Applications of synchrotron X-ray imaging in bone research

Murielle Salomé
Outline

1. Bone

2. Relevant X-ray Imaging techniques

3. Bone micro-structure
   - 3D bone micro-architecture in (µCT)
   - 3D mineral bone density (quantitative µCT)
   - In vivo µCT

4. Bone mineralisation
   - Apatite maturation (µXANES)
   - Mineral crystallites, collagen fibers (µSAXS)

5. Biomechanics
   - Bone under compressive stress (µCT)

6. Metals accumulation in bone
   - Localisation of La in bone (µXRF)

7. Orthopedics and biomaterials
   - DEI imaging of bone implants
   - Scaffold osseo-integration (µCT, µXRD)

8. FTIR micro-spectroscopy

9. Perspectives
1. Bone
Bone

- Functions
  - Support and protection of the soft tissues and organs
  - Body movement
  - Mineral storage (Ca, P...) and homeostasis
  - Hematopoiesis

- Cortical bone
  - Hard outer layer of bone
  - Closely packed osteons

- Cancellous bone
  - Less dense than cortical bone
  - Honeycomb-like 3D structure
  - Network of plate and bar shaped trabeculae

X-ray μCT image of human trabecular bone


X-ray Imaging techniques at ESRF, 5-6 February 2007
Bone tissue – A composite nano-material

- Composite nano-material
  - Collagen fibrils reinforced with mineral platelets
  - Mechanical competences (stiffness and toughness)

- Organic matrix
  - Mainly type I collagen forming a triple helical structure arranged in fibrils (Ø100 nm)
  - Template for mineral phase
  - Some non-collagenous proteins

- Mineral phase
  - Poorly crystalline carbonated apatite
  - Many ion substitutions
  - 2-5 nm thick apatite crystallites
  - Crystals aligned along fibril axis

- Bone formation and remodeling
  - Bone continuously resorbed and replaced by new bone
  - Inhomogeneous bone mineralisation: bone regions with different mineral content depending on tissue age
2. Relevant X-ray Imaging techniques
SR X-ray imaging and bone research

Bone micro-structure

- Alteration with age, pathology (osteoporosis, osteoarthritis ...)
- Effects of therapy

High resolution imaging in 2D and 3D (µm / nm)

Spatially resolved information

Metals accumulation in bone

Pb, Al, Sr, La ...

Detection of trace elements with high sensitivity (ppm)

Bone mineralisation

- Identification of mineral phases
- Apatite maturation
- Crystallinity
- Alteration with age, pathology (rickets, osteogenesis imperfecta, osteopetrosis ...)

Chemical speciation
Molecular information
Crystallographic properties

Bone mechanical properties

- Bone micro- / nano-structure behavior under strain

Orthopedics and Biomaterials

- Micro-structure
- Bone integration

X-ray Imaging techniques at ESRF, 5-6 February 2007
### SR X-ray imaging and bone research

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In blue: examples developed hereafter

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3. Bone micro-structure
Quantification of the 3D micro-architecture of trabecular bone using x-ray microtomography

- 3D Non-destructive imaging technique

- High spatial resolution, tunable from 10 µm to <1 µm

- Access to 3D histomorphometric parameters
  - Bone volume fraction (BV/TV)
  - Bone trabecular thickness (Tb.Th)
  - Trabecular spacing (Tb.Sp)
  - Connectivity parameters
  - Anisotropy
  - ...

- Quantitative reconstruction of linear attenuation coefficient in monochromatic beam
  - 3D Bone mineral density

- Dedicated sample environments for In situ studies
  - Bone structure deformation and cracks propagation under compressive stress

- In vivo studies
Evolution of bone trabecular structure with age

- Decrease of trabecular bone volume with age
- Increase of mean trabecular spacing
- Decrease in connectivity
- No significant thinning of the trabeculae

10 vertebral spongiosa samples from women with different ages.

F. Peyrin et al., Cellular and Molecular Biology 46(6), 1089-1102, 2000.
Quantitative measurement of the degree of mineralization in bone

Monochromatic beam -> Reconstruction of linear attenuation coefficient $\mu$

Quantification of 3D mineral content provided a suitable calibration

S. Nuzzo, ESRF ID19
F. Peyrin, CREATIS INSA Lyon & ESRF ID19

S. Nuzzo et al., *Journal of Bone Mineral Research* 17(8), 1372-1382, 2002.
Quantitative μCT: Calibration procedure

Phantoms: homogeneous water solutions of various known $K_2HPO_4$ concentrations

Calibration curve
Reconstructed linear attenuation coefficient $\rightarrow$ Bone mineral concentration


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Comparison with micro-radiography: qualitative results

Human iliac crest biopsies

μ-radiography

SR μCT: virtual 100μm thick slice

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Comparison with micro-radiography: quantitative results

Degree of mineralization of bone (DMB) distribution measured by the two techniques

Repeated on 4 biopsies

Mean difference = 4.7 %

Slightly higher in trabecular than in cortical bone

Effects of a sequential Etidronate therapy in post-menopausal osteoporosis

- Iliac crest biopsies from 14 patients, before (baseline), after 1 year and after 2 years of treatment.
- Sequential 13 week therapy repeated 4 times:
  - Etidronate (Procter and Gamble) 400 mg/day for 2 weeks
  - Ca (Sandoz) 1g/day for 11 weeks
- Measurement of the 3D microarchitecture parameters and bone mineralization

![3D µCT image of an iliac crest biopsy](image)

~7mm

3D µCT image of an iliac crest biopsy

- Baseline → After 2 years:
  - HA concentration 12% (cortical)
  - HA concentration 8% (trabecular)

In vivo imaging of bone micro-architecture in mice

ID19 µCT setup

In vivo imaging of bone micro-architecture in mice

Transverse slice μCT image in the femur of a B6 mouse

voxel size : 10.13 µm
5 mn scan time

2.3 Gy
SNR : 8.3 (18.4dB)

7.5 Gy
11.2 (24.2dB)

14.5 Gy
24.3 (27.9dB)

S. Bayat, ESRF
F. Peyrin, CREATIS INSA Lyon & ESRF ID19


X-ray Imaging techniques at ESRF, 5-6 February 2007
4. Bone mineralisation
Bone mineral and possible substitutions

- Apatites

\[
\text{Me}_{10} (XO_4)_6 Y_2
\]

<table>
<thead>
<tr>
<th>Stoichiometric hydroxyapatite</th>
<th>(\text{Ca}^{2+})</th>
<th>(\text{PO}_4^{3-})</th>
<th>(\text{OH}^-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main possible substitutions</td>
<td>(\text{Sr}^{2+}, \text{Pb}^{2+}, \text{Cd}^{2+}, \text{Mn}^{2+}, \text{Na}^+, \text{La}^{3+}, \text{Mg}^{2+} \ldots)</td>
<td>(\text{HPO}_4^{2-}, \text{CO}_3^{2-}, \text{SO}_4^{2-}, \text{SiO}_4^{3-} \ldots)</td>
<td>(\text{F}^-, \text{Cl}^-, \text{Br}^-, \text{CO}_3^{2-}, \text{O}_2^{2-} \ldots) vacancy:</td>
</tr>
</tbody>
</table>

- Many forms of non-stoichiometric apatites
- Bone mineral: carbonated apatite \(\text{Ca}_{8.3} \square_{1.7} (\text{PO}_4)_{4.3} (\text{HPO}_4, \text{CO}_3)_{1.7} (\text{OH})_{0.3} \square_{1.7}\)
- Adsorption and exchanges at the surface of the crystals
- Synthetic calcium phosphates are good models of bone mineral and offer similar physico-chemical properties.
Bone apatite maturation state using P and Ca K-edge μ-XANES

- Maturation of bone mineral and possible precursors of bone apatites are not well known yet.

- Better knowledge of bone mineralisation helpful for treatment of diseases like rickets, osteogenesis imperfecta or osteopetrosis.

- Local assessment of bone apatite maturation state using μ-XANES
XANES spectra of synthetic calcium phosphates at the Ca K-edge

- HA Ca$_{10}$(PO$_4$)$_6$(OH)$_2$
- β-TCP Ca$_3$(PO$_4$)$_2$
- α-TCP Ca$_3$(PO$_4$)$_2$
- ACP Ca$_3$(PO$_4$)$_2$, n H$_2$O
- OCP Ca$_8$H$_2$(PO$_4$)$_6$, 5 H$_2$O
- DCPA CaHPO$_4$
- DCPD CaHPO$_4$, 2 H$_2$O

Follow-up of the maturation of poorly crystalline apatites using XANES at Ca K-edge

D. Eichert, M. Salomé, ESRF ID21
C. Rey, CIRIMAT-ENSIACET, Toulouse

X-ray Imaging techniques at ESRF, 5-6 February 2007
Follow-up of the maturation of poorly crystalline apatites using XANES at Ca K-edge


D. Eichert, M. Salomé, ESRF ID21
C. Rey, CIRIMAT-ENSIACET, Toulouse

X-ray Imaging techniques at ESRF, 5-6 February 2007
Follow-up of the maturation of poorly crystalline apatites using XANES at Ca K-edge

Correlation between maturation and modification of the XANES spectra
Technique sensitive to the modification of calcium and phosphorus environments


D. Eichert, M. Salomé, ESRF ID21
C. Rey, CIRIMAT-ENSIACET, Toulouse

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X-ray diffraction and bone

- X-ray diffraction
  - Crystal lattice in mineral crystallites
  - Identification of mineral phases

- Small Angle X-ray Scattering (SAXS):
  - Orientation of the collagen fibers
  - Distribution of mineral crystallites
    - Shape
    - Thickness of the smallest crystallite dimension
    - Orientation, degree of alignment of the particles

- μ-SAXS mapping with micro-beams
  - 2D maps of bone crystallites properties

Please refer to:
P. Fratzl et al., Connective Tissue Research 34(4), 247-254, 1996.

X-ray Imaging techniques at ESRF, 5-6 February 2007
Micro-focus SAXS: 2D mapping of bone crystallites properties

Raster scan $\mu$-SAXS mapping

Crystallite shape $\eta$

Crystallite thickness $T$

Transmission radiograph

Osteons

Modern human bone, 200 $\mu$m thick section

Applications in archaeological bone studies

5. Biomechanics
In situ microtomography study of human bone under compressive stress

- Development of a micro-compression device compatible with µCT setup
- Analysis of microcracks origination, growth and propagation in 3D
- Study of bone biomechanical properties under dynamic strain

In situ µCT study of human bone under compressive stress

1. Excision

Human metacarpus cortical bone

2. Data acquisition

Rotation

Strain

X-Ray beam

3. 3D Reconstruction and segmentation


0 Mpa 20 Mpa 60 Mpa 80 Mpa

P. Bleuet, ESRF ID22
J.P. Roux, G. Boivin, INSERM 403, Lyon

X-ray Imaging techniques at ESRF, 5-6 February 2007
Micro-compression system for bone samples

- Compatibility with µCT setup
- High accuracy rotation controlled by precision bearings
- Fatigue testing within 0 to 1% strain
  - Sinusoidal motion with 5-10 µm amplitude using a piezoelectric actuator
- Measurement of (F,D): 2 motion gauges + 1 force gauge

**Sample cell**

*Ex-vivo physiological conditions*

\[ T=37\,^\circ C \quad H=100\% \]

Design by Y. Dabin, ESRF Technical Services

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P. Bleuet, ESRF ID22
J.P. Roux, G. Boivin, INSERM 403, Lyon
In situ microtomography study of human bone under compressive stress

Biomechanical parameters

\[
E = 3.5 \text{ Gpa} \\
\nu = 0.33
\]

P. Bleuet, ESRF ID22
J. P. Roux, G. Boivin, INSERM 403, Lyon
6. Metals accumulation in bone
 Metals accumulation in bone

- Easy storage and exchange of metals in bone
- May induce mineralisation defects
- Effects often dose-dependent

- Metals issued from
  - Food
  - Therapy (La, Sr, …)
  - Environment contamination (Pb, Cr, …)
  - Release from metallic implants (Ti, Co, Fe, Cr, …)

- Assessment of trace metals accumulation sites and potential toxicity

- μ-fluorescence (μXRF) mapping
  - High sensitivity (a few ppm)
  - Chemical speciation in μ-XANES
µ-XRF mapping of La in bone of chronic renal failure rats

- Accumulation of phosphate in Chronic Renal Failure patients (CRF)

- To decrease serum phosphate level: use of phosphate binders
  - Aluminum hydroxide Al(OH3)
  - Calcium carbonate CaCO3
  - Side effects: Al mineralization defects, Ca extra-osseous calcification

- Alternative: Lanthanum carbonate

- Check possible effect on bone mineralization, possible accumulation in bone tissue, tissue localization is important
µ-XRF mapping of La in bone of chronic renal failure rats

Goldner stained adjacent slice (500x)
Osteoid tissue: red, Mineralised bone: blue

10μm thick trabecular bone section.
E=6.3 keV, 36 μm x 32 μm, 1 μm step size, 5s/pixel

Chronic renal failure rat model (5/6 nephrectomy) loaded with daily oral doses of lanthanum carbonate (2g/kg/day) over a 12 week period.

Localisation of La in different types of renal osteodistrophy

**Animal with normal bone histology**
Bulk La concentration 2.68 µg/g

**Animal showing hyperparathyroid bone disease**
Bulk La concentration 2.71 µg/g

**Animal with a mineralization defect**
Bulk La concentration 2.81 µg/g

**Conclusions:**
- La generally at the outer edge of mineralized bone both at active and quiescent sites
- No obvious relationship between osteoid amount or type of renal osteodistrophy
- After a 2-4 weeks washout period: same localization of La, no more mineralization defect

7. Orthopedics and Biomaterials
Diffraction Enhanced Imaging (DEI) of bone

Diffraction Enhanced Imaging of joint cartilage

Identification of the quality of bone ingrowth into HA layer of implant

Human hip head
DEI, ESRF ID17
30keV, 50% rocking curve

Histological section
DEI, ESRF ID17
50keV, 15% rocking curve

Titanium implant integration in sheep, 9 week post-surgery


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Kinetics of bone deposition into porous calcium phosphate scaffolds

Highly porous hydroxyapatite scaffolds seeded with bone marrow stromal cells, implanted in immunodeficient mice.

8 weeks 16 weeks 24 weeks implantation


V.S. Komlev, Universita Politecnica delle Marche and CNISM, Ancona, Italy & Institute for Physical Chemistry of Ceramics, Russian Academy of Sciences, Moscow, Russia

F. Peyrin, CREATIS INSA Lyon & ESRF ID19

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Kinetics of bone deposition into porous calcium phosphate scaffolds

Histogram of absorption coefficient in the 3D µCT image

Scaffold before implantation

Scaffold + newly formed bone after:
- □ 8 weeks,
- ● 16 weeks,
- Δ 24 weeks implantation


V. S. Komlev, Universita Politecnica delle Marche and CNISM, Ancona, Italy & Institute for Physical Chemistry of Ceramics, Russian Academy of Sciences, Moscow, Russia

F. Peyrin, CREATIS INSA Lyon & ESRF ID19

X-ray Imaging techniques at ESRF, 5-6 February 2007
Osseo-integration of silicon-stabilized tricalcium phosphate bioceramics

- Silicon-stabilized tricalcium phosphate bioceramics (Millenium Biologix Corp., Canada) 67% Si-TCP, 33% HA/β-TCP, seeded with bone marrow stromal cells.
- Implanted in immuno-deficient mice for 8 or 24 weeks

Decrease of scaffold thickness, increase of bone thickness

μ-CT images (ESRF ID19) of scaffold before and after 24 weeks implantation


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Osseo-integration of silicon-stabilized tricalcium phosphate bioceramics

- Mapping of the variation of the scattered intensity of different phases:
  - HA in newly formed bone
  - HA in scaffold
  - TCP in scaffold

μ-diffraction images (ESRF ID13) of scaffold after 8 weeks implantation in immuno-deficient mouse


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8. FTIR micro-spectroscopy
A brief reminder about FTIR spectroscopy

Absorbance = -log(I/I₀)

Wave-numbers (cm⁻¹)

Molecular and structural information

Excited level
Fundamental level

Stretching
Symmetrical
Asymmetrical

Angle deformation
In the plane
Rocking
Scissoring
Out of plane
Wagging
Twisting

Courtesy of M. Cotte, ESRF ID21

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Fourier-Transform Infra-Red Spectroscopy of bone

- Physico-chemical characterization of bone apatite
- Nature of the mineral phases
- Determination of relative amount of mineral / matrix
- Mineral crystallinity and maturity
- Nature of substituents and sites in apatite structure
- Environment of the ions
- Matrix composition
Bone FTIR spectrum

\[ \text{Amide I, Amide II, Amide III, } \nu_1, \nu_3 \text{ PO}_4^{3-}, \nu_2 \text{ CO}_3^{2-}, \nu_4 \text{ PO}_4^{3-} \]

FTIR spectrum of trochanter bone

D. Eichert, ESRF ID21

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Main FTIR absorbance bands in bone

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<th>Wavenumbers</th>
<th>Information</th>
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<td>ν₁,ν₃ PO₄³⁻ bands</td>
<td>900 – 1200 cm⁻¹</td>
<td>Crystal maturity</td>
</tr>
<tr>
<td>Amide I bands</td>
<td>1590 - 1720 cm⁻¹</td>
<td>Organic phase</td>
</tr>
<tr>
<td>ν₄ PO₄³⁻ bands</td>
<td>500 – 650 cm⁻¹</td>
<td>Crystallinity</td>
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<tr>
<td>ν₂ CO₃²⁻ bands</td>
<td>878 cm⁻¹</td>
<td>Type A carbonate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Substitution OH⁻ site</td>
</tr>
<tr>
<td>ν₂ CO₃²⁻ bands</td>
<td>871 cm⁻¹</td>
<td>Type B carbonate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Substitution PO₄³⁻ site</td>
</tr>
<tr>
<td>ν₂ CO₃²⁻ bands</td>
<td>866 cm⁻¹</td>
<td>Labile surface carbonate</td>
</tr>
</tbody>
</table>

Please refer to:
H.M. Kim et al., Calcified Tissue International 59, 58-63, 1996.
FTIR Micro-spectroscopy mapping of biomaterial integration

Visible light image

Biomaterial
\(v_L\) OH band of HA
630 cm\(^{-1}\)

Bone
\(v_2\) CO\(_3^{2-}\) bands
878 cm\(^{-1}\), 871 cm\(^{-1}\)

Organic matrix

HA cement implanted in rat tibia for 3 weeks


ID21 FTIR microscope
Aperture: 10x10 \(\mu\)m; Step size: 3 \(\mu\)m, 128 scans

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9. Perspectives
Future – Pushing further the spatial resolution …

- Development of imaging techniques at nano-scale
  - Nano-tomography

- Development of nano-probes
  - Nano-fluorescence
  - Nano-spectroscopy
  - Nano-diffraction

- Development of dedicated sample environments for in situ experiments
  - Under stress/strain

- Multi-modal approach combining techniques