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Polyacrylamide Hydrogels Produce Hydrogen Peroxide from Osmotic Swelling in Aqueous Media

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Abstract

This work demonstrates that hydrogen peroxide (H₂O₂) is generated in weak polyacrylamide hydrogels due to mechanochemical reactions to osmotic swelling. Hydrogels are important tools and materials for many biomedical applications, particularly for growth of stem cells. However, swollen gels are under constant tension, which makes their individual chains susceptible to mechanochemical bond breakage. In this work, an assay was developed to measure the generation of H₂O₂ as a result of hydrogel swelling. Polyacrylamide hydrogels with both weak disulfide and strong PEG-diacrylate crosslinkers were synthesized and swelled. H₂O₂ generation increased in the presence of weaker crosslinkers, up to 30 μ M H₂O₂, whereas stronger crosslinkers reduced this to 5 μ M H₂O₂. H₂O₂ levels decreased when swelled in the presence of dextran to reduce osmotic stress or increased if the gels were conjugated to an acrylated surface. Finally, H₂O₂ continued to form for days after the gels had reached their equilibrium sizes, independently of dissolved oxygen. The results of this work impact those working in the 3D cell culture community and demonstrate that even well-characterized systems undergo mechanochemical processes in mild environments.

Graphical abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information

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Calibration curves, resorufin diffusion test, comparison to elastic modulus, dextran controls, additional constrained swelling tests, and precompression tests (PDF).

The authors declare no competing financial interest.

INTRODUCTION

Hydrogels are elastic polymer networks that contain a large water fraction. Due to their general biocompatibility, hydrophilicity, and tunable properties, hydrogels are ubiquitous in applications such as tissue engineering, biomedical implants, and injectable materials; examples include contact lenses,^{1–6} wound dressings,^{7–12} drug delivery vehicles,^{4,13–16} and others. In addition, hydrogels have been utilized extensively as 3D scaffolds for tissue engineering because of how cell growth in hydrogels mimics morphologies and phenotypes found in living systems. These advances have been vital for controlling stem cell differentiation into specific phenotypes, generally through careful design of hydrogel primary structure, polymerization chemistry, culture apparatus, and growth conditions.^{17–19} In particular, control over levels and types of reactive oxygen species (ROS) are vital for not only maintaining cell health, but also obtaining a desired phenotype.^{20–24} Hydrogen peroxide, in particular, is an important molecule for cell signaling for its ability to modulate thiol redox chemistry and resulting protein structure.^{25–27}

While considerable effort has been devoted to the design and control of hydrogel properties, there are inherent limitations to hydrogel structure that may affect cell culture and other biological applications. For example, hydrogels are usually synthesized in a concentrated state, then exposed to aqueous media to wash away contaminants, establish an equilibrium with growth media, or simply replenish nutrients for cell growth. However, after a hydrogel reaches its swollen state up to several times its initial volume, the polymer network experiences the osmotic stress of polymer chains exposed to a miscible solvent but prevented from dissolving into the medium. Each chain is thus under a constant mechanical tension, as described by the Flory–Rehner equation.^{28,29} This effect has been elegantly demonstrated by utilizing anisotropic swelling constraints to generate wrinkles or creases.^{30–33}

If mechanical tension is applied to macromolecules, the individual chains may start to break in response to the applied tension.³⁴ While small numbers of bond breakages should not affect bulk mechanical properties, covalent bond breakage in aqueous media has been shown to produce ROS. For example, Grzybowski et al. demonstrated that manually compressing cylindrical hydrogels of PDMS caused the release of enough hydrogen peroxide to initiate

chemical reactions.³⁵ Tavazzi and co-workers designed a silicone contact lens that could release a small amount of hydrogen peroxide under mechanical pressure as low as could be produced by an eyelid.⁶ Similarly, our group showed that small amounts of compressive force could produce hydroxyl radicals and hypochlorite ions that could activate masked fluorophores.³⁶ ROS generation occurred even without notable signs of gel damage, such as void formation or a change in modulus. In terms of monitoring such bond tension, most mechanophores capable of localizing strain or damage have been evaluated in tougher elastomers such as crosslinked poly(methyl methacrylate)^{37–44} or multiple network hydrogels.⁴⁵ However, hydrogels, particularly when simulating extracellular matrix, are generally mechanically weak, with typical shear moduli <40 kPa,⁴⁶ so it is possible that they could be subject to bond breakage within the gel.

Here, we show that even the simple act of swelling a polyacrylamide hydrogel in buffer results in the generation of quantifiable, micromolar levels of hydrogen peroxide. To measure this response, we developed an enzymatic biochemical assay that could quantify small amounts of H₂O₂ in hydrogels using a visible, chromogenic readout. Inspired by common enzyme-linked assays, horseradish peroxidase (HRP) was utilized to convert Amplex Ultrared (AUR, ThermoFisher) to pink-colored and fluorescent resorufin in the presence of hydrogen peroxide (Figure 1).⁴⁷ This assay was utilized to show that hydrogen peroxide levels increase in polyacrylamide hydrogels over several days. H₂O₂ generation was found to be higher when weaker disulfide crosslinkers were used instead of stronger, PEG-based crosslinkers. However, increasing tension by covalently attaching gels to their glass molds essentially eliminated this difference. This work shows that hydrogel structure by itself is responsible for ROS generation, and these effects should be considered when performing 3D cell culture.

EXPERIMENTAL SECTION

General Procedures and Materials.

Acrylamide (AAM) and *N,N'*-bis(acryloyl)cystamine (BAC) were purchased from Alfa Aesar. Tetramethylethylenediamine (TEMED) and Amplex UltraRed reagent (AUR) were purchased from ThermoFisher Scientific. Peroxidase from horseradish (HRP), 3-(trimethoxysilyl) propyl methacrylate, dextran from *Leuconostoc* spp ($M_r \sim 100000$), and anhydrous (99.9%) dimethyl sulfoxide were purchased from Sigma-Aldrich. Ammonium persulfate (APS) was purchased from CalabrioChem Inc. Polyethylene(glycol) diacrylate (PEGDA; $M_w = 400$) was purchased from PolySciences Inc. Ethanol (EtOH) was purchased from Decon Laboratories Inc. Glacial acetic acid was purchased from Macron Fine Chemicals. Water (dd-H₂O) was ionized using Milli-Q Advantage A-10 water purification system (Millipore, U.S.A.). Microscopic glass slides (3" × 1" × 1.2 mm) and micro cover glass (18 mm × 18 mm) were purchased from VWR. Rain-X water repellent manufactured by ITW (Global Bands, TX) was purchased from Home Depot. All UV–vis absorbance spectra were acquired on a Safire2 UV–vis microplate reader (TECAN, Switzerland). The absorbance for the samples were measured at 568 nm in a Nunclon 96-clear Microwell plate. Mechanical characterization tests were performed on a TA Instruments RSA-G2 Solids Analyzer.

Gel Synthesis.

Prior to preparing the gel solution, glass slides were coated with Rain-X by spraying it onto the slide and wiping the excess solution. This was repeated twice to ensure proper coating. Stock solutions of BAC (5 mg/mL), TEMED (0.1 M) were prepared and used for each experiment. A 10 mg/mL stock of APS was prepared fresh before every experiment to avoid redox degradation.

A general procedure for gelation is as follows, adjusting where appropriate for loadings given in the text. AAm was dissolved in Millipore water in a 1.5 mL centrifugation tube. The crosslinker (depending on the experiment) was added to the solution and vortexed for 30 s. Then, APS and TEMED were added in succession and vortexed for 30 s. A total of 162 μL of the gel solution was pipetted onto the glass slide and a coverslip was placed over it using a tweezer. The slips were placed gently to avoid trapping air bubbles. The solutions were left to gel for 2.5 h at room temperature.

Mechanical Characterization of Hydrogels.

Static uniaxial tensile tests were performed on a rectangular specimen of the hydrogel samples. Gels were allowed to swell in 3 mL phosphate buffer solution and taken out at the reported time points. Rectangular specimens of fixed dimensions were sliced using a razor blade and loaded onto the machine. The samples were strained up to 10% of the original length at a strain rate of 0.01 mm/s. At the end of each experiment, the modulus was calculated from the stress–strain plot derived from the machine-generated data.

Hydrogen Peroxide Assay.

A general swelling solution was prepared by mixing 1.5 mL of 0.05 mg/mL HRP (in PBS) with 1.5 mL of 0.1 mM AUR (in PBS) to make final concentrations of 0.025 mg/mL HRP with 0.05 mM AUR. For dextran experiments, the same amounts were used, with 1.25% (w/v) 100 kDa dextran added. The 3 mL solution was added to each of the wells in a 6-well plate. For each experiment, a gel was added to each of 5 wells, while one well was left without a gel as a control sample. The 6-well plate was agitated using a platform shaker at room temperature between measurements under foil to keep dark.

For measurements, 100 μL of PBS was pipetted into each of the border wells of a 96-well plate as a thermal barrier. Next, a set of wells was devoted to a calibration curve containing 0.025 mg/mL HRP and 0.05 mM AUR and a series of H_2O_2 concentrations (0, 0.3, 1.0, 3.0, 5.0 μM). At each time point indicated in the text, 100 μL of each of the gel-swelling solution and control solution in the 6-well plate was transferred to an empty well in the 96-well plate. The absorbance was measured in the plate reader at 568 nm. Absorbance values were converted to H_2O_2 concentrations using the calibration curves in the 96-well plate.

Coverslip Functionalization.

The 18×18 mm coverslips were first treated with UV/ozone for 1 h. The coverslips were then placed in polypropylene coverslip holders. Next, 90 mL of 95% ethanol was added to a polypropylene beaker with a magnetic stir bar. Five drops of glacial acetic acid were added to reach a pH of 4.5–5, and the mixture was stirred for 5 min, after which 0.5 mL of 3-

(trimethoxysilyl)propyl methacrylate was added and stirred for another 5 min. Stirring was then turned off, and the coverslip holder was wholly immersed into the mixture for 1 h. The holder was then removed and rinsed with 70% ethanol, DI water, and isopropyl alcohol. The coverslips were dried under a gentle airstream.

RESULTS AND DISCUSSION

In order to quantify small levels of H_2O_2 using techniques compatible with hydrogels, a colorimetric assay was developed. The inspiration for this assay was the common enzyme-linked immunosorbent assay (ELISA), which often utilizes a HRP-linked antibody to quantify a biomarker via comparison with a simultaneously run calibration curve; in this case, the HRP was used to quantify H_2O_2 rather than the reverse. Polyacrylamide was chosen as the test hydrogel because of its simple synthesis, ubiquity, and the ability to switch crosslinkers. Finally, a thin, square hydrogel geometry was chosen both to simplify the application of osmotic stress and to eliminate any lateral strain due to gravity during the curing process. In a typical procedure, acrylamide (AAM), one or more crosslinkers (BAC and PEGDA, as indicated below), APS, and TEMED were mixed in a tube. The mixture was placed on a glass slide treated with Rain-X, which prevented adhesion by the gel during the polymerization. A cover glass was placed on top, which caused flattening of the aqueous mixture, and the mixture was allowed to cure for 2.5 h in the dark at room temperature. When Rain-X was applied to the glass, the gels detached easily from their mold and showed little topological damage (Figure 1d).

Initial studies were run with gels containing a weak crosslinker, which was most likely to produce mechanically derived ROS.³⁶ AAM was copolymerized with 0.01 mol % bis(acryl)cystamine (BAC), which contains a disulfide. Each gel was placed in 50 mM PBS containing both HRP and Amplex Ultrared (AUR). Gels typically swelled to 1.3–5.5 $\times$ their original mass when subjected to these conditions, depending on the formulation. Both the gel and the solution became noticeably pink up through 90 h swelling (Figure 1). By comparing the UV–vis absorbance at 568 nm to a simultaneous H_2O_2 /AUR/HRP calibration curve (Figure S1), it was found that, after 90 h, hydrogels crosslinked with 0.01 and 0.05 mol % BAC produced $11.58 \mu\text{M} \pm 3.53 \mu\text{M}$ and $12.8 \mu\text{M} \pm 4.7 \mu\text{M}$ H_2O_2 , respectively, in the swelling solution. These values corresponded to 34.7 and 38.4 nmol of hydrogen peroxide generated. Greater addition of BAC crosslinker produced more H_2O_2 : 0.1% BAC and 0.5% BAC produced $28.45 \pm 5.66 \mu\text{M}$ and $25.38 \pm 6.85 \mu\text{M}$, respectively (Figure 2). We attribute the increased H_2O_2 production to increased amounts of disulfide crosslinker and its weak covalent bond.⁴⁸ Despite the apparent bond breakage, however, the gel did not appear to show damage or defects except for some wrinkling. Without a gel present, only a background of $2 \mu\text{M}$ H_2O_2 formed, which was likely due to ambient reactions of dissolved oxygen in the aqueous medium.⁴⁹ In addition, the exchange of resorufin from the gel to the swelling solution reached equilibrium within 10 min, indicating that diffusional effects were not significant (Figure S2).

In order to test the effect of crosslinker strength, the BAC-cross-linker gels were compared to polyacrylamide hydrogels crosslinked with poly(ethylene glycol) diacrylate (PEGDA, M_w = 400). PEGDA only contains C–C and C–O bonds, which have dissociation energies of 83

and 85 kcal mol⁻¹, respectively, as compared to ~60 kcal mol⁻¹ for disulfide.⁴⁸ For these gels, the opposite trend was shown, in which increasing PEGDA loading decreased H₂O₂ generation. This result shows that stronger crosslinkers such as PEGDA are better able to withstand the osmotic stress on the hydrogel, leading to lower levels of H₂O₂. Finally, in order to control for mechanical properties of the gel, a series of gels was prepared in which the weak crosslinker (BAC) was coinorporated with the stronger crosslinker (PEGDA). Crosslinker loading was held at 0.5 mol % while varying the ratio of BAC to PEGDA (Figure 3). As the amount of BAC increased from 0 to 0.10 mol %, the production of H₂O₂ increased from 5.5 to 11 μ M through 90 h of incubation. In addition, the smaller error bars indicate that mixed crosslinkers yielded hydrogels with a more consistent mechanochemical response. We attribute this effect to the BAC crosslinker acting as a sacrificial crosslinker prior to PEGDA breakage, which allows the local gel mechanical properties to be preserved even as the BAC crosslinkers break. In addition, there appeared to be no correlation between gel elastic modulus and hydrogen peroxide generation, so clearly the effect was based on the local bond strength of each of the crosslinkers (Figure S3).

Next, we sought to understand the effect of mechanical strain on hydrogen peroxide generation. First, osmotic tension was decreased by incubating these same gels with a 1.25 wt % solution of 100 kDa dextran.^{50–52} As a result, while dextran itself did not appear to affect the background signal, reducing osmotic pressure resulted in decreased levels of H₂O₂, underscoring the effect of mechanical tension on hydrogen peroxide production (Figure S4). To increase tension within the polymer chains, constraints were applied to the hydrogel. The same gel formulations were prepared, but one of the coverslips was functionalized with acrylate groups (see Experimental Section). Upon swelling, these gels could not detach from the acrylated substrate, creating wrinkle patterns on the top surface.³³ For gels with the weaker BAC crosslinks, the difference in H₂O₂ generation for bound and unbound gels was insignificant (Figure S5). However, the increase in H₂O₂ generation was significant for gels containing the stronger PEGDA crosslinker, to the point that crosslinker identity became insignificant for H₂O₂ generation (Figure 4). Thus, the effect of confined swelling appeared to affect stronger gels more strongly than weaker gels, possibly due to the increased strain in the bound gels being able to overcome the stronger C–C and C–O bonds in the PEGDA gels. This strain can be observed visually through the generation of wrinkles in the gel topography (Figure S5). Gel swelling alone proved to be sufficient to generate hydrogen peroxide, likely because swelling affects all the bonds in the network while tearing and crushing mainly affects the bonds near the tearing interface.^{39,45} For example, if the gel were compressed prior to swelling, similar absorbances were measured (Figure S6).

We hypothesized that mechanical tension in the polymer chains was the primary driving force for bond breakage. Thus, we would expect H₂O₂ to be produced after swelling equilibrium had been achieved. To test this hypothesis, at various time points both the mass of the gel and the absorbance of the swelling solution were recorded. As seen in Figure 5, each hydrogel reached its equilibrium mass within 4 h. However, the majority of the H₂O₂ was produced from 8–90 h. These results indicate that the bond breakages occur after the chains reach their equilibrium extension. Tensile tests showed a corresponding decrease in elastic modulus over the course of the same time period, despite the overall appearance of gels maintaining their structure (Figure S7). Finally, studies were performed to address the

mechanism of these mechanochemical reactions in the hydrogels. In previous work, we had posited that bond breakage leads to the generation of radicals, which reacts with ambient water to produce ROS such as hydroxyl radicals³⁶ or hydrogen peroxide.^{6,35} However, radicals can also react with dissolved oxygen gas, which is known as an inhibitor for free radical polymerization.⁵³ Surprisingly, however, similar levels of resorufin were obtained whether the assay was run in degassed or air-saturated buffer (Figure S8). Thus, while dissolved oxygen is likely to produce small levels of background H₂O₂, it is not necessary for hydrogen peroxide formation by strained gels. Unfortunately, this also means that the hydrogen peroxide will continue to be generated, even after the initial buffer is exchanged, thus, requiring continuous washing to ensure low levels of accumulation.

CONCLUSIONS

This work shows that even the act of exposing a weak polyacrylamide hydrogel to osmotic swelling generates hydrogen peroxide by mechanochemical bond breakage. First, an assay was developed to monitor H₂O₂ levels based on the peroxidase-mediated conversion of colorless Amplex Ultrared to pink resorufin. Next, the effect of osmotic swelling on hydrogel structure was determined. When weaker, disulfide-containing crosslinkers were used, >10 μ M H₂O₂ was produced. Addition of stronger, PEG-based crosslinkers reduced but did not eliminate H₂O₂ generation. However, constrained swelling of hydrogels with PEG-based crosslinkers increased H₂O₂ levels. Finally, this effect was found to be generated mostly after the gel reached its full extension and did not depend on dissolved oxygen. This work has considerable implications for those utilizing hydrogels for cell culture, particularly with stem cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Schematic of bond breakage in hydrogels under mechanical tension. As gels swell their chains extend, applying mechanical tension to short crosslinkers. (b) The mechanochemical breakage of thiols results in abstraction of a hydrogen radical from ambient water, producing hydrogen peroxide. The hydrogen peroxide converts colorless Amplex Ultrared (AUR) to pink resorufin. (c) Hydrogel components utilized in this study: polyacrylamide (AAm), N,N' -bis(acryloyl)cystamine (BAC), and polyethylene(glycol) diacrylate (PEGDA, $M_w = 400$). (d) Photographs of process. The polyacrylamide hydrogel is prepared as a thin square, then placed in buffer containing AUR and HRP. The resorufin equilibrates with the external fluid, which is sampled and its absorbance measured.

Figure 2.

Hydrogen peroxide concentration measured from solution after (left to right) 2, 24, and 90 h swelling for each gel formulation indicated. H/A = HRP and AUR only, B = bis(acryl)cystamine (BAC) crosslinker, P = poly(ethylene glycol) diacrylate (PEGDA) crosslinker. (a) Gels with BAC crosslinker. (b) Gels containing PEGDA crosslinker.

Figure 3.

Hydrogen peroxide concentration measured from solution after (left to right) 2, 24, and 90 h swelling for each gel formulation indicated, where [X–Y] = [%BAC–%PEGDA]. H/A = HRP and AUR only.

Figure 4.

Hydrogen peroxide concentration measured from solution after 90 h swelling. Gels are paired by nonbinding mold (left) and acrylate-functionalized mold (right, with A suffix), where each bar represents a two-tailed *t* test and * *p* < 0.05. Key: H/A = HRP and AUR only, B = bis(acryl)cystamine crosslinker, P = poly(ethylene glycol) diacrylate crosslinker, [X–Y] = [%BAC–%PEGDA].

Figure 5. Measured hydrogen peroxide concentration (left axis) and gel swelling ratio (by mass, relative to preswell mass, right axis) for various gel formulations as a function of time: (a) 0.1% PEGDA; (b) 0.5% PEGDA; (c) 0.01% BAC–0.49% PEGDA; (d) 0.05% BAC–0.45% PEGDA. Error bars represent standard deviation from three replicates.