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Complete List of Authors:	Yapor, Janet; Colorado State University, Department of Chemistry Lutzke, Alec; Colorado State University, Pegalajar-Jurado, Adoracion; Colorado State University, Neufeld, Bella; Colorado State University, Chemistry Damodaran, Vinod; Colorado State University, Chemistry Reynolds, Melissa; Colorado State University, Chemistry

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J. P. Yapor,[a] A. Lutzke,[a] A. Pegalajar-Jurado,[a] B. H. Neufeld,[a] V. B. Damodaran,[a†] M. M. Reynolds*[a,b]

Nitric oxide (NO) is a biologically-active free radical involved in numerous physiological processes such as regulation of vasodilation, promotion of cell proliferation and angiogenesis, and modulation of the inflammatory and immune responses. Furthermore, NO has demonstrated the ability to mitigate the foreign body response that often results in the failure of implanted biomedical devices. Although NO has promising therapeutic value, the short physiological half-life of exogenous NO complicates its effective delivery. For this reason, the development of NO-releasing materials that permit the localized delivery of NO is an advantageous method of utilizing this molecule for biomedical applications. Herein, we report the synthesis and characterization of biodegradable NO-releasing polyesters prepared from citric acid, maleic acid, and 1,8-octanediol. NO release was achieved by incorporation of *S*-nitrosothiol donor groups through conjugation of cysteamine and ethyl cysteinate to the polyesters, followed by *S*-nitrosation with *tert*-butyl nitrite. The extent of NO loading and the release properties under physiological conditions (pH 7.4 PBS, 37 °C) were determined by chemiluminesence-based NO detection. The average total NO content of poly(citric-*co*-maleic acid-*co*-1,8-octanediol)-cysteamine was determined to be 0.45 ± 0.07 mol NO g^{-1} polymer, while the NO content for poly(citric-*co*-maleic acid-*co*-1,8-octanediol)-ethyl cysteinate was 0.16 ± 0.04 mol NO g^{-1} polymer. Continuous NO release under physiological conditions was observed for at least 6 days for the cysteamine analog and 4 days for the ethyl cysteinate analog. Cell viability assays and morphological studies with human dermal fibroblasts indicated an absence of toxic leachates at a cytotoxic level, and suggested that these citrate-based polyesters may be suitable for future biomedical applications.

1. Introduction

Current polymeric materials used for applications such as tissue engineering and the fabrication of biomedical devices are frequently exposed to adverse phenomena, including bacterial infections and the foreign body response.[1-3] These complications often require medical intervention and may ultimately result in the rejection and failure of biomedical implants.[4-6] In response to such harmful outcomes, consistent progress has been made toward the development of new polymeric materials for biomedical applications, and many recent efforts have focused on the incorporation of biomolecules or other therapeutic agents within polymer matrices to minimize adverse reactions. The combination of the bulk properties of the polymeric matrix with the controlled delivery of a therapeutic agent aims to enhance the overall material performance, and extend the lifetime of implantable devices.

Nitric oxide (NO) can be highlighted among the biomolecules that are the focus of study in the biomaterials community. The identification of NO in the late 1980s as a biologically-active molecule was an important development in the field of medicine.[7, 8] Since then, it has been established that NO plays a crucial role in numerous biological processes such as cardiovascular homeostasis, immune response, neurotransmission, and inhibition of cancer cell growth.[9-12] From a biomaterials perspective, previous research has shown that NO has the ability to mitigate the foreign body response and reduce device fouling by simulating the natural NO release from the endothelium.[13] However, the relatively short biological half-life of NO under physiological conditions (0.09 to >2 s in extravascular tissue) has limited the use of exogenous NO as a method of addressing implant failure.[14] Therefore, one strategy to improve the performance of biomaterials has been the development of polymers that contain NO-releasing functional groups that serve as an NO source with greater stability. Two broad categories of NO-releasing functional groups are *N*-diazeniumdiolates and *S*-nitrosothiols (RSNOs), which are coupled to polymeric substrates to afford materials that release NO under physiological conditions (pH 7.4, 37 °C).[15] Such materials permit the release of therapeutic NO directly at biological interfaces, and may lead to enhanced performance in

[a]*Department of Chemistry and* [b]*School of Biomedical Engineering, Colorado State University, Fort Collins, CO 80523, USA.*
†Current address: New Jersey Center for Biomaterials, Rutgers University, Piscataway, NJ 08854, USA.
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polysaccharides, polyurethanes, and biodegradable polyesters.[16-20] Polyesters have been investigated as platforms for the development of NO-releasing materials due to the well-established biocompatibility and biodegradability of polyesters such as poly(lactic-*co*-glycolic acid) (PLGA), among others, and their common use in the development of degradable biomedical implants.[21] Coneski *et al.* have reported the synthesis of NO-releasing polyesters from glycerol or pentaerythritol and diacids.[22] NO release was achieved by the conjugation of thiols such as cysteamine and penicillamine to the polymers, followed by the formation of RSNO functional groups by reaction with sodium nitrite under acidic conditions. NO release was continuous for as many as 6 days, and it was demonstrated that this release reduced bacterial adhesion (*Pseudomonas aeruginosa*) by up to 80% compared with controls. The Ameer group has also reported the development of promising NO-releasing polyester systems based on low-toxicity citric acid and diols, where NO release was achieved by either blending the *N*-diazeniumdiolate derived from *N*,*N*-diethyldiethylenetriamine (DEDETA/NO) into the materials or by preparing a copolymer with *N*,*N*′-bis(2-hydroxyethyl)ethylenediamine that contained secondary amine groups, followed by subsequent *N*-diazeniumdiolation.[23, 24] However, the NO release from *N*-diazeniumdiolates under oxygenated conditions is known to produce nitrosating species such as N_2O_3, that subsequently react with secondary amines to form carcinogenic secondary *N*-nitrosamines, which may pose a considerable health risk.[25-29]

Herein, we present an alternative approach to the synthesis of citrate-based NO-releasing polyesters that is intended to avoid any potential complications associated with secondary *N*-nitrosamine formation. Our work focuses on the development of biodegradable NO-releasing polyesters from citrate-based poly(citric-*co*-maleic acid-*co*-1,8-octanediol) (PCMO). Due to the inherently low toxicity of citric acid, polyesters that incorporate citric acid as a component of their structure have been investigated for a variety of biomedical uses including cardiac tissue engineering, drug delivery, and bio-imaging.[30-32] Furthermore, the inclusion of maleic acid provides a site for further chemical modification of the polymers. To avoid the use of *N*-diazeniumdiolates, we achieved NO release through covalent incorporation of the bioavailable thiols cysteamine and cysteine (as the ethyl ester), followed by *S*-nitrosation to yield the corresponding RSNOs.[33, 34] Unlike *N*-diazeniumdiolates, RSNOs are known to occur naturally in the form of endogenous *S*-nitrosoglutathione and macromolecular *S*-nitrosoalbumin, among others.[35] RSNOs serve as NO donors under physiological conditions through thermal or metal-catalyzed decomposition, and are understood to form disulfide as their major decomposition product.[36] The NO loading and release properties of the *S*-nitrosated citrate polymers was characterized using chemiluminescence-based NO detection, demonstrating that these materials were able to sustain NO release over 4 to 6 days under physiological conditions. The cytotoxicity of both *S*-nitrosated PCMO derivatives was assessed using LIVE/DEAD and CellTiter-Blue® viability assays, in addition to morphological studies, to evaluate the suitability of these materials for biomedical applications.

2. Materials and methods

2.1 Materials

Citric acid (99.5%), maleic acid (98%), 1,8-octanediol (98%), and triethylamine (TEA) were obtained from Alfa Aesar (Ward Hill, MA, USA). Cysteamine hydrochloride (97%) and *tert*-butyl nitrite (*t*-BuONO, 90%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-Cysteine ethyl ester hydrochloride (99%) and 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 99%) were from Chem-Impex International (Wood Dale, IL, USA). *N*-Hydroxysuccinimide (NHS, 98%) was obtained from Acros Organics (Somerville, NJ, USA). Phosphate buffered saline (PBS) tablets were procured from EMD Chemicals (Gibbstown, NJ, USA). DL-Dithiothreitol (DTT, 99%) was obtained from AMRESCO (Solon, OH, USA). Bis(2,4-dinitrophenyl) disulfide (80%) was obtained from TCI (Tokyo, Japan). Human dermal fibroblasts (HDF) were purchased from ZenBio, Inc. (Durham, NC, USA). Amphotericin B, gentamicin, and Media 106 fetal bovine serum supplement were purchased from Gibco (Grand Island, NY, USA). Alexa Fluor® 568 Phalloidin, DAPI, propidium iodide (PI), SYTO9, and trypsin-EDTA (0.25%) were purchased from Life Technologies (Grand Island, NY, USA). CellTiter-Blue® was obtained from Promega (Madison, WI, USA). 25T tissue culture (TC) plates were purchased from VWR (Denver, CO, USA). All chemicals were used as received.

2.2 Characterization techniques

^{1}H NMR spectra were obtained in dimethylsulfoxide-d_6 (DMSO-d_6) using an Agilent (Varian) Inova 400 MHz FT-NMR (Agilent Technologies, Inc., Santa Clara, CA, USA). Chemical shifts for ^{1}H NMR spectra were recorded in parts per million (ppm), and were referenced relative to tetramethylsilane (TMS, 0.00 ppm) as an internal standard. FTIR-ATR spectra were recorded in the range of 650-4000 cm^{-1} using a Nicolet 6700 FTIR spectrometer (Thermo Electron Corporation, Madison, WI, USA). UV-Vis absorption studies were performed using a Nicolet Evolution 300 UV-Vis spectrophotometer (Thermo Electron Corporation). Polymer molecular weight was characterized by gel permeation chromatography (GPC) using a Waters University 1500 GPC instrument (Waters, Milford, MA, USA). Thermal transitions were detected by differential scanning calorimetry (DSC) with a TA modulated 2920 DSC and the decomposition temperature of the materials was determined by thermogravimetric analysis (TGA) utilizing a TA thermogravimetric analyzer 2950 (TA Instruments, New Castle, DE, USA).

Ellman's assay for thiol quantification. Quantification of incorporated thiol was performed using a modified Ellman's assay as described elsewhere.[37] Briefly, samples of each polymer ($n \geq 3$) were suspended in dimethylformamide (DMF) and treated with 100 µL of 4.00 mM bis(2,4-dinitrophenyl)

disulfide. The samples were then agitated for 1 h at room temperature. Aliquots were transferred to a 1 cm quartz cuvette and absorbance values were acquired at λ_{max} = 478 nm. Thiol content was calculated using *N*-acetyl cysteine standards.

Polymer degradation under physiological conditions. In order to confirm the hydrolytic degradability of the materials, gravimetric analysis was performed to assess changes in weight after incubating the materials under physiological conditions.[38] PCMO and *S*-nitrosated PCMO derivatives were placed in 5 mL phosphate buffered saline (PBS) at pH 7.4 and incubated at 37 °C for up to 4 weeks. Samples were collected every 7 days, washed with Millipore water (18.2 MΩ·cm), and lyophilized for 24 h prior to measurement. For samples incubated longer than 1 week, the buffer solution was replaced at the end of each week.

Chemiluminescence-based NO analysis. NO release from *S*-nitrosated citrate polyester samples was evaluated using Sievers chemiluminescence NO analyzers (NOA 280i, GE Analytical, Boulder, CO, USA), following our earlier reported procedure.[39] The instruments were calibrated prior to each analysis using nitrogen (zero gas) and 45 ppm NO/nitrogen, and the nitrogen sweep gas flow during analysis was maintained at 200 mL/min. Total NO content was obtained by heating polymer samples and appropriate controls ($n \geq 3$) to 120 °C, which initiated the NO-releasing thermal decomposition of the RSNO groups.[18] The NO emission from this process was measured and used to quantify the amount of thermally releasable NO present in each material. To determine NO release under physiological conditions, polymer samples ($n \geq 3$) were suspended in deoxygenated 10 mM PBS (pH 7.4) at 37 °C, and the NO release was measured for 4 to 6 days, depending on the release properties of the material. All samples were shielded from direct exposure to light in order to prevent photodecomposition of the light sensitive RSNO.[40]

2.3 Synthesis of materials

Poly(citric-*co*-maleic acid-*co*-1,8-octanediol) (PCMO) (1). The biodegradable citrate-based polymer was prepared by a melt-phase polycondensation between maleic acid (2.32 g, 20.0 mmol), citric acid (5.76 g, 30.0 mmol), and 1,8-octanediol (7.31 g, 50.0 mmol). The polycondensation was carried out without an exogenous catalyst following a modification of a previously reported procedure.[41, 42] The reagents were added to a vented 250 mL flask equipped with a nitrogen inlet and the reaction mass was stirred under a high flow of nitrogen for 30 min, then heated to 140 °C with constant stirring to initiate the polycondensation. The colorless melted mass was maintained at 140 °C for 2 h under nitrogen flow to obtain the polymer. The crude polymer was then dissolved by sonication in absolute ethanol (35 mL) and added over 30 min to Millipore water (200 mL) containing Tween-80 to remove any unreacted materials. The mixture was stirred for 1 h and allowed to settle at 4 °C. The supernatant was decanted, and the polymer was washed 3 times with Millipore water (200 mL) and lyophilized for 4 days. ^{1}H NMR δ_H/ppm (400 MHz, DMSO-d_6): 1.20-1.65 (-$(CH_2)_6$-), 2.66-2.76 (-CH_2CO_2-), 3.95-4.03 (-OCH_2-), 6.35 (-HC=CH-), 12.6 (-CO_2H); IR ν_{max}/cm^{-1}: 3600-3200 (O-H alcohol, carboxylic acid), 2930-2856 (C-H), 1724 (C=O, carbonyl), 1167 (C-O). A synthetic scheme that illustrates the synthesis of polyester analogs is given in Figure 1.

PCMO-cysteamine (PCMO-CysAm) (2a). PCMO (12.4 g) was dissolved by sonication in DMF (100 mL). Free carboxylic acid groups of PCMO were reacted with EDC·HCl (3.06 g, 16.0 mmol) and NHS (2.81 g, 24.0 mmol) at 0 °C, and the reaction was maintained at this temperature for 1 h. The mixture was then allowed to warm to RT and was stirred overnight to activate the carboxylic acid groups. A solution of dry cysteamine hydrochloride (3.26 g, 28.7 mmol) in DMF (20 mL) and was added to the NHS activated polymer with 6.0 mL of TEA. The polymer solution was protected from direct exposure to light and stirred under nitrogen for 48 h. The polymer was then isolated after filtration through Celite and evaporation of the solvent under vacuum. The crude material was subsequently redissolved in absolute ethanol (40 mL) and treated with DTT (0.200 g, 1.30 mmol) and TEA (0.18 mL). The polymer solution was added over 30 min to Millipore water (200 mL) containing Tween-80 and stirred for 1 h, then allowed to settle at 4 °C and washed as previously described for PCMO. The purified material was recovered by lyophilization.

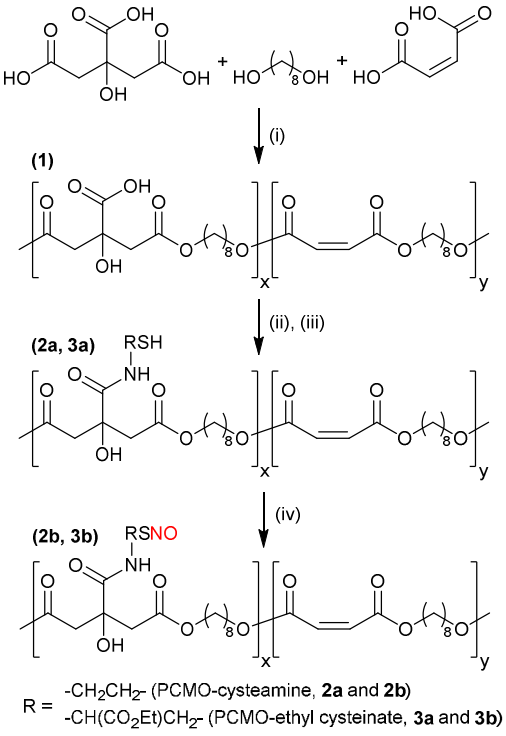

Figure 1. Synthesis of PCMO and both thiolated (2a, 3a) and *S*-nitrosated (2b, 3b) derivatives. (i) 140 °C, 2 h, (ii) NHS, EDC·HCl, 0 °C to RT, overnight, cysteamine hydrochloride/ethyl cysteinate hydrochloride, RT, 48 h, (iii) DTT, TEA, RT, 1 h, (iv) *t*-BuONO, EtOH, RT, 4h.

^{1}H NMR δ_H/ppm (400 MHz, DMSO-d_6): 1.20-1.65 (-$(CH_2)_6$-), 2.66-2.76 (-CH_2CO_2-), 2.98 (-CH_2SH), 3.22 ((CO)NHCH-), 3.95-4.03 (-OCH_2-), 6.35 (-HC=CH-), 7.82-7.92 (-(CO)NH-); IR ν_{max}/cm^{-1}: 3600-3200 (O-H), 2930-2856 (C-H), 1724 (C=O, carbonyl), 1656 (amide I), 1531 (amide II), 1167 (C-O).

***S*-nitrosated PCMO-cysteamine (PCMO-CysAm-NO) (2b).** A mixture of PCMO-cysteamine/ethanol (2a) (25 mg/mL) and *t*-BuONO (0.500 mL) was stirred at RT for 4 h in a vial protected from light. After *S*-nitrosation, the material was stirred under vacuum to remove solvent, residual *t*-BuONO, and volatile byproducts. IR ν_{max}/cm^{-1}: 3600-3200 (O-H alcohol, carboxylic acid), 2930-2856 (C-H), 1728 (C=O, carbonyl), 1653 (amide I), 1529 (amide II), 1173 (C-O). UV-Vis λ_{max}/nm: 335 (RSNO, $n_O \rightarrow \pi^*$), 550 (RSNO, $n_N \rightarrow \pi^*$).

PCMO-ethyl cysteinate (PCMO-EtCys) (3a). An analog was prepared following the general method provided for 2a using cysteine ethyl ester hydrochloride (5.33 g, 28.7 mmol). ^{1}H NMR δ_H/ppm (400 MHz, DMSO-d_6): 1.18-1.22 (-CH_3) 1.20-1.65 (-$(CH_2)_6$-), 2.66-2.76 (-CH_2CO_2-), 3.70 (-CH_2SH), 4.42 (-(CO)NH-CH_2-), 3.95-4.03 (-OCH_2-), 6.35 (-HC=CH-), 7.84-7.94 (-(CO)NH-); IR ν_{max}/cm^{-1}: 3600-3200 (O-H alcohol, carboxylic acid), 2930-2856 (C-H), 1724 (C=O, carbonyl), 1673 (amide I), 1518 (amide II), 1167 (C-O).

***S*-nitrosated PCMO-ethyl cysteinate (PCMO-EtCys-NO) (3b).** An analog was prepared from 3a using the procedure described for 2b. IR ν_{max}/cm^{-1}: 3600-3200 (O-H alcohol, carboxylic acid), 2930-2856 (C-H), 1733 (C=O, carbonyl), 1684 (amide I), 1517 (amide II), 1176 (C-O). UV-Vis λ_{max}/nm: 336 (RSNO, $n_O \rightarrow \pi^*$), 549 (RSNO, $n_N \rightarrow \pi^*$).

2.4 Cell studies

For cell studies, HDF were cultured in media 106 supplemented with 2% v/v fetal bovine serum, 1 µg/mL hydrocortisone, 10 ng/mL human epidermal growth factor, 3 ng/mL basic fibroblast growth factor, 10 µg/mL heparin, 125 µg/mL amphotericin B, and 5 mg/mL gentamicin in a 25T flask. HDF were incubated at 37 °C in a humid atmosphere with 5% CO_2. Prior to the experiments, cells were collected from a 25T flask by addition of 1 mL of a trypsin-EDTA (0.25%) solution, and re-suspended at different concentrations (cells/mL) depending on the specific experiment.

Preparation of extracts. All cytotoxicity experiments were performed according to ISO standards.[43] Extracts from the two *S*-nitrosated PCMO derivatives were obtained at physiological temperature (37 °C in a humid atmosphere with 5% CO_2). To prepare extracts, each material was incubated in the above described cell media (concentration of 0.5 mg material/mL cell media) for 24 h in sterilized 20 mL amber vials. After 24 h, the resulting extracts were used for cell experiments. As a control, cell media was kept under the same conditions and used to replace media in the positive and negative control samples.

Extract experiments. To perform experiments with extracts, 5.0 x 10^4 cells per well were seeded in a 24-well plate for assessing the morphology of the cells, 2.5 x 10^4 cells per well were seeded in a 24-well plate for CellTiter-Blue® viability assays and 2.5 x 10^4 cells per well were seeded in a 24-well plate for LIVE/DEAD assay. HDF cells were incubated for 48 h prior to exposure to the different extracts (37 °C in a humid atmosphere with 5% CO_2) followed by 24 h in the presence of the extracts at 37 °C in a humid atmosphere with 5% CO_2. All biological assays were repeated $n \geq 9$.

Morphological studies. Each time, three wells were used for positive control and three wells were used for each material extract. After rinsing all samples twice with PBS, cells were fixed by exposing them to 3.7% methanol-free formaldehyde (300 µL) in PBS for 10 min at RT. All wells were rinsed twice with PBS and exposed to acetone (200 µL), cooled to 0 °C for 3 min, rinsed twice again with PBS, and stained using 200 µL of staining solution for 20 min at RT. Staining solution consisted of DAPI and Alexa Fluor® 568 Phalloidin at concentrations of 7.60 µM and 6.60 µM, respectively, and was prepared in PBS. All wells were finally rinsed twice with DI water and imaged. Fluorescence microscopy images were taken using an Olympus IX73 fluorescence microscope. An excitation wavelength of 358 nm was used for DAPI (461 nm emission observed), and a wavelength of 578 nm was used for Alexa Fluor® 568 Phalloidin (600 nm emission observed). Images of cells are composed of two overlaid images (DAPI and Alexa) combined using the Olympus CellSens software.

CellTiter-Blue® viability assay. For each experiment, three wells containing cells were used as a positive control (labeled TC), three wells were used as a negative control, and three wells containing cells were exposed to each material extract. For the negative control, wells were rinsed twice with PBS and cells were exposed to methanol (100 µL per well) for 45 min to reduce viability. Methanol was removed and all wells were rinsed twice with PBS and 300 µL of CellTiter-Blue® solution was added. CellTiter-Blue® solution was prepared by mixing 20 µL of CellTiter-Blue® solution for each 100 µL of warm cell media. After 4 h incubation at 37 °C in a humid atmosphere with 5% CO_2, absorbance readings were measured with a plate reader (BioTek Synergy 2) at 570 nm and 600 nm.

LIVE/DEAD assay. Each time, three wells were used for positive control, three were used for negative control, and three wells were used for each material extract. For the negative control, cells were exposed to methanol as described above. In this case, the PBS was removed after the second wash and 200 µL of LIVE/DEAD solution was added to each well. Samples were incubated for 1 h at RT. Subsequently, the LIVE/DEAD solution was removed and DI water was added to each well. To prepare the LIVE/DEAD solution, PI and SYTO9 were added to 0.85% w/v NaCl solution at concentrations of 3 µM and 0.5 µM, respectively. Fluorescence microscopy images were obtained using an Olympus IX73 fluorescence microscope. An excitation wavelength of 543 nm was used for PI (617 nm emission observed), and a wavelength of 488 nm was used for SYTO9 (500 nm emission observed). Images of cells used for LIVE/DEAD assay are composed of two overlaid images (PI and SYTO9), combined using the Olympus CellSens software.

3. Results and discussion

3.1 Synthesis and characterization of PCMO

PCMO was synthesized from citric acid, maleic acid, and 1,8-octanediol following a modified literature protocol to yield an amorphous polyester.[41, 42] Citric acid is generally regarded as non-toxic and occurs endogenously as citrate, where it participates in several crucial metabolic pathways.[44, 45] As a tri-functional carboxylic acid, citric acid permits both crosslinking through the formation of multiple ester linkages and the conjugation of pendant groups using carbodiimide-mediated coupling reactions.[46] Furthermore, it has been established that certain citrate-based polymers exhibit excellent hemocompatibility and such materials have frequently been proposed for blood-contacting applications.[47, 48] It was previously demonstrated that the incorporation of maleic acid as a component of polyesters allows post-polymerization crosslinking to occur through exposure to UV light, and the hydrophobic 1,8-octanediol acts to balance the inherent hydrophilicity of citrate.[49] The catalyst-free polycondensation was carried out in the melt-phase at 140 °C for 2 h with a constant nitrogen flow to remove water, and the structure of PCMO was independently confirmed by ^{1}H NMR (Fig. S1) and compared to previous reports.[23] The multiplet at 2.66-2.76 ppm corresponds to the methylene protons from citrate units, while peaks at 1.20-1.65 (-CH_2-) and 3.95-4.03 (-OCH_2-) ppm confirmed incorporation of 1,8-octanediol. The alkenyl protons of the maleate group appeared at 6.35 ppm. The broad feature centered at 12.6 ppm is attributable to remaining free carboxyl groups from either the citrate or maleate units. The FTIR-ATR spectrum (shown in Fig. S4 and S5) features an ester carbonyl C=O stretching band at 1724 cm^{-1}, which confirmed the ester linkages between the monomers, while the contribution of carboxylic acid O-H stretching to the broad feature between 3600-3200 cm^{-1} supported the preservation of free carboxylic acids groups for further post-polymerization modifications. GPC analysis with polystyrene standards showed a weight average molecular weight (M_w) of 175,000 g mol^{-1}, a number average molecular weight (M_n) of 33,100 g mol^{-1} and a polydispersity index (PDI) of 5.29. TGA indicated an onset decomposition temperature of 178 °C, and the glass transition temperature of -16 °C was determined by DSC.

3.2 Synthesis and characterization of thiolated PCMO

The thiols cysteamine and ethyl cysteinate were conjugated to PCMO by formation of amide linkages between the primary amine of the thiols and the remaining free carboxylic acid groups of the polyester. This was accomplished *via* a carbodiimide-mediated coupling reaction in a modification of a previously reported literature protocol.[39] This process results in the covalent incorporation of thiol groups that permit the subsequent formation of NO-releasing RSNO groups.[50] Incorporation of the thiols was confirmed by ^{1}H NMR, where the appearance of additional multiplets at 2.98 and 3.22 ppm was attributable to the methylene protons of cysteamine, while features at 1.18 – 1.22 (-CH_3), 3.70 (-CH_2SH), and 4.42 (-(CO)NHCH-) ppm corresponded to ethyl cysteinate (Fig. S2 and S3). The feature at approximately 7.8 – 7.9 ppm for both derivatives indicated the formation of amide bonds between the thiol and the polymer. In addition, the development of characteristic amide absorbance bands in the FTIR-ATR spectrum of the thiolated polymers further supported the successful formation of amide linkages.[51] In the case of PCMO-CysAm, these prominent features occur at 1656 (amide I) and 1531 cm^{-1} (amide II), while PCMO-EtCys displays equivalent features at 1673 and 1518 cm^{-1} (Fig. S4 and S5). These features are absent in the FTIR-ATR spectrum of unmodified PCMO. Although the comparative weakness of the IR features associated with thiol groups prevented their identification in the spectra of the thiolated polymers, the presence of thiol groups was confirmed using a modified Ellman's assay protocol with bis(2,4-dinitrophenyl) disulfide in DMF (Table 1).[52] Use of the more common Ellman's reagent 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) in aqueous base was not possible due to the lack of water solubility exhibited by PCMO derivatives and the general hydrophobicity of the polymer, which limited water permeation and reduced the accessibility of the thiol groups.[53] GPC analysis of the thiolated derivatives was complicated by crosslinking from disulfide formation and was limited to characterization of the parent polyester.

3.3 Synthesis and characterization of *S*-nitrosated PCMO

S-Nitrosation of the thiolated polymers was carried out in ethanol under anhydrous conditions using the alkyl nitrite *t*-BuONO as the nitrosating agent.[54] This comparatively mild procedure avoids the use of sodium nitrite and aqueous acid that could lead to parallel hydrolysis of the polymeric ester linkages, and permits removal of unreacted *t*-BuONO and volatile byproducts under vacuum.[18, 55] The successful conversion of thiol groups to the corresponding RSNO was supported by UV-Vis spectrophotometry in DMSO, where characteristic RSNO absorptions at 335 ($n_O \rightarrow \pi^*$) and 550 nm

Table 1. Summarized thiol and NO content data for PCMO derivatives

Material	Thiol Content[a] (mmol g^{-1})	Total NO Content[b] (mmol g^{-1})	Cumulative NO Release[c] (mmol g^{-1})	% NO/Thiol Content
PCMO-Cysteamine (2a, 2b)	0.856 ± 0.030	0.45 ± 0.07	0.29 ± 0.05	52 ± 8
PCMO-Ethyl cysteinate (3a, 3b)	0.797 ± 0.038	0.16 ± 0.04	0.066 ± 0.003	13 ± 3

[a]Values determined for 2a and 3a using a modified Ellman's assay protocol in DMF. [b]Values determined by NO analysis of 2b and 3b through thermal decomposition of the RSNO. [c]Release measured under physiological conditions (pH 7.4, 37 °C) in 10 mM PBS over 6 days (2b) and 4 days (3b). Measurements were ended when the detected NO release was below 3 ppb. For all experiments, n ≥ 3 and results are reported as the mean ± SD.

($n_N \rightarrow \pi^*$) for PCMO-CysAm-NO and equivalent absorptions for PCMO-EtCys-NO at 336 and 549 nm were observed (Fig. S6 and S7).[36] Identification of the $n_N \rightarrow \pi^*$ transition near 550 nm was supported by the use of diffuse reflectance UV-Vis, since the low molar extinction coefficient of that absorbance feature complicated its detection in DMSO due to solubility limitations. No features developed that could be attributable to the formation of undesirable *N*-nitrosamide or alkyl nitrite species, which typically result in pronounced and distinctive absorptions in the range of 260 - 400 nm.[54, 56] The FTIR-ATR spectra of both *S*-nitrosated materials indicated that the primary structural features of the polyester were preserved, with bands at 3600-3200 (O-H), 2930-2856 (C-H), 1728 (C=O, carbonyl), 1653 (amide I), 1529 (amide II), and 1173 cm^{-1} (C-O) for PCMO-CysAm-NO that are consistent with those of the parent thiolated polyester (Fig. S4). These features appear at 3600-3200, 2931-2856, 1733, 1684, 1517, and 1176 cm^{-1} in the case of PCMO-EtCys-NO (Fig. S5). For both thiolated PCMO derivatives, the efficacy of the *S*-nitrosation process (as evaluated by UV-Vis) was greatest using ethanol as a solvent, where the polymers exhibited appreciable solubility. The conversion of thiol to RSNO (% total NO/thiol content) under identical conditions was 52 ± 8% for PCMO-CysAm-NO, and 13 ± 3% for PCMO-EtCys-NO. A previous report identified a similar reduction in *S*-nitrosation efficiency in the case of a PLGA derivative bearing cysteine pendant groups, where this outcome was attributed to the influence of the additional neighboring carbonyl group that was not present in cysteamine derivatives.[50] In addition, the extent of *S*-nitrosation may be limited due to parallel decomposition of the RSNO.[36]

The total NO content of each polymer was directly quantified using highly selective chemiluminescence-based NO detection.[57, 58] Samples of each *S*-nitrosated material were heated to 120 °C in a stepwise manner under deoxygenated conditions in custom gas-flow cells with a constant flow of nitrogen, following a previously reported method.[17] The thermal decomposition of the RSNO groups resulted in the quantifiable release of NO, which was detected by the instrument as it occurred. The instrument output (NO concentration) was converted to total NO release using a calibration constant obtained from gases of known NO concentration and used to calculate the amount of thermally-releasable NO present in the polymer samples. In all cases, the samples were heated until no further NO emission was detected. Controls consisted of thiolated PCMO derivatives heated under identical conditions, a process that was not observed to result in statistically relevant NO release.[59] NO release under physiological conditions was determined in gas-flow cells by immersing the materials in pH 7.4 PBS at 37 °C while protected from direct exposure to light (Fig. 2a). The gradual decomposition of the RSNO functional groups resulted in the release of NO. Experiments were performed in triplicate and an average of 0.29 ± 0.05 mmol NO g^{-1} was released under these conditions over the course of 6 days from PCMO-CysAm-NO, and 0.066 ± 0.003 mmol NO g^{-1} was released from PCMO-EtCys-NO in 4 days (Fig. 2b). The experiments were terminated when continuing NO release fell below 3.0 ppb. In a prior report, Zhao et al. calculated total NO release (by Griess assay) from an *N*-diazeniumdiolated copolymer consisting of citric acid, 1,8-octanediol, and *N*,*N*′-bis(2-hydroxyethyl)ethylenediamine coated on expanded poly(tetrafluoroethylene) vascular grafts in the range of 0.4 - 0.5 μmol/g polymer. In comparison, PCMO-CysAm-NO and PCMO-EtCys-NO demonstrate greater total NO release under physiological conditions and do not exhibit the concomitant formation of potentially hazardous *N*-nitrosamine that was observed for a citrate-based polymer system containing blended DEDETA/NO.[23] The overall quantity of NO released under physiological conditions was largely proportional to the total NO content of the materials. In addition, the general similarity of the physiological NO release properties for both *S*-nitrosated PCMO derivatives indicated that the morphology and hydrophobicity of the polymer itself was responsible for the release characteristics of the materials. The duration of NO

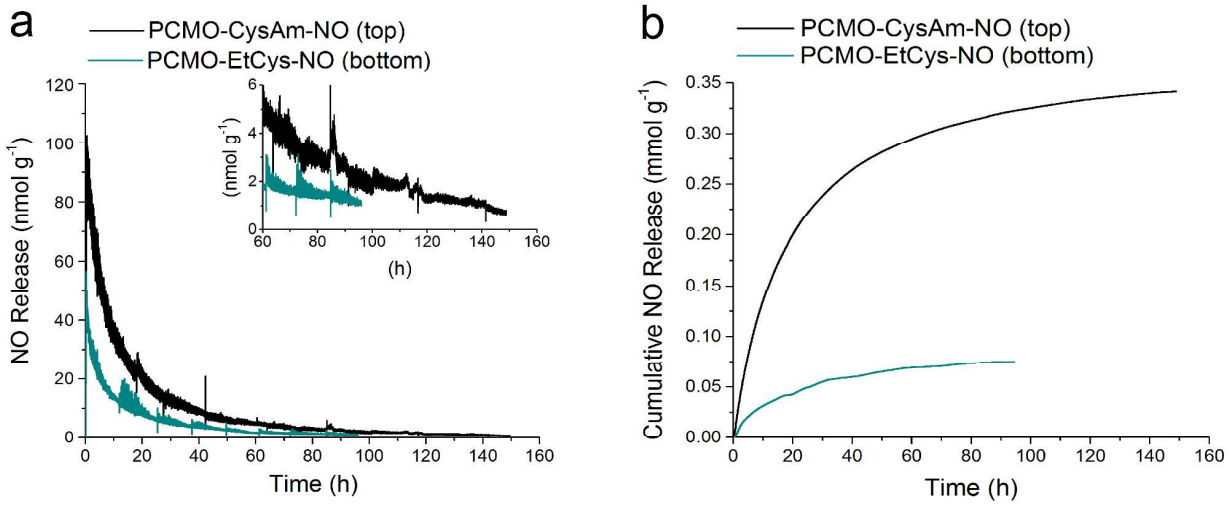

Figure 2. (a) Representative real-time NO release profiles for PCMO-CysAm-NO (top) and PCMO-EtCys-NO (bottom) under physiological conditions (pH 7.4, 37 °C) in 10 mM PBS. Inset: NO release after 60 hours. (b) Representative cumulative NO release profiles under physiological conditions.

release in *S*-nitrosated materials is frequently attributable to the stability of the RSNO, a property that is greatly dependent upon the prevailing conditions.[35, 50, 60] It has been previously observed that greater hydrophilicity and solvent permeation under physiological conditions promoted rapid RSNO decomposition, while isolation within hydrophobic polymer matrices improved the duration of NO release.[17] For this reason, the 4 to 6 day release from hydrophobic *S*-nitrosated PCMO derivatives is primarily attributable to reduced solvent accessibility, which inhibits the NO-releasing decomposition of RSNO to NO and disulfide (2RSNO → 2NO + RSSR).[36]

3.4 Polymer degradation under physiological conditions

The potential biodegradability of the polymers was assessed by immersing samples (n = 3) of PCMO, PCMO-CysAm-NO, and PCMO-EtCys-NO in 10 mM pH 7.4 PBS buffer at 37 °C in the absence of light for up to 4 weeks (Fig. 3). The results indicate relatively rapid degradation for the unmodified PCMO during this time period. A dramatic decrease in polymer weight occurring between week 2 and 3 was observed, where 65% of the polymer mass was lost (Table S1). The rapid degradation of the parent PCMO was largely attributable to the exposure of additional surface area as the degradation proceeded. In comparison, the *S*-nitrosated derivatives exhibited only a 50% reduction in initial weight at the end of the 4 week period. The reduced degradation rate of the *S*-nitrosated polymers can be attributed to crosslinking arising from the formation of disulfide linkages.

3.5 Cytotoxicity studies

The relative cytotoxicity of potential leachates from the two *S*-nitrosated citrate-based polyesters (PCMO-CysAm-NO and PCMO-EtCys-NO) with respect to HDF was evaluated using an extract approach, based on the commonly accepted ISO regulation 10993 part 5.[43] Morphological studies and two cytotoxicity tests (CellTiter-Blue® viability and LIVE/DEAD) were performed to ensure the suitability of these materials for biomedical applications. Figure 4a reveals cells with the anticipated morphology for HDF fixed to a TC plate after reaching 90% confluency. Both *S*-nitrosated PCMO derivatives show comparable morphology to the positive control sample, confirming that neither of the extracts induces any changes in cell morphology (Fig. 4b and 4c).

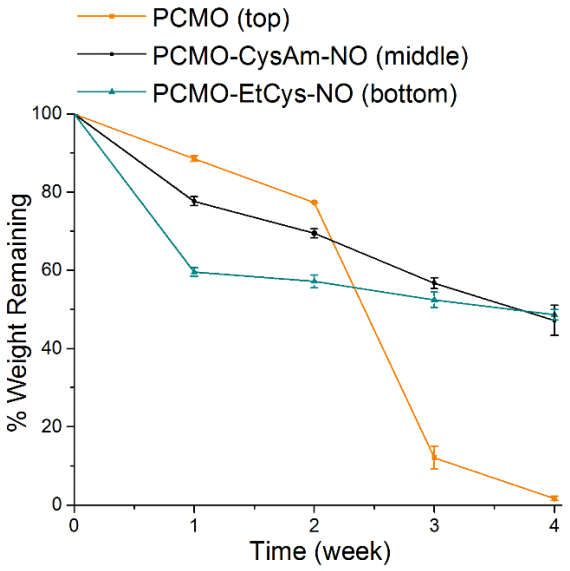

Figure 3. Degradation profiles of PCMO and *S*-nitrosated derivatives under physiological conditions (pH 7.4 PBS, 37 °C).

CellTiter-Blue® viability assay was used to assess the percentage of viable cells present in the control samples as well as in the presence of the different extracts. CellTiter-Blue® viability assay functions similarly to the commonly used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, with the advantage that only one step is required as the reduced product is water soluble. In both cases, the mitochondrial activity in live cells is tested, therefore this assay is an excellent indicator of the cell viability.[61] Figure 5 shows the viability results for TC and cells exposed to extracts from both *S*-nitrosated PCMO derivatives. Negative control samples (values not included in the graph) indicated that there were no viable cells after exposure to methanol for 45 minutes, and the absorbance from these samples was equivalent to a CellTiter-Blue® solution. For PCMO-CysAm-NO and PCMO-EtCys-NO extracts, the percentage of viable cells was very similar to the positive control indicating that there was no decrease in cellular viability (normalized with the positive control sample) when cells were exposed to the extracts. These results suggest that there were no toxic leachates and/or toxic degradation products at a cytotoxic concentration present in either of the extracts.

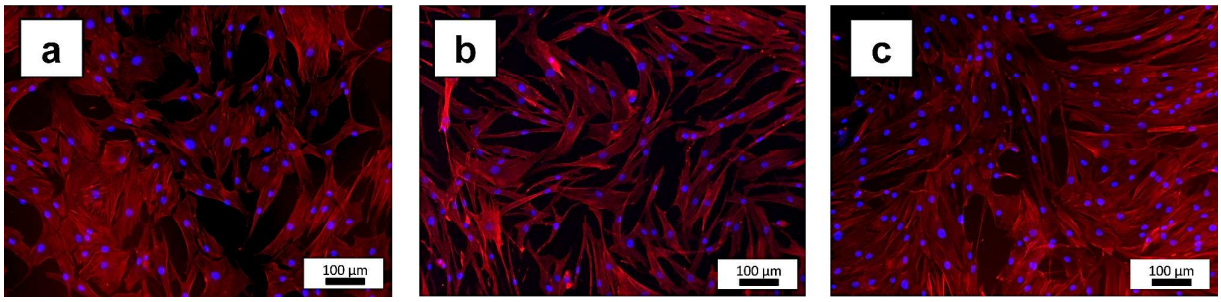

Figure 4. *Morphological studies.* Overlaid fluorescence microscopy images of human dermal fibroblasts attached to a TC plate for 72 h using: (a) cell media (positive control), (b) extract from PCMO-CysAm-NO, and (c) extract from PCMO-EtCys-NO. Blue areas are cell nuclei and red areas are cell actin cytoskeletons. For all experiments, $n \geq 9$.

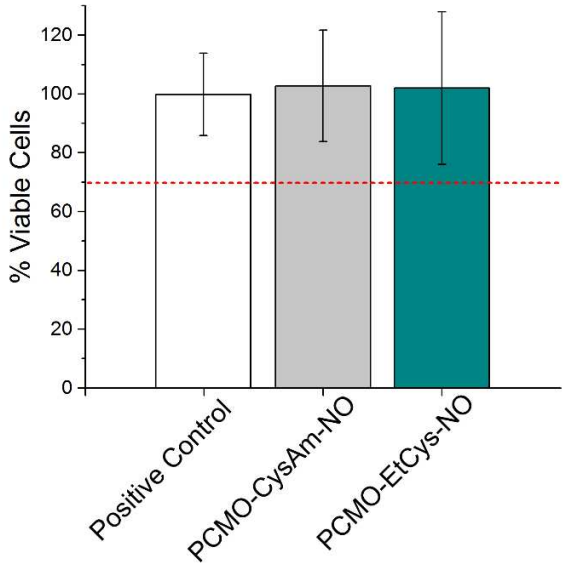

Figure 5. *CellTiter-Blue® viability assays.* % Viable human dermal fibroblasts cultured in cell media (positive control), in extract from PCMO-CysAm-NO, and in extract from PCMO-EtCys-NO. Red line indicates the 70% threshold for viability assessment. For all experiments, $n \geq 9$.

Similar results to CellTiter-Blue® viability assay were observed when the LIVE/DEAD assay was performed. Results from LIVE/DEAD assay are presented in Figure 6. HDF cells attached to TC plates were used as positive control and cells exposed to methanol prior to staining were used as negative control. A similar area covered with HDF was observed in the positive control sample and the wells exposed to PCMO-CysAm-NO and PCMO-EtCys-NO extracts. Figure 6a shows positive control cells where the majority of the cells are observed in green indicating that the cell membrane is intact and the cells are considered alive.

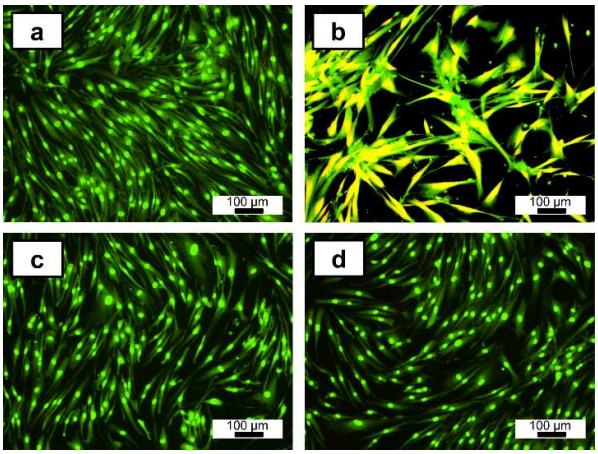

Figure 6. *LIVE/DEAD studies*. Overlaid fluorescence microscopy images of human dermal fibroblasts attached to a TC plate for 72 h using: (a) cell media (positive control), (b) cell media and exposure to methanol prior to staining (negative control), (c) extract from PCMO-CysAm-NO, and (d) extract from PCMO-EtCys-NO. Green cells are considered alive and red cells are considered dead. For all experiments, $n \geq 9$.

Figure 6b illustrates cells that were exposed to methanol prior to staining. The yellow color indicates that the cell membrane was disrupted, therefore, a mixture of red and green staining is observed. Figure 6c and 6d display HDF that have been exposed to extracts from PCMO-CysAm-NO and PCMO-EtCys-NO, respectively. Both images display green cells indicating that neither of the material extracts damage the cell wall. Cytotoxicity results were consistent with those performed by Wang *et al.* where it was demonstrated that the citrate-based parent material did not hinder cell growth.[23]

4. Conclusions

The work presented here is the first, to our knowledge, that reports the synthesis and characterization of *S*-nitrosated citrate-based polyesters with quantified extended NO release. In the case of PCMO-CysAm-NO, total NO content of 0.45 mmol g^{-1} was obtained, while the NO content of PCMO-EtCys-NO was found to be 0.16 mmol g^{-1}. The polymers released quantifiable NO over the course of at least 4 (PCMO-EtCys-NO) to 6 days (PCMO-CysAm-NO) with respective average cumulative NO releases of 0.066 and 0.29 mmol g^{-1}. In both cases, *S*-nitrosation was not found to alter the backbone structure of the polymer and no toxic *N*-nitroso species were detected by UV-Vis or FTIR-ATR. Cell viability studies with HDF indicated viability above the cytotoxicity threshold as compared to a positive control, and morphological studies confirmed that the polymer extracts did not induce changes to cell morphology. Taken together, these results suggest that PCMO derivatives may be candidates for potential biomedical applications.

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