Design and Synthesis of Hydroxyapatite Composites Containing an mPEG−Dendritic Poly(L-lysine) Star Polycaprolactone

Keri A. Boduch-Lee,[†] Toby Chapman,*[,†] Sarah E. Petricca,[‡] Kacey G. Marra,[§] and Prashant Kumta[‡,⊥]

Department of Chemistry and Department of Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, and Department of Biomedical Engineering, Institute for Complex Engineered Systems, and Department of Materials Science Engineering, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213

Received March 31, 2004; Revised Manuscript Received August 9, 2004

ABSTRACT: The design and synthesis of star polycaprolactone−hydroxyapatite films for use as a biodegradable matrix for bone tissue engineering will be presented. mPEG-dendritic-poly(L-lysine) terminated with glycolic acid residues serve as the star polymer cores for attachment of polycaprolactone by ring-opening polymerization of ϵ-caprolactone. Initial studies involved testing physical blends of the *tert*-butoxycarbonyl (Boc) and glycolamide terminated dendrons, generations zero through six, with varying concentrations of hydroxyapatite (HA). The star polymer had not only drastically different solubility but also greatly improved film quality. Further improvements were made in film quality and homogeneity by utilizing the star polymers in the presence of a chemical synthesis of hydroxyapatite. The improved films were then tested for their mechanical strength properties. The star polymer from generation 6.5 and 20 wt % of hydroxyapatite exhibited good mechanical properties with an average Young's modulus of 1.77 MPa, tensile strength of 4.19 MPa, and ultimate elongation of 19%. The details of the chemical synthesis of hydroxyapatite in the presence of the star polymer are discussed.

Introduction

As our population ages, the need for bone substitutes is increasing rapidly. The National Osteoporosis Foundation in their 2002 publication, *America's Bone Health*, estimates that over 10 million people already have osteoporosis while 50% of all women and 25% of all men over the age of 50 will suffer from an osteoporotic fracture in their lifetime. The current therapies include autografting, allografting, and the use of some synthetic materials. Current synthetic materials are often made of plastics or metals which are molded and implanted to replicate the needed tissue. These can invoke an immune response by the recipient and often pose blood compatibility problems. In addition, most of these materials are subject to fatigue and wear. They are not capable of remolding, growing, and regenerating with time. They often have a limited lifetime and so are problematic for young patients.

The goal in tissue engineering seems to have shifted to the development of materials that will not only be viable substitutes for tissues but will restore, maintain, and even possibly enhance the material's function over time, making it possible to incorporate the new material and lead to permanent repair. (For reviews of tissue engineering see refs 1 and 2.) Specifically, much of the new research in this field has been centered on finding a scaffold that, while maintaining the structure and strength, will also allow for natural tissue to develop, heal, and eventually replace the scaffold.

Currently, hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$, is a commonly used ceramic in bone tissue engineering. This inorganic ceramic is similar to the apatite found in natural bone. Natural apatite, which makes up approximately 45% of total bone mass, is a poorly crystalline material because of the incorporation of additional ions such as magnesium and carbonate.[3] In this way, natural apatite differs from commercially available HA which is relatively crystalline. HA can be implanted without any adverse reactions as it is protein-free and it also participates in bone bonding through osteogenesis.[4] It is osteoconductive;[5] large quantities are easily obtainable and can be fabricated into a porous or fully dense ceramic. However, by itself, its poor tensile strength makes it impossible to sustain the rigors of load-bearing applications. Additionally, the chalky powder forms brittle films making it difficult to process into complex shapes, causing it to crumble under stress.[6]

HA, while having poor strength on its own,[5] when combined with polymers provides a composite with improved strength.[5,7] Degradable polymers have an added advantage because they do not have to be removed, eliminating the need for additional surgeries. The polymeric part of the composite is then metabolized and excreted, while the HA is assimilated in the body.[8] Scaffolds provide temporary mechanical support until the tissue has healed. The scaffold must then gradually transfer stress back to the healing tissue, while degrading at a rate similar to the rate of healing. Many polymers have been utilized to attempt to accomplish these goals for scaffolding, including peptides and hydrogels, but the two most widely used polymers have been poly(glycolic acid), PGA, and poly(lactic acid), PLA.[9−16]

While numerous polymer−HA composites have been explored, little has been conducted with dendrimers and HA. As demonstrated, dendrimers have multiple func-

[†] Department of Chemistry, University of Pittsburgh.
[‡] Department of Biomedical Engineering, Carnegie Mellon University.
[§] Department of Surgery, University of Pittsburgh, and Institute for Complex Engineered Systems, Carnegie Mellon University.
[⊥] Department of Materials Science Engineering, Carnegie Mellon University.
* To whom all correspondence should be addressed. E-mail Tchapman@pitt.edu.

10.1021/ma0493630 CCC: $27.50
Published on Web 10/29/2004

tionalities, especially at the termini which are all available for chemical reaction or designed interactions. This can be advantageous in tissue engineering. The multiple functionalities of the dendrimer should allow for increased interaction with the HA ceramic. This symbiotic interaction would strengthen the matrix and could aid in limiting the crystallization of the HA. This amorphous version of HA would more closely resemble the apatite naturally found in bone. The increased number of interactions would also allow for the increased strength of the HA within the composite. HA is normally a brittle chalky ceramic, and the interactions with the dendrimer could allow the formation of a tough and flexible film. There are two recent examples of dendrimers synthesized for possible tissue engineering applications. Carnahan and Grinstaff synthesized a poly(ether−ester) dendrimer from glycerol and lactic acid.[17] The same group also synthesized a dendrimer based on glycerol and succinic acid for the same purposes.[18]

Star polymers as well as dendrimers have an architecture which may also be useful in tissue engineering. These polymers begin with a multifunctional core to which multiple linear arms are attached. One example of a star polymer incorporating poly(ϵ-caprolactone) was reported by Hedrick and co-workers.[19] In his first report, ϵ-caprolactone was polymerized on the ends of a multifunctional core. More recently, other groups have also worked on synthesizing star polymers for various applications.[20] In one recent paper, Sanda et al. reported three- and four-armed star-shaped poly(ϵ-caprolactone) polymers.[21] Fréchet and co-workers performed a ROP of ϵ-caprolactone on a dendritic core to yield a star polymer.[22] Additionally, a multifunctional core was used to form a star polymer with block copolymers of PCL and poly(DL-lactic-*co*-glycolic acid) arms.[23] Finally, Zhao et al.[24] have effected star polymer formation by ROP of L-lactide onto hydroxyl terminated PAMAM dendrimers. Because of the biocompatibility and biodegradation of these molecules, all of these structures could have applications in drug delivery or tissue engineering.

Dendrimers alone are not known to make good films because they lack the entanglements seen in linear polymers.[25] These entanglements allow for the film flexibility and strength. By attaching linear segments to the ends of the dendrimer, the entanglements should increase, leading to better film formation. The addition of PCL would retain hydroxyl terminated groups on the polymer structure, which would allow the potential for strong HA interactions, such as hydrogen bonding to the phosphate groups. PCL has already proven to be useful in many medical applications such as surgical sutures[26] and drug delivery.[27] In addition, PCL is not water-soluble while it is biodegradable, making it possible to use in the body as a matrix for tissue engineering. The amine and alcohol terminated PEG−poly(L-lysine) dendrimer are water-soluble and therefore not able to be used during the chemical synthesis of HA. The procedure calls for a water washing during the purification process during which any water-soluble polymers would simply be washed away. This would also occur in aqueous media inside the body. By adding the linear PCL arms to the dendrimer, the resulting star polymer becomes insoluble in water, making it possible to explore the chemical synthesis of HA in the presence of the polymers.

Previously, Stupp and co-workers synthesized hydroxyapatite in the presence of polypeptides and polyelectrolytes.[10] Poly(sodium acrylate), poly(L-lysine), and poly(L-sodium glutamate) were dissolved in the media while nucleation and growth of apatite crystals occurred. The dissolved polymers may have induced mineral nucleation but also quenched crystal growth by adsorbing the mineral onto the surfaces. They showed the ability to intimately incorporate apatite with a more poorly crystalline nature directly in the polymer.

This project focuses on the synthesis of new materials to be used as scaffolds to support natural bone growth. In this work, we are focusing on a previously reported mPEG−poly(L-lysine) dendrimer hybrid.[28] Poly(L-lysine) dendrimers were chosen partly because of their biocompatibility and biodegradability. It can be thought the degradable products of the polymer would be the amino acid L-lysine, essential for the biosynthesis of proteins. This dendrimer was then altered to incorporate hydroxyl termini by capping the dendrimer as a glycolic acid amide. To introduce the star polymer, these dendrimers were used as the initiators for caprolactone polymerization. These dendrimer and star polymers were tested for their ability to make films with HA by physical mixing as well as chemically synthesizing HA in the presence of the polymer.

Experimental Section

Materials. Diisopropylethylamine (DIEA) and tin(II) ethylhexanoate were purchased from Aldrich Chemical Co. ϵ-Caprolactone (Aldrich) was distilled under vacuum. Dimethylformamide (Fisher) was distilled over P_2O_5. Methylene chloride (Fisher) was distilled under N_2 from CaH_2. Calcium nitrate tetrahydrate (99% A.C.S. Reagent) and phosphoric acid (85 wt % solution in water, A.C.S. Reagent) were purchased from Aldrich. Ammonium hydroxide (assay 29.6% m) was used from Fisher with no special preparation. Ethylenediaminetetraacetic acid (Sigma), PBS solution (Gibco, Invitrogen Corp.), and CellTiter 96 AQ_{ueous} One Solution Reagent (Promega) were used as received. MG63 cells were obtained from ATCC (Manassas, VA). All other reagents were used as received.

pH Monitoring. The monitoring of the pH was done with an Accumet 916 pH meter and the AccuTupH+ electrode from Fisher Scientific.

Mechanical Testing. Samples prior to the tensile strength measurements were cut into thin strips, approximately 12 mm in length and 5 mm in width. Tensile strength tests were performed on an Instron (model 5500-R) employing a crosshead speed of 2 mm/min with gauge dimensions 7 mm in length and 5 mm in width. An Instron static load of 100 N was utilized for each test.

Phase Analysis. To verify that the calcium phosphate synthesized was in fact HA, phase analysis of the composites was performed using X-ray diffraction (XRD). The Philips X'Pert PRO X-ray diffraction system was operated at 45 kV and 40 mA at a 2θ range of 10−80°, using a step size of 0.05 with a 2 s exposure time.

Size Exclusion Chromatography (SEC). SEC analyses were performed on a SEC line consisting of a 510 pump, a U6K universal injector, and a 410 differential refractometer (all Waters). Separations were achieved at 35 °C across a set of two 5 μm columns (linear and 500A) from Phenogel with THF (HPLC grade, Fisher) eluting at 0.400 mL/min.

Synthesis of Poly(L-lysine) Dendrimer (1). The synthesis of poly(L-lysine) was carried out as previously reported by our group.[28]

Synthesis of *N*-Hydroxysuccinimidyl Glycolate (2). This synthesis was accomplished utilizing a DCC to couple *N*-hydroxysuccinimide to glycolic acid according to the procedure of Flouret and co-workers.[29]

Synthesis of Glycol Amide-Capped Dendrimer (3). (Generation 4.5 is used as a general synthesis; all other generations are synthesized in a similar manner.) A stirred solution of **1** (4.50 g, 0.51 mmol) and **2** (2.83 g, 16.35 mmol) in DMF (45 mL) was purged with N_2. The solution was then stirred as DIEA (5.70 mL, 32.70 mmol) was added via syringe. The solution was allowed to stir under N_2 for 12 h. The solvent was then removed in vacuo to yield a viscous oil. Approximately 5 mL of deionized water was added to the oil. The solution was then placed in dialysis tubing (MWCO 3500) and stirred in deionized water for 4 days, changing the water bath every 24 h. The tube was then emptied and the solvent removed in vacuo. The pale yellow solid was then dissolved in minimal DMF and precipitated into anhydrous ether. Drying yielded a white powdery product. Yields relative to previous generation: generation 4.5 (58%), generation 5.5 (87%), generation 6.5 (51%). ^{1}H NMR (300 MHz, MeOD): generation 4.5, δ 4.63 (d, C**H**−H), 4.55 (d, CH−**H**), 4.18 (s, CH), 3.95 (s, C**H**−H), 3.89 (s, CH−**H**), 3.56 (s, OCH_2), 3.07 (s, CH_2), 1.61 (br s, CH_2), 1.36 (br s, CH_2), 1.20 (br s, CH_2) Found area ratios, relative to PEG (averaged for three spectra): 0.0135:0.0135:0.0337:0.0127:0.0127:1:0.0433:0.0445:0.0481:0.0372; respectively. Calculated area ratios, relative to PEG: 0.0176:0.0176:0.033:0.0176:0.0176:1:0.066:0.066:0.066:0.066; respectively. ^{1}H NMR (300 MHz, MeOD): generation 5.5, δ 4.63 (d, C**H**−H), 4.55 (d, CH−**H**), 4.16 (s, CH), 3.95 (s, C**H**−H), 3.88 (s, CH−**H**), 3.53 (s, OCH_2), 3.13 (s, CH_2), 1.58 (br s, CH_2), 1.30 (br s, CH_2), 1.16 (br s, CH_2). Found area ratios, relative to PEG (averaged for three spectra): 0.0421:0.0421:0.0819:0.0486:0.0486:1:0.1387:0.1645:0.1673:0.1592; respectively. Calculated area ratios, relative to PEG: 0.0352:0.0352:0.0682:0.0352:0.0352:1:0.1364:0.1364:0.1364:0.1364; respectively. ^{1}H NMR (300 MHz, MeOD): generation 6.5, δ 4.56 (br s, CH_2), 4.19 (s, CH), 3.91 (br s, CH_2), 3.55 (s, OCH_2), 3.14 (s, CH_2), 1.61 (br s, CH_2), 1.31 (br s, CH_2) Found area ratios, relative to PEG (averaged for three spectra): 0.1778:0.1641:0.1881:1:0.2561:0.2707:0.2852:0.2671; respectively. Calculated area ratios, relative to PEG; 0.1408:0.1386:0.1408:1:0.2772:0.2772:0.2772:0.2772; respectively.

Synthesis of Poly(ϵ-caprolactone) Star Dendrimer (4). (Generation 4.5 is used as the general synthesis; all other generations are synthesized in a similar manner.) A flask containing **3** (0.50 g, 0.063 mmol) was purged with N_2. ϵ-Caprolactone (5.21 mL, 48.88 mmol) was then added via syringe, and the mixture was heated to 110 °C. Tin octoate (0.015 mL, 0.049 mmol) was then added via syringe. The solution was allowed to stir for 24 h. The reaction mixture was dissolved in CH_2Cl_2 to produce a viscous solution. The gel was added slowly to cold methanol to produce a white solid. The solid was dried. The solid was then reprecipitated by dissolving in CH_2Cl_2 and adding it slowly to cold methanol again. This solid was dried to yield the white product. ^{1}H NMR (300 MHz, $CDCl_3$): generation 4.5; δ 4.04 (t, CH_2), 3.62 (s, CH_2), 2.29 (t, CH_2), 1.65 (m, CH_2), 1.36 (m, CH_2). Found area ratios, relative to PEG; 8.6:1:8.6:18.8:9.2; respectively. Generation 5.5; δ 4.04 (t, CH_2), 3.63 (s, CH_2), 2.29 (t, CH_2), 1.63 (m, CH_2), 1.38 (m, CH_2). Found area ratios, relative to PEG; 11.9:1:11.8:26.7:12.7; respectively. Generation 6.5; δ 4.04 (t, CH_2), 3.62 (s, CH_2), 2.28 (t, CH_2), 1.62 (m, CH_2), 1.36 (m, CH_2). Found area ratios, relative to PEG; 27.5:1:26.2:60.6:28.6; respectively.

Dendrimer−Hydroxyapatite Physical Blends. Each generation of the dendrimers, **1** and **3**, was dissolved in CH_2Cl_2. This solution was then placed into a Teflon-coated dish. While stirring, commercial HA powder was added. This was then covered with a Kimwipe, and the solvent was allowed to evaporate, leaving a composite blend. The weight percent of HA was varied between 0%, 20%, and 40% for each moiety. The dendrimer mass was adjusted to maintain a total mass of 0.5 g for each blend.

Star Polymer−Hydroxyapatite Physical Blends. The star polymer, **4**, of each generation was dissolved in THF. This solution was then placed into a Teflon-coated dish. While stirring, HA powder was added. This was then covered with a Kimwipe, and the solvent was allowed to evaporate. The star polymers films were made with no HA present as well as at 20% by mass at each generation. The star polymer mass was adjusted to maintain a total mass of 0.5 g for each blend.

Chemical Synthesis of Star Polymer−Hydroxyapatite Composites. In a small beaker, H_3PO_4 (50 μL, 0.746 mmol) was added to THF (15 mL). Enough NH_4OH (~0.310 mL) was then added to achieve a pH of 8.0. This solution was allowed to stir, covered, for 30 min followed by addition of $Ca(NO_3)_2 \cdot 4H_2O$ (294 mg, 1.244 mmol). This mixture was stirred for approximately 4 h. The generation 6.5 poly(ϵ-caprolactone) star (0.50 g, 0.674 μmol) dissolved in THF (15 mL) was then added to the reaction mixture. After stirring covered for an additional 2 h, the solvent was allowed to evaporate to form a viscous solution. During this process, the pH is maintained at ~7.4, adding additional NH_4OH if necessary. Once the solution had become fairly viscous, the composite solution was poured into a Teflon beaker and allowed to dry overnight. These composites were then removed from the beaker and immersed in water for 3 days to remove any traces of ammonium nitrate prior to testing. The final composites contained 20 wt % HA.

Cell Adhesion Studies. MG63 cells were cultured using F12 media, supplemented with 5% fetal calf serum and 1% penn/strep. The MG63 cells were incubated at 37 °C and 5% CO_2. Once confluency was reached, the cells were trypsinized using 0.25% trypsin solution with 1 mM ethylenediaminetetraacetic acid for 5 min. Once counted using a hemacytometer (Hausser Scientific Co.), 100 000 cells were added to each individual well, which contained a sample of composite material; generation 6.5 PCL star, straight chained PCL or no sample, which formed the positive control.

The cells were incubated on the scaffold material for either 4 or 24 h, at which point the media and nonattached cells were aspirated. Each well was washed three times with PBS solution. Media (200 mL) and CellTiter 96 AQ_{ueous} One Solution Reagent (20 mL) were pipeted into each well of the 96 well assay plate containing the samples. The plate was then incubated in a 37 °C and 5% CO_2 atmosphere for 2 h. The absorbance was measured at 490 nm using a Tecan SpectraFluor.

Results and Discussion

Synthesis. The dendrimer synthesized is based on the amino acid L-lysine which was originally synthesized by Denkewalter.[30] In our synthesis it is assembled on a commercial poly(ethylene glycol), PEG, support.[28] This support allows for a straightforward isolation of the resulting dendrimer by precipitation in diethyl ether. The dendrimer, **1**, is constructed by stepwise addition of the lysine-based monomer using typical peptide coupling reactions (Figure 1).

Each layer is assembled by coupling an activated ester of the lysine monomer to the free amine on the termini of the previous generation. The amines of the monomer are protected by *tert*-butoxycarbonyl (Boc) groups to ensure only one layer is coupled at a time. Once the coupling is complete and the molecule is isolated, the new amine termini are deprotected by acid treatment. Thus, the molecule is ready for the next generation of monomer to be coupled, and the process is repeated until the desired number of generations has been added. For ease, dendrimers are named in whole and half generations. Each generation is the addition of a layer of di-*t*-Boc-lysine and thus represents a doubling of end groups. The half generations, then, are the deprotected generations and will have the same number of end groups as the whole generation counterpart. So, while the whole generation dendrimer, in this case, is terminated by the Boc protecting group, the dendrimer half generations are terminated by the trifluoroacetate ammonium salt (**1**).

For the synthesis of hydroxyl terminated dendrimers, the activated ester, **2**, was coupled to the dendrimer

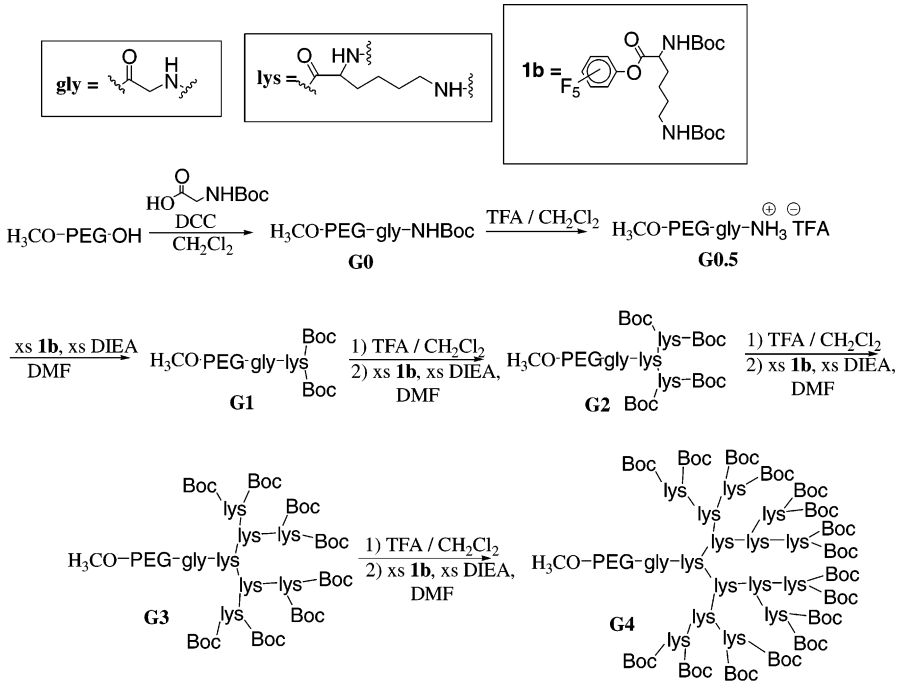


Figure 1. Synthesis of poly(L-lysine) dendrimer.

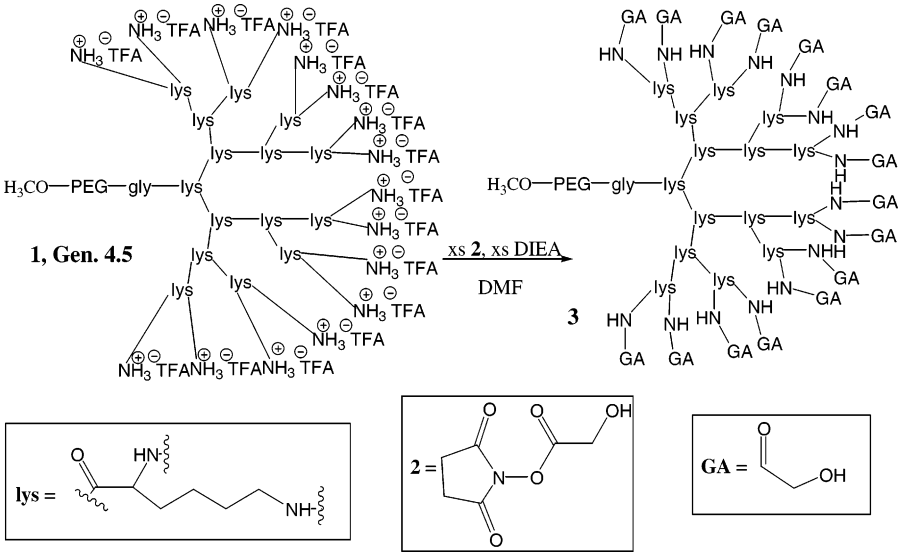


Figure 2. Synthesis of glycol amide-capped dendrimer (generation 4.5 shown).

(generations 4.5, 5.5, and 6.5) to produce **3** after purification (Figure 2). ^{1}H NMR was used to check for complete addition of the glycol amide termini to the dendrimer. Since the relative peak size compared to the corresponding PEG peak was rather small, the averaged integration value for three spectra was reported. In addition, to ensure the reaction went to completion, ninhydrin was used to test for free amines present due to unreacted dendrimer termini. All reactions were shown to be negative to the ninhydrin, supporting complete addition of the glycol amide termini.

The poly(ϵ-caprolactone) star polymer **4** was synthesized by ring-opening polymerization with tin octoate as the catalyst[8] (Figure 3), initiated by the hydroxyl termini of **3**. The polymerization was carried out in the bulk due to the ability to solubilize the dendrimer in ϵ-caprolactone at 110 °C. A simple methanol precipitation allowed for the purification of **4** from any unreacted dendrimer and ϵ-caprolactone monomer.

The degree of polymerization of the PCL was determined by ^{1}H NMR. The area represented by the methylene hydrogens within the poly(ethylene glycol), PEG, was compared to internal methylene groups of poly(ϵ-caprolactone). Since the average molecular weight of the PEG, determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS),[30] is known to be 5036 g/mol, the average number of methylene protons in the PEG is 454. This comparison allowed us to determine the number of repeat units present in the PCL. Assuming that all termini have reacted equally, an average degree of polymerization for each chain can also be determined. Using this information, the total mass of PCL per molecule as well as the mass of the entire star can be found. These values for each generation are shown in Table 1.

Physical Blends. The film quality was determined by its visual composition, flexibility, and the solubility

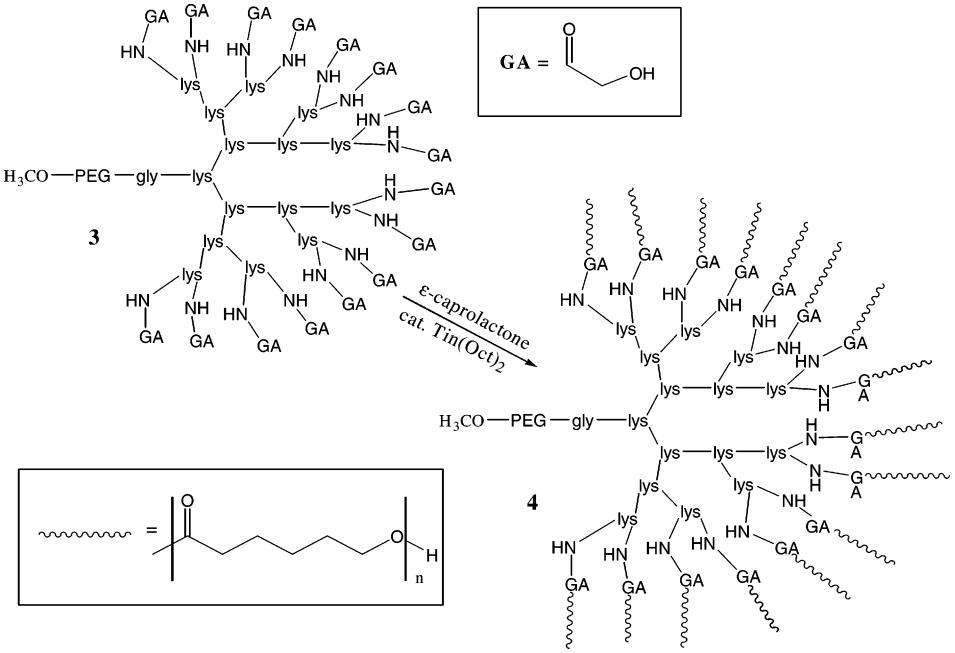


Figure 3. Synthesis of poly(ϵ-caprolactone) star polymer (generation 4.5 shown).

Table 1. Molecular Weight of the PCL Star Determined by ^{1}H NMR

generation	PEG:PCL[a]	DP[b]	M_n of PCL[c]	M_n of star
4.5	1:8.6	121.5	225 931	233 859
5.5	1:11.9	84.3	313 515	324 437
6.5	1:27.5	97.5	725 213	742 029

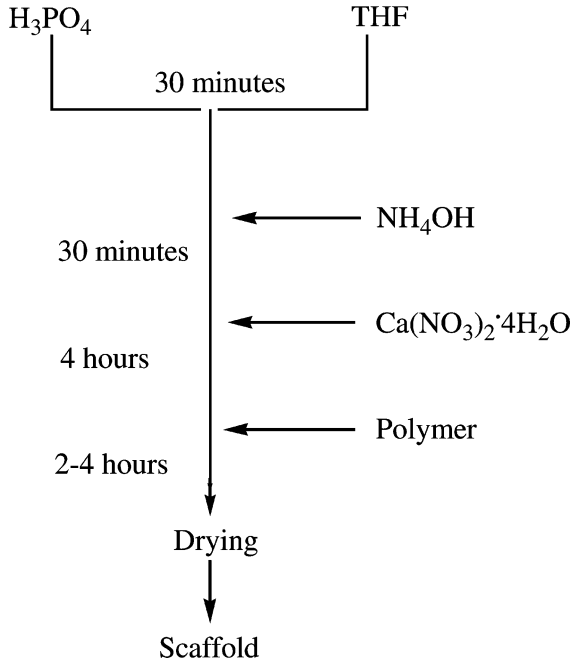
[a] PEG methylene protons at 3.62 ppm vs PCL methylene protons at 4.04 ppm. [b] Average number of units per chain assuming all termini reacted equally. [c] Total mass of all PCL chains in the entire molecule.

of the film when placed in deionized water. The simple amine and Boc terminated dendrimers, when physically mixed with hydroxyapatite, could be made into semiflexible films at higher generations (Gen. 4−6). The lower generation blends were brittle and soluble in water. The glycol amide terminated dendrimers (Gen. 4−6) could also be processed into semiflexible films. These films, as expected, were water-soluble. All of the films, however, were heterogeneous as it appeared that the HA, which was insoluble in the solvent conditions, aggregated, and settled quickly during the evaporation process. The PCL star polymers, as they contained high PCL weight percentages, were insoluble in water and were able to form flexible films without the presence of hydroxyapatite. At 20 wt % HA content, the films remain flexible; however, when the wt % is increased to 40 wt %, the films become brittle and chalky.

Chemically Derived HA Composites. The PEG-dendrimers terminated as amines or glycol amides were not suited for the chemical synthesis of HA because of their water solubility. The chemical synthesis of HA was conducted in an organic solvent in which the dendrimer was also soluble, followed by a washing with water to remove any ammonium nitrate salts that were formed as byproducts during the procedure. This aqueous washing would have also dissolved the dendrimer and glycol amide-capped dendrimer, making the procedure impossible on such materials.

The water-insoluble PCL star, however, was an excellent candidate for such a procedure (Scheme 1). The star polymer dissolved easily in THF and afforded an easy way to introduce the polymer during the HA synthesis.

Scheme 1. Schematic of Chemical Synthesis of HA Dendrimer Composite



It was imperative that the relative amounts of each component added during the HA synthesis ensure that a 1.67 ratio of calcium to phosphorus is achieved. If these ratios were altered, the formation of HA was not seen and instead other calcium-containing ceramics would be formed. During our synthesis, however, as determined by the X-ray diffraction analysis of the polymer composite compared to HA by itself, the diagnostic peaks from HA as well as those from PCL are clearly visible (Figure 4). The characteristic signature peaks seen at 2θ values of 31.79° ((121) plane), 46.73° ((222) plane), 49.53° ((123) plane), and 53.28° ((004) plane) are associated with the formation of HA, while the diagnostic PCL peaks can be found at 2θ values of 21.12°, 23.41°, 43.78°, 51.17°, and 72.61°, respectively. These peaks are readily seen in the composite spectrum,

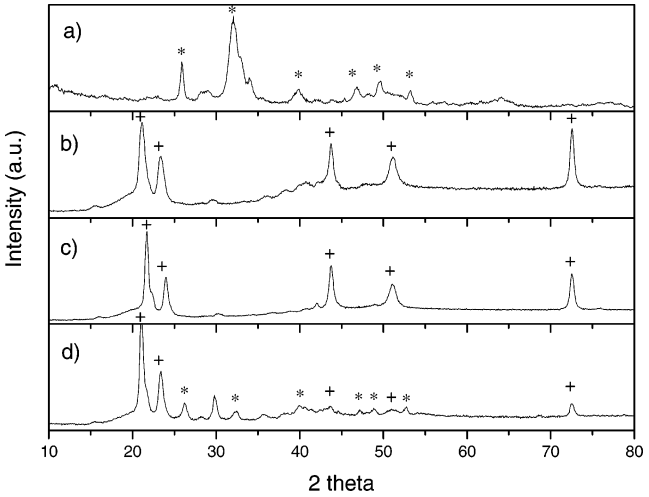


Figure 4. X-ray diffraction patterns: (a) HA alone, (b) PCL alone, (c) generation 6.5 PCL star polymer, and (d) generation 6.5 star polymer/HA composite from chemical synthesis (∗ indicates characteristic HA peak, while + denotes that of PCL).

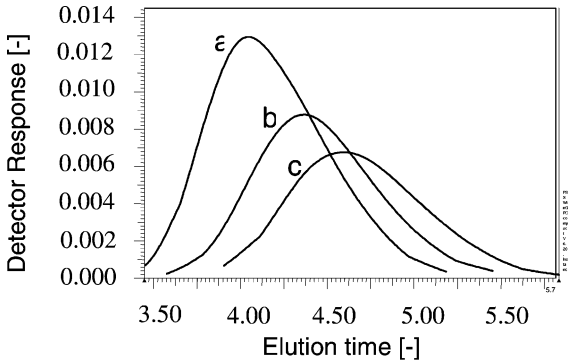


Figure 5. GPC traces from degraded generation 6.5 PCL star polymer: (a) generation 6.5 PCL star control, (b) generation 6.5 PCL star after 15 min (pH = 3.3), and (c) generation 6.5 PCL star after 60 min (pH = 2.0).

although the HA peaks in the composite are reduced in intensity and somewhat broadened. The composite has only 20% HA, and the broadened peaks may reflect microcrystallinity.

During initial studies of the chemical synthesis of HA, the pH was not being monitored, and the results from the composites were variable, sometimes failing to give flexible films, forming primarily brittle materials. It was thought the polymer star might be sometimes degrading during the synthesis of HA. It was decided to analyze the polymer using GPC as the HA reaction progressed. In addition, if the polymer was degrading, the thought was that it was pH dependent. Aliquots of the reactions mixture were removed at specific time intervals, and the pH at each time was also measured. The aliquots were dried in vacuo, dissolved in HPLC grade THF, filtered, and chromatographed. The pH was found to quickly drop from the initial pH of ~9 to an acidic pH. The pH continued to drop as the reaction continued, and the HA was formed, eventually reaching as low as a pH of ~1.5. Therefore, as expected and seen by GPC, there was significant degradation seen in the polymer stars, presumably ester hydrolysis of the PCL (Figure 5). The initial generation 6.5 star polymer showed a GPC M_n of 143 510. After only 15 min the molecular weight had decreased to 57 337, as the HA synthesis conditions had decreased the pH to 3.3. At the 1 h mark, the M_n was found to be 26 988 and the pH was at 2.0. The actual

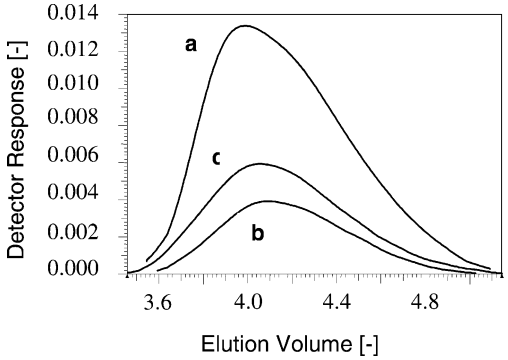


Figure 6. GPC traces from generation 6.5 PCL star polymer in controlled pH environment: (a) generation 6.5 PCL star control, (b) generation 6.5 PCL star after 30 min (pH = 7.0), and (c) generation 6.5 PCL star after 60 min (pH = 6.5).

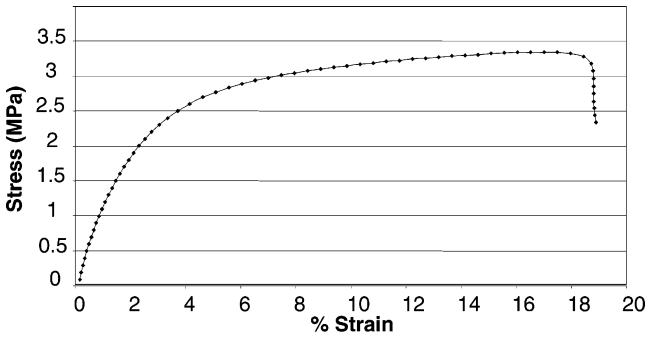


Figure 7. Sample Instron measurement from generation 6.5 PCL star polymer composite.

M_n values determined by GPC are not accurate as the polymer architecture has a large effect on the resulting M_n, and there are no ideal standards available. Since GPC separates samples based on hydrodynamic size, branched polymers such as dendrimers and star polymers which have a more compact structure compared to that of a linear PEG would appear to have a smaller M_n for an equal mass. These values, therefore, are lower than those shown in Table 1 as determined by ^{1}H NMR. While these GPC M_n values are not absolute, they are conclusive that a dramatic molecular weight loss could be seen as the chemical synthesis of HA progressed. This weight loss was seen throughout the 120 min that the aliquots were removed from the reaction mixture. However, after 1 h the rate of degradation had somewhat abated. By the end of the 120 min, the M_n value had decreased to approximately 25 000 and the pH was 1.6. Reproducible, good films are obtained with careful control of the pH as described in our procedure.

The chemical synthesis procedure was altered to monitor the pH during the entire reaction and maintain the pH of approximately 7 by addition of ammonium hydroxide, as needed. The results became much more reliable, and no degradation was seen in the polymer during the course of the entire reaction (Figure 6); the resulting films were opaque and flexible.

Mechanical Tests. The chemically synthesized composites made using the PCL star polymers containing 20 wt % HA were tested for tensile strength on an Instron model 5500-R. The samples were each run four times with a typical run shown in Figure 7. The average Young's modulus was found to be 1.77 MPa, and the ultimate tensile strength was seen as 4.19 MPa.

Values for all four Instron measurements including the three additional measurements used in determining

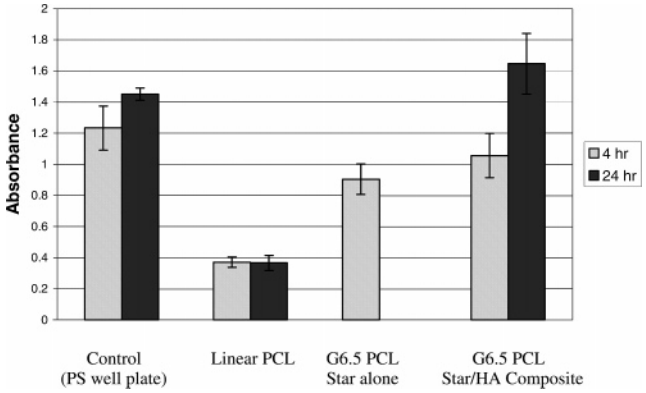


Figure 8. MG63 cell adhesion studies.

Table 2. Mechanical Data for Generation 6.5 PCL Star Polymer Composite Samples

	Young's modulus (MPa)	ultimate tensile strength (MPa)
sample 1	1.69	3.34
sample 2	1.59	3.59
sample 3	1.77	4.39
sample 4	2.02	5.42
average	1.77	4.19

Table 3. Mechanical Data for Comparison

material	Young's modulus (MPa)	ultimate tensile strength (MPa)
generation 6.5 PCL star composite	1.77 ± 0.18	4.19 ± 0.94
linear PCL composites[32]	106.35 ± 5.81	2.81 ± 0.23
porous PCL[33]	11.8 ± 4.0	1.1 ± 0.1
trabecular bone[34]	∼50−100	1.2
normal bone[34]	11.5	49

the average values for the Young's modulus, and the ultimate tensile strength can be found in Table 2.

The Young's modulus and tensile strength of other materials found in the literature are shown in Table 3 for comparison. The linear PCL composites were generally stiff but extremely brittle and do not absorb much energy before failure showing poor toughness characteristics, providing a % strain between 0.08% and 0.09% at break. However, the introduction of the dendritic core star polymer provided flexible films with a higher ultimate tensile strength and tougher materials with an ultimate strain of about 19% at break. The large degree of extension is a good indication of a large amount of energy that the composite can absorb before undergoing failure, thus indicating its toughness characteristics. The large difference in elongation between the composites made with linear caprolactone and the star form is not understood but may be a function of the high molecular weight of the star, yet containing a large number of end groups which can serve to plasticize the material. The PEG chain could add to this effect; however, since it is only 5000 g/mol, it is a very small percentage of the whole molecule and therefore we believe its effect would be minimal. The structure−property relationships are currently being investigated.

Cell Adhesion Studies. The results from the cellular adhesion study can be seen in Figure 8. After 4 h, no significant difference was seen in the attachment of MG63 osteoblast-like cells to the generation 6.5 PCL star/HA composite compared to the generation 6.5 PCL star alone, but both were well above that of linear PCL alone. After 24 h, the adhesion on the composite material significantly increased. There was no significant difference between the polystyrene surface of the culture plate, which served as a positive control, and the composites after 24 h.

As expected, there were significantly more cells attached to the HA containing composites than the straight chain PCL alone; however, it was interesting that the cell attachment was much higher in the generation 6.5 PCL star without HA than the linear PCL alone. The PCL star/HA composite clearly provides a favorable surface for osteoblast-like cell attachment, which may be assisted by the dendritic-poly(L-lysine) core.

Conclusions

The use of these dendrimers and their functionalized partners shows potential for their use in the biomedical applications. Since dendrimers possess multiple termini that are easily functionalized, the polymer can be tailored for specific purposes. For example, the incorporation of PCL at the termini eliminated the water solubility problem and allowed the use of these polymers during the in-situ chemical synthesis of the HA. The flexibility of these chemically synthesized composites and their favorable cell attachment properties show promise for use in bone tissue engineering. Further investigation is underway to investigate the effect of PCL chain length on film quality while the hydroxyl termini can be further functionalized for additional interaction with the hydroxyapatite. These possibilities and others are currently being explored in our laboratories.

Acknowledgment. The researchers thank the National Science Foundation Award #DMR-0073586 for financial support of this research. We especially thank Daiwon Choi from the Department of Materials Science Engineering at Carnegie Mellon University for his help with the linear PCL composite data.

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MA0493630