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Reducible Disulfide Poly(beta-amino ester) Hydrogels for Antioxidant Delivery

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Abstract

Recently, biomaterials have been designed to contain redox-sensitive moieties, such as thiols and disulfides, to impart responsive degradation and/or controlled release. However, due to the high sensitivity of cellular redox-based systems which maintain free-radical homeostasis (e.g. glutathione/glutathione disulfide), if these biomaterials modify the cellular redox environment, they may inadvertently affect cellular compatibility and/or oxidative stress defenses. In this work, we hypothesize that the degradation products of a poly(β -amino ester) (PBAE) hydrogel formed with redox sensitive disulfide (cystamine) crosslinking could serve as a supplement to the environmental cellular antioxidant defenses. Upon introduction into a reducing environment, these disulfide-containing hydrogels cleave to present bound-thiol groups, yet remain in the bulk form at up to 66 mol% cystamine of the total amines. By controlling the molar fraction of cystamine, it was apparent that the thiol content varied human umbilical vein endothelial cell (HUVEC)

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viability IC_{50} values across an order of magnitude. Further, upon introduction of an enzymatic oxidative stress generator to the cell culture (HX/XO), pre-incubated thiolated hydrogel degradation products conferred cellular and mitochondrial protection from acute oxidative stress, whereas non-reduced disulfide-containing degradation products offered no protection. This polymer may be an advantageous implantable drug delivery system for use in acute oxidative stress prophylaxis and/or chronic oxidative stress cell therapies due to its solid/liquid reversibility in a redox environment, controlled thiolation, high loading capacity through covalent drug-addition, and simple post-synthesis modification which bound-thiols introduce.

Graphical Abstract

Keywords

Oxidative Stress; Biocompatibility; Hydrogel; Disulfide; Antioxidant

1 Introduction

The redox status of cells is highly regulated, with shifts in the balance of pro- and antioxidant signals and compounds, potentially resulting in a state of cellular stress [1]. This can occur in the case of an acute oxidative burden (e.g., radiation and reperfusion injury) or oxidative stress-based disease (e.g., pulmonary pneumonitis, atherosclerosis, and rheumatoid arthritis) [2–6]. Through carefully timed delivery of antioxidants, the redox buffering capacity may be temporarily increased, shifting the cytosolic capacity to a more reduced state, alleviating transient oxidative injury [7, 8]. Implantable biomaterials eluting antioxidants may increase biocompatibility through suppressing pro-inflammatory signals during the foreign body response (i.e. acute inflammation pathway), improving implant integration [9]. On the contrary, biomaterials eluting or generating oxidants may induce inflammatory response and fibrous capsule formation, and may cause detrimental effects such as implant failure via degradation, and patient discomfort [10–12], or conversely, protect the implant via inhibition of host cells in avascular capsules [13].

Disulfide bonds are a common responsive crosslinking system used in polymeric biomaterials [14–17] and may be utilized as a means of biodegradation via reduction for drug release to the cytosol [18, 19]. Human cells typically contain from 1 to 10 mM glutathione (GSH) and roughly 1/100 that content of glutathione disulfide (GSSG) [16, 20, 21]. This ratio of GSH to GSSG is maintained through the action of a recycling system, mainly glutathione reductase/NADPH⁺ [7, 22, 23]. Unfortunately, cellular entry of disulfides may result in reduced antioxidant capacity through oxidation of glutathione in the cell via

disulfide cleavage within the biomaterial or degradation products, and create mixed disulfides unable to participate in enzymatic regeneration of glutathione [24]. Delivery of reduced disulfide (thiol-containing) materials may be utilized to not only re-balance cellular response under oxidative stress conditions, but also enhance cell viability of biomaterials.

Originally studied as a less-cytotoxic cationic polymer for use in gene delivery [25, 26], poly(β -amino esters) (PBAEs) have also been increasingly researched in the bulk state as biocompatible implantable hydrogels, which may be readily functionalized for a variety of applications [27–29]. Using a disulfide-containing PBAE hydrogel, which may be reduced to contain bound-thiols within the bulk matrix for antioxidant delivery, we found that a large differential existed between the cytotoxicity of the degradation products which contained disulfides and those containing thiols (reduced disulfides). Further, oxidative stress was found to be significantly less with the thiol containing hydrogel degradation products, which indicated the importance of biomaterial redox state and the potential for high biocompatibility compared to other antioxidant containing biomaterials.

2 Materials and Methods

2.1 Materials

All reagents were used as received without further purification steps. Cystamine dihydrochloride, 4,7,10-trioxa-1,13-tridecanediamine (TTD), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB/Ellman's Reagent), diethyleneglycol diacrylate (DEGDA), and 2-mercaptoethanol (2-ME) was purchased from Polysciences Inc. (Warrington, PA). Immobilized tris(2-carboxyethyl) phosphine (TCEP) beads were purchased through Thermo Scientific (Waltham, MA). EGM-2 cell media and HUVEC were purchased through Lonza (Hopkinton, MA). Hypoxanthine and xanthine oxidase were purchased through Sigma-Aldrich (St. Louis, MO). Calcein-AM was obtained from Life Technologies (Grand Island, NY). All solvents were purchased through either Fisher Scientific (Waltham, MA) or Pharmco-AAPER (Brookfield, CT).

2.2 Cystamine PBAE Hydrogel Synthesis

Throughout hydrogel synthesis, the total acrylate to total amine proton ratio was kept at a stoichiometric ratio of 1 to 1. First, cystamine was mixed with anhydrous dimethylsulfoxide (DMSO) at 133 mg/mL during sonication to aid in solubilization. Afterwards TDD was added and then introduced into DEGDA. The mixture was then dispensed onto a casting ring upon a Teflon plate, and kept for 24 hours at 60°C. After removal from the oven, thin film hydrogels were washed with DMSO to remove unreacted reagent, solvent removed under vacuum for 48 hours (-80°C , <0.1 mBar), and stored with desiccant in argon at $2-5^{\circ}\text{C}$.

2.3 Hydrogel Swelling and Mesh Size Calculations

Swelling studies proceeded via weighing the dry material before and after swelling in DMSO for at least 8 hours to achieve equilibrium. For reversible swelling steps, a new dry mass was calculated after freeze drying the material to account for any loss of mass washed away. The swelling ratios, q , were found by dividing the swollen polymer, W_s by the weight of the dry polymer, W_d , in equation 1. The polymer volume fractions, v_2 , were calculated by

equation 2, where ρ_p and ρ_s are the densities of the dry polymer (1.3 g/cm³) and DMSO solvent (1.1 g/cm³) respectively [30].

$$q = \frac{w_s}{w_d} \quad (1)$$

$$v_2 = \frac{1}{1 + \frac{\rho_p}{\rho_s}(q-1)} \quad (2)$$

Mesh size (ξ) calculations were performed using solvent interaction calculations using Flory-Rehner [31–33] equation 3 to find the number average molecular weight between crosslinks, M_C , required for equation 4 [31, 34]. M_N is the number average molecular weight of the free chain polymer (from 217–194 Da for 0–66 mol% cystamine of the amines in the disulfide state, and 217–169 Da in the reduced), v_2 is the specific volume of the swollen amorphous hydrogel, V_1 is molar volume of DMSO in the hydrogel (71.03 mL/mol), l is the repeat polymer bond length (1.54 Å for C-C), C_n is the Flory characteristic ratio (constant 4.0 used for ethylene glycol systems as an approximation [35, 36]), and M_r is the molecular weight of the repeating unit (44 Da for ethylene glycol assumed).

$$\frac{1}{M_C} = \frac{2}{M_N} - \frac{\bar{v}_2}{V_1} \frac{[\ln(1-v_2) + v_2 + \chi v_2^2]}{v_2^{\frac{1}{3}} - \frac{v_2}{2}} \quad (3)$$

$$\xi = l v_2^{-\frac{1}{3}} \left(\frac{2C_n M_C}{M_r} \right)^{\frac{1}{2}} \quad (4)$$

To determine the Flory-Huggins parameter, χ , equation 5 was used where χ_H is the enthalpic contribution and χ_S is the entropic contribution, typically used as 0.34 [37]. Equation 6 finds the enthalpic contribution, R is the universal gas constant, T is the absolute temperature, δ_2 and δ_1 are the Hildebrand solubility parameters of the polymer (equation 7) and solvent (12.0 cal/cm³ [27]) respectively.

$$\chi = \chi_H + \chi_S \quad (5)$$

$$\chi_H = \frac{V_1}{RT} (\delta_2 - \delta_1)^2 \quad (6)$$

To calculate the Hildebrand solubility parameters, equation 7 was used, where E_{coh_i} is the cohesive energy of each bond type in the crosslink, and V_i is the molar volume of the bond type in the crosslink.

$$\delta_2 = \left(\frac{\sum E_{coh_i}}{\sum V_i} \right)^{0.5} \quad (7)$$

Cohesive energies and molar volumes were used from Fedors values in Van Krevelen et al. [37]. The Flory-Huggins parameter for the initial oxidized state was used as an estimation in calculating the mesh size of the reduced, and then re-oxidized hydrogel.

2.4 Fourier Transform Infrared Spectroscopy

Using Fourier transform infrared spectroscopy (FTIR) (Varian 7000e) and a heated (60°C) attenuated total reflectance (ATR) stage, the starting unreacted materials were added to the stage for real-time FTIR analysis during polymerization. A spectrum was taken at each time point, and using Varian Resolutions Pro, peak areas were calculated for the carbonyl (C=O) and carbon double bonds (C=C) of acrylates. Reaction was measured by plotting the C=C/C=O ratio as a function of time, where the C=O bonds would be conserved during reaction.

2.5 X-ray Photoelectron Spectroscopy

A Thermo Scientific K-Alpha x-ray photoelectron spectrometer (XPS) was used with an EX06 ion source. Hydrogel samples were freeze dried overnight, then transferred to a high vacuum chamber for 24 hours. After this, the samples were scanned looking at the S2p binding energy range, and analyzed with Thermo Scientific Advantage Data System.

2.6 Hydrogel Degradation

To determine hydrogel degradation, 10 mM phosphate buffered saline (PBS) was used under sink conditions (volume 10x material solubility limit). Samples were stored in a shaken 37°C water bath for the indicated time periods, whereupon the total liquid supernatant was collected, and fresh PBS was added. To study degradation rate of reduced hydrogels, a 50 mol% CA/TTD hydrogel was first reduced in 4x excess DMSO for 16 hours using 50 mM free TCEP. Due to the swelling ratio of the 50/50 mol% CA/TTD hydrogel being 4.2 wet/dry, and 11% of mass solubilized in reducing conditions, the gels were then washed 3 times with PBS to remove residual TCEP down to below 10 µM. Degradation samples were also taken of a control gel made of 50/50 mol% CA/TTD, not exposed to reducing conditions, and a 0/100 mol% CA/TTD hydrogel exposed to reducing conditions to control for residual free TCEP that may not be fully removed from the assay solution.

2.7 Sample Reduction and Thiol Detection

To create reducing conditions, either TCEP or 2-ME reducing agents were used. Soluble hydrogel degradation product samples were reduced with TCEP immobilized agarose beads at a concentration at or below 2 mM disulfides (Pierce™ TCEP beads contain 8 mM

reducing power) for 30 minutes while well-mixed on a vortexer. To detect free/bound thiol, Ellman's reagent (DTNB) solution was first created at 0.0784 mg/ml using Ellman's buffer, and was kept out of light. After dilution of 17.8 μ L of sample into 182.2 μ L of Ellman's reagent solution, the mixture was incubated for 15 minutes prior to sample analysis using a UV-vis spectrophotometer (412 nm). Thiol content was calculated based upon a calibration curve of reduced cystamine with TCEP beads and Ellman's assay.

2.8 Mass Spectrometry

50/50 mol% CA/TTD hydrogel degradation products were degraded in DI water to full solubility. To detect mass shifts due to disulfide cleavage, two samples were analysed using direct injection into a Varian 1200 mass spectrometer in positive ion mode, with or without presence of TCEP reducing agent, in a 50/50 mixture of Milli-Q water and methanol with 0.1% formic acid.

2.9 Cytotoxicity

Human umbilical vein endothelial cells (HUVECs) were seeded into 96 well plates (Costar) at 9,000 cells/well and grown to confluence. Cells were treated with neutralized (pH 7.4) degraded hydrogel materials in both oxidized and reduced states that were serially diluted in EGM-2 Endothelial Cell Growth Media (Lonza). Similar conditions were used when treating cells with glycolic acid and lactic acid in PBS (Figure S2), and the pH was adjusted to 7.4 to mimic PLGA degradation products in a buffered physiological environment. Cells were incubated for 24 hours at 37°C and 5% CO₂. Material was removed after 24 hours and cells were stained with Calcein-AM. HUVEC viability percentage was measured using a UV-vis spectrophotometer (Biotek SynergyMX) at excitation/emission wavelengths of 485/528 nm. Data was normalized to live controls.

2.10 Reduction of Oxidative Stress in HUVEC

HUVECs were seeded into 96 well plates at 9,000 cells/well and grown to confluence. Cells were incubated in neutralized hydrogel degradation products at concentrations between 2.5 and 10 mg/mL in EGM-2 endothelial cell growth media. After one hour, 18.75 mU/mL of xanthine oxidase (XO) was added followed by 200 μ M hypoxanthine (HX) to cause injury via hydrogen peroxide and superoxide production. Cells were incubated with material and HX/XO for 24 hours at 37°C and 5% CO₂. HUVEC viability was measured by Calcein-AM intensity using UV-vis spectrophotometry. Data was normalized to live controls.

2.11 Mitochondrial Respiratory Response in HUVEC

A Seahorse Biosciences XF flux analyzer was used to monitor mitochondrial bioenergetics with the SS/SH hydrogel degradation products $\pm$ HX/XO by recording oxygen consumption rates (OCR) in pmol/min as well as extracellular acidification rates (ECAR) in terms of pH change. In this analysis, the assay parameters/treatments were set to determine changes in OCR to study the effect of HX/XO generated oxidative stress, and not changes in ECAR glycolytic flux. A 50/50 mol% CA/TTD hydrogel degradation product concentration was used which was below the Calcein-AM derived IC₅₀ for both the SH and SS systems (both SS and SH used 0.156 mg/mL with SH at 0.363 ± 0.006 mM thiol, n=3, standard deviation).

HX/XO (200 μ M/18.75 mU/mL) was set at the same concentration as the Calcein-AM studies such that mitochondrial response may be deviated from non-treated controls. HUVECs were seeded at a density of 35,000 cells/well in a 96-well tissue culture plate and were allowed to adhere overnight. Media without cells was used as a background during XF flux analysis. After overnight incubation, cells were treated with the hydrogel degradation product sample groups for 1 hour, whereupon HX/XO was added and incubated for 24 hours in total at 37°C and 5% CO₂. After 24 hours of treatment, the well plate was prepared for the mitochondrial stress assay using an XF flux analyzer.

The cell growth media was then washed with XF Assay Modified DMEM media (5.5 mM glucose, 1 mM pyruvate, 2 mM glutamine). After washing, wells were further incubated in the same FX media for 1 hour at 37°C in a non-CO₂ chamber. The well plate was then subjected to the mitochondrial stress assay, which includes series addition of electron transport inhibitors i.e. oligomycin (1 μ M), FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) (1 μ M) and a mixture of rotenone (1 μ M) and antimycin A (1 μ M) in a serial manner to study mitochondrial bioenergetics response. Each inhibitor serves the function of determining at least one bioenergetic component of the mitochondrion. After initial incubation to determine basal respiration, oligomycin is added, which acts as ATP synthase inhibitor and determines the ATP production rate linked to the mitochondria. FCCP is then added, a proton gradient uncoupling agent, which gives determination of the maximum respiration. The mitochondrial spare respiration capacity is calculated as the difference between the maximal respiration and the basal respiration. Rotenone/antimycin A completely shuts down the mitochondrial function by inhibiting complex I and II function in electron transport chain and is added to give an estimate of any non-mitochondrial OCR which is baseline subtracted in calculating basal respiration and maximum respiration. In Gupta, 2016, see Fig. 3B for graphical explanation of a typical Seahorse XF output chromatogram [38].

OCR and ECAR were measured every 3 minutes including addition of inhibitor drugs in series. Post XF analyzer measurement, cells in each well were fixed for BCA protein assay to estimate the protein content of the adhered cells. To proceed for the assay, FX media was removed and cells in each well were lysed by adding 25 μ L of cell lysis buffer (0.32 mM sucrose, 2 mM EDTA, 2 mM EGTA, 20 mM HEPES at pH 7.4, containing protease inhibitors 4 μ g/mL leupeptin, 4 μ g/mL pepstatin, 5 μ g/mL aprotinin, and 0.2 mM PMSF). The plate was incubated at -20°C overnight. The next day, the plate with lysed cells was analyzed for protein levels using BCA protein assay kit (Thermo Scientific, Rockford IL). The OCR (pmol/min) values were then normalized using the protein content values, and data was also obtained as pmol/min/ μ g of protein or %OCR from the third basal values of measurement for each group. Hydrogel degradation products, either thiolated or disulfide containing, were not expected to interfere with the Seahorse Biosciences XF assay analysis.

2.12 Statistical Methods

Significance testing was performed using ANOVA two factor with replication followed by the Bonferroni correction factor for multiple comparisons with a significance threshold of

$p < 0.05$. Figures 1–6 are reported as mean $\pm$ standard deviation. Figures 7 and 8 are reported as mean $\pm$ standard error.

3 Results

3.1 Hydrogel Formation

Disulfide cystamine-containing hydrogels were formed through aza-Michael addition of cystamine (CA) with diethylene glycol diacrylate (DEGDA) (Figure 1A). The relative content of thiol was controlled through the addition of another di-primary amine, 4,7,10-trioxa-1,13-tridecanediamine (TTD). To verify the reaction between the acrylates and amines, real-time ATR-FTIR (attenuated total reflectance Fourier transform infra-red spectroscopy) analysis of the polymerization at 60°C was performed, where the acrylate conversion was detected (Figure 1B). The peak intensity of the C=C found in the DEGDA acrylate group decreased with time relative to that of the carbonyl peak intensity, indicating that the Michael addition reaction took place over the 24 hour period. Post-reaction, hydrogels were washed in dimethylsulfoxide (DMSO) to remove unreacted materials. This resulted in a mass loss of less than 7% (Figure 2A), suggesting that at least 93% of reactants added were incorporated into the hydrogel. Further, only 4 wt% of the total added cystamine was washed out for the 50/50 mol% CA/TTD hydrogel, demonstrating a high reactivity of cystamine (Figure 2B). Detection of the surface chemistry using X-ray photoelectron spectroscopy (XPS) revealed consistent proportionality of sulfur S2p orbitals (Figure 1C). Although disulfides and thiols contain overlapping binding energies, the presence of bound sulfur is nonetheless detected, with 163 eV hypothesized to be in disulfide configuration. Slight asymmetry may be due to 2p spin-orbit doublets of 1/2 and 3/2. Importantly, there are no oxidant shifts reflecting sulfones or sulfonates, which would appear at binding energies greater than 163 eV, around 168 eV.

Being a PBAE, the rate of hydrogel degradation in PBS was expected to be dependent upon the relative hydrophilicity. As cystamine is highly hydrophilic, it should increase the degradation rate with increasing content. By exposing CA/TTD hydrogels to PBS, hydrogels indeed degraded faster with increased cystamine content; the 75/25 mol% CA/TTD hydrogel degraded over 24 to 48 hours, compared to 48 to 72 hours for those with the lower cystamine content gels (50/50 mol% CA/TTD) (Figure 3A). Since these disulfide hydrogels hydrolytically degrade into a distribution of acid fragments with and without cystamine and TTD, these fragments should be released with intact disulfide bonds. Therefore, we gathered and reduced the supernatants of hydrolytically degraded hydrogels and performed Ellman's thiol assay to determine if the rate of disulfide release matched that of the degradation mass loss, or if there was degradative preference to the disulfide regions due to their hydrophilicity. Release rates of disulfides were found over the same periods as the degradation products (Figure 3B). By plotting the disulfide release vs. the degradation, M_t/M_∞ , it is apparent how cystamine release kinetics compare to the bulk system. As seen, the closeness to the $y=x$ line in Figure 3C indicates that release was controlled at the same degradative rate as the bulk material.

3.2 Effect of a Reducing Environment

As the hydrogels contained disulfide crosslinks, it would be expected that exposure to a reducing environment would cleave these disulfides, decreasing the crosslinking density of the hydrogel. Depending upon the extent of cystamine incorporation, this reduced crosslinking density could result in the dissolution of the gel. Using 2-ME reducing agent in excess, we measured the mass remaining of a range of cystamine content hydrogels after 12 hours of vortexing in PBS covered with argon (Figure 3D). We found that of hydrogels formed, a threshold existed, where at 66.6/33.3 mol% CA/TTD and lower cystamine content, hydrogels did not fully dissolve into soluble degradation products. However, at concentrations above 66.6/33.3 mol% CA/TTD cystamine (e.g. 75/25 mol% CA/TTD), complete solubilization occurred. This indicates the ability for delivery of this material in a reduced state with antioxidant thiol groups that are likely exposed at a CA/TTD content less than or equal to 66.6 mol% cystamine of the amines.

3.3 Drug Release of Pre-reduced Hydrogels

For reduced hydrogels below the 66.6/33.3 mol% CA/TTD cystamine content threshold, hydrogels should contain antioxidant moieties as endcaps where disulfides previously existed. Upon introduction to an aqueous environment, the ester-cleaved fragments would thereby contain these thiol endcaps, and allow for antioxidant release proportional to the rate of degradation. To determine this, we first reduced a 50/50 mol% CA/TTD hydrogel in excess TCEP, and then after washing away free reducing agent, the thiol content was measured in degradation supernatants as a function of incubation time in PBS. As anticipated, a 50/50 mol% CA/TTD hydrogel, reduced prior to degradation, showed release of thiol groups in the supernatant solution (Figure 4A). The degradation time decreased substantially from a previous 48–72 hours in the oxidized state to 10–24 hours in the reduced form. Similarly, for a 75/25 mol% CA/TTD hydrogel, only 2 hours were required for complete solubilization in TCEP containing DMSO (Figure 4B) compared to 24–48 hours in the aqueous disulfide state. With 50/50 mol% CA/TTD reduced degradation products, disulfide formation occurred rapidly in the first 24 hours, but slowed over a period of 11 days (Figure 4C) in 37°C PBS for a half-life of roughly 7 days.

Mass spectrometry of 50/50 mol% CA/TTD degradation products, with and without presence of excess TCEP reducing agent, showed formation of several species of interest, with an overall shift to lower molecular weight species due to disulfide cleavage (Figure 4D). Mass to charge ratios (m/z) of 150, 222, 238, 310, and 398 are all potentially thiol-containing. Species with $m/z = 292$ is thought to be a fragmentation of a hydroxyl group from 310. Species with m/z 251 and 267 are residual excess TCEP and oxidized TCEP respectively.

3.4 Hydrogel Swelling and Mesh Size

To attain equilibrium swelling, hydrogels of varying cystamine content were placed in anhydrous DMSO for 8 hours in reducing conditions (Figure 5A). While the 75/25 mol% CA/TTD hydrogel solubilized completely, the lower content gels produced a large spike in swelling ratio in DMSO, indicating backbone cleavage. Hypothetically, as disulfide crosslinking groups can be reversibly reduced and oxidized, it should be possible to shift the

equilibrium swelling ratio based upon thiol oxidation to disulfide. To see if a margin of the crosslinking could be recovered, we re-oxidized these hydrogels in air after freeze drying. Since soluble fragments were washed away, the only thiols able to oxidize to disulfides were those bound to the hydrogel. It was found that the swelling ratio was decreased slightly from the reduced form ($p=0.003$ for 50/50 mol% CA/TTD, $p=0.13$ for 66.6/33.3 mol% CA/TTD).

Flory-Huggins parameter (χ) calculation (Figure 5C) showed a decrease in value with increasing cystamine content, for both the oxidized and reduced materials. The mesh size calculation found sizes from $8.2 \pm 0.2 \text{ \AA}$ to $9.0 \pm 0.6 \text{ \AA}$ for the oxidized, and $8.0 \pm 0.15 \text{ \AA}$ to $18.2 \pm 2.8 \text{ \AA}$ for the reduced hydrogels, with increasing mesh size with increasing cystamine content (Figure 5B). After re-oxidation of the initially reduced hydrogel, the mesh size decreased, but was not significantly recovered at $16.7 \pm 2.9 \text{ \AA}$. $n=3$, standard deviation.

3.5 Cytotoxicity of Degradation Products on HUVEC

To evaluate cellular toxicity of the cystamine hydrogel degradation products, human umbilical vein endothelial cells (HUVEC) were exposed to serial dilutions of hydrogel degradation products for 24 hours, and viability was measured using Calcein-AM live stain. Degradation products were either reduced using TCEP beads or kept neat (disulfide state) and quantified for their thiol content in PBS. It was found that hydrogel degradation products containing cystamine showed large differential toxicity dependent upon their redox state, whereas treatment with TCEP reducing agent had only a small effect on cell response to the control hydrogel without cystamine (Figure 6A–C). Degradation products with reduced cystamine resulted in a cellular toxic concentration (IC_{50}) between 13.5 and 15.5 times higher than their respective disulfide containing samples. IC_{50} values are found in Table 1. While a small IC_{50} shift was observed on the control with reducing agent (Figure 6A), it was likely due to a low level of leached reducing agent from the TCEP beads, a value less than 1 mM thiol.

3.6 Protection from Oxidative Stress on HUVEC

To demonstrate suppression of oxidative stress, HUVECs were exposed to a hydrogen peroxide and superoxide-producing hypoxanthine and xanthine oxidase (HX/XO) reaction in the presence of hydrogel degradation products in both redox states [39]. A significant increase in cell viability was demonstrated with the reduced sample compared to a blank control containing only HX/XO and cells at two concentrations of 2.5 and 5 mg/mL (Figure 6D). In contrast, the oxidized samples showed no viability with or without superoxide insult at the same concentrations tested (Figure 6E). This result exemplifies the differential toxicity between the two material types in that the reduced form lowers oxidative injury, yet does not prevent injury in the oxidized form at equal concentrations.

3.7 Mitochondrial Response to Oxidative Stress on HUVEC

In order to further our mechanistic understanding behind the viability differences found between oxidized (SS) and reduced (SH) hydrogel degradation products, we performed a Seahorse Bioscience XF assay. This assay is useful to determine changes in mitochondrial oxygen consumption rate, as under cellular distress, mitochondrial dysfunction may ultimately cause cell death. Using an equal hydrogel degradation product concentration

(0.156 mg/mL), which was below the IC₅₀ for both the SS and SH containing 50/50 mol% CA/TTD, we subjected the hydrogel degradation products to HX/XO injury for testing with the Seahorse Biosciences XF assay to analyze effects of mitochondrial oxygen consumption rate (OCR).

3.7.1 Total OCR—Without HX/XO treatment, the disulfide-containing hydrogel degradation products (SS) showed several significant differences in viability in total OCR values (Figure 7A–D) for ATP production, total respiration, and max respiration compared to live controls. The decreases in OCR in the SS groups is similar to the viability differences found in the Calcein-AM viability-based HX/XO injury study of Figure 6.6E. Also similar to Figure 6E, with HX treatment, there were no statistical changes comparing the live control to the SS material, indicating no protection. Comparing the cellular protein content of Figure S1, there is an average decrease, but non-significant difference between the control and SS groups without HX treatment. This small decrease may be due to mitochondrial stress due to reduced ATP production, as can be seen by the significant decrease in SS total OCR without HX treatment compared to the control without HX treatment (Figure 7B).

Looking at the reduced thiol-containing hydrogel degradation products (SH) for total OCR without HX treatment, results are again similar to the prior HX/XO viability assay (Figure 6D) in that there are no statistical changes in viability compared to the -HX live control for all four tests. Also, with HX treatment, SH viability remains statistically higher than the respective control for basal respiration and ATP production. These results indicate higher cellular viability after SH material addition compared to SS, and also a protective effect against HX/XO induced oxidative stress. Comparing the protein normalized % OCR may be more insightful to remove confounding protein differences that indicate cellular viability, and not necessarily mitochondrial function directly.

3.7.2 % OCR (Protein Normalized)—After normalizing for protein content (Figure S1), the statistical differences found in Figure 7A–D were not present in Figure 7E–H for both the SS and SH materials, with the only exception being for -HX SS spare respiration, and, while insignificant, lower averages were found for the SS material in ATP production and max respiration with HX treatment. Spare respiration determines how well mitochondria respond when there is increased cellular energy demand, defined as the difference between the basal and maximal OCR in the assay. The disulfide hydrogel degradation products responded poorly to spare respiration compared to the SH material, perhaps indicating that the SS material is causing mitochondria to shift ATP energy towards detoxification of the SS material, thus lowering the respiratory reserve energy. Total ATP production for the SH hydrogel degradation products with HX treatment remained very high, further identifying potential protection. Testing multiple concentrations could identify if we are able to replicate the dose-response found in the HX/XO viability assay (Figure 6D, E).

4 Discussion

Through variation of cystamine content as a di-primary amine containing crosslinker, we were able to produce hydrogels that degraded either hydrolytically or in a reducing environment due to the presence of ester bonds in the PBAE backbone. Cystamine

functionality provided the utility to deliver these materials in either a disulfide-containing oxidized state or thiol-containing reduced state. Interestingly, with a disulfide content lower than 75/25 mol% CA/TTD, hydrogels were shown to only partially degrade in reducing conditions, leading to a thiol-containing bulk material. Due to physico-chemical changes of the hydrogels upon redox state of disulfide bonds, disulfide hydrogels showed degradation periods from 48–72 hours, whereas the reduced material degraded more rapidly from 10–24 hours. This decrease in degradation period was perhaps due to a combination of larger void volume, allowing for greater solvent interaction, and enhanced hydrophilicity with the extra thiol endcap content. Due to the relatively quick degradation period found *in vitro*, it could be expected *in vivo* that the macrophage healing response would have only just begun, aiding in biocompatibility and diffusion of the reduced thiol system degradation products to the surrounding region [11]. However, the *in vitro* hydrogel degradation rate is only an approximation of the potential rate *in vivo* [40], as biological degradation is dependent upon additional factors, such as implant site and enzymatic/immune activity.

An increased cystamine content produced both an increased swelling ratio and degradation rate, and thus it was hypothesized that if these hydrogels were exposed to reducing conditions, their swelling ratio would increase additionally from the increase in mesh size due to reductive decoupling of disulfide bonds. After placing hydrogels of varying cystamine content in DMSO $\pm$ reducing agent until equilibrium swelling occurred, large swelling ratios were produced in reducing conditions compared to without, as predicted. We then sought to test the reversibility of the reduced crosslinks via re-oxidation of the reduced hydrogel, which would be indicated via decreased swelling ratios. Indeed, after re-oxidation, the swelling ratio was decreased. Full recovery of the oxidized swelling ratio was not expected due to washing of solubilized products which diffused from the hydrogel upon initial reduction, and thus only bound-thiols could re-couple. Corresponding to swelling studies, Flory-Huggins (χ) parameter calculation showed a decreased value with increasing cystamine content, similarly for both the oxidized and reduced materials, indicating increased miscibility with DMSO. This parameter behaviour correlated with the observed increase in swelling ratio. Since χ values were greater for the reduced thiol backbone compared to oxidized disulfide, the increase in swelling was not due to an increased polymer-solvent miscibility, a conclusion corroborated by disulfide cleavage.

Comparing the disulfide hydrogel degradation products in the thiol and disulfide form, cytotoxicity spanned over an order of magnitude difference (Table 1). The reduced degradation products containing thiols produced very high cytocompatibility concentrations (6.8–8.5 mg/mL), but containing physiologically relevant concentrations of thiols, (8.7–11 mM). Considering how there is higher thiol content at a lower bulk material concentration for the 75/25 mol% CA/TTD hydrogel, yet nearly equal for the 50/50 mol% CA/TTD, there may be a combination of effects occurring to determine cytotoxicity, with DEGDA and TTD being cytotoxic factors and thiols being cytoprotective. Impressively, these reduced materials produced about half the IC_{50} of PLGA as a biocompatible material control (IC_{50} = 16.6 mg/mL, Figure S2). Comparing these IC_{50} values to literature of other PBAE hydrogel supernatants, the unloaded 0/100 mol% CA/TTD and oxidized samples show comparable IC_{50} values to PEGDA with TTD crosslinked hydrogels of Wattamwar et al. [28]. In

contrast, the thiol-containing samples show low cytotoxicity, similar to Lakes et al. [27] using DEGDA/PEGDA/isobutylamine and vancomycin hydrogels, and lower cytotoxicity than the bioactive poly-antioxidant PBAE from Wattamwar et al. (1 mg/mL) [41]. It was originally hypothesized that the presence of disulfides would increase cytotoxicity compared to the unloaded control, but this was not observed as IC₅₀ values of the disulfide containing hydrogels were similar to those for the unloaded 0/100 mol% CA/TTD, near 0.5 mg/mL.

Under adverse oxidative conditions, mitochondrial damage from excess reactive oxygen species (ROS) can lead to further release of ROS into the cytoplasm via increased mitochondrial permeability (ROS-induced ROS-release) [42]. Therefore, the maintenance of mitochondrial function is imperative for cellular health, as when dysregulated it can be a factor in chronic inflammatory diseases and chronic wounds. Through prophylactic delivery of antioxidant capacity to the cellular environment, cellular protection was hypothesized to occur against oxidative stress assay on HUVEC. HX/XO reaction was used to initiate an oxidative stress response as it mimics the disease-based inflammatory response system common in ischemia/reperfusion injury [43]. The concentration of HX was selected such that it would produce the same concentration of superoxide as described by ischemia/reperfusion injury [44]. The HX/XO reaction produces roughly a 4:1 ratio of cell membrane-permeable hydrogen peroxide to non-permeable but highly reactive superoxide, creating a combination of extracellular and intracellular oxidation [45]. By adding the hydrogel degradation products 1 hour in advance, this assay could model a scenario of prophylaxis to a general oxidative stress injury, perhaps from radiation effects producing water-based reactive oxygen species, or to combat chronic ROS generation in the penumbral tissue adjacent to ischemia/reperfusion injury.

Compared to the untreated controls, the thiol-containing materials indeed showed oxidative protection, protecting the cellular viability from 0% viability untreated to roughly 75% viability treated at both the 2.5 and 5.0 mg/mL material concentrations. Controlled for the same concentration, the disulfide materials showed no protective effect as anticipated being above the IC₅₀. Without facilitated diffusion or active transport, negatively charged fragments (Figure 4D) would likely remain at the cellular exterior as the phospholipid bilayer is generally impermeable to charged molecules larger than ions [46]. Interestingly, these zwitterionic fragments are perhaps reminiscent to several actively transported metabolites, such as glutamate and aspartate, and the dicarboxylic acid, succinate. However, due to the molecular weight of PBAE degradation products ranging from 150 to 800 Da, it is likely that the majority of protective effects of the thiolated degradation products seen are due to extracellular scavenging of ROS produced by the HX/XO assay. Nonetheless, it is intriguing that even without ROS induction, there is such a great divide in cytocompatibility between the disulfide and thiolated degradation products. And thus, future investigation into cellular uptake mechanisms would garner elucidation to these aspects.

To determine if there were additional complex effects occurring on mitochondrial health obscured in the general cytotoxicity assays, we then performed a similar oxidative stress experiment measuring mitochondrial oxygen consumption rates (OCR), but using lower hydrogel degradation product concentrations below the IC₅₀ concentrations for both the thiolated and disulfide degradation products. Comparing the thiolated and disulfide materials

mitochondrial response, several statistical differences appeared in the total OCR values between the two material states as reported in the results section. In general, the disulfide hydrogel degradation products produced less than half the total OCR average values than that of the thiolated hydrogel degradation products under non-stressed conditions, indicating some innate toxicity of the material towards mitochondrial health, whereas the thiolated material showed no statistical differences from the live control. Total OCR values take into account effects of viable cell counts, as cell protein content is not normalized into the values (separation of cells measured for cell protein content numbers was based upon those cells remaining adhered to the well plate post washing). Therefore, if cell death or viability shifts occur, total OCR numbers would show differences compared to protein normalized %OCR. However, while there were no significant differences in cell protein content (Figure S1) with the material concentrations being well below the respective IC_{50} , most significant differences found in total OCR were not found after protein normalization.

With the viable cell protein normalized %OCR, significant differences remained in spare respiratory capacity, which measures how well mitochondria are able to continue energy production under stressed conditions. Comparing the two materials, the thiolated degradation products conserved both the total OCR and protein normalized %OCR $\pm$ HX/XO compared to the live controls, whereas the disulfide hydrogel degradation products depleted it. This lowered spare respiratory capacity from the disulfide hydrogel degradation products may be indicative of mitochondria shifting energy towards detoxification of the material. This lack of energy could perhaps be from the DEGDA/TTD content itself that shows much lower cytocompatibility than the thiol material, and/or recovery from the high disulfide content altering the overall cellular redox ratio, resulting in less energy to be available for stressed condition reserves. To further identify this, a total and GSH/GSSG ratio analysis would be insightful.

To further understand the complexity of mitochondrial health, future work may investigate whether or not these hydrogel degradation products provide regional protection from oxidation in the extracellular space only, or also in the cytoplasmic space, as could be determined via uptake studies. The greater the tertiary amine content of the degradation product, the more overall positively charged it will be, whereas the greater the number of acid residues, there will be a net negative charge at physiological pH, which could perhaps create selective species uptake. Additionally, future studies of implant biocompatibility and efficacy could be tested with relevant oxidative stress injury models *in vivo* to provide more insight into the quickly degrading and highly-thiolated reduced PBAE hydrogel produced.

5 Conclusions

The outcome of converting the redox state of cystamine PBAE hydrogels is reminiscent to the effects of cellular GSH:GSSG ratio. With slight offset of this ratio, antioxidant protection is enhanced or depleted, requiring mitochondrial metabolic activity to shift the redox ratio back to a homeostatic environment and away from pathophysiological conditions. The presence of thiol groups within the degradation products affected the cytocompatibility greatly, with up to a 15.5 times greater IC_{50} for the thiolated material than the disulfide, nearing 1 wt% concentration and 10 mM thiol content, the same order of

magnitude as cellular GSH (1–10 mM). With such high thiol content available at sub IC₅₀ concentrations, the thiolated degradation products significantly defended cells against oxidative stress whereas the disulfide products did not affect viability. Upon pre-incubation, the thiolated material also showed the ability to protect mitochondrial metabolism under oxidative stress conditions, unlike the disulfide material. This material in the thiolated form gives rise to potential applications of cellular antioxidant fortification, relevant to eliminating acute oxidative stress events and avoiding chronic inflammatory diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Statement of Significance

In this work, we demonstrate a unique property of disulfide containing degradable biomaterials. By changing the state of the oxidation state of the degradation products (From oxidized to reduce) it is possible to increase the LD50 of the material by an order of magnitude. This dramatic shift is linked directly to the oxidative stress response of the cells and suggests a possible mechanism by which one can tune the cellular response to degradable biomaterials.

Figure 1. Disulfide PBAE and thiolated nanoparticle reaction scheme. A) PBAE disulfide hydrogel reaction scheme and degradation products upon complete hydrolysis and reduction. Products 1–3 are inclusive of potential speciation. Product 4 is an example of a fully hydrolyzed thiolated degradation product of 1 containing groups R^1 , R^2 , R^3 . B) Carbon double bond conversion using real-time FTIR polymerization on a heated ATR crystal, where the dashed abscissa line indicates the endpoint C=C/C=O ratio after 24 hours. C) XPS S2p spectra comparing sulfur (disulfide/bound thiol) binding energy at 163 eV. n=1 B,C.

Figure 2. Hydrogel washing with variation of cystamine content. A) Mass of hydrogels remaining after washing with DMSO and freeze drying (mass after wash / mass before wash), n=3, standard deviation. B) Thiol content of total lost in DMSO washing, n=1.

Figure 3.

Disulfide hydrogel degradation characteristics. A) Mass remaining of 50/50 and 75/25 mol% CA/TTD hydrogels via degradation in PBS at 37°C in sink conditions. B) Release rate of 50/50 and 75/25 mol% CA/TTD hydrogel degradation products in the disulfide form, post-reduced for thiol detection. C) Comparison of disulfide release rate vs. bulk hydrogel degradation. D) Fraction of insoluble hydrogel mass remaining after washing with 2-mercaptoethanol in DMSO vs. cystamine molar content.

Figure 4.

Thiolated hydrogel degradation and oxidation. A) Rate of thiol-containing species eluting from 50/50 mol% CA/TTD, or 0/100 mol% CA/TTD hydrogels in PBS. These hydrogels were either pre-reduced with TCEP in DMSO (16 hr), washed with PBS 3 times, and then degraded (designated as 'Pre-reduced then Degraded'), or were degraded in PBS in the disulfide state, but reduced post-degradation (designated as 'Degraded then Reduced'). Sample 'No Reducing Agent' did not see externally supplied reducing agent at any point in the assay as a background control. n=3 each for A–D, standard deviation. B) Kinetics of reduction for a 75/25 mol% CA/TTD hydrogel in 2-mercaptoethanol and DMSO. C) Percent of solubilized 50/50 mol% CA/TTD hydrogel degradation products remaining in the thiol state over time in PBS at 37°C. D) ESI mass spectrometry of 50/50 mol% CA/TTD degradation products with and without free TCEP. Upon complete solubilisation of the hydrogel, TCEP-HCl was added to the 'w/ TCEP' sample in excess to ensure reduction of disulfide bonds. Inset lists potential degradation products. n=3 for A–C, standard deviation, n=1 for D with a representative sample.

Figure 5. Equilibrium swelling of various cystamine content hydrogels in DMSO. A) Swelling ratio, B) calculated mesh size, and C) calculated Flory-Huggins parameter. $n=3$, standard deviation.

Figure 6.

Cellular viability of HUVEC in the presence of degradation products. A–C) Cellular viability in the presence of hydrogel degradation products without (disulfide form) or with (reduced form) TCEP reducing agent beads with A) 0/100 mol% CA/TTD (unloaded), B) 50/50 mol% CA/TTD C) 75/25 mol% CA/TDD. D–E) HX/XO free radical generation assay comparing cell viability of 50/50 mol% CA/TTD hydrogels in the reduced state and oxidized state, with and without oxidative injury with dose response (200 μ M HX, 18.75 mU XO). $n=5$ A–E, standard deviation. * $p<0.05$ with Bonferroni correction to the “No Hydrogel +HX”.

Figure 7.

Seahorse Biosciences XF mitochondrial response assay on HUVEC. 50/50 mol% CA/TTD degradation products, $\pm$ HX/XO (200 μ M/18.75 mU/mL). A–D) OCR results. E–H) %OCR results normalized for protein content. Details of bioenergies determinations may be found in the Mitochondrial Respiratory Response methods section. $n=5$ for treatments, $n=7$ for live control. Standard error. * $p<0.05$ with Bonferroni correction compared to respective controls.

Table 1Comparison of IC₅₀ values on HUVEC

Mol% CA/TTD	IC ₅₀ (mg/mL)		IC ₅₀ (mM)	
	–Reducing Agent	+Reducing Agent	–Reducing Agent (Disulfide)	+Reducing Agent (Thiol)
0/100	0.56	1.0	0.00	1.0 [*]
50/50	0.63	8.5	0.32	8.7
75/25	0.44	6.8	0.35	11
PLGA	16.6	—	0.00	—

^{*}
Contains residual TCEP, no thiol