Enzymatic Synthesis of Unique Thymidine-Containing Polyphenols

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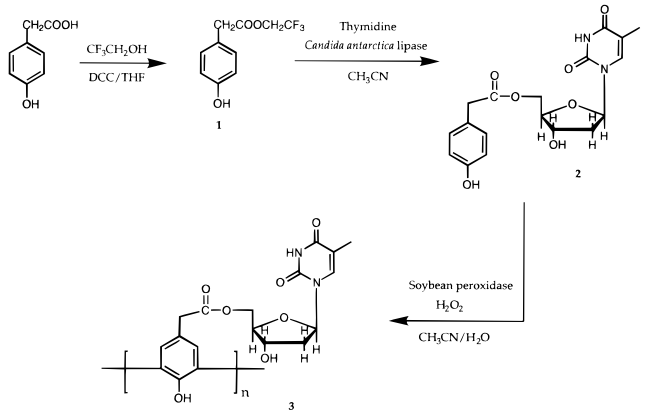
Polynucleotides, in addition to their natural role as carriers of genetic information, offer a wide array of unique chemistries that can be exploited for use as therapeutic and diagnostic reagents,[1] and as highly selective materials for biorecognition[2] where specific binding to target molecules is desired. Unfortunately, native polynucleotides are susceptible to ubiquitous endo- and exonucleases present in many biological systems. In addition, the natural phosphodiester linkage is particularly labile to harsh conditions often found in abiotic environments including extremes in pH and temperature. Conversely, replacing the phosphodiester linkage with unnatural bonds can result in a wide array of functionalized polymers. This strategy has been exploited to some extent for the chemical derivatization of thymine and related bases and nucleosides with vinyl groups to give functionalized vinyl polymers for use as selective absorbents.[3,4] Polynucleotide analogues have also been synthesized using a variety of other polymer backbones including poly(trimethyleneimine),[5] poly(vinyl alcohol)s,[6] poly(ethylenimine),[7] and poly(vinylamine).[8] Nucleic acid analogues possessing high biostability and affinity based on polypeptide backbones have also been developed.[9] In all cases, polymers with a broad range of sizes (10^3 to 10^5 Da) and controlled hydrophobicity/hydrophilicity, chain flexibility, and stability were produced for specific applications including aptamers that selectively bind organic ligands[10] and polymeric drugs for chemotherapy.[6,11]

Nearly all of the aforementioned synthetic schemes require multiple chemical steps that provide little selectivity in the attachment of the nucleoside or nucleoside derivative to the polymeric backbone. Selectivity, if necessary, is achieved by complex site-specific chemistries involving blocking and deblocking steps. Such selectivity may be critical in the preparation of polymers with side chains occupying a specific orientation around the polymer backbone, thereby resulting in unique properties. In the present work, we have developed a unique, simple, and effective technique to prepare novel polynucelosides with unnatural polymeric backbones and with high inherent selectivity. Specifically, we have used a two-enzyme coupled reaction system to generate nucleoside-based polyphenols. The resulting polymers may provide highly selective and stable materials for therapeutic, diagnostic, and materials applications.

The synthetic approach is shown in Scheme 1. Thymidine was used as a model nucleoside and a trifluoroethyl ester derivative of *p*-hydroxyphenylacetic acid

Scheme 1



(**1**)[12] was used as the phenolic derivative. Regioselective acylation of thymidine at the 5′-hydroxyl was achieved in nearly anhydrous CH_3CN using the lipase from *Candida antarctica*. This was followed by polymerization of the phenolic nucleoside derivative catalyzed by the peroxidase from soybean hulls (SBP).

The lipase-catalyzed formation of the thymidine 5′-*p*-hydroxyphenylacetate (**2**) was facile and over 95% of a 50 mM solution of **1** in CH_3CN was consumed in 18 h to give almost entirely monoester **2**.[13] Purification of the monoester resulted in two distinct products in a ratio of ca. 5:1 with overall 70% isolated yield.[13] ^1H and ^{13}C NMR confirmed that the major product was the 5′-ester (1.10 g, 59% isolated yield based on conversion of **1**). The minor product was presumably the 3′-ester (0.21 g, 11% isolated yield); thus, the enzymatic transformation in CH_3CN was highly regioselective. Similar specificity was observed for the *Pseudomonas cepacia* lipase-catalyzed acylation of thymidine with vinyl butyrate in tetrahydrofuran.[14] The resulting thymidine monoesters were soluble in CH_3CN and dimethyl sulfoxide (DMSO). The reaction was essentially catalyzed in a solid phase; the concentration of thymidine dissolved in CH_3CN did not exceed 3 mM. Hence, *C. antarctica* lipase is effective in catalyzing the efficient regioselective acylation of a poorly organic solvent-soluble nucleoside in CH_3CN.

SBP-catalyzed polymerization of **2** was carried out in the presence of H_2O_2 in a solution of aqueous buffer (50 mM Na phosphate, pH 7.0) containing 60% (v/v) CH_3CN following a procedure developed previously for polyhydroquinone synthesis.[15] Conversion of **2** reached 65% (as determined by reversed-phase HPLC) and the reaction was terminated by centrifuging the suspension and separating the reaction components into soluble and insoluble fractions.

The soluble fraction was precipitated by adding to neat CH_3CN. Extensive washing with CH_3CN followed by water gave an isolated yield of 65 mg (28% of reacted **2**) of poly(thymidine 5′-*p*-hydroxyphenylacetate) (**3**). ^1H NMR in DMSO-d_6 showed a clear presence of the thymidine (essentially identical with that in **2**); however, the phenolic peaks were significantly broadened, indicating polyphenol synthesis (data not shown). Molecular weight determination[16] provided an $M_n = 21\,700$ and an $M_w/M_n = 1.2$. DSC[17] of the polymer indicated the presence of a T_g at 60 °C and a broad melting point

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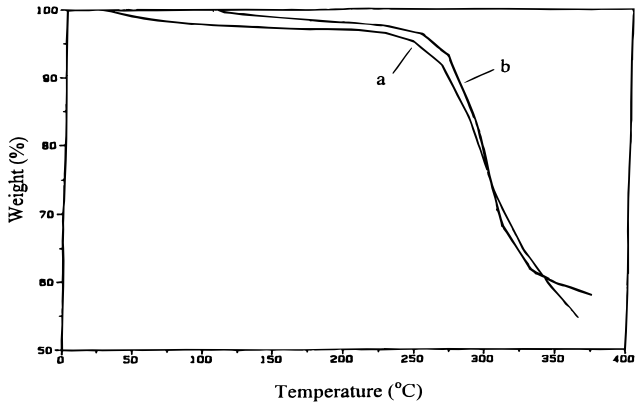

Figure 1. TGA profiles of poly(thymidine 5′-*p*-hydroxyphenylacetate) from the CH_3CN/H_2O-containing enzymatic reaction mixture: (a) soluble fraction; (b) insoluble fraction.

at 140−160 °C. The polymer was thermally stable to ca. 250 °C, as determined by TGA (Figure 1).

The insoluble fraction from the polymerization reaction was extensively washed with water (to remove entrained enzyme) and then CH_3CN to remove unreacted **2** to yield 45 mg (18% of reacted **2**). This fraction was partially soluble in DMSO. GPC analysis of the DMSO-soluble fraction (using the same value of d*n*/d*c* as that determined for the CH_3CN-H_2O soluble fraction) provided an estimate of $M_w > 10^5$. The material was subjected to DSC; however, no clear T_g was obtained nor did the material undergo a defined melting, suggesting that the polymer was both amorphous and likely polydisperse. Nonetheless, the higher molecular weight fraction of **3** was stable to over 250 °C, much as with the lower molecular weight fraction (Figure 1).

In summary, we have employed a two-enzyme reaction sequence to generate novel phenolic polymers containing a thymidine pendent group. It is anticipated that this approach is not limited to either the nucleoside or the phenol and that many natural or unnatural nucleosides and phenols can be used. It is important to note that both lipases[18] and peroxidases[19] are among the most broadly selective enzymes and can accept a tremendous variety of nucelophilic and phenolic substrates, respectively. The former is expected to provide significant structural and chemical diversity, while the latter is expected to provide polymer backbone structure and stability. We are presently investigating further the influence of reaction regioselectivity (controlled by the lipase) and the polymer size (controlled by the peroxidase) on the physicochemical properties of the polymer. A potential advantage of the coupled enzymatic approach is the high degree of control that can be exerted over both reactions that comprise the synthetic scheme for the generation of these materials. As such, these materials may be useful as artificial matrices and as selective adsorbents of nucleosides and their polymeric nucleic acids and derivatives in bioseparations.

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(12) *p*-Hydroxyphenylacetic acid (38 g, 0.25 mol), 1,3-dicyclohexylcarbodiimide (DCC, 52 g, 0.25 mol), and trifluoroethanol (90 mL, 1.24 mol) were mixed in 0.41 L of THF at a concentration of 0.5, 0.5, and 2.5 M, respectively. The mixture was magnetically stirred at 250 rpm and 25 °C for 24 h. The *p*-hydroxyphenylacetic trifluoroethyl ester (**1**) was purified using silica gel flash chromatography with a mobile phase consisting of ethyl acetate:hexane (1:3). The isolated yield of ester was ca. 30% based on the weight of added acid. ^{1}H NMR (DMSO-d_6, δ, ppm): 3.41 (2H, s, $-CH_2-$ of the acid), 4.50−4.63 (2H, 4 peaks, $-CH_2-$ of the alcohol), 6.60 (2H, d, aromatic), 6.90 (2H, d, aromatic), 9.21 (1H, s, phenyl-OH). ^{13}C NMR (DMSO-d_6, δ, ppm): 38.35 (C-7), 59.27−60.44 (5 peaks, CF_3), 115.2 (C-2, 6), 119−128 (4-peaks, CF_3), 123.6 (C-4), 130.3 (C-3, 5), 156.5 (C-1), 170.3 (C=O).

(13) Thymidine (2.42 g, 10 mmol) and 1.17 g (5.0 mmol) of **1** were mixed in 0.1 L of CH_3CN. The solubility of thymindine in CH_3CN was ca. 3 mM at 25 °C. Thus, excess thymidine was present in suspension to ensure saturation in the reaction solution throughout the course of enzymatic reaction. The reaction was initiated by the addition of 200 mg of *C. antarctica* lipase (Novo Nordisk, Baegsvaerd, Denmark), and the reaction mixture was stirred at 250 rpm and 25 °C. Following the reaction (18 h), the enzyme was removed by centrifugation, and **2** was purified by silica gel flash chromatography with neat CH_3CN as the mobile phase. ^{1}H NMR (DMSO-d_6, δ, ppm): 1.82 (3H, d, CH_3- of thymidine), 2.07 (2H, s, 2′-H of sugar moiety of thymidine), 3.60 (2H, s, $-CH_2-$ of the acid), 3.95 (1H, m, 4′-H), 4.15−4.30 (3H, m, 5′- and 3′-H), 5.41 (1H, 3′-OH), 6.18−6.24 (1H, m, 1′-H), 6.71 (2H, d, aromatic), 7.06 (2H, d, aromatic), 7.36−7.45 (1H, d, 3-H of the base), 9.30 (1H, s-broad, phenyl-OH), 11.29 (1H, s, NH of the base). ^{13}C NMR (DMSO-d_6, δ, ppm): 12.23 (C-5, CH_3- group), 38.67 (C-2′-sugar moiety), 40.0 ($-CH_2-$ of the ester), 64.30 (C-5′), 70.4 (C-3′), 83.70 (C-5′), 83.88 (C-1′), 109.9 (C-3 of the base), 115.2 (C-2,6 of phenyl), 124.3 (C-4 of phenyl), 130.3 (C-3,5 of phenyl), 135.9 (C-4 of thymidine), 150.5 (C-1 of thymidine), 156.4 (C-1 of phenyl), 163.8 (C-2 of thymidine), 171.6 (C=O).

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(16) GPC was performed with a Waters HR 5E Styrogel column (M_w 2000 to 1×10^6), connected with a Wyatt miniDawn (Wyatt Technology, CA) light scattering detector and Waters 410 refractometer. DMSO was used as the solvent for

polymers, and the d*n*/d*c* of the poly(phenolic thymidine) was determined to be 0.08 mL/g.

(17) Thermal analysis of the polymer was performed on a DSC 2910 and TGA 2950 (DuPont Instruments, Wilmington, DE). The heating rates were all at 10 °C/min, and the sample size was ca. 4 mg. For thermal analyses the flow rate of nitrogen gas was 100 mL/min.

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