EUROPEAN COOPERATION
IN SCIENCE AND TECHNOLOGY

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

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Collaboration with
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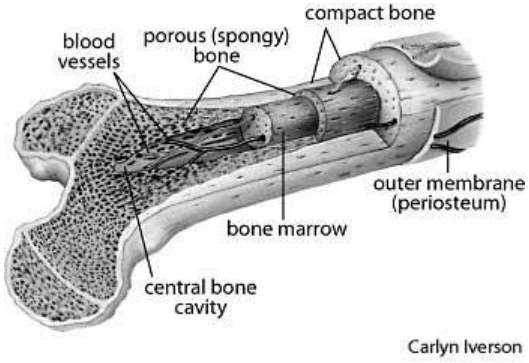
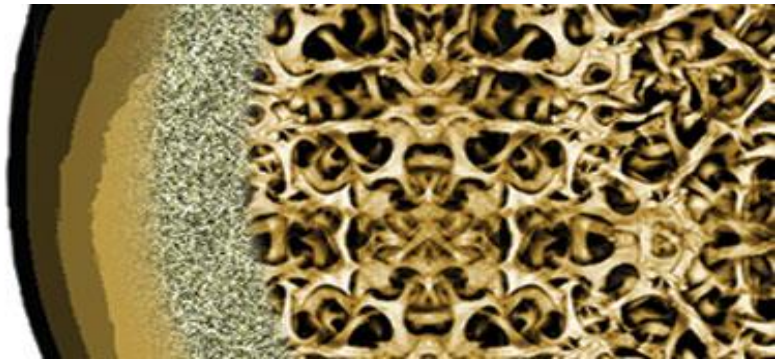
Introduction





Os compact

Os spongieux

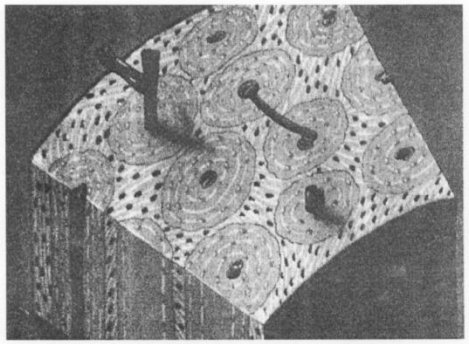
Compact bone

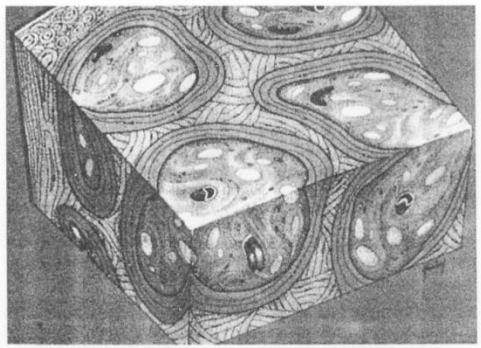
Porosity

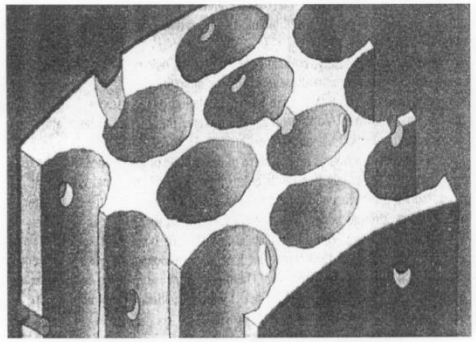
Φ : 190-230 µm

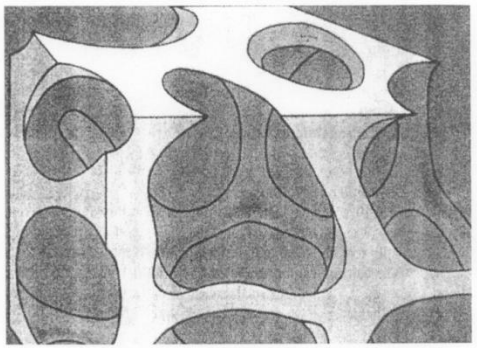
V : 65%

σ_c = 80-200 MPa









Spongy bone

Porosity

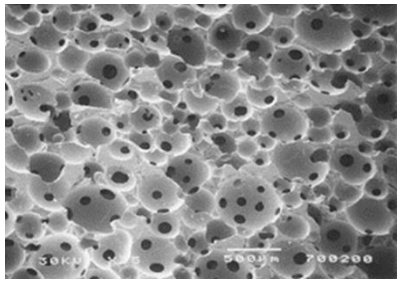
Φ : 500-600 µm

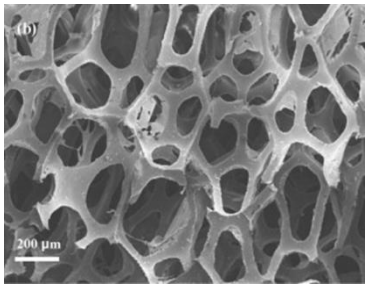
V : 80%

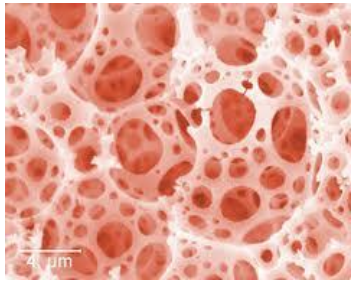
σ_c = a few tenths MPa

Introduction

- **Porous ceramics :** **wide range of structures**
- **Foams with cell walls randomly oriented in space**

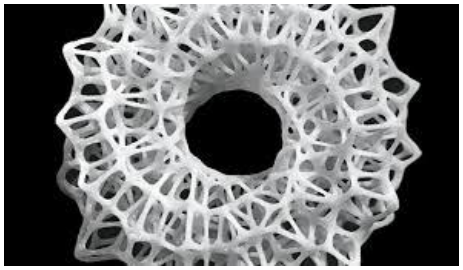


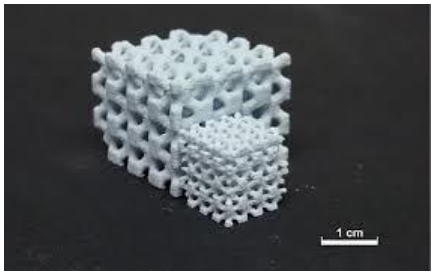


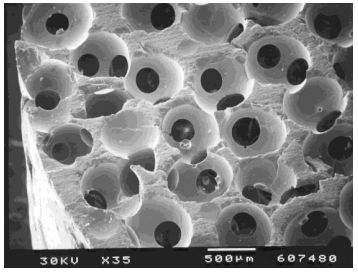


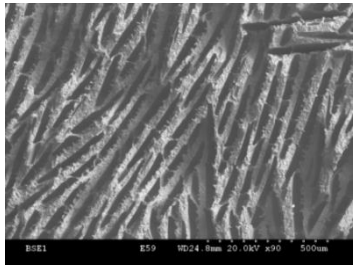
- **Honeycombs with polyhedral cells 3D arranged**

- **Connected hollow spheres or rods or fibres…**





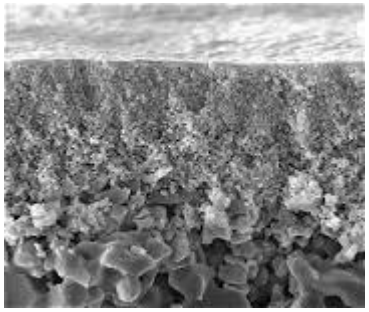


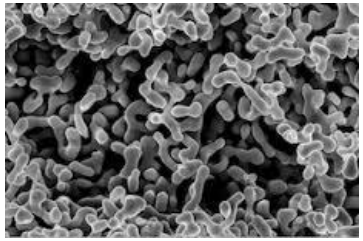


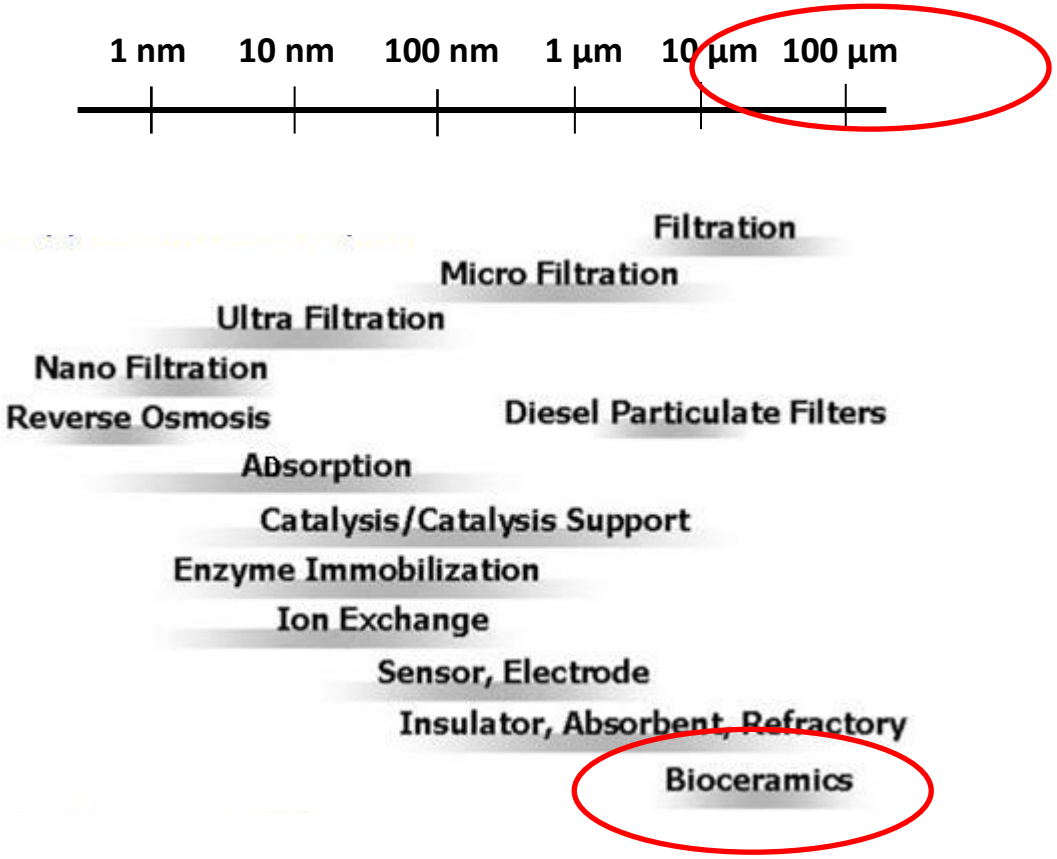
P. Colombo, Phil.Trans.R.Soc.A 2006

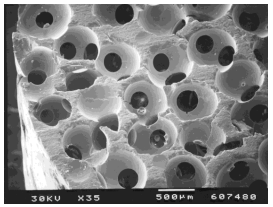
Introduction

- **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.**









Ohji and M.Fukushima. International Material Reviews 57, 2, 2012

Introduction

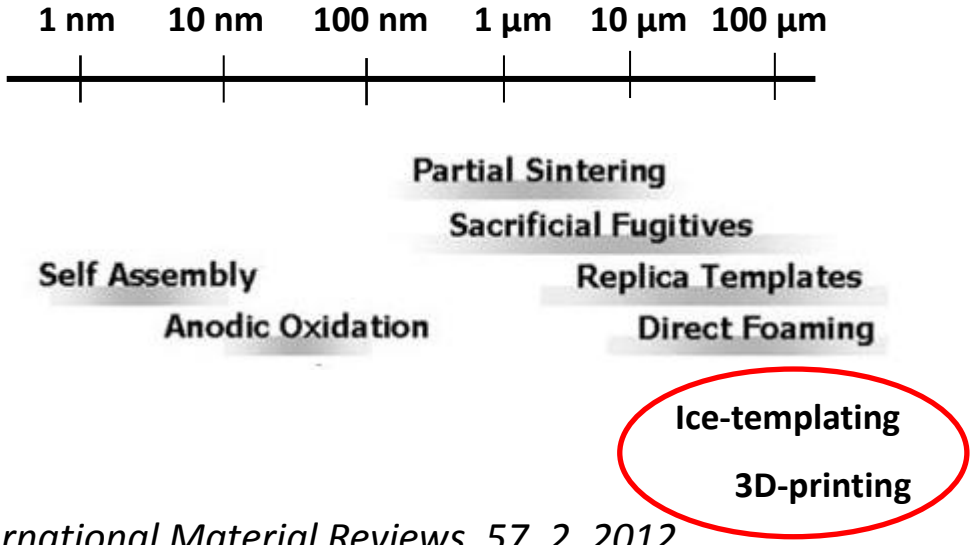
➢ **Various processing routes :**

Conventional methods :

partial sintering, sacrificial fugitives, replica templates, direct foaming , extrusion, aerogels…

Innovative methods:

ice-templating, additive manufacturing methods as 3D-printing,…



Ohji and M.Fukushima. International Material Reviews 57, 2, 2012

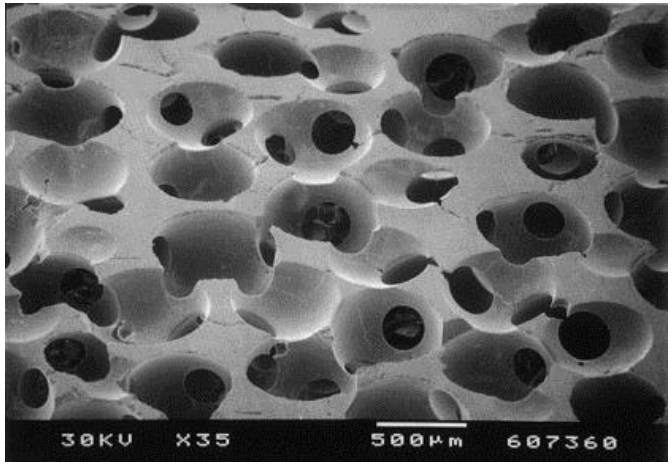
Introduction

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

M. Descamps et al., J. Eur. Ceram. Soc. 28 (2008) 149



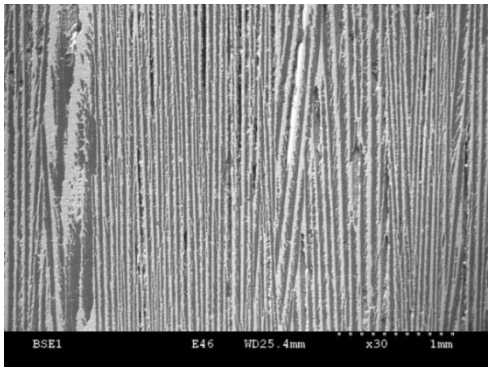
This technique type has been industrially applied by SOFAMOR – DANEK, BIOCETIS and BIOLU since a few years.

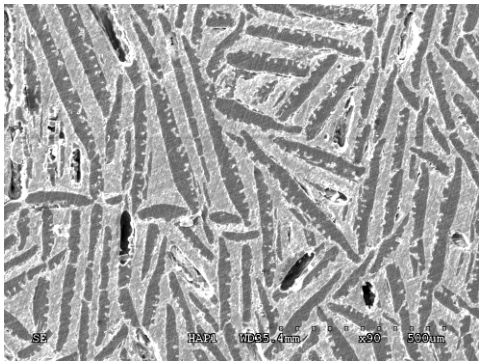
Introduction

Three methods to manufacture macroporous and microporous ceramic scaffolds:

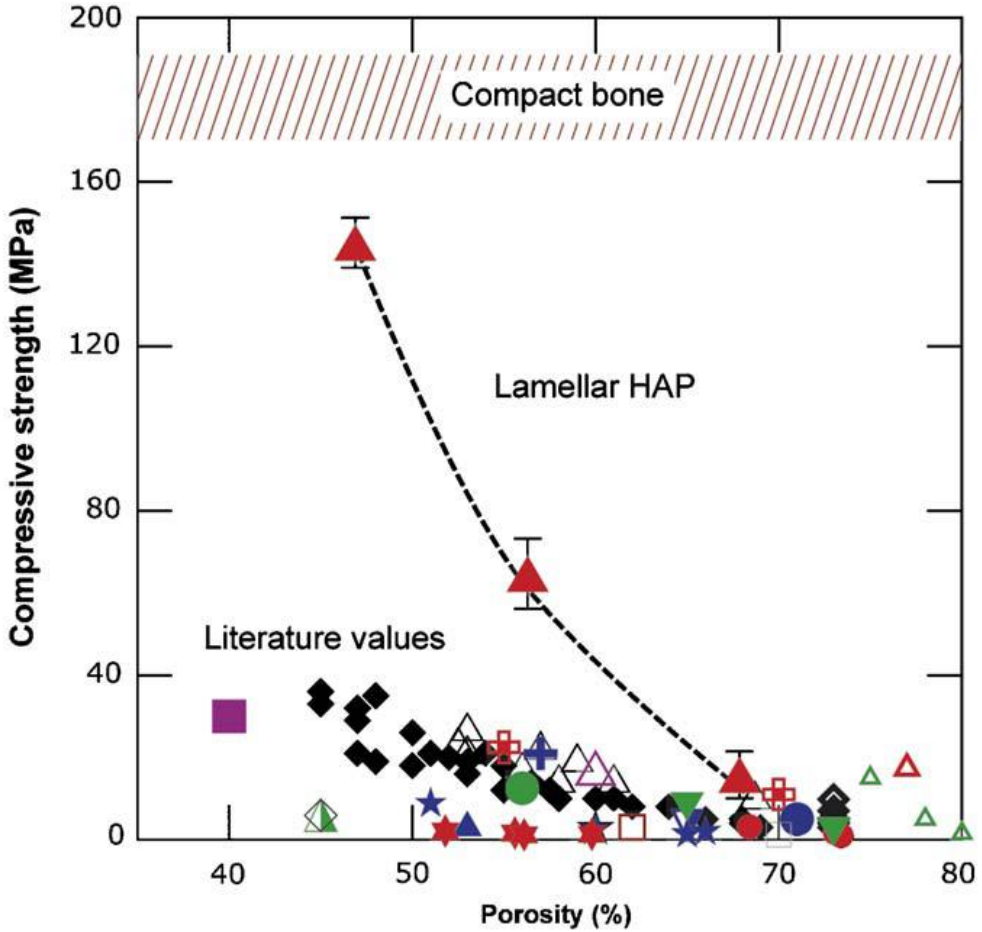
- The first one by ceramic slurry infiltration of organic bead skeleton, permits to have an isotropic structure with close control of the pore size and the interconnection size but relatively low mechanical resistance.

- 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 σ_c and pore size (S. Deville).

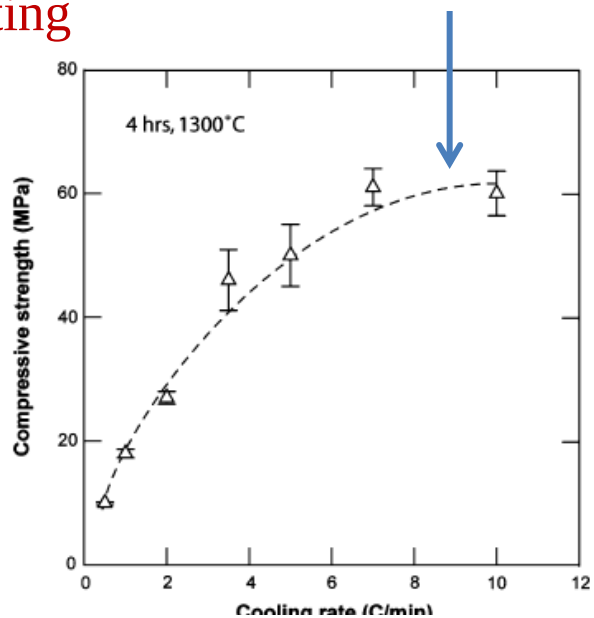


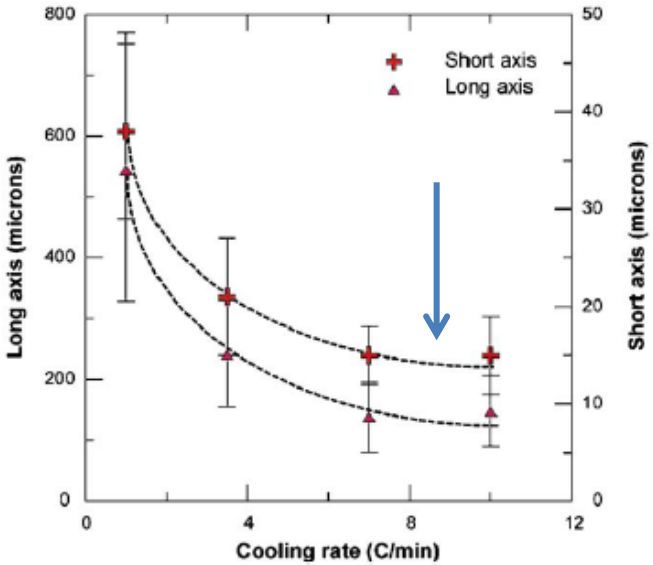


Ceramic casting by ice templating



S. Deville et al,, Biomaterials 27 (2006) 5480–5489





Up today substitutes present lower σ_c values than compact bone one's, the freeze casting excepted.

But high σ_c values correspond to smaller pore sizes than usually used for bone substitutes.

Introduction

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.

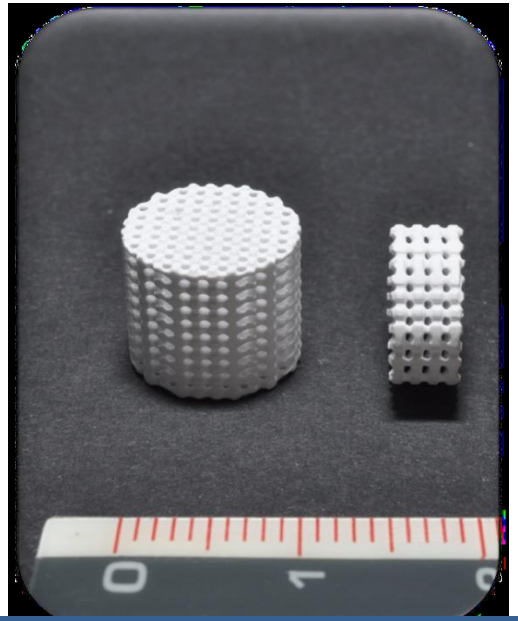
- The second one by ceramic slurry cast using ice templating, permits to develop anisotropic structure which allows higher compressive strength but compromise has to be found between σ_c and pore size.

-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.

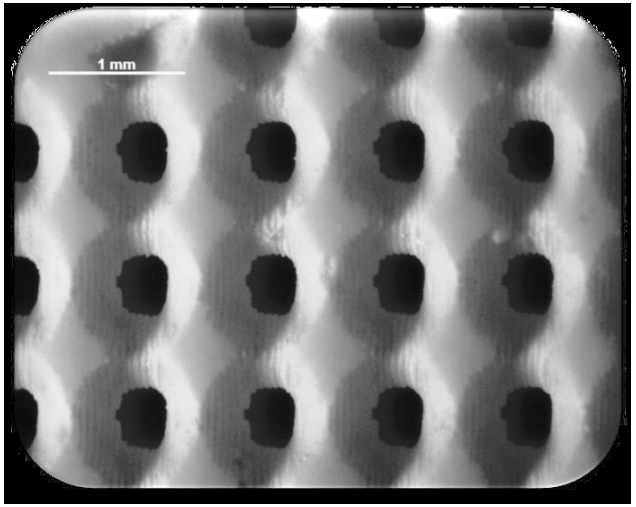
3D Printing of ceramic slurry in UV sensitive resin

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
Additive Manufacturing of Ceramics: A Review J.Ceram.Sci.Tech 05 [04] 245-260 (2014)



Lithoz GmbH



By courtesy of Dr Johannes Homa

Introduction

The objectives of our study is to compare the mechanical properties and cell colonization ability of these different structures

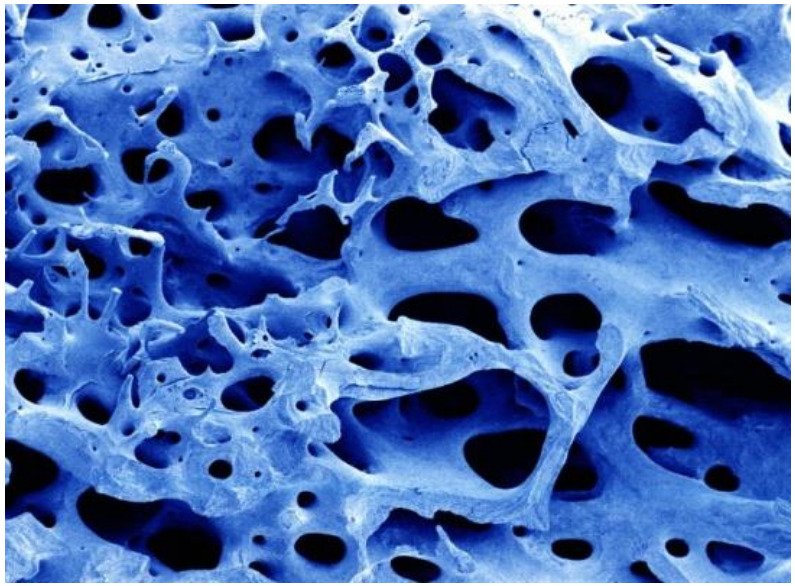
Part I: Presentation of the three shaping methods applied to calcium phosphate materials and the material structural properties

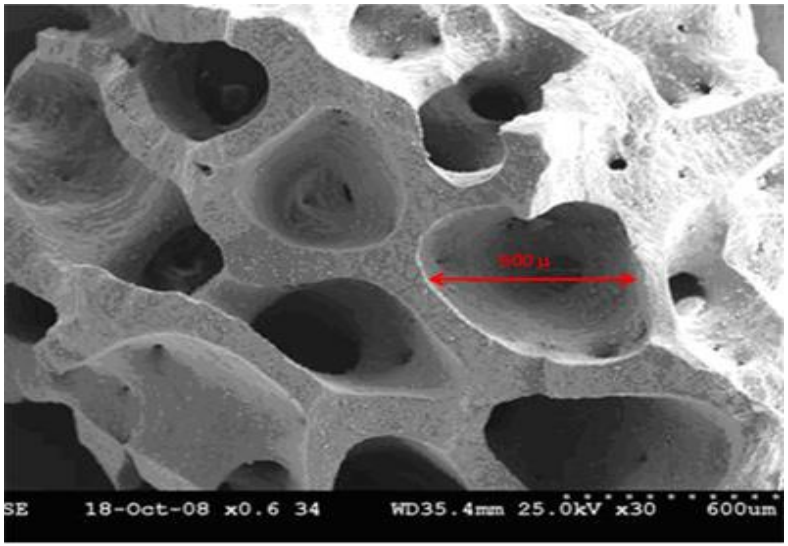
Part II: Comparison of cell colonization ability by static in vitro tests for the two methods: replica and freeze casting

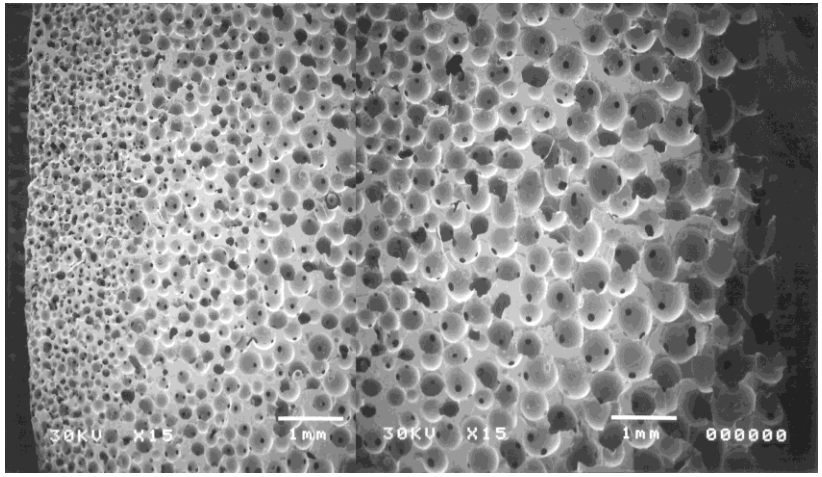
Part III: Functionalization of as-prepared scaffolds by drug and phage impregnation through microporosity

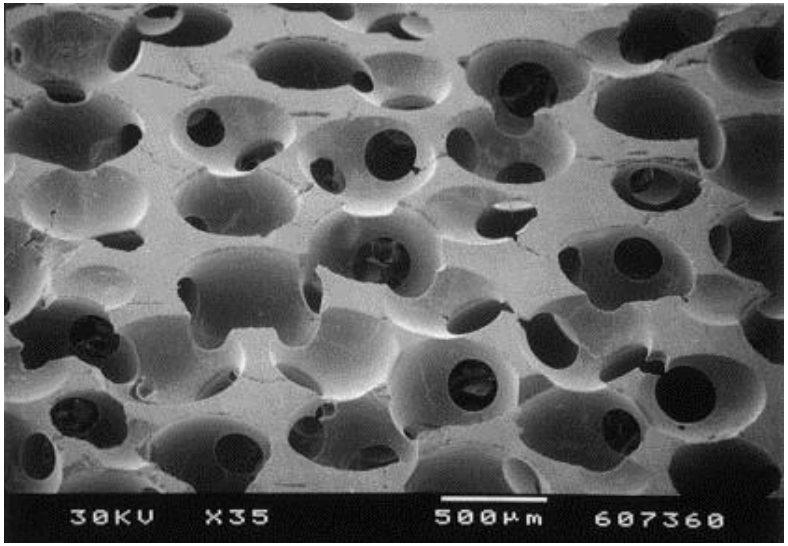
First method: Ceramic slurry infiltration of organic skeleton

Human bone









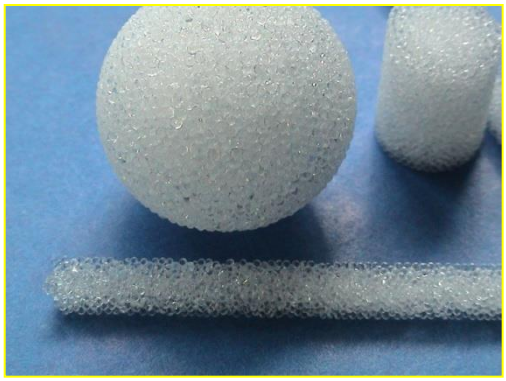
Patent FR2823305

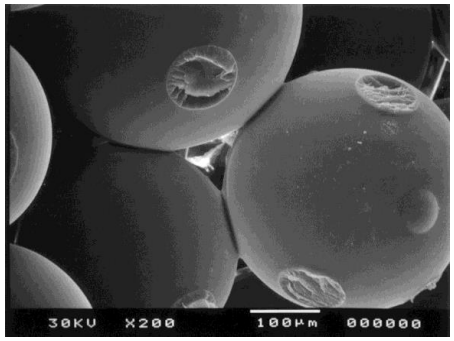
First method: Ceramic slurry infiltration of organic skeleton

Organic skeleton preparation

Chemical forming with acetone

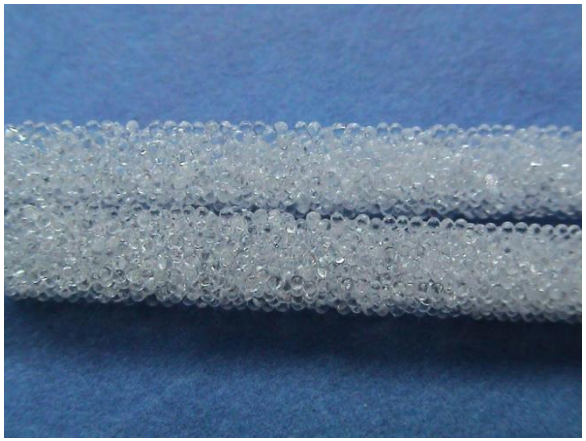
under pressure



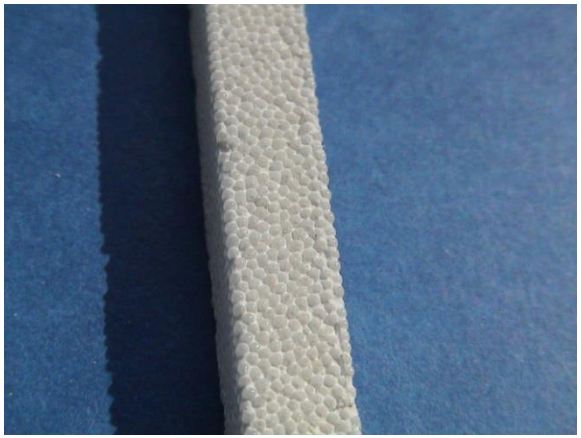


- Bonding between PMMA beads (scaffold)
- Controlled diameter bonding (Interconnection) depends on time, temperature, pressure

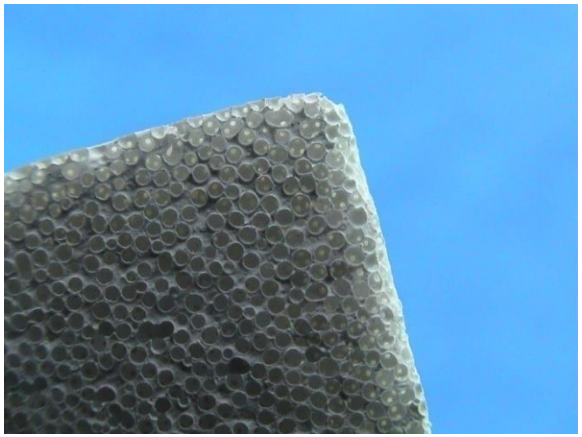
First method: Ceramic slurry infiltration of organic skeleton



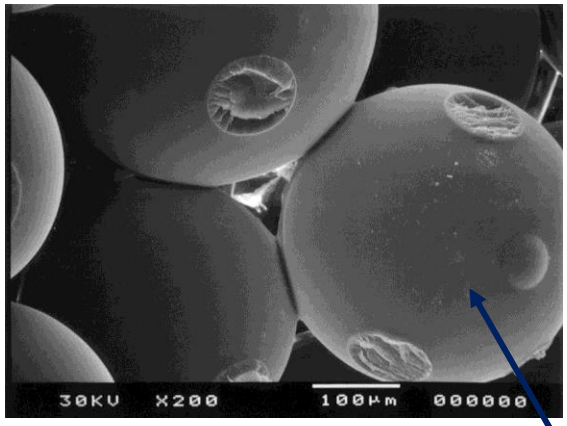
Shaping

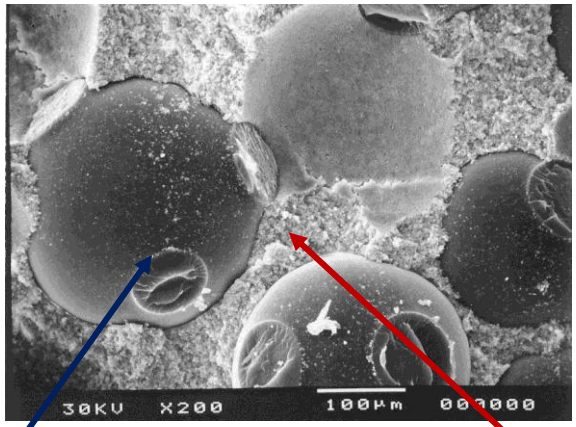


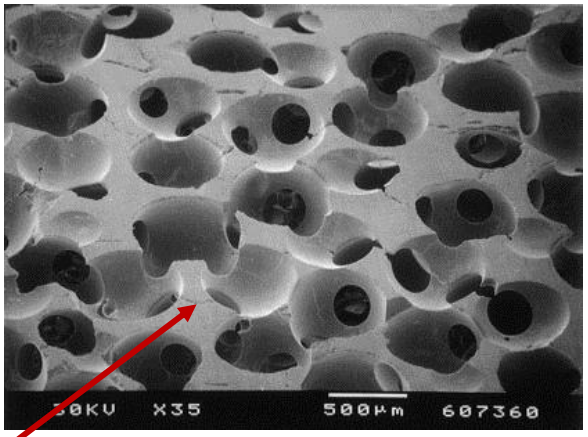
Impregnation



Debinding







PMMA beads

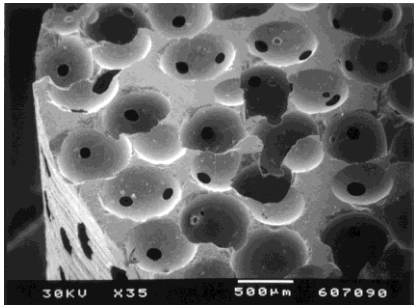
ceramic

First method: Ceramic slurry infiltration of organic skeleton

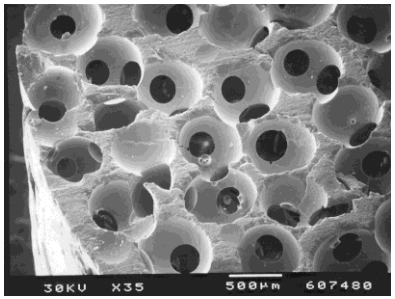
- Control of pore size depending on PMMA beads size
- Control of interconnection diameters: Id

PMMA beads (500 - 600 µm)

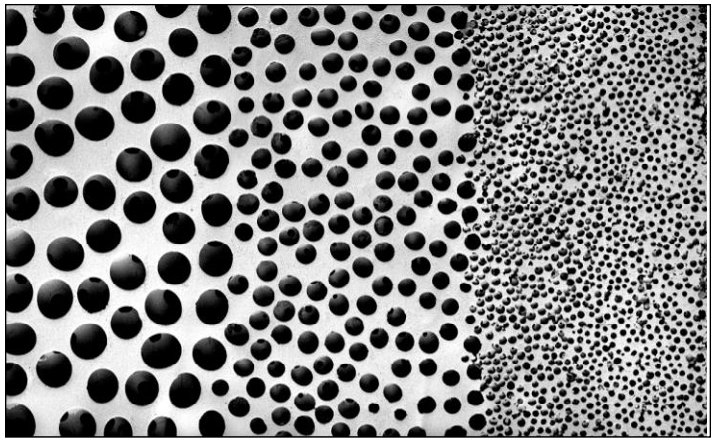
Id : 60 µm

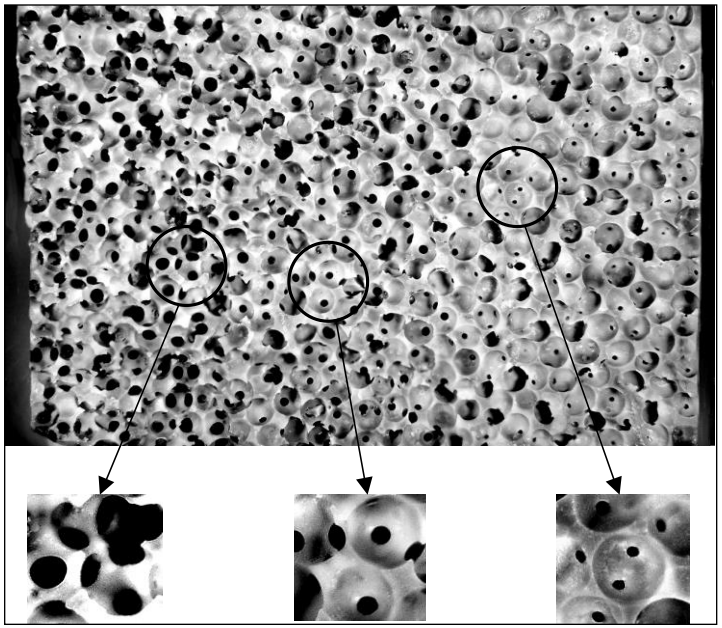


Id: 260 µm



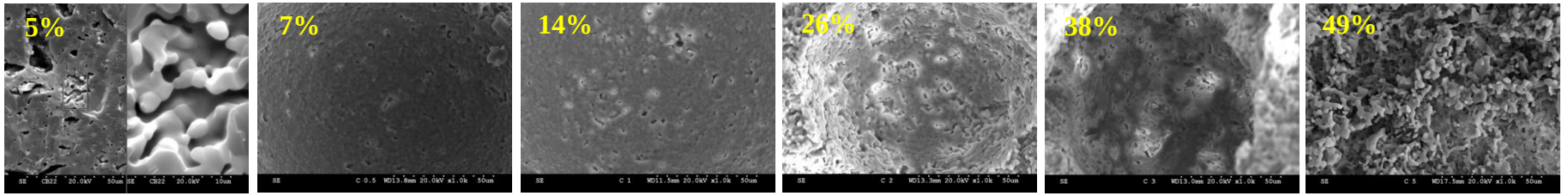
- Control of porosity gradient in pore size and interconnection size

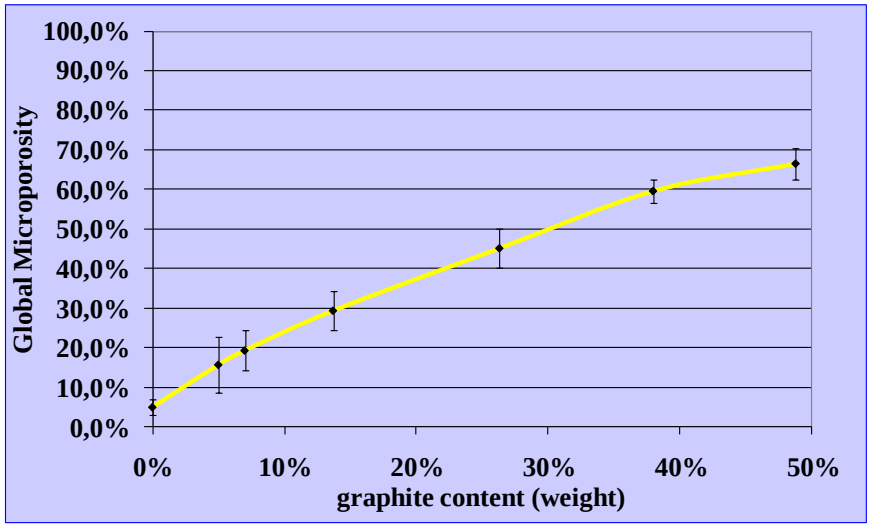


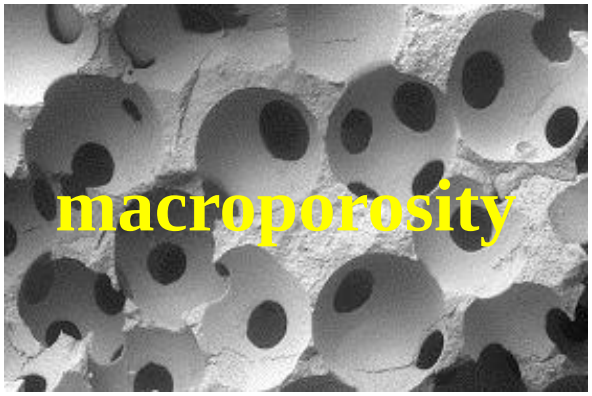
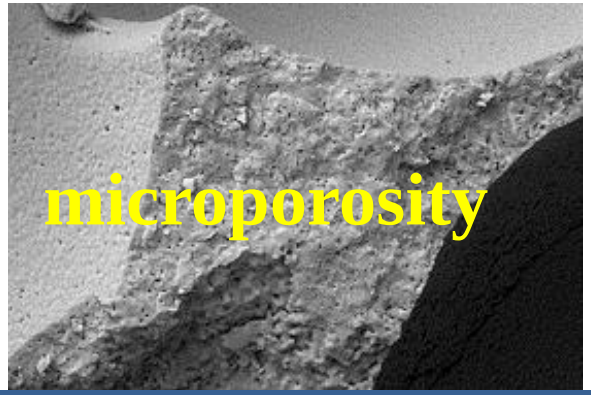


First method: Ceramic slurry infiltration of organic skeleton

Possibility to add microporosity by mixing graphite as micropore forming agent.

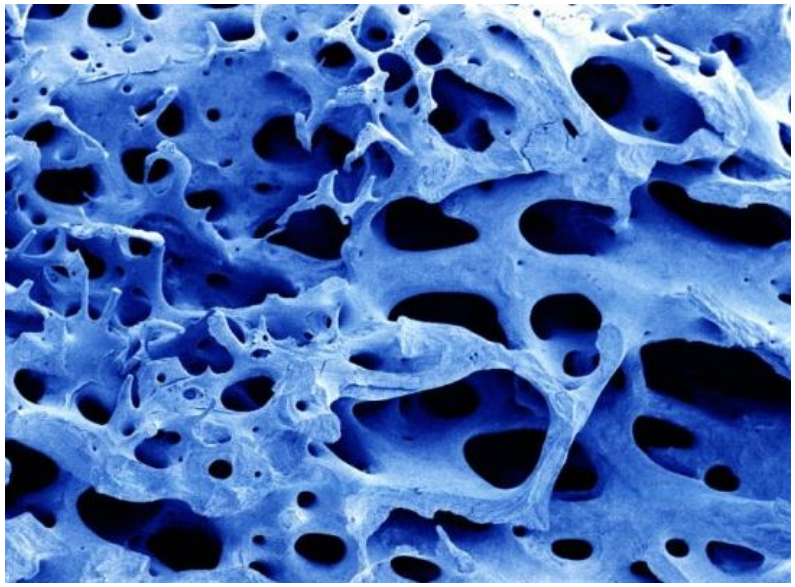


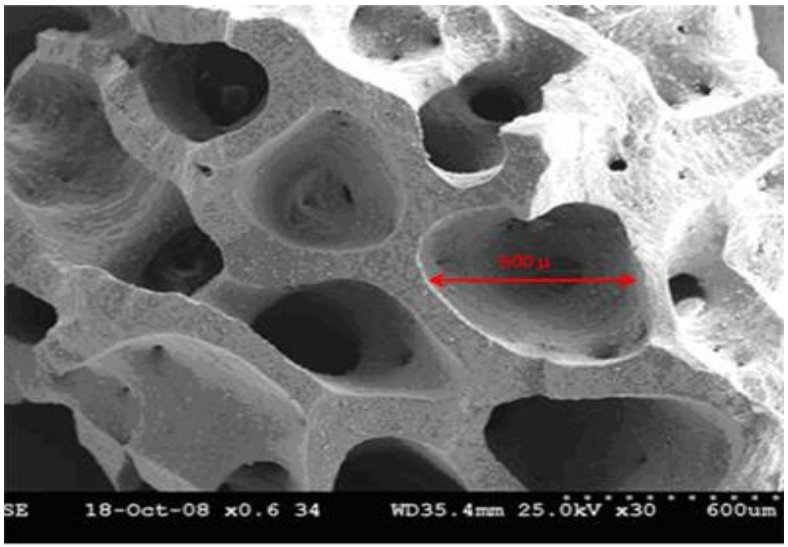


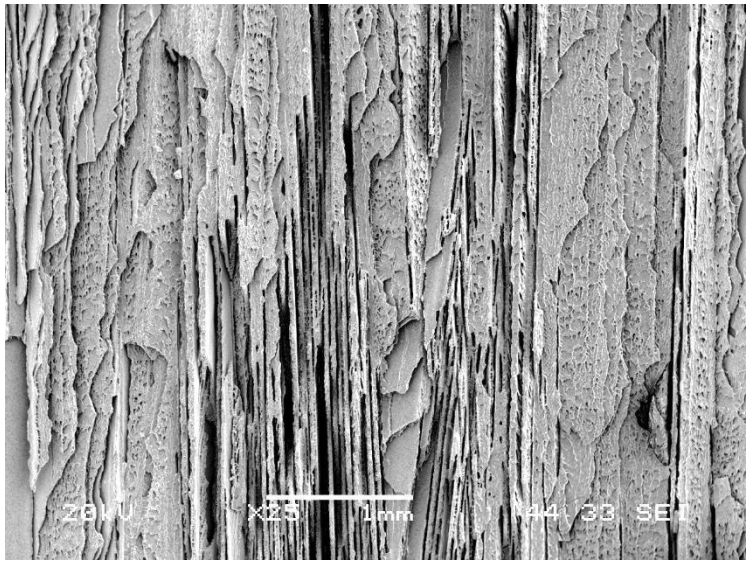
Controlled  and 

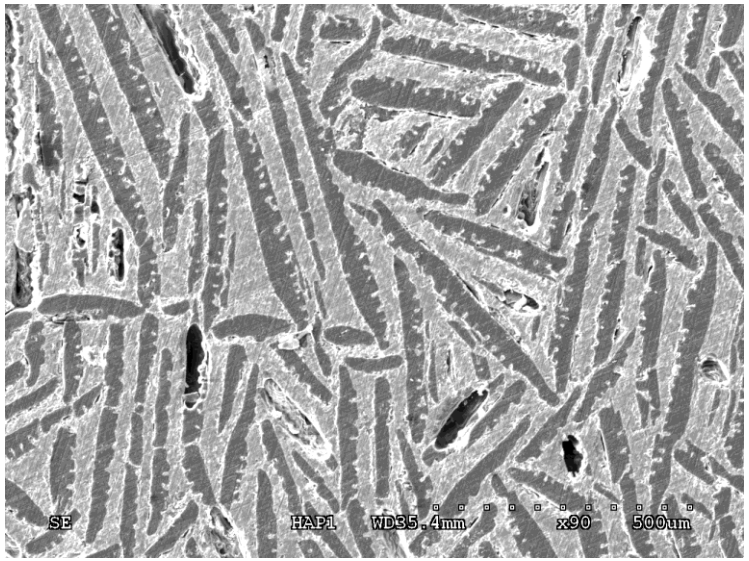
Second method: Ceramic slurry ice templating

Human bone



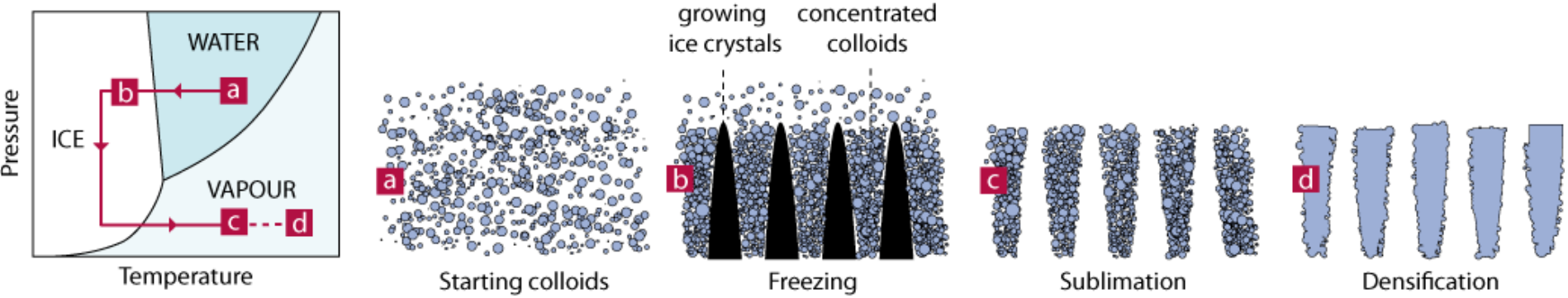






D. Hautcoeur Ph D UMons-BCRC Nov 2014

Second method: Ceramic slurry ice templating

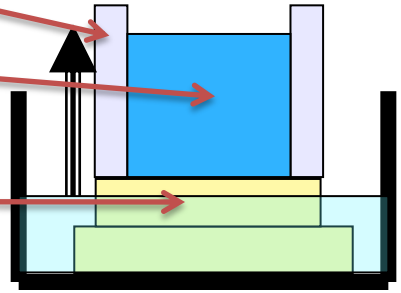


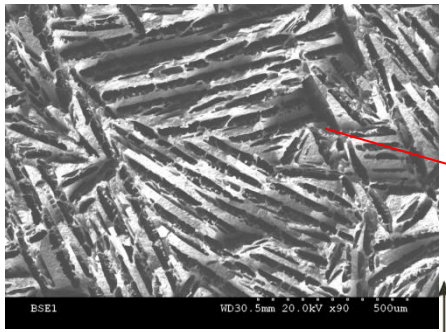
Mold
insulating wall

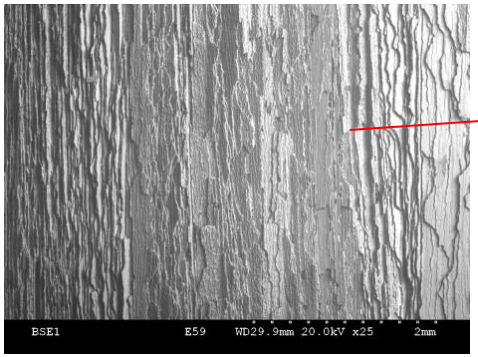
Slurry

Conductive material

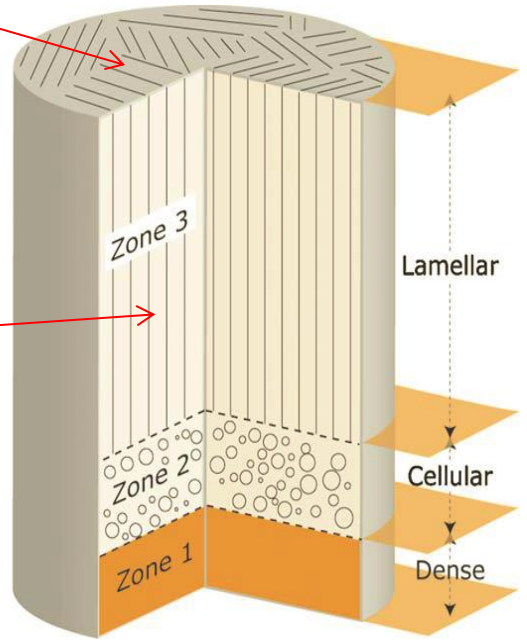
Cooling liquid







Ice growth direction



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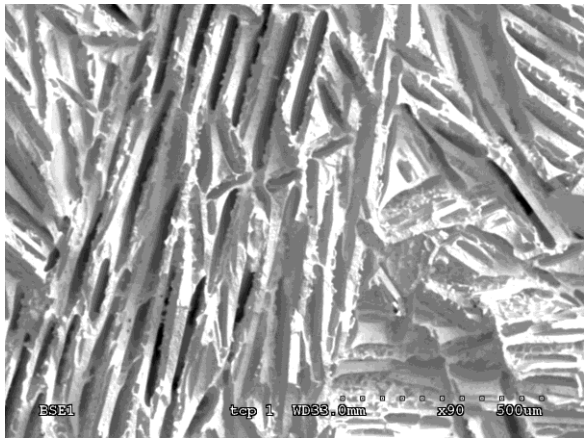
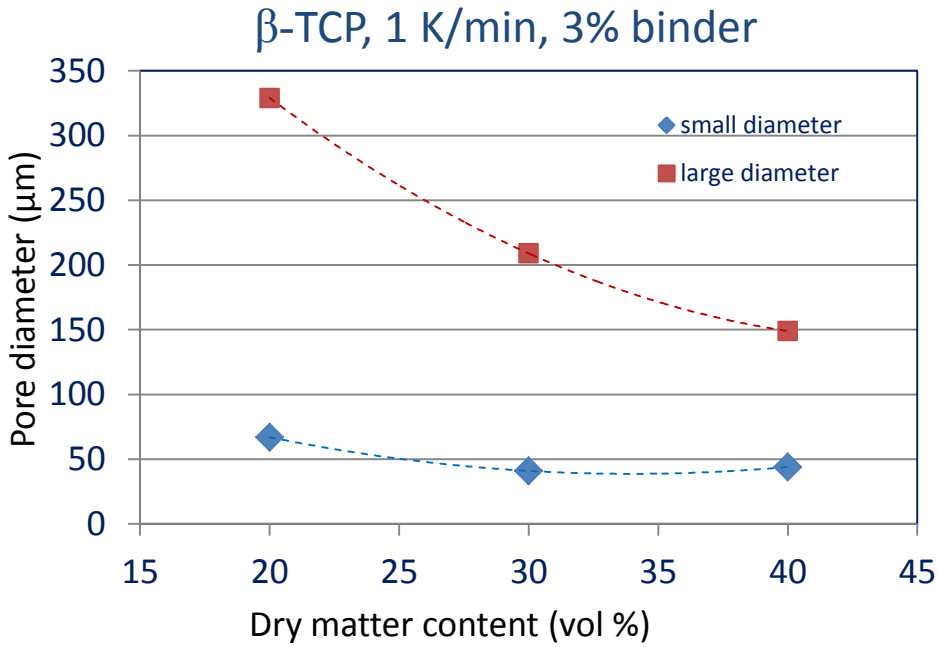
S. Deville et al,, Biomaterials 27 (2006) 5480–5489

Second method: Ceramic slurry ice templating

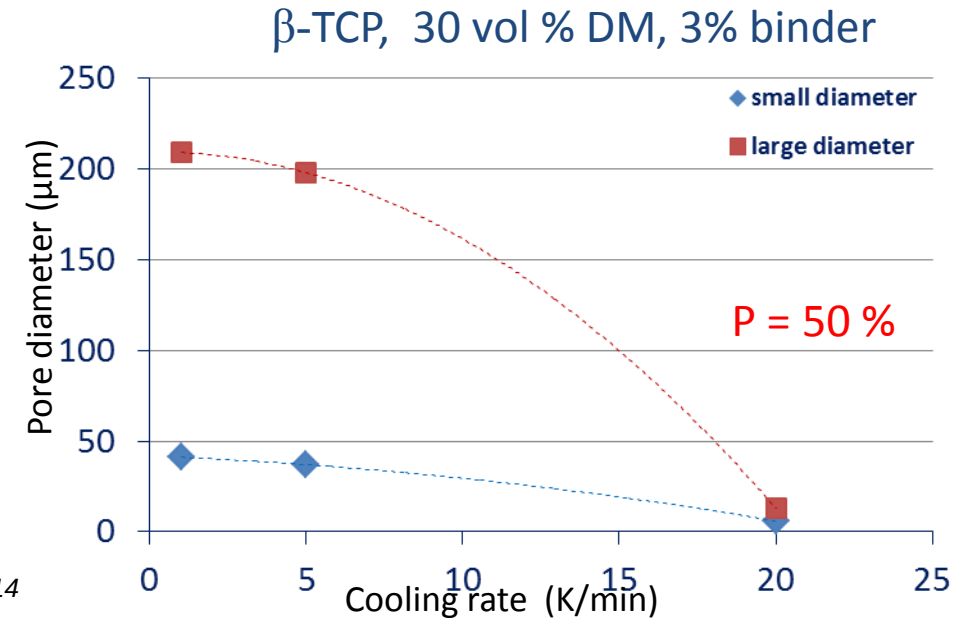




Pore long axis size:

- **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 %



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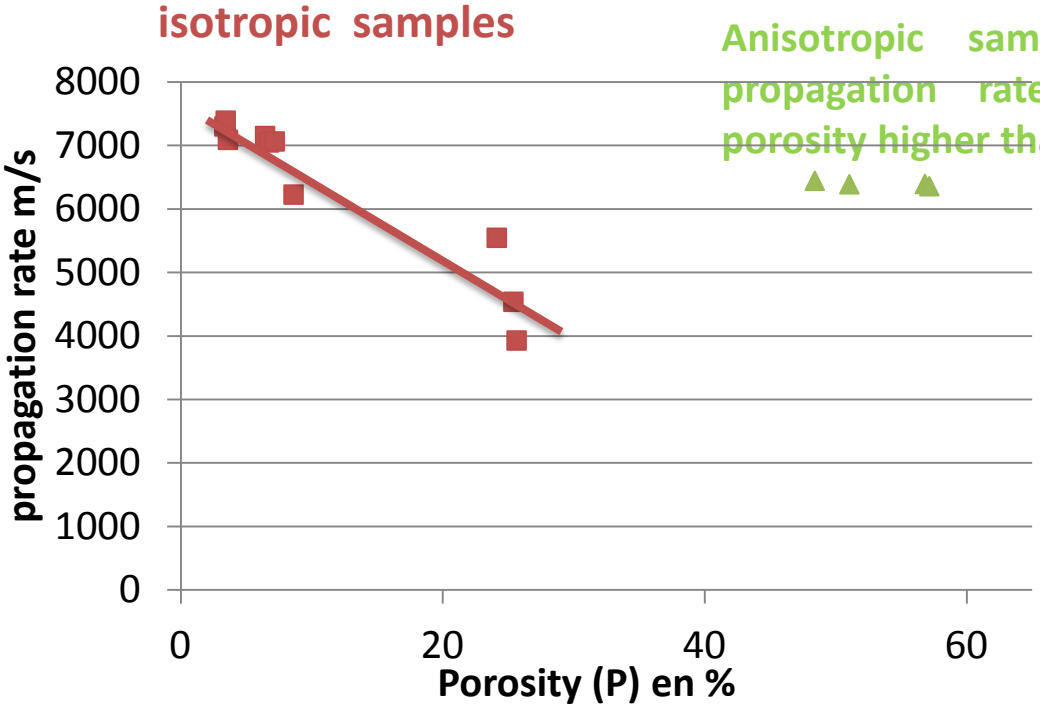
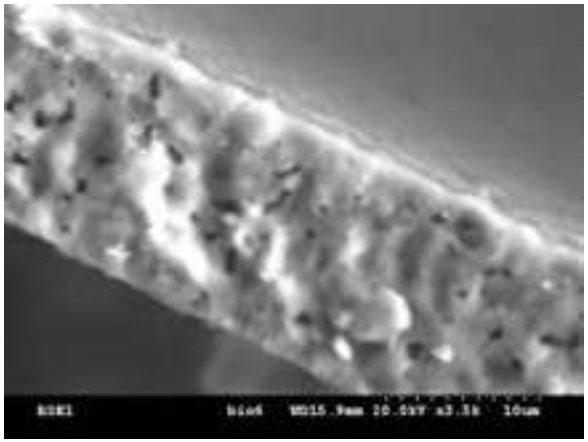
Second method: Ceramic slurry ice templating

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



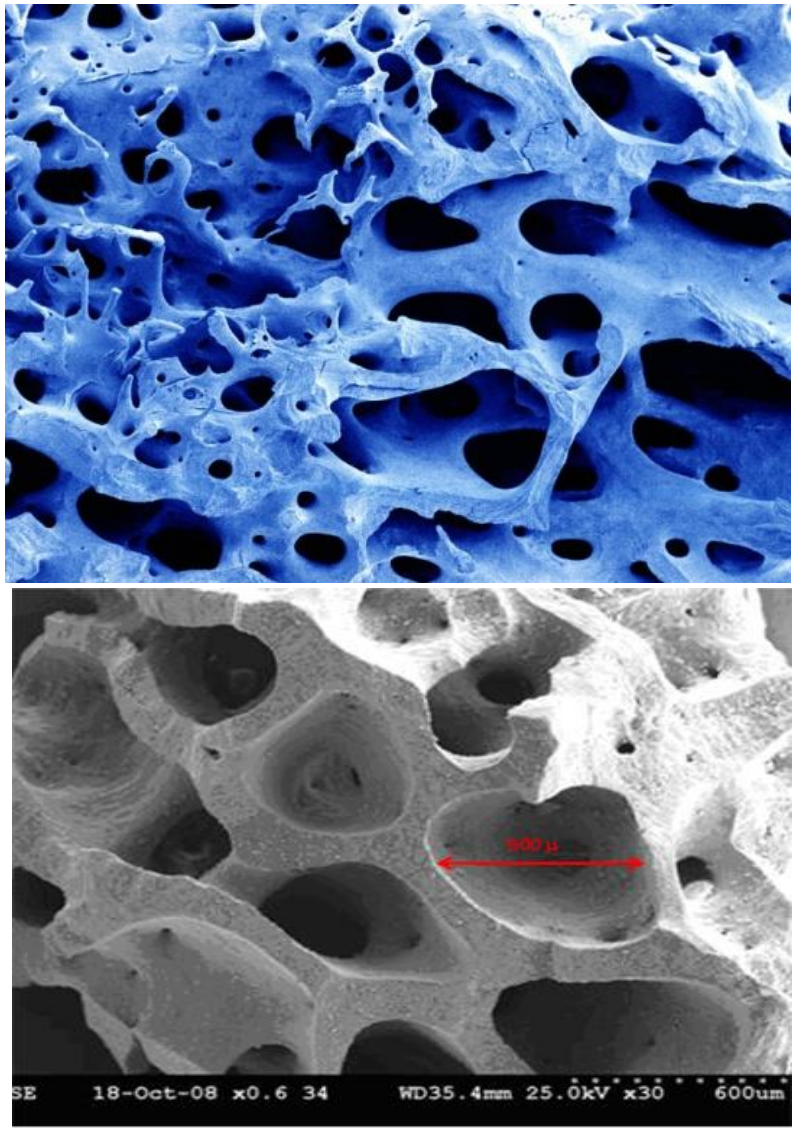


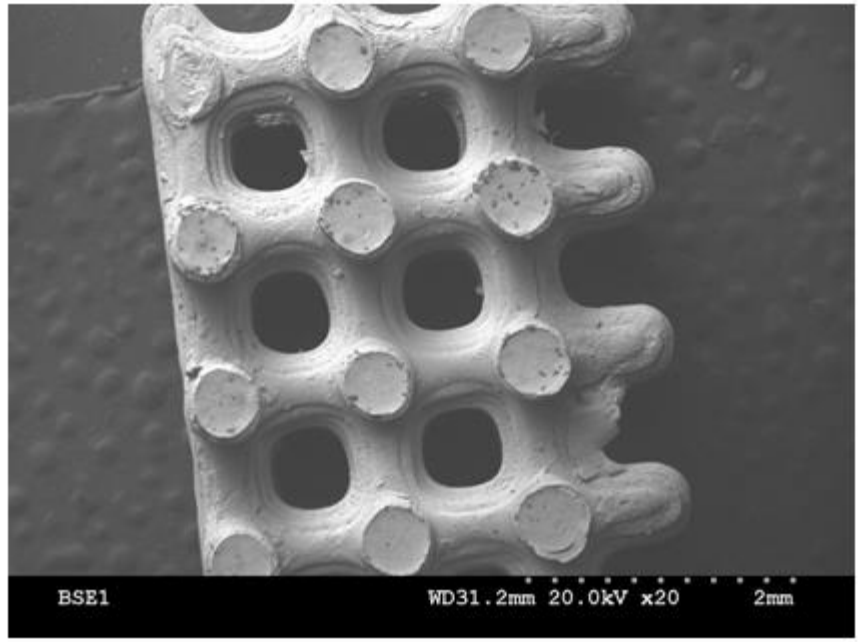
10 % microporosity

Slurry composition and freezing rate	Ceramic walls porosity % (average)
28% Vol.+ 3% PEG1000 and 1.3°C/min	11.5± 0.2
33% Vol.+ 3% PEG1000 and 1.3°C/min	11.2 ± 0.2

Third method: 3D printing of ceramic slurry

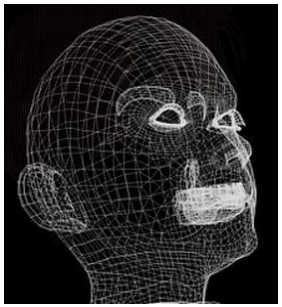
Human bone



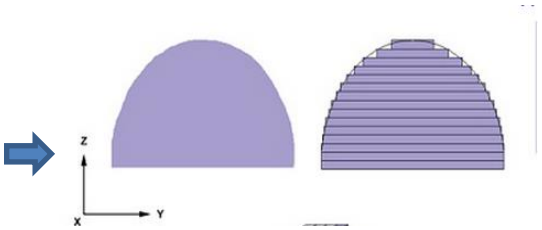


JC Hornez LMCPA January2015

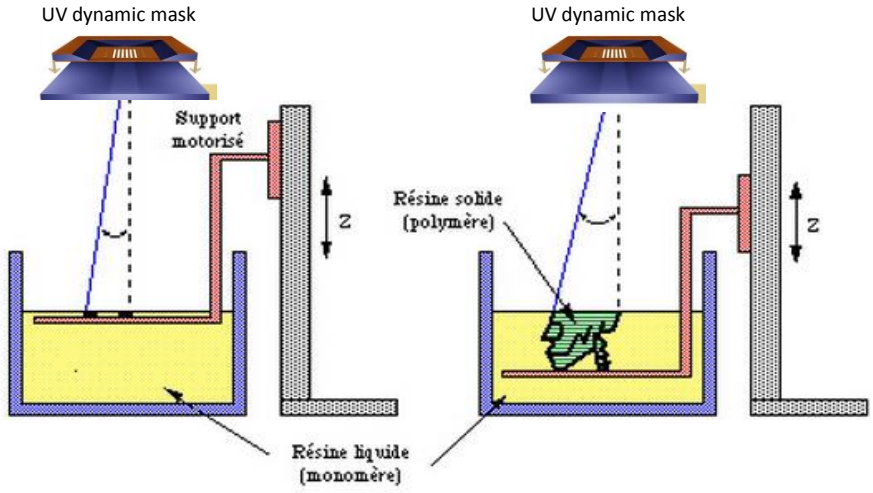
Third method: 3D printing of ceramic slurry



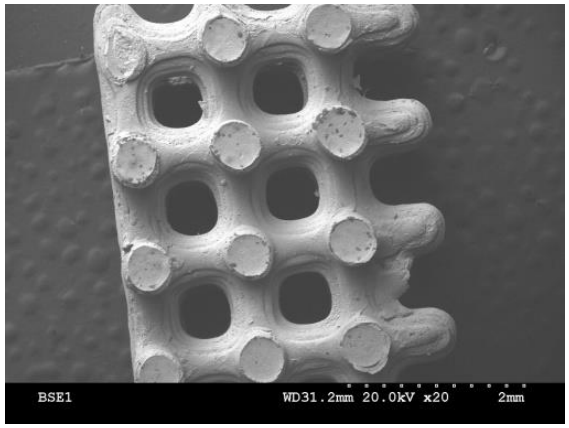
CAO

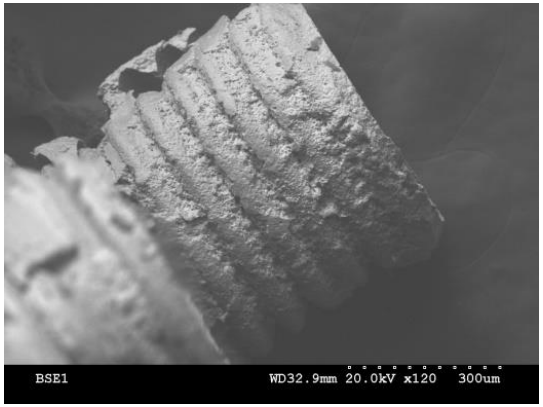


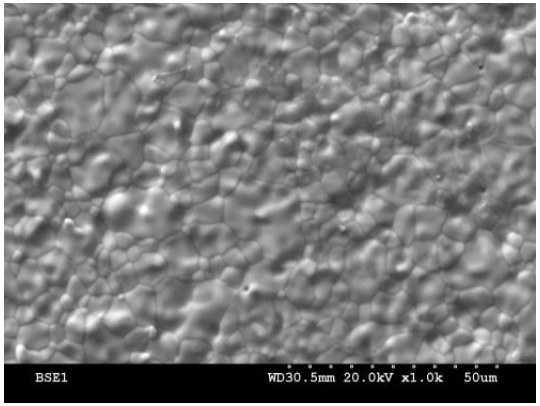
3D slicer



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

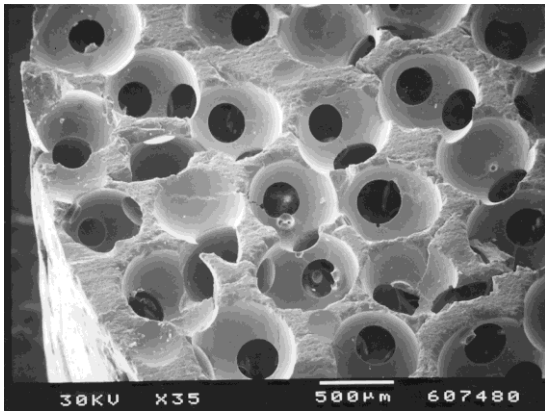






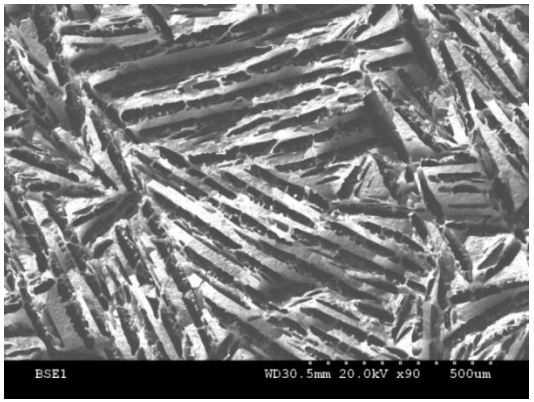
Comparison of the three different macroporosities

Isotropic porosity



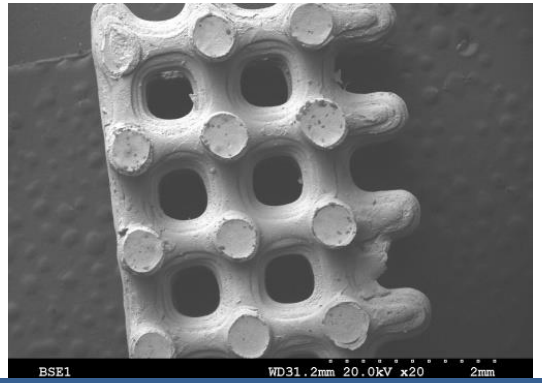
- 65% porosity (limits 65-75%)
- 100 µm interconnection
- 500-600 µm spherical pore diameter
- HA: 15 MPa, TCP: >15 MPa
- Possibility for pore size and content gradient

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
  σ_c : 21 MPa
  β-TCP: 40% porosity, 280/35 µm pore Ø
  σ_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

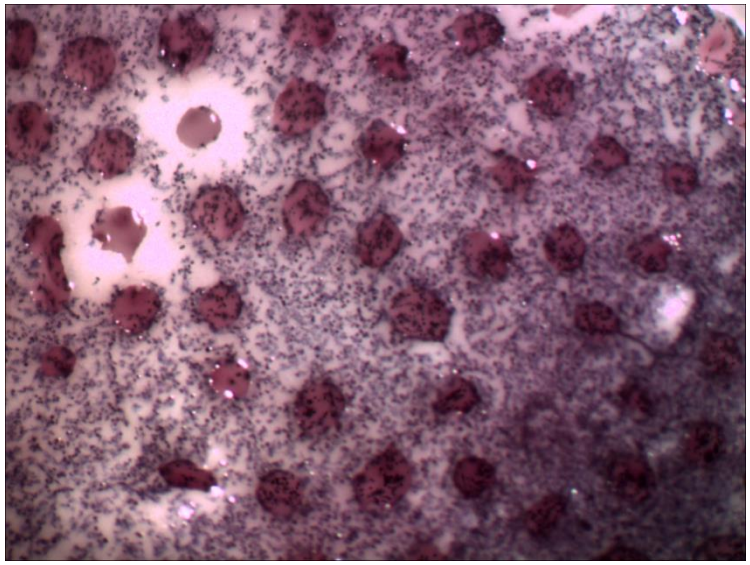
Colonization tests with MG63 osteoblasts

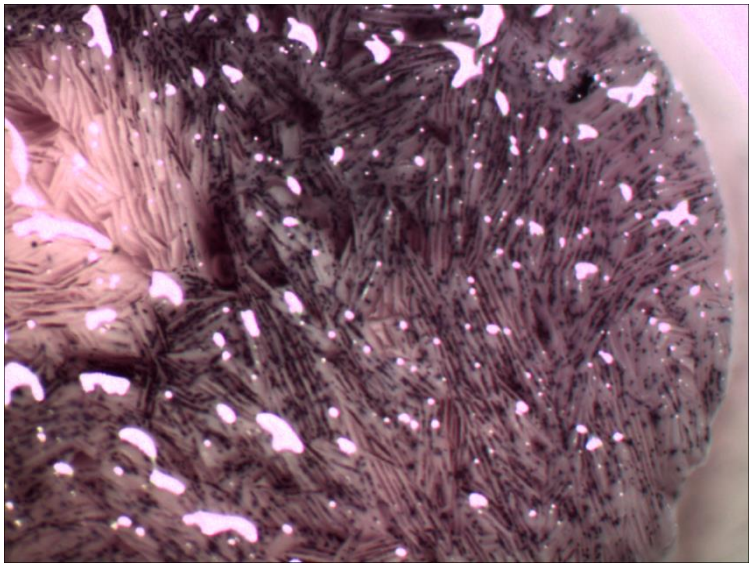
1 day

Organic bead skeleton infitration

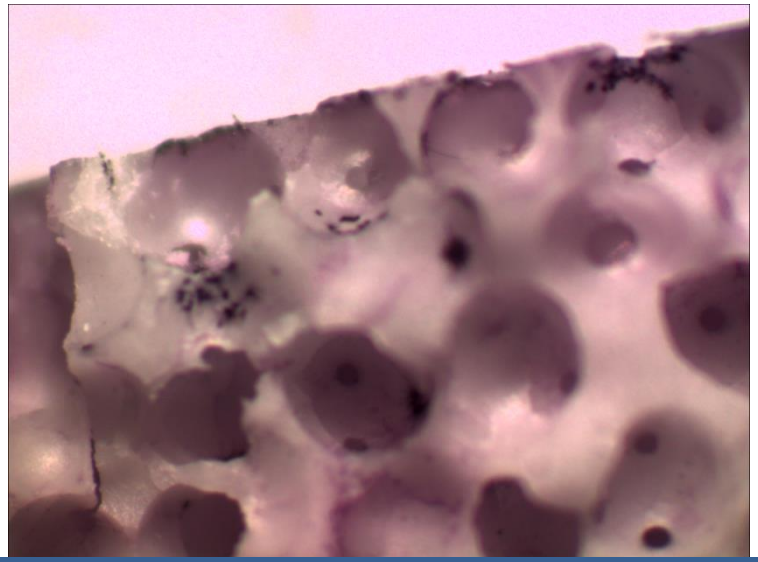
Ice - templating

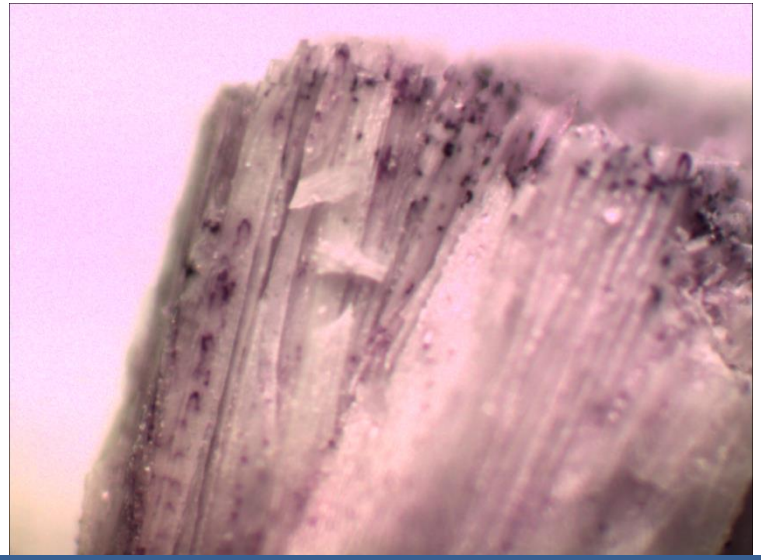
Top





Slice



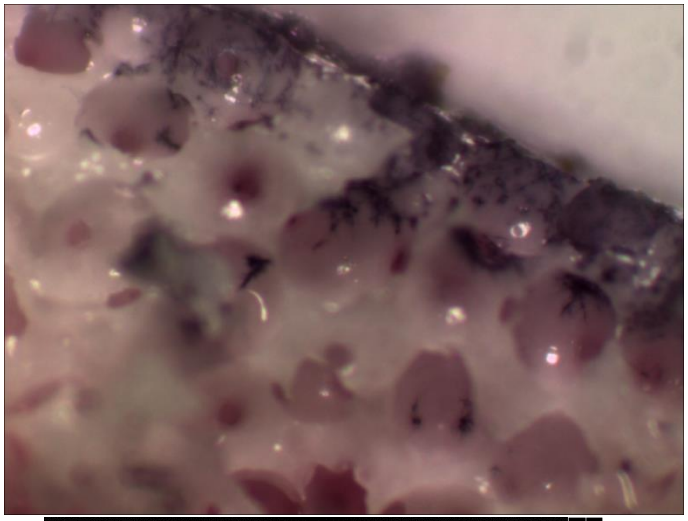


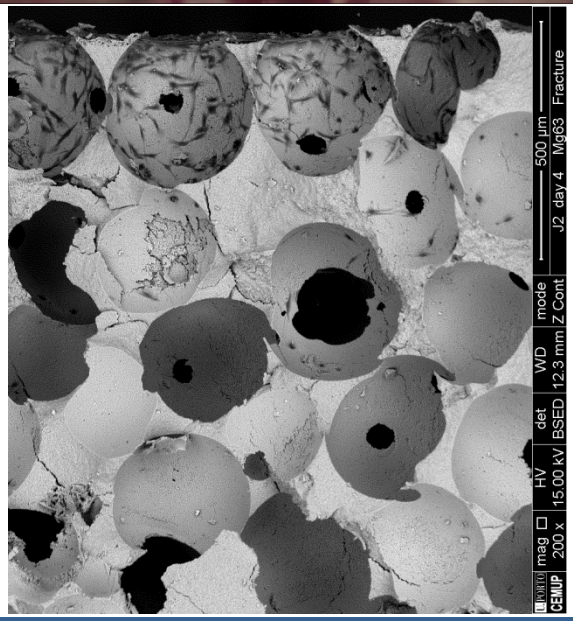
Colonization tests with MG63 osteoblasts

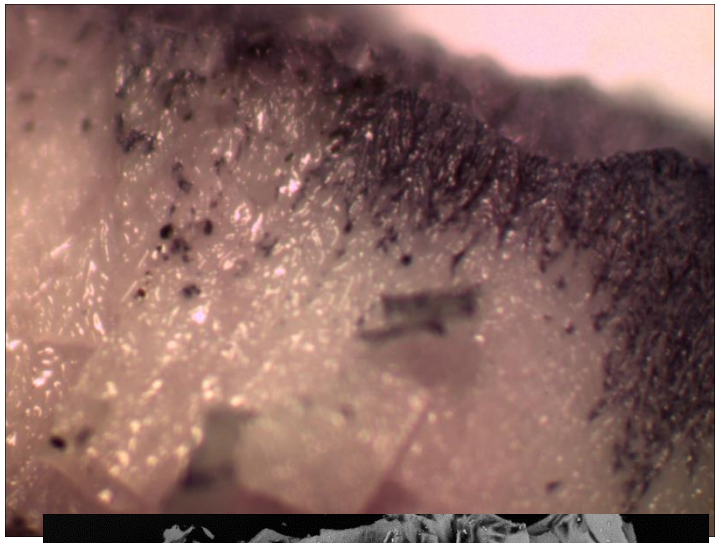
Organic bead skeleton infitration

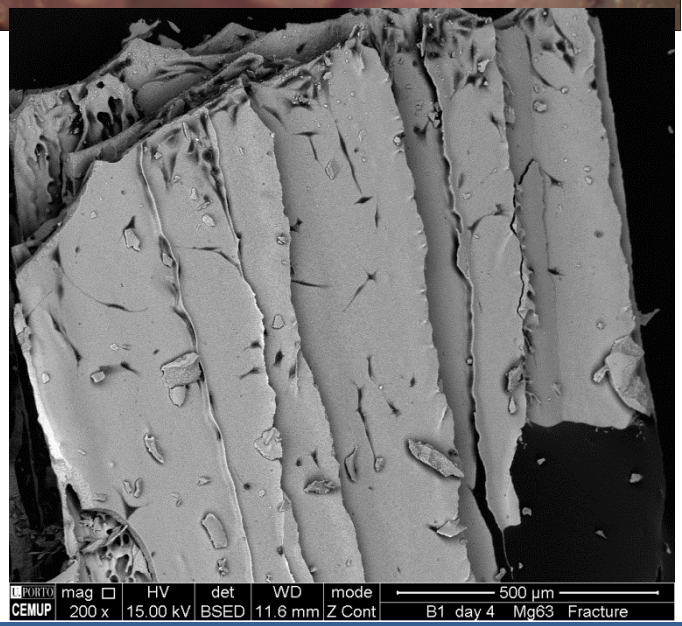
4 days

Ice - templating









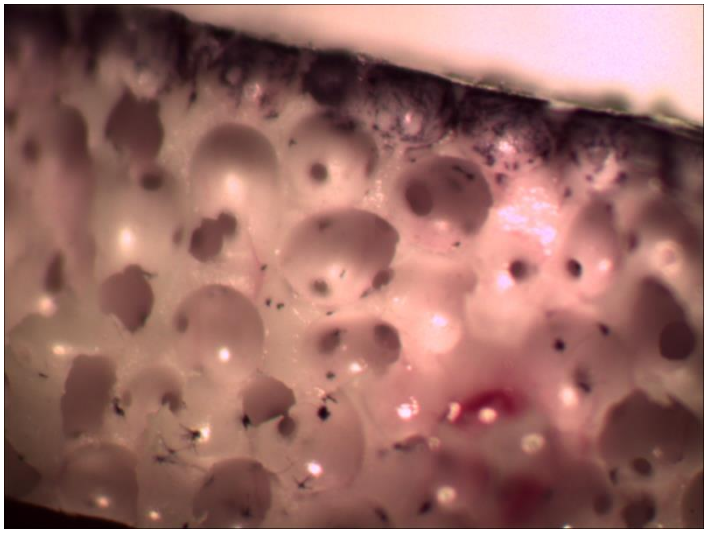
Colonization tests with MG63 osteoblasts

Organic bead skeleton infitration

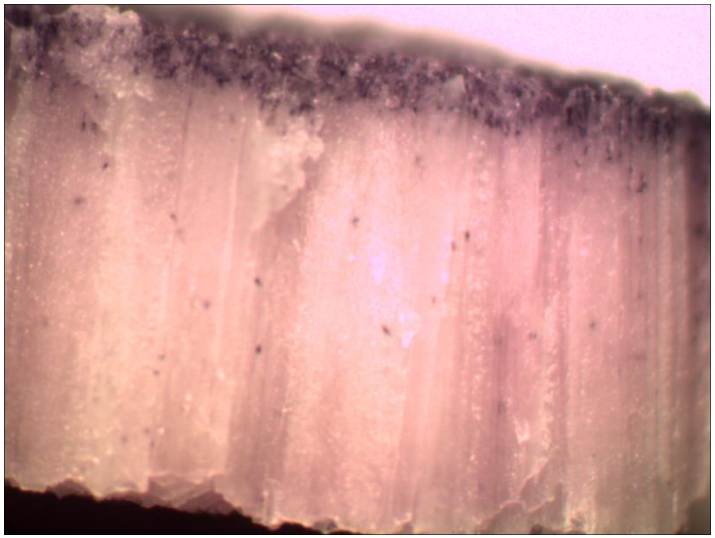
7 days

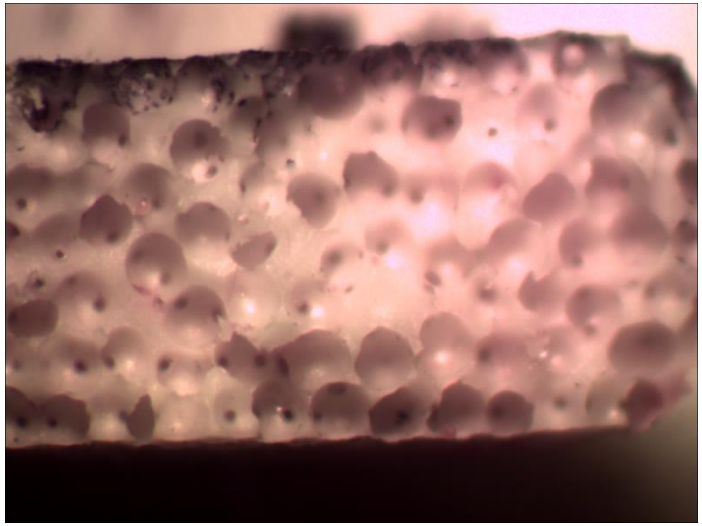
Ice - templating

Beads 750 µm



37/200 µm



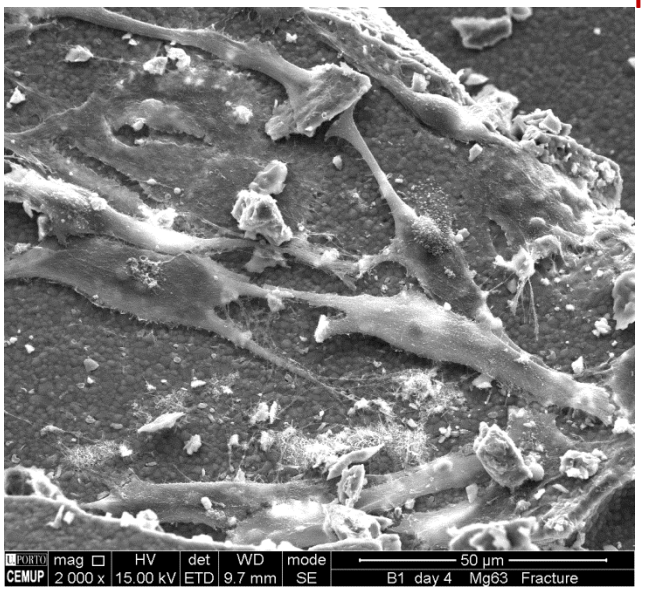


Beads 350 µm

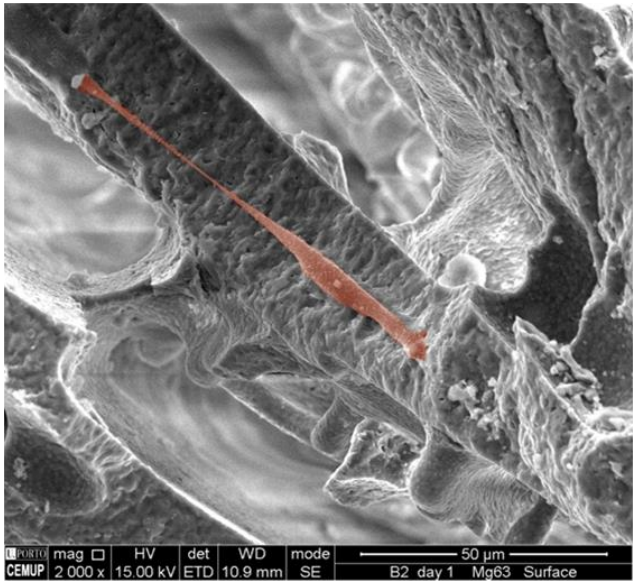
Colonization tests with MG63 osteoblasts

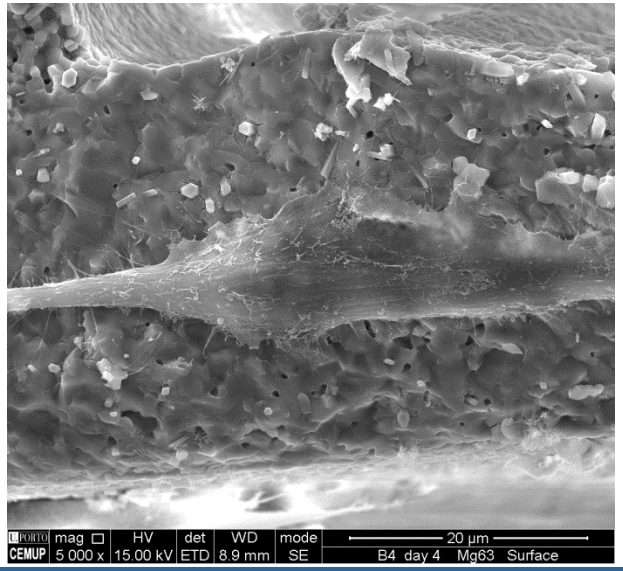
Ice - templating

198 µm/37 µm

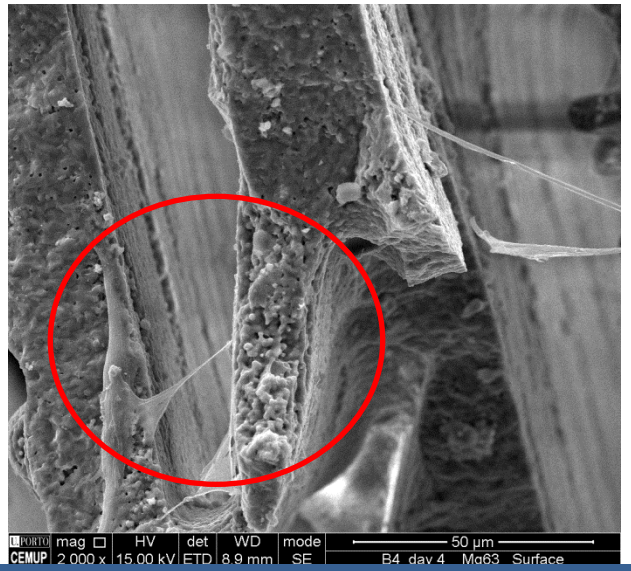


1 day

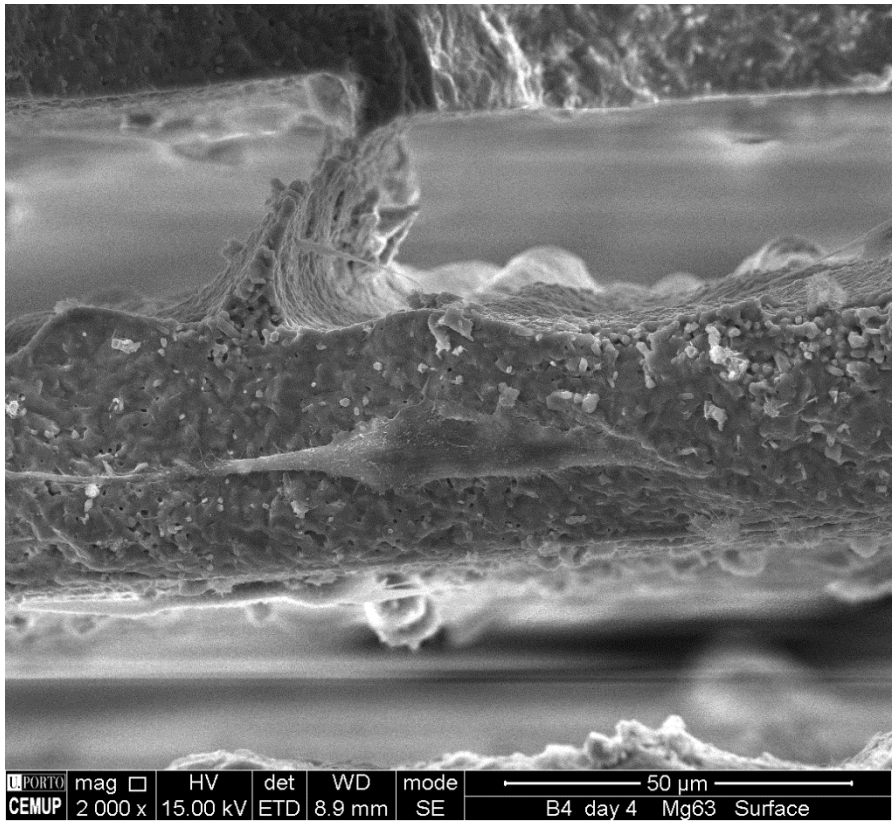


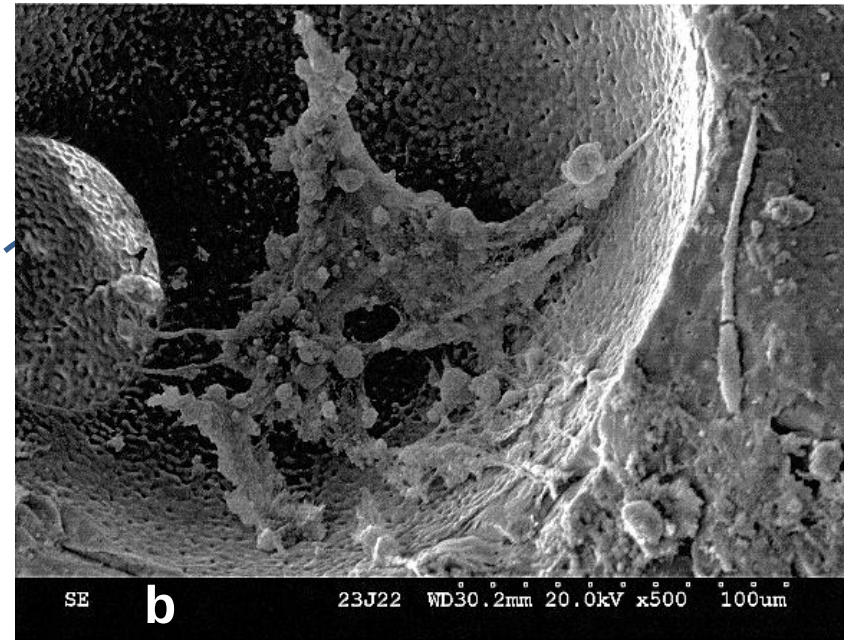


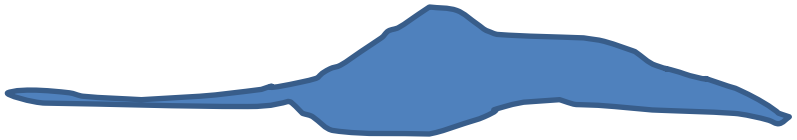
4 days



Colonization tests with MG63 osteoblasts







Moving osteoblast morphology

Static osteoblast morphology

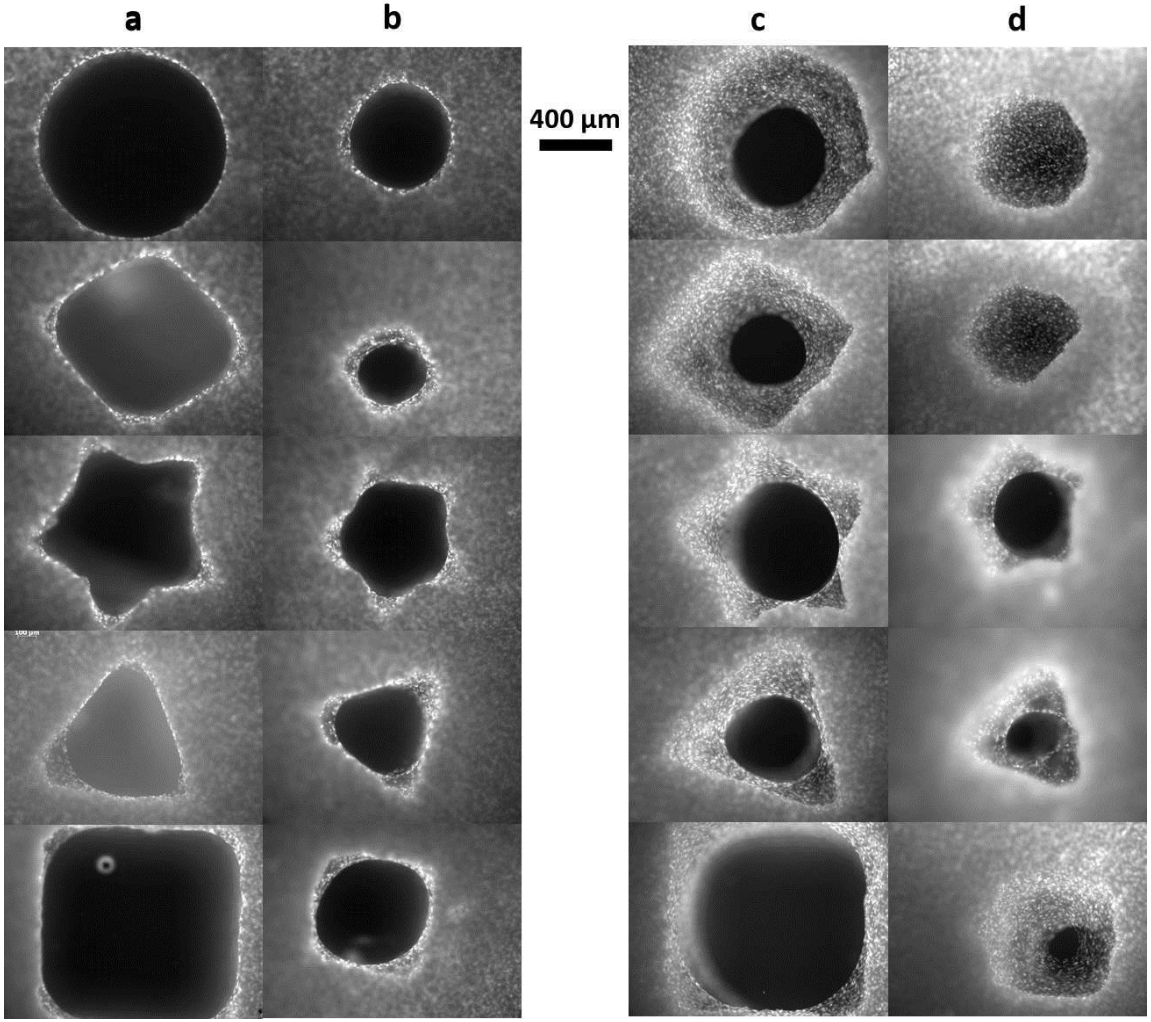
- What will be the impact of these two different macroporosities (size and morphology) on human cell invasion?

  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 immunoflurescence 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

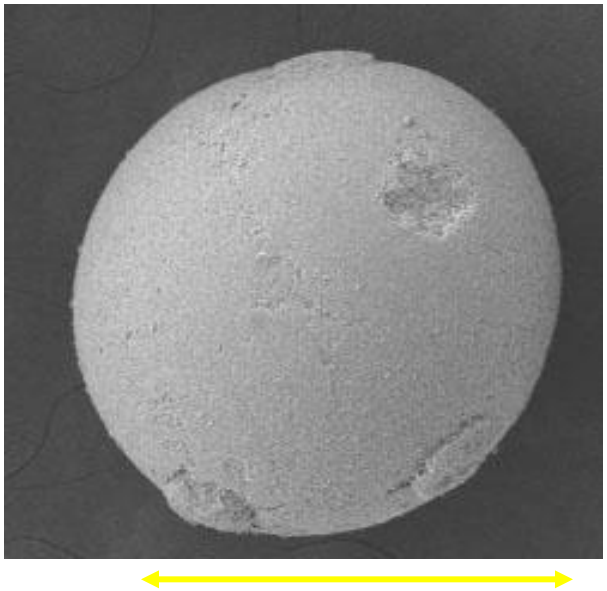
Part III Functionalization of as-prepared scaffolds by drug and phage impregnation through microporosity

Chemical functionalization

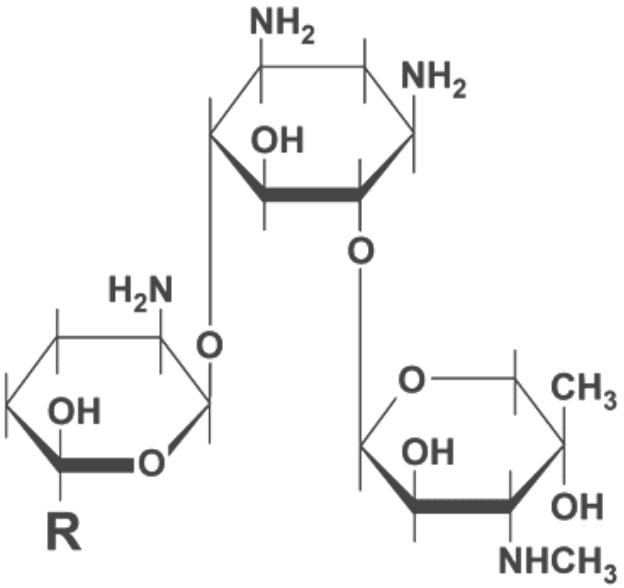
Biological functionalization

Chemical functionalisation

20% µ-porosity



1 mm

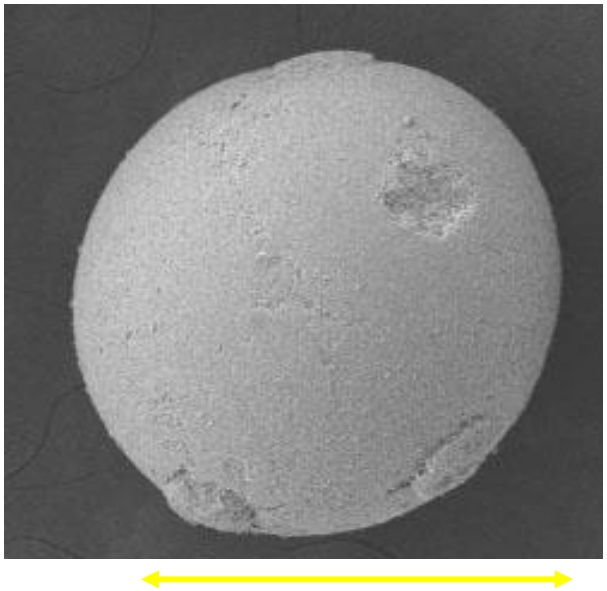


gentamicine :	R :
C1A	—CH_2NH_2
C2	—$CH(CH_3)NH_2$
C1	—$CH(CH_3)NHCH_3$

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

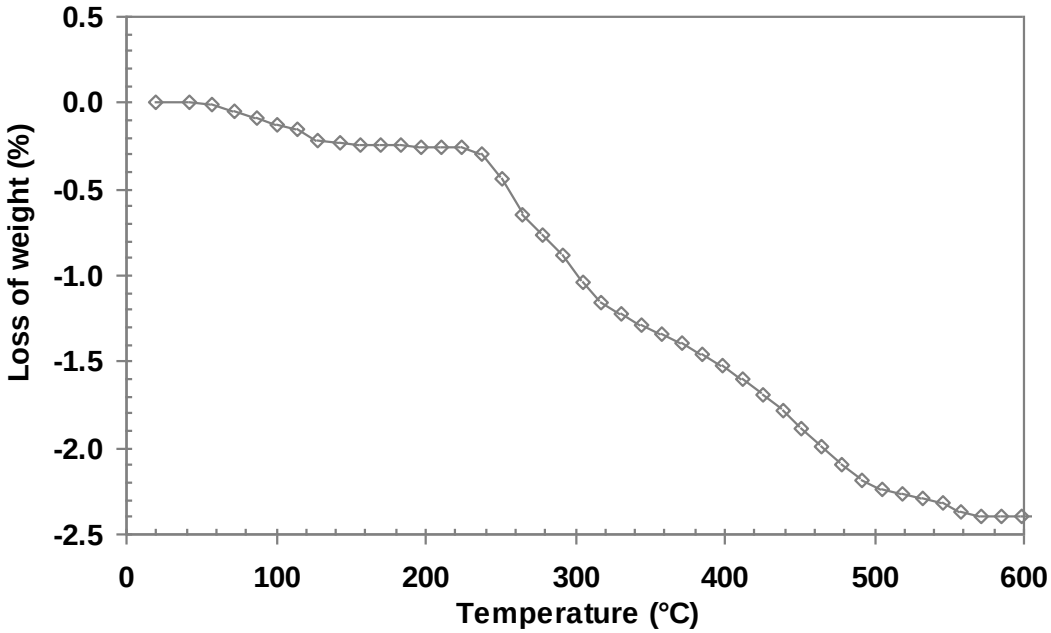
Chemical functionalisation

20% µ-porosity



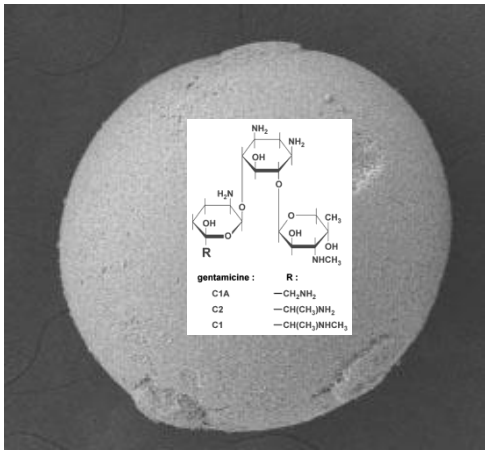
1 mm

TGA of gentamicin loaded HA microporous beads

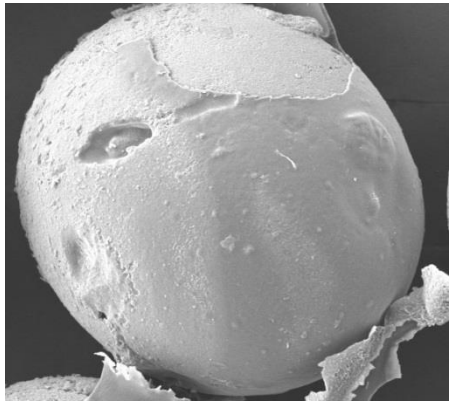


The HA beads are loaded with 40 mg gentamicine /g HA

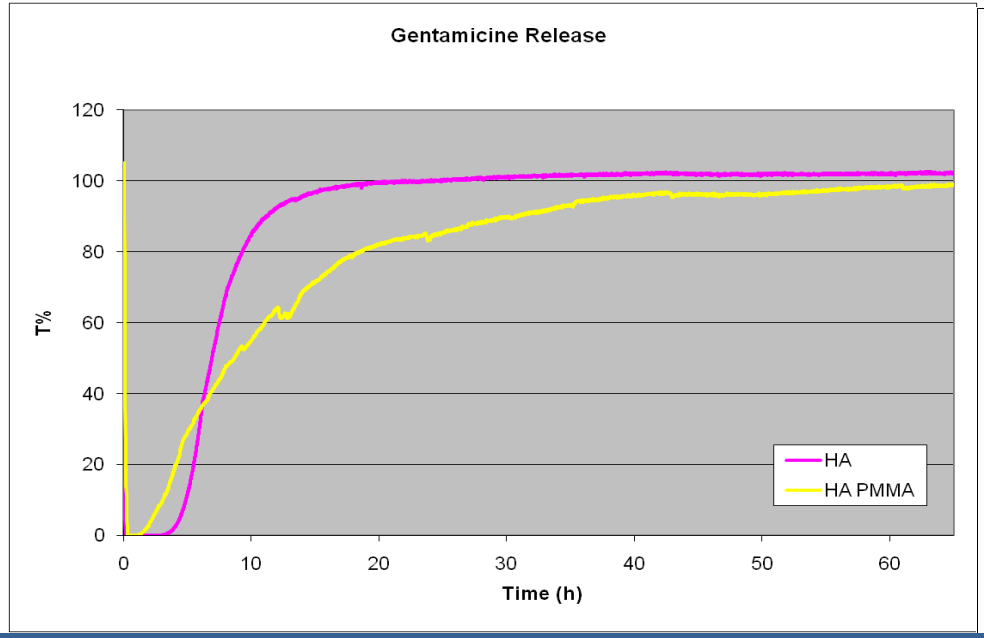
Chemical functionalization

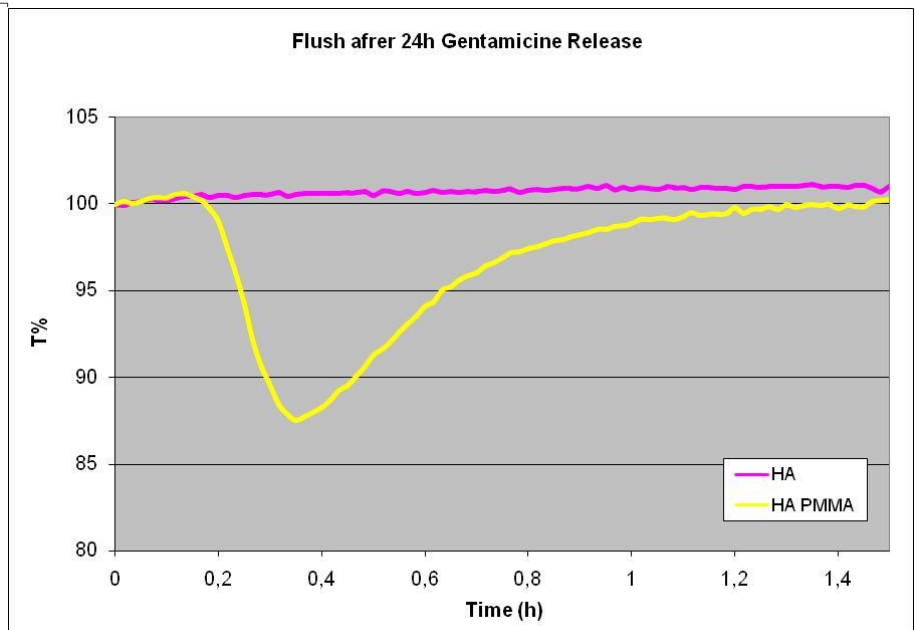


Polymer coating

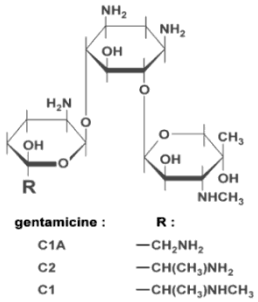


By protecting the surface, it is possible to control the drug delivery

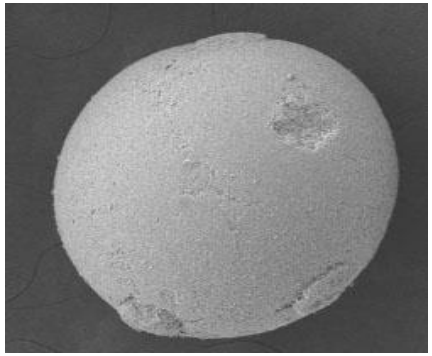


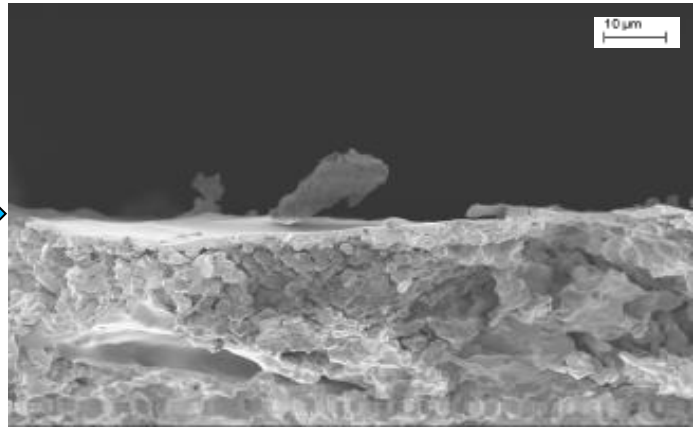


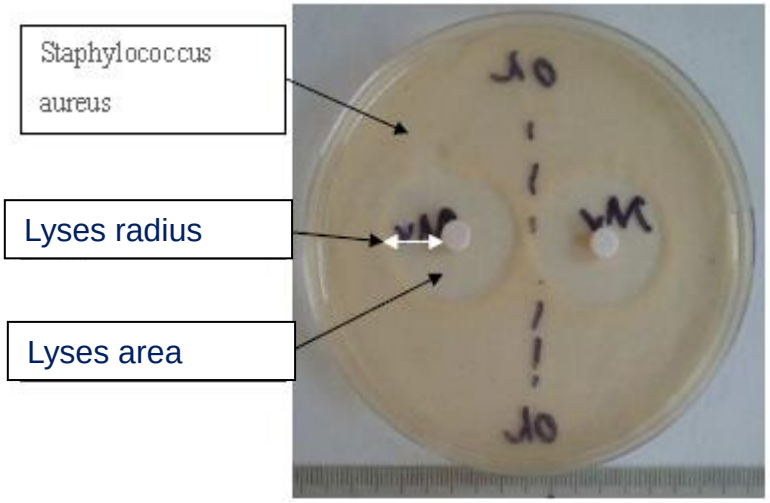
Chemical functionalization



+ polymer



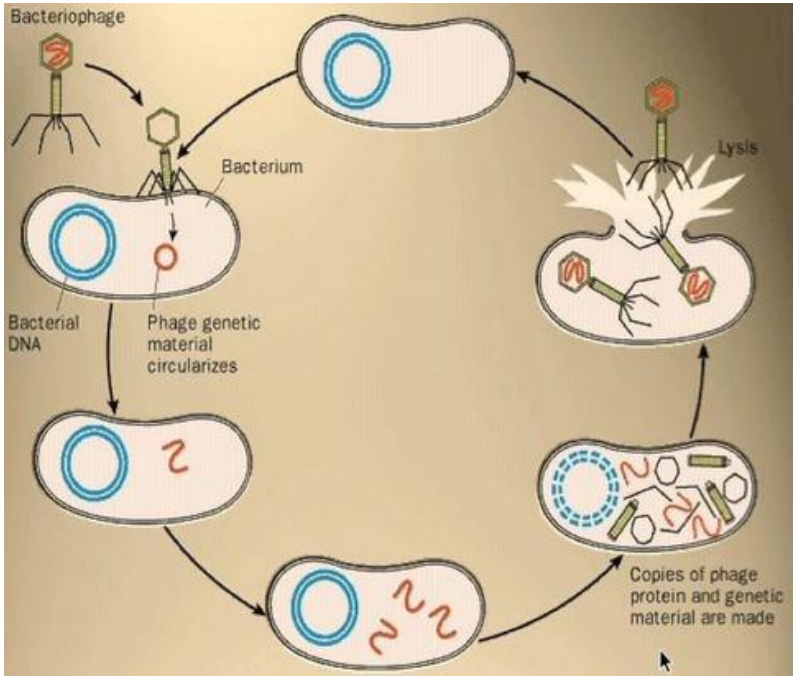




« Functionalisation of porous HA for bone substitutes » E.Meurice et al *JECS 32 (2012) 2673-2678*

Biological functionalization

A bacteriophage (phage) is a virus that infects and replicates only within a specific bacterium



Bacteriophage lytic cycle

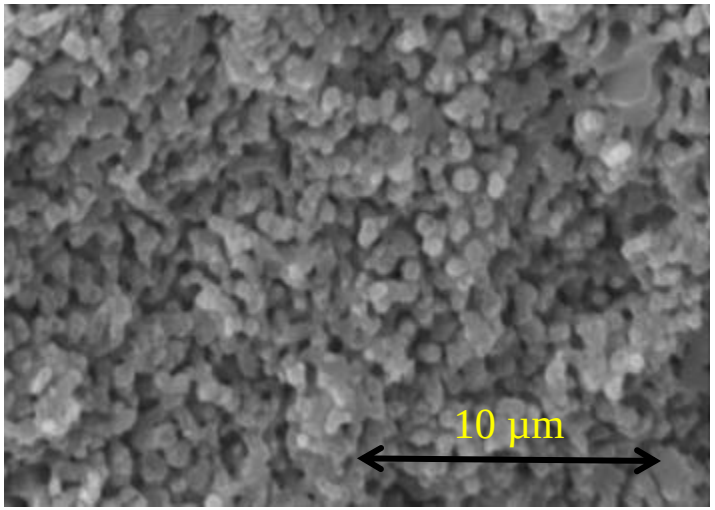
Diameter
24 to 200 nm

The antibacterial activity of ceramics loaded with λ phage was tested on the bacterium Escherichia Coli K12.

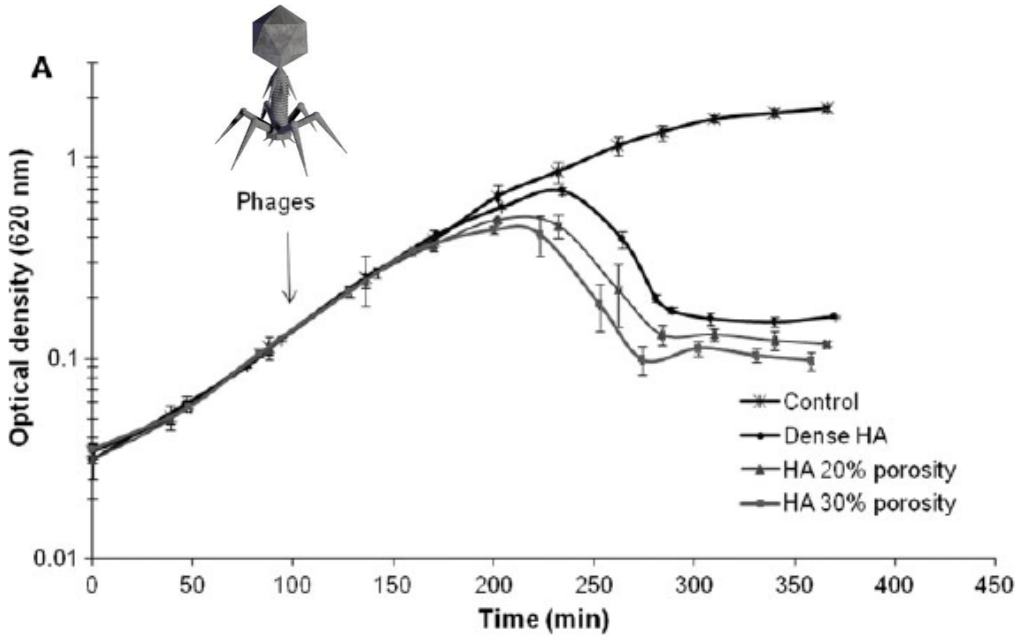
Biological functionalization

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.



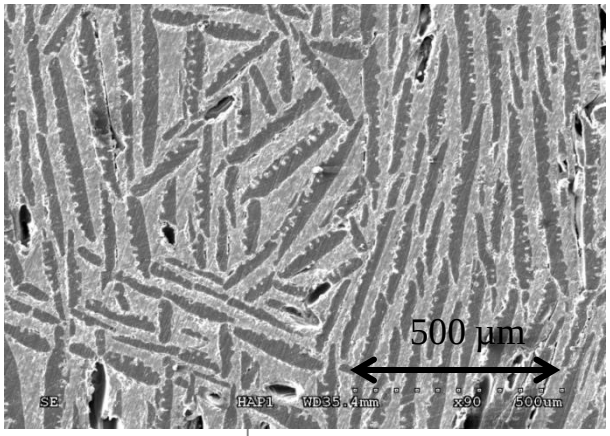
HA 30% µporosity

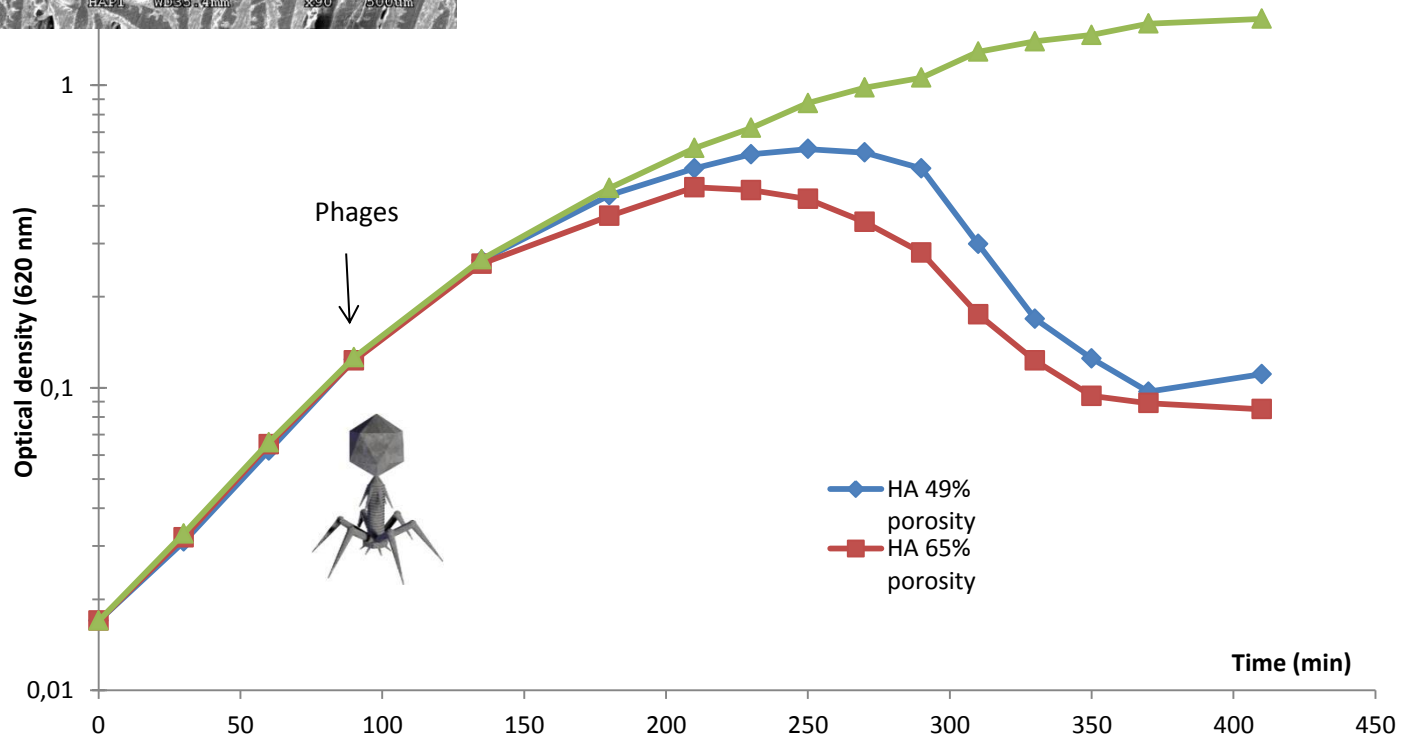


A slow down of bacterial growth kinetic was noted after 80 min (HA) followed by the death of bacteria. This phenomenon appears sooner as the microporosity is higher.

« New antibacterial microporous CaP materials loaded with phages for prophylactic treatment in bone surgery » E.Meurice et al. Journal of Materials Science: Material of Medecine 23,10, 2012, 2445-2452.

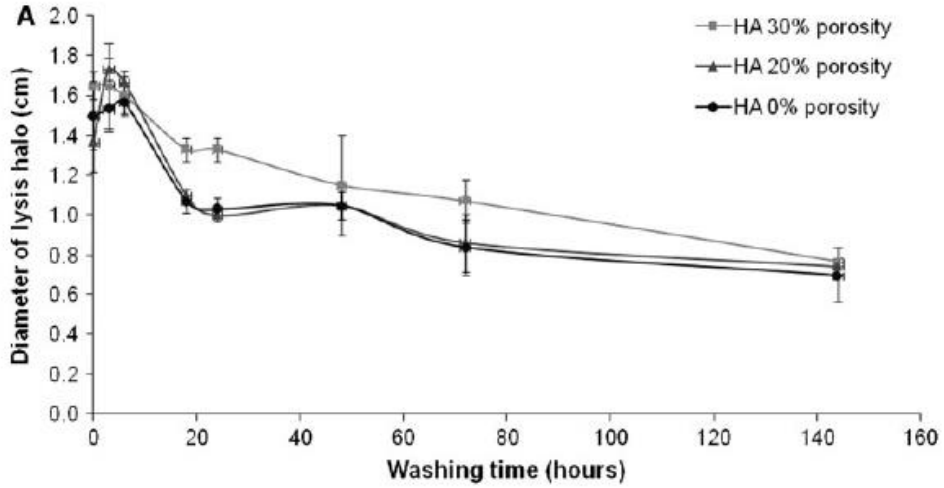
Biological functionalization

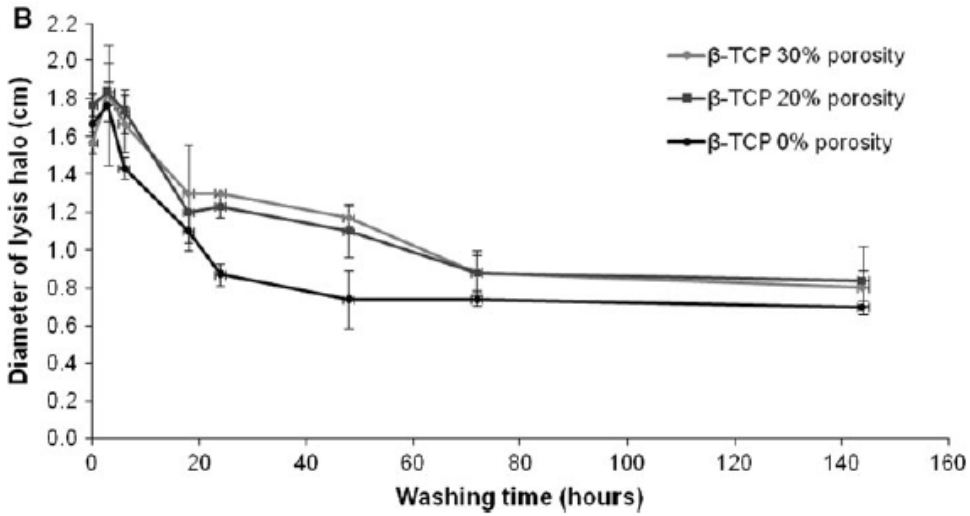


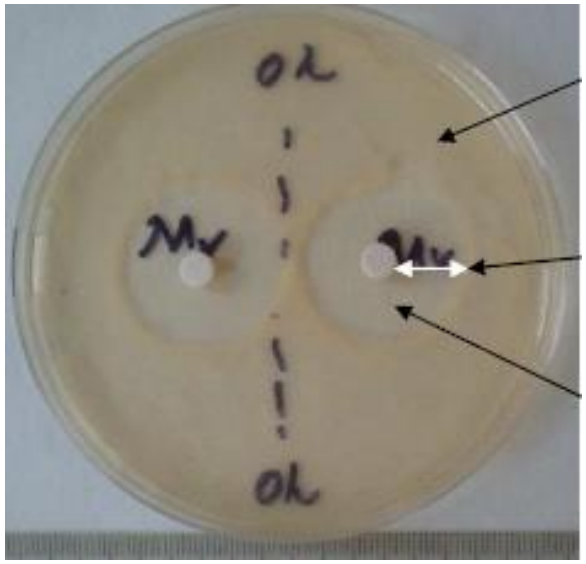


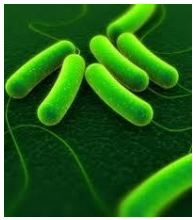
Same effect with ice-templated samples with higher porosity level

Biological functionalization









E coli

phage

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

Conclusion

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

♦ 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.

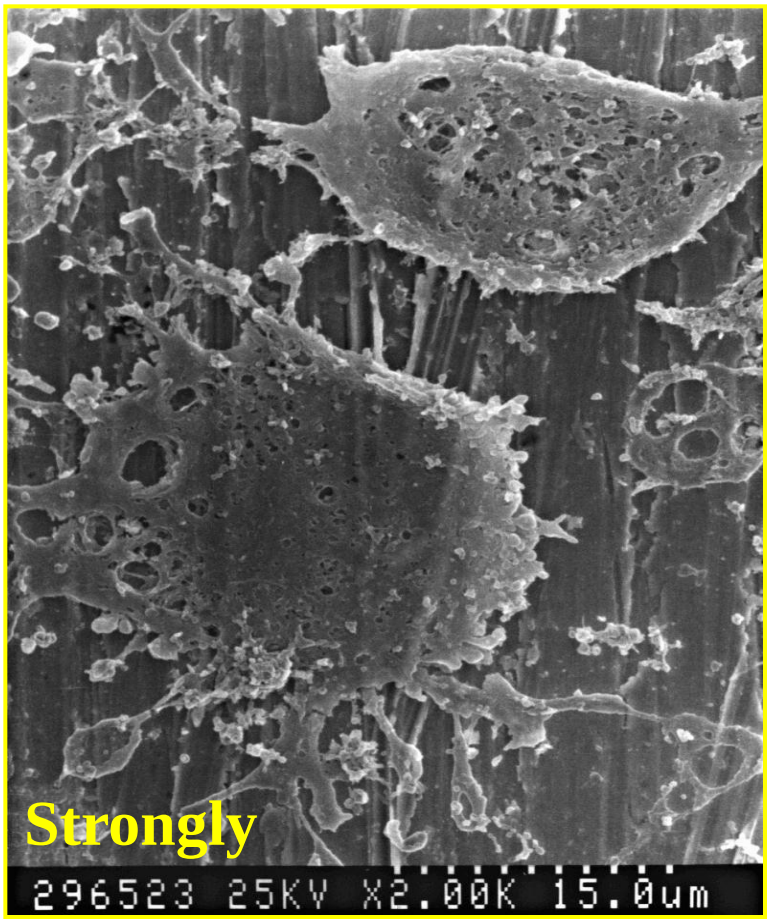
♦ The microporosity allowing a better control of resorbability can be also used as biological substances and phage supports.
This study has shown that phage loaded ceramics could be used in bone prophylactic treatments.


Thank you
for your attention
U.PORTO
CEMUP
mag
1 000 x
HV
15.00 kV
det
ETD
WD
11.3 mm
mode
SE
100 µm
B4 day 1 Mg63 Surface

Osteoblasts MG63

- 2 multi-well plates (48 wells)
- 2 10^5 cell/ml α-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.

NIH 3T3 Fibroblasts (6 days)



Biological functionalization

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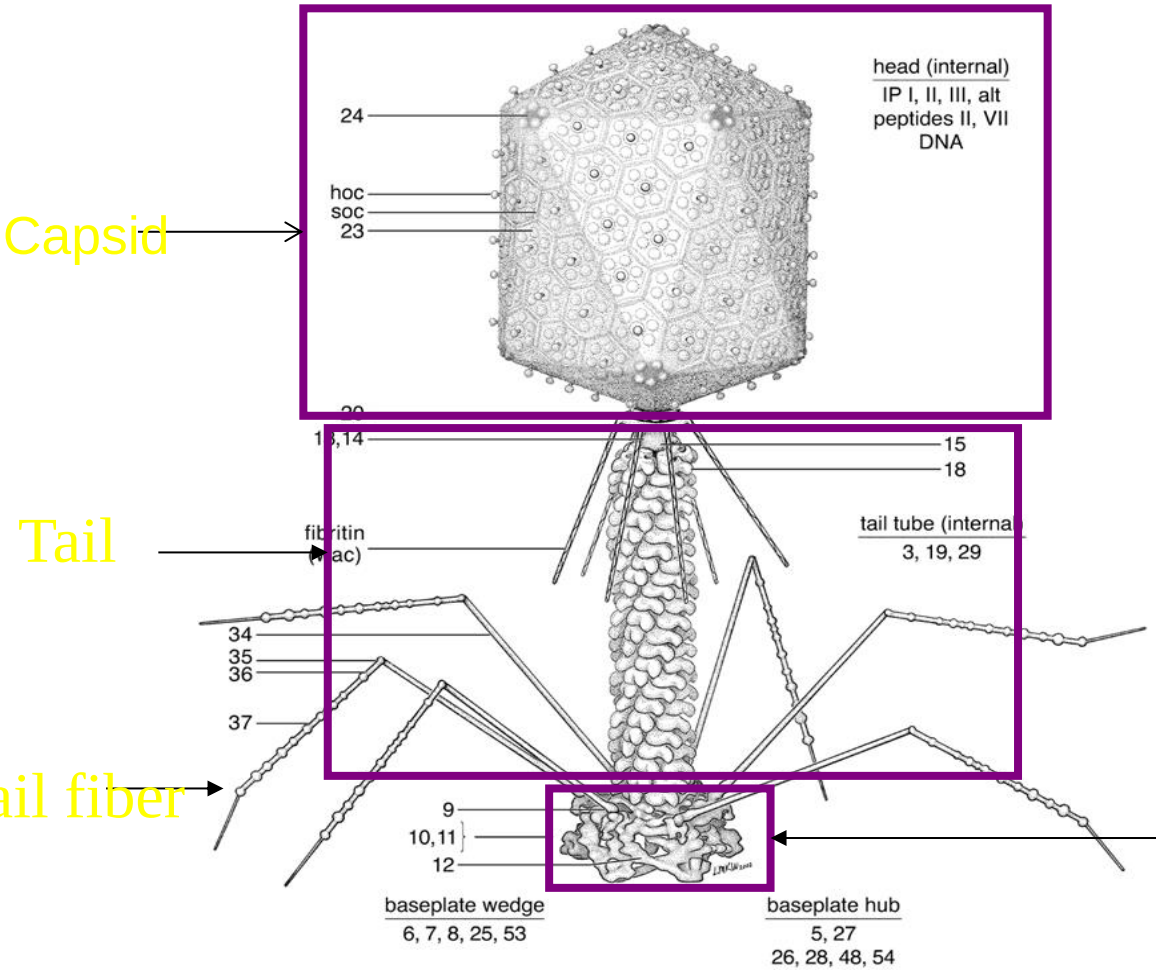
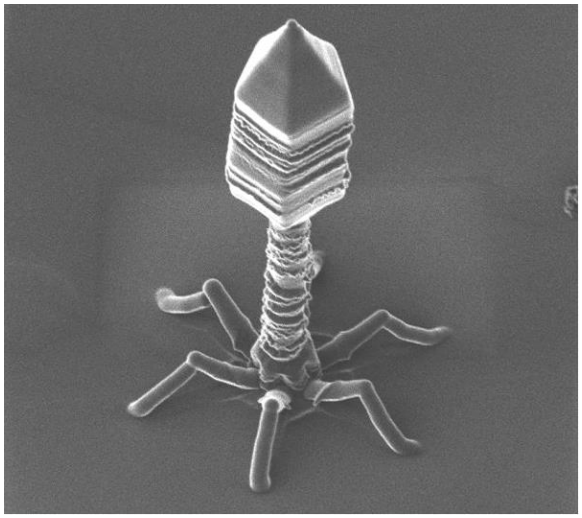




Diameter
24 to 200 nm

Discovered in 1915 by Frederick W. Twort and Felix d'Hérelle

Biological functionalization

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).

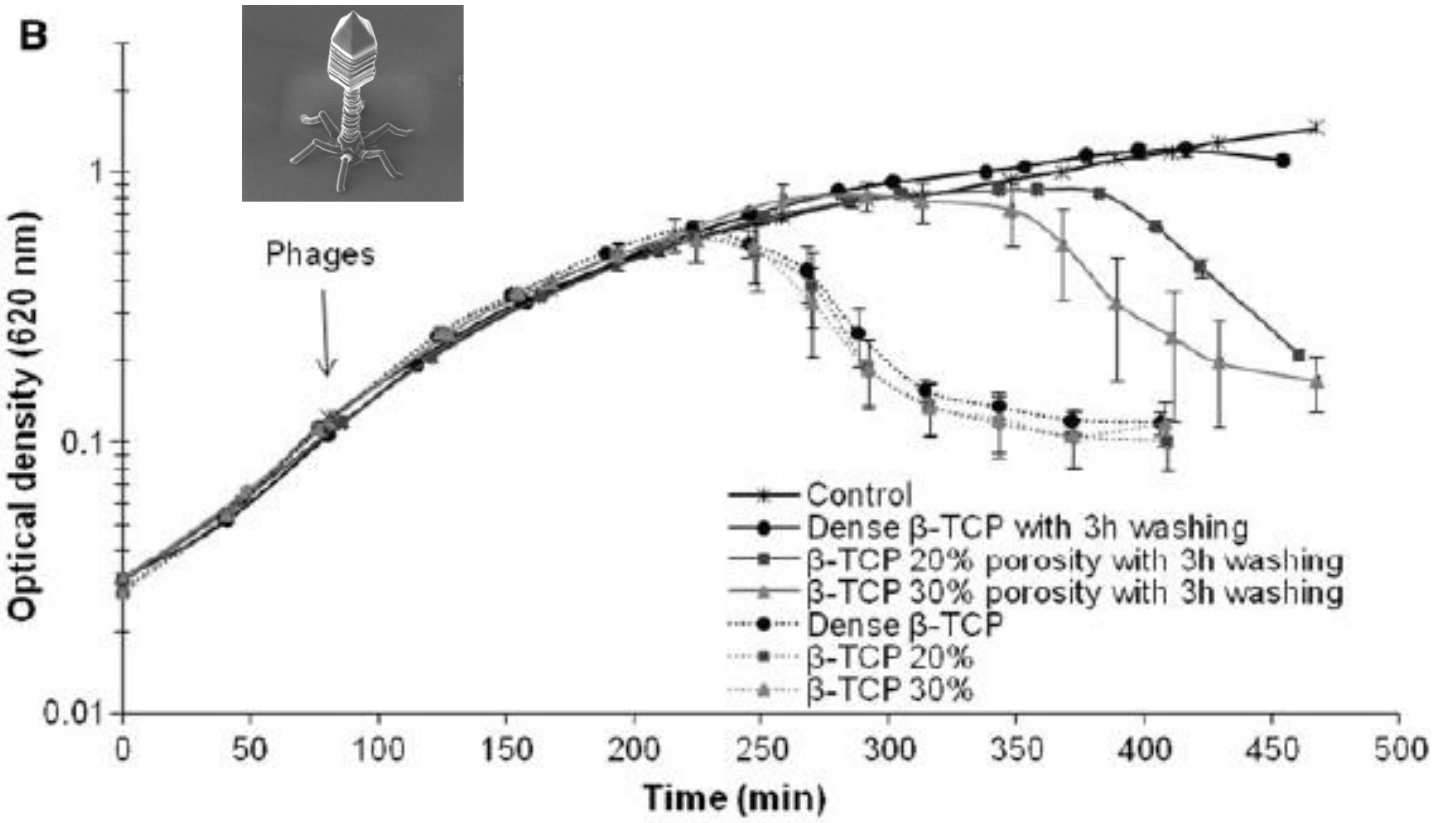
-Phage λvir stock was prepared by infecting *Escherichia coli K12 strain (A324).*

-*HA and TCP* samples with different porosity level (0, 20 and 40%) were incubated for 24 h with 5 ml of stock λ phage.

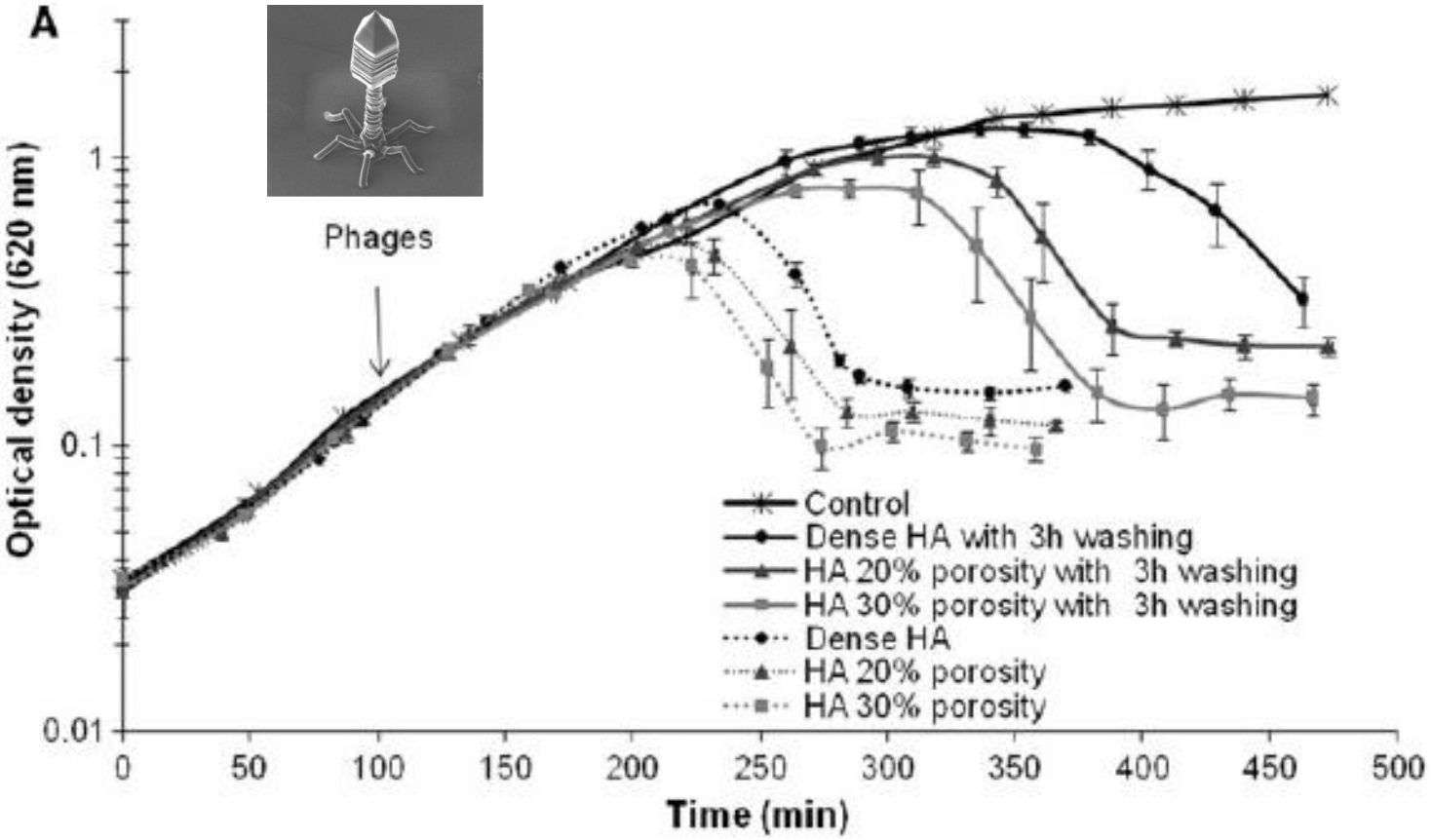
-After incubation, the sample was washed once with LB medium to remove excess phage suspension.

-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.

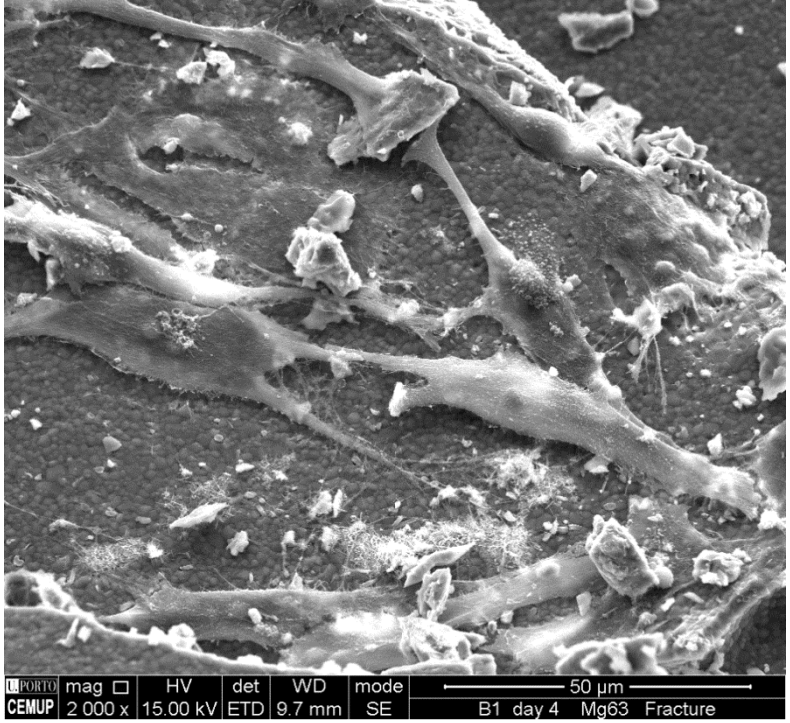
Biological functionalisation



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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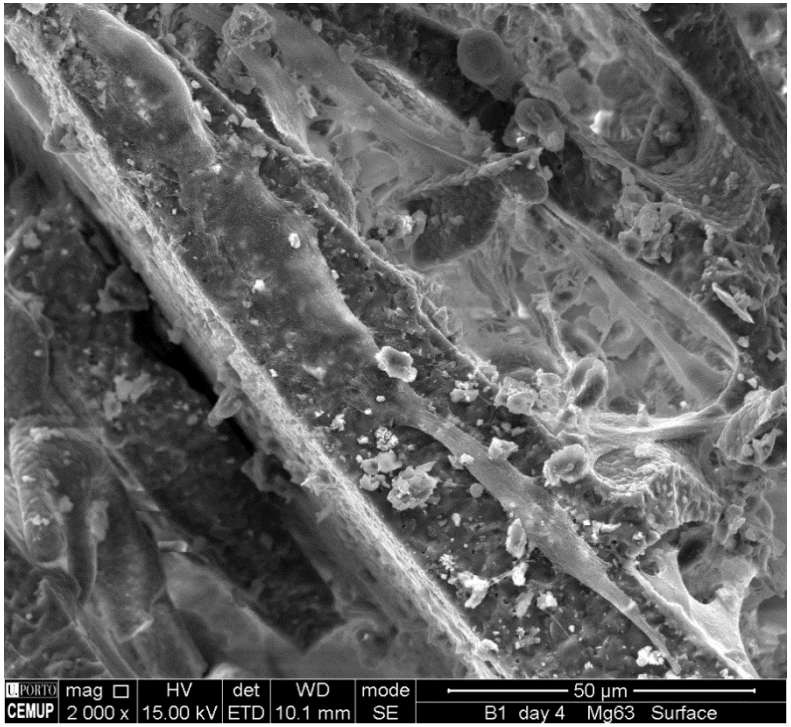
B1 day 4 Mg63 Fracture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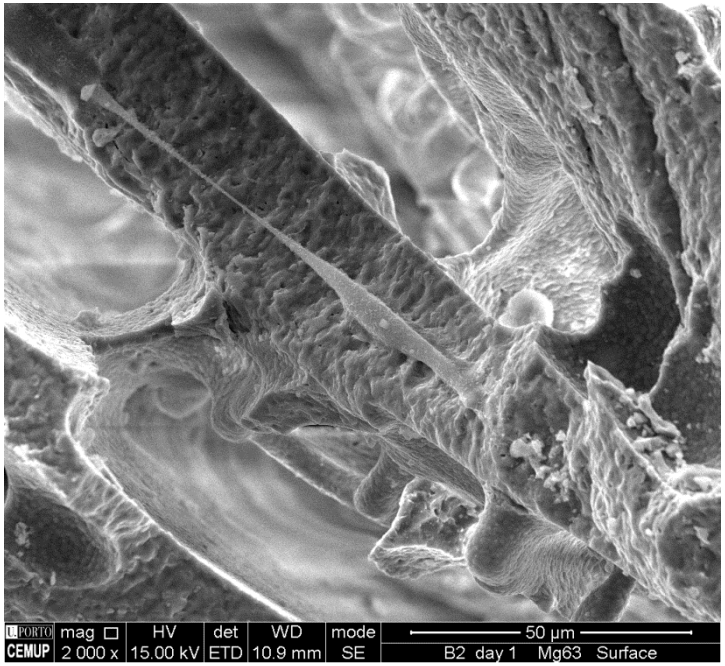
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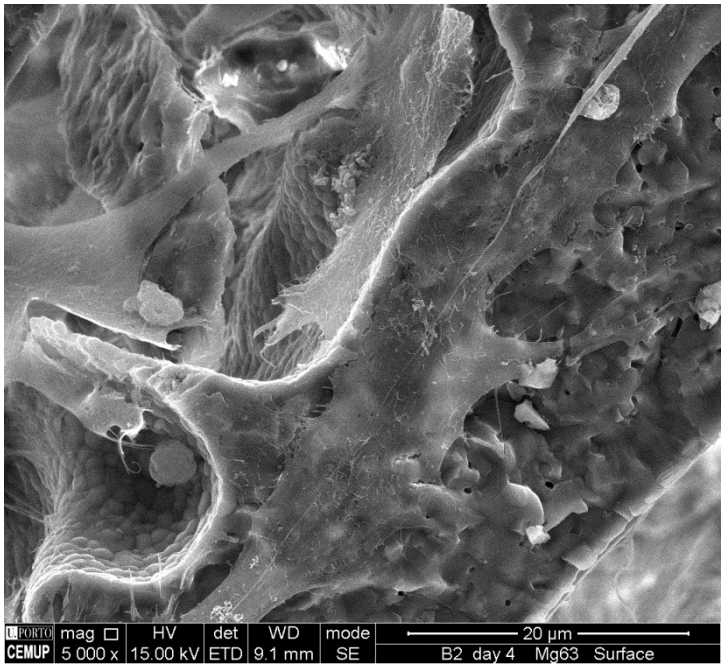
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20 µm
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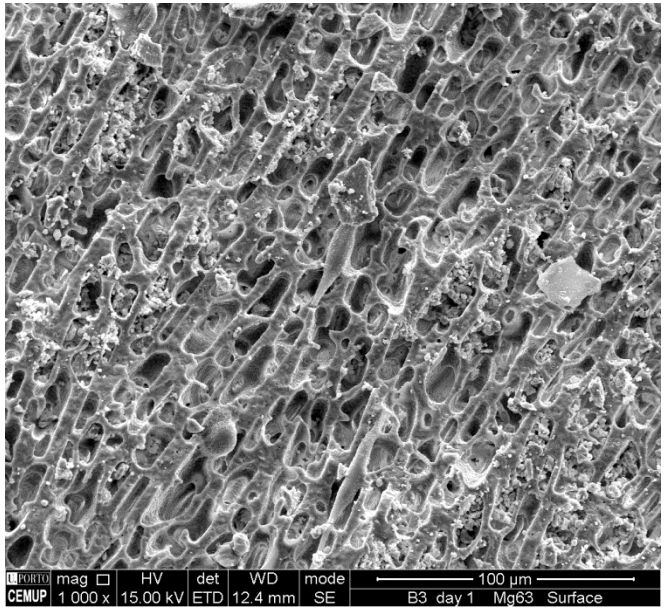
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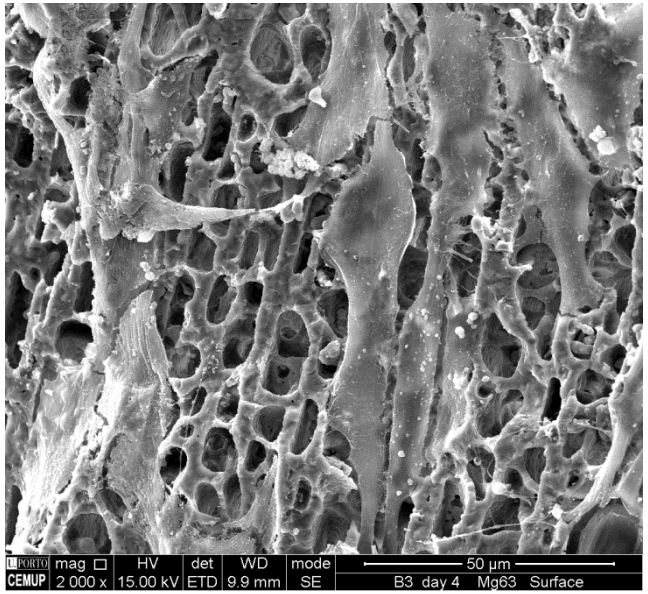
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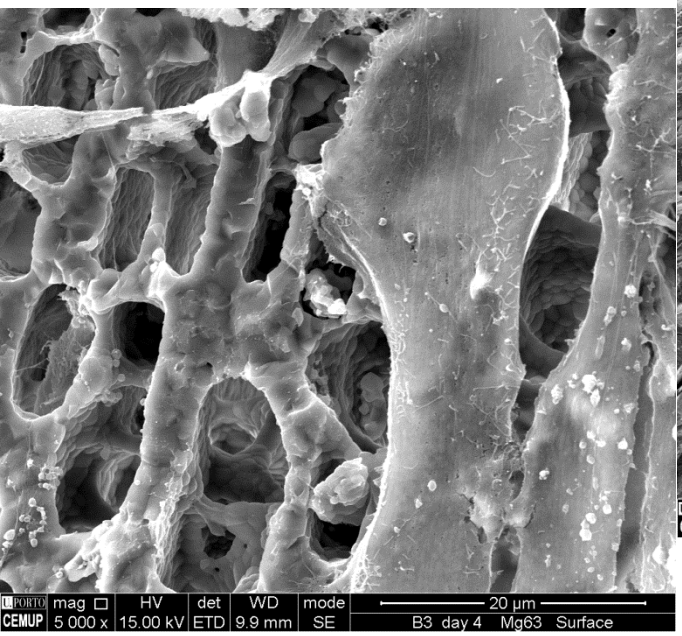
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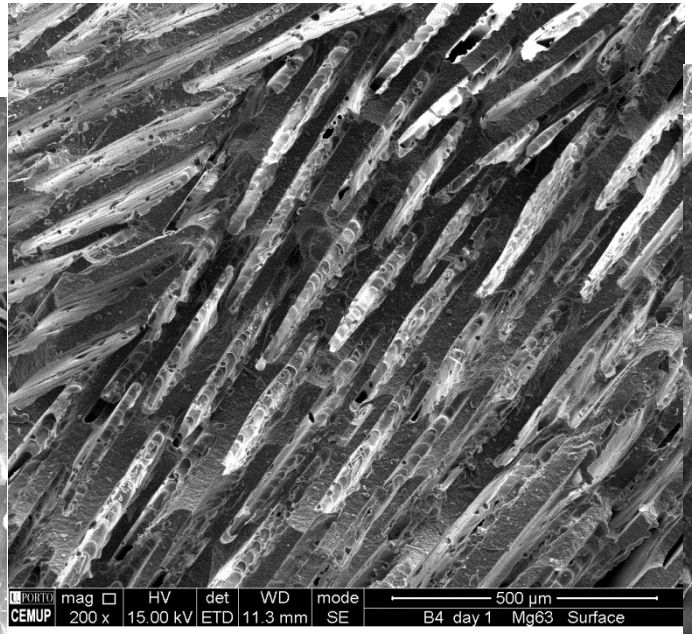
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500 µm
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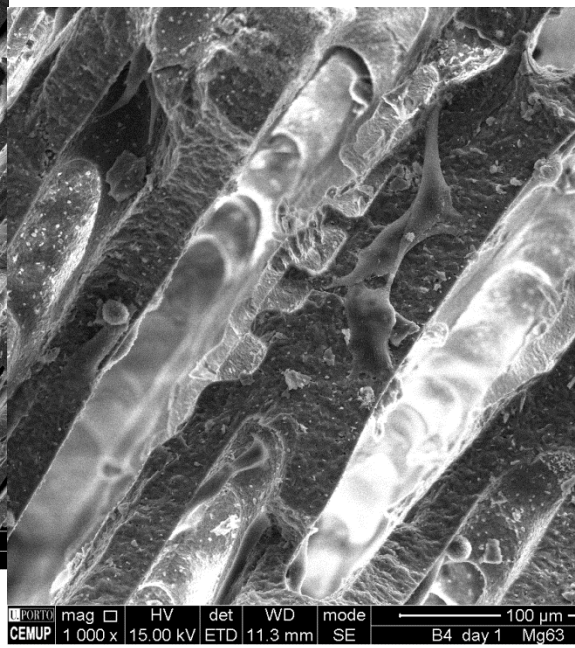
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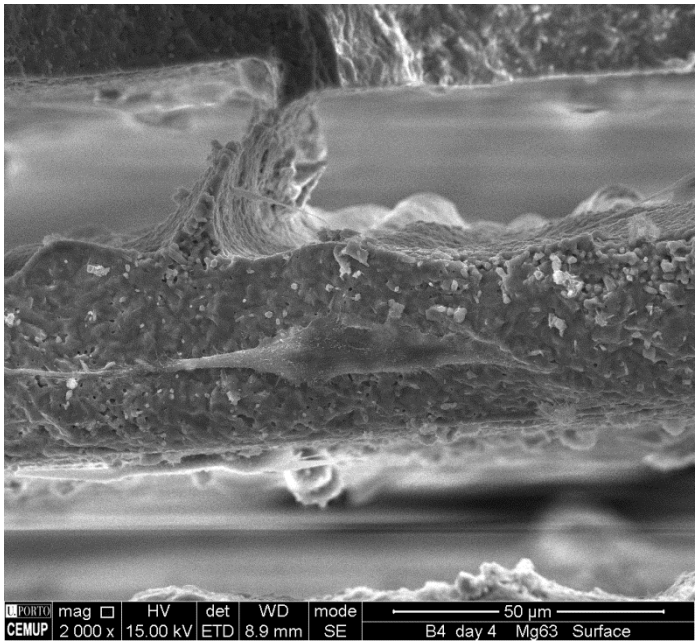
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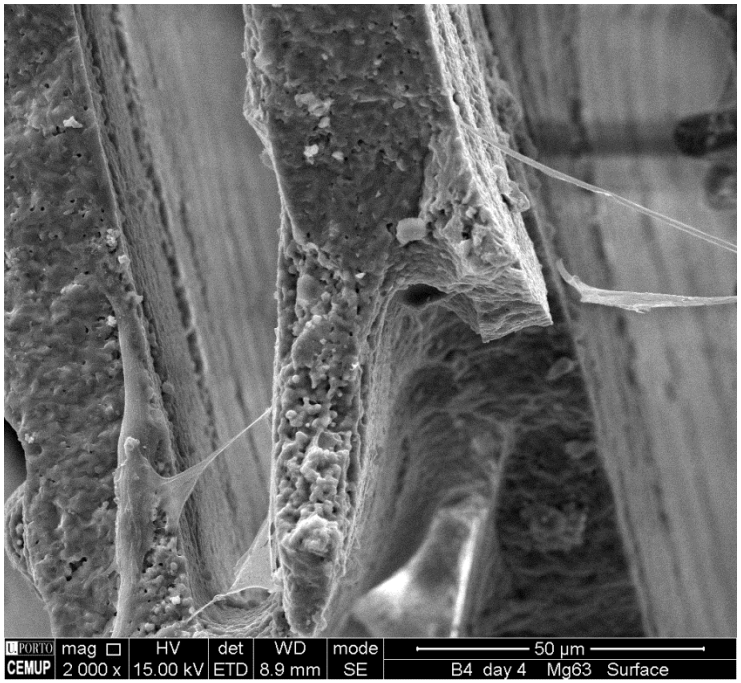
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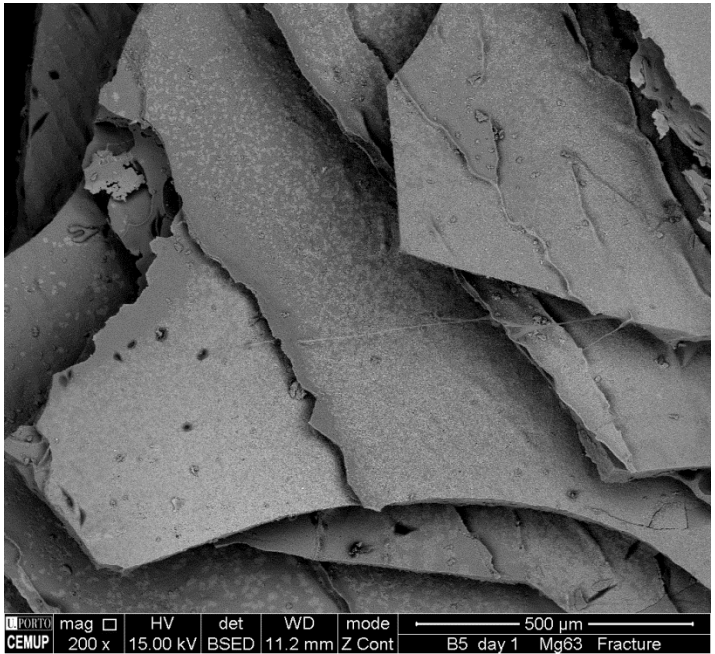
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mode
Z Cont
500 µm
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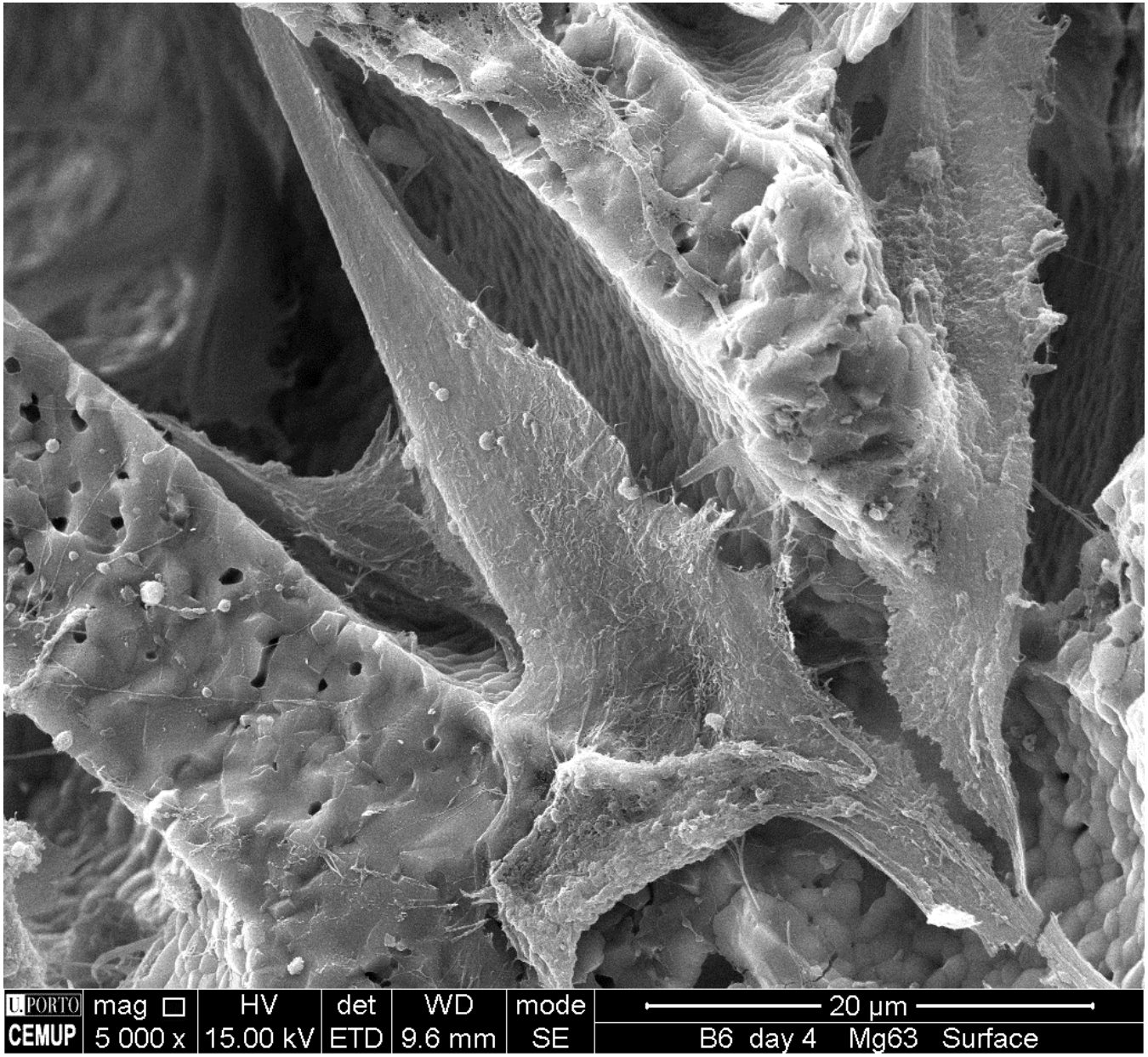
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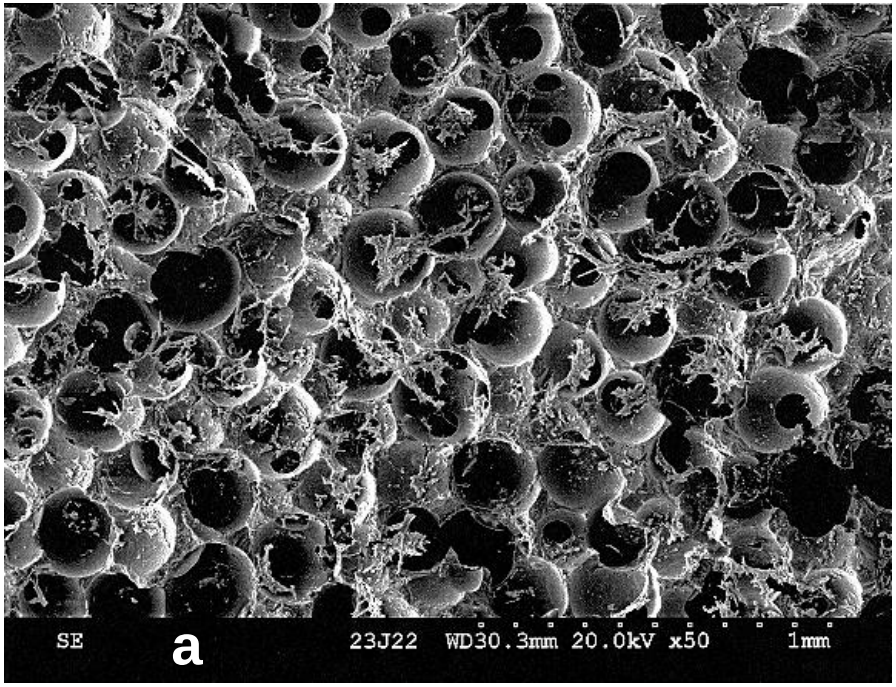
9.6 mm
mode
SE
20 µm
B6 day 4 Mg63 Surface

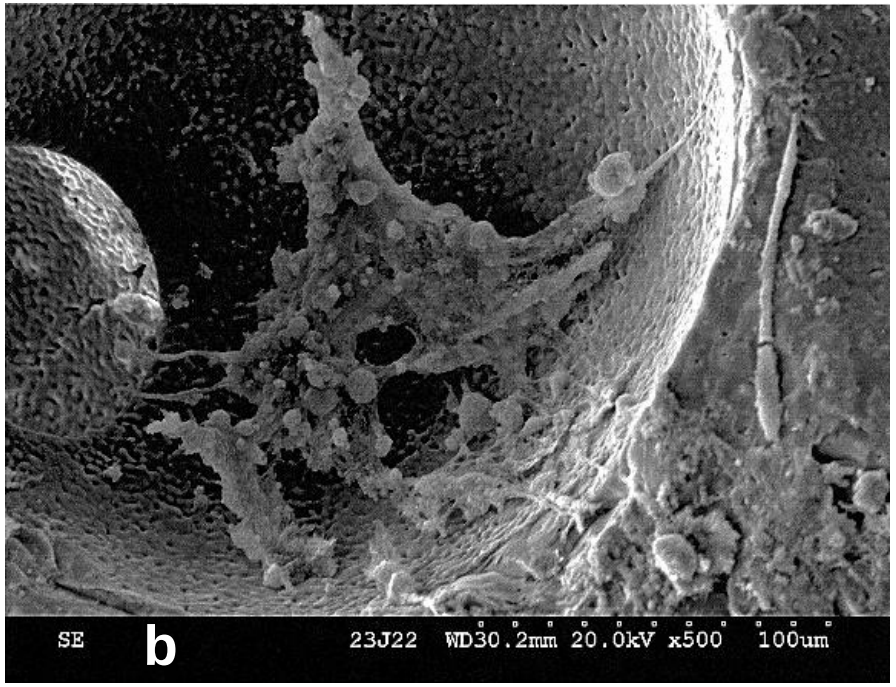
First method

Ceramic slurry infiltration of organic skeleton

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

- **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)