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The Influence of Side Group Modification in Polyphosphazenes on Hydrolysis and Cell Adhesion of Blends with PLGA

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Abstract

Polyphosphazenes have been synthesized with tris(hydroxymethyl)amino methane (THAM) side groups and with co-substituents glycine ethyl ester and alanine ethyl ester. The THAM side group was linked to the polyphosphazene backbone via the amino function. The three pendent hydroxyl functions on each THAM side group were utilized for hydrogen bonding association with poly (glycolic-lactic acid) (PLGA). Co-substitution of the polyphosphazene with both THAM and glycine or alanine ethyl esters was employed to avoid the insolubility of the single-substituent THAM - substituted polyphosphazenes. Both poly[(tris(hydroxymethyl)aminomethane)(ethyl glycinate) phosphazene] and poly[(tris(hydroxymethyl)aminomethane)(ethyl alanato)phosphazene] (1:1 ratio of side groups) were blended with PLGA (50:50) or PLGA (85:15). DSC analysis indicated miscible blend formation, irrespective of the detailed molecular structure of the polyphosphazene or the composition of PLGA in the blend. Hydrolysis studies of the polyphosphazene:PLGA (50:50) blends indicated that the PLGA component hydrolyzed more rapidly than the polyphosphazene. Primary osteoblast cell studies showed good cell adhesion to the polymer blends during 14 days, but subsequent limited cell spreading due to increased surface roughness as the two polymers eroded at different rates.

Keywords

polyphosphazenes; amino acid; bioerodible; THAM; polymer blends; osteoblast cells

Introduction

Poly(lactide-co-glycolide) (PLGA), poly(L-lactide) (PLA), and poly(glycolide) (PGA) have received considerable attention for many biomedical applications based on their good thermal stability [1–3], biocompatibility[4,5], adequate mechanical properties [4,6,7], and cell adhesion [8]. These polyesters are also bioerodible [9]. The hydrolysis rate is lowered by

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increased ratios of L-lactide in the co-polymer. These are some of the necessary properties for bioerodible implantable devices such as bioerodible sutures [10], drug delivery vehicles [11, 12], and tissue engineering applications [13,14]. However, one of the complications for biomedical uses of lactide- and glycolide-based polyesters is the acidity of the hydrolysis products. This can be detrimental to cell growth in tissue engineering research. For this reason, new materials are being developed in attempts to combine the beneficial properties of PLGA and minimize its disadvantages.

Polyphosphazenes are hybrid polymer systems with a backbone of alternating phosphorus-nitrogen atoms and with two organic side groups covalently linked to each phosphorus atom. The most common synthesis method for these polymers uses macromolecular substitution reactions to replace chlorine atoms in poly(dichlorophosphazene) by organic groups. Because more than 250 different organic nucleophiles are known to participate in this reaction, a large degree of tailorability is possible. The properties of the final material depend on the side group substituents [15]. Polyphosphazenes with amino acid ester side groups are generally hydrolytically sensitive [16,17]. The hydrolysis of amino acid ester substituted polyphosphazenes results in the formation of phosphates, ammonia, and the corresponding amino acid and alcohol [16,18]. The formation of phosphates and ammonia generates a possible buffer system that helps to maintain the near neutral pH of the media. Poly(amino acid ester phosphazenes) are also known to be effective surfaces for cell adhesion and cell growth [19, 20].

Attempts have been made to combine the useful properties of PLGA and polyphosphazenes through the formation of miscible polyphosphazene/PLGA blends. Miscibility requires that the polyphosphazene should possess side groups that are capable of hydrogen bonding with the ester oxygens atoms of PLGA. Amino acid ester based side groups have this ability via the amino proton. However, many of the composite systems studied in previous work suffer from incompatibility because, of steric protection by groups at the α -carbon substituent site of the amino acid esters. This decreases the ability of the polyphosphazene to form miscible blends with PLGA [21,22]. Dipeptide ethyl ester side groups have also been linked to the polyphosphazene backbone and these show good miscibility with PLGA [18]. Nevertheless, alternative side groups linked to a polyphosphazene chain are still needed in order to maximize blend compatibility and encourage cell adhesion and proliferation in tissue engineering applications.

In the present work we have examined the possibility that tris(hydroxymethyl)aminomethane (THAM) side groups linked to a polyphosphazene may offer biomedical advantages for polymer-polymer composite formation. THAM is a reagent that is used to buffer aqueous solutions from pH 7.0 to pH 9.0 for biological research. The primary amino unit of THAM was utilized for chlorine replacement with poly(dichlorophosphazene) (**1**). However, polyphosphazenes with the THAM substituent only became insoluble during synthesis, possibly by cross-linking, and could not be purified by the normal reprecipitation or dialysis techniques. Thus, the synthesis of co-substituted polymers with both THAM and glycine ethyl ester or alanine ethyl ester in a 1:1 ratio was attempted. The molecular structures of these co-substituted polyphosphazenes were determined by ^1H and ^{31}P NMR techniques. The phosphazene polymers were then blended with PLGA (50:50) and PLGA (85:15) with weight percentages of 25%, 50%, and 75% polyphosphazene. The blend miscibility was monitored by differential scanning calorimetry, and the absence of micro-level phase separation was confirmed by scanning electron microscopy. Heterophase hydrolysis studies of blends that contained 50%, by weight, of the two polyphosphazenes with PLGA 50:50 were conducted. Osteoblast cell adhesion and proliferation at the surface was also analyzed for these blends.

Experimental

Reagents and Equipment

All synthetic reactions were carried out with the reactants under a dry argon atmosphere using standard Schlenk line techniques. Tetrahydrofuran and triethylamine (EMD) were dried using solvent purification columns [23]. Alanine ethyl ester hydrochloride (Chem Impex), glycine ethyl ester hydrochloride, tris(hydroxymethyl)amino methane (Alfa Aesar), and poly(lactide-co-glycolide) (50:50 and 85:15) (Ethicon) were used as received. 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 ^1H NMR spectra were obtained with use of a Bruker 360 WM instrument operated at 145 MHz and 360 MHz, respectively. Glass transition temperatures were measured with a TA Instruments Q10 differential scanning calorimetry 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.

Synthesis of poly[(tris(hydroxymethyl)amino methane)₁(ethyl glycinato)phosphazene] (2)

Poly(dichlorophosphazene) (5.00 g, 43.2 mmol) was dissolved in THF (400 mL). Glycine ethyl ester hydrochloride (6.27 g, 44.9 mmol) was suspended in THF (250 mL) and triethylamine (14.4 mL, 104 mmol) was added to the solution. The glycine ethyl ester mixture was refluxed overnight, after which time the triethylamine hydrochloride salts were filtered from the solution. The glycine ethyl ester solution was then added drop-wise to the polymer solution. The resultant solution was stirred at room temperature for eight hours, and then tris(hydroxymethyl)amino methane (9.52 g, 60.4 mmol) and triethylamine (12.0 mL, 86.3 mmol) were added. The reaction mixture was stirred at reflux for 24 hours, then filtered and concentrated to dryness. The product was dialyzed versus methanol (three days) and isolated as an opaque, waxy solid. The purified polymer was characterized by ^1H and ^{31}P NMR techniques (Table 1). Polymer yields were in the range of 70–75% based on the initial amount of $(\text{NPCl}_2)_n$.

Synthesis of poly[(tris(hydroxymethyl)amino methane)₁(ethyl alanato)₁phosphazene] (3)

A similar process was used, with the exception that alanine ethyl ester was utilized in place of glycine ethyl ester. The crude product was dialyzed versus methanol (three days) and isolated as a white, brittle polymer. The purified polymer was characterized with ^1H and ^{31}P NMR techniques (Table 1). The yields were in the range of 76–78 %.

Blend miscibility studies of polymers 2 and 3 with PLGA

Three different compositions were studied for each blended system consisting of 25%, 50%, and 75% (wt/wt) of polyphosphazene with the appropriate molecular composition of PLGA. PLGA (50:50) and PLGA (85:15) were used in the polymer blends. A total of 100 mg of a sample formed by combination of the polyphosphazene and PLGA) was used to prepare each blend. The parent polymers were dissolved individually in 1 mL of chloroform. The solutions were then combined and mixed for one hour. No solution-phase separation was observed. The solutions were then placed in casting trays and air dried for 24 hours, followed by vacuum drying for one week. The resultant solid films were analyzed by DSC and SEM techniques to determine the final solid-phase blend miscibility.

Hydrolysis studies of the polymer blends

Films for hydrolysis studies were solution-cast from chloroform (as described above) to contain 400 mg of total sample in 4 mL of solvent. The blend systems were composed of 200 mg of each polyphosphazene (polymers 2 or 3) with 200 mg of PLGA 50:50. The parent polymers were dissolved individually, then the polyphosphazene solution was added to the PLGA solution (no solution phase separation was observed). Films were solution cast and dried for 24 hours, then dried under vacuum for seven days. The films were cut into 1 cm × 1 cm squares, weighed, and immersed in deionized water (pH = 6.0). The study was conducted for six weeks with three samples removed after each week. The water uptake of each film was measured immediately after removal of the film from the aqueous medium. The films were then dried under vacuum for two weeks to ensure that all the water was removed from the sample. The pH of the aqueous medium was recorded for each sample following removal of the film. The dry films were analyzed by measuring the weight loss, molecular weight decline (by GPC), and surface analysis via SEM techniques.

Primary rat osteoblast cell seeding

Primary rat osteoblast cells were isolated from calvaria of 2–3 day old neonatal Sprague-Dawley rats by adopting a previously reported protocol.²⁵ Before cell seeding, polymer films (0.1 mm thickness and 10 mm diameter) were sterilized by treatment with 70% ethanol for 30 min, and exposure to UV radiation for 10 minutes. These films were placed in 48 well plates and seeded with 50,000 osteoblast cells of passage number 3. The scaffolds were incubated for 2h without media to promote cell attachment. Later, cell seeded films were submerged in 1 ml of Ham's F-12 media supplemented with 12% FBS and 1% PS. The culture was maintained for 21 days in an incubator at 37°C, 5% CO₂ and 95% humidified air with media changing every two days.

Cell morphology

At days 7 and 14, the cell seeded scaffolds (n=2) were removed from the culture and washed with PBS to remove any unattached cells. Cells on the scaffolds were fixed at 4 °C in 1% and 3% glutaraldehyde for 1 and 24 h, respectively. These scaffolds were subjected to sequential dehydration for 10 min each with ethanol series (30%, 50%, 70%, 90% and 100%). At the end, cell-fixed scaffolds were coated with Au/Pd and visualized under SEM (JEOL-JSM840) cell morphology on polymer films.

Osteoblast cell activity

The osteoblast cell activity on the polymer films was measured using a colorimetric MTS assay (Promega, Madison, WI). The assay works on the basis that metabolically active cells reduce tetrazolium-based MTS reagent to a purplish formazan product. At days 3, 7 and 14, polymer films with cells were taken out of culture and washed twice with PBS, then placed into a new well plate with 0.5ml of media in each well. A 100 µL sample of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) reagent was added to each well and incubated at 37°C for 2 h. At the end of the incubation period, the reaction was stopped by adding 125 µL of 10% sodium dodecyl sulfate (SDS) solution. Subsequently, the solution was diluted in a 4:1 ratio using distilled water, and the absorbance was read at 490 nm using a Tecan SpectroFluo Plus plate reader (TECAN, Boston, MA). The measured absorbance is correlated to the activity of osteoblasts on a particular polymer film. A sample number n=3 was used for this study. The quantitative data for cell activity was reported in the form of mean ± standard deviation. Statistical analysis was performed using a one-way analysis of variance (ANOVA) using Tukey test to determine the statistical significance between the two means evaluated at p<0.05.

Results and Discussion

Synthesis of 1:1 cosubstituted poly[(tris(hydroxymethyl)amino methane)(ethyl glycinato) phosphazene] (**2**) and poly[(tris(hydroxymethyl)amino methane)(ethyl alanato) phosphazene] (**3**)

Tris(hydroxymethyl)amino methane (THAM) has an amino functional group that is capable of replacing chlorine atoms in poly(dichlorophosphazene). Polymers **2** and **3** were synthesized using the THAM side group together with the co-substituents glycine ethyl ester or alanine ethyl ester. The amino acid ester side groups were linked to the backbone before the THAM substituent was added, as outlined in Scheme 1. There was no evidence that any of the hydroxyl functions of the THAM units reacted to form P-O-C bonds. Thus, the ^{31}P NMR data shown in Table 1 showed no P-O-C chemical shifts between -5 ppm and -12 ppm. The glass transition temperatures (T_g) of polymers **2** and **3**, at 23.3 °C and 23.6 °C respectively, are appreciably higher than the transitions for the single-substituent poly[bis(ethyl glycinato)phosphazene] and poly[bis(ethyl alanato)phosphazene] (-40 °C and -10 °C, respectively) [16] and [17]. These T_g values are consistent with the interpretation that polymers **2** and **3** have restricted backbone motion, probably due to hydrogen bonding between the side groups. No crystalline regions were detected by DSC analysis. The GPC-determined molecular weights were estimated to be 151 kg/mol and 210 kg/mol for polymers **2** and **3**, respectively. This corresponds to 565 repeat units for polymer **2** and 715 repeat units for polymer **3**.

The choice of side groups for covalent linkage to the polyphosphazene backbone was based on the perceived need for hydrogen bonding sites, such as hydroxyl or amino units to interact with the oxygen atoms of PLGA. These interactions could develop micro-scale blend miscibility between the two polymer systems. Each THAM side group has three pendent hydroxyl functions that can participate in hydrogen bonding. The amino acid ester side groups also aid hydrogen bonding while, at the same time, controlling the hydrolysis rate of the polymer. Previous studies demonstrated that increased hydrogen bonding between polyphosphazenes and other polymers leads to complete micro-scale blend miscibility.

Fabrication of polyphosphazene/poly(lactide-co-glycolide) blends

The fabrication of different blend compositions was used to determine which weight ratios provided the most miscible systems. Two different compositions of PLGA were used, 50:50 lactide:glycolide and 85:15 lactide:glycolide. PLGA 50:50 offers less steric hindrance to polymer-polymer interactions than the 100% lactide polymer with its α -methyl groups. Thus the carbonyl function of the glycolic repeat unit is more easily accessible for hydrogen bonding with the polyphosphazene side groups. PLGA 85:15 has a slower hydrolysis rate than PLGA 50:50 due to the increased concentration of lactic acid units.

DSC analysis of the solution-cast, blended films indicated the formation of completely miscible blends, regardless of the composition of the blend or the composition of the PLGA. Representative examples of the thermal transitions for the blends formed from polymer **2** and PLGA are shown in Figure 1. When polymer **2** was blended with PLGA 50:50, a single thermal transition was detected for each composition of the blend. Thus, a single glass transition temperature was detected for Blend A (25% polymer **2**: 75% PLGA 50:50) at 24.1 °C, for Blend B (50% polymer **2**: 50% PLGA 50:50) at 30.9 °C, and for Blend C (75% polymer **2**: 25% PLGA 50:50) at 41.5 °C. The melting transition temperature of PLGA was not detected in the blends, as shown in Figure 1. A similar effect was found for the blends of polymer **2** with PLGA 85:15. The single glass transition temperature detected for Blend D (75% polymer **2**: 25% PLGA 85:15) was at 34.7 °C, for Blend E (50% polymer **2**: 50% PLGA 85:15) at 32.9 °C, and for Blend F (75% polymer **2**: 25% PLGA 85:15) at 39.6 °C. SEM analysis of the surfaces of all the polymer blends showed no micro-scale phase separation.

Based on DSC analysis, the blends of polymer **3** with PLGA were completely miscible, irrespective the blend ratio and the PLGA composition. Figure 2 shows a representative example of the detected thermal transitions of blends comprised of polymer **3** and PLGA 50:50 (Blend H) or PLGA 85:15 (Blend K). Blends that contained 25% polymer **3** and 75% PLGA 50:50 (Blend G) had a single glass transition temperature at 23.7°C, while the blend that contained 75% polymer **3** with 25% PLGA 50:50 (Blend I) had a glass transition temperature at 31.6°C. Blend H (50% polymer **3**, 50% PLGA 50:50), shown in Figure 2, had a single glass transition temperature at 28.2°C. Polymer blends of polymer **3** with PLGA 85:15 also had single thermal transitions at 29.9°C (Blend J; 25% polymer **3**: 75% PLGA 85:15), 34.8°C (Blend K; 50% polymer **3**: 50% PLGA 85:15), and 40.6°C (Blend L; 75% polymer **3**: 25% PLGA 85:15). SEM analysis of the surfaces supported the DSC data since no phase separation was detected.

All the blends (Blends A – L) were studied by ATRIR spectroscopy to examine the hydrogen bonding between the polyphosphazene and the polyester carbonyl functions. The pristine polymers (polyphosphazenes and PLGA) had a C=O vibrational peak at 1750 cm⁻¹, while the blends had a similar peak, but at 1735 cm⁻¹. This change in the vibrational frequency is evidence for hydrogen bonding. Hydrogen bonding probably provides the driving force for blend miscibility at the micro-scale.

The increased hydrogen bonding character that was introduced by the THAM side groups probably significantly assists the blend miscibility. The presence of the three hydroxyl protons on each THAM side group generates multiple sites for hydrogen bonding to the PLGA carbonyl groups than is found in amino acid ester side units. These hydroxyl sites are also in close molecular proximity and more able to generate good interactions with the more sterically hindered PLGA 85:15.

Hydrolysis of polymer blends comprised of polymer **2** or polymer **3** with PLGA 50:50

The hydrolysis of Blends B and H in aqueous media (pH=6.0) were studied at 37.0°C for six weeks. PLGA 50:50 was utilized because this composition of PLGA degrades faster (6–12 weeks) than its counterpart, PLGA 85:15. The pH of the solutions was not buffered for two reasons. First, buffer solutions that contain phosphate could not be used because they would mask the ³¹P NMR spectrum of the phosphazene hydrolysis products. Second, an objective was to determine if the hydrolysis of polymers **2** or **3** can compensate for the acidic pH generated by the hydrolysis of PLGA 50:50. In practice, it was found that the pH decreased from pH 6 to 2.8, because of the more rapid hydrolysis of the PLGA component of the blends. The polyphosphazene component showed no significant molecular weight decline, even after six weeks in the hydrolysis medium, and this indicates that the phosphazene did not hydrolyze at a fast enough rate to buffer the medium.

The analysis of the pH of the aqueous media for the hydrolysis of Blends B and H are shown in Figure 3. The pH decreased dramatically between week 2 and week 3, and declined steadily thereafter until the study was concluded at week 6. This decrease in pH is a consequence of the hydrolysis of PLGA 50:50 to lactic acid and glycolic acid. The hydrolysis of PLGA 50:50 was probably assisted by water uptake by the films. Blend B had absorbed 20% water, even after only one week in the hydrolysis medium. Blend H absorbed 17% water during the same time period. The water absorbed water remained at 20% and 17% throughout the six week period. The weight of each film was then recorded after drying under reduced pressure for two weeks. The weight losses for Blends B and H are shown in Figure 4. This weight loss is probably due to the lactic and glycolic acids diffusing into the bulk aqueous medium.

The acidity of the hydrolysis medium differs from previous hydrolysis studies of polyphosphazene:PLGA blends in which a distinct buffering effect was detected. A reason for

this loss of buffering ability by the polyphosphazene component was evident after a molecular weight analysis. As the films degraded, the polyphosphazene component underwent only a slight decrease in molecular weight, with a maximum molecular weight loss of 20% for polymer **2** and 14% for polymer **3**. On the other hand, the PLGA component showed an immediate molecular weight decline and, after the six week period, the molecular weight loss was approximately 90%. Therefore, it was concluded that the polyphosphazene component did not hydrolyze at a fast enough rate to counteract the acidity and, thus, did not affect the pH of the media. This was confirmed by ^{31}P NMR analysis of the aqueous media, because no phosphorous signals were detected during the course of the study. The surface hydrolysis was monitored with the use of SEM techniques, as shown in Figure 5 and Figure 6. Interestingly, the blend miscibility was monitored at each analysis time and it was found that the blends had phase separated after week 1. This suggests that the rapid hydrolysis of PLGA disrupts the inter-polymer hydrogen bonding and leads to phase separation. Such behavior raises the possibility that hydrolysis of these blends can generate a porous structure which would facilitate three-dimensional colonization of the construct by mammalian cells.

Osteoblast cell morphology and activity

Primary osteoblast cell growth on PLGA and Blend E were visualized using SEM and shown in Figure 7. At day 7, well spread morphology was observed on both PLGA (Fig. 7a) and Blend E (Fig. 7b). It is clear that osteoblasts adhere and grow on the blend surfaces. Figure 8 shows the absorption recorded (MTS assay) on blends E, K and PLGA 85:15 films cultured for days 1, 3 and 7. The recorded absorption is a direct measure of the metabolic activity of the primary rat osteoblast cells present on the particular polymer film. At days 1, 3 and 7, the cell activity for blend films was significantly lower than the activity recorded on PLGA films. However, the osteoblast cell activity on both the blends was observed to progressively increase with increase in the culture time. This short term cell study indicates the good osteocompatibility of the blend films and therefore has potential to be used as scaffold materials for bone tissue engineering applications.

Conclusions

Polyphosphazenes with both tris(hydroxymethyl)amino methane (THAM) and glycine or alanine ethyl ester side groups were synthesized via macromolecular replacement of the chlorine atoms in poly(dichlorophosphazene). Blends of these polymers with PLGA were determined to be completely miscible irrespective of the blend ratios or the composition of PLGA. SEM analysis of the films showed no phase separation in the solution-cast films. Hydrolysis of films at 37°C in deionized water indicated that the polyphosphazene did not hydrolyze significantly during six weeks, but that the PLGA (50:50) was completely hydrolyzed. DSC analyses of the hydrolyzed films showed a completely phase-separated blend structure after one week in aqueous media at 37°C. These studies suggest that the blends may be useful for applications in which the initial hydrolytic breakdown of PLGA generates a porous structure with some residual strength. The porous framework would then break down over a much longer period of time. Thus, fine-tuning of the system might yield materials with tissue engineering properties superior to those of PLGA. Osteoblast cell adhesion experiments showed cell proliferation on the films through day 7, indicating the osteocompatibility of the blends.

Acknowledgments

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Figure 1. DSC traces of polymer 2, Blend B, Blend E, PLGA 50:50, and PLGA 85:15. The arrows indicate the detected glass transition temperatures.

Figure 2. DSC traces of polymer 3, Blend H, Blend K, PLGA 50:50, and PLGA 85:15. The arrows show the glass transition temperatures.

Figure 3.
pH of the hydrolysis media from Blend B (◇), Blend H (■), and pristine PLGA 50:50 (▲).

Figure 4.
Hydrolytic weight loss of Blend B (▲) and Blend H (■).

Figure 5.
SEM images of the surface of Blend B after A) week 1, B) week 2, and C) week 6.

Figure 6.
SEM image of Blend H after A) week 1, B) week 2, and C) week 6.

Figure 7.
SEM micrographs of primary rat osteoblast cells cultured after 7 days on PLGA (a) and Blend E (b).

Figure 8.
Cell activity measured using MTS assay.

Scheme 1.

Synthesis of co-substituted polyphosphazenes 2 and 3.

Table 1

Structural and physical characterization of polymers 2 and 3.

Polymer	¹ H NMR (ppm)	³¹ P NMR (ppm)	T _g (°C)	MW ^a (g/mol)	PDI
2	1.2 (3H), 3.6 (2H), 3.8 (6H), 4.2 (2H)	-0.7	23.3	151 000	1.9
3	1.3 (6H), 3.7 (1H), 3.9 (6H), 4.1 (2H)	-0.6	23.6	210 000	2.1

^aThe GPC-derived molecular weight approximate to M_w.