



Oxygen-Mediated Enzymatic Polymerization of Thiol–Ene Hydrogels

Journal:	<i>Journal of Materials Chemistry B</i>
Manuscript ID:	TB-ART-12-2013-021794.R1
Article Type:	Paper
Date Submitted by the Author:	11-Feb-2014
Complete List of Authors:	Zavada, Scott; University of Michigan, Macromolecular Science and Engineering McHardy, Nicholas; University of Michigan, Material Science and Engineering Scott, Timothy; University of Michigan, Chemical Engineering; University of Michigan, Macromolecular Science and Engineering

Cite this: DOI: 10.1039/x0xx00000x

Oxygen-Mediated Enzymatic Polymerization of Thiol–Ene Hydrogels

S.R. Zavada,^a N. R. McHardy,^b and T. F. Scott^{a,c}Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Materials that solidify in response to an initiation stimulus are currently utilized in several biomedical and surgical applications; however, their clinical adoption would be more widespread with improved physical properties and biocompatibility. One chemistry that is particularly promising is based on the thiol–ene addition reaction, a radical-mediated step-growth polymerization that is resistant to oxygen inhibition and thus is an excellent candidate for materials that polymerize upon exposure to aerobic conditions. Here, thiol–ene-based hydrogels are polymerized by exposing aqueous solutions of multi-functional thiol and allyl ether PEG monomers, in combination with enzymatic radical initiating systems, to air. An initiating system based on glucose oxidase, glucose, and Fe²⁺ is initially investigated where, in the presence of glucose, the glucose oxidase reduces oxygen to hydrogen peroxide which is then further reduced by Fe²⁺ to yield hydroxyl radicals capable of initiating thiol–ene polymerization. While this system is shown to effectively initiate polymerization after exposure to oxygen, the polymerization rate does not monotonically increase with raised Fe²⁺ concentration owing to inhibitory reactions that retard polymerization at higher Fe²⁺ concentrations. Conversely, replacing the Fe²⁺ with horseradish peroxidase affords an initiating system that is not subject to the iron-mediated inhibitory reactions and enables increased polymerization rates to be attained.

Introduction

Materials that are polymerizable *in situ*, such as composite dental restoratives,^{1, 2} suture-less wound closures,^{3, 4} and hemostatic sealants,^{5–7} are currently used in a wide range of medical procedures. However, although several materials and approaches for tissue adhesives and sealants have emerged, substantial research attention is still required to fulfill the potential of polymeric materials that can be applied to a wound site in liquid form and solidify *in situ*, alleviating the need for sutures or bandages. The development of new biocompatible materials that polymerize rapidly upon application, demonstrate excellent adhesion to tissue, and are readily tailored to suit the requirements dictated by the wound site, is essential to fully realize this potential.

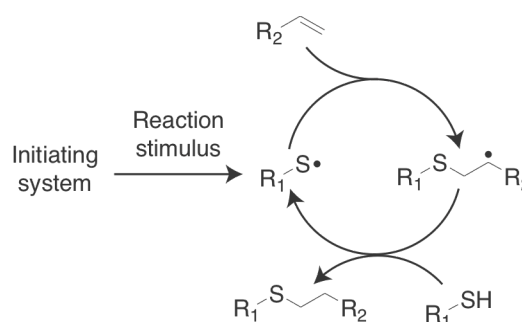
Many of the currently available systems are packaged as two liquid formulations that solidify via an *in situ* polymer network forming reaction upon combination. For example, the albumin/glutaraldehyde system, where glutaraldehyde serves as a difunctional cross-linker that reacts with the amine functionalities present in both albumin and the substrate tissue, has been successfully used to the seal tears in blood vessels.^{5, 8}

However, glutaraldehyde poses a host of safety concerns, including the potential for inducing nerve injury, mutagenicity, and tissue necrosis.⁵ Fibrinogen/thrombin preparations similarly react *in situ*, mimicking the final stages of the blood clot-forming coagulation cascade,⁹ to yield a cross-linked fibrin network. These fibrin-based materials are typically considered safer than albumin/glutaraldehyde formulations;⁵ however, the resultant proteinaceous polymer network exhibits limited mechanical robustness, failing under moderate pressures.^{5, 10, 11} Moreover, as both the albumin/glutaraldehyde and fibrinogen/thrombin systems are derived from animal sources, there exists a perceived risk of viral infection or allergic reaction to the patient.^{5, 7} This potential risk is readily eliminated by utilizing formulations comprised of wholly synthetic materials such as poly(ethylene glycol) (PEG)-based hydrogels. Several reaction mechanisms have been employed to effect the cross-linking and gelation of aqueous telechelic PEG solutions including succinimide/amine additions⁵ and catechol cross-linking upon addition of sodium periodate, a strong oxidant.¹² The primary deficiency of these binary-formulation systems is that they require mixing, typically performed within

the delivery device itself, to induce polymerization;⁵ consequently, inadequate mixing or improper mixing ratios prevents complete polymer network development, yielding incompletely polymerized regions throughout the material and adversely affecting its mechanical robustness and adhesion to the substrate tissue.

An alternative to systems that require mixing immediately prior to application are those that are packaged as a single formulation and that rely upon some external stimulus, such as light, elevated temperature, or exposure to an environmentally-borne chemical reactant, to initiate polymerization. For example, (meth)acrylate monomers formulated with a photoinitiator will readily polymerize, *via* a chain-growth mechanism, upon exposure to light.¹³ While the use of light permits fine spatial and temporal reaction control, the necessary availability of an intense light source is undesirable for many medical procedures. Conversely, cyanoacrylate adhesives require no preparation or additional equipment to effect polymerization; rather, they polymerize immediately upon exposure to atmospheric water or weak nucleophiles such as hydroxyl and amine functional groups on skin, yielding a solid polymer.^{4, 5} Owing to their rapid reaction kinetics, capacity for environmentally-triggered cure, and the high strength and excellent tissue adhesion typically exhibited by the generated polymers,⁴ cyanoacrylates have found widespread use as suture replacements for incision closure. Additionally, the application of cyanoacrylates to incision sites is far simpler and faster than suture placement, and subsequent clinician visits for suture removal is obviated by the hydrolytic degradability of poly(cyanoacrylates). Nevertheless, serious cytotoxicity concerns persist over the hydrolytic degradation products of these materials (e.g., formaldehyde),^{4, 14, 15} limiting their clinical utilization exclusively to external applications. Furthermore, measures necessary to reduce chronic exposure to toxic cyanoacrylate vapour experienced by health professionals are burdensome.¹⁶ Finally, cyanoacrylate resins are not amenable to chemical modification owing to the reactivity of the cyanoacrylate functional group; this lack of extensibility severely curtails the attainable property range which, as poly(cyanoacrylates) tend to be relatively brittle,¹⁷ hinders their broader adoption.

Despite their limitations, cyanoacrylates illustrate the tremendous utility of employing an environmentally-borne initiation stimulus, rather than light or the co-reaction upon combination of two precursor formulations, to effect polymerization. Although water is a common stimulus to induce the polymerization of cyanoacrylates and other materials,^{18, 19} oxygen poses as a convenient environmentally-borne polymerization initiation stimulus for radical-mediated polymerizations as it is both ubiquitous, being present at 21% of Earth's atmosphere, and highly reactive, being able to participate in redox reactions and generate free radicals. For example, oxygen has been used as a reactant in enzyme-mediated monosaccharide oxidations to ultimately yield radicals and initiate (meth)acrylate polymerizations of aqueous monomeric formulations.^{20, 21} Thus, the aqueous meth(acrylate)



Scheme 1. The radical-mediated thiol-ene reaction mechanism. Upon radical generation, thiol-ene reactions proceed *via* alternating propagation and chain-transfer reactions, where a thiyl radical propagates to an electron-rich carbon-carbon double bond generating a thioether and carbon-centered radical. This radical subsequently abstracts a thiol hydrogen to yield a stable thioether product and regenerate a thiyl radical. Thus, this reaction is an alternating copolymerization where thiol and vinyl groups are consumed at equal rates.

formulations were stable in the absence of oxygen but, upon exposure to air, reacted to generate polymeric materials and, for cross-linking systems, hydrogels. A limitation of this approach is the typically inhibitory influence oxygen has on radical-mediated chain-growth polymerizations, leading to incomplete cure and a high concentration of unreacted, leachable monomers. These enzyme-mediated polymerizations could thus be improved through the use of mechanisms that are resistant to oxygen inhibition such as the radical-mediated thiol-ene addition.²²

Thiol-ene polymerizations proceed *via* alternating propagation and chain transfer events between thiols and electron-rich carbon-carbon double bonds (e.g., allyl ether or vinyl ether) (Scheme 1), leading to step-growth evolution of the molecular weight.²² Unlike radical-mediated meth(acrylate) chain-growth polymerizations, the thiol-ene reaction mechanism is extraordinarily resistant to oxygen-inhibition,²² owing to the facile abstractability of the thiol hydrogen, and thus is well-suited as the polymerization mechanism for an oxygen-mediated hydrogel formation. Furthermore, many thiol- and ene-functionalized monomers exist, offering versatility and fine control over the physical properties of the resultant polymer and potential degradation products. Additionally, the thiol-ene mechanism is tolerant to the presence of a wide range of functional groups that may be present on cell-binding epitopes or growth factors incorporated in the formulation. Here, we examine aqueous thiol-ene solutions formulated with oxidoreductase-based enzymatic radical initiation systems that remain stable under anaerobic conditions but polymerize rapidly upon exposure to atmospheric oxygen, combining the desirable features of thiol-ene chemistry with the convenience of an environmentally-borne initiation stimulus.

Experimental

Materials and synthesis

D-Glucose (Fisher Scientific), iron(II) sulfate heptahydrate (Sigma-Aldrich), glucose oxidase from *Aspergillus niger* (GOx, 147.9 U/mg, Sigma-Aldrich), horseradish peroxidase (HRP,

Type VI, 261 purpurogallin U/mg, Sigma-Aldrich), and acetylacetone (AA, Sigma-Aldrich) were used as received. Cupferron (*N*-nitroso-*N*-phenylhydroxylamine ammonium salt), used as a radical inhibitor to prevent premature polymerization, was obtained from Wako Chemicals. 2-(*N*-Morpholino)ethanesulfonic acid (MES) aqueous buffer (0.1 M MES, pH 4.5) was obtained from Teknova.

A model thiol-ene formulation, comprised of PEG-based trithiol and diallyl ether monomers, was used for all experiments. Ethoxylated trimethylolpropane tri(3-mercaptopropionate) (ETTMP 1300, molecular weight (MW) ~1300 g/mol) was donated by Evans Chemetics and used without further purification. Poly(ethylene glycol) diallyl ether (PEGDAE) was synthesized using a modified method from the literature.²³ Briefly, dihydroxylated PEG (MW ~600 g/mol) was dissolved in toluene (~18 g PEG in 400 mL toluene) under nitrogen and excess sodium hydride was added slowly. After the gas evolution ceased, a large excess of allyl bromide was added slowly and the reaction mixture was stirred and heated to 70°C. After 24 hours, the mixture was filtered and the toluene and residual allyl bromide were removed under vacuum from the filtrate. Proton nuclear magnetic resonance (NMR) spectroscopy (Varian MR400, 400 MHz), with DMSO-*d*₆ as solvent, was used to confirm the complete substitution of hydroxyl groups, characterized by the absence of a peak at 4.56 ppm.²⁴ The resulting product was a slightly yellow liquid at room temperature. The monomers and buffer were subjected to at least six freeze-pump-thaw cycles to ensure rigorous deoxygenation prior to use.

Polymerization kinetics

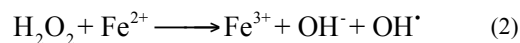
Under anaerobic conditions, the thiol- and ene-functionalized PEG monomers, in a 1:1 thiol:ene stoichiometric ratio, were dissolved in MES buffer to afford a final combined monomer concentration of 35 wt% (15.4 wt% PEGDAE, 19.6 wt% ETTMP) and stabilized by the addition of cupferron (3.2 mM) (see Figure S1), to which stock solutions of the other formulation components (e.g., glucose, GOx, iron(II) sulfate heptahydrate, horseradish peroxidase, and acetylacetone) in MES buffer were added. The pH 4.5 for buffer was selected to ensure slightly acidic conditions for optimum GOx activity²⁵ and to minimize the thiolate concentration, owing to the dissociation of the thiol proton (mercaptopropionate p*K*_a is approximately 10.4),²⁶ ensuring minimal disulfide or thiol oxidation by the generated hydrogen peroxide.²⁷ Glucose solutions were prepared 24 hours prior to use to ensure mutarotation equilibrium,²⁸ while enzyme solutions were used within 48 hours of dissolution. All stock solutions and thiol-ene formulations used were precipitate-free. For oxygen-mediated polymerization experiments, the thiol-ene formulations were removed from the anaerobic chamber, bubbled with air (21% oxygen) for 20 seconds, and immediately pipetted into a glass-bottomed cylindrical sample cell, affording 2.5 mm thick samples, and placed in a Thermo Scientific Nicolet 6700 FTIR spectrometer. The polymerization reaction commenced upon exposure to aerobic conditions, and functional group

conversions were determined in real-time using FTIR spectroscopy²⁹ by monitoring the disappearance of the peak area centered at 6139 cm⁻¹, corresponding to the vinyl-CH stretch.³⁰ Spectra were collected at a rate of approximately three every two seconds. Anaerobic control experiments were similarly performed using FTIR spectroscopy on samples that remained unexposed to oxygen. All experiments were performed in triplicate at ambient temperature and the average and standard error reported.

Results and discussion

Coupled enzymatic-Fe(II)/Fe(III) radical initiation

Aqueous monomer solutions of ETTMP and PEGDAE in MES buffer were formulated with a three-component, enzymatic, radical initiating system, comprised of GOx, glucose, and FeSO₄, exposed to air (ca. 21% oxygen), and the polymerization reaction was monitored by FTIR spectroscopy for 30 minutes. In the presence of oxygen, GOx oxidizes glucose to generate gluconolactone and hydrogen peroxide which is subsequently reduced by Fe²⁺, (i.e., the Fenton reaction) according to the reactions²¹



thus generating hydroxyl radicals capable of initiating the thiol-ene polymerization. As the radical-initiating system is composed of three components (excluding oxygen), studies were performed where the initial concentration of one component was varied while the initial concentrations of the other two were maintained. Previous work has shown that the Fe²⁺-mediated production of hydroxyl radicals from H₂O₂ is significantly enhanced by the presence of physiological thiol-bearing compounds such as glutathione and cysteine,³¹ suggesting a particular suitability for Fenton chemistry to effect rapid polymerization of thiol-ene materials owing to their ubiquitous thiol functional groups.

We first investigated the influence of Fe²⁺ concentration on the thiol-ene hydrogel polymerization by varying the initial concentration of Fe²⁺ from 0 to 720 μM while the initial concentrations of glucose and GOx were maintained at 56 mM and 14.8 kU/L, respectively (see Figure 1). At low Fe²⁺ concentrations (≤ 72 μM), the maximum rate of polymerization and extent of reaction both increased as the Fe²⁺ concentration was raised. This initiating system relies on the Fe²⁺-mediated Fenton reaction to generate hydroxyl radical initiating species from hydrogen peroxide afforded by the action of GOx on glucose in the presence of oxygen. The reaction depicted in Equation 2 suggests a simple relation between the Fe²⁺ concentration and the rate of hydroxyl radical generation; however, the several concurrent interactions between Fe²⁺, Fe³⁺, H₂O₂, and radical species convolute this process. For

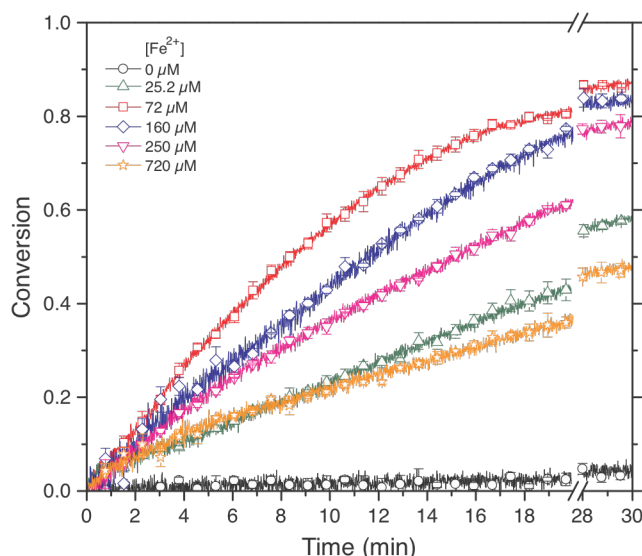
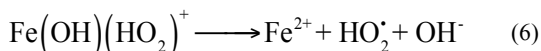
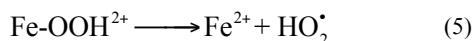
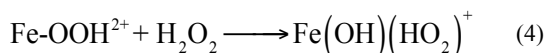
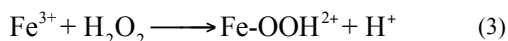
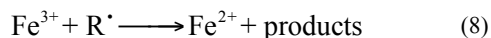
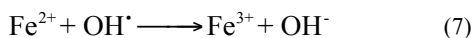


Figure 1. Influence of initial Fe^{2+} concentration. Allyl ether functional group conversions, monitored by FTIR spectroscopy, are shown for aqueous thiol-ene solutions formulated with glucose, GOx, and Fe^{2+} (56 mM, 14.8 kU/L, and concentrations as shown in legend, respectively) upon exposure to air.

example, several other reactions that lead to the generation of potential initiating radical species include^{32,33}



where the Fe^{3+} generated in Equation 2 is ultimately reduced back to Fe^{2+} which is thus catalytically decomposing H_2O_2 . As seen in Figure 1, the extent of polymerization after 30 minutes monotonically decreases at initial Fe^{2+} concentrations in excess of 72 μM . Although this inhibitory effect of Fe^{2+} on radical polymerizations is not readily explained by the reactions described in Equations 2–6, both Fe^{2+} and Fe^{3+} are known to participate in oxidative termination reactions with some radical species,^{21,32} such as



where R^{\cdot} is a propagating radical. Consequently, radicals are rapidly consumed in these competing reactions and are thus unavailable to participate in the hydrogel polymerization, leading to the observed Fe^{2+} -dependent polymerization retardation and ultimately limiting the maximum attainable polymerization rate and extent. The inhibitory effect of raised Fe^{2+} concentrations has previously been observed in the radical-

mediated polymerization of an acrylate-based hydrogel that employed a GOx-glucose- Fe^{2+} radical-generating initiation system at similar Fe^{2+} concentrations.²¹ The inhibition of radical polymerizations by Fe^{2+} and Fe^{3+} has been shown to be monomer dependent where, for example, the polymerization rate of methyl(methacrylate) is unaffected by Fe^{3+} ,³⁴ nevertheless, similar resistance to Fe^{2+} -dependent retardation is clearly not exhibited by the current thiol-ene formulation. Thus, while Fe^{2+} is necessary to induce polymerization, reaction rates do not scale with initial Fe^{2+} concentration. Notably, the polymerization proceeded under aerobic conditions even in the absence of added Fe^{2+} , albeit at an extremely slow rate. (Figure 1, 0 μM Fe^{2+}). As the generation of H_2O_2 does not require Fe^{2+} to proceed and it is stable in acidic media at ambient temperature, we ascribe the small amount of polymerization observed under aerobic conditions at 0 μM Fe^{2+} to the presence of trace amounts of iron impurities in the formulation precursors.

The influence of the initial glucose concentration on the polymerization rate, final conversion, and substrate saturation of GOx was examined by monitoring polymerizations of aqueous thiol-ene solutions formulated with initial Fe^{2+} and GOx concentrations of 72 μM and 14.8 kU/L, respectively, and initial concentrations of glucose varied from 0 to 170 mM upon exposure to oxygen (see Figure 2). As expected, the polymerization rate and final conversion increase monotonically as the glucose concentration is raised at moderate glucose concentrations; however, at 28 mM and above, a zero-order dependence of glucose concentration on the polymerization rate is observed, demonstrating saturation of the GOx by its glucose substrate at these concentrations. At raised glucose concentrations, the allyl ether conversions approach 90% after 30 minutes of air exposure indicating the complete consumption of one of the reagents. The oxidation of glucose

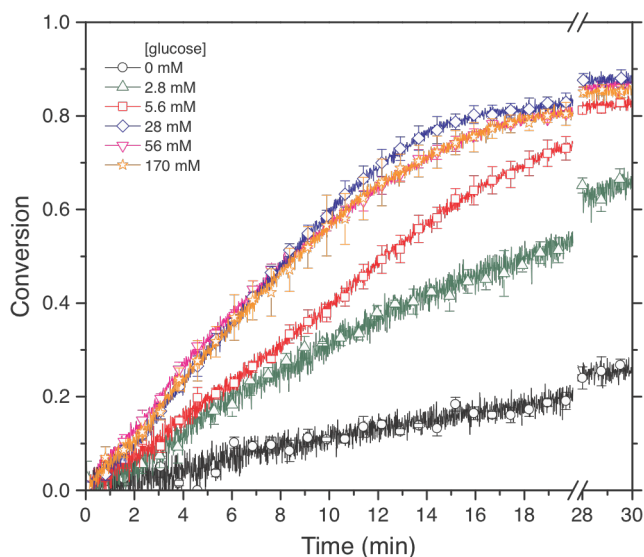


Figure 2. Influence of initial glucose concentration. Allyl ether functional group conversions are shown for aqueous thiol-ene solutions formulated with GOx, Fe^{2+} , and glucose (14.8 kU/L, 72 μM , and concentrations as shown in legend, respectively) upon exposure to air.

by GOx is initially accompanied by the reduction of flavin adenine dinucleotide (FAD) to FADH₂ which is subsequently reoxidized by O₂ to afford H₂O₂.³⁵ As both GOx and Fe²⁺ act catalytically to generate initiating species, the incomplete polymerization suggests complete consumption of either glucose or dissolved oxygen during the reaction. The consistent reaction extents after 30 minutes for glucose concentrations from 28 to 170 mM indicates that, rather than glucose being the limiting reagent, the dissolved oxygen initially introduced by bubbling the formulation with air is depleted by the oxidation of glucose faster than it can be replenished by diffusion from the atmosphere.

As the enzyme GOx is the most expensive of the formulation components in this coupled radical initiation scheme, we varied the GOx concentration to determine the minimum amount that would afford a reasonable polymerization rate and reaction extent. The concentration of GOx in the aqueous thiol-ene solutions was varied from 0 to 14.8 kU/L while maintaining initial glucose and Fe²⁺ concentrations at 56 mM and 72 μM, respectively (see Figure 3), and both the reaction rate and extent increased as the GOx concentration was raised. An important parameter to consider for the clinical utilization of hydrogel sealants is the gelation time. Too short a gel time results in gelation prior to application at the wound site and hence prevents adequate adhesion and sealing; conversely, too long a gel time results in excessively delayed solidification of the sealing material. The theoretical gel point of an ideal step-growth polymerization, described by classical Flory-Stockmayer theory,³⁶ depends upon the average number of functional groups on each monomer and the stoichiometric ratio between functional groups. As the model thiol-ene formulation, used throughout this study was comprised of trithiol and diallyl ether monomers in a 1:1 thiol:ene stoichiometric ratio, the theoretical gel point is calculated as approximately 70% conversion. Thus, the formulations containing 0 and 1.5 kU/L GOx did not gel over the course of 30 minute air exposure, while the 7.4 and 14.8 kU/L GOx formulations gelled after ~15 and ~13.5 minutes (excluding the initial air bubbling period and concomitant polymerization), respectively. Currently, these gel times are not clinically relevant; however, the gel point conversion can be conveniently decreased by increasing the monomer functionality. For example, replacing the PEG diallyl ether for a tetraallylated PEG in the current formulation would decrease the gel point conversion to ~41% corresponding to a gel time of a more useful ~6.5 minutes for the 14.8 kU/L GOx formulation.

Air exposure of a glucose-free, GOx- and Fe²⁺-containing formulation (Figure 2, 0 mM glucose) and a GOx-free, glucose- and Fe²⁺-containing formulation (Figure 3, 0 kU/L GOx) unexpectedly afforded slow polymerization; notably, the polymerization rates of these two formulations were, within the experimental error, identical and significantly faster than the Fe²⁺-free, glucose- and GOx-containing formulation (Figure 1, 0 μM Fe²⁺). Although an aqueous thiol-ene solution formulated with 14.8 kU/L GOx, 56 mM glucose, and 72 μM Fe²⁺ proved stable under anaerobic conditions (see Figure S2),

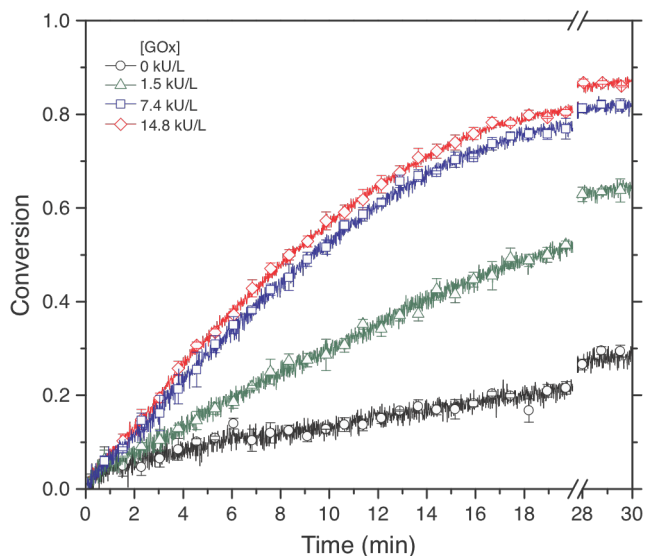
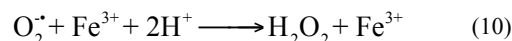
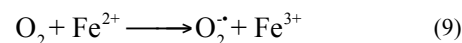
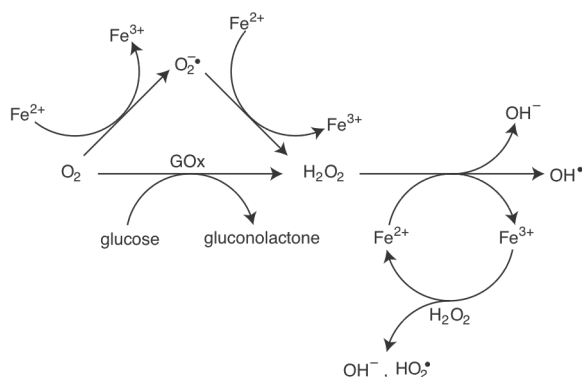


Figure 3. Influence of GOx concentration. Allyl ether functional group conversions are shown for aqueous thiol-ene solutions formulated with glucose, Fe²⁺, and GOx (56 mM, 72 μM, and concentrations as shown in legend, respectively) upon exposure to air.

demonstrating the necessity for oxygen exposure to initiate the polymerization, an aqueous thiol-ene solution containing none of the initiating system components proved similarly stable under aerobic conditions (Figure S1). As GOx exhibits high substrate specificity,²⁵ enzymatic generation of H₂O₂ does not proceed in the absence of glucose. Moreover, there is no mechanism for glucose to generate initiating species by itself upon oxygen exposure at ambient temperature. Generation of radicals necessary for the observed polymerization is thus attributable to direct oxygen reduction by Fe²⁺ according to the reaction equations^{37, 38}



where the generated H₂O₂ subsequently oxidizes Fe²⁺ to Fe³⁺ *via* the Fenton reaction (Equation 2) to yield hydroxyl radical initiating species. Notably, previously described acrylate systems polymerized by GOx-glucose-Fe²⁺ initiating systems did not demonstrate any evidence of polymerization in the absence of glucose.^{20, 21} Given the strong inhibition of radical-mediated chain-growth acrylate polymerizations by oxygen, the low radical generation rate may not have been adequate to sufficiently deplete the oxygen concentration to overcome this inhibition and allow the polymerization to proceed. In contrast, the radicals formed upon air exposure *via* this non-enzymatic mechanism are able to effect polymerization here, albeit at a low polymerization rate, owing to the resistance of the thiol-ene reaction to oxygen inhibition. Nevertheless, although the radicals afforded by this pathway do effect thiol-ene polymerization under aerobic conditions, all components of the combined GOx-glucose-Fe²⁺ system are necessary for useful polymerization rates.



Scheme 2. The generation of hydroxyl radicals upon exposure of the GOx-glucose- Fe^{2+} system to aerobic conditions. The primary hydroxyl radical-generating mechanism is the GOx-mediated reduction of oxygen to hydrogen peroxide that is further reduced to hydroxyl radicals by Fe^{2+} . Alternatively, the reduction of oxygen by Fe^{2+} yields superoxide radicals that are similarly reduced to hydrogen peroxide. The Fe^{3+} generated during these reactions is recycled back to Fe^{2+} via reaction with hydrogen peroxide.

The several redox reactions that lead to hydroxyl radical generation by the GOx-glucose- Fe^{2+} initiating system are summarized in Scheme 2. The introduction of oxygen leads to the generation of hydrogen peroxide both through the GOx-catalyzed oxidation of glucose and accompanying reduction of oxygen, or *via* a non-enzymatic route where oxygen is directly reduced by Fe^{2+} . The *in situ* generated hydrogen peroxide is further reduced by Fe^{2+} to yield hydroxyl radicals, a species capable of initiating the thiol-ene polymerization, and Fe^{3+} which in turn can be recycled back to Fe^{2+} . This GOx-glucose- Fe^{2+} system is deficient as a radical initiating system for the fabrication of thiol-ene hydrogels primarily because raised Fe^{2+} concentrations significantly retard, rather than enhance, thiol-ene polymerization rates; indeed, the 720 μM Fe^{2+} system (Figure 1) did not gel even after exposure to aerobic conditions for thirty minutes. In clinical settings, it may be desirable to perform the polymerization at physiological pH values where the thiolate ion concentration is non-negligible. Under these conditions, the Fe^{3+} generated from Fe^{2+} -mediated H_2O_2 reduction would oxidize thiolate anions to thiyl radicals,^{37, 38} potentially augmenting thiol-ene polymerization rates, and rapidly recovering the Fe^{2+} species which are then available to participate in further H_2O_2 decomposition events. Regardless, given the complexity of $\text{Fe}^{2+}/\text{Fe}^{3+}$ redox chemistry, the development of an approach that enables radical generation from H_2O_2 decomposition, but avoids the utilization of Fe^{2+} and its associated inhibition of radical polymerizations, is attractive.

Horseradish peroxidase radical initiation

Horseradish peroxidase (HRP) is an oxidoreductase capable of generating radicals by catalysing the action of a suitable oxidant, commonly hydrogen peroxide, on a reductant.³⁹ The utility of HRP for the initiation of polymerization reactions is well-documented and has been used to synthesize polymers through the oxidative polymerization of phenols⁴⁰ and the free-radical, chain-growth polymerization of acrylamides⁴¹ and (meth)acrylates.⁴² For example, poly(methyl methacrylate) (PMMA) was synthesized from a formulation of methyl

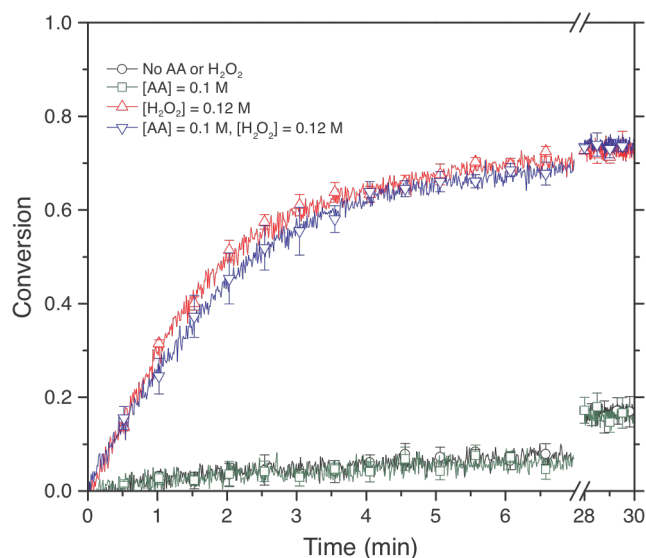


Figure 4. Influence of added oxidant and reductant. Allyl ether functional group conversions are shown for aqueous thiol-ene solutions formulated with HRP, H_2O_2 , and acetylacetone (AA) (261 kU/L and concentrations as shown in legend, respectively) upon exposure to air.

methacrylate, HRP, hydrogen peroxide, and acetylacetone as a substrate for HRP-mediated oxidation.⁴² The polymerization did not proceed in the absence of acetylacetone, indicating that the methacrylate monomer itself was a poor HRP substrate; however, in the presence of acetylacetone, oxidation by HRP generated radical species able to initiate the polymerization. As H_2O_2 was generated in our initial enzymatic polymerization approach by the action of GOx on glucose in the presence of oxygen, we investigated the potential for HRP in conjunction with H_2O_2 to generate radical initiation species to effect thiol-ene polymerization.

Aqueous solutions of our thiol and ene monomers in MES buffer were formulated with HRP and the influences of H_2O_2 and/or acetylacetone, a common reductant substrate utilized with HRP-mediated radical polymerizations, on the polymerization upon atmospheric exposure were examined (Figure 4). In the presence of H_2O_2 , the polymerization proceeds rapidly, attributable to the generation of radicals by HRP-mediated substrate oxidation. Notably, the presence of acetylacetone in the formulation did not influence the polymerization rate or extent. Low molecular weight thiols have previously been shown to act as HRP-mediated oxidation substrates,^{43, 44} demonstrating not only that the thiol component of the polymerizable formulation is acting as the HRP substrate, but that it is saturating the enzyme. Although no HRP-mediated thiol-ene polymerization was observed under anaerobic, H_2O_2 -free conditions (see Figure S3), interestingly, the polymerization of a thiol-ene solution formulated with HRP did proceed slowly upon air exposure, even in the absence of added H_2O_2 (Figure 4, no AA or H_2O_2 , and 0.1 M AA). Under this H_2O_2 -free condition, the addition of acetylacetone again did not influence the reaction rate. HRP has previously been observed to use oxygen as a substrate to oxidize organic reductants, although at far lower efficiency than its utilization of H_2O_2 .⁴⁴

Thus, we attribute the generation of adequate radicals to effect the exogenous H_2O_2 -free thiol-ene polymerization observed in Figure 4 to this HRP-mediated aerobic oxidation of thiols.

Coupled bienzymatic GOx-HRP radical initiation

The H_2O_2 generation observed for the GOx-glucose formulations under aerobic conditions, evidenced by its oxidation of Fe^{2+} and concomitant radical generation (Figure 1), and the successful and rapid thiol-ene hydrogel polymerization by HRP and H_2O_2 , even in the absence of an additional reductant such as AA (Figure 4), suggests that HRP may be a convenient, drop-in replacement for Fe^{2+} in a coupled, oxygen-mediated, thiol-ene radical initiation system. Here, the H_2O_2 generated by the GOx-glucose system upon exposure to oxygen would serve as an oxidant source for the action of HRP on the thiol functional groups to yield hydroxyl and thiyl initiating radicals (Scheme 3). This coupled GOx-glucose-HRP initiating system would not require Fe^{2+} for radical generation and consequently would not suffer from the polymerization retardation resulting from high Fe^{2+} concentrations. Such bienzymatic approaches are not without precedent. For example, Uyama *et al.* used the GOx-glucose system for the *in situ* generation of hydrogen peroxide to effect the oxidative polymerization of phenols by HRP,⁴⁵ circumventing potential HRP deactivation by high H_2O_2 concentrations.^{39, 46} In contrast to our approach where polymerization proceeds upon exposure to atmospheric oxygen, Uyama *et al.* initiated polymerization by the addition of glucose.

We investigated the coupling of these two enzymatic reactions by examining the influence of the HRP concentration on the polymerization rate and final conversion of aqueous thiol-ene solutions formulated with initial glucose and GOx concentrations of 56 mM and 14.8 kU/L, respectively, and HRP concentrations varied from 26.1 to 261 kU/L upon exposure to

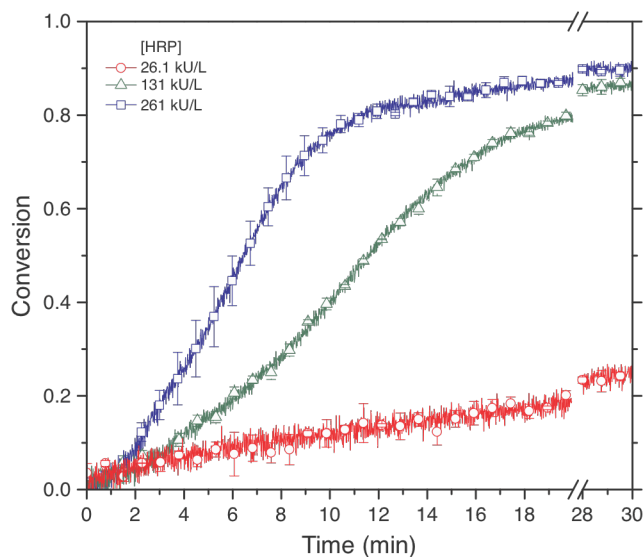
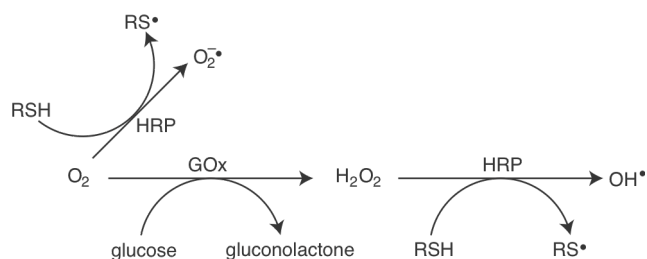


Figure 5. Influence of HRP concentration on a coupled GOx-HRP thiol-ene polymerization initiation. Allyl ether functional group conversions are shown for aqueous thiol-ene solutions formulated with glucose, GOx, and HRP (56 mM, 14.8 kU/L and concentrations as shown in legend, respectively) upon exposure to air.



Scheme 3. The generation of radicals upon exposure of the GOx-HRP system to aerobic conditions. The radical species generated by this coupled bienzymatic system are capable of initiating thiol-ene polymerization. The primary radical generation mechanism proceeds *via* the GOx-mediated oxidation of glucose to yield H_2O_2 , from which thiyl and hydroxyl radicals are generated by the action of HRP on a substrate thiol. The direct, HRP-mediated oxidation of thiols by oxygen is a minor radical generation contributor.

oxygen (see Figure 5). As expected, no polymerization was noted when the formulation was kept under anaerobic conditions; however, upon oxygen exposure, polymerization successfully proceeded at rates that monotonically increased with raised HRP concentration (see Figure 5). Importantly, the polymerization rate at 261 kU/L HRP exceeded that of the fastest GOx-glucose- Fe^{2+} system, reducing the gel time from ~13.5 minutes for the Fe^{2+} -based initiating system to ~8.5 minutes (again excluding the air bubbling period) when HRP was employed. Moreover, this gel time could be further reduced, as required for clinical utility, either by increasing the monomer functionality or by further raising the HRP concentration. Finally, the addition of acetylacetone again did not afford any significant polymerization rate or extent variation (Figure S4), demonstrating the HRP saturation by thiol and hence ineffective incorporation of additional reductant species.

Conclusions

Aqueous solutions of multi-functional thiol and allyl ether monomers were polymerized into solid hydrogels through *in situ* enzymatic generation of radical initiating species. The first enzymatic system uses GOx, in the presence of glucose and oxygen, to generate hydrogen peroxide that, upon reduction by Fe^{2+} , forms hydroxyl radicals capable of initiating thiol-ene polymerization. In addition to this enzyme-mediated process, a non-enzymatic route involving the direct reduction of oxygen to hydroxyl ions by Fe^{2+} is also capable of initiating thiol-ene polymerization. While this second route is a minor contributor compared to the GOx-mediated mechanism, it is of interest as this process is seemingly unique to thiol-ene chemistry and is attributed to the resistance of the radical thiol-ene addition reaction to oxygen inhibition. However, although the ternary system of GOx, glucose, and Fe^{2+} proved capable of initiating polymerization of thiol-ene-based formulations, the presence of Fe^{2+} limits polymerization rates through several reactions that consume radicals. Consequently, increasing the concentration of Fe^{2+} beyond 72 μM lead to decreased polymerization conversions where the monomer formulations did not gel even after thirty minutes air exposure. As an alternative to the

utilization of Fe^{2+} , HRP was used as to catalyse the production of radicals from GOx-generated H_2O_2 . Interestingly, the thiol–ene system did not require the incorporation of an additional reductant for polymerization to proceed, indicating that the thiol monomer is capable of being oxidized directly by HRP, using either oxygen or hydrogen peroxide as oxidant, to form initiating radical species. After successful demonstration of HRP to mediate thiol–ene polymerization, HRP was used in combination with GOx and glucose to afford an alternative three-component initiating system that only generates radicals upon oxygen exposure. The GOx-glucose-HRP system was not subject to the polymerization retardation caused by high Fe^{2+} concentrations and yielded polymerization rates that exceeded those of the GOx-glucose- Fe^{2+} system.

Acknowledgements

The authors gratefully acknowledge funding from the National Institutes of Health (NIH R21, award number R21 EB011761) and from the National Aeronautics and Space Administration (NASA Space Technology Research Fellowship, award number NNX12AM31H).

Notes and references

^a *Macromolecular Science and Engineering Program, University of Michigan, Ann Arbor, MI 48109-2136, USA*

^b *Department of Materials Science and Engineering, University of Michigan, Ann Arbor, MI 48109-2136, USA.*

^c *Department of Chemical Engineering, University of Michigan, Ann Arbor, MI 48109-2136, USA. E-mail: tfscott@umich.edu*

Electronic Supplementary Information (ESI) available:

Figure S1. Stability of the thiol–ene solution in 0.1 M MES buffer.

Figure S2. Stability of the thiol–ene solution formulated with the GOx-glucose- Fe^{2+} radical initiating system.

Figure S3. Stability of the thiol–ene solution formulated with HRP.

Figure S4. Influence of acetylacetone on a coupled GOx-HRP initiated thiol–ene polymerization.

See DOI: 10.1039/b000000x/

- 1 A. Peutzfeldt, *Eur. J. Oral. Sci.*, 1997, **105**, 97-116.
- 2 N. Moszner and U. Salz, *Prog. Polym. Sci.*, 2001, **26**, 535-576.
- 3 A. Lauto, D. Mawad and L. J. R. Foster, *J. Chem. Technol. Biotechnol.*, 2008, **83**, 464-472.
- 4 S. Thennarasu, N. Krishnamurti and K. L. Shantha, *J. Adhes. Sci. Technol.*, 1989, **3**, 237-260.
- 5 W. D. Spotnitz and S. Burks, *Transfusion*, 2008, **48**, 1502-1516.
- 6 W. D. Spotnitz and S. Burks, *Clin. Appl. Thromb. Hemost.*, 2010, **16**, 497-514.
- 7 J. C. Wheat and J. S. Wolf, Jr., *Urol. Clin. North Am.*, 2009, **36**, 265-275.
- 8 J. E. Bavaria, D. R. Brinster, R. C. Gorman, Y. J. Woo, T. Gleason and A. Pochettino, *Ann. Thorac. Surg.*, 2002, **74**, S1848-1852; discussion S1857-1863.
- 9 D. H. Sierra, *J. Biomater. Appl.*, 1993, **7**, 309-352.
- 10 F. H. Silver, M. C. Wang and G. D. Pins, *Biomaterials*, 1995, **16**, 891-903.
- 11 J. R. Eriksen, J. I. Bech, D. Linnemann and J. Rosenberg, *Hernia*, 2008, **12**, 483-491.
- 12 C. E. Brubaker, H. Kissler, L. J. Wang, D. B. Kaufman and P. B. Messersmith, *Biomaterials*, 2010, **31**, 420-427.
- 13 J. S. Choi and H. S. Yoo, *J. Biomed. Mater. Res. A*, 2010, **95**, 564-573.
- 14 B. J. Vote and M. J. Elder, *Clin. Experiment Ophthalmol.*, 2000, **28**, 437-442.
- 15 M. Radosevich, H. A. Goubran and T. Burnouf, *Vox Sang.*, 1997, **72**, 133-143.
- 16 P. A. Leggat, U. Kedjarune and D. R. Smith, *Ind. Health*, 2004, **42**, 207-211.
- 17 R. A. Chivers and R. G. Wolowacz, *Int. J. Adhes. Adhes.*, 1997, **17**, 127-132.
- 18 A. Kowalewska, *J. Mater. Chem.*, 2005, **15**, 4997-5006.
- 19 H. Ni, A. D. Skaja and M. D. Soucek, *Prog. Org. Coat.*, 2000, **40**, 175-184.
- 20 H. Iwata, Y. Hata, T. Matsuda and Y. Ikada, *J. Polym. Sci. Pol. Chem.*, 1991, **29**, 1217-1218.
- 21 L. M. Johnson, B. D. Fairbanks, K. S. Anseth and C. N. Bowman, *Biomacromolecules*, 2009, **10**, 3114-3121.
- 22 C. E. Hoyle and C. N. Bowman, *Angew. Chem. Int. Ed.*, 2010, **49**, 1540-1573.
- 23 T. F. Scott, C. J. Kloxin, R. B. Draughon and C. N. Bowman, *Macromolecules*, 2008, **41**, 2987-2989.
- 24 J. M. Dust, Z. H. Fang and J. M. Harris, *Macromolecules*, 1990, **23**, 3742-3746.
- 25 S. B. Bankar, M. V. Bule, R. S. Singhal and L. Ananthanarayan, *Biotechnol. Adv.*, 2009, **27**, 489-501.
- 26 C. F. Bernasconi and R. B. Killion, *J. Am. Chem. Soc.*, 1988, **110**, 7506-7512.
- 27 C. C. Winterbourn and D. Metodiewa, *Free Radical Biol. Med.*, 1999, **27**, 322-328.
- 28 N. Le Barc'H, J. M. Gossel, P. Looten and M. Mathlouthi, *Food Chem.*, 2001, **74**, 119-124.
- 29 N. B. Cramer and C. N. Bowman, *J. Polym. Sci. Pol. Chem.*, 2001, **39**, 3311-3319.
- 30 N. B. Cramer, C. L. Couch, K. M. Schreck, J. A. Carioscia, J. E. Boulden, J. W. Stansbury and C. N. Bowman, *Dent. Mater.*, 2010, **26**, 21-28.
- 31 A. J. Nappi and E. Vass, *BBA-Gen. Subjects*, 1997, **1336**, 295-302.
- 32 E. M. Siedlecka, A. Wieckowska and P. Stepnowski, *J. Hazard. Mater.*, 2007, **147**, 497-502.
- 33 C. Walling and A. Goosen, *J. Am. Chem. Soc.*, 1973, **95**, 2987-2991.
- 34 F. S. Dainton and P. H. Seaman, *J. Polym. Sci.*, 1959, **39**, 279-297.
- 35 S. Witt, G. Wohlfahrt, D. Schomburg, H. J. Hecht and H. M. Kalisz, *Biochem. J.*, 2000, **347**, 553-559.
- 36 C. W. Macosko and D. R. Miller, *Macromolecules*, 1976, **9**, 199-206.
- 37 R. Munday, *Free Radical Biol. Med.*, 1989, **7**, 659-673.
- 38 S. Wadhwa and R. J. Mumper, *Cancer Lett.*, 2013, **337**, 8-21.
- 39 F. Hollmann and I. W. C. E. Arends, *Polymers*, 2012, **4**, 759-793.
- 40 J. S. Dordick, M. A. Marletta and A. M. Klivanov, *Biotechnol. Bioeng.*, 1987, **30**, 31-36.
- 41 Z. Q. Cai, W. C. Wang, G. Ruan and X. F. Wen, *Int. J. Chem. Kinet.*, 2012, **44**, 475-481.
- 42 B. Kalra and R. A. Gross, *Biomacromolecules*, 2000, **1**, 501-505.
- 43 H. Pichorner, A. Couperus, S. A. A. Korori and R. Ebermann, *Phytochemistry*, 1992, **31**, 3371-3376.

Journal Name

- 44 U. Burner and C. Obinger, *Febs Lett.*, 1997, **411**, 269-274.
- 45 H. Uyama, H. Kurioka and S. Kobayashi, *Polym. J.*, 1997, **29**, 190-192.
- 46 R. Taboada-Puig, C. Junghanns, P. Demarche, M. T. Moreira, G. Feijoo, J. M. Lema and S. N. Agathos, *Bioresource. Technol.*, 2011, **102**, 6593-6599.