The Biomimetic Future of Bone & Soft Tissue Regeneration

The Body Knows The Difference®
Citrate-Based Biomaterial Review

- Novel class of biodegradable elastomer
- Biomaterial design mimics extracellular matrix crosslinking to enhance tissue regeneration
- Modular chemical design offers unique material properties to fit a wide variety of regenerative engineering applications
Citrate is a natural substance that is required for life.

• As a strong chelator of metal ions, citrate is involved in many biological functions

• Regulator of cell metabolism and energy production.

• Abundant in bone and plays a large role in native apatite formation

• Natural stimulant of metabolic activity.

• Accelerates mesenchymal stem cell differentiation
How Does **Citrate** Promote Stem Cell Differentiation into Osteoblasts and Matrix Mineralization Leading to Bone Tissue Regeneration

**Citrate provides the following advantages:**

- Chemical sites to enhance polymer-bioceramic interactions
  - The ability to load physiologic bioceramic concentrations (65 wt.-%) into the polymer while maintaining elasticity
  - Controls bioceramic mineralization to mimic physiologic nanoapatite
  - Chelation with ions to enhance biomaterial strength

- Chemical sites to mimic extracellular matrix polymer chain crosslinking
- Increases cell energy
- Accelerates stem cell differentiation
How do Calcium ions Promote Stem Cell Differentiation into Osteoblasts and Matrix Mineralization Leading to Bone Tissue Regeneration

Calcium ions (Ca\(^{2+}\))
- Calcium begins to elute from Citregen samples in as little as 24 hours
- “Bone formation associated with a hydroxyapatite coating is believed to begin with surface dissolution of the hydroxyapatite, which releases calcium and phosphate ions into the space around the implant. Reprecipitation of carbonated apatite then occurs…”
- Extra-cellular calcium plays an important role in the regulation of the osteoblast/osteoclast relationship, with higher Ca levels reducing the activity of osteoclasts and increasing that of osteoblasts
- Calcium released by hydroxyapatite accelerates osteoblast differentiation
- Calcium signaling is an important aspect to consider; calcium is transported into cells through specific calcium channels and activates multiple processes to produce proteins such as osteopontin (OPN) that are associated with osteoblast differentiation

1 Hydroxyapatite-Coated Prostheses in Total Hip and Knee Arthroplasty; doubleton and Manley; JBJS 86-A no. 11, November, 2004
2 The effect of calcium ion concentration on osteoblast viability, proliferation and differentiation in monolayer and 3D culture; Shinichi Maeno, Yasuo Niki, Hideo Matsumoto, Hideo Morioka, Taku Yatabe, Atsushi Funayama, Yoshiaki Toyama, Tetsushi Taguchi, Junzo Tanaka; Biomaterials 26 (2005) 4847–4855
How do Phosphate ions Promote Stem Cell Differentiation into Osteoblasts and Matrix Mineralization Leading to Bone Tissue Regeneration

Phosphate ions (PO$_4^{3-}$)

- Phosphate begins to elute after one day of soaking in PBS
- Extracellular phosphate plays an important role in osteogenic differentiation through transport into the mitochondria to support ATP synthesis in the Citric Acid Cycle
- CaP matrices serve as reservoirs for bone growth factors
- Supplementation of phosphates in vitro increase the expression of BMP2 much like the addition of strontium and magnesium do
- Phosphates interact in the Citric Acid Cycle to create ATP (adenosine triphosphate) that is transported outside the cell through a specific transport channel, where it degrades into adenosine and signals stem cell differentiation into osteoblasts

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Citregen Chemistry

Pre-Polymer Synthesis

\[
\begin{align*}
\text{Citric Acid} & \quad + \quad \text{1,8-Octanediol} \\
\end{align*}
\]

\[\Delta, N_2 \rightarrow \text{Poly(octamethylene citrate) (POC)} \quad \text{Pre-Polymer}\]

\[
\begin{align*}
R &= H, \text{ or} \\
R' &= H, \text{ or} \\
\end{align*}
\]

Hydrolytic Degradation

\[
\begin{align*}
\text{POC} & \quad \rightarrow \quad \text{Citric Acid} \quad + \quad \text{1,8-Octanediol} \\
\end{align*}
\]
Biomaterial Design - Crosslinking

Extracellular Matrix

Molecular ECM-Like Structure

- Collagen
- Laminin
- Proteoglycan
- Entacin

Crosslinking
- Confers elasticity
- Mechanotransduction
- Completely amorphous
- Surface erosion
- Homogeneous degradation

Pendant Functionality
- Carboxylic acid and hydroxyl
- Located in bulk material
- Sites for small molecule attachment

Ester Bonds
- Hydrolysable sites
- Monomeric degradation products
- Between monomers and crosslinking sites

Ionic Interaction
- Chelation with ions
- Additional crosslinking
- Interaction with bioceramics
Bioceramic Interaction

The citrate-based composite is evenly filled with hydroxyapatite (HA) particles (60 wt.-%).

- Bioceramic Interaction – uniform surface can be seen.
- Chelation of Calcium Ca2+ to the negative charge of Citrate.
- Bone is made up of 60% HA which is why there is 60% HA in Citregen-V.
Role of Citrate in Bone

- Stimulates mineralization (growth of HA crystals)
- Stabilizes and regulates HA crystal size
- Binds to apatite for stronger, flexible construct

Bone heals in layers – a bunch of nano HA plates stacked upon one another. This is what gives bone its flexibility.

Citrate is responsible for forming HA layers.

Citregen’s Unique Position in Orthopedics

**Tissue Citrate Levels**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Citrate (µmol/g)</th>
<th>Zinc (µmol/g)</th>
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<tr>
<td>Bone/Teeth</td>
<td>20-80</td>
<td>2-5</td>
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<tr>
<td>Cartilage</td>
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<tr>
<td>Blood Plasma</td>
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<td>0.014</td>
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<tr>
<td>Soft Tissues</td>
<td>0.2-0.4</td>
<td>0.2-0.6</td>
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**Osteoblasts are Citrate Producers**

![Citrate Chelation](image1)

**Native Nanoapatite Formation**

![Bioceramic Dispersion](image2)

**Nutrient Rich Degradation Products**

![Citregen](image3)
Citregen’s Unique Position in Orthopedics

• In its current form, citrate enables biomimetic concentrations of hydroxyapatite to be incorporated
  • Citrate dissolves HA into constituent calcium and phosphate ions that are eluted from the material to the local environment in vivo
  • HA buffers the release citric acid (cross-linking is never fully complete, free acid is released)
  • Citrate that is released induces genetic signaling to stimulate biologic cascades of action
  • Citrate that remains in the HA helps to maintain shape and structure of the device for functionality during the healing process
  • Citrate, calcium, and phosphate participate in the biological process of bone formation and regeneration
    • Citrate naturally resides in bone and acts to organize mineralization, strength, and flexibility of the bone
    • Calcium induces multiple biologic functions including mineralization of surfaces to induce bone regeneration
    • Phosphates are key to the formation of bone and the action of osteoblasts and osteoclasts
Composite Material Properties

- Left graph shows that a composite with less HA degrades faster.
- Right graph shows the pH solution as the composite is degrading. pH is neutral after 7 days.
Composite Material Properties

- Composites were eroded in accelerated conditions and dried.
- Mass loss is linear meaning it degrades at a constant rate.
- Volume decrease is linear meaning the size shrinks at the same rate.
- Volume to Mass is also linear meaning 20% volume = 20% mass.
- Surface erosion hydrolysis (degradation).

No bulk degradation as seen with polylactides
Biocompatibility and Safety
Testing Preformed by Nelson Labs

- ISO 10993-5
- All Test done per FDA GMP Regulations CFR Parts 210, 211 & 820

Results:

<table>
<thead>
<tr>
<th>Test Article:</th>
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<th>Extraction Ratio</th>
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<td>Results Pass/Fail</td>
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<td>#2</td>
<td>#3</td>
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<td>Pass</td>
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Controls:

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<th>Identification</th>
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<tr>
<td>Negative Control - Polypropylene Pellets</td>
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<td>Media Control</td>
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<tr>
<td>Positive Control - Latex Natural Rubber</td>
<td>4</td>
<td>4</td>
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</table>

- Standard test required by the FDA.
- If a material is leeching out anything toxic, this test will find it.
- Top Line is Citregen and it passed with all Zeros.
Citregen Monomer Safety

- Human acute monocyte leukemia cells (THP-1) were used to study inflammatory response in vitro.
- Production of pro-inflammatory tumor necrosis factor-α (TNF-α) for both monomers was significantly lower than positive control LPS.
- Both monomers did not stimulate the release of IL-1β from monocytes.
- Both monomers were not cytotoxic (viability above 70%).

Citregen monomers (citrate and octanediol) were added to monocytes.
Monocytes are responsible for inflammation.
Citrate and octanediol did not cause monocytes to release chemicals that initiate inflammation.
Citregen and all its constituents (monomers and HA) are safe and biocompatible.
Biocompatibility and Safety

- Porous composite plugs (5 x 12 mm)
- Implanted into sheep vertebral body

- Shows tissue growing into the specimen
The left graph shows that stem cells take in citrate. This is important because stem cells are doing something with citrate.

The right graph shows Enhanced Gene Expression which means that bone is continually remodeling.

- Runx2 is responsible to turn stem cells into osteoblasts.
- COL 1a1 is needed for collagen production.
- SPP1 is required to recruit osteoclasts to the site for remodeling.
- Citric acid turns on the switch to make more of these genes.
hMSC Mineralization

• Calcium Quantification means stem cells are mineralizing more on Citregen. This effect still occurs in normal media (media designed for cell growth not differentiation)
• Alkaline Phosphatase (ALP) is a protein needed for mineralization.
• Stem cells on Citregen produce more ALP which is why stem cells mineralize more.
Antimicrobial Properties

• Over a 90% reduction in E. coli and S. aureus CFUs after incubation with citrate-based polymers
• Citrate shows a synergistic antimicrobial effect with cefazolin against S. aureus (100% kill)

• Citregen (POC) kills more E. coli and S. Aureus than PLGA polymers.
• Citrate combined with Cefazolin demonstrated a 100% kill
Current Preclinical Studies

• Ovine tendon anchor biological response

• Ovine functional ACL repair
  • Dr. Ross Palmer (Colorado State University)

• Ingenuity pathway analysis and protein analysis
  • Dr. Manus Biggs Curam (Galway, Ireland)
Potential Orthopedic Applications

- Osteotomy wedges
  - MMP & TTA procedures
- Loading bone void filler

8 Weeks Post-Op
Potential Orthopedic Applications

- Ligament/tendon attachment
  - Suture anchors

- Ligament/tendon attachment
  - Interference screws for CCL reconstruction

- Cross-section of Citregen screw shows great bone apposition along left side of image, proposed tendon on right side of implant

- Predicate is not the same opacity as Citregen. Bone apposition is good, tendon pathway is not as defined
Potential Orthopedic Applications

- **Thermoresponsive Osteoinductive Injectable Composite**
  - This composite has the ability to rapidly induce localized bone formation, is shape conforming and has antioxidant properties.  
  - Early return to function following traumatic fractures and surgically created osteotomies is based on the healing of the fracture or osteotomy site without clinically significant complications.  
  - Earlier return to function for the individual patient, potentially decreasing morbidity, and could shorten the convalescent period, allowing for an earlier return to full function.  
  - Decreasing complications associated with bone healing and allowing early active mobilization could also decrease cost of treatment.

- **Applications**
  - TPLO & TTA Osteotomies
  - Corrective Osteotomies
  - Craniomaxillofacial
  - Dental to fill defects after tooth extractions for both Canine & Equine
  - Pan Carpal & Pan Tarsal Arthrodeses
  - Comminuted Fractures
  - SubChondral Bone Cysts in Horse

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1 A thermoresponsive, citrate-based macromolecule for bone regenerative engineering; Simona Morochnik et al, Journal of Biomedical Research 2018.
Potential Orthopedic Applications

• Spine Spacers

• Applications
  • Cervical fusion for disc associated wobbler syndrome (DAWS)
  • Lumbar fusion for lumbo-sacral instability
Summary

- **Fully synthetic biomimetic biodegradable polymer**
  - Multifunctional citrate monomer cornerstone
  - Polymer chain crosslinking
    - Mimic crosslinked structure of native extracellular matrix
    - Homogeneous surface erosion degradation mechanism
  - Thermoset elastomer
  - Chemistry allows compositions with up to 60 wt.-% bioceramic to match native bone mineral content
Citrate Bibliography

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Ovine ACL Reconstruction

0 Month Baseline

CITREGEN

PLDLA - βTCP
Ovine ACL Reconstruction

3 Month Baseline

CITREGEN

PLDLA - βTCP
Ovine ACL Reconstruction

6 Month

CITREGEN

PLDLA - βTCP
UPenn Ovine Metaphyseal Study – Suture Anchor  
Thomas Schaer, BS, DVM, University of Pennsylvania

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<tr>
<th>Material</th>
<th>Irritancy Score</th>
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<td></td>
<td>6 Weeks</td>
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<tr>
<td>Citregen</td>
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<tr>
<td>Predicate</td>
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- **Citregen Anchor**
- **New bone formation**
- **Fibrous Tissue**
- **Predicate**