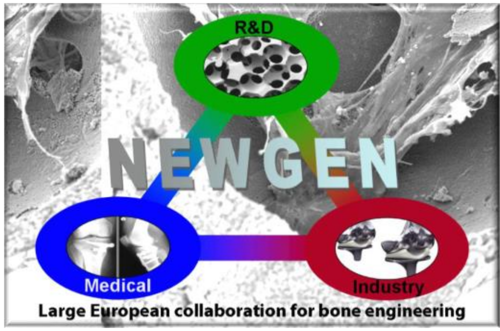
MP1301 – NEWGEN
Work Group 4



- *In vitro* studies

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Torino, March 2014

In vitro models to address the cell response to biomaterials for bone tissue applications

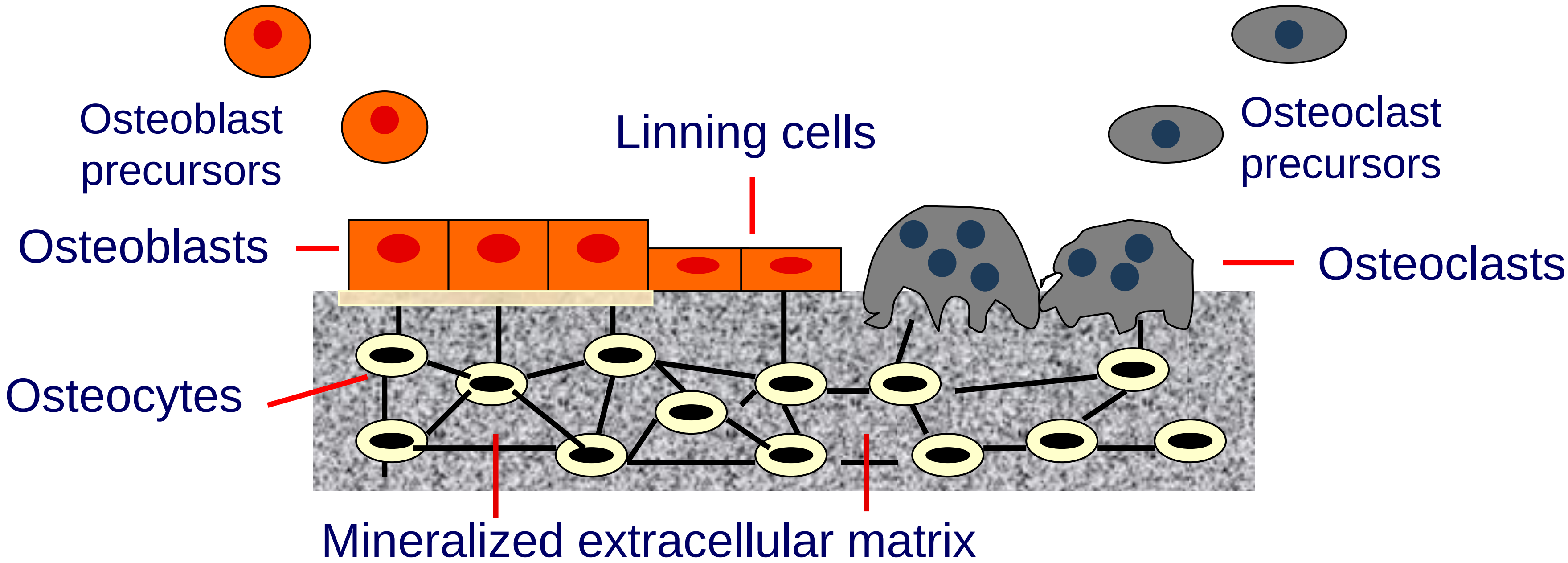
Overview of representative *in vitro* models to address the cellular and molecular response to materials for bone related applications: Cultures of (i) osteoblastic cells, (ii) osteoclastic cells, (iii) endothelial cells, and (iv) co-cultures of endothelial and osteoblastic cells and of osteoblastic and osteoclastic cells.

……which is the first stage of the biocompatibility testing

Cell culture models of bone metabolism

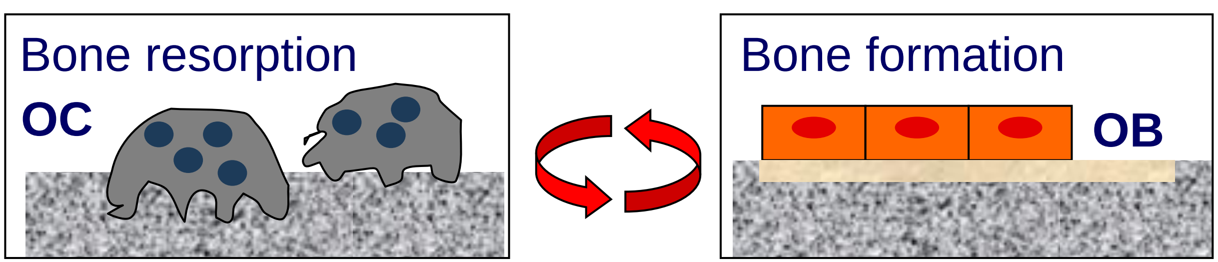
- Cultures of human Osteoblastic cells
  (Bone formation)
- Cultures of human Osteoclastic cells
  (Bone resorption)
- Cultures of human Endothelial cells
  (Angiogenesis)
- Co-cultures of human osteoblastic/osteoclastic cells
  (Bone remodeling)
- Co-cultures of human endothelial cells/osteoblasts
  (Relationship angiogenesis/osteogenesis)

The bone microenvironment:
The bone cells and the extracellular matrix

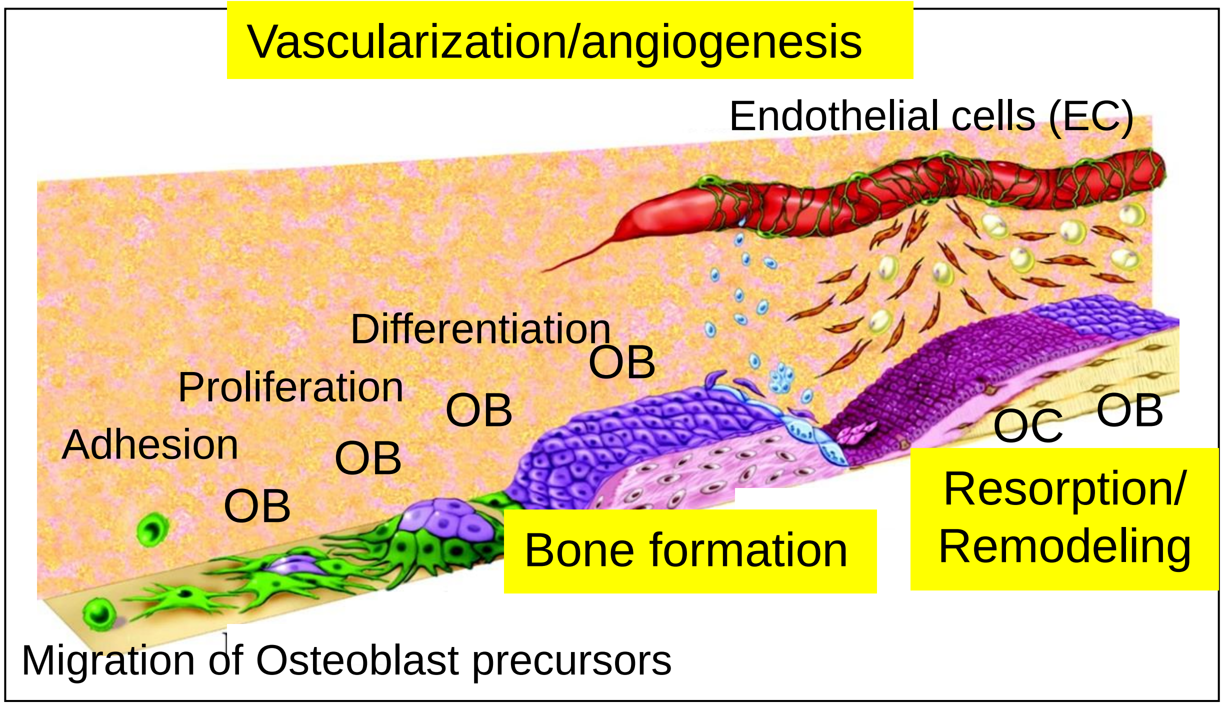


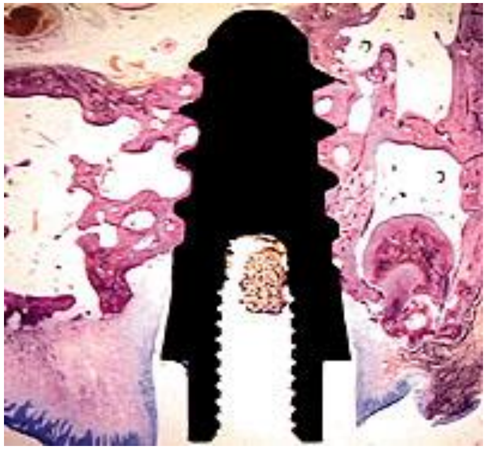
Collagen type I (~ 90%)
Glycoproteins, Proteoglycans, Growth factors, cytokines,
……Hydroxyapatite

Remodeling



The bone microenvironment: Cellular events



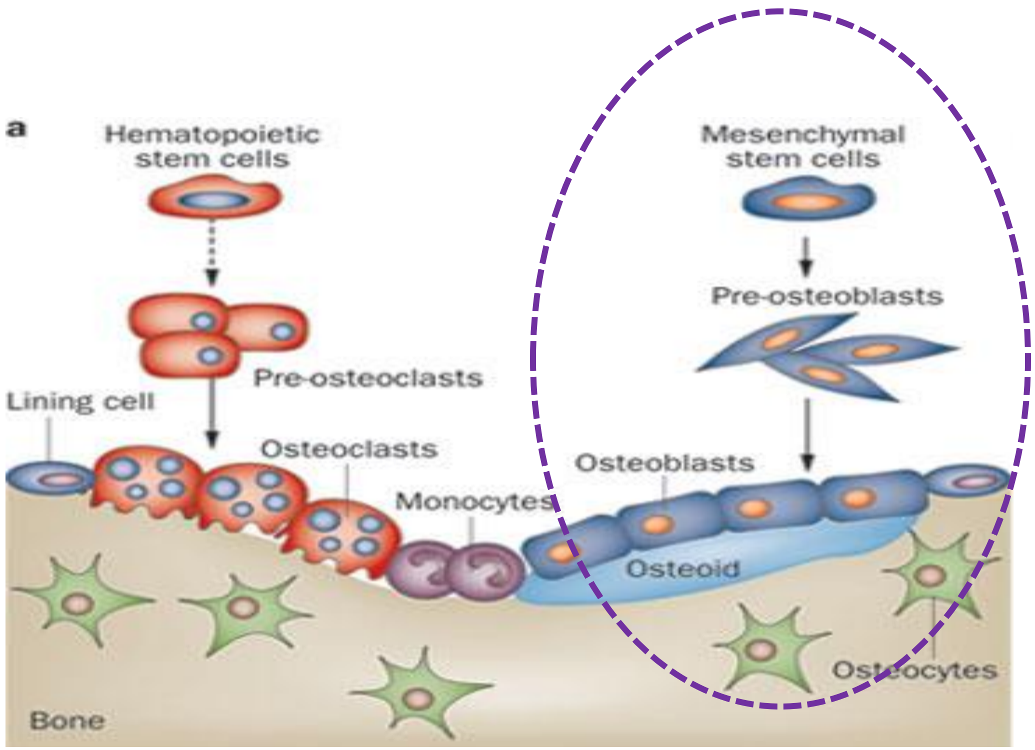


bone regeneration process relies in key timely cellular interactions at the bone/biomaterial interface. Initial events involve the recruitment of osteogenic precursors and their differentiation leading to an active bone formation process.

Cultures of human Osteoblastic cells

Representative model of the proliferation/differentiation sequence during the development of the Osteoblastic phenotype, including the formation of the mineralized matrix

Osteoclastic cells



Osteoblastic cells

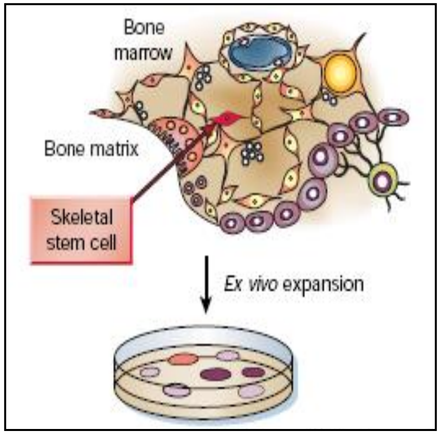
Human osteoblastic cell cultures

Primary culture

Established from Mesenchymal stem cells present in the bone marrow, trabecular bone fragments

Standard culture conditions:

α-MEM
10% Fetal bovine serum
Penicillin/strptomycin
Fungizone (2.5 µgmL^{-1})
Ascorbic acid (50 μg/ml)
37ºC, 5% CO_2/ar



Proliferation of Mesenchymal stem cells (70 – 80% confluence)

Subculture

β-glicerophosphate (10 mM)
Dexamethason (10 nM)

Induction of the osteoblastic phenotype

Human osteoblastic cell cultures

Characterization of the cell behaviour:

- Cell adhesion to the material substrate
- Cell viability/Proliferation (MTT, DNA, Protein)
- Apoptosis
- Cell cycle
- Morphology/F-actin cytoskeleton
- Focal adhesion points
- Expression of osteoblastic genes
  (Runx-2; Col-1; ALP; OC; RUNKL; OPG; …)
- Functional activity
  Alkaline phosphatase activity
  Formation of a mineralized matrix
- Intracellular signalling pathways

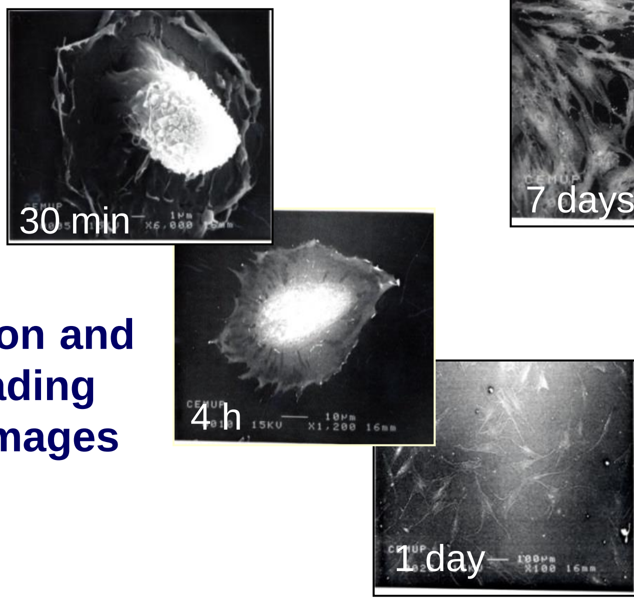
Biochemical, histochemical, immunohistochemical and molecular methodologies

Scanning electron microscopy (SEM)

Confocal laser scanning microscopy (CLSM)

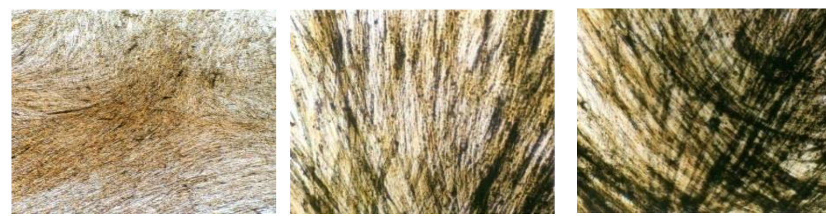
Cultures of human Osteoblastic cells

Characterization of the cell behaviour



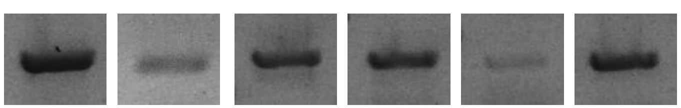
Adhesion and spreading SEM images

Cell proliferation (SEM images

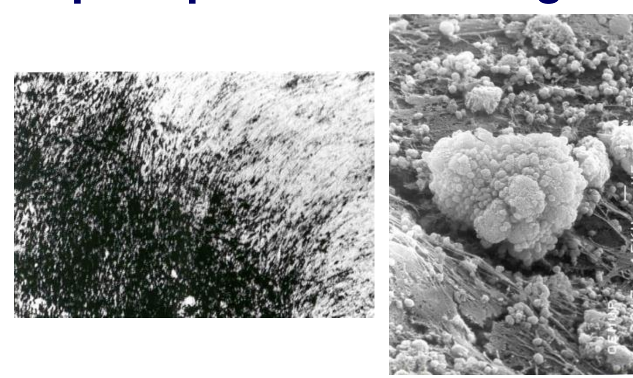


Alkaline phosphatase staining

Gene expression profile

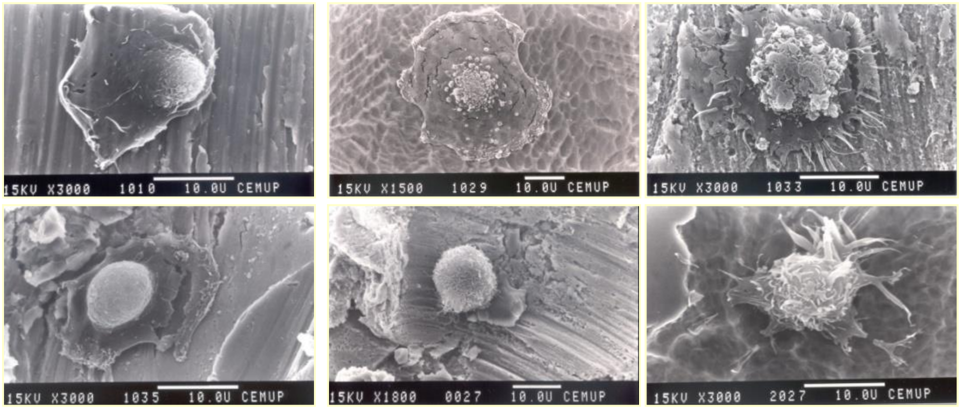


Runx-2; Col-1; ALP; OC; OPG; RANKL; …..



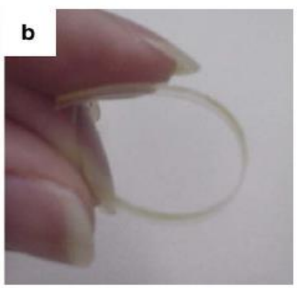
Matrix mineralization (SEM)

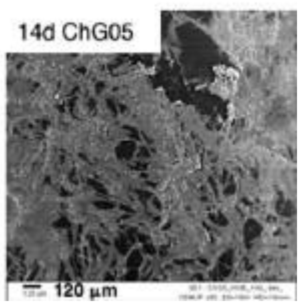
Osteoblastic cell response to Biomaterials for bone tissue applications

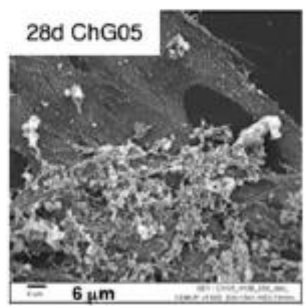


Cell adhesion to Titanium substrates with different surface treatments; 30 min

J. Mater. Sci.: Mater. Med., 13: 421-431 (2002)



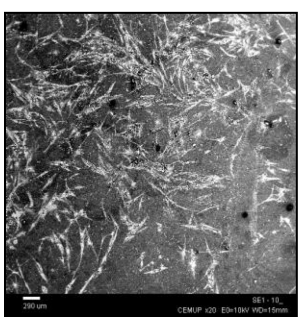


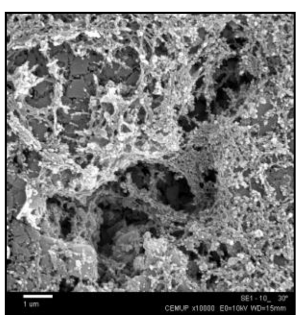


Chitosan-organosiloxane Hybrid Membranes

Biomaterials 26: 485-493 (2005)

Acta Biomaterialia 5: 346-355 (2009)

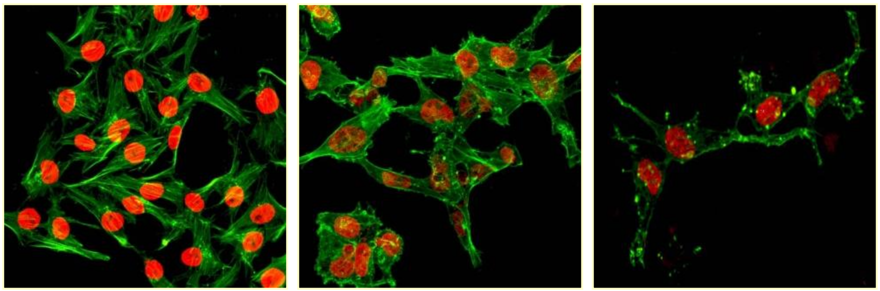




Glass/Si3N4 composites

Biomaterials 23: 4897-4906 (2002)

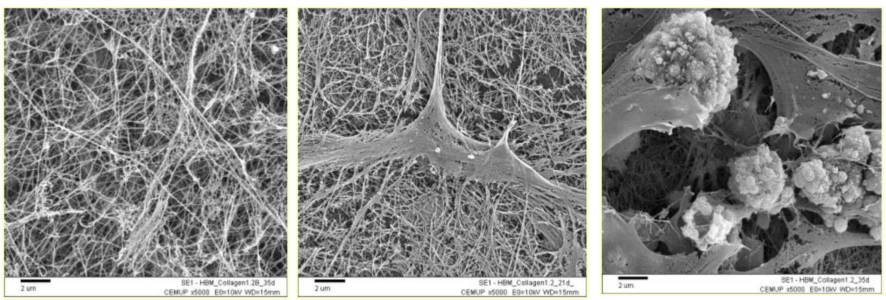
Osteoblastic cell response to Biomaterials for bone tissue applications



Glass/HA composites with different degradation rates

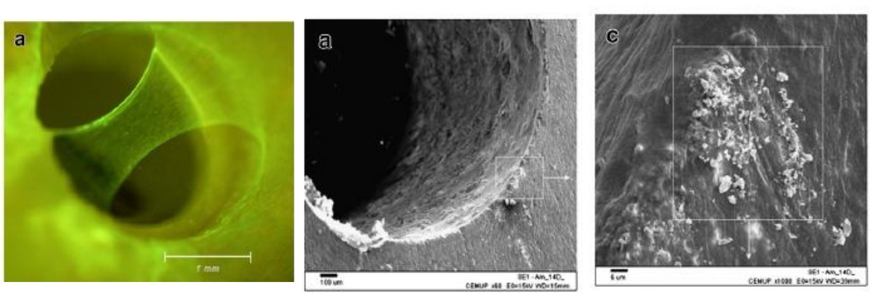
Biomaterials, 26: 485-493 (2005)

J. Biomed. Mat. Res., 74: 347-355 (2005)



Collagen matrix

Connective Tissue Research, 50: 336-346; 2009

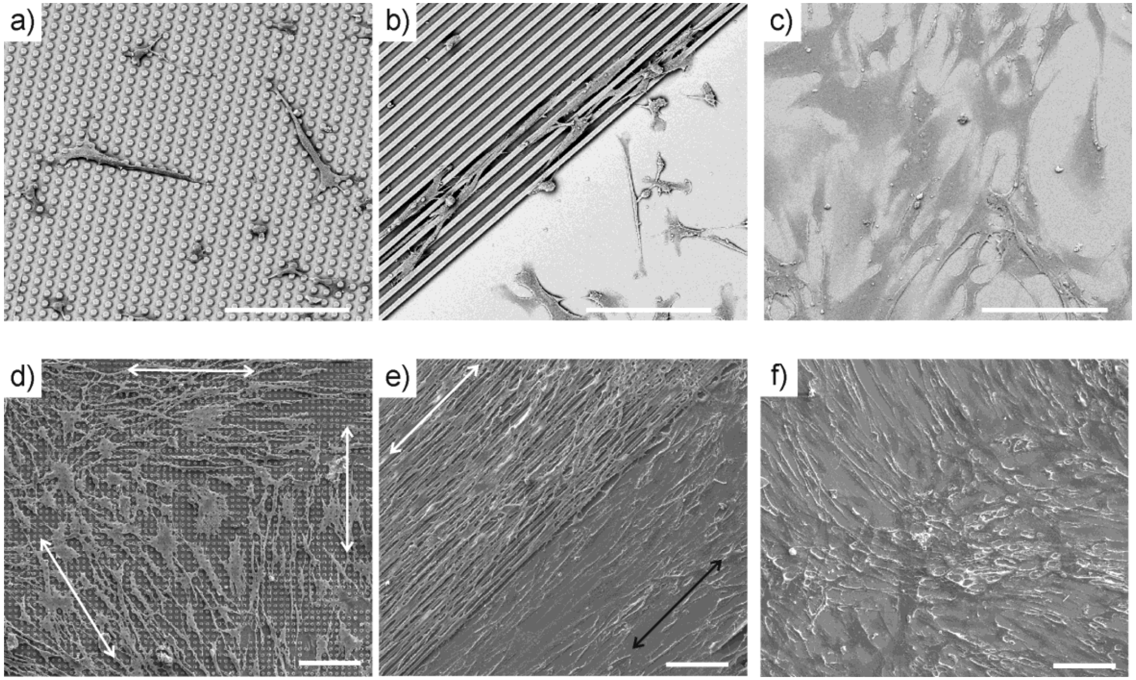


Macroporous ceramics

Materials Science and Engineering C 29: 930-935 (2009)

Osteoblastic cell response to Biomaterials for bone tissue applications

Guided proliferation of osteoblastic cells on patterned surfaces



J Biomed Mater Res B, 101: 762-9 *(2013)*

Dental Materials, 28:1250-1260 *(2012)*

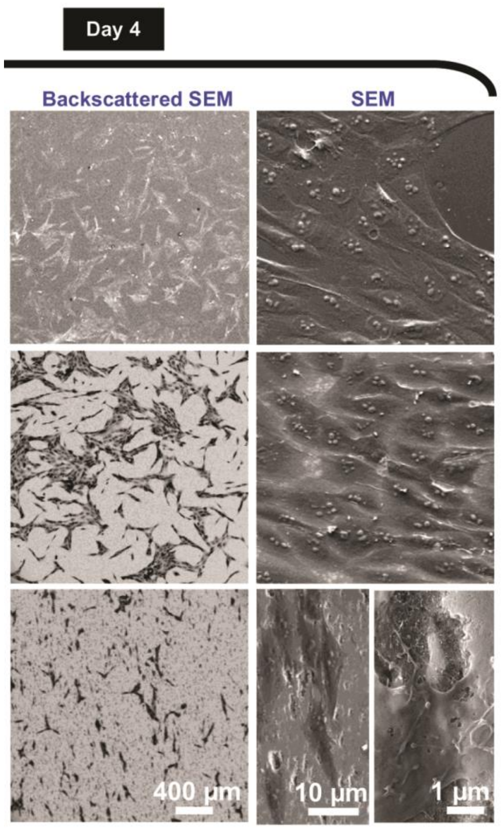
Dental Materials 27: 581-589 (2011)

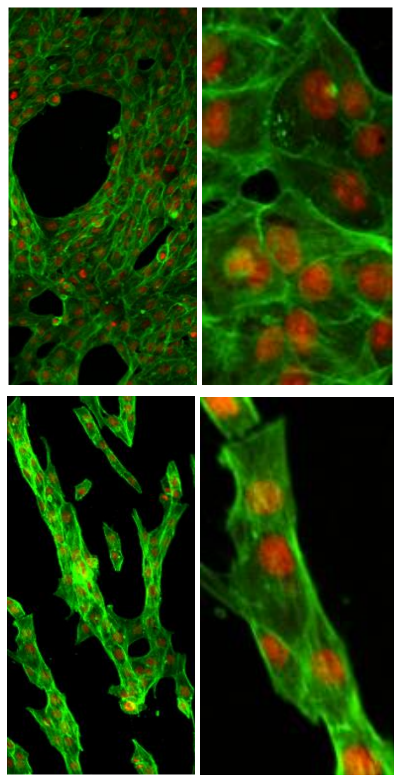
Microsc Microanal 16:670-67 (2010)

Osteoblastic cell response to Biomaterials for bone tissue applications

Carbon nanotubes/Glass/HA composites

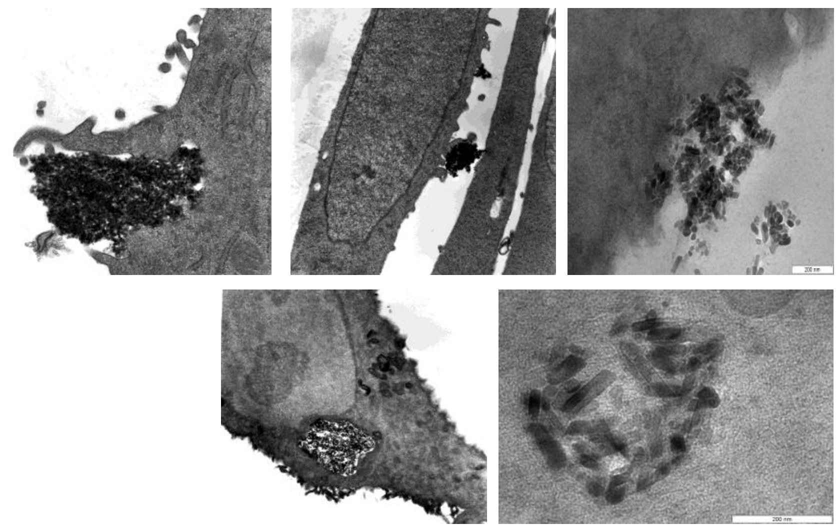
Electrical stimulation of Carbon nanotubes/Glass/HA composites





J Biomedical Nanotechnology, 10:725-743 (2014)

Osteoblastic cell response to Hydroxyapatite nanoparticles

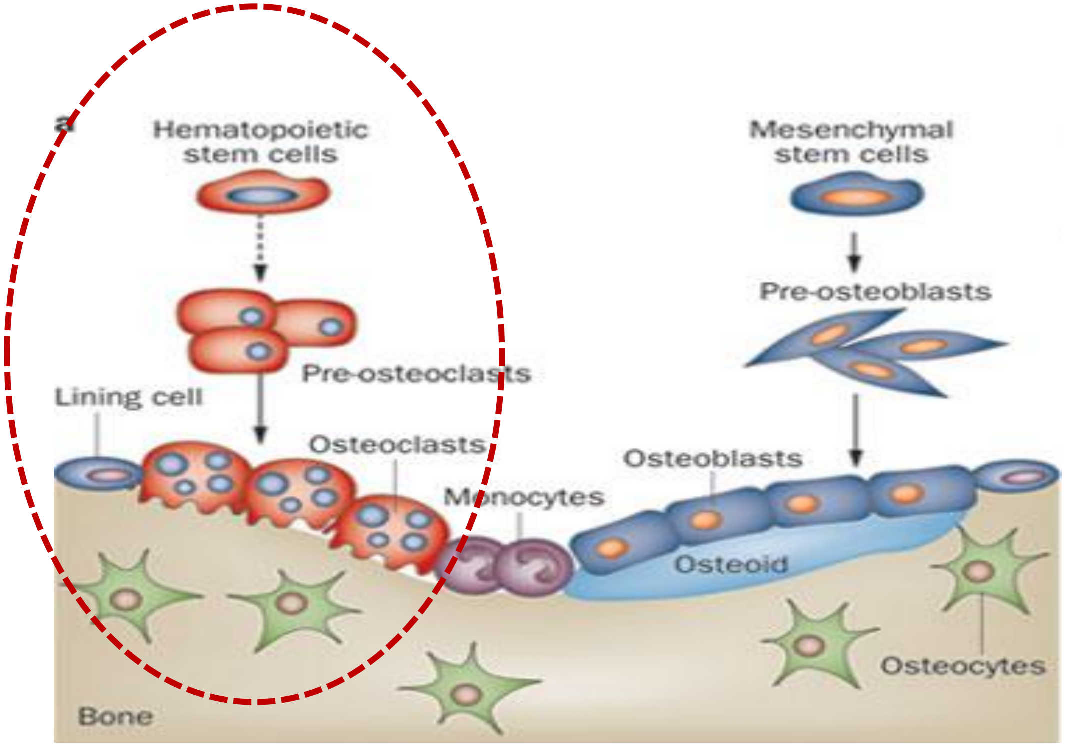


Journal of the Royal Society Interface, 9: 3397-3410 (2012)

Cultures of human Osteoclastic cells

Representative model of the proliferation/differentiation sequence during the development of the Osteoclastic phenotype, including the resorption of the mineralized matrix

Osteoclastic cells



Osteoblastic cells

Human osteoclastic cell cultures

Characterization of the cell behaviour:

- Cell adhesion to the material substrate
- Total protein content
- Apoptosis
- Morphology
- Formation of actin rings
- Immunostaining of Calcitonin and Vitronectin receptors
- Expression of osteoclastic genes
  (c-myc; c-src; TRAP; CATK, CA; …)
- Functional activity
  - TRAP activity
  - Formation TRAP+ multinucleated cells
  - Resorption activity
- Intracellular signaling pathways

Biochemical, histochemical, immunohistochemical and molecular methodologies

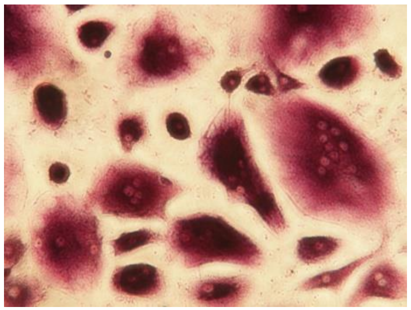
Scanning electron microscopy (SEM)

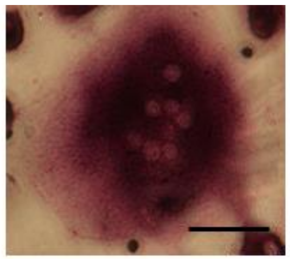
Confocal laser scanning microscopy (CLSM)

Human osteoclastic cell cultures

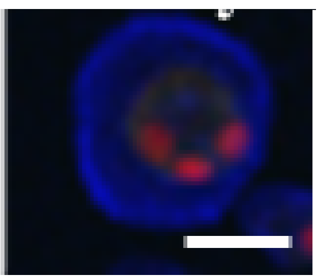
Characterization of the Osteoclastic response

Formation of multinucleated cells
TRAP staining

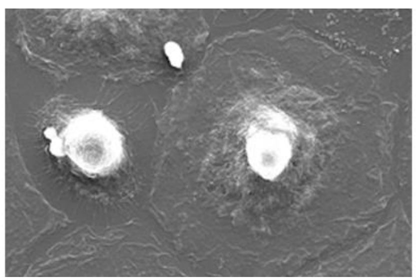




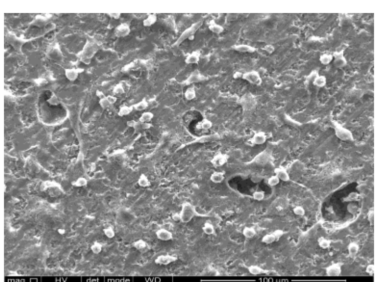
Actin ring

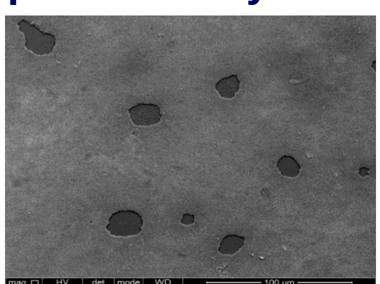


SEM

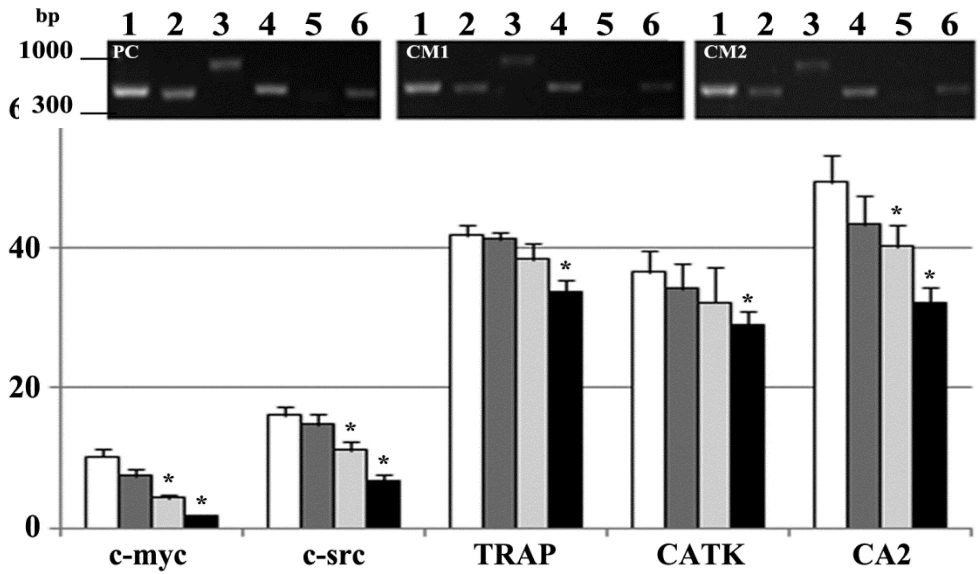


SEM: resorption activity





Expression of osteoclastogenic genes



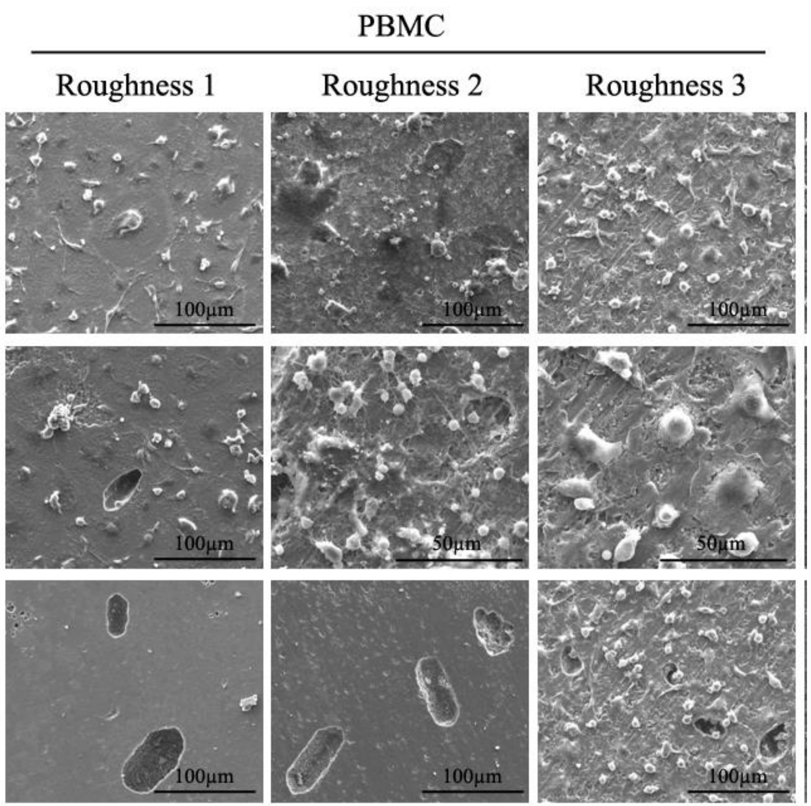
J Cellular Biochemistry 109: 205-216 (2010)

Cell Proliferation; 44: 410-419 (2011)

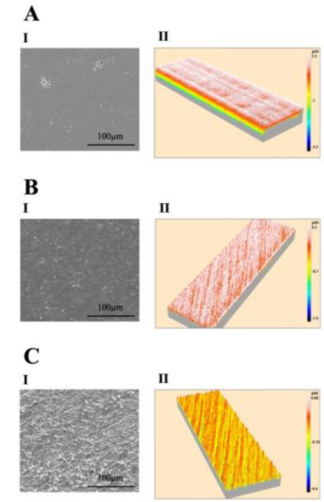
Cell Proliferation; 44: 264-73 (2011)

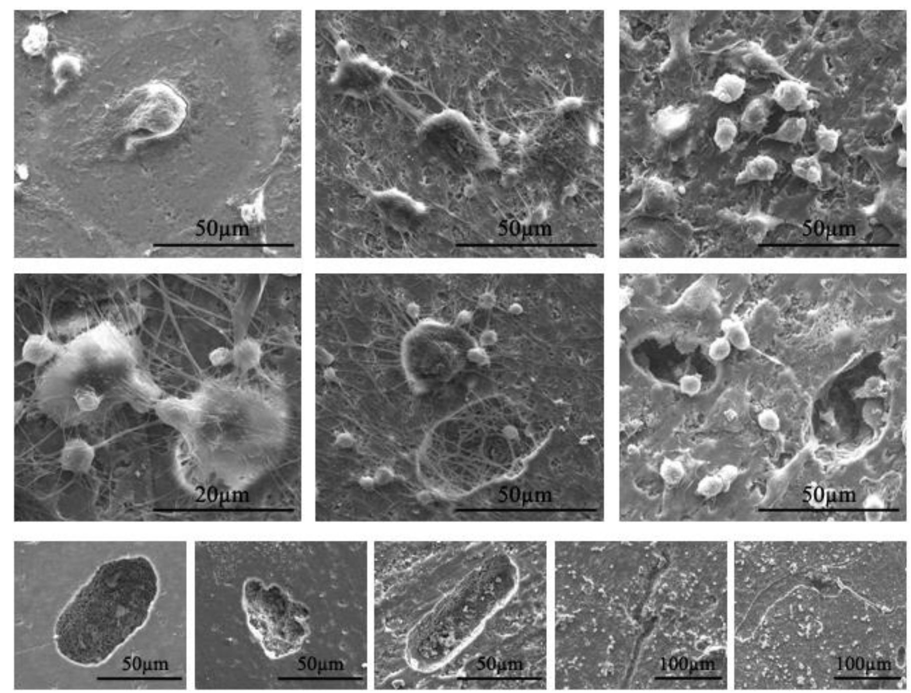
Osteoclastic cell response to Biomaterials for bone tissue applications

Osteoclastic activity on Hydroxyapatite substrates with different roughness



Acta Biomaterialia, 8:1137-45 (2012)





Culture of endothelial cells

Microvascular endothelial cells (commercially available: HDMEC)

Characterization of the cell behaviour:

- Cell adhesion to the material substrate
- Cell viability/proliferation. Pattern of cell growth
- Apoptosis
- Cell cycle
- Morphology/ F-actin cytoskeleton
- Immunostaining of PECAM-1, VE-caderin, factor vWB
- Expression of endothelial genes
  (PECAM-1, VE-caderin, factor vWB)
- Functional activity
  Production of NO
  Formation of tubular-like structures

Biochemical, histochemical, immunohistochemical and molecular methodologies

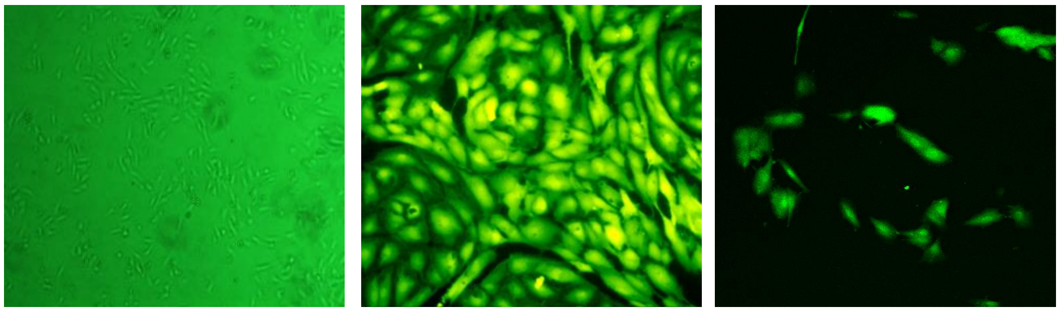
Scanning electron microscopy (SEM)

Confocal laser scanning microscopy (CLSM)

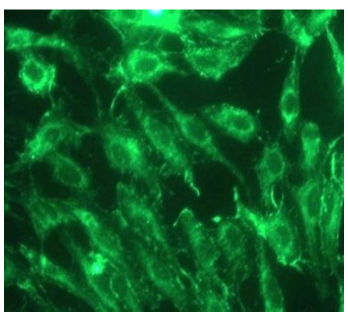
Culture of endothelial cells

Microvascular endothelial cells (HDMEC)

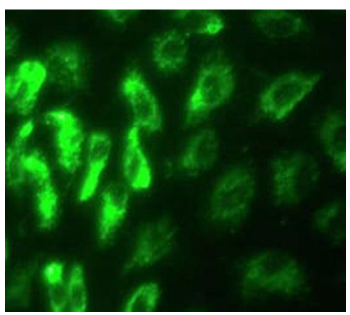
Pattern of cell growth



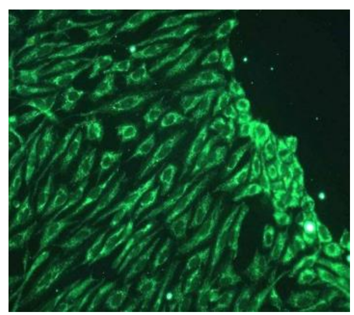
PECAM-1

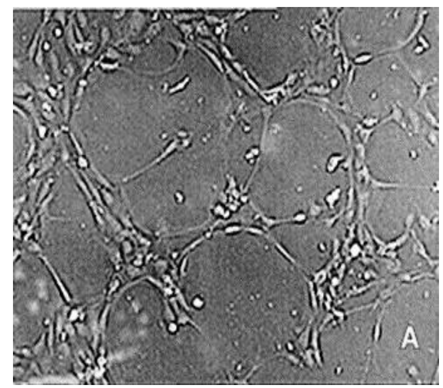


factor vWB



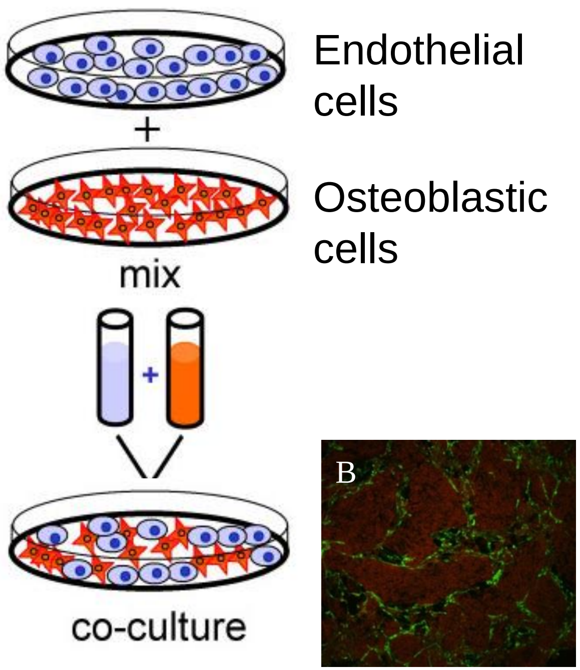
VE-caderin





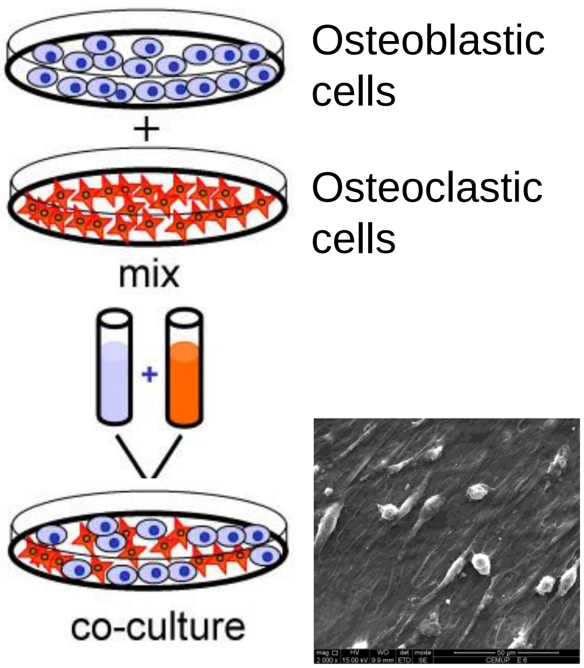
Formation of tubular-like structures

Co-cultures
endothelial cells /osteoblastic cells



Characterization for
Osteoblastic and endothelial parameters

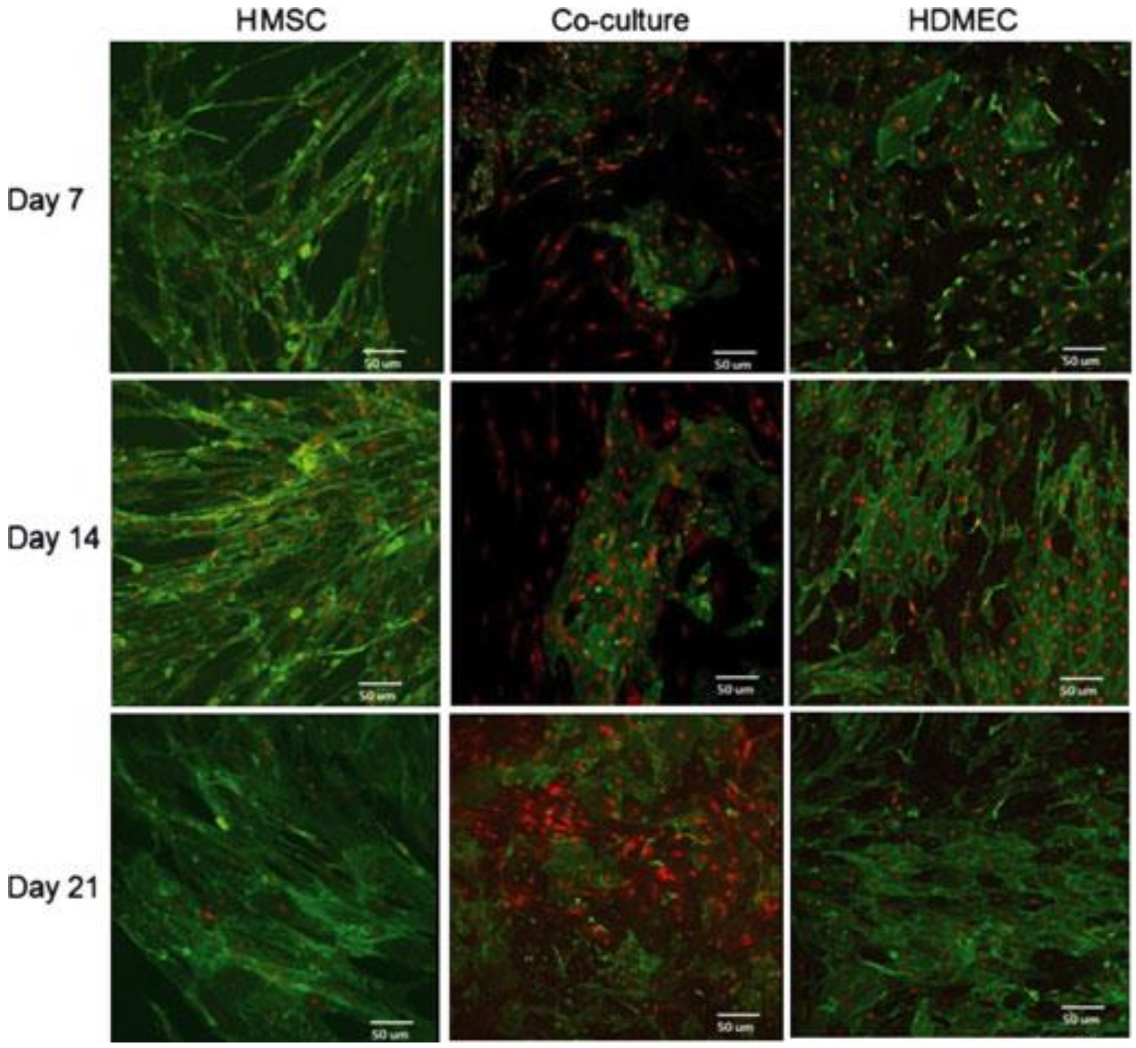
Co-cultures
Osteoblastic /osteoclastic cells



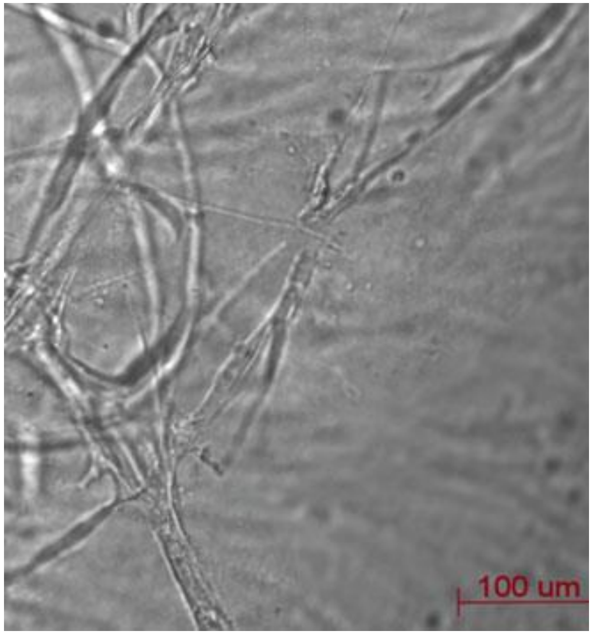
Characterization for
Osteoblastic and osteoclastic parameters

Co-cultures of endothelial cells /osteoblastic cells

Immunostaining of osteoblast cells, endothelial cells and co-cultures



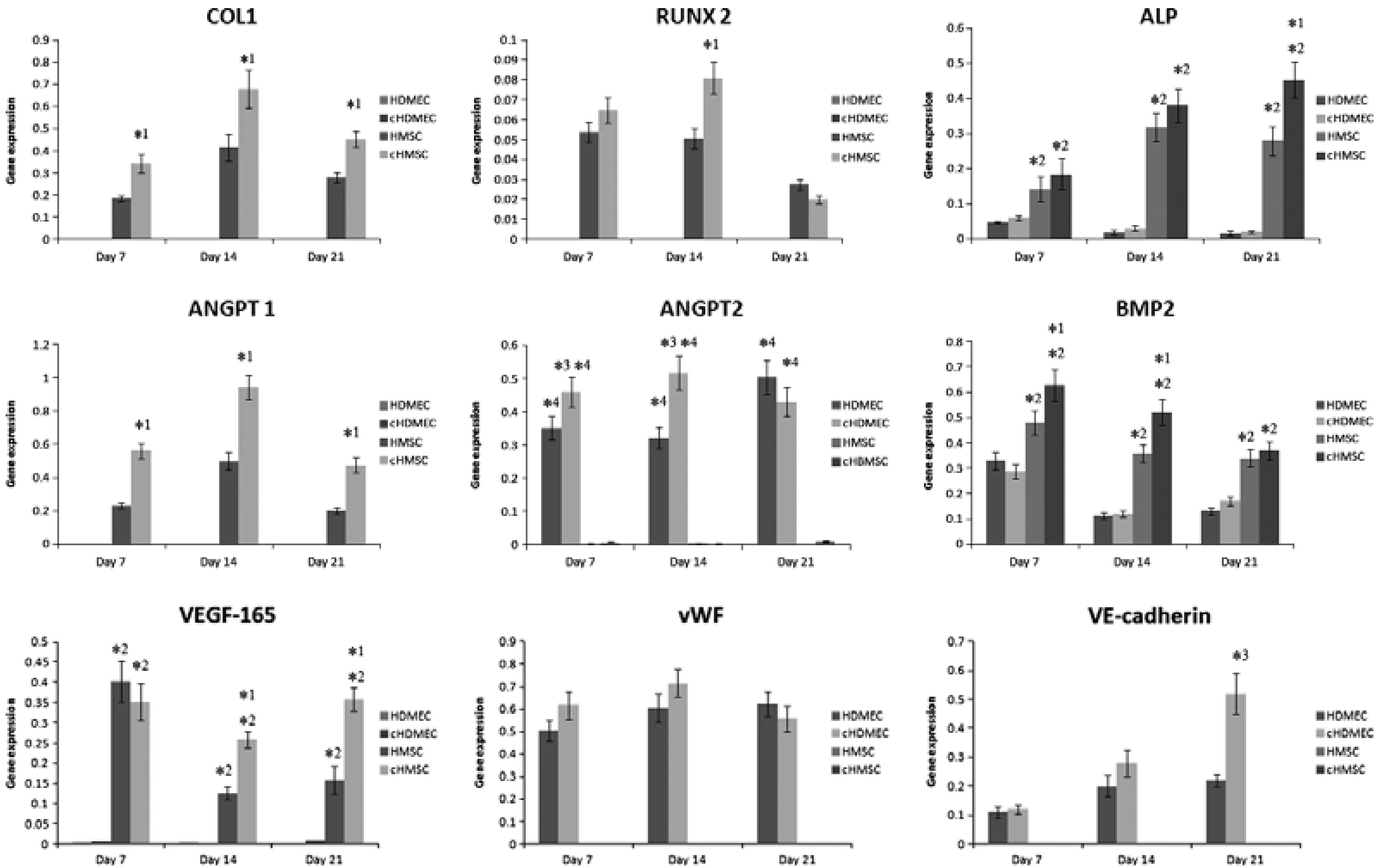
Cell proliferation; 45:320-334 (2012)



Formation of tubular-like structures

Co-cultures of endothelial cells /osteoblastic cells

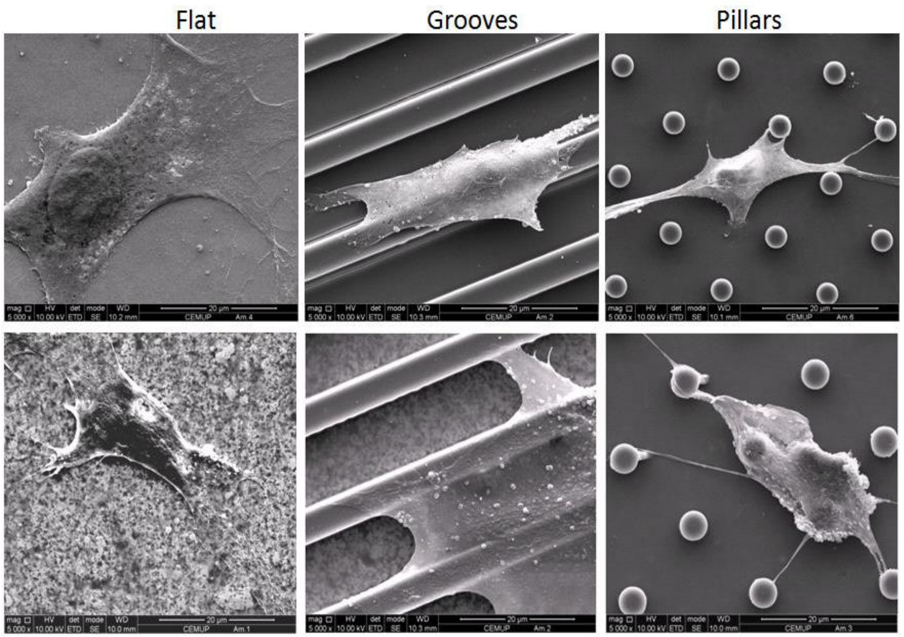
Expression of osteoblastic and endothelial genes in monocultures and co-cultures of endothelial cells and osteoblasts



Cell proliferation; 45:320-334 (2012)

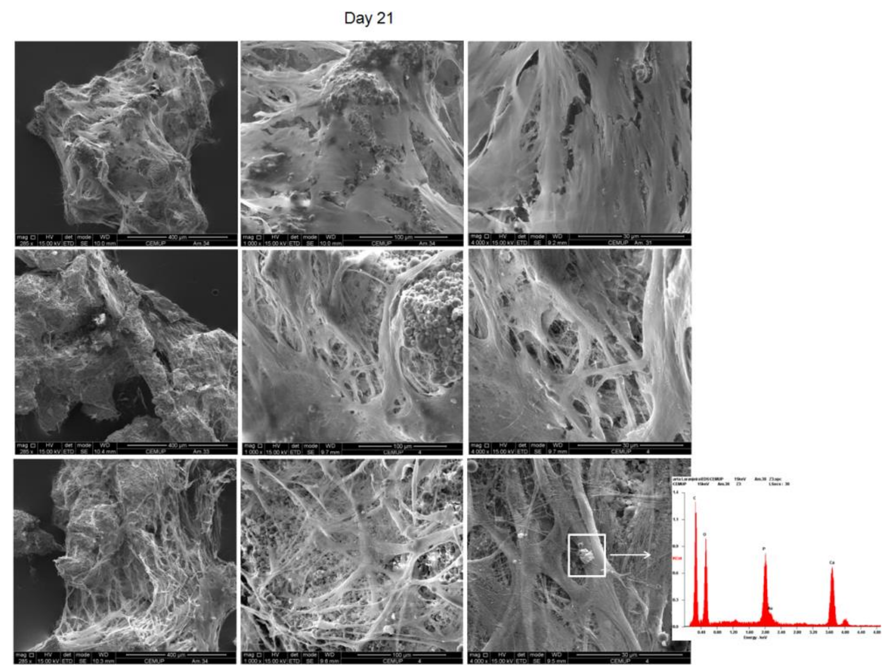
Cell response to co-cultures of osteoblastic and endothelial cells cells

Silica coating surfaces for zirconia implants
Endothelial cells



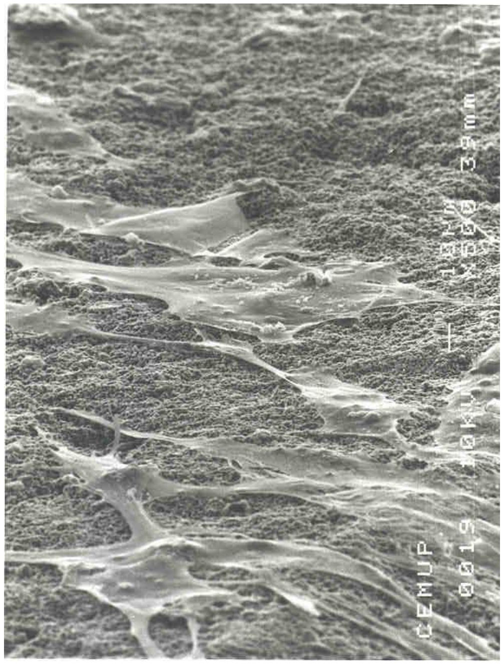
J Biomedical Nanotechnology, submitted (2013)

Macroporous granules of nanostructured-hydroxyapatite agglomerates
Co-cultures of osteoblastic and endothelial cells



J Biomedical Nanotechnology, in press (2013)

Cellular models of bone metabolism



J Biomat App
14: 113-168; 1999

Advantages

Information on the molecular and cellular response in a controlled environment

Selection of the experimental conditions to analyse specific aspects of the cell response, in the absence of the complex *in vivo* conditions

Limitations

• Alteration of the cell phenotype with the culture time

• Absence of the in vivo 3D environment, the matrix organization and the complex chemical and mechanical factors

- *In vitro* observations can not be extrapolated to *in vivo*

First stage of biological response to biomaterials

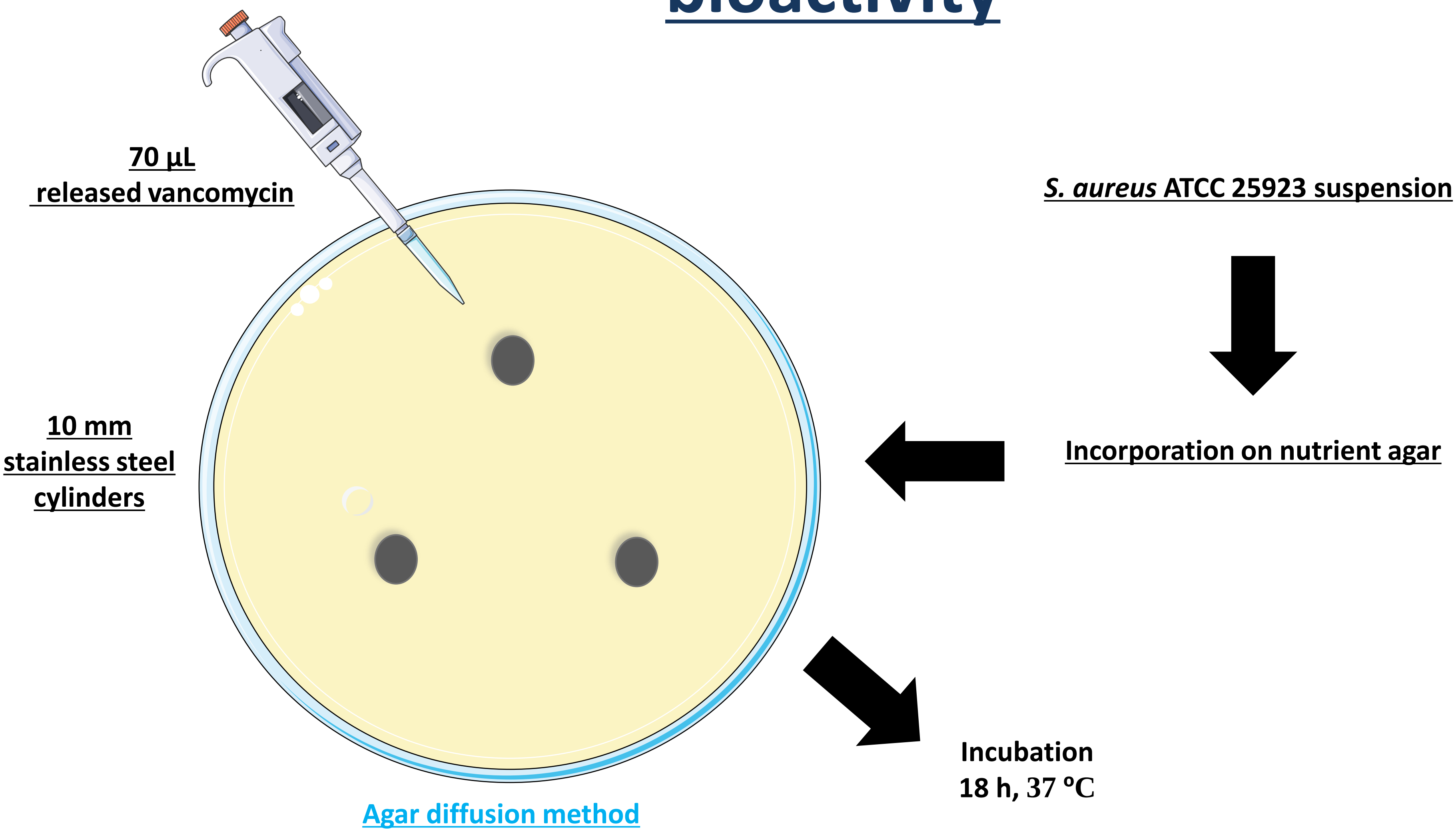
Bacterial adhesion

- Streptococcus mutans
- *Streptococcus gordonii*
- Staphylococcus aureus
- Staphylococcus Epidermidis
- Pseudomonas aeruginosa
- E..Coli
- ....

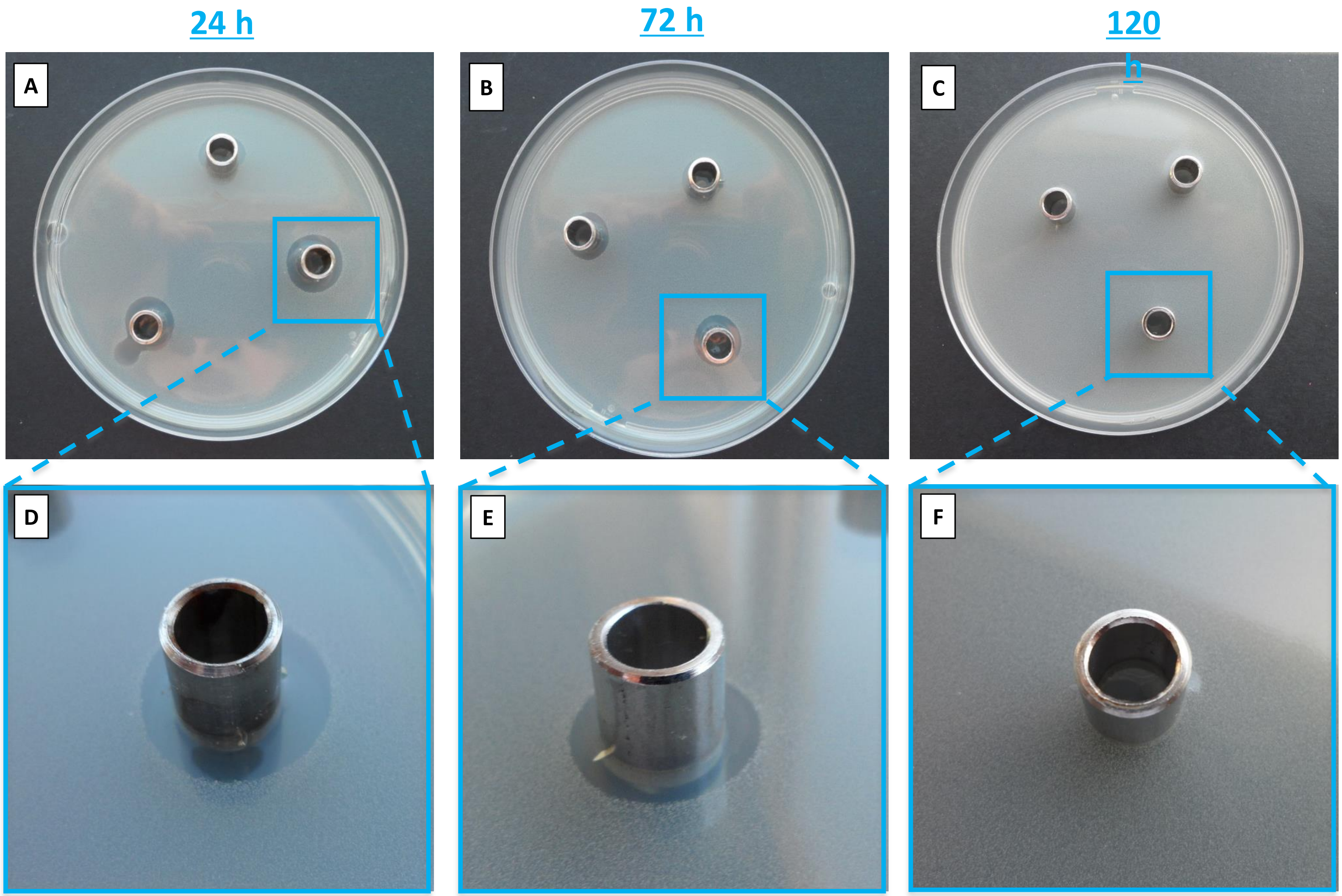
- Quantification of bacterial adhesion- Colony forming unit counting (CFU).
- SEM,
- Confocal laser scanning microscopy

- Biofilm analysis
- Quorum sensing studies

Drug release studies bioactivity

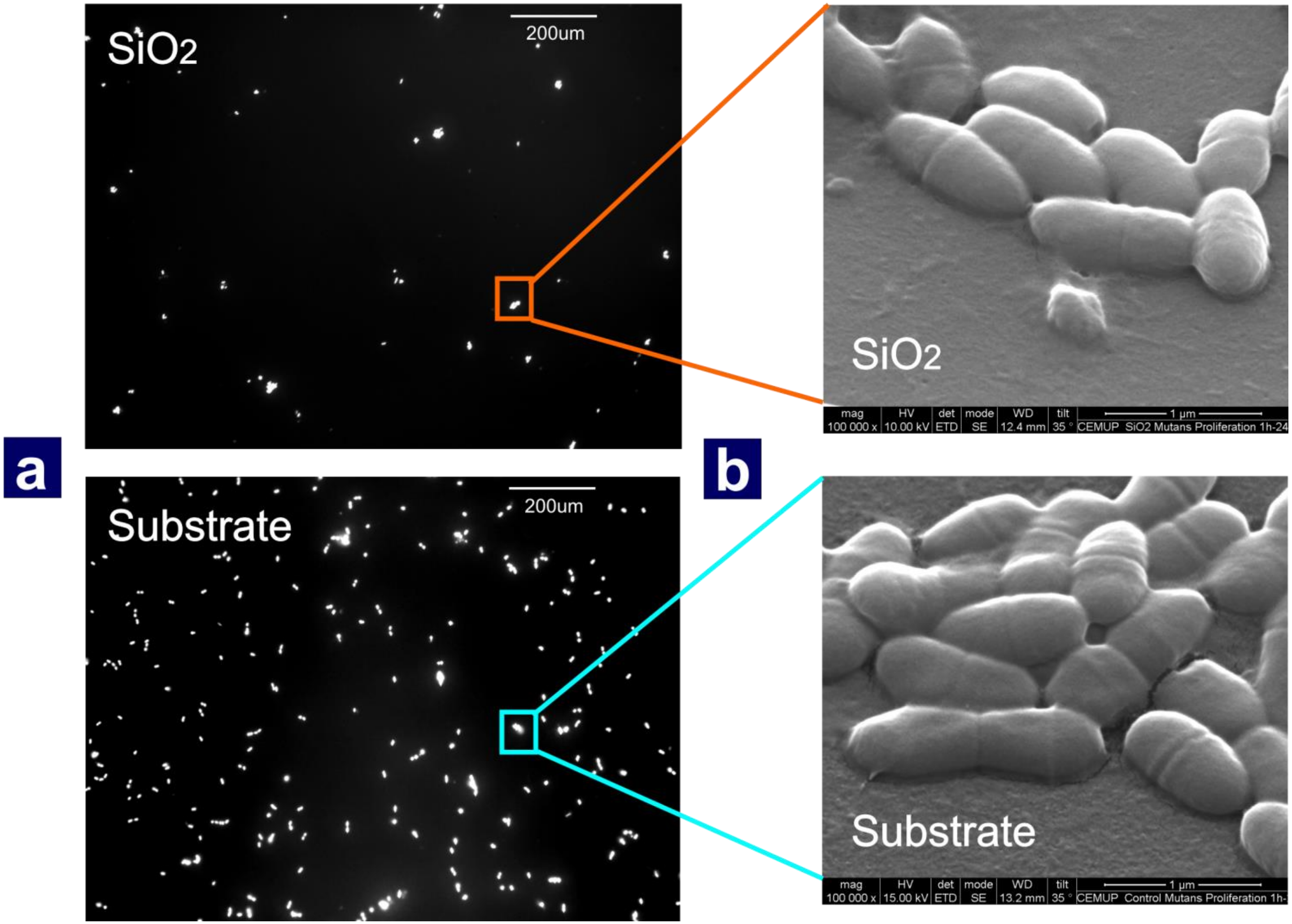


Drug release studies: bioactivity



Lost bioactivity? Limit of detection?

Bacterial Adherence (St. Mutans – 4h)



<u>**Peláez-Vargas A,**</u> Fernandes MH, Ferraz MP, Monteiro FJ. **St. mutans and osteoblast adhesion on silica coatings.** J Dent Res , **2009.**

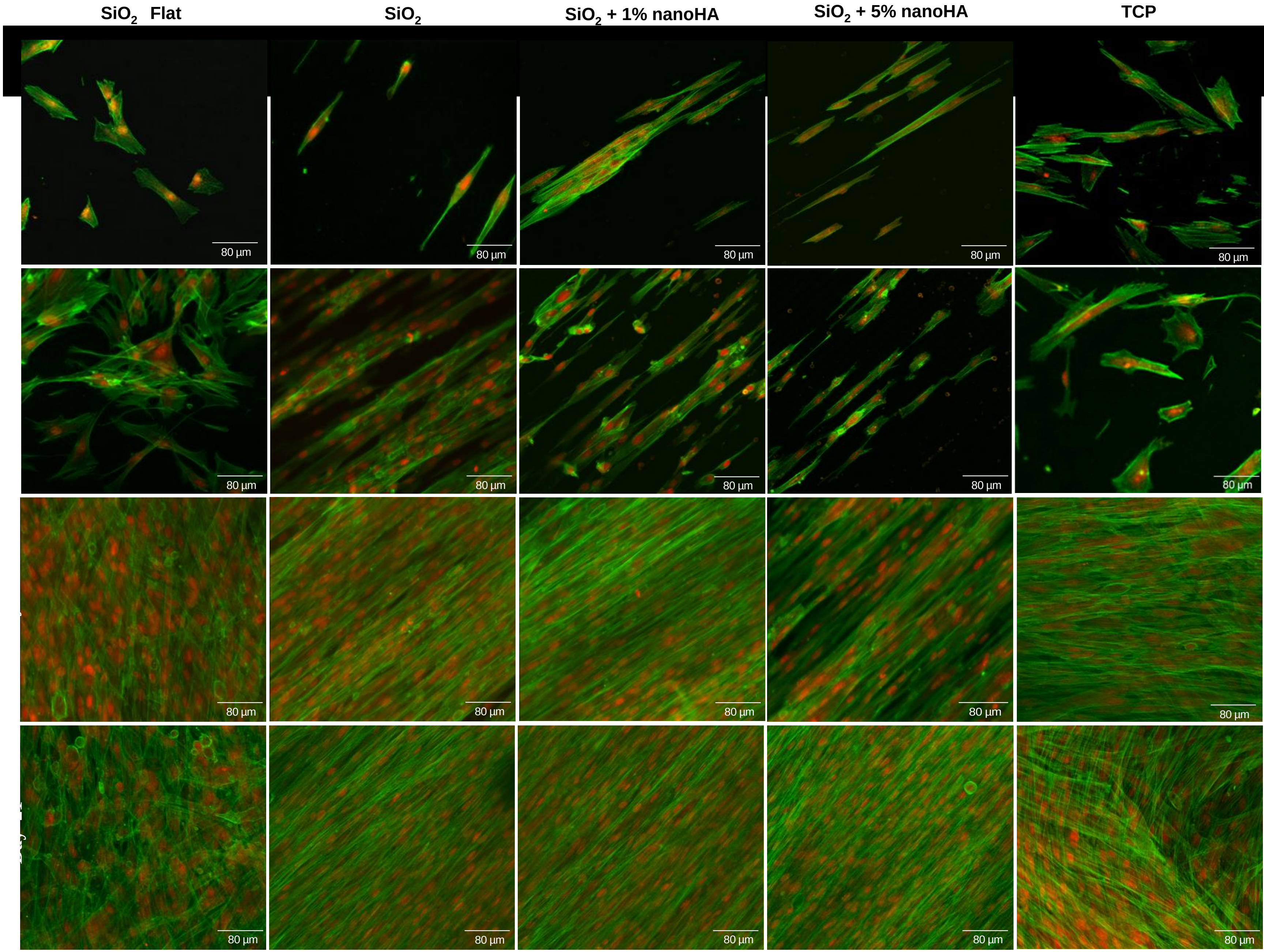
Microscopy techniques

- Epifluorescence Microscopy

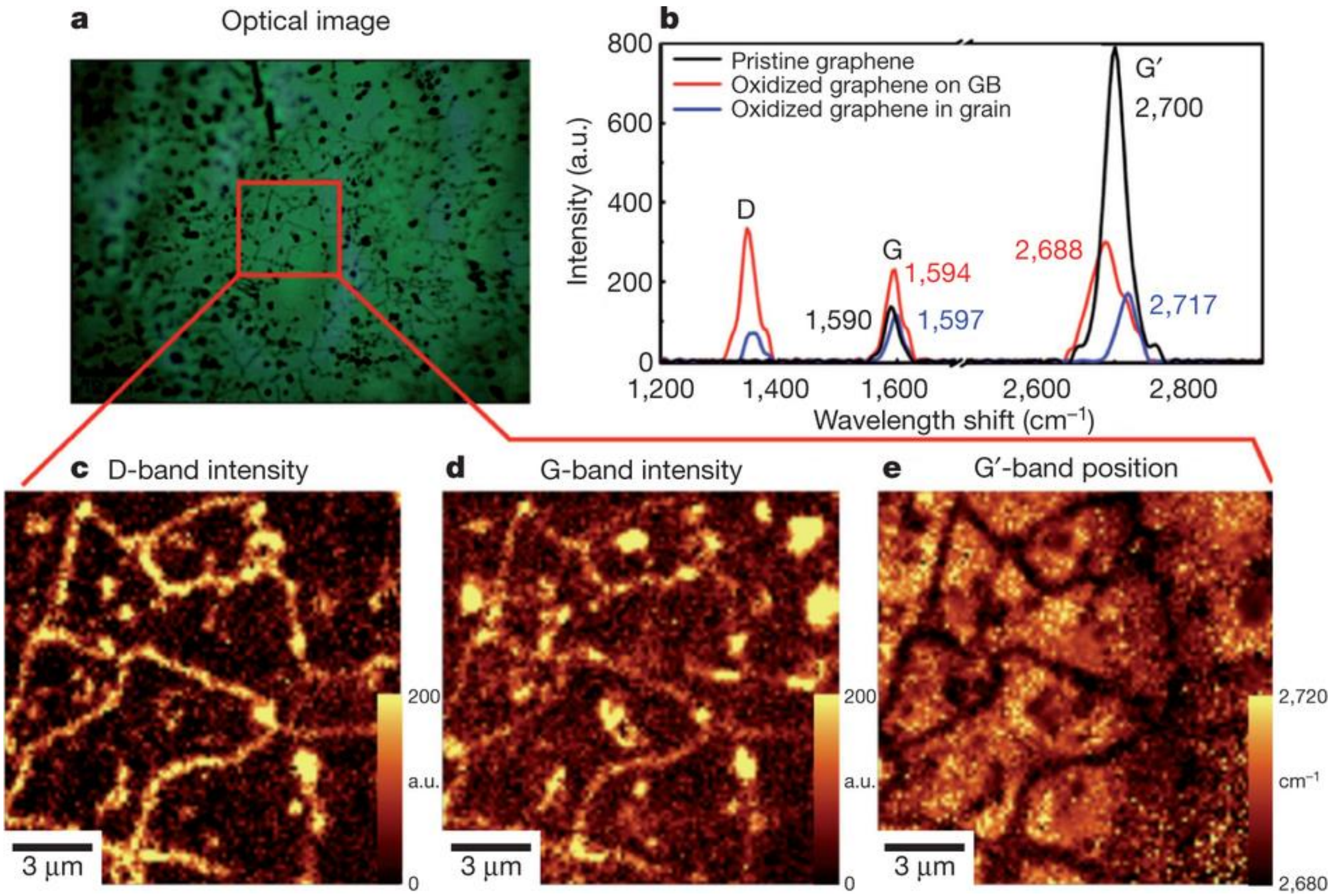
- time lapse microscopy – (Epifuorescense)

for Motility behaviour

Morphology and motility

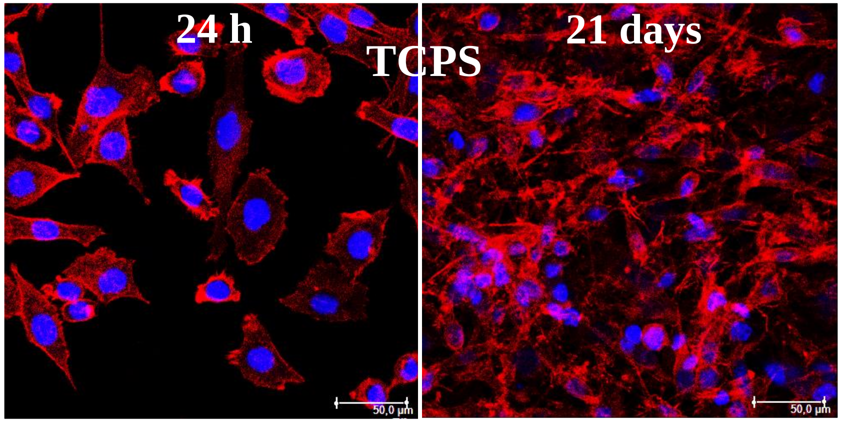


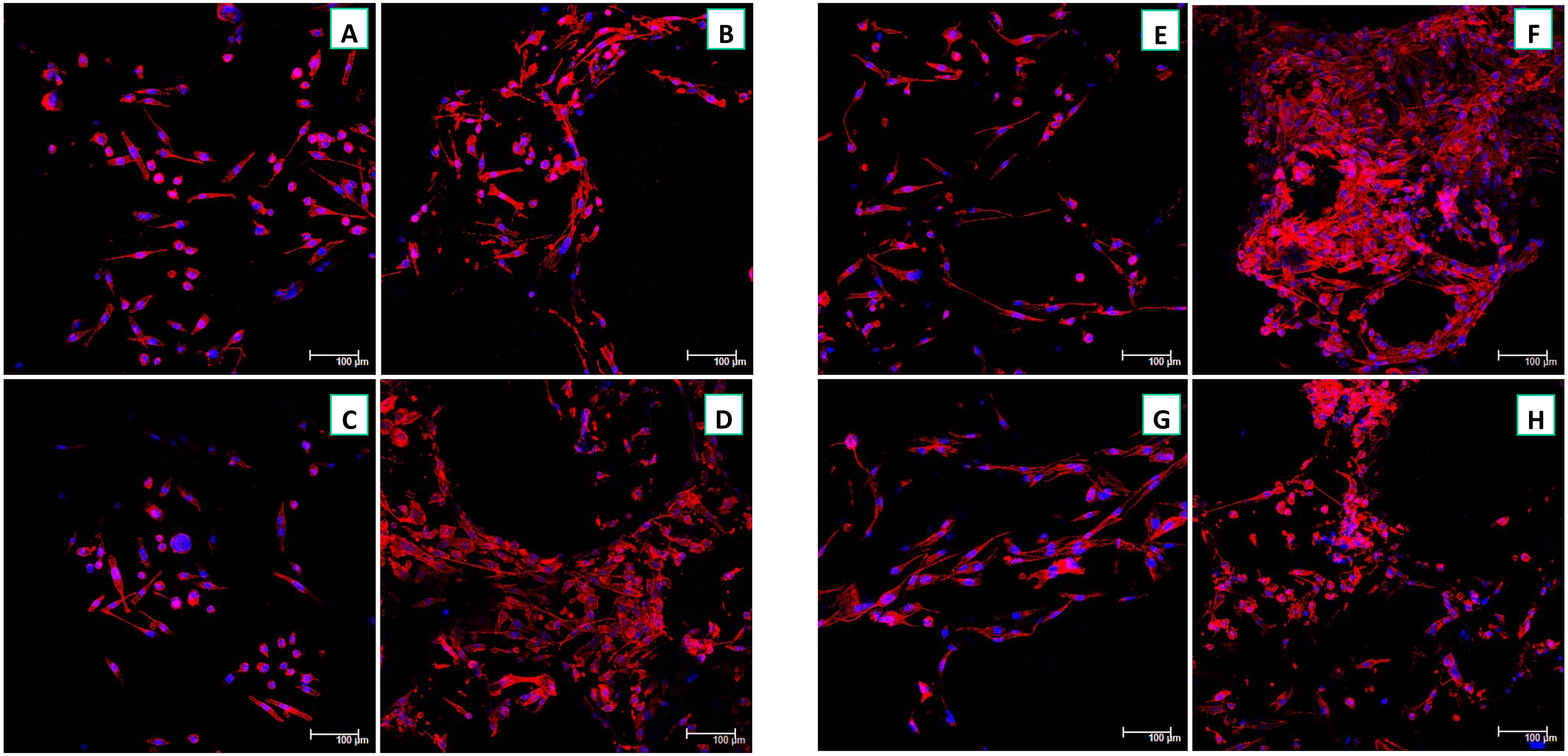
Confocal Laser Scanning Microscopy (CLSM) particularly relevant in the case of 3D scaffolds



CLSM

Red – Cell cytoskeleton
Blue – Cell nuclei





Microscopy techniques

- Confocal Raman microscopy-” chemical” imaging functional groups on substrates, or on proteins (other macromolecules)

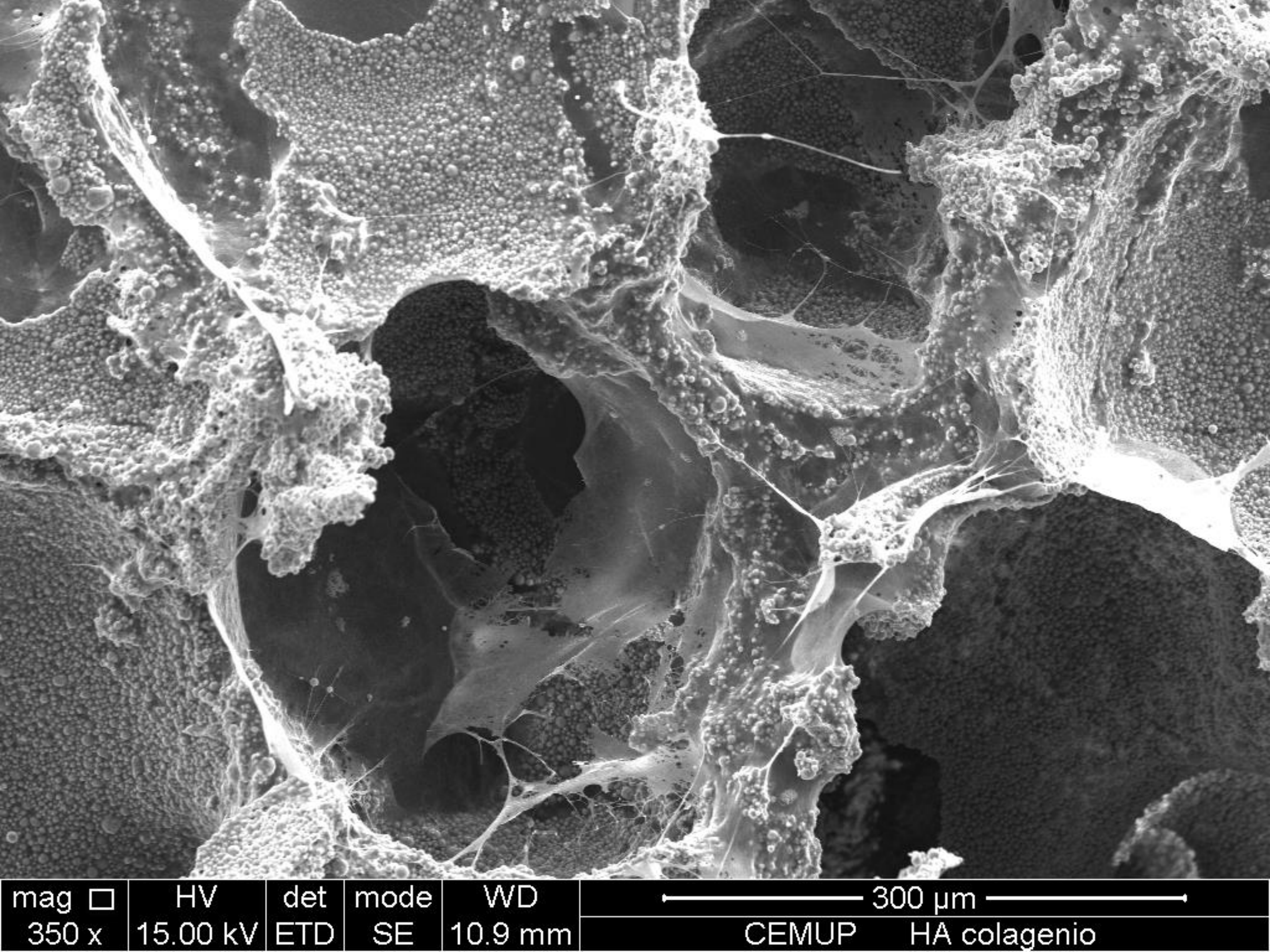
S.E.M. techniques

- SEM(high Vacuum)
- ESEM
- CryoSEM
- for surface morphology, and topography cell adhesion, distribution and morphology

(SE electrons image)

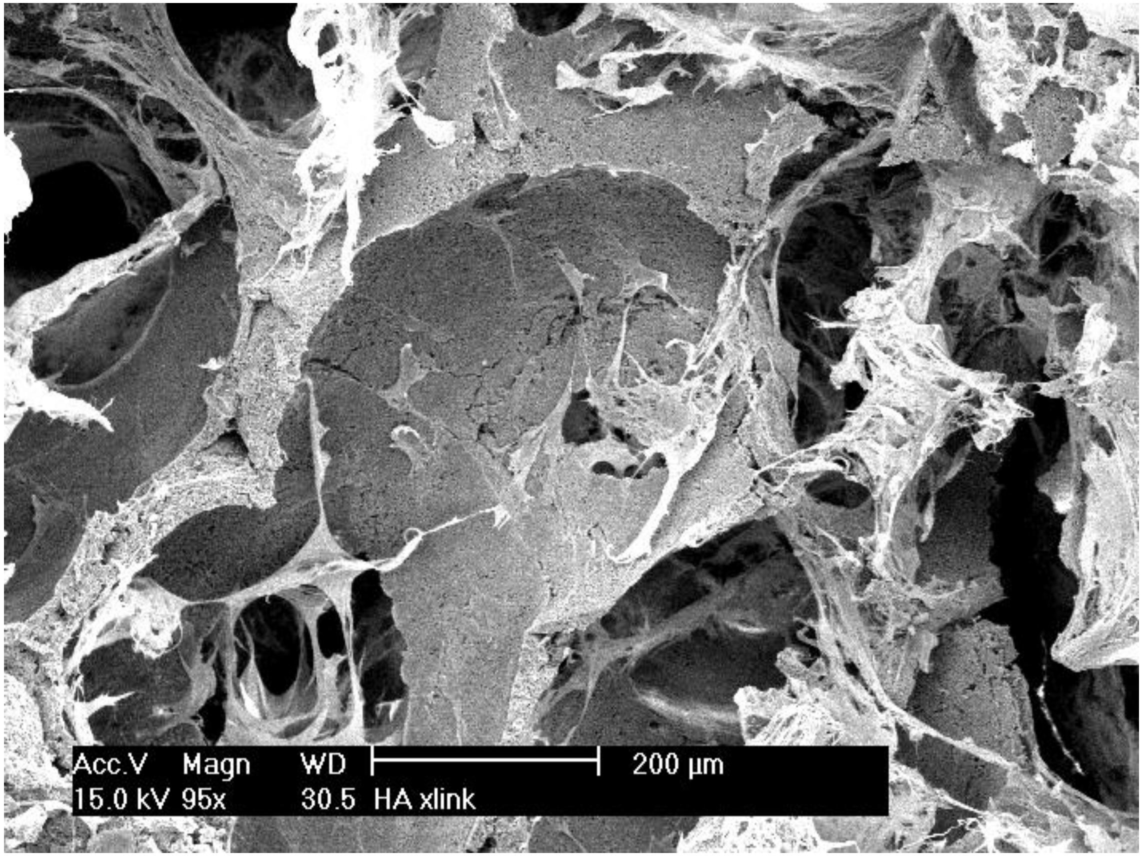
- Atomic number contrast (BS electrons Image)

- EDS analysis


mag □
350 x
HV
15.00 kV
det
ETD
mode
SE
WD
10.9 mm
300 µm
CEMUP
HA colagenio

Environmental scanning electron microscopy (ESEM)

The bioceramics were loaded with RMSC
for a week and their structures were visualised.

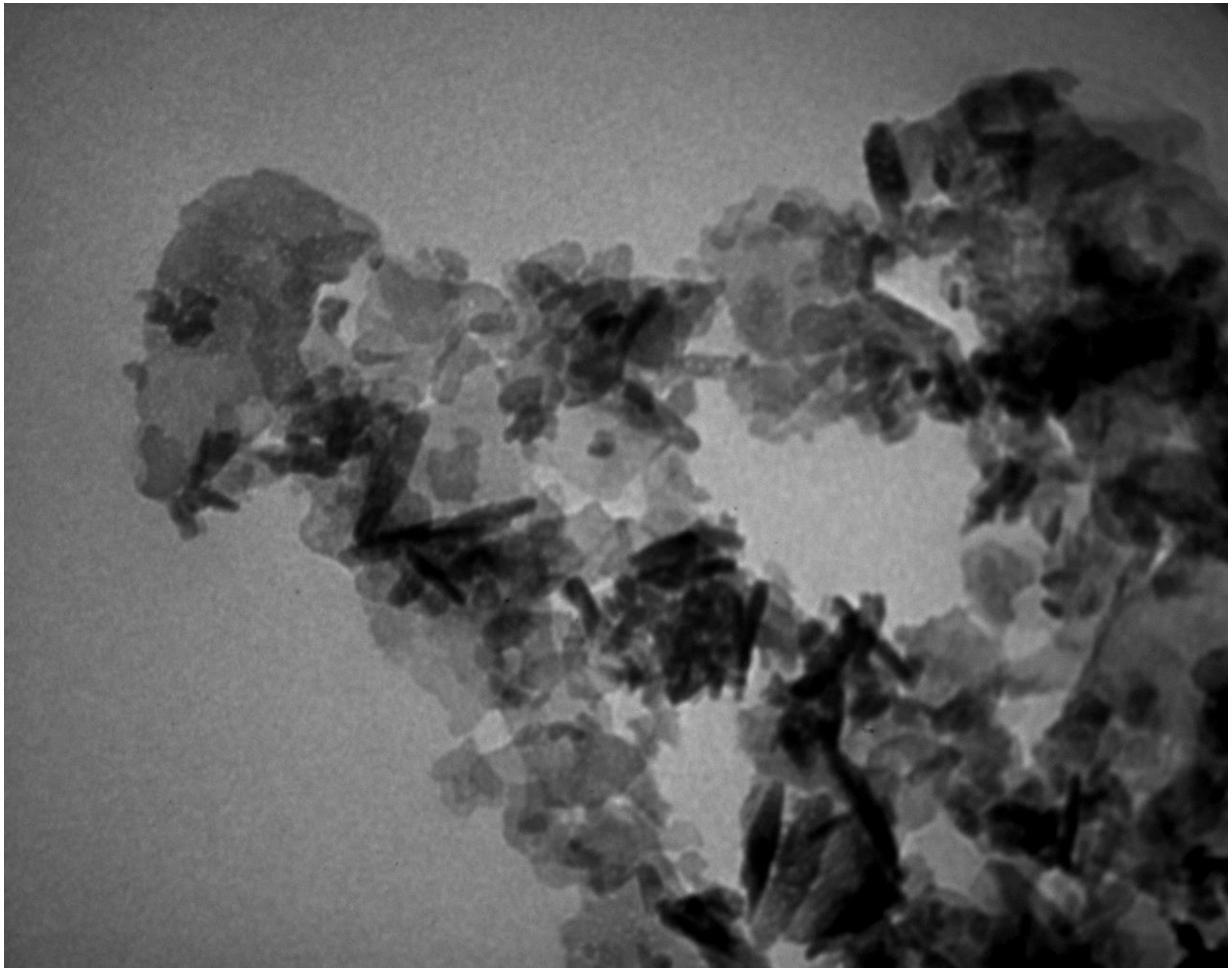


(ESEM) of the bioceramics: HA/crosslinked collagen.

Microscopy techniques

- TEM
- morphology (and EDS analysis) at nanoscale

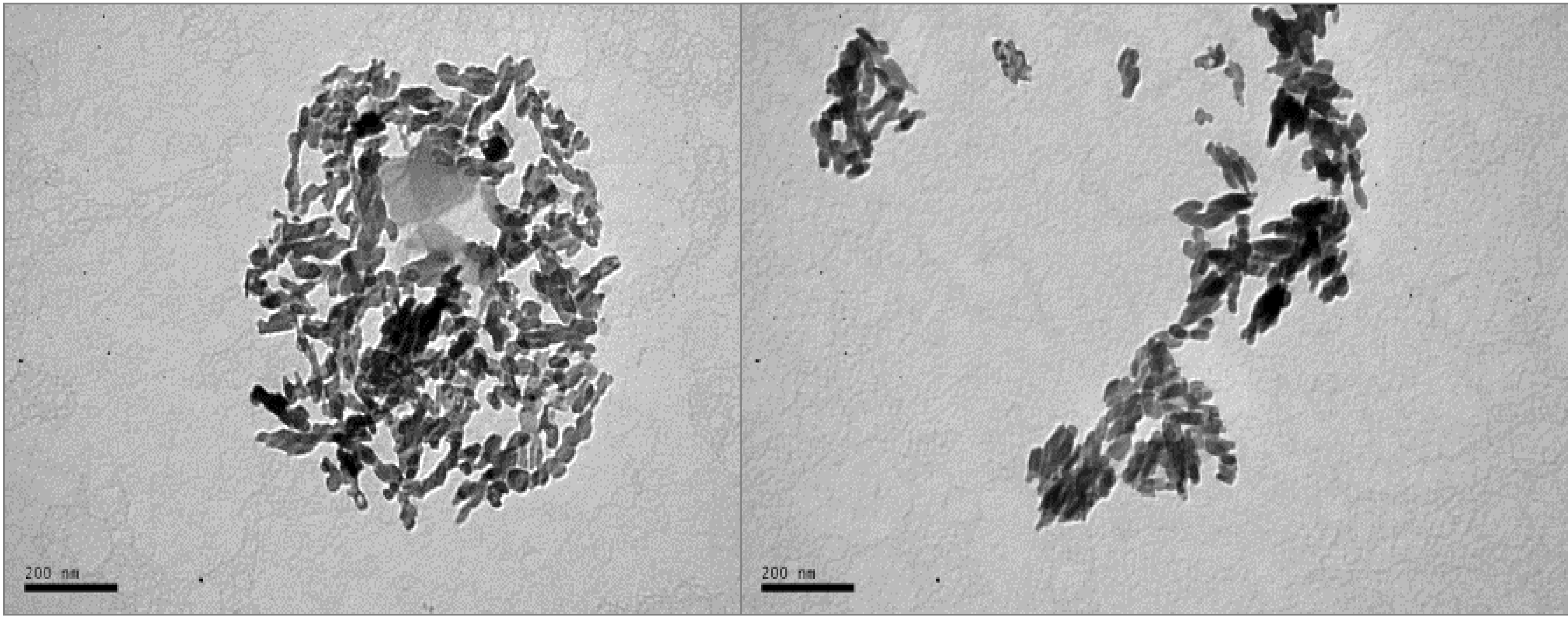
TEM

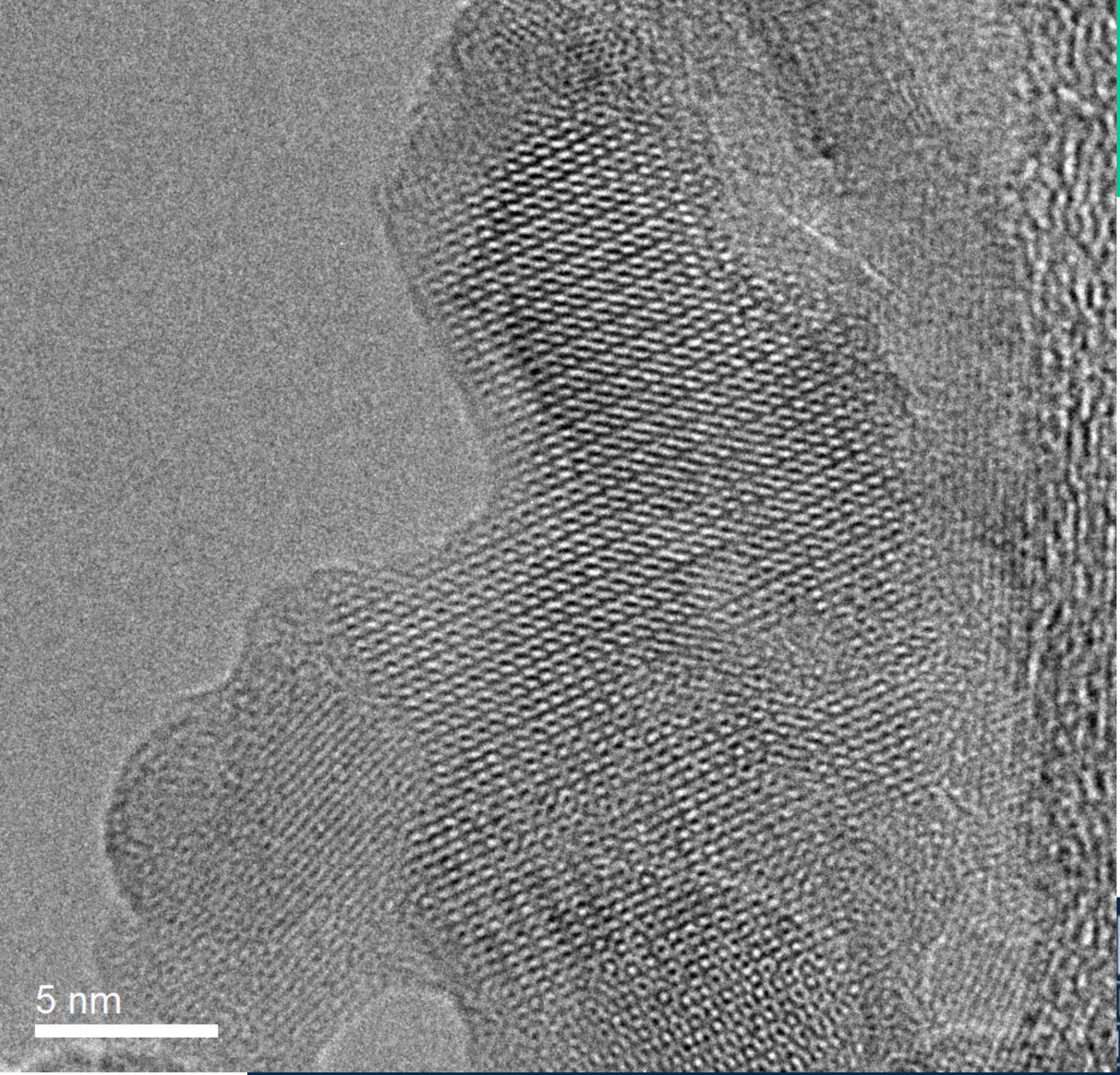


Amostra COM x50000, x5.7

TEM

Size and morphology



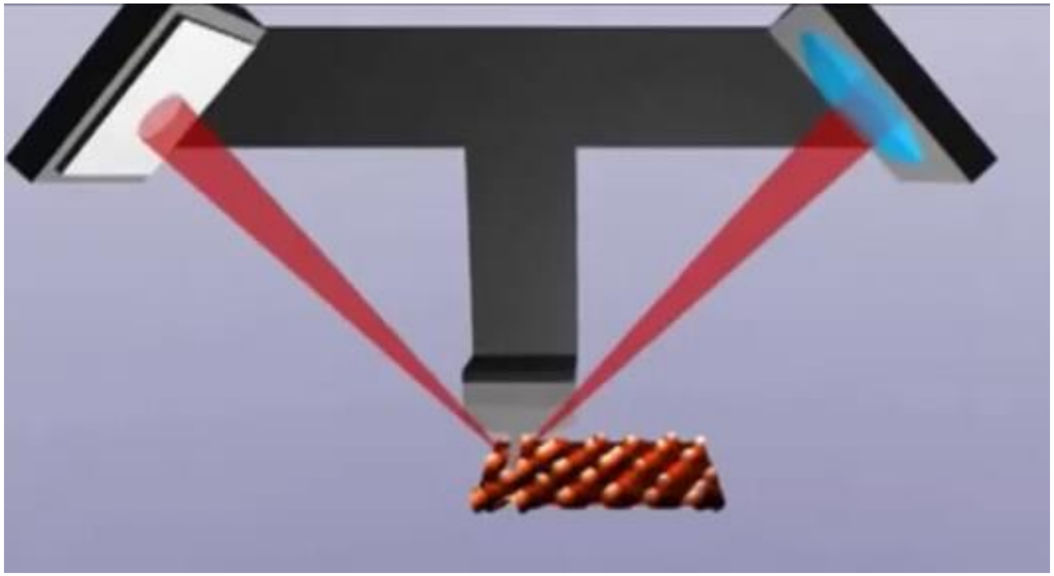


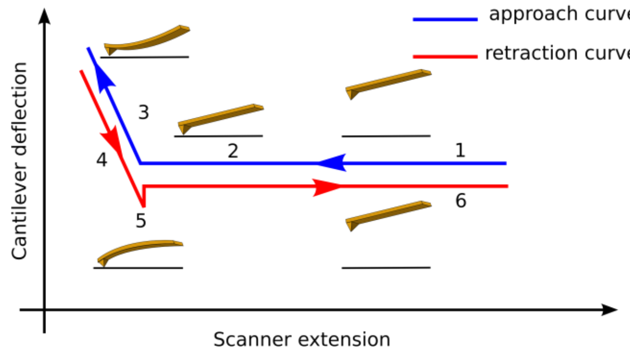
HRTEM
Nanophased
hydroxyapaptite
gel

AFM

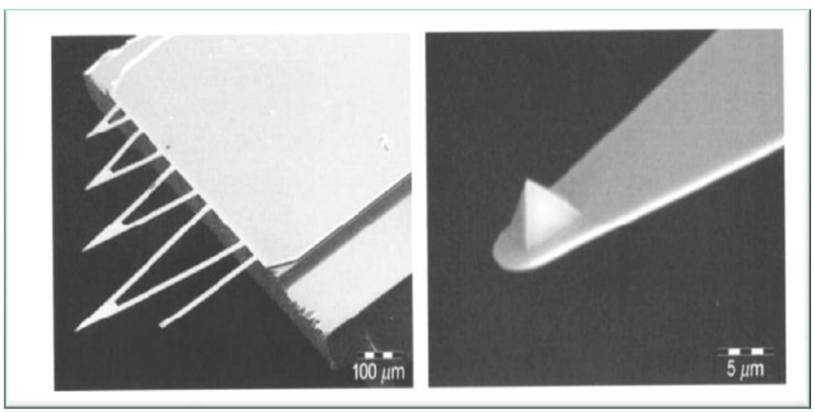
- surface nanotopography
- AFM/ Epifluorescent microscope associated studies in liquid environments

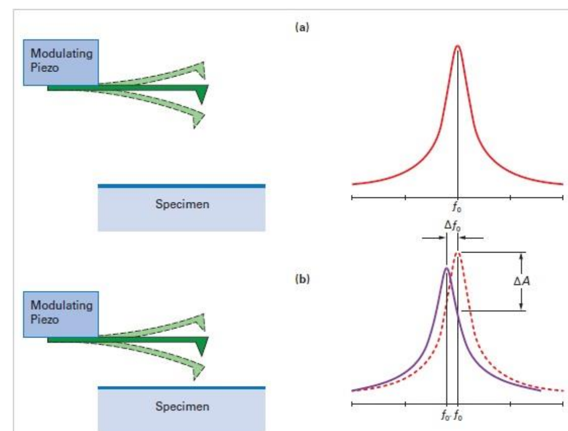
ATOMIC FORCE MICROSCOPY (AFM)



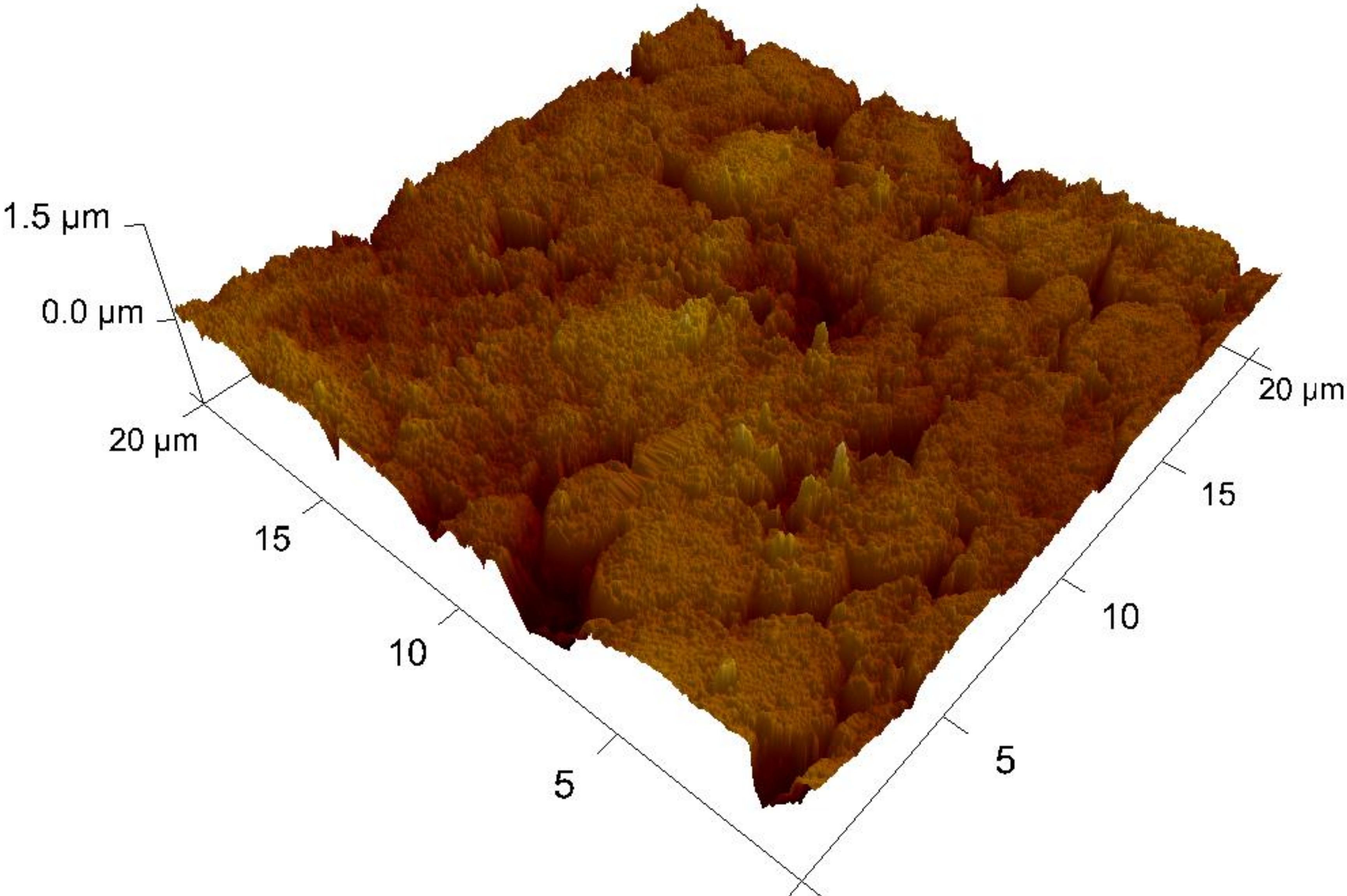


Contact mode

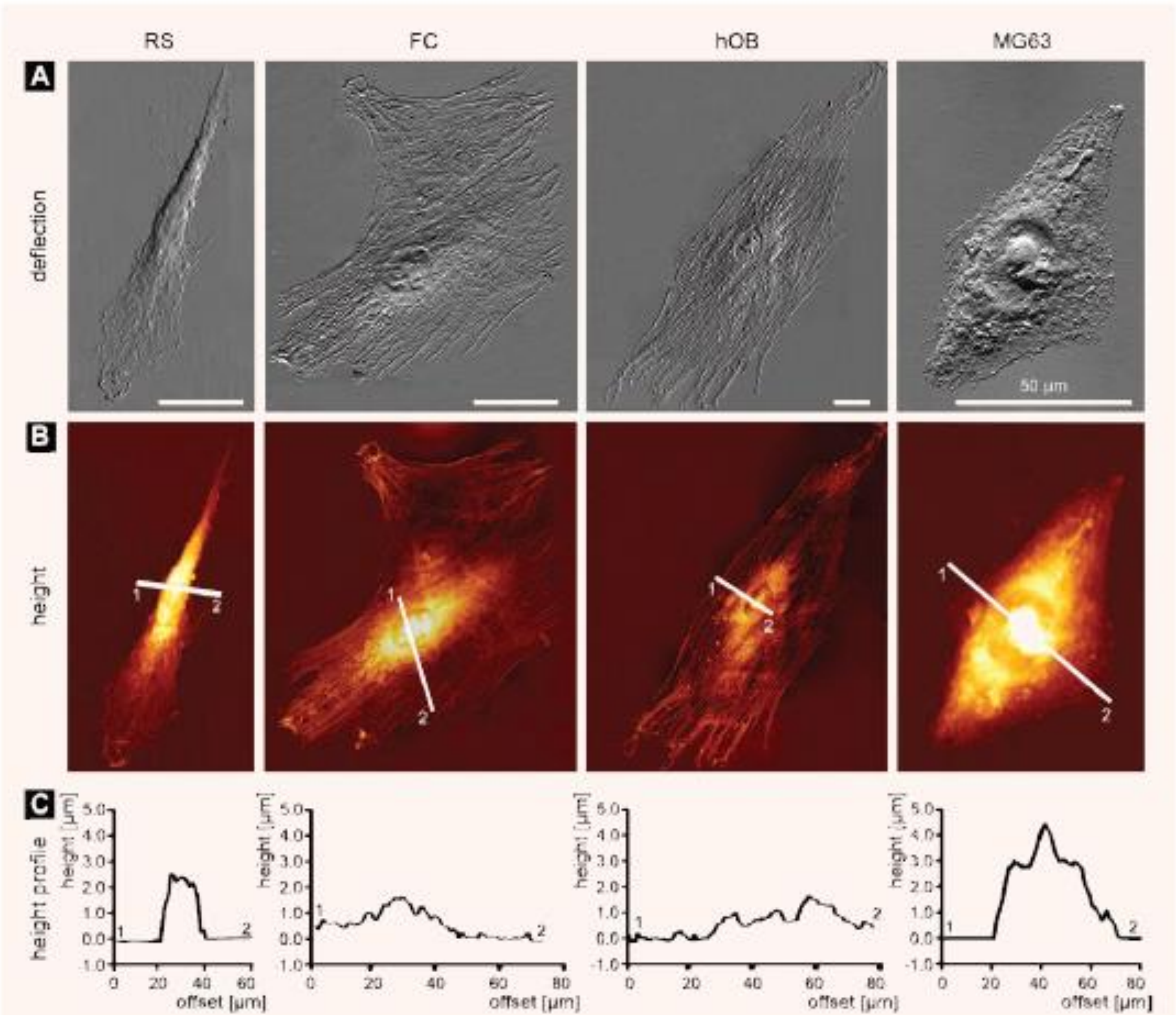




Oscillating mode


1.5 µm
0.0 µm
20 µm
15
10
5
20 µm
15
10
5

CELL MORPHOMETRY



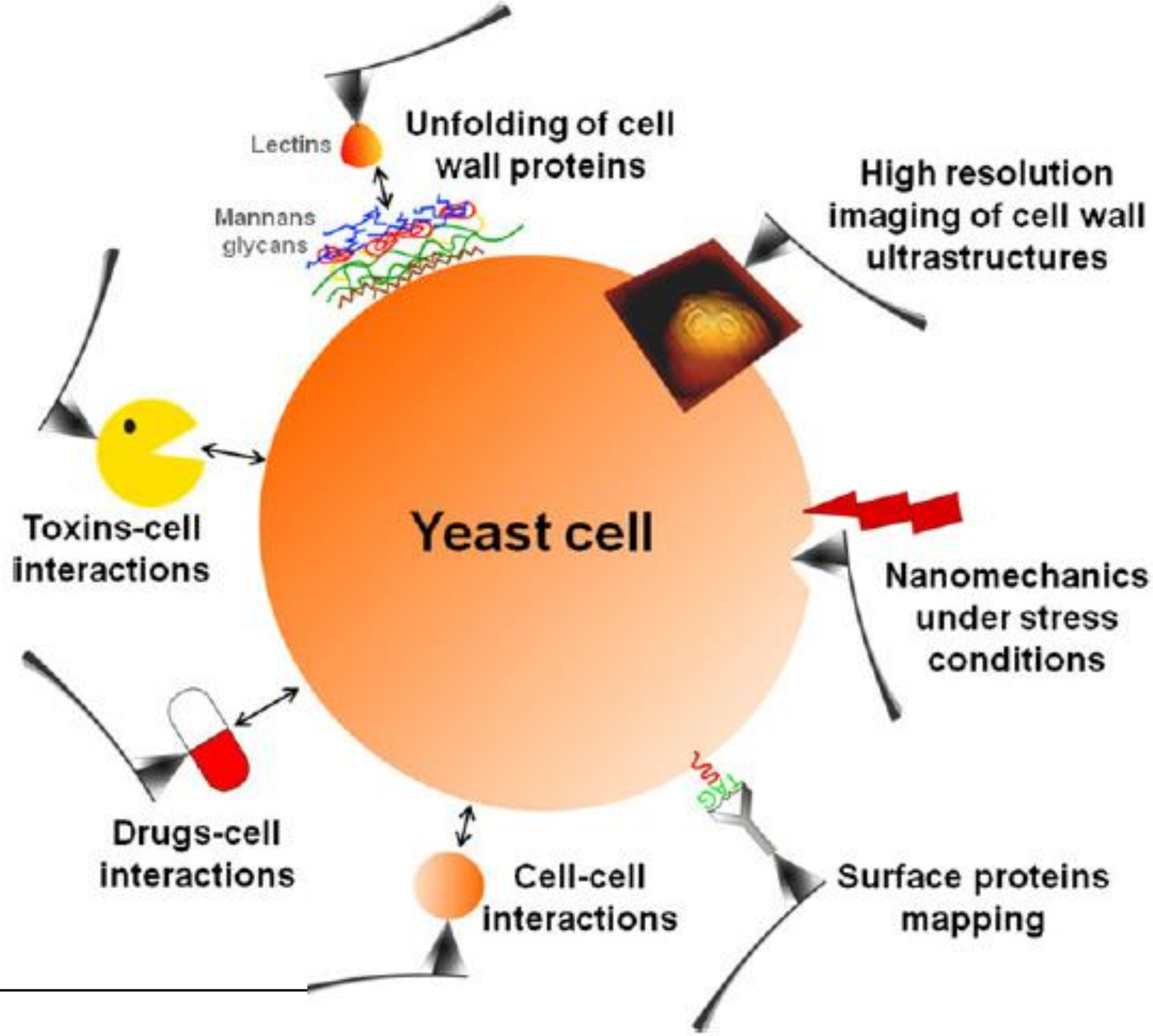
MSC - Mesenchymal Stem cells -→ rapid self-renewal (RS)

MSC - Mesenchymal Stem -→ slowly replicate flat cells (FC)

hOB – Osteoblasts

MG63 – Osteosarcoma cells line

Docheva D, *et al*, **J. Cell. Mol. Med.** Vol 12, No 2, 2008 pp. 537-552



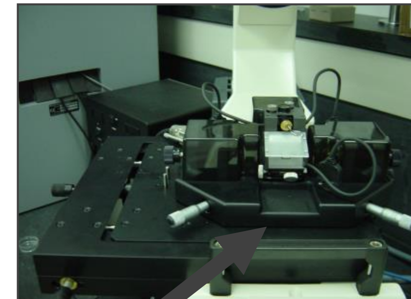
Curr Genet (2013) 59:187 196
DOI 10.1007/s00294 013 0411 0

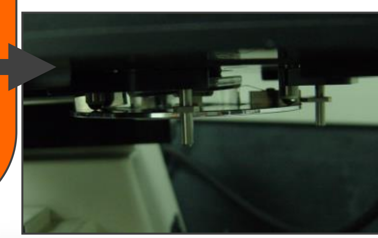
RESEARCH ARTICLE

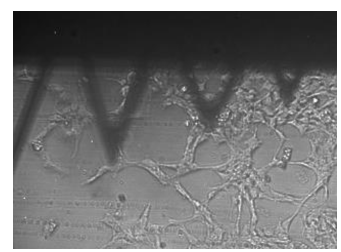
Use of atomic force microscopy (AFM) to explore cell wall properties and response to stress in the yeast *Saccharomyces cerevisiae*

Jean Marie Francois · Cécile Formosa ·
Marion Schiavone · Flavien Pillet · Hélène Martin-Yken ·
Etienne Dague

AFM COUPLED WITH OM



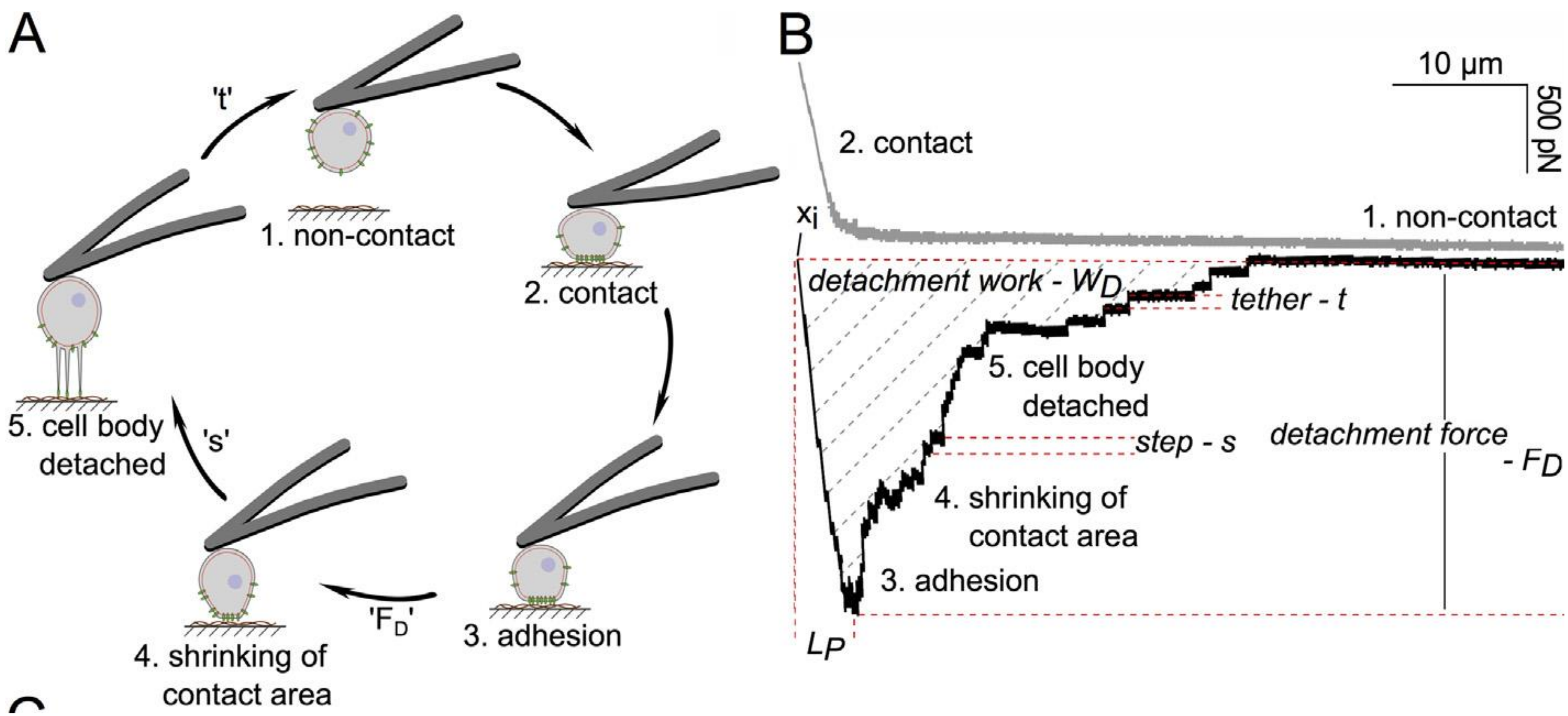




MSTC-AU Tip

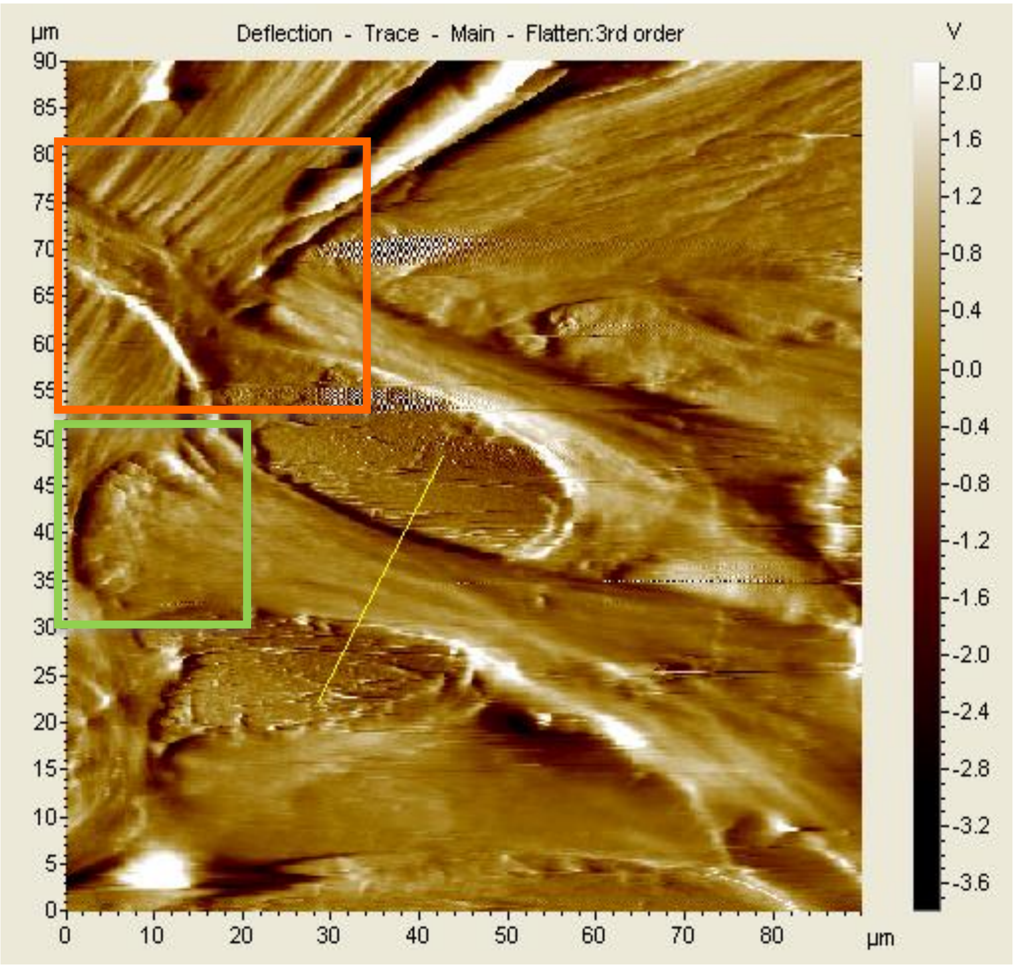
Sample plates

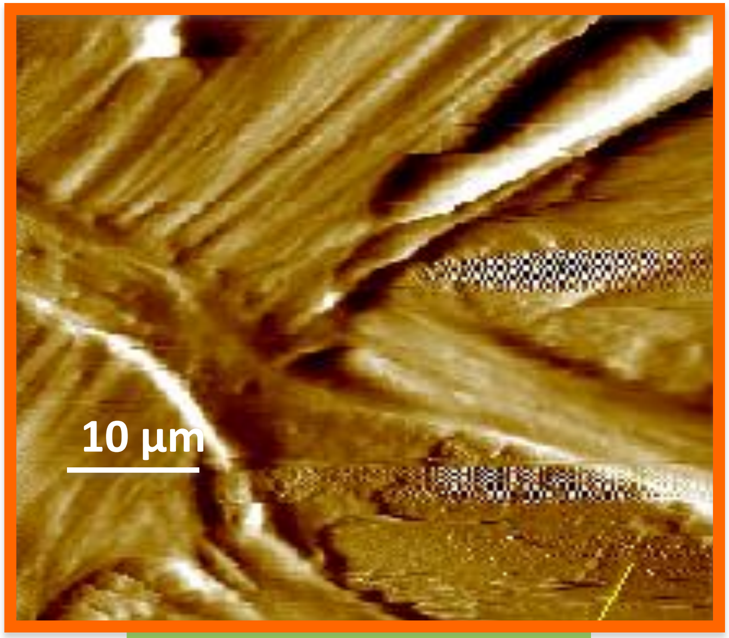
NANOINDENTATION MODELS

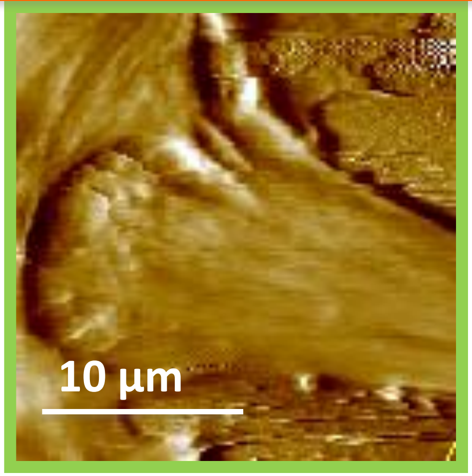


J. Friedrichs et al. / Methods 60 (2013) 169–178

OSTEOBLASTS CELL LINE



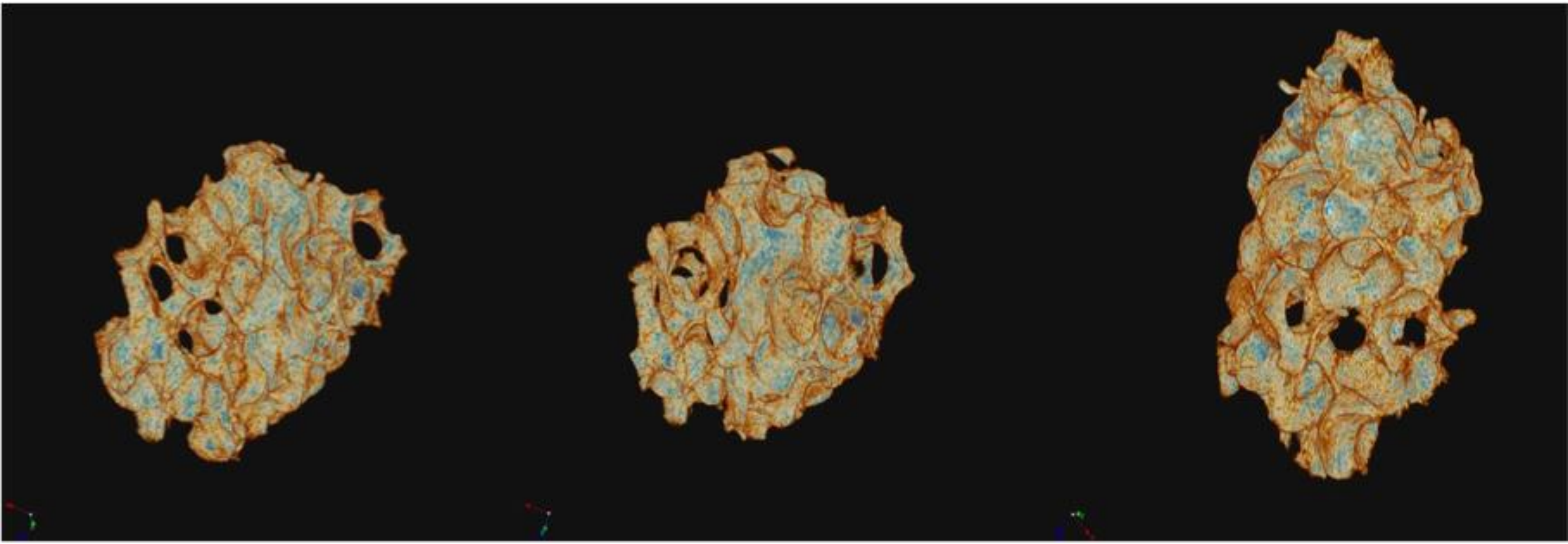




<u>Complementary techniques</u>

- Some examples:
- Electron probe microanalysis - X rays wavelength dispersive spectroscopy
- XPS
- FTIR spectroscopy
- Contact angle measurements (hydrophobicity)
- Zeta potential for surface charge assessment

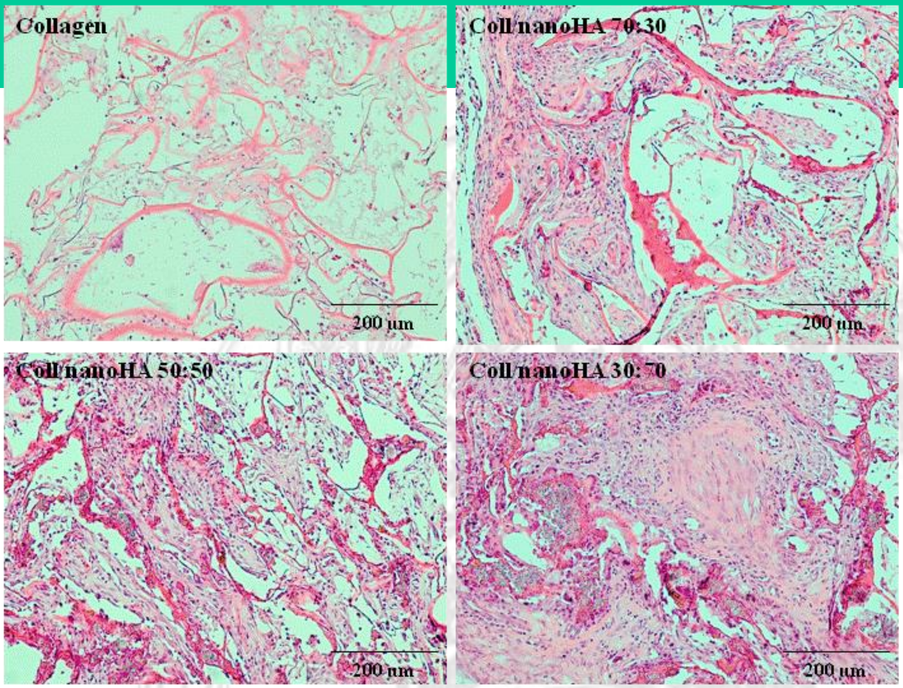
MicroCT



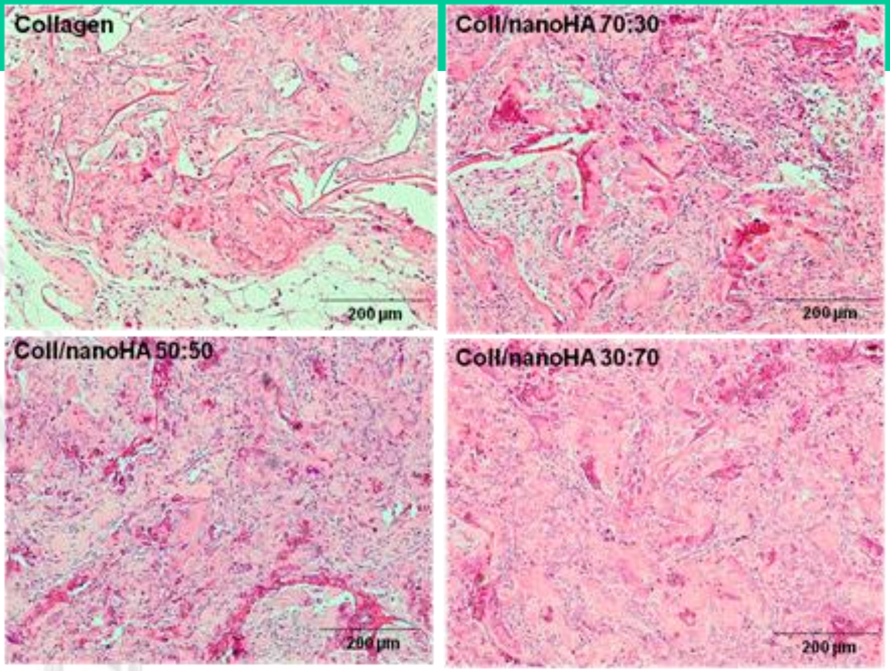
•

- In this analysis we left out a crucial component that are the *in vivo* experiments

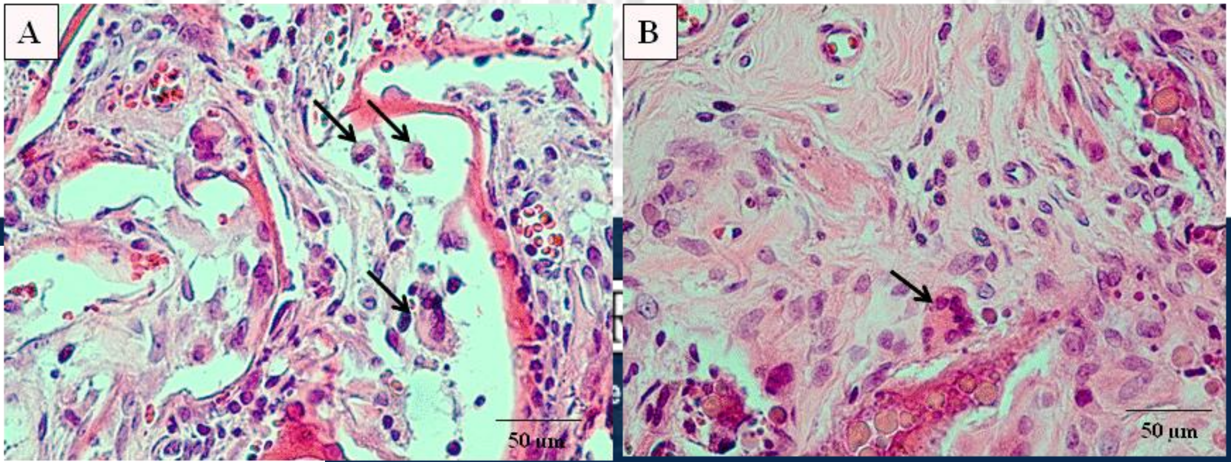
In vivo Characterization



H&E stained scaffold sections after 7 days of *in vivo* implantation.



after 30 days of *in vivo* implantation.



associate laboratory
IBMC
INEB

Questions that may be raised in this project in terms of WG4

- What will be the full project objectives?
- What shall be the main lines of research in WG 4?
- How are the several groups linked in terms of common objectives ?
- What are the specificities and major capabilities of the participating partners?
- What actions can we start to generate a collaboration momentum?

Comments from colleagues

- I feel that the most important parameter to be discussed is how to compare results of in vitro studies with those from in vivo. It is difficult to replicate the clinical scenario. Thus most of the in vitro work cannot be replicated in vivo. Particularly with acellerated studies where any changes noted are exaggerated

Josette Camilleri

- a full suite/programme of in vitro testing to assess biological performance of the projects new materials should include:
- 1) A range of acellular physiological tests which analyse phenomena such as dissolution/re-precipitation based bioactivity either with or without the presence of amino acids and serum proteins – where studies are performed on porous scaffolds as they are intended to be used as these phenomena are highly surface area and morphology dependant. There should be two groups of tests:
- · Static tests – for comparison with SBF type tests performed in the literature and also
- · Dynamic tests: as these will provide information regarding the materials response to a dynamic environment, more closely resembeling the physiological conditions in vivo and providing guidance as to how to set up a 3D perfusion culture system in step (5).

- (2) Basic acellular biological testing of materials to characterise protein interactions – we have developed in-house techniques to attach fluorophore probes to target proteins/growth factors to enable monitoring of protein adsorption/desorption under competitive and dynamic conditions. Again these should be ideally performed under static and dynamic conditions to help with the final design of the system in (5).

K. Hing

Thank you for your attention!

PORTO – World Heritage (**UNESCO)**