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Title: Towards the development and characterisation of an easy handling sheet-like biohybrid bone substitute.

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Abstract

We designed a sheet-like bone substitute capable of adapting to different geometries and becoming a standard tissue-engineered process for bone surgery. Preosteoblastic cells were seeded on to a monolayer of calcium phosphate granules and cultured in a flat parallelepipedic cell culture chamber for one month. From the various diameters of the granules examined, the 80 – 200 μm group exhibited the most homogeneous performances regarding both biological (cell morphology, viability, differentiation and simple metabolic activity) and mechanical (cohesion and stress-strain behaviour) properties. This sheet was easy to handle after extraction from the culture chamber and showed versatile geometry and flexibility, making it easy to use for surgeons, especially for small defects of the maxillofacial area.

1. Introduction

Tissue engineering is considered to be the most powerful alternative to autologous grafts in bone surgery. It consists of culturing autologous cells from the patient on a biomaterial with a specific shape and composition, known as a scaffold, and leading to *in vitro*-built bone tissue that can then be implanted^{1,2}. This approach should allow clinicians to circumvent the limitations both of allografts, namely rejection and the number of donors³, and of pure biomaterial implants that cannot be colonised^{4,5}.

Despite a tremendous number of studies with a variety of cells, materials, shapes or bioreactors, to date there is still no consensus on the best process leading to adequate bone substitutes based on tissue engineering⁶⁻⁹. Various materials have been studied and considered as possible scaffolds for *in vitro* bone ingrowth, such as ceramics^{10,11}, polymers^{12,13}, natural biomaterials such as silk or chitosan¹⁴⁻¹⁶ or to a lesser extent metals⁷, as well as composites of these different groups¹⁷. Relevant shapes for the scaffold have also been debated, with different studies on granules and microspheres¹⁸, fibres^{19,20}, hydrogels²¹ or engineered complex structures^{22,23}. In addition, a bioreactor's ability to mimic *in vivo* environmental conditions is still an unsolved issue, with a wide variety of systems using mechanical stretching, flow perfusion, spinner flasks, rotating wall vessels²⁴⁻²⁶ or specifically designed chambers²⁷.

Targeted applications may be helpful for making choices from among all the possibilities. Besides intrinsic mechanical and biological properties (micro- and macroporosity^{28,29}, pore interconnectivity³⁰, osteoconduction and osteoinduction³¹), specific clinical requirements also have to be satisfied by the target substitute: it must be adaptable, and fit with all geometries of the bone mass loss in the surgical area, for instance the complex

geometry of the oral and maxillofacial area ; it must have optimal cell viability and differentiation, and be stretched or folded during surgery without damage, thanks to the relevant mechanical properties³². From this point of view, current solutions have limitations in terms of their mechanical and biological behaviour⁷, the vascularisation of the scaffold³³ and the cost for a standard bioreactor process³⁴.

Following a previous study of these clinical requirements³⁵, we selected a set of parameters for a new *in vitro*-built bone substitute to be applied in bone surgery. A monolayer of calcium phosphate (CP) granules was selected as the scaffold, inside a specific flat cell culture chamber with a large culture surface. Calcium phosphate ceramics have been widely used for bone and dental tissue engineering³⁶ thanks to their relevant properties, especially osteoconduction and osteoinduction¹⁰. In addition, stacking granules may create the required porous interconnected structure and high surface-to-volume ratio², and it showed better results than CP blocks³⁷. This biomaterial was colonised by MC3T3-E1 preosteoblastic cells, a usual cell line to determine the potential of new scaffolds for proliferation, adhesion and differentiation of bone tissue³⁸⁻⁴⁰. No growth factor was added to the culture medium as we expected the system culture chamber + biomaterial to be efficient in promoting cell growth and differentiation. After one month of culture, both the biological and mechanical properties of the substitute were examined, depending on the CP granules' mean diameter. We focused particularly on the morphology and shape relevance of the bone substitute for clinical applications, especially the small geometry of defects in the maxillofacial area.

2. Materials and methods

2.1 Cell culture chamber

We used the Clinicell25® culture cassette (Mabio-International, France) as a specific flat cell culture chamber in order to obtain the *in vitro*-built tissue. The geometry is a parallelepipedic chamber with a 24 cm²-culture surface for a 10 mL-volume filled with medium, with 2 Luer Lock connections (Fig. 1). It is gas permeable and plasma treated to promote cell adhesion. After one month of culture, the upper side of the chamber was opened to remove the tissue and to perform the analysis.

2.2 Biomaterial

Calcium phosphate granules (20 % hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, 80 % β -tricalcium phosphate $\text{Ca}_3(\text{PO}_4)_2$, Biomatlante, France) were inserted as the scaffold material inside the culture chamber in order to obtain a single monolayer covering the whole surface. The layer was stable enough after 2 days of culture to prevent displacements of the biomaterial when the culture medium was changed. Different mean diameters of granule were investigated: <80 μm , 80-200 μm , 500-1000 μm and mixed composition (Table 1). Hereafter we refer to these groups as the S (small), M (medium), L (large) and Mix groups, respectively. A group without CP granules inside the culture chamber was studied as a control.

The cell culture chambers with CP granules were preincubated with complete medium for 48 hours before cell seeding.

2.3 Cell culture

The murine preosteoblastic cell line MC3T3-E1 subclone 4 (ATCC® CRL-2593) was cultured in α -MEM Eagle medium with stabilised glutamine (Pan-Biotech, Germany) supplemented with 10 % FBS (Gibco Invitrogen, USA), 1 % Penicillin-Streptomycin (Gibco Invitrogen, USA), 1 % sodium pyruvate (Gibco Invitrogen, USA) and maintained at 37 °C

and 5 % CO₂. To develop the substitute, cells were harvested at P24 and seeded in a specific cell culture chamber (10 million cells in each chamber) for one month with the same supplemented medium and 50 mg/L of ascorbic acid (Sigma Aldrich, USA). The medium was changed every 2 or 3 days.

2.4 Scanning Electronic Microscopy (SEM) observations

After the substitute was removed from the culture chamber, tissue morphology and cell adhesion were observed using SEM (Philips XL30 ESEM-FEG, the Netherlands). The samples were immersed in Rembaum solution (as described in Rajaraman *et al*⁴¹) for 24 hours, rinsed with de-mineralised water and then coated with gold before observation.

2.5 Viability test

Cell viability was estimated by a Live/Dead® kit (Invitrogen, USA) according to the manufacturer's protocol. Briefly, Calcein AM (1µM) and Ethidium homodimer-1 (EthD-1, 1 µM) fluorescent dyes were respectively employed to stain viable and dead cells. The samples were observed using fluorescence microscopy (Leica microsystems, Germany), allowing us to qualitatively determine cell viability and morphology of leaving cells. Holes in the CP granule monolayer were observed with phase contrast. Cell nuclei were counterstained by Hoechst 33342 dye (Invitrogen, USA, 0.5 µg/mL) to estimate the viability rate. Image processing was carried out with the free software CellProfiler (www.cellprofiler.org). Briefly, colour images were converted to greyscale images, then erosion was applied (a morphological operation to separate close nuclei from each other) and cells were counted based on size and intensity of the nuclei. At least 5 areas were randomly chosen for image acquisition on the sheet-like substitute for each group.

2.6 Metabolic activity test

The cell metabolic activity was quantitatively measured with the AlamarBlue® Assay (Invitrogen, USA). One day per week, the culture medium was removed from the culture chamber in a dark room and replaced with culture medium complemented with 10 % of AlamarBlue® solution. The culture chamber was incubated for 45 minutes at 37°C. The complemented medium was then removed and fluorescence intensity was measured (excitation wavelength: 560 nm, emission wavelength: 595 nm). The changes in fluorescence correspond to the biochemical reduction of the AlamarBlue® solution in the culture medium resulting from cell metabolic activity (redox reaction in the mitochondria). The fluorescence intensity of the complemented medium without contact with the cells was subtracted from the results before the analysis.

2.7 Differentiation test

Alkaline phosphatase (ALP) activity, an early marker of osteoblast differentiation, was assessed by a histochemical semi-quantitative approach, using an Alkaline phosphatase kit (Sigma-Aldrich, USA) according to the manufacturer's protocol. Samples were observed with a binocular magnifier (Leica Microsystems, Germany).

An Enzyme-linked Immunosorbent Assay Kit (ELISA kit, Cloud-Clone Corp., USA) for osteocalcin (OCN) was also used according to the manufacturer's protocol to study the late-stage differentiation of samples. Briefly, the culture medium removed from the chambers at day 30 after 24h of incubation was added in a plate pre-coated with an antibody specific to OCN. After incubation with the kit's reagents, color changes were measured with spectrofluorimetry at 450nm and linked to the concentration of OCN with a standard curve. The results were compared to the protein total mass using a standard Bradford assay. Lysis was performed with ripa buffer (Fisher Scientific, USA), then Coomassie blue was added

(Interchim, France) and absorbance was read at 570nm. The standard curve was obtained with bovine serum albumin (Interchim, France).

2.8 Stress-strain tests

Using the Electroforce 3200® system (Bose, USA), conventional tensile tests were conducted on preloaded tissue strips (1 cm x 3 cm) at the speed of 0.1 mm/s until failure. Forces vs displacement were recorded. *In situ* tests were performed with SEM observations and a specific tensile testing machine (Deben Microtest 300, UK), with the same speed and sample's size as standard tensile tests, as a tool for understanding the relationship between the tissue's microstructure and its mechanical properties. In order to spot easily the region of interest, a cutout was made in the middle of the strip before mounting. Displacement was regularly stopped to perform image acquisition of the crack propagation and evaluate damage mechanism.

2.9 Statistic tests

At least six independent experiments were performed for each group (S, M, L, Mix or control), then means and standard deviations were calculated. Two-way analysis of variance with Tukey's test (metabolic activity) and unpaired t-test (viability, OCN production) were used to define the significance of the results.

3. Results

Cell culture chambers were filled with CP granules of a specific size (S, M, L, Mix) inserted as a monolayer. Ten million MC3T3-E1 cells were seeded in the medium-filled chamber. Cell colonisation was assured via the follow-up of metabolic activity. After a 4-week incubation, each culture chamber was opened to collect the reconstructed tissue.

3.1 Tissue Cohesion

After one month of culture in the chamber, we investigated the possibility of extracting a biohybrid sheet-like substitute made of bone cell tissue and CP granules from the chamber. The most cohesive sheet was the M group: it was possible for the substitute to be stretched or folded and for it to stay cohesive (Fig. 2). The S group offered the most heterogeneous results, with, in some tissue areas, the worst handling (no possibilities for removing the tissue from the chamber) and in other areas a tissue as cohesive as that of the M group. The other groups (L and Mix) had a sheet-like morphology inside the chamber, but the substitute lost its integrity once removed. In the **control group (chamber without CP granules)**, cells organised in a layer that we were not able to remove.

To explain these different tissue cohesions, SEM observations were conducted. The control group (Fig. 3A) showed a dense and continuous cell tissue. **CP granules without cells can be seen on fig. 3B.** We found the same homogeneous tissue covering the granule monolayer for the M and Mix groups (Fig. 3D, 3F). Concerning the L group, the cells were more likely to attach and grow inside the macroporosity of the granules (Fig. 3E). There was only heterogeneous tissue with cell spots when samples from the S group were observed (Fig. 3C).

3.2 Cell Viability

We visualised granules colonisation by living cells of each group under fluorescence microscopy using Calcein AM. As seen above with SEM observations, the **control group** showed a continuous cell tissue (Fig. 4A) that we found also on the CP granule layer for M and Mix groups (Fig. 4D, 4F, CP granules' autofluorescence can be seen on fig. 4B). The S

group's tissue was heterogeneous with only spots of living cells (Fig. 4C). We noticed few cell bridges between granules for the L group (Fig. 4E).

Analysis of fluorescence microscopy images allowed us to evaluate the viability percentage from the ratio between dead cells' nuclei (visualised with EthD-1 dye) and all existing nuclei (Hoechst 44432 dye) (Fig. 5). The S and L groups showed the highest viability rates ($76\pm 24\%$ and $66\pm 25\%$ respectively), but with relatively high standard deviations due to the heterogeneity of the tissue as seen above. Viability dropped to $59\pm 18\%$ for the M group and to $50\pm 17\%$ for the Mix group. Standard deviations were lower than in the S and L groups, due to better homogeneity. However, the viability rates of each group showed values not statistically different from control condition ($62\pm 15\%$), suggesting that the CP granules did not induce specific cell mortality.

3.3 Metabolic activity

The metabolic activity of the cells in each group was investigated once a week throughout the culture period, using the AlamarBlue® assay to evaluate mitochondrial reduction activity. The results, expressed with arbitrary units of the spectrofluorimeter, allowed us to estimate the cell viability and global proliferation of the rebuilt tissue.

In order to show the variation profiles during the culture, data were normalised by the fluorescence intensities recorded on day 7 (Fig. 6). We were so able to highlight changes in the metabolic activity throughout the month of culture and to perform a statistical study. Two main profiles were noticed : on the one hand, S and L groups showed increasing metabolic activity week after week, with the highest increase for the S group (statistically different from the control group at day 14, $p < 0.05$ and day 21, $p < 0.0001$). On the other hand, stable profiles were obtained for M and Mix groups, where metabolic activity appeared to remain constant, as it was noticed for the control too.

To explain these two profiles, untreated data were compiled to show the evolution in metabolic activity between the first (Y axis) and the last (X axis) measurements, ie between 7 and 28 days (Fig. 7). For each chamber, data points were included in elliptic domains where the major axis was defined using the linear regression line and the minor axis using the corresponding correlation coefficient (MS Excel software). It appeared that groups previously described with stable metabolic profiles (M and Mix groups) showed higher values of metabolic activity, except for the control group. Especially, most of the points of the M group were located near the diagonal, which means that metabolic activity was stable over time of culture, despite the variability.

3.4 Differentiation

Subclone 4 cells from the preosteoblastic MC3T3-E1 line are not able to produce ALP in conventional culture while they are not totally differentiated into osteoblast cells³⁸. This enzyme was therefore a suitable marker for investigating the influence of CP granules' size on cell differentiation after one month of culture in the chamber. In addition, staining for ALP activity on the whole sheet-like substitute allowed us to identify the high differentiation areas and to characterise the homogeneity of the tissue.

After staining, the red areas were representative of significant ALP activity, whereas CP granules themselves remained white (Fig. 8B). The control group showed a homogeneous medium staining (Fig. 8A). The differentiation was higher with M group where the presence of 800-200 μ m CP granules did not create heterogeneity compared to the control (Fig. 8E). A heterogeneous repartition of differentiated cells areas was obtained with the Mix group, in which ALP activity appeared at the edges of the substitute, where the cells were in contact with the chamber's edges (this area can be seen on fig. 8F). The S group's substitutes also showed heterogeneous areas, with red spots in various locations (Fig. 8C), but without

specific repartition. The L group (Fig. 8D) did not show any red areas, indicating that this size of CP granules did not promote the ALP activity.

We investigated then the stage of differentiation of M group's samples, which showed the best results in terms of ALP activity as well as mechanical cohesion. Using ELISA kit and Bradford assay, we were able to detect the production of OCN by the cells inside the culture chamber (485 ± 97 pg/ μ g/24h, Fig. 9). This showed that the substitute reached the latest stage of osteoblast differentiation⁴². The same range was found, without significant difference, for the control group (554 ± 120 pg/ μ g/24h, Fig. 9), which offered no cohesion as seen above.

3.5 Summary of biological characterisation

Macroscopic and microscopic observations, along with the biological behaviour described above, are summarised in Table 1. From all the groups, only the M group's substitutes remained cohesive and could be handled without breaking outside of the culture chamber (Fig. 2). The M group showed same levels of ALP activity, OCN production and viability as the control group, and the CP granules homogeneously covered over the whole surface of the monolayer by the cell tissue as well (Fig. 4D). Its stable metabolic activity between the first and fourth week was coherent with the high differentiated state of cells (Fig. 6, 7). Concerning the other groups, it was found that (1) the CP granules were not efficiently bound together by the cell tissue in the L group, because the cells grew inside the pores of the biggest granules (Fig. 3D), that (2) the Mix group's samples revealed heterogeneous ALP activity, with a gradient from the centre of the substitute (no differentiation) to its edges (medium differentiation) (Fig. 8D), and (3) the cell proliferation of the S group's samples was heterogeneous with spots of living cells (Fig. 4C).

Because the sheet-like substitute from the M group was cohesive and could be correctly handled, further studies were performed on this sample to investigate its mechanical behaviour and to establish the relationship between biological response, tissue microstructure and its macroscopic mechanical properties. **Despite results close to M group's ones for some biological properties, the other groups were dismissed from the mechanical characterization due to their consistent lack of cohesion.**

3.6 Mechanical behaviour

The sheet-like substitute covered a surface area of approximately 24 cm² (as seen in Fig. 1), so at least three strips (3 cm by 1.5 cm) of this tissue could be obtained from each culture chamber dedicated to the mechanical study. Conventional tensile tests were performed as described above (2.8) on 13 samples, leading to an analysis of the maximum stress as a function of strain, the mean breaking strain and the Young's modulus (Fig. 10). As the substitute developed from a single layer of 80-200 µm CP granules, a thickness of 200 µm was hypothesised to estimate the generated stress. The strips reached a maximum mean stress of **34±15 kPa for 28%** of strain. The mechanical behaviour appeared to be linear elastic and we could estimate a mean Young's modulus of **1.6±0.3 kPa** for the reconstructed tissue (Fig. 10).

In situ tensile tests were also performed under SEM, using the specific module Deben Microtest 300 mounted on the SEM device (Philips XL30 ESEM-FEG), in order to observe failure propagation and local damage mechanisms, and more specifically the interactions between the CP granules and bone tissue. This analysis led to the conclusion that the reconstructed tissue presented a strong adhesion between the cellular matrix and the CP granules. Hence, the extracellular matrix (ECM) was identified as the part of the tissue which

broke, without any displacement of the granules on either side of the tear (Fig. 11). There was no loosening of the granules within the substitute when tensed.

4. Discussion

Bone can be affected by a lot of diseases⁴³ or damage caused by accidents. As previously stated, tissue engineering is considered to be a promising method for improving healthcare and repairs⁴⁴. Our choices in terms of biomaterial, cell culture chamber, and tissue geometry were determined by the clinicians' expectations. Calcium phosphate ceramics are well known for their osteoconduction and osteoinduction properties¹⁰, especially the hydroxyapatite / tricalcium phosphate compounds⁴⁵. Used as a granule monolayer in a parallelepipedic cell culture chamber, they led to a hybrid bone substitute with a flexible geometry which could be relevant for several clinical fields, for instance maxillofacial surgery: it would be suitable for the **small** different complex shapes needed in this area. The culture chamber, able to be completely filled with medium and with an adequate surface, was specifically chosen to obtain such a geometry of rebuilt tissue. Moreover, when HA powders are used to fill bone defects, they are usually mixed with other matrices to avoid migration out of the implant region⁴⁶. Because the granules are bound together by the cell tissue, our process could avoid this need of an additional substance.

In this study, we did not expect to mimic the exact mechanical properties of native bone, but we aimed to obtain a cohesive substitute with a relevant shape for clinical issues. The biological and mechanical properties of the *in vitro*-built tissue with different mean diameters of CP granules used as a scaffold were therefore investigated to determine their optimal size. Biological and mechanical behaviours are strongly bound together, with mechanical stimuli playing an important role in bone cell development, known as

mechanotransduction⁴⁷. These induced biological modifications in turn modify the mechanical properties of bone with changes in the production of tissue components and cell alignment⁴⁷.

The goal of the global process was to meet clinicians' expectations for both aspects. Changing the mean diameter of the CP granules modified the macroporosity of the monolayer, known to be an important factor for cell attachment and cell viability⁴⁸⁻⁵¹. In particular, for HA/TCP particles, Mankani *et al* investigated *in vivo* bone formation for several size ranges⁵². Bone marrow stromal cells and HA/TCP particles were transplanted into mice for at least 4 weeks, and bone formation was estimated by semi-quantitative observations and histomorphometric methods. The behaviour of the different groups of rebuilt tissues appeared to be clearly different, showing a strong link between particle size and the properties of the tissue. We observed the same tendency: the M group (80 – 200 μm) was more suitable for the target shape of a sheet-like substitute than the other ones. It showed homogeneous cell tissue, with CP granules bound together by the ECM, and high and homogeneous differentiation. In addition, it was the only group providing these positive results for all of the biological properties investigated (Table 1), with results closed to control's ones in terms of viability, cell tissue morphology and OCN production, but with higher ALP activity and metabolic activity. After removal from the culture chamber and manipulations, M group's substitutes remained stable and cohesive. The lack of early development and/or homogeneous renewal noticed in the other groups led to a heterogeneous, non-differentiated and non-cohesive tissue. These results were coherent with the work of Mankani *et al*⁵², who used the same size range as the M group in order to obtain the greatest bone formation.

It should be highlighted that, for the M group, the homogeneous and high differentiation shown by the ALP staining test was achieved with only acid ascorbic, but without other traditional added factors (such as beta-glycerophosphate or dexamethasone^{53,54}) or specific differentiation medium, which is actually a common method^{2,8,15,18}. Our process, based on a parallelepipedic cell culture chamber and a CP granule monolayer, was thus efficient enough to promote the differentiation of the MC3T3 subclone 4 cells. It has been shown that the use of additional growth factors, such as transforming growth factor beta (TGF- β), bone morphogenetic protein (BMP) or fibroblast growth factors (FGF), may create complications during the clinical step⁵⁵, with the need for accurate and precautionary validation for the patient's safety⁵⁶. For these reasons, our factor-free process seems relevant for both industrial and clinical validations, with further studies needed concerning the use of fetal bovine serum.

Because the M group's substitutes were stable and cohesive, we were able to investigate the mechanical properties of the biohybrid tissue. The study of breaking mechanisms under SEM observations showed a complete break but no loosening of the CP granules, which were maintained together by the ECM on both sides of the failure. Quantitatively, it was difficult to estimate which range of maximal stress and strain needs to be targeted. Native bone tissue effectively shows high variability in its mechanical properties *in vivo*, especially due to the constant remodeling⁵⁷ and the composite nature of the tissue: Guilak *et al*⁵⁸ found a Young's modulus of 0.6kPa for the cartilage ECM, and Rho *et al*⁵⁹ estimated it at 10.4 ± 3.5 GPa for trabecular bone with a mechanical method. During the tensile tests on tissue strips, low stress values (mean stress < 35kPa) and estimated Young's modulus (< 2kPa) were measured. These results showed thus an improvement compared to the ECM alone and the M group's substitutes remained cohesive during manipulations, but these values

would not be adequate to avoid failures during clinical operations. For these reasons, the main goal of further studies should be the investigation of a new biomaterial with inherent mechanical resistance before cell growth. Sun *et al*⁶⁰ found a Young's modulus of around 15 MPa for a commercial skin tissue matrix. This value could thus be a target threshold for a substitute which would be resistant to handling by the clinician.

The mechanical cohesion showed that our sheet-like bone substitute could meet clinicians' expectations regarding the easy handling issue. The biological behaviour should be confirmed with future *in vivo* trials (subcutaneous then intramuscular implantations on mice to investigate mineralisation and bone integration). Recent *in vivo* studies about calcium phosphate granules used alone without *in vitro* tissue engineering step suggest a relevant response of our substitute^{61,62}.

5. Conclusion

In this study, we proposed a new bone tissue engineering method based on a CP granule monolayer and a specific cell culture chamber. We showed that our process, with an optimal mean diameter for the granules (80 – 200 µm), allowed us to generate a sheet-like bone substitute with properties close to clinicians' expectations, especially for maxillofacial applications, such as versatile geometry, flexibility, mechanical resistance and elastic behaviour, without the use of traditional growth factors. It has to be improved in order to guarantee better mechanical cohesion during surgery, but it is a promising construct for *in vivo* trials, which should confirm that it is an adaptable, autologous and factor-free alternative to grafts and biomaterials used alone.

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Author Disclosure Statement

No competing financial interests exist.

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