EVALUACIÓN DEL POTENCIAL BIOMÉDICO DE NUEVOS BIOMATERIALES PARA APLICACIÓN EN CIRUGÍA ORTOPÉDICA Y TRAUMATOLOGÍA

BIOMEDICAL POTENTIAL EVALUATION OF NEW BIOMATERIALS FOR APPLICATION IN ORTHOPEDIC SURGERY AND TRAUMATOLOGY.

by

Daniel Amat Trujillo

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AUTOR: Daniel Amat Trujillo

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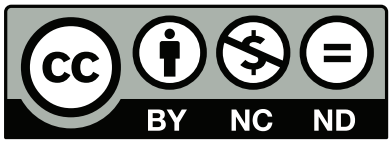

ABSTRACT

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BIOMEDICAL POTENTIAL EVALUATION OF NEW BIOMATERIALS FOR APPLICATION IN ORTHOPEDIC SURGERY AND TRAUMATOLOGY

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Under the Supervision of Drs. Leonor Santos-Ruiz and Mª José Mora Huzman

ABSTRACT

Over the last decades, bone disorders have steeped up due to the aging of the population associated with lifestyle risk factors such as obesity and reduced physical activity. Besides, among the young, healthy population the prevalence of complex fractures has also increased due to factors like locomotive accidents or extreme sports. Clinical situations related to complex fractures, non-union fractures; osteoporosis and fragility fracture; osteoarthritis; rheumatoid arthritis, and so on, demand the development of therapeutic elements with the ability to induce tissue regeneration, or to replace damaged or loss bone and restore organ function. Such is the objective of biomedical research. Within this, tissue engineering has emerged as a promising alternative to current bone augmentation and bone replacement therapies, which include the use of autografts and allografts. The aim of bone tissue engineering is to produce *ex vivo* implantable osseous tissue that can replace missing tissue or stimulate native bone

regeneration. These implants would consist of the right combination of cells, osteoinductive molecules and biocompatible materials.

Biomaterials are demanded in Orthopaedics for either assisting bone repair or replacing bone function. The former application is fulfilled by bone graft substitutes, characterized by their resorbability; while the latter is fulfilled by prosthetic implants made up of metals. In any case the ideal biomaterial for bone repair should mimic the bone extracellular matrix as to its physical, mechanical and chemical properties.

The aim of the present work was to evaluate the biomedical potential, in the context of bone therapeutic repair, of different types of materials: the mesoporous silica-based ceramics SBA-15 and HA-SBA-15; the ICIE and ICIE-derived Nitru bioglasses; and a macroporous Ti_6Al_4V alloy sintered by electron beam melting (EBM) into 3D macroporous scaffolds. Resorbable materials (ceramics and bioglasses) were evaluated *in vitro* and *in vivo* to assess for their biocompatible and osteconductive properties. *In vitro* studies were meant to evaluate the cellular response to the materials, and included culture and osteoinduction of osteoblastic and bone marrow-derived mesenchymal stem cells (BM-MSC) on the materials. *In vivo* studies looked further into the biocompatibility and osteoconductivity of the materials and included ectopic and orthotopic implantation of the materials in adult rats, followed by histopathological analysis of the implants and their surrounding host's tissues. Although known to be biocompatible, the Ti_6Al_4V alloy scaffolds were evaluated *in vitro* to test out if EBM sintering renders scaffolds with good superficial properties, i.e., surfaces where cells can attach, grow and differentiate. Osseointegration of metal prostheses requires not only mechanical but also tissular integration which, in turn, requires a favourable cell response to the prosthesis surface. In this case, osteoblastic and BM-MSC cells were

used to evaluate osteoconductivity, and endothelial cells were used, alone and cocultured with osteoblasts, were used to evaluate angioconductivity.

I. Silica-based mesoporous ceramics: SBA-15 and HA-SBA-15

Particulate mesoporous silica-based ceramics SBA-15 and HA-SBA-15 were manufactured by Dr. Díaz-Cuenca at *Instituto de Ciencia de Materiales* (Sevilla, Spain). Two kind of particulate ceramics were used on this work: SBA-15 and HA-SBA-15. The main difference between them is that HA-SBA-15 contains hydroxyapatite crystals grown onto the SBA-15 matrix in order to combine the properties of silica-based ceramics with those derived from calcium phosphates.

Osteoblastic cells and BM-MSC cells, either cell lines or cells from primary cultures, were used to evaluate the biocompatibility and osteoconductivity of SBA-15 and HA-SBA-15 materials. To study the interaction of cells with the particulate materials flat, standard 2D cell culture and 3D micromass culture were used. Results showed that cells could grow in the presence of the materials, but there seemed to exist a certain cytotoxic effect of the particles. Culture on OptiCell^{TM} chambers showed that at a first stage, cells attached to the SBA-15 and HA-SBA-15 particles forming aggregates, but afterwards all particles disappeared from the culture coinciding with a certain decrease in cell density. Actin cytoskeleton visualization by phalloidin-TRICT staining and histochemical staining of alkaline phosphatase revealed altered cellular morphology, thus confirming a certain deleterious effect of particles on the cells.

To further characterize the effect of particulate SBA-15 and HA-SBA-15, nuclei manual counting and methylene blue assay were used to measure cell population growth in the presence of growing concentrations of the particulate materials. No significant

differences were found as to culture growth between controls with no particles and cultures containing up to 0.1 mg/ml of particles in the culture medium. A concentration-dependent decrease in cell growth was found for particle concentrations above that, more remarkable for HA-SBA-15.

To figure out if the cytotoxic effect was due to dissolution products from the SBA-15 and HA-SBA-15 particles, culture media were conditioned by one week incubation with 0.8 mg/ml of SBA-15 or HA-SBA-15, and these conditioned media were used to culture osteoblastic and BM-MSC cells. Results showed no effect on cell growth, suggesting cytotoxicity depended on the direct interaction between the cells and the SBA-15 and HA-SBA-15 particles. Time-lapse confocal microscopy suggested particles were being internalized by the cells. Culture in the presence of Celltracker Green^{TM}-loaded particles and confocal imaging of the cells in the z-axis confirmed internalization. Upregulation of *Lamp2* gene expression in cells cultured with particulate materials suggested a possible relation between particle internalization and cell death.

In order to elucidate if cell cytotoxicity depended on particle size, cells were cultured in the presence of materials with particle sizes below and above 45 nm (maximum size = 100 nm). Methylene blue assay showed no significant difference as to cell growth, and time-lapse microscopy showed no difference as to cell behaviour.

Altogether, these results suggested particulate materials SBA-15 and HA-SBA-15 might have a potential in intracellular delivery of drugs or genes if used at low doses, but are not appropriate as cell carriers.

As the particulate format of SBA-15 and HA-SBA-15 showed a potential in delivering molecules but not cells, they were not suitable for bone tissue engineering. To overcome this limitation, SBA-15 was sintered into monolithic scaffolds by Dr. Díaz-Cuenca's group at ICMS-CSIC (Seville-Spain). Sintered scaffolds were called "compact" and

"porous" according to their sintering method and inner structure. The former were solid while the latter contained not-inteconnected micro and macroporosity.

In vitro studies were carried out seeding mouse osteoblastic cells and rat BM-MSCs on the sintered materials to assess cell adhesion and spreading on them. Cell staining with CellTracker Green™ allowed visualization and monitoring of cells grown onto the materials over time. Results showed that cells could attach and populate SBA-15 sintered materials, preferring the porous and irregular inner surfaces of the scaffolds to their outer, smooth surfaces. SEM imaging confirmed cells had reached confluency on the surface of the materials, confirming *in vitro* biocompatibility.

Then the sintered monolithic SBA-15 scaffolds were tested *in vivo*, implanting an orthotopic location between the calvaria and the periosteum of adult rats. Implants were recovered at different time points, fixed, embedded, sectioned and stained with haematoxilin-eosin, picrosirius-haematoxilin and Masson-Goldner trichrome. Histopathological analysis revealed a heavy inflammatory response of the host tissues against both compact and porous SBA-15 monoliths. At early time points, polymorphonuclear cells and lymphocytes surrounded the implants and, in the case of porous SBA-15, filled the poruses of the material. Later, the lymphoid tissue was substituted by fibroblastic cells, and a capsule of dense fibres was visible around the implant, forming a fibrotic cyst. On these late sampling times, the materials had been partially resorbed being porous SBA-15, as expected, the more resorbed of the two.

In order to check the ability of sintered porous SBA-15 to deliver osteogenic molecules, the monolithic scaffolds were loaded with BMP2 and implanted ectopically between the dorsal musculature of adult rats. An already well-known bone substitute, hidroxiapatite (HA), was used as control material. Implants were dissected out after 7 and 21 days and processed for histopathological analysis as previously described. In this case, porous

SBA-15 implants also elicited host rejection, but BMP2-loaded SBA-15 implants did not. The material appeared surrounded by fibroblasctic cells and no fibrotic cyst formation occurred. This different performance of BMP-loaded and unloaded materials confirmed the ability of sintered SBA-15 to deliver BMP2, although it did not perform as well as the HA controls, where some ossification nodules could be found when loaded with the same dose of BMP-2.

In conclusion, sintered SBA-15 material, although biocompatible *in vitro*, lacks the needed *in vivo* biocompatibility pursued in a bone substitute or in a tissue-engineering scaffold. However, it is resorbable *in vivo* and can be functionalized with osteinductive molecules as BMP-2. These characteristics encourage its modification by material researchers in order to make it more biocompatible.

II. ICIE and ICIE-derived nitrided bioglass

The goal of this chapter was to evaluate the biocompatibility of a nitrided ICIE-derived bioglass, named Nitru. ICIE, ICIE-SBN and Nitru bioglasses were provided by Dr. Orgaz's group, at Instituto de Cerámica y Vidrio de Madrid (ICV-CSIC). The murine osteoprogenitor cell line MC3T3-E1, a well known *in vitro* model of osteogenic differentiation, was used to *in vitro* evaluate the biocompatibility and osteoconductivity of the bioglass scaffolds. MC3T3-E1 cells were seeded in the hydrophilic OH (ICIE and ICIE-SBN) and NH_2-functionalized bioglasses (Nitru), and kept in culture for seven days. Nuclei were labelled with Hoechst 33352 and monitored daily in a dissecting microscope. Photographs were taken at different time points to assess cell presence, viability and distribution within the glass surfaces. Cell proliferation within the scaffolds was assessed by MTT assay. Scanning electron microscopy was used to analyse cell morphology and cell-bioglass contacts. Progression of osteogenic

differentiation was assessed by alkaline phosphatase activity assay (pNPP substrate), and by alkaline phosphatase staining with *Fast Red Violet* LP/Naftol AS-BI phosphate. Cells attached to and grew on all three bioglass types, and their morphology was indicative of a good interaction with the bioglasses surface. MTT assay confirmed cell growth an all the bioglasses, being this growth significantly higher in the Nitru bioglass. ALP assays confirmed quantitatively and qualitatively that osseous differentiation occurred upon proper stimulation. Interestingly, cells seeded on ICIE bioglass needed a period of adaptation to the substrate prior to being able to respond to the differentiating stimulus. This adaptation was not needed in cells seeded in Nitru bioglass, suggesting a better relationship of the cells with the nitrided surface.

To characterize the *in vivo* performance of Nitru bioglasses, chips of this material were implanted within the dorsal muscles of adult rats and the implants were recovered after 7 and 21 days. A set of chips was implanted as-sinthesized, and a second set was impregnated in BMP2 (suboptimal dose) prior to implantation. Histopathological analysis showed that both nude and BMP2-impregnated Nitru chips were biocompatible and ressorbable, as host cells were found to have entered and populated the materials. All the bioglasses pores were filled with cells, extracellular matrix and blood vessels. The tissue inside the bioglass pores changed with time to a less cellular, and more fibrous, likely more mature connective tissue type. Although no bone was formed in any of the implants (BMP2 was at suboptimal concentration), population and differentiation were more advanced in BMP2-impregnated Nitru chips, confirming that Nitru bioglasses can retain and deliver BMP2.

In conclusion, the new nitrided ICIE-derived bioglass has shown to be biocompatible and osteoconductive both *in vitro* and *in vivo*, although not osteoinductive *per se*. Nevertheless, it can be functionalized with osteoinductive molecules like BMP2. These

data suggest nitrided ICIE-derived bioglass may become an acceptable therapeutic option for bone tissue repair, either as a bone substitute or as tissue engineering scaffold, in combination with stem cells and osteogenic molecules.

III. Ti_6Al_4V alloy sintered by Electron Beam Melting (Ti-EBM)

Titanium scaffolds were designed and manufactured by Donato Monopoli's group, at the Department of Mechanical Engineering of *Instituto Tecnológico de Canarias* (ITC). Our goal was to confirm the superficial properties of the EBM-sintered scaffolds were appropriate to favour the osseointegration of such scaffolds, i.e., cell response to EBM-sintered titanium was favourable so that the different cell types present in bone would be able to adhere, spread and differentiate on the EBM-Ti surface. For this purpose, osteoblastic cells, BM-MSCs and endothelial cells were cultured, alone or in coculture, on the titanium scaffolds, and their behaviour monitored.

Both flat perforated discs and 3D macroporous cylinders were used. Osteoblastic and BM-MSC were seeded on the titanium and monitored by Hoescht 33342 nuclei staining and periodic observation and imaging. Cells attached to and grew on the Ti-EBM scaffolds, and with time they bridged the pores of the scaffold, as if trying to fill in these pores. Cell growth was confirmed by MTT assay, and cell phenotype was further observed by SEM, confirming that cells had spread all over the titanium surface, covering it up completely. Osseous differentiation was assessed by alkaline phosphatase activity, and by inmunodetection of collagen type I and bone sialoprotein.

In order to evaluate the potential of Ti-EBM to become vascularised, endothelial cells (HuVEC and HDMEC) were cultured on flat and 3D Ti-EBM scaffolds, either nude or fibronectin-coated. Endothelial cells attached to both coated and nude titanium

scaffolds, and grew on it, as confirmed by Calcein AM staining and expression of the endothelial marker PECAM-1. To evaluate if endothelial cells adapted well to the Ti-EBM surface, E-Selectin expression was studied. Endothelial cells were seeded onto nude and fibronectin-coated Ti-EBM scaffolds and E-selectin expression was detected by immunofluorescence. E-selectin expression was observed only in positive controls, which had been exposed to *E. coli*'s LPS, but it was not observed on the endothelial cells in contact with Ti-EBM scaffold. Therefore, this material does not elicit an inflammatory response in endothelial cells.

To *in vitro* mimic a bone environment cocultures of osteoblastic and endothelial cells were carried out. Ti-EBM scaffolds were seeded with human osteoblastic and endothelial cells and the progression of the cultures followed up by ALP fluorescent staining and PECAM-1 immunofluorescence to check osteoblastic and endothelial differentiation, respectively. Osteoblastic cells grew and spread over the titanium forming a cell layer to which endothelial cells attached. Endothelial cells grew over the osteoblastic layer for three weeks, forming a layer themselves, and afterwards rearranged themselves into tubes. Tube formation coincided with an upregulation of VEGF protein secretion, as confirmed by ELISA measures of the culture medium.

In summary, titanium scaffolds sintered by EBM allow adhesion, growth and differentiation of osteoblastic, BM-MSC and endothelial cells. These osteoconductive and angioconductive properties would not only favour osseointegration of prostheses made up of EBM-Ti, they also open up the possibility of implanting the prosthesis in combination with cells to promote prosthesis interaction with host bone, and to stimulate bone formation inside the scaffold macropores, to further accelerate and increase prosthesis integration.

CONCLUSIONS / CONCLUSIONES

Conclusions

1- SBA-15 and HA-SBA-15 particulate materials exert/produce/have a concentration-dependent a cytotoxic effec on osteoblastic cells and BM-MSC. Cytotoxicity is higher for HA-SBA-15.

2- Cytotoxic effect of SBA-15 and HA-SBA-15 does not depend of dissolution products released by the particles, but on the direct interaction between the cells and the material

3- Cells internalize SBA-15 and HA-SBA-15 particles. It is possible, though, that other factos add up to the cytotoxic effects of these particulate materials.

4- Sintered SBA-15 materials, either as compact or porous monoliths, are biocompatible in vitro (they can be populated by osteoblastic cells).

5- Sintered SBA-15 materials, either as compact or porous monoliths, are resorbable in vivo, being in porous form the faster resorbed.

6- Sintered SBA-15 materials, either as compact or porous monoliths, are not biocompatible in vivo, as they trigger a foreign-body rejection reaction of the host's tissues that involves granuloma and fibrous-cyst formation .

7- SBA-15 sintered into porous monoliths can adsorbe and deliver active BMP2. Impregnation of SBA-15 with BMP2 makes it biocompatible (foreign body rejection stops to occur), but it does not stimulate bone formation at the implant site.

8- ICIE bioglasses (ICIE-16, ICIE-SBN and Nitru) are biocompatible and osteoconductive in vitro, being Nitru the most favorable to cell growth and differentiation.

9- Nitrided bioglass (Nitru) is biocompatible and resorbable in vivo, and it can adsorb and deliver biologically active BMP2.

10- Ti_6Al_4V ELI alloy sintered by Electron Beam Melting are osteoconductive and angioconductive in vitro.