Influence of porous architecture of scaffolds obtained from different routes on the cell colonization.

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Collaboration with
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Introduction

Compact bone

Porosity
$\Phi: 190-230 \text{ } \mu\text{m}$

$V: 65\%$

$\sigma_c = 80-200 \text{ MPa}$

Spongy bone

Porosity
$\Phi: 500-600 \text{ } \mu\text{m}$

$V: 80\%$

$\sigma_c = \text{a few tenths } \text{MPa}$

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Introduction

- **Porous ceramics**: wide range of structures
  - Foams with cell walls randomly oriented in space
  - Honeycombs with polyhedral cells 3D arranged
  - Connected hollow spheres or rods or fibres...

*P. Colombo, Phil.Trans.R.Soc.A 2006*
Introduction

- Large applications fields in advanced engineering such as filtering liquids and particles in gas streams, porous burners, lightweight load-bearing structures, ... and biomedical devices such as bone substitutes.

Introduction

- Various processing routes:

**Conventional methods:**
- partial sintering, sacrificial fugitives, replica templates, direct foaming, extrusion, aerogels...

**Innovative methods:**
- ice-templating, additive manufacturing methods as 3D-printing,...

Three methods to manufacture macroporous and microporous ceramic scaffolds:

- The first one by ceramic slurry infiltration of organic bead skeleton, permits to have an isotropic structure with close control of the pore size and the interconnection size but relatively low mechanical resistance.

Patent FR2823305, Biocetis SARL, M. DESCAMPS, P HARDOUIN, J LU, F MONCHAU


This technique type has been industrially applied by SOFAMOR – DANEK, BIOCETIS and BIOLU since a few years.
Three methods to manufacture macroporous and microporous ceramic scaffolds:

- The first one by ceramic slurry infiltration of organic bead skeleton, permits to have an isotropic structure with close control of the pore size and the interconnection size but relatively low mechanical resistance.

- The second one by ceramic slurry casting using ice templating, permits to develop anisotropic structure which allows higher compressive strength but compromise has to be found between $\sigma_c$ and pore size (S. Deville).
Up today substitutes present lower $\sigma_c$ values than compact bone one’s, the freeze casting excepted. But high $\sigma_c$ values correspond to smaller pore sizes than usually used for bone substitutes.
Three methods to manufacture macroporous and microporous ceramic scaffolds:

- The first one by ceramic slurry infiltration of organic bead skeleton, leads to an isotropic structure with close control of the pore size and the interconnection size but relatively low mechanical resistance.

- The second one by ceramic slurry cast using ice templating, permits to develop anisotropic structure which allows higher compressive strength but compromise has to be found between $\sigma_c$ and pore size.

- The third one by 3D printing of ceramic slurry in UV sensitive resin permits to develop anisotropic structure with regularly continuous channels with bigger size and square shape.
Ceramic stereolithography is an additive manufacturing process which employs a ceramic slurry in a liquid ultraviolet curable photopolymer and an ultraviolet dynamic mask to build parts’ layers one at a time. This layer by layer deposition technology is today commercialized by various companies such as 3DCERAM, SIRRIS (high-viscosity paste slurries) and Admatec Europe BV and Lithoz GmbH (low-viscosity slurries).

J. Deckers, J. Vleugels, J. P. Kruth
The objectives of our study is to compare the mechanical properties and cell colonization ability of these different structures

Part I: Presentation of the three shaping methods applied to calcium phosphate materials and the material structural properties

Part II: Comparison of cell colonization ability by static in vitro tests for the two methods: replica and freeze casting

Part III: Functionalization of as-prepared scaffolds by drug and phage impregnation through microporosity
First method: Ceramic slurry infiltration of organic skeleton

Human bone

Patent FR2823305
First method: Ceramic slurry infiltration of organic skeleton

Organic skeleton preparation

Chemical forming with acetone under pressure

- Bonding between PMMA beads (scaffold)
- Controlled diameter bonding (Interconnection) depends on time, temperature, pressure
First method: Ceramic slurry infiltration of organic skeleton

Shaping  Impregnation  Debinding

PMMA beads  ceramic

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First method: Ceramic slurry infiltration of organic skeleton

- Control of pore size depending on PMMA beads size
- Control of interconnection diameters: $I_d$
  PMMA beads (500 - 600 µm)

Control of porosity gradient in pore size and interconnection size

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First method: Ceramic slurry infiltration of organic skeleton
Possibility to add microporosity by mixing graphite as micropore forming agent.

Controlled macroporosity and microporosity
Second method: Ceramic slurry ice templating

Human bone

D. Hautcoeur Ph D UMons-BCRC Nov 2014
Second method: Ceramic slurry ice templating

D. Hautcoeur Ph D UMons-BCRC Nov 2014

S. Deville et al., Biomaterials 27 (2006) 5480–5489
Second method: Ceramic slurry ice templating

Pore long axis size:
- between 150 and 340 µm versus dry matter content
- between 13 and 210 µm versus cooling rate.

Total porosity:
36 to 67 % versus dry matter %

D. Hautcoeur Ph D UMons-BCRC Nov 2014

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Relationship between porosity and propagation rate for isotropic samples (calibration curve) and anisotropic samples.

Anisotropic sample = high propagation rate despite a porosity higher than 40%.

### Slurry composition and freezing rate

<table>
<thead>
<tr>
<th>Slurry composition and freezing rate</th>
<th>Ceramic walls porosity % (average)</th>
</tr>
</thead>
<tbody>
<tr>
<td>28% Vol.+ 3% PEG1000 and 1.3°C/min</td>
<td>11.5 ± 0.2</td>
</tr>
<tr>
<td><strong>33% Vol.</strong>+ 3% PEG1000 and 1.3°C/min</td>
<td>11.2 ± 0.2</td>
</tr>
</tbody>
</table>
Third method: 3D printing of ceramic slurry

Human bone

JC Hornez LMCPA January 2015
Third method: 3D printing of ceramic slurry

- The thickness of a single layer, typically 20 µm to 100 µm
- Close control of porosity shape, size and orientation

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Comparison of the three different macroporosities

| Isotropic porosity | 65% porosity (limits 65-75%)  
|                   | 100 µm interconnection  
|                   | 500-600 µm spherical pore diameter  
|                   | HA: 15 MPa, TCP: >15 MPa  
|                   | Possibility for pore size and content gradient |
| Anisotropic porosity | 55% porosity (limits 40-75%)  
|                     | Ellipsoidal porosity  
|                     | 13-400 µm pore large diameter  
|                     | 6 - 70 µm pore small diameter  
|                     | 10 - 50 µm width of wall  
|                     | HA: 51% porosity, 180/35 µm pore Ø  
|                     | $\sigma_c: 21$ MPa  
|                     | $\beta$-TCP: 40% porosity, 280/35 µm pore Ø  
|                     | $\sigma_c: 35$ MPa |
| Controlled porosity | 500-700 µm tubular porosity  
|                    | Up to 80% porosity  
|                    | Any sizes and shapes are achievable |
Part II Comparison of cell colonization ability by static in vitro tests for the two methods: replica and freeze casting

- What will be the impact of these two different macroporosities (size and morphology) on human cell invasion?

- Is the ice-templated samples porosity size enough large to permit the human cell invasion?

Colonization tests with MG63 osteoblasts

STSM E.Meurice at INEB, Porto July 2014
Colonization tests with MG63 osteoblasts

Organic bead skeleton infiltration

1 day

Ice - templating

Top

Slice

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Colonization tests with MG63 osteoblasts

Organic bead skeleton infiltration  4 days  Ice - templating
Colonization tests with MG63 osteoblasts

Organic bead skeleton infiltration
Beads 750 µm

Ice - templating
37/200 µm

Beads 350 µm
Colonization tests with MG63 osteoblasts

Ice-templating

1 day

4 days

198 µm/37 µm
Colonization tests with MG63 osteoblasts

Moving osteoblast morphology

Static osteoblast morphology

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What will be the impact of these two different macroporosities (size and morphology) on human cell invasion?

The columnar porosity seems to be preferable for osteoblast mobility inside the substitute.

Is the ice-templated samples porosity size enough large to permit the human cell invasion?

Yes

What could be the benefit of 3D printing technique for cell invasion? Marie Lasgorceix Ph thesis (SPCTS Limoges)
Micrographs by immunofluorescence of porous substrates in SiHA obtained by micro stereolithography: PhD of Marie Lasgorceix SPCTS Limoges 2014

large pores (a) and small pores (b), after 7 days of incubation with MC3T3 cells, large pores (c) and small pores (d) after 14 days
Part III  Functionalization of as-prepared scaffolds by drug and phage impregnation through microporosity

Chemical functionalization

Biological functionalization
Chemical functionalisation

The micro-porosity allows functionalisation of the ceramic by loading the microstructure with various active substances.

20% µ-porosity

1 mm

![Diagram of chemical structures](image)

gentamicine :  
C1A  — CH₂NH₂  
C2  — CH(CH₃)NH₂  
C1  — CH(CH₃)NHCH₃

R :
20% μ-porosity

The HA beads are loaded with 40 mg gentamicine /g HA
By protecting the surface, it is possible to control the drug delivery

Chemical functionalization

Polymer coating

Gentamicine Release

Flush after 24h Gentamicine Release

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Chemical functionalization

Chemical functionalization of porous HA for bone substitutes

Staphylococcus aureus

Lyses radius

Lyses area

« Functionalisation of porous HA for bone substitutes » E. Meurice et al. *JECS 32 (2012) 2673-2678"
A bacteriophage (phage) is a virus that infects and replicates only within a specific bacterium.

Bacteriophage lytic cycle

The antibacterial activity of ceramics loaded with \( \lambda \) phage was tested on the bacterium Escherichia Coli K12.
Biological functionalization

Bacterial growth kinetics (*Escherichia coli K12*) was measured by optical density at 620 nm in presence of λ vir phage in ceramic supports with various porosity.

HA samples with different microporosity level (0, 20 and 30%) were incubated for 24 h with 5 ml of λ phage stock and added into culture tubes after obtaining a growth of bacterial two generations.

A slow down of bacterial growth kinetic was noted after 80 min (HA) followed by the death of bacteria. This phenomenon appears sooner as the microporosity is higher.

Same effect with ice-templated samples with higher porosity level
Biological functionalization

Antibiogram (Escherichia coli K12) with lyses diameter measured in presence of phage in ceramic supports with various porosity

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The structure of bone substitute obtained by different processes have been compared:

♦ Process using a PMMA bead skeleton allows to obtain an isotropic spherical porosity with a close control of size:
  • microporosity: 0 to 40 vol %, µm sized  
  • macroporosity: 60 to 75 vol %, 200 to 3000 µm pore size and 20 - 400 µm interconnection size.
It is possible by this method to build porosity gradient to mimic the natural bone structure.

♦ Process using ceramic slurry ice templating leads to an oriented porosity structure:
  • microporosity: 10 vol %
  • macroporosity: 40 to 75 vol %, 180 - 300 µm size (large diameter).

♦ Process using 3D printing should allow to build up continuous pore channels with control of shape and size with gradient from piece core up to the surface.
  • macroporosity: up to 80 vol %, customized pore size

Conclusion

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Concluding

♦ Concerning the human cell invasion tests, the columnar porosity obtained by ice-templating method seems surprisingly very favorable for the mobility of osteoblasts inside the substitute. The shape and size of pores would influence also the cell colonization kinetic. These results have to be confirmed by dynamic tests which will be performed in the following months by our partners from INEB Instituto de Engenharia Biomedica, University Porto. This STSM has initiated a collaborative research with INEB. The following actions are a co-direction with F. Monteiro of a starting PhD thesis and a financial support for the PhD student has been obtained from JECS Trust for a 2 month stay at INEB.

♦ The microporosity allowing a better control of resorbability can be also used as biological substances and phage supports. This study has shown that phage loaded ceramics could be used in bone prophylactic treatments.
Thank you for your attention
Osteoblasts MG63

- 2 multi-well plates (48 wells)
- $2 \times 10^5$ cell/ml $\alpha$-MEM + 10% FBS, 1% ascorbic acid, 1% penicillin, 1% fungicide.
- 37°C under 5% CO$_2$.
- After 24 hours or 4 days if incubation: coloration with MTT (0.5 mg/ml) during 3 hours.
A bacteriophage (phage) is a virus that infects and replicates only within bacteria. Discovered in 1915 by Frederick W. Twort and Felix d'Hérelle.
The bacterium Escherichia Coli K12 strain is lytic λ phage sensitive. -The bacterium was grown at 150 rpm and incubated at 37°C, in agitation 170 rpm in Luria-Bertani broth -Solid media used is the R-medium (added agar at 15 g/L). -Phage λvir stock was prepared by infecting Escherichia coli K12 strain (A324). -HA and TCP samples with different porosity level (0, 20 and 40%) were incubated for 24 h with 5 ml of stock λ phage. -After incubation, the sample was washed once with LB medium to remove excess phage suspension. -Cultures of 30 ml of Escherichia coli K12 (A324) in LB liquid medium were performed for each condition. The growth of E. coli was performed at 37°C with agitation at 170 rpm.
Biological functionalisation

Part 3: Functionalisation of as-prepared scaffolds
**Biological functionalisation**

Bacterial growth kinetics (*Escherichia coli K12*) measured by optical density at 620 nm in presence of phage in ceramic supports with various porosity.
Part 1: Mimic bone structure

Ceramic slurry infiltration of organic skeleton

- 65% porosity (limits: 65-75%)
- 100 µm interconnections
- 500-600 µm pore diameter

First method

- HA 15 MPa
- TCP >15 MPa

Macro/meso-porous HA samples colonized by MC3T3-E1 osteoblasts after a 6-days culture (a).

First mineralized bone particles can already be detected (b).

With the collaboration of GRB University Lille2 (Prof Hildebrand’s team)