

A Melt Manufacturing Route for Biocatalytic Thermoset Hydrogels

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Abstract – Hydrogels are a staple of polymeric research, with a wide array of applications ranging from bioelectronics, biosensors, drug delivery, separations, and many others. A hydrogel can be envisioned as a swollen polymer matrix, usually containing large amounts of water, making them useful for applications involving biological material, such as cells and enzymes. Traditionally, these materials are manufactured from solution-based crosslinking or 3D-printing, making them difficult to produce at scale. Herein, we demonstrate a technique capable of scalable production of poly(ethylene oxide) hydrogels using melt extrusion followed by photo-initiated crosslinking in the melt state. Additionally, we demonstrate control of the hydrogel network parameters, allowing for entrapment of active agents, (carbonic anhydrase enzyme), producing functional hydrogel films.

1. Introduction –

Hydrogels are a burgeoning area of polymer research in which crosslinked polymer matrices are swollen with water to yield a highly-hydrated material with a continuous liquid phase. Such an environment is favorable for supporting biological matter, such as cells and enzymes, making hydrogels a staple in drug-delivery, biosensing, and bioseparations (Rodrigo-Navorro 2021, Altin-Yavuzarslan 2024). The functionality of these materials is often derived from immobilized or entrapped enzymes that catalyze reactions with biotechnological importance (Meyer Meyer 2022, Teixeira 2012, Tan Bilal 2021). Carbonic anhydrase, CA, is one such enzyme that can quickly convert aqueous CO_2 and HCO_3^- , opening pathways for carbon capture, utilization, and storage, (Dordick 2022). There have been several implementations of CA immobilization for CO_2

separation in recent years. Fu et al. developed ultra-thin liquid membranes consisting of nanometer-sized channels containing immobilized CA, (Fu Jiang Brinker 2018). Additionally, Zhang et al and Shen et al. 3D-printed poly(ethylene oxide), PEO, hydrogels containing CA to create hydrogel sorbents, (Salmon group). In all cases, CO₂ separation performance was found to increase with incorporation of CA.

These applications, and others, demonstrate the value of designing biocatalytic materials to address industrial problems. However, it is challenging to manufacture biocomposite hydrogels on a scale that matches the scope of these ~~problems~~ challenges. For instance, traditional polymer membranes used for CO₂ separations are manufactured via fiber spinning techniques which can achieve 50 meters per min or higher. (Koros Clausi 2000). By contrast, biocomposite hydrogels are usually fabricated through low throughput 3D-printing or batch mode casting, require expensive cross-linking reagents, and cannot be made continuously to meet the demands of large scale problems like carbon capture.

Producing hydrogels via melt extrusion and post-extrusion crosslinking may be a way to improve the scalability of manufacturing biocatalytic materials. Melt extrusion is a scalable polymer processing technique used to make products like plastic bags, pipes, and various polymer coatings. During this process, polymer powders or pellets are continuously fed upstream of a heated screw-based extruder where the polymers are heated and sheared to produce a melt. The melt is then conveyed by rotating screws towards the exit, where it is pushed through an exit die to form its shape. There has been growing interest in developing protein composite materials using melt extrusion (Zheng Pokorski 2021). Combining high throughput manufacturing and high product encapsulation would allow for scalable production of enzyme-based materials.

In this work, we demonstrate the feasibility of producing biocatalytic hydrogels via melt extrusion and post-extrusion crosslinking. ~~The key to producing defect-free hydrogels was performing cross-linking in the melt state to maintain a homogeneous reaction medium.~~ An intuitive first consideration in manufacturing is polymer selection and crosslinking mechanism. Solid- or melt-phase crosslinking must be employed, as traditional solvent-based crosslinking would lead to dissolution of the extruded form. In this work, we used PEO as it is known to have an affinity for CO₂, can be processed at low ~~temperatures, and~~ temperatures, and is a readily available commodity polymer often used in hydrogel research. Solid-state crosslinking of PEO via incorporation of pentaerythritol tetraacrylate, (PETRA), and benzophenone, (BP), has been demonstrated previously, (Doytcheva work, Ashton 2015, Korley Jordan 2017) however, in our hands these protocols led to phase separated and inhomogeneous materials. The key to producing defect-free hydrogels was performing cross-linking in the melt state to maintain a homogeneous reaction medium.

Here we highlight rapid melt-based crosslinking of PEO using PETRA and BP as a crosslinker and photoinitiator, respectively. By varying crosslinker concentration, we demonstrate strong control of final gel properties. Mesh sizes as a function of crosslinker concentration ~~varied~~ varied from less than 1 nm up to 60 nm, within the range of common monomeric enzymes. We then manufacture these hydrogels in the presence of CA, immobilizing ~~them-CA~~ within the hydrogel matrix and. ~~Leaching and activity studies demonstrated~~ high enzyme active-CA. Additionally, these biocatalytic gels are stable over a period of weeks, allowing them to be used ~~in more realistic settings for extended time periods.~~ Lastly, we scale up this process to a lab-scale twin-screw extruder, demonstrating similar swelling, ~~leaching-enzyme retention~~ and activity behaviors compared to batch-scale samples.

2. Materials and Methods –

2.1 Materials –

- PEO 100,000
- PETRA
- Carbonic anhydrase
- Chloroform
- Benzophenone
- PNPA and PNP
- Acetonitrile
- BCA components

2.2 Batch-scale Hydrogel Design

2.2.1 Making of PEO + PETRA powders

To make the powders of PEO, BP, and PETRA, all 3 components were dissolved in CHCl_3 and mixed for 72 hours to create a homogeneous solution. The solution was then cast into a petri dish, and CHCl_3 was ~~slowly~~ evaporated ~~away~~ over 24 hours at room temperature to produce a solid polymer film, the ~~film was then~~ vacuum ~~dried~~ ed for an additional 24 hours to remove any residual chloroform. The dried film was then blended to produce a fine powder.

Commented [JP1]: Blended how? In a blender - model, duration, speed?

2.2.2 Crosslinking Conditions of Control Hydrogels

~~The produced p~~ Powders were ~~next~~ melt pressed for 2 minutes at 80 °C to produce PEO films of uniform thickness. Films were then loaded between two borosilicate slides and placed on a hot

Commented [JP2]: Pressure?

plate set to the intended crosslinking temperature. Samples were allowed roughly a minute to ~~thoroughly~~ melt before crosslinking was started. Crosslinking was done using an OmniCure s1500 with a 320-500nm ~~filter~~. The ~~now-crosslinked~~ film was then removed from the hot plate and allowed to cool back to room temperature before initial mass and dimensions were collected.

Commented [JP3]: UV fluence?

2.2.3 Crosslinking Conditions of Enzyme-loaded Hydrogels

To create crosslinked films containing enzyme, the same powders for control hydrogels were physically mixed with lyophilized enzyme powder and vortexed. Samples were then melt pressed and crosslinked in the same method as the control ~~hydrogels~~.

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2.3 Hydrogels ~~Designed-Fabricated~~ via Melt Extrusion

The same process was used to create powders as described for batch process gel design. ~~4.4 g~~ ~~P~~ powder mixtures of 1.5 wt% enzyme ~~powder~~ were used in all melt extrusion conditions ~~(4.4 g)~~. A Haake™ MiniCTW Micro-Conical Twin Screw Compounder was used to perform lab-scale extrusion. Samples were processed ~~in recirculation mode~~ at 100 °C for 5 min with a screw speed of 20 rpm before being extruded from the ~~instrument~~. Samples were then crosslinked under the same conditions described above.

Commented [JP5]: Extruded using what die?

2.4 Swelling Studies

All swelling studies were performed using DI water. Samples were given 24 hours to reach equilibrium in water, although samples appeared to reach equilibrium in a matter of minutes. Samples were then weighed and measured, then dried at 50 °C under vacuum until constant mass was observed, which typically occurred within 48 hours, and their final mass was recorded. These values were then used to calculate gel fraction, (GF), and equilibrium water content, (EWC).

Gel fraction ~~can be thought of as the percentage of polymer retained following crosslinking and~~ is defined in Eq 1.

$$GF = \frac{M_f}{M_i} \times 100\% \quad \text{Eq. 1}$$

Commented [JP6]: Generally there is discussion in these methods - just the procedures here

EWC is ~~the percentage of equilibrium mass attributed to water, demonstrated in~~ calculated using Equation 2.

$$EWC = \frac{(M_s - M_f)}{M_s} \times 100\% \quad \text{Eq. 2}$$

Here, M_i is initial mass post-crosslinking, M_s is mass at equilibrium swelling, and M_f is the mass of the dried film following equilibrium swelling.

2.5 Mesh Size calculations using Equilibrium Swelling Theory

Mesh sizes of PEO hydrogels were estimated using Flory and Rehner's equilibrium swelling theory, (EST). Wong et al. produced PEO hydrogels using a similar method to this work, and their mesh size estimation approach was used in this work, (cite).

After determination of GF and EWC, polymer volume fraction, $V_{2,s}$, was calculated using Eq. 3.

$$V_{2,s} = \left(1 + \frac{\rho_p}{\rho_p} \left(\frac{M_s}{M_f} - 1 \right) \right)^{-1} \quad \text{Eq. 3}$$

This value can then be used to estimate the molecular weight of polymer between crosslinks, M_c , Eq. 4.

$$\frac{1}{M_c} = \frac{2}{M_n} - \frac{\ln(1 - V_{2,S}) + V_{2,S} + \chi V_{2,S}^2}{\rho_p V_1 (V_{2,S}^{1/3} - V_{2,S}/2)} \quad \text{Eq. 4}$$

Mesh size can then be calculated using Eq. 5.

$$\xi = V_{2,S}^{-1/3} \left(2 C_n M_c / M_r \right)^{1/2} l \quad \text{Eq. 5}$$

2.6 Rheology and Mesh Size Estimations using Rubber Elasticity Theory

A Discovery HR30 rheometer from TA instruments was used to measure storage moduli, (G') of various hydrogels. All tests were performed using the Environmental Testing Chamber attachment and a 25 mm parallel plate geometry, (ETC), with the temperature set to 25 °C. Oscillation frequency tests were performed using constant 0.1% strain with angular frequency ranging from 1 to 500 rad/s. All samples were run in triplicate.

Commented [JP7]: Will G' be in the SI somewhere?

Rubber elasticity theory was ~~also~~ used to estimate mesh size of hydrogels. Karvinen et al. used rheology to measure hydrogel storage moduli, and correlated the elastic response to a mesh size, (cite). Following the same approach, mesh size was calculated using Eq. 6.

$$\xi = \left(\frac{G' N_A}{RT} \right)^{-1/3} \quad \text{Eq. 6}$$

Here, N_A is Avogadro's number, R is the gas constant, and T is absolute temperature.

2.7 Scanning Electron Microscopy

Debika work

2.8 Leaching Studies

All crosslinked films were produced using the procedure described above. Initial masses were recorded and enzyme content was estimated based on the initial powder wt%. Crosslinked films were loaded into known volumes of 0.04 M phosphate buffer, pH 7.4, constituting Day 0. Buffer was extracted on Days 1, 3, and 7, with new buffer being added to each film sample. A bicinchoninic acid, (BCA), assay was used to measure enzyme concentration in the extracted buffer. BCA assay solution was created by mixing 50 parts Reagent A and 1 part Reagent B, by volume. Three dilutions were used for each sample, (1x, 0.75x, and 0.5x), with all samples also run in triplicate. 25 μ L of each dilution was loaded into a 96-well plate. 200 μ L of BCA solution was then added to each well. The plate was then covered and loaded into a 37 °C oven to sit for 30 minutes. Following the incubation period, the plate was directly loaded into a Synergy | HT microplate reader and absorbance was measured at 562 nm. Concentration was then calculated using a pre-measured calibration curve (Fig. S#).

2.9 Hydrogel Activity Studies

CA-loaded hydrogel activity was measured by monitoring the rate of *p*-nitrophenyl acetate, (PNPA), hydrolysis. As the enzyme-catalyzed reaction occurs within the hydrogel structure, and is much faster than the free solution reaction, what is realistically being measured is the rate of *p*-nitrophenol, (PNP) release from the gel.

Phosphate buffer and PNPA in acetonitrile were added to each well in a 6-well plate, and initial absorbance values were measured at 410 nm using the same microplate reader mentioned above. Pre-leached gels were then added to each well, and the plate was placed onto an orbital mixer for continuous mixing. Every 5 minutes, the plate was removed from the mixer and absorbance readings were taken. Readings were taken for at least 30 minutes. All samples were run in triplicate against control hydrogels containing no enzyme, (Fig. S#).

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3. Results and Discussion

3.1 Crosslinking and Swelling of PEO Hydrogels

A solid-phase crosslinking procedure was developed to enable continuous manufacturing in the melt-phase. Crosslinking in solution would require dissolution of the extrudate, negating the benefits of melt-processing. Solid-state crosslinking of PEO has been accomplished previously using benzophenone (BP) and pentaerythritol triacrylate, (PETA), and PETRA at room temperature, which was used as a starting point to screen reaction conditions (CITE). ~~Conditions were initially screened by dissolving all~~ All crosslinking components, (PEO, PETRA, and BP) were first dissolved in chloroform, (CHCl₃), followed by solution casting to generate solid films to develop cross-linking protocols prior to scaling to extrusion, (Fig. 1a). This approach was chosen during the initial stages of this work because it enabled rapid screening ~~many different of reaction~~ conditions rapidly and with minimal use of reagents.

The film was placed under ~~the~~ UV light and allowed to crosslink for 5 minutes at room temperature. The crosslinked film was then swelled in DI H₂O and for 24 hours. The resulting hydrogel displayed large heterogeneous regions and was quite fragile, (Fig. 1b). This was hypothesized to be due to crystallinity and phase separation of crosslinking components and PEO matrix at room temperature. This was surprising, as room temperature cross-linking of PEO films has been achieved by us and others in the past, however, this system was unamenable to solid state cross-linking at ambient temperature. Doytcheva et al. demonstrated an increased gel fraction for PEO hydrogels when crosslinked above the melting temperature of PEO. Such an approach would eliminate the crystalline regions as well as improve the mobility of all components. Additionally, as the intent is to manufacture these materials via melt processing, the polymer will already be in the melt-phase upon exiting the extruder.

Commented [JP9]: Fluence?

Commented [JP10]: Put this in an overview figure, schematic style

Commented [SH11R10]: Done

Commented [SH12R10]: Oh do you mean show how this would be done at scale? Like with heated rollers and UV curing?

Commented [JP13R10]: No, just the whole process - can you actually send me the files, I'd like to tinker with the overview figure

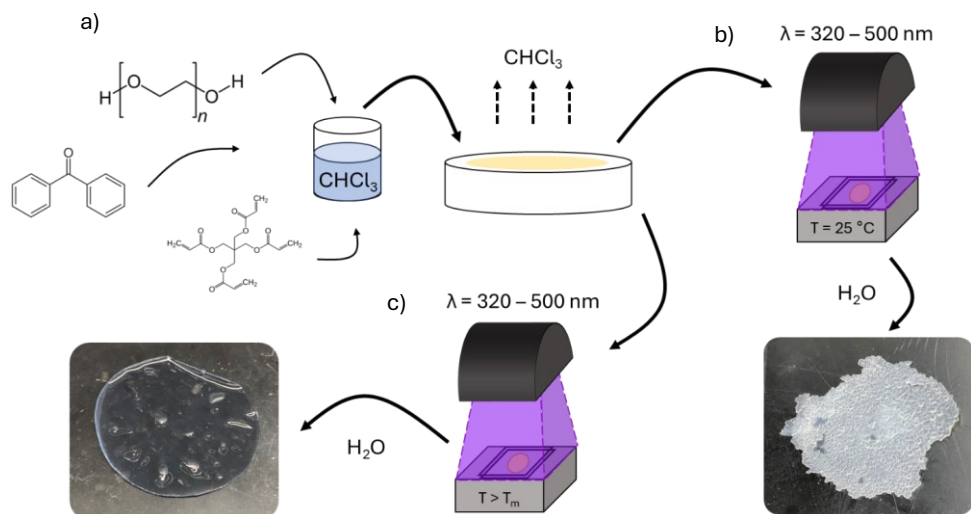


Figure 1. Schematic of batch-scale gel manufacturing. a) PEO, BP, and PETRA were dissolved in CHCl_3 then cast into a petri dish. CHCl_3 was then evaporated to produce a solid film. b) Initial samples were crosslinked at room temperature, producing heterogenous hydrogels once swollen. c) Powderizing, melt-pressing, and crosslinking in the melt state produced a much more homogeneous hydrogel.

The crosslinking setup was adapted to be performed on a hotplate, (Fig. 1c). A powderizing and melt-pressing step were also added. Powderizing the uncrosslinked film allows for easier loading into a twin-screw extruder setup, while melt-pressing creates a more uniform film like what one would expect upon exiting the extruder. Three temperatures, (70, 85, and 100°C), three crosslinking times, (1, 3, and 5 min), and three amounts of crosslinker, (5, 10, and 20 wt%), were examined to understand each variable's role on gel properties, (Fig 2a).

Commented [JP14]: Do you have film thicknesses?

Commented [JP15R14]: As compared to extrusion?

Commented [SH16R14]: I do, but I forgot to grab them this past week. I know generally the extruded samples get to be between 0.9-1mm, while batch gels are ~0.4mm. I will remeasure the extruded gels this next week

Commented [JP17]: Figures 2b and 2c are very hard to decipher.

Can they be split up? Or zoomed in - say Y-axis from 40-100%?

Commented [JP18R17]: I guess if I zoom in, I can get the take away - time and temperature play little role, only xlink %

Commented [JP19R17]: I still think this is a little too busy - maybe a figure that is an overview with images of gels - includes batch and continuous processing, both above and below T_m crosslinking? This could be Figure 1 - then data in figure 2?

Commented [JP20R17]: All structures should have same font size, bond width/length, etc

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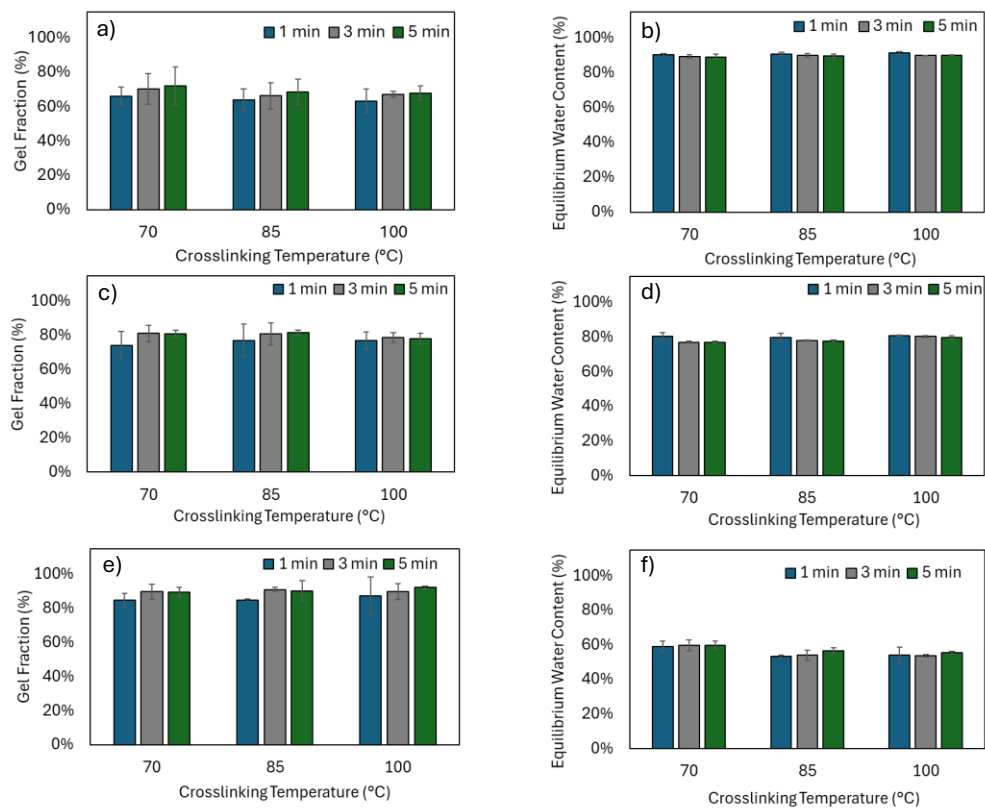


Figure 2. Effects of time, temperature, and crosslinker concentration on gel fraction and equilibrium water content. Gel fractions for hydrogels made with crosslinker amounts of a) 5 wt%, c) 10 wt%, and e) 20 wt%. Equilibrium water contents for hydrogels made with crosslinker amounts of b) 5 wt%, d) 10 wt%, and f) 20 wt%.

Both time and temperature had minimal impact on GF and EWC. This suggests that no further energetic barriers are overcome by increasing the temperature within the range investigated. This also suggests complete crosslinking is observed over a very short time, (<1 minute), which can provide insight into required crosslinking conditions during eventual scale up.

Commented [JP24]: It's minimal but I would say there is a trend - especially at low X-link % vs time

Commented [JP25R24]: This trend make sense but I think it should be mentioned

One variable that did change as irradiation time varied was an unexpected curling of the hydrogel after swelling. Samples crosslinked for 1 minute curled much more than samples crosslinked for 5 minutes, an indication of heterogeneous crosslinking. As a result, further studies were crosslinked on top of a piece of aluminum foil, which can reflect UV light, creating flatter hydrogels upon swelling.

Commented [JP26]: Do you have a reference for this?

Having developed a technique to produce flat, reproducible hydrogels, more detailed studies were performed to better understand the role of crosslinker content of gel properties. In particular, we were most interested in controlling mesh size, with the intention of encapsulating enzymes within the gel structure. Nine different samples containing varying crosslinker concentrations, ranging from 5 w/w% to 20 w/w%, were created and crosslinked as described above. Initial masses were recorded and then given 24 hours to equilibrate in DI H₂O. Samples were then placed in fresh DI H₂O for another 24 hours. This final state was taken as equilibrium. All samples were then dried under vacuum at 50 °C, with measurements taken every 24 hours until a stable value was reached. This value was taken as the final dried mass. Gel fraction and EWC were then calculated for each sample, (Fig. 3a).

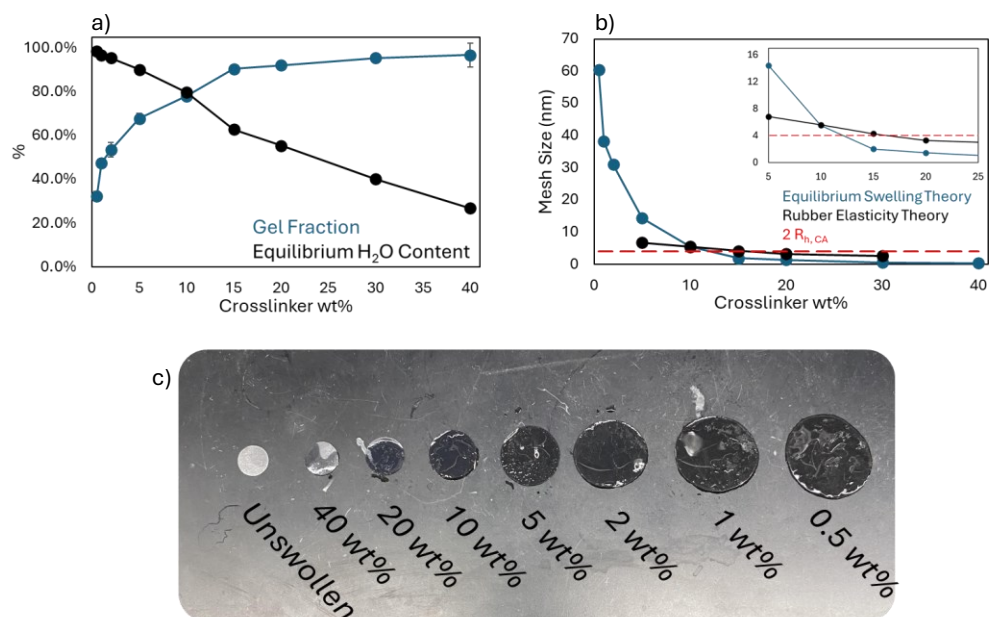


Figure 3. Crosslinker concentration studies. a) Gel Fraction and Equilibrium H₂O content vs. crosslinker wt%. b) Mesh size calculated using EST and RET vs. crosslinker wt% compared to twice the hydrodynamic radius of bovine CA enzyme. c) Images of PEO hydrogels compared to the initial unswollen film.

Large increases in gel fractions are observed at small crosslinker concentrations, eventually stabilizing at a value of around 95%. By this point, the maximum number of polymer strands have been added to the gel network, preventing further increases in gel fraction. However, equilibrium water content continued to decrease as gel fraction stabilized. Here, although no more network strands are added, additional links are created between network strands, continuing to decrease M_c . This further restricts chain flexibility, lowering the thermodynamically favorable water concentration in the gel.

3.2 Mesh Size Estimations of PEO Hydrogels

Two techniques were implemented to estimate the mesh size of the hydrogel matrices. Perhaps the most common method is equilibrium swelling theory, (EST). In EST, there is a thermodynamic tradeoff between the mixing of the polymer and solvent, resulting in swelling, and the extension of the polymer matrix, restricting the number of available conformations. Peppas has derived the key equations from Flory and Rehner's work (cite). Several authors have used this technique to estimate the molecular weight between crosslinks, and from that value calculate average mesh size (cite).

There was roughly a 120x difference in mesh size between the smallest and largest gels. 0.5 wt% crosslinker samples produced mesh sizes of approximately 60 nm. At the other end of the spectrum, 40 wt% crosslinker samples were estimated to have mesh sizes less than 0.5 nm. A point of interest is the change between 10 wt% and 15 wt% gels. ξ calculations showed a decrease from 5.5 nm to 2 nm over this range. CA is known to have a hydrodynamic radius, (R_h), of 2 nm, so ξ must be smaller than $2 R_h$ for efficient entrapment of the enzyme. These calculations suggest that 10 wt% samples should leach notably more CA than 15 wt% samples.

Additionally, rubber elasticity theory, (RET), was used to obtain a mesh size estimate. Author et al. proposed relying solely on the elastic contributions to the observed viscoelastic behavior, (storage modulus, G'), to obtain a characteristic length which correlates to mesh size. Comparable mesh size values were obtained for high crosslinker concentrations. However, as crosslinker content decreased, mesh size calculations differed wildly from EST estimations. This is most likely due to the increased viscous contributions to gel behavior, essentially meaning the gel is behaving less like a rubber. Regardless, this technique also suggested that roughly 15 wt% crosslinker should produce a hydrogel with the proper mesh size.

Additionally, to confirm that there were no large defects within the gels, scanning electron microscopy, (SEM), was also used. As SEM cannot detect ~nm pores, this was simply used to provide reassurance that the estimated nm mesh sizes are appropriate. All examined images of lyophilized hydrogels did display any large defects, suggesting the mesh size estimations were in the appropriate range, **Fig. S#**.

3.3 Production of Batch-scale Biocatalytic Hydrogels

Leaching studies were performed next to investigate if the produced hydrogels could retain large percentages of enzyme-loading. To produce enzyme-loaded gels, PEO powders containing varying amounts of crosslinker were created, as mentioned above. These powders were then physically mixed with lyophilized CA powder, melt pressed at 80 °C, then crosslinked for 5 minutes at 100 °C. Initial masses were recorded, and protein content was estimated from initial weight percents corrected by recovery rates obtained from previous work **(cite us)**. Films were then loaded into known volumes of phosphate buffered solutions. The films were allowed to equilibrate, and the free solution was recovered. The volume of recovered solution was noted and the enzyme concentration within each batch was calculated using a BCA assay. Gels were then loaded into fresh buffer. This was repeated on Day 3 and Day 7. By Day 7, all gels had reached a relatively stable enzyme concentration based on no CA being detected from the BCA assay.

CA concentrations remaining in the gel correlated strongly with mesh size estimations obtained from swelling and rheological studies, **(Fig. #)**. Both 5 and 10 wt% crosslinker gels leached the majority of CA into buffer, with 7% and 21% remaining in the hydrogel by Day 7. Both samples were estimated to have mesh sizes that were larger than 4 nm. Once the 15 wt% threshold was crossed, the majority of CA remained in the gel following equilibrium swelling. 20 wt% samples were estimated to retain roughly 80% of CA, while 30 wt% gels retained 70%. One would predict

Commented [JP27]: Why do 100C if 70C works?

Commented [JP28R27]: And 5 min?

Commented [SH29R27]: This approach guaranteed flat gels, so I just made all of them this way

a flipped result, with 30 wt% gels holding more enzyme, based on mesh size. It is not necessarily believed by the authors that 20 wt% samples retain more enzyme, as factors like imperfect mixing early in the gel manufacturing process can lead to these discrepancies. Both samples were able to retain very large percentages of CA, suggesting both have mesh sizes that are within the appropriate range for enzyme entrapment.

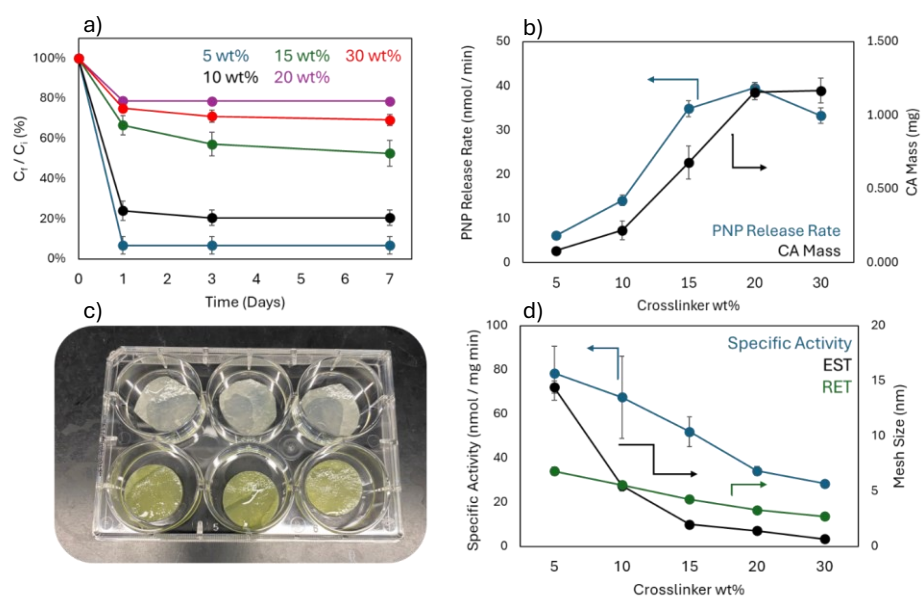


Figure 4. Biocatalytic hydrogel leaching and activity studies. a) The ratio of CA remaining in the gel, (C_t), to CA in the initial, dry, crosslinked film, (C_i). b) PNP release rate and CA mass vs. hydrogel crosslinker concentration. c) Image showing control hydrogels, (top), and biocatalytic hydrogels, (bottom), following an activity assay. d) Specific activity of CA vs. crosslinker concentration compared to mesh size estimations.

Knowing that large fractions of CA can be captured in the hydrogel matrix, the next step was to determine if the immobilized CA is active. A well-known assay used to measure CA activity is *p*-nitrophenyl acetate hydrolysis to *p*-nitrophenol and acetate, with *p*-nitrophenol showing high absorbance at 405 nm. This assay was used extensively in the previous work demonstrating thermal stability of lyophilized bovine CA, (cite us).

In this set of experiments, PEO hydrogels were created using the same approach as in the leaching studies. Samples were allowed to leach over 7 days, with BCA assays being performed throughout to monitor enzyme release. Once no enzyme could be detected in free buffer, gels were loaded into a solution of *p*-nitrophenyl acetate in acetonitrile and buffer, and absorbance measurements were taken every 5 minutes. PNP release rates were of comparable order to previous work, (Shen Salmon 2023), given similar mesh sizes. As one may expect, there was a sizeable increase in measured *p*-nitrophenol release when going from 5 wt% to 30 wt% crosslinker content due to the higher concentration of enzyme. Leaching studies suggested that there was a small concentration of enzyme still remaining in the 5 wt% gels even with a mesh size of 3-4 times larger than the enzyme diameter. This is confirmed via the activity assays, which show an accelerated rate of product formation. From 20 wt% to 30 wt%, there is a slight decrease in PNP release, even with comparable masses of enzyme between the two samples. This suggests the presence of diffusion limitations on PNPA into the gel and PNP out. Specific activity calculations suggest ~~s~~-further restrictions on the reaction rate. Contribution of CA to the rate of PNP release was measured to significantly decrease as mesh size decreased.

3.4 Scale-up Studies of Biocatalytic Hydrogels Produced using Melt Extrusion

With clear demonstration of active CA within the hydrogel, scale up studies were performed using the ~~a~~ Haake™ MiniCTW Micro-Conical Twin Screw Compounder. Here, only 20 wt% crosslinker

Commented [JP30]: Tell me something about this instrument?

hydrogels were used, as they had the optimal combination of swelling behavior and protein retention. 15 wt% gels had good swelling behavior and were easily handled, however they leached a significant amount of enzyme following swelling. 30 wt% gels retained almost all CA, however they were quite brittle and ripped easily.

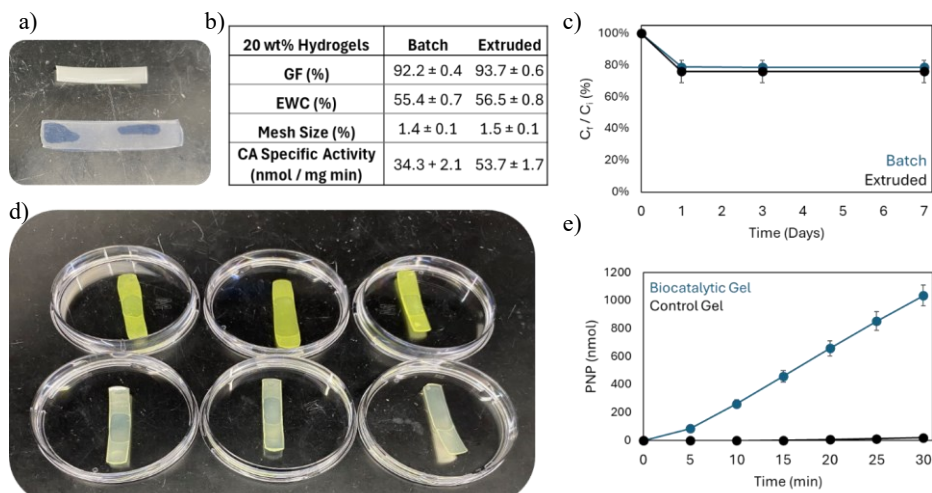


Figure 5. Biocatalytic hydrogel membranes produced via a lab-scale twin-screw melt extruder. a) Extrudate, (top), and crosslinked, swollen hydrogel. b) Swelling studies and activity assay results for 20 wt% gels designed in the batch process and using melt extrusion. c) Leaching studies of batch biocatalytic hydrogels vs. extruded biocatalytic hydrogels. d) Image showing biocatalytic, (top), and control, (bottom), hydrogels following an activity assay. e) PNP release rate of biocatalytic hydrogels vs. control hydrogels.

Like in previous studies, powders were formed containing PEO, crosslinker, and photoinitiators. Half of the powder was then combined with lyophilized CA, while the other half was used for the control. Both samples were melt processed and cycled in the twin-screw extruder for 5 minutes at 100 °C and 20 rpm. Following the cycling period, the extruder exit was opened and the extrudate was collected. Samples were then cut into 1-inch segments, loaded between two pieces of borosilicate glass, and crosslinked at 100 °C for 5 minutes.

Commented [JP31]: Add the degree sign in all cases

Commented [SH32R31]: Done

All extruded samples appeared homogeneous, implying that the extrusion process was adequate in allowing for proper [mixing](#). Additional 1-inch samples were also cut from the control extrudate and placed into H₂O. All samples dissolved, demonstrating that they are not crosslinked upon exit of the extruder.

With the crosslinked control samples, swelling studies were performed to confirm that similar mesh sizes were obtained from extruded gels. GF and EWC were calculated, and mesh size was estimated using EST. All values were essentially equal to batch scale PEO hydrogels, suggesting equivalency in processing, [\(Figure 5a\)](#).

Initial enzyme content in the CA-loaded extruded was estimated using recovery percentages from previous work [\(cite us\)](#). Leaching studies were then performed in the same way as in batch studies described above. Both batch and extruded gels showed very similar enzyme retention, again confirming that mesh size estimations are appropriate. By Day 7, no further enzyme was detected in solution, so activity studies were performed next to confirm the presence of active enzyme in the gel.

Activity assays were performed in the same way as batch studies. Pre-leached gels were loaded into a solution of PNPA and absorbance was measured every three minutes. Visually it is quite clear that there is active CA within the gel, given by the bright yellow coloring of enzyme-loaded gels [\(Figure 5d\)](#). Additionally, the measured reaction rate is significantly higher than that of the control gel containing no enzyme. Estimated specific activity of CA was measured to be quite comparable to the activity in batch gels. The discrepancy is most likely attributed to small errors during the estimation of protein concentration. Regardless, the results demonstrate similar, strong activity in samples produced via melt extrusion.

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Commented [SH34R33]: I don't have a super easy way to do this. PETRA and BP are not super water soluble, so I'd have to do some sort of solvent exchange to pull the protein out. And since you're always going to lose some in that process, I figured it wouldn't be the best way. Leaching and activity assays from different sections of the extrudate showed similar values though

4. Conclusions

This work has demonstrated a novel approach for manufacturing hydrogel materials at scale using melt extrusion and melt-phase crosslinking. Incorporation of PETRA and BP in the PEO powder allows for simple loading and extrusion. Removed from the extruder, the sample was shown to be uncrosslinked. By crosslinking in the melt phase, high GF gels are produced, (> 80%), within 1 minute of light exposure, (320-500 nm). Additionally, hydrogel mesh size was shown to be easily controlled by simply altering the amount of crosslinker present during the melt processing step. Batch scale studies suggest mesh size can range from less than 1 nm to greater than 60 nm, depending on crosslinker concentration. Additionally, mesh sizes within this range are ideal for entrapment of many different enzymes.

To illustrate, we demonstrated immobilization of bovine carbonic anhydrase within the hydrogel matrix. Leaching studies of batch-scale hydrogels showed strong correlation between mesh size and protein retention. If the estimated mesh size was significantly higher than the diameter of the enzyme, almost all of the enzyme was shown to leach out into free buffer. Producing hydrogels with mesh sizes lower than enzyme diameter allowed for high protein retention, in the range of 70-80%. Additionally, activity assays using enzyme-loaded hydrogels demonstrated highly active CA present in the gel.

Combining, we demonstrate production of biocatalytic hydrogels, containing active CA, produced via melt extrusion and melt-phase crosslinking. Resulting swelling and leaching studies showed similar behavior to batch-scale gel manufacturing, suggesting equivalency in the process. Finally, activity assays demonstrate active CA present in the hydrogel.

Commented [JP35]: 1 minute?

Commented [SH36R35]: Yeah you're right. I used 5 min for most of the studies but that statement is correct and it sounds a lot better

Collectively, what has been demonstrated is a simple, scalable manufacturing route for design of biocatalytic materials. Bovine CA was selected to demonstrate feasibility for several reasons. First, bovine CA is a monomeric enzyme, which is known to have improved stability, especially when lyophilized, (cite). One thing to consider is the presence of the metal cofactor, (Zn^{2+}), which is a vital component of the CA activity mechanism. Loss of this metal would render the enzyme inactive. However, previous work has also demonstrated the thermal stability of CA during melt processing conditions, showing the enzyme retains Zn^{2+} following melt extrusion (cite us again).

Perhaps the largest motivation for incorporating CA into the hydrogel is its usefulness for CO_2 capture. CA possesses a high turnover rate converting CO_2 to HCO_3^- , which can then be easily converted to MgCO_3^- or CaCO_3^- , which are useful in many applications. Many authors have demonstrated the effectiveness of incorporating active CA into a hydrogel for improved mass transfer, (cite).

Broadly, this work highlights a manufacturing route to produce biocatalytic hydrogels at scale. Although not installed for this work, one can employ a heated UV light-curing conveyor system attached to the end of the extruder for continuous extrusion and crosslinking. This work focused on developing biocatalytic hydrogels for CO_2 capture. However, this approach should be more broadly applicable, capable of producing biocatalytic materials for many other important applications at scale.

Commented [JP37]: Now that it is here I don't like it so much - move to the conclusion