Surface Coated Polyurethane with Improved Bioactivity and Cytocompatibility

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ABSTRACT

Polyurethane (PU) has outstanding mechanical properties that make it an ideal candidate for certain biomedical implant applications, however it lacks bioactivity. Bioactivity allows for direct tissue attachment at the bio-interface, enabling implant fixation, while preventing fibrous encapsulation. To induce bioactivity, PU was coated with a layer of hydroxyapatite (HA) by a solvent-compression method. After nine days in simulated body fluid (SBF), bioactivity of the HA coated substrates was confirmed by the formation of an apatite layer on top of the HA layer, which was not observed on the uncoated substrates. The apatite layer contained Na and Mg ions which were absent in the HA layer and higher carbonate levels. Both Ca and P ions were reduced in the SBF containing HA coated substrates, indicating that apatite formation proceeded by immediate uptake of both these ions from the SBF. To determine cytocompatibility of the HA coated PU substrates towards Graham 293 fibroblasts, MG-63 osteosarcoma, and MCF-7 epithelial cells, crystal violet DNA staining and the MTT assay were employed. After 24 and 72 hours, the HA coated surfaces displayed significantly higher cell numbers and metabolically active cells, compared to the virgin uncoated PU surfaces for all three cell lines (n=5, $p<0.05$).

INTRODUCTION

Fibrous encapsulation of biomaterials still remains one of the major challenges facing biomaterial scientists. Encapsulation of a biomaterial with non-adherent fibrous tissue is believed to be due to a lack of cell attachment to the implant surface, which invariably triggers the host immune response, preventing tissue regeneration and repair. Bioactive materials generally do not undergo fibrous encapsulation, and cells directly attach to them. Hydroxyapatite (HA) is known to be bioactive and host tissue would directly bond to it without formation of fibrous tissue. HA, however, is brittle, exhibiting low fracture strength and fatigue resistance and is therefore commonly reinforced with polymers or applied as coating thereon to broaden its application. In this study, polyurethane (PU) was investigated as a polymeric substrate for HA coatings since it is biocompatible and offers a number of important properties such as toughness, strength, flexibility, and easy processing. PU was coated with HA by a simple and efficient solvent-compression method. The bioactivity of the HA coated substrates was investigated by *in vitro* testing in SBF. Cell attachment and proliferation onto the substrates was determined to identify the relative *in vitro* cytocompatibility of these surfaces towards Graham 293 (fibroblasts), MG-63 (osteosarcomas) and MCF-7 (epithelial) cell lines.

METHODOLOGY

1 Coating of hydroxyapatite onto polyurethane

The HA powder was prepared in-house by solid state reaction of ß-tricalcium phosphate and calcium hydroxide at 1020°C. The Ca/P ratio of the HA was 1.63±0.03. Polycarbonate thermoplastic PU (PC 3575A Carbothane, Noveon) was dried at 60°C for 4 hours and then compression moulded into sheets with heating at 215°C. 20mm x 3mm disks were immersed in reagent grade cyclohexanone for 30-40 seconds, and then immediately compressed with HA powder contained in a die assembly in a Centorr Hand Press (13 KPa for 5 minutes). The coated substrates were dried at 60°C for 2 hours, and unbound HA powder was removed by vigorous scrubbing and ultrasonic agitation. To extract the residual solvent, water leaching was performed in distilled water (190 mℓ/mm² of sample area) at 60°C for 48 hours, with 10 water changes.

2 *In vitro* bioactivity testing with SBF

HA coated and uncoated PU samples were incubated for 9 days in simulated body fluid (SBF) at 37.5°C and pH 7.4, with the SBF being refreshed every 2 days. SBF replicates blood plasma in ionic composition and pH. The sample's surface morphology was investigated with a scanning electron microscope (SEM) and the ionic composition of the surface was analysed by energy dispersive x-ray spectroscopy (EDS). Before every buffer change, the Ca and P ion concentrations in the SBF were determined by inductively coupled plasma optical emission spectroscopy (ICP-MS).

3 Cytocompatibility studies

MCF-7 (Human breast epithelial carcinoma), Graham 293 (Human embryonic kidney "fibroblastoid") and MG-63 (Human Osteosarcoma cells) were used. Cells were seeded in 24-well cell culture trays onto HA coated and uncoated PU substrates and cell attachment and proliferation was studied by allowing cells to attach (24 hours) and grow (72 hours) respectively. The cell culture plates without any substrate served as the 100% control. **Crystal violet DNA staining assay**: Seeded cells were exposed to 1% crystal violet solution for 30 mins., followed by thorough washing. The amount of absorbed dye was measured at 570 nm with a universal microplate reader. The topographical morphologies induced during cell attachment and growth was assessed by employing SEM. **Mitochondrial dehydrogenase activity (MTT) assay**: Seeded cells were incubated with the MTT (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay at 37°C for 3½ hours. Formazan production was monitored at 570 nm with a universal microplate reader. Statistical analysis of experimental data was performed by means of ANOVA and students' t-test (for unpaired samples), with n = 5 in each experiment.

RESULTS

1 Characterisation of HA coatings

From SEM analysis of the HA coating, a microrough topography was revealed that was fairly homogenous (**Figure 1A**). The coatings on the PU disks were confirmed to be crystalline HA as determined by x-ray-diffraction spectroscopy (XRD) (**Figure 1B**). The HA layer appeared well bonded to the underlying PU surface as the coating remained intact even after vigorous scrubbing and sticky tape adhesion testing. Average HA coating thickness observed by SEM cross-sections was approximately 115 -165 μm.

2 SBF testing for bioactivity

A dune-like layer developed on the HA coated surfaces after 9 days in SBF that was absent on the uncoated PU substrate (**Figure 2**). From the EDS analysis (**Figure 3**) the presence of Na, Mg and a higher level of carbonate ions in addition to Ca and P ions, confirmed the presence of bone-apatite which has the following formula: $[(Ca,Mg,Na)_{10}(PO_4,CO_3)_6(OH)_2]$. Both Ca and P levels decreased significantly in the SBF containing HA coated PU substrates while both ions remained relatively stable for the control samples (SBF itself and SBF containing uncoated PU substrates) (**Figure 4**). The high rate of Ca and P loss from the SBF for HA coated PU substrates, suggest apatite formation and growth on the HA coating, via precipitation and ion exchange between the SBF and the HA layer.

3 Cytocompatibility testing

The crystal violet assay was employed to stain cellular DNA, which is directly related to the number of cells attached onto the substrates. The MTT assay was utilised to determine the number of viable and metabolically active cells. The crystal violet assay and the MTT assay revealed higher cell numbers and cell metabolic activity respectively following attachment and growth on the HA coated PU surfaces compared to the virgin PU surfaces with all three cell lines (**Figures 5A, 5B**). The order of cell number and metabolic activity on the HA coated PU substrates appears in **Table 1**.

	24 hours	72 hours
Crystal violet	MCF-7 > MG-63 > Graham 293	MG-63 > MCF-7 > Graham 293
MTT	Graham 293 > MG-63 > MCF	Graham 293 > MG-63 > MCF

Table 1: Order of cell preference on the HA coated surface after 24 and 72 hours as detected by the crystal violet and MTT assays.

The increase of the osteosarcoma cells on the HA coating after 72 hours was expected since it has been well established that HA is bioactive and would have a high affinity for bone cells. Although the Graham 293 fibroblasts showed relatively poor attachment and growth with regard to cell number, they were found to be the most metabolically active cell line on the HA coated PU surfaces after 24 and 72 hours.

4 Cell morphology

Healthy cells are generally flattened, well spread, adhere firmly to surfaces, and display large focal adhesions. Aggregated cells, with a rounded morphology however generally indicate poor adhesion, and less cell viability. Cell morphology was used as a qualitative measure of cell viability. Graham 293 fibroblast cells appeared morphologically flat, promising good proliferation on both surfaces, although fewer focal adhesions and less migration were observed on HA coated PU surfaces compared to the uncoated PU surfaces (**Figures 6a-d**). MG-63 osteosarcoma cells attached to the uncoated PU surface with characteristic lacunae formations, and displayed a rounded morphology (**Figure 6e,f**), while the cells appeared flatter and more integrated with the HA coated surface (**Figure 6g,h**). MCF-7 epithelial cells assumed a flat morphology on both the uncoated PU and HA coated surfaces (**Figures 6i – l**), with cells following the contours of the HA coating, indicative of stronger adhesion.

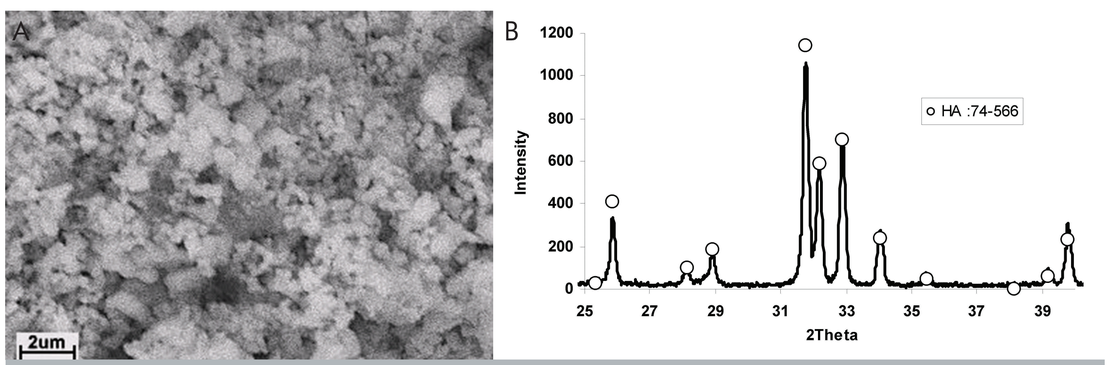

Figure 1: HA coating (**A**) on PU prepared by a solvent-compression method and XRD spectrum of coating (**B**)

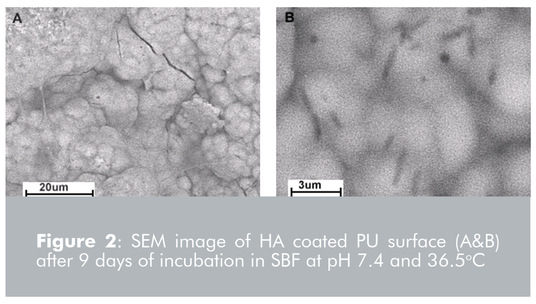

Figure 2: SEM image of HA coated PU surface (A&B) after 9 days of incubation in SBF at pH 7.4 and 36.5°C

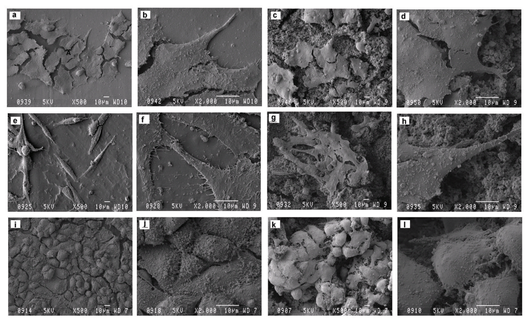

Figure 6: Topographical morphologies of Graham 293 fibroblasts (a-d), MG-63 osteosarcomas (e-h) and MCF-7 epithelial cells (i-l) on uncoated and HA coated PU surfaces by SEM. Figures 6 a, b, e, f, i and j are cells seeded on the uncoated PU surfaces and Figures 6 c, d, g, h, k and l are cells seeded on the HA coated PU surfaces

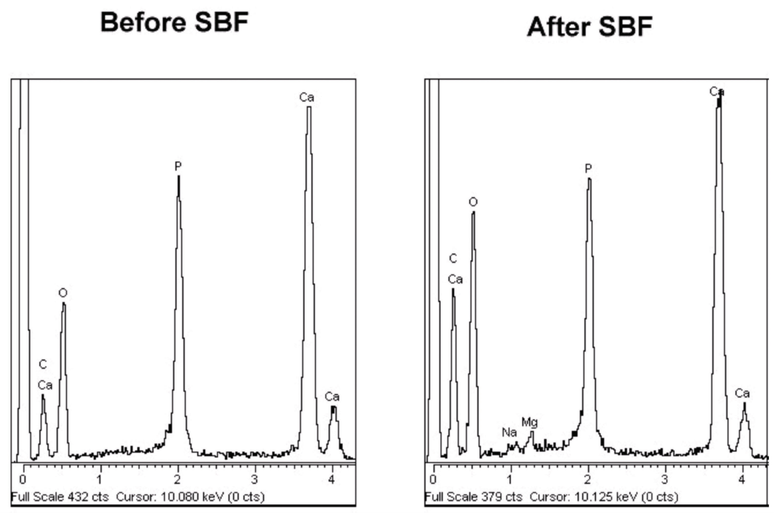

Figure 3: EDS spectra of HA coated PU before and after SBF treatment showing presence of the apatite layer in the latter

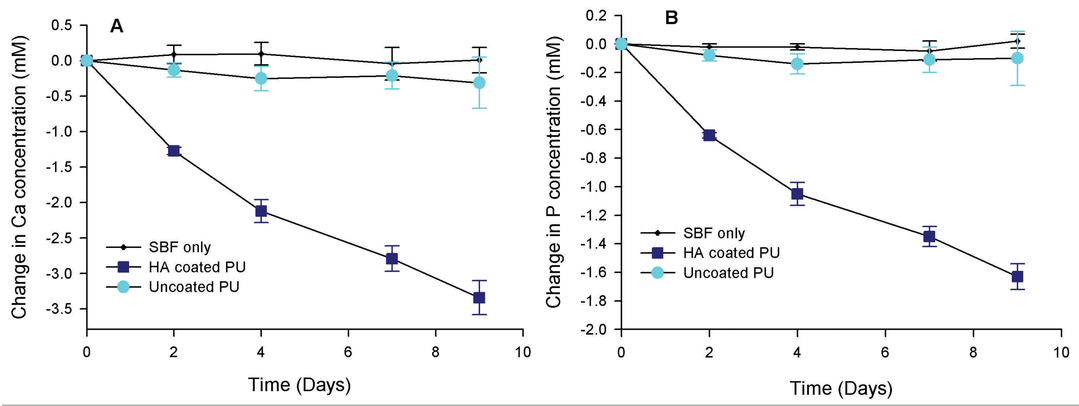

Figure 4: Cumulative calcium (**A**) and phosphorus (**B**) loss in SBF containing no samples (control), uncoated PU substrates and HA coated PU

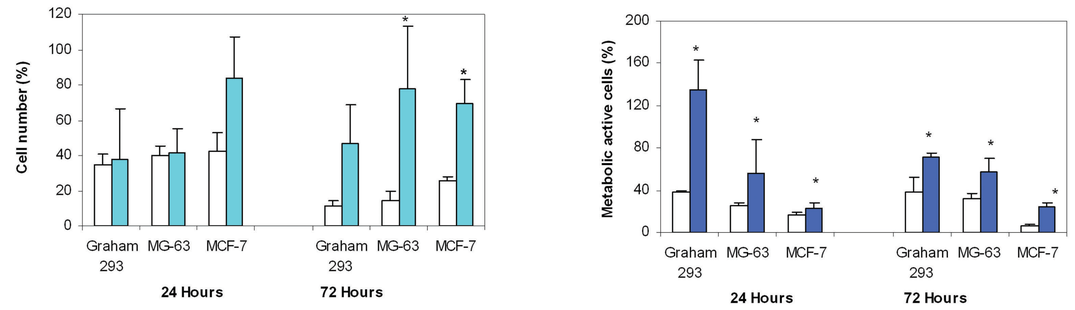

Figure 5: DNA crystal violet assay (**A**) and MTT assay (**B**) for cell number and metabolic activity respectively following cell attachment (24 hours) and proliferation (72 hours) on HA coated and uncoated PU surfaces using Graham 293 (fibroblasts), MG-63 (Osteoblasts) and MCF-7 (epithelial) cell lines. The filled and unfilled blocks refer to the HA coated and uncoated PU substrates respectively. The cell culture plate was used as the 100% control. The asterisk indicate statistically significant P-values of $P< 0.05$ with n=5

CONCLUSION

HA coatings prepared by a solvent-compression method, displayed a microrough, relatively homogenous topography that was strongly bound to the underlying PU surface. HA coated PU substrates showed bioactivity when incubated *in vitro* in SBF for 9 days, while the virgin uncoated PU substrates were non-bioactive. The HA coated PU substrates have demonstrated cytocompatibility towards fibroblasts, osteosarcoma, and epithelial cells as determined by the crystal violet assay for DNA staining and the MTT assay for metabolic activity. The Graham 293 fibroblasts were found to be the most metabolically active cell line on the HA coated PU surfaces after 24 and 72 hours indicating that if this material were to be implanted in soft tissue, it should anchor well under the skin surface and prevent movement of the implant. In addition preference of the HA coating towards bone cells has also been confirmed. Since cell lines were used for these cell culture studies, it can only be considered as an early stage investigation of cytocompatibility. These cytocompatibility studies must be confirmed with primary isolated cells before application in soft or hard tissue is considered. These HA coated PU substrates could find application in soft tissue engineering, including cartilage replacement and maxillofacial implants, as well as in ID tags, biosensors, etc.