 0.014	<	0.520 ± 0.067
Mineral phase/ Or-	Mineral-to-matrix (Pro 853)	*	9.298 ± 0.534	<	15.605 ± 1.006
	Mineral-to-matrix (Pro 853 + Hyp)	*	6.758 ± 0.434	<	9.983 ± 0.482
	Mineral-to-matrix (Pro 853 + Hyp + Pro 920)	*	4.932 ± 0.322	<	6.742 ± 0.064
ganic	Mineral-to-matrix $(\delta(CH_2))$	*	2.873 ± 0.292	<	5.707 ± 1.115
pnase	Mineral-to-matrix (Phe)	*	2.083 ± 0.007	<	3.139 ± 0.079
	Mineral-to-matrix (Tyr)	*	5.085 ± 0.478	<	7.213 ± 1.360
	Mineral-to-matrix (Amide III) "Calcium content"	NS	0.406 ± 0.036	≈</td <td>0.421 ± 0.026</td>	0.421 ± 0.026
	Carbonate-to-matrix	*	2.780 ± 0.320	>	1.560 ± 0.104
Nanoin- dentation	Young's modulus E	**	$12.8 \pm 1.8 \text{ GPa}$	<	15.7 ± 2.3 GPa

3.4 Discussion

In this study, bone tissue compositional analysis conducted by Raman spectroscopy has been complemented with nanoindentation measurements to evaluate the effects of nanoscopic compositional and structural changes on elastic properties at the microscale. The two techniques have been performed in site-matched locations within newly formed bone, isolated thanks to a bone chamber model, and compared to reference mature cortical bone tissue. The present study has evidenced differences between newly formed and mature bone with higher carbonate-to-phosphate ratio within the mineral phase of newly formed bone, lower phenylalanine-to-proline, hydroxyproline-to-proline and amide I/

amide III ratios within its organic phase as well as lower mineral-to-matrix ratios and higher carbonate-to-matrix ratio. In addition to these compositional properties, lower values of the Young's modulus have been measured in newly formed bone tissue.

3.4.1 Composition and mechanical properties within newly formed and mature bone

Young's modulus values measured in this study are of the same order of magnitude as those obtained in previous studies on rabbit bone [10, 22, 210]. The Young's modulus of newly formed bone found herein $(12.8 \pm 1.8 \text{ GPa})$ is slightly lower than the values obtained in [210], which may be due to the following reasons. First, the nanoindentation measurement technique is different since Vayron et al. [210] utilised a dynamic mode using a continuous stiffness measurement (CSM) technique, while the static mode is considered here. Second, we assume a Poisson's ratio of bone tissue equal to 0.3, while Vayron et al. [210] used a value of 0.25. Note that assuming a value of 0.25 for the Poisson's ratio would have led to values of Young's modulus of newly formed bone tissue of 13.2 ± 1.9 GPa, closer to those found in [210]. Based on the literature [8, 105, 165, 220, 228], assuming a value of 0.3 for the Poisson's ratio seems to be more realistic compared to 0.25. In mature bone, Young's modulus is equal to 15.7 ± 2.3 GPa, which is in agreement with other nanoindentation studies on rabbit bone [10, 22], which have reported Young's moduli of mature bone between 9 and 17 GPa. Concerning Raman spectroscopy, all measured Raman ratios are of the same order of magnitude as in previous studies [4, 91, 106, 162, 187, 188] as detailed hereafter.

3.4.2 Bone mineral phase of newly formed tissue under remodelling

During osseointegration, ongoing bone remodelling occurs within newly formed bone tissue made of small mineral crystals. The sample being harvested 10 weeks after the implantation, remodelling is indeed likely to occur within the bone chamber since bone healing time is a few weeks for rabbits (see chapter 1, subsection 1.3.1) [143]. Ongoing remodelling is suggested by bone tissue mineral characteristics. Carbonate-to-phosphate ratio is an indicator of the substitution of the carbonate groups into the phosphate groups of hydroxyapatite $Ca_5(PO_4)_3OH$ crystals, sign of new mineral deposition and remodelling. The higher ratio measured in newly formed bone tissue indicates that carbonate ions from the mineral environment are incorporated into the apatite lattice [4]. Such a phenomenon changes the atomic structure of crystals, affecting their size, solubility and thermal stability, enabling easier apatite remodelling [223]. Likewise, active bone tissue remodelling has been previously observed from high carbonate-to-phosphate ratio in bone tissue healing of a subcalvarial defect [4]. Such ongoing remodelling process, less active in mature tissue, was expected around an implant, where newly formed bone tissue adapts its structure to its new mechanical environment. Moreover, the higher carbonate-to-matrix ratio supports this hypothesis as it describes the evolution of carbonate minerals relative to amide I from the collagen matrix. The carbonate-to-matrix ratio's significantly higher value in the newly formed bone tissue with a high standard deviation could also be explained by carbonate substitution and different remodelling stages [41].

Furthermore, a decrease in carbonate substitution has been linked to an increase in mineral crystallinity in synthetic carbonated apatites [14] and the mineral crystallinity parameter is representative of the mineral crystal structure. At the atomic scale, a low mineral crystallinity corresponds to small mineral crystals [4]. In newly formed bone tissue, measured mineral crystallinity is slightly lower but not significantly different compared to mature bone tissue, which is consistent with other Raman spectroscopy studies, suggesting smaller crystals in healing bone [4] and at the BII [187]. The higher variation of the mineral crystallinity in newly formed bone tissue indicates more heterogeneity in crystal sizes, which can be explained by different mineralisation stages inside the gap. This trend is also in agreement with another study of our group, analysing mineral crystal size on similar coin-shaped implant samples using SAXS measurements (chapter 1, subsubsection 1.4.1.4) at Synchrotron beamlines (SLS, PSI, Villigen, Switzerland). Indeed, the average crystal thickness found in the bone chamber is significantly lower than in mature bone with larger standard deviations [118].

3.4.3 Immature organic phase within newly formed bone

The difference between the composition of the organic phases highlights an immature organic phase for newly formed bone with fewer collagen crosslinkings. The bone organic phase has been investigated via the three parameters based on ratios of different collagen components: the hydroxyproline-to-proline ratio (Hyp/Pro), the amide I/amide III ratio and the phenylalanine-to-proline (Phe/Pro) ratio. First, hydroxyproline (Hyp) and proline (Pro) are two major amino acids composing collagen molecules [162], Hyp obtained from Pro transformation is involved in hydrogen bonds stabilising the collagen triple helix in the mature organic phase. Second, amide I is an indicator of collagen crosslinking [188], so the amide I/amide III ratio is an indicator of collagen conformational changes [208]. Thus, both lower Hyp/Pro and amide I/amide III ratios obtained in newly formed bone tissue indicate a less mature tissue, with fewer collagen crosslinkings. Third, phenylalanine (Phe) is another amino acid commonly measured in Raman analysis as high Phe levels in newly formed bone's extracellular matrix is a sign of non-mature tissue [187, 188]. Besides, with the lower mineral-to-matrix (Phe) ratio for newly formed bone, the present analysis reveals a larger quantity of Phe compared to mature bone tissue. As the Phe level is reduced during osseointegration, the lower Phe/Pro ratio in young bone tissue suggests a faster decrease in Pro during osseointegration, which could be explained by Pro transformation into Hyp during collagen maturation. However, this transformation needs to be further investigated to better characterise the evolution of phenylalanine and proline contents at the BII.

3.4.4 A lower mineral content in newly formed bone

While newly formed bone is a non-mature tissue, its mineral content is low relatively to its organic content. To evaluate the mineral-to-matrix ratios, the $\nu_1 PO_4^{3-}$ phosphate band is generally accepted as the best indicator for the mineral phase. However, different organic constituents have been previously used to describe the matrix phase [128]: proline (Pro) bands at 853 cm⁻¹ and 920 cm⁻¹ as well as the hydroxyproline (Hyp) band at 872 cm⁻¹ [21, 187, 188], but also amino acids such as phenylalanine (Phe) [187, 188] and tyrosine (Tyr) [187] or proteins with CH₂ deformation (δ (CH₂)) band [4, 130, 162]. In the present study, all mineral-to-matrix ratio combinations presented in Table 3.1 appear to be consistently lower in newly formed than in mature bone tissue, indicating lower mineralisation and potential bone tissue maturation in process at the BII [4, 21, 130, 162, 187, 188].

3.4.5 Effects of compositional and structural changes on bone elastic properties

The difference in properties of newly formed and mature cortical bone tissues can be related to the difference in mineralisation obtained with Raman analysis, since a lower elastic modulus has already been linked to a less mineralised tissue [181, 182, 210, 220]. In particular, the lower amount of mineral (low mineral-to-matrix ratios) and lower crystallinity (crystal size and lattice order) have been related to lower elastic modulus [91, 226] and reduced strength [188, 226] of bone. Moreover, the bone tissue remodelling activity evidenced in newly formed bone tissue (high carbonate-to-phosphate and carbonate-to-matrix ratios) could create vacancies eventually resulting in microstrains within the matrix of mature bone [21].

3.4.6 Limitations

Only one sample has been considered in the present study, which is justified for the following reasons. First, the coin-shaped implant model used here has already been validated in previous studies [136, 210, 211], which showed that this model allowed the clear distinction between newly formed bone and mature bone. In a previous nanoindentation study [210], the standard deviation of Young's modulus of newly formed bone tissue within the bone chamber was equal to 1.81 GPa (respectively 1.55 GPa and 2.10 GPa) after 4 weeks of healing (respectively 7 and 13 weeks). A similar standard deviation equal to 1.8 GPa is obtained herein. Although interspecimen variability is expected, we assume that the relative difference between newly formed and mature bone is expected to be similar for distinct samples. Note that only one sample per healing time was also considered in [210]. Second, the sample has been stained with van Gieson picro-fuchs to confirm that the three lines of measurements have actually been performed in newly formed bone tissue. Third, the nanoindentation apparatus has been carefully calibrated with fused silica, and the Raman spectroscopy instrument has been used in previous studies [160, 194] so that both techniques have been validated. Fourth, a high number of Raman measurements have been done to ensure a reliable description of bone compositional changes. Specifically, six lines of interest and 400 Raman spectra per line have been considered, whereas other Raman studies on bone characterisation [4, 21, 125, 162, 188] only considered tens of spectra for each sample. In the future, more samples could be analysed to further investigate the correlation between mechanical and compositional parameters and the effect of healing time.

Another limitation lies in the resin embedding using formalin and dehydrating the sample which may alter bone nanostructure [58] and hence affect bone mechanical properties. In particular, the elastic stiffness is known to increase with bone dehydration [82]. However, comparative studies can be performed when all samples are embedded in a similar way because all samples or regions of interest are similarly affected [93]. The analyses carried out herein rely on relative variations among specimens. Furthermore, such embedding procedure is essential in order to slice specimens containing metal without damaging

the BII, and represents a common procedure conducted in the literature [42, 99, 105, 210].

3.5 Conclusion

Bone tissue composition and structure at the nanoscale and their impact on the microscopic elastic properties have been investigated with combined Raman and nanoindentation experimental analyses. Newly formed bone tissue around an implant has been isolated thanks to an *in vivo* chamber model, and it is found to present lower mineral content, lower crystallinity, lower collagen crosslink ratio, and higher remodelling rate compared to mature bone. These characteristics explain the lower elastic properties compared to mature bone tissue. The results indicate that the presence of an implant affects bone composition and nanostructure during osseointegration, which impacts the mechanical properties.

The observed lower mechanical properties close to the implant might impact the implant anchorage and ultimately the surgical success. Further analyses, to investigate if differences in biomechanical properties can be distinguished within the bone chamber, may help to better understand how osseointegration affects periprosthetic bone tissue.

3.6 Key points

- This study performs a site-matched evaluation of composition and mechanical properties of newly formed bone tissue at the BII compared to existing mature cortical bone.
- The *in vivo* coin-shaped implant model ensures measurements in newly formed bone isolated from pre-existing mature bone tissue thanks to the designed bone chamber below the implant surface.
- Raman spectrocopy can probe variations in composition and nanostructure in the mineral and organic phases of bone through the relative evolution of the intensity and amplitude of the Raman bands of interest.
- In comparison with pre-existing mature bone, newly formed bone presents lower mineralisation, smaller apatite crystals, fewer crosslinks within the organic collagen phase and higher remodelling rate.
- Such nanoscopic composition and structure of newly formed bone tissue are associated with lower microscopic elastic properties with Young's modulus of 12.8 ± 1.8 GPa compared to 15.7 ± 2.3 GPa within mature bone, retrieved from the load-displacement curve of nanoindentation measurements.

Chapter 4. Spatio-temporal variations of bone elastic properties at the BII

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4.1 Introduction

In chapter 3, the quality of new bone tissue at the BII, in comparison with mature bone, has been approached through its composition and its local mechanical properties. To further understand osseointegration phenomena and thus implant stability (chapter 1, subsection 1.2.2), the spatial and temporal variations of mechanical properties within isolated newly formed bone around an implant are important and have not been clearly investigated in the literature [36, 104, 228].

Taking advantage of the benefits of the standardised model previously used in chapter 2 and chapter 3, this study investigates bone quality at the BII with a special focus on its mechanical properties, aiming at a refined spatial analysis together with a temporal evolution of the bone tissue over healing time to better understand the osseointegration process.

Local microscopic elastic properties and bone spreading of newly formed bone have been assessed on eight coin-shaped implant samples using a multi-modal approach: nanoindentation (chapter 1, subsubsection 1.4.3.1) to estimate the indentation modulus, micro-Brillouin scattering (chapter 1, subsubsection 1.4.3.2) to measure the wave velocity and extract the mass density by combining it with the indentation modulus, and histological analysis (chapter 1, subsubsection 1.4.1.2) to quantify 2D bone distribution. The spatiotemporal variations of all properties within newly formed bone have been investigated through the definition of subregions of interest within the bone chamber, and considering two healing times (7 and 13 weeks, n=4 per time point). The combined analysis has enabled us to clarify how osseointegration progresses at the implant surface.

The previous collaboration with clinical surgeons from Hôpital Henri Mondor AP-HP (Créteil, France) and veterinary experts from ENVA (Maisons-Alfort, France) enabled the *in vivo* study. Moreover, new international collaborations were set up with ARTORG Center for biomedical engineering research (University of Bern, Switzerland) for the nanoindentation measurements and with the Laboratory of Ultrasonic Electronics (Doshisha University, Japan) for the micro-Brillouin scattering. The collaborative design of the study and analysis have resulted in the following paper [62], currently under minor revision: Fraulob M., Le Cann S., Voumard B, Yasui H., Yano K., Vayron R., Matsukawa M., Zysset P., and Haïat G., "Multimodal evaluation of the spatio-temporal variations of periprosthetic bone properties", *Journal of Biomechanical Engineering*, submitted.

4.2 Methods

4.2.1 Sample preparation

Identically to the samples of chapter 2, eight Ti6Al4V coin-shaped implants (Fig. 2.1 (chapter 2)) were inserted into the proximal-medial part of the right tibia in eight rabbits (chapter 2, subsection 2.2.3). The implants had previously been sandblasted (chapter 2, subsection 2.2.1) to reach an implant surface roughness of $S_a=3.46 \pm 0.25 \ \mu\text{m}$, which resulted in enhanced bone growth (see chapter 2, subsection 2.4.3 and [64]). Four samples were obtained after 7 weeks of healing time and the four others after 13 weeks of healing time.

The harvested samples were embedded (chapter 2, subsection 2.2.6) and could then be cut in the transverse plane. Two slices were produced from each of the eight samples. The first slice was 800 µm-thick and was cut approximately in the middle of the sample. It was glued on a Permanox slice (Thermo Scientific Nunc, Waltham, USA) and its surface was polished (PM5, Logitech[®], Glasgow, UK) with abrasive paper, SiC Foil with grain size of 1200 and 2000 grit and polishing cloths with 0.3 µm aluminium oxide powder until the sample reached a thickness of 500 µm. The slice was finally rinsed in an ultrasonic bath with 0.9 % NaCl for 20 min. The prepared slice was first analysed by nanoindentation (see subsection 4.2.3), before being stained for histological analysis (see subsection 4.2.2). The second consecutive slice was 400 µm-thick and both sides were manually polished (Minitom, Struers[®], Ballerup, Denmark) using abrasive paper, SiC Foil with grain size of 1200 grit until the thickness was reduced to approximately 200 µm thanks to a sample holder controlling the removal of material (AccuStop, Struers[®], Ballerup, Denmark). This thinnest slice was analysed by micro-Brillouin scattering (see subsection 4.2.4).

4.2.2 Histological analysis

For the histological analysis (detailed protocol in subsection 2.2.6 (chapter 2)), the slices were stained with van Gieson picro-fuchsin to colour bone tissues in red, as shown in Fig. 4.1A. Four ROIs were defined within the bone chamber filled with newly formed bone without any mature bone: two lateral ROIs (L1 and L2) and two central ROIs (M1 and M2).



Figure 4.1 – A) Microscopic image obtained after van Gieson picro-fuchsin staining of a 500 μ m slice after 13 weeks of healing time. Bone tissue is coloured in red and the implant in black. The red rectangular ROI corresponds to the bone chamber colonised with newly formed bone, which is divided into 4 subregions of interest: lateral (L1, L2) and central (M1, M2). The black rectangular ROI corresponds to the region where measurements were taken in mature cortical bone. B) Cropped image of the chamber after segmentation where the bone pixels are in black and the rest of the bone chamber is in white pixels.

Each 500 µm-thick slice was imaged by light microscopy with Stemi 305 (Zeiss[®], Jena, Germany). Using ImageJ (NIH, Bethesda, USA) [184], on the microscope image of each sample, the bone chamber part was cropped and the cropped image was visually segmented to distinguish bone pixels from the rest of the chamber (Fig. 4.1B). For all 4 ROIs (Fig. 4.1A), the ratio between the bone area (i.e. segmented bone pixels BA) and the total area (i.e. all pixels in the ROI TA) (BA/TA) was calculated using the BoneJ plugin [51].

4.2.3 Nanoindentation measurements

Nanoindentation analyses were performed during a six-week stay at ARTORG Center for biomedical engineering research (University of Bern, Switzerland). Measurements were carried out along the bone axis with a nanoindenter (UNHT, CSM Instrument, Switzerland) equipped with a Berkovich diamond indentation tip under dry conditions. A sapphire sphere was used to precisely detect the sample surface. Following the principle of Fig. 3.5B,C (chapter 3), the indentation tip reached a maximum depth of 1 μ m during a 30 s holding time after a loading phase (loading rate: 100 mN/min) and before an unloading phase (unloading rate: 400 mN/min).

Prior to all measurements, the device was calibrated by performing measurements in fused silica. In each lateral (L1 and L2) and central (M1 and M2) ROI, nanoindentation measurements were carried out in sixteen locations that were manually selected, when enough bone was present. Moreover, forty control values were measured below the chamber in mature cortical bone with corresponding anatomical location, at a distance from the bone chamber comprised between 40 μ m and 400 μ m (black rectangular ROI in Fig. 4.1A). All locations were chosen in order to avoid indenting PMMA and were separated from each other (respectively from the implant surface) by a minimum distance of 14 μ m (respectively 7 μ m).

Identically to the analysis of subsection 3.2.3 (chapter 3) using the Oliver and Pharr method [156], for each measurement, the reduced modulus E_r was retrieved from the load-displacement curve (Eq. 3.1 (chapter 3)) and the indentation modulus E^* could then be determined following Eq. 4.1:

$$\frac{1}{E_r} = \frac{1}{E^*} + \frac{1 - \nu_i^2}{E_i} \tag{4.1}$$

where $E_i=1141$ GPa and $\nu_i=0.07$ are the Young's modulus and the Poisson's ratio of the diamond indenter tip.

Note that the relationship between the indentation modulus E^* and the Young's modulus E used in subsection 3.2.3 (chapter 3) is given by:

$$E = E^* (1 - \nu_h^2) \tag{4.2}$$

with $\nu_b=0.3$ is the Poisson's ratio of bone tissue [8, 105, 228]. Considering the indentation modulus E^* rather than the Young's modulus E avoids any assumption about Poisson's ratio of bone.

4.2.4 Micro-Brillouin scattering measurements

Micro-Brillouin scattering measurements were conducted in the Laboratory of Ultrasonic Electronics (Doshisha University, Japan). A six-pass tandem Fabry-Pérot interferometre (JRS Scientific Instruments, Zurich, Switzerland) (Fig. 4.2) using a 532 nm (λ_i) solid state laser (Spectra Physics, Excelsior, Santa Clara, CA) was used. The 200 µmthick slices were fixed on a flat metal reflector and placed with a controlled angle of $\theta/2$ between the sample's normal and the incident laser beam (Fig. 4.2A,B). The laser spot had an approximate resolution of 10 µm and a power of 12 mW avoiding any heat effect.



Figure 4.2 – A) Sample placed on a reflector with an angle $\theta/2=45^{\circ}$ under the microscope. B) Schematic representation of reflection induced θ angle micro-Brillouin scattering measurement configuration. C) Schematic pathway of the incident laser beam reflected by the specimen before being detected by the photomultiplier tube (PMT) after the Tandem Fabry-Pérot interferometer to be analysed by the photon counter.

The incident laser beam (vector k_i) propagated through the studied sample following Snell-Descartes laws (Fig. 4.2B) and phonons (vector k_B) were created because of Brillouin scattering effects arising from the interaction between light and bone tissue, leading to a frequency shift of the scattered beam (vector k_s). The scattered beam was detected with a photomultiplier (Hamamatsu, R464s, Shizuoka, Japan) (Fig. 4.2C) and averaged from Sandercock Type Tandem Fabry-Pérot interferometre (The table stable, Mettmenstetten, Switzerland) scans with a photon counter (Fig. 4.2C). After a Voigt fitting on the obtained spectrum (Fig. 4.3), the frequency shifts Δf between the incident laser beam and the resultant scattered beam were used to access to the wave velocity in the plane of the sample surface, v, following Eq. 4.3 [110]:

$$v = \frac{\Delta f * \lambda_i}{2 * \sin(\theta/2)} \tag{4.3}$$

where $\lambda_i = 532$ nm is the laser wavelength and $\theta/2 = 45^{\circ}$ is the angle between the sample's normal and the incident laser beam.

The measurement locations were selected thanks to an optical microscope (Photon design, Mercure, Tokyo, Japan) (Fig. 4.2A), to fit as much as possible the nanoindentation locations. Within the bone chamber, two measurements were gathered for each of the subregions (Fig. 4.1A), when enough bone was present. Moreover six measurements were taken in the mature region for each sample. For micro-Brillouin measurements, the ultrasonic waves propagate in the plane of the sample, which corresponds to the plane perpendicular to the bone axis. For each location, the measurement was repeated five times to limit the impact of noise, and led to an average value of five velocities.



Figure 4.3 – Micro-Brillouin spectrum and bone frequency shifts (denoted by Δf). The spectrum represents the filtered (1D median filter) and smoothed data for one given measuring point corresponding to a wave velocity equal to 4994 m/s.

4.2.5 Statistical analysis

Differences between ROIs were used to evaluate the spatial distribution of bone properties within the chamber. Because of the low number of measurements in the ROIs, often related to limited bone tissue, values were averaged among larger groups as presented in Table 4.1.

Table 4.1 – Definition of the groups used in the statistical analysis and derived from the bone chamber regions of interest defined in Fig. 4.1A

Groups	Regions		
Newly formed bone	L1 + L2 + M1 + M2		
Close	L1 + M1		
Far	L2 + M2		
Lateral	L1 + L2		
Central	M1 + M2		

For all groups mentioned in Table 4.1 and for mature bone, the average and standard deviation values of E^* (nanoindentation), v (micro-Brillouin scattering) and BA/TA (histology) were determined for all samples corresponding to a healing time of 7 weeks and of 13 weeks. The results were compared using non-parametric Mann-Whitney U-tests (Matlab R2017b, MathWorks[®], Natick, USA) to assess the statistical significance between the groups.

4.2.6 Local density estimation

Combining nanoindentation and micro-Brillouin scattering results could provide an estimation of the relative variations of bone mass density ρ at the scale of several micrometres, since ρ is linked to wave velocity v and Young's modulus E assuming an isotropic elastic constitutive law following [211]:

$$v = \sqrt{\frac{E}{\rho}} \tag{4.4}$$

The differentiation of Eq. 4.4 led to:

$$\frac{\Delta\rho}{\rho_0} = \frac{\Delta E}{E_0} - 2\frac{\Delta v}{v_0} \tag{4.5}$$

where ΔE , Δv and $\Delta \rho$ (respectively, E_0 , v_0 and ρ_0) represent the differences between the values (respectively, the average values) of E, v and ρ for two studied groups.

4.3 Results

4.3.1 Histological analysis

After 13 weeks of healing time, the bone ratio BA/TA is significantly lower for the far group compared to the close group (Fig. 4.4A) as well as for the central group compared to the lateral group (Fig. 4.4B). The same trend is observed after 7 weeks of healing time without being significant. For all the groups, BA/TA after 13 weeks of healing time is higher than after 7 weeks without being significant. Moreover, the value of the standard deviations decreases as a function of healing time for all groups.



Figure 4.4 – 2D bone distributions (BA/TA) presented as average and standard deviation values after 7 weeks (7w) and 13 weeks (13w) of healing time for A) the close and far groups and B) the lateral and central groups. The Mann-Whitney U-test analysis leads to: * = p-value ≤ 0.05 , NS = p-value > 0.05 (non-significant difference).

4.3.2 Nanoindentation measurements

After both healing times (7 and 13 weeks), the indentation modulus E^* is significantly higher in mature than in newly formed bone tissue (Fig. 4.5A). Moreover, E^* is significantly higher after 13 weeks of healing time compared to 7 weeks of healing time in newly formed bone tissue, whereas it does not vary significantly in mature bone tissue.
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Within the bone chamber, the indentation modulus E^* is significantly lower in the far group than in the close group after 7 weeks of healing time, while no significant differences are observed after 13 weeks (Fig. 4.5B). When comparing lateral group to central groups (Fig. 4.5C), E^* is significantly lower in the center after 7 weeks of healing time, with differences decreasing and becoming non-significant after 13 weeks. Moreover, for all groups, a significant increase in E^* with healing time is observed after 13 weeks of healing time compared to 7 weeks of healing time (Fig. 4.5B,C).



Figure 4.5 – Distribution of the indentation modulus E^* presented as average and standard deviation values after 7 weeks (7w) and 13 weeks (13w) of healing time, based on the groups defined in Table 4.1. A) Mature vs newly formed bone tissue, B) close vs far within the bone chamber and C) lateral vs central within the bone chamber. The Mann-Whitney U-test analysis leads to: * = p-value ≤ 0.05 , ** = p-value ≤ 0.01 , *** = p-value ≤ 0.001 , NS = p-value > 0.05 (non-significant difference).



Figure 4.6 – Distribution of the wave velocity v presented as average and standard deviation values after 7 weeks (7w) and 13 weeks (13w) of healing time. A) Mature vs newly formed bone tissues, B) close vs far groups and C) lateral vs central groups. The Mann-Whitney U-test analysis leads to: * = p-value ≤ 0.05 , ** = p-value ≤ 0.01 , *** = p-value ≤ 0.001 , NS = p-value > 0.05 (non-significant difference).

4.3.3 Micro-Brillouin scattering measurements

After both healing times, the wave velocity v is significantly higher in mature than in newly formed bone tissue (Fig. 4.6A). Moreover, the wave velocities are significantly higher for both newly formed and mature bone tissues after 13 weeks of healing time compared to 7 weeks of healing time.

Within the bone chamber, no significant differences in wave velocities are observed between the far and the close group after 7 or 13 weeks of healing time (Fig. 4.6B). Similarly, no significant differences are obtained between the lateral and central groups (Fig. 4.6C). All groups depict higher wave velocities after a longer healing period, all being significant except for the central group (Fig. 4.6B,C).

4.3.4 Local relative variations and density estimation

The positive values in Table 4.2 indicate that all three parameters $(E, v \text{ and } \rho)$ are i) lower in newly formed compared to mature bone, ii) lower far from the implant compared to close regions and iii) lower in the centre of the chamber compared to lateral sides, except for velocity after 7 weeks of healing time. For all compared groups, the variations of v are smaller than those of E and ρ . Furthermore, the variations of E and ρ are larger after 7 weeks than 13 weeks of healing time for all groups.

Table 4.2 – Relative variations of E, v and ρ obtained when comparing i) newly formed and mature bone tissues, ii) the far and close groups and iii) the central and lateral groups after 7 and 13 weeks of healing time. The data corresponding to the relative variation of E are obtained from Fig. 4.5 using Eq. 4.2. Positive values indicate an increase in the parameters from newly formed to mature bone, from far to close groups and from central to lateral groups.

Compared groups	Parameters	7 weeks of healing	13 weeks of healing
i) newly formed bone vs mature bone	$\Delta E/E_0$	30.5~%	18.3~%
	$\Delta v/v_0$	8.1 %	6.1~%
	$\Delta ho / ho_0$	14.3~%	6.1~%
ii) far vs close	$\Delta E/E_0$	12.3~%	3.2~%
	$\Delta v/v_0$	0 %	0.3~%
	$\Delta ho / ho_0$	12.3~%	2.5~%
	$\Delta E/E_0$	14.4 %	2.8~%
iii) central vs lateral	$\Delta v/v_0$	-1.2 %	0.2~%
	$\Delta \rho / \rho_0$	16.8 %	2.4~%

4.4 Discussion

The present multi-modal and multi-physics study combines nanoindentation, micro-Brillouin scattering and histology, on specimens obtained with experimental *in vivo* surgery to investigate the spatio-temporal variations of periprosthetic bone biomechanical properties. Within the bone chamber divided into subregions, the two elastic parameters, indentation modulus E^* and wave velocity v, as well as the 2D bone distributions and the estimated variations of bone mass density have suggested higher bone properties close to the implant surface and to the chamber lateral edges, but the differences seem to fade out for longer healing times.

4.4.1 Bone elastic parameters

The average indentation modulus E^* obtained herein is equal to 11.2 GPa (respectively 12.84 GPa) within newly formed bone and to 15.23 GPa (respectively 15.42 GPa) in mature cortical bone after 7 weeks (respectively 13 weeks) of healing time. The averaged Young's modulus E (derived from the indentation modulus E^* using Eq. 4.2) obtained herein is equal to 10.19 GPa (respectively 11.68 GPa) in newly formed bone and to 13.86 GPa (respectively 14.03 GPa) in mature cortical bone after 7 weeks (respectively 13 weeks) of healing time. Here, a bone Poisson's ratio of 0.3 was chosen based on the literature but different values have also been considered [230]. Note that a variation of the bone Poisson's ratio from 0.2 to 0.5 (a bone Poisson's ratio equal to 0.5 represents incompressible materials such as soft tissues) leads to a decrease of 25 % in the bone Young's modulus, according to the data of Table 4.3, and does not affect the relative variation of the bone Young's modulus obtained between the different groups in Table 4.2. The observed Young's moduli are of the same order of magnitude than values obtained in chapter 3 and in previous studies in the literature. In particular in rabbit femurs [10, 22], the obtained elastic parameters are in the range of 6-12 GPa for newly formed bone and of 9-17 GPa for mature bone tissue.

Table 4.3 – Sensitivity study on the averaged Young's modulus E (GPa) within newly formed (NF) and mature (M) bone and its relative variation $\Delta E/E_0$ between NF and M bone after 7 and 13 weeks of healing, for Poisson's ratio equal to 0.2, 0.3, 0.4 and 0.5.

Poisson's	7 weeks			13 weeks		
ratio	NF	M	$\Delta E/E_0$	NF	Μ	$\Delta E/E_0$
0.2	10.75	14.62	30.5	12.33	14.80	18.3
0.3	10.19	13.86	30.5	11.68	14.03	18.3
0.4	9.41	12.79	30.5	10.79	12.95	18.3
0.5	8.40	11.42	30.5	9.63	11.57	18.3

The values of wave velocity v are comprised between $4.77.10^3$ and $5.41.10^3$ m/s and are in agreement with the previous study on the same *in vivo* model [136] and in the same range as the results obtained in other studies characterising wave velocities within bone trabeculae [102, 204].

4.4.2 Effect of healing time on bone elastic properties

For most groups, the two studied elastic parameters E^* and v are higher after 13 weeks than after 7 weeks of healing time and in mature than in newly formed bone. Consequently, since constant values are obtained in mature bone, the increase in elastic parameters with healing time and maturation leads to larger differences in E, v and ρ (see Table 4.2) after 7 weeks of healing, when only limited remodelling has occurred among newly formed bone, than after 13 weeks of healing time, when most bone has been remodelled and properties are closer to mature bone properties. For example, ρ increases by 14.3 % (respectively 6.1 %) between newly formed and mature bone tissue after 7 weeks (respectively 13 weeks) of healing time, which is comparable to the increase of 12.2 % (respectively 2.2 %) found in [211].

The gradual increase in E, v and ρ from 7 to 13 weeks of healing time and from newly formed to mature cortical bone tissue may be explained by the increase in mineralisation occurring during osseointegration phenomena [67]. Previous nanoindentation studies have shown that the bone elastic modulus increases as a function of healing time using the same *in vivo* model [210], a titanium plate model [42], around dental implant [99] and within bone defects [10]. The study of chapter 3 combining nanoindentation and Raman spectroscopy measurements also suggests that a higher bone elastic modulus is related to a more advanced bone mineralisation stage. Furthermore, the aforementioned experimental results are in agreement with a homogenisation model showing a non-linear increase in the Young's modulus as a function of bone tissue mineralisation [181, 182]. Moreover, wave velocity also increases as a function of bone tissue mineralisation, in line with results of previous micro-Brillouin scattering studies [5, 65, 102, 136].

The increase in bone mass density as a function of healing time and between newly formed and mature bone can be explained by the increasing degree of mineralisation, since mineral, which is more present in more mature tissue (chapter 3, subsection 3.4.4), is denser than other bone components (collagen fibres and water) [210], consistently with bone tissue maturation [92]. Note that since the velocity v can be modelled by a ratio between E and ρ (Eq. 4.4), its relative variations are lower compared to E. Therefore, only variations of E and ρ will be considered in what follows.

4.4.3 Spatial variations of bone elastic properties at the BII



Figure 4.7 – Schematic representation of the spatial variation of E and ρ in each ROI of the bone chamber. The black (respectively white) symbols correspond to a comparison between the close and far (respectively lateral and central) groups. The '+' (respectively '-') sign indicates the ROI where E and ρ are higher (respectively lower). The large arrows correspond to the direction of bone growth occurring initially and the small arrows represent later bone spreading.

Analysing the spatial variations of E and ρ helps understand the course of osseointegration phenomena in the bone chamber. Since elastic properties increase with mineralisation increasing itself with healing time, the increase in E and ρ as a function of healing time (Fig. 4.5 and Table 4.2) can be considered as a marker of tissue mineralisation and of osseointegration (results from chapter 3). The schematic comparison of the values of E and ρ as a function of the position in the bone chamber shown in Fig. 4.7 emphasises that bone tissue in the close group (black '+' signs) and in the lateral group (white '+' signs) is more mineralised and thus older compared to bone tissue located in the far group (black '-' signs) and in the central group (white '-' signs). Combining the close-to-far and the lateral-to-central evolutions of bone properties leads to the conclusion that the highest bone properties are obtained in the upper corners of the bone chamber (i.e. L1 region in Fig. 4.1A), which suggests that bone creation initiates there. The upper corners' region might indeed be the site of important local stresses, which could explain the start of bone formation. Mechanical loads have besides been observed to trigger bone formation [76] such as within the threading of dental implants. The bone elastic properties measured using nanoindentation around a dental implant were higher in the upper parts of implant threading, explained by the local stress concentration [228].

After being created in the bone chamber's upper corners, bone tissue then grows along the implant surface and towards mature bone tissue before spreading to the most central region of the bone chamber (arrows in Fig. 4.7). Such scenario is consistent with the variation of BA/TA values shown in Fig. 4.4 indicating a larger amount of bone close to the implant and in the lateral region than in the far and central regions.

4.4.4 Contact osteogenesis within the bone chamber

The aforementioned osseointegration scenario may be discussed in the light of the description of "contact" and "distance" osteogenesis presented in subsection 1.2.2 (chapter 1). Contact osteogenesis corresponds to bone growth from the implant surface to the pre-existing bone tissue whereas distance osteogenesis corresponds to bone growth from the pre-existing bone to the implant surface. Our results showing a bone formation starting in the upper corners of the bone chamber seem in better agreement with the contact osteogenesis scenario and could be explained by blood accumulation in such regions after surgery, providing the osteogenic cells needed for bone formation [45, 112]. A predominant contact osteogenesis is expected with rougher implant surfaces [39], as such surfaces offer more space for cells to attach to the implant surface, thus promoting bone cell migration [77] and resulting in more osseointegrated implant, as shown in chapter 2. Furthermore, bone does not only grow in contact with titanium, but also along PTFE on the lateral sides of the bone chamber. This second material may interfere with the spatial evolution of bone tissue and future studies should investigate bone attachment on it and the effect of changing PTFE into another biomaterial.

To characterise bone nanostructure at the BII, sequential slices of this same specimen group have also been investigated with SAXS measurements (chapter 1, subsubsection 1.4.1.4) performed at Synchrotron beamlines (SLS, PSI, Villigen, Switzerland) for another study in our group. A similar spatial division within the bone chamber (Fig. 4.1A) has been considered to probe crystal thickness, quantify bone content and assess the corresponding spatio-temporal evolution [118]. The bone content is found slightly higher in upper corners, confirming the present histological results with more bone quantified in the corners too, which also suggests the hypothesis of contact osteogenesis. Moreover, the SAXS measurements evidence ongoing distance osteogenesis starting from pre-existing mature bone with thicker crystals, linked to more mature tissue [132], found in regions close to mature bone. The SAXS measurements support the present results and also give a more precise insight of bone healing kinetics revealing the presence of distance osteogenesis.

4.4.5 Limitations

This study has several limitations. First, as already stated and discussed in the chapter 3 (subsection 3.4.6), the samples were embedded in PMMA, which is mandatory to be able to cut them and access the BII without damaging it, in spite of possible effects on bone properties. Second, the sample size remains relatively low (n=4 per group) because of the *in vivo* model and of time-consuming measurements. However, the present study has doubled the number of samples previously analysed [211] and a refined analysis per specimen, through multiple measurements and ROIs, has allowed the study of spatial variations. Third, nanoindentation and micro-Brillouin scattering analyses were performed on two consecutive slices because the thickness of the slices needed to be different. Fourth, although bone tissue is known to have an anisotropic mechanical behaviour [33, 34], the isotropic assumption was made herein for the bone tissue constitutive law. Similar assumption was made for nanoindentation analyses in the literature [8, 105, 220, 228] and in chapter 3, which is particularly justified for newly formed bone because woven bone structure is disordered [189] and its mineral crystals are distributed isotropically [195, 202]. The isotropic assumption is particularly important in order to derive the variation of mass density. However, nanoindentation and micro-Brillouin scattering were performed in two orthogonal directions. Here, we have assumed similar relative variations of all components of the stiffness tensor. Future studies should consider the anisotropic behaviour of the bone tissue (discussed in the following chapter). Fifth, the coin-shaped implant model leads to a relatively low level of mechanical loading, which limits osseointegration phenomena but the aim of this implant model is to differentiate mature and newly formed bone tissue and to work under standardised conditions.

4.5 Conclusion

This study combining nanoindentation, micro-Brillouin scattering, histology and a dedicated *in vivo* model has allowed the investigation of newly formed bone tissue properties compared to mature cortical bone, evidencing lower elastic properties for newly formed bone. Moreover, the spatio-temporal evolution of newly formed bone properties has been evaluated by considering different ROIs within the bone chamber filled with newly formed bone. Bone elastic properties and estimated bone mass density are found to be higher close to the implant surface and in the lateral ROIs than in the far and central ROIs, as well as after longer healing times suggesting a higher degree of mineralisation consistent with bone tissue maturation. These results suggest a bone spreading path governed by contact osteogenesis, confirmed by 2D bone distribution measured by histology. Characterising mechanical bone properties at the BII has enabled us to identify how bone develops around the BII providing a better understanding of osseointegration phenomena, which is likely to improve implant stability and surgical outcomes.

4.6 Key points

- This microscale study on the coin-shaped implant model consists in a combined analysis of nanoindentation and micro-Brillouin scattering to assess bone elastic properties, and histology to visualise bone formation.
- Elastic properties are found lower in newly formed bone than in mature bone explained by a lower degree of mineralisation (see chapter 3) and the observed difference decreases with healing time. For example, the Young's modulus is lower of 30.5 % in newly formed than in mature bone after 7 weeks of healing and of 18.3 % after 13 weeks.
- By combining the results from nanoindentation and micro-Brillouin scattering, the evolution of bone mass density could be evaluated showing an increase between newly formed and mature bone of 14.3 % after 7 weeks of healing and of 6.1 % after 13 weeks.
- Spatial variations of elastic properties in the bone chamber, confirmed by histology, suggest a bone spreading path starting in the upper corners and spreading along implant and towards pre-existing bone, consistent with contact osteogenesis.
- Studying bone mechanical properties at the BII informs about how bone develops around an implant, leading to a better understanding of osseointegration and implant stability determining surgical success.

Chapter 5. Perspectives

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5.1 Further characterisation of the BII

5.1.1 Towards a better quantification of bone growth at the BII

As presented in chapter 1 and discussed in chapter 2, bone quantity and bone-implant contact are determinants of implant stability (chapter 1, subsection 1.2.4). Moreover, investigating the evolution of bone contact and quantity at the BII provides information about the kinetics of bone healing such as how bone spreads at the implant surface, as studied in chapter 4 where histology confirms the observed bone path. Such study of bone growth kinetics is unfortunately not possible around clinical implant as newly formed and mature bone are intertwined.

The coin-shaped implant model described in this thesis is an interesting tool to characterise exclusively newly formed bone tissue at the BII. However, further studies are necessary to quantify bone ingrowth at the BII with more precision by increasing the number of histological slices and the resolution of QUS scans, considered in chapter 2.

Furthermore, as introduced in chapter 1, high-resolution imaging techniques such as X-ray μ CT (chapter 1, subsubsection 1.4.1.3) or electron tomography (chapter 1, paragraph 1.4.1.5) can provide a 3D visualisation of the bone tissue structure and bone ingrowth around an implant. Compared to the study of bulk bone, the choice of such methods is limited by the presence of titanium implants impacting sample preparation and resulting images. Indeed with X-ray based imaging techniques, metal implants create artefacts hampering precise observation of the BII. To solve this problem, neutron μ CT (chapter 1, subsubsection 1.4.1.3) has recently been used as a new 3D imaging technique to study microscopically periprosthetic bone tissues at the BII [94], with no observed artefact from the metallic components. Analysing the coin-shaped implant samples with neutron μ CT could give a detailed visualisation of tissues in the bone chamber. Further analyses could compare the resulting images with histology slices as a validation. The comparison of neutron and QUS scans (similarly to the Fig. 2.10 (chapter 2) but for the whole implant surface) would enable better understanding and identification of the contribution of bone quality in the QUS results presented in subsection 2.4.6 (chapter 2).

5.1.2 Further biomechanical characterisation of the BII

5.1.2.1 Taking into account bone anisotropy and viscoelasticity

In nanoindentation analyses of chapter 3 and chapter 4, bone has been assumed as an isotropic and linear elastic material. If such assumptions are commonly assumed in the literature [8, 105, 220, 228], they remain strong as bone's mechanical behaviour has been known to be anisotropic [34] and viscoelastic[224]. Taking into account such constitutive laws would lead to a more realistic characterisation of the BII and its mechanical response.

Multiple experimental measurements in perpendicular directions could help better identify mechanical properties and evaluate bone anisotropy. In a trabecula, micro-Brillouin scattering, assessing wave velocity in the plane of the sample surface, has been used in combination with scanning acoustic microscopy, measuring acoustic impedance perpendicularly to the sample surface [102]. At the BII, such analyses in site-matched regions and in perpendicular directions could evaluate microscopic elastic anisotropy. Nanoindentation can also be further performed in several directions as it has been done in osteonal measurements [34]. Such protocol would be an interesting way to pursue the started experimental characterisation of bone elastic properties, but with our implant model, multi-directional indentation is challenging as the sample preparation should give access to the BII from different orientations whereas the BII is delicate during cutting.

To limit the assumptions made on bone mechanical behaviour, bone viscoelasticity could be taken into account with dynamic mechanical analyses (DMA). For example, dynamic nanoindentation has assessed the viscoelastic response of bone depending on its collagen content [164]. Even if for the moment only the static mode has been considered, such a DMA unit is available on the nanoindentation device used in chapter 3, so a study of viscoelasticity at the BII could be designed in our future collaboration with ICMPE. However, bone's viscoelastic response is sensitive to the experimental conditions (such as sample preparation, temperature and wet or dry samples) [224], and further embedding procedures need to be investigated to limit the effect on bone mechanical properties.

5.1.2.2 Investigating the rupture of the BII under mechanical loading

Fracture is a common cause of implant failure. Studying the BII strength and how rupture occurs is necessary to link it with bone-implant contact at the BII and bone properties in order to understand and prevent implant loosening. Mechanical testing such as push-out or pull-out tests are commonly used (see chapter 1, paragraph 1.4.3.3) but, in torsion test of paragraph 1.4.3.3 (chapter 1), crack propagation is more stable. Such macroscopic mechanical test would investigate bone tissue's strength in response to torsion creating shear stress at the BII. In our group, a cleavage device has been developed [137] to assess torsional rupture on standard coin-shaped implants as a function of healing time. Such macroscopic characterisation could be added to our multi-modal experimental approach to correlate the strength to rupture of the BII and its local microand nanoscopic properties.

In situ loading experiments could help investigate the response of the developed BII by coupling simultaneously bone characterisation and mechanical loading of the ex vivo sample in a multi-scale approach to investigate the strength of the BII and how tissues respond to stress in terms of orientation and alignment for instance. Bone microstructure and multi-scale deformation could be analysed during tensile tests to understand bone fracture [75], similarly to the study of soft tissues undergoing macroscopic traction tests while imaging the evolution of their microstructure with multi-photon microscopy [16, 88]. Such approach has also been used to study the BII with implanted metallic screws imaged with X-ray [117] and neutron tomography [116] during in situ pull-out tests. Conducting similar studies on the coin-shaped implant model of this thesis would give specific information about the BII formed with new bone of the bone chamber. For example, a mechanical load could be applied on the coin-shaped implant until rupture and, in the meantime, to evaluate induced deformations of the micro- and nanostructure of newly formed bone, the sample would be imaged with neutron μCT (as suggested in subsection 5.1.1), with nonlinear optical or multi-photon imaging to describe the microstructure [18, 127] or with SAXS to characterise the nanostructure [118]. However, such combined analyses may be difficult to implement as they would necessitate compatible techniques and setups.

To be able to understand the influence on the BII strength of each parameter such as bone-implant contact, bone quantity and bone material properties, the study of bone rupture could be completed using numerical simulation with FE models (chapter 1, section 1.5). For example, as introduced in subsubsection 1.5.1.2 (chapter 1), mechanical macrotests such as torsion, push-out and pull-out tests may be numerically modelled monitoring the stress levels at the BII and following the crack propagation, which are not accessible experimentally.

5.1.2.3 Characterisation of bone-implant friction

Immediately after surgery, the amplitude of micromotions (chapter 1, subsection 1.2.3) determines implantation success through primary stability. Micromotions between the implant and bone tissue at the implantation site depend on the frictional behaviour, so studying the friction between an implant and bone tissue is essential for the understanding of implant stability. The cleavage device in development at MSME mentioned in subsubsection 5.1.2.2 can be used as a friction test (chapter 1, paragraph 1.4.3.3) as well, when considering bone samples and non-osseointegrated coin-shaped implants. Such friction test would be performed in torsion under a chosen normal force and being careful of misalignment errors. The impact of implant surface roughness (see below subsubsection 5.2.1.1) on the friction coefficient could then be studied.

5.1.3 Effect of mechanical loading during osseointegration

The mechanical environment has been shown to affect osseointegration during the healing phase, inducing changes in bone structure and composition [76].

5.1.3.1 Bone adaptation

Bone adaptation has been observed since early biomechanical studies with Wolff's law in 1892. Wolff's law states that bone structure (trabeculae for instance) adapts and aligns with the direction of the principal stress [29, 222]. The bone shape, material properties and structure are thus optimised in response to mechanical loading and exercise [206, 222]. In the presence of implants, studies have investigated the effect of the loading frequency and magnitude [229] and of insertion torque [55] on the BIC values, as well as the loading effect on elastic parameters around dental implants [9] and within thread regions with higher stress concentration [228]. Mechanical adaptation of bone involves its nanoscopic structure as well. For example, under mastication forces, the biological apatite crystals near the tooth preferentially align following the local stress distribution [95, 151].

No mechanical stimulus is applied on the current *in vivo* implant model of this thesis, similarly to the healing phase in dental surgery. However, some inherent mechanical stress are generated by the design of the implant model. It would be interesting to evaluate those mechanical constraints by creating a FE model of a coin-shaped implant surrounded by the PTFE cap, inserted and osseointegrated within mature cortical bone. A mapping of stress and strains could be drawn depending on the healing time modelled with different bone-implant contact, bone quantity in the bone chamber and material properties of newly formed bone. Note that before building such a model, the force generated by the fixation elastic string needs to be assessed. Such mapping could explain the bone spreading path (Fig. 4.7 (chapter 4)) obtained in subsection 4.4.3 by verifying if stress are concentrated in the upper corners of the bone chamber where bone formation seems to initiate in agreement with a previous study [228].

Yet, to take full advantage of the implant model, adding controlled mechanical stimulation during osseointegration could improve the understanding of bone formation within the bone chamber in response to the mechanical environment. This would need the design of a device stimulating the implantation site *in vivo*, in accordance with the guidelines for animal experimentations and with the ethical committee.

5.1.3.2 Mechanotransduction

Mechanotransduction is the biological process under Wolff's law, converting mechanical stimuli into biomechanical signals [57] thanks to osteocytes and perhaps bone lining cells too [107] (chapter 1, subsection 1.1.2). Strains applied to bone affect the fluid flow shear stress circulating within bone lacunocanalicular porosity and irrigating osteocytes with cell signalling molecules and nutrients. Consequently, osteocytes are informed about the mechanical environment and the mechanosensing cells will then adapt bone remodelling by recruiting necessary bone cells (osteoblasts and osteoclasts) [29, 107, 222].

The bone chamber implant model could be useful to identify mechanotransduction actors such as osteocytes, cell walls, cytoskeletons, plasma membrane and ion channels [60] and to understand how they affect bone growth. Cellular experiments following cell proliferation or marking some specific mechanotransduction actors within the bone chamber, for instance, would be interesting to understand the initiation of osseointegration and better control implant stability.

Such experimental work could be coupled with a multi-scale computational model of bone remodelling (developed in MSME in a recently defended PhD thesis) [131]. It could be adapted to the BII and to our bone chamber model to better understand bone spreading observed in subsection 4.4.3 (chapter 4).

5.2 Clinical applications and medical devices

All studies in this thesis have been designed with one clinical goal: understanding the implant stability to ensure surgical success.

5.2.1 Optimising clinical implants and treatments

5.2.1.1 Optimisation of the implant surface properties

By identifying newly formed bone's properties, research studies about osseointegration allow the optimisation of the orthopaedic and dental clinical implants' materials [120]. For example, elastic moduli of periprosthetic bone tissue have been evaluated for dental implants made of titanium and of other metals [8, 104]. As suggested in subsection 4.4.4 (chapter 4), considering other metals than Ti6Al4V for the coin-shaped implant and even other material categories such as polymers or ceramics would be of great interest for the clinic using such materials as well [80, 219]. Implant material properties can also be modified with a surface coating and, in an ongoing study of our group in collaboration with the laboratory Chimie Structures et Propriétés de Biomatériaux et d'Agents Thérapeutiques UMR 7244 (Université Paris 13, Paris, France), the coin-shaped titanium implants have been grafted with bioactive polymers [40] which have an antibacterial effect [142] to evaluate the effect on implant anchorage. Concerning implant surface design, implant surface roughness, obtained by surface treatment such as sandblasting or acid etching, also plays an important role in the osseointegration process (chapter 1, subsection 1.2.3). In this thesis, the investigation of the effects of implant surface roughness has been started in chapter 2 considering sandblasted implants of two roughness levels. Further studies of the impact of implant surface roughness could take advantage of numerical simulation enabling to control independently each parameters (chapter 1, section 1.5). In MSME, a 2D FE model has been created with a sinusoidal interface modelling microroughness (Fig. 5.1) [171] similarly to the model of ultrasound propagation validating the study of chapter 2. Already implemented under tensile loading (Fig. 5.1c), such model is currently being adapted to investigate the effect of shear loading on shear stress at the BII, and then broaden the knowledge of stress shielding inducing implant loosening (chapter 1, subsubsection 1.5.1.3). The influence of implant surface roughness, BIC value and bone and implant material properties, for example, could be investigated through a parametric study.



Figure 5.1 – Multi-scale description of the BII under tensile loading. (a) Macroscopic view of a femoral stem as an example, (b) mesoscopic sinusoidal description of the BII and (c) microscopic model of the implant surface roughness (taken from [171]).

5.2.1.2 Optimisation of medical treatments

Implant stability could also be optimised through medical treatments and research studies investigate various clinical options to optimise osseointegration. For example, in fracture treatment, the impact of drugs on bone remodelling [133] as well as the influence of bone substitute on bone strength [108] have been considered. In orthopaedic surgery, patients' stem cells are used to treat osteoarthritis for instance since stem cells are known to have regenerative effects on cartilage and bone tissues [96]. Such treatment is investigated in an ongoing study of the research group in collaboration with Etablissement Français du Sang EFS (Créteil, France) as stem cell solutions have been placed at the surface of the coin-shaped implant model to encourage the osseointegration process.

5.2.2 Developing medical devices for stability assessment

The present PhD work develops methods to characterise osseointegration that can be implemented in medical devices to help surgeons in their daily practice by monitoring implant stability *in vivo* [174] in order to detect when an implant can be loaded or anticipate implant loosening. Surgeons need to find a compromise between early (or even sometimes immediate) loading to trigger the osseointegration process and long healing times before loading to ensure a strong BII after bone adaptation (subsubsection 5.1.3.1) [212]. Surgeons often make their decisions based on empirical measurements of the implant's stability, and thus there is a clinical need for medical devices to reliably quantify osseointegration *in vivo*.

To assess *in vivo* primary and secondary implant stability (chapter 1, subsection 1.2.2) in clinical practice, imaging techniques such as X-ray μ CT [183] and MRI based approaches [71] have been suggested for the evaluation of the implant osseointegration, but their performances remain limited because of imaging artefacts due to the presence of titanium [190] and limited image resolution. To overcome those limitations, different biomechanical methods have been developed, in particular for dental implants. The Periotest[®] method (Bensheim, Germany) [185] is based on the mechanical response of the implant to an impact, monitoring the induced contact duration. However, the reproducibility of the measurements has been questioned [144, 145]. The resonance frequency analysis (RFA), used in the commercialised Osstell[®] device (Gothenburg, Sweden), records the implant first bending resonance frequency [146], but it is related to the stiffness of the whole boneimplant structure [166] rather than to the local properties of the BII. The RFA method thus remains limited for a direct evaluation of the biomechanical properties of the BII, independently from the larger bone environment or anatomy [12, 173].

Since ultrasonic waves are sensitive to the bone elastic properties [115], QUS techniques represent an attractive approach to evaluate implant stability, which is the focus of the start-up WaveImplant, currently developed in the research group. The QUS method used in this thesis in chapter 2 has been adapted into a clinical QUS device consisting in a monoelement transducer directly screwed within a dental implant to measure its echographic response (Fig. 5.2). The results gathered in chapter 2 can help the development of such clinical methods, like previous *in vitro* [135], *in silico* [134, 213, 214] and *in vivo* [212] studies. More recently, the results obtained for dental implants *in vitro* [216] and *in vivo* [87, 215] have been shown to be more reproducible and more sensitive to implant stability compared to data obtained using a RFA-based approach. During my PhD, such *in vivo* comparative study led to a submitted paper [87]:

Hériveaux Y., Vayron R., Fraulob M., Albini Lomami H., Lenormand C., and Haïat G., "Assessment of dental implant stability using resonance frequency analysis and quantitative ultrasound methods", *Journal of Prosthodontic Research*, submitted.

Further work could pursue *in silico* the comparison between the QUS and RFA techniques to evaluate the sensitivity of the two medical devices to dental implant stability without experimental artefact.

Using similar signal processing to QUS analysis, the start-up WaveImplant also develops a surgical hammer instrumented with a force sensor to estimate the primary stability



Figure 5.2 – A) Schematic view of the *in vivo* ultrasonic device setup (taken from [212]). B) Picture of *in vivo* QUS measurements with the ultrasonic transducer screwed in a dental implant inserted in rabbit tibia (taken from [87]).

of femoral stem [52, 200] and acetabular cup [23, 147, 148, 199] implants in hip arthroplasty to assist orthopaedic surgeons in their decisions in the operating room. Such device has been validated *in vitro* [200], *ex vivo* [23, 148] and in cadaveric studies [52, 147]. This hammer can also be used to retrieve bone properties during osteotomies and prevent fracture during rhinoplasty for instance [90].

5.3 Key points

- Bone growth contact and structure close to the implant surface could be detailed with neutron μCT and, in combination with QUS scans, it would enable more accurate interpretation of the BII's ultrasonic response.
- More detailed mechanical properties of the BII such as bone anisotropy and viscolelasticity, the rupture of the BII and implant-bone frictional behaviour would deepen the knowledge of the biomechanical behaviour at the BII.
- The effect of mechanical loading during osseoint egration on the coin-shaped implant should also be considered *in vivo* as bone adapts its structure following Wolff's law thanks to the mechanotrans duction process, which could be introduced through a computational model.
- The bone chamber implant model is an interesting tool to evaluate the impact of implant material and surface properties as well as of medical treatments on osseoin-tegration in order to improve clinical outcomes.
- The results of this PhD work can help develop medical devices such as QUS-based devices to assess dental implant stability in clinical practice.

Synthesis and conclusion

Implant failure still often occurs and remains difficult to anticipate. This PhD work aimed at investigating the evolution of the bone properties of the bone-implant interface (BII) which determine the implant stability and thus the success of orthopaedic and dental surgeries. The originality of this PhD work lies in a multi-scale, multi-modal and multi-physics experimental approach (synthesised in Fig. 5.3) to characterise the evolution of newly formed bone tissue at the BII over healing time. An *in vivo* bone chamber implant model has been used to clearly distinguish newly formed bone from pre-existing mature cortical bone and investigate the osseointegration phenomena in standardised and reproducible conditions. Through site-matched measurements, we have characterised bone compositional, structural and mechanical properties. Various experimental methods across several disciplines including mechanics, acoustics, physics, high-resolution imaging, image analysis, material engineering, tissue biology and medicine have been implemented to assess bone tissue properties and how they are intertwined across the different scales of bone (macro-, micro- and nanoscale). These multi-disciplinary studies required the set up of multiple collaborations with French and international research groups.

After an orthopaedic or dental implantation, osseointegration occurs during the healing period, so bone grows at the surface of the implant and its amount is a marker of stability. Within the bone chamber of the coin-shaped implant inserted in rabbit, the amount of bone in direct contact with the implant surface, the bone-implant contact (BIC), increases at the micro- and macroscale as measured by histological and quantitative ultrasound (QUS) analyses between 7 and 13 weeks of healing (chapter 2). Such evolution has been validated by the finite element (FE) numerical model of the microroughness sinusoidal profile.

The observed increase in bone quantity at the BII during healing comes along with differences in local bone properties. In chapter 3, bone tissue formed during healing close to the BII is analysed with Raman spectroscopy evidencing that it is composed of less mineralised content, with fewer crosslinks within the organic collagen phase and higher remodelling rate compared to pre-existing mature bone tissue. An insight on nanostructure has also been obtained with smaller apatite crystals. These nanoscopic compositional and structural properties of less mature bone tissue have been associated with lower microscopic elastic moduli measured in site-matched locations with nanoindentation.

Bone mechanical properties are therefore strongly determined by its nanoscopic composition and structure. The structure of its constituents, for instance hydroxyapatite crystal orientation and size as well as collagen fibre arrangement, are likely to be responsible for bone strength and mechanical behaviour. Therefore, mechanical properties, which may be experimentally more accessible to characterise bone tissue and osseointegration, act as indicators of bone nanoscale composition and structure, at larger microand even macroscale.

In chapter 4, the elastic properties of newly formed bone within the bone chamber, and particularly their spatio-temporal evolution, have been further investigated with nanoindentation and micro-Brillouin scattering. The elastic properties increase with healing time, and higher elastic properties have been measured close to the implant surface and to the bone chamber edges than in its centre. Those spatio-temporal variations within bone chamber, related to the composition of newly formed bone indicating the mineralisation stage and thus bone tissue age, have allowed a better understanding of the kinetics of bone growth and demonstrate a bone spreading path starting in the bone chamber upper corners before spreading along the implant surface and towards mature bone, in agreement with bone chamber content measured with histology.

The multi-physics analyses conducted at site-matched locations have furthermore proven that all properties defining bone quantity and quality are interdependent. This interdependency is the result of mechanotransduction (chapter 5, subsubsection 5.1.3.2), the common process at the origin of every studied bone properties. Mechanotransduction may help explain the signalling pathway involved in bone cell recruitment for bone formation, leading for example to contact osteogenesis suggested by the bone spreading path found in chapter 4.

Osseointegration can be affected by several factors, one of them being the implant surface properties. The influence of implant surface roughness has been considered in chapter 2 with implants of two surface roughness levels. The experimental acoustic and histological analyses, validated by the corresponding FE model, show that higher surface roughness encourages osseointegration at a given healing time. The effect of implant roughness could be related to the effect of the mechanical environment on osseointegration since rough implants induce a higher friction coefficient at the BII reducing micromotion. Furthermore, the impact of the mechanical environment has also been noticed in this thesis in chapter 4 since the obtained bone spreading path starts in the upper corners of the bone chamber where the stress concentration is supposed to be the highest.

In future studies, mechanical loading should be introduced in the implant model to quantify its effects within the bone chamber and evaluate bone adaptation and mechanotransduction process. Moreover, to complete this PhD work and further characterise the BII and the osseointegration process, bone growth at the BII could be quantified more precisely in 3D. Biomechanical characterisation of the BII could also be deepened by considering bone's anisotropic and viscoelastic behaviour, the BII rupture and bone-implant friction.

Such characterisation developed in this thesis constitutes an important support for clinical practice as bone quantity and quality at the BII, results of the osseointegration process, are key to implant secondary stability, enabling long-term surgical success. These research studies then aim at optimising clinical implants and treatments, and represent a first basis to identify the optimal implant surface properties for example. The research techniques may also lead to new medical devices as the ultrasonic probe evaluating dental implant stability, based on the QUS technique (chapter 2).



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Collaborations

• ARTORG Center for biomedical engineering research, University of Bern, Switzerland

Collaborators: Prof. Philippe Zysset, Benjamin Voumard

<u>Role:</u> nanoindentation measurements for the study of chapter 4 during two stays (1 month and 2 weeks)

• Centre de Recherche BioMédicale, Ecole Nationale Vétérinaire d'Alfort, France

Collaborators: Dr. Thomas Lilin, Benoît Lécuelle

<u>Role</u>: hosting the whole *in vivo* phases of all studies including implantation surgery and animals' care and housing

• Department of Mechanical Science and Engineering, University of Illinois at Urbana-Champaign, USA

Collaborators: Prof. Iwona Jasiuk, Siyuan Pang

Role: Raman spectroscopy analyses on the sent samples for the study of chapter 3

• Institut de Chimie et des Matériaux Paris-Est, UMR7182, Thiais, France <u>Collaborator:</u> Dr. Mathilde Laurent-Brocq

<u>Role:</u> nanoindentation measurements for the study of chapter 3

• Laboratory of Ultrasonic Electronics, Applied Ultrasonic Research Center, Doshisha University, Kyotanabe, Japan

Collaborators: Prof. Mami Matsukawa, Hirokazu Yasui, Keita Yano

<u>Role:</u> micro-Brillouin scattering measurements on the sent samples for the study of chapter 4 and results' analysis during a one-week stay

• Service de Chirurgie Orthopédique et Traumatologique, Hôpital Henri Mondor AP-HP, Créteil, France

<u>Collaborators:</u> Dr. Charles-Henri Flouzat-Lachaniette, Dr. Arnaud Dubory, Hugues Albini Lomami, Victor Housset

<u>Role:</u> implantation surgery for all studies

Personal publications and presentations

Peer-reviewed publications

- 1. Gao X., <u>Fraulob M.</u>, and Haïat G., "Biomechanical behaviours of the bone-implant interface: a review", *Journal of the Royal Society Interface*, 16: 20190259, 2019.
- Hériveaux Y., Vayron R., <u>Fraulob M.</u>, Albini Lomami H., Lenormand C., and Haïat G., "Assessment of dental implant stability using resonance frequency analysis and quantitative ultrasound methods", *Journal of Prosthodontic Research*, accepted (in press).
- <u>Fraulob M.</u>, Pang S., Le Cann S., Vayron R., Laurent-Brocq M., Todatry S., Soares J. A.N.T., Jasiuk I., and Haïat G., "Multimodal characterization of the boneimplant interface using Raman spectroscopy and nanoindentation", *Medical Engineering and Physics*, 84:60-67, 2020.
- Fraulob M., Le Cann S., Voumard B, Yasui H., Yano K., Vayron R., Matsukawa M., Zysset P., and Haïat G., "Multimodal evaluation of the spatio-temporal variations of periprosthetic bone properties", *Journal of Biomechanical Engineering*, accepted, 2020.
- 5. <u>Fraulob M.</u>, Vayron R., Le Cann S., Lécuelle B., Hériveaux Y., Albini Lomami H., Flouzat-Lachaniette C. H., and Haïat G., "Quantitative ultrasound assessment of the influence of roughness and healing time on osseointegration", *Scientific Reports*, submitted (under revision).
- Le Cann S., Törnquist E., Silva Barreto I., <u>Fraulob M.</u>, Albini Lomami H., Verezhak M., Guizar-Sicairos M., Isaksson H., and Haïat G., "Spatio-temporal evolution of hydroxyapatite crystal thickness at the bone-implant interface", *Acta Biomaterialia*, accepted, 2020.

International congress presentations

 Fraulob M., Pang S., Le Cann S., Vayron R., Laurent-Brocq M., Jasiuk I., and Haïat G., "Biomechanical characterisation of the bone-implant interface with nanoindentation an Raman spectroscopy", 25th Congress of the European Society of Biomechanics, 7th-10th July 2019, Vienna, Austria. Oral presentation.

Seminars and other oral presentations

- Fraulob M., "Biomechanical characterisation of the bone-implant interface", 9th November 2018, PhD students day, Laboratoire Modélisation et Simulation Multi-Echelle, Université Paris-Est Marne-La-Vallée (UPEM).
- Fraulob M., "Biomechanical characterisation of the bone-implant interface", 21st May 2019, PhD students day, Ecole Doctorale Sciences Ingénierie et Environnement, Université Paris-Est Créteil (UPEC).
- 3. Fraulob M., "Biomechanical characterisation of the bone-implant interface", 18th October 2019, Acoustics and Vibration Lab, Hanyang University, Seoul.
- Fraulob M., "Biomechanical characterisation of the bone-implant interface", 23rd October 2019, Department of Electrical Engineering, Faculty of Science and Engineering, Doshisha University, Kyotanabe.

Résumé substantiel

L'os est un matériau biologique composite fortement organisé et structuré de l'échelle nanoscopique à l'échelle macroscopique. Composant de base du squelette, l'os s'adapte à son environnement grâce à un remodelage constant, induit et contrôlé par de multiples mécanismes biologiques, chimiques et mécaniques. En particulier, lors du processus de cicatrisation après une implantation orthopédique ou dentaire, par l'action de cellules osseuses (ostéoblastes, ostéoclastes et ostéocytes), du tissu osseux se forme et se remodèle autour de l'implant au cours du phénomène d'ostéointégration. En contact direct avec la surface de l'implant, ce nouveau tissu constitue une interface os-implant dont les propriétés déterminent la stabilité de l'implant à long terme, et qui est alors essentielle au succès chirurgical. En effet, les échecs relativement fréquents ne sont pas encore clairement expliqués mais ils semblent liés à un manque de stabilité menant à la perte de l'implant. La stabilité implantaire résulte de la combinaison de nombreux paramètres interconnectés dont l'étendue du contact direct formé entre la surface de l'implant et l'os néoformé, les diverses propriétés définissant la qualité du tissu à l'interface et la quantité d'os environnant. Caractériser l'ensemble des propriétés de l'interface os-implant et comprendre la complexité de leurs interactions nécessite une approche multi-échelle, multi-modale et multi-physique, primordiale pour comprendre le processus d'ostéointégration et les mécanismes menant à la stabilité des implants en vue d'anticiper et de limiter les échecs encore trop souvent observés. Une telle caractérisation est l'objectif de ce travail de thèse qui s'inscrit dans le contexte clinique actuel où les implantations sont de plus en plus courantes en chirurgie orthopédique et dentaire.

Dans ce travail, une caractérisation expérimentale de l'interface os-implant a été menée grâce à un modèle d'implant standardisé constitué d'un implant en alliage de titane (Ti6Al4V), en forme de pastille, entouré d'une couronne de téflon dont la géométrie permet la formation d'une chambre osseuse entre la surface de l'implant et l'os cortical mature préexistant. Cette chambre osseuse, initialement vide après la procédure chirurgicale, assure que l'ensemble des tissus osseux présents après la période de cicatrisation correspond à de l'os néoformé afin de mieux comprendre le processus d'ostéointégration. Dans les études menées dans cette thèse, de tels implants ont d'abord été insérés *in vivo* dans des fémurs et des tibias de lapins sous diverses conditions (temps de cicatrisation, rugosité de surface) pour comprendre les variations biologiques observées. Ensuite, des mesures *ex vivo* interdisciplinaires (mécanique, acoustique, physique, biologie, imagerie) ont été menées en combinant plusieurs techniques d'analyses par échantillon afin de caractériser et relier entre elles les variations des propriétés mécaniques, de composition et de structure de l'os néoformé à l'interface aux échelles nano-, micro- et macroscopiques, tout en limitant le nombre d'animaux considérés.

Dans une première étude (chapitre 2), l'influence du temps de cicatrisation sur l'étendue du contact os-implant est évaluée par l'analyse macroscopique de la réponse ultrasonore de l'interface os-implant et par des analyses histologiques à l'échelle microscopique. Après 13 semaines de cicatrisation, plus d'os est présent en contact avec l'implant et ce contact est plus homogène qu'après 7 semaines. Basée sur la réflexion des ondes ultrasonores qui dépend de l'écart d'impédance acoustique entre les matériaux en contact à l'interface os-implant, l'analyse non-destructive par ultrasons quantitatifs démontre une corrélation négative entre la valeur du contact os-implant et le coefficient de réflexion à l'interface. Ces résultats ont été validés par un modèle aux éléments finis simulant la surface de l'implant par un profil sinusoïdal. De plus, la comparaison des deux méthodes met en évidence la meilleure sensibilité des ultrasons quantitatifs comparés à l'histologie.

Cette première étude a mis en évidence l'évolution de la quantité de tissu au contact direct de l'implant, mais suggère également l'impact des propriétés matérielles, notamment les propriétés acoustiques de l'os à l'interface os-implant, sur les scans ultrasonores obtenus. Ceci a motivé la réalisation de nouvelles études pour caractériser la qualité du nouveau tissu osseux en comparaison avec l'os mature existant.

A l'échelle nanoscopique, la composition des phases minérales et organiques du tissu osseux nouvellement formé a été analysée par spectroscopie Raman (chapitre 3) et comparée à celle de l'os mature de référence. Le tissu nouvellement formé de la chambre osseuse présente une minéralisation moins avancée avec moins de liaisons entre les molécules de collagène de la phase organique et un taux de remodelage plus élevé que dans l'os mature préexistant. De plus, la spectroscopie Raman permet également d'accéder à la nanostructure via le taux de cristallinité qui suggère une taille de cristal réduite dans la phase minérale du tissu osseux dans la chambre osseuse. Pour observer dans quelles mesures ces différences de structure et de composition se répercutent sur les propriétés mécaniques du tissu, des mesures mécaniques localisées ont été réalisées aux mêmes endroits par nanoindentation. Des modules d'élasticité plus faibles (de valeur moyenne 12.8 ± 1.8 GPa) ont ainsi pu être associés à la composition et à la structure moins mature de l'os néoformé par rapport à l'os mature (de module d'élasticité moyen égal à 15.7 ± 2.3 GPa).

Cette approche bi-modale et bi-échelle révèle que les propriétés mécaniques du tissu osseux résultent de la composition et de la structure du tissu à des échelles plus faibles puisque, par exemple, les dimensions, l'orientation et l'arrangement des cristaux d'apatite et des fibres de collagène déterminent la résistance et le comportement mécanique du tissu aux échelles supérieures. Le comportement mécanique du tissu aux échelles microet macroscopiques, souvent plus accessible expérimentalement, peut alors être considéré comme un indicateur de la composition et de la structure nanoscopique de l'os.

Le lien entre le comportement mécanique d'un tissu et son état de minéralisation a été exploité dans une étude spatio-temporelle de l'os néoformé de la chambre osseuse (chapitre 4). Pour cette troisième étude, les évolutions spatiale (position relative dans la chambre osseuse) et temporelle (deux temps de cicatrisation) du module d'élasticité et de la vitesse de propagation des ultrasons dans le tissu nouvellement formé au contact de l'implant ont été analysées conjointement par nanoindentation et diffusion micro-Brillouin. La vitesse de propagation des ondes dans le tissu osseux dépend à la fois de l'élasticité et de la densité massique du milieu, ce qui a motivé la combinaison des résultats de mesures de diffusion micro-Brillouin et de nanoindentation pour extraire les variations relatives de densité massique de l'os à l'échelle du tissu. Cette étude a révélé que les propriétés élastiques augmentent entre 7 et 13 semaines de cicatrisation tout en restant plus faibles que dans l'os mature. Par exemple, l'écart de densité massique entre l'os néoformé et l'os mature est de 14.3 % après 7 semaines de cicatrisation puis de seulement 6.1~% après 13 semaines. Les variations relatives du module d'élasticité, de la vitesse des ondes ultrasonores et de la densité massique entre les différentes régions de la chambre osseuse permettent de déduire des valeurs plus élevées proches de la surface de l'implant (de 12.3 % pour la densité massique, par exemple, après 7 semaines de cicatrisation) et des surfaces latérales (de 16.8 %) que dans les régions centrales. En lien avec les observations du chapitre 3 où les faibles propriétés élastiques du tissu dans la chambre osseuse ont été reliées à une minéralisation moins avancée et donc à un tissu plus jeune, ces variations spatio-temporelles suggèrent un profil de propagation de l'os permettant d'appréhender sa cinétique de croissance. L'os se formerait d'abord dans les coins de la chambre osseuse avant de se propager le long de ses bords vers l'os mature et le long de la surface de l'implant. Cette évolution est en accord avec la répartition du contenu de la chambre osseuse observée par histologie.

Effectuant des analyses multiples sur les mêmes échantillons, cette thèse prouve l'interdépendance de toutes les propriétés définissant la quantité et la qualité de l'os à l'interface.

Par ailleurs, le phénomène d'ostéointégration peut être modulé par différents facteurs, comme la rugosité de surface de l'implant. Cet aspect a été étudié dans le chapitre 2 où deux groupes d'implants différenciés par leurs rugosités ont été insérés. Après un temps de cicatrisation donné (7 ou 13 semaines), une surface d'implant plus rugueuse favorise l'ostéointégration comme observé au travers d'un contact os-implant plus élevé mesuré par des ultrasons quantitatifs et les analyses histologiques et validé par la simulation numérique. Cette influence de la rugosité de surface de l'implant suggère un impact de l'environnement mécanique sur le processus d'ostéointégration. En effet, les implants rugueux génèrent un coefficient de frottement plus élevé à l'interface os-implant limitant les micromouvements, qui peuvent être responsables d'un échec d'intégration. Quantifier l'effet de la friction entre la surface de l'implant et le tissu osseux sur la stabilité de l'implant fait partie des pistes d'étude pour le futur.

Bien que le modèle *in vivo* n'inclut pas de stimulation mécanique, l'environnement mécanique imposé par la géométrie du modèle semble tout de même influencer la croissance osseuse. D'après les résultats du chapitre 4, la propagation de l'os serait initiée aux coins de la chambre osseuse, où la concentration des contraintes est susceptible d'être la plus forte, avant de progresser en direction de l'os mature. Cette évolution pourrait être expliquée par le processus de mécanotransduction, qui gère la formation osseuse en réponse aux stimulations mécaniques environnantes, et confère ainsi au nouveau tissu toutes ses propriétés étudiées au cours de cette thèse. Appliquer un chargement mécanique au modèle *in vivo* pourrait alors compléter l'étude des phénomènes d'ostéointégration menant à la stabilité osseuse. De plus, la caractérisation de la quantité de tissu osseux (chapitre 2) à l'interface os-implant pourrait être encore précisée en quantifiant sa répartition 3D autour de la surface de l'implant par des techniques d'imagerie à haute résolution. Lors des analyses de nanoindentation (chapitres 3 et 4), le tissu osseux a été considéré comme un matériau isotrope et élastique. En prenant en compte son anisotropie et sa viscoélasticité, une meilleure description du comportement mécanique de l'interface pourrait être obtenue. Enfin, l'approche multi-échelle, multi-modale et multi-physique pourrait être étendue à l'étude de la rupture de l'interface sous l'effet d'un chargement mécanique *ex vivo*. Comprendre ainsi les propriétés osseuses et les mécanismes entraînant la rupture de l'interface os-implant est aussi important car il s'agit d'une cause fréquente de perte d'implants.

L'approche multi-échelle, multi-modale et multi-physique de cette thèse a permis de mieux comprendre les phénomènes d'ostéointégration en évaluant la quantité d'os néoformé à l'interface os-implant ainsi que sa composition et sa structure associées à ses propriétés mécaniques. De tels travaux de recherche sur l'évolution de l'interface osimplant et sa stabilité en rapport avec différents facteurs (comme la rugosité de la surface de l'implant, par exemple) sont une aide précieuse pour optimiser la conception des implants et le traitement des patients. En particulier, les ultrasons quantitatifs utilisés dans l'étude du chapitre 2 ont permis le développement d'un nouveau dispositif de mesures de la stabilité d'implants dentaires, illustrant le potentiel de ces études et des méthodes utilisées pour le développement de dispositifs médicaux destinés aux chirurgiens.

Résumé

Titre: Caractérisation biomécanique de l'interface os-implant.

Les interventions chirurgicales impliquant des implants orthopédiques ou dentaires échouent encore souvent pour des raisons liées à un manque de stabilité. Pendant la phase de cicatrisation suivant la chirurgie, du tissu osseux se forme et se remodèle directement à la surface de l'implant grâce au processus d'ostéointégration créant l'interface os-implant. La quantité et les propriétés biomécaniques de l'os néoformé entourant l'implant déterminent sa stabilité à long terme et donc le succès de la chirurgie. Afin d'appréhender la complexité de l'interface organisée hiérarchiquement de l'échelle nano- à macroscopique avec des propriétés évolutives témoignant de son caractère vivant, ce travail de thèse a mis en place une approche expérimentale multi-échelle, multi-modale et multi-physique. Un modèle d'implant *in vivo* avec une chambre osseuse distinguant clairement l'os néoformé de l'os cortical mature préexistant a été utilisé pour se placer dans des conditions standardisées et contrôlées.

Dans une première étude portant sur la quantité d'os à l'interface, le contact osimplant augmente avec le temps de cicatrisation et la rugosité de surface de l'implant, comme en témoignent les mesures par histologie et ultrasons quantitatifs à l'échelle microet macroscopique. Cette évolution a aussi été validée avec un modèle numérique aux éléments finis simulant la surface microscopique de l'implant par un profil sinusoïdal.

L'augmentation observée de la quantité d'os à l'interface os-implant pendant la cicatrisation s'accompagne de différences de composition et structure osseuses. Dans une seconde étude, des mesures par spectroscopie Raman ont mis en évidence des cristaux d'apatite de la phase minérale plus petits et des composants moins minéralisés dans l'os néoformé comparé à l'os mature, avec moins de liaisons entre les molécules de collagène de la phase organique et un taux de remodelage plus élevé. Grâce à une analyse conjointe avec des mesures de nanoindentation, ces différences de composition et structure nanoscopiques de l'os périprothétique ont été reliées à de faibles modules élastiques microscopiques.

Puisque les propriétés élastiques augmentent avec la minéralisation et l'âge du tissu, afin d'étudier plus précisément la cinétique de la croissance osseuse à l'interface os-implant, une troisième étude s'est penchée sur l'évolution spatio-temporelle des propriétés élastiques microscopiques au sein de la chambre osseuse en combinant nanoindentation et diffusion micro-Brillouin. Les résultats suggèrent que l'os commence à se former dans les régions de la chambre osseuse où les contraintes mécaniques sont susceptibles d'être les plus élevées, avant de se développer le long de la surface de l'implant et en direction de l'os mature. Ce profil de propagation osseuse, en accord avec les mesures histologiques du contenu de la chambre osseuse, est cohérent avec le phénomène d'ostéogenèse de contact.

Ces analyses multi-physiques combinées sur les mêmes échantillons démontrent que toutes les propriétés caractérisant la quantité et la qualité osseuse sont interdépendantes à travers les différentes échelles de l'os. De tels travaux de recherche, étudiant l'évolution simultanée des propriétés de l'os périprothétique, sont essentiels pour mieux comprendre le phénomène d'ostéointégration et la stabilité implantaire. Ces études pourraient aider à l'amélioration du succès chirurgical à long terme suite à la pose d'implants.

Mots-clés: interface os-implant, ostéointégration, modèle d'implant *in vivo*, caractérisation multi-échelle et multi-modale combinée sur même échantillon, composition et structure de l'os, propriétés élastiques de l'os

Abstract

Title: Biomechanical characterisation of the bone-implant interface.

Implant failures still often occur in orthopaedic and dental surgeries and are related to a lack of biomechanical stability. During the healing period following surgery, bone tissue forms and remodels in direct contact with the implant surface, creating a bone-implant interface (BII) thanks to the osseointegration process. The amount and properties of bone tissue surrounding the implant determine the long-term implant stability and thus its surgical success. To tackle the complexity of this BII hierarchically organised from the nano- to the macroscale and its living nature inducing evolving properties, this PhD work has implemented a multi-scale, multi-modal and multi-physics experimental approach. To work in standardised and controlled conditions, an *in vivo* coin-shaped implant model was used, including a bone chamber to clearly distinguish between newly formed bone and pre-existing mature cortical bone.

Focusing first on the amount of bone at the BII, the bone-implant contact increases with healing time and implant surface roughness at the micro- and macroscale, as measured by histological and quantitative ultrasound analyses and validated by a finite element numerical model simulating the microscopic implant surface with a sinusoidal profile.

The observed increase in bone quantity at the BII during healing comes along with differences in bone composition and structure. Raman spectroscopy has evidenced smaller apatite crystals and less mineralised content in newly formed bone compared to mature bone, with fewer crosslinks within the organic collagen phase and higher remodelling rate. The different nanoscopic composition and structure of periprosthetic bone induce lower microscopic elastic moduli, measured in site-matched locations with nanoindentation.

As elastic properties increase with mineralisation and tissue ageing, the spatio-temporal evolution of microscopic elastic properties within the bone chamber has also been evaluated with nanoindentation and micro-Brillouin scattering to investigate the kinetics of bone growth at the BII. Results suggest that bone starts to form in bone chamber's specific regions, where stresses are likely to be the highest, before spreading along the implant surface and towards mature bone. Such bone spreading path, in agreement with the bone chamber content measured by histology, is consistent with contact osteogenesis phenomena.

The multi-physics analyses performed at site-matched locations have proven that all properties defining bone quantity and quality are interdependent across bone scales. Such research studies are essential to better understand osseointegration phenomena and implant stability, by investigating how periprosthetic bone properties evolve simultaneously. The present studies are likely to provide support to improve long-term surgical success of clinical implants.

Keywords: bone-implant interface, osseointegration, *in vivo* implant model, multi-scale and multi-modal site-matched characterisation, bone composition and structure, bone elastic properties

