

Synthesis and Characterisation of a Degradable Poly(lactic acid)–Poly(ethylene glycol) Copolymer with Biotinylated End Groups

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Poly(lactic acid)–poly(ethylene glycol)–biotin (PLA–PEG–biotin) is a degradable polymer with protein resistant properties that can undergo rapid surface engineering in aqueous media to create biomimetic surfaces. Surface engineering of this polymer is dependent on biomolecular interactions between the biotin end group and the protein avidin. Given the vigorous conditions of synthesis, it is essential that the manufacture of the polymer does not alter the biotin structure or its molecular recognition. Equally, it is important that the incorporation of biotin does not adversely affect the physicochemical properties of the polymer. ¹H NMR provides evidence of biotin attachment and structural integrity. ¹H NMR, gel permeation chromatography (GPC), and differential scanning calorimetry (DSC) analysis shows there is no significant effect on bulk properties induced by the biotin end group. Surface plasmon resonance (SPR) and fluorescent spectroscopy studies using the 2-(4'-hydroxyazobenzene) benzoic acid (HABA)/avidin complex show that the biotin moieties binding capabilities are not impaired by the synthesis.

Introduction

With recent advances in tissue engineering and drug delivery,^{1–5} there has been an increasing need for new biomaterials that can present biological molecules at their surfaces.⁶ Peptide and carbohydrate motifs are used in cell signaling and extensive studies have elucidated that specific amino acid sequences such as RGD or IKVAV in extracellular matrices that are responsible for cell adhesion.^{7–10} The ability of a material to immobilize these adhesion ligands has been clearly demonstrated, by the use of self-assembling monolayers, to be a useful strategy in controlling cell-material interactions in both tissue engineering and drug delivery.^{11–16}

The main strategy to date for attaching peptide sequences to polymers has been covalent coupling.¹¹ This technique is not however applicable if polymers lack reactive side groups or if reaction conditions damage the polymer or biomolecular ligand.^{17,18} Poly(lactic acid)–poly(ethylene glycol)–biotin (PLA–PEG–biotin) is a polymer that can immobilize different biomolecules under mild aqueous conditions on a biodegradable surface (Figure 1).¹⁹

PLA–PEG copolymers and their use as an effective biomaterial in drug delivery and tissue engineering have been well characterized.^{20–26} PLA is the backbone of the polymer and provides structural integrity to any scaffold.^{26–29} Poly(ethylene glycol) (PEG) has long been considered an attractive biomaterial because of its ability to resist protein adsorption.^{30–33} In PLA–PEG–biotin, PEG acts as a spacer group for the biotin thus improving biotin-binding capabilities

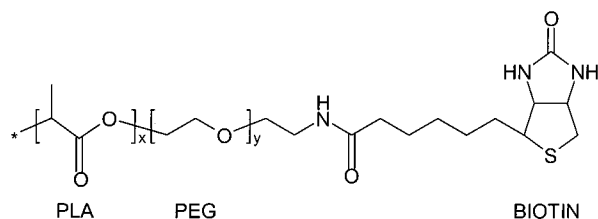


Figure 1. Structure of PLA–PEG–biotin.

by reducing steric hindrance. Each biotin moiety on the PLA–PEG–biotin attaches to one of the four biotin binding sites on the tetrameric protein avidin leaving available binding pockets for any biotinylated peptide sequences or ligands.^{34–39} PLA–PEG–biotin has been shown to have favorable responses from endothelial cells when presenting RGD sequences to the surface using this technique.¹⁹ This, therefore, has the potential to be a valuable system for the manufacture of tissue engineering scaffolds or matrices.

An important factor in the design of new biomaterials is reproducibility in terms of chemical, physical and biological properties. PLA–PEG–biotin can be synthesized in two easy steps: biotin attachment to bifunctional PEG followed by PLA attachment to PEG–biotin by ring-opening polymerization of the lactide. The polymerization is possible using two methods; solution polymerization or melt polymerization. Surface plasmon resonance (SPR) and fluorescent spectroscopy prove to be useful methods of assessing the surface engineering process and whether the biotin unit is degraded or the biological activity reduced by synthesis. Much of the surface properties of this polymer have been studied; however, the bulk properties of this biotin-containing PLA–PEG copolymer have yet to be characterized.^{19,40}

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Polymer end groups have been shown to affect the bulk properties of a polymer.^{41–48} An example of this was found in a poly(propylene glycol)–poly(ethylene glycol) copolymer where a change from a methoxy-terminated end group to a hydroxyl end group lead to changes in the glass transition temperature ranging from 12 to 20 °C. This was attributed to interactions with the hydroxyl end group and the ether oxygens on the backbone of the polymer and end-to-end hydrogen-bonding interactions.⁴⁹ Given that the biotin interaction with avidin involves establishing 36 hydrogen bonds (excluding water molecules) and the presence of both ether and ester oxygens along the backbone of the polymer, it is feasible that biotin as an end group could influence the bulk properties of PLA–PEG in a similar manner.⁵⁰ Therefore, we undertook a systematic study to assess the impact of the biotin end group on the PLA–PEG.

Experimental Section

Materials. α -Hydroxy- ω -amine PEG (M_w 3400) and methoxy-terminated PEG (M_w 3000) were purchased from Shearwater Polymers. DL-Lactide was purchased from Purac and stored at 0 °C under nitrogen. *N*-Hydroxy succinimide (NHS) biotin was purchased from Pierce & Warriner. Stannous octanoate [Sn(Oct)₂] and all solvents were purchased from Sigma/Aldrich. Stannous octanoate was stored under vacuum and used as received following a nitrogen purge and ¹H NMR checks for impurities.

Synthesis of PLA-PEG–Biotin. Synthesis of Biotin–PEG–OH. α -Hydroxy- ω -amine PEG (1 g) was dissolved into acetonitrile (2 mL). Methylene chloride (1 mL) and Et₃N (80 μ L) were added and the mixture then stirred for 1 min. After addition of NHS–biotin (0.250 g), the reactants were stirred overnight under argon. The reaction was worked-up by the slow addition of diethyl ether (40 mL) to precipitate the polymer, which was then filtered on a Buchner funnel and washed with diethyl ether. The isolated material was then dissolved in hot 2-propanol (70 °C) to give an opaque-cloudy solution. The polymer was reprecipitated on cooling; this product was then analyzed for biotin attachment by ¹H NMR spectroscopy.

Removing Water Impurities and Aqueous Impurities from Biotin–PEG–OH. The polymer (350 mg) was dissolved into toluene (70 mL) and refluxed with a Dean–Stark trap and a condenser. Then 70% of the toluene was removed by distillation. The polymer was isolated on a rotary evaporator. To remove residual solvent, the polymer was dried under vacuum (Edwards RV8) for 2 days.

Preparation of PLA–PEG–Biotin. The Graft Polymerization of Lactide on Biotin–PEG–OH by Solution Polymerization. Glassware was silanized by rinsing with a 5% methyltrichlorosilane solution in toluene, rinsed with acetone, and left overnight to dry at 130 °C. A 50 mL round-bottom flask was charged with biotin–PEG–OH (0.35 g). Lactide (2 g) was transferred into the round-bottom flask and diluted with 10 mL of toluene, and the round-bottom flask was capped and placed in the desiccator. The round-bottom flask was then taken out of the desiccator and heated to 60 °C until the contents went into solution. Sn(Oct)₂/

toluene (0.1 g in 1 mL) was then added, and the reaction was then brought to reflux at 110 °C for 4 h under argon. Following this, the reaction vessel was equipped with a Dean–Stark trap and any remaining solvent was removed by vacuum rotary evaporator. The remaining viscous material was heated to 140 °C, giving a melt, which was left for 1 h under argon. The reaction mixture was allowed to cool after which it was dissolved in approximately 10 mL of dichloromethane. This polymer solution was then added dropwise to a stirring solution of 100 mL of diethyl ether. The final product was isolated by vacuum filtration and lyophilized overnight. Approximately 2.0 g of white powder was isolated.

The Graft Polymerization of Lactide on Biotin–PEG–OH by Melt Polymerization. This method was as described for the solution polymerization with the exception that the contents were not diluted with toluene. The round-bottom flask with lactide (2 g) and biotin–PEG–OH (0.35 g) was sealed under argon and heated at 140 °C for 16 h before being reprecipitated as previously described.

Preparation of PLA–PEG by Melt and Solution Polymerization. Synthesis was performed as described for PLA–PEG–biotin, substituting biotin–PEG–OH with Me–PEG–OH. Synthesis of low-content lactide PLA–PEG–biotin by melt and solution polymerization were completed as described for PLA–PEG–biotin using 0.2 g lactide.

Instrumentation Methods. Nuclear Magnetic Resonance Spectroscopy (NMR). Proton ¹H NMR spectra were recorded on a Bruker ARX-250 spectrometer at 250 MHz. ¹H NMR chemical shifts were measured in parts per million (ppm) relative to CHCl₃ in CDCl₃ and DMSO in DMSO-*d*₆.

Gel Permeation Chromatography (GPC). GPC studies were conducted using degassed chloroform as the eluent with a sample concentration of 0.2% w/v, an injection volume of 100 μ L, columns consisting of 2 \times Plgel 5 μ m (300 \times 7.5 mm), and a flow rate of 1.0 mL/min. Studies were carried out at ambient temperature and a pressure of 70 bar. The detector used DRI, and polystyrene (Easical PS-2) was used to calibrate the system.

Differential Scanning Calorimetry (DSC). DSC thermograms were recorded with a Perkin-Elmer CCA7 analyzer and a Perkin-Elmer TAC 7/DX. The heating rate was 10 °C a minute with a quenching rate of 200 °C a minute. Values of glass transition temperatures (T_g) were taken from the midpoint of the transition region.

Surface Plasmon Resonance (SPR). The SPR equipment (Ortho Clinical Diagnostics, Chalfont, St-Giles, U.K.) employs a Kretschmann configuration with a monochromatic laser source of 780 nm wavelength. The glass slides used had a 50 nm silver layer on one side. These were coated with samples from a 3 mg/mL chloroform solution spun-cast with 100 μ L at 2000 rpm. This resulted in films that gave time-dependent SPR angle (ϕ_{SPR}) (in millidegrees of mDA) which is the angle at which the minimum in light intensity occurs. Avidin (Sigma) was made up to 5 \times 10^{–7} M in dibasic phosphate buffer (10 mM, pH 7.4), which also acted as the medium for washing. The flow rate was 0.24 mL/min and binding of avidin was determined over 1000 s time periods. Injection of the avidin solution took place

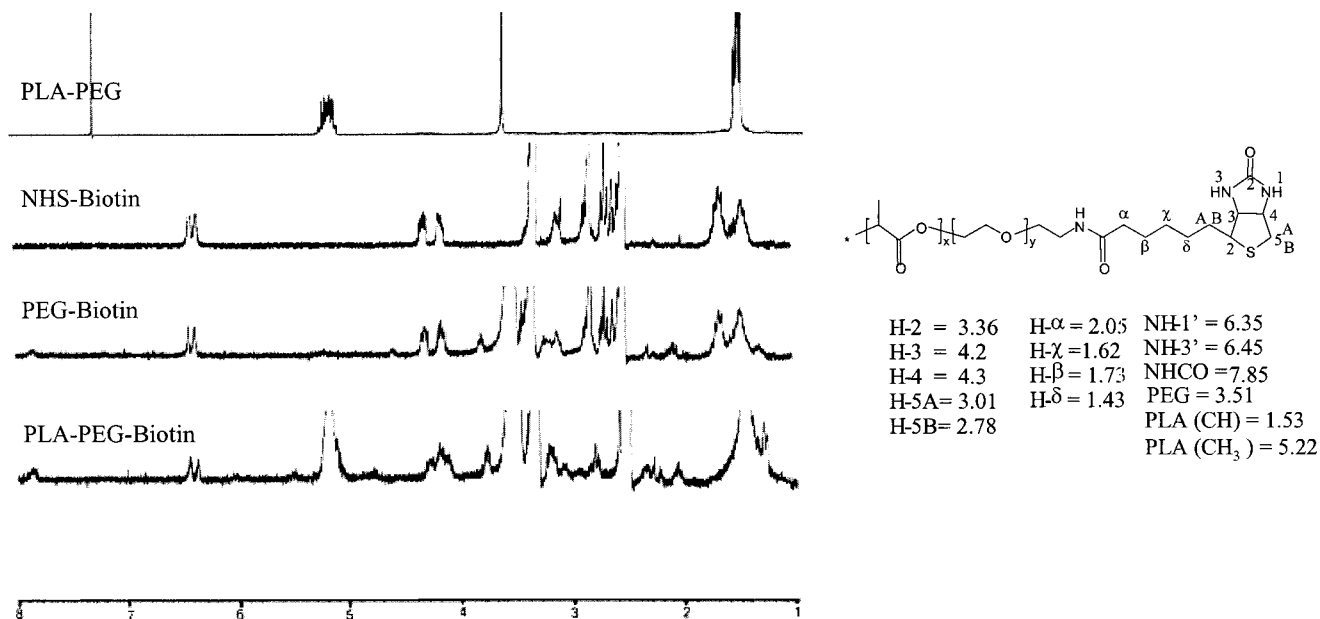


Figure 2. ^1H NMR spectra of PLA–PEG, NHS–biotin, biotin–PEG–OH, and low molecular weight PLA–PEG–biotin.

approximately 100 s after the start of each run. Results were taken from at least eight repeats.

Fluorescence Spectroscopy Studies Using the [2-(4'-Hydroxyazobenzene)benzoic acid] (HABA)/Avidin Reagent. Spectroscopy studies were completed on a Beckman DU-600 spectrometer at a fixed wavelength of 500 nm at 37 °C. The average read time was 0.5 s and the read mode [Abs]. Three blanks were read followed by three readings of the HABA/avidin in 1 mL cuvettes. Each polymer sample was recorded three times over a subset of four repeats. The assay was completed by measuring the absorbance of the avidin–HABA complex at A_{500} before and after it has been placed over a film of PLA–PEG–biotin of known surface area. The absorption decreases proportionately to the biotin present on the surface because biotin displaces HABA due to its higher affinity for avidin. The change in absorbance can then be used to calculate the amount of biotin present.³⁴

Results and Discussion

The attachment of biotin to H_2N –PEG–OH was achieved through *N*-hydroxy-succinimide chemistry. The graft polymerization of lactide to the HO–PEG–biotin proceeds through a ring-opening polymerization mediated by the $\text{Sn}(\text{Oct})_2$ complex suggested by Kricheldorf.⁵¹

Evidence of Attachment of Biotin. Analysis of PEG–biotin by ^1H NMR spectroscopy showed the appearance of a triplet at 2.05 ppm that can be assigned to the methylene from the biotin chain α to the amide and the appearance of a broad singlet belonging to the free amido proton at 7.85 ppm. These signals were not present on the NMR spectra of NHS–biotin. The biotin group was identified through the two methine protons (H-3, H-4) from the cyclic biotin structure at 4.3 and 4.2 ppm and two urea protons (H-1', H-3') from the cyclic biotin structure at 6.45, 6.35 ppm.^{52,53} In NMR spectra's of PLA–PEG–biotin (M_w 22 210), biotin signals could not be seen as the signal from the PLA overwhelmed the biotin signal. (CH; 1.53 ppm, CH₃; 5.22

ppm). For this reason a low-content lactide PLA–PEG–biotin was synthesized (0.2 g of lactide, 0.35 g of PEG–biotin). ^1H NMR studies on the low-content lactide PLA–PEG–biotin obtained by solution and melt polymerization showed that biotin integrity and attachment was maintained after graft polymerization of lactide to the PEG–biotin, with the biotin protons (H-3, H-4, H-1', H-3') at 4.2, 4.3, 6.35, and 6.45 ppm and the amide bond at 7.85 ppm still clearly detectable (Figure 2). However due to degeneracy in the 1.53 and 5.22 ppm regions from the PLA signals, SPR and fluorescent spectroscopy using the HABA/avidin complex were necessary to confirm complete biotin binding integrity.

Structural analysis and comparisons of PLA–PEG and PLA–PEG–biotin by melt and solution polymerization were undertaken to see the effect biotin attachment had on the PLA–PEG bulk properties. NMR spectroscopy and GPC studies were completed to ensure that the structural characteristics and molecular weight of the polymer were consistent so that any change in thermal properties were not due to variations other than the end group. Analysis showed very little difference when characterization was completed by NMR spectroscopy and GPC (Table 1). NMR spectroscopy analysis gave similar integral ratios of PLA to PEG for all four polymers with characteristic peaks for PLA being a doublet (CH) at 1.53 ppm and a quartet (CH₃) at 5.22 ppm and a singlet for PEG (CH₂) at 3.51 ppm. GPC studies gave a mean molecular weight of 24000 ± 2370 confirming that molecular weight differences should not be responsible for any large differences in bulk properties. DSC data showed little difference in thermal properties between the biotinylated and nonbiotinylated PLA–PEG and the melt and solution polymerized products suggesting that the biotin end group does not change the bulk properties of the polymer (Figure 3). This was consistent with the work carried out by Kricheldorf et al. on the variation of end groups and synthesis of block copolymers with PEG by anionic polymerization of lactide where DSC studies showed that thermal properties

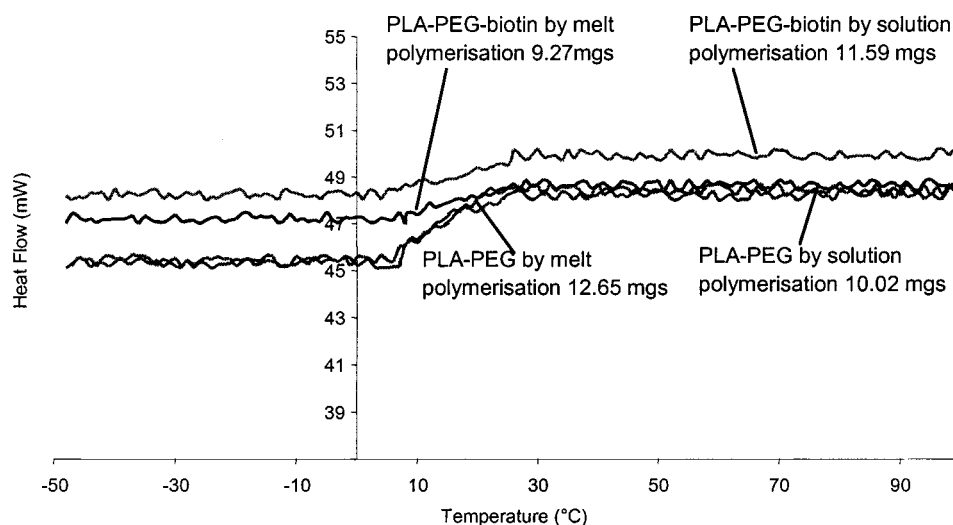
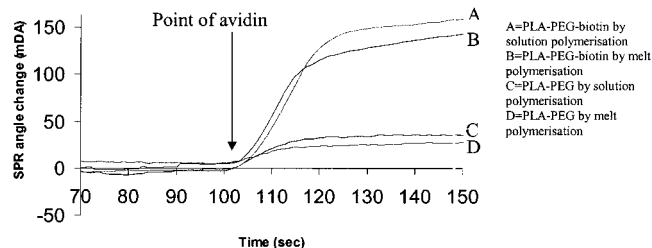
Table 1. Bulk Properties of PLA-PEG and PLA-PEG-Biotin by ^1H NMR, GPC, and DSC Analysis

polymer	LA/EG		DP_{PEG}^b	DP_{PLA}^b	M_w	M_n^c	T_g ($^{\circ}\text{C}$)	^1H NMR shifts (ppm) CDCl ₃ as ref		
	in	in						CH	CH ₂	CH ₃
	feed	product						(PLA)	(PEG)	(PLA)
PLA-PEG-biotin by melt polymerization	6.1	5.5	76	418	22 680	33 440	16	1.56	3.53	5.24
PLA-PEG-biotin by solution polymerization	6.2	5.3	76	403	22 210	32 290	17	1.53	3.51	5.22
PLA-PEG by melt polymerization.	6.2	6.8	68	462	27 440	36 260	14	1.55	3.51	5.20
PLA-PEG by solution polymerization	6.1	5.8	68	394	23 690	31 360	16	1.54	3.52	5.23

^a Determined by using the integration ratio of resonances due to PEG blocks at 3.5 ppm ($-\text{O}-\text{CH}_2-\text{CH}_2-$) and to PLA blocks at 1.55 ppm (CH_3) in the ^1H NMR spectra. ^b $\text{DP}_{\text{PEG-B}} = 3350/44 = 76$ and $\text{DP}_{\text{PEG}} = 3000/44 = 68$, $\text{DP}_{\text{PLA}} = \text{DP}_{\text{PEG}}(\text{LA}/\text{EG})$. ^c $M_n = 44\text{DP}_{\text{PEG}} + 72\text{DP}_{\text{PLA}}$.

Table 2. Surface Analysis of Biotin Integrity and Binding Capabilities by SPR and Fluorescent Spectroscopy with the HABA/Avidin Complex

polymer	SPR shifts (mDA)	absorbance value after addition to polymer film	
		in buffer	in DME tissue culture media
PLA-PEG-biotin by melt polymerization	170.46 ± 19.57	1.056 ± 0.002	1.3911 ± 0.006
PLA-PEG-biotin by solution polymerization	181.58 ± 12.19	1.052 ± 0.003	1.3749 ± 0.006
PLA-PEG by melt polymerization	40.90 ± 5.07	1.111 ± 0.010	1.4219 ± 0.005
PLA-PEG by solution polymerization	42.75 ± 6.48	1.105 ± 0.003	1.4197 ± 0.008

Scan Rate $10^{\circ}\text{C}/\text{Minute}$ **Figure 3.** DSC thermograms for PLA-PEG-biotin and PLA-PEG by melt and solution polymerization.**Figure 4.** SPR traces measuring avidin immobilization on PLA-PEG and PLA-PEG-biotin by melt and solution polymerization.

are predominantly dependent on the PLA:PEG ratio and not the end group.⁵⁴⁻⁵⁶

Having shown that the presence of the biotin group did not affect bulk properties significantly, SPR and fluorescent spectroscopy studies with HABA/avidin were completed to assess whether the biotin moiety or its binding capabilities with avidin were impaired during synthesis (Table 2). Assessment was also undertaken to resolve whether the route of synthesis showed any effect on the biotin attachment or its binding capabilities, given that melt polymerization of

this polymer had not been completed before and had been synthesized at a temperature 30°C higher than solution polymerization. SPR analysis measured avidin binding with the PLA-PEG-biotin and PLA-PEG by both synthetic routes. The increase in ϕ_{SPR} from the injection of avidin for the melt polymerized PLA-PEG was 40.90 ± 5.07 mDA and for the solution polymerized PLA-PEG was 42.75 ± 6.48 mDA. The PLA-PEG-biotin by melt and solution polymerization after injection of avidin gave ϕ_{SPR} shifts of 170.46 ± 4.89 mDA and 181.58 ± 12.19 mDA, respectively. There is a clear difference between the PLA-PEG and the PLA-PEG-biotin (Figure 3) that is consistent with previous work.⁴⁰ The shifts noted with the PLA-PEG surface can be attributed to nonspecific interactions, which have been well characterized by Green et al.^{57,58} There was no significant statistical difference in comparisons of polymers synthesized by either melt or solution polymerization.

Fluorescent spectroscopy studies using the HABA/avidin complex supported SPR data with no decrease in absorbance for the PLA-PEG and a decrease from 1.100 ± 0.004 to

1.052 (equivalent to 625 260 molecules of biotin/ μm^2) \pm 0.003 and 1.056 (539 010 molecules of biotin/ μm^2) \pm 0.002 for PLA–PEG–biotin by solution polymerization and PLA–PEG–biotin by melt polymerization respectively. A modification of the HABA/avidin protocol to assess PLA–PEG–biotin binding under cell culture conditions was to prepare the HABA/avidin reagent in Dulbecco's modified eagle medium (DMEM) with high glucose, L-glutamine, HEPES buffer, and pyrodoxine hydrochloride. The effect of the media was to increase the absorbance from 1.100 ± 0.004 to 1.4254 ± 0.013 . This value did not decrease when HABA/avidin reagent in DMEM was added to a film of PLA–PEG. A decrease was, however, detected from 1.447 ± 0.0026 to 1.3911 ± 0.0059 (625 190 molecules of biotin/ μm^2) for a film of PLA–PEG–biotin by solution polymerization and a decrease from 1.4190 ± 0.0055 to 1.3749 ± 0.0069 (514 670 molecules of biotin/ μm^2) for a film of PLA–PEG–biotin by melt polymerization. The results show clear evidence of HABA displacement with avidin by the biotin moieties with the PLA–PEG–biotin but no displacement with the PLA–PEG, which is consistent with expectations. This in conjunction with SPR data, which also showed clear differences in ϕ_{SPR} shifts between the PLA–PEG–biotin and the PLA–PEG, suggests that biotin structural integrity had been maintained together with binding capabilities during synthesis.

Conclusion

Novel biomaterials that integrate biological activity into their surface chemistries are of importance in the advancement of tissue engineering and drug delivery systems. It is necessary that in the synthesis of these materials, such as PLA–PEG–biotin, the integrity of the functionalized end group be maintained without affecting the bulk properties. Here we have shown that the synthesis of PLA–PEG–biotin can be carried without any degradation to the biotin end group. ^1H NMR spectroscopic analysis has confirmed biotin attachment and integrity while ^1H NMR, GPC and DSC characterization have shown that bulk properties are virtually unaffected by a biotin end group and are primarily dependent on the PLA:PEG ratios and molecular weights. SPR analysis and fluorescent spectroscopy studies with the HABA/avidin complex have shown that biotin's binding abilities have not been impaired during synthesis by either melt or solution polymerization.

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