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My scientific short term mission took place in the premises of the Laboratory for Bone Metabolism and Regeneration located at the Faculty of Dental Medicine of the University of Porto, Portugal. I was under the supervision of Pr. Maria Helena Fernandes and Pr. Fernando Jorge Monteiro. This scientific mission took place over March and April of 2016 and is in the continuity of my last scientific mission.

Bio-ceramics such as calcium phosphate have been used for decades in the treatment of bone defect. The quality of the bone substitute integration is tributary of many parameters. For instance, pore size, shape and porosity levels are important factors in osteogenesis. By developing new processing and shaping techniques, these parameters can be controlled in order to generate novel and original architectures. The latter will influence cell invasion and activity. This study sets out to explore the links between cell invasion and the porous architecture of a bone substitute made out of Beta Tricalcium Phosphate (β-TCP) and obtain by freeze casting. Freeze casting or ice templating generates elliptical tubular pores that are oriented and interconnected. This peculiar morphology could enhance cell invasion and colonization. Comparison will be made with other structures achieved by techniques such as slip casting (Slurry impregnation of an organic scaffold) and stereo-lithography.

Previous biological assessment of the samples indicated that the processing methods did not affect the material's biocompatibility. Over a period of 4 days we assessed osteoblasts (MG63) adhesion, proliferation and penetration in the samples. Preliminary results show that the material is suitable for cell culture after 24 h of pre-incubation with culture medium. Over the testing period cell growth was steady for every single sample. Osteoblasts were adherent to the material and exhibited adequate cell morphology. Cell penetration in the material was noticed and varied according to the pore architecture.

This time human mesenchymal stem cells were chosen (H-MSC). They are found in the bone marrow but are known to be non-hematopoietic stem cells. H-MSCs are able to generate bone tissue, cartilage, adipocytes and fibrous tissue. Cases of ectopic bone formation following bone marrow implantation in a heterotropic site can be found in literature. The future of those cells is determined by external stimuli that can be of a chemical or a physical nature. This leg of the study, aims to show that the material used is suitable for human mesenchymal stem cells and that the porous network might enhance the chances of osteogenic differentiation.

Material/methods

Powder fabrication and shaping took place at the LMCPA, France and BCRC, Belgium. β-TCP powder was obtained by low temperature, aqueous precipitation. A phosphate solution is introduced in a reactor containing a calcium solution under mechanical stirring. Temperature and pH are closely

controlled as they are key parameters to yielding a good quality product. This reaction takes place over 20 h. The powder is then collected, dried up, undergoes a thermal treatment and is finally ground up.
Processing techniques were optimized in order to produce sample exhibiting 30 to 50 % porosity with interconnected pores ranging from 30 to 500 µm in diameter. Five different samples were made and compared: BIO1, BIO4 and BIO7 (Freeze casting); PS (Slip casting) and 3D (Stereo-lithography).

- Freeze casting: a stable slurry is frozen in a Teflon mould from top to bottom. The composition of the slurry (Powder content, water content and additives) and the freezing rate will determine the architectural structure of the sample.
- Slip casting: a polymeric structure made out of partially fused PMMA beads is impregnated with an aqueous ceramic suspension. Beads of known dimensions are chemically welded together in a controlled manner. The operator is allowed by the technique to choose the dimensions of the contact points between beads thus determining pore interconnections size. The PMMA beads are eliminated via an adapted thermal treatment.
- Stereolithography: a photosensitive slurry is selectively polymerized according to a tridimensional computer model.

The following table is a recap of the characteristics of each sample:

Sample denomination	**Shaping Method**	**Porosity (%)**	**Pore and interconnections diameter (µm)**
3D	Stereolithography	50%	500 / 100
PS	Impregnation of a polymeric skeleton	65%	500 / 100
BIO1	Freeze casting	50	150 / 40
BIO4		50	360 / 55
BIO7		36	150 / 45

Every single sample was sterilized at 121 °C for 30 min.

Prior to seeding, every single sample was incubated in 1 ml of α-MEM for 24 h. The medium was renewed once. After this step, the samples were incubated in 1 ml of complete medium (α-MEM + 10 % Foetal Bovine Serum + 1 % Penicillin) for 3 h. Incubation took place in a humidified atmosphere of 5 % of CO2 at 37°C. This procedure aims to create an adequate environment for cell culture.
The amount of cells is 3 x 10^4 cells/cm. Culture medium was renewed twice a week.

Tests:

- Metabolic activity and cell proliferation
    - MTT: Cell metabolic activity was assessed via MTT. 1% of MTT was added to the culture medium and incubated for 3 h. The samples were then removed from their culture plates and placed onto a new one. The samples were observed through a stereomicroscope. Cell membrane destruction was caused by incubating samples in 1 ml of DMSO for 15 min. a 100 µl of medium was then transferred to a 96 well microplate destined to a plate reader. Absorption values were measured at 550 nm.
    - Resazurin: the samples were transferred to a new culture plate. 1 ml of fresh medium with 10 % resazurin was added to the samples. Incubation lasted for 3 h. A 100 µl of medium in then transferred to a microplate. The fluorescence was

measured with a microplate reader at 530 nm for excitation and 530 nm for emission.

- ALP activity: Cells were permeabilized with 1 ml of a 0.1 % triton solution for 30 min at room temperature. Cell lysate were then frozen (- 20 °C) until the next step. The total amount of protein was determined by the Lowry method and by using BSA as standard. Alp activity was determined by substrate hydrolysis (Nitrophenol phosphate) in an alkaline buffer solution. The plate was incubated with 15 µl of Folin's phenol reagent for 1 h at room temperature in the dark. The product, P-nitrophenol was measured with a plate reader at 405 nm. Results were normalized to total protein content and are expressed in nanomoles of product yielded by 1 g of protein. After the assay, the culture medium is renewed.
- Cell morphology
    - Cell morphology was assessed by observation through a confocal microscope of immunostained, fixed cells (3.7 % formaldehyde for 15min). Immunostaining took place as follows: the samples were incubated for 30 min in 0.1 % triton and 30 min in BSA. In order to mark actin filaments, samples were incubated for 30 min with phalloidin. Their nucleus were stained after the samples were washed with PBS with DAPI.
    - In order to prepare samples for SEM, they were fixed with a 1,5 % glutaraldehyde solution for 15 min.

Results

1. Metabolic activity and cell proliferation

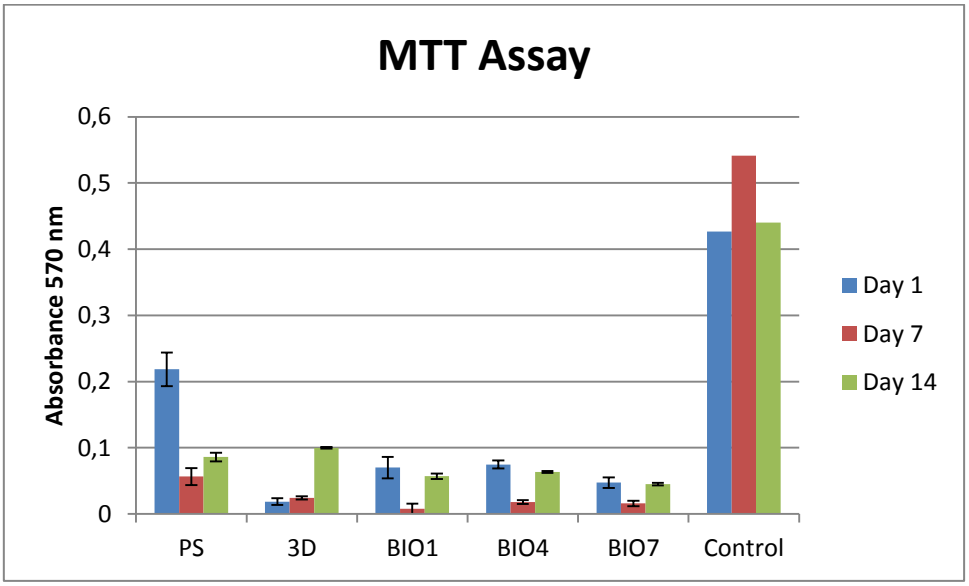
Observation of MTT stained cells:

Day 1: Observation shows that cells can be seen spread out over the surface of every single sample. They can also be seen inside the samples, in the inner pores. 3D and PS exhibit more cells in the inner pores than the frozen samples. This is probably due to the sizes of the pores and the interconnection. The latter assumption must be confirmed through more observations.

Day 7: An important decrease in cell population was observed. Very few cells could be observed on and inside the samples. At this point in the experimentation, these observations suggest adaptability problems that could be linked to the material itself or the environment the material is creating (Heavy ion leaching). Positive control well shows healthy cells covering the bottom of the well.

Day 14: An increase in cell population was noticed. Observation suggests that there are more cells on the materials surface than day 7. This assumption can only be confirmed by the quantitative results.

MTT quantitative assay:



The quantitative results reflect the observations conclusions. Cell population decreased steadily until day 7. An increase can then be noticed at day 14.
Day 1: PS had the most cells, followed by the frozen samples. BIO 1 and BIO 4 have no significant differences. BIO 7 is significantly inferior to BIO 4. The frozen samples exhibit very low absorbance reading compared to PS, as their values are inferior to a third of the value associated with PS. 3D did not perform well at all, as it has the lowest absorbance value of all.

Day 7: A steady decrease in cell population can be noticed for samples PS, BIO 1 BIO 4, and BIO7. In the case of sample 3D, its cell population was stable. PS is the sample that has the most cells. At day 7 the control well's cell population reached its peak.

Day 14: An increase in cell population is noticed. Every single samples show significant cell growth compared to day 7. In the case of 3D, cell population reached higher values than day 1, making it the best performing sample at day 14. Control well indicates that confluence was probably met hence the decreased absorbance value.

Conclusions: BIO 1 and BIO 4's performance are very similar with no significant differences. Their higher absorbance values suggest that these 2 samples are better than BIO 7. However, the best performing samples overall are PS and 3D.

MTT quantitative and qualitative results are very similar and tend to the same conclusions: PS and 3D exhibit the highest cell metabolic activity at day 14. The frozen samples did not perform as well as the other samples. No significant differences can be noticed between the absorbance values at day 14. A decrease in cell population is visible around day 7 for every sample except sample 3D.

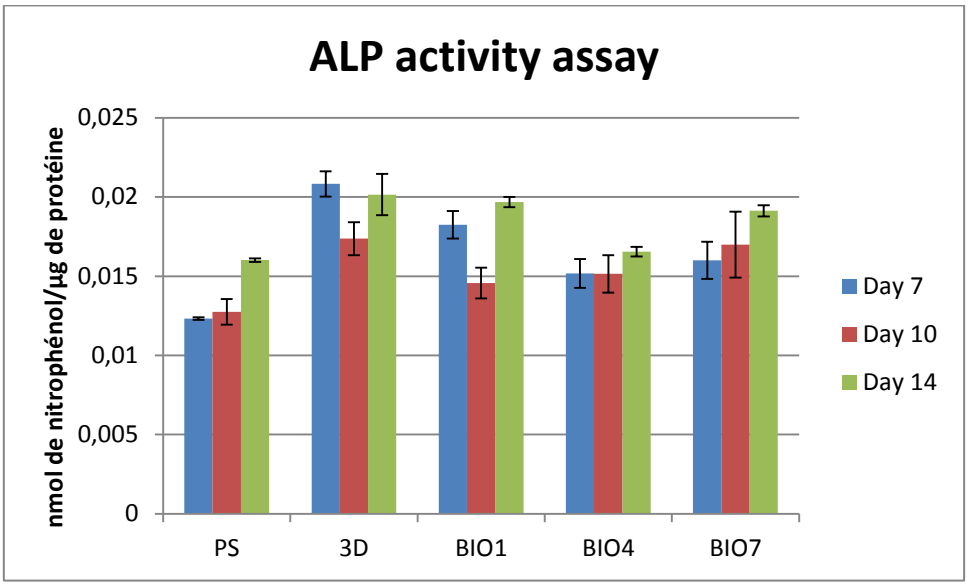
Resazurin assay

Data not shown

Day 1: Cells are present on every single sample except for BIO4. PS exhibits the higher value of fluorescence followed by 3D. Concerning the frozen samples, BIO1 exhibits the best fluorescence value.
Day 3: A decrease in cell population is noticed for every single sample except for BIO4, where an increase in cell population was noticed. BIO7 exhibits the lowest fluorescence value with 0. In the case of 3D and PS, the fluorescence value measured at day 3 is the lowest over the testing period.
Day 7: PS and 3D show a recovery of cell activity. The frozen samples show fluorescence values of 0.
Day 10: Fluorescence values are the same for PS and 3D. However, the frozen samples show a slight increase in fluorescence. The values are still very low compared to 3D and PS.
Day 14: PS and 3D show a slight decrease in fluorescence value but no significant differences can be found. BIO1 and BIO4 show some fluorescence without any significant difference with day 10. BIO7 does not show any fluorescence.

This assay was performed over 14 days on the same samples.
The control well results are very similar to the ones measured during the MTT assay. An increase in metabolic activity is noticed from day 1 to 7, when it peaks. The following time points (Day 10 and day 14) show a steady decrease in cell population. This can be attributed to cell confluence.
On the whole, frozen samples did not perform well. They exhibit fluorescence values that are lower than 3D and PS. A decrease in cell population can be seen on day 3 for every single sample. On day 7, PS and 3D show an increase in cell population. This is not the case with the frozen samples where the fluorescence values reached 0. Beyond day 7, cell population increased in PS and 3D. However no significant differences can be found between day 7 and day 10. On day 14, the last day, a slight decrease in the fluorescence was noticed. As can be seen on the graph, the frozen samples did not perform well. Cell activity can be seen past day 7 but the validity of these very low values can be questioned.

2. ALP activity



PS, BIO4 and BIO7 show a similar pattern. The values measured on day 7 and days 10 are very close. They then increase on day 14. In that case, only PS exhibit significant differences between day 14 and day 10 and 7.
3D and Bio 1 display a different behavior. It can be seen on the graph that on day 10 a decrease in protein activity occurs before increasing to levels equivalent to day 7.
These values are very low and can be correlated to the low cell count found on the material.

3. Cell morphology

SEM Pictures (Ongoing: more pictures will be available shortly)

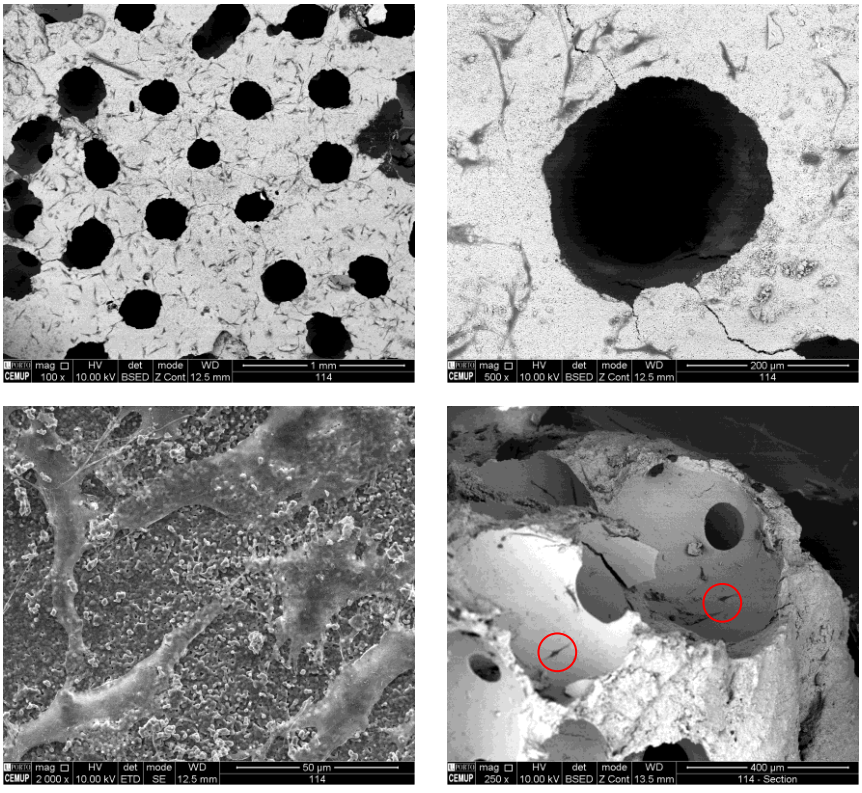


Figure 1: SEM pictures of sample PS - Day 14

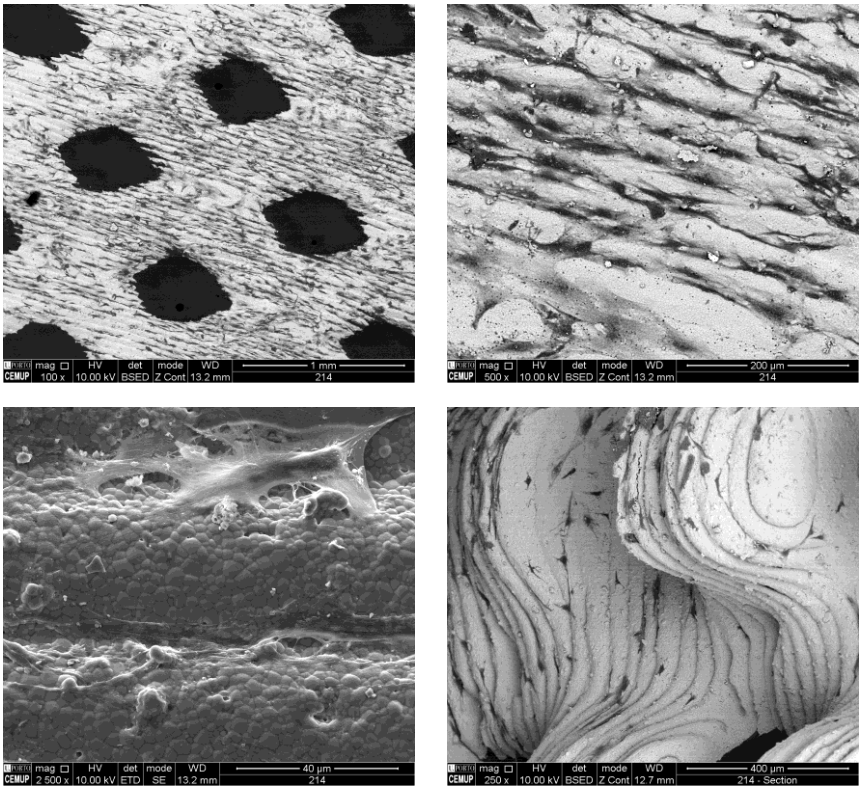


Figure 2: SEM pictures of 3D sample - Day 14

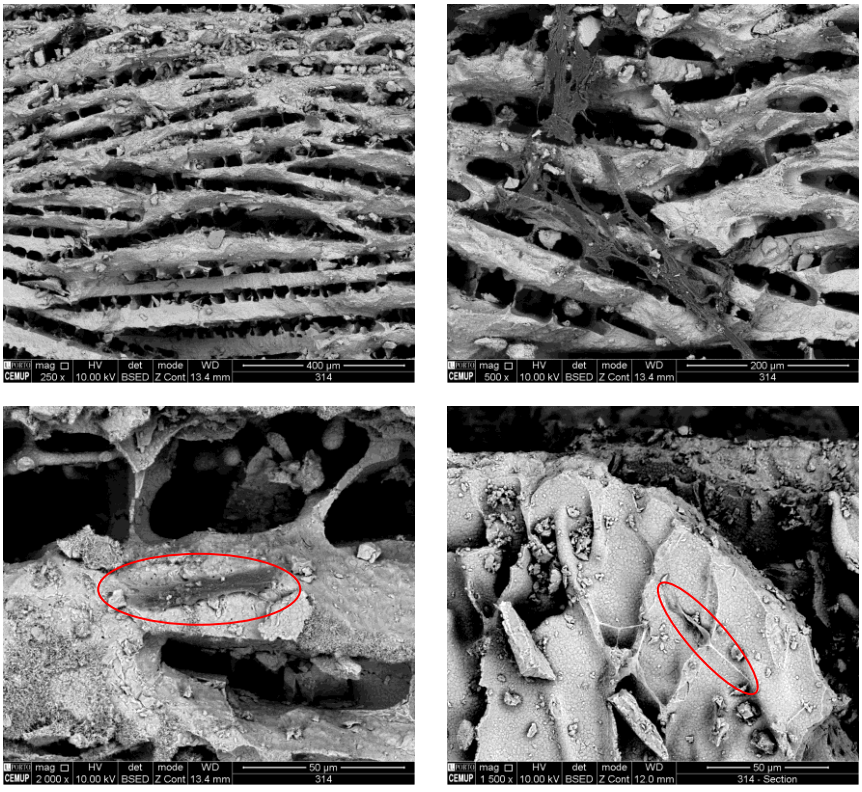


Figure 3: SEM pictures of BIO 1 sample – Day 14

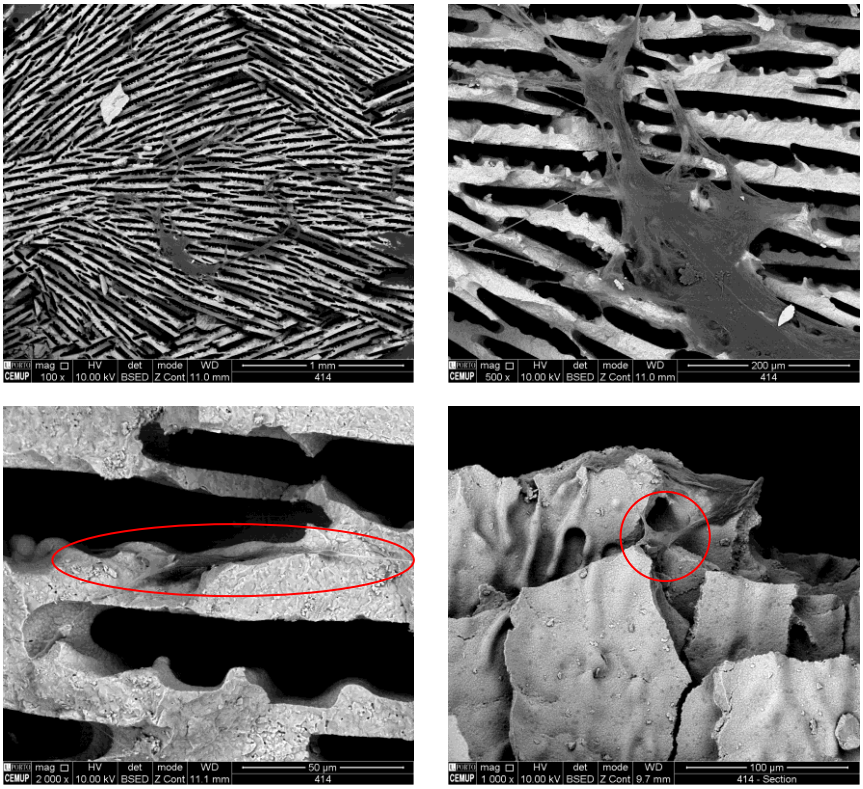


Figure 4: SEM pictures of BIO 4 sample - Day 14

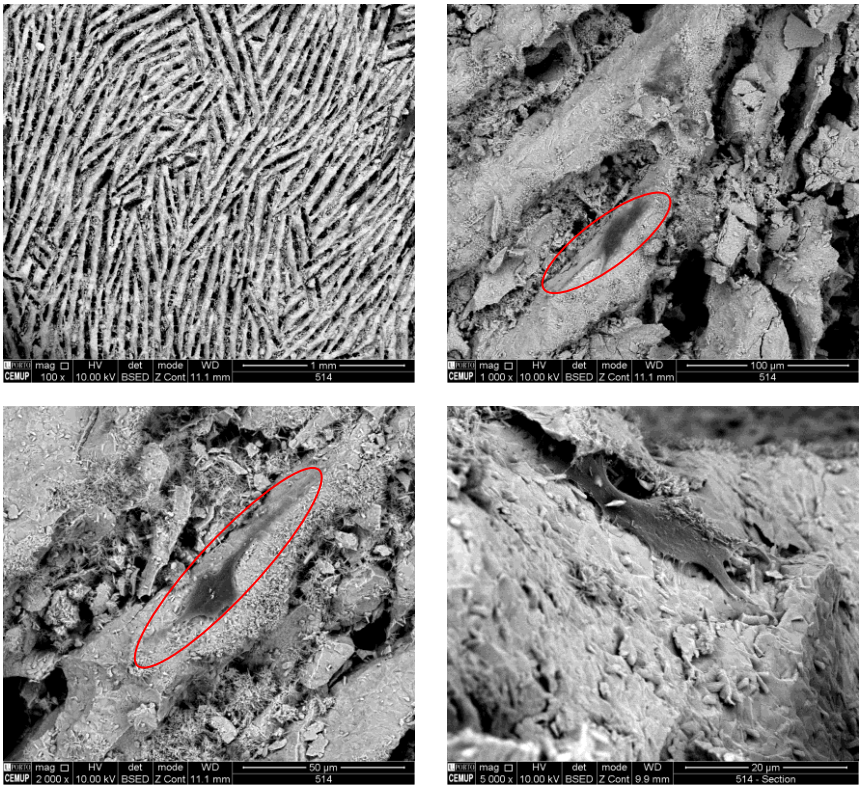


Figure 5: SEM picture of BIO 7 sample - Day 14

SEM pictures were taken at day 14. Cells can be found on every single sample. However population density is not the same and varies from sample to sample. PS and 3D seems to have more cells than the frozen samples. The cells exhibit morphological characteristics such as an elongated flat body with cytoplasmic extensions thus indicating that the cells are adherent. Pictures of sections of the samples show cells inside the pores. They exhibit the same morphological attributes than the cells on the surface.

Due to the shaping process used to make 3D, grooves can be seen on the samples. They are 50 µm deep and are separated by the same distance. As can be seen on the pictures the cells are organized according to the grooves. It is clear here that the cells reacted to the topography of the material. However with the current results it is difficult to assess the extent of that influence.
BIO 1 and BIO 7 have on their surfaces, crystal like structures that could be apatite deposits.

Confocal microscopy picture

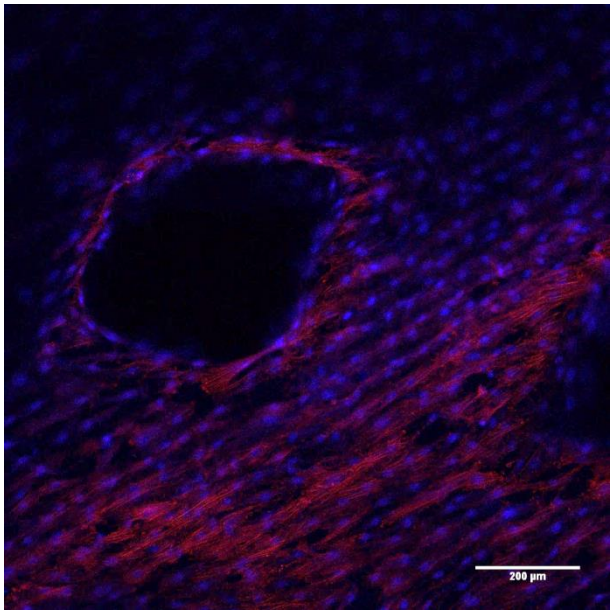


Figure 6: Immunostaining of actin filament and nucleus on day 14 - 3D

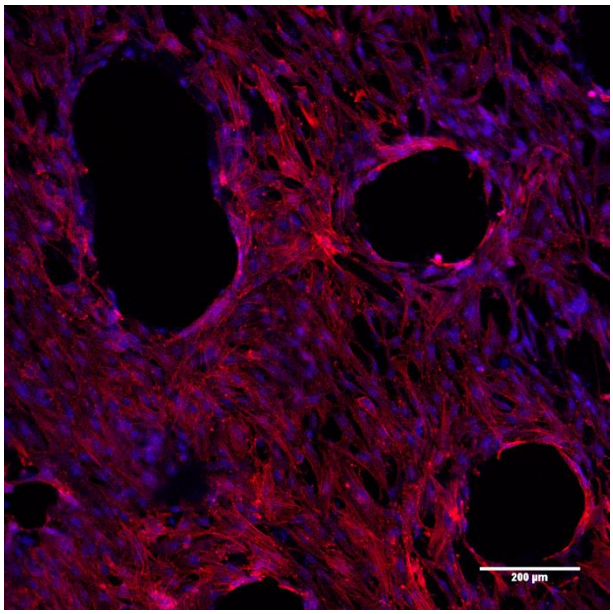


Figure 7: Immunostaining of actin filament and nucleus on day 14 - PS

Actin filaments were stained by phalloidin (Red) and the nucleus by DAPI (Blue). On every single sample, cells were seen. The cells exhibited morphological characteristics such as cytoplasmic extensions and adhesion focal points. Pictures were taken on day 14.

As seen on SEM pictures, cells on 3D are organized. They are parallel to the grooves. This pattern can be seen in the way actin filaments are aligned. Even the major axis of most of the nucleus is aligned to the grooves (Picture not shown). It seems that cells on sample 3D has less cytoplasmic extension than cells found on PS sample. On the latter, cells tend to be “star shaped” with more than 2 cytoplasmic extensions.

Discussion

Positive control wells exhibit higher number of cells than the samples because they are being grown on a two-dimensional support. Cells grown on tridimensional scaffolds are subjected to growth regulation mechanism. The latter explains partially the difference between samples and control results. It can also be hypothesize that the material is not adequate for this cell type. Heavy ion leaching might be at fault. In order to confirm that assumption, further experiments should be carried out. One of them will be to study the ion leaching in SBF over a period of at least 21 days. This experiment will also allow the assessment of apatite precipitation over the material.

Observations of the MTT stained cells can be correlated to the quantitative MTT results. Literature seems to indicate that between 1 and 7 days, mesenchymal stem cells exhibit a lag phase characterized by a low growth rate and thus a low metabolic activity. In order to confirm that assumption, tests periods should be prolonged. The resazurin assays were not conclusive. Extremely low levels of absorbance were measured compared to the controls. Overall, mesenchymal stem cells did not perform well on the samples. Metabolic activity tests and vitality tests seem to indicate that cells are slightly better on samples 3D and PS compared to the frozen samples BIO 1, BIO 4 and BIO 7. Thus their validity can be questioned. No significant differences were noticed between samples concerning ALP activity. But 3D seems to exhibit higher levels of protein activity that the other samples.

SEM pictures show cells with morphological characteristics of adherent cells. Their bodies are elongated and flat with cytoplasmic extensions. Some samples (BIO 1 and BIO 7) display structures, on their surface, that look like apatite crystals. But this assumption needs to be confirmed. This early apatite deposit might be a result of specific surface and pore size. BIO 1 and BIO7 share the same pore size. They are also the sample with the largest specific surface.

The topography of the 3D sample allows the cells to organize themselves. This can be seen on the SEM pictures, with aligned cells in the grooves. This phenomenon is confirmed by the confocal pictures of the immunostained 3D sample. The actin filaments (in red) are parallel to the grooves. Cells react to topographic stimuli. This phenomenon is known as mechanotransduction. When subjected to such stimuli, the cell conveys signals through its cytoskeleton to its nucleus where cell function is changed. In this case we don’t know to which extent the topography influenced cell activity. Further investigations are needed.

Preliminary results suggest that PS and 3D were better suited for the mesemchymal stem cells. Frozen samples yielded poor results over a period of 14 days. These tests need to be supported with additional assay over a period of 21 days at least. Test such as a decomposition kinetic in Simulated Body Fluid over a period of 21 days is considered. This test will enable us to study the state of the pores and the interconnections after the deposition of an apatite layer.