Tissue Regeneration under Ectopic Conditions

Tissue Regeneration Engineering

with(out)

Stem Cells

STRANGER THINGS type of lecture

Tissue Repair (Healing)

• Regeneration of injured tissue (replacement by normal cells of the same kind)

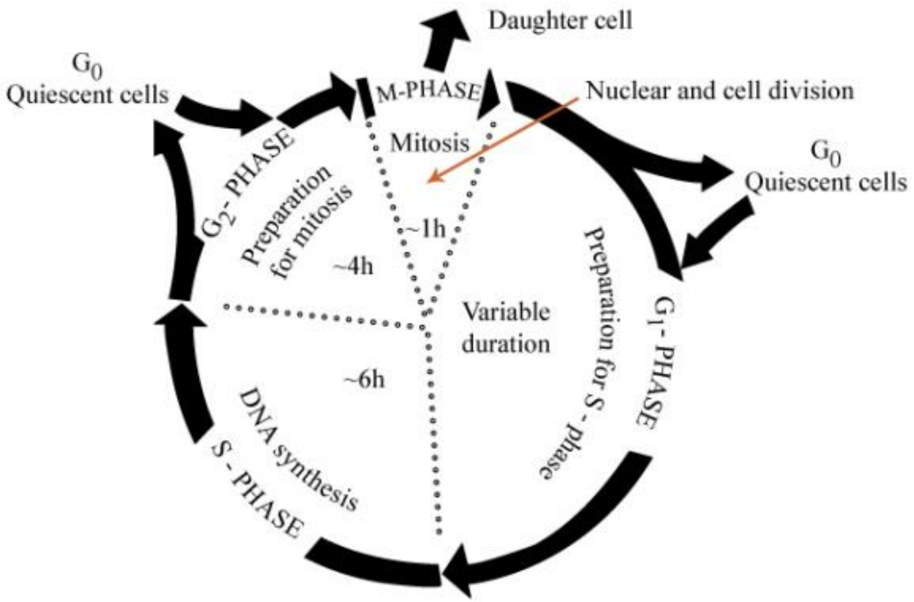
• Replacement by fibrous tissue (fibrosis, scarring)

It may start early after tissue damage

- regeneration
  by parenchymal cells of the same type
- reparation
  replacement by connective tissue (fibrosis)
  result - scar

Normal Cell Proliferation

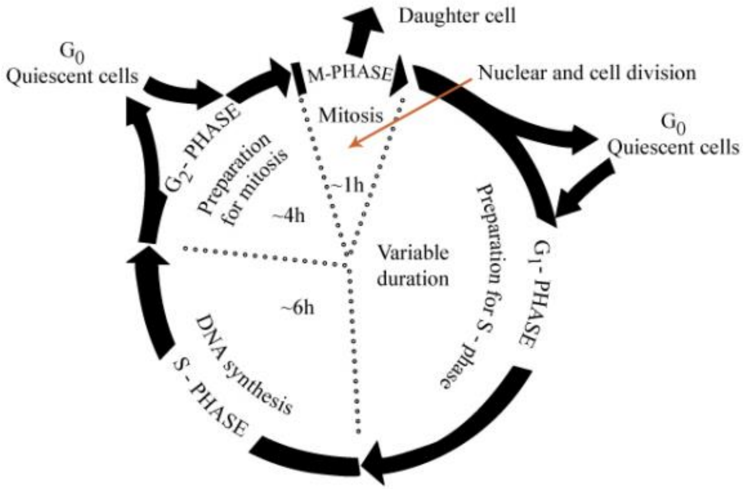
Proliferating cells progress through a series of defined phases and checkpoint, collectively call the *cell cycle*



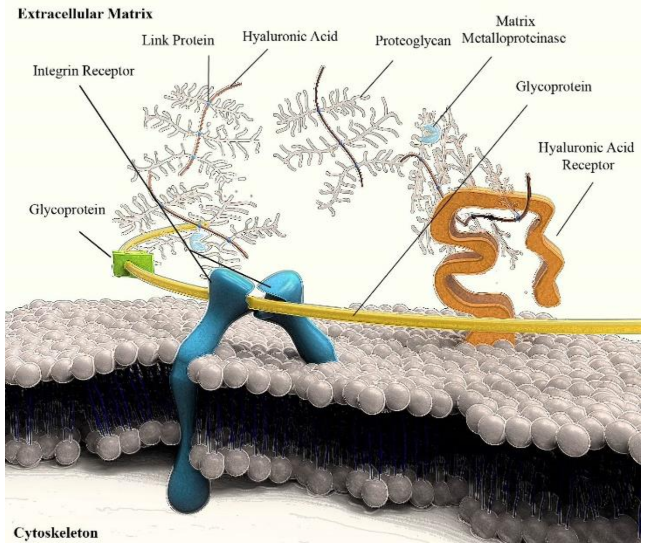
Karlinski, M., & Reiner, O. (2018). Unfolding the folds: how the biomechanics of the extracellular matrix contributes to cortical gyrification. *Opera Medica et Physiologica*, *4*(2).

Control of Cell Cycle

• Progression through the cell cycle is controlled at specific checkpoints (restriction point in G 1, mitosis entry and mitosis exit)
• Transition between stages of mitosis is triggered by increased activity of cyclin-dependet kinases (CDK)
• Each CDK modulates the activity of a subset of cellular targets specific for progression through individual transitions with the cell cycle



Cell-ECM interactions



components

collagen (18 types) – I, III, IV, V; tensile strength
elastin (+ fibrillin) – return to normal structure after stress
glycoproteins - adhesion, binding ECM to cells (fibronectin, laminin)
proteoglycans and hyalouronans – lubrication (gels)

Roles of the ECM

• Mechanical support • Determination of cell polarity • Control of cell growth • Control/maintenance of cell differentiation • Scaffolding for tissue renewal • Establishment of tissue microenvironment • Storage and presentation of regulatory proteins

← Cell growth and differentiation are dependent on extracellular signals from soluble polypeptide growth factors and the ECM. BUT NOT EXCLUSIVELY!

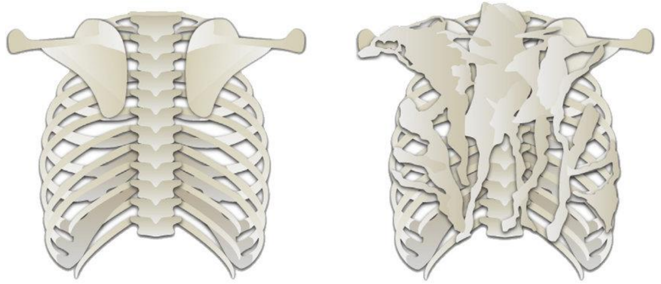
Karlinski, M., & Reiner, O. (2018). Unfolding the folds: how the biomechanics of the extracellular matrix contributes to cortical gyrification. *Opera Medica et Physiologica*, *4*(2).

...because one size does **not** fit all...

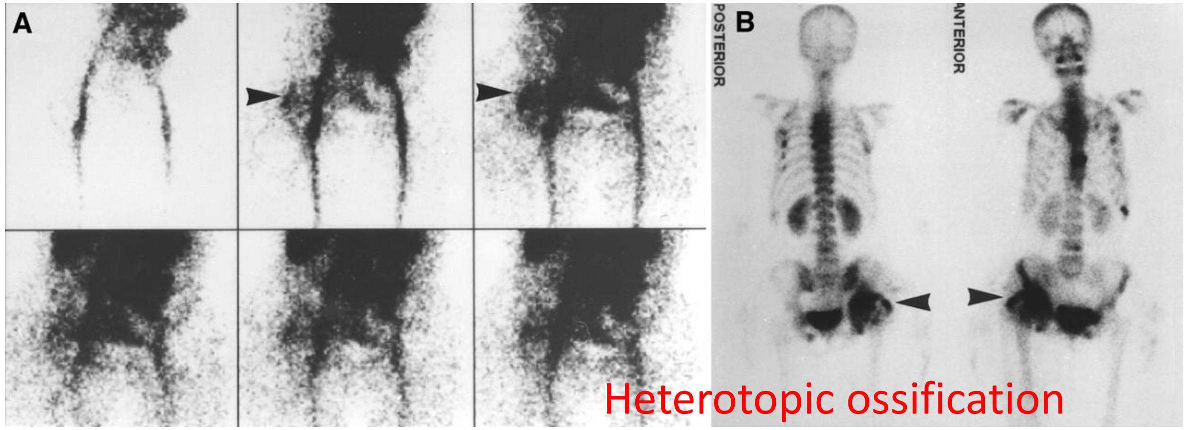
Ectopism

In tissue engineering, ectopic (human) tissue formation (from the Greek word ektopos or "far from a place"), refers to tissue that forms or is located where it does not belong or to structures that form within scaffolds implanted in non-specific sites.

From the point of view of clinical diagnoses, the term (referring to the same tissue phenotype) most often covers the ossification of tissues outside their usual origins.

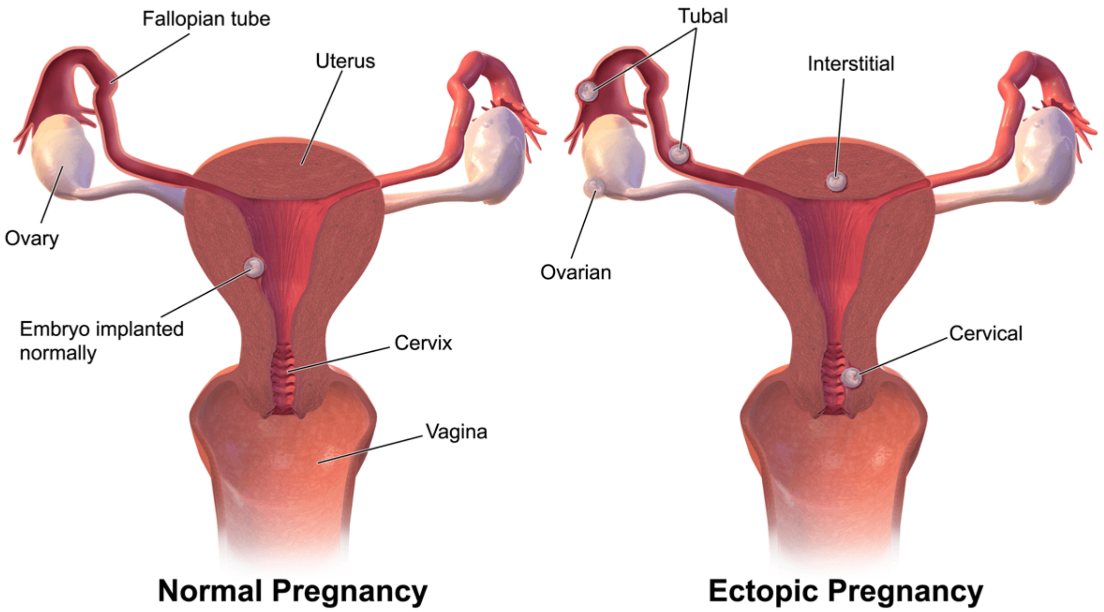


Nakajima, T., & Ikeya, M. (2019). Insights into the biology of fibrodysplasia ossificans progressiva using patient-derived induced pluripotent stem cells. *Regenerative therapy*, *11*, 25-30.

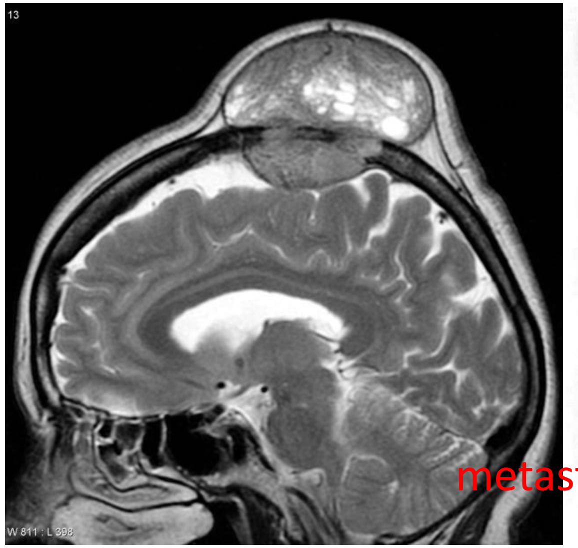


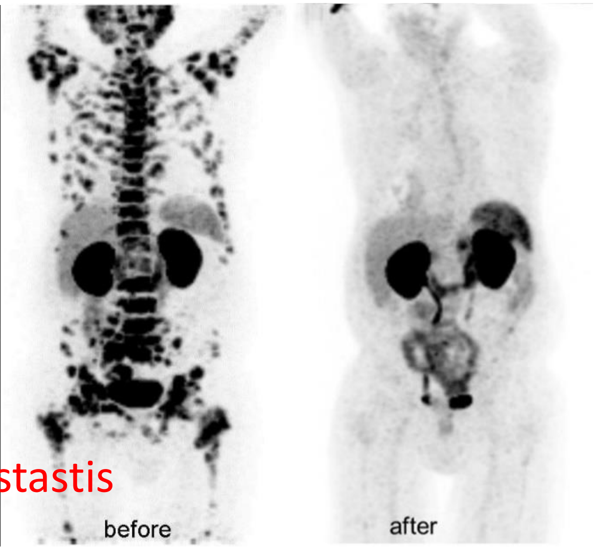
Heterotopic ossification

Shehab, Dia, Abdelhamid H. Elgazzar, and B. David Collier. "Heterotopic ossification." *Journal of Nuclear Medicine* 43, no. 3 (2002): 346-353.



https://en.wikipedia.org/wiki/Ectopic_pregnancy





metastastis

https://www.wikidoc.org/index.php/Follicular_thyroid_cancer_MRI

https://ec.europa.eu/jrc/en/news/prostate-cancer-alpha-therapy-shows-impressive-results

In tissue engineering, ectopic bone tissue is the result of ossification of scaffolds implanted in sites not specific to bone formation.

Subcutaneous implantation

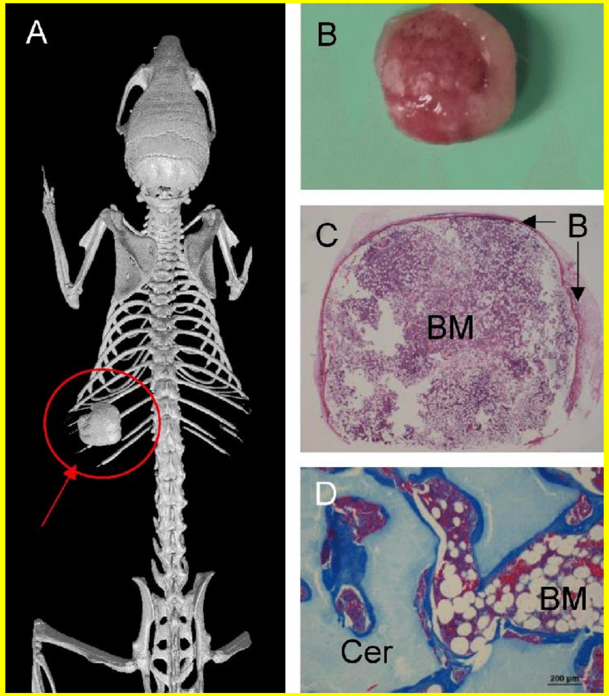


Fig.1 Ectopic bone "ossicle." (A) Whole body micro–computerized tomography image showing bone tissue in a mouse. (B) Gross morphology of a mouse-harvested ossicle. (C) Hematoxylin/eosin histological staining of an ossicle based on an implant of hMSC carrier gelatin sponge with BMP-2. (D) Masson's trichrome histological staining of an ossicle based on hMSC carrier ceramic implant. Note the remaining ceramic material in pale blue (Cer), newly formed bone in the surface of the ceramics in dark blue, and mature BM tissue with hematopoietic cells, adipocytes, and vascular structures with erythrocytes in red.

Abarrategi, A., Mian, S. A., Passaro, D., Rouault-Pierre, K., Grey, W., & Bonnet, D. (2018). Modeling the human bone marrow niche in mice: From host bone marrow engraftment to bioengineering approaches. *Journal of Experimental Medicine*, *215*(3), 729-743.

Intramuscular implantation

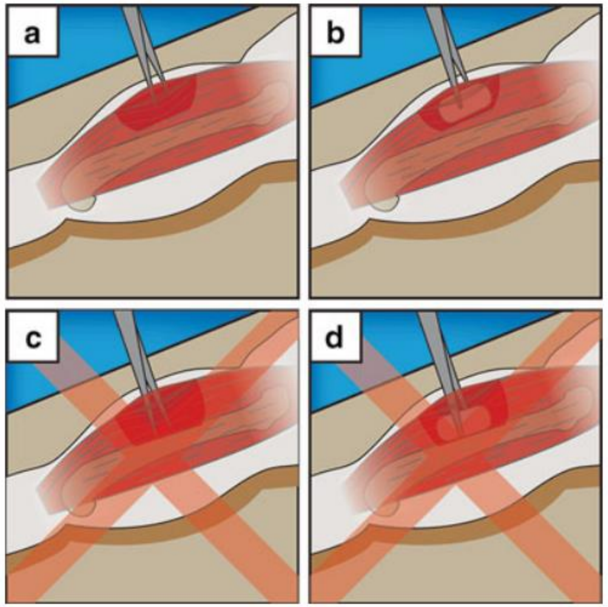
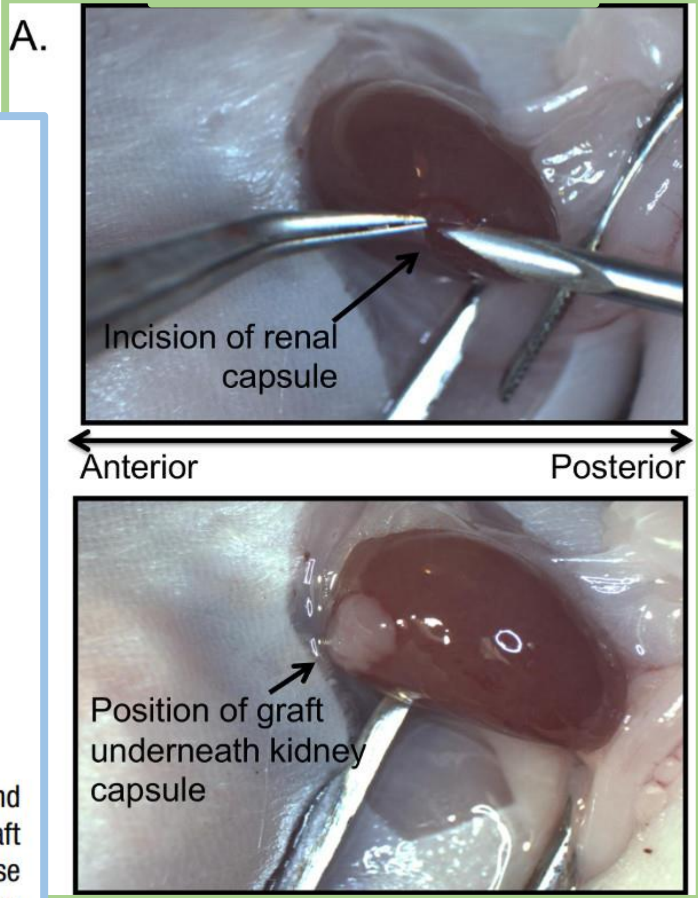


Fig. 2 Illustrated Depth of Muscle Pouch Creation. Axial view of mouse left hind limb. (**a**) Proper surgical creation of muscle pouch and (**b**) implantation of graft material. (**c**) One must be mindful as to not create a pocket so deep as to expose the periosteum. (**d**) Graft placed too close to the periosteum will render new bone indistinguishable from the femur

Asatrian, G., Chang, L., & James, A. W. (2014). Muscle pouch implantation: an ectopic bone formation model. In *Animal Models for Stem Cell Therapy* (pp. 185-191). Humana Press, New York, NY.

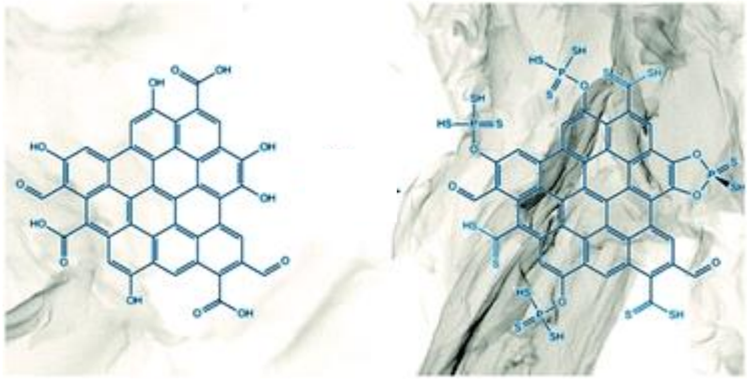
Renal capsule model



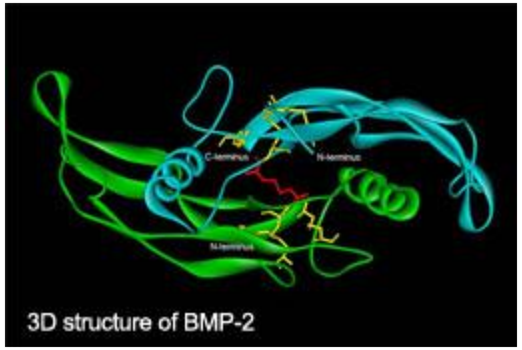
Morillon II, Y. M., Manzoor, F., Wang, B., & Tisch, R. (2015). Isolation and transplantation of different aged murine thymic grafts. *JoVE (Journal of Visualized Experiments)*, (99), e52709.

OSTEOINDUCTION

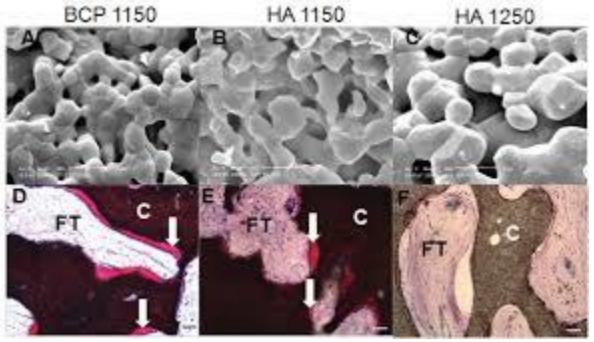
Material chemistry



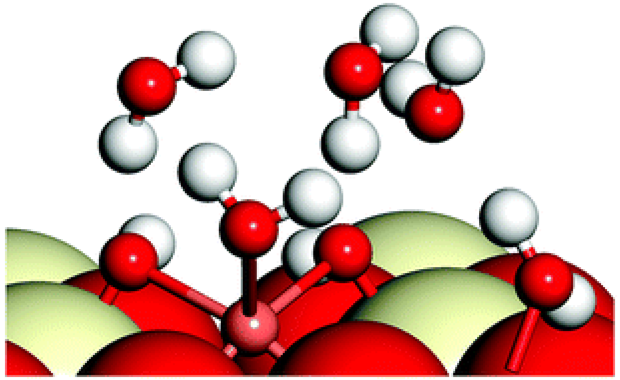
BMP / other protein coating / mixture



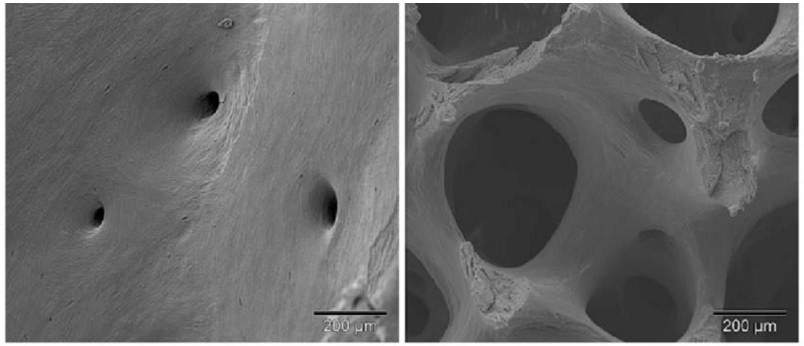
Calcium – based ceramics

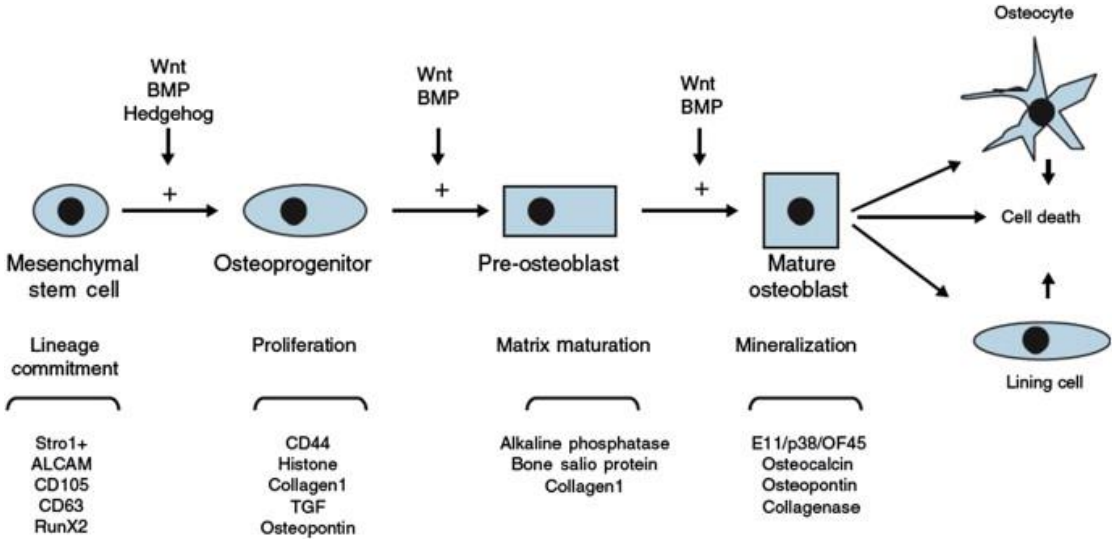


Adsorption

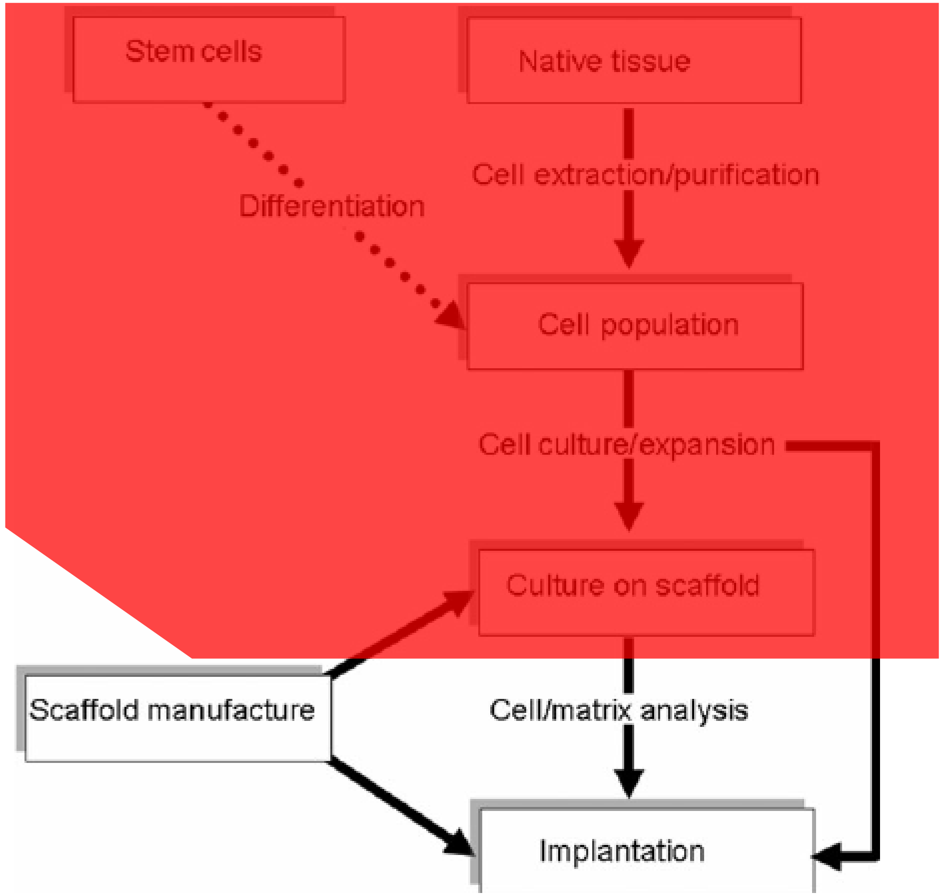


Morphological features





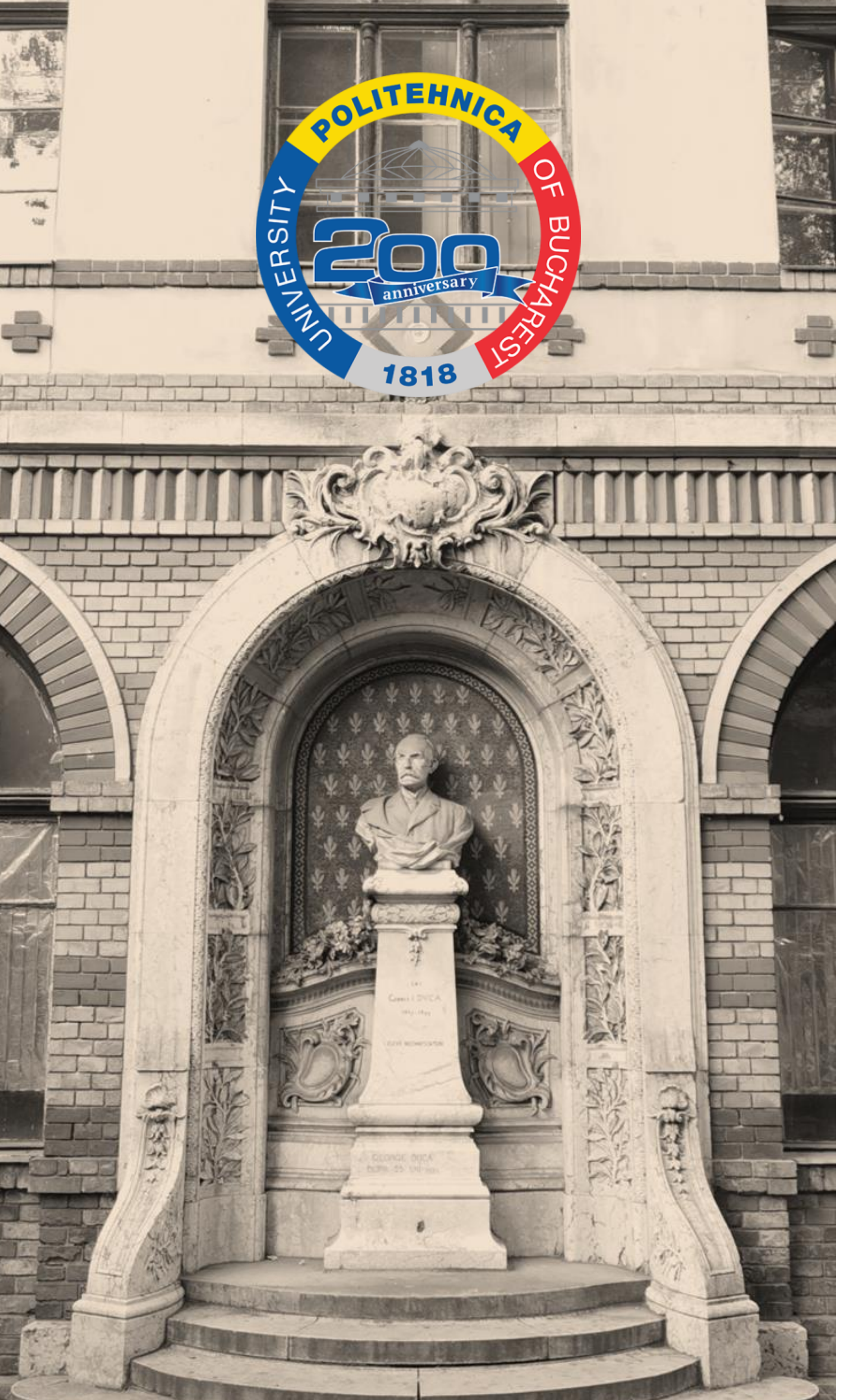
STIMULI / MODIFICATIONS



COMMON OSTEOINDUCTIVE AGENTS

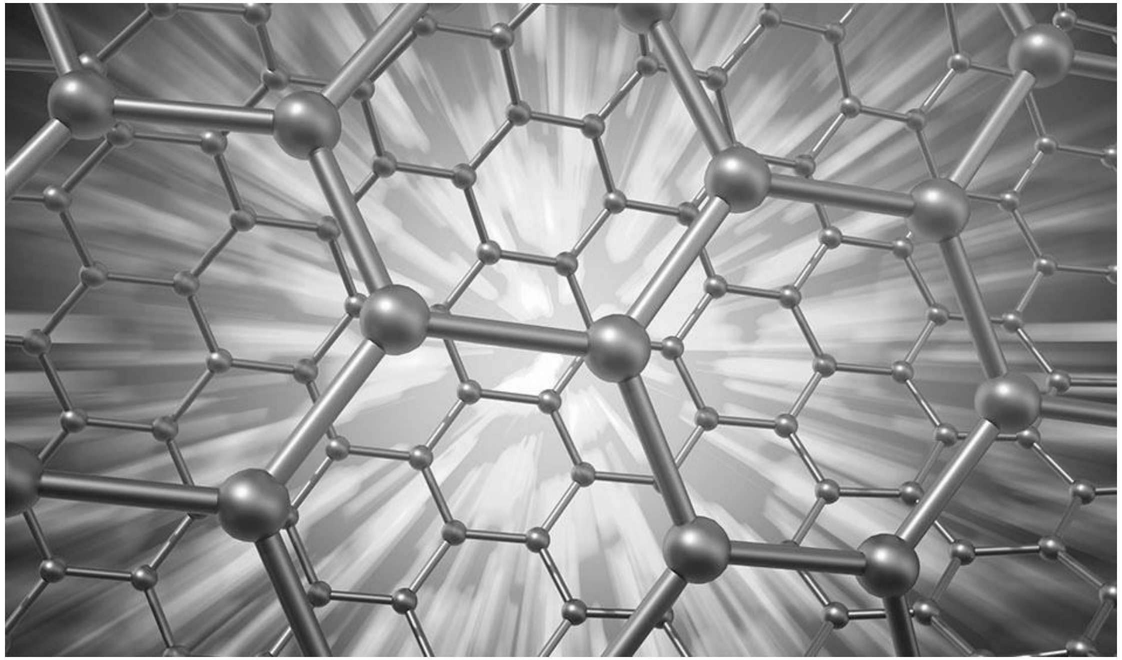
STEM CELLS

OSTEOPROGENITOR LINEAGES

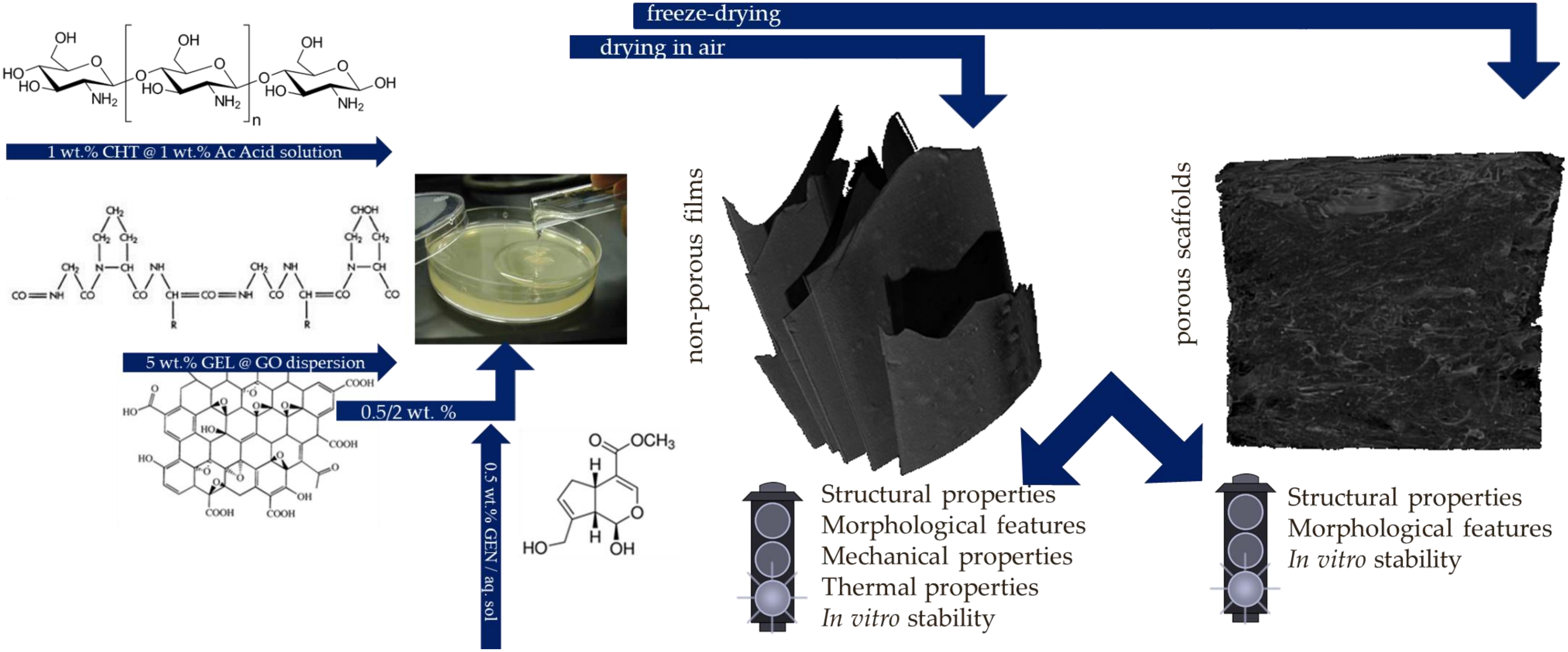


APMG
Advanced Polymer Materials Group

THE ATTRIBUTES OF ECTOPIC OSTEOINDUCTION IN GRAPHENE OXIDE-INLAYED BIOPOLYMER BLENDS



CHITOSAN/GELATIN blends @ GRAPHENE OXIDE # GENIPIN



2017 2018 2019 **2020**

Graphene Oxide Reinforcing Genipin Crosslinked Chitosan-Gelatin Blend Films

by George Mihail Vlasceanu [1,2], Livia Elena Crica [2], Andreea Madalina Pandele [2,3] and Mariana Ionita [1,2,*]

[1] Faculty of Medical Engineering, University Politehnica of Bucharest, Gh Polizu 1-7, 011061 Bucharest, Romania
[2] Advanced Polymer Materials Group, University Politehnica of Bucharest, Gh Polizu 1-7, 011061 Bucharest, Romania
[3] Faculty of Applied Chemistry and Materials Sciences, University Politehnica of Bucharest, Gh Polizu 1-7, 011061 Bucharest, Romania
[*] Author to whom correspondence should be addressed.

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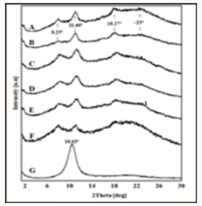
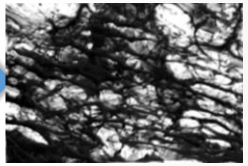
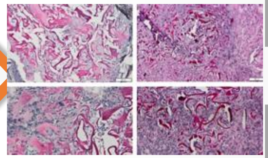
Comprehensive Appraisal of Graphene–Oxide Ratio in Porous Biopolymer Hybrids Targeting Bone-Tissue Regeneration

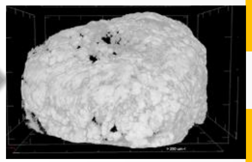
by George Mihail Vlasceanu [1,2], Aida Șelaru [3,4], Sorina Dinescu [3,5], Cornel Balta [6], Hildegard Herman [6], Sami Gharbia [3,6], Anca Hermenean [3,6], Mariana Ionita [1,2,*] and Marieta Costache [3,5]

[1] Faculty of Medical Engineering, University Politehnica of Bucharest, Gh Polizu 1-7, 011061 Bucharest, Romania
[2] Advanced Polymer Materials Group, University Politehnica of Bucharest, Gh. Polizu 1-7, 011061 Bucharest, Romania
[3] Department of Biochemistry and Molecular Biology, University of Bucharest, Spl. Independentei 91-95, 050095 Bucharest, Romania
[4] Department of Immunology, National Institute for Research and Development in Biomedical Pathology and Biomedical Sciences "Victor Babes", Spl. Independentei 99-101, 050096 Bucharest, Romania
[5] Research Institute of the University of Bucharest, 050095 Bucharest, Romania
[6] Aurel Ardelean Institute of Life Sciences, Vasile Godis Western University of Arad, Rebreanu 86, 310414 Arad, Romania
[*] Author to whom correspondence should be addressed.









01

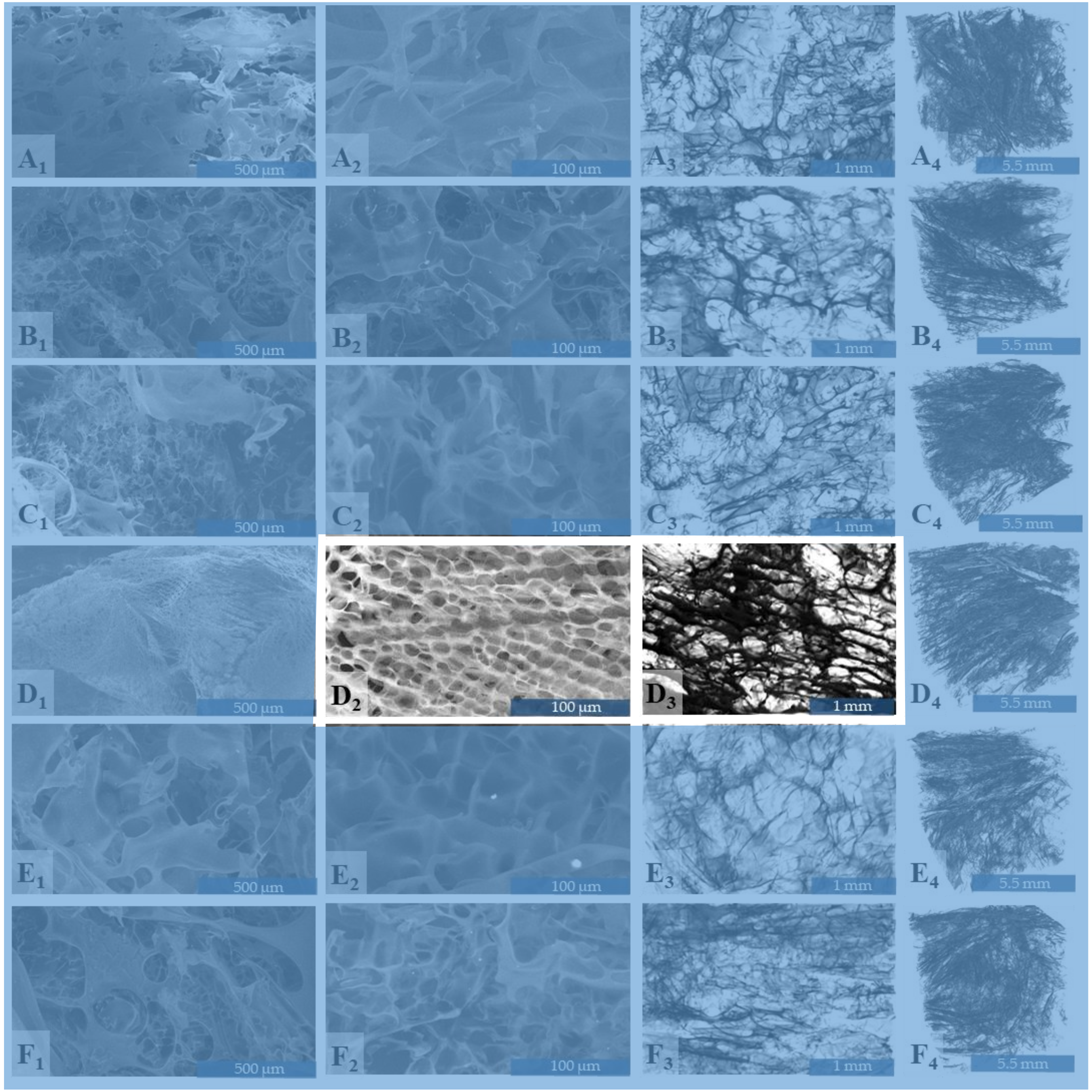
Graphene Oxide Reinforcing Genipin Crosslinked Chitosan-Gelatin Blend Films

02

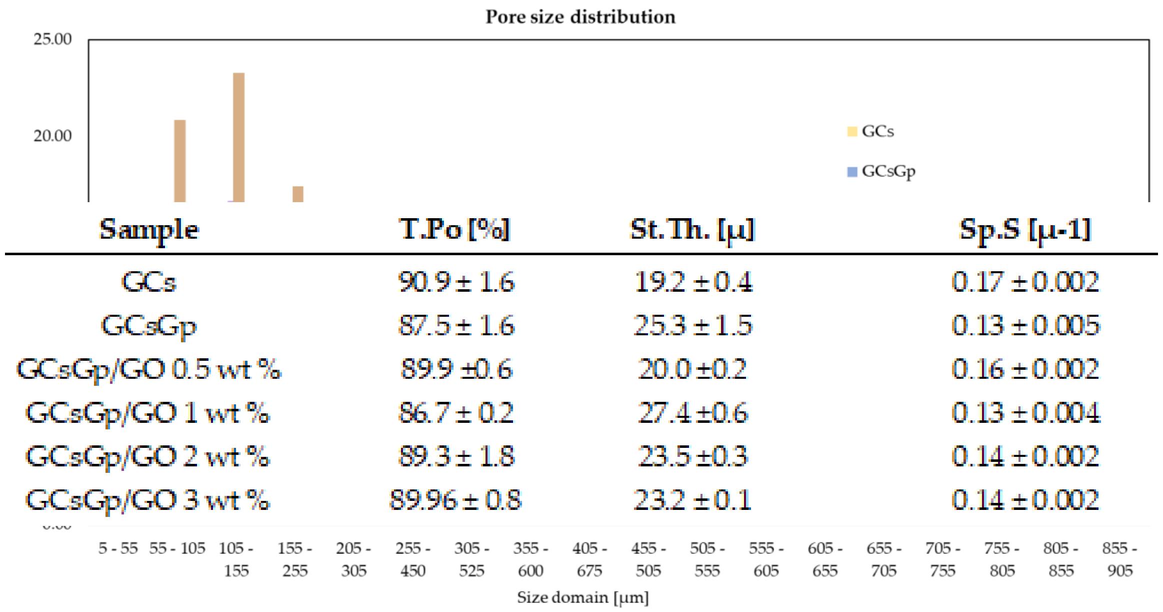
Comprehensive appraisal of graphene oxide ratio in porous biopolymer hybrids targeting bone tissue regeneration

03

Graphene oxide-inlayed polymer blends: ectopic osteogenesis attributes *manuscript in preparation*



SEM and µCT analyses



Sample	T.Po [%]	St.Th. [µ]	Sp.S [µ-1]
GCs	90.9 ± 1.6	19.2 ± 0.4	0.17 ± 0.002
GCsGp	87.5 ± 1.6	25.3 ± 1.5	0.13 ± 0.005
GCsGp/GO 0.5 wt %	89.9 ±0.6	20.0 ±0.2	0.16 ± 0.002
GCsGp/GO 1 wt %	86.7 ± 0.2	27.4 ±0.6	0.13 ± 0.004
GCsGp/GO 2 wt %	89.3 ± 1.8	23.5 ±0.3	0.14 ± 0.002
GCsGp/GO 3 wt %	89.96 ± 0.8	23.2 ± 0.1	0.14 ± 0.002

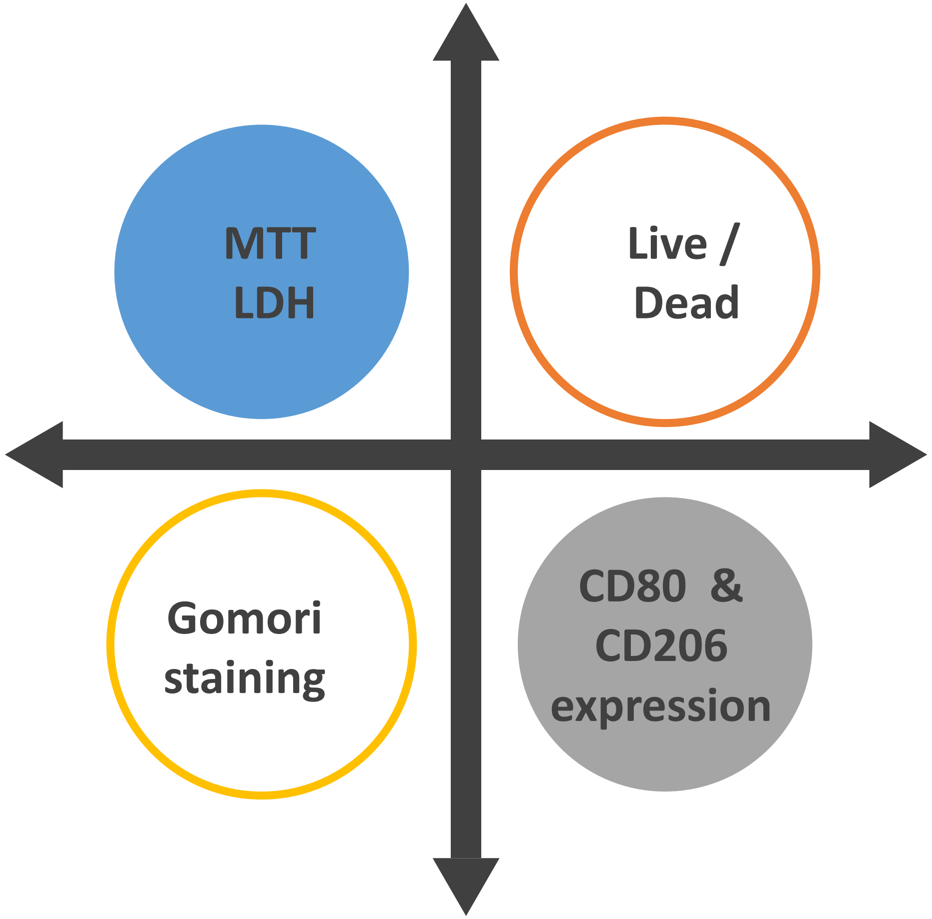
01 Broad pore size domain

02 GO enabled pore patterning

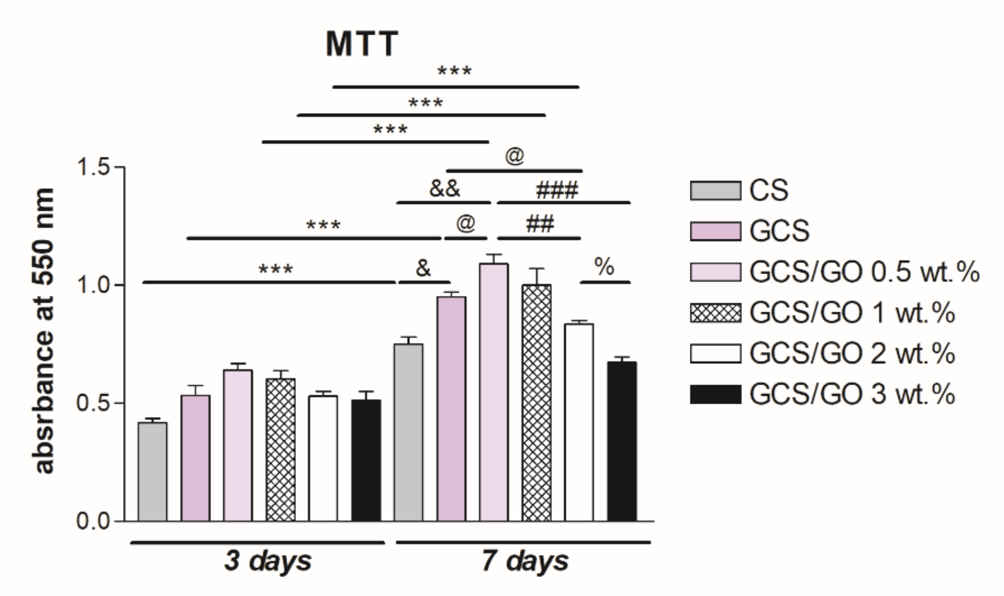
03 Bone-like morphology

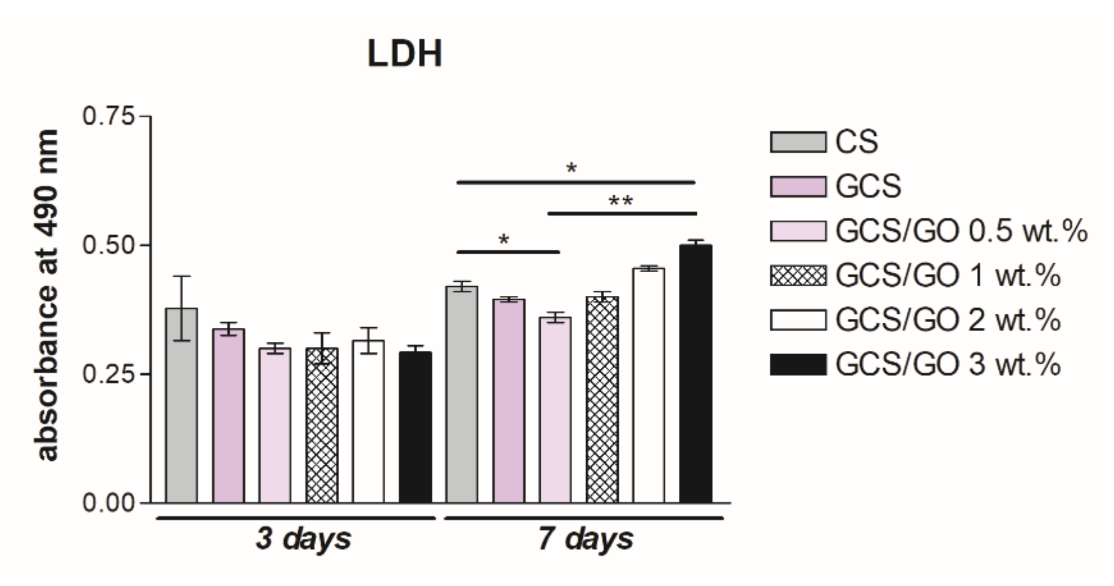
04 Cell affinity

COMPOSITES BIOCOMPATIBILITY



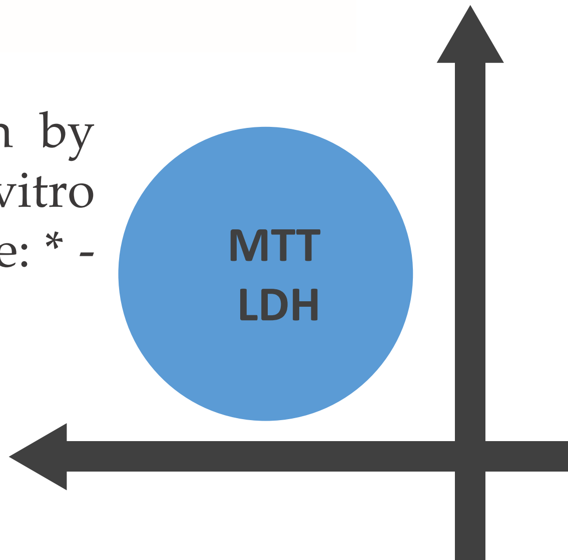
In vitro BIOCOMPATIBILITY



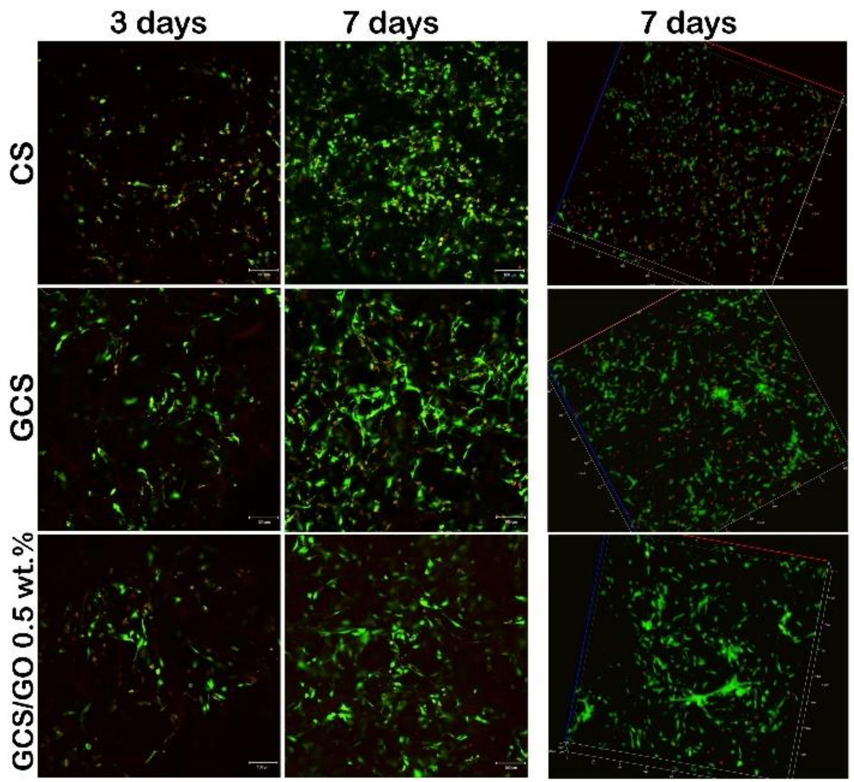


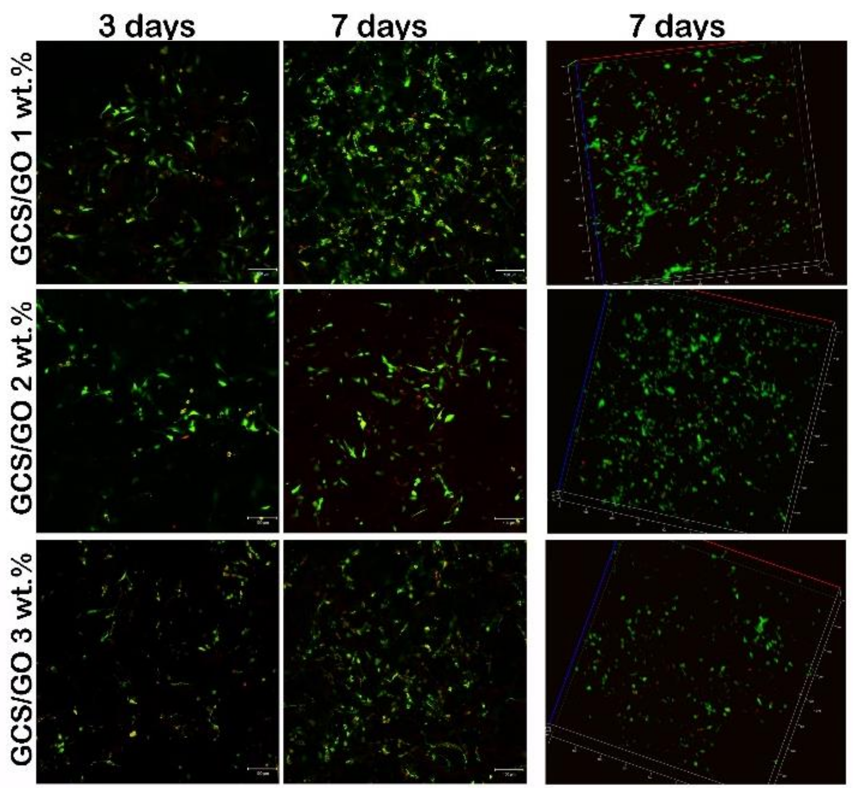
Murine pre-osteoblasts viability and proliferation profile as resulted from quantitative evaluation by MTT assay after 3 and 7 days of in vitro cell culture; Statistical significance: @,& and % - $p<0.05$; ## and && - $p<0.01$; *** and ### - $p<0.001$.

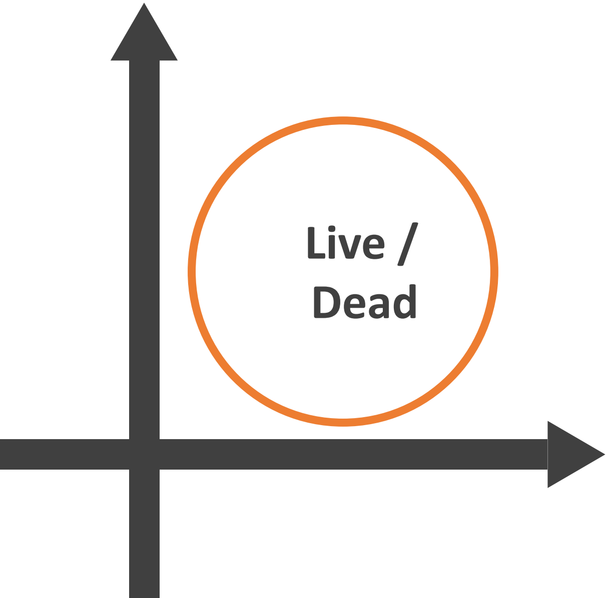
Scaffolds' cytotoxicity evaluation by LDH assay during 7 days of in vitro cell culture. Statistical significance: * - $p<0.05$; ** - $p<0.01$.



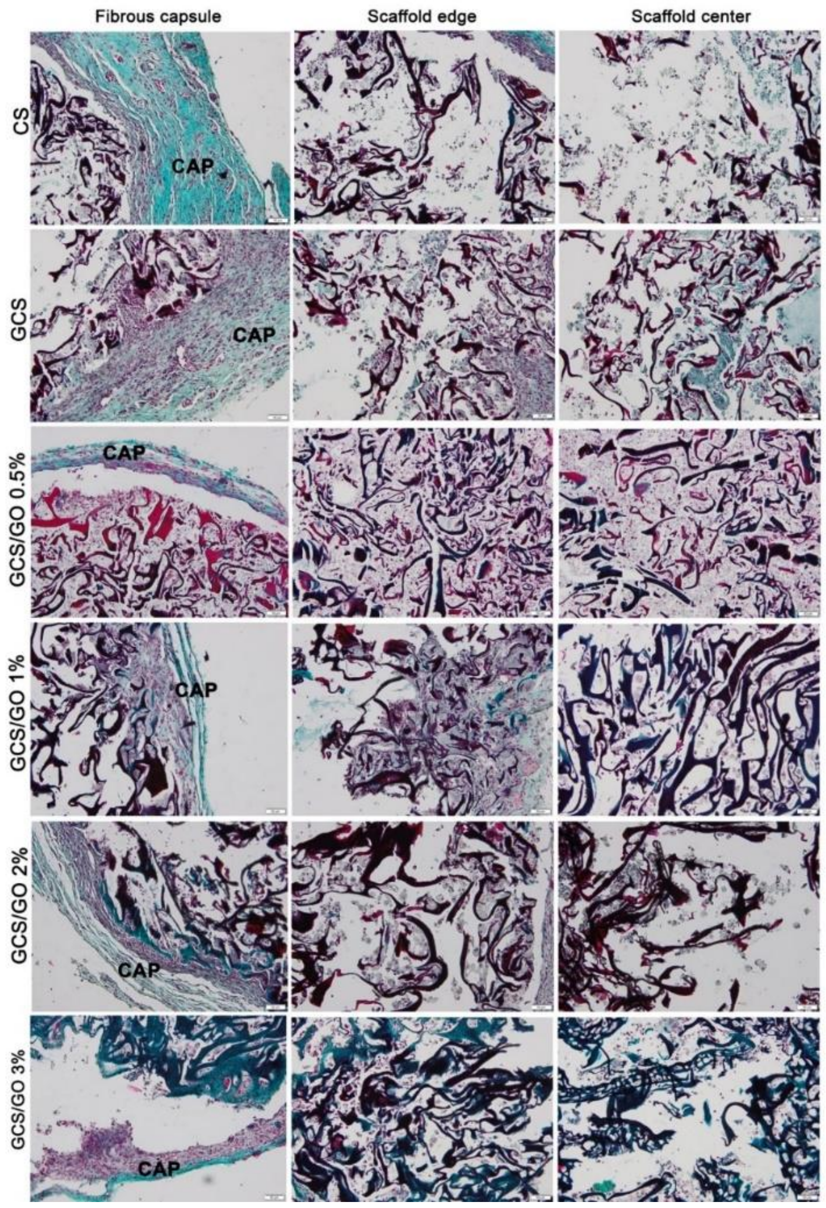
In vitro BIOCOMPATIBILITY







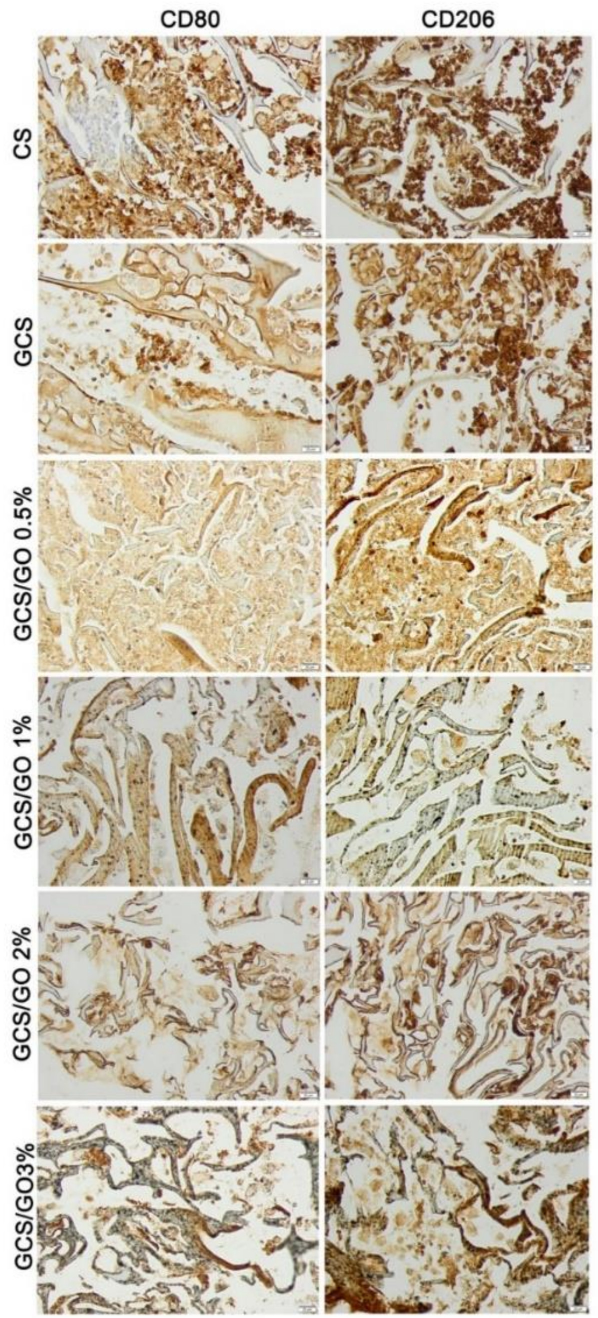
Fluorescence microscopy evaluation of living (green-labeled) and dead (red-labeled) cells in contact with GCS and GCSGp/GO scaffolds during one week of *in vitro* cell culture.



Gomori staining

CD80 & CD206 expression

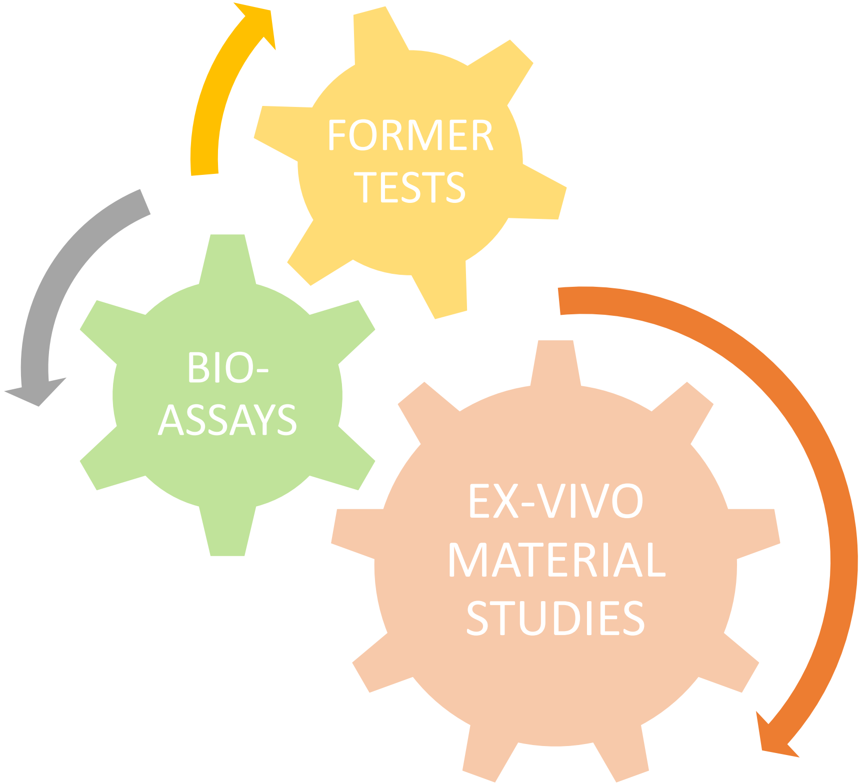
Light images of Gomori trichrome stained scaffolds at week 4 post-implantation showing the varying thickness of the capsules "CAP" surrounding the different scaffolds (first column), the histological aspect of the edge and center of scaffolds (second and third column).

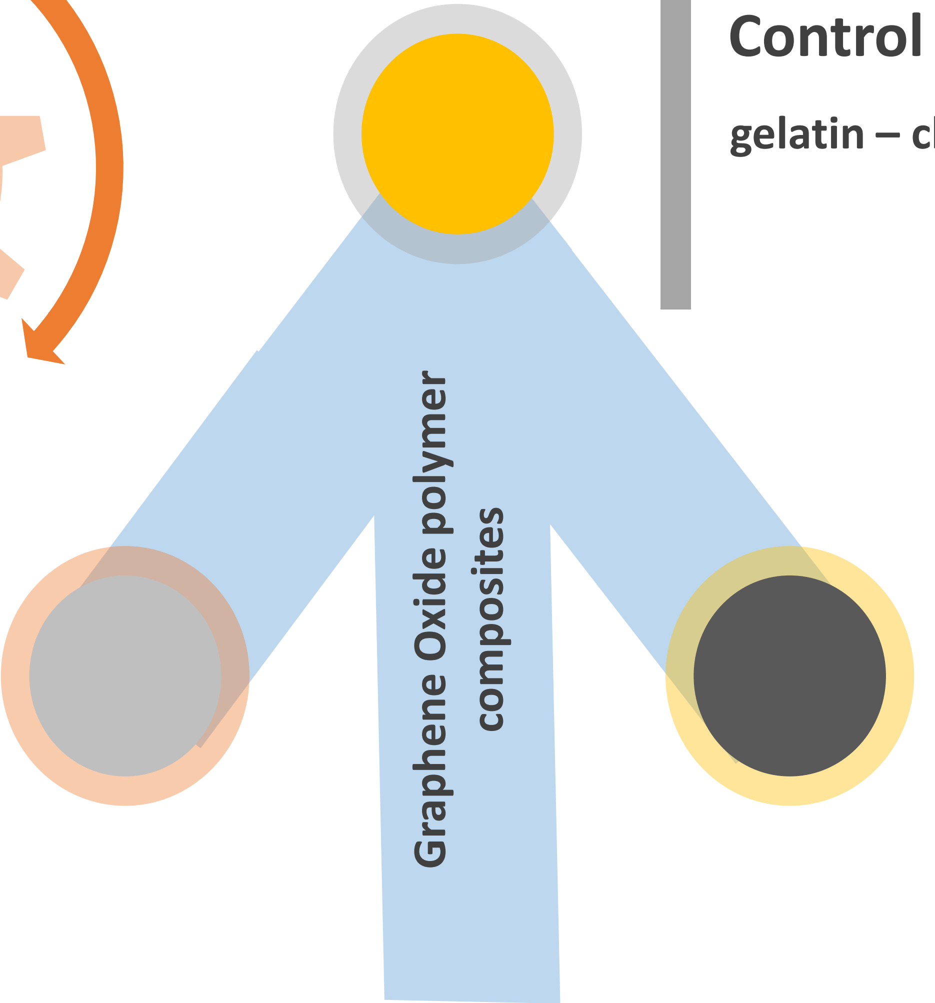


Immunohistochemical expression of CD80 and CD206 at week 4 post-implantation.

In vivo BIOCOMPATIBILITY

Global objective





Control

gelatin – chitosan (GCs)

Crosslinking

gelatin – chitosan – genipin (GCsGp)

Low GO %

gelatin – chitosan – genipin – graphene oxide (GCsGp-GO)

I. Initial characterization

durotaxis

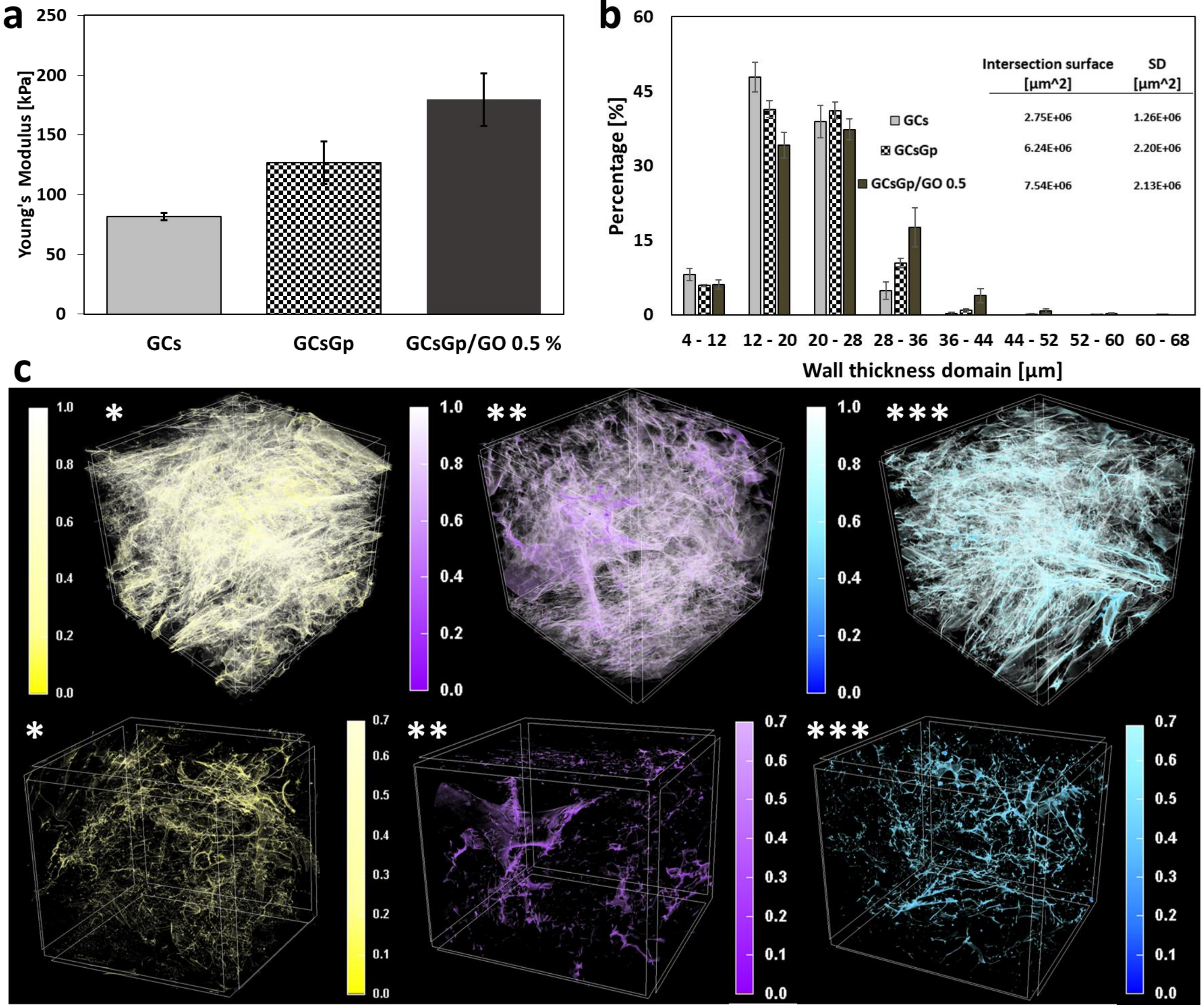


Fig. 1. (a) Plotting of the compression modulus of hidrated materials, before implantation; (b) Histogram depiction of the wall thickness size domain calculated in CTAn (Bruker); (c) Color-highlighted 3D renderings of (*) GCs, (**) GCsGp and (***) GCsGp/GO 0.5% scaffold captured in CTVox.

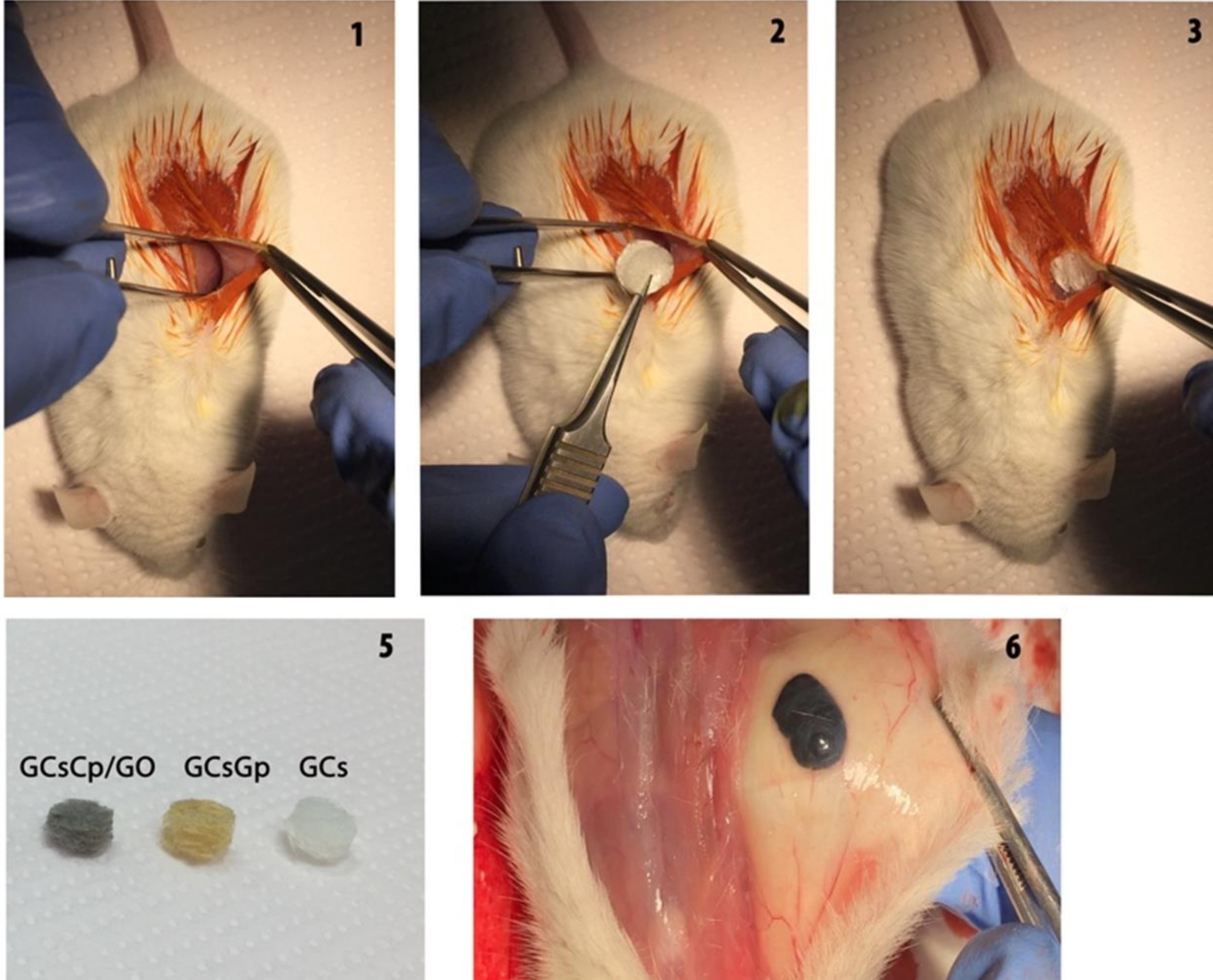


Fig. 2. Experimental design. (1) Preparation of subcutaneous pocket in the dorsum of mice, (2, 3) Ectopic subcutaneous implantation of the scaffold (4) Closure of the overlaying skin (5) Scaffolds before implantation, (6) GCsGp/GO 0.5% wt. scaffold at 4 weeks after subcutaneously implantation to mice.

II. Biological and immunohistochemical characterization // *in vitro*

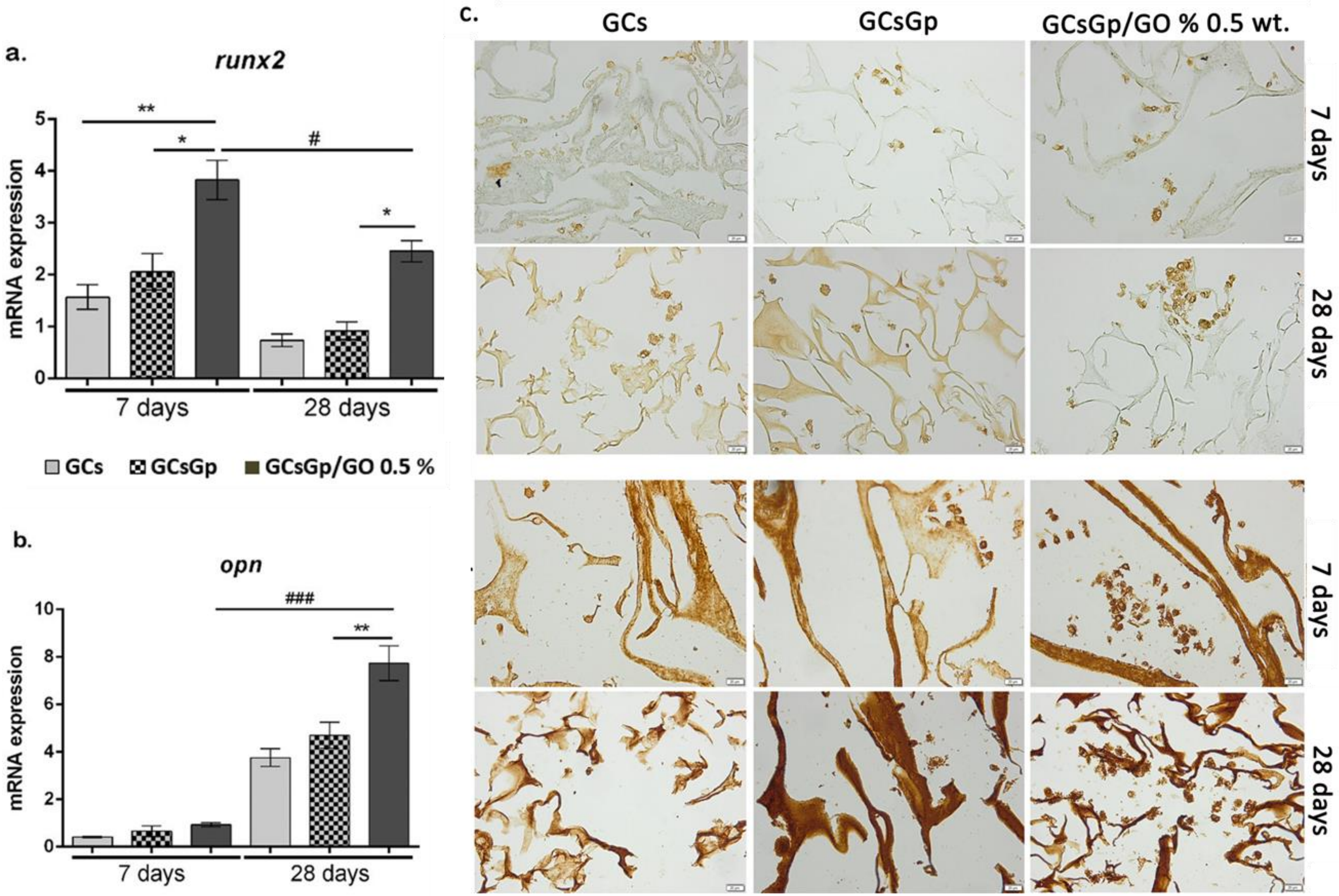


Figure 3. *In vitro* osteogenic profile analyses a) *runx2* (a.) and *opn* (b.) gene expression in differentiated 3T3-E1 cells in contact with GCsGp/GO materials. Statistical significance $^{###}p<0.001$; $^{**,##}p<0.01$; $^{\#,*}p<0.05$; b) immunohistochemical runx2 and opn expression in differentiated 3T3-E1 cells in contact with GCsGp/GO materials.

II. Biological and immunohistochemical characterization *in vitro*

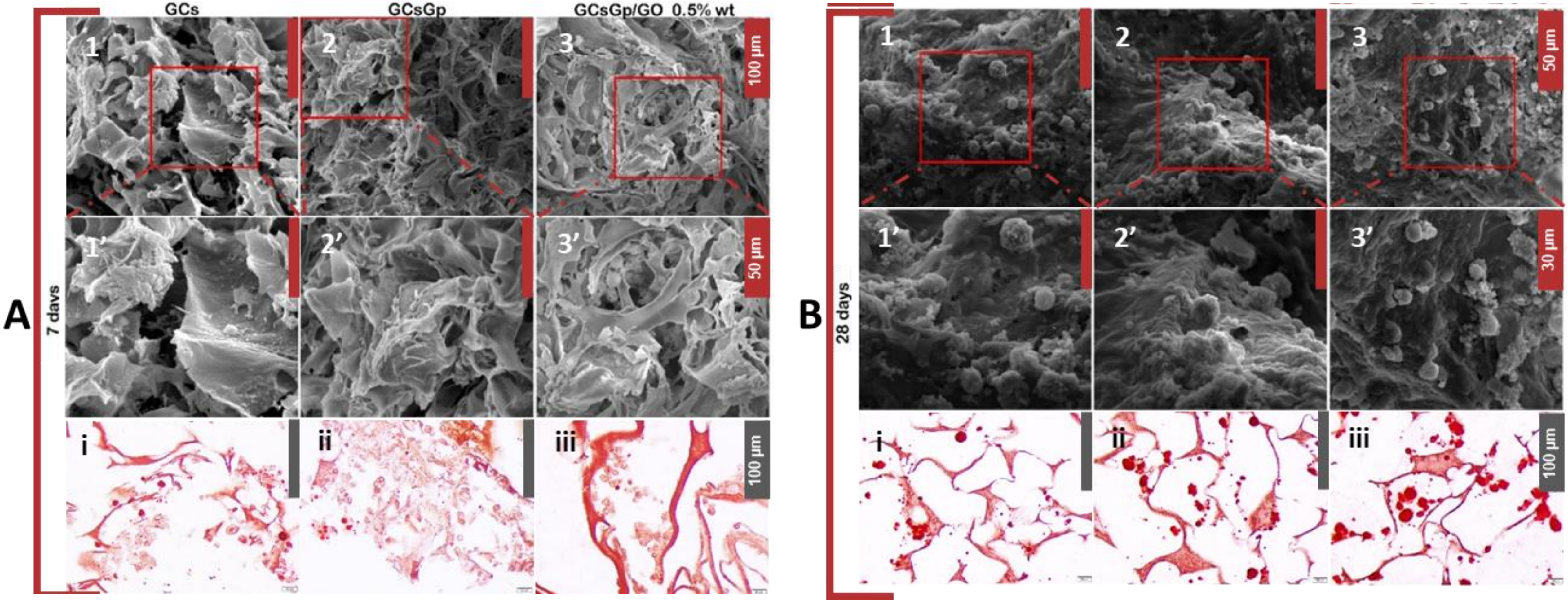


Figure 4. Qualitative evaluation of cellular distribution and morphology in GCsGp/GO scaffolds during 7 (A1-3) and 28 (B1-3) days of osteogenic differentiation using SEM. Qualitative evaluation of *in vitro* calcium accumulation in bECM using ARS histological staining at after 7 (A i-iii) and 28 (B i-iii) days.

II. Biological and immunohistochemistry *in vivo*

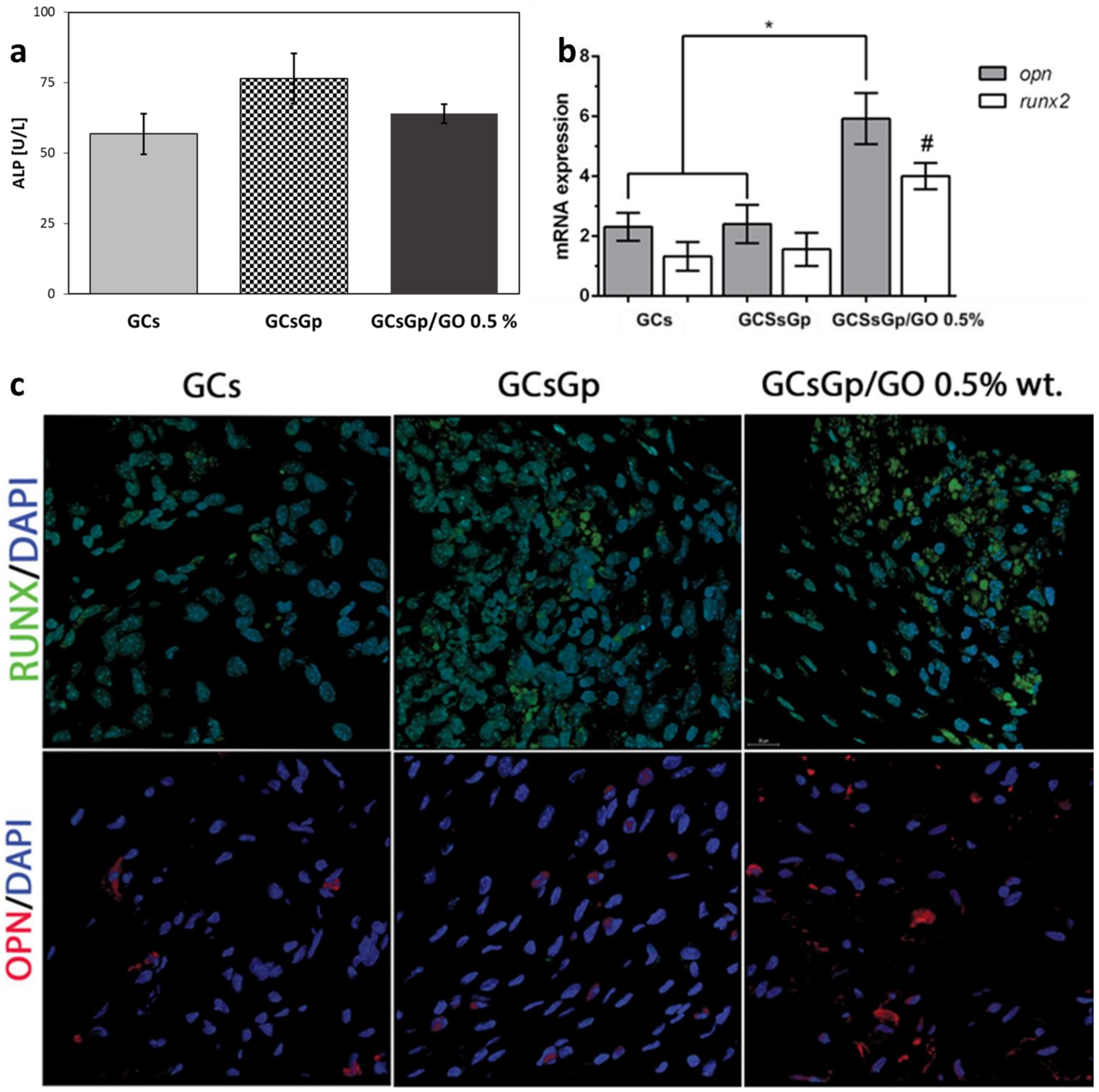


Figure 5. (a) Seric ALP activity 4 weeks post-implantation of GCs, GCsGp and GCsGp/GO 0.5% wt. scaffolds to mice. *In vivo* osteogenic profile analyses (b) mRNA expression of *opn* and *runx2* four weeks post-implantation (statistical significance $^{\#,*}p<0.05$); (c) confocal microscopy protein expression of *opn* and *runx2* four weeks post-implantation.

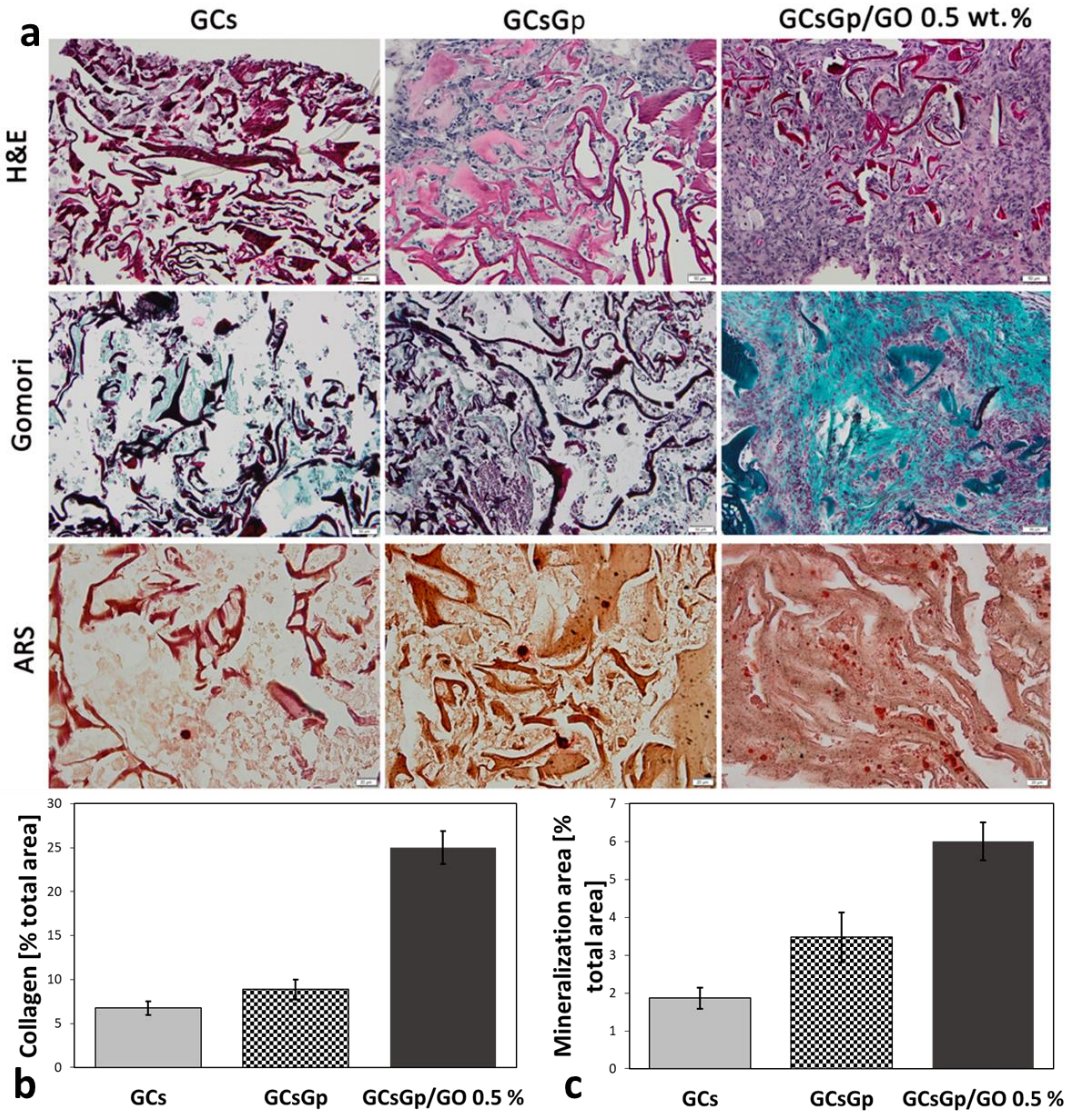


Figure 6. Histological analysis of the ectopic bone occurence in GCs, GCsGp and GCsGp/GO 0.5% wt. scaffolds at 4 weeks post-implantation. **a**) Representative H&E, Gömöri trichrome and ARS stainings. Scale Bar 20µm; **b)** The analysis of the area of collagen domains according to Gömöri staining indicated that significantly more collagen was secreted within GCsGp/GO 0.5% wt. group as opposed to GCs group ($*p < 0.001$); **c)**. ARS staining indicates that significantly more calcium mineral deposits are present in GCsGp/GO 0.5% wt. group than GCs group ($*p < 0.001$).

III. Ex-vivo characterization

morphology

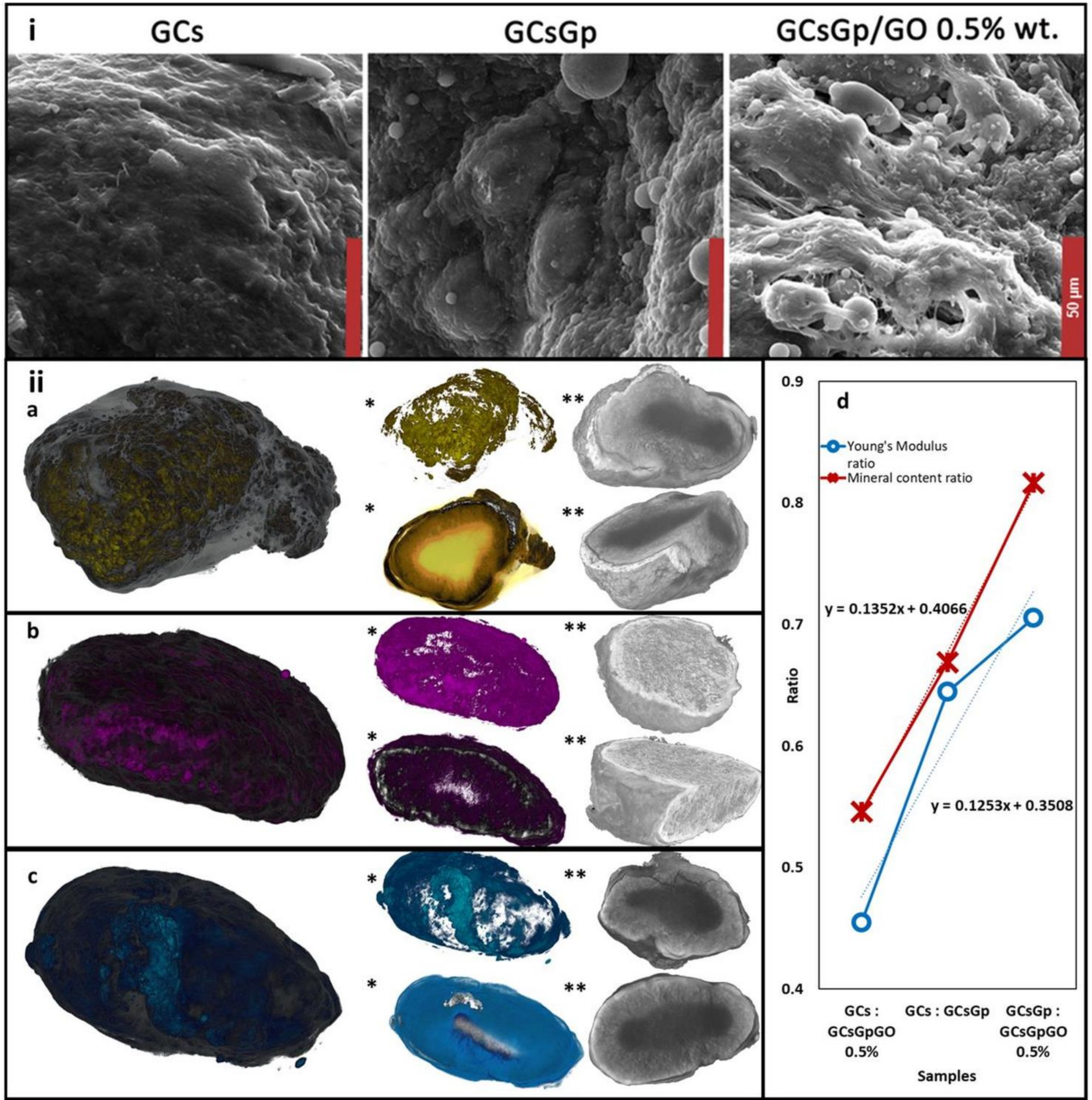
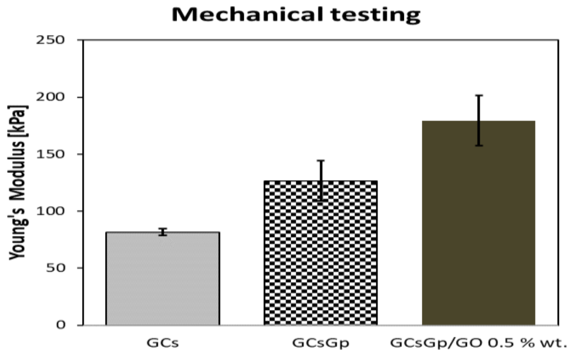


Figure 7. Post-explantation morphological characterization by means of i) SEM micrograps of GCs, GCsGp and GCsGp/GO 0.5% wt. scaffolds 28 days post-implantation; ii) Colorized μCT images of (a) GCs, (b) GCsGp and (c) GCsGp/GO 0.5% wt. scaffolds explanted 28 days; (*) marks indicate captures whereby the bi-phasic nature of the samples was separately highlighted and (**) marks indicate sectional views of the central morphology of the samples. (d) Charted data correlating mechanical properties and mineral formation based on the constitutional nature of the composites

III. Ex-vivo study

Table 1. Quantitative evaluation of de novo bone formation within the explanted specimens, by means of micro-CT analysis.

Sample	GCS	GCsGp	GCsGP/GO 0,5
BMP [%]	21.4	32	39.2



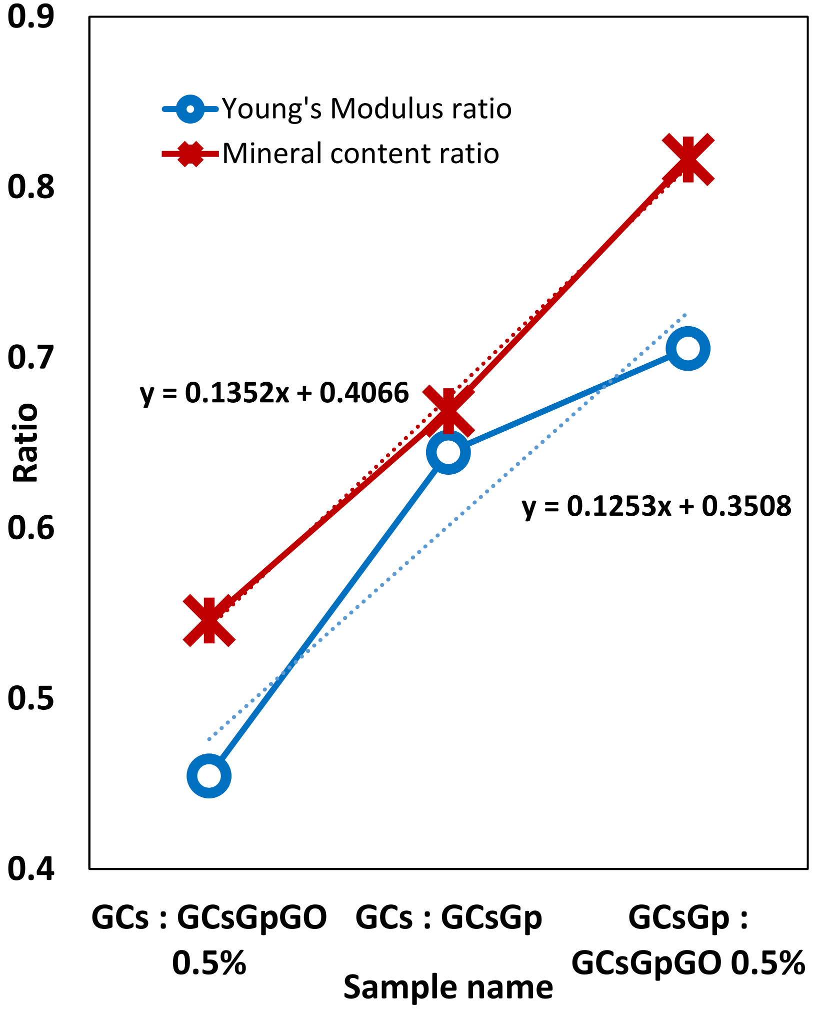


Figure 8. Correlation between the ratios of Young's modulus and the mineral content's of GCs, GCsGp and GCsGp/GO.5

III. Ex-vivo characterization *structure*

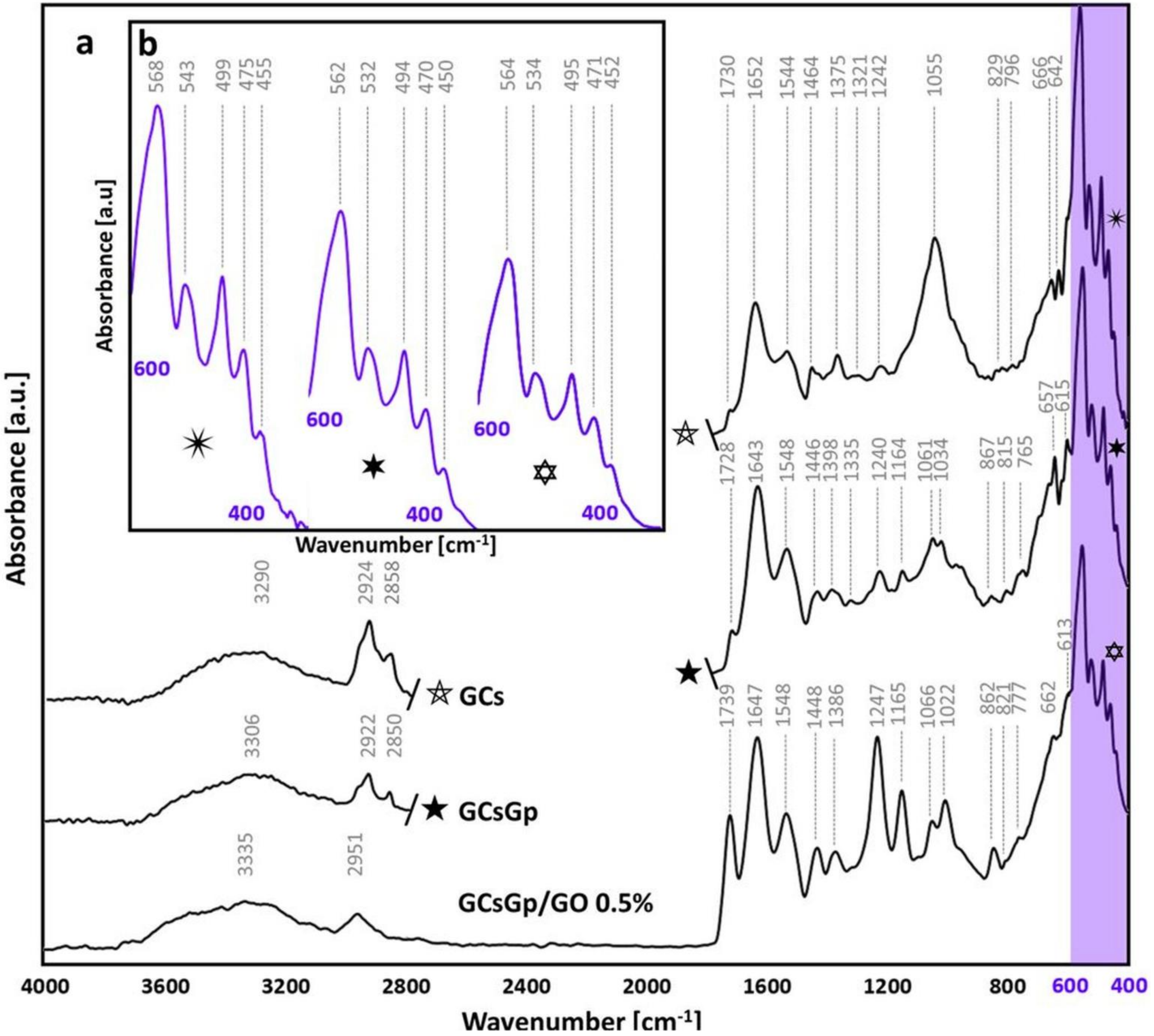
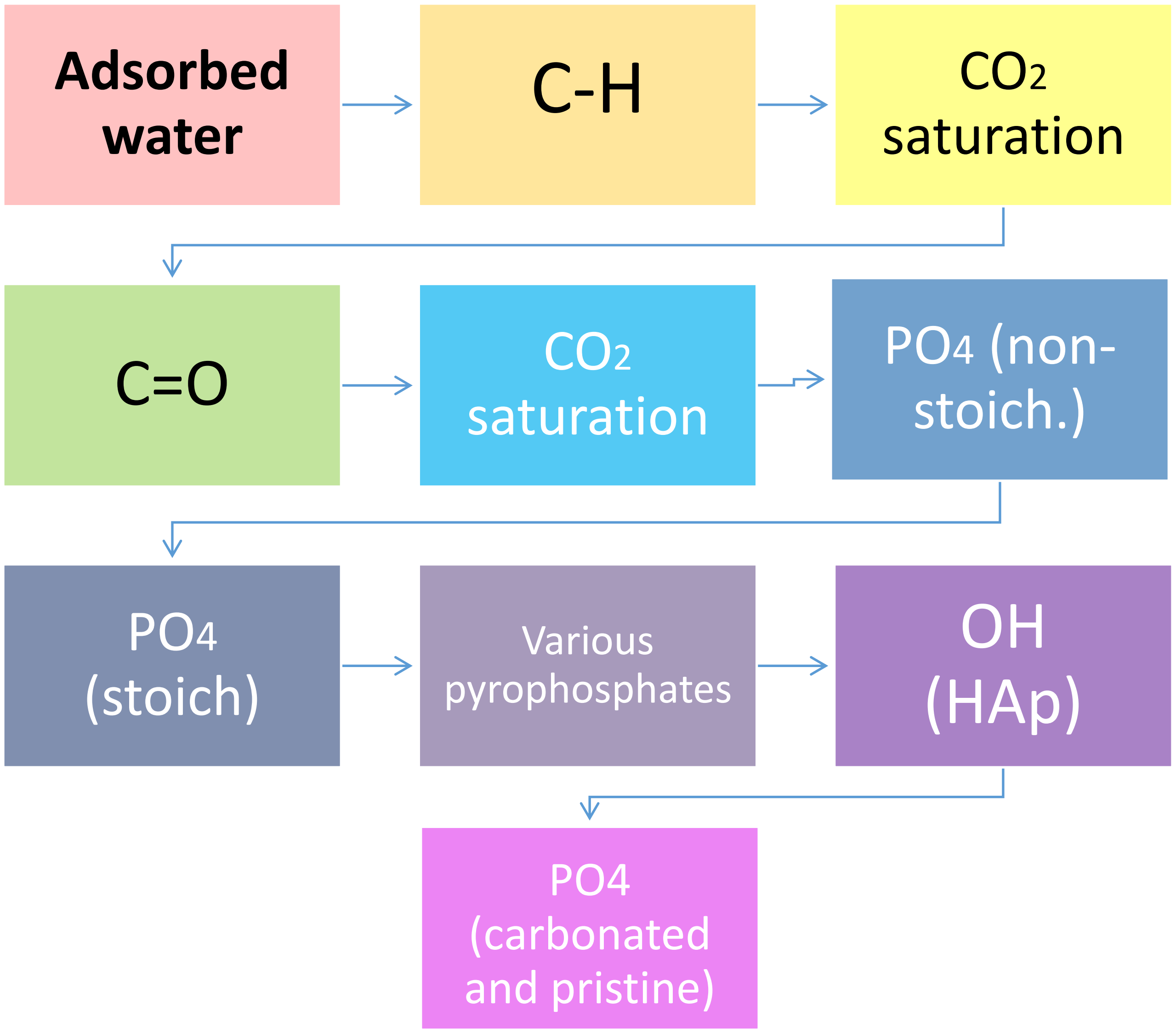


Figure 9. FTIR spectra of GCs, GCsGp and GCsGp/GO.5 ex-vivo.

III. Ex-vivo characterization *structure*



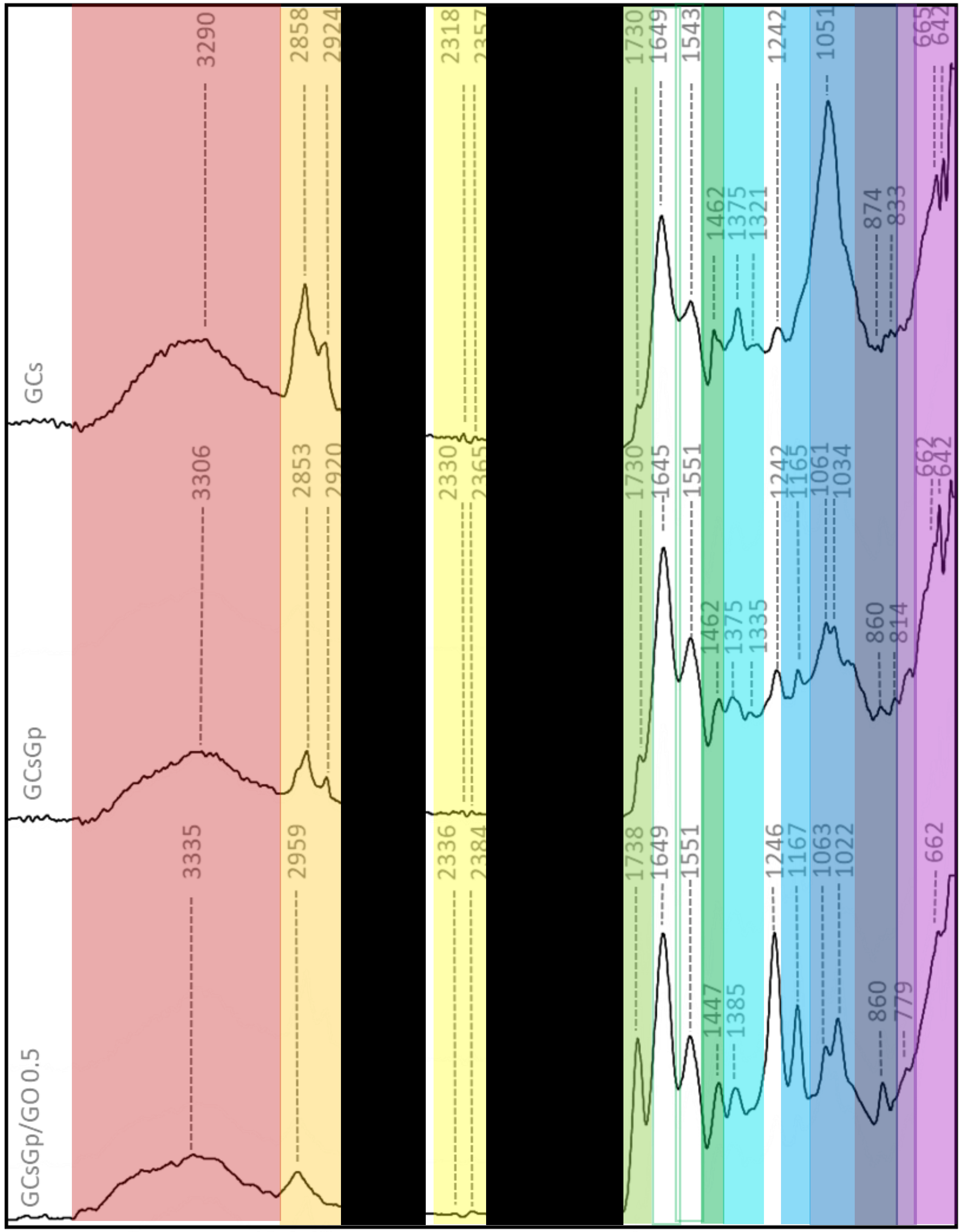


Figure 9. FTIR spectra of GCs, GCsGp and GCsGp/GO.5 ex-vivo.

III. Ex-vivo characterization *structure*

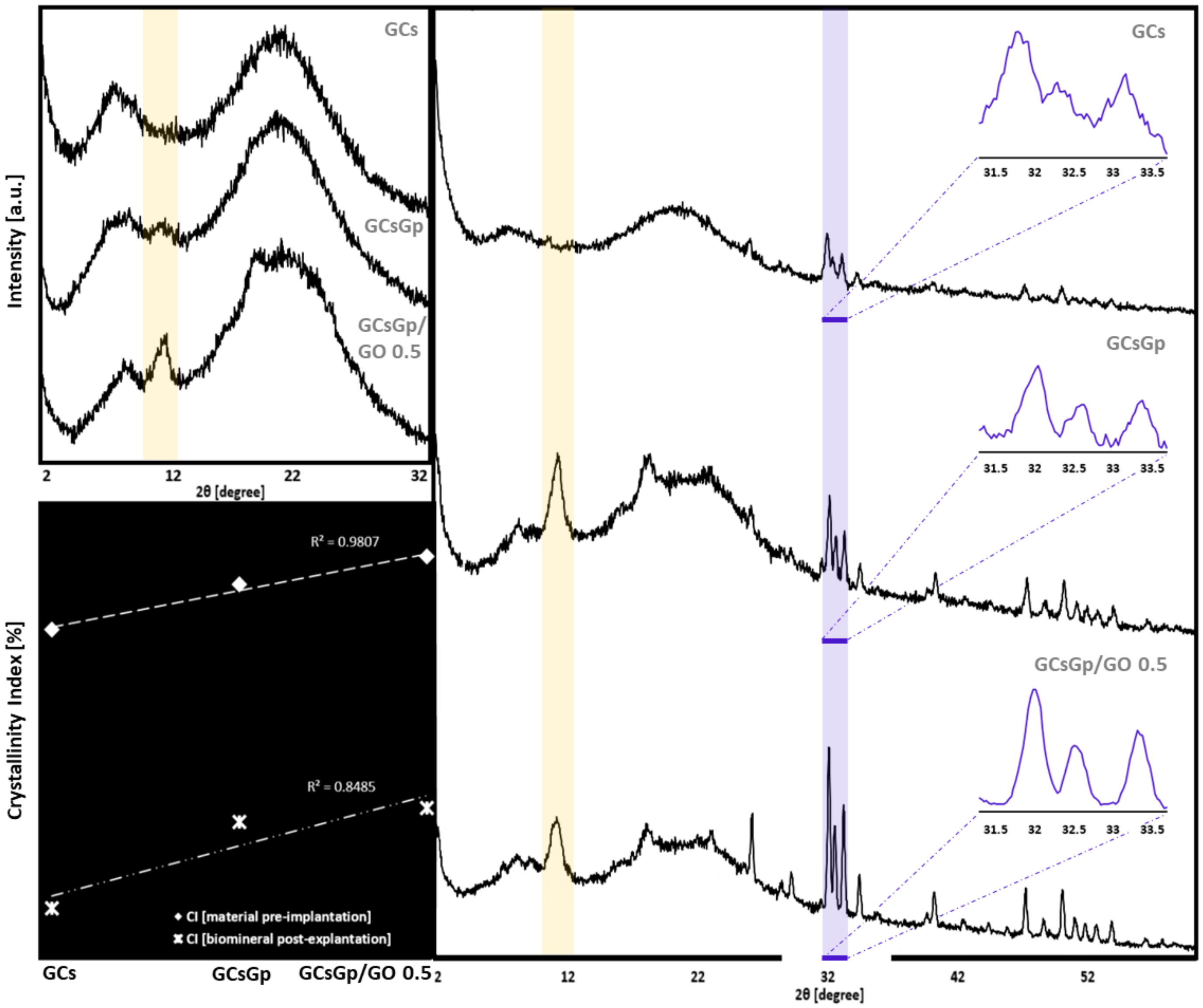
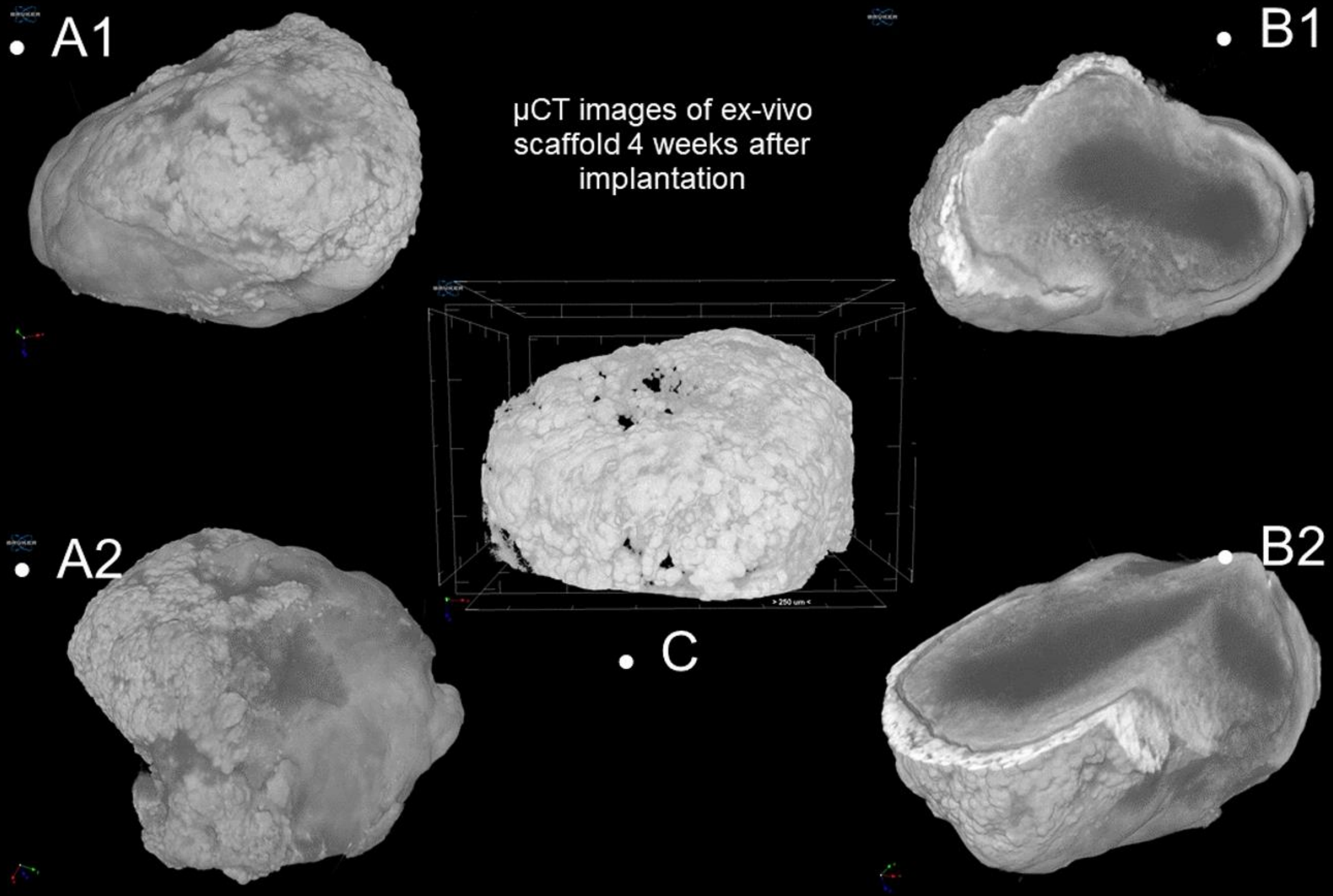


Figure 10. XRD spectra of GCs, GCsGp and GCsGp/GO.5 before (upper-left corner) and after explantation.. Plotting of cristalinity index variations of the three specimens before/after implantation

Conclusions

- **Genipin and GO effect onto the osteogenesis and osteoinduction**
- **GO fine tunes durotacticity in an all-inclusive manner**
- **Material characterization of ex-vivo specimens can provide new insights with respect to classic *in vivo* and *in vitro* bio-assays (validation).**
- **GO composites manifested ectopic osteogenic behavior**
- **Results concur on the fact that 0.5 wt. % GO load provided the most suitable support for osteoinductivity**
- **Ectopic ostegenesis investigation ongoing of superior GO supplementation**



- Fig. 1.4w. Exterior side views (A1, A2), cross sections (B1, B2) and mineral close-up (C) in CHT-GEL. Scale bar = 250μm

[Click here for video]

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