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Development of Novel Biodegradable Amino Acid Ester Based Polyphosphazene–Hydroxyapatite Composites for Bone Tissue Engineering

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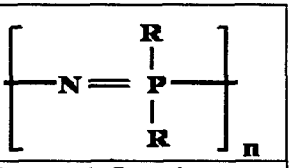
ASTRACT

Hydroxyapatite formed from low temperature setting calcium phosphate cements (CPC) are currently been used for various orthopaedic applications. CPCs are attractive candidates for the development of scaffolds for bone tissue engineering, since they are moldable, resorbable, set at physiological temperature without the use of toxic chemicals, and can be processed in an operating room setting. However they may have mechanical disadvantages which seriously limit them to non-load bearing orthopaedic applications. The aim of the present study was to develop composites from polyphosphazenes and calcium deficient hydroxyapatite precursors to form poorly crystalline hydroxyapatite-polymer composites. Composites were formed from calcium deficient hydroxyapatite precursors (Ca/P – 1.5, 1.6) and biodegradable polyphosphazenes, poly[bis(ethyl alanato)phosphazene] (PNEA) and poly[(50%ethyl alanato) (50%methyl phenoxy)phosphazene] ($PNEA_{50}mPh_{50}$) at physiological temperature. The results demonstrated that poorly crystalline hydroxyapatite that resembled the mineral component of bone was formed in the presence of biodegradable polyphosphazenes. The surface morphology of all the four composites was identical with a porous microstructure. The composites supported the adhesion and proliferation of osteoblast like MC3T3-E1 cells making them potential candidates for bone tissue engineering.

Keywords: Low temperature setting calcium phosphate cements, Polyphosphazenes, Composites, Bone tissue engineering

INTRODUCTION

Hydroxyapatite (HA) is a major mineral component in bone and it has been extensively used as a bone graft substitute due to its biocompatibility, and its ability to bond directly with bone [1, 2]. However the resorption rate of HA is slow. They are available in hardened form and require machining before use. Recently low temperature setting calcium phosphate cements (CPC) have emerged as attractive candidates for a variety of orthopaedic applications due to its similarity to the mineral component of bone. Brown et al. [3] developed self setting CPC, which set at body temperature and does not require high temperature for setting or use of toxic reagents [4]. CPCs can be mixed with the appropriate solution to form a paste and can be sculpted into a bone defect of any shape. Hence these materials can be prepared under operating room setting. In spite of all these advantages CPCs suffer from brittleness, low tensile strength, and low resistance to impact loading which seriously limits their applications [1, 2]. However studies have shown that the mechanical properties of CPCs can be improved by preparing composites with appropriate polymers. Thus gelatin, poly(vinyl alcohol), and poly(acrylic acid) have been used to improve the mechanical properties of tetracalcium phosphate-dicalcium phosphate dehydrate cement [5, 6]. Biodegradable polymers are attractive candidates for developing composites for transient applications such as tissue engineering. Durucan et al. [7, 8] synthesized composites of α-tricalcium phosphate and poly(lactic acid) and α-tricalcium phosphate and poly(lactide-*co*-glycolide). These composites showed improved mechanical properties when compared to the pure cement. Though these systems showed improved mechanical properties, a reduction in workability and setting time was observed [5, 6].



Structure 1. General structure of polyphosphazene

Polyphosphazenes are high molecular weight, inorganic polymers with alternate phosphorus and nitrogen atoms in the backbone (Structure 1) and each phosphorus atom is attached to organic, inorganic or organometallic side groups [9]. Amino acid ester polyphosphazenes are potential candidates for various biomedical applications due to their well controllable degradation rate, non toxic and neutral degradation products, and biocompatibility [9]. Further, the high flexibility of polyphosphazenes combined with the availability of active groups along the polymer chain make polyphosphazenes potential candidates for developing self reinforced composites with hydroxyapatite precursors. The mechanism of degradation of amino acid ester polyphosphazenes mainly involves the hydrolysis of ester moieties resulting in the formation of active carboxylic groups followed by backbone cleavage. The active groups in biodegradable polyphosphazenes can provide potential sites for interaction with the calcium ions of hydroxyapatite precursors resulting in the formation of an intimate composite when reacted with low temperature apatite precursors.

The aim of the present study was to develop composites from biodegradable polyphosphazenes and calcium deficient hydroxyapatite precursors to form poorly crystalline hydroxyapatite-polymer composites and to evaluate the osteocompatibility of the novel composites using osteoblast like MC3T3-E1 cells.

Materials and Methods

Biodegradable polyphosphazenes used in the present study were poly[bis(ethyl alanato)phosphazene] (PNEA) and poly[(50%ethyl alanato) (50%methyl phenoxy)phosphazene] ($PNEA_{50}mPh_{50}$). These polymers were synthesized by a two step process which involved the

thermal ring opening polymerization of hexachlorocyclotriphosphazene to form poly(dichlorophosphazene), followed by the simultaneous or sequential substitution of the chlorine atoms of the poly(dichlorophosphazene) by appropriate side groups. The structure of the polymers was confirmed by multinuclear magnetic resonance spectroscopy (Bruker 360MHz Spectrometer), and the molecular weight was determined using a gel permeation chromatography (GPC, HP1090, Agilent Technologies). The glass transition temperature of the polymers was measured using a differential scanning calorimeter (TA Instruments, Q10 DSC) with a heating rate of 10°C per minute under nitrogen atmosphere.

Synthesis of PNEA

Briefly, poly(dichlorophosphazene) (8.0g; 0.069mol) was dissolved in THF and was reacted with L-alanine ethyl ester (42.4g; 0.276mol) in the presence of triethylamine (86.6mL, 0.622mol). The polymer (PNEA) was isolated and purified by successive precipitations into hexane (3×) and pentane (2×). **^{31}P NMR** ($CDCl_3$), ppm: δ -1.1; **^{1}H NMR** ($CDCl_3$), ppm: δ 4.4 (1H), 4.1 (2H), 1.6 (3H), 1.3 (3H). M_n = 202,000, M_w = 408,000, PDI = 2.0. Tg = -3°C

Synthesis of $PNEA_{50}mPh_{50}$

Poly(dicholorophosphazene) (8.0g, 0.069mol) was allowed to react with the sodium salt of p-methyl phenol (8.21g, 0.076mol) in THF, to yield a partially substituted polymer. The remaining chlorine atoms in the poly(dichlorophosphazene) were replaced with excess of L-alanine ethyl ester (63.7g, 0.414mol) in the presence of triethylamine (58ml, 0.414mol). The reaction mixture was refluxed for two days to ensure complete substitution. The polymer was isolated by precipitation in hexanes and purified by repeated precipitations in hexanes and ethanol. **^{31}P NMR**: ($CDCl_3$), ppm: δ -5.0672; **^{1}H NMR** ($CDCl_3$), ppm: δ 6.8 (4H), 3.9 (3H), 2.2 (3H). 1.8 (3H), 1.1 (3H). M_n = 2,219,000, M_w = 4,608,000, PDI = 2.076. Tg = -6°C

Preparation of CDHA (Ca/P – 1.5) and CDSHA (Ca/P – 1.6) precursors

Tetracalcium phosphate (TetCP) used for the preparation of calcium deficient hydroxyapatite precursors was obtained from the reaction between calcium carbonate (Osram, Sylvania, PA) and monocalcium phosphate monohydrate (MCPM, FMC, NY). TetCP, with a calcium to phosphate ratio of 2.0 and dicalcium phosphate anhydrous were reacted in varying molar ratios to obtain two different calcium deficient hydroxyapatite precursors.

$$3\,CaCO_3 + Ca(H_2PO_4)_2.\,H_2O \longrightarrow Ca_4(PO_4)_2O$$

Tet CP - Ca/P = 2.0

$$\text{Tet CP} + CaHPO_4 \longrightarrow Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$$

$$x = 1.0 \Longrightarrow \text{CDHA}$$

$$x = 0.4 \Longrightarrow \text{CDSHA}$$

Preparation of Calcium Deficient Hydroxyapatite – Polyphosphazene Composite Precursors

The composite precursors were synthesized by an emulsion technique. Briefly, 1.5g of the polymers PNEA and $PNEA_{50}mPh_{50}$, were dissolved in 30ml of methanol and dimethyl formamide respectively. The solution was added drop wise to a vigorously stirred suspension of

15g of the calcium deficient hydroxyapatite in 1 liter of heptane and 50ml of methanol or 50 ml of DMF at room temperature. After the polymer solution was added, the suspension was stirred for 10 minutes and the excess solvent was evaporated to dryness using a rotary evaporator. The resultant solid was dried under vacuum at 50°C for 72 hours.

Preparation of Calcium Deficient Hydroxyapatite – Polyphosphazene Composites

The precursors of each of the composites were mixed with 0.5% phosphoric acid (Acros 201140010) in the ratio 1:1 (w/v) to form a paste. The pastes were injected in an appropriate mold and were incubated for 24 hours at 37°C in a humidified atmosphere.

X-ray Diffraction Analysis

X-ray diffraction (XRD) was used to confirm the formation of poorly crystalline hydroxyapatite in the presence of polyphosphazenes after incubation for 24h at 37°C The hydroxyapatite-polymer composites formed from the precursors were finely ground using a mortar and pestle. The fine powder was mounted on a glass slide and analyzed using a XRD. The analysis was performed using an XRD (Scintag Inc., Sunnyvale, CA) from 20° to 40° (2-theta) with a step size of 0.02° and scan rate of 2° per minute.

Surface Morphology

The surface morphology of the hydroxyapatite formed from the composite precursors was analyzed using scanning electron microscopy (JEOL 6700F, USA). The samples were sputter coated with Gold / Palladium and were viewed under the SEM.

MC3T3-E1 cell adhesion on composites

Cell adhesion on the surface of the hydroxyapatites was evaluated to assess the viability of these biomaterials for bone regeneration. MC3T3-E1 cells obtained from ATCC (USA) were used in these studies. For seeding cells, composite matrices were prepared by placing the precursor paste in 48 well plates and gently pressed to form a uniform smooth surface. The matrices were incubated for 24 hours at 37°C in a humidified atmosphere. After incubation, the matrices were incubated with minimal essential media (MEM, Gibco, USA) for half an hour. Fifty thousand (50,000) MC3T3-E1 cells from the subcultures were plated onto the scaffolds and fed with MEM supplemented with 10% fetal bovine serum (FBS, Gibco, USA), and 1% penicillin-streptomycin (Gibco, USA). The media was changed every other day up to 3 days.

The adhesion of MC3T3-E1 cells on the surface of hydroxyapatite formed were evaluated qualitatively using a SEM (JEOL 6700F, USA) at 3 days. After 3 days in culture the matrices were washed with phosphate buffer and the cells were fixed 3% gluteraldehyde for 24 hours, and then washed with distilled water followed by air drying. The samples were sputter coated with Gold / Palladium and were viewed under the SEM.

RESULTS AND DISCUSSION

The formation of hydroxyapatite from the composite precursors after 24 hours incubation at 37°C was confirmed using a XRD. Figure 1a and b shows the XRD spectra of the hydroxyapatite from composites of CDHA and CDSHA in the presence of PNEA and $PNEA_{50}mPh_{50}$. Two peaks at 26° (2θ) and 32° (2θ) were observed. Normally crystalline hydroxyapatite exhibits three distinct peaks at about 31.83°, 32.10°, and 32.90° (2θ) [10]. The broad peak at 32° (2θ) indicates the formation of poorly crystalline hydroxyapatite from the

composite precursors at 37°C after 24 hours. Thus HA formed from the composite precursors are found to be poorly crystalline resembling the mineral component of bone [10].

The surface of the composites formed from the precursors were examined using a SEM. Figure 2 a, b, c, and d shows the surface morphology of PNEA-CDHA, PNEA-CDSHA, $PNEA_{50}mPh_{50}$-CDHA, and $PNEA_{50}mPh_{50}$-CDSHA respectively. The hydroxyapatite formed from all the four composite precursors was similar with no significant differences in gross morphologies. The connectivity of the spherical agglomerates as evidenced from the SEM leads to setting and rigidity of the cement [3].

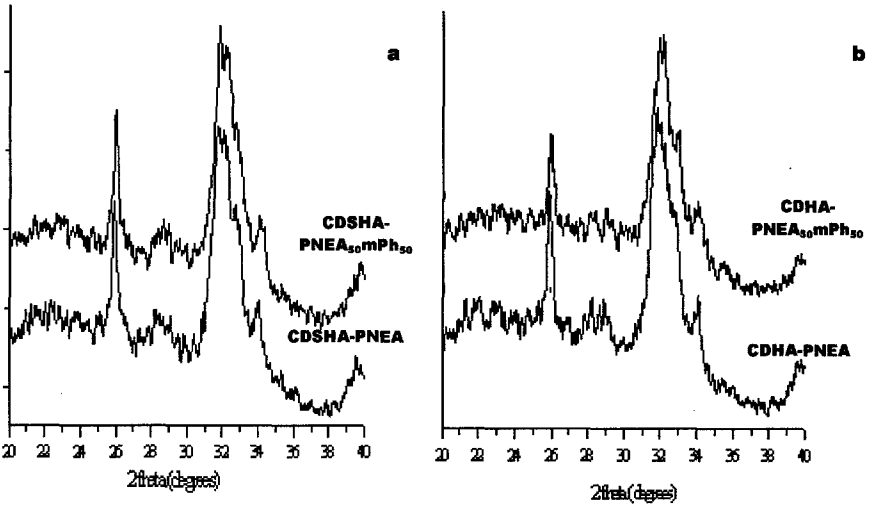


Figure 1 a, b. XRD of CDSHA-PNEA and CDSHA-$PNEA_{50}mPh_{50}$ (a), CDHA-PNEA and CDHA- $PNEA_{50}mPh_{50}$ (b) after 24 hours at 37°C.

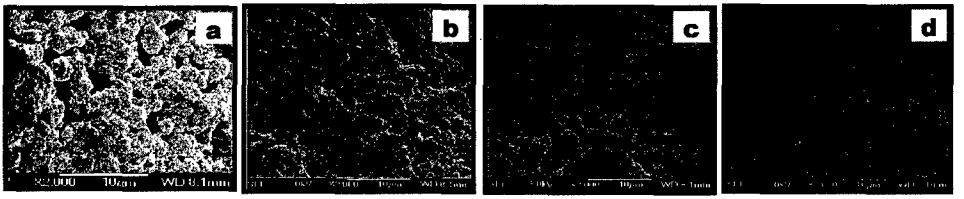


Figure 2. Surface morphology of poorly crystalline hydroxyapatite formed from composite precursors without cells (Magnification – X2000). (a) PNEA-CDHA; (b) PNEA-CDSHA; (c) $PNEA_{50}mPh_{50}$-CDHA (d) $PNEA_{50}mPh_{50}$-CDSHA.

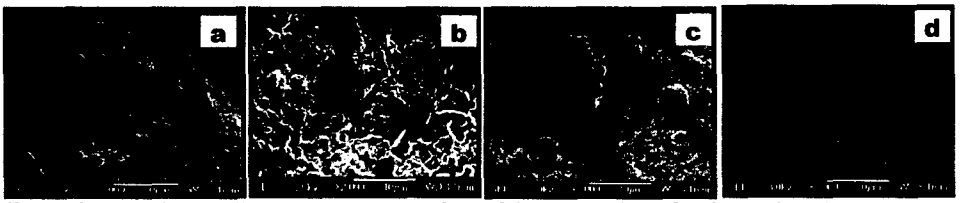


Figure 3. Osteoblast cell adhesion on the surface of the composites after 3 days in culture (Magnification – X2000). (a) PNEA-CDHA; (b) PNEA-CDSHA; (c) $PNEA_{50}mPh_{50}$-CDHA; (d) $PNEA_{50}mPh_{50}$-CDSHA

Figure 3 a, b, c, and d shows the adhesion of MC3T3-E1 cells on the surface of PNEA-CDHA, PNEA-CDSHA, $PNEA_{50}mPh_{50}$-CDHA, and $PNEA_{50}mPh_{50}$-CDSHA respectively after 3 days in culture. Osteoblast cells adhered and a monolayer of well spread cells were found on the surface of all the composites after 3 days in culture. Studies to quantitatively assess the cell proliferation and gene expression of MC3T3-E1 cells on the surface of the composites are underway.

CONCLUSIONS

The aim of the present study was to develop novel low temperature self setting composites using amino acid ester based polyphosphazenes and calcium deficient hydroxyapatites. The results showed that poorly crystalline hydroxyapatite resembling the mineral component of bone was formed. All the composites supported the adhesion of MC3T3-E1 cells showing that they are potential candidates for bone tissue engineering.

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