Increasing the Processability of Pullulan for Biological Applications by Changes in Molecular Weight

Increasing the Processability of Pullulan for Biological Applications by Changes in Molecular Weight

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Abstract

Previous studies have shown that pullulan films are able to stabilize enzymes and other labile molecules from thermal and oxidative degradation. Solutions made with commercially available pullulan are extremely viscous and difficult to process limiting the ability to use low-cost printing systems, such as inkjet printers, to format pullulan-containing. In this work, we show that pullulan can be made printable by decreasing its chain length by acid hydrolysis. The acid hydrolysis reaction was modelled using statistical software; the molecular weight of pullulan decreased with increasing reaction time, temperature and acid concentration. Interactions between time and temperature, and temperature and acid concentration were determined to be significant to the reaction as well.

The mechanical properties and oxygen permeability of films made from pullulan with different molecular weights were also measured. The films were found to have similar tensile properties and oxygen permeabilities to each other and to those obtained using native pullulan. Using a thermally unstable enzyme (acetylcholinesterase) and an easily oxidizable small molecule (indoxyl acetate) as test materials, it was found that these films have the same ability to stabilize the enzyme and to serve as an oxygen barrier, as the films made with native pullulan.

It was also found that pullulan is inkjet printable as long as the molecular weight is 56 kDa. Poor jetting and clogging of the printhead was observed when pullulan with a molecular weight higher than this threshold was used. Microarray printing was also demonstrated by a printing acetylcholinesterase/pullulan in nano-sized volumes using a

Dimatix inkjet printer and showing activity of the enzyme after printing and storage at ambient conditions. Proof of concept of microarray printing opens up the potential for future applications of pullulan in other high throughput applications.

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Chapter 1 Introduction

Biosensors are important analytical tools used to detect, quantify or monitor specific chemical species in a variety of environments (Setti, 2005). They are used in a wide variety of industries such as, agriculture, biotechnology and medicine; in fact, a common application of biosensors is used in the detection of blood glucose for screening and treating diabetes (Mohanty, 2006). Biosensors consist of two parts: a biological element that recognizes a specific chemical species and a physical sensor that transduces the recognition of the biological element into a signal (Scheller, 2001; Mohanty, 2006). Enzymes are often used as the recognition element because of their high chemical specificity (Scheller, 2001). However, enzymes are fragile and may be denatured by a variety of conditions; for example: high temperatures, pressure, mechanical stress, humidity, extreme pH (either too acidic or basic), ionic strength or presence of denaturing substances such as, alcohols, heavy metals and detergents (Di Risio, 2007). Enzymes may be covalently or non-covalently modified leading to conformational changes, and aggregation may result as well (Arakawa, 2001). A common method of stabilizing enzymes, and proteins in general, is to include an additive in the solution so enzymes will maintain its native conformation during chemical handling and storage (Arakawa, 2001). For example, cryoprotectants are used to prevent the formation of ice crystals during freezing processes and an antimicrobial agent may be used to inhibit microbial growth (Nishioka, 2004). Generally, additives used to stabilize enzymes are specific to each enzyme and the environmental stresses it may experience (Arakawa, 2001).

1.1 Pullulan

Pullulan, a linear polysaccharide made up of repeating units of maltrotriose, has been found to be a good stabilizer of enzymes and other labile molecules. Due to the periodic presence of α-1,6 glycosidic linkages which allows the backbone structure to be highly flexible, pullulan has greater aqueous solubility compared to other polysaccharides, such as amylose, which are stable over a broad range of pH (Buliga, 1987; Farris, 2014). Pullulan also has other unique properties including, adhesiveness, film and fibre formability, and enzyme mediated degradability (Farris, 2014). Its films have been demonstrated to have good mechanical strength, and good oxygen barrier properties (Farris, 2014; Domań-Pytka, 2004).

Pullulan has been used in a variety of applications, largely in biomedical and food industries since pullulan has been found to be non-toxic, non-mutagenic and edible (Farris, 2014). In the biomedical industry, pullulan has been used in drug therapies, tissue engineering, wound healing and to replace gelatin in the manufacturing of soft and hard shell capsules for pharmaceuticals. In the food industry, pullulan has been used as a thickening, texturizing and gelling agent, and as a filler for low calorie foods since pullulan is resistant to digestive enzymes (Farris, 2014; Domań, 2004). There are also many potential applications to use pullulan in food packaging or coatings due to its oxygen barrier properties and it has been demonstrated to extend the shelf life of certain foods. In a study by Wu et al., a pullulan coating was shown to extend the shelf life of fresh cut apples by reducing moisture and solute loss, and by mitigating gas exchange and

oxidative reaction rates (2013). The coating was also able to reduce the browning of the apple slices since pullulan was able to reduce the permeability of oxygen to the fruit surface (Wu, 2013). The most commercially known application of pullulan is its use in Listerine breath strips. Some applications of pullulan may be limited due to its affinity to water and the neutral charge of the pullulan backbone, as it does not allow for ionic interactions between pullulan and charged polymers or small molecules (Farris, 2014). In addition, some commercial applications may be hindered by the high cost of pullulan, which ranges between $ 25 – 30 USD/kg (Farris, 2014).

Recently, our group has demonstrated that pullulan is able to stabilize a variety of enzymes and labile molecules, in solution and in solid tablet formulations. In 2014, Jahanshahi-Anbuhi et al. created pullulan tablets containing acetylcholinesterase (AchE) and indoxyl acetate (IDA) to facilitate an enzymatic reaction involved in the detection of pesticides. It was demonstrated that the enzyme and labile molecule could retain its activity when stored in pullulan tablets at room temperature for two months. AchE tablets were also stable when exposed to heat up to at temperature of 50 °C for 30 minutes (Jahanshahi-Anbuhi, 2014). In addition, *Taq* polymerase was stabilized in pullulan tablets and retained 90% of its initial activity after storage at room temperature for 50 days, compared to only 40% of activity retained for the non-immobilized enzyme (Jahanshahi-Anbuhi, 2014). Another research group also demonstrated that the thermostability of *Taq* polymerase by pullulan in cross-priming isothermal amplification (Wang, 2014). The addition of pullulan resulted in increased enzymatic activity leading to improved

amplification efficiency and increased nucleic acid yields (Wang, 2014). Kannan et al. used pullulan to stabilize lactic acid and other reagents used for colorimetric detection, to make a point-of-care paper-based sensor for the detection of lactate dehydrogenase (LDH) (2015). The paper-based sensors were stable at 4 ℃ for up to 5 weeks, compared to the assay solution which was only stable for 1 day at 4 ℃. The LDH sensors were not stable at room temperature after 3 weeks of storage (Kannan, 2015). In 2016, Jahanshahi-Anbuhi et al. demonstrated the ability to stabilize luciferase and luciferin in pullulan tablets; all reagents and cofactors of the luminescence assay was incorporated into a single "all inclusive" tablet which could be used to detect ATP (Jahanshahi-Anbuhi, 2016). The detection limit of the tablets for ATP was found to be comparable to that of a highly sensitive commercially available assay kit. It was proposed that pullulan restricts the rotational motion of entrapped proteins so the protein is unable to denature; pullulan has a high glass transition temperature, resulting in restricted protein mobility even at high temperatures (Jahanshahi-Anbuhi, 2016). Most recently, pullulan was used to make another point of care diagnostic paper-based sensor for the detection of DNA or microRNA, using target-induced rolling circle amplification, by encapsulating reagents required for rolling circle amplification in pullulan tablets (Liu, 2016).

Besides pullulan, there are many other additives that have demonstrated stabilization properties. However, they may not have the other unique properties of pullulan such as film formability, aqueous solubility at a broad range of temperatures and adhesiveness. Some of these additives include amino acids, sugars, polymers, polyols, organic and

inorganic salts (Arakawa, 2001). The addition of charged amino acids, such as L-arginine and L-glutamic acid to protein solutions have shown to prevent protein aggregation and precipitation (Golovanov, 2004). Long term stability of the protein solutions was increased because the excess charged amino acids acted as competitive inhibitors to proteases and the free amino acids bound to the surface of the protein which interfered with protein binding (Golovanov, 2004). Glycerol and some sugars such as sucrose, lactose, glucose, trehalose and maltose, are common additives used to stabilize proteins by directly interacting with the protein via hydrogen bonding (Arakawa, 2001). These additives also prevent protein dehydration, which is known to induce conformational changes; it has been shown that native conformation of proteins can be maintained in a dried state if the protein was dehydrated in the presence of a stabilizing sugar (Arakawa, 2001). Compared to lactose, maltose, sucrose and glucose, trehalose was found to be the most effective in stabilizing proteins during dehydration and storage (Sun, 1998). It was proposed that this was due to extensive hydrogen bonding by trehalose which stabilizes proteins, and because it has the highest glass transition temperature, on the basis of mole% of glucose rings, compared to the other studied sugars. This allows for trehalose to remain in its glassy state at ambient temperatures, thus restricting protein mobility, even under these conditions (Sun, 1998).

1.2 Microarrays

One potential application of pullulan, which takes advantage of its stabilization properties, is to stabilize proteins in microarrays. As previously stated, enzymes, and

proteins in general, are delicate and may denature in extreme environmental conditions; they may be more sensitive to non-optimal conditions in microarrays because the proteins are present in small volumes, high concentrations and are typically immobilized onto a solid support (Lee, 2002). An ideal microarray printing system can create a microarray of uniform, dense, small droplets using a minimal volume of solution while preventing solution contamination and protein damage (Barbulovic-Nad, 2006). Additives can be used to prevent biomolecular damage during printing, and solution properties are modified to allow for uniform droplet formation by the printing system.

Microarrays are a two dimensional arrangement of specific biological probes, deposited in onto a surface, which may have binding activity against a library of target molecules (Wu, 2008). Typically, the probes are arrayed in a high density format, with thousands of molecules immobilized in a small area that could range in 50-300 μm in diameter (Barbulovic-Nad, 2006; Wu, 2008). In protein microarrays, the biological probes or target molecules may be enzymes or proteins, which are probed to determine protein function, interactions between proteins and DNA, substrates or other proteins (Lee, 2002). They can be used to screen for protein markers, disease diagnostics and prevention, and drug discovery (Barbulovic-Nad, 2006; Lee, 2002). Nucleic acids, cells and tissues may also be used as biological probes in the microarray. Proteins can be more difficult to print than DNA as the three dimensional structure of proteins must be kept in order to maintain its functionality and proteins are more sensitive to environmental conditions during sample preparation (Barbulovic-Nad, 2006). In addition, protein analytes may exist in very small

concentrations, which may make them difficult to detect, and there are no direct amplification methods for these less abundant proteins (Wu, 2008).

Additives are frequently used in ink solutions when printing microarrays in order for the printed solutions to have ideal rheological properties; viscosity and surface tension need to be optimized to make a solution ejectable (Di Risio, 2007). Additives must also be able to stabilize the protein solution against stresses that would cause denaturation and reduced enzymatic activity. Some additives may be used in high concentrations in order to increase a solution viscosity to a desired value. However high concentrations of additives and high viscosity solutions may be difficult to print due to pin clogging, spot smearing and may result in adsorption of the solution to nozzle walls which may contaminate future printing solutions (Wu, 2008). In addition, high additive concentrations may also result in decreased rates of diffusion of the substrate and products to and from enzyme active sites (Di Risio, 2007).

Common additives used in printing include polyethylene glycol (PEG), ethylene glycol, glycerol, polyvinyl alcohol and carboxymethyl cellulose (CMC). A study by Di Risio and Yan compared the effectiveness of these additives at stabilizing horseradish peroxidase (HRP) and found CMC to be the best viscosity modifier due to its structure and physicochemical properties which allowed for a lower concentration of CMC to be used to achieve optimal solution viscosity and stabilize HRP (2007). 10 wt% glycerol was also used as a humectant to reduce first drop problems, in which ink composition and rheological properties change in the printing nozzle due to solvent evaporation during idle

periods which increases the driving energy needed for drop ejection, potentially leading to nozzle clogging (Di Risio, 2007). Glycerol is frequently used to reduce solvent evaporation of the printed nano-sized droplets (Wu, 2008). It is also able to stabilize some enzymes; Setti et al. used glycerol to print a glucose biosensor containing glucose oxidase in a buffer, with EDTA as an antimicrobial agent, and found that the additive did not affect the activity of the enzyme (2004). The use of glycerol was also demonstrated to decrease mean spot diameter, decrease intra-spot coefficient of variation and allow for increased spotting density of the array (Olle, 2005).

Other additives, that have demonstrated stability properties for enzymes, have also been reported to be suitable for printing microarrays. Nishioka et al. reported using a mixture of 10% trehalose and 1% glucose stabilized their model enzyme, peroxidase, against pressure-induced denaturing during the printing process due to the hydrogen bonding between the sugars and enzyme (2004). One study found PEG, with an average molecular weight of 200 kDa, improved the activity and stability of FITC-BSA by preferential hydration and pertinent hydrophobic interactions with the protein surface (Lee, 2002). Another study used polyvinyl alcohol to print anti-human IL-1B, IL4, and TNFα antibody microarrays (Wu, 2008). The immobilized antibodies were able to retain their activity after one month of storage at 4 °C. Some studies have reported enhanced enzyme activities when low concentrations of polymers or carbohydrates, such as PEG and dextran, are added to enzyme solutions (Di Risio, 2007).

There are a variety of technologies that can be used to print microarrays; they are classified based on whether or not there is physical contact between the printing device and substrate: contact printing and non-contact printing. In contact printing, microarrays are formed by direct contact between a printing device and substrate. The printing device may be a pin, microstamp or nano-tip. Pins may be solid, in which the pin must be dipped into a reservoir after a few spots are deposited, or split, in which the pin has a microchannel that can load samples so more spots can be printed per sample load (Barbulovic-Nad, 2006). With split pin printing, the sample is loaded into microchannel by capillary forces and in order to overcome surface tension forces to dispense the sample, the pin is accelerated towards the substrate and decelerated when the pin touches the substrate surface, printing picolitre- to nanolitre-sized sample volumes (Barbulovic-Nad, 2006). Although the split pin allows for higher throughput of sample printing compared to a solid pin, it is susceptible to tip deformation, spot variations and pin clogging, especially for high viscosity solutions (Barbulovic-Nad, 2006; Rose, 1999). Wider pins tend to be used to reduce clogging, however spot sizes will be larger, resulting in less dense arrays. Nano-tip printing is similar to that of pin printing, but spots are printed on a submicron scale using atomic force microscopy tip. In microstamp printing, samples are adsorbed to a stamp surface and transferred to the substrate by physical contact, allowing for a large number of proteins to be deposited in a parallel fashion (Barbulovic-Nad, 2006). Despite the simplicity and robustness of contact printing, these printers are generally slow, expensive and prone to problems with contamination (Barbulovic-Nad, 2006).

Non contact printing may be more advantageous over contact printing because the printing device and substrate remain separated which allows for reduced contamination and higher printing throughput, especially when many microarray devices can be printed in parallel (Barbulovic-Nad, 2006). One of the most prominent technologies is inkjet printing, due to its commercial usage in office printers. With inkjet printing, droplets are deposited by a pressure pulse, generated within the small orifice of the printhead nozzle, which reduces the space for ink, pushing the solution out of the nozzle. The pressure pulse can be generated by a piezoelectric actuator, in which crystalline materials undergo mechanical stress when an electrical field is applied to it, as is seen in piezoelectric inkjet printers (Setti, 2005). In thermal inkjet printers, the pulse is generated by a vapour bubble forming on the surface of a heating resistor; with increased heating the bubble grows bigger until it bursts, ejecting the ink (Setti, 2005). This technology exposes the biological elements to extreme thermal stresses, as the heating resistor can heat solutions to temperatures ranging from 200 – 300 °C (Setti, 2004). In 2004, Setti et al. demonstrated that printing β-galactosidase (βGAL) with a thermal inkjet printer resulted in a 15% loss of enzymatic activity. They later demonstrated the printing of glucose oxidase with no considerable degradation by depositing the enzyme with a conductive polymer blend of poly(3,4-ethylenedioxythiophene) and polystyrene sulfonic acid (Setti, 2005). In both inkjet printing technologies, proteins undergo shear stresses as they pass through the nozzle and upon impact with the substrate; Setti et al. estimated the shear stress from a thermal inkjet printer was about 10 ms^{-1} (2004). Droplets are ejected at high speeds, about

10 m/s, in order to improve printing resolution and enhance penetration of the ink into paper (Nishioka, 2004). In addition, commercial printers are usually operated under resonance conditions, in which pressure waves are timed to reinforce each other, thus ink solution would undergo even higher shear stresses (Nishioka, 2004). Nishioka et al. demonstrated that more enzymatic activity was retained after piezoelectric printing by decreasing the compression rate of the liquid (2004). They also demonstrated the use of a sugar solution, of 10% trehalose and 1% glucose, in order to stabilize the enzyme, peroxidase, during printing.

Other non contact printing technologies exist, despite the relative inexpensiveness of inkjet printing. Examples of these technologies include: screen printing, photolithography, motion controlled pin printing and electrospray deposition (ESD). With screen printing, parts of a screen are blocked off, forming the negative image of what is printed, which prevents the sample solution from passing through (Setti, 2004). It is a commonly used approach due to its high precision, high speed and relative low cost; however, a screen must be designed for every biosensor that is produced, and inks must be highly specialized (Setti, 2004; 2005). In photolithography, the sample is bound to micrometer-sized open regions, on a glass surface, made from exposure to UV light in a positive photoresist layer (Barbulovic-Nad, 2006). Motion controlled pin printing technology is similar to that of pin printing with a split pin, however the pin does not touch the substrate and the printing system is typically more sophisticated and expensive (Barbulovic-Nad, 2006). Samples are deposited by momentum when the pin is accelerated towards the substrate and stopped before contact with the substrate

(Barbulovic-Nad, 2006). ESD is commonly used to deposit thin films of polymers, semiconductive ceramics and radioactive sources (Barbulovic-Nad, 2006). In this technology, an electric field is activated between the printer nozzle and substrate which drives the sample out of the nozzle; the sample is attracted to the substrate through holes in a dielectric mask (Barbulovic-Nad, 2006).

There are many different types of printing technologies for printing microarrays and biological elements. Each type of technology has its benefits and disadvantages, and the printing technology of choice may be specific to each enzyme solution, the additives used and its ultimate application. Solution concentration and viscosity largely govern its printability, and each printer will have ideal solution characteristics (Xu, 2007). For example, piezoelectric inkjet printers typically require higher ink viscosity, ranging from 5 – 15 cps, compared to thermal inkjet printers, capable of printing solutions with viscosities ranging from 1- 1.5 cps (Di Risio, 2007). The use of additives helps to modify the ink viscosity to achieve optimum conditions for printing and to stabilize the printed biological molecules.

1.3 Degradation of pullulan and other polysaccharides

Ink solutions that contain high molecular weight polymers tend to have reduced printability, due to increased shear viscosities (de Gans, 2004). Intrinsic viscosity is determined by chain length and chain stiffness (Lazaridou, 2003). Pullulan solutions, from long chain pullulan, are highly viscous; they are not a suitable additive for

microarray printing despite its good stabilization properties. Therefore, in order to reduce viscosity of pullulan but maintain the same concentration required to stabilize enzymes and proteins within the pullulan films, the length of pullulan chains would need to be reduced for pullulan solutions to be printable. Some methods of polysaccharide degradation, which are further discussed, include: acid hydrolysis, enzymatic hydrolysis, and thermal degradation. It is also possible to control the production of pullulan at a specific molecular weight by selecting an optimal strain of *Aureobasidium pullulans*, which produces pullulan as an extracellular polysaccharide, and varying the fermentation conditions (Buliga, 1987; Lazaridou, 2003).

Acid hydrolysis of pullulan has not been studied in the literature, but this degradation mechanism has been used to decrease the chain length of other polysaccharides, such as dextran, pectin, starches, and cellulose (Basedow, 1978; Thibault, 1993; Swanson, 1948; Kim, 2012; Fagan, 1971; Torget, 2000). By comparing the glucose content during acid hydrolysis with hydrochloric acid, at 30 °C, of amylose, amylopectin, glycogen, dextran and other starches, Swanson and Cori found that α-1,4 glycosidic linkages were more easily hydrolyzed compared to α-1,6 linkages, since glycogen and dextran had lower rates of hydrolysis (1948). They also found that the location, in the chain backbone or in a branch, and the length of the chain, did not affect the availability of the α-1,4 linkages to acid hydrolysis, since it was determined that the rate of hydrolysis for maltose was the same as that for amylose (Swanson, 1948). By comparing hydrolysis rates for amylose and Schardinger dextrans, cyclic structures which have α-1,4 linkages, it was concluded

that α-1,4 linkages in open chains were more likely to be hydrolyzed compared to the linkages in a cyclic structure (Swanson, 1948). Kim et al. studied the hydrolysis of starches with various amylases content, by sulfuric acid. It was found that acid hydrolysis initially occurs in amorphous regions of polymers, and crystalline regions will degrade later (Kim, 2012). Since pullulan is amorphous, acid hydrolysis should occur throughout the polysaccharide at the same rate (Lazaridou, 2003; Farris, 2014).

Acid hydrolysis can be affected by time, temperature, acid concentration, reactor shape and polysaccharide concentration (Aguilar, 2002; Torget, 2000; Basedow, 1978). Aguilar et al. used three different temperatures and acid concentrations to hydrolyze sugar cane bagasse with sulfuric acid (2002). In each hydrolysis, different concentrations of xylose, glucose, acetic acid and furfural were obtained at various time points; the rate at which the products were obtained also differed between each hydrolysis (Aguilar, 2002). Torget et al. used three different reactor setups, batch, percolation, and continual shrinking bed reactors, to hydrolyze cellulose to yield glucose (2000). The rate of hydrolysis differed for each reactor, and only the continual shrinking bed reactor was able to obtain a glucose yield above 70% (Torget, 2000). A study on the kinetics of acid hydrolysis on dextran by sulfuric acid determined that the hydrolytic degradation of dextran followed first order reaction kinetics with respect to the concentration of dextran (Basedow, 1978).

Thermal degradation of pullulan has also been demonstrated by Strlič et al. In this study, they degraded pullulan standards at 180 °C in oxidative and inert atmospheres, and found that the degradation process was not random (Strlič, 2003). Rather, a multi-modal

distribution was observed and after long periods of time, similar fragment lengths were observed. Thermal degradation was also observed at a lower temperature of 80 °C, in air, in which the degradation appeared to be random. Thermal degradation has also been performed with cellulosic materials (Strlič, 2003).

Pullulan may also be degraded by enzymatic hydrolysis by specific enzymes called pullulanases. There are many different enzymes, capable of degrading pullulan and other polysaccharides. The degradation products made from pullulanases are used in a variety of industries, such as food, chemical and pharmaceutical. For example, they are used to in starch hydrolysis to produce maltose syrups which have high purity of glucose and fructose (Domań-Pytka, 2004; Singh, 2010). One study used pullulanases to break down pullulan to compare the digestibility of long chain pullulan to medium chain pullulan to maltodextrin (Peters, 2011).

There are four classifications of pullulan-degrading enzymes. The first are glucoamylases, which hydrolyze α-1,4 glycosidic bonds at non reducing ends to release glucose (Domań-Pytka, 2004). The second group are called type 1 pullulanases, or true pullulanases. They specifically hydrolyze α-1,6 glycosidic linkages to produce maltotriose (Domań-Pytka, 2004; Singh, 2010). However, type 1 pullulanases are unable to hydrolyze α-1,6 linkages in glycogen or panose (Domań-Pytka, 2004). The third and fourth classification include isopullulanases and neopullulanases, which both hydrolyze α-1,4 glycosidic linkages (Domań-Pytka, 2004). Isopullulanses have been only found in fungi, but are able to degrade pullulan to form isopanose and have not been found to hydrolyze starch.

Neopullulanases degrade pullulan to form panose, and have been found to hydrolyze α-1,6 linkages in some other polysaccharides (Domań-Pytka, 2004). Two other enzymes can be used for pullulan degradation. Amylopullulanase, also known as type II pullulanase, has the ability to break α-1,4 and α-1,6 glycosidic linkages in starch, but in pullulan, this enzyme will only cleave α-1,6 bonds to produce maltotriose (Domań-Pytka, 2004). Lastly, there is pullulan hydrolase type III, which has enzymatic activities to cleave α-1,4 and α-1,6 glycosidic bonds in pullulan to produce maltotriose, panose or maltose (Domań-Pytka, 2004).

The study of the relationship of the molecular weight of a polysaccharide to its functional properties need to be done in order to determine the functionality and potential end use applications of the polysaccharide (Lazaridou, 2003). However, studies using pullulan typically only use pullulan at a high molecular weight; it is unknown if low molecular weight pullulan also have the same physical and chemical properties. Some research that has previously worked with low molecular weight pullulan, include a paper by Buliga and Brant. Working with pullulan of various molecular weights, obtained from Hayashibara, they found a linear relationship between molecular weight and solution properties, when plotted on a double logarithmic plot (Buliga, 1987). The solution properties include intrinsic viscosity, root-mean-square z-average radii of gyration and second virial coefficient. It was also determined that for high molecular weight pullulan, the dimension characteristic ratio reaches an asymptotic value of 4.3 (Buliga, 1987). Previous research done by our group studied the lens formation ability of different molecular weight

pullulan within capillary tubes. Jahanshahi-Anbuhi et al. found that 70 kDa was the lowest molecular weight in which pullulan could form a methanol resistant lens (2015).

A paper by Lazaridou et al. studied the solution rheology of different molecular weight pullulan and mechanical properties of its films (2003). Using a solvent-gradient technique, they were able to obtain three different molecular weight fractions, ranging from 60 – 560 kDa. As expected, low molecular weight pullulan samples had a lower intrinsic viscosity, compared to high molecular weight samples, due to the higher chain flexibility and reduced chain length. As such, the critical concentration, the point where individual pullulan chains start to entangle and onset of significant coil overlap and interpretation occurs, increased with increasing molecular weight. They did not determine a relationship between pullulan molecular weight and glass transition temperatures, although it was observed that all the pullulan samples demonstrated similar water plasticization responses (Lazaridou, 2003). For mechanical testing, Lazaridou et al. only worked with pullulan films with a molecular weight from 99 kDa and above, due to extreme brittleness from its low molecular weight films. From tensile testing, it was determined that tensile modulus, tensile strength and elongation values all increased with increase molecular weight. However, it was proposed that the polydispersity index may also influence mechanical properties (Lazaridou, 2003).

1.4 Objectives

The main purpose of this work was to increase the processability of pullulan in order to print a protein microarray in which the enzymes or substrates were stabilized in pullulan

films. Previous work by our group has demonstrated that pullulan is able to stabilize a variety of enzymes and labile molecules; however, preparation of these tablets was done by hand, which may be difficult due to the viscous nature of concentrated pullulan resulting in variability between tablets. The use of an automated system allows for the deposition of droplets that are uniform, small and in a dense array, which is unachievable if the deposition was performed manually. It also allows for the required volume of enzyme solution to be minimized leading to decreased costs (Barbulovic-Nad, 2006).

First, acid hydrolysis was the mechanism used to break down pullulan into shorter molecular weight fragments. The hydrolysis reaction was statistically modelled using a design of experiments in order to generate a predictive model. In this manner, hydrolysis conditions could be controlled in order to achieve pullulan fragments of desired molecular weight instead of performing acid hydrolysis and seeing what molecular weight fragments are obtained.

Next, the printability of pullulan fragments was evaluated using a piezoelectric printer, and the ability to print a microarray of enzymes in pullulan solution was demonstrated. Here, pullulan was not only used as a stabilizer for enzymes or labile molecules, but it also served as a viscosity modifier for printing. Lastly, the mechanical and enzyme stability properties of the pullulan fragments were evaluated, and compared to the properties of native pullulan. Acetylcholinesterase and indoxyl acetate were used as the model enzyme and labile molecule, respectively, when studying the stabilization properties of pullulan.

Chapter 2 Methods

2.1 Acid Hydrolysis

2.1.1 Experimental setup

Reagents: Food grade pullulan was obtained from Hayashibara. 1-propanol and sodium hydroxide pellets were obtained from Sigma-Aldrich. Hydrochloric acid was acquired from Caledon Laboratories.

Acid hydrolysis: 25 g of pullulan was dissolved into MilliQ water at a concentration of 10 wt% and the pH was adjusted with hydrochloric acid to the specified hydrolysis conditions. Samples were taken at 1, 2, 4, 6, 8 and 24 hours after the addition of acid and the pH was adjusted to 7. Pullulan samples were then precipitated with 1-propanol, in a pullulan to propanol ratio of 3:2. The pullulan/propanol mixtures were centrifuged at 3750 rpm, at 25 ºC for 15 minutes. The propanol was decanted and the remaining precipitate was dried on a Petri dish (Fisherbrand) at room temperature for 3 days in a fume hood and then dried in a heater at 60 ºC for 7 days to evaporate the remaining propanol.

2.1.2 Design of experiments

Preliminary experiments were performed to determine the best method for temperature and pH control and to determine optimal sampling time points, pullulan concentrations and reaction volumes to perform the acid hydrolysis. From these experiments, it was determined that temperature, pH and time were the most important factors to affect the molecular weight of pullulan fragments from acid hydrolysis. A full factorial was set up

to determine the effect of pH and temperature. The effect of time was studied by taking multiple time points for each set of hydrolysis conditions studied. The levels for each variable can be seen in Table 2.1.1 and the order of experiments is seen in Table 2.1.2. A fifth experiment, the center point was also performed, at 45 ºC and pH 1.5. Stir speed, pullulan concentration and reaction volume were kept constant at 200 rpm, 10 wt% and 250 mL, respectively.

Table 2.1.1 Low and high levels for each factor studied in the first full factorial

Factor	Low Level	High Level
Temperature	25 ºC	70 ºC
pH	1.0	2.0

Table 2.1.2 First full factorial experimental design

Experiment Number	Order of Experiments	Temperature	pH
1	3	-1	-1
2	2	+1	-1
3	1	-1	+1
4	4	+1	+1
5	5	-0.1	0

From the results of the initial factorial, another full factorial was performed within the space of the first factorial using the centre point condition from the half factorial. The modified high and low levels for temperature and pH can be seen on Table 2.1.3 and the experimental design is shown on Table 2.1.4.

Table 2.1.3 Low and high levels for each variable studied in the second full factorial

Factor	Low level	High Level
Temperature	30 ºC	55 ºC
pH	1.0	1.5

Table 2.1.4 Second full factorial experimental design

Experiment Number	Order of experiments	Temperature	pH
6	2	-1	-1
7	3	-1	+1
8	1	+1	-1
9	Using data from DOE experiment #5	0.2	+1

2.2 Pullulan Characterization

2.2.1 Viscosity

Viscosity measurements were performed by tuning-fork vibration method using a SV-10 sine-wave vibro viscometer (A&D Company, Limited). Single point calibration was done using water at room temperature. Pullulan solutions, at a concentration of 10 wt%, were analyzed at room temperature.

2.2.2 Gel permeation chromatography

The molecular weight of hydrolyzed pullulan was determined using gel permeation chromatography (GPC). The GPC system consisted of a Waters 515 HPLC pump, Waters plus 717 autosampler, three columns and a Waters 2414 refractive index detector. The mobile phase was 0.5 M $NaNO_3$, 25 mM CHES buffer at pH 10. The system was

calibrated to polyethylene glycol standards so a broad pullulan standard (PolyAnalytik) with a M_w of 30 kDa and a PDI of 1.74 was used to adjust the calibration for pullulan solutions. 20 µL of pullulan samples at a concentration of 5 mg/mL were injected and allowed to run through the system for 60 minutes.

2.2.3 Mechanical testing

Film formation: Pullulan films were formed by casting 7.5 mL of 10 wt% pullulan of various molecular weights into 8.5 cm diameter Petri dishes (Fisherbrand), and allowed to dry overnight at ambient conditions. Once dry, films were cut into test specimens 8.5 cm in length, with a shoulder width of 2 cm and gauge width of 1 cm, as seen in Figure 2.2.1. The thickness of the specimen gauge was measured with a digital micrometer (Testing Machines Inc.) at three different points and an average thickness was obtained.

Mechanical testing: Prior to tests, specimens were stored at 23 °C, 50% RH for two weeks. Tensile tests were also performed at these conditions, using an Instron 4411 universal testing system (Instron ®) with a 50 N load cell. The tensile modulus and maximum tensile strength were calculated from the initial slope of the stress-strain curve and the maximum load.

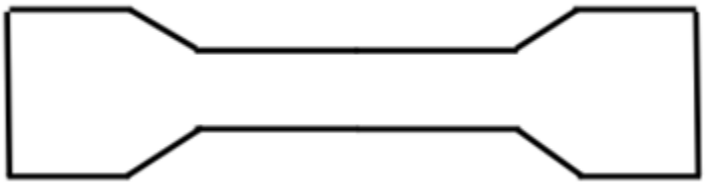

Figure 2.2.1. Tensile testing specimen template

2.2.4 Oxygen permeability

Film formation: 1.5 mL of 10 wt% pullulan of various molecular weights were cast into Falcon tissue culture dishes, 35 mm in diameter, and allowed to dry overnight under ambient conditions, forming a film at the bottom of the dish. The thickness of the film was measured with a digital micrometer (Testing Machines Inc.) at three different points and an average thickness was obtained.

Permeability testing: For permeability measurements, the circular film was attached to the end of one neck, 24 mm in diameter, of a three-neck round bottom flask, taking care to seal the film to the neck using aluminum foil adhesive and electrical tape. Nitrogen was bubbled into 100 mL of water in the flask, to reduce the oxygen concentration (below 0.5 mg/L). The pullulan film covering an opening of the flask was left at ambient conditions over 24 hours to allow the oxygen in the atmosphere to return to the flask and dissolve into the water. The concentration of dissolved oxygen was monitored using an Orion 083005D probe connected to an Orion 850 dissolved oxygen meter (Thermo Fisher Scientific) while the water was stirred at 200 rpm.

2.2.5 Printability

Pullulan printability was evaluated on 10 wt% solutions of pullulan at various molecular weights. The solutions were filtered with a 0.2 µm membrane prior to printing. A Dimatix Materials Printer 2800 series (Fujifilm) was used to evaluate the printability of the pullulan solutions from a piezoelectric inkjet printer. 1.5 mL of the pullulan solution was

loaded into the Dimatix 11610 cartridge and solutions were fired at room temperature, with a voltage of 40 V. The DropWatcher camera was used to view and image the number of nozzles that pullulan was ejected through.

2.3 Labile Molecule Stabilty

Reagents: Acetylcholinesterase (AchE) from *Electrophorus electricus* and indoxyl acetate (IDA) were obtained from Sigma Aldrich. Tris(hydroxymethyl)aminomethane (Tris) was acquired from BioShop Canada Inc and used at a concentration of 0.1 M, pH 8.9. Methanol (MeOH) was from Riedel-de Haën. Pullulan of various molecular weights was used at a concentration of 10 wt%, dissolved into Tris.

2.3.1 Acetylcholinesterase

Pill Preparation: 1 µL of 250 U/mL AchE in Tris was dissolved into 9 µL of pullulan at 8 different molecular weights. The AchE/pullulan mixture was dried at the bottom of a 96 well plate, forming pullulan pills encapsulating the enzyme, and stored at 23 ºC, 50% RH. The enzymatic activity of the pills was evaluated over a period of four months to determine the amount of residual active AchE encapsulated in the pills. Negative controls were also done, in which AchE was dried to wells without pullulan.

Enzymatic Activity Testing: Residual AchE activity was tested for every molecular weight pullulan studied at every time point. 99 µL of Tris buffer was added to each well to dissolve the pullulan encapsulated enzyme and the 96 well plate was shaken for 5

minutes prior to addition of IDA. 1 µL of fresh IDA at a concentration of 125 mM in MeOH was added to each well and the absorbance of each well was evaluated at 605 nm by an Infinite M1000 spectrophotometer (Tecan) every minute for one hour. Samples were shaken for 30 seconds, 2 cm amplitude, between every absorbance reading. The reaction rate was evaluated by the change of absorbance over time and the initial rate of reaction was compared between the various pullulan molecular weights studied.

Paper Preparation: 250 U/mL AchE in Tris buffer was mixed with 10 wt% pullulan with a molecular weight of 56 kDa in a ratio of 1:9 AchE to pullulan. 1.5 mL of the AchE/pullulan mixture is filtered with a 0.2 µm membrane and loaded into a DMC-116010 cartridge. The mixture was ejected from the Dimatix cartridge onto Whatman No. 1 paper, in a 5 by 5 array of squares. Each square was 1 mm by 1 mm in size with a distance of 5 mm between each square, as seen in Figure 2.4.1. Each array of squares was cut into a square measuring 30 mm by 30 mm.

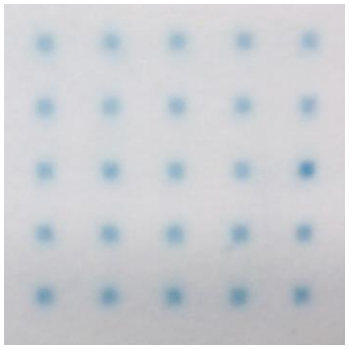

Figure 2.3.1. A template of the arrangement of the AchE/pullulan mixture printed onto Whatman No. 1 paper from a Dimatix printer.

Enzymatic Activity of AchE on Paper: The paper arrays were stored at room temperature or in a heater at 30 ℃ or 40 ºC. To react the AchE, the paper arrays was submerged in a 1 mL mixture of 125 mM IDA in methanol and 0.1 M Tris buffer at a ratio of 1:100. The paper arrays were removed from the IDA mixture and allowed to dry while photos were taken every 2 minutes, for 1 hour, using an iPhone 4S camera. Images were analyzed with ImageJ to determine the rate of reaction of AchE and IDA.

2.3.2 Indoxyl acetate

Solution & Pills Preparation: 125 mM IDA in MeOH dissolved into 10 wt% pullulan, at a ratio of 1:9 IDA to pullulan, was stored in Eppendorf tubes or cast onto an overhead transparency (Xerox). Only pullulan of 150, 106, 75, 56 and 43 kDa were used in pill stability (because at 23C, 50% RH, 43 kDa was the lowest MW at which films can form). Pills were allowed to dry overnight and then removed from the transparency and stored in glass vials.

Self-Hydrolysis Tests: IDA solutions were prepared in 10 wt% pullulan or in Tris buffer or kept in methanol and stored into Eppendorf tubes at ambient conditions. Eppendorf tubes were imaged with an iPhone 4S camera every hour for 24 hours. Images were analyzed for the rate of colour change using ImageJ to determine the rate of self hydrolysis by IDA.

Room Temperature Stability Tests: IDA solutions and pills were stored on the bench top at 23 ºC, 50% RH. 10 µL of IDA-pullulan mixtures was reacted with 1 µL of fresh AchE at 250 U/mL and each well was topped with Tris for a total volume of 100 µL. Pills were dissolved with 100 µL Tris and then reacted with 1 µL fresh AchE. Absorbance values were evaluated at 605 nm for one hour with an Infinite M1000 spectrophotometer (Tecan) to determine the reaction rate. IDA solutions were tested over a period of 1 week and IDA pills were tested over a period of 4 months.

Relative Humidity Tests: IDA pills were stored in the fridge in a desiccant container until use. Prior to tests, the IDA pills were stored in a humidity chamber (Espec) for 24 hours at 40 ºC and the specified relative humidity. Pills were then dissolved in 100 µL of Tris buffer and reacted with 1 µL of fresh AchE at 250 U/mL. Absorbencies were evaluated at 605 nm for one hour using an Infinite M1000 spectrophotometer (Tecan) to determine the reaction rate.

Chapter 3 Results and Discussion

3.1 Design of Experiments

3.1.1 DOE results

From plotting the molecular weight of pullulan fragments obtained from the acid hydrolysis design of experiments, trends regarding the effect of temperature, time and acid concentration on molecular weight were observed. In general, pullulan fragments tend to have increased molecular weights with decreased acid hydrolysis times, lower temperatures and increased pH, or decreased acid concentration, as seen in Figure 3.1.1. In fact, molecular weight tends to decrease exponentially with increasing time and temperature. This is consistent with the kinetic model proposed in 1936 by af Ekenstam to describe the degradation of linear polymers which are of high molecular weight, where the degradation is a first order process (Strlič, 2003). First order kinetics have also been observed for acid hydrolysis of other polysaccharides. Aguilar et al. studied the use of sulfuric acid hydrolysis to produce xylose from sugar can bagasse (Aguilar, 2002). A kinetic model, in which the reaction was pseudo first order, was proposed to explain the variation of time on the production of xylose and the by-products, glucose, and the reaction inhibitors, acetic acid and furfural. The model allowed for the optimization of reaction time in order to maximize production of xylose and glucose, and minimize acetic acid and furfural production. This work also performed hydrolysis at three temperatures and three different acid concentrations, but modelling and optimization was not performed for these factors (Aguilar, 2002). Acid hydrolysis of cellulose from paper

refuse, using sulfuric acid, was also demonstrated to follow pseudo first order reaction kinetics (Fagan, 1971).

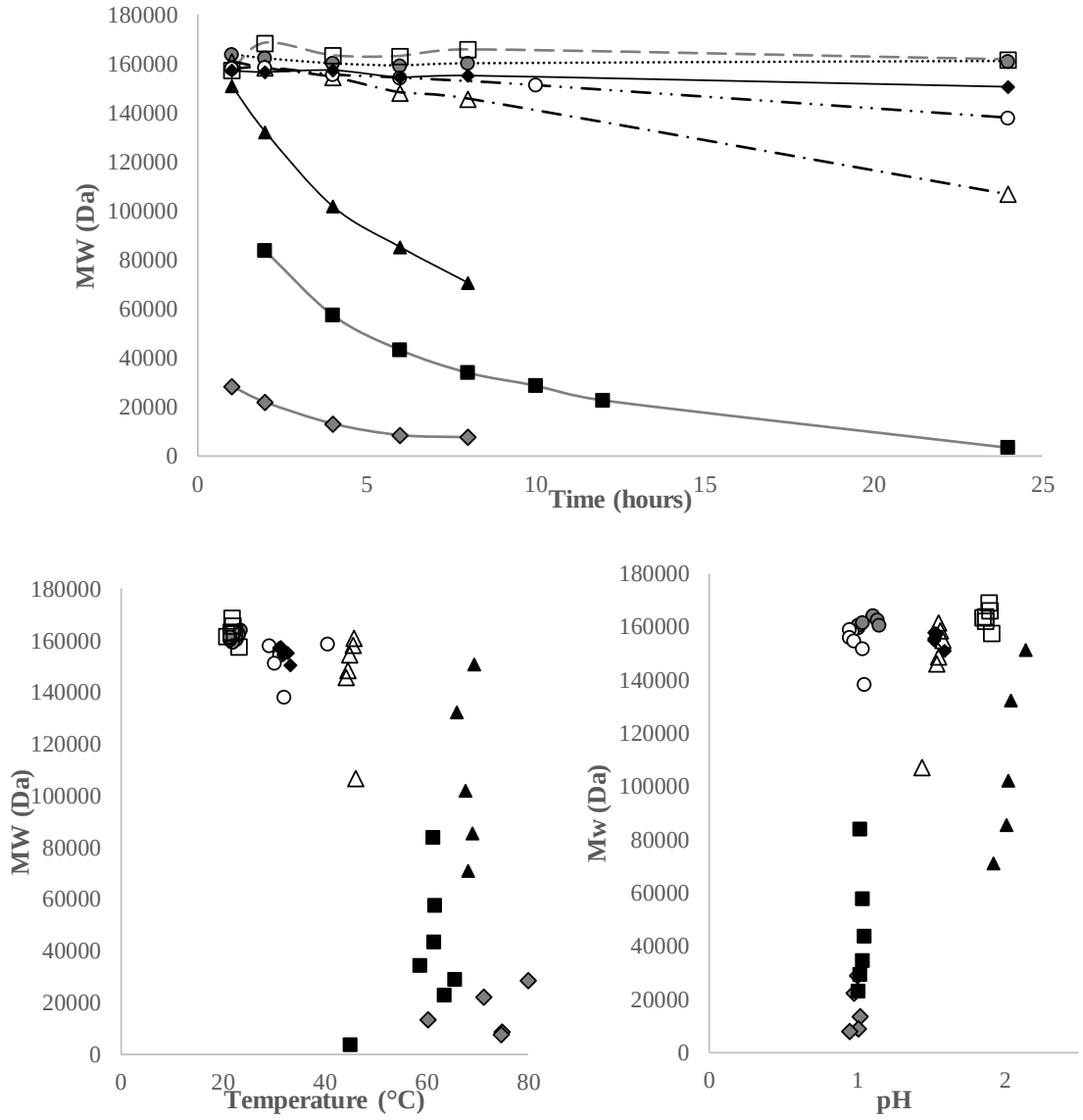

Figure 3.1.1 Molecular weight of pullulan fragments with respect to time, temperature and acid concentration from a design of experiments of acid hydrolysis: DOE 1 (25°C, pH 1,), DOE 2 (70°C, pH 1,), DOE 3 (25°C, pH 2,), DOE 4 (70°C, pH 2,), DOE 5 (45°C, pH 1.5,), DOE 6 (30°C, pH 1,), DOE 7 (30°C, pH 1.5,) and DOE 8 (55°C, pH 1,)

It can be noted that for DOE 1 and DOE 3, when the hydrolysis was performed at 25 ℃, pullulan was not hydrolyzed by HCl since the molecular weight of the fragments obtained were the same as the initial molecular weight of pullulan. In contrast, the length of pullulan chains was reduced after 24 hours of acid hydrolysis at 30 ℃, in DOE 6 and 7. This may indicate the existence of a lower boundary for temperature (at 25 ℃) in order to achieve acid hydrolysis.

3.1.2 Modelling in R

In order to verify the effect of time, temperature and pH on the molecular weight of pullulan fragments from acid hydrolysis, and investigate any two factor or three factor interactions between the three factors, the results from the design of experiments were modelled with R. Time and temperature were converted to coded variables corresponding to the second design of experiments. pH was adjusted to acid concentration before being converted to coded units. Data from DOE 1 and 3 were not included in the R model since the pullulan was not hydrolyzed. Based on trends observed in Figure 3.1.1, it was suspected that molecular weight follows a negative exponential trend with respect to time and temperature. For ease of coding, it was assumed that molecular weight also follows a negative exponential trend with respect to acid concentration. As such, a hypothesized model of pullulan acid hydrolysis, with capital letters denoting coefficients or constants and not including interactions between factors is seen in Equation 3.1.1.

$$\mathrm{MW} = \mathrm{A}e^{-\mathrm{B}*(\mathrm{time})} + (-\mathrm{C})e^{\mathrm{D}*(\mathrm{temperature})} + \mathrm{F}e^{[\mathrm{H}^+]} + \mathrm{G} \qquad \text{Eq. 3.1.1}$$

Linearizing the equation, simplifying the constants and including all the interactions, gives Equation 3.1.2.

$$\ln(\mathrm{MW}) = \mathrm{A(time)} + \mathrm{B(temperature)} + \mathrm{C}[\mathrm{H}^+] + \mathrm{D(time)(temperature)} + \mathrm{E(time)}[\mathrm{H}^+] + \mathrm{F(temperature)}[\mathrm{H}^+] + \mathrm{G(time)(temperature)}[\mathrm{H}^+] + \mathrm{H} \quad \text{Eq. 3.1.2}$$

The equation modelled with R, including all two factor and three factor interactions, demonstrated that the three factor interaction and the interaction between time and acid concentration were not important, because the estimated coefficient was not statistically significant, since the 95% confidence interval spanned zero, indicating that the coefficient was not distinctly positive or negative. The estimated coefficients from Equation 3.1.2, with the 95% confidence interval, is shown in Figure 5.1 in the supplementary data. To improve the estimate of the model coefficients, the equation was refitted to only include the main factors and two factor interactions between time & temperature and temperature & acid concentration. The estimated coefficients from the design of experiments for pullulan acid hydrolysis is shown in Figure 3.1.2. Equation 3.1.3 denotes specifically what the intercept and coefficients are estimated to be for the main factors and their interactions:

$$\ln(\mathrm{MW}) = -0.253(\mathrm{time}) - 0.658(\mathrm{temperature}) - 0.200[\mathrm{H}^+] - 0.312(\mathrm{time})(\mathrm{temperature}) - 0.177(\mathrm{temperature})[\mathrm{H}^+] + 11.5 \quad \text{Eq. 3.1.3}$$

The coefficients for the main factors, time, temperature and acid concentration were negative, which follows the trends observed in Figure 3.1.1. In addition, the statistical significance, 95% confidence level that the coefficient exists in its respective bounds, confirmes the relationship between decreasing pullulan molecular weight and decreasing

time, temperature and acid concentration. It also verifies that the exponential relationship that was assumed does give a good fit to the acid hydrolysis model. It is interesting to note that of the main factors, increased temperature was the most significant in decreasing molecular weight of pullulan fragments, followed by time and acid concentration.

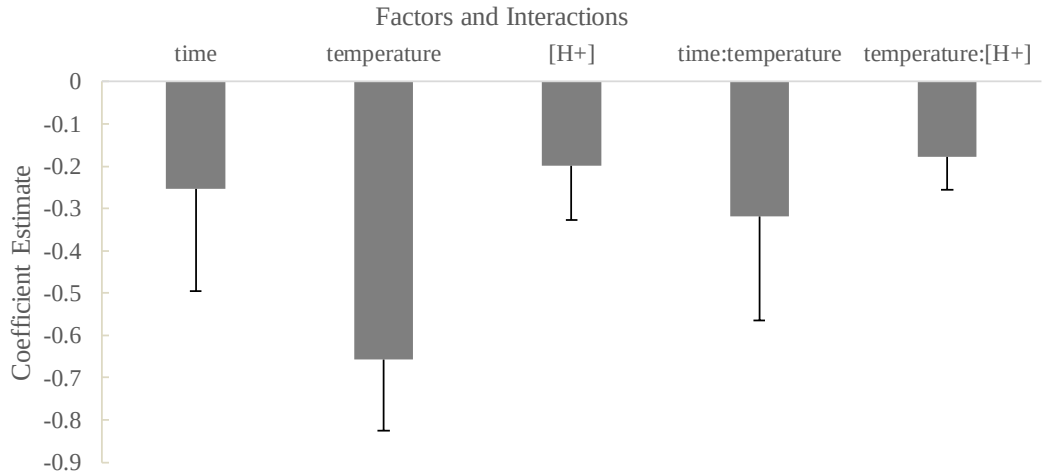

Figure 3.1.2 Estimated coefficients of factors and interactions of a pullulan acid hydrolysis model as determined by R, using a design of experiments. Colons represent the interaction between the two factors. Error bars represent the 95% confidence interval for each coefficient.

By modelling the hydrolysis in R, two factor interactions can also be noted. From Figure 3.1.2, it can be observed that there was a significant interaction between time and temperature, and temperature and acid concentration. Since the coefficient for the interaction between time and temperature is negative, this indicates that having the coded variables represented with opposite signs, i.e. decreased time and increased temperature or vice versa, would result in increased molecular weight of pullulan fragments. However, the most sensible interpretation of this coefficient would be that if both time

and temperature variables were coded positively, i.e. increased time and increased temperature, since the interaction coefficient is negative, a decrease in molecular weight is expected. The decrease in molecular weight from the interaction is in addition to the individual effect of increased time and temperature on decreasing molecular weight. A similar interpretation can be noted for the two factor interaction between temperature and acid concentration: if temperature was coded negatively and acid concentration was coded positively, or vice versa, increased pullulan molecular weight would be expected. However, the logical explanation would be that a significant interaction exists when increased temperatures and increased acid concentrations are used in acid hydrolysis, which would result in a further decrease in pullulan molecular weight.

To evaluate the fit of the model, Equation 3.1.3 was used to predict the molecular weight of the pullulan fragments obtained from the acid hydrolysis experiments. A comparison of the predicted molecular weights against the data determined from GPC, as seen in Figure 5.2a in the supplementary data, demonstrates the model is reasonable in fitting the data from the design of experiments. In general, the data could be found along the line of ideal model prediction, in which the predicted pullulan molecular weight is equal to the actual molecular weight. The slope of the line of best fit of the data is 0.980 ± 0.058 (the error represents the 95% confidence interval); the interval spans 1, the slope of the ideal line, indicating a goodness of fit of the model.

The model was also tested against data obtained from a preliminary acid hydrolysis experiment that was done at 60 °C, pH 1.2, with 9 samples taken over 24 hours. A

comparison between predicted molecular weight values and the actual molecular weight as determined by GPC is seen in Figure 5.2b. From the figure, it can be seen that the model was reasonable in estimating the molecular weight of pullulan fragments below 55 kDa; for higher molecular weight pullulan, the model tended to underestimate the molecular weight. The underestimation is also reflected by the slope of the line of best fit, 1.368 ± 0.181, (the error represents the 95% confidence interval) which demonstrates the actual molecular weight of the pullulan fragments was found to be larger than the predicted molecular weight. Despite the goodness of fit of the model as determined by the data from the DOE, there is a light lack of fit for high molecular weight pullulan so the model would be best used to predict molecular weight of shorter pullulan fragments.

3.2 Pullulan Fragment Characterization

Molecular weights and viscosity of all pullulan fragments obtained from preliminary acid hydrolyses and acid hydrolyses done as part of the design of experiments were determined by gel permeation chromatography and a viscometer, respectively. Further experimentation of the pullulan fragments (i.e. tensile testing, oxygen permeability and labile molecule stabilization) was done for seven groups of pullulan fragments. Non-hydrolyzed, native, pullulan was also tested and compared against the pullulan fragments. The groups of pullulan fragments are represented by the average weight-average molecular weight. Their molecular weights, polydispersity indexes (PDI) and viscosities are shown in Table 3.2.1.

Table 3.2.1 Molecular weight groups of pullulan fragments used for testing of mechanical and stabilization properties

Pullulan MW Groups	Average Mn (kDa)	Average Mw (kDa)	Average Mp (kDa)	PDI	Viscosity at 10 wt% (mPa·s)
160 (native)	100.58 ± 5.88	161.54 ± 3.75	153.55 ± 2.57	1.61	57.4 ± 4.5
106	63.57 ± 2.75	105.99 ± 0.37	116.03 ± 3.50	1.67	24.47 ± 1.84
75	47.99 ± 2.46	74.89 ± 0.71	73.66 ± 1.89	1.56	13.11 ± 1.21
56	33.20 ± 1.69	56.19 ± 0.45	56.86 ± 0.34	1.70	8.49 ± 0.61
43	26.06 ± 1.44	43.40 ± 0.58	44.73 ± 0.57	1.67	6.66 ± 0.59
30	18.07 ± 0.88	30.14 ±1.04	30.51 ± 1.16	1.67	4.50 ± 0.34
19	12.26 ± 1.08	19.38 ± 0.54	19.61 ± 0.73	1.59	3.54 ±0.37
10	6.77 ± 0.75	10.95 ± 0.87	12.41 ± 0.91	1.62	2.53 ± 0.12

3.2.1 Mechanical testing

From tensile tests, pullulan films made from various molecular weight fragments were demonstrated to have similar mechanical properties, since no significant trend could be observed in Figure 3.2.1 between tensile modulus, maximum tensile strength, and percentage of elongation with respect to the molecular weight of pullulan. The observed range of modulus, 1500 – 2500 MPa, was the same as to what was reported for other pullulan films by Kawahara et al. (2003) and similar to pullulan films with different water content, which range from less than 500 to 2500 MPa (Lazaridou et al, 2003). The values for maximum tensile strength, 30 – 50 MPa, and percentage elongation, 3 – 7 %, also fall into the range observed by Lazaridou et al., 10 – 80 MPa and 3 – 10%, respectively (2003).

As stated in the introduction, a study was previously done that compared the effects of molecular weight on mechanical properties of pullulan films (Lazaridou, 2003). In this paper, a general trend was observed in which the maximum force of break, modulus, and elongation at break decreased with decreasing molecular weight, signifying a gradual transition from brittle to ductile failure. This trend has been observed in other polymers, such as methyl cellulose and hydroxypropyl cellulose (Lazaridou, 2003). It has been proposed that a possible mechanism is due to the increasing number of chain entanglements with increasing polymer chain length, which leads to an apparent increase in modulus when the chains slip past entanglements (Lazaridou, 2003). It is worth mentioning that this paper only studied pullulan with molecular weights of 100, 260, 360 and 560 kDa, whereas this study predominantly worked with pullulan fragments below 160 kDa, so the trends observed in the paper by Lazaridou et al. may not apply to lower molecular weight pullulan fragments. A detailed comparison of tensile properties of the high molecular weight pullulan samples reveals that the modulus, maximum tensile strength and percentage elongation of 360 kDa pullulan tended to be lower than the values for 260 and 560 kDa pullulan. The only apparent difference between the three samples would be the high PDI of the 360 kDa pullulan sample (2.4) compared to the PDIs of the 260 and 560 kDa pullulan sample (1.6 and 1.9, respectively) (Lazaridou, 2003). Therefore, it was proposed that polydispersity index, in addition to molecular weight, may also affect tensile properties. PDI has been observed to influence the elongation at break in thermoplastic starches (Lazaridou, 2003). In this manner, since all

the pullulan samples in this work have similar PDI, it may be reasonable for all the pullulan fragments to have similar PDI.

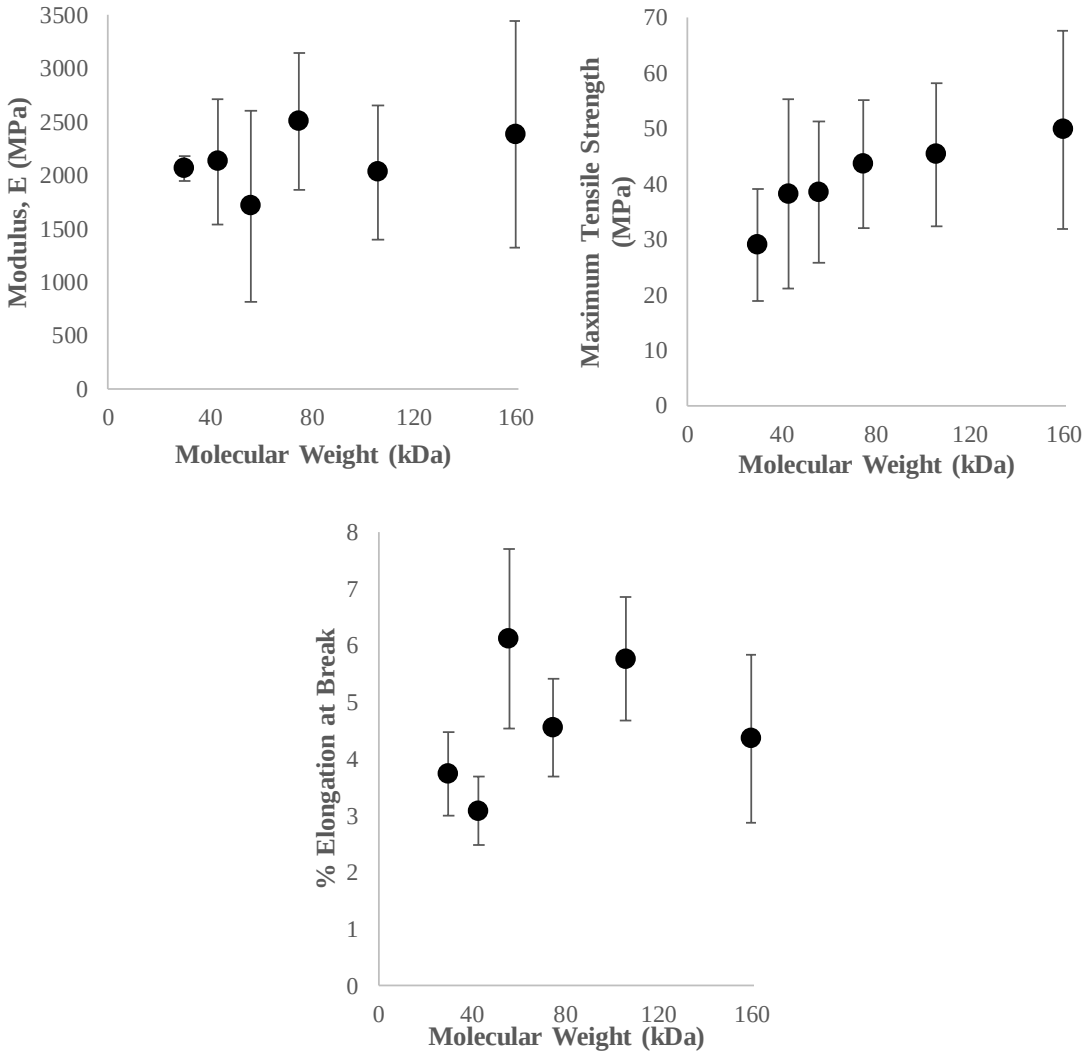

Figure 3.2.1 Modulus, maximum tensile strength and percentage elongation of various molecular weight pullulan films. Error bars represent standard deviation of at least three experiments.

The difference in observed trends, between this work and the research by Lazaridou et al., may be due to other factors as well. Firstly, water content of the pullulan film will affect

tensile properties. Increasing water content will decrease the tensile modulus, maximum tensile strength and increase the percentage elongation (Lazaridou, 2003; Kawahara, 2003). The presence of a plasticizer, including water or other compounds such as, sorbitol, is known to affect mechanical properties of polymers by increasing intermolecular distances, increasing backbone chain mobility, and decreasing local viscosity (Lazaridou, 2003). This does not explain the difference in observed trends because the water content was estimated to range between 10 – 15% when the pullulan films were stored at 50% RH (Zhang, 2015), which was within the range of RH tested by Lazaridou et al. (2003). In addition, it is assumed that the pullulan films did not contain any plasticizers or contaminants in significant concentrations.

Another factor that would affect tensile properties is the temperature at which the pullulan films were prepared (Kawahara, 2003). Kawahara et al. determined that pullulan films dried at high temperatures, 40 – 60 °C, had a statistically significant decrease in reported tensile modulus and maximum tensile strength compared to pullulan films cast at 4 - 25 °C (2003). In this work and the paper by Lazaridou et al., pullulan films were dried at room temperature, so this also does not explain the difference in observed trends.

3.2.2 Oxygen permeability

Many reviews have cited the good oxygen barrier properties of pullulan films (Farris, 2014; Domań-Pytka, 2004). In this work, oxygen permeability was evaluated by sealing a three-neck round bottom flask with a pullulan film, purging water in the flask with nitrogen, and allowing the oxygen in the atmosphere to re-enter the system via the

pullulan film. Pullulan of various molecular weights were used to make films and compared to not using a pullulan film. Figure 3.2.2 demonstrates that the rate of increasing oxygen concentration inside the flask was slower when pullulan films were used to seal the opening compared to having no pullulan barrier. As such, the water within the flask could achieve oxygen saturation after only 60 minutes for an open flask, whereas it took 12 – 24 hours for oxygen saturation to be achieved with a pullulan barrier. The initial flow rate of oxygen into the flask was found to be comparable between the different pullulan molecular weight films. The flux of oxygen (*J*) across the opening of the flask can be determined using the initial oxygen flow rate, which was determined from the linear portion of the curve in Figure 3.2.2., as seen in Equation 3.2.1.

$$J = \frac{\text{flow rate}}{\text{surface area}} = \frac{P\ (p_2 - p_1)}{\delta} \qquad \text{Eq 3.2.1}$$

In addition, permeability of a film can be found by Fick's law. Using the thickness of the film (δ) and overall pressure difference of oxygen ($p_2 - p_1$), an experimental value for oxygen permeability (P) was determined, as seen in Equation 3.2.1. It is assumed that oxygen follows Henry's law, which is reasonable because the gas pressure is at 1 atm, and the diffusion of oxygen is independent of other gases (Geankoplis, 2003). It is also assumed that the limiting factor in oxygen permeability of pullulan was the flow of oxygen across the pullulan film and not the diffusion of oxygen across the air-water interface or the diffusion of oxygen through the water to the probe. A comparison of the oxygen permeabilities of pullulan films with respect to the pullulan molecular weight can be seen in Figure 3.2.3, in which the pullulan films have similar oxygen permeabilities with respect to other films of different molecular weights.

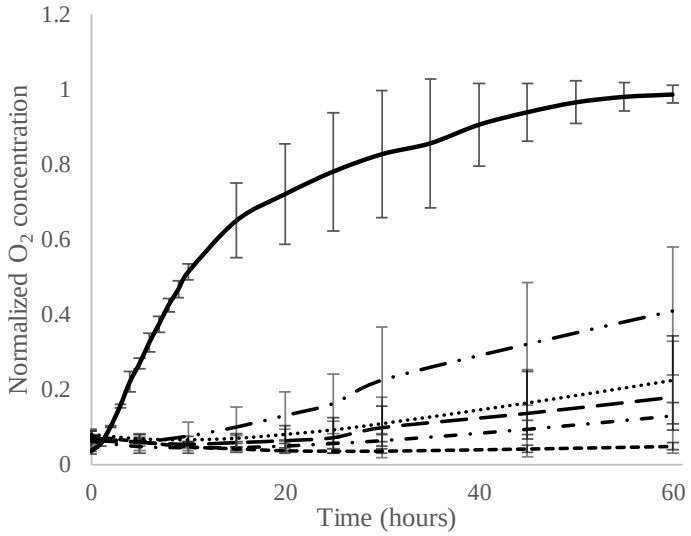

Figure 3.2.2 A comparison of the changing oxygen concentration, normalized to the initial oxygen concentration prior to nitrogen purging, over one hour for pullulan films of various molecular weights: 160 kDa (·····), 106 kDa (-----), 75 kDa (— · ·), 56 kDa (- · - ·), and 43 kDa (– –); and no pullulan film (——). Error bars represent the standard deviation of triplicate experiments.

The pullulan film oxygen permeabilities were compared to another value found in literature. Gontard et al. found that pullulan films had an oxygen permeability of 17 amol/(m·s·Pa), equivalent to 1.03 $x10^{-10}$ mmol/(min·mm·atm), which is 2 – 3 orders of magnitude smaller than the values found here, which range from 2 $x10^{-8}$ to 2 $x10^{-7}$ mmol/(min·mm·atm) (1996). The large discrepancy may be due to the differences in humidity at which the experiments were performed, since increased RH tends to increase permeability of a film (Gontard, 1996). Gontard et al. determined the oxygen permeability at 30% RH, because the pullulan film became viscous and they were unable to obtain measurements at high relative humidities (1996). In contrast, in this work, one side of the pullulan films was exposed to water-saturated air which is a greater RH than

30% RH; thus it is reasonable for the oxygen permeabilities for all the molecular weight pullulan films determined by this experiment to be greater than what was determined at 30% RH.

Therefore, in order to assess the validity of this method for finding oxygen permeability, pectin films were made using the same method as described by Gontard et al. (1996). The permeability of the pectin film was found to be 4296 amol/(m·s·Pa) compared to the permeability value, 1340 amol/(m·s·Pa), at 96% RH, as reported by Gontard et al. (1996) Given that the experimental permeability for pectin films found in this study was of the same order of magnitude as the value reported in literature, it is assumed that the method performed in this study can give a good estimate for oxygen permeability of pullulan films. Figure 5.3 in the supplementary data shows the changing oxygen concentration with respect to time for the pectin film tested.

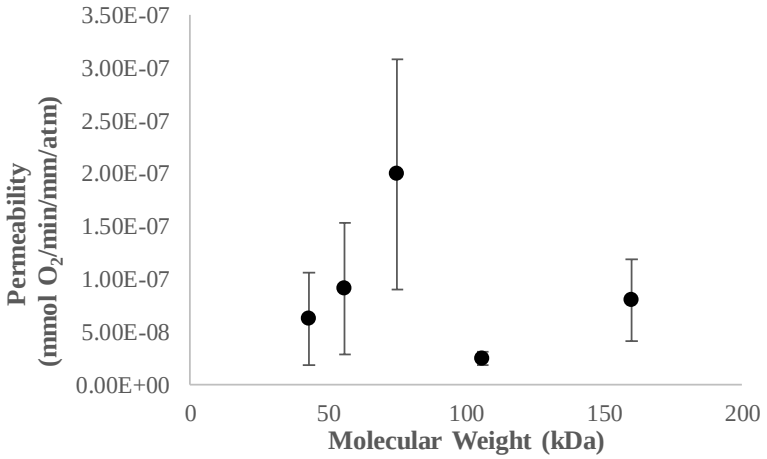

Figure 3.2.3 Permeability of pullulan films of various molecular weights to gaseous oxygen. Error bars represent the standard deviation of triplicate experiments.

3.2.3 Printability

Lower molecular weight pullulan is more printable compared to higher molecular weight pullulan; as seen in Figure 3.2.4, all 16 nozzles of the Dimatix cartridge were able to eject pullulan at 10 kDa. Pullulan is considered ejectable when the nozzle is able to dispense the solution forming a droplet or a long filament as seen in Figure 3.2.4a. With increasing molecular weight, the nozzles will pulse the pullulan solution but no droplets detach from the nozzle; an example of a pulsing nozzle is seen in Figure 3.2.4b. When printing high molecular weight pullulan, solid pullulan plugs form and are ejected from the nozzle, as seen in Figure 3.2.4c or there is no movement of the pullulan solution, resulting in a clogged nozzle. The maximum pullulan molecular weight that was consistently printable is 56 kDa, with a viscosity of 8.49 ± 0.61 mPa·s. This is slightly lower than the viscosity of an ideal fluid of 10 mPa·s (Fujifilm, 2008). The Dimatix printer is advertised to be able to jet fluids with viscosities ranging from 2-30 mPa·s; however, in this experiment, pullulan solutions with a viscosity greater than 8.49 mPa·s were unable to be ejected from the Dimatix cartridge.

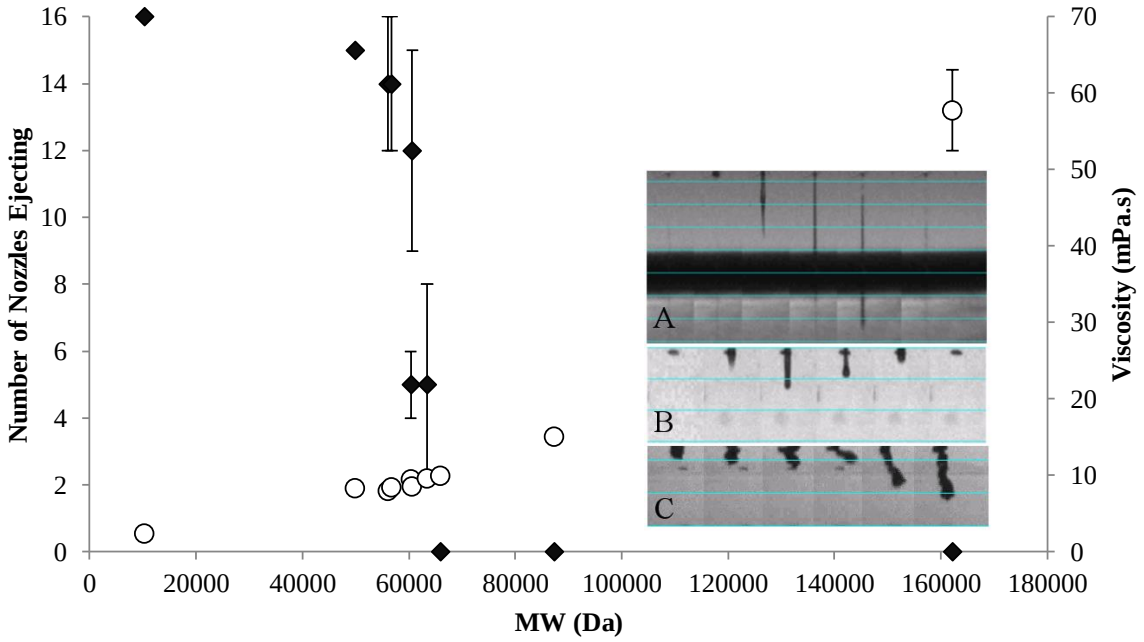

Figure 3.2.4. The number of nozzles that eject (◆) 10 wt% pullulan , out of 16 nozzles total, with respect to the pullulan molecular weight compared to the viscosity of the solution (○) (on the secondary axis). Error bars represent the standard deviation of at least three experiments.
Inset: pictures from DropWatcher showing images of droplet formation or no formation for: (a) a "jetting" set of nozzles; (b) a "pulsing" set of nozzles where droplet formation does not occur; (c) Solid formation on the cartridge head resulting in clogged nozzles

As stated previously, the printability of polymer solutions is largely affected by the solution concentration and viscosity. Printability is reduced for high molecular weight polymers due to increased shear viscosity (Xu, 2007). In a study done by de Gans et al., the maximum printable mass fraction of dilute polydisperse polystyrene in acetophenone decreased with increasing molecular weight (2004). It was proposed that the polymer undergoes elastic stresses due to elongational flow in the nozzle and at high molecular weights, the elastic stresses undergone by the polymer is greater than the kinetic energy generated by the nozzle so ink droplets cannot be formed. When the strain is removed

from the polymer, it returns to its thermodynamically stable coiled state, resulting in the droplet pulsing from the nozzle hole (de Gans., 2004). Lack of movement of pullulan from the Dimatix cartridge nozzles can be due to the high viscosity of high molecular weight pullulan leading to high elastic stress that the nozzle kinetic energy cannot overcome or due to solid formation by evaporation of water from the pullulan solution (Xu., 2007). Solid pullulan plug formation has been previously observed by other members of our group; Jahanshahi-Anbuhi et al. formed methanol-blocking pullulan lenses by allowing 2 µL of 9.1 wt% pullulan solution to form a plug and dry inside 1.15 mm i.d. Fisherbrand glass capillaries (2015). In comparison, the Dimatix cartridge nozzle orifice has a smaller diameter, 21.5 µm, and a smaller volume of pullulan solution, 10 pL, so it is reasonable that the evaporation of water from the pullulan solution to form solid plugs can occur within minutes. The continuous flow of pullulan from the cartridge reservoir results in “long/continuous” plug formation as seen in Figure 3.2.4c.

3.3 Labile Molecule Stability

Research previously done by our group has demonstrated that pullulan is capable of protecting labile molecules that are encapsulated in the dried pullulan against thermal denaturation and chemical modification, such as oxidation (Jahanshahi-Anbuhi, 2014; 2016). It is advantageous to be able to print pullulan with commercially available printers in order to increase its processability, for example in the application of producing microarrays. In section 3.2.3, it has been demonstrated that pullulan printability can be achieved by using lower molecular weight pullulan. However, it is necessary to

investigate whether the lower molecular weight pullulan also has good stabilization properties; whether it is capable to protect labile molecules against thermal denaturation and chemical modification.

Acetylcholinesterase and indoxyl acetate were the chosen studied enzyme and labile molecule, respectively, because the assay is colorimetric and only involves these two reagents. AchE catalyzes the hydrolysis of IDA to indoxyl, which is oxidized by air to indigo, which produces a blue colour detectable to the eye (Pohanka, 2011). The rate of colour change is analogous to the enzymatic activity for a specific amount of active IDA. The initial rate of reaction, as depicted by the linear portion of the curve prior to 20 minutes in Figure 3.3.1, was used to quantify the residual amount of active AchE or IDA compared to the amount of active AchE or IDA on the first day of tests.

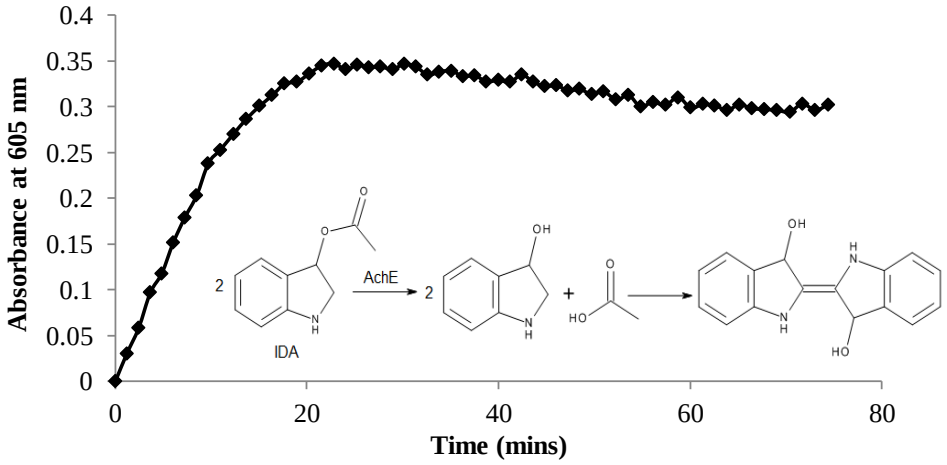

Figure 3.3.1 An example of reaction kinetics between AchE and IDA based on absorbance values from the spectrophotometer; Inset: chemical reaction mechanism of AchE and IDA

3.3.1 Acetylcholinesterase stability

The stability of acetylcholinesterase was studied using two methods: encapsulating AchE into pullulan pills and printing AchE onto a paper substrate with the use of printable pullulan.

AchE, when encapsulated into pullulan and dried to form pills, was able to retain its enzymatic activity over four months. Figure 3.3.2a illustrates that dried AchE in 160, 56, and 10 kDa pullulan demonstrated about 100% enzymatic activity at all times tested; compared to not using pullulan, in which the dried AchE could only maintain 16.9% of its activity over one month. Stabilization properties of pullulan fragments at seven molecular weights (Table 3.1) were studied in addition to native pullulan (which was previously studied by Jahanshahi-Anbuhi in 2014). All the pullulan molecular weights studied retained similar amounts of active enzyme at every time point so only three molecular weights, 160, 56 and 10 kDa, are shown in Figure 3.3.2a for ease of reading. Figure 3.3.2b demonstrates the similarity in AchE activity between the various pullulan fragments since the residual activity of AchE encapsulated in each of the eight pullulan fragments are similar, within error, after 1 week and 4 months of encapsulation.

Pullulan has good stabilization properties because it is able to maintain the native protein conformation in a dehydrated state, similar to trehalose (Jahanashahi-Anbuhi, 2016). Pullulan chains are able to restrict the mobility of proteins through hydrogen bonding (Arakawa, 2001; Sun, 1998; Nishioka, 2004). It is proposed that pullulan fragments had similar stabilization properties to that of native pullulan, in this study, because all pullulan solutions used had the same concentration, on a weight basis. In this manner, low

molecular weight pullulan solutions would contain greater amounts of pullulan chains compared to high molecular weight pullulan solutions, despite the pullulan chains being shorter. Therefore, it is likely that the number of interactions between the enzyme and pullulan solutions to be similar for all pullulan solutions, resulting in the observation that all the pullulan fragments tested have similar stabilization properties to native pullulan. It is suspected that the increase in enzymatic activity (greater than 100% residual activity reported) is due to macromolecular crowding which results in enzyme self-association (Di Risio, 2007).

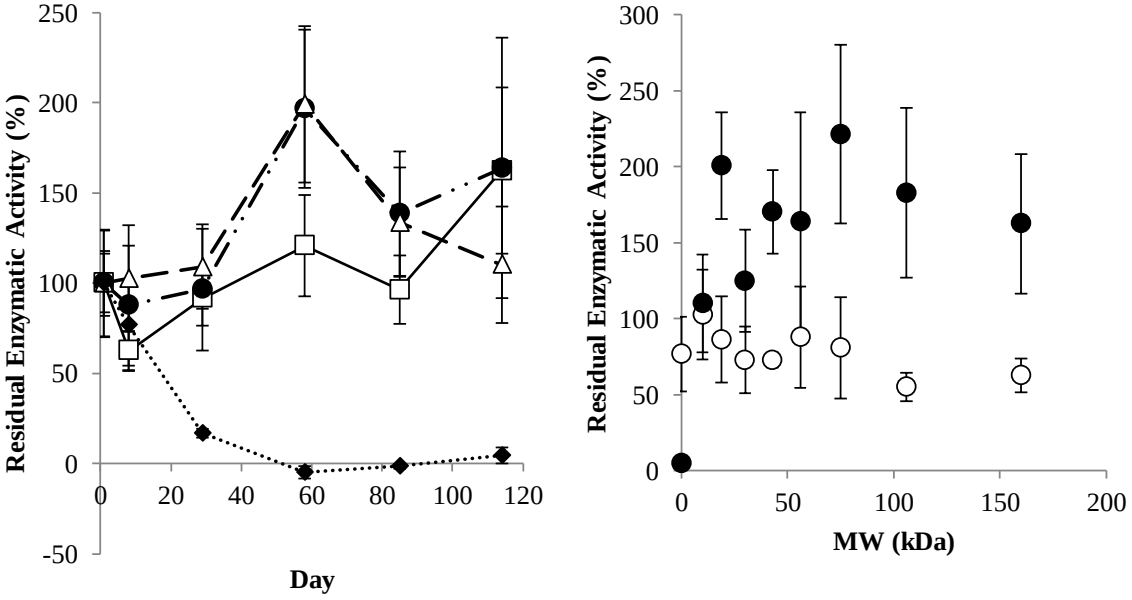

Figure 3.3.2. (a) Residual activity of AchE in pullulan pills made of 160 kDa (—□—), 56 kDa (- ●- ·), and 10 kDa (−△−), and no pullulan (···◆···) at room temperature over 4 months; (b) Residual enzymatic activity of AchE encapsulated in pullulan at various molecular weight after 1 week (○) and after 4 months (●); For both plots, data is normalized with respect to the activity of encapsulated AchE 24 hours after pill preparation. Error bars represent a standard deviation of triplicate experiments.

To demonstrate the potential application of using pullulan to stabilize enzymes in microarrays, AchE in 56 kDa pullulan was printed onto Whatman No. 1 paper using a Dimatix piezoelectric printer. Since AchE was able to react with IDA after printing, it indicates that the printing process did not cause any damage to the AchE in pullulan. In fact, paper arrays stored at ambient conditions (23 ℃, 50% RH) were stable for up to 7 weeks, as seen in Figure 3.3.3, which is expected since AchE in 56 kDa pullulan pills were stable over 4 months.

It was also of interest to test the stability of AchE in pullulan at high temperatures since external temperatures may fluctuate to above 40 °C. When stored at 30 ℃ and 40° C, the printed arrays were stable for two weeks, with the residual activity of AchE dropping to 48.6% and 43.9%, respectively for 30 ℃ and 40 ℃, at three weeks. It has been proposed that pullulan has good stabilization properties at high temperatures because pullulan has a high glass transition temperature, so the polymer chains are not disrupted, even at extreme temperatures (Jahanshahi-Anbuhi, 2016; Sun, 1998). Although it was not studied in this work, it is expected that all lower molecular weight pullulan fragments are able to stabilize enzymes at high temperatures since glass transition temperature is not affected by polymer molecular weight. (Lazaridou, 2003)

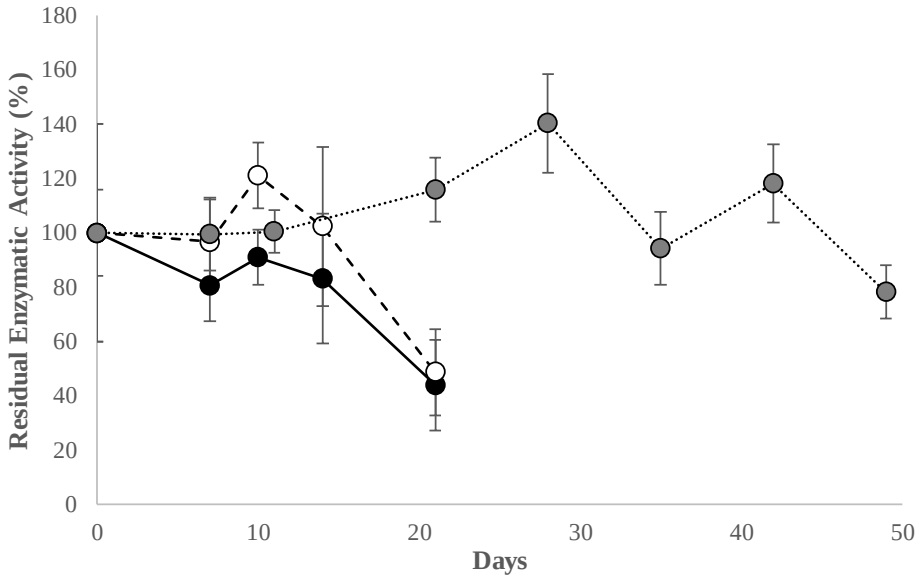

Figure 3.3.3. AchE printed in microarrays on Whatman No. 1 paper with 56 kDa pullulan stored at 23 ℃ (·····●·····), 30 ℃ (- -○- -), and 40 ℃ (—●—); Data points are normalized with respect to the AchE activity of an arrayed paper immediately after being printed. Error bars represent a standard deviation of 25 printed spots.

3.3.2 Indoxyl acetate stability

The stability of indoxyl acetate using various molecular weights of pullulan was studied using 3 methods: stability of IDA in solution with pullulan, stability of IDA encapsulated in pullulan pills and the use of pullulan to reduce the self-hydrolysis of IDA when not it was not dissolved in methanol.

Pullulan solutions, at all molecular weights tested, were able to retain some active IDA over two days, with all solutions having no remaining active IDA after three days as seen in Figure 3.3.4a. Similar to AchE, all molecular weights tested demonstrated similar stabilization properties to each other, compared to IDA dissolved in Tris buffer without pullulan, since the percentage of remaining active IDA in each pullulan molecular weight

was similar to each other, within error, after one and three days of storage under ambient conditions, as seen in Figure 3.3.4b.

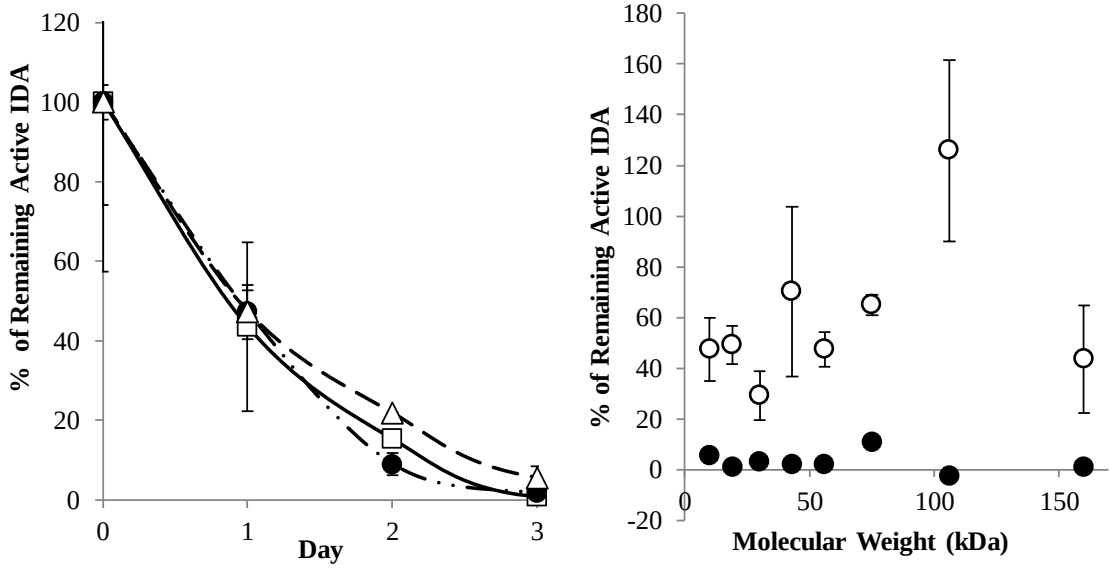

Figure 3.3.4 (a) The amount of active IDA remaining, as a percentage of the initial amount of active IDA, in pullulan solutions with a molecular weight of 160 kDa (—□—), 56 kDa (- -●- ·), and 10 kDa (– –△– –) stored under ambient conditions over 3 days; (b) Amount of active IDA remaining, with respect to initial amount of active IDA, stored in 9 different molecular weight pullulan solutions at ambient conditions, after 1 day (○) and 3 days (●); Error bars represent a standard deviation of triplicate experiments.

The amount of active IDA decreases with respect to time, when stored in pullulan solutions, is likely because IDA has the ability to undergo self hydrolysis (Müller, 1997). Self hydrolysis of IDA has been observed to occur faster under basic conditions; which was seen in this study since all reactions a\were carried out in Tris buffer at pH 8.9 (Müller, 1997). As such, the self hydrolysis of IDA in pullulan was studied and it was found that the rate of colour change of IDA stored in pullulan solutions was slower than the rate of colour change of IDA diluted in Tris buffer, as seen in Figure 3.3.5a. Figure

3.3.5b demonstrates that for all pullulan solutions of various molecular weights, the rate of self hydrolysis of IDA is similar. This may explain why the amount of remaining active IDA in the pullulan solutions were similar, irrespective of the molecular weight.

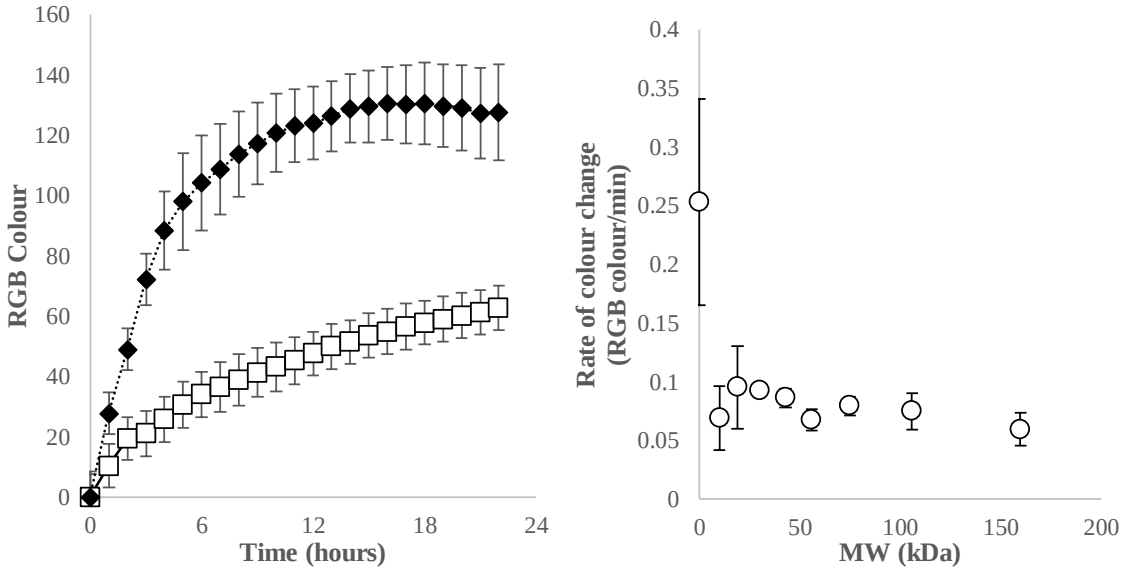

Figure 3.3.5 (a) RGB colour, analyzed by ImageJ, of self hydrolyzed IDA in 160 kDa pullulan solutions (—□—) and without pullulan solution (···◆···) over 24 hours (b) Rate of colour change of self hydrolyzed IDA in pullulan solutions at various molecular weights. Error bars represent the standard deviation of triplicate experiments.

Pullulan pills have previously been demonstrated to have stabilize IDA for long times compared to pullulan solutions (Jahanshahi-Anbuhi, 2014). In this work, only four molecular weights were used to make pullulan pills, in addition to native pullulan, because pullulan fragments of 30 kDa and lower were unable to form a film that was removable from a transparency film at ambient conditions. Figure 3.3.6 demonstrates that pullulan pills, of all five molecular weights tested, were able to retain active IDA in the dried pullulan pills for 30 days, after which only about 30% of active IDA remains. All

the molecular weights tested have similar stabilization properties, especially when compared to IDA dried without pullulan as there was no residual active IDA even after 24 hours.

The stabilization properties of pullulan on IDA are suggested to be due to the reduction of oxidation, because pullulan acts as an oxygen barrier (Jahanshahi-Anbuhi, 2014). This is confirmed by oxygen permeability tests, which demonstrated that pullulan films were able to reduce the flow rate of oxygen compared to no pullulan film barrier. It may also be inferred that pullulan of various molecular weights can stabilize IDA similarly because all pullulan films of different molecular weights had similar oxygen permeabilities, as noted in section 3.2.2.

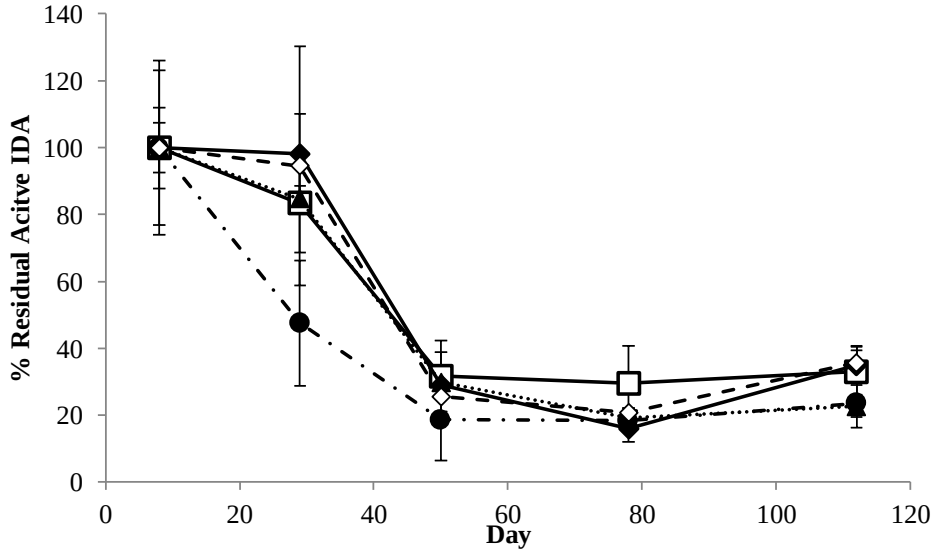

Figure 3.3.6 Percentage of active IDA encapsulated in pullulan pills at a molecular weight of 160 kDa (—□—), 106 kDa (—◆—), 75 kDa (···▲···), 56 kDa (- ● - ·), and 43 kDa (- ◇ -) over four months; Data is normalized with respect to the residual amount of IDA on the first time point of sampling. Error bars represent a standard deviation of three trials.

It is known that relative humidity influences the oxygen permeability of films. Gontard et al. studied the influence of relative humidity on oxygen and carbon dioxide permeabilities for various films and observed that permeabilities increased at increased relative humidity (1996). Since IDA is sensitive to oxygen and previous studies have suggested that pullulan acts as an oxygen barrier to stabilize IDA over long periods of time, the oxygen permeability of pullulan at different relative humidities could be tested with IDA encapsulated in pullulan pills and exposure of the pills to different humidities. The oxygen permeability could be inferred from the amount of active IDA remaining in the pullulan pill when tested with AchE.

From Figure 3.3.7, pullulan, at all molecular weights, was able to stabilize IDA at 40 °C, for 24 hours, with increasing relative humidities up to 80% RH. At 100% RH, on average across the five molecular weights tested, only about 14% ± 8% of active IDA was retained after 24-hour storage, indicating that the pullulan film became highly oxygen permeable. The high permeability of the film would also reflect the high oxygen permeability values, determined in section 3.2.2. Since more active IDA could be retained at conditions with lower relative humidity, the oxygen permeability of pullulan could be comparable to the value determined by Gontard et al. (1996). Similar to previous experiments, there was no difference, within error, in observed trends between the various molecular weights of pullulan tested, which may be due to all molecular weights of pullulan having similar oxygen permeabilities.

In a study done by Gontard et al., the oxygen permeability of wheat gluten and other hydrophilic films was found to remain relatively constant at relative humidities below

50% and at RH levels above 50%, oxygen permeability values increased exponentially with increasing RH (1996). This could also be seen in Figure 3.3.7, where the amount residual active IDA is relatively similar at 20%, 40% and 60% RH; at increased RH, the amount of residual active IDA decreases due to increased oxygen permeability. It was suggested that the increase in permeability was partially due to the increase in oxygen solubility in the film due to increased water content in the film (Gontard, 1996). At high relative humidity, pullulan films, which are highly sensitive to moisture content, were likely to absorb water to the point at which the film becomes dissolved, which would also affect the stability of IDA. This would explain why there was no remaining active IDA after storage of the IDA pills at 100% RH.

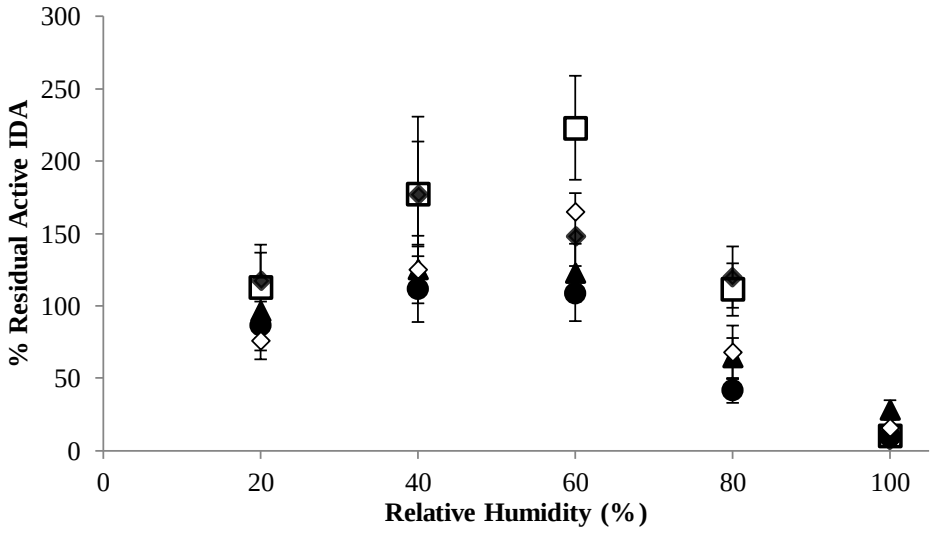

Figure 3.3.7 Amount of residual active IDA encapsulated in pullulan pills at 160 kDa (□), 106 kDa (◆), 75 kDa (▲), 56 kDa (●), and 43 kDa (◇). Pullulan pills were stored at 40 ℃, and different relative humidities for 24 hours. Data points are normalized with respect to the amount of active IDA in pullulan pills that did not undergo high RH storage conditions. Error bars represent a standard deviation of three samples

Chapter 4 Conclusions

4.1 Summary of work

From the design of experiments of pullulan acid hydrolysis, it was determined that there was a decrease in the molecular weight of pullulan fragments with increasing time, temperature and acid concentration. There were also two significant interactions in the model, between time and temperature, and between temperature and acid concentration. A predictive model was also determined, but it was found to have a light lack of fit for long pullulan fragments as the model tended to underestimate their molecular weight. More acid hydrolysis could be done within the DOE parameters to refine the model and determine the reproducibility of the experiments. Even so, acid hydrolysis is an effective method of shortening the length of high molecular weight pullulan.

A study of the pullulan fragments obtained from acid hydrolysis determined that low molecular weight pullulan fragments had similar tensile properties and oxygen permeabilities to native pullulan since no trend was observed for these properties with respect to molecular weight. The printability of pullulan was determined to depend on molecular weight and viscoelastic properties of solutions; droplet formation was achieved for pullulan fragments of molecular weight 56 kDa and lower. In addition, low molecular weight pullulan was demonstrated to be as effective as native pullulan in stabilizing AchE and IDA over long periods of time. Dried pullulan pills were able to stabilize the enzyme and labile molecule for 4 months and 1 month, respectively, at ambient conditions. Pullulan films were confirmed to be good oxygen barriers, as they were able to stabilize

oxygen-sensitive IDA up to 40 ℃, 80% RH, for 24 hours. Microarray printing, onto Whatman No. 1 paper, was demonstrated with AchE, using a Dimatix materials printer; pullulan was able to stabilize the printed AchE during the printing process and in storage, at 40 ℃, for up to 2 weeks.

4.2 Future directions

The main objective of this work was achieved since it was demonstrated that pullulan, at a molecular weight of 56 kDa, was able to be printed from a piezoelectric printer. These pullulan fragments were able to be printed in a microarray format on paper, with uniform and reproducible spots. However, more work will need to be done for pullulan to be used to stabilize mass-produced commercial biosensors.

Firstly, a study could be done investigating the printing of protein microarrays onto a glass substrate, since microarrays are usually done on chemically-modified glass slides in order to immobilize the enzyme probes. It would be interesting to note whether pullulan would interfere with the immobilization of proteins onto the substrate, which may cause the proteins to wash away when pullulan becomes dissolved. If pullulan was immobilized onto the surface along with the protein, it may hinder the detection of the target molecule due to the viscous nature of pullulan which would slow diffusion of the target molecule to the enzyme.

It may also be ideal to modify the surface tension of the pullulan solutions, to within the optimal parameters of the Dimatix printer, to obtain an ink that would print perfect droplets. For a perfect droplet, only a single sphere of solution would be ejected, without

a tail and with minimal satellite droplet formation (de Gans et al., 2004). This would help with printing resolution and allow for the printing of microarrays with decreased interspot distances. Optimization of surface tension properties would also help obtain reproducible printed spots by other printing techniques, such as pin printing. It is suggested to use a neutral surfactant in order to not interfere with the activity of the enzymes or labile molecules being stabilized.

Lastly, it would be interesting to test the thermal stabilization properties of the pullulan fragments using more labile enzymes. While AchE is a good model enzyme, AchE solutions, without pullulan encapsulation, have been demonstrated to be active after storage at 40 °C for 30 minutes (Jahanshahi-Anbuhi, 2014). Luciferase, a thermally sensitive enzyme which has been used in assays to detect ATP and has been previously used by our group, would be a good candidate for future studies.

With continued improvement to pullulan-based inks, there is potential for the usage of pullulan in industrial applications. One such application to research or the pharmaceutical industry would be commercial protein microarrays, where microarrays can be printed at a central location, en masse, and stabilized during shipment to a consumer without need for temperature or humidity control.

Chapter 5 Supplementary Figures

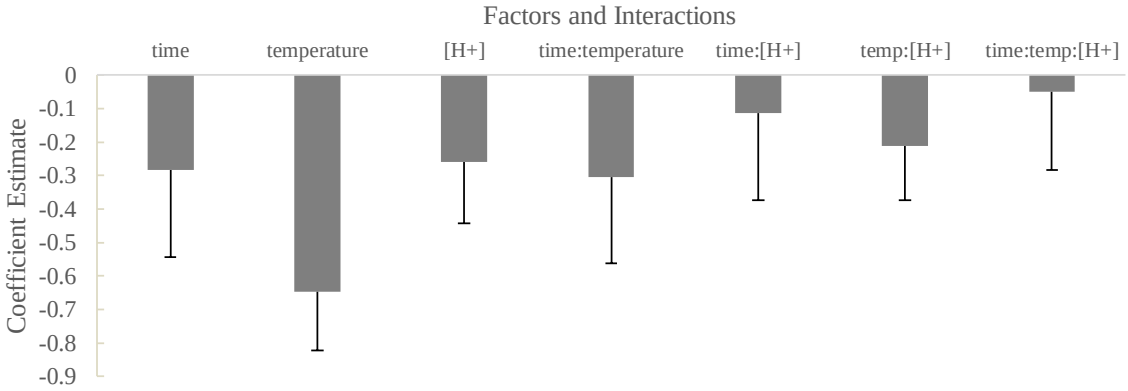

Figure 5.1 Estimated coefficients of all factors and interactions in the model of pullulan acid hydrolysis, as determined by R, using a design of experiments. Colons represent the interaction between two or three factors. Error bars represent the 95% confidence interval for each coefficient.

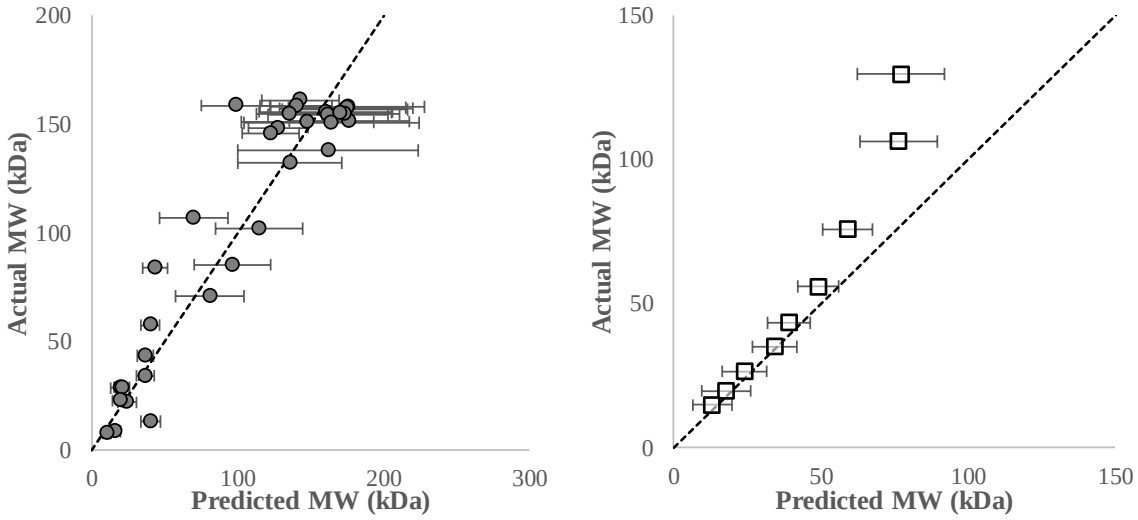

Figure 5.2 Molecular weight of pullulan fragments obtained from acid hydrolysis experiments determined by GPC plotted against the predicted molecular weight from the acid hydrolysis model (a) with data from the DOE experiments, which were used to come up with the model; (b) with data from a preliminary hydrolysis, done at 60 °C, pH 1.2. The dashed line denotes ideal outcome, where the predicted molecular weight is the same as the actual molecular weight of pullulan fragments. Error bars denote the 95% confidence interval of the predicted molecular weight.

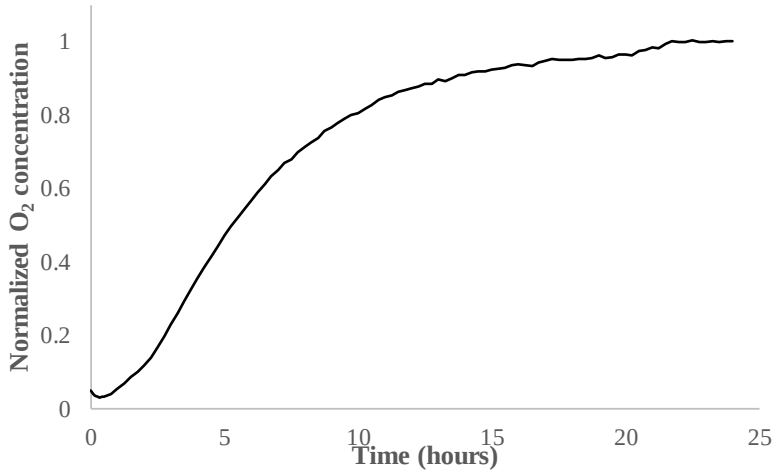

Figure 5.3 The change of oxygen concentration, normalized to the initial oxygen concentration prior to nitrogen purging, over 24 hours for a pectin film.

From this graph, the oxygen flux was determined to be 1.07 $x10^{-7}$ mmol O_2/(min·mm^2), and the oxygen permeability of the pectin film was determined to be 2.61 $x10^{-8}$ mmol O_2/(min·mm·atm) (or 4296 amol/(m·s·Pa)).

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