In vitro and in vivo investigation of a range of phosphate glass-reinforced polyhydroxybutyrate-based degradable composites

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A completely degradable melt-processed composite was produced using a phosphate-based glass in the soda–lime–phosphorus pentoxide ternary phase system. In vitro degradation studies showed that the mass loss and mechanical property change could be closely correlated with the solubility rate of the reinforcing glass. The in vivo studies showed a slight inflammatory reaction, but good compatibility. With time, this inflammatory reaction disappeared and the initial reaction was interpreted as being due to a high solubility rate of the glass. The high solubility of the glass was derived not from the composition, but because the glass was in particulate form and therefore had a high surface area. This was of interest, as it showed that the polymer was highly permeable and did not encapsulate the glass as expected with a melt-processing method.

1. Introduction
Polyhydroxybutyrate (PHB) and its associated copolymers with polyhydroxyvalerate (PHV) are attracting much attention from a variety of fields [1–3]. The mechanical properties of the polymers are adequate, but the polymer has two highly exploitable properties: it is piezoelectric [4] and hydrolytically degradable [5]. The development of this material for surgical use has been undertaken because the material has also proved to be biologically acceptable in trials conducted [6]. After degradation the product breaks down to β-hydroxybutyric acid (β-HBA), a natural constituent of human blood.

Research has also been undertaken to enhance the mechanical properties [7], e.g. by the simple process of melt-forming with another phase to produce a composite, and this has proved successful. In this paper we describe the development of a composite using a phosphate-based glass as the reinforcing phase. The development of this composite was undertaken to try to develop a material with a more predictable degradation pattern, using the solubility rate of the glass filler to control the overall composite degradation.

The first part of this paper describes an in vitro trial that was undertaken, to determine the degradation pattern and its relationship to the glass solubility. The second stage was an in vivo implantation study, to determine both the biocompatibility and the degradation pattern in soft and hard tissue.

2. Materials and methods
PHB–7% PHV (Mw 580000) was obtained from Marlborough Biopolymers. A 7% incorporation of PHV enables a lower processing temperature to be used, thus ensuring less degradation during thermal processing. Glass compositions were chosen from previous work [8] and glasses with four solubility rates were used. Table I shows the mol% of each oxide required to make the particular solubility glass. The raw materials used to make the glass were mixed thoroughly and placed in a platinum crucible. This mixture was sintered at 1000 °C for 30 min. Following removal from the furnace, the glass was poured on to a steel table to cool. Subsequently, the glass was ground in a vibratory mill to obtain a powder of particle size <14 µm. To produce a composite, the glass and polymer (both in powder form) were automixed at a glass addition up to 40 wt% and injection moulded to form standard tensile test pieces.

2.1. In vitro studies
To produce specimens for the in vitro studies, composites with a glass filler addition of 40 wt% were produced for injection moulding to form standard dumbbell-shaped tensile test pieces. These injection moulded specimens for in vitro degradation were placed in phosphate-buffered saline (PBS) at pH 7.4. This was maintained at 37 °C. At the appropriate time

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specimens were removed from the solution for testing. To identify the four composites they are referred to by the solubility rate of the glass filler used, i.e. 0.5, 0.25, 0.17 and 0.065 (Table I).

All mechanical testing of specimens was performed on an ESH hydraulic testing machine. The specimens were tested at a displacement of 5 mm min⁻¹ (strain rate 5%) until failure and the subsequent load-extension outputs were recorded on a flat-bed plotter. From this graph the slope of the linear part of the curve was used to calculate the Young's modulus (E). The ultimate tensile strength (UTS) and percentage elongation at failure (%e) were also determined. To determine the wet and dry weights, the samples were, at the appropriate time, removed from solution and the surface was wiped clean of excess moisture. Following weighing, the samples were dried for 24 h at 50 °C and then weighed again. The weight was plotted as a percentage of the initial weight using

% wet weight
\[ \frac{\text{wet weight at time } t - \text{initial dry weight}}{\text{initial dry weight}} \times 100 \]

% dry weight
\[ \frac{\text{dry weight at time } t - \text{initial dry weight}}{\text{initial dry weight}} \times 100 \]

2.2. In vivo studies
The in vivo study was in two parts, a subcutaneous-type (SC) implant and a non-load-bearing femoral (NL) implant. Both the SC and the NL implants were machined from the central section of tensile test pieces produced by injection moulding. Three solubility rate implants were produced for both the SC and the NL: a rapidly dissolving implant using glass type 0.5, a medium-rate dissolving implant using glass type 0.25 and a slowly dissolving implant using glass type 0.065.

For the SC implant the composite was machined to produce an implant of dimensions 10 mm × 8 mm × 2 mm. Using 10-week-old male Wistar rats and under halothane anaesthesia, an incision was made in the skin overlying the muscles of the hindlimb. The implant was sterilized with alcohol and inserted into the wound. Closure was by skin clips. For the NL implants, 16-week-old male Wistar rats were used. Again, using halothane anaesthesia, an incision was made through the skin and the muscles overlying the femur were retracted to expose the midshaft. A hole 2 mm in diameter was made in the lateral wall of the femur and a rivet-shaped implant 2 mm in diameter was inserted. Closure was performed subcutaneously with Vicryl 4/0 sutures and then the skin was closed with skin clips.

2.3. Specimen processing: SC implant
For the SC implants, at 2, 4, 8 and 12 weeks, the appropriate rats were killed by exsanguination under anaesthesia and the blood collected with a heparin anticoagulant. The blood was centrifuged at 1500 r.p.m. for 5 min to obtain the plasma. Three cuvettes were used for each sample: reagent blank, control and sample. To each of these tubes 3.0 ml β-HBA reagent and 0.05 ml [3-hydroxybutyrate (β-HBDH) was added and warmed to 37 °C. To the blank tube 0.05 ml deionized water was added, 0.05 ml β-HBA calibrator to the calibrator cuvette (containing 50 mg dl⁻¹ β-HBA) and finally 0.05 ml plasma to the sample cuvette. The cuvettes were warmed at 37 °C for 10 min and then the absorbance at 340 nm was measured.

2.4. Determination of β-HBA concentration
\[ sA = A(\text{sample}) - A(\text{blank}) \]

\[ \text{serum } \beta\text{-HBA (mg dl}^{-1}\text{)} = sA \times 104 \]

To convert to SI units (mmol l⁻¹), the β-HBA concentration (mg dl⁻¹) is multiplied by 0.096. A kit obtainable from Sigma Chemical Co. (procedure no. 310-UV) was used for determination of the PHB levels in the plasma. Utilizing the reaction

\[ \beta\text{-hydroxybutyrate} + \text{NAD} \rightarrow \text{acetoacetate} + \text{NADH} \]

A catalyst β-HBDH catalyses the oxidation of β-HBA to acetoacetate. During this reaction an equimolar amount of nicotinamide–adenine dinucleotide (NAD) is reduced to NADH. At wavelength 340 nm NADH absorbs light, hence the increase in absorbance in light is directly proportional to the β-HBDH concentration in the serum sample.

For the implant processing, the skin overlying the implant, and the implant and surrounding tissue were removed. The sample was placed in buffered formalin and processed using standard soft-tissue techniques. The sections were stained using haemotoxylin and eosin (H & E).

2.5. Specimen processing: NL implants
The NL implants were also processed by similar methods, but the bone specimens were decalcified before sectioning. H & E staining was again used.

3. Results
3.1. In vitro studies
Figs 1 and 2 show the wet and dry weight loss with time. The curves show a very characteristic initial