Biomaterials 31 (2010) 8507–8515

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Miscibility of choline-substituted polyphosphazenes with PLGA and osteoblast activity on resulting blends

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ARTICLE INFO

Article history:
Received 11 May 2010
Accepted 27 July 2010
Available online 25 August 2010

Keywords:
Polyphosphazenes
Choline chloride
Biomaterials
Amino acid
Osteoblast
Poly(lactic-co-glycolic acid)

ABSTRACT

The preparation of phosphazene tissue engineering scaffolds with bioactive side groups has been accomplished using the biological buffer, choline chloride. Mixed-substituent phosphazene cyclic trimers (as model systems) and polymers with choline chloride and glycine ethyl ester, alanine ethyl ester, valine ethyl ester, or phenylalanine ethyl ester were synthesized. Two different synthetic protocols were examined. A sodium hydride mediated route resulted in polyphosphazenes with a low choline content, while a cesium carbonate mediated process produced polyphosphazenes with higher choline content. The phosphazene structures and physical properties were studied using multinuclear NMR, differential scanning calorimetry (DSC), and gel permeation chromatography (GPC) techniques. The resultant polymers were then blended with PLGA (50:50) or PLGA (85:15) and characterized by DSC analysis and scanning electron microscopy (SEM). Polymer products obtained via the sodium hydride route produced miscible blends with both ratios of PLGA, while the cesium carbonate route yielded products with reduced blend miscibility. Heterophase hydrolysis experiments in aqueous media revealed that the polymer blends hydrolyzed to near-neutral pH media (~5.8 to 6.8). The effect of different molecular structures on cellular adhesion showed osteoblast proliferation with an elevated osteoblast phenotype expression compared to PLGA over a 21-day culture period.

1. Introduction

A large fraction of biomaterials research is focused on the use of long existing polymers such as polylactic acid (PLA) [1], polylactic-co-glycolic acid (PLGA) [2,3], and polycaprolactones (PCLs) [4]. These polymers are used mainly for their biodegradability and biocompatibility that allows them to be employed as hard tissue engineering scaffolds [1,4], drug delivery vehicles [3], bioerodible sutures [2], and fixation devices [2]. The primary concern is that no single polymer can address all the required properties needed for these applications. For example, biomaterials based solely on PLA, PLGA, or PCLs degrade into acidic byproducts that can cause tissue necrosis and cellular delamination at or around the implant site [5]. To address this problem, incorporation of polymers that buffer the acidic hydrolysis products of polyesters can generate biomaterials that have the required properties. In addition, polymer blends have the potential to produce materials that have mechanical stability greater than the individual polymers [6].

Biological buffers are an important class of molecules that perform many essential functions necessary for normal cellular activity. However, existing polymers that contain or are derived from biological buffers are low molecular weight water-soluble materials used for drug or gene delivery [7,8]. These compounds could be useful in tissue engineering materials if they were components of high molecular weight water-insoluble polymers. Choline is one such biological buffer that could be useful if incorporated into a high molecular weight polymer [9]. Choline is a naturally-occurring quaternary amino alcohol that has many important functions such as a source for methyl groups used in biological methylation [10,11]. It is a nutritional supplement [12,13], and is also a significant structural component in cell walls [14,15]. In addition, choline chloride has been shown to promote cell growth [16]. In the case of hard tissue engineering, the positive charge would provide a nucleation site for hydroxyapatite formation. It could also be used as an ionic cross-linking site in the presence of phosphates to improve the mechanical performance, and should be

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doi:10.1016/j.biomaterials.2010.07.094

able to buffer the acidic hydrolysis products of polyesters when used in tissue engineering scaffolds.

Polyphosphazenes are highly tunable polymers that in principle could serve as a platform to produce novel choline biomaterials. The high degree of molecular tunability of phosphazenes allows for facile changes in the side group structure. Different side groups can generate properties that are appropriate for drug delivery [17], hydrophobic surfaces [18], or biomaterials [19–23]. These changes in side group structure allow polyphosphazenes to form miscible blends with polyesters that can buffer the acidic hydrolysis products of polyesters [28,30]. For example, polyphosphazenes that contain dipeptide esters have better blend miscibility with PLGA (50:50) compared to poly(amino acid ester phosphazenes) blends with PLGA (50:50) [26,27]. This is due to the extra hydrogen bonding sites provided by the amide linkage in the dipeptide ester side groups [24,25,30]. However, these polymers cause rapid osteoblast cellular delamination and necrosis due the acidity generated when the polyphosphazene/PLGA blended system is hydrolyzed *in vitro* over a period of 21 days. Therefore, alternative polymers developed from choline should help to promote cell growth by buffering the hydrolysis products of PLGA in tissue engineering scaffolds.

Here, we describe the synthesis of a series of small-molecule model cyclic-trimeric phosphazene species with choline co-substituted with glycine ethyl ester, alanine ethyl ester, phenylalanine ethyl ester, or valine ethyl ester. This was followed by the synthesis of the corresponding polyphosphazenes using two different synthetic pathways: (1) the use of sodium hydride to reduce the choline chloride to the choloxide before addition to poly (dichlorophosphazene) and (2) the *in situ* reaction with cesium carbonate. The hydrolytic stability and unique thermal properties of the resultant polymers were then examined. These choline-based polyphosphazenes were then blended with PLGA (50:50) and PLGA (85:15) via solution-casting techniques. The blends were studied by differential scanning calorimetry (DSC), scanning electron microscopy (SEM), and FT-IR spectroscopy to determine the extent of blend miscibility. The hydrolysis rates and products were determined along with the osteoconductivity of the blended polymer materials.

2. Materials and methods

2.1. Reagents and equipment

All synthetic reactions were carried out under a dry argon atmosphere using standard Schlenk line techniques. Tetrahydrofuran and triethylamine (EMD) were dried using solvent purification columns [29]. Alanine ethyl ester hydrochloride (Chem Impex), valine ethyl ester hydrochloride (Bachem), phenylalanine ethyl ester hydrochloride (Chem Impex), glycine ethyl ester hydrochloride (Alfa Aesar), 60% sodium hydride dispersion in mineral oil (Sigma Aldrich), Sephadex G-25 (VWR), PLGA (50:50) (Ethicon Division of Johnson and Johnson; weight-average molecular weight 2,000,000), PLGA (85:15) (Ethicon Division of Johnson and Johnson; weight-average molecular weight 4,800,000), and anhydrous diglyme (Sigma Aldrich) were used as received. Cesium carbonate (Sigma Aldrich) and choline chloride was dried under vacuum at 100 °C for 1 week before being stored in an inert atmosphere glove box before use. Poly(dichlorophosphazene) was prepared by the thermal ring-opening polymerization of recrystallized and sublimed hexachlorocyclotriphosphazene (Fushimi Chemical Co., Japan) in evacuated Pyrex tubes at 250 °C. ^{31}P and ^{1}H NMR spectra were obtained with the use of a Bruker 360WM instrument operated at 145 MHz and 360 MHz, respectively. Glass transition temperatures were measured with a TA Instruments Q10 differential scanning calorimetry (DSC) apparatus with a heating rate of 10 °C/min and a sample size of ca. 10 mg. Gel permeation chromatograms were obtained using a Hewlett–Packard HP 1100 gel permeation chromatograph equipped with two Phenomenex Phenogel linear 10 columns and a Hewlett–Packard 1047A refractive index detector. The samples were eluted at 1.0 mL/min with a 10 mM solution of tetra-*n*-butylammonium nitrate in THF. The elution times were calibrated with polystyrene standards. Mass spectrometric analysis data were collected using turbo spray ionization technique on an Applied Biosystems API 150EX LC/MS mass spectrometer. Scanning electron microscopy (SEM) was obtained using a Philips FEI Quanta 200 Environmental Scanning Electron Microscope. The SEM samples were prepared by placement of a polymer sample onto carbon tape, followed by insertion into the SEM equipment. The use of low vacuum mode was used for imaging under the following conditions: 20 KeV source voltage, pressure approx. 0.88 Torr, and a working distance of approx. 10 mm. ATRIR scans of the films were analyzed with a Digilab (Randolph, MA) FTS 7000 spectrometer with a zinc selenide ATR crystal with 32 scans per sample. pH values were measure using a VWR Symphony SB70P pH meter.

2.2. Synthesis of model cyclic trimers *1*–*4*

The preparation of the model compound cyclic trimers **1**–**4** followed similar procedures. The synthesis of **1** is given as a representative example. Hexachlorocyclotriphosphazene (1.00 g, 2.88 mmol) was dissolved in tetrahydrofuran (150 mL) and cesium carbonate (9.18 g, 28.2 mmol) was added to the hexachlorocyclotriphosphazene solution. Glycine ethyl ester hydrochloride (1.20 g, 8.63 mmol) and triethylamine (1.24 mL, 8.92 mmol) in THF (100 mL) were refluxed for 24 h, filtered, and then added to the chlorophosphazene solution drop-wise over a period of 1 h and the mixture was stirred for 24 h at room temperature. Choline chloride (2.41 g, 17.3 mmol) and diglyme (100 mL) were added to the reaction mixture, which was stirred for 48 h at room temperature, 24 h at 40 °C, and was then refluxed for 24 h. The absence of a ^{31}P NMR signal after this sequence indicated that the product had precipitated from the THF solution. The recovered precipitate was soluble in de-ionized water. Purification by column chromatography using Sephadex G-25 (size exclusion chromatography) was completed. Cyclic trimers **1**–**4** contained equal molar amounts of amino acid ester and choline, and were soluble in de-ionized water. Physical and structural characterization data are presented in Table 1. The yields were 40–55% based on the initial amount of hexachlorocyclotriphosphazene.

2.3. Synthesis of polymers *5*–*8*

The preparation of the polyphosphazenes **5**–**8** using sodium hydride to form the sodium salt of choline followed similar procedures. The synthesis of **5** is given as a representative example. Poly(dichlorophosphazene) (5.00 g, 43.1 mmol) was dissolved in THF (500 mL). Glycine ethyl ester hydrochloride (6.02 g, 43.1 mmol) was suspended in THF (200 mL), and triethylamine (24.0 mL, 172 mmol). This suspension was refluxed for 24 h, filtered, and then added to the poly(dichlorophosphazene) solution over a 1 h period. The polymer mixture was stirred for 24 h at room temperature. Choline chloride (18.1 g, 129 mmol) was reacted with sodium hydride (3.45 g, 86.2 mmol) in diglyme (250 mL) at 75 °C for 48 h. This suspension was added to the polymer solution. The resultant solution was stirred at room temperature for 24 h, and was then refluxed for an additional 48 h. The solution was concentrated and dialyzed against methanol for 3 days in 12–14,000 MWCO dialysis tubing. The solution was removed from the dialysis tubing and was dried under reduced pressure for 1 week to produce a yellow–orange powder. The yield was 41–50% based on the amount of poly(dichlorophosphazene) used.

2.4. Synthesis of polymers *9*–*12*

The preparation of the polyphosphazenes **9**–**12** made use of cesium carbonate to prepare the sodium salt of choline. The synthesis of **12** is given as a representative example. Poly(dichlorophosphazene) (5.00 g, 43.1 mmol) was dissolved in THF (400 mL). Cesium carbonate (Cs_2CO_3) (61.9 g, 190 mmol) was added to the poly (dichlorophosphazene) solution. Phenylalanine ethyl ester hydrochloride (9.912 g, 43.1 mmol) was suspended in THF (300 mL), and was treated with triethylamine (6.62 mL, 47.5 mmol). This suspension was refluxed for 24 h, filtered, and then added to the polymer solution over a 1 h period, before the polymer solution was stirred for 24 h at room temperature. Choline chloride (18.1 g, 129 mmol) and diglyme (200 mL) were added to the polymer solution. The resultant solution was stirred at room

Table 1
Characterization data for small-molecule model [(amino ethyl ester)$_3$(choloxy chloride)$_3$ cyclotriphosphazenes].

Trimer	^{31}P (ppm)[a]	^{1}H (ppm)[a]	*m*/*z*
(**1**)	3.3	1.21 (3H, t, gly), 3.43 (9H, s, chol), 3.53 (2H, t, chol), 3.61 (2H, s, gly), 3.86 (2H, t, chol), 4.09 (2H, q, gly)	249
(**2**)	1.8	1.18 (3H, t, ala), 1.30 (3H, d, ala), 3.43 (9H, s, chol), 3.51 (2H, t, chol), 3.59 (1H, q, ala), 3.83 (2H, t, chol), 4.06 (2H, q, ala)	264
(**3**)	2.3	0.91 (6H, d, val), 1.21 (3H, t, val), 3.34 (1H, m, val), 3.51 (9H,s, chol), 3.60 (2H, t, chol), 3.66 (1H, d, val), 3.83 (2H, t, chol), 4.12 (2H, q, val)	292
(**4**)	3.3	0.93 (3H, t, phe), 3.05 (2H, d, phe), 3.39(9H, s, chol), 3.62 (2H, t, chol), 3.72 (1H, t, phe), 3.86 (2H, t, chol), 4.24 (2H, q, phe), 7.09 (5H, m, phe)	340

[a] All NMRs were taken in D_2O.