IN VITRO CYTOTOXICITY ASSESSMENT OF CARBON FABRIC COATED WITH CALCIUM PHOSPHATE

Hongmei Han, Biomedical Materials Research Group, University of Brighton, Brighton, UK, BN2 4GJ
Gary J. Phillips, Biomedical Materials Research Group, University of Brighton, Brighton, UK, BN2 4GJ
Sergey V. Mikhalovsky, Biomedical Materials Research Group, University of Brighton, Brighton, UK, BN2 4GJ
Andrew W. Lloyd, Biomedical Materials Research Group, University of Brighton, Brighton, UK, BN2 4GJ

Abstract

The success or failure of medical implants often depends on the cell-surface behaviour after implantation of the device. Carbon fabrics have previously been suggested as an implant material for the restoration of articular surfaces as a consequence of their porosity and flexibility. This study investigated the use of woven carbon fabric which had been sonoelectrochemically coated with calcium phosphate to enhance bone cell attachment and proliferation *in vitro*. Human osteoblast-like cells (MG63) were used to study the interactions between cells and material and assess the cytotoxicity of the substrates. The cytotoxicity of the materials was assessed using an MTS assay to determine the viability of the osteoblast-like MG63 cells in direct contact with the carbon fabric or calcium phosphate coated carbon fabrics, and to assess the cytotoxicity of extracts from these materials. The morphology of the surface adherent cells was assessed by scanning electron microscopy (SEM). Experimental results showed that neither carbon fabrics nor calcium phosphate coated materials were cytotoxic. Furthermore, cell attachment and proliferation were enhanced by coating carbon fabrics with calcium phosphate. Scanning electron micrographs showed that cells had a normal morphology and were well spread similar to those seen in the tissue culture plate control. These flexible calcium phosphate coated fabrics may, therefore, have uses in the reconstruction of bone for orthopaedic and dental surgery.

Introduction

Carbon, in all its forms (carbonaceous materials), has been demonstrated to be uniquely biocompatible with living bone and other tissue and therefore is well accepted by the biological environment (Jenkins, G.M., 1980; Kazakov, M.E., 1989; Minns, R.J., 1982). Carbon fabrics have previously been suggested as an implant material for the restoration of articular surfaces as a consequence of their porosity and flexibility (Muckle, D.S., 1989; Blazewicz, M., 2001; Aichroth, P. M., 1991; Minns, R.J., 1989) and it has been known for more than thirty years that calcium phosphate ceramics can be used successfully for replacing and augmenting bone tissue. The most important properties of calcium phosphates are their bioresorption and bioactivity that attributed to the similarity between their composition and structure and the mineral phase of bone (Citeau, A., 2005; Julien, M., 2007; Pioletti, D.P., 2000; Van der Wal, E., 2006). Attempts have been made in this study to combine the advantages of carbon with the bioactive characteristics of calcium phosphates to result in an array of new calcium phosphate coated carbon biomaterials, for the repair and re-construction of bone and tissue defects.

Biomaterials, regardless of whether they are permanent or biodegradable, naturally occurring or synthetic, need to be biocompatible. In addition materials for use in bone tissue engineering should ideally be osteoinductive, osteoconductive, integrative, porous and mechanically compatible with native bone (El-Ghannam A, 2005). Clearly, the success or failure of the biomedical implants depends on the control and consequences of cell-surface behaviour after implantation of the device. Thus, *in vitro* tests using osteoblast cells are essential for bone tissue-implant interface study (Balani,K., 2007; Knabe, C., *et al*., 2002; Morrison, C., 1995; Olivares, R., Rodil, S.E., and Arzate, H., 2004).

Human osteoblast-like MG63 cells have been well characterized and display numerous osteoblastic traits that are typical of a comparatively immature osteoblast, therefore were commonly used as the cell culture model (Montanaro, L., *et al*., 2002; Ramires,P.A. *et al*., 2001). In this study, the interaction between a range of calcium phosphate (CaP) coated carbon fabrics and human osteoblast-like cells (MG63) were investigated to assess the biocompatibility of this new range of biomaterials.

Experimental

Calcium phosphate (CaP) was coated onto carbon fabric (C-Tex Carbon fabrics produced by MAST carbon Ltd, UK and an activated carbon cloth from Ukraine) surfaces by sonoelectrodeposition at 50ºC, 20 mA/cm^2 current density, 40 KHz ultrasonic frequency and 2.16 W/cm^2 ultrasonic intensity for 45 minutes in an electrolyte solution containing 10 mM Ca^{2+} at pH 5.5.

MG63 cells were routinely cultured at 37ºC in a humidified atmosphere of 5% CO_2 in air, in Minimum Essential Medium with Earle's salt (EMEM, PAA E15-825, UK), 10% fetal bovine serum (FBS, PAA A15-144), 0.2% penicillin and streptomycin solution (100×, PAA P11-010) and 1% non-essential amino acids (100×, PAA

M11-003) and supplemented with 100μM 2-phospho-L-Ascorbic Acid (Sigma 49752, UK) and 10mM β–Glycerophosphate (Sigma G9891, UK) to activate the osteoblasts.

Four kinds of samples, C-Tex (I-T), CaP coated C-Tex (II-T), activated carbon cloth (I-A) and CaP coated activated cloth (II-A), of diameter ~Φ15mm were autoclaved at 163ºC for 2hr (n=3) and immersed in 4 ml osteogenic media and incubated at 37ºC in a humidified atmosphere of 5% CO_2 for 24 hours to prepare material extracts. The supernatants were diluted in osteogenic media in twofold concentrations: 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0% of the original.

In vitro cytotoxicity tests were carried out using an MTS assay. MG63 cells (5,000) were seeded in 100 μl of each material extract per well of 96-well plate in 4 replicates and control wells containing only osteogenic media (no material extracts). The media were aspirated after 24, 48 and 72 hr incubation. Diluted MTS reagent (120 μl) (CellTiter 96 Promega Corp., USA, 1:5 MTS reagent to osteogenic culture medium) was added to the wells and after incubation at 37°C for 2 hours, the plates were read at 492nm using a microplate reader (Thermo Multiskan Ascent). Cell viability (%) was calculated according to the following equation: cell viability (%) = OD492(sample)/OD492(control).

To determine the direct interaction of MG63 cells with materials, the samples were sterilized prior to use using UV irradiation for 1 h and preconditioned for 24 h with 1 ml osteogenic medium. The media was then removed and the cells (20,000 cells/well) were plated directly on the polystyrene surface of the 24-well plate that served as a control or on the sample surfaces in 1 ml osteogenic medium. Following the removal of the media after 72 hr incubation, 300 μl of diluted MTS reagent (CellTiter 96 Promega Corp., USA, 1:5 MTS reagent to osteogenic culture medium) were added to each sample and incubate at 37°C for 2 hours. Supernatant (120 μl) was transferred from each well to a fresh 96-well plate in two replicates and the absorbance was measured at 492 nm using a microplate reader (Thermo Multiskan Ascent).

To observe cell morphologies, after 72 h incubation at 37ºC in 5% CO_2 atmosphere, the cells attached on the surfaces were fixed with 2.5% glutaraldehyde (Sigma, UK) in Cacodylate buffer (Sigma, UK) for 1 h at room temperature. After thorough washing with 0.1M Cacodylate buffer, the cells on the surfaces were dehydrated in ethanol graded series (25%, 50%, 75% and 2×100%) for 15 min each and allowed to freeze dried. The samples were coated with palladium and examined by JEOL JSM 6310 scanning electron microscopy (SEM).

Results and Discussions

Coating carbon fabrics with calcium phosphate clearly enhanced the cell viability (Fig.1), although the improvement on two different carbon substrates was significantly different. A thicker, more uniform layer of CaP coating was produced on activated A-type carbon fabrics than on C-Tex carbon, under the same sonoelectrodeposition condition. However, the absorbance reading of CaP coated activated carbon was dramatically lower than that of CaP coated C-Tex.

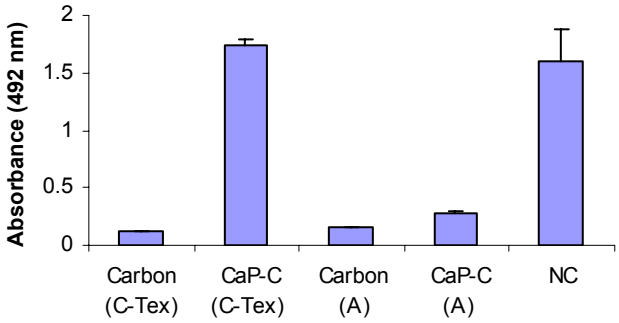

Figure 1 OD values of MTS assay after 72 hour cell interaction directly with different carbon and CaP coated carbon materials.

The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. The quantity of formazan product as measured by the absorbance at 490nm is directly proportional to the number of living cells in culture. As a result, the MTS assay is normally useful in *in vitro* cytotoxicity investigations. However in the present study, because of the strong adsorption of activated A-type carbon fabrics, with or without CaP coating, the supernatant became absolutely colorless at every treatment stage (pre-soaking, cell incubation, and MTS assay). The carbon fabrics may, therefore, adsorb the viability marker dyes, reducing the absorbance reading and giving a false result indicating reduced viability and thus cytotoxicity. Therefore, particular care should be taken when assessing the toxicity of carbon materials using colorimetric methodologies.

A further consideration that should be taken into account is the fact that carbon may adsorb some of the constituents of the growth media including ions and protein, thereby essentially starving the cells. Our previous work has demonstrated that pre-soaking carbon materials with the media reduces this interference and results in a corresponding increase the cell viability.

Due to the limitations of the MTS-based assay, an extract-based method has been employed to assess the cytotoxic potential of the carbon materials and eliminate the carbon adsorption effect. MG63 cells were exposed to extracts from each of the materials, prepared as a series of dilutions. Figure 2 demonstrates the cell viabilities were above 0.7 in most cases and occasionally higher than that of the control. Thus it is reasonable to deduce that the extracts are not toxic compared with the control and some appear superior to the polyethylene tissue culture plate control.

It is also clear from Figure. 2 that CaP coating resulted in different effects on MG63 cell viability for the different carbon fabrics. For non-activated C-Tex, the CaP increased the cell viability in the first 48h incubation and afterwards showed a reduced viability. In the activated carbon fabrics, the CaP exhibited positive effects after 72h interaction with cells. However, after 48h incubation, differences in cell viability were apparent on both types of coated and uncoated carbon cloth. Greater cell viability was seen on both the uncoated and coated C-Tex samples compared with the activated (A-type) cloth. In addition, after 72h incubation, improved cell viability was seen on the CaP coated activated carbon cloth compared with the uncoated activated sample.

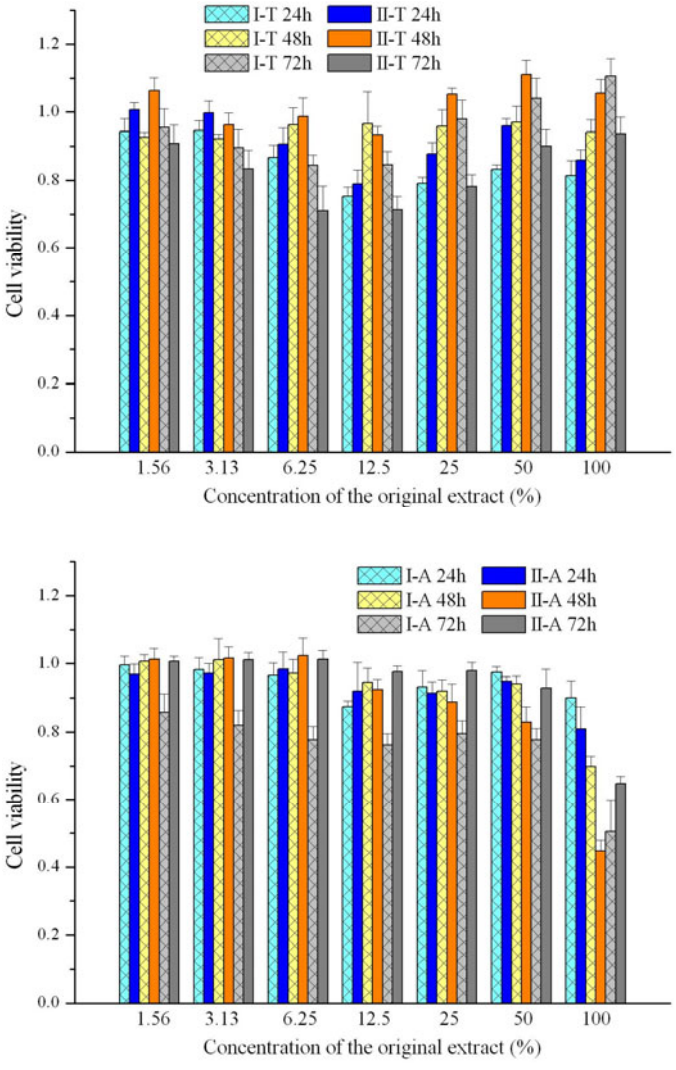

Figure 2 Cell viability after certain period of interaction with different carbon, CaP-carbon extracts. The control value was set 1.

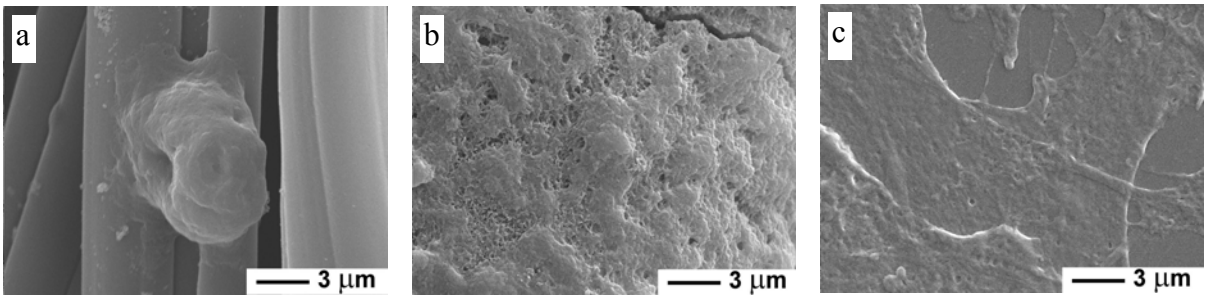

Fig.3 Cell morphologies after 72 hour interaction with carbon and CaP coated carbon materials. (a) C-Tex carbon fabric, (b) CaP coated C-Tex carbon, and (c) tissue culture plate control.

Cell adhesion and morphology investigated with scanning electron microscopy demonstrated clearly that both carbon fabrics and CaP coated carbon fabrics supported the osteoblast adhesion. After 72 h interaction with carbon fabrics, osteoblast-like MG63 cells showed a rounded morphology (Fig.3a) on the carbon fibre surfaces, inferring their survival when interacting with carbon. Highly spread cells were visible on the CaP coated, but not non-coated carbon fabrics. It is therefore conceivable that the carbon materials lack cyto-activity. On the other hand, more spread cell morphology in Fig.3b suggested that the calcium phosphate coatings dramatically change the cell/material behaviour. Cell attachment and proliferation were enhanced enormously by coating carbon fabrics with calcium phosphate, and cells had a normal morphology and were well spread similar to those seen in the tissue culture plate control (Fig.3c).

Conclusions

The present study demonstrates that, in our experimental conditions, human sarcoma derived osteoblast-like MG63 cells are appropriate to investigate interactions with biomaterials in vitro for bone tissue engineering.

Experimental results showed that neither carbon fabrics nor calcium phosphate coated materials were cytotoxic. Cell attachment and proliferation were enhanced by coating carbon fabrics with calcium phosphate. Scanning electron micrographs showed cells had a normal morphology and were well spread similar to those seen in the tissue culture plate control.

Different carbon fabrics induced calcium phosphate coatings with different features, and triggered different cell/material interactions. Future work will examine the impacts of such factors on cell/material interaction.

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References

Ahmed El-Ghannam. 2005. Bone reconstruction: from bioceramics to tissue engineering. *Expert Review of Medical Devices, 2*(1): 87-101.

Aichroth, P. M., Patel, D. V., Jones, C. B., and Wand, J. S. 1991. A combined intra- and extra-articular reconstruction using a carbon-dacron composite prosthesis for chronic anterior cruciate instability. *International Orthopaedics, 15*: 219-227.

Balani, K., Anderson, R., Laha, T., Andara, M., Tercero, J., Crumpler, E., et al. 2007. Plasma-sprayed carbon nanotube reinforced hydroxyapatite coatings and their interaction with human osteoblasts in vitro. *Biomaterials, 28*(4): 618-624.

Blazewicz, M. 2001. Carbon materials in the treatment of soft and hard tissue injuries. *European cells and materials, 2*: 21-29.

Citeau, A., Guicheux, J., Vinatier, C., Layrolle, P., Nguyen, T. P., Pilet, P., et al. 2005. In vitro biological effects of titanium rough surface obtained by calcium phosphate grid blasting. *Biomaterials, 26*(2): 157-165.

Jenkins, G M. 1980. Biomedical applications of carbons and graphites. *Clin . phys . Physiol . Meas., 1*: 171-194.

Julien, M., Khairoun, I., LeGeros, R. Z., Delplace, S., Pilet, P., Weiss, P., et al. 2007. Physico-chemical-mechanical and in vitro biological properties of calcium phosphate cements with doped amorphous calcium phosphates. *Biomaterials, 28*(6): 956-965.

Knabe, C., Klar, F., Fitzner, R., Radlanski, R. J., and Gross, U. 2002. In vitro investigation of titanium and hydroxyapatite dental implant surfaces using a rat bone marrow stromal cell culture system. *Biomaterials, 23*(15): 3235-3245.

Kazakov, M. E., Bizyakina, N. G., Prokimnov, V. V., Lebedev, V. S., Movshovich, I. A., Gavryushenko, N. S. et al. 1989. Application of carbon materials in traumatology and orthopedics. *Journal Fibre Chemistry, 21*: 135-137.

Minns, R.J., Muckle, D.S., and Donkin, J.E. 1982. The repair of osteochondral defects in osteoarthritic rabbit knees by the use of carbon fibre. *Biomaterials, 3*: 81-86.

Minns, R.J., and Muckle, D. S. 1989. Mechanical and histological response of carbon fibre pads implanted in the rabbit patella. *Biomaterials, 10*: 273-276.

Montanaro, L., Arciola, C. R., Campoccia, D., and Cervellati, M. 2002. In vitro effects on MG63 osteoblast-like cells following contact with two roughness-differing fluorohydroxyapatite-coated titanium alloys. *Biomaterials, 23*(17): 3651-3659.

Morrison, C., Macnair, R., MacDonald, C., Wykman, A., Goldie, I., and Grant, M. H. 1995. In vitro biocompatibility testing of polymers for orthopaedic implants using cultured fibroblasts and osteoblasts. *Biomaterials, 16*(13): 987-992.

Muckle, D.S. and Minns. R.J. 1989. Biological response to woven carbon fibre pads in the knee. *Journal of bone and joint surgery, 71-B*: 60-62.

Olivares, R., Rodil, S. E., and Arzate, H. 2004. In vitro studies of the biomineralization in amorphous carbon films. *Surface and Coatings Technology, 177-178*: 758-764.

Pioletti, D. P., Takei, H., Lin, T., Van Landuyt, P., Jun Ma, Q., Yong Kwon, S., et al. 2000. The effects of calcium phosphate cement particles on osteoblast functions. *Biomaterials, 21*(11): 1103-1114.

Ramires, P. A., Romito, A., Cosentino, F., and Milella, E. 2001. The influence of titania/hydroxyapatite composite coatings on in vitro osteoblasts behaviour. *Biomaterials, 22*(12): 1467-1474.

Van der Wal, E., Vredenberg, A. M., Ter Brugge, P. J., Wolke, J. G. C., and Jansen, J. A. 2006. The in vitro behavior of as-prepared and pre-immersed RF-sputtered calcium phosphate thin films in a rat bone marrow cell model. *Biomaterials, 27*(8): 1333-1340.