J Mater Sci: Mater Med (2008) 19:855–859
DOI 10.1007/s10856-007-3005-x

Biocompatibility of highly macroporous ceramic scaffolds: cell adhesion and morphology studies

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Received: 5 June 2006 / Accepted: 15 March 2007 / Published online: 1 August 2007

Abstract Hydroxyapatite porous scaffolds can be used for tissue engineering applications since they can serve as templates for cell adhesion, proliferation and ultimately for tissue repair. One way to address this issue is to evaluate the cell adhesion using several characterization techniques namely, cytotoxicity assays and cell visualization. On the other hand, when using highly macroporous scaffolds some techniques may not be adequate for evaluation, such as MTT. In this work, cytotoxicity assay (MTS), scanning electron microscopy (SEM) and Confocal laser scanning microscopy (CLSM) were used to evaluate cell adhesion in highly macroporous hydroxyapatite scaffolds. It was possible to observe that some techniques were not suitable to evaluate cell adhesion. In addition, it was shown that for this kind of scaffolds, confocal laser scanning microscopy is a powerful tool for cell adhesion and proliferation evaluation.

Introduction

Tissue engineering is an interdisciplinary research field that seeks the development of techniques/materials to repair and/or reconstruct damaged or lost tissue and organs.

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Porous materials offer a promising strategy since they can act as templates for cell adhesion, proliferation and ultimately for tissue formation.

However, to serve as a scaffold, the material must follow certain criteria: be biocompatible, osteoconductive, osteointegrative, and have enough mechanical strength to provide structural support during bone growth and remodelling [1].

In addition, scaffolds can serve as delivery vehicles for cytokines such as bone morphogenetic proteins (BMPs), transforming growth factors (TGFs) that can transform recruited precursor cells from the host into bone matrix producing cells, thus providing osteoconduction.

New centers for bone formation such as osteoblasts and mesenchymal cells that have the potential to commit to an osteoblastic lineage can be established when seeding the scaffolds before implantation with cells, leading to enhanced osteogenesis.

Combining scaffolds, cytokines and cells to generate ex vivo tissue-engineered constructs is the next step in tissue engineering since it may provide more effective bone regeneration in vivo, when compared to biomaterial matrices alone [2]. The first question to be raised is how to achieve an optimal scaffold with adequate porosity.

Porosity is defined as the percentage of void space in a solid and it influences the bone ingrowth, since it allows migration and proliferation of osteoblasts and mesenchymal cells, as well as vascularization [3, 4].

The minimum pore size required to regenerate mineralized tissue is generally considered to be ~100 μm. However, pore sizes greater than 300 μm were observed to have a greater penetration of mineralised tissue in comparison with smaller pore sizes.

At pore sizes of 75 μm, hardly any mineralised tissue is found within the scaffold. It is believed that for smaller

pore sizes the penetration of neovascularisation, and nutrient supply, to the growing cells is slowed down [1].

Without a doubt there are several critera in the design of scaffolds for tissue engineering that need to be obeyed, such as: highly porous structure to support cell attachment proliferation and extra-cellular matrix production; an interconnected pore network to promote nutrient and waste exchange; a biocompatible and bioresorbable substrate with controllable degradation rates. This will lead to a suitable surface chemistry for cell attachment, proliferation, and differentiation; mechanical properties to support, or equal, those of the tissues at the site of implantation and a reproducible architecture of clinically relevant size and shape.

Nevertheless, the scaffolds should mimic cancellous bone morphology, structure and function in order to optimize integration in the surrounding tissue.

Bone is a structure composed of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) crystals deposited within an organic matrix (~95% is type I collagen). Hydroxyapatite, has been used in medicine and dentistry for over 20 years and is biocompatible and osteoconductive, and has excellent chemical and biological affinity with bony tissues [5–9].

In this work, highly porous hydroxyapatite samples are subjected to an in vitro evaluation to follow the cells behaviour in such environment. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) are useful visualisation techniques of cell adhesion and proliferation. In this work, these techniques will be applied and their adequacy to three-dimensional materials will be evaluated.

Materials and methods

The scaffolds were prepared using polyurethane sponges kindly provided by Recticel (Belgium). These polyurethane sponges were impregnated with ceramic slurry constituted by water, hydroxyapatite and a tensioactive. The ceramic slurry was prepared using several HA: Water: Tensioactive ratios, being the best ratio 6:4:0.2. The polyurethane sponge was squeezed to remove slurry excess and submitted to the following sintering cycle: heating at 1 °C/min followed by a 1 h stage at 600 °C; heating at 4 °C/min followed by another stage of 1 h at 1350 °C and then the samples were cooled inside the oven.

Afterwards, the sponges were cut with a sharp razor to the desired shape [10–12].

Cell culture procedures

MG63 cells, derived from human osteosarcoma, express a number of features characteristic of osteoblasts and were used in these experiments. They were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air, in 75 cm^2, flasks containing 10 mL of alpha minimum essential medium (α-MEM) (Gibco), 10% foetal calf serum (Gibco), 0.5% gentamicin (Gibco) and 1% fungizone (Gibco). The medium was changed every third day.

The scaffolds were autoclaved (120 °C, 20 min), placed in non-treated 96-well plates (14 mg/well, ca. 1 cm^2 surface area) to avoid cell adhesion to the bottom of the wells.

The cells were seeded at 10^4 cells on the top of each scaffold and incubated at 37 °C overnight to allow cell adhesion. Afterwards, fresh medium was added until it reached a final volume of 150 μL per well.

The MG63 osteoblasts cells were cultured onto hydroxyapatite scaffolds for periods of 1, 3 and 7 days. Control cultures were performed on tissue culture polystyrene under the same conditions as the scaffolds.

At each time point, the samples were treated and cell adhesion, distribution and morphology were assessed by cytotoxicity assay (MTS), scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM).

Data are presented as the average of five replicates (mean ± standard deviation) and duplicate culture experiments were performed.

All assays are described in detail below.

MTS cytotoxicity assay

The MTS cytotoxicity assay (Cell Titer 96 Aq Non-Radioactive Cell Assay, Promega), an alternative method of the widely used MTT assay, was used to screen the viability of the cells cultured on the hydroxyapatite scaffolds. The MTT test, in which the reaction product is water insoluble formazan, could not be used because the formed formazan was strongly attached to the surface of the scaffolds being impossible to obtain a correct measurement. In the MTS test, using a novel tetrazolium compound, the reaction product formed was soluble in the culture medium.

Briefly, the MTS was added to the wells and incubated for 3 h; the blank used was culture medium with MTS and absorbance was measured at 492 nm in an ELISA 96 well-plate reader. The results obtained were treated and represented by their mean value and standard deviation parameters.

SEM

Hydroxyapatite scaffolds were washed twice with PBS and fixed in 1.5% v/v glutaraldehyde in 0.14 M sodium cacodylate (pH 7.4) for 30 min at room temperature.

Dehydration was performed by sequential immersion in serial diluted ethanol solutions of 50, 60, 70, 80, 90, and 100% v/v. The samples were kept in absolute alcohol and taken to critical point, using CO_2. The samples were then sputtered with a thin gold film for SEM analysis.

CLSM

Hydroxyapatite scaffolds were washed twice with PBS, fixed in 4% v/v formaldehyde (methanol-free; Polyscience) for 15 min, permeabilized with 0.1% v/v Triton ×-100 for 5 min, and incubated in 10 mg/mL bovine serum albumin and 100 g/mL RNAse for 45 min at room temperature. F-actin filaments were stained with Alexafluor-conjugated phalloidin (Molecular Probes) for 20 min and nuclei were counterstained with 10 g/mL propidium iodide (Sigma) for 10 min. Finally, samples were washed with PBS and mounted in Vectashield®. CLSM images were acquired on a BioRad MRC 600 microscope and the images were treated with Leica software.

Results

MTS cytotoxicity assay

The cytotoxicity/cell viability of the hydroxyapatite sponges was assessed by the MTS cytotoxicity assay (Fig. 1).

By analyzing the results obtained, it can be observed that the hydroxyapatite scaffolds are biocompatible and that the cells adhere and proliferate well when compared to the control.

In addition, neutral red assay was performed and also revealed that the cells were able to grow and proliferate quite well. These data was not shown, since it does not give any additional information [13].

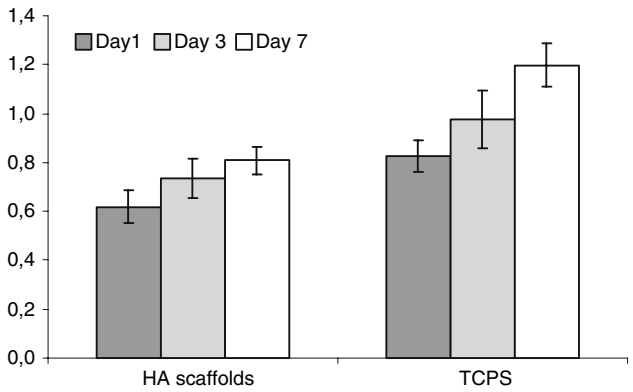

Fig. 1 MTS assay performed on hydroxyapatite scaffolds (HA) and tissue culture polystyrene (TCPS) samples cultured with MG63 cells ($n = 5$)

SEM and CLSM analysis

The SEM micrographs of the hydroxyapatite scaffolds can be seen in Fig. 2.

Analysing the SEM micrographs it is possible to observe the presence of macropores (Fig. 2A and B) and micropores (Fig. 2C and D) as well as interconnected porosity (Fig. 2A). This kind of structure provides favourable conditions for cell adhesion and proliferation since it offers sites for cell adhesion as well as interconnectivity necessary for the nutrient and macromolecules transport.

This matrix is characterized by macropores which average size is higher than 100 μm and micropores with an average size of 1 μm. In addition, the macroporosity was determined in a previous work [12] by the liquid displacement method; leading to porosity values of 67%.

In Fig. 3, it may be seen in detail, the cell adhesion and morphology to the hydroxyapatite in day 1, 3 and 7 of cell culture.

On the third day of cell culture, the cells are completely spreaded along the available surface of the hydroxyapatite scaffold inner pores.

Also, the cells philopodia are strongly attached to several points of the pores as it can be observed in Fig. 3B and C.

SEM is very helpful technique to observe cell morphology and distribution along the scaffold surface and its inner pores. In the case of the samples without cells the scaffolds were well characterized by SEM. It was possible to observe the microporosity in detail and also the pores interconnectivity, showing that this technique is adequate for such studies.

However, in the case of the samples with cells, one issue that was difficult to address was the marking of the samples.

When preparing the samples for cell culture it was possible to detect that the sample marking was quite difficult to achieve due to the porosity of the samples. Several methods were tested bearing in mind that for cell culture, extra care should be provided due to the sensitivity of the cells to some components. In addition to the difficulty to identify the cell seeded side, complications in determining the correct side to perform the SEM analysis where raised.

On the other hand, this problem was overcomed by the use of confocal laser scanning microscopy. With this technique, it was possible to observe the cell adhesion, proliferation and distribution along the surface and inner pores of the scaffold without the need of marking the samples side.

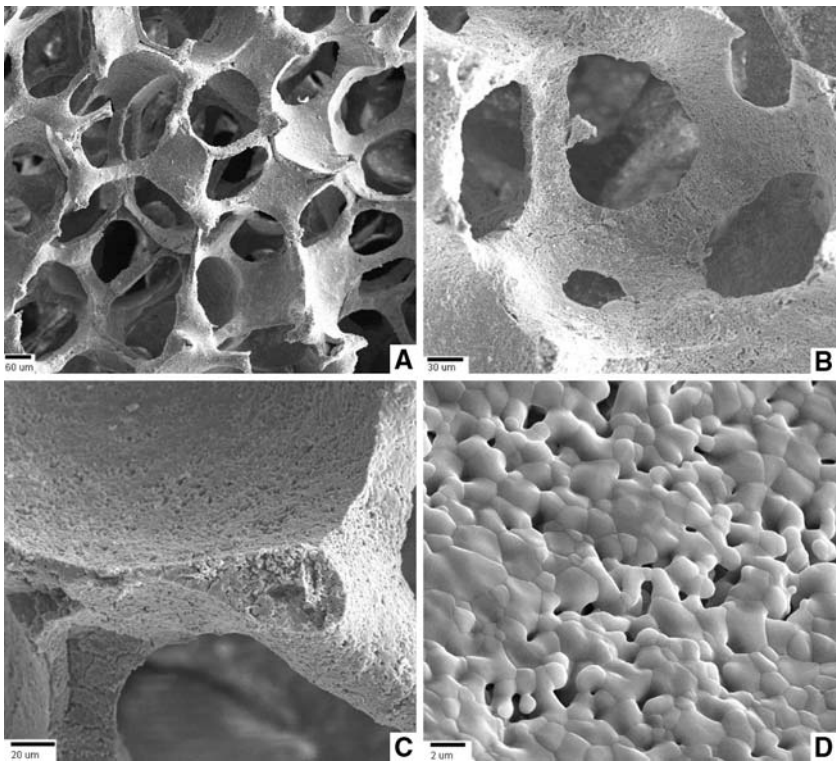

Fig. 2 SEM micrographs of the hydroxyapatite scaffold at different magnifications: 100× (**A**), 270× (**B**), 1500× (**C**) and 4000× (**D**)

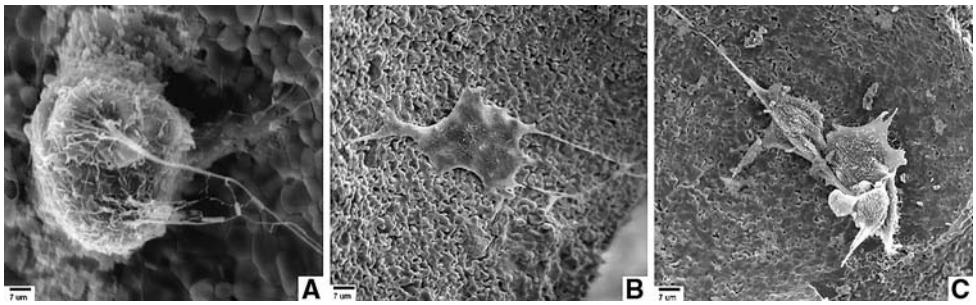

Fig. 3 SEM micrographs of the hydroxyapatite scaffold seeded with MG63 cells at day 1 (**A**), day 3 (**B**) and day 7 (**C**). Detail of cell attached to an inner pore of the scaffold

The confocal laser scanning microscopy images of scaffolds seeded with MG 63 cells can be visualised in Figs. 4 and 5.

With this technique, it may be seen that the cells in the first day of cell culture are already proliferating through the inner pores of the scaffold. In the next days of culture, the first layers of the scaffold are completely full of cells attached to their pore walls (Fig. 4).

With the software it was possible to edit the previous images and observe in more detail the distribution of cells

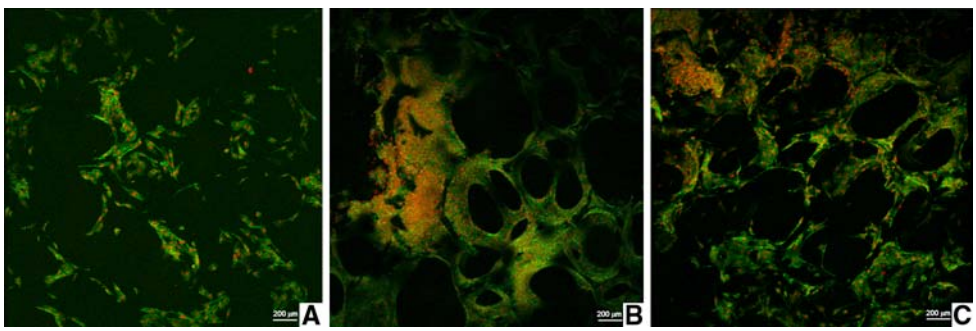

Fig. 4 CLSM of the hydroxyapatite scaffolds on day 1 (**A**), day 3 (**B**) and day 7 (**C**). The cells actin filaments are stained with green colour and the nuclei counterstained with red colour

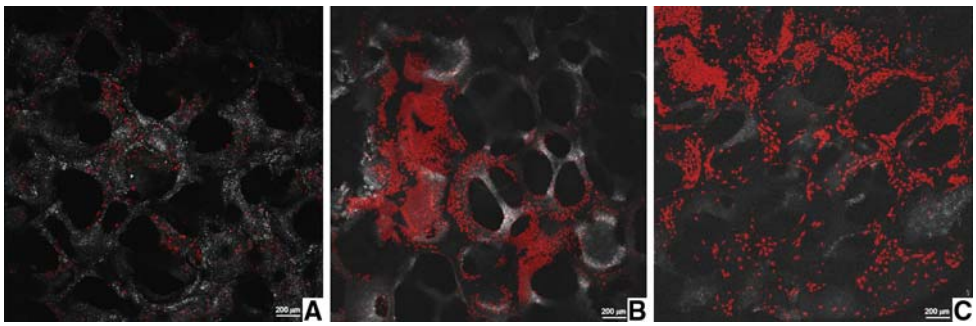

Fig. 5 CLSM of the hydroxyapatite scaffolds on day 1 (**A**), day 3 (**B**) and day 7(**C**)

along the scaffold surface (Fig. 5). As a result, the nuclei appear stained in red and the matrix in grey allowing a more accurate observation.

On day 1, the cells are spreaded along the pores and on day 3 they are starting to concentrate in a location forming a cell cluster. On day 7 it is possible to see that the proliferation increased, corroborating the previous data presented.

Conclusions

In this work it was possible to obtain structures that were highly macroporous as well as microporous.

Furthermore, these hydroxyapatite scaffolds also possess interconnected porosity, which is a fundamental requirement for bone replacement substitutes.

The method used provides reproducible matrices that can be used also in other fields such as drug or protein delivery.

The results also demonstrate that the matrix is not cytotoxic and that the cells strongly adhere to the substrate in the first hours of cell/ substrate contact. In addition, MTS assays reveal that the cells were able to grow and proliferate quite quickly.

It was demonstrated that SEM is a very good technique to assess the scaffolds porosity and surface. However, it was not the best technique to evaluate the cells proliferation and distribution. When working with 3D materials and if there is the need to visualise specific subjects, some techniques do not provide enough information and need to be complimented, as this was the case. CLSM is clearly a more appropriated tool to visualize the cells distribution and proliferation along the surface and subsequent inner pores.

References

1. A. C. JONES, *Biomaterials* **25** (2004) 4947
2. V. KARAGGEORGIOU, *Biomaterials* **27** (2005) 5474
3. C. A. LEON and Y LEON, *Adv. Colloid. Interface Sci.* **76–77** (1998) 341
4. Y. KUBOKI, H. TAKITA, D. KOBAYASHI, E. TSURUGA, M. INOUE and M. MURATA et al. *J. Biomed. Mater. Res.* **39**(2) (1998) 190
5. U. HEISE, J. F. OSBORN and F. DUWE, *Int. Orthop.* **14** (1990) 329
6. R. W. BUCHOLZ, A. CARLTON and R. E. HOLMES, *Orthop. Clin. North. Am.* **18** (1987) 323
7. P. A. HILL, *Br. J. Orthod.* **25** (1998) 101
8. R. B. MARTIN, *Mater. Sci. Forum* **293** (1999) 5
9. C. R. NUNES, S. J. SIMSKE, R. SACHDEVA and L. M. WOLFORD, *J. Biomed. Mater. Res.* **36** (1997) 560
10. A. C. QUEIROZ, S. TEIXEIRA, F. J. MONTEIRO and J. D. SANTOS, *Key Eng. Mater.* **254–256** (2004) 997
11. A. C. QUEIROZ, S. TEIXEIRA, F. J. MONTEIRO and J. D. SANTOS, *Mater. Sci. Forum*, **455–456** (2004) 358
12. A. C. QUEIROZ, J. D. SANTOS, F. J. MONTEIRO and H. A. POROUS, *Key Eng. Mater.* **284–286** (2005)
13. S. M. TEIXEIRA, A. C. QUEIROZ, F. J. MONTEIRO and M. P. FERRAZ, "In vitro analysis of macroporous ceramic matrices: preliminary results." *19th European Conference on Biomaterials*. PP-P173; Naples, Italy, Sept **11–15** (2005)