Title

Phenolic Acid-based Poly(anhydride-esters) as Antioxidant Biomaterials

Journal

Macromolecular Bioscience, 16(2)

ISSN

1616-5187

Authors

Prudencio, Almudena
Faig, Jonathan J
Song, MinJung
et al.

Publication Date

2016-02-01

DOI

10.1002/mabi.201500244

Peer reviewed

HHS Public Access

Author manuscript

Macromol Biosci. Author manuscript; available in PMC 2017 February 01.

Published in final edited form as:

Macromol Biosci. 2016 February ; 16(2): 214–222. doi:10.1002/mabi.201500244.

Phenolic acid-based poly(anhydride-esters) as antioxidant biomaterials[a]

Almudena Prudencio,
Department of Chemistry and Chemical Biology, Rutgers University, 610 Taylor Road, Piscataway, NJ 08854-8087, USA

Jonathan J. Faig,
Department of Chemistry and Chemical Biology, Rutgers University, 610 Taylor Road, Piscataway, NJ 08854-8087, USA

MinJung Song, and
Department of Biomedical Engineering, Rutgers University, 599 Taylor Road, Piscataway, NJ 08854-8087, USA

Prof. Kathryn E. Uhrich
Department of Chemistry and Chemical Biology, Rutgers University, 610 Taylor Road, Piscataway, NJ 08854-8087, USA

Kathryn E. Uhrich: keuhrich@rutgers.edu

Abstract

Poly(anhydride-esters) comprised of naturally occurring, non-toxic phenolic acids, namely syringic and vanillic acid, with antioxidant properties were prepared via solution polymerization methods. Polymer and polymer precursor physiochemical properties were characterized, including polymer molecular weight and thermal properties. *In vitro* release studies illustrated that polymer hydrolytic degradation was influenced by relative hydrophobicity and degree of methoxy substitution of the phenolic acids. Further, the released phenolic acids were found to maintain antioxidant potency relative to free phenolic acid controls as determined by a 2,2-diphenyl-1-picrylhydrazyl assay. Polymer cytotoxicity was assessed with L929 fibroblasts in polymer-containing media; appropriate cell morphology and high fibroblast proliferation were obtained for the polymers at the lower concentrations. These polymers deliver non-cytotoxic levels of naturally occurring antioxidants, which could be efficacious in topical delivery of antioxidant therapies.

Keywords

biodegradable; drug delivery systems; antioxidants; poly(anhydride-esters); phenolic acids

Correspondence to: Kathryn E. Uhrich, keuhrich@rutgers.edu.

Supporting Information

Proliferation of L929 fibtroblasts in 0.01 mg/mL Phenolic acid-based PAEs (**4a**–**d**) and fluorescent images showing cell morphology at 0.1 and 0.01 mg/mL Phenolic acid-based PAEs (**4a**–**d**). Supporting Information is available from the Wiley Online Library.

1. Introduction

Increased life expectancies have led to a rise in individuals predisposed to aging-associated chronic diseases. Contributing to this development of aging-associated chronic diseases is oxidative stress, a condition in which the accumulation of free radicals (e.g., reactive oxygen species) overcomes the body's natural ability to remove them.[1–3] Oxidative stress has been shown to induce DNA, protein, and lipid oxidation,[3, 4] impacting a myriad of cellular functions and leading to cardiovascular disorders,[4, 5] cancer,[2] and neurodegenerative diseases such as Parkinson's disease[6] and Alzheimer's disease.[3, 7] While the human body employs antioxidants to mitigate reactive oxygen species,[3] additional measures are often necessary to inhibit oxidative stress. Thus, considerable research has focused on attenuating oxidative stress-induced damage through the use of external antioxidants.[5, 8, 9]

Numerous therapeutically relevant antioxidants have shown promise in combatting oxidative stress. Syringic acid (SGA) and vanillic acid (VA), two such therapeutics, are naturally occurring, bioactive phenolic acids commonly found in biorenewable resources such as herbs, fruits, and vegetables.[10, 11] Research over the past decade has illustrated the therapeutic potential of VA and SGA as a prophylactic treatment for cardiovascular[12] and neurodegenerative disease.[13] Additionally, SGA and VA have been shown to possess hepatoprotective capabilities,[14] which is beneficial in curtailing chronic liver disease, and have been effectively utilized as antihypertensive agents against L-NAME-induced hypertensive rats.[15] Furthermore, SGA and VA have been found to possess topically relevant properties. *In vitro* studies suggest VA to be an effective antimelanogenic agent via inhibition of tyrosinase activity,[16] and SGA to be successful in diminishing cyclooxygenase-2,[17] and enzymes associated with extracellular matrix degradation,[18] activity.

Owing to VA and SGA's efficacious properties, researchers have investigated their incorporation into biomaterials.[19, 20] For instance, VA-loaded zinc/aluminum-based layered double hydroxides were successfully formulated for bioactive delivery but suffered rapid release rates (81% in ~ 8 h), which could result in increased toxicity.[21] Additionally, VA and SGA have been incorporated into polymeric systems. Miller *et al.* synthesized VA- and SGA-containing polyesters; however, the bioactive in these systems was conjugated through water-stable ether bonds.[22] Similarly, biodegradable VA-containing copolyesters as well as VA- and SGA-containing random copolyanhydrides have been synthesized, but their bioactive release and biological activity have not been evaluated.[23–26]

While antioxidants can efficaciously combat oxidative stress, delivery vehicles and/or formulations are often required to provide sustained delivery[27] and overcome antioxidant instability.[28–30] We utilized an approach developed in our lab, namely the chemical incorporation of bioactives and biocompatible linker molecules to generate biodegradable, biocompatible polymers. In a recent example, hydroxycinnamates (i.e., ferulic acid and *p*-coumaric acid) were incorporated into poly(anhydride-ester) (PAE) and shown to protect the bioactives from decomposition while retaining bioactivity upon hydrolytic release.[28, 31, 32]

In this paper, we describe the two-step synthesis of biodegradable VA- and SGA-containing PAEs with adipic and sebacic linker molecules that release antioxidants in a sustained manner. The hydrolytic degradation profiles of VA- and SGA-containing PAEs are influenced by both polymer hydrophobicity and steric effects surrounding the ester bond. Bioactive is measured using a 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay to assess the antioxidant activity of the polymer degradation media. Additionally *in vitro* cytotoxicity experiments will be investigated using L929 fibroblasts to determine if these polymers are appropriate for topical use.

2. Experimental Section

2.1. Materials

1 N hydrochloric acid (HCl), 1 N sodium hydroxide (NaOH), polytetrafluoroethylene (PTFE) and poly(vinylidine fluoride) syringe filters, and Wheaton glass scintillation vials were obtained from Fisher Scientific (Fair Lawn, NJ). All other chemicals and solvents were acquired from Sigma-Aldrich (Milwaukee, WI) and used as received unless mentioned otherwise.

2.2. Structural Characterization

Proton (^{1}H) and carbon (^{13}C) nuclear magnetic resonance (NMR) spectroscopies were recorded on a Varian 400 or 500 MHz spectrometer by dissolving samples (5–10 mg for ^{1}H NMR, 40 mg for ^{13}C NMR) in deuterated dimethyl sulfoxide (DMSO-d_6), which also served as internal reference. Fourier transform infrared (FTIR) spectra were acquired from a Thermo Nicolet/Avatar 360 spectrophotometer, by pressing polymer precursors (1 wt. %) with KBr into discs or solvent-casting polymers with dichloromethane (DCM) onto sodium chloride plates. Each sample spectrum was subjected to 32 scans.

2.3. Molecular Weight

Polymer weight-averaged molecular weight (M_w) and polydispersity index (PDI) data were determined using gel permeation chromatography (GPC). A Waters (Milford, MA) system consisting of a 1515 Isocratic HPLC pump, a 717plus autosampler, and a 2414 refractive index detector was used. An IBM ThinkCentre CPU with Waters Breeze Version 3.20 software was used for data acquisition and analysis. Samples were dissolved in DCM (10 mg/mL) and filtered through 0.45 um PTFE syringe filters prior to injection. Aliquots (10 μL) were injected and resolved on a Jordi divinylbenzene mixed-bed GPC column (7.8 × 300 mm, Alltech Associates, Deerfield, IL) at 25 °C, with DCM as the mobile phase at a flow rate of 1.0 mL/min. Molecular weights were calibrated relative to broad polystyrene standards (Polymer Source Inc., Dorval, Canada). Elemental analyses were provided by QTI (Whitehouse, NJ).

2.4. Thermal Analysis

Differential scanning calorimetry (DSC) measurements were performed on a Thermal Advantage (TA; New Castle, DE) DSC Q200 and processed using an IBM ThinkCentre computer equipped with TA Universal Analysis 2000 software, version 4.5A. Samples (4–6 mg) were heated from −10 – 200 °C at a rate of 10 °C/min and cooled at a rate of 10 °C/min

to −10 °C with a minimum of two cycles for each sample set to determine polymer precursor melting (T_m) and polymer glass transition (T_g) temperatures.

Thermogravimetric analysis (TGA) was used to obtain polymer sample decomposition temperatures (T_d). TGA analysis was conducted using a PerkinElmer (Waltham, MA) TGA7 analyzer with TAC7/DX controller equipped with a Dell OptiPlex Gx 110 computer operating Perkin Elmer Pyris software. Polymer samples (5–10 mg) were heated under nitrogen at a rate of 10 °C/min from 25 – 400 °C. T_d was defined as the start of decomposition, illustrated by the beginning of a sharp slope on the thermogram.

2.5. Phenolic Acid-based Diacid (3) Synthesis

Phenolic acid-based diacids were synthesized following a modified protocol (Scheme 1).[33, 34] In brief, phenolic acid (**1**, VA or SGA, 2 eq.) was dissolved in anhydrous tetrahydrofuran (THF, 50 mL) under nitrogen, followed by pyridine (6 eq.) addition. A solution of diacyl chloride (**2**, 1.1 eq.) diluted in anhydrous THF (10 mL) was then added drop-wise over 1 h, producing an off-white suspension. After stirring an additional 3 h, the reaction was quenched with deionized (DI) water and acidified to pH ~ 2 using concentrated HCl. Diacid (**3**) was then isolated via vacuum filtration, washed with DI water (3 × 100mL), and subsequently dried overnight under vacuum at 60 °C to acquire pure **3.2**.

VA (adipic) diacid (3a)—Yield: 4.65 g, 93% (pale yellow powder). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.59 (m, 4H, ArH), 7.22 (d, 4H, ArH), 3.83 (s, 6H, -OCH_3), 2.67 (t, 4H, CH_2), 1.78 (t, 4H, CH_2). ^{13}C NMR (DMSO-d_6): δ 171.4, 167.3, 151.5, 143.7, 130.2, 123.7, 122.9, 113.8, 56.5 33.5, 24.4. IR (KBr, cm^{-1}): 3600-3100 (OH, COOH), 1756 (C=O, ester), 1694 (C=O, COOH). Anal. Calcd.: C, 59.2%; H, 5.0%; O, 35.8%. Found: C, 58.8%; H, 4.8%; O, 36.3%. T_m: 205–206 °C.

VA (sebacic) diacid (3b)—Yield: 4.07 g, 81% (pale yellow powder). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.56 (m, 4H, ArH), 7.18 (d, 4H, ArH), 3.80 (s, 6H, -OCH_3), 2.57 (t, 4H, CH_2), 1.64 (t, 4H, CH_2), 1.35 (m, 8H, CH_2). ^{13}C NMR (DMSO-d_6): δ 171.5, 167.3, 151.5, 143.7, 130.2, 123.7, 122.8, 113.8, 56.5, 33.8, 29.3, 28.9, 25.1. IR (KBr, cm^{-1}): 3650-3100 (OH, COOH), 1759 (C=O, ester), 1698 (C=O, COOH). Anal. Calcd.: C, 62.2%; H, 6.0%; O, 31.8%. Found: C, 61.8%; H, 6.1%; O, 32.1%. T_m: 195–197 °C.

SGA (adipic) diacid (3c)—Yield: 4.53 g, 90% (white powder). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.28 (s, 4H, ArH), 3.81 (s, 12H, -OCH_3), 2.64 (t, 4H, CH_2), 1.77 (t, 4H, CH_2). ^{13}C NMR (DMSO-d_6): δ 171.0, 167.4, 152.4, 132.3, 129.6, 106.6, 56.8, 34.0, 24.5. IR (KBr, cm^{-1}): 3650 (OH, COOH), 1764 (C=O, ester), 1690 (C=O, COOH). Anal. Calcd.: C, 56.9%; H, 5.2%; O, 37.9%. Found: C, 56.6%; H, 5.1%; O, 38.3%. T_m: 238–240 °C.

SGA (sebacic) diacid (3d)—Yield: 4.26 g, 85% (white powder). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.26 (s, 4H, ArH), 3.79 (s, 12H, -OCH_3), 2.55 (t, 4H, CH_2), 1.64 (t, 4H, CH_2), 1.39 (m, 8H, CH_2). ^{13}C NMR (DMSO-d_6): δ 171.1, 167.4, 152.4, 132.4, 129.5, 106.6, 56.8, 33.7, 29.3, 28.9, 25.3. IR (KBr, cm^{-1}): 3600-3100 (OH, COOH), 1763 (C=O, ester), 1694

(C=O, COOH). Anal. Calcd.: C, 59.8%; H, 6.1%; O, 34.1%. Found: C, 59.5%; H, 6.2%; O, 34.3%. T_m: 193–195 °C.

2.6. Phenolic Acid-based PAE (4) Synthesis

Phenolic acid-based diacids (**3a**–**d**) were polymerized via solution polymerization.[28, 31, 33] Diacids (**3a**–**d**, 1 eq.) were suspended in anhydrous DCM (25 mL) under nitrogen followed by anhydrous triethylamine (TEA, 4.4 eq.) addition. The reaction was cooled to 0 °C and a 20% w/v solution of triphosgene (1 eq.) in anhydrous DCM added drop-wise over 45 min. The reaction was allowed to stir until complete CO_2 evolution ceased. Crude polymer was precipitated over chilled diethyl ether and isolated via vacuum filtration. The crude product was then dissolved in DCM (50 mL), washed with 1 N HCl (150 mL), and the organic layer was concentrated *in vacuo* to obtain pure polymer (**4a**–**d**).

VA (adipic) PAE (4a)—Yield: 1.22 g, 61% (off-white powder). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.78 (m, 4H, ArH), 7.34 (d, 4H, ArH), 3.86 (s, 6H, -OCH_3), 2.68 (t, 4H, CH_2), 1.77 (t, 4H, CH_2). IR (NaCl, cm^{-1}): 1787, 1721 (C=O, anhydride), 1766 (C=O, ester). M_w = 85.0 kDa, PDI = 1.8. T_d = 293 °C, T_g = 59 °C. Contact Angle = 60°.

VA (sebacic) PAE (4b)—Yield: 1.46 g, 49% (off-white powder). ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.74 (m, 4H, ArH), 7.31 (d, 4H, ArH), 3.85 (s, 6H, -OCH_3), 2.58 (t, 4H, CH_2), 1.64 (t, 4H, CH_2), 1.34 (m, 8H, CH_2). IR (NaCl, cm^{-1}): 1783, 1724 (C=O, anhydride), 1767 (C=O, ester). M_w = 90.0 kDa, PDI = 1.4. T_d = 344 °C, T_g = 36 °C. Contact Angle = 71°.

SGA (adipic) PAE (4c)—Yield: 1.44 g, 72% (white powder). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.47 (s, 4H, ArH), 3.85 (s, 12H, -OCH_3), 2.67 (t, 4H, CH_2), 1.77 (t, 4H, CH_2). IR (NaCl, cm^{-1}): 1791, 1723 (C=O, anhydride), 1766 (C=O, ester). M_w = 26.2 kDa, PDI = 1.5. T_d = 317 °C, T_g = 109 °C. Contact Angle = 61°.

SGA (sebacic) PAE (4d)—Yield: 0.79 g, 52% (white powder). ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.44 (s, 4H, ArH), 3.84 (s, 12H, -OCH_3), 2.58 (t, 4H, CH_2), 1.64 (t, 4H, CH_2), 1.37 (m, 8H, CH_2). IR (NaCl, cm^{-1}): 1790, 1721 (C=O, anhydride), 1763 (C=O, ester). M_w = 33.9 kDa, PDI = 1.5. T_d = 354 °C, T_g = 80 °C. Contact Angle = 75°

2.7. Relative Polymer Hydrophobicity

Sessile-drop contact angles of DI water on polymer discs were measured using an automated Ramé-hart goniometer (Model 250, Netcong, NJ) with DROPimage advanced software. Angles were measured in triplicate for each polymer and an average value obtained. Polymer discs were prepared by pressing finely ground polymers (55 ± 5 mg) into 8 mm diameter × 1 mm thick discs in an IR pellet die (International Crystal Laboratories, Garfield, NJ) with a bench-top hydraulic press (Carver model M, Wabash, IN) by applying a pressure of 10,000 psi for 10 min at room temperature.

2.8. *In Vitro* Phenolic Acid Release from Polymer and Accelerated Hydrolytic Degradation

In vitro degradation in phosphate-buffered saline (PBS, pH 7.4) was used to determine the release of phenolic acids from polymers. Polymer discs (n=3) were prepared by pressing

ground polymers (55 ± 5 mg) into 8 mm diameter × 1 mm thick discs in an IR pellet die (International Crystal Laboratories, Garfield, NJ) with a bench-top hydraulic press (Carver model M, Wabash, IN). A pressure of 10,000 psi was applied for 10 min at room temperature.

Hydrolytic degradation was performed by placing polymer discs in 20 mL Wheaton glass scintillation vials (Fisher Scientific, Fair Lawn, NJ) with 10 mL of PBS. Polymer discs were then incubated at 37 °C with mild agitation (60 rpm) using a controlled environment incubator-shaker (New Brunswick Scientific Co., Edison, NJ). Degradation media (5 mL) was removed at predetermined time-points and replaced with fresh PBS (5 mL) to maintain sink conditions. Spent media was examined using high-performance liquid chromatography (HPLC) with an ultraviolet-visible (UV-Vis) spectroscopy detector. The degradation products were analyzed and quantified via HPLC using an XTerra RP18 5 μm 4.6 × 150 mm column (Waters, Milford, MA) by a Waters 2695 Separations Module equipped with a Waters 2487 Dual λ Absorbance Detector. All samples were filtered through 0.22 μm poly(vinylidine fluoride) syringe filters prior to autoinjection (10 μL). Samples were then separated at 25 °C using a 50:50 v/v mobile phase comprised of 50 mM KH_2PO_4 in DI water with 1% formic acid (pH = 2.5) and methanol at a flow rate of 0.7 mL/min. Degradation media absorbance was monitored at λ = 252 and 261 nm for VA and SGA, respectively. VA and SGA release was quantified based on previously developed calibration curves from known VA and SGA standard solutions.

To confirm polymers (**4a**–**d**) fully degrade into their respective phenolic acids, (VA and SGA) an accelerated hydrolytic degradation study was conducted. Polymer powder (n = 3, 20 ± 5mg) was suspended in 5 mL 1 N NaOH in 20 mL Wheaton glass scintillation vials and incubated at 37 °C in a controlled incubator-shaker (60 rpm). After incubating two days, samples were removed and neutralized using 1 N HCl (5 mL). Media was then examined and quantified using the methods described above.

2.9. Antioxidant Radical Scavenging Activity

Following previously established protocols, a 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was utilized to evaluate the antioxidant activity of VA and SGA in the degradation media.[28, 35] Antioxidant activity was evaluated by incubating degradation media (0.1 mL) from day 30 of *in vitro* release studies in a DPPH solution (0.024 mg/mL in 3.9 mL methanol) for 1 h at room temperature, monitoring the change in absorbance at 517 nm relative to a reference sample (0.1 mL PBS in 3.9 mL DPPH solution) using a Perkin Elmer Lambda XLS spectrophotometer (Waltham, MA). Free VA and SGA solutions were made fresh at concentrations corresponding to the day 30 release media and analyzed under identical conditions to that of the degradation media. DPPH % radical reduction was determined by $[(Abs_{t0} - Abs_t)/Abs_{t0}] \times 100$, in which Abs_{t0} represents the reference absorbance and Abs_t represents the sample absorbance after 1 h. All radical scavenging assays were conducted in triplicate. Student's *t* tests were utilized to ascertain any significant difference in antioxidant activity between free VA and SGA and VA and SGA degradation media (significantly different if $p < 0.05$).

2.10. Cytotoxicity Studies

VA and SGA-based polymers' cell cytotoxicity was evaluated by culturing NCTC clone L929 mouse areolar/adipose fibroblasts in media containing dissolved polymer. Polymers were first sterilized via UV irradiation for 300 s and subsequently dissolved in DMSO (40 mg/mL stock solution). Polymer stock solutions were then serially diluted to obtain three different concentrations (1 mg/mL, 0.1 mg/mL, and 0.01 mg/mL), following standard polymer cytotoxicity protocols.[28, 36, 37] Cell culture medium consisted of Dulbecco's Modified Eagle's Medium with 10% v/v fetal bovine serum, 1% glutamine and 50 U/mL of penicillin and streptomycin. Cells were seeded onto the wells of a 24-well plate containing differing polymer solutions at a density of 5×10^4 cells per well and cultivated for 72 h. Medium containing an identical concentration of DMSO as in the three polymer solution concentrations was used as a control. All experiments were performed in triplicate for each polymer solution.

Fibroblast morphology was observed using a fluorescent microscope (IX81, Olympus, Japan) equipped with 10X phase-contrast objective at 24, 48, and 72 h. Cell cytotoxicity was measured using a Live/Dead assay, in which a Calcein AM stain served as a fluorescent marker for only live cell cytoplasms.[38] At each time point, medium was removed and fibroblasts washed with PBS (pH 7.4, MP Biomedical, Aurora, OH) and incubated with 200 μL of Calcein AM staining solution (8 μM, Molecular Probe, Carsbad, CA) for 40 min at 4 °C. Fluorescence intensity was measured using a fluorescent plate reader (Cytofluor® Series 4000, Applied Biosystems, Woodinville, CA) at 485 nm excitation and 530 nm emission. Live cell numbers were calculated against a standard curve. All polymers were assayed in triplicate.

3. Results and Discussion

3.1. Synthesis and Characterization

VA and SGA-based diacids (**3a**–**d**) were synthesized following previously established protocols,[26, 37, 39] in which pyridine was first reacted with an acyl chloride to generate a pyridinium ion that selectively reacted with the phenol of VA or SGA (**1**, Scheme 1). Diacids were isolated in high yields (84 to 93%), and their chemical structures and composition were confirmed by NMR spectroscopy, FTIR spectroscopy, and elemental analysis. Using **3a** as an example, ^{1}H NMR spectra indicated successful diacid synthesis as illustrated by the disappearance of the phenolic proton at 9.81 ppm (**1**, Figure 1A) and appearance of the methylene protons at 2.68 and 1.77 ppm (Figure 1B). Successful diacid synthesis was further demonstrated through the ester bond formation, as indicated by the FTIR carbonyl ester stretch (1756 cm^{-1}, Figure 2A).

Isolated diacids were subsequently polymerized using triphosgene in the presence of TEA to acquire PAEs **4a**–**d**. Solution polymerization was chosen after melt-condensation polymerization attempts with SGA (adipic) diacid resulted in rapid vitrification, yielding oligomers. This occurrence is consistent with previous literature using similar SGA-based diacids.[26] ^{1}H NMR spectra signified successful polymerization through the upfield shift and broadening of the aromatic peaks (Figure 1C), while FTIR spectra revealed the

disappearance of the C=O acid stretch and appearance of the anhydride peaks at 1783 and 1724 cm^{-1} (Figure 2B). The chemical incorporation of VA and SGA into PAE backbones enabled drug loading ranging from 67 to 78 wt. % (Table 1).

VA and SGA-based PAEs displayed molecular weights ranging from 26.2 to 90.0 kDA, with higher methoxy-substituted phenolic acid-based PAEs (**4c**–**d**) producing lower molecular weight polymers relative to lower methoxy-substituted phenolic acid-based PAEs (**4a**–**b**). Additionally, methoxy-substitution was found to influence both glass transition (T_g) and decomposition (T_d) temperatures (Table 1). Higher methoxy-substituted PAEs (**4c**–**d**) displayed higher T_g values compared to lower methoxy-substituted PAEs (**4a**–**b**), likely due to the additional methoxy moiety reducing flexibility about the ester bond. Increasing methoxy content when comparing different bioactives with the same linker molecule (i.e., **4a** vs. **4c**) also resulted in an increased T_d. The impact of methoxy substitution on T_g via increasing chain rigidness has previously been observed in polymer systems,[26] while methoxy-substitution trends regarding M_w, T_g, and T_g are concurrent with previous PAEs.[28, 31]

Two different aliphatic chain linker molecules (adipic acid and sebacic acid, C6 and C10, respectively) were investigated as previous research has demonstrated that the linker molecule composition influences thermal properties and degradation profile.[39] Indeed, the aliphatic chain length of the linker molecule influenced polymer molecular weights, with increasing methylene content resulting in a higher molecular weight when comparing polymers comprised of the same phenolic acids (i.e., **4a** vs. **4b**). Additionally, aliphatic linker chain length affected thermal properties by decreasing T_g and increasing T_d with increasing aliphatic content (Table 1), consistent in similar PAE systems.[39]

3.2. Polymer Hydrolytic Degrdation

Phenolic acid-based PAE hydrolytic degradation was studied using polymer discs, immersed in PBS under physiological conditions (pH = 7.4, 37 °C) for 30 days. Polymer degradation was investigated via HPLC, monitoring the release of free phenolic acid (VA or SGA) from PAE systems. VA and SGA concentrations in the degradation media were quantified at each time point based on standardized calibration curves.

In agreement with previous work, all PAEs experienced lag periods (1–2 days) in which no bioactive or precursor (i.e., diacid, short chain oligomer, etc.) was released.[40, 41] Also consistent with previous research,[41] the highly labile anhydride bonds degraded first, generating diacid (**3a**–**d**), which subsequently underwent further ester hydrolysis to yield free VA and SGA as confirmed by HPLC. HPLC chromatograms revealed peaks at retention times 4.52 and 4.30 min for VA and SGA, respectively, while additional peaks were observed at later retention times, which corresponded to other degradation products (i.e., diacid and/or short-chain oligomers, Figure 3). Cumulative percent release of free VA and SGA from their respective polymer systems is presented in Figure 4. At the completion of the 30-day study, cumulative free phenolic acid release ranged from 2.6–11.1 %. The remaining VA and SGA were either released in the form of diacid and/or short chain oligomer (Figure 3) or present as non-degraded polymer. To verify that polymers fully degrade into their respective phenolic acids, an accelerated hydrolytic degradation study was

conducted. Polymer samples (20 ± 5mg) were placed in 1 N NaOH and incubated for two days, after which media was neutralized. HPLC chromatographs revealed free phenolic acid (VA or SGA) with no additional degradation products. Additionally, the amount of free phenolic acid present in the degradation media was not be statistically different from the calculated amount that **4a**–**d** could release.

In comparing the degradation profiles of PAEs containing the same phenolic acid but different linker molecules, it was observed that longer aliphatic linker molecules promoted a slower degradation. As water penetration is a significant factor influencing polyanhydride degradation,[42] it is plausible that the relative phenolic acid-based PAE hydrophobicity impacts PAE degradation and resulting VA and SGA release. As such, phenolic acid-based PAEs' relative hydrophobicity was determined by static contact angle measurements. Static contact angle measurements of PBS on polymer disc surfaces revealed phenolic acid-based PAEs to be hydrophobic with contact angles ranging from 60 to 75° (Table 1). As the length of the aliphatic chain in the linker molecule increased, relative hydrophobicity increased as denoted by the increase in contact angle, which is consistent with published linker influences on PAEs.[39] Therefore, when comparing PAEs comprised of the same phenolic acid (e.g., **4c** vs. **4d**), cumulative degradation rates correlated to polymer hydrophobicity, with longer linker molecules slowing phenolic acid release.

Additionally, comparing the degradation profiles of PAEs containing the same linker molecule but different phenolic acid revealed slower phenolic acid release with increasing methoxy substitution. We hypothesize that the increased methoxy substitution around the ester bond reduces the rate of bond hydrolysis through steric effects.[39]

3.3. Antioxidant Radical Scavenging Activity

Degradation media antioxidant activity was assessed using a DPPH radical scavenging assay to confirm that polymer synthesis did not detrimentally impact VA and SGA's antioxidant activity (Figure 5). Polymer release media (**4a**–**d**) showed no statistical difference compared to free bioactive (**1**) of identical concentration. The percentage of DPPH reduction correlated with concentration as increasing concentration of respective bioactives (**1**) resulted in increased DPPH reduction.[43]

3.4. Cytotoxicity Studies

Phenolic acid-based PAE (**4a**–**d**) cytotoxicity studies were conducted with L929 fibroblasts, standard cells recommended by the American Society for Testing and Materials (ASTM) and representative of the predominant tissue in the body.[45] For this study, polymer solutions of 0.1 mg/mL and 0.01 mg/mL were prepared, while solutions of media containing DMSO at identical volumes to the polymer solutions were utilized as a control. These concentrations were chosen based upon the bioactive release media concentration in the *in vitro* polymer release studies. Cellular morphology and fibroblast proliferation were analyzed every 24 h over a three day period. In general, no significant morphological changes were observed between cells incubated with polymers (**4a**–**d**) at 0.01 mg/mL and 0.1 mg/mL (Figures S1 and S2) and the controls. For both polymers and controls, cells

successfully attached to the well plate and exhibited cell with rounded as well as stellate morphologies over three days.

Fibroblast proliferation was monitored over the same three-day period, which allowed enough time to guarantee cell-polymer contact and complete cell cycles. Cells displayed positive growth cycles in all polymer solutions at both concentrations. At 0.01 (Figure S3) and 0.1 mg/mL (Figure 6) polymer concentrations cell populations experienced a two-fold growth from day 1 to 3, indicative of at least one cell growth cycle, for all polymers. Additionally, at these concentrations cell numbers were within standard deviation of the control and thus displayed no cytotoxic behavior.

Correlations between cell proliferation and phenolic acid-based PAE hydrophilicity were evident throughout the study. Polymers **4a** and **4c**, the most hydrophilic as indicated by contact angle measurements, were also the most cytocompatible. This data suggests that the wettability of materials significantly promotes healthy cell morphology and proliferation by promoting cell attachment.[46, 47] Overall, VA and SGA-containing antioxidant-based PAEs were shown to be cytocompatible at concentrations 0.1 mg/mL and below, concentrations high enough to exhibit antioxidant properties as indicated by DPPH studies.

4. Conclusions

A series of VA- or SGA-containing, antioxidant-based PAEs was successfully synthesized using solution polymerization methods. Through the chemical incorporation of these bioactives into a polymer backbone, a controlled release system with high drug-loading efficiency (67% to 78%) was developed. Antioxidant-based PAEs were found to be hydrolytically degradable with released bioactives possessing comparable antioxidant activity to that of the free antioxidant bioactives. Bioactive release is a function of polymer degradation and is thus influenced by polymer hydrophobicity and steric hindrance around the ester bond (i.e., mono-substituted vs. di-substituted). Additionally, all polymers were cytocompatible with mouse fibroblasts at 0.1 mg/mL and 0.01 mg/mL concentrations, indicating their potential use *in vivo*. The physical and cytocompatible properties displayed by these antioxidant-based PAEs provide an effective means for controlled and sustained delivery of antioxidants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the National Institutes of Health and industrial collaborators for financial support.

References

1. Li Y, Kong D, Ahmad A, Bao B, Sarkar FH. Antioxidants & redox signaling. 2013; 19:139. [PubMed: 23391445]
2. Srinivasan K. Critical reviews in food science and nutrition. 2014; 54:352. [PubMed: 24188307]

3. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. The international journal of biochemistry & cell biology. 2007; 39:44. [PubMed: 16978905]
4. Elahi MMK, YX, Matata BM. Oxidative Medicine and Cellular Longevity. 2009; 2:259. [PubMed: 20716913]
5. Madamanchi NR, Vendrov A, Runge MS. Arteriosclerosis, thrombosis, and vascular biology. 2005; 25:29.
6. Smeyne M, Smeyne RJ. Free radical biology & medicine. 2013; 62:13. [PubMed: 23665395]
7. Su C, Sun F, Cunningham RL, Rybalchenko N, Singh M. Age. 2014; 36:9685. [PubMed: 25015774]
8. Svobodova AP, Walterova JD. Biomed Pap. 2003; 147:137.
9. Halliwell. Drugs & Aging. 2001; 18:685. [PubMed: 11599635]
10. Zheng WWS. J Agric Food Chem. 2001; 49:5165. [PubMed: 11714298]
11. Alves MJ, Ferreira IC, Froufe HJ, Abreu RM, Martins A, Pintado M. Journal of applied microbiology. 2013; 115:346. [PubMed: 23510516]
12. Prince PS, Dhanasekar K, Rajakumar S. Cardiovascular toxicology. 2011; 11:58. [PubMed: 21161433]
13. Rekha KR, Selvakumar GP, Sivakamasundari RI. Biomedicine & Aging Pathology. 2014; 4:95.
14. Itoh A, et al. Biol Pharm Bull. 2010; 33:983. [PubMed: 20522963]
15. Kumar SSM, Raja B. Journal of Pharmacy Research. 2012; 5:2942.
16. Chou TH, Ding HY, Hung WJ, Liang CH. Experimental dermatology. 2010; 19:742. [PubMed: 20482617]
17. Stanikunaite R, Khan SI, Trappe JM, Ross SA. Phytotherapy research : PTR. 2009; 23:575. [PubMed: 19067382]
18. Maity N, Nema NK, Abedy MK, Sarkar BK, Mukherjee PK. Journal of ethnopharmacology. 2011; 137:1300. [PubMed: 21843618]
19. Ravichandran M, Hettiarachchy NS, Ganesh V, Ricke SC, Singh S. Journal of Food Safety. 2011; 31:462.
20. He Q, Yin S, Sato T. Journal of Physics and Chemistry of Solids. 2004; 65:395.
21. Silion M, Hritcu D, Lisa G, Popa MI. Journal of Porous Materials. 2011; 19:267.
22. Mialon L, Vanderhenst R, Pemba AG, Miller SA. Macromolecular rapid communications. 2011
23. Wilsens CHRM, Verhoeven JMGA, Noordover BAJ, Hansen MR, Auhl D, Rastogi S. Macromolecules. 2014:140505135134005.
24. Lovell CS, Montes de Oca H, Farrar D, Ries ME, Ward IM. Polymer. 2010; 51:2013.
25. Lovell CS, Ries ME, Ward IM, Montes de Oca H, Farrar D. Macromolecules. 2013; 46:1201.
26. Fan J, Jiang HL, Chen D, Zhu KJ. Journal of Applied Polymer Science. 2006; 100:1214.
27. Cheng YH, Yang SH, Lin FH. Biomaterials. 2011; 32:6953. [PubMed: 21774981]
28. Ouimet MA, Griffin J, Carbone-Howell AL, Wu WH, Stebbins ND, Di R, Uhrich KE. Biomacromolecules. 2013; 14:854. [PubMed: 23327626]
29. Han L, Du LB, Kumar A, Jia HY, Liang XJ, Tian Q, Nie GJ, Liu Y. Biomaterials. 2012; 33:8517. [PubMed: 22922024]
30. Merrell JG, McLaughlin SW, Tie L, Laurencin CT, Chen AF, Nair LS. Clinical and experimental pharmacology & physiology. 2009; 36:1149. [PubMed: 19473187]
31. Ouimet MA, Stebbins ND, Uhrich KE. Macromolecular rapid communications. 2013; 34:1231. [PubMed: 23836606]
32. Carbone-Howell AL, Stebbins ND, Uhrich KE. Biomacromolecules. 2014; 15:1889. [PubMed: 24702678]
33. Carbone AL, Uhrich KE. Macromol Rapid Commun. 2009; 30:1021. [PubMed: 20161638]
34. Schmeltzer RCJM, Griffin J, Uhrich KE. J Biomater Sci, Polym Ed. 2008; 19:1295. [PubMed: 18854123]
35. Scherer R, Godoy HT. Food Chemistry. 2009; 112:654.
36. Schmalenberg KE, Frachiger L, Nikkhouy-Albers L, Uhrich KE. Biomacromolecules. 2001; 2:851. [PubMed: 11710041]

37. Schmeltzer SKE, RC, Uhrich KE. Biomacromolecules. 2005; 6:359. [PubMed: 15638540]
38. Cutler S. Biomaterials. 2003; 24:1759. [PubMed: 12593958]
39. Prudencio SRCA, Uhrich KE. Macromolecules. 2005; 38:6895. [PubMed: 23976793]
40. Gopferich ATJ. Advanced Drug Delivery Reviews. 2002; 54:911. [PubMed: 12384315]
41. Erdmann LU, KE. Biomaterials. 2000; 21:1941. [PubMed: 10941915]
42. Goepferich A. Biomaterials. 1996; 17:103. [PubMed: 8624387]
43. Hua D, Zhang D, Huang B, Yi P, Yan C. Carbohydrate polymers. 2014; 103:143. [PubMed: 24528712]
44. Cai YZ, Mei S, Jie X, Luo Q, Corke H. Life sciences. 2006; 78:2872. [PubMed: 16325868]
45. Duncan, EL.; Mayesh, J.; Saha, P.; Saha, S.; Scranton, M. New Products and Standards in Biomaterials Science. Elsevier Academic Press; New York: 2004.
46. van Kooten TS, van der Mei J, Busscher H. Biomaterials. 1993; 13:897. [PubMed: 1477258]
47. van Wachem PB, Feijein T, Bantjes J, Detmers A, van Aken JW. Biomaterials. 1985; 6:403. [PubMed: 4084642]

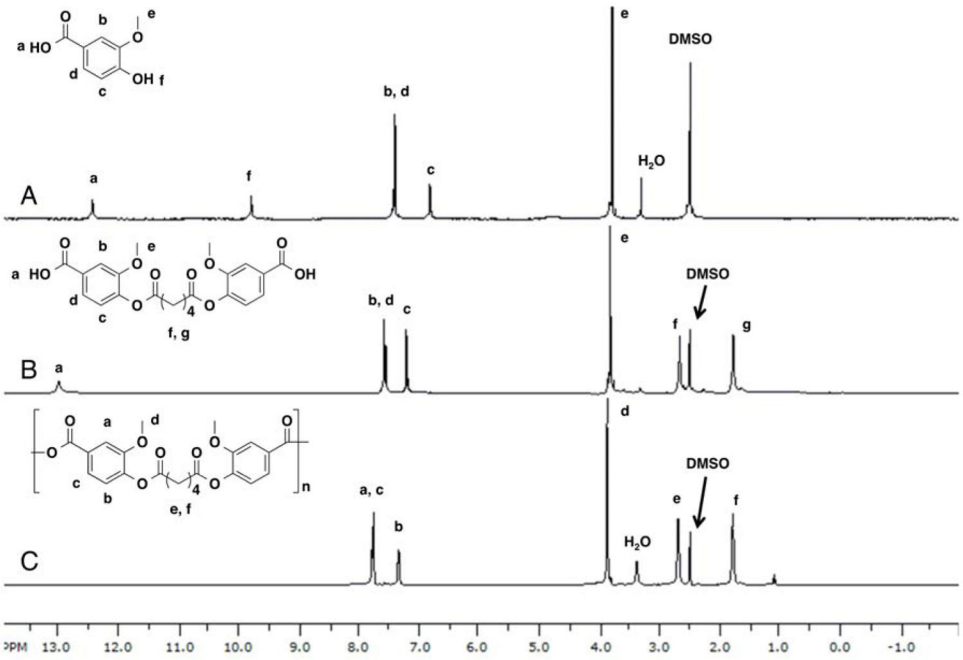


Figure 1.

Representative ^{1}H NMR spectra comparing VA (adipic) PAE and polymer precursors. VA, **1** (A, top), VA (adipic) diacid, **3** (B, middle), and VA (adipic) PAE, **4** (C, bottom) spectra. Coupling of VA to adipoyl chloride confirmed via appearance of methylene protons (B, f and g), while successful polymerization (B) is evident by the upfield shift in aromatic protons (C).

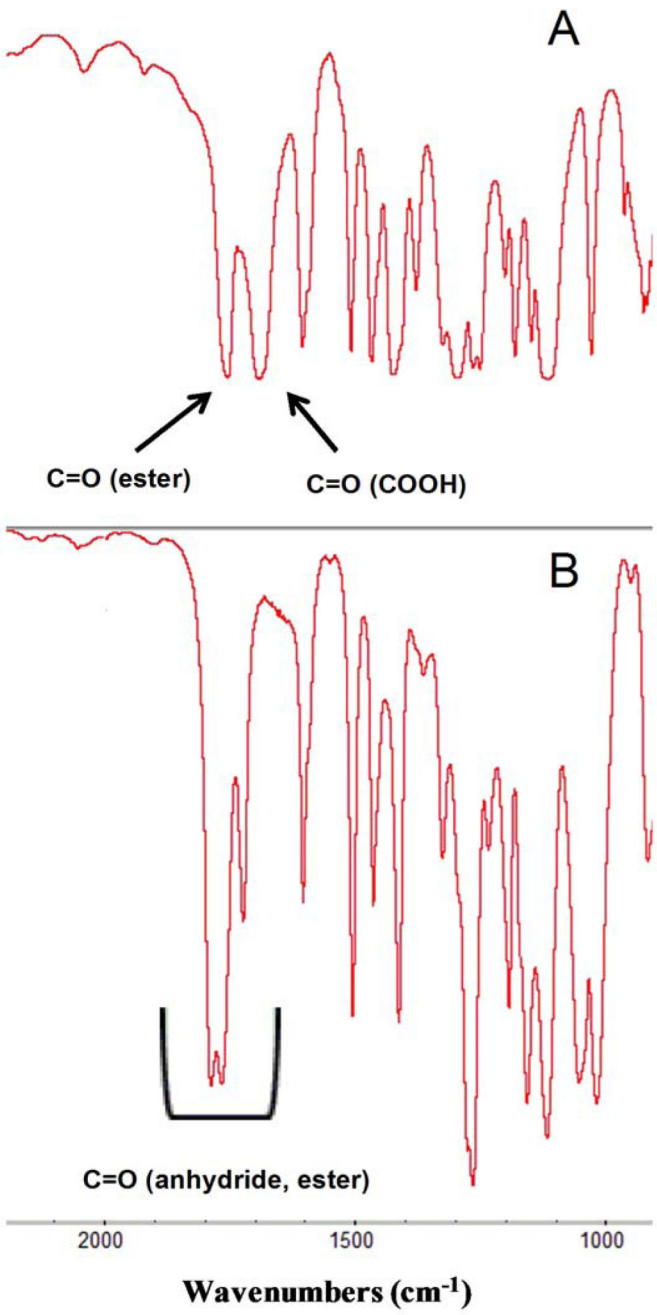


Figure 2.

Representative FTIR spectra of VA (adipic) diacid, 3 (A, top) and VA (adipic) PAE, **4** (B, bottom). Presence of C=O (COOH) at 1698 cm^{-1} in A verifies VA (adipic) diacid synthesis whereas the absence of the C=O (COOH) and appearance of the C=O anhydride (1787, 1721 cm^{-1}) confirms successful polymer synthesis.

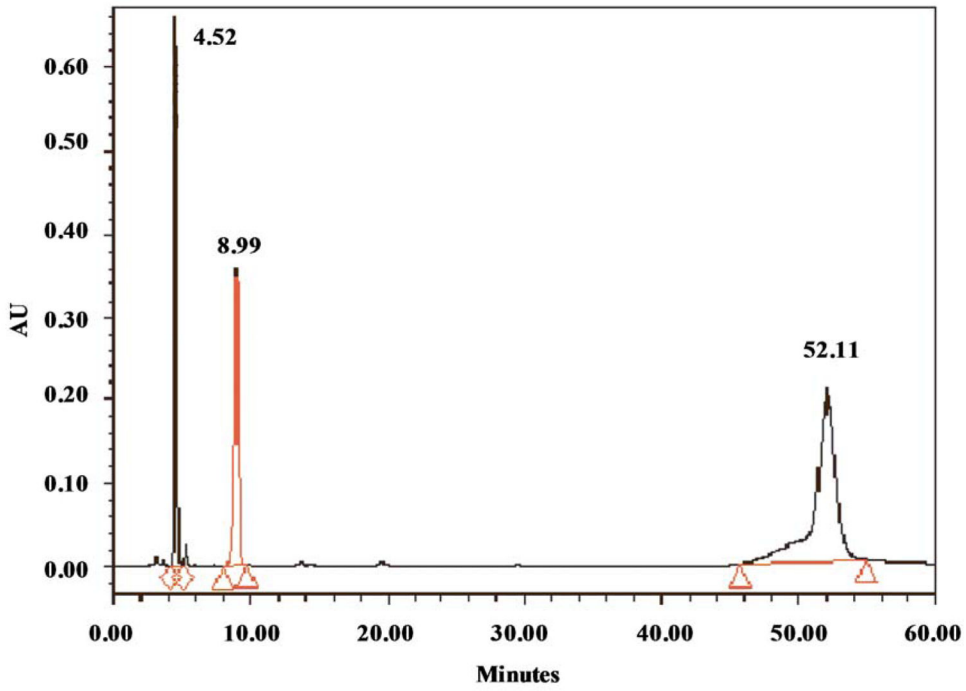


Figure 3.

HPLC chromatograph of VA (adipic) PAE disc degradation on day 7 showing VA at 4.52, VA (adipic) diacid at 8.99, and short-chain oligomers at 52.11 min retention time.

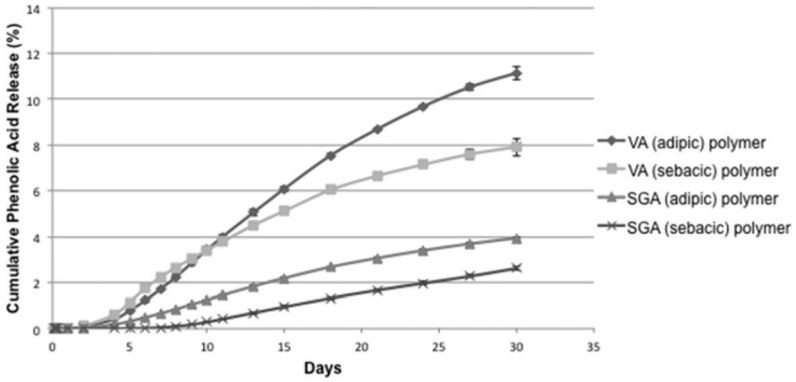


Figure 4.

Release curve showing the cumulative release of free bioactive (free VA or SGA) over 30 days, based upon known bioactive concentration from VA- and SGA- PAE discs incubated under physiological conditions.

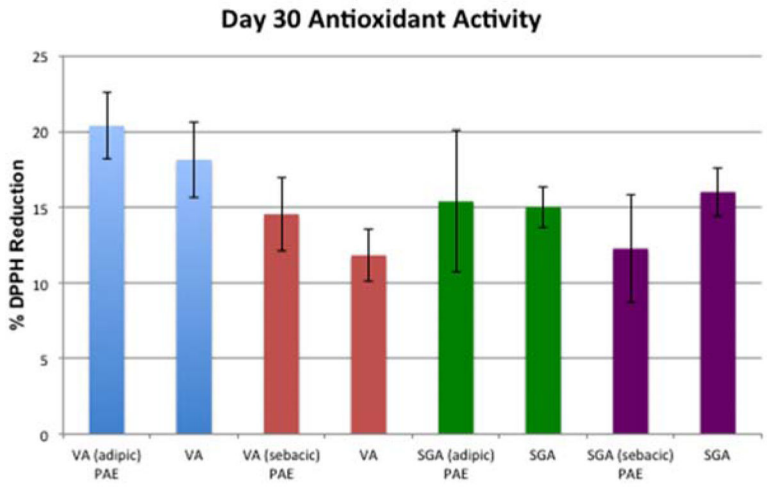


Figure 5.

Antioxidant activity of VA and SGA-based PAEs release media compared to free bioactive (VA or SGA) of identical concentration. Study reveals that antioxidant activity was maintained in released bioactives.

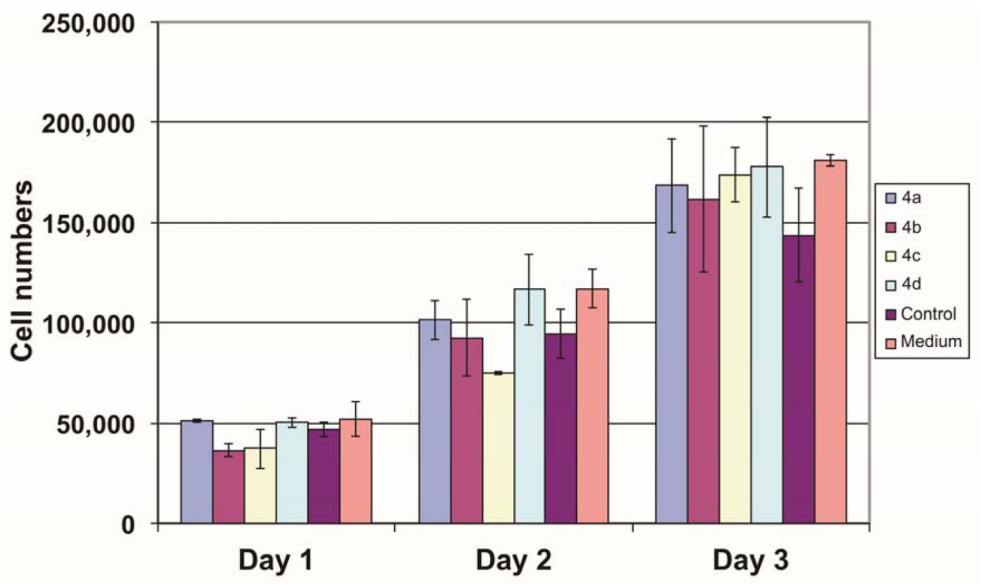
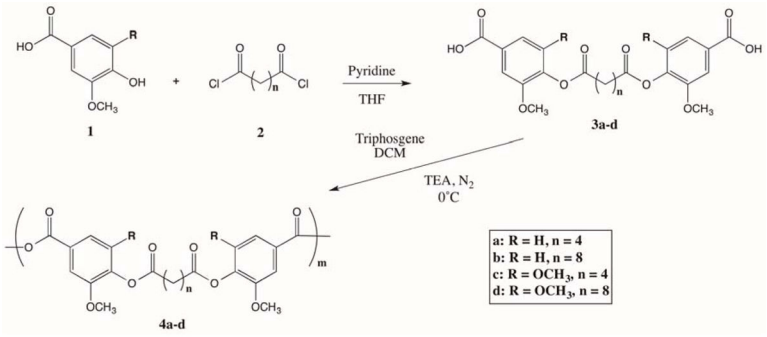


Figure 6.

Proliferation of L929 fibroblasts in 0.1 mg/mL Phenolic acid-based PAEs (**4a–d**) at days 1–3. Control is DMSO-containing media with an identical concentration DMSO as polymer samples.



Scheme 1.

Synthetic scheme for phenolic acid-based PAE (**4**)

Table 1

Properties of Phenolic acid-based PAEs (**4a**–**d**). Polymer drug loading was calculated from the repeat unit structure. GPC was used to ascertain polymer Mw and PDI.

Polymer	Drug Loading (%)	Mw (kDa)	PDI	T_g (°C)	T_d (°C)	Contact Angle (°)
4a	75.3	85.0	1.8	59	293	60 ± 1.5
4b	66.9	90.0	1.4	37	344	71 ± 0.58
4c	78.3	26.2	1.5	109	317	61 ± 2.9
4d	70.5	33.9	1.5	80	354	75 ± 1.5