Fluorescence Microscopy and Glass Scaffolds in Tissue Engineering

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-- I cut myself on glass!
And you propose to use glass as a bone-replacement material ?!
• The emergence of bioactive glass—L. Hench, 1960s
  - Melt-quench CaO-Na$_2$O-P$_2$O$_5$-SiO$_2$ glass (on the market now) and
  - Sol-gel SiO$_2$-CaO-(P$_2$O$_5$) glass

Bioactive Glass Formulation

White portion is the proper composition of phosphorous, calcium, sodium, and silicon found in bioactive glasses.
Nano-Macro Dual-Porous Bioglass produced by two novel techniques in Dr. Jain’s group at IMI-NFG, LU:

(1) Melt-Quench Heat-Edge Procedure:

Macro-porosity (10-200 μm)  Nano-porosity (5-50 nm)

45S5 formulation (45%SiO₂ - 24.5%CaO - 24.5%Na₂O - 6%P₂O₅)
Nano-Macro Dual-Porous Bioglass produced by two novel techniques in Dr. Jain’s group at IMI-NFG, LU:

(2) Sol-gel Procedure:

70% SiO$_2$ + 30% CaO formulation
Our bioglass scaffolds are very different from conventional 45S bioglass. They are next generation bioscaffolds!

“Tailored Amorphous Multi-Porous (TAMP) Bioscaffolds” for Hard Tissue Regeneration
Unique benefits of TAMP bioglass bone-replacement scaffolds not shared by any other currently used bone-replacement implants (titanium, Teflon®, ceramics, dead bone):

-- Dual porosity allows cells to colonize the surface AND the inside of the scaffolds

-- Bioglass is resorbed over time in the body and will be replaced by normal, natural bone

-- Ions (silicon, calcium) leaching from the bioglass promote the differentiation of bone precursor cells into mature, calcified-matrix secreting bone cells (osteoblasts)
Animal connective tissues consist largely of extracellular matrix (ECM):
the 4 major protein types of extracellular matrix

4 basic types of tissues

- Epithelial
- Nervous
- Muscular
- Connective

- Bone
  (hard, dense)
- Skin, Tendons
  (flexible)
- Cartilage
  (shock-absorbing)
- Eye-interior
  (jelly-like)

Protein Components:

- (1) Collagens
- (2) Fibronectin
- (3) Laminins
- (4) Proteoglycans
The bone matrix (calcium phosphate and collagen fibrils) is secreted by osteoblasts and by fibroblasts in other connective tissues.

Osteoblasts = Bone-forming cells
(A) Light micrograph of a section of forming bone stained with toluidine blue. Osteoblasts with abundant, blue-stained rER lay down bone matrix (green) on the surface of calcified cartilage (pink).
(B) Schematic of osteoblasts
The bone matrix (calcium phosphate and collagen fibrils) is secreted by osteoblasts and by fibroblasts in other connective tissues.
FDA (U.S. Food and Drug Administration) requires drugs, implants, etc. potentially used in humans to be tested on 3 levels:

1) Cell Culture Models

2) Animal Models

3) Trials in Humans
Cell-Lines used so far:

**ATCC® Number:** CRL-1427™

**Designations:** MG-63

**Biosafety Level:** 1

**Medium & Serum:** See Propagation

**Organism:** Homo sapiens (human)

**Source:** Organ: bone

**Disease:** osteosarcoma

**Price:** $244.00

**Depositors:** A Billiau

**Shipped:** frozen

**Growth Properties:** adherent

**Morphology:** fibroblast

**ATCC® Number:** CRL-2593™

**Designations:** MC3T3-E1 Subclone 4

**Biosafety Level:** 1

**Medium & Serum:** See Propagation

**Organism:** Mus musculus (mouse)

**Source:** Organ: bone

**Tissue:** calvaria

**Cell Type:** preosteoblast;

**Strain:** C57BL/6

**Price:** $294.00

**Depositors:** RT Franceschi

**Shipped:** frozen

**Growth Properties:** adherent

**Morphology:** fibroblast

Note: The Subclone 4 will secrete calcified extracellular matrix when grown in the presence of ascorbic acid and phosphate.
Challenge 1: Due to porosity, bioglass scaffolds are not transparent!
Solution: Fluorescence Microscopy!
Fluorescent Dyes and their Excitation and Emission Wavelengths

DAPI

Fluorescein

Rhodamine
Green Fluorescent Protein (GFP) and Derivatives

Nobel Prize in Chemistry, 2008
-- Fluorescence microscopy allows us to investigate the structure, dynamics and functions of cells;
-- to determine what cellular function might went wrong in disease;
-- and to detect cells growing on non-transparent surfaces.
Detection of Cells Growing on Non-Transparent Objects:
MC3T3 bone-precursor cells growing on non-transparent bio-glass,
(nuclei stained with DAPI, combined fluorescence and reflection illumination)
Challenge 2: Scaffold texture and surface topology; large pores create a very rough, uneven scaffold surface!
Solution: Long working-distance objectives that have a larger focal depth.
Challenge 3: Bioglass scaffolds dissolve over time. How do we deal with increasing, potentially harmful calcium and silicon ion concentrations in the cell culture medium and the culture vessels (washed away in the body by blood and other extracellular fluids)?

(A) Pre-incubation of the scaffolds

(B) Culture conditions during incubation time

(C) Handling of the scaffolds in preparation for cell-seeding
**Result 1:** Cells colonize the scaffolds on the surface AND deep within the macro-pores

Two fluorescence images of the same area of a sol-gel glass scaffold taken at different focal depth; MC3T3 bone cells, cell nuclei stained with DAPI
Result 1: Cells colonize the scaffolds on the surface AND deep within the macro-pores

MC3T3 bone-precursor cells growing on non-transparent bio-glass,
(cell nuclei stained with Propidium Iodide, Actin filaments decorated with Alexa488-Phalloidin)
Result 2: Pre-Impregnation of bio-glass scaffolds with extracellular matrix proteins results in faster scaffold colonization.
Result 3: Bio-glass Scaffolds (sol-gel and melt-quench) promote cell proliferation

**Figure:** Average number of MC3T3 cells observed per time point. Cell nuclei were visualized with Hoechst stain, and 9 images were taken per sol-gel sample using a 10X lens. The number of cells per image was averaged for each sample, and then these values were averaged again to provide a cell number per time point representative of all samples. The average number of cells per mm² increased from 13.2 to 630 over the 26 day study, equaling 5.5 cell cycles, doubling every 4.5 days.
Result 4: Scaffold surface texture influences cell attachment and proliferation: Smooth surfaces increase cell attachment and proliferation; rough surfaces decrease cell attachment and proliferation.
Result 4: Scaffold surface texture influences cell attachment and proliferation:
On smooth (A-C), but not on rough bioglass surfaces (D, E) cells form typical actin stress fibers (A-C), as well as fibrillar (A) and focal (B, C) cell adhesions.

**Smooth Surfaces:**
\[ R_a \sim 0.05 - 0.1 \ \mu m \]

Stains (A):
- Actin
- Fibrillar Adhesions (\( \alpha 5 \) Integrin)
- DAPI

**Rough Surfaces:**
\[ R_a \sim 0.5 - 2 \ \mu m \]

Stains (B-E):
- Actin
- Focal Adhesions (Vinculin)
- DAPI
**Result 5:** Ions (silicon, calcium) leaching from the bio-glass scaffolds promote the differentiation of bone precursor cells into mature, calcified-matrix secreting bone cells (osteoblasts).

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**Figure:** Schemes of bone-precursor cell differentiation into mature bone cells. (A) Describes the possible fates of bone progenitor cells, ranging from apoptosis to complete differentiation into mature osteocytes. (B) Represents the activation cascade that occurs during differentiation. (C) Describes the relative order in which specific genes are expressed as bone-precursor cells differentiate.

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Cbfa1 = Runx2  
ALP = alkaline phosphatase  
COL = collagen  
OSN = Osteonectin  
OPN = Osteopontin  
OSC = Osteocalcin/bglap  
BSP = bone sialoprotein
**Result 5:** Ions (silicon, calcium) leaching from the bioglass scaffolds promote the differentiation of bone precursor cells into mature, calcified-matrix secreting bone cells (osteoblasts).

Quantitative Reverse Transcription - Polymerase Chain Reaction (qRT-PCR) analysis:
Result 5: Ions (silicon, calcium) leaching from the bioglass scaffolds promote the differentiation of bone precursor cells into mature, calcified-matrix secreting bone cells (osteoblasts).

Heat-Maps

Scatter-Plots

qRT-PCR analysis using 96-well bone-cell differentiation arrays:
Result 6: Sol-gel bioglass scaffolds implanted under the skin (subcutaneously) of a rabbit dissolve with minimal inflammation and are replaced by normal connective tissue.

Overall view of a tissue section 1 week after implantation (subcutaneous 3D porous bioglass disc, asterisk). Tissue section was stained with Stevenel's blue and Vangeison. Only minimal inflammation and encapsulation (scare-formation) of the scaffold is visible.
Histological micrographs of 1 week ectopically placed porous bioglass disc stained with Stevenel's blue and Vangeison revealing only a thin fibro-vascular encapsulation with minimal cell infiltration.

(a) Thin fibrovascular capsule (greenish color) (arrows) surrounding bioglass disc (asterisk). Note the peripheral blue zone of the infiltrating inflammatory cells. (b) Center of the disc material without cells (asterisk). (c) Higher magnification of the periphery of the disc with minimal cell infiltration.

Results: M. Marei et al.
Histological micrographs for 3 weeks ectopically placed porous bioglass disc stained with Stevenel's blue and Vangeison revealing cells, collagen and neo-vasculature inside the porous bioglass material.
(a) Thin fibro-vascular capsule (arrow) surrounding the bioglass disc (asterisk) with apparent increase in granular tissue invasion inside. (b) Neo-vasculature (arrows) deeper inside the disc material (asterisk). (c) Higher magnification of the apparent neo-vasculature.

Results: M. Marei et al.
Histological micrographs at higher magnification of 5 week ectopically placed porous bioglass disc stained with Stevenel's blue and Vangeison showing neo-vascularity, bioglass scaffold structure, interaction with the invading granulation tissue in the center of the disc, and replacement of the scaffold by connective tissue.

(a) Only islands of partially degraded bioglass material (arrows) inside the disc core remain. (b) Higher magnification of the irregular shaped material islands (arrow). (c) Granular tissue invading and interacting with the porous bioglass disc at higher magnification (asterisk).

Results: M. Marei et al.
Biocompatibility and Bioactivity of nano-macro dual-porous glass bone-replacement scaffolds

Project Team:

Prof. Himanshu Jain, Diamond Chair of Materials Science & Engineering, Director International Materials Institute for New Functionality in Glass (IMI-NFG), Lehigh U.

Prof. Matthias M. Falk, Department of Biological Sciences, Lehigh U.

Prof. Jutta Y. Marzillier, Department of Biological Sciences, Lehigh U.

Prof. Mona K. Marei, Tissue Engineering Labs, Faculty of Dentistry, Alexandria U., Egypt

Students: (PhD) Shaojie Wang, Susan Baker
(Undergraduates) Regina McBarb, Laura Bowen, Sean White, Andrew Salim, Stephanie Eider, Samantha Golden, Pauline Krzyszczyk, Leslie Smith, Freedom High School student: Raina Jain
First Place, $7,500.00

Raina Jain
11th Grade,
Freedom High School,
Bethlehem, Penn.

Project Title: Engineering Glass Bone Implants to Enhance the Adhesion of Precursor Osteoblast Cells

Jain (center) accepts the first place award from Allan Jarvis of sanofi-pasteur (left) and Paul Hanle of the Biotechnology Institute.
Raina Jain, a Bethlehem Freedom High-School Student performing research in Dr. Falk’s laboratory won this year’s BioGENEius Challenge (one of the major high-school student science competitions). She was invited to the 1st Science Fair held at the White House on Monday, Oct. 18, 2010 to present her research to US President Obama.

First Place, $7,500.00

Raina Jain
11th Grade, Freedom High School, Bethlehem, Penn.

Project Title: Engineering Glass Bone Implants to Enhance the Adhesion of Precursor Osteoblast Cells

Jain (center) accepts the first place award from Allan Jarvis of sanofi-pasteur (left) and Paul Hanle of the Biotechnology Institute.
Thank you for listening,

… and THINK BIG!