Simple Fabrication of Organophosphorus Hydrolase-Chitosan Non-covalent Conjugate Films for Facile Degradation of Organophosphorus Compounds

A thesis

submitted by

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Abstract

Organophosphorus hydrolase (OPH) is an extensively researched enzyme for several decades for its capability to catalyze the hydrolysis of organophosphorus (OP) compounds. OP compounds have been used as neurotoxic chemical warfare agents such as sarin and also as pesticides such as paraoxon. While being the most potent catalyst for the degradation of OP compounds, OPH faces critical limitations, such as the loss of activity in dry conditions, limited working environment, and long term storage. By combining OPH with a hydroxyl rich biopolymer chitosan, I demonstrate a simple fabrication method which sustains OPH activity in a dry film format. OPH activity is determined by quantitative analysis of the enzyme kinetic. While the loss of activity of dry OPH was observed overnight, OPH embedded in chitosan displayed prolonged activity for over a month. This prolonged stability of chitosan-embedded OPH can be utilized toward facile remediation of contaminated water.

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1. Introduction

Organophosphorus (OP) compounds are neurotoxins commonly used in pesticides and insecticides, and have similar structures as chemical warfare agents such as sarin. OP compounds function as pesticides by inhibiting acetylcholinesterase and causing an accumulation of acetylcholine. The buildup of acetylcholine interferes with muscular responses and may cause death at high concentrations.(Donarski et al.) Its neurotoxic effects impact animals and humans the same way it affects insects. In the United States, OP compounds are the most widely used pesticide, causing surface water contamination in densely agricultural areas. Such surface water contamination runs off into other surface water, stream systems, and urban areas, inhibiting the use of that body of water.(Pedersen, Yeager and Suffet) As an effective biocatalyst that degrades OP compounds, organophosphorus hydrolase (OPH) enzyme has been a major research topic for several decades.(Dumas et al.; Lei et al.; Cao et al.) For example, Chen uses genetic engineering to increase enzymatic kinetics and to express the enzyme on the cell surface to increase the efficiency (Singh et al.) and Constantine entraps chitosan by layer-by-layer technique(Constantine, Mello et al.). While these approaches address the improved use of OPH as a biocatalyst, the processes involved are quite difficult and labor intense.

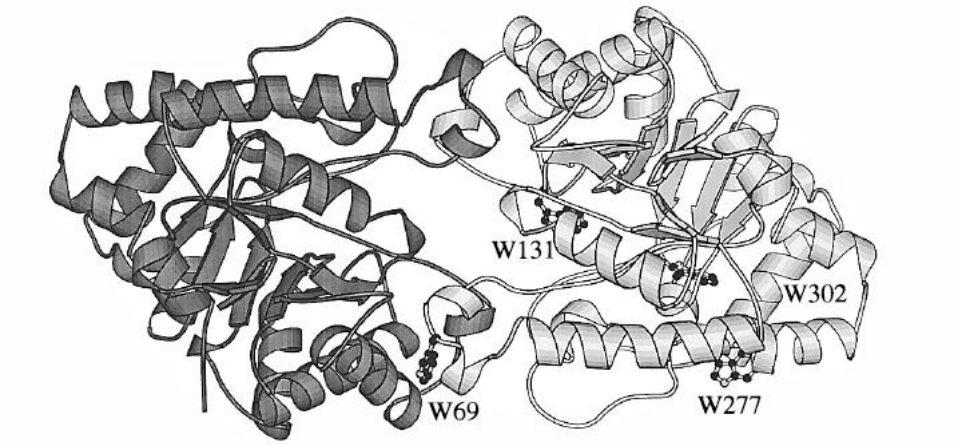


Figure 1. The ribbon drawing of homodimeric OPH generated from crystal structure. (Grimsley et al.)

OPH is a protein (Figure 1); therefore it is prone to thermal degradation at high temperatures and inactivation after long term storage. As a protein that functions in aqueous environments, drying OPH into useful formats such as fibers or films leads to the loss of enzymatic activity. Upstream and downstream processes for the production of protein and the formulation required for storage are expensive. Therefore, there is a need for a novel method to stabilize OPH for long and economical storage in useful formats.

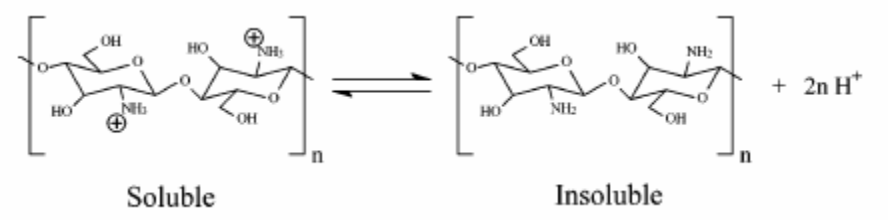


Figure 2. The transition of chitosan from soluble to insoluble form by adjusting the pH. At low pH, the primary amines are protonated and chitosan is soluble. At high pH, the primary amines are deprotonated and chitosan is insoluble. (H. Yi et al.)

The proposed solution is to entrap OPH with biopolymer chitosan to improve stability. Chitosan is a deacetylated form of chitin, a compound found in the shells of crustaceans and in fungus. The N-acetylglucosamine is deacetylated to glucosamine, providing chitosan with ample primary amines. The primary amines allow chitosan to be soluble or insoluble depending on the pH as shown in Figure 2. The pKa of the glucosamine is approximately 6.3 (Park et al.), therefore the transition between insoluble and soluble chitosan happens between pH of 6.0 and 6.5. The choice of chitosan as the biopolymer for embedding OPH is therefore ideal, since the chitosan film is insoluble at the pH of 9.0 for OPH enzymatic activity. As shown in Figure 3, chitosan has two hydroxyl groups per repeating unit, providing a hydrophilic environment even when dry. There are other polymers that have abundant hydroxyl groups such as polyvinyl alcohol and polyethylene glycol, however due to their solubility in water, the produced film will eventually dissolve in the reaction mixture. Due to the pH sensitivity of chitosan, the chitosan film will remain unsoluble under the reaction condition of the OPH enzyme activity assay. Chitosan as a biopolymer also provides a mild environment compared to other polymers, preventing OPH degradation prior to the drying process. By simply mixing OPH and chitosan and drying into a thin film, chitosan should provide a structural support which allows for the reuse of the biocatalyst and provide OPH a hydrophilic environment to sustain enzymatic activity under long term storage. Shown in Scheme 1 is the fabrication method of non-covalent OPH-Chitosan film.

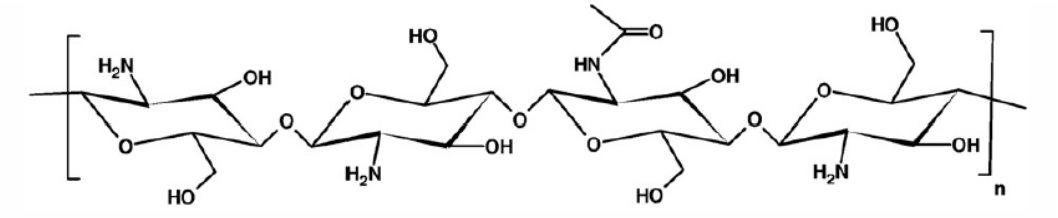
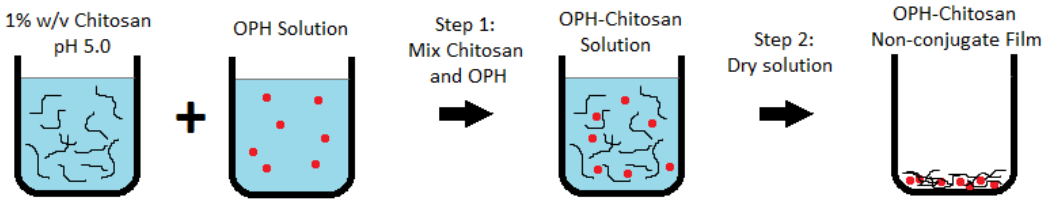


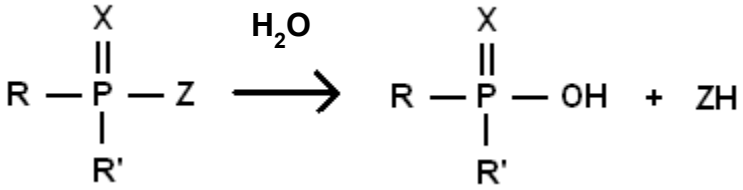
Figure 3. The structure of 75% deactylated chitosan and its deprotonated insoluble form at high pH. There are four repeating units shown with three glucosamines and one N-acetylglucosamine. (Mao, Sun and Kissel)



Scheme 1. The fabrication method of non-conjugate OPH-Chitosan film.

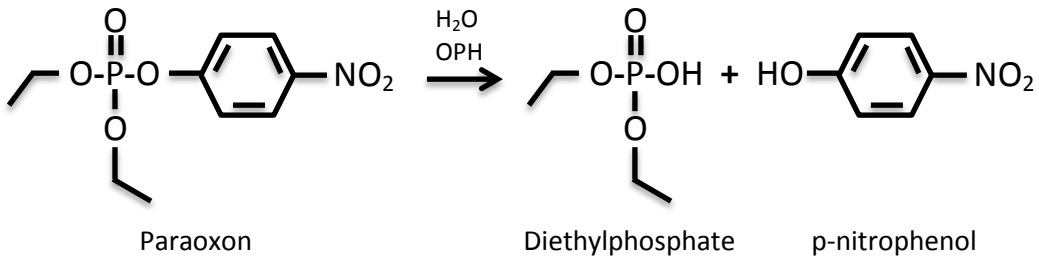
2. OPH Kinetics

Organophosphorus Hydrolase (OPH) is an enzyme that hydrolyzes organophosphorus compounds (OPC). The reaction of the hydrolysis of OPCs with OPH is as follows:



(1)

where X is an oxygen or a sulfur, R is an alkoxy group, R' is an alkoxy or a phenyl group, and Z is a phenoxy group or a fluorine.(Dumas et al.) For all experiments conducted to determine OPH kinetics in my research, the substrate used was paraoxon, where X is oxygen, R and R' are ethoxy, and Z is p-nitrophenoxy. Therefore, the products formed are p-nitrophenol and diethylphosphate as shown in Scheme 2.



Scheme 2. The hydrolysis of the P-O bond in paraoxon by Organophosphorus Hydrolase.

The reaction mixture is a clear solution with some varying level of turbidity, which turns into a yellow solution as the product p-nitrophenol is formed. The formation of the yellow colored p-nitrophenol can be detected using an UV-visible spectrophotometer by reading the sample's absorbance at 400nm. To calculate the rate of product being formed, the change in absorbance of the reaction solution was measured using an UV-visible spectrophotometer over some time interval. The change in absorbance is directly related to the change in product concentration by the definition of the Beer-Lambert Law:

$$Abs = \varepsilon cl \qquad (2)$$

where ε is the molar extinction coefficient 17,000 M^{-1} cm^{-1}, c is the concentration of the product p-nitrophenol, and l is the path length of the UV-visible spectrophotometer beam through the sample.

The rate at which the product is being produced can then be written as:

$$r_P = \frac{dC_P}{dt} = \frac{1}{\epsilon l}\frac{dA}{dt} \qquad (3)$$

By the definition of Michaelis-Menten enzyme reaction, the reaction rate can also be identified as:

$$r_P = \frac{dC_P}{dt} = \frac{v_{max}C_R}{K_M + C_R} = \frac{k_{cat}C_E C_R}{K_M + C_R} \qquad (4)$$

where k_{cat} is the turnover rate in s^{-1}, C_E is the concentration of enzyme in µM, C_R is the concentration of substrate in µM, and K_M is the Michaelis constant in µM.(Fogler) Dumas has determined the k_{cat} to be 2100 s^{-1} and K_M to be 0.09 mM in the case of the hydrolysis of paraoxon(Dumas et al.); however, Omburo reports K_M values ranging from 45±5 to 400±60 µM and k_{cat} values ranging from 1,520±40 to 7,750±270 s^{-1} depending on the metal present in the bimetallic center of the OPH(Omburo et al.). As these values suggest, the k_{cat} and K_M values vary in a wide range with large errors and therefore a single value cannot be assigned as the kinetic parameters for the hydrolysis of paraoxon by OPH.

In the following thesis, the activity of OPH in different conditions and formats will be investigated and compared. The enzymatic activity is defined as the number of product moles produced per unit time; therefore it can be calculated by multiplying the reaction rate by the reaction volume as follows:

$$Activity = \frac{1}{\epsilon l}\frac{dA}{dt} \times Reaction\ Volume \qquad (5)$$

3. Materials and Method

3.1. Chitosan Solution

Chitosan solution (1% w/v) was prepared by dissolving 75-85% deacetylated chitosan (Aldrich) in water by mixing on a stir plate for several days while adjusting the pH to 5.0. Then the solution was filtered and stored at 4˚C.

3.2. Organophosphorus Hydrolase (OPH)

OPH was purchased from Lybradyn (Illinois) for the 96 well plate studies. OPH was produced in the lab for the chitosan films made on polydimethylsiloxane (PDMS) molds. Escherichia coli cell strain DH5α with the expression plasmid pUC19-opd cloned into pTrcHisB (Invitrogen) vector was grown in batch. Luria Bertani (LB) media with agar (Fisher Scientific) with 100 µg/ml ampicillin (Fisher Bioreagents) was poured in petri dishes. E. coli were first grown on LB agar medium supplemented with 100µg/ml of ampicillin using serial dilution streaking method. A single isolated colony was inoculated into a 3 ml tube and incubated for approximately 10 hours at 37ºC and then expanded to 50 ml. All suspension cell cultures were performed in LB media (Difco) containing 50 µg/ml ampicillin at 37˚C.

Cells were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) (Fisher) to initiate production of OPH when cell growth was in the exponential phase, which corresponds to an OD660nm ~0.4-0.8. Cells were ruptured using

primary amine free Bugbuster (Novagen) with an addition of Halt™ Protease Inhibitor Single Use Cocktail EDTA-free (Thermo Scientific). The ruptured cell solution was centrifuged for 10 minutes at 10,000 rpm and the supernatant which includes the OPH was retained and filtered with 0.22 μm filter.

After centrifugation and filtration, OPH in the cell extract was purified using an Immobilized Metal Affinity Chromatography (IMAC) column (GE Healthcare) loaded with copper sulfate. The column was first loaded with 2 ml of 0.1 M copper sulfate and rinsed using 20 mM phosphate buffer saline (PBS). Cell extract was loaded into the column. Loosely bound proteins were eluted with 10 mM imidazole in PBS and OPH was eluted with 50 mM imidazole in PBS. Purified OPH was used immediately or stored at 4˚C.

3.3. OPH Quantification

A Coomassie protein assay kit (Pierce) was used for OPH quantification. 90 μl of 2 mg/ml bovine serum albumin (BSA) standand was added to 4.5 ml of Coomassie blue. The prepared standard was used to serially diluted to create standards of BSA concentrations 2, 1, 0.8, 0.6, 0.5, 0.4, 0.2, 0.1, and 0.01 mg/ml. Test samples were prepared by adding 30 μl of sample to 1.5 ml of Coomassie blue. Mixtures were left at room temperature for 5 minutes to allow reaction. Absorbances of the samples were taken with a spectrophotometer at 595 nm. Concentrations of the samples were calculated by using the standard curve obtained by measuring the BSA standards.

3.4. OPH Chitosan Films in 96 well plate

OPH (Lybradyn, Illinois) was reconstituted to 10 mg/ml with DI water. OPH chitosan solutions were prepared with various OPH to chitosan mass ratios of 1:9, 1:19, and 1:99. Reconstituted OPH and chitosan solution were mixed vigorously by vortexing. 100 µl of OPH and chitosan solution were deposited into the wells. The 1:9 samples were placed in wells B1-B12, the 1:19 samples were placed in wells C1-C12, and the 1:99 samples were placed in wells D1-D12. The 96 well plate was placed in a 30˚C incubator for 24 hours to dry.

3.5. OPH Chitosan Films on Polydimethylsiloxane (PDMS)

To create OPH chitosan films without support, the films were prepared on PDMS molds and peeled off. PDMS molds were created using the Sylgard® 184 Silicone Elastomer Kit (Dow Corning) and curing at 65˚C. The OPH prepared in lab and purified were mixed with chitosan solution with varying OPH to chitosan mass ratios of 1:10, 1:100, 1:200, and 1:400. The OPH and chitosan solution were vortexed and placed on a rotator to mix thoroughly for a couple of hours. The OPH chitosan solution was placed under the vacuum for degassing. Each sample dilution was deposited into the PDMS mold so that each film contained 0.02 mg of OPH. The solutions were left on the bench top overnight to dry. The 1:200 and 1:400 samples were not dry and were placed in a 30˚C incubator overnight. Once all films were dry, the films were peeled off from the PDMS mold and placed in microcentrifuge tubes to prevent films from accumulating moisture from the air.

The concentration of the purified product was determined by Coomassie Blue protein assay.

3.6. OPH Enzyme Activity Assay

The activity of the produced OPH enzyme was determined by a colorimetric assay using a UV-vis spectrophotometer. Using paraoxon as the substrate, OPH hydrolyzes the phosphorus-ester bonds in paraoxon, producing a yellow colored p-nitrophenol that can be detected by measuring the absorbance change at 400 nm. The reaction mixture for OPH enzyme activity assay includes 1 part sample, 1 part 250 mM 2-(cyclohexylamino) ethanesulfonic acid (CHES) buffer (Sigma), and 6 parts DI water. 2 parts 1 mM paraoxon (Sigma-Aldrich) was added to the reaction mixture last and the activity assay proceeded immediately after the addition of paraoxon. The UV visible spectrophotometer was used to detect the absorbance change of the reaction mixture at 400 nm at a given time interval.

4. Results and Discussion

4.1. Stability of OPH in Solution

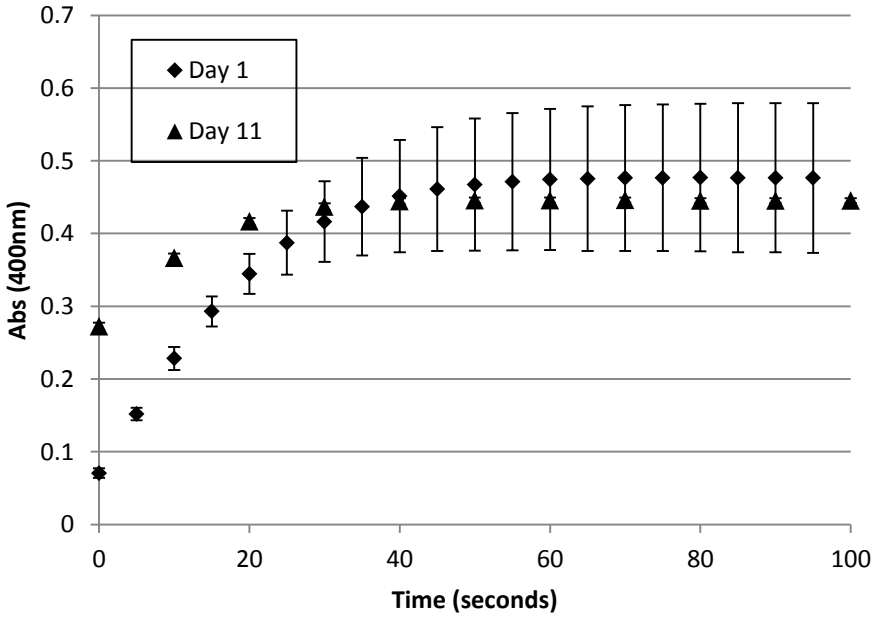


Figure 4. Stability of purified OPH made in the lab over a course of 11 days stored at room temperature.

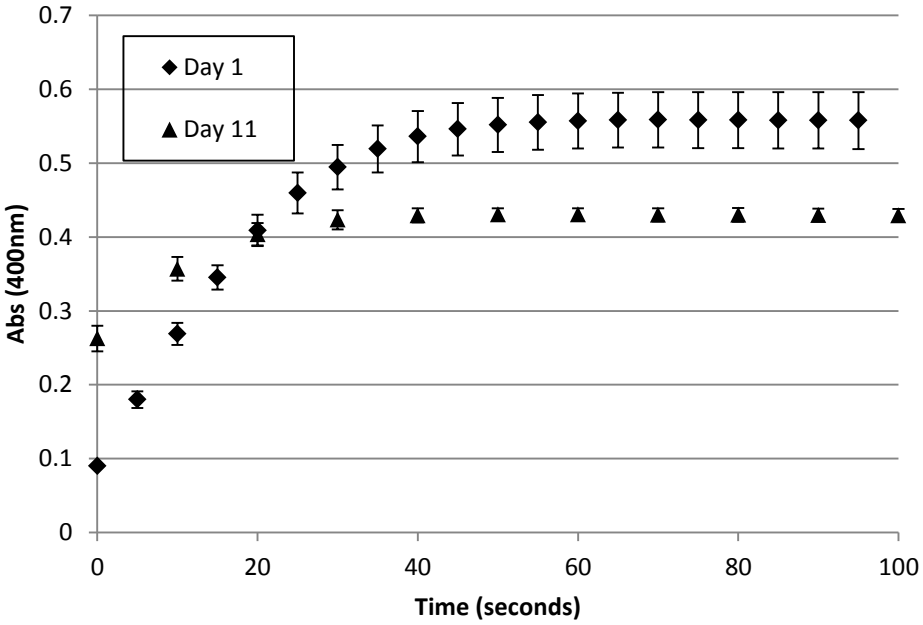


Figure 5. Stability of purified OPH made in the lab over a course of 11 days stored at 30˚C.

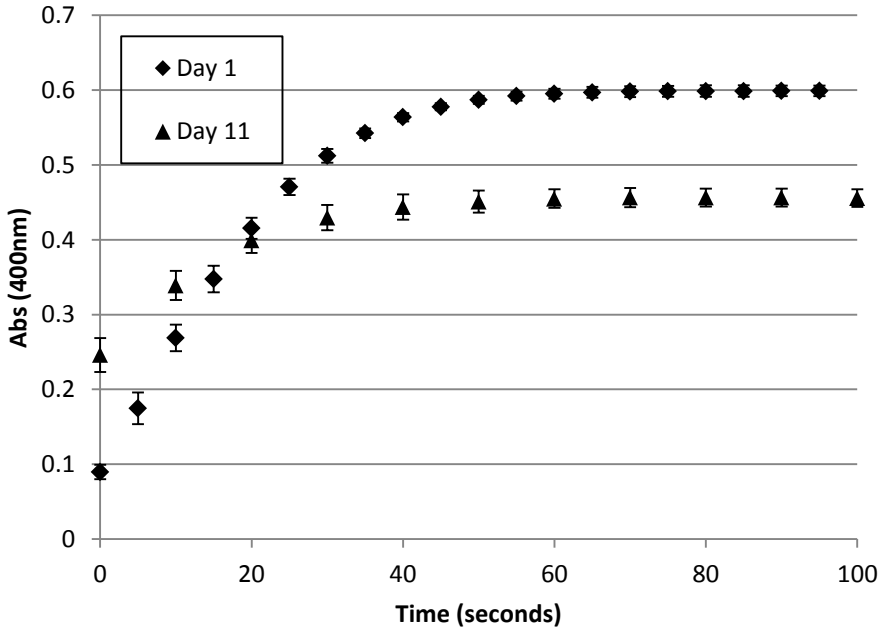


Figure 6. Stability of purified OPH made in the lab over a course of 11 days stored at 37˚C.

In Figure 4, Figure 5, and Figure 6 I demonstrate the stability of organophosphorus hydrolase (OPH) stored in solution at varying temperatures over an extended period of time. To do this, I purified OPH from a batch of cell culture, divided the solution into three microcentrifuge tubes, which were then stored at room temperature or in incubators set at 30˚C and 37˚C for one and 11 days. Activity was measured by adding 10 μg of OPH and paraoxon into a cuvette with a stir bar and the absorbance was measured at varying time intervals in triplicates. First, as shown in Figure 4, Figure 5, and Figure 6, the absorbance increases linearly with time until the absorbance reaches a maximum for all samples. Second, the Day 11 samples start at a slightly higher absorbance than the Day 1 samples. Third, the absorbance for Day 1 samples increase at a greater rate than Day 11 samples. Finally, the maximum absorbance achieved for 30˚C and 37˚C for Day 11 samples is lower than Day 1 in Figure 5 and Figure 6.

Table 1 Activity of OPH in solution stored for 1 and 11 days at room temperature, 30 degrees Celsius, and 37 degrees Celsius.

	Room Temperature		30 Celsius		37 Celsius	
Sample	Day 1	Day 11	Day 1	Day 11	Day 1	Day 11
Activity (nmol/min)	97.4	50.8	113.6	50.1	116.5	54.4

These overall results indicate enzymatic activity of OPH at various temperatures stored for extended period of time. The high initial absorbance of the Day 11 samples is due to the turbidity of samples observed at all

temperature ranges. The turbidity may be due to aggregation of the protein under unfavorable storage conditions, which in this case are the high storage temperatures. The aggregation of the protein seems to have hindered its enzymatic capabilities, as shown in the decrease in enzymatic activity between Day 11 samples compared to Day 1 samples in Table 1. Table 1 shows that at Day 11, roughly half the initial activity is observed for all storage temperatures, yet it is on the same order of magnitude. The higher rate of change in absorbance in Day 1 samples observed in Figure 4, Figure 5, and Figure 6 also reflect the higher activity due to equation (5).

The low maximum absorbance achieved by the 30˚C and 37˚C samples in Figure 5 and Figure 6are most likely due to the paraoxon having a solubility of 2.4 mg/ml and the paraoxon stock available in the lab is approximately 27.8 mg/ml.(Williams) Since the paraoxon is not completely miscible at the condition, the amount of paraoxon measured may differ from batch to batch. As shown in equation (4), the reaction rate is dependent on the substrate concentration and the usage of partially immiscible substrate will affect with obtaining consistent data. In order to prevent inconsistencies due to non-optimal paraoxon concentrations, paraoxon stock solutions should be made lower than 2.4 mg/ml in future experiments.

Using Dumas's reported values of k_{cat} of 2100 s^{-1} and K_M of 0.09 mM and calculating activity according to equation (5), the expected activity is 12.07 µmol/min.(Dumas et al.) This value is 3 orders of magnitude higher than the

calculated activity for 10 µg of OPH via experimental values. As previously mentioned, the catalytic parameters for an enzyme can range widely depending on the reaction condition. Additionally, although I have optimized OPH purification by Immobilized Metal Affinity Chromatography (IMAC), the purified enzyme is not highly pure and the 10 µg of protein may not be purely of OPH. As shown in Equation (4), a lower than expected enzyme concentration due to impurities decrease the rate of reaction. The non-optimal concentration of paraoxon stock solution will also affect the reaction rate as indicated by the role of substrate concentration in the rate equation (4).

Although activity is observed at wide range of temperature and storage time in solution, the OPH will be more easily managed if it can be stored in a light and dry format without the solution. The next step will be to study the activity of OPH in various dried designs in order to achieve high activity OPH that is in readily accessible formats that will be easy to use and store. In summary, OPH in solution displays high activity through 11 days of storage at room temperature, 30˚C, and 37˚C with some loss of activity with time.

4.2. Stability of Dried OPH

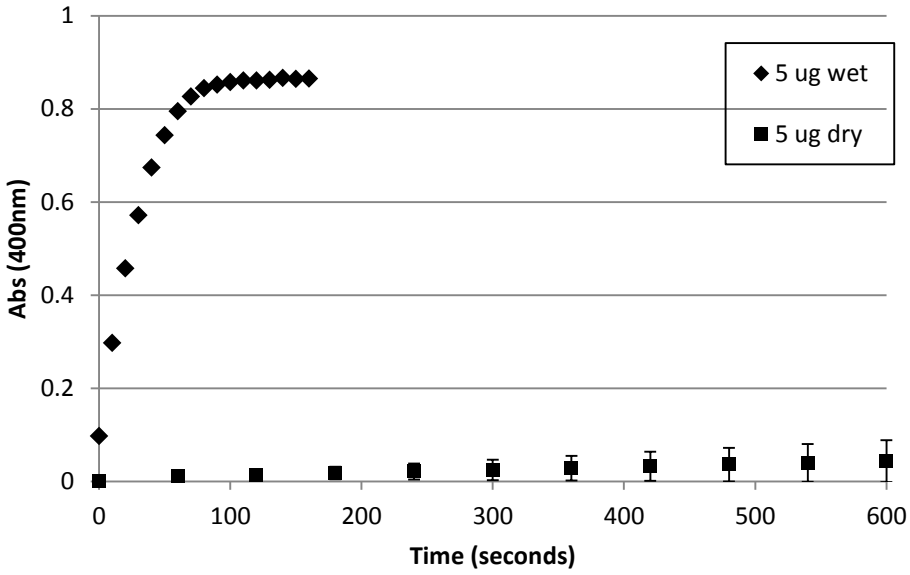


Figure 7. The activity assay results for purified OPH made in the lab and after drying overnight.

Table 2. Calculated enzymatic activity for 5 µg of wet and dried OPH

Sample	Activity (nmol/min)
5 µg Wet	111.5
5 µg Dry	0.4235

In **Figure 7. The activity assay results for purified OPH made in the lab and after drying overnight.**Figure 7, I demonstrate the difference in enzymatic activity of wet and dry organophosphorus hydrolase (OPH) in bulk solution. For this, I used 5 µg of OPH in the wet and dry form. The wet sample is OPH made in the lab stored in the purification buffer. To prepare the dry OPH sample, I measured 5 µg of the wet OPH into microcentrifuge tubes and placed them in a 30˚C incubator overnight until the OPH was dry. The activity was measured in triplicates by adding paraoxon and the sample to a cuvette with a stir bar and the absorbance at 400 nm was read at various time intervals. First, the wet sample shows a steep linear increase in absorbance and reaches a maximum. Second, the dry sample shows a linear increase, but at a very slow rate compared to the wet sample.

The steep change in absorbance for the wet sample indicates a high activity rate at which the substrate is being consumed rapidly. As the substrate is being consumed, the activity slows down until most substrate is converted to product and the absorbance reaches a maximum. The dry samples display an increase in absorbance at a very low rate, indicating the loss of activity in OPH that occurred during the drying procedure. The faster conversion of substrate to product for the wet sample versus the dry sample is reflected on the activity calculated listed in Table 2. The wet sample has four orders of magnitude higher activity than the dry sample. Using reported k_{cat} of 2100 s^{-1} and K_M of 0.09 mM,

the enzymatic activity for 5 μg of OPH is 6.036 μmoles/min.(Dumas et al.) In Table 2, the wet sample has an activity of 0.112 μmoles/min which is relatively close to the expected value of 6.036 μmoles/min. As previously discussed, kinetic parameters for a reaction have a wide range of values and the obtained activity is within accordance to the expected value. On the other hand, the dry sample has five orders of magnitude less activity than the expected value. Evidently, the drying of OPH has resulted in an irreversible conformational change that has permanently affected its ability to catalyze the hydrolysis of paraoxon.

In the attempt of keeping OPH in a dry format, it is evident that there is a need for a stabilizing agent that increases the shelf life, maintains high activity, and even possibly serves as a matrix for reusability of the valuable enzyme. Chen's group has genetically engineered bacterial cells to express OPH on cell surfaces as a support(Mulchandani et al.; Wang, Mulchandani and Chen), Leblanc's group has used layer-by-layer assembly of chitosan and poly(thiophene-3-acetic acid)(Constantine, Gatts-Asfura et al.; Constantine, Mello et al.), and Ackerman's group has entrapped OPH in nanoporous supports(Lei et al.). In the future, I will be developing a quick procedure for entrapping OPH in chitosan in a simple film format. In summary, the results show the significant loss of activity when OPH is in a convenient dry format compared to the enzyme in a buffered solution.

4.3. Fabrication of Organophosphorus Hydrolase (OPH) Embedded Chitosan Films in 96-well Plate

4.3.1. Varying OPH Amounts in Chitosan Films

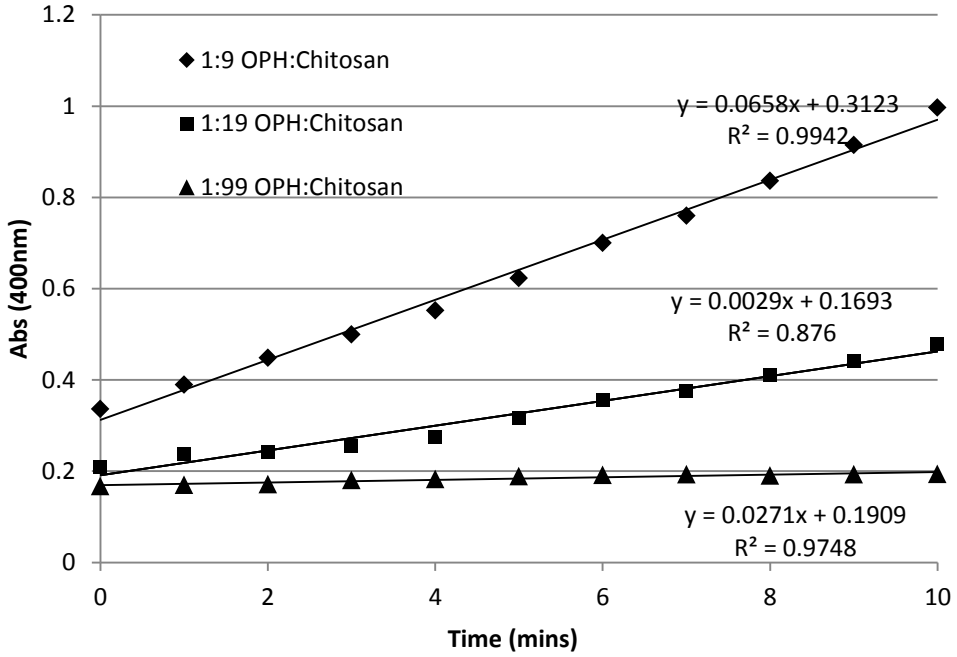


Figure 8. The activity assay of OPH embedded in chitosan film in a 96 well plate with varying amounts of OPH. Films were prepared with OPH to chitosan mass ratios of 1:9, 1:19, and 1:99.

Table 3. Calculated enzymatic activity for chitosan film with varying amounts of OPH in 96 well format.

Sample	Activity (nmol/min)
1:9	7.74
1:19	3.19
1:99	0.34

As shown in Figure 8, I demonstrate the enzymatic activity of chitosan embedded organophosphorous hydrolase (OPH) dry films in a 96 well plate format. To fabricate the films, I mixed OPH and chitosan in varying mass ratios of OPH to chitosan of 1:9, 1:19, and 1:99. 100 µL of each sample preparation was deposited in each well and samples were dried overnight in a 30˚C incubator. This yielded films with varying amount of OPH, with 1:9 films having the greatest amount of OPH and the 1:99 films containing the least amount of OPH. The activity was measured in duplicates by adding the substrate paraoxon to the film and the absorbance at 400nm was recorded at one minute intervals on a plate reader.

First, Figure 8 shows that the absorbance increases quite linearly for all conditions. Second, the absorbance increases more rapidly with higher OPH to chitosan mass ratio. These results indicate catalytic activity of the chitosan embedded OPH and activity increases with increasing amount of OPH. Importantly, OPH maintains activity under dry conditions. Using reported k_{cat} of 2100 s^{-1} and K_M of 0.09 mM, the activities for the 1:9, 1:19, and 1:99 samples are 298 µmoles/min, 148 µmoles/min, and 29.8 µmoles/min, respectively.(Dumas et al.) The expected activity values are five orders of magnitude higher than the obtained enzyme activity listed in Table 3. This can be due to a number of reasons, including mass transfer limitations of the substrate diffusing into the

chitosan film, the activity of the OPH decreasing in the dry format due to insufficient hydroxyl groups provided by the chitosan, and that the reported k_{cat} and K_M values are for bulk solution reactions and the film activity assay format differs significantly from the traditional assay method.

The mass transfer limitation can be investigated by comparing the activity of OPH in chitosan film when one or both sides of the film are exposed to the reaction solution. If the activity assay is hindered by mass transfer limitation, the activity assay completed with both sides of the film exposed to the reaction solution should have higher activity than when only one side is exposed. To determine whether there are sufficient hydroxyl groups provided by chitosan or not, the amount of OPH can be held constant while varying the amount of OPH. If mass transfer limitation does not play a big role, the effect of increasing the amount of hydroxyl groups will be confirmed if activity also increases. In summary, results shown in Figure 8 demonstrate catalytic activity and stability of chitosan embedded OPH in a dry film.

4.3.2. 5 Day Stability of OPH Embedded Chitosan Films

Table 4. Activity values of chitosan embedded OPH over 5 days with varying OPH to chitosan mass ratio of 1:9, 1:19, and 1:99.

	Activity (nmol/min)		
Day	1:9	1:19	1:99
1	6.770	2.204	0.519
2	16.036	8.022	1.397
3	4.571	4.870	0.360
4	2.553	1.083	0.048
5	3.597	1.200	0.481
Average	6.705	3.476	0.561
Standard Deviation	5.443	2.964	0.503

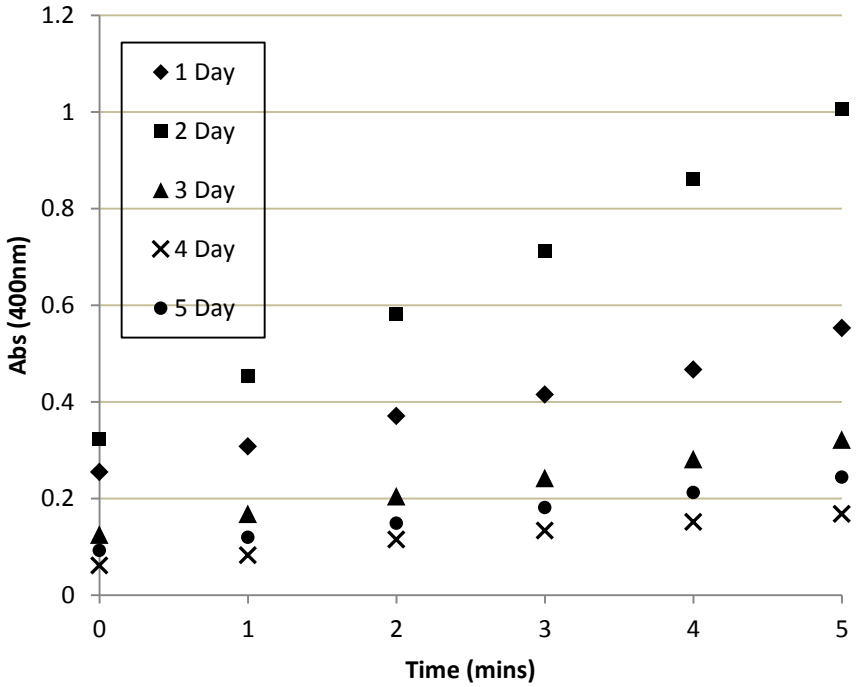


Figure 9. Enzymatic activity assay of chitosan embedded OPH in a 96-well plate stored at room temperature over 5 days with OPH to chitosan mass ratio of 1:9.

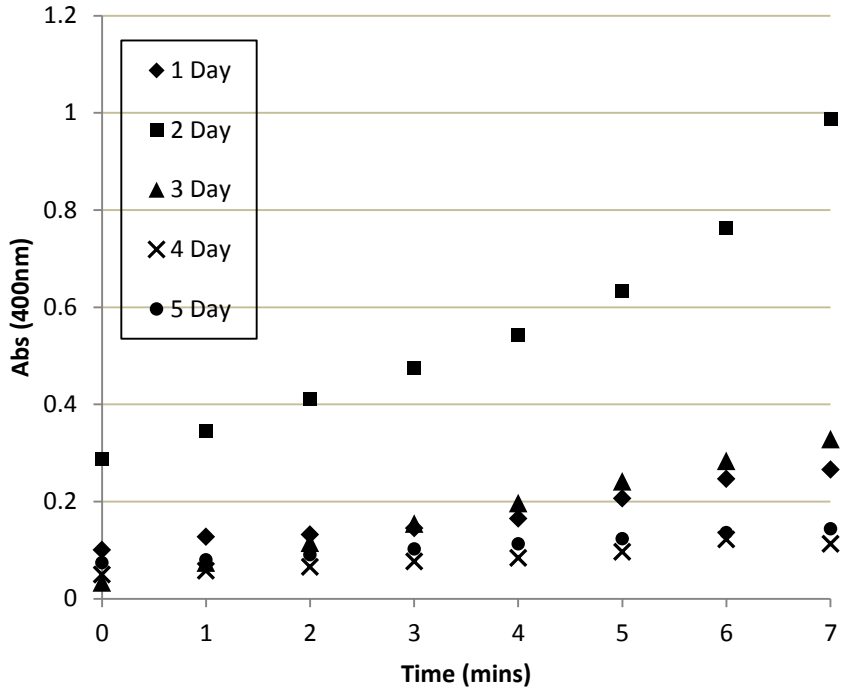


Figure 10. Enzymatic activity assay of chitosan embedded OPH in a 96-well plate stored at room temperature over 5 days with OPH to chitosan mass ratio of 1:19.

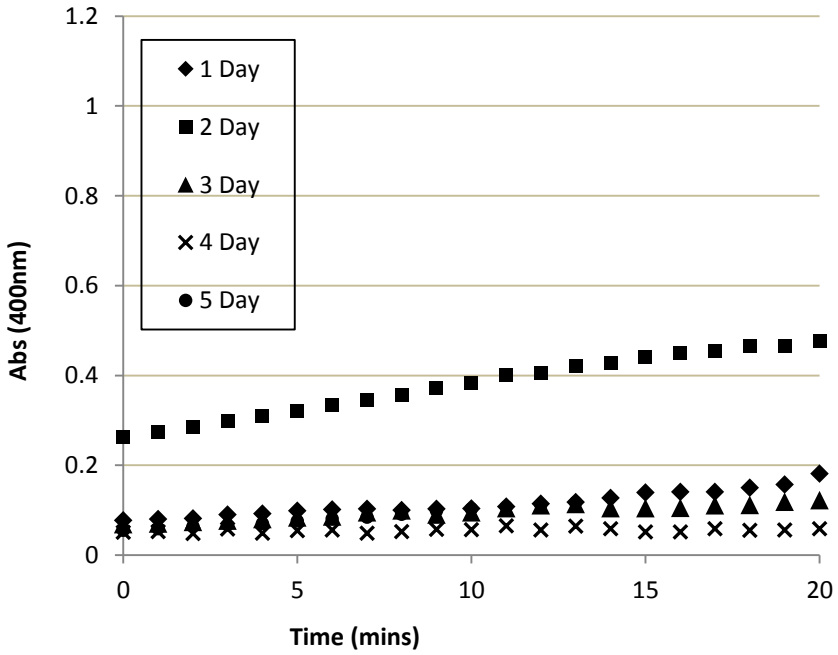


Figure 11. Enzymatic activity assay of chitosan embedded OPH in a 96-well plate stored at room temperature over 5 days with OPH to chitosan mass ratio of 1:99.

In Figure 9, Figure 10, and Figure 11, I show the prolonged activity of OPH in a dry chitosan film in a 96-well plate format. For this, I prepared solutions with OPH to chitosan mass ratios of 1:9, 1:19, and 1:99 and dispensed 100 µl into each well. After overnight drying in a 30˚C incubator, the samples were stored at room temperature. Activity was measured in duplicates by adding paraoxon to the well and the absorbance was measured at 400 nm at 1 minute time intervals. First, absorbance increases quite linearly with time. Second, the change of absorbance over time is highest for the 1:9 sample in Figure 9 and lowest for the 1:99 sample in Figure 11. Third, as depicted by the square data points in Figure 9, Figure 10, and Figure 11, the initial absorbance and the rate of absorbance increase is much higher on Day 2 than on the other days.

The linear increase of absorbance with time indicates prolonged activity of the dry OPH in chitosan film over the course of five days. As indicated by Equation (5), the change in absorbance with time correlates with the reaction rate and enzyme activity. By Equation (5), a higher rate of change in absorbance results in a greater reaction rate and activity, as listed in Table 4. Since the same volume was pipetted into each well, the lower ratio of OPH to chitosan has a higher quantity of OPH in the film than the higher ratio of OPH to chitosan. Therefore, higher activity of 1:9 samples correlates to a greater amount of OPH present in the film compared to 1:19 and 1:99 films and is as expected.

The high absorbance reading for Day 2 in Figure 9, Figure 10, and Figure 11 shown in the square data points may be due to error in setting up the UV-visible spectrophotometer or preparing paraoxon solution. Since every measurement taken on the same day shows the same inconsistency, it is highly unlikely that the measurement is accurate. The error with the UV-visible spectrophotometer may be due to inadequate bulb warming or perhaps the wrong blank was used. The alternate error could be due to the fact the paraoxon stock has a higher concentration than the solubility of paraoxon in water, which was only determined after the experiments were completed. Since the stock solution of paraoxon is greater than the maximum solubility in water, the stock solution is not a homogeneous solution. Pipetting the same volume of stock solution may not yield the same quantity of paraoxon every single time.

In previous studies, the stability of OPH in solution and dried OPH was investigated. The dried OPH lost most activity overnight, whereas the solution OPH maintained half its activity over 11 days. As seen in Table 4, dried chitosan films with OPH maintain activity over five days. Although some loss of activity is observed, the same magnitude of activity is maintained. As dry formats, the OPH in chitosan film are light weight compared to OPH in solution and are easy to store large quantities. In summary, chitosan serves as a stabilizing agent, prolonging the activity of OPH in dry chitosan films over several days.

4.3.3. Reusability of Chitosan Embedded OPH in 96-well Plate

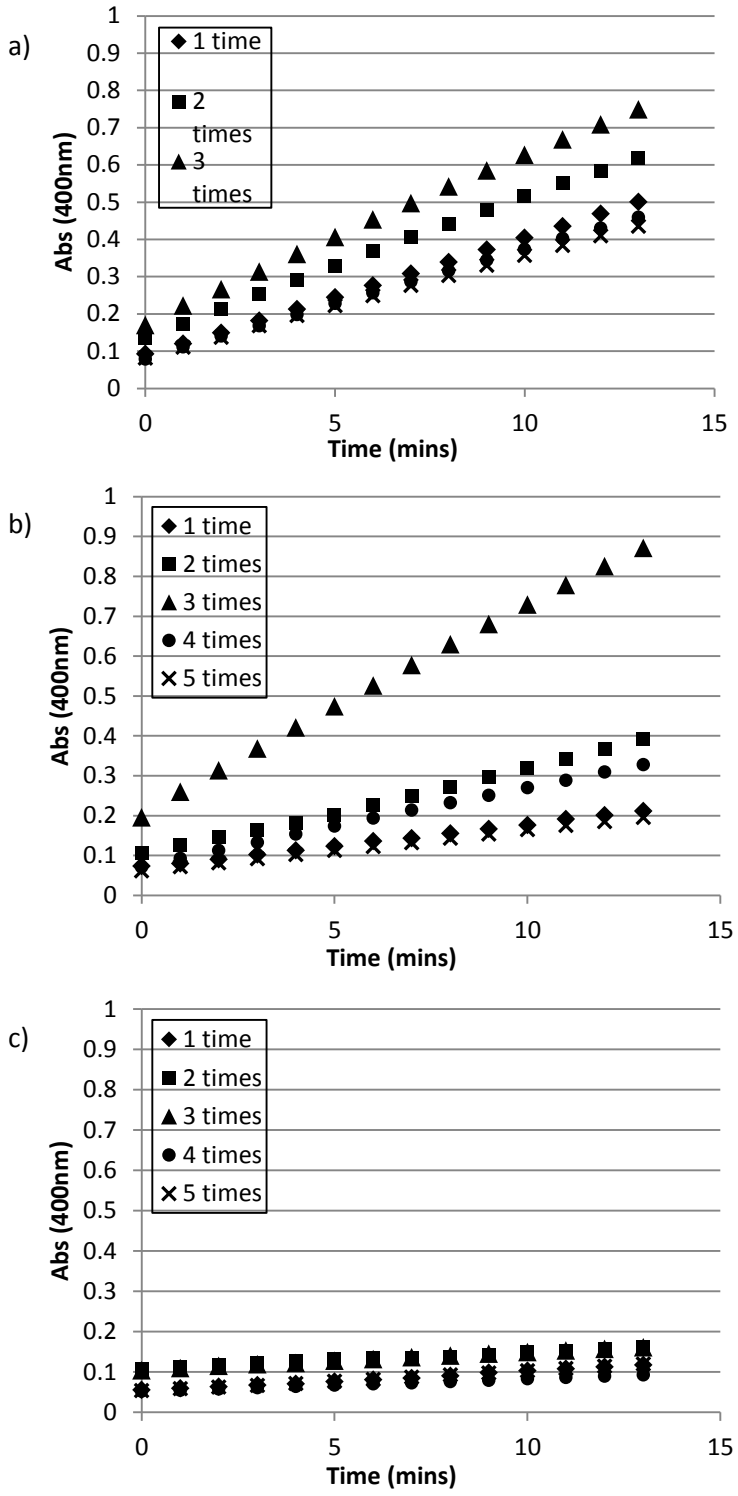


Figure 12. Enzymatic activity assay of chitosan embedded OPH reused once every day over the course of 5 days with varying OPH to chitosan mass ratio of a) 1:9, b) 1:19, and c) 1:99.

Here I demonstrate the reusability of the dried OPH entrapped in chitosan film in a 96 well plate. To do this, I prepared 1:9, 1:19, and 1:99 mass ratio of OPH to chitosan solutions and dispensed 100 µl into each well of a 96 well plate. After overnight drying in a 30˚C incubator, the samples were stored at room temperature. The activity was measured in duplicates by adding paraoxon to the wells and measuring the change of absorbance in between one minute time intervals. Once a film completed an activity assay, I triple rinsed the well with DI water by pipetting up and down and allowed it to dry to repeat the activity assay the following day. Figure 13 shows pre and post activity assay images. In Figure 12, I show the change of absorbance of the reaction mixture over time for the 1:9, 1:19, and 1:99 samples after reusing the film 1-5 times. In Table 5, I show the activity of each sample calculated from the change of absorbance with time with Equation (5). First, the absorbance changes linearly with time. Second, the slope of the absorbance change over time is steeper with the 1:9 samples and flatter with the 1:99 samples. Third, even after multiple uses, all samples with the same OPH concentration display similar activity as indicated by the slopes in Figure 12 and by the activity values calculated from the slopes in Table 5.

Table 5. Enzymatic activity of dried OPH in chitosan films with varying OPH to chitosan mass ratios used 1-5 times.

Number of times used	Activity (nmoles/min)		
	1:9 OPH:Chitosan	1:19 OPH:Chitosan	1:99 OPH:Chitosan
1	3.72	1.27	0.58
2	4.40	2.58	0.48
3	5.24	6.08	0.51
4	3.43	2.32	0.37
5	3.19	1.22	0.60
Average	4.00	2.69	0.51
Standard Deviation	0.830	1.989	0.092

a)

b)

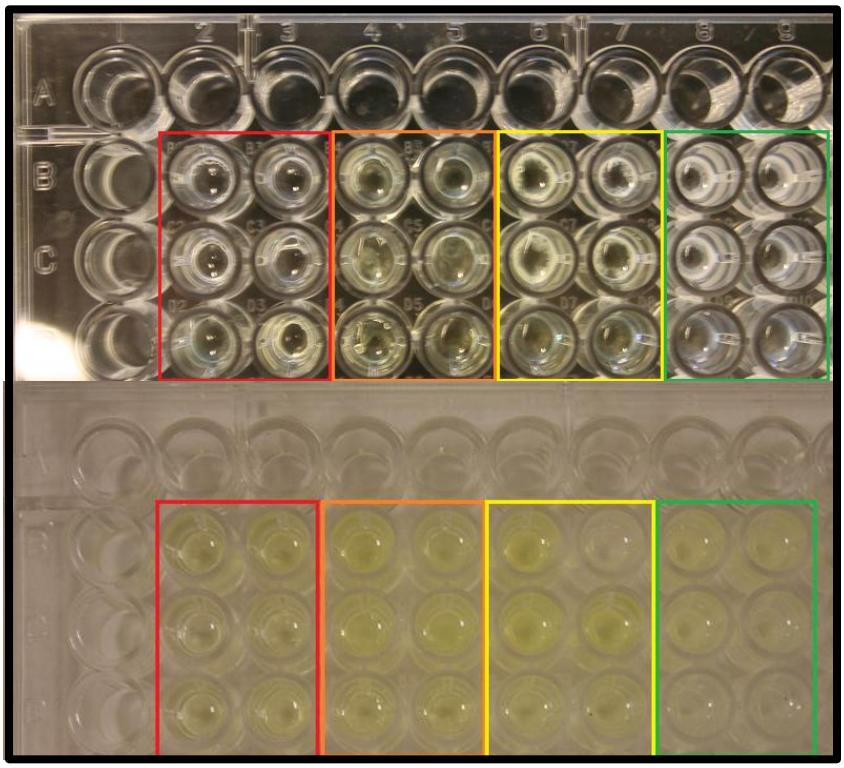


Figure 13. 96 well plate format OPH-chitosan film. (a) Films with reaction mixture minus the substrate. Some wells have varying hue of yellow due to previous usage in activity assay. (Red – 4th, Orange – 3rd, Yellow – 2nd, Green – 1st) (b) Wells after addition of substrate. Each well has an increased hue of yellow, showing that the films can be washed and reused and still demonstrate activity.

The linear increase in absorbance with time in Figure 12 indicates preservation of enzymatic activity as indicated by Equation (5), where the change in absorbance correlates to the amount of product being formed. The decrease in the activity from the 1:9 samples to 1:99 in Table 5 is due to the decrease in slopes from Figure 12a to Figure 12c. Since the same volume of varying concentrations of OPH were used in the study, the 1:9 sample contains the highest amount of OPH and the 1:99 sample contains the least amount of OPH. Therefore, the 1:9 samples are expected to have more catalytic activity due to greater presence of enzyme and the experimental data correspond to the expected outcome.

The similar slopes in Figure 12a, Figure 12b, and Figure 12c for each of the sample reused multiple times indicate that activity is conserved. This is confirmed by the calculated activity values tabulated in Table 5. The 1:9 samples maintained an activity of 4.00 ± 0.830 nmoles/min, the 1:19 samples maintained an activity of 2.69 ± 1.989 nmoles/min, and the 1:99 samples maintained an activity of 0.51 ± 0.092 nmoles/min. The 1:19 sample has a greater standard deviation due to the sample that was reused three times shown in Figure 12b as the triangular data set. The high change in absorbance for that sample is most likely due to insufficient washing of the film after its previous use. The reaction rate's dependency on substrate concentration is described by equation (4), where the substrate concentration appears in both the numerator and

denominator. If unreacted paraoxon is left on the film from insufficient washing and dried so that further reaction cannot occur, the subsequent activity assay will result in a reaction mixture with a greater substrate concentration than intended. The small variation within sample sets maybe due to washing of the film removing some OPH from the film and also the paraoxon stock solution prepared higher than its maximum solubility in water. In Figure 13, the pre-assay wells have a yellow hue from residual product from previous assays. Although product from previous assay is present, the key factor in calculating activity is the change in absorbance. Therefore, the initial presence of the product contributes to higher initial reading and not to the change in absorbance. Another reason will be physical obstruction of the beam path by the chitosan film. As seen in Figure 13, the chitosan film can be observed as opaque white color that is occasionally found in the middle of the well where the UV-vis beam passes through. The 96-well plate format has demonstrated the extended shelf life of OPH in chitosan film as well as the reusability, however addresses an additional concern of measuring the change of absorbance of the reaction mixture by shooting the UV-vis beam through the film.

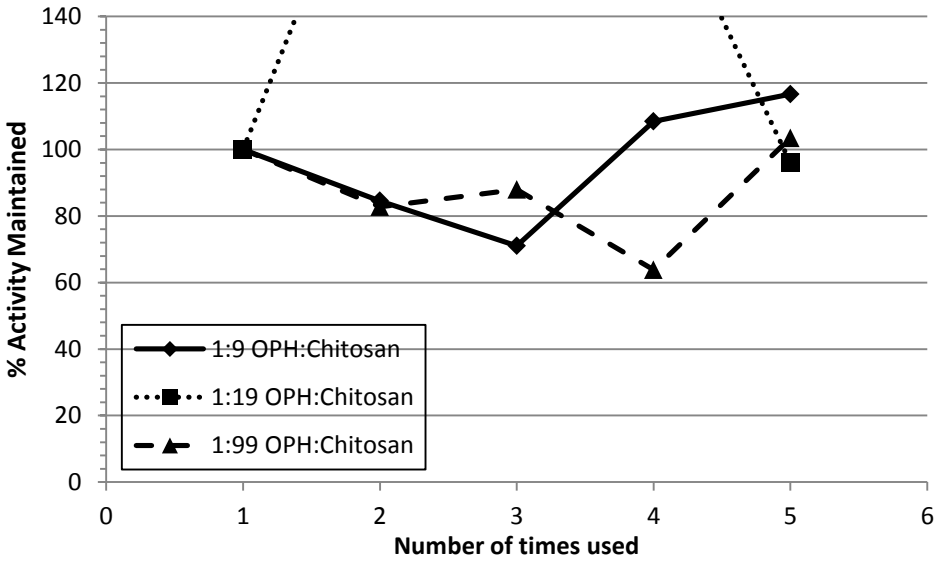


Figure 14. The percent of enzymatic activity maintained for each sample as film was reused.

Taking the activity calculated for the samples used once as the original activity of the film, Figure 14 shows the change in activity as films are reused. With the exception of the 1:19 sample represented by the square data points, the films maintained more than 60% of the original activity after several repeated use and washing. The increasing activity of 1:9 films is probably due to insufficient washing and the UV-vis beam being detected through the chitosan film as described in the previous paragraph. In order to increase accuracy of measuring activity by maintaining a consistent substrate amount, a thorough washing method should be developed. Also, there is an evident need to develop a film and activity assay procedure in which the film is not obstructing the absorbance reading in the future. This finding illustrate that OPH can be stored dried and embedded into a film that allows recovery of the enzyme for repeated use even after washing.

To investigate the effect of washing, a couple of studies can be completed in the future. The water used for washing can be stored to complete an activity assay. If OPH is present in significant quantity in the wash, activity will be observed. If the reaction rate of the wash is of interest, I suggest leaving the wash for several hours prior to an activity assay to ensure full conversion of remaining substrate to product. An alternate method will be to measure activity of a film after a wash and compare activities between pre and post washing. This method might be difficult to identify whether a decrease in activity is due to the

loss of OPH from washing or loss of the enzyme's catalytic ability over repeated use. In summary, OPH dried in chitosan films prepared at various concentrations maintained over 60% of its original activity after multiple uses and washes.

4.4. Organophosphorus Hydrolase (OPH) Embedded Chitosan Films Prepared on Polydimethylsiloxane (PDMS) Molds

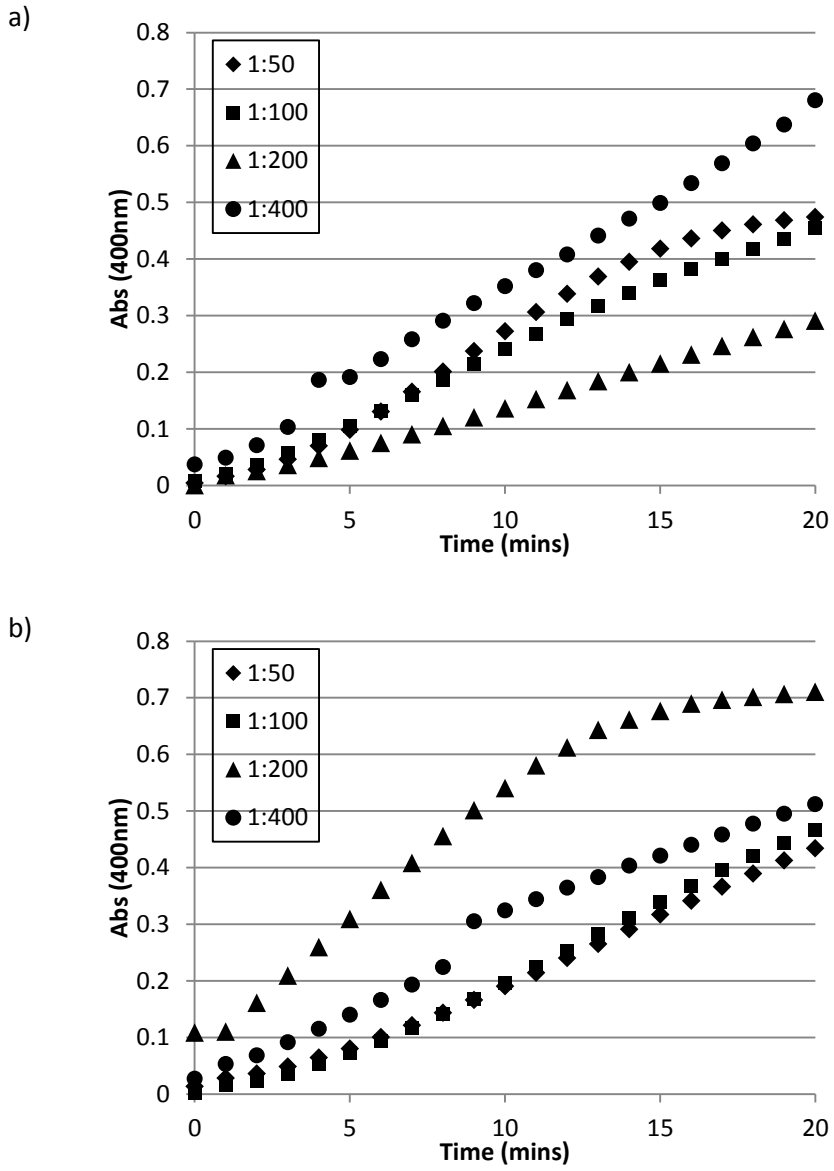


Figure 15. Activity assay of dried OPH in chitosan film prepared with various OPH to chitosan mass ratio. Films were placed in cuvette for activity assay after a) 2 days of storage and b) 33 days of storage.

In Figure 15, I demonstrate the prolonged activity of dried OPH in chitosan film with the constant OPH amount and varying amounts of chitosan. To do this, *Escherichia coli* genetically engineered to produce OPH were grown in flask cultures and OPH was harvested by an Immobilized Metal Affinity Column (IMAC). Varying mass ratio solutions of OPH to chitosan mixtures of 1:50, 1:100, 1:200, and 1:400 were made. After thorough mixing and degassing under vacuum, solutions were dispensed into polydimethylsiloxane (PDMS) molds so that each film had the 0.02 mg of OPH. Once dried the films were removed from PDMS molds and stored in microcentrifuge tubes to prevent films absorbing moisture. The films were held in place in a cuvette by a brass mesh to allow solution to freely mix with a stir bar while preventing the film from blocking the beam path of the UV visible spectrophotometer. Activity was measured by adding the film and paraoxon into cuvettes and absorbance was measured every minute. Dried chitosan films are shown in Figure 16 and post activity assays of the films are depicted in Figure 17. First, within the first five minutes of the activity assay, a lag in the increase of absorbance is detected in Figure 15a and Figure 15b. Second, after the lag the absorbance increases linearly with time. Finally, the enzyme activity values listed in Table 6 are all similar except for the 1:200 samples on Day 2 and on Day 33, where the activity was low and high compared to the other samples respectively.

Table 6. Enzymatic activity of various OPH to chitosan mass ratio on day 2 and day 33 of storage at room temperature.

Sample OPH:Chitosan	Activity (nmol/min)	
	Day 2	Day 33
1:50	3.92	2.74
1:100	3.11	3.12
1:200	1.74	5.80
1:400	3.73	2.85
Average	3.12	3.63
Standard deviation	0.98	1.46

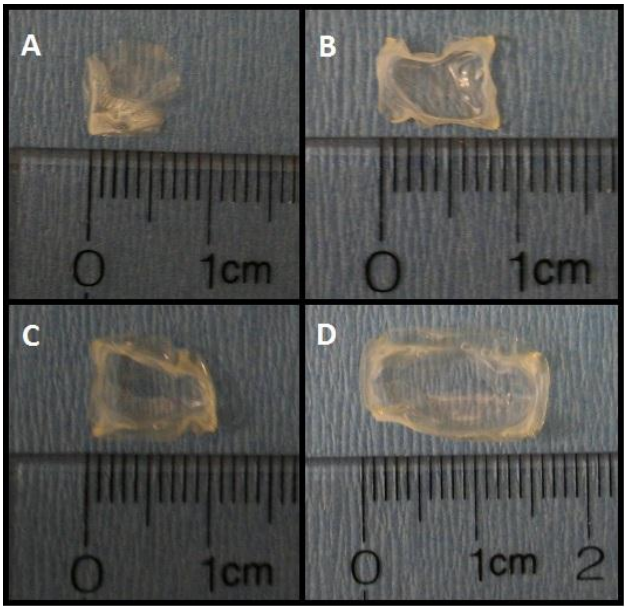


Figure 16. OPH embedded chitosan films with varying OPH to chitosan ratios of a) 1:50, b) 1:100, c) 1:200, and d) 1:400.

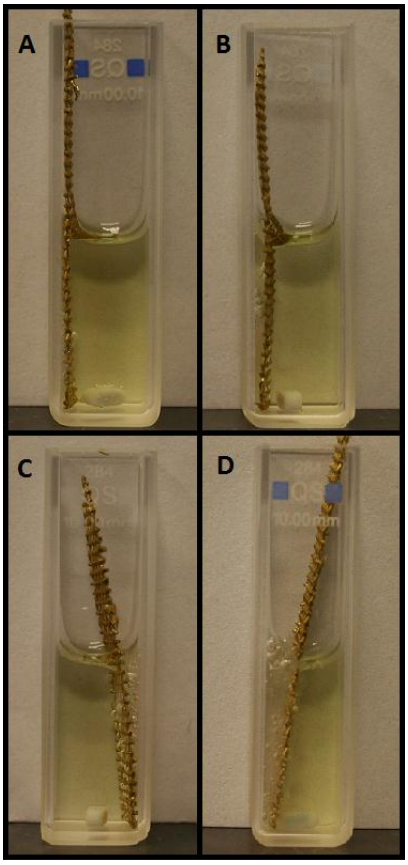


Figure 17. Post activity assay cuvettes with OPH embedded chitosan films with varying OPH to chitosan ratios of a) 1:50, b) 1:100, c) 1:200, and d) 1:400.

The lag in the change of absorbance in the first five minutes of Figure 15 indicates the time taken for the chitosan film to rehydrate. Since molecules cannot readily transport in a dry matrix, the chitosan film must be rehydrated in order for the substrate to come into contact with the enzyme in the chitosan film. As the film rehydrates, the chitosan biopolymer chain expands, the entrapped enzymes become more easily accessible, and substrate is allowed to travel anywhere liquid exists,. As the reaction solution slowly penetrates the film, the absorbance increases non-linearly with time due to the increase in available enzyme. Once the film is fully saturated with the reaction solution, the rate of absorbance change with time becomes linear and constant because the amount of enzyme available to convert substrate into product is constant.

The calculated activity in Table 6 shows that all the films have similar activities, with an average of 3.12 ± 0.98 nmoles/min and 3.63 ± 1.46 nmol/min for samples stored for 2 and 33 days, repectively. This shows that there are sufficient amounts of hydroxyl groups on the chitosan for 1:50 samples to maintain the activity of OPH through 33 days of storage. The 1:400 sample also have similar activity to the 1:50 sample, which indicate that the bloated chitosan film does not interfere with the diffusion of the paraoxon once the film is fully hydrated.

Since the same amount of OPH is present in each film, I expect the activity to be constant. However, I observed some fluctuation in OPH activity,

namely the 1:200 sample on both Day 2 and Day 33. The low activity of the Day 2 1:200 sample may be due to the loss of OPH activity that is sometimes observed as in Figure 18. For some unknown reason, the chitosan film sometimes expands and deforms and OPH activity is lost. A plausible reason maybe the change in pH of the reaction solution assay to assay. Although the reaction solution is kept constant, there may be a different amount of salt present in each film that was used for purification that can change the pH. As previously mentioned in Section 1, chitosan becomes soluble at low pH and insoluble at high pH. The excessive bloating of the chitosan film may be due to lower than ideal pH that allowed the chitosan to go in solution. Further studies on the interactions of chitosan film with the reaction solution should be completed to determine optimal reaction conditions of the film to maintain OPH activity. Because the cause of the excessive swelling of the chitosan film is unknown, there was inconsistent performance of the OPH film from batch to batch. The chitosan films are also very brittle when dry. Peeling the film off the PDMS mold, the film breaks and some pieces of the film is lost. The loss of the pieces may also contribute to the loss of activity. The increase in activity may indicate that a greater amount of OPH is present in the film. This is quite possible, since the chitosan solution prior to drying is very viscous and difficult to manage. Solutions may not be mixed homogeneously despite vigorous mixing, creating films that contain less or more than expected amounts of OPH. However, for practical uses of these films for the bioremediation of water contaminated with organophosphorus compounds, the

precise distribution of enzyme in the film is not critical and should not be an issue.

Once hydrated, the chitosan film also is not structurally sound and the reuse of the film is difficult. OPH may also be lost when the film breaks when it is removed from the PDMS mold. For these reasons, chitosan films should be fabricated on a surface for support if intended to be reused. Since the results indicate that chitosan films require about 5 minutes for the films to rehydrate, the equilibration of the chitosan film in the reaction mixture prior to substrate addition for a minimum of 5 minutes is recommended in order to avoid the lag in the change of absorbance as observed in Figure 15. In summary, I demonstrated the sustained activity of dried OPH embedded in varying amounts of chitosan over a period of time in a film format.

Figure 18. Completed activity assays of 1:50 mass ratio of OPH to chitosan film. Left: Chitosan film expands and OPH activity is not observed. Right: Chitosan film did not expand as much compared to the left figure and activity was observed.

5. Conclusions

In this thesis, I have demonstrated that chitosan can be used as a stabilizing agent to prolong the activity of dried organophosphorus hydrolase (OPH). The observations presented in this thesis clearly show chitosan's ability to stabilize OPH and extend its activity under dry environments by providing a hydrophilic environment through the ample hydroxyl groups present on the chitosan chain. While other researches demonstrate similar results through complex processes, such as OPH expression on the cell surface(Wu, Mulchandani and Chen), the covalent immobilization of OPH to carbon nanotubes(Pedrosa et al.), and entrapping in mesoporous silica supports(Chen et al.), the method I demonstrate is a simple two step preparation of 1) mixing chitosan and OPH and 2) drying the solution.

Without the presence of chitosan, OPH activity declines significantly over a few days. In section 4, the stability of OPH stored in solution without the presence of chitosan at room temperature, 30˚C, and 37˚C shows the activity decreased to 50% of the day 1 activity after 11 days of storage as shown in Table 1. While the OPH in solution maintains some degree of activity, significant activity loss is observed and the activity is expected to keep declining. The dried OPH solution shows less stability, as shown in Figure 7. After overnight drying, the dry OPH loses almost all activity compared to the OPH in solution measured the day before.

OPH embedded in chitosan film clearly shows the extension of OPH activity over long storage. With the proposed simple fabrication method of mixing chitosan and OPH followed by drying, a similar activity value was maintained through storage and there was no sign of sharp decline of activity during the storage. In Table 4 of section 6.2, the chitosan films with OPH in a 96-well plate format maintains activity over a course of five days. In Table 6 of section 7, the chitosan films with OPH have the same average activity after 2 days and 33 days of storage. The dry OPH in chitosan films clearly show the prolonged activity of OPH over a long storage period while the OPH in solution lost 50% of its activity over 11 days and the dried OPH solution completely lost its activity overnight.

Not only does the OPH in chitosan film demonstrate the enhanced stability of OPH at long term storage, it also demonstrates the use of chitosan as a structural support which allows the OPH to be reused repeatedly. In section 6.3, the chitosan embedded OPH films were made in 96-well plates and were repeatedly used after washing. In Table 5, the films maintained its activity after multiple washes, indicating the successful entrapment of OPH in the chitosan film and minimal loss of OPH through washing.

Being the most potent catalyst available for the hydrolysis of neurotoxic organophosphorus compounds, there is a critical need for stabilizing OPH for extended period of time. The agricultural industry uses organophosphorus

compounds as an effective pesticide and therefore it is common to find traces of these neurotoxic compounds in nearby surface water. Also, with increasing threats of terrorist acts, it is critical to be able to have a potent remediation catalyst available and ready for use at any time. The results shown in this thesis demonstrate the ability to store OPH in a lightweight dried chitosan film without the loss of its activity.

6. Future Research

There is a clear need to study the reaction condition of Organophosphorus Hydrolase (OPH) in chitosan film in order to achieve consistent activity. As shown in Figure 18, the success of OPH enzyme activity in chitosan films varies. In some cases, the chitosan film bloated and OPH activity was not observed. Because of chitosan's pH sensitivity described in Figure 2, where chitosan is described as insoluble at high pH and soluble at low pH, the amount of CHES buffer of pH 9.0 used might need to be optimized. Perhaps the insufficient amount of CHES buffer used might be contributing to a lower pH of the reaction mixture, allowing chitosan to be soluble and occupy the entire reaction volume. Since the chitosan solution is very viscous, the lack of activity may be due to the mass transfer limitation of the paraoxon substrate to the OPH enzyme entrapped in the chitosan solution.

The entrapment of OPH in chitosan clearly shows the enhanced enzymatic stability of OPH at dry conditions over long term storage and also the mechanical stability through the reusability of the films. The enhanced enzymatic and mechanical stability of OPH with chitosan is a very exciting development because of the fabrication potentials of chitosan. Chitosan has been used in various fabrication methods such as electrospinning(Desai et al.), forming mats out of fibers(Vondran, Sun and Schauer), and electrodeposition of chitosan(H. M. Yi et al.). The electrospinning of chitosan can provide a high surface area to

volume ratio, therefore the accessibility of substrate to OPH will be greatly increased. The fabrication of mats can be used as filter membranes or textiles for clothing. Biosensors can be made by the electrodeposition of OPH and chitosan solution for the detection of organophosphorus compounds. Utilizing the findings of enhanced stability of OPH by chitosan discussed in this thesis, the chitosan and OPH formulation can be used to fabricate formats other than films in the future.

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