Novel bioassay to evaluate biocompatibility of bioactive glass scaffolds for tissue engineering

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The aim of the present study was to investigate a novel *ex ovo* bioassay for the first time using the chick embryo chorioallantoic membrane (CAM) for testing tissue engineering bioceramic scaffolds. Bioglass based scaffolds with porosity in the range of 90–95% were fabricated using the foam replica technique and sintering at 1100°C for 1 h. Scaffolds ($5 \times 5 \times 2$ mm^3) were placed on the CAM at 10 days of total incubation. The embryos were killed 5 days after implantation. The scaffolds and CAM were explanted, fixed in formalin solution and processed for embedding in methyl methacrylate. Histological analysis using ground sections showed that the scaffolds were surrounded by CAM. There was no occurrence of macrophages or related inflammatory cells. The results described in this paper indicate that the developed bioassay is an appropriate approach as an alternative to conventional animal models to evaluate the biocompatibility of scaffold biomaterials for tissue engineering and regenerative medicine.

Keywords: Tissue engineering, Bioglass, Biocompatibility, Scaffolds, Chick embryo

Introduction

The need for new *in vivo* experimental models as alternatives to the experimental animal models currently in use, has recently been emphasised.[1] In this context, several authors have proposed the use of chick embryos as an experimental model to study the biocompatibility of materials, given its advantages over mammalian systems. Among these advantages are universal availability of eggs, relatively low cost, speedy development and rapid growth of the embryos, easy handling, and compliance with the replacement, reduction and refinement (3Rs) principles on using alternative methods to animal experiments in biomaterials and tissue engineering research.[2–11]

The chick embryo chorioallantoic membrane (CAM) assay was originally conceived as an alternative *in vivo* method for angiogenesis, toxicity and irritation studies.[6,7] It has been also suggested that the CAM assay has potential for general evaluation of tissue responses to biomaterials and implants since it provides a natural environment for growing blood vessels and all the components of the complex host interactions.[2–11] The CAM assay involves a straightforward procedure of either windowing the eggshell to have access to the CAM *in ovo* or preparation of shell less cultures of chick embryos *ex ovo*, which increases the CAM area available for multiple tests on a single CAM. This technique involves the transfer of the entire egg contents to a container simulating the eggshell after 72 h of *in ovo* incubation.[12,13]

Bone tissue engineering requires a suitable scaffold material, which has to be both bioactive and bioresorbable and fulfill a number of other prerequisites, including suitable mechanical strength and stiffness, interconnected pore structure, the ability to enable cell attachment and cell proliferation as well as ease of manufacture.[14,15] In recent studies,[16,17] the well known and cost effective foam replica technique has been adapted to fabricate bioactive glass–ceramic scaffolds based on the 45S5 Bioglass composition.[18] These scaffolds fulfill all essential requirements for bone tissue engineering applications and they have been shown to support osteoblast-like cells (MG 63) attachment as well as promote cell proliferation.[17] Moreover the mechanical competence and bioactivity of the scaffolds have been confirmed, which depend on the phase transformation of the crystalline phase ($Na_2Ca_2Si_3O_9$) present in the glass–ceramic struts into a biodegradable amorphous calcium phosphate phase during cell culture, in simulated body fluid or when the scaffolds are immersed in cell free culture medium.[16,17] The use of Bioglass to fabricate scaffolds has the additional advantage of

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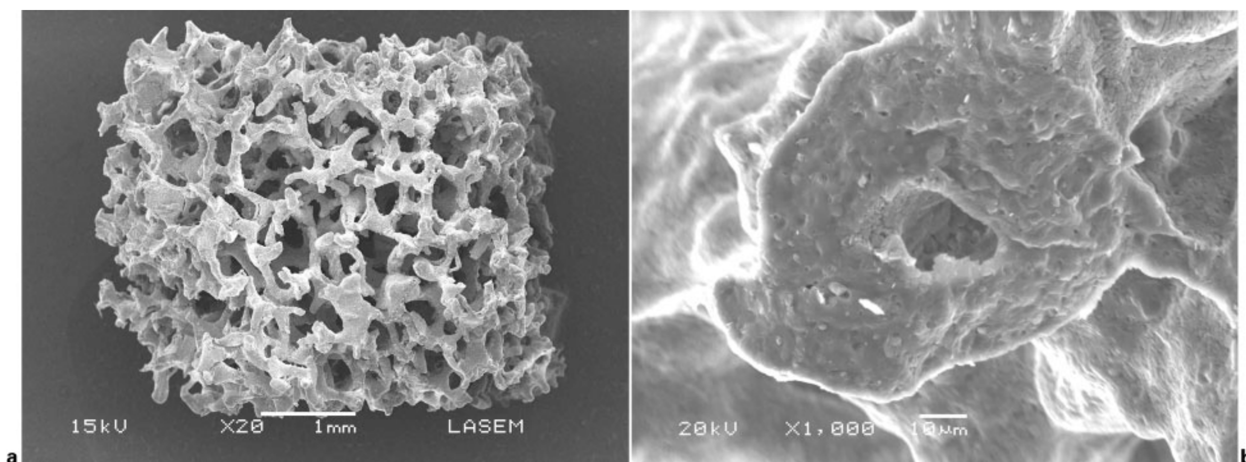


a low magnification; *b* high magnification
1 Scanning electron microscopy images of 45S5 Bioglass derived glass ceramic scaffold sintered at 1100°C

potential rapid bone formation according to the demonstrated direct effect of Bioglass dissolution products (ion release) on human osteoblast cell proliferation.[19] The foam derived Bioglass based glass–ceramic scaffolds however have not yet been investigated *in vivo*.

The aim of this work was therefore to evaluate the *in vivo* biocompatibility of Bioglass derived glass–ceramic scaffolds for the first time using the chick embryo CAM as the experimental model.

Materials and methods

Scaffolds

45S5 Bioglass (nominal composition: $45SiO_2$–24·5CaO–24·$5Na_2O$–$6P_2O_5$, wt-%) derived glass–ceramic scaffolds with porosity in the range 90–95% were fabricated using the foam replica technique and sintering at 1100°C for 1 h, as described in detail elsewhere.[16] Briefly, a polymer (e.g. polyurethane) foam, which serves as a sacrificial template, is coated with a Bioglass slurry, which infiltrates the pore structure and Bioglass particles (<5 μm in size) adhere on the polymer surface forming a homogeneous coating. After drying, the polymer foam is burned out slowly at 400°C to minimise damage to the Bioglass coating. Once the polymer sacrificial template has been removed, the Bioglass scaffold is sintered to the desired density and partially crystallised using a predermined and optimised heat treatment schedule.[16]

In vivo bioassay

Fertile eggs (*Gallus sp*) were used. These were incubated *in ovo* at 37°C with 60% relative humidity in a standard laboratory incubator for 72 h. The *ex ovo* embryo development was carried out in a polystyrene container (4×7 cm diameter, 0·2 mm thick, Huhtamaki Argentina) placed inside another high density polyethylene container with a screw top (8·5×9 cm diameter, TE&T) containing 50 mL of distilled water for humidification. Neither tissue culture medium nor antibiotics were added to the cultures.[20] All culture chambers were maintained at 37°C in humidified air atmosphere for the desired length of time. Prismatic scaffolds of dimensions 5×5×2 mm^3 were placed on the CAM at 10 days of total incubation. All embryos were killed at 15 days of total incubation. Each experiment included ten embryos per group and was repeated twice.

Histologic processing

The scaffolds and surrounding CAM were explanted, fixed in 4% paraformaldehyde in PBS, and processed for embedding in methyl methacrylate. Ground sections were obtained and stained with silver methenamine[21] for histological evaluation by light microscopy (Zeiss Axioskop 2 MOT, Carl Zeiss, Jena, Germany).

Results and discussion

Macroscopic evaluation

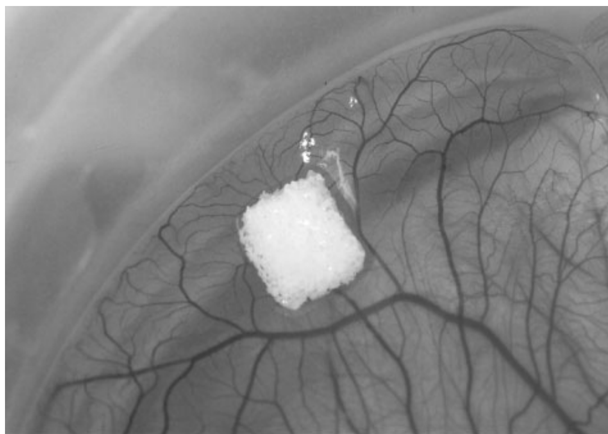
The typical macrostructure and microstructure of the Bioglass derived scaffolds are shown in Fig. 1*a* and *b* respectively. The image in Fig. 1*a* shows the highly interconnected pore structure resembling the morphology of cancellous bone. Moreover, extensive sintering of the 45S5 Bioglass particles by a viscous flow sintering mechanism has occurred leading to fully densified and microcracking free struts containing fine crystalline grains of size ~0·5 μm.[22] The presence of a central hole in the struts, as observed in Fig. 1*b*, is inherent to the foam replica method used to fabricate the scaffolds, as also discussed in detail elsewhere.[16,22] The combination of extensive densification and the presence of a crystalline phase in the struts are expected to lead to improved mechanical properties of these scaffolds.

Figure 2 shows the location of a scaffold placed on the CAM at 10 days of incubation. Macroscopic evaluation revealed that all biomaterials implanted on the CAM remained *in situ*. At 48 h post-implantation scaffolds were fully incorporated by the CAM and did not interfere with normal CAM development. No vascular reaction was detectable around scaffolds. Moreover no opaque granulomatous tissue surrounding the scaffolds in any of the cases was found.

Histological evaluation

At 5 days post-implantation, histological analysis revealed that the epithelial tissue of CAM had developed a continuous interface all around the scaffold, in intimate contact with the surface of the scaffold (Fig. 3). No tissue penetration in the pores of the biomaterial or inflammatory infiltrate was observed. In addition, no angiogenic response was detectable.

Previous studies have demonstrated that the CAM response to biomaterials depends on the chemical



2 Image of Bioglass scaffold placed on chick embryo CAM at 10 days of total incubation

composition and the physical structure of the material.[2,3,10] For example, Zwadlo-Klarwasser *et al.*[2] observed that materials with a smooth surface, such as PVC and Tecoflex (a polyurethane) appear to be antiangiogenic, mostly because of the positive charge in its surface. Angiogenesis was found to be more readily induced by rough materials, such as filter paper and collagen/elastin membrane.[2,3] It was also observed by Oates *et al.*[10] that angiogenesis and inflammation are dependant on the pore size of a matrix. For example, angiogenesis and inflammation have been demonstrated to be affected by altering the porosity of polylactic acid scaffolds.[10]

The present study evaluated, for the first time, the biological response of the CAM to a bioactive glass–ceramic material with a tridimensional porous structure, derived from 45S5 Bioglass. The Bioglass derived glass–ceramic scaffolds proved to be biocompatible in terms of the absence of inflammatory response at the implant site (CAM) as demonstrated by macroscopic and histological evaluation. A more detailed investigation on the effect of incorporating bioactive inorganic scaffolds on epithelial tissue development utilising different compositions of the bioactive glass matrix as well as quantification of the extent of tissue infiltration into the pores and angiogenesis effects in the CAM model is being carried out.

Conclusion

The results described in this paper indicate that the developed bioassay based on chick embryo CAM constitutes an appropriate alternative animal model to evaluate the biocompatibility of scaffold biomaterials for tissue engineering and regenerative medicine. The bioactive glass–ceramic three-dimensional scaffolds investigated were confirmed to be biocompatible and exhibited no adverse effects on normal CAM development.

It is hoped that the results of this study will contribute to document the advantages of the chick embryo CAM model as a novel bioassay with the potential to reduce the use of conventional animal testing.

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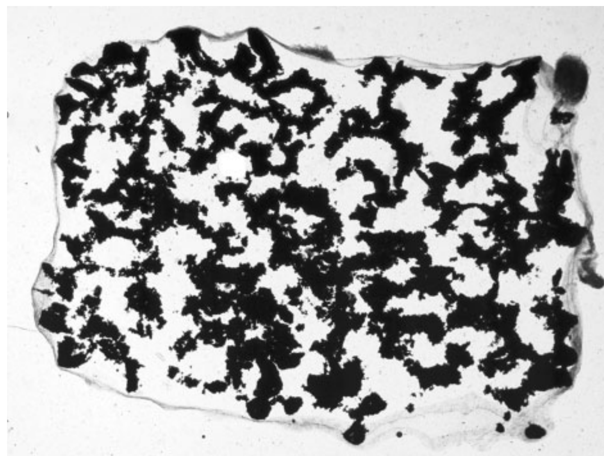

3 Photomicrograph of representative ground section of scaffold and surrounding CAM at 5 days post-implantation: notice that epithelial tissue of CAM has developed continuous interface all around Bioglass scaffold; silver methenamine; original magnification ×20

References

1. F. P. Gruber and T. Hartung: *ALTEX*, 2004, **21**, (Suppl. 1), 3–31.
2. G. Zwadlo-Klarwasser, K. Gorlitz, B. Hafemann, D. Klee and B. Klosterhalfen: *J. Mater. Sci. Mater. Med.*, 2001, **12**, 195–199.
3. T. I. Valdes, D. Kreutzer and F. Moussy: *J. Biomed. Mater. Res.*, 2002, **62**, 273–282.
4. U. Klueh, D. I. Dorsky, F. Moussy and D. L. Kreutzer: *J. Biomed. Mater. Res.*, 2003, **67**, 838–843.
5. J. Borges, F. T. Tegtmeier, N. T. Padron, M. C. Mueller, E. M. Lang and G. B. Stark: *Tissue Eng.*, 2003, **9**, 441–450.
6. E. Falkner, C. Eder, B. Kapeller, W. Froschl, C. Schmatz, K. Macfelda and U. M. Losert: *Altern. Lab. Anim.*, 2004, **32**, 573–580.
7. C. Eder, E. Falkner, S. Nehrer, U. M, Losert and H. Schoeffl: *ALTEX*, 2006, **23**, 17–23.
8. M.W. Laschke, Y. Harder, M. Amon, I. Martin, J. Farhadi, A. Ring, N. Torio-Padron, R. Schramm, M. Rucker, D. Junker, J. M. Haufel, C. Carvalho, M. Heberer, G. Germann, B. Vollmar and M. D. Menger: *Tissue Eng.*, 2006, **12**, 2093–2104.
9. J. Azzarello, M. A. Ihnat, B. P. Kropp, L. A. Warnke and H. K. Lin: *Biomed. Mater.*, 2007, **2**, 1–7.
10. M. R. Oates, C. M. Duncan and J. A. Hunt: *Biomaterials*, 2007, **28**, 3679–3686.
11. L. Dreesmann, M. Ahlers and B. Schlosshauer: *Biomaterials*, 2007, **28**, 5536–5543.
12. M. Richardson and G. Singh: *Curr. Drug Targets Cardiovasc. Haematol. Disord.*, 2003, **3**, 155–185.
13. A. C. Tufan and N. L. Satiroglu-Tufan: *Curr. Cancer Drug Targets*, 2005, **5**, 249–266.
14. V. Guarino, F. Causa and L. Ambrosio: *Expert Rev. Med. Dev.*, 2007, **4**, (3), 405–418.
15. K. Rezwan, Q. Z. Chen, J. J. Blaker and A. R. Boccaccini: *Biomaterials*, 2006, **27**, 3413–3431.
16. Q. Z. Chen, I. D. Thompson and A. R. Boccaccini: *Biomaterials*, 2006, **27**, 2414–2425.
17. Q. Z. Chen, A. Efthymiou, V. Salih and A. R. Boccaccini: *J. Biomed. Mater. Res. Part A*, 2008, **84**, (4), 1049–1060.
18. L. L. Hench: *J. Am. Ceram. Soc.*, 1998, **81**, 1705–1728.
19. I. D. Xynos, A. J. Edgar, L. D. K. Buttery, L. L. Hench and J. M. Polak: *J. Biomed. Mater. Res.*, 2001, **55**, 151–159.
20. A. Gorustovich, L. Haro, G. Vargas and R. V. Mesones: *Dev. Biol.*, 2007, **306**, (1), 407.
21. P. Frayssinet, J. S. Hanker, N. Rouquet, I. Primout and B. Gianmmara: *Biotech. Histochem.*, 1999, **74**, 10–15.
22. A. R. Boccaccini, Q. Z. Chen, L. Lefebvre, L. Gremillard and J. Chevalier: *Faraday Discuss.*, 2007, **136**, 27–44.