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Enzymatic Activity Preservation and Protection through Entrapment within Degradable Hydrogels[†]

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Abstract

This work aims to develop a repeatable enzyme entrapment method that preserves activity within an amicable environment while resisting activity reduction in the presence of environmental challenges. Advances in such methods have wide potential use in biosensor applications. In this work β-galactosidase (lactase) enzyme was entrapped within hydrogel matrices of acrylamide (ACR) crosslinked with *N,N'*-methylenebisacrylamide (BIS, non-degradable) or poly(ethylene glycol) diacrylate (PEGDA, degradable) to create "biogels." Diffusivity studies of control, enzyme free, hydrogel constructs showed near-Fickian swelling behavior in PBS regardless of crosslinker type or density. As expected, the swelling rate, K_s, decreased when increasing the crosslink density from 78.6 to 14.7 min^{-1} over a range of 1 to 20 mole % PEGDA indicating that diffusivity into the matrix is dependent on crosslink density. Fabricated biogels were evaluated for maintained enzyme activity in the 7-8 pH range. PEGDA crosslinked gels consistently showed improved enzymatic activity retention as compared to BIS crosslinked gels. As PEGDA crosslink density increased from 5 to 10 mole %, enzymatic activity retention post initial entrapment increased. Higher PEGDA crosslink densities between 15 and 40% decreased enzymatic activity due to assumed steric hindrance of the entrapped enzyme and also decreased substrate and product diffusion. Increased enzymatic stability was observed in 40 mole % PEGDA crosslinked gels. The biogels were pH challenged to 8.0 and stability, measured as retention of activity, was observed to be 91%. Free, non-entrapped, solution based enzyme conversion only retained 23% activity under the same pH challenge conditions. No significant loss of active enzyme was determined to elute out of the biogels during storage in PBS or during biogel wash and recycling. This entrapment method illustrates the potential to sterically hinder and diffusively impede enzymes from performing their function. Degradation of the network crosslinks can then potentially enable the reactivation of the enzyme at a site and time dictated by the user.

Keywords: enzyme entrapment, hydrogel, degradable, PEGDA

Introduction

This work investigates the use of an abiotic entrapment method with potential to suspend enzyme activity for extended durations and then enable reactivation on-demand – at site and time dictated by the user. Functional polymer hydrogels can create a stabilizing cavity around the enzyme, allowing for on-demand enzymatic activity through crosslink degradation. The enzyme/hydrogel complex, herein referred to as a biogel, acts to maintain enzymatic activity, even when the surroundings are perturbed from the optimum environment. Such biogels can be used in biosensors where maintaining enzymatic activity is pertinent (Hernandez and Fernandez-Lafuente 2011), applications where small molecules are required but hard to produce in high purity, such as wound healing (Montesinos et al. 1997), and possibly for use in the degradation of small molecular hazards for decontamination (Kim et al. 2002). Determining the presence of pathogens or toxins using on-demand enzymatically active biogel biosensors could provide rapid screening and detection, reducing the number of infectious outbreaks. This work also takes advantage of natural enzymatic processes to enable the repeatable synthesis of high purity biomolecules when compared to other published synthesis methods.

The ability to produce, degrade, or detect small molecules through the use of naturally occurring enzymes within living cells is often hindered by an excess of cellular byproducts. Currently, synthesis of desired small molecules is performed *in vitro*, *in vivo,* and by chemical synthesis. Each method has its own challenges. Typically, *in vitro* synthesis requires the amplification of genes to produce large quantities of the required enzymes. These enzymes must then be extracted from cells and purified. Final product formation requires the addition of substrate, followed by enzymatic action on the substrate to construct the product, and prior to purification (Sperandio et al. 2003). *In vivo* techniques require further purification as a result of rigorous whole organ digestion protocols in order to isolate the desired small molecule (Tannock et al. 2005). These additional byproducts vastly add to the complexity of the purification process. Further, chemical synthesis of biomolecules typically requires the use of many volatiles and purification methods are inefficient at removing chemical reactants (Vilchez et al. 2007).

Chemical synthesis and degradation of molecules is both expensive and often leads to the production of undesirable hazardous byproducts (Wagner et al. 2009). Solution based enzymatic reactions, similar to *in vitro* synthesis, waste active enzyme. Once substrate and product molecules have reached a state of equilibrium, substrate diffusion towards the active sites of the enzyme diminishes and natural enzymatic high conversion rates cannot be continuously exploited. Product must be filtered out or additional substrate must be added for use before high conversion rates return. To combat this issue, expensive single point alterations to enzymes have been used to allow for cycling or continuous substrate flows over enzymatic active sites, but this method is currently not economical for large-scale biomolecule production (Rao et al. 1998). Contrarily, physical entrapment of the enzyme will allow for the protein to easily be filtered out and reused by depositing it into fresh substrate, thus continuing rapid conversion. These challenges have lead to an increased interest in immobilizing enzymes in a stabilizing environment to continuously utilize natural high conversion rates.

Previously used techniques to stabilize enzymes include but are not limited to: genetic point alteration, chemical modification, and the supplementation of additives such as sugars (Eijsink et al. 2005; Iyer and Ananthanarayan 2008). In depth reviews of both these techniques as well as the selection process of supports, enzymes, and conditions describe many difficulties and advantages to the various techniques (Garcia-Galan et al. 2011; Mateo et al. 2007; Sassolas et al. 2012; Sheldon 2007). Often immobilization methods structurally deform enzymes leading to reduced activity (Betancor and Luckarift 2008). For example, single, multipoint alterations as well as covalent attachment methods alter the enzyme itself, typically creating more rigid segments of enzyme that resist overall structural degradation. This process can cause unnatural tertiary structures to form, leading to the destruction of the active site or substrate diffusion issues (Dean et al. 1997). During gluteraldehyde fixation, for example, immobilization is achieved by crosslinking the enzyme's peripheral lysine residues. Alonso et al. recorded an average loss of activity of 20% due to this immobilization process (Alonso et al. 2005). To strengthen hydrophobic interactions between non-polar amino acids, additives such as dimethyl sulfoxide and glycerol have also been used to create rigid enzyme segments as well as reduce solvent

enzyme interactions. These techniques have demonstrated increased protein longevity within a sub zero atmosphere (Iyer and Ananthanarayan 2008; Marsac Y 2006). Another method, biopolymer entrapment by alginate, is inexpensive and can be performed in mild conditions. However, drawbacks include low mechanical strength and high enzyme leakage due to large uncontrolled pore size (Santagapita et al. 2011). Polymers have also been used in combination with enzyme alteration; Marsac et al., determined that site-specific attachment of polyethylene glycol (PEG) improves enzyme solubility, which, similar to the discussed additives, aids in enzymatic resistance to degradation (Marsac Y 2006). Similarly, Bellusci et al. combined polymer chemistry and biological attachment of enzymes by adsorbing lipase enzyme onto ethylene-vinyl alcohol polymers functionalized with acyl chlorides (Mariangela Bellusci 2012).

In addition to these methods, synthetic polymers including PEG and polyvinyl alcohols (PVA), have been shown to increase enzyme stability by limiting solvent enzyme interactions through non-specific polymer-enzyme interactions (Brady and Jordaan 2009; Eijsink et al. 2005; Iyer and Ananthanarayan 2008). Immobilizations by protein entrapment within various hydrogel and polymeric networks increased stability and lifetime of enzymes (Bolivar et al. 2008; Elnashar et al. 2008; Gill and Ballesteros 2000a; Gill and Ballesteros 2000b; Gupta and Chaudhury 2007; Mateo et al. 2000; Wang et al. 2008).

This research aims to extend and improve current methods to create an easy, reliable, and repeatable enzymatic immobilization technique. Biogels were synthesized which can be easily tailored to a desired enzyme based on its size and isoelectric point. Initial studies were successful with lactase, and the biogel immobilization method in the present study may be extended for use with highly valuable multimeric biosensing enzymes such as glucose oxidase and horseradish peroxidase (Sheppard et al. 2012), though each new enzyme will require individual optimization. Our method has the potential to be a generic, bioinspired, economical technique specifically designed for the synthesis of high-purity biomolecules with long-term enzymatic stability. Additionally, the comprehensive swelling, degradation and initial activity retention data presented here will aid researchers looking to utilize

biogels for biosensing platforms. Biogel activity optimization was carried out by varying crosslink density of the entrapping matrix, eliminating the need for genetic or covalent enzyme modification. The resulting biogel complexes have the enzymatic ability to produce desired biological small molecules. A library of hydrogel matrices was prepared and subsequently compared in order to create favorable environmental conditions for any enzyme of interest. Included in this study are various crosslink density biogels with either degrading or non-degrading crosslinkers. These methods fulfill the need for an easy, reliable, and repeatable enzymatic immobilization technique for potential use in production of high purity biomolecules, the natural degradation of molecular hazards, or for future biosensor applications.

Materials and Methods

Materials

Acrylamide (ACR), *N,N'*-methylenebisacrylamide (BIS, non-degradable), poly(ethylene glycol)diacrylate (Mn=700 PEGDA, degradable), ammonium persulfate (APS), *N,N,N'N'*-tetramethylethylenediamine (TEMED), ortho-nitrophenyl- -D-galactopyranoside (ONPG), phosphate buffered saline (PBS) and β-galactosidase (lactase, 476kDa) from *kluyveromyces lactis* were purchased from Sigma Aldrich and were used as received except for PEGDA which was first filtered through an inhibitor removal column prior to application. NHS-Rhodamine, borate buffer, and buffer exchange spin columns were purchased from Pierce® and used as described in the Pierce® NHS-Rhodamine Antibody Labeling Kit for confocal microscope imaging of biogels.

Equipment

A SpectraMax(R) M5 plate reader, from Molecular Devices (set on 6 well plate, absorbance at λ = 420 nm, to measure ONP concentrations every 20 seconds for 10 minutes) was used to quantify retained enzymatic activity through degradation of substrate ONPG into product ONP. This was also used to optimize enzymatic protection through entrapment using highly crosslinked networks. A Nipkow

(spinning) disk-equipped Olympus IX81 confocal microscope with a 10X objective was used to determine protein localization and aggregation within gels.

Hydrogel Synthesis

Biogels and hydrogel controls, with and without enzyme respectively, were synthesized using free radical polymerization of ACR monomer crosslinked at various densities with BIS (non-degradable) or PEGDA (degradable via hydrolysis). Gelation was initiated at room temperature with APS and stabilized with TEMED; gels were allowed to cure in a well ventilated area for 25 minutes, until solid. To determine enzyme activity within gels, 15 μl of lactase enzyme, rated at •3000 units/ml, was added to 500 μl blank hydrogel slurry just prior to the initiation of gelation. 30 μl of the solutions (blank hydrogels and various biogel fabrication precursor slurries) were drop-cast onto activated cover slips to form thin films approximately 75 μm in height.

The validation enzyme, β-galactosidase (lactase), a digestive enzyme which degrades lactose substrate into glucose and galactosidase, was entrapped within the matrix to create the active biogel. Lactase is a multimeric enzyme and is most likely to become de-activated through the dissociation of the subunits. It relies on cations to maintain its native and active tertiary and quaternary structure (Fernandez-Lafuente 2009). It was chosen for these studies due to its application in biosensing (Sassolas et al. 2012; Sharma et al. 2004a; Sharma et al. 2004b) and activity in the pH 7-8 range.

Films were washed in PBS for 2 hours, then placed in absorbance plates with ortho-nitrophenyl- -D-galactopyranoside (ONPG), an analogue substrate to lactase which, when catalyzed, yields one galatosidase molecule and one ortho-nitrophenol (ONP) molecule. ONP is used to report lactase activity as it is readily absorbs light at λ= 420 nm, making it a simple model enzyme system to utilize during crosslinker type and density optimization studies. Substrate was introduced to the biogels at concentrations of 0.1, 0.2, 0.4, 1, and 10 mg ONPG/ml PBS and conversion was measured. Kinetic studies were performed at t = 2, 24 and 48 hours to determine retention of enzymatic activity and the reliability of repeated product formation. When not actively being tested these samples were stored at

4°C in PBS. The calculation of the Michaelis-Menten constant, K_m, and the maximum reaction velocity, V_{max}, required the use of time-lapse enzymatic kinetic conversion curves of biogels and a constructed Lineweaver-Burke plot (Supporting Information Figure S3a and S3b, respectively). Subsequent analysis from the Lineweaver-Burke plot derives the enzyme kinetics, namely, the y-intercept is equal to $1/V_{max}$ and the slope is equal to K_m/V_{max}.

Swelling and Degradation Studies

Swelling studies were performed on blank hydrogels with 1, 5, 10, 13, 15 and 20 mole % crosslink density using BIS or PEGDA crosslinkers. Three 500 µl gels of each crosslink density were cast in 24 well plates. Gels were first dried in air at room temperature for 24 hours from their synthesized state obtaining their original dry mass (M_0). Endpoint of drying was determined to be 24 hours after subsequent time points revealed no further mass loss. Gels were then placed in 5 ml aqueous PBS for swelling studies. Measurements were taken every 20 minutes for the first 4 hours, when the swelling factor is rapidly increasing, and then periodically up to the 24 hour mark to indicate no change in the maximum swelling factor. Degradation studies were commenced from the maximum PBS swelling point by submerging each sample in 2 ml of a 500 µM NaOH (pH 10.7) degradation solution. Samples were weighed daily until the gels became unable to handle and mechanically weak. The degradation solution was refreshed after each measurement.

Positive Control Studies

Positive controls provide proof of efficacy of the enzyme entrapment and protection procedures. Solution based and chemical fixation methods were used to determine maximum enzyme kinetics. Solution based kinetics without gels were determined by diluting lactase with PBS to match quantities utilized in entrapped biogel studies. Samples were refrigerated at 4 °C in PBS for 2, 24, and 48 hours as well as 8 and 15 days to determine the enzyme activity retention over extended periods of time. These studies were performed with and without the addition of gluteraldehyde activated coverslip testing solution based controls in the presence and absence of coverslips used in subsequent experiments.

Dissimilar from the gel entrapment studies, no wash steps were used as the enzyme would have been removed during the removal of the wash buffer.

Chemical fixation enzyme kinetics were determined by dropping 30 μl of diluted enzyme solution onto gluteraldehyde activated coverslips. The solution was evenly distributed over the surface with the addition of a top glass coverslip. After 20 minutes the top coverslip was removed and the bottom coverslip was washed in PBS for 2 hours before measuring the kinetic parameters. The calculated conversion rates, specifically measuring the Michaelis–Menten constants, related only to enzyme adsorbed and chemically fixed to the glass slide.

Further control study methods determining the enzyme diffusion out of the biogels during wash steps as well as comparative biogel studies using alginate as the entrapment matrix are presented in the supporting information available in the online.

Confocal Microscopy

β-galactosidase was diluted to 2 mg/ml using a NanoDrop ND-1000 Spectrophotometer. The PBS buffer was replaced with borate buffer, pH 8.5, using buffer exchange spin columns. 500 μl of the 2 mg/ml lactase in borate buffer was then added to the vial of NHS-Rhodamine dye reagent and incubated for 1 hour for protein labeling. The labeled enzyme was purified by running the samples through spin columns at 1000 rpm. The collected samples were stored at 4^{o}C until used for confocal imaging. The labeled enzyme was entrapped within drop cast biogels in dilutions as previously described for enzymatic activity studies. The films were washed for two hours in a PBS solution and then imaged on the confocal microscope using the 10X objective. Three 10μm depth images were taken of each slide and analyzed in ImageJ for bright spots and intensity measurements. There were a total of 3 slides per crosslink density (5, 20, and 40 mole %) studied.

Protection through Entrapment

Biogels were prepared as described in the hydrogel synthesis section and subjected to increasing pH to determine the protection provided by the surrounding polymer matrix. A dilute NaOH solution at pH

8.0 was utilized to alter the biogel pH and determine activity retention. The synthesized thin film biogels were washed in PBS for 2 hours, tested for reference initial kinetic values, $V_{max}(I)$ and $K_m(I)$, and then evaluated after the addition of 200 μL of a dilute NaOH solution titrated to a pH of 8.0. The biogel activity was determined after the pH increase for 1 minute yielding final kinetic values for $V_{max}(F)$ and $K_m(F)$. Conversion studies investigated the percentage of retention in kinetic activity values ($V_{max}(F)/V_{max}(I) * 100$) to determine retention of activity after the pH fluctuation challenge.

Results and Discussion

Swelling studies performed on BIS and PEGDA crosslinked hydrogels aided in the understanding of intrinsic diffusion parameters of blank hydrogels. Crosslinking affected the maximum velocity of enzymatic substrate conversion into product in fabricated biogels. Degradation studies of the networks have led to insight involving rates of construct decay and its effect on the protection of enzymes. Highly crosslinked PEGDA hydrogels are expected to scavenge oxidative OH^- molecules leading to hydrolysis of the ester group and crosslink degradation leading to enhanced protection of the enzymes. Positive controls in this work aim to show that negligible enzyme is lost through the wash cycles and that the fabricated biogels show promise in being an abiotic, generic, and economical entrapment matrix when compared to alginate gels. Confocal imaging was used to determine the distribution of enzyme within the gel in order to compare clustering phenomena with varying crosslink densities of PEGDA crosslinked biogels. Enzymatic kinetic studies performed at 2, 24, and 48 hours proved repeatable synthesis and enzyme stability over the 2 day study. Protection studies using a pH challenge of 8.0 proved the efficiency of highly crosslinked networks over solution based activity retention.

Swelling

Swelling studies were performed for blank hydrogels crosslinked at 1-20 mole % with BIS (non-degradable) or PEGDA (degradable through hydrolysis) and are shown in Figures 1a and 1b, respectively. Data is represented as a swelling factor, calculated from the ratio of $(M_t-M_0)/M_0$, where M_0 is the mass of samples after drying at room temperature for 24 hours and M_t is the swollen mass at time t. Typical to Fickian swelling behavior, the first 50% of experimental swelling data increased linearly

indicating that the swelling reactions were controlled exclusively by the chemical potential gradient of water into the matrix. Swelling ratios (SR(%)), 100X the swelling factor, were fitted to the following Fickian swelling relation (Equation 1) as described by Peppas et al. assuming no polymer relaxation term (Mariangela Bellusci 2012; Peppas et al. 2006; Tasdelen et al. 2004).

$$\mathrm{SR}(\%) = 100 \times \frac{(M_t - M_0)}{M_0} = K_s e^{0.5t} \qquad (1)$$

where, $(M_t\text{-}M_0)/M_0$ is the swelling factor as previously described, and the constant K_s is the swelling rate of the polymer sample.

Swelling studies of 1-20 mole % BIS (non-degradable) and PEGDA (degradable through hydrolysis) crosslinked gels in Figures 1a and 1b, respectively show equilibrium of swelling is reached within five hours and is maintained without mass loss for the remainder of the study. Gel swelling is visually represented in Figure 1c. Increasing crosslink density reduced maximum swelling as shown in both Figures 1a and 1b. Swelling data was modeled by the Fickian equation and calculated values for K_s, are shown in Table I. K_s, values were inversely related to crosslink density as expected for Fickian swelling.

Primary diffusion related issues encountered were: difficulties with substrate reaching the enzyme and difficulty for product molecules to diffuse out of the gel for separation. Typically, gels with higher PBS diffusion coefficients are not subject to these issues, allowing for relatively free movement of substrate and product molecules into and out of the matrix. Biogels were fabricated through a drop cast method leading to films approximately 75 μm thick. At low crosslink densities, diffusion of dissolved substrate and product molecules was concluded not to be the rate limiting factor of enzyme conversion. The rate limiting step was determined to be enzyme conversion of substrate into product. Lumped into this factor is the binding of substrate, the conversion of substrate to product, and the enzyme release of the product molecule. However, at high crosslink densities, diffusion may become a factor in the perceived enzyme activity.

The fabricated blank hydrogels crosslinked with BIS or PEGDA both showed Fickian swelling in PBS media. PEGDA crosslinked gels swelled more than BIS crosslinked gels, especially at high crosslink densities. Increased crosslink density decreased the maximum swelling and also decreased the swelling rate. Thus, slower diffusion may affect the enzyme's ability to convert substrate into product at high crosslink densities. At low crosslink densities the rate limiting factor is assumed to be the process of enzyme conversion into substrate.

Degradation

Hydrogel degradation, similar to swelling studies, was monitored through mass increase and the calculated swelling factor. As the PEGDA crosslinker hydrolyzed, the mesh size of the polymer matrix increased, allowing for an influx of water without considerable shape deformation or measurable surface erosion. The swelling ratio was determined experimentally as described in Equation 1. BIS crosslinked matrixes did not degrade with the addition of NaOH as there are no hydrolyzable bonds. BIS crosslink control studies were performed at 1, 2, 3, 5, 10, and 15 mole % for 7 days. PEGDA crosslink degradation studies were performed at 1, 2, 3, 4, 5, 6, 7, 8, and 10 mole % for up to 12 days for as long as the samples maintained mechanical stability and were able to be handled. Results of these studies are shown in Figures 2a and 2b, respectively.

Swelling and degradation results from the non-degradable, BIS crosslinked gel controls, revealed that size and structure was maintained throughout the experiment. As expected, no measurable mass gain or loss was recorded, demonstrating that crosslinks were not degraded. Figure 2c shows no degradation or erosion of the specimen when left in the 500 μM NaOH degradation media for one week, allowing for comparison with similar crosslink density PEGDA gels.

In contrast to the control (BIS crosslinked) gels, PEGDA crosslinked gels degraded when the crosslinker was hydrolyzed by the degradation media. The gels maintained overall aspect ratios and shape despite becoming highly swollen as the crosslinks degraded (Figure 2c). When the PEGDA molecules degraded, cavities within the matrix became larger, allowing for water molecules to diffuse more easily into the gel. Increasing the matrix crosslink density decreased the degradation rate of the

hydrogel matrices, as expected. Extensive hydrolysis was necessary to create the cavities for water diffusion in these blank hydrogels. Degradation of all of the crosslinks led to complete dissolution of the gel into the media. The early stages of complete degradation can be seen in the 2, 3, and 4 mole % crosslinked hydrogels in Figure 2b. Large quantities of water invade the gel, swelling it to the point where the gel is drastically degraded in a short amount of time. Similar results were observed when the strength of the degradation media was increased tenfold to a 5 mM NaOH solution. 1-10 mole % PEGDA gels were studied and rapidly swelled in the media with swelling factors up to 40 and 50 and then the gels swiftly degrading into the solution within a single day making results difficult to quantify.

The fabricated ACR gels crosslinked with BIS and PEGDA compared the degradation of PEGDA gels with BIS gels. At high crosslink densities PEGDA gels had less swelling, and therefore, less degradation when compared to low PEGDA crosslink density gels which rapidly decayed over time. The increased PEGDA density, as described in the swelling section of this work, decreased the diffusivity constant D and making it more difficult for solution to diffuse into the gel. A large number of these crosslinks must be degraded to increase the diffusion of degradation media into the gel. With fewer hydroxyl molecules reaching the hydrolysable PEG bonds, the degradation is slowed.

Positive Control Studies

Solution based kinetics were determined for lactase diluted in PBS to match quantities utilized in biogel studies. To determine the solution based activity retention over extended periods of time, samples were "aged" by storing diluted samples at 4 ^{o}C for 2, 24, and 48 hours as well as 8 and 15 days. V_{max} (μM/L/min) and K_m (μM) were calculated through the application of Lineweaver-Burke plots of the aged enzyme solution based kinetic conversion studies performed at 2, 24, and 48 hours as well at 8 and 15 days. The maximum, V_{max}, determined at 2 hours was 1520 ± 180 μM/L/min. V_{max} decreased as the sample was aged to the 8th day to 1070 ± 200 μM/L/min. Increased aging time had an inverse relationship to V_{max} while K_m remained relatively unchanged at 2600 ± 260 μM. Tests with and without

gluteraldehyde activated glass slides showed no significant differences in the solution based kinetics caused by the addition of the slides used in subsequent studies.

Chemical fixation enzyme kinetic parameters were determined using lactase crosslinked onto glass coverslips and washed in PBS for 2 hours. The determined V_{max}, 230 ± 140 μM/L/min, and K_m, 2190 ± 420 μM, was related only to enzyme adsorbed and chemically fixed to the glass slide. As expected, these values were less than solution based enzymatic activity, as much of the enzyme did not crosslink onto the gluteraldehyde activated slide and was washed away.

Additional controls (See online Supporting Information) confirmed that no significant loss of active enzyme eluted out of the biogels during storage in PBS or during biogel wash and recycling.

Confocal Microscope

Fluorescence images were analyzed with ImageJ for gels with PEGDA crosslink densities of 5, 20, and 40%. Three images were taken over a depth of 10 μm in order to ensure that the fluorescently tagged enzymes were entrapped within the network matrix and not adsorbed to the surfaces. The depth images were complied into a single image and analyzed to obtain average enzyme fluorescence intensities and clustering within the biogels. Enzyme clusters were located and determined by bright spot analysis.

The average intensity measured for all biogels were statistically equal, denoting that an equal amount of enzyme was entrapped within each gel regardless of crosslinker concentration, as seen in Figure 3a. Control, enzyme free, hydrogels were tested to determine the background autofluorescence of fabricated ACR/PEGDA biogels. The only statistical difference in average intensity, determined through the used of a two-tailed paired student t-test (p= 0.05), was determined to be between the biogels, gels with active fluorescently labeled enzyme entrapped, and the blank control hydrogels.

Clustering increased with increased crosslink density as depicted in Figure 3b. A statistically significant increase (two-tail paired student t-test, p = 0.05) in clustering was found for 40 mole % crosslinked gels when compared to the 5 mole % crosslinked gels. There was a large standard error

among to the 20 mole % crosslinked data due to film lift off, therefore this piece of data cannot be used to determine nor mitigate an aggregation trend. It is also extrapolated from this data that the increase in crosslinks per volume of gel forces the enzyme to cluster into small groups prior to complete polymerization, instead of being distributed evenly within the biogel.

Enzyme Entrapment and Wash Studies

Lactase enzyme was entrapped within the following biogel network: 5 mole % BIS crosslinked, and 5, 7, 10, 13, 20, 25, 30, 35, 40 mole % PEGDA crosslinked as discussed in the methods section. After washing, these gels were tested for enzymatic activity through kinetic conversion studies. V_{max} and K_m were derived for each synthesized gel immediately and at various "long term" time points including 3, 24, and 48 hours post entrapment depending on the study. V_{max} and K_m were calculated using these plots as described. Representative data of long term conversion kinetic parameters are reported in Figure 4.

As shown in Figure 4, initial responses to entrapment decreased the V_{max} by at least 50% from free enzyme, depending on the density and type of crosslinker utilized for the biogel. This initial loss of activity was due to the gelation and initial wash process. The majority of the initial velocity loss is due to the loss of active enzyme in the initial wash buffer. This buffer contained enzyme that remained free throughout the biogel fabrication process as well as any unreacted reagents. The other lost activity is assumed to be an undesirable effect of the free radical initiator, APS. APS is a strong oxidizer that will react with many molecules, not just the monomer and crosslinker in solution. The chemical reaction between APS and the enzyme leads to its subsequent deactivation. BIS crosslinked biogels maintained the least activity upon initial entrapment when compared with PEGDA crosslinked biogels. Similarly a large initial loss can be seen in the 7 mole % crosslinked PEGDA where subsequent aging of the gel only slightly reduced the activity in comparison to a large drop in activity seen in the 5 and 10 mole % PEGDA gels. It is thought that this phenomenon resulted from the additional protection provided by the hydrolyzable PEG linkages of the PEGDA biogels. These bonds allow for extra molecular sites for APS to be consumed before coming into contact with enzyme. To further verify this assumption, Figure 4 also illustrates increased density of PEGDA crosslinks increased V_{max} retention over 48 hour time trials.

PEGDA crosslink densities above 10% show an initial decrease in V_{max} as well as diminished enzymatic activity retention over time as can be seen in Figure 5. It is assumed that PEGDA crosslinker creates a more habitable environment for the enzyme, allowing for increased retention in activity until steric hindrances caused by dense crosslinks affect the enzyme's ability to change its conformation.

K_m was recorded for all experiments and resulted in $K_m = 3070 \pm 1710$ μmoles. Ideally, K_m should remain constant through the use of the same enzyme and same batch. Variation was most likely due to affinity changes caused by random enzymatic conformational changes over time and through the entrapment procedure, notably adverse APS/enzymatic reactions. No commonalities or trends were seen between BIS or PEGDA crosslinked gels or through variations of crosslink density.

Protection through Entrapment

Entrapped enzymes showed retention of activity compared to controls as shown in Table II. The enzyme activity was tested initially and compared to post entrapment activities measured after the pH was raised from 7.4 to 8.0. The positive control tested free enzyme challenged to a pH 8.0 fluctuation. Solution based kinetics were widely affected by the pH showing native enzyme sensitivity. Local pH within the biogel is similarly affected causing the reduced activity. Increasing the crosslink density increased the enzyme stability through the pH challenge. It is presumed that the PEGDA bonds absorb a number of the OH^- molecules leading to partial hydrolysis of the PEGDA crosslinker and stabilizing the pH toward more favorable levels. Relatively large errors can be seen in the data due to the random nature of entrapment being utilized; homogeneously mixed enzyme within the matrix may be more protected from this pH alteration whereas clusters close to the surface may be perturbed to the point of denaturation.

Conclusion

This work demonstrates a repeatable, effective entrapment method that preserves enzyme activity within a favorable environment and resists activity reduction due to pH fluctuations in the system. Lactase entrapped within ACR/BIS showed poor retention of initial and extended enzymatic activity as described by V_{max} retention as compared to ACR/PEGDA hydrogel matrices. PEGDA acts to protect the

enzyme from overall structural degradation, and as PEGDA crosslink density increased between 5-10%, enzymatic retention increased. It is accepted that crosslink densities of 15-40% decrease enzymatic retention due to steric hindrance of the enzyme and decreased diffusion of substrate to the active sites of the enzyme. The optimal crosslink density for lactase activity preservation depends on the anticipated environment, such as extended lifetime or resistance to pH disturbances, and specific application. Future research is aimed toward synthesizing protective matrices to combine multiple protective measures. This entrapment method shows marked improvement upon enzyme entrapment compared to alginate gels and shows promise for use in on-demand applications.

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Figure Captions

Figure 1. Swelling factor as a function of time. (a) Non-degradable gel: 1-20 mole % BIS crosslinked swelling factor over 24 hours. Crosslink density is inversely proportional to initial swelling rate and maximum swelling. b) Degradable gel: 1-25 mole % PEGDA crosslinked swelling factor over 24 hours. Crosslink density is inversely proportional to initial swelling rate and maximum swelling. c) Time lapse imaging show morphological changes of 10 mole % crosslinked PEGDA/ACR and BIS/ACR crosslinked gels as they swell in PBS over a 24 hour period.

Figure 2. Degradation studies in 500 μM NaOH hydrolysis media reported as swelling factor as a function of time. a) 1-20 mole % crosslinked blank hydrogels crosslinked with BIS. Crosslinks did not degrade, thus gels maintain original 24 hour PBS swollen state. N=3 for all samples and all time points. b) 1-10 mole % crosslinked blank hydrogels with PEGDA. N=3 for each sample at each time point. Outliers at 2-4 days are due to rapid degradation of crosslinks leading to high swelling factors before complete dissolution of 1-4% PEGDA samples. c) Time lapse imaging of morphological changes of 10 mole % crosslinked PEGDA/ACR and BIS/ACR gels degrading in 500 μM NaOH over a 6 day study period.

Figure 3. Enzyme distribution characteristics within 5, 20 and 40 mole % crosslinked gels. a) Enzyme distribution as measured through intensity in ImageJ software. b) ImageJ measurements to determine bright intensity segments related to groups of labeled enzymes within the gel matrices and clustering characteristics of the samples. The cutoff value in ImageJ was set to 50 to determine fluorescent enzyme aggregates from the background. Blank gels of each crosslink density contained no bright spots.

Figure 4. Enzymatic V_{max} in (μM/L/min) properties of low crosslink density gel compositions. PEGDA gels outperformed BIS gels. Increasing PEGDA crosslink density enhanced retention up to 10 mole % crosslink density. Positive control is free enzyme kinetic behavior in like conditions.

Figure 5. Enzymatic V_{max} in (μM/L/min) properties of high crosslink density gel compositions. Decreased V_{max} retention is shown in gels with greater than 10% crosslink density.

Table I. Calculated Fickian swelling constant, K_s, for BIS and PEGDA crosslinked gels.

Cross-linker	Mole %	K_s (min^{-1})
BIS	1*	31
	2*	50
	3*	45
	4*	38
	5	41
	10	34
	15	17
	20	-
PEGDA	1*	79
	2*	69
	3*	70
	4	63
	5	45
	10	39
	15	18
	20	15

–R^2 values are below 0.80.

*Gels are mechanically weak leading to large variations

Table II. Enzymatic activity retention.

Sample	V_{max} Retention	Standard Deviation
30% PEGDA	48%	± 18%
35% PEGDA	85%	± 4%
40% PEGDA	91%	± 10%
Control	23%	± 8%

Retention is reported as described in the method as a percentage of initial kinetic activity post 1 minute application of a NaOH solution pH 8.0 and the control is a solution based free enzyme kinetic response to the pH fluctuation challenge.

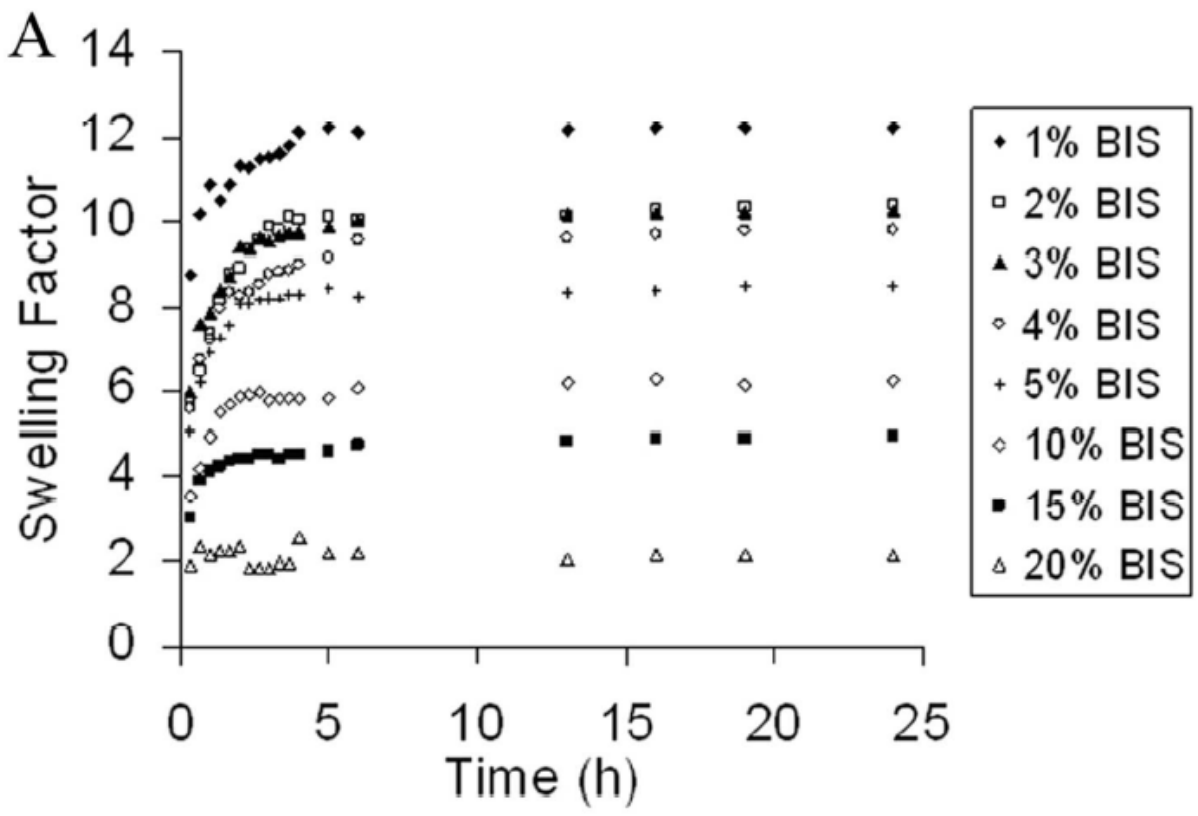


Figure 1a

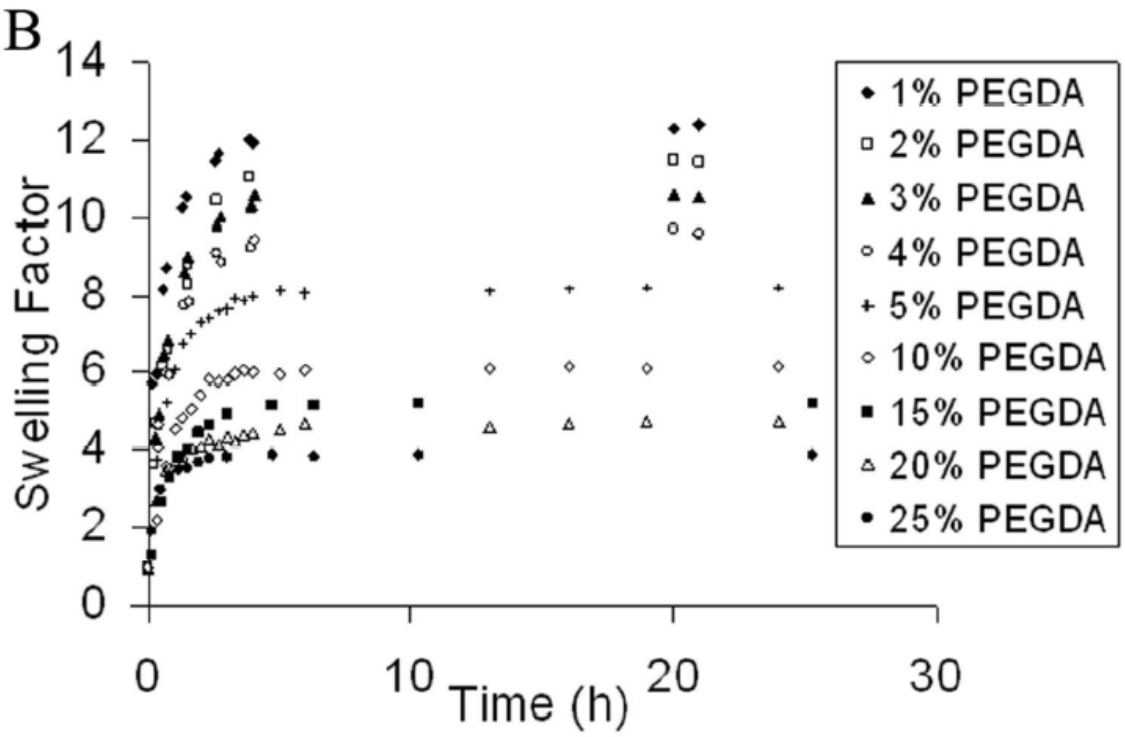


Figure 1b

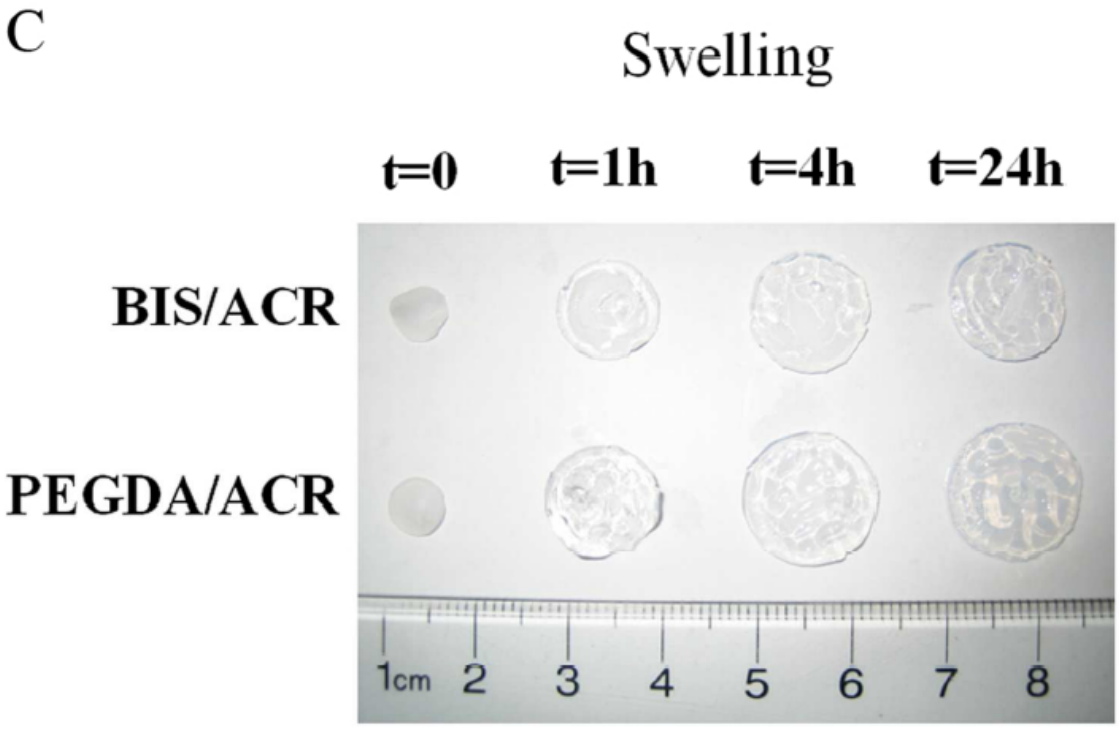


Figure 1c

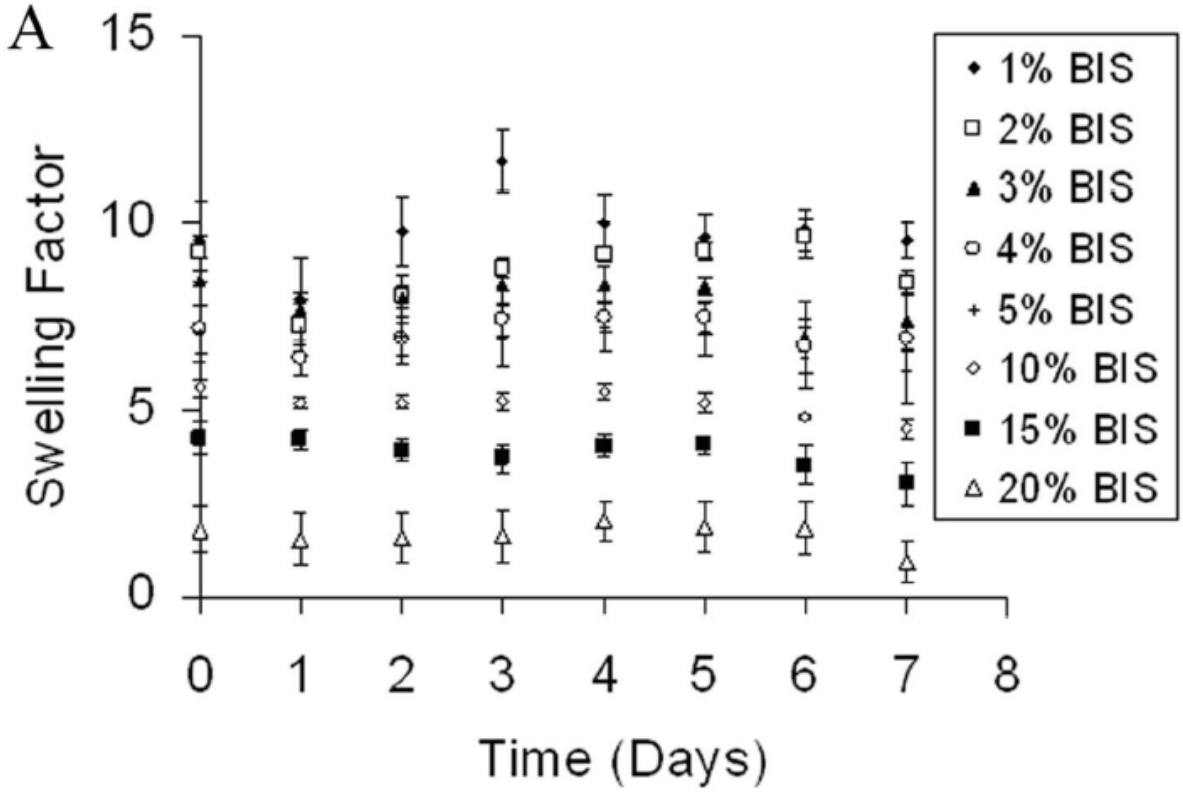
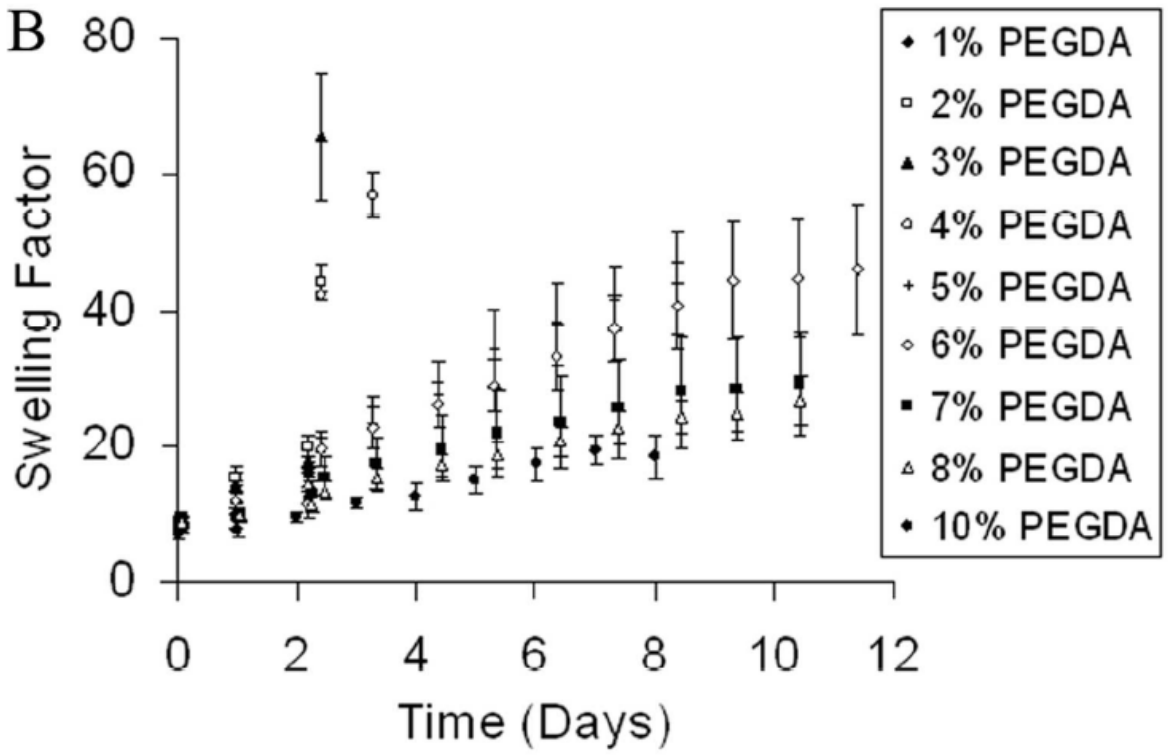


Figure 2a



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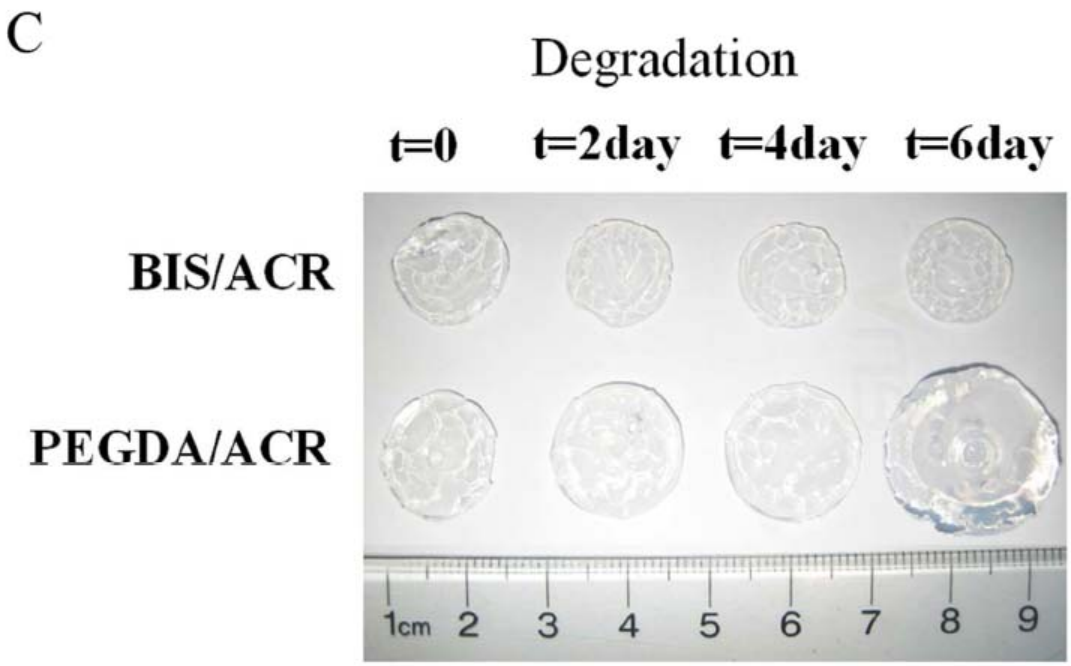

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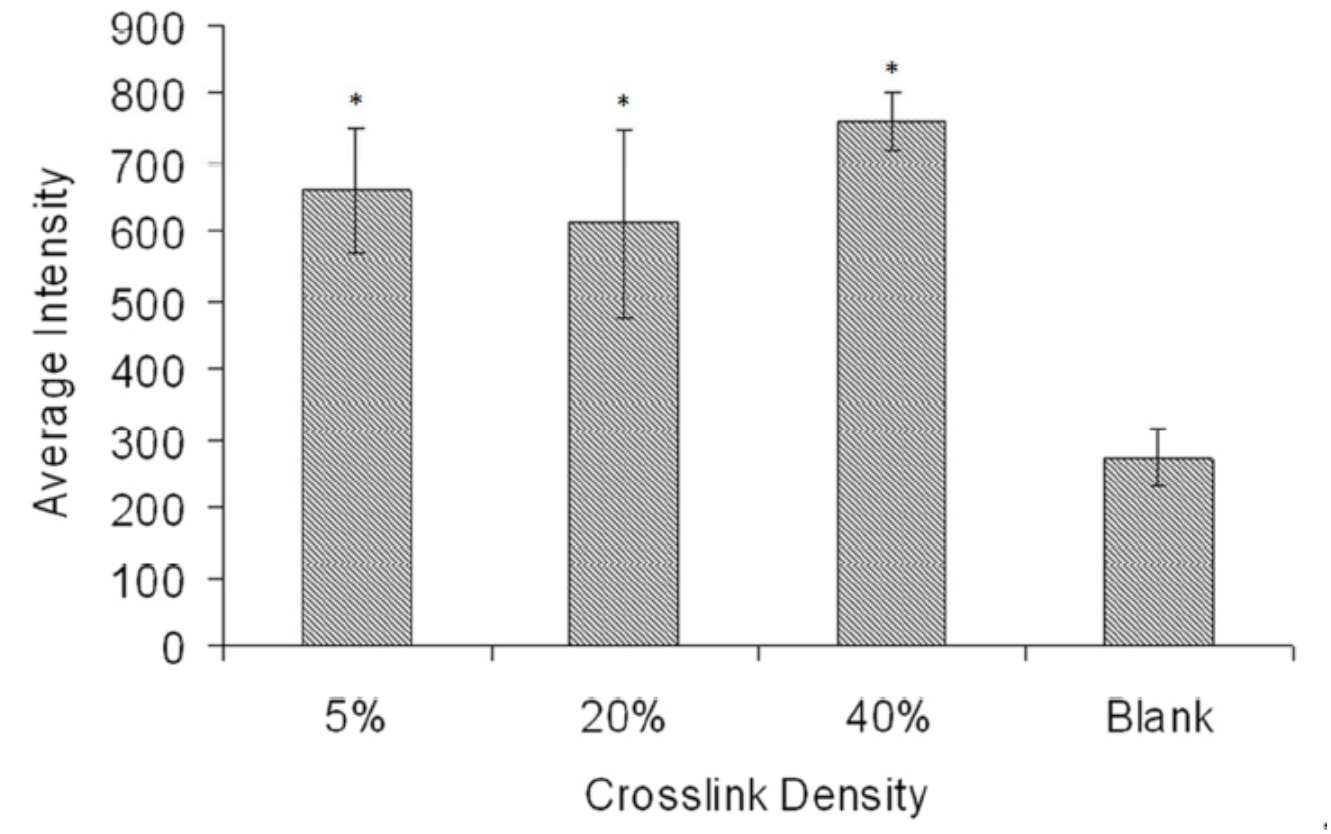

Figure 3a

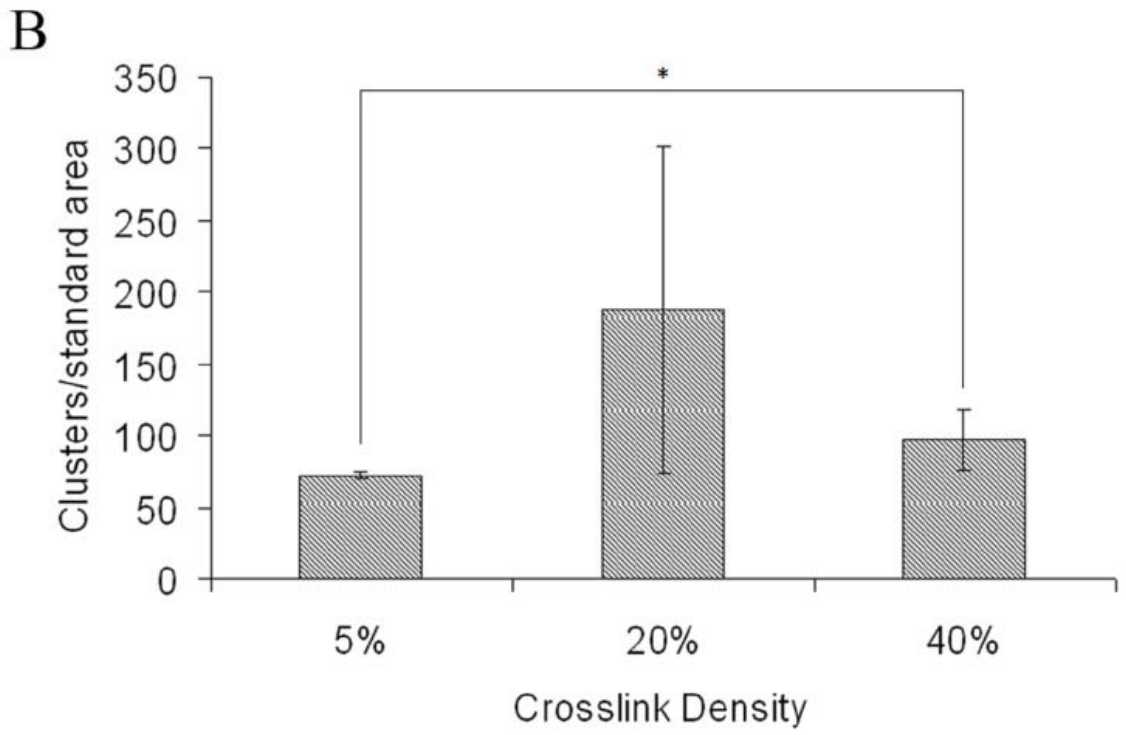

Figure 3b

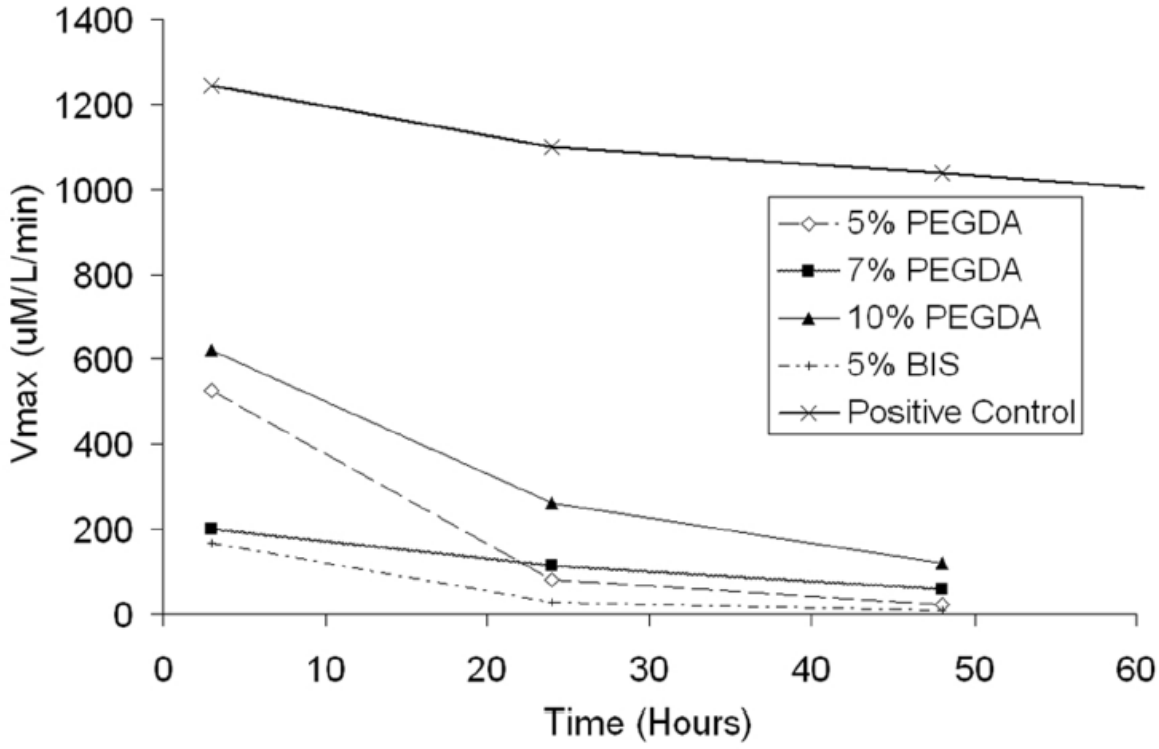

Figure 4

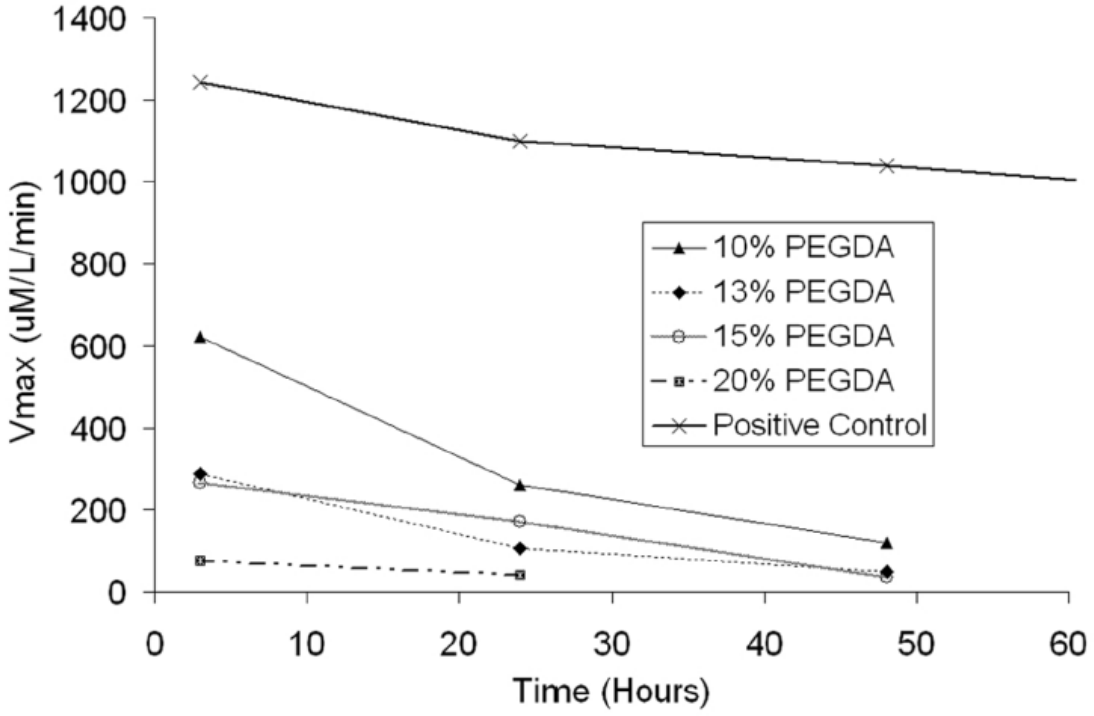

Figure 5