VIBRATIONAL SPECTROSCOPIC METHODS: FTIR, FTIRI & RAMAN.

E. P. Paschalis
HOW DOES VIBRATIONAL SPECTROSCOPY RECOGNIZE BONE?

FRACTURES vs COMMON WISDOM

SHOWCASE THE INFORMATION THAT VIBRATIONAL SPECTROSCOPY CAN PROVIDE IN RELATION TO BONE

HOW DOES THIS INFORMATION AGREE/DISAGREE WITH OSTEOPOROSIS MANTRA OF BMD AND TURNOVER

HOW CAN IT HELP US IMPROVE EXISTING DEFINITIONS

WHAT IS ITS PLACE IN THE BIGGER PICTURE?
HOW DOES VIBRATIONAL SPECTROSCOPY RECOGNIZE BONE?

Bone = Collagen/Mineral Nano-Composite

Mineral (~70%) Organic Matrix (~20%)
# FRACTURES vs COMMON WISDOM

**Contribution of Post-Treatment BMD Increases to Vertebral Fracture Risk Reduction over 3 Years**

<table>
<thead>
<tr>
<th>Author</th>
<th>Treatment</th>
<th>Analysis Method*</th>
<th>% Fx Reduction Explained by BMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cummings</td>
<td>Alendronate (FIT)</td>
<td>IPD</td>
<td>16%</td>
</tr>
<tr>
<td>Sarkar</td>
<td>Raloxifene (MORE)</td>
<td>IPD</td>
<td>4%</td>
</tr>
<tr>
<td>Watts</td>
<td>Risedronate (VERT and HIP)</td>
<td>IPD</td>
<td>18%</td>
</tr>
</tbody>
</table>

* Analysis of individual patient data (IPD)

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Sarkar et al, JBMR 2002  
Watts et al, J Clin Densitometry 2004
Cortical and cancellous bone were independently evaluated in iliac crest biopsies from 54 women (32 with fractures, 22 without) who had significantly different spine but not hip BMDs.

The ONLY “universal” correlation between fracture and bone properties was collagen cross-link ratio.
FRACTURES vs COMMON WISDOM

Bone strength & quality

Structural Properties:
- Geometry
- Size
- Shape
- Microarchitecture
  - Trabecular
  - Cortical / porosity

Material Properties:
- Mineral
  - Mineral : Matrix
  - Crystallite maturity/size
- Collagen
  - Type
  - Cross-links
- Microdamage

Bone Turnover

P. Chem
Lathyrogens

F
BPs
Sr
## Features of Bone Tissue That Reduce Strength and Fracture Toughness

<table>
<thead>
<tr>
<th>Feature</th>
<th>Modulus</th>
<th>Ultimate strain</th>
<th>Ultimate stress</th>
<th>Toughness</th>
<th>Cause/Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poorly mineralized bone</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>Osteomalacia</td>
</tr>
<tr>
<td>Hypermineralized bone</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>Reduced turnover</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Increased mean tissue age</td>
</tr>
<tr>
<td>Increased crystallinity:</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>Reduced turnover</td>
</tr>
<tr>
<td>morphology of apatite crystal</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>Increased mean tissue age</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Fluoride accumulation</td>
</tr>
<tr>
<td>Denaturation of collagen molecule</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>Unclear</td>
</tr>
<tr>
<td>Debonding of mineral collagen</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
<td>Fluoride accumulation</td>
</tr>
</tbody>
</table>

Biological Apatites

*Derived from Greek word meaning "to deceive"*

- Crystallite chemical composition is changing
- Maturity
  - *phase purity, stoichiometry, solubility*
- Concomitantly/as a result, crystallite size changes
- Crystallinity

white -- Hydrogen, Red -- Oxygen, purple - Phosphorous, Blue - calcium

http://www.ecs.syr.edu/faculty/schwarz/project1.htm
Mineral Crystallinity matters

Theoretical Considerations

- Study of biological composites at the nano level reveals that optimal mechanical properties are associated with a definite plateau in mineral crystallinity. Crystallite sizes larger than the plateau value result in inferior mechanical performance.

Animal model data

- Plenty of animal models with altered mineral crystallinity & bone mechanical properties

- Fluoride treatment resulted in increased BMD & bone fragility. Upon analysis, fluoride-treated bone had higher crystallinity compared to normal.

Human Clinical data

- X-ray powder diffraction
- Fourier transform infrared
- Small angle x-ray scattering
- Fourier transform infrared imaging

Compromised Mechanical Properties

- Ovariectomized animals: larger
- Osteogenesis Imperfecta: smaller
- Hyp mice: smaller
- Osteopontin KO mice: larger
- Osteocalcin KO mice: smaller

COLLAGEN

- SCAFFOLD
- MECHANICAL PROPERTIES
- INITIATION OF MINERALIZATION?
- Cross-links are tissue- rather than collagen type-specific
How Do Collagen Molecules Form a Functional/Stable Fibril?

Covalent Intermolecular Cross-linking
**Importance of collagen cross-links**

- **Theoretical Considerations**
  - The matrix is proposed to play an important role in alleviating impact damage to mineral crystallites, and to matrix/mineral interfaces.

- **Animal model data**
  - Animal models with altered collagen cross-links & bone mechanical properties

- **Human Clinical data**
  - Osteogenesis Imperfecta
  - Lathyrism
  - Vitamin B6 deficiency

**Animal Models**
- Osteogenesis Imperfecta: mouse
- Lathyrism: rat
- Vitamin B6 deficiency: chicken
Bone mineral crystallites are capable of sustained growth when bathed in aqueous media, even in the absence of cellular activity.

Rey C., Glimcher M. et al; Cells & Mat. 5, 345-356, 1995

Post-translational modifications may take place in biological fluids, even in the absence of cellular and/or enzymatic activity.

BONE STRENGTH

CRYSTAL SIZE

NORMAL RANGE

COLLAGEN x-links

• OI
• Hyp
• Osteocalcin KO

• OI
• Lathyrism
• Vitamin B6-deficiency

• Fluoride
• Ovx
• Osteopontin KO
INFRARED SPECTROSCOPY

BASIS

TWISTING - BENDING - ROTATION - VIBRATION

ATOMS IN A MOLECULE

ABSORPTION AT PARTICULAR WAVELENGTHS

CHARACTERISTIC OF FUNCTIONAL GROUPS

OVERALL CONFIGURATION OF ATOMS

SUBTLE INTERACTIONS WITH SURROUNDING ATOMS OF MOLECULE

STAMP OF INDIVIDUALITY ON THE SPECTRUM OF EACH COMPOUND
TYPICAL FTIRM SPECTRUM OF BONE

- CO$_3^-$
- PO$_4^{2-}$
- AMIDE III
- AMIDE II
- AMIDE I
**Fourier Transform Infrared Spectroscopy**

**Major Bone Relevant Outcomes**

**MINERAL DENSITY** (mineral : matrix)
May or may not agree with BMD or BMDD as it expresses mineral with respect to volume AND matrix

**CARBONATE CONTENT & TYPE**
Important for crystal lattice considerations and solubility

**MINERAL MATURITY**
Direct measure is chemistry. Crystallinity may be extrapolated, but caution should be exercised when interpreting results

**COLLAGEN CROSS-LINKS**
UNIQUE capability to describe them as a function of tissue age AND surface metabolic activity
RELEVANCE OF 1020 / 1030 RATIO

CTI, 59:480-487, 1996
Statistically significant correlation of the FT-IRM parameters with the SAXS parameter of crystal thickness.

Open and full symbols represent data from cortical and cancellous bone, respectively.
Table 1. Global averages of FTIR parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal (n = 10)</th>
<th>SD</th>
<th>High Turnover (n = 6)</th>
<th>SD</th>
<th>Low Turnover (n = 6)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral: matrix</td>
<td>3.95</td>
<td>0.52</td>
<td>2.91</td>
<td>0.35**</td>
<td>3.67</td>
<td>0.27</td>
</tr>
<tr>
<td>Carbonate: phosphate</td>
<td>0.0097</td>
<td>0.005</td>
<td>0.0105</td>
<td>0.0019</td>
<td>0.0094</td>
<td>0.0017</td>
</tr>
<tr>
<td>Carbonate: amide I</td>
<td>0.034</td>
<td>0.018</td>
<td>0.0300</td>
<td>0.006</td>
<td>0.0350</td>
<td>0.0005</td>
</tr>
<tr>
<td>1030:1020 peak area ratio</td>
<td>1.069</td>
<td>0.091</td>
<td>1.585</td>
<td>0.18**</td>
<td>1.313</td>
<td>0.270*</td>
</tr>
<tr>
<td>1112:960 peak area ratio</td>
<td>3.82</td>
<td>1.70</td>
<td>4.94</td>
<td>1.6</td>
<td>3.32</td>
<td>1.20</td>
</tr>
</tbody>
</table>

% CHANGES IN MINERAL QUALITY WITHIN THE FIRST 60 um AT BONE FORMING SURFACES

![Image of boneforming surfaces](image)

![Graph showing % changes in mineral quality](image)
How Do Collagen Molecules Form a Functional / Stable Fibril?

I: soft tissues
II: skin and cornea
III: skeletal tissues

1. dehydro-dihydroxylysinonorleucine (deH-DHLNL),
2. dehydro-hydroxylysinonorleucine (deH-HLNL),
3. dehydro-histidinohydroxymerodesmosine (deH-HHMD),
4. pyridinoline (Pyr),
5. deoxypyridinoline (lysyl analog of Pyr, d-Pyr),
6. histidinohydroxylysinonorleucine (HHL),
7. pyrrole
INFRARED IMAGING
In case of collagen analysis, histologically stained sections may be employed.
Match spatial distribution of collagen cross-links with mineralization
Collagen cross-links & bone turnover

Mild Primary Hyperparathyroidism

HyperPT

J Clin Endocrinol Metab. 2008 Sep;93(9):3484-9.
HOW IS COLLAGEN QUALITY IN OSTEOPOROSIS?
HOW IS COLLAGEN QUALITY IN OSTEOPOROSIS?

Normal N = 10
Low-turnover (LTOP) = 10
High-turnover (HTOP) = 10
HOW IS COLLAGEN QUALITY IN FRAGILE BONE?

JBMR (2004)
What is the clinical evidence / suggestions?

- J Bone Miner Res. 2007 May;22(5):747-56.
- J Bone Miner Res. 2007 Jan;22(1):127-34
- JAMA. 2005 Mar 2;293(9):1121-2

HOMOCYSTEINE & FRACTURE
### PLASMA HOMOCYSTEINE LEVELS & COLLAGEN CROSS-LINKS

#### Patient data

<table>
<thead>
<tr>
<th>Patient Age (yrs)</th>
<th>Hcys level (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>20</td>
</tr>
<tr>
<td>80</td>
<td>30</td>
</tr>
<tr>
<td>90</td>
<td>40</td>
</tr>
<tr>
<td>100</td>
<td>50</td>
</tr>
</tbody>
</table>

**Spearman r**: 0.3715  
**95% confidence interval**: -0.1139 to 0.7138  
**P value (two-tailed)**: 0.1173  
**P value summary**: ns

#### Unpaired t test

<table>
<thead>
<tr>
<th>Are means signif. different? (P &lt; 0.05)</th>
<th>One- or two-tailed P value?</th>
<th>t, df</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>Two-tailed</td>
<td>t=4.129 df=17</td>
</tr>
<tr>
<td>ns</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Bone. 2009 May;44(5):959-64.

#### Graphical Data

- **Hcys level vs Patient Age**:
  - High Hcys
  - Low Hcys

- **Hcys level vs collx form**:
  - Collagen cross-links form
  - High Hcys
  - Low Hcys

- **Hcys level vs collx res**:
  - Collagen cross-links resolution
  - High Hcys
  - Low Hcys

- **Anatomical Location (µm)**
  - Normal
  - HTOP
  - LTOP

- **Pyr / divalent**
  - High Hcys
  - Low Hcys

- **Pyr / divalent vs Anatomical Location (µm)**
  - Normal Hcys
  - HTOP Hcys
  - LTOP Hcys
**PLASMA HOMOCYSTEINE LEVELS & COLLAGEN CROSS-LINKS**

Unpaired t test

- P value: 0.0007
- P value summary: ***
- Are means signif. different? (P < 0.05): Yes
- One- or two-tailed P value?: Two-tailed
- t, df: t=4.129 df=17

The graph shows the relationship between plasma homocysteine levels (µM) and the area of the 1660 band and 1690 band. The area decreases as the homocysteine levels increase.

Bone. 2009 May;44(5):959-64.
<table>
<thead>
<tr>
<th></th>
<th>FTIRI</th>
<th>RAMAN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spatial resolution</td>
<td>7 um</td>
<td>0.6 um</td>
</tr>
<tr>
<td>Tissue preparation</td>
<td>Dehydration &amp; embedding</td>
<td>None</td>
</tr>
<tr>
<td>Background literature</td>
<td>Solid</td>
<td>Chaotic</td>
</tr>
<tr>
<td>Acquisition times</td>
<td>5 minutes</td>
<td>Seconds – 2 minutes</td>
</tr>
<tr>
<td>Signal to noise</td>
<td>Superior</td>
<td>Inferior</td>
</tr>
<tr>
<td>Mineral information</td>
<td>A lot of information</td>
<td>Some</td>
</tr>
<tr>
<td>Carbonate information</td>
<td>A lot of calculations involved</td>
<td>Straightforward</td>
</tr>
<tr>
<td>Collagen</td>
<td>A lot of information</td>
<td>Some</td>
</tr>
<tr>
<td>Orientation</td>
<td>Natively none</td>
<td>A lot of information</td>
</tr>
</tbody>
</table>

P045 CORTICAL BONE ORIENTATION AND COMPOSITION IN A MOUSE MODEL AS A FUNCTION OF TISSUE AGE VS ANIMAL AGE S. Gamsjaeger, P. Roschger, K. Klaushofer, E. P. Paschalis, P. Fratzl
RAMAN & CELL CULTURES

phenylalanine

amide I

DNA

amide III
FTIRI & Raman vibrational spectroscopic techniques are capable of providing information on material properties

Information on all tissue components at the same time

No probe molecule required. Non-destructive

Employed parameters have been / are validated against “golden standard” techniques such as XRD, and Biochemical analyses

To date, mineral maturity & the ratio of two of the major mineralizing collagen cross-links, may be deduced in thin tissue sections with optimal spatial resolution of ~ 0.6-6.3 um. Moreover, structure vs orientation effects may be discerned

Its major strength is that it may provide answer to the question: **what is the difference between normal and diseased/treated tissue at EQUIVALENT tissue age anatomical locations?**

As a result, effects due to alterations in turnover and/or other factors may be readily discerned
Information provided in spatially resolved manner thus can compare material properties as a function of tissue age and bone surface activity (SF).

Not likely to be used in everyday clinical practice as biopsy is required.

Excellent tool in cases where BMD does not fully account for fracture risk (SF).

Excellent tool in animal model experiments.

Excellent tool for determining material properties (clinical trials).

Excellent tool for establishing effect of therapies on bone material properties.