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Novel synthesis and characterization of a collagen-based biopolymer initiated by hydroxyapatite nanoparticles

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ABSTRACT

In this study, we developed a novel synthesis method to create a complex collagen-based biopolymer that promises to possess the necessary material properties for a bone graft substitute. The synthesis was carried out in several steps. In the first step, a ring-opening polymerization reaction initiated by hydroxyapatite nanoparticles was used to polymerize D,L-lactide and glycolide monomers to form a poly(lactide-co-glycolide) co-polymer. In the second step, the polymerization product was coupled with succinic anhydride, and subsequently was reacted with N-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide as the cross-linking agent, in order to activate the co-polymer for collagen attachment. In the third and final step, the activated co-polymer was attached to calf skin collagen type I, in hydrochloric acid/phosphate buffer solution and the precipitated co-polymer with attached collagen was isolated. The synthesis was monitored by proton nuclear magnetic resonance, infrared and Raman spectroscopies, and the products after each step were characterized by thermal and mechanical analysis. Calculations of the relative amounts of the various components, coupled with initial dynamic mechanical analysis testing of the resulting biopolymer, afforded a preliminary assessment of the structure of the complex biomaterial formed by this novel polymerization process.

1. Introduction

Bone grafting is a standard surgical practice, involving the replacement of deficient bone in order to repair bone fractures and bone defects caused by congenital disorders, traumatic injury or surgery of bone tumors [1,2]. It has been estimated that 6.8 million fractures occur annually in the USA [3–8], resulting in an estimated expenditure of US$21 billion [5,6]. More than half a million bone graft procedures are performed each year in the USA, which makes bone grafts the second most frequently transplanted material [5,8]. Bone grafts can serve as scaffolds onto which natural bone can grow and regenerate in order to repair the fracture or defect. Autogenous bone graft sources have been widely used since the early 1900s [9,10], involving the harvest of bone tissue from the patient's own body. Autografts have less probability of being rejected by the immune system and induce complications such as pathogen transmission. However, the shortcomings of autografts include limited availability and donor site morbidity [9–13], which give rise to various complications that result from obtaining tissue from the patient's own body. Complications include chronic pain, scarring, bleeding and infection at the site of bone tissue retrieval. An alternative is allogeneic bone grafting, obtained from human cadavers or living donors. There are issues associated with this treatment as well, such as immunogenicity and rejection reactions and the possibility of infection transmission [11–13].

In an attempt to circumvent the difficulties associated with either autologous or allogeneic bone tissue replacement, extensive current research is aimed at the development of a suitable scaffold made of synthetic or natural biomaterials. Such a scaffold should be able to promote the migration, proliferation and differentiation of bone cells for bone regeneration [14–25], and at the same time shield the patient from the perils of bone tissue harvesting and/or tissue rejection. At present, there are no synthetic bone graft substitute materials available that have similar or superior biological

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and mechanical properties compared to bone itself. Hydroxyapatite (HAP) bioceramic is currently used clinically as a bone graft substitute material. However, pure HAP bioceramic is only suitable for repairing small fractures or non-load-bearing bone defects because of its brittleness, low mechanical strength and weak fatigue resistance [16,19–20]. In order to overcome these limitations, resorbable polymers such as poly(L-lactide) (PLLA) were compounded with the ceramic materials such as HAP, resulting in a biocomposite that would exhibit improved mechanical properties [17–26]. However, the incompatibility between the polymer and the ceramic material due to the lack of bonding between the polymer and the inorganic moiety resulted in a phase separation at the polymer–filler interface. Such filler–polymer de-bonding and separation have been typically overcome by inducing covalent bonding between the filler phase and the polymer matrix [27–29]. Hong et al. [30] used this concept to successfully graft short chains of PLLA (PLLA oligomers) directly to the hydroxyl groups on the surface of nanocrystalline HAP and then blend up to 40 wt.% of the surface-modified particles into the PLLA matrix. The resulting composite material retained its biocompatibility, but became more ductile, most likely due to the plasticizing effect of the oligomer moiety. A more attractive system for bone repair applications is the biodegradable poly(D,L-lactide-co-glycolide) (PLGA) polymer, generated by the polymerization of lactide and glycolide monomers, because its degradation rate can be adjusted by altering the ratio of lactic to glycolic acids to match the rate of bone formation [31–33].

Clearly, the fabrication of synthetic bone graft substitutes that would match the exceptional ability of natural bone to self-repair is very challenging. It is most likely that a single phase system would not possess all the necessary materials characteristics, and hence modifications of the polymer matrix may be needed to modulate the properties of the composite and generate the required bioactive material. A possible strategy would consist of the surface modification of the polymer matrix in order to enhance certain surface properties such as hydrophilicity or chemical compatibility. A more efficient strategy would be to tether to the polymer a biological macromolecule, which, if properly chosen, would assist with both the compatibilization between the polymer matrix and the HAP moiety and with the integration with the surrounding tissue. Collagens, being the most abundant proteins in the body, and having suitable properties such as biodegradability, bioabsorbability with low antigenicity, high affinity to water and the ability to interact with cells through integrin recognition, are a very promising candidate for such a modification of the polymer matrix [14,34–36].

With the above preamble, it is clear that there is still a need for a resorbable polymer system with sufficient strength and toughness for bone tissue engineering applications. Separation at the composite filler–polymer interface suggests that bonding between all the components of the multi-phase system is required to obtain a mechanically and thermally stable material. In this study, the novel synthesis of a nano-HAP-PLGA-collagen (nHAP-PLGA-col) multiphase biomaterial was developed using HAP nanoparticles (nHAP) as initiators and eventually as the reinforcing inorganic phase. The synthesis process was carried out in several steps and was monitored by proton nuclear magnetic resonance (^{1}H NMR), Fourier transform infrared (FTIR) and Raman spectroscopies. The products after each step were also characterized by thermogravimetric analysis (TGA) and and differential scanning calorimetry (DSC). The estimation of the ratios of the components in the resulting complex biopolymer, coupled with its initial dynamic mechanical analysis testing, afforded a preliminary assessment of the structure of the complex biomaterial formed by this novel polymerization process.

2. Experimental

2.1. Materials

D,L-lactide and glycolide monomers were obtained from Ortec, Inc. (Easley, SC). HAP nanopowder (⩽200 nm) was purchased from Sigma Aldrich. Lyophilized calf skin collagen type I (M_w = 1,000,000 g mol^{-1}) was purchased from Elastin Products Company, Inc. (Owensville, MO). Stannous octoate, succinic anhydride (99%), N-hydroxysuccinimide (98%) (NHS), N,N-dicyclohexylcarbodiimide (DCC), N,N-dimethylformamide (DMF), ethyl acetate (99.5%), diethyl ether anhydrous, methylene chloride, toluene (99.8%), phosphate buffer (pH 7.4) and hydrochloric acid (HCl) were purchased from Fisher Scientific. All the solvents used (DMF, ethyl acetate, methylene chloride) were stored over molecular sieves (4 Å).

2.2. Polymerization of PLGA initiated by nHAP

The amount of 0.84 g nHAP was dried overnight in a vacuum oven at 80 °C to get rid of moisture. nHAP was further dried in a glass reactor placed in an oil bath for 30 min at 100 °C. 39.42 g of D,L-lactide (75 wt.%) and 10.58 g (25 wt.%) of glycolide monomers were added to the reactor and stirred with a stainless steel rotator while the oil bath temperature was set at 150 °C, which was chosen based on a previous study by Bendix [37]. The reactor was purged with nitrogen gas three times and when the monomer melted, the molten monomer and nHAP mixture was sonicated with a sonicator probe for 5 min. 0.05 g stannous octoate catalyst was dissolved in 10 ml toluene and added to the reactor when the oil bath temperature reached 150 °C. Samples were collected at different time intervals during the polymerization process to be analyzed by ^{1}H NMR. After 2 h of polymerization, the reaction was placed under vacuum (25 in Hg) for 30 min and then 0.2 g succinic anhydride was added subsequently to obtain a carboxyl end group, for the functionalization of the PLGA product. After an overall period of 4.5 h of polymerization, the reactor was taken out of the oil bath and the product at this stage was denoted as nHAP-PLGA co-polymer, which was collected with a spatula and dried overnight in vacuum at room temperature.

2.3. Activation of nHAP-PLGA with N-hydroxysuccinimide

10 g of previously prepared nHAP-PLGA co-polymer was dissolved in 200 ml dried methylene chloride and 0.39 g NHS (molar ratio of NHS/co-polymer = 10) and 0.09 g DCC (30% excess) was added according to the method followed by Porjazoska et al., who successfully attached collagen with poly(ethylene glycol)-poly(D,L-lactide-co-glycolide)-poly(ethylene glycol) (PEG-PLGA-PEG) tri-block co-polymer [14]. The reaction was allowed to continue for 20 h under a nitrogen atmosphere. The polymer solution was then dissolved in ethyl acetate and precipitated in anhydrous diethyl ether. The precipitated polymer was collected with a spatula and dried in vacuum at room temperature overnight. Dried nHAP-PLGA-NHS polymer was stored at 4 °C until it was used in subsequent steps.

2.4. Attachment of collagen to the PLGA co-polymer

160 mg calf skin collagen type I was dissolved in 223.8 ml 1 mM HCl in an ice bath and subsequently diluted with 300 ml 50 mM phosphate buffer (pH 7.4). 0.96 g nHAP-PLGA-NHS polymer was dissolved in 26.6 ml anhydrous DMF and added dropwise to the collagen solution in the ice bath according to the method followed

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