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Development of Biodegradable Polyphosphazene- Nanohydroxyapatite Composite Nanofibers Via Electrospinning

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Abstract

Biodegradable polymeric nanofibers are of great interest as scaffolds for tissue engineering and drug delivery due to their extremely high surface area, high aspect ratio and similarity in structure to the extracellular matrix (ECM). Polyphosphazenes due to their synthetic flexibility, wide range of physico-chemical properties, non-toxic and neutral degradation products and excellent biocompatibility are suitable candidates for biomedical applications. The objective of the present study was to develop and evaluate composite nanofibers of a biodegradable polyphosphazene, poly[bis(ethyl alanato)phosphazene] (PNEA) and nanocrystals of hydroxyapatite (nHAp) via electrospinning. A suspension of nHAp in dimethyl formamide (DMF) sonicated with PNEA solution in tetrahydrofuran (THF) was used to develop composite nanofiber matrices via electrospinning at ambient conditions. In the present study the theoretical loading of nHAp was varied from 50%-90% (w/w) to PNEA. The nHAp content (actual loading of nHAp) of the composite nanofibers was determined by gravimetric estimation. The composite nanofibers were characterized by transmission electron microscopy (TEM), gravimetry and energy dispersive X-ray mapping. This study demonstrated the feasibility of developing novel composite nanofibers of biodegradable polyphosphazenes with more than 50% (w/w) loading of nHAp on and within the nanofibers.

Keywords: Polyphosphazenes, Bone tissue engineering, Electrospinning, Nanofiber, Nanohydroxyapatite.

Introduction

Tissue engineering is defined as the application of biological, chemical and engineering principles toward the repair, restoration or regeneration of living tissues using biomaterials, cells and factors, alone or in combination [1]. This approach is emerging as an alternative therapeutic strategy in the treatment of a number of injuries including those of bone. In natural bone tissue, collagen nanofibrills constitute the extracellular matrix (ECM). The effort is to develop a biomaterial based scaffold which can mimic the structural properties of ECM [2]. These synthetic, biocompatible matrices should degrade within the body at a rate similar to the rate of tissue regeneration resulting in non-toxic degradation products [3]. Biodegradable polymeric nanofibers, due to their extremely high surface area, high aspect ratio and structural similarity to the ECM are generating considerable interest as scaffolds for tissue engineering [4].

Various processes are currently being investigated to fabricate fibers with diameters in the nanometer range such as template synthesis, self assembly, drawing, phase separation and electrospinning [5]. Among these, the process of electrospinning seems to be very promising due to the ease of fabrication, reproducibility, control over the process and comparatively lower cost [6]. In electrospinning, a polymer solution of suitable viscosity is subjected to an intense electrical potential which then undergoes a bending instability to afford fibers having diameter in the nanometer range.

We have recently demonstrated the fabrication of nanofibrous matrices from a novel class of polymer called polyphosphazene as potential candidate for tissue engineering applications [5,7]. Polyphosphazenes are polymers with an inorganic backbone of phosphorus and nitrogen atoms with two side groups attached to the phosphorus atom. These polymers can be rendered biodegradable by replacing the chlorine atoms of the macromolecular precursor poly(dichlorophosphazene), by suitable side groups such as alkoxides or with amines of low pKa [6].

The aim of the present study was to develop composite nanofibers from biodegradable polyphosphazenes and nano-sized hydroxyapatite (nHAp) crystals as candidates for bone tissue engineering applications. The drive for using nHAp as the reinforcing phase of a bone tissue engineering composite scaffold stems from the fact that natural bone has a composite structure, with hydroxyapatite (HAp) as a primary inorganic component composing approximately 70% of bone's dry weight. This apatite (calcium phosphate) resides within the bone as plate-like nanocrystals which are about 20-80 nm in length, along with other organic materials (principally collagen I nanofibrills), conferring the bone its structural rigidity and high modulus [8]. Earlier studies have shown HAp to be a bioactive material [9] and the presence of nHAp crystals in a composite could act as heterogeneous nucleators of the apatite, circumventing the large energy requirement of initial apatite formation required for the mineralization process to begin [8]. Moreover, physiological remodeling of the bone substitute materials *in vivo* might be facilitated by the smaller size of nHAp crystallites [9]. Hence, a composite tissue engineering scaffold with PNEA along with nHAp crystals may lead to an ideal tissue engineering scaffold which would impart biocompatibility, biodegradability and also bioactivity to the scaffolds.

Our current research involves development of novel nanofibrous composite scaffolds of biodegradable polyphosphazenes and nHAp crystals. The objective of the present study was to evaluate the feasibility of incorporating nHAp crystals into a biodegradable polyphosphazene (poly[bis(ethyl alanato) phosphazene]) (PNEA) nanofiber matrix and to determine the maximum loading of nHAp, to develop composite nanofiber scaffolds for applications in bone tissue engineering.

Experimental Section

Materials: Hexachlorocyclotriphosphazene (Nippon Fine Chemical Co. Tokyo, Japan) was purified by recrystallization from hexane followed by vacuum sublimation (55°C and at 0.05 mm Hg). All the solvents were pre-dried before use. Tetrahydrofuran (THF), (EM Science, Gibbstown, NJ) was distilled from sodium/benzophenone immediately before use in the reactions. Ethylalanine ester was obtained from Aldrich Chemical Co, Milwaukee, WI. N, N-dimethylformamide (DMF) (Sigma St. Louis, MO) was stored over molecular sieves (4°A) before use. A hydroxyapatite nanocrystal (10-30 nm) suspension in ethanol was obtained from Berkeley Advanced Biomaterials Inc., Berkeley, CA.

Characterization: NMR spectra were obtained at 298 K using a Bruker AMX-360 NMR spectrometer resonating at 360.23 MHz for ^{1}H, 145.81 MHz for ^{31}P, and 90.56 MHz for ^{13}C. All ^{1}H and ^{13}C NMR samples were prepared with deuterated THF (Isotec, 99.5 %) and referenced to tetramethylsilane (TMS). ^{31}P NMR shifts are relative to 85 % phosphoric acid as an external reference with positive shift values downfield from the reference. The molecular weight of the polymer was estimated using a Hewlett-Packard HP 1090 gel permeation chromatograph equipped with an HP 1047A refractive index detector and calibrated against polystyrene standards (Polysciences). The samples were eluted at a flow rate of 1 mL/min at 40°C with solution of 0.1% tetrabutyl ammonium bromide (Aldrich) in THF (OmniSolv). A 1 % solution of the polymer in THF was used for molecular weight determination. Energy Dispersive X-ray Mapping was done by Philips XL30 environmental scanning electron microscope with field emission gun equipped with energy dispersive x-ray spectroscope (FEI Company, Hillsboro, OR).

Synthesis of poly[bis (ethylalanato)phosphazene] (PNEA): Briefly, the macromolecular intermediate poly(dichlophosphazene) was prepared by the thermal ring opening polymerization of hexachlorocyclotriphosphazene (Nippon Fine Chemical Co.) at 250°C in an evacuated Pyrex tube. The chlorine atoms of poly(dichlorophosphazene) (8.0 g; 0.069 mol) were then replaced with L-alanine ethyl ester (42.4g; 0.276 mol) in the presence of triethylamine (86.6 mL, 0.622 mol). The polymer (PNEA) was isolated and purified by successive precipitations into hexane (3×) and pentane (2×). The product was an off-white fibrous material. **^{31}P NMR**: ($CDCl_3$), ppm : δ -1.1. **^{1}H NMR** ($CDCl_3$), ppm : δ 4.4 (1 H), 4.1 (2 H), 1.6 (3 H). 1.3 (3 H). Mn = 99282, Mw = 450967, PDI = 4.542; Tg = -3 ^{0}C

Hydroxyapatite loading: Nanocrystals were obtained by drying and lyophilizing commercially obtained 10-30 nm nanocrystals in ethanolic solution. The nHAp was then suspended in DMF by sonication at 50-60 Hz in a Fisher Scientific FS 21H sonicator. The suspension of nHAp in DMF was added to the polymer solution in THF and sonicated for another 30 minutes. The concentration in the suspension was varied from 50%-90% (w/w) of nHAp to the polymer (theoretical loading).

Electrospinning: The electrospinning apparatus used in the present study consisted of a 20 mL glass syringe fitted with a blunt end needle of 18 gauge and a grounded electrode. The grounded electrode consists of a copper plate covered with aluminum foil coated substrate placed at a 30 cm constant working distance from the needle tip. The syringe was fixed parallel to a grounded collection screen and the sonicated polymer/nHAp suspension was allowed to flow at a constant flow rate of 2.0 mL/hr using a syringe pump from Kent Scientific Corporation, Torrington, CT. A Gamma High Voltage Supply ES40P-20W (0-40 kV, 20 Watts, Gamma High Voltage Research, Florida) was used as the power source. A positive voltage was applied to the polymer solution in the glass syringe by attaching an alligator clip to the needle from the positive

lead. The electrospinning was carried out at ambient temperature and pressure. The spun composite nanofiber matrices were dried under vacuum at room temperature for 24 h.

X-ray mapping: Energy dispersive x-ray mapping of the fibers was performed to evaluate the morphology and presence of calcium within the nanofibers as an indication of hydroxyapatite loading on and within the fiber.

Transmission Electron Microscopy: To ascertain the different phases (nHAp and polymeric) present in the fiber and their disposition in the fiber, the composite nanofibers were embedded in an epoxy polymer and baked overnight at a temperature of 60^0C in a Fisher Scientific Isotemp 725G oven, Pittsburgh, PA. The embedded composite fibers in the hardened epoxy matrix were microtomed in 60-80 nm sections with an Ultracut E, Lieca Inc., Deerfield, IL instrument mounted on nickel grid with a coating of Formvar and stained with iodine/potassium iodide to stain the polymer phase. The diluted suspension of the DMF dispersed nHAp were mounted on platinum grids directly and dried. They were examined under a JEOL 100CX transmission electron microscope (JEOL, Boston, MA).

Gravimetry: To evaluate the actual loading of nHAp in the nanofiber matrix, a known weight of the matrix was collected in a pre-weighed crucible and heated at 800^0C overnight in a high temperature ceramic lined oven from Barkmeyer, Ney 6-160A, Yucaipa, CA, to remove the polymer phase of the composite fiber. The final weight of the crucible was taken after cooling down to room temperature and percentage loading of nHAp was calculated by the weight difference (actual loading). All the measurements were done in triplicate and reported as mean ± standard deviation.

Results

Mapping of calcium on the nanofibers: Fig. 1a shows the low resolution SEM of the composite nanofiber matrix loaded with nHAp. Fig. 1b clearly demonstrates the presence of the calcium along the same fibers indicating the incorporation of nHAp on and within the nanofibers.

Fig 1a) PNEA composite nanofibers with 70% nHAp

Fig 1b) PNEA composite nanofibers with 70% nHAp loading mapped for Calcium

1) X-Ray mapping of the composite nanofibrous matrix of PNEA loaded with 70% (w/w) nHAp

Transmission electron microscopy: Fig 2a shows the TEM of nHAp dispersed in DMF showing the presence of needle like crystals of about 10-30 nm diameter. Fig 2b shows the presence of the nHAp crystals aligned together to form a string like feature within the PNEA nanofiber matrix .

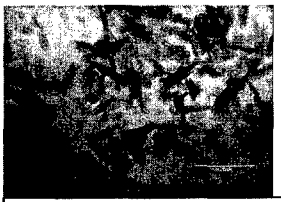


Fig 2a) TEM of nHAp suspended in DMF

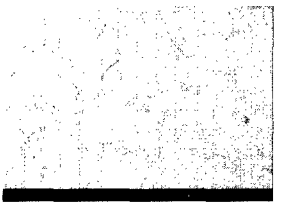


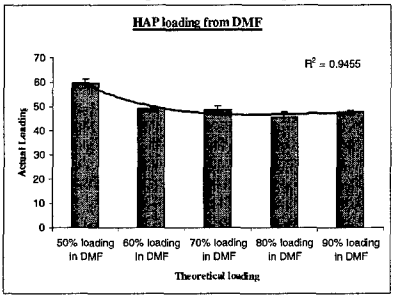
Fig 2b) TEM of composite PNEA nanofibers

Gravimetric Estimation of nHAp:

Fig. 3 shows the actual nHAp loading in the composite nanofibers as obtained from the gravimetric study. It has been found that a theoretical loading of 50% (w/w) nHAp yielded the maximum incorporation of nHAp within the fibers using DMF as a medium in the range of theoretical loading studied (50%-90% w/w). Higher percentages in theoretical loading of nHAp (60-90%) did not result in appreciable increment in the actual loading of nHAp in the fibers as evidenced from Fig. 3.

Discussion

Biodegradable amino acid ester substituted polyphosphazenes are excellent candidates for biomedical application due to their demonstrated biocompatibility, non-toxic and neutral degradation products. We are currently investigating alanine ethyl ester substituted homopolymer, PNEA, as a candidate polymer for developing bone tissue engineering scaffolds. We have previously demonstrated the excellent *in vitro* and *in vivo* osteocompatibility of PNEA. Recently, the feasibility of developing nanofibers of PNEA by the process of electrospinning has been demonstrated by us. The objective of the present study was to develop PNEA composite nanofibers with nHAp as a biodegradable scaffold that could better emulate the microstructure of bone which is composed of collagen nanofibrills and hydroxyapatite nanocrystals.



In the present study DMF was used as the suspension medium for nHAp as it is known to stabilize the nHAp crystals effectively because of the polarity and possibly due to its specific molecular structure [10]. The low resolution SEM (Fig 1a). shows the location of fibers in the composite matrix developed.

Fig 3) nHAp loading of Composite nanofibers

The presence of nHAp on and within these fibers was confirmed by the presence of calcium along the fiber (Fig. 1b) as the nHAp is the only source of calcium in the composite nanofibers.

Transmission electron microscopy was used to determine the morphology of the nHAp as well as the presence of the same within the fibers. The thickness of the needle shaped nHAp was found to be 10-30 nm (Fig. 2a). This size range should be very effective for incorporation of nHAp within PNEA fibers having a diameter of 100-310 nm, as in the present study. This has been confirmed by the TEM of nanofibers which clearly showed evidence of well dispersed nHAp crystals within and along the fibers (Fig. 2b).

Quantitative estimation of nHAp loading within the nanofibrous matrix was determined by gravimetry. Theoretical loading of 50% (w/w) of the nanofibers showed an actual loading of 59% (w/w) of nHAp in the nanofiber matrix. This could be due to the preferential electrostatic pull on nanocrystals by the grounded electrode compared to the polymeric phase. Further

increase in theoretical loading did not result in an incremental changes in actual loading presumably due to the instability of the nHAp suspension at higher concentration. We are currently investigating the effect of different suspension media on the actual loading of nHAp in PNEA nanofibers.

Conclusions

In this study we demonstrated successfully the fabrication of biodegradable PNEA/nHAp composite nanofiber matrix. The biodegradability and biocompatibility of PNEA along with the bioactivity of nHAp makes them potential candidates as scaffolds for bone tissue engineering and other biomedical applications.

Acknowledgements

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