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**ACTIVATION OF LACTOPEROXIDASE SYSTEM IN MILK BY GLUCOSE
OXIDASE IMMOBILIZED IN ELECTROSPUN POLYLACTIDE MICROFIBERS**

A Thesis

Presented to

The Faculty of Graduate Studies

of

The University of Guelph

by

Yuting Zhou

In partial fulfillment of requirements

For the degree of

Master of Science

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ABSTRACT

ACTIVATION OF LACTOPEROXIDASE SYSTEM IN MILK BY GLUCOSE OXIDASE IMMOBILIZED IN ELECTROSPUN POLYLACTIDE MICROFIBERS

Yuting Zhou

Advisor: Dr. Loong-Tak Lim

University of Guelph, 2009

In this study, glucose oxidase (GOX) was immobilized in electrospun polylactide (PLA) fibers to activate the lactoperoxidase (LP) system in milk. The GOX-containing microfibers were electrospun from emulsions prepared by dispersing aqueous GOX in PLA dissolved in a chloroform and N,N-dimethylformamide blend, using sorbitan monopalmitate as an emulsifier. The enzymatic activity of GOX-in-PLA fibers (1100 ± 400 nm diameter) was more than 19 times higher than that of the GOX-in-PLA membrane formed by direct casting, due to the larger surface area of the electrospun fibers. The activation of LP system in model solutions using GOX-in-PLA fibers provided a more sustained generation of antimicrobial OSCN⁻ than direct activation using H₂O₂. Preliminary evaluation on milk samples showed that the electrospun GOX-in-PLA microfibers are capable of activating the naturally present LP system. Microbial analysis of LP system activated in milk exerted antimicrobial effect, indicating that they may be promising for active food packaging applications to extend the shelf life of milk.

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1 INTRODUCTION

Bovine milk is a nutrient-rich liquid food which is an important component of the human diet. However, the nutrient-rich characteristic of milk is also ideal for microbial growth. In order to prolong the shelf-life and expand the consumption of milk, methodologies to improve its microbial keeping quality are needed. Currently, refrigeration is used to stabilize raw milk during transportation. Moreover, milk is pasteurized (e.g., high temperature/short time) to extend its shelf-life up to about two weeks when stored at under refrigeration. Nevertheless, temperature abuses (5-60°C) that occur in milk before and after pasteurization can induce microbial spoilage and decrease its keeping quality. This is more so in under-developed countries where proper sanitation and temperature control are lacking (Fuglsang *et al.* 1995).

Although preservatives are commonly available to the food industry for reducing milk product spoilage, their use is not ideal because of consumer's preference for natural over the preserved milk. In milk, there exists a natural antimicrobial mechanism, known as the lactoperoxidase (LP) system, which has been demonstrated to be effective for controlling the growth of microorganisms (Korhonen 1980; Reiter 1985; Chakraborty *et al.* 1986; Garcia-Garibay *et al.* 1995; Barrett *et al.* 1999). The use of this naturally occurring antimicrobial, in conjunction with active food packaging, may be favorable since direct addition of non-native compounds into the milk can be avoided.

Active packaging contains subsidiary constituents that have been deliberately included in or on either the packaging material or the package headspace to enhance the performance of the overall system (Robertson, 2006). Antimicrobial, antioxidant, bioactive compounds and other functionalized agents have been incorporated with packaging materials (Brody *et al.* 2001; Suppakul *et al.* 2003; Min *et al.* 2005; Del Nobile *et al.* 2008). The development of antimicrobial active packaging has been mainly focused on the incorporation of antimicrobial agents in continuous film/sheet packaging structures, which has limited contact surface area with the food product (Brody and Budny 1995; Brody *et al.* 2001).

Over the past few years, there has been an increasing research interest on exploiting fibers that are submicron in diameter as carriers for various bioactive agents (Aussanwasathien *et al.* 2005; Sawicka *et al.* 2005; Li *et al.* 2006) (Ye *et al.* 2005) and as medical tissue scaffolds (Mo *et al.* 2004; Vaz *et al.* 2005; Yang *et al.* 2005). Besides forming fibers from a single polymer material, polymeric fibers incorporated with carbon nanotubes, gold/ZnO, TiO₂, and silica have been prepared to obtain better properties (Reneker *et al.* 2001; Ziegler *et al.* 2001; Ko *et al.* 2002; Huang *et al.* 2003; Shao *et al.* 2003). Non-woven electrospun fibers have been commercialized as filtration materials for contaminant removal in air (eSpin Technologies).

Such electrospun fibers may be ideal for active packaging applications due to their large surface-to-volume ratio. Some of them have a porous structure,

which may allow reactant and product molecules to diffuse in and out the fiber. In this study, electrospun fibers were used to activate the LP system by immobilizing the enzyme, glucose oxidase (GOX), in electrospun polylactide (PLA) fibers. The active fibrous membrane, when attached to the interior surface of a milk package, is hypothesized to be suitable for extending the shelf-life of milk.

2 LITERATURE REVIEW

2.1. LACTOPEROXIDASE (LP) SYSTEM

The LP system is a natural antimicrobial mechanism in milk involving LP and other compounds, some of which could be influenced by intrinsic and extrinsic factors of milk. This well documented system was reported several decades ago when Hanssen first suggested that, in raw milk, the antimicrobial activity of LP against *Bacillus typhosa* and *B. paratyphosa* was associated with the presence of oxidases and peroxidases in milk (Hanssen 1924). Moreover, Wright and Tramer pointed out that the inhibition of microorganisms could only be caused by an oxidation product formed by LP in the presence of hydrogen peroxidase (H_2O_2) (Wright and Tramer 1958). Morrison *et al.* established a simple method to isolate and purify LP (Morrison *et al.* 1957), which enabled the role of LP to be studied by using the pure enzyme. The role of H_2O_2 was substantiated by the discovery that the inhibition of streptococci was prevented by the addition of catalase at a very high concentration that does not occur physiologically in milk or other biological fluids, and which decomposes H_2O_2 to H_2O and O_2 (Reiter 1985). The third factor required in the LP system was identified as thiocyanate, the oxidation product of which inhibits some strains of lactic acid producing streptococci (Oram and Reiter 1966). Depending on the bacterial strain concerned, the oxidation reaction would lead to the temporary growth-inhibition or death of the bacterial cell (Korhonen 1980).

It is generally agreed that the LP system is comprised of three components, namely, LP, thiocyanate and H₂O₂. The enzyme LP can catalyze the oxidation of thiocyanate ion (SCN⁻; acts as electron donor) by H₂O₂ to hypothiocyanite anion (OSCN⁻; in equilibrium with hypothiocyanous acid HOSCN), which is capable of inhibiting many microorganisms. The major antimicrobial agent, OSCN⁻ or HOSCN, exerts its action by oxidizing the sulphydryl (-SH) groups of proteins to disulphides (RSSR). Milk proteins contain very few accessible sulphydryl groups, whereas microorganisms contain many, so the reaction of OSCN⁻ is targeted at the microorganisms present in milk (Barrett *et al.* 1999). In short, the possible mechanisms of the LP system are shown in Figure 1.

Reactions catalyzed by lactoperoxidase:

Generation of higher oxyacids:

Oxidation of protein sulphydryls:

Figure 1. Antimicrobial activity of lactoperoxidase: possible mechanism. Adapted from Fuglsang *et al* 1995.

LP concentrations in milk range from 10 to 30 µg/ml, and milk has a variable SCN⁻ concentration, up to 15 ppm and can be higher in summer than in

winter (Lawrence 1970; Reiter 1985; Vasavada and Cousin 1993). On the other hand, H_2O_2 is generally absent in milk of good quality due to its own protective mechanism against the toxicity of H_2O_2 . The H_2O_2 generated by xanthine oxidase, Cu^{2+} , ascorbic acid, and sulfhydryl oxidase are either bound to LP or reduced by catalase (Reiter 1985). Also, Pruitt and Reiter found that usually it is the availability of H_2O_2 that determined the magnitude of antibacterial activity of peroxidase systems (Pruitt and Reiter 1985). Therefore, excess H_2O_2 needs to be present in order to activate the LP system and, in turn, delay milk spoilage caused by microorganisms.

Several studies have investigated the activity of LPS in raw milk (Chakraborty *et al.* 1986; Gupta *et al.* 1986; Sarkar and Misra 1994) and milk with mild treatments such as pasteurization (Sarkar and Misra 1994; Barrett *et al.* 1999). In some developing countries, where milk is susceptible to temperature abuse and poor sanitary conditions during transportation and storage, H_2O_2 has been added to raw milk to activate the LP system to preserve the milk during transportation (IDF 1988). Activation of the LP system prior to pasteurization significantly retarded the recovery and growth of the surviving natural milk microflora (Kamau *et al.* 1990). However, the LP is denatured to various extents when milk is heated, and consequently the index of quality decreases with increasing treatment temperature. Previous work by Korhonen has shown that heating at 60°C for 15 min resulted in complete destruction of LP (Korhonen 1980), whereas a 70% residual activity was reported when HTST pasteurization

conditions of 72°C for 15 s were used (Barrett *et al.* 1999). Extended heating at 73°C for 10 min reduces the activity of LP to approximately 1% (Vasavada and Cousin 1993). Griffiths pointed out that LP activity was reduced by ~40% at 80°C for 15 s through laboratory studies while almost destroyed at 78°C for 15 s through a plate heat exchanger (Griffiths 1986). According to the previous study, treatment temperature will affect the antimicrobial efficacy of the LP system. Potentially, the use of LP may offer an opportunity to decrease the thermal requirement for pasteurization or reduce the refrigeration requirement during storage.

2.2. GLUCOSE OXIDASE AND GLUCOSE (GOX/GLUCOSE) SYSTEM

As discussed in the previous section, the activation of the LP system requires the presence of H₂O₂. To provide the required H₂O₂ for activating the antimicrobial system, we are proposing to use GOX to catalyze the oxidization of the naturally present glucose in milk.

GOX/glucose is a typical oxidoreductase system often used for generating H₂O₂. Glucose oxidase (β -D-glucose: oxygen-oxidoreductase, EC1.1.3.4) from *Aspergillus niger* is a flavoprotein that catalyzes the oxidation of β -D-glucose to D-glucono- β -lactone and hydrogen H₂O₂, using O₂ as the electron acceptor. D-glucono- β -lactone later exists in equilibrium with D-gluconic acid spontaneously

in aqueous solution. The equations below show the reaction scheme (Fuglsang *et al.* 1995).

Due to the antimicrobial properties of H_2O_2 and pH lowering effect caused by gluconic acid, the GOX/glucose system itself is potent against microorganisms. However, long-term exposure of a food product to H_2O_2 can cause rancidity due to lipid oxidation. This may explain why direct addition of H_2O_2 to milk is not used in practice and that the LP system is more effective than the direct addition of H_2O_2 (Fuglsang *et al.* 1995). Not only owing to higher potency of OSCN^- , the damage of milk caused by H_2O_2 could be avoided through the depletion of H_2O_2 by the LP system.

Milk lacks GOX and the content of glucose is variable due to the different species of bovine cows, feed, and climates (Jensen 1995). Therefore, in order to use glucose to activate the LP system, it is necessary to evaluate the effects of glucose content in milk. GOX is thermolabile; in phosphate buffer (pH 6.0), its activity is almost completely lost after heating at 64°C for 15 min (Gouda *et al.* 2003). Hence, direct addition of GOX to milk prior to pasteurization may not be optimal. Immobilizing GOX in a carrier that is an integral part of packaging may be a more feasible solution.

2.3. ACTIVE PACKAGING

Today, consumers are more demanding for foods that are healthy, wholesome and of high quality. With increasing exposable household incomes, consumers are more willing to purchase high quality packaged food at an increased cost, such as minimally processed and preservative-free foods. This trend is the key driver for the use of active packaging, which is capable of maintaining food quality during storage.

By and large, active packaging can be divided into two categories. One category involves containing active agents in sachets or pads, which are then placed inside the food packages. By contrast, the second approach incorporates the active agents directly into or on the surface of packaging materials (Robertson 2006). Oxygen scavengers and moisture absorbers, two of the examples of the former category, are by far the most commonly used active packaging available commercially. Other examples of active packaging systems include CO₂ absorbers/emitters, ethylene scavengers, odor removers, ethanol emitters, antioxidants and antimicrobial emitters, microwave susceptors, self-heating and self-cooling packages and packages that exhibit variable barrier properties (Robertson 2006).

With the advent of new polymer materials and antimicrobials, research and development of antimicrobial materials for food applications such as packaging

and other food contact surfaces is expected to grow in the next decade, particularly in the following areas (Appendini and Hotchkiss 2002):

- (1) Addition of pads/sachets carrying volatile antimicrobial agents;
- (2) Incorporation of volatile and non-volatile antimicrobial agents directly into packaging polymers;
- (3) Coating or adsorbing antimicrobials onto packaging surfaces;
- (4) Immobilization of antimicrobials to packaging surfaces by ionic or covalent linkages;
- (5) Use of packaging polymers that are inherently antimicrobial.

2.4. ENZYME IMMOBILIZATION

Many active agents have been immobilized onto the surface of packaging polymers or into a sachet contained inside active packages for bactericidal or bacteriostatic purposes, including synthetic peptides (Haynie *et al.* 1995; Appendini and Hotchkiss 2001), lysozyme (Appendini and Hotchkiss 1997; Mermelstein 1998; Conte *et al.* 2007), GOX, catalase and lactase (García and Galindo 1990; Hsiue and Wang 1990; Garcia-Garibay *et al.* 1995; Goddard *et al.* 2007). For instance, a number of years ago, PharmaCal, Ltd. developed a two-enzyme system involving GOX and catalase to intercept oxygen and has applied the technology in active packaging to improve oxygen removal (Brody and Budny 1995).

Enzymes have been immobilized in different polymer carriers. In general, the immobilization of enzyme in polymer can be based on three main methods (Figure 2), namely covalent binding, crosslinking and entrapment (Cantarella *et al.* 1997; Koudelka-Hep *et al.* 1997)

Figure 2. Approaches of enzyme immobilization.

Covalent binding is frequently used to immobilize enzymes to a supportive carrier. Covalently attaching an enzyme to a polymer film surface is an attractive method since the enzyme is unlikely to migrate into the food and will retain activity as long as the substrate is present (Goddard *et al.* 2007). Many

researchers reported that immobilizing GOX to a polymer substrate via a covalent bond tends to reduce its activity as compared to the free enzyme. This may result from the reduced availability of the active sites of the enzyme due to conformational change during the immobilization process. However, immobilized enzymes tend to be more stable during storage and end-use as compared to the free counterparts (García and Galindo 1990; Wang and Hsiue 1993; Li *et al.* 1998; Suye *et al.* 1998; Vartiainen *et al.* 2005). For example, Tiller *et al.* found that the affinity for substrate of immobilized GOX coupled to a positively functionalized surface was higher than the free enzyme (Tiller *et al.* 2002). In addition, it has been reported that the substrate affinity of immobilized GOX was directly proportional with the immobilization density (Klei *et al.* 1978). Moreover, one disadvantage of carrier-bound enzymes is that they may have reduced catalytic activity resulting from introduction of a large proportion of noncatalytic mass, around 90-99% of the total mass (Sheldon *et al.* 1997).

The second type of immobilization method is by cross-linking the enzymes via bifunctional compounds that have a negligible molecular weight compared with that of the enzyme. This method can overcome the loss of activity due to direct covalent bonding to the carrier (Sheldon *et al.* 1997). In many cases, the two methods are combined together for the application of enzyme immobilization on polymer materials.

Polyolefins, such as PE (polyethylene) and PP (polypropylene) are widely used as packaging materials in the food industry due to their good chemical and water resistance and simple manufacturing processes. In a milk carton package, a thin layer of PE is always coated on the inner surface to provide a moisture barrier and enable heat sealing. The inner surface may serve as a carrier for enzyme attachment; however, because PE polymer lacks active groups, functionalization of the film surfaces is needed. For example, carboxyl groups can be introduced onto the PE surface by graft polymerization with acrylic acid (Wang and Hsiue 1993) or acrylamide (Sano *et al.* 1993) using argon plasma pretreatment. Grafting of amino and carboxylic groups onto PE can be achieved by employing anhydrous ammonia and oxygen as reactive gases (Sipehia 1988-89). Vartiainen *et al.* employed direct N₂-plasma with NH₃ and CO₂ as reactive gases for introducing amino and carboxyl groups to PP surface (Vartiainen *et al.* 2005). Besides plasma treatments, UV irradiation and chemical modification have also been used to incorporate active groups onto the surface of PP and PE (Nahar *et al.* 2001; Naqvi *et al.* 2002; Goddard *et al.* 2007).

The immobilization of enzyme to packaging surfaces often requires the use of mediator reagents. By far, glutaraldehyde and carbodiimide are the most widely used bifunctional reagents for immobilizing enzyme to activate polymer surfaces. Carbodiimide is used to achieve amide bonding between carboxyl group and primary amines of the enzyme; whereas glutaraldehyde is utilized to bridge two amino groups (Vartiainen *et al.* 2005). Diglycidyl ether has also been

used as a mediator, functioning the same way as glutaraldehyde (Sano *et al.* 1993). In addition, a “spacer” arm may be used to link the functional surface and enzyme to increase the mobility of the immobilized enzyme, which improves the enzymatic activity (Bulmus *et al.* 1997). The most common “spacers” used for enzyme immobilization are dextrans, polyethyleneglycol (PEG), ethylenediamine and polyethyleneimine due to their low toxicity. For GOX, when it is immobilized to poly(acrylic acid) grafted PE using poly(ethylene oxide) as a spacer, its pH and thermal stability are both lowered when compared both to free enzyme and the enzyme immobilized to the polymer without inserting a spacer (Wang and Hsiue 1993).

The entrapment of enzyme, also known as encapsulation, is based on the localization of an enzyme either in a lattice or a micro capsule of polymers (Brena and Batista-Viera 1997). There is no covalent association between the network and the biomolecule. Among those enzyme immobilization methods, encapsulation is of particular interest because of the simplicity of preparation and biomolecule freedom (Campas and Marty 1997). Natural polymers such as gelatin, agarose, carrageenan, cellulose, and albumin, are extensively used in gel entrapment of enzymes. However, because of their poor mechanical properties, low temperature tolerance and weak bacterial resistance, synthetic polymers are more widely used (Cantarella *et al.* 1997). The mass transfer through membrane and gel limits the application of this method. To overcome this, fiber and micro entrapments are of increasing interest (Wadiak and Carbonell 1975; Dinelli *et al.* 1976). The structure of fibers or micro-carriers

allows the diffusion of substrate and product molecules across the polymer to participate in the reaction, while still entrapping the enzyme. This approach would be expedient when enzyme migration to the applied products needs to be avoided.

2.5. ELECTROSPUN FIBERS

The academic community generally agrees to the < 100 nm criterion as the benchmark for nanotechnology (Ko 2004), while broader flexibility, even up to 500 nm, is classified as sub-microtechnology (Ramakrishna *et al.* 2005). As shown in Figure 2, surface area of fibers increases dramatically as their diameter decreases, thereby resulting in higher activity of biomaterials attached to the fiber surface (Ko 2004). Also, the smaller diameter of conductive fibers plays an important role in increasing their electrical activity (EL-Aufy *et al.* 2003). In general, it has been established that as fiber diameter decreases, the strength of fiber tends to increase exponentially (Tomanek *et al.* 2008).

There are many methods for forming submicron fibers, including chemical vapor deposition (CVD) (Manafi and Badiie 2008), template synthesis (Moon and MacCarthy 2003), phase separation (Lim and Tan 2003), and electrospinning (Doshi and Reneker 1995; Jia *et al.* 2002; Herricks *et al.* 2005; Ye *et al.* 2005; Ren *et al.* 2006). In CVD process, substrate is exposed to volatile precursors which react and/or decompose on the surface of substrate to produce desired deposit. Nanocylinders of desired materials could be synthesized within the pores of a porous membrane through template synthesis. Among these methods, electrospun fibers have received the most research interest due to a

relatively simple production process. Electrospun fibers have been explored for a number of applications, including tissue scaffolds, carriers for bioactive agents, protective clothing, high performance filters, biosensors, wound dressing, drug delivery, etc. (Huang *et al.* 2003; Katti *et al.* 2003; Zheng *et al.* 2003; Li and Xia 2004; Teo and Ramakrishna 2006; Goldberg *et al.* 2007).

Figure 3. Effect of fiber diameter on surface area. Adapted from Ko 2004.

2.6. ELECTROSPINNING

Electrospinning is a process capable of forming ultrafine fibers with diameters in the range of 10 to several hundred nanometers by spinning a polymer solution or melt using electrostatic force. The principle of electrospinning is straightforward: the electrical force applied on a deformed drop of polymer solution or melt overcomes a critical value, surface tension of the drop, and stretches it in the electrical field (Huang *et al.* 2003). In a typical electrospinning process (Figure 3) the polymer solution or melt first forms a shape known as a Taylor cone by increasing the voltage applied to the spinneret. When the voltage increases beyond the critical value, the polymer solution eventually ejects from the tip of the Taylor cone and is directed towards the grounded collector. A typical polymer jet exhibits an initial linear region as it leaves the Taylor cone, but undergoes whipping instability as it travels further away from the spinneret (Li and Xia 2004; Ramakrishna *et al.* 2005).

Figure 4. Schematic representation of setup for electrospinning of PLA and GOX-in-PLA microfibers.

Vega Lugo has summarized the effects of electrospinning conditions on the properties of electrospun fibers (Table 1). As shown in this Table, the properties of solution, electrospinning conditions and ambient parameters all contribute to the properties of the final electrospun fibers such as morphology, diameter, and their mechanical characteristics. For example, as the viscosity of solution increases, the diameter of fibers becomes larger, the beads become bigger and their shape changes from spherical to spindle-like (Hong *et al.* 1999; Ramakrishna *et al.* 2005; Vega Lugo 2008).

Table 1. Factors affecting properties of electrospun fibers. Adapted from Vega Lugo, 2008.

Electrospun properties/characteristics	fibersFactors effects	References (polymeric system)
Morphology	Rapid rate of evaporation resulted in ribbon like fibers.	(Fong <i>et al.</i> 2002) (6 Nylon in HFIP and DMF)
Diameter	Larger diameter is related to higher solution concentration Higher dielectric constant is related to thinner diameter Higher applied voltage related to larger diameter. Higher temperature produced more uniform fibers	(Ramakrishna <i>et al.</i> 2005) (Huang <i>et al.</i> 2003) (Son <i>et al.</i> 2004) (PEO in chloroform, ethanol, DMF and water) (Demir <i>et al.</i> 2002) (polyurethane in DMF) (Demir <i>et al.</i> 2002) (polyurethane in DMF)
Presence of beads	Increase in viscosity and conductivity avoided the formation of beads	(Fong <i>et al.</i> 2002) (PEO in water) (Ramakrishna <i>et al.</i> 2005)
Deposition rate of micro-nanofibers	Higher temperature related to higher deposition rate of micro-nanofibers	(Demir <i>et al.</i> 2002) (polyurethane in DMF)
Formation of droplets	Low viscosity in the polymer solution promoted the formation of droplets (the viscosity range in which the polymer solution is spinnable depends on the polymer)	(Fong <i>et al.</i> 2002) (PEO in water) (Huang <i>et al.</i> 2003)

As long as no splitting of the jet occurs, viscosity, which is related to polymer molecular weight and solution concentration, is one of the most important factors influencing the diameter of electrospun fibers. Demir *et al.* (2002) found that fiber diameter of electrospun polyurethane fibers is proportional

to the third power of solution concentration. Furthermore, increased concentration favors the formation of curly fibers instead of beads. The voltage applied to the spinneret also plays a role in affecting the morphology of electrospun fibers. It has been reported that increasing the applied voltage results in decreased stability of the electrospinning jet, which, in turn, increases the amount of beads formed and fiber diameter (Deitzel *et al.* 2001). The longer the spinneret-collector distance the greater is the occurrence of beads on the collector because the field strength then exceeds its optimal value (Megelski *et al.* 2002; Jarusuwannapoom *et al.* 2005). In some circumstances, the increased distance leads to smaller diameter fibers since there is a longer flight time for the solution to be stretched before it is deposited on the target (Reneker *et al.* 2000; Zhao *et al.* 2004; Ayutsede *et al.* 2005).

Besides the process parameters discussed above, ambient conditions, such as temperature and relative humidity (RH), will also affect the morphology of electrospun fibers. In the case of electrospinning of a polyoxymethylene solution, the change of RH in the environment influenced fiber structure, pore size and the distribution of fibers of different pore diameter (Kongkhleng *et al.* 2008). It has also been pointed out that elevated temperature can bring about a decrease of bead density and slight increase of fiber diameter, when a chitosan and poly(acrylamide) blend is electrospun (Desai and Kit 2008).

Besides forming fibers of mono-material, the electrospinning process also can be adapted for spinning composite fibers with enhanced material and functional properties. For instance, carbon nanotubes (Ko *et al.* 2002), silver particles (Xu *et al.* 2006) and nanoclays (Lee *et al.* 2004) have been incorporated into polymer solutions to produce composite fibers for various applications. Electrospun fibers have also been exploited as an enzyme carrier to enhance enzyme stability and reusability (Jia and others 2002; Kim and others 2005a; Herricks and others 2005; Ye and others 2006; Kim and others 2005b). According to their large surface area and easy production, electrospun nanofibers could be potentially explored as enzyme carriers in the field of enzyme immobilization.

In general, two techniques have been used to immobilize enzymes in electrospun fibers. The first approach involves activating the surface of electrospun fibers such that the enzyme of interest is attached to the fiber surface, directly or indirectly through a linker, via covalent attachment (Jia and others 2002; Kim and others 2005a; Wang and others 2006). The second approach involves the direct incorporation of enzyme into a polymer solution followed by electrospinning. This approach has the advantage of embedding the enzyme within the fibers to achieve optimal stability, and the porous morphology of the fiber carrier to facilitate the mass-transfer of reaction substrates (Xie and Hsieh 2003; Wu and others 2005; Sawicka and others 2005; Ren and others 2006; Nakane and others 2007). Although the second approach is

straightforward, because the enzyme is not covalently attached to the polymer, it has a propensity to leach out from the fiber carrier. To circumvent this problem, several studies have successfully obtained stable and bioactive fibrous mats by electrospinning polymer emulsions, wherein the enzyme-containing aqueous phase is dispersed in the hydrophobic continuous phase with the aid of a surfactant (Herricks and others 2005; Yang and others 2008).

3 JUSTIFICATIONS AND OBJECTIVES

Unlike conventional food packaging systems, which only provides passive protection of food, active packaging actively facilitates the protection of food to maintain or enhance its quality. In many recent studies, antimicrobial agents have been incorporated in packaging materials. For example, cinnamon essential oil was immobilized in wax paraffin as an active coating to preserve bread (Rodriguez *et al.* 2008); and thymol was loaded in zein films and its release exerted an antimicrobial effect (Del Nobile *et al.* 2008). Although a number of studies reported the activation of the LP system as an antimicrobial preservative, most of them involved direct addition of the necessary components. Only a few studies using immobilized enzymes in whey protein film and alginate film to activate LP have been reported (Min *et al.* 2005; Yener *et al.* 2009). Unfortunately, their applications are not optimum because the polymer materials will swell in an aqueous phase. Hence, enzyme immobilization on polymer materials of mechanical resistance should be explored.

Electrospinning is a fast and convenient approach for producing micro- to nanofibers with large surface area. Applying this technique, an enzyme may be incorporated into the fiber-forming solution and spun into a fibrous membrane, which could then be attached to the interior of a package. We envisage that an active packaging system which is capable of extending the shelf-life of milk can be achieved. Here, GOX will be immobilized in electrospun fibers to trigger the

generation of H_2O_2 in order to constitute a complete LP system. To prepare the GOX-immobilized fiber, PLA was chosen as the carrier polymer for electrospinning because it is relatively hydrophobic, environmentally benign, and it can be electrospun readily to form mono-material, nanocomposite, and bioactive fibers (Dersch and others 2003; Ogata and others 2006; Zhou and others 2006a; Zhou and others 2006b; Li and others 2006). Furthermore, preliminary studies showed that the electrospun PLA fibers are porous, which is an important feature to enable the diffusion of reactants and products through the fibers (Bognitzki *et al.* 2001).

In this study, we will explore the use of electrospun fibers as a carrier to immobilize GOX for activating LP system in milk. The objectives of this project are:

- Development of methodology to immobilize GOX in electrospun PLA fibers:
 - ❖ Development of the formulation for PLA solution with GOX emulsion.
 - ❖ Determination of appropriate electrospinning process conditions.
 - ❖ Evaluation of microstructure of the electrospun GOX-in-PLA fibers.
- Evaluation of the enzymatic activity of the immobilized GOX.
- Evaluation of the storage stability and reusability of the immobilized GOX.
- Evaluation of the efficacy of immobilized GOX in milk.

4 ELECTROSPINNING OF GOX IN PLA MICROFIBERS

4.1. MATERIALS

PLA resins (6201D) were donated by Natureworks LLC (Minnetonka, MN, USA). Low-density polyethylene (LDPE) film (Sclairfilm S291) was donated by DuPont Canada Inc. (Whitby, Ontario). Chloroform, dimethylformamide (DMF), sorbitan monopalmitate (SMP), and GOX from *Aspergillus niger* (155.6 U/mg, 75% protein) were all purchased from Sigma-Aldrich Inc. (Oakville, Ontario).

4.2. METHODS

4.2.1. PREPARATION OF PLA SOLUTION AND GOX-IN-PLA EMULSIONS

The procedures for preparing GOX-in-PLA emulsion are summarized in Figure 4. PLA solution (10% w/w) was prepared by dissolving PLA resin in a chloroform:DMF solvent blend (9:1 by weight). DMF was added to depress the vapor pressure of chloroform to prevent the premature drying of PLA solution, which otherwise tended to cause spinneret blockage during electrospinning.

Aqueous GOX-in-chloroform emulsions containing 5% (w/w) SMP and different aqueous GOX concentrations (1%, 2%, and 10% w/w) were prepared. The emulsification of polymer solutions was carried out using an ultrasonic processor (Vibra-Cell™ VC 505, Sonics & Materials Inc., Newtown, CT, USA) for 1 min (2-s on and 2-s off pulses). The emulsification step was conducted in an ice bath to prevent thermal denaturation of the enzyme. To determine the droplet size of the dispersed phase, the emulsions were diluted in chloroform and their particle size distributions were measured using dynamic light scattering (DLS)

(Zetasizer Nano-ZS, ZEN3600, Malvern Instruments Ltd., UK) in triplicate (Liu *et al.* 2007). To prepare the final aqueous GOX-in-PLA emulsions, the aqueous GOX-in-chloroform emulsions were mixed with PLA solution at a ratio of 20:80 by weight. Six different formulations were prepared to evaluate the effect of emulsifier and aqueous phase contents. The formulations of the aqueous GOX-in-PLA emulsions (A, B, C, D, E, and F) and pure PLA (G) solution are listed in Table 2. All formulations were adjusted such that they all had the same final GOX concentration of 0.343 mg/g in the spinning emulsion, although the amounts of aqueous phase were different.

Figure 5. Flow diagram for preparation of GOX-in-PLA emulsion.

Table 2. Aqueous GOX and SMP contents of various emulsion formulations (A to F) for electrospinning into GOX-in-PLA microfibers. Formulation G is the control without GOX and SMP.

Dispersed aqueous GOX phase, % w/w	SMP, % w/w		
	0	1	2
0	G	—	—
0.2	—	A	D
0.4	—	B	E
2.0	—	C	F

4.2.2. ELECTROSPINNING AND CASTING OF GOX-CONTAINING PLA

PLA microfibers were electrospun using a setup shown schematically in Figure 5. PLA solution or aqueous GOX-in-PLA emulsion was loaded in a 10-mL glass syringe (SGE Analytical Science Pty Ltd., Ringwood, Victoria, Australia) connected to a 20-gauge blunt end stainless steel needle spinneret (Hamilton Co., Reno, NV, USA) via Teflon tubing (Hamilton Co.). The polymer solution was pumped to the spinneret at a constant flow rate (1 mL/hr) by means of an infusion pump (Model 780100, Kd Scientific Inc., Holliston, MA, USA). The spinneret was attached to a positive electrode maintained at 10 kV using a DC power supply (Model 210-30R, Bertan, NY, USA), while the 25-cm diameter circular steel plate was grounded to zero potential. A piece of square LDPE film (900 cm²) was attached to the collector to act as a backing material to support the electrospun fibers. The distance between the tip of the spinneret and the collector was fixed at 16 cm and a fixed spinning time of 10 min was used

throughout the entire study. The entire setup was housed within an environmental chamber (MLR- 350H, Sanyo Electric Co., Ltd., Japan) to control the relative humidity (RH) and temperature. Three RHs (10%, 20%, 30%) and three temperatures (10°C, 20°C, 30°C) were selected to optimize electrospinning conditions.

In this study, as in a typical electrospinning process, the polymer solution first formed a pendant droplet as it exited from the spinneret tip. By increasing the voltage applied to the spinneret, the droplet deformed into a shape known as a Taylor cone (Doshi and Reneker 1995). When the voltage increased beyond the critical 8 kV, the polymer solution eventually ejected from the tip of the Taylor cone and was directed towards the grounded collector. The typical polymer jet exhibited an initial linear region as it left the Taylor cone, but whipped chaotically as it traveled further away from the spinneret. The drawing effect due to this whipping and the rapid evaporation of solvent from the polymer jet resulted in continuous solidified GOX-in-PLA fiber accumulation on the collector. By allowing the spinning process to continue, a nonwoven fibrous mat was obtained. For comparison, three portions of aqueous GOX-in-PLA emulsion (6.0 mg) were also cast on LDPE film to form GOX-in-PLA membrane of 1cm × 1cm, 1.5 cm × 1.5 cm, and 2 cm × 2 cm of different thicknesses. The membrane thickness was measured by a digital micrometer (Testing Machines Inc., Ronkonkoma, NY, USA).

4.2.3. SCANNING ELECTRON MICROSCOPY (SEM)

The microstructure and morphology of microfibers were evaluated using a scanning electron microscope (Model S-570, Hitachi High Technologies Corp., Tokyo, Japan). A small piece of sample (1 cm²) was cut and mounted on an aluminum holder and coated with gold using a sputter coater (Model K550, Emitech, England). The fibers were scanned at an accelerating voltage of 10 kV. The diameters of the fibers were measured using Image-Pro Plus 6.0 software from Media Cybernetics Inc. (Bethesda, MD, USA)

4.3. RESULTS AND DISCUSSIONS

4.3.1. PARTICLE SIZE OF GOX-IN-CHLOROFORM EMULSION

DLS data showed an increasing particle size for GOX-in-chloroform emulsions (Figure 6), ranging from 90, 110, to 130 nm (Table 3), as the dispersed aqueous GOX phase increased from 1%, 2%, to 10% w/w, respectively. Due to the limiting emulsification capability of SMP, the dispersed aqueous phase aggregated as its amount increased. The increased droplet size may have also contributed to the spinning instability and poor consistency in fiber morphology.

Figure 6. Size distribution of GOX aqueous solution in chloroform at three concentrations: 1%, 2%, and 10% w/w.

Table 3. Zeta average size of GOX solution in chloroform at three concentrations: 1%, 2%, and 10% w/w.

Type	Sample Name	T (°C)	Z-Ave (d.nm)	Pdl	Pk 1 Mean Int (d.nm)	Pk 1 Area Int (%)
Size	1% 1	25	92.69	0.175	106.8	100
Size	1% 2	25	93.46	0.13	108.6	100
Size	1% 3	25	92.76	0.113	105.6	100
Size	2% 1	25	106.2	0.158	122.4	100
Size	2% 2	25	104.9	0.125	121.6	100
Size	2% 3	25	104	0.112	118.1	100
Size	10% 1	25	134.1	0.148	158.6	100
Size	10% 2	25	133.8	0.143	158.5	100
Size	10% 3	25	134.5	0.112	153.2	100

4.3.2. ELECTROSPINNING OF GOX-IN-PLA MICROFIBERS

Electrospinning behaviors of GOX-in-PLA emulsions and PLA solution were different. At 10 kV, the linear jet region was approximately 2 cm for the PLA solution, and about 1 cm for GOX-in-PLA emulsions. This observation suggested that the electrostatic force imposed on the polymer jet was lower for the GOX-in-PLA emulsion than the PLA solution, probably due to the higher electric conductivity of the emulsion and the propensity of water to dissipate electrostatic charges. Increasing aqueous GOX loadings in the emulsion also caused the Taylor cone to vibrate sporadically, which may be caused by the perturbation of static charge due to the presence of more conductive aqueous droplets.

Figure 7 shows GOX-in-PLA microfibers electrospun on LDPE films under nine experimental conditions. Although minimal differences were observed in the SEM images for these nine microfiber samples (SEM images not shown), it was observed that higher RH caused more dense fiber distribution on the target. By contrast, the temperature had little effect on the morphology of electrospun fibers.

The effect of RH may be related to the ability of moisture in the air to dissipate electrostatic charges on the polymer jet, which in turn diminish the electrostatic force. Based on the electrospinning mechanism, the linear jet portion is shorter as the electrostatic force reduces and this resulted in more whipping motion of the polymer jets, which caused the spread of electrospun fibers. However, the phenomenon mentioned above occurs when the collector is comprised of a conductive metal rather than an insulated PE film as used in current study. On the other hand, the higher RH dissipates electrostatic charges on the PE film, which results in an increase of electrostatic force on polymer jets and in turn a more centralized position of the fibers on the target was achieved. In this study, the effect of RH on insulative film may outweigh that on the polymer jet. Hence, the electrospun fibers obtained at higher RH are more concentrated. Based on the consistency of fiber morphology and stability of electrospinning process, 30% RH and 20°C were chosen as the electrospinning conditions for subsequent experiments.

Figure 7. Distribution GOX-in-PLA microfibers obtained at 9 conditions. The white patches are electrospun microfibers collected on PE film.

4.3.3. SEM ANALYSIS

All aqueous GOX-in-PLA emulsions tested (Table 1) could be electrospun, with the exception of formulation F (highest SMP and aqueous phase contents), which produced microfibers that were wet and carried a strong chloroform odor after 10 min of electrospinning. As shown in Figure 8D and 8E, microfibers spun from emulsions with higher SMP content (2%, w/w) produced wavy microfibers that tended to form fused junction zones, indicating that the microfibers were not

completely dry when they landed on the collector. Thus, although SMP can be used to stabilize the GOX-in-PLA emulsion, excessive surfactant can hamper the solidification of microfibers during spinning due to the vapor pressure depression effect of SMP on chloroform. In contrast, microfibers spun from formulations containing lower SMP content (1%, w/w; Figure 8A to 8C) were well separated from each other. Formulations B and C resulted in microfibers with smaller diameters than formulation A, but irregular remnants were also evident from the SEM micrograph.

These remnants may be caused by coalesced aqueous phase droplets due to the limited amount of surfactant used. Microfibers electrospun from PLA solution alone (Figure 8G) exhibited the most uniform morphologies but were larger in diameter than the other formulations tested. The larger fiber diameter observed for pure PLA fiber can be attributed to a more rapid evaporation of chloroform, quicker solidification of polymer jet, and reduced drawing imposed on the fiber.

Based on these observations, it can be concluded that the emulsifier content played an important role in affecting fiber morphology. To facilitate the electrospinning process, the amount of SMP used and the aqueous GOX loading should be minimized. Accordingly, in the subsequent experiments, formulation A was chosen for the electrospinning of GOX-in-PLA microfibers.

Figure 8. SEM micrographs of microfibers electrospun from PLA solution and various aqueous GOX-in-PLA emulsions (see Table 2 for formulation code).

4.4. CONCLUSIONS

In this study, GOX was successfully immobilized in PLA fibers by electrospinning GOX-in-PLA emulsions prepared with the aid of SMP emulsifier. Environmental conditions, temperature and RH, of the electrospinning process had no significant effect on the morphology of microfibers under SEM. However, RH affected the distribution of microfibers on the target. In order to obtain evenly distributed microfibers, 30% RH was selected as the final electrospinning RH and room temperature (20 °C) was used for all subsequent experiments.

More SMP could help better dissolution of aqueous phase in chloroform, but at the same time it prevented the volatilization of chloroform during the electrospinning process. Therefore, minimizing the use of SMP and enzymatic aqueous phase (1%, w/w and 0.2%, w/w, respectively) resulted in a stable electrospinning process, which produced well-isolated GOX-in-PLA fibers.

5 ENZYME ACTIVITY OF GOX-IN-PLA MICROFIBERS

5.1. MATERIALS

D-(+)-glucose, sodium thiocyanate, catalase suspension in $(\text{NH}_4)_2\text{SO}_4$ solution from *Aspergillus niger* (164 U/ μL), 5,5'-dithiobis(2-nitrobenzoic acid) (Nbs_2), 2-mercaptonethanol, H_2O_2 solution, phosphate dibasic and monobasic were obtained from Sigma-Aldrich Inc. (Oakville, Ontario). GOX-in-PLA fibers were prepared as described in a previous section,

5.2. METHODS

5.2.1. ASSAY OF OSCN^-

The assay of OSCN^- in a model system (deionized distilled water, LP and SCN^-) was based on measuring the decrease of absorbance at 409 nm due to the oxidation of 5-thio-2-nitrobenzoic acid (Nbs) to the colorless product 5, 5'-dithiobis (2-nitrobenzoic acid) (Nbs_2) (Bosch *et al.* 2000). Milk protein, which may affect spectrophotometric measurement, was precipitated and removed as described by Kamau and others (Kamau *et al.* 1990). After protein precipitation, 0.5 mL of the OSCN^- -containing supernatant was mixed with 3.5 mL of pH 7.2 potassium phosphate buffer, followed by the addition of 0.5 mL Nbs . The absorbance at 409 nm was measured using a spectrophotometer (Ocean Optics, Winter Park, FL, USA). The calculation for OSCN^- content was based on the method described by Bosch *et al.* (2000):

$$[\text{OSCN}^-] = \frac{\text{Final Volume} \times (A_{\text{control}} - A_{\text{sample}})}{14.05 \times 2 \times \text{Sample Volume}} \text{ mM}$$

All the reagents were prepared fresh daily except for the Nbs, which was kept at 4°C overnight before use.

5.2.2. ENZYME ACTIVITY OF GOX-IN-PLA MICROFIBERS AND CAST MEMBRANES

To evaluate the enzymatic activity of GOX-in-PLA microfibers, a piece of GOX-in-PLA fibrous mat (2 × 2 cm) was placed in a vial containing the model LP system described in the previous section. The LP system reaction end product, OSCN⁻, was determined using the Nbs assay described in the previous section. One unit (U) of enzymatic activity of GOX-in-PLA fiber was calculated based on the production of 1 μmol of OSCN⁻ per minute. The GOX activity was normalized by the weight of enzyme and expressed as units per milligram GOX. The initial OSCN⁻ generation rate was used for enzymatic activity calculation. Enzymatic activity was compared between GOX-in-PLA microfibers and GOX-in-PLA cast membrane, both of which were prepared from the same emulsion.

To evaluate the stability of immobilized GOX during storage, free enzyme and GOX-in-PLA microfibers were kept at 4°C in hermetically sealed glass jars containing desiccant. The enzymatic activity of both specimens was tested at day 1, 4, 16, and 26.

In addition, in order to evaluate the retention of enzyme in fiber, GOX-in-PLA fibrous mats were reused multiple times by rinsing them twice with deionized distilled water and retesting them for enzymatic activity.

5.3. RESULTS AND DISCUSSIONS

5.3.1. ENZYME ACTIVITY OF GOX-IN-PLA MICROFIBERS VERSUS CAST MEMBRANES

To elucidate the effect of fiber versus membrane carriers, the enzymatic activity for GOX-in-PLA microfibers and cast membrane were evaluated. As shown in Figure 9, GOX encapsulated in the microfibers exhibited enzymatic activity of more than one order of magnitude than in the cast membrane. The effect of thickness is also evident from Figure 9, which shows that thinner cast membranes exhibited higher GOX specific activity. These observations suggest that enzyme species immobilized near the carrier surface were more available to participate in the reaction than those embedded in the core due to mass transport limitation for the reaction substrates and products.

Figure 9. Comparison of GOX activity between GOX-in-PLA microfibers and cast membranes of

different thicknesses. Standard deviations are represented by the error bars.

GOX activity of the microfibers was 13.05 ± 1.26 U/mg GOX, which was significantly higher ($P < 0.05$) than that of the cast membranes. This result was expected due to the larger surface area and more porous structure of the electrospun fibers compared to the cast membranes. Higher enzymatic activities for electrospun fibers than for cast membranes were also reported in the literature for lipase immobilized in polyvinyl alcohol (PVA) fibers (Nakane *et al.* 2007), lipase in cross-linked casein fibers (Xie and Hsieh 2003), and GOX in PVA fibers (Ren *et al.* 2006).

5.3.2. STABILITY OF ENZYME WITHIN GOX-IN-PLA MICROFIBERS

GOX enzymatic activity of the prepared GOX-in-PLA microfibers was about 2 times lower than for the free enzyme (Figure 10). This can be attributed to the limited diffusion of reactants and steric hindrance on the active sites of the immobilized GOX. However, the immobilized enzyme was more stable under the storage condition tested (4°C, 0% RH). After storage for 26 d, GOX immobilized in fiber retained 65% of the original activity, while the activity of the free GOX was reduced to less than 10%.

Figure 10. Storage stability of free GOX and GOX immobilized in PLA microfibers. Data points are mean values based on triplicate determination. Data points are mean values based on triplicate determination. Standard deviations are represented by the error bars.

Figure 11 shows the retention of enzymatic activity during multiple dipping tests with the fiber mats. The enzyme activity decreased by about 37% during the first reuse, suggesting significant leaching losses had occurred during the test and the washing step. Thereafter, repeated reuse of the GOX-in-PLA fibers resulted in a gradual loss of enzymatic activity. However, this result showed that a substantial proportion of GOX was immobilized and remained active in the PLA fibers.

Figure 11. Enzymatic activity of GOX-in-PLA microfibers after repeated assays (20 min per test cycle in LP model system at $21 \pm 2^\circ\text{C}$). Data points are mean values based on triplicate determination. Standard deviations are represented by the error bars.

5.4. CONCLUSIONS

The enzymatic activity increased as the thickness of cast GOX-in-PLA membranes decreased. However, GOX-in-PLA microfibers had significantly higher enzymatic activity than the cast GOX-in-PLA membranes. This may be due to the larger surface area of microfibers and their porous structure, which allow the enzyme greater availability to reactants. At 4°C and 0% RH, the GOX-in-PLA microfibers retained ~62% of their initial enzyme activity, while the free GOX exhibited a ~94% activity loss at the end of day 26. The gradual loss of enzyme

activity in microfibers as they were reused indicated that a substantial amount of GOX, although not all, was encapsulated within PLA microfibers.

6 ACTIVATION OF MODEL LP SYSTEM

6.1. MATERIALS

D-(+)-glucose, sodium thiocyanate, catalase suspension in $(\text{NH}_4)_2\text{SO}_4$ solution from *Aspergillus niger* (164 U/ μL), Nbs₂, 2-mercaptonethanol, H_2O_2 solution, phosphate dibasic and monobasic were obtained from Sigma-Aldrich Inc. (Oakville, Ontario). GOX-in-PLA fibers were prepared as described in a previous section,

6.2. METHODS

6.2.1. TEMPERATURE EFFECT ON OSCN⁻ ACCUMULATION IN LP MODEL SYSTEM

Free GOX (1.82 mg/L) and glucose (1.42 mM) were added in a model LP system consisting of 4.74 mg/L LP and 0.47 mM SCN^- . These GOX and glucose concentrations provided an excess of H_2O_2 as a substrate for LP enzyme reaction. To understand the temperature effect on the end product OSCN^- , two temperatures, 4°C and 23°C, were applied to the model LP system.

6.2.2. DIRECT COMPARED WITH INDIRECT ACTIVATION OF LP SYSTEM

Three methods were tested to elucidate the OSCN^- generation profile in the model LP system: (1) direct activation with 1.41 mM H_2O_2 to act as an oxidizer for SCN^- ; (2) indirect activation with 1.42 mM glucose and 1.41 mg/L free GOX; and (3) indirect activation with 1.46 mM glucose and GOX-in-PLA fibrous mat (2 × 2 cm; 0.345 mg). The model LP system used here was made up of 9.71 mg/L LP and 1.46 mM SCN^- . In method (2), the glucose content was

adjusted such that the amount of H_2O_2 generated was theoretically the same as in method (1). In methods (2) and (3), since the activation of LP is governed by two reactions (that is, the oxidation of glucose catalyzed by GOX to produce H_2O_2 , followed by the oxidation of SCN^- by H_2O_2 catalyzed by LP), excess quantities of LP, SCN^- , and glucose were used to ensure that both the GOX- and LP-catalyzed reactions were not reactant limited. These conditions were established based on previous studies reported in the literature (Min *et al.* 2005; Min and Krochta 2005). To follow the kinetics of OSCN^- formation, the reaction was allowed to proceed for up to 30 h at $21 \pm 2^\circ\text{C}$ under continuous agitation.

6.3. RESULTS AND DISCUSSIONS

6.3.1. TEMPERATURE EFFECT ON MODEL LP SYSTEM

Figure 12. Indirect activation of LP system by 1.42 mM glucose and 1.82 mg/L free glucose oxidase (GOX) at 4°C and 23°C . Data points are mean values based on triplicate determination. Standard deviations are represented by the error bars.

From Figure 12, after its peak, OSCN^- depleted faster at 23°C than at 4°C . The presence of a maximum for these profiles can be attributed to the competing OSCN^- formation and decomposition reactions. OSCN^- is relatively unstable; it decomposes spontaneously in a few hours after it is formed in milk (Korhonen 1980). Aune and Thomas (1977) reported that, although OSCN^- decomposed relatively slowly at 25°C (second-order rate constant of $0.1 \text{ M}^{-1}\text{s}^{-1}$), when the amount of H_2O_2 added was greater than thiocyanate, the accumulation of OSCN^- declined due to the oxidation of OSCN^- by H_2O_2 . According to the phenomenon in Figure 12, the lower temperature might slow down the OSCN^- autologous degradation and mitigate the influence of excessive H_2O_2 .

6.3.2. DIRECT COMPARED WITH INDIRECT ACTIVATION OF LP SYSTEM

In the model LP system, direct addition of H_2O_2 resulted in a rapid initial increase of OSCN^- concentration during the first 15 min, followed by an exponential decay (Figure 14; method (1)). In contrast, the initial rate of OSCN^- formation was slower when the LP system was activated by indirect H_2O_2 generation using the GOX and glucose reaction (Figure 14; method (2)); here, OSCN^- concentration peaked at 2.5 h. When GOX was immobilized in PLA fibers, the OSCN^- concentration maximum was further delayed to 7.1 h (Figure 13; method (3)), which may be caused by the limited reactant diffusion into the PLA microfibers. Activation of the LP system through enzyme generated H_2O_2 also

maintained a higher OSCN^- concentration during the test as compared to the direct H_2O_2 addition method.

Figure 13. Comparison of OSCN^- accumulation profiles for model LP system activated by different methods. Method (1) - direct addition of 1.41 mM H_2O_2 solution; method (2) - indirect H_2O_2 generation by adding 1.42 mM glucose and 1.41 mg/L GOX solution; method (3) - adding 1.46 mM glucose and GOX-in-PLA microfibers. Data points are mean values based on triplicate determination. Standard deviations are represented by the error bars.

The presence of a maximum for these profiles can be attributed to the competing OSCN^- formation and decomposition reactions. OSCN^- is relatively unstable; it decomposes spontaneously in a few hours after it is formed in milk (Korhonen 1980). Dionysius and others (1992) observed that when H_2O_2 was generated using GOX, the low $\text{H}_2\text{O}_2/\text{SCN}^-$ ratio resulted in OSCN^- as the oxidation product. However, when H_2O_2 was generated rapidly by sodium

carbonate peroxyhydrate, the accumulation became strongly dependent on SCN^- concentration. A low $\text{H}_2\text{O}_2/\text{SCN}^-$ ratio resulted in OSCN^- as the predominant product, but a high $\text{H}_2\text{O}_2/\text{SCN}^-$ ratio reduced the accumulation of OSCN^- substantially (Dionysius and others, 1992). Another possible reason for the observed rapid depletion of OSCN^- in method (1) is the deactivation of LP by the direct H_2O_2 addition. It has been reported that excess H_2O_2 can lead to the formation of a ferrylperoxidase adduct, resulting in the irreversible inactivation of LP (Seifu *et al.* 2005).

Figure 14. The OSCN^- concentration profiles for methods (1) and (2) (mentioned in Figure 13) in the presence of equal amounts of H_2O_2 produced by direct addition and oxidation of glucose, respectively. Data points are mean values based on triplicate determination. Standard deviations are represented by the error bars.

Based on these experimental observations and the reported data from the literature, we can conclude that the controlled release of H_2O_2 using GOX-in-PLA fibers is more effective than direct addition of H_2O_2 for achieving a sustained generation of antimicrobial OSCN^- in milk, presumably due to reduced oxidative effect of H_2O_2 on LP and OSCN^- .

6.4. CONCLUSIONS

When free GOX and glucose were used as the source of H_2O_2 , the depletion of OSCN^- after its peak accumulation was slower at 4°C than 23°C . This might be due to the instability of OSCN^- at the higher temperature. Compared to direct activation of LP using H_2O_2 , the GOX-in-PLA microfibers provided a more sustained production of antimicrobial OSCN^- , presumably due to the oxidation of OSCN^- and deactivation of LP by the excess H_2O_2 present. Theoretically, a higher content of OSCN^- will provide a greater antimicrobial effect.

7 LP SYSTEM IN BOVINE MILK

7.1. MATERIALS

D-(+)-glucose, sodium thiocyanate, catalase suspension in $(\text{NH}_4)_2\text{SO}_4$ solution from *Aspergillus niger* (164 U/ μL), Nbs₂, 2-mercaptonethanol, H₂O₂ solution, ABTS and glycerol ReagentPlus™ were obtained from Sigma-Aldrich Inc. (Oakville, Ontario). GOX-in-PLA fibers were prepared as described in a previous section. Raw milk was obtained from Elora Research Station (Elora, Ontario). All media Difco™ Lactobacilli MRS agar, Difco™ Pseudomonas Agar F, Difco™ Plate Count Agar (PCA) and BD GasPak™ EZ Anaerobe Container System were purchased from Becton Dickinson (Sparks, MD). Fisher Bioreagents® peptone was obtained from Thermo Fisher Scientific Inc. (Fairlawn, NJ).

7.2. METHODS

7.2.1. LP CONTENT IN BOVINE MILK

Determination of LP activity in milk was based on LP catalyzed oxidation of ABTS to its oxidation product (ABTSO) (IDF 1993). The activity, one unit U_{LP}, was expressed as the amount of enzyme that oxidizes 1 μmol ABTS/min.

To prepare skim milk samples for LP assay, raw milk was centrifuged at 6000 rpm (6371 G) for 25 min at 4°C and then filtered through glass microfiber filter paper (934-AH, Fisher Scientific Inc., Whitby, Ontario, Canada) to remove the lipid phase (Dalglish *et al.* 2005). To prepare heated milk samples, raw milk samples (3 mL) were placed in a 72°C water bath for 15 s to simulate a

conventional pasteurization process. All the milk samples were kept at 4°C temporarily before determination. To eliminate interference by protein during spectrophotometric measurements, the proteins were precipitated and removed from all the milk samples as described by Kamau and others (1990).

7.2.2. PRELIMINARY STUDY ON ACTIVATION OF LP SYSTEM IN MILK BY GOX-IN-PLA MICROFIBERS

To test the efficacy of GOX-in-PLA fibers in milk and the possible limiting LP components in milk, GOX-in-PLA fibrous mats (4 cm × 4 cm; ~3 mg) were placed in four different milk samples (30 mL): (a) milk as is; (b) milk fortified with 0.49 mM SCN⁻; (c) milk fortified with 1.46 mM glucose; and (d) milk fortified with 0.47 mM SCN⁻ and 1.42 mM glucose. The non-fortified milk sample without GOX-in-PLA fibers was used as a control. The OSCN⁻ generated was monitored for up to 12 h at 21 ± 2°C by analyzing three 0.5 mL aliquots of sample at predetermined time intervals. All sample analyses were carried out in triplicate.

7.2.3. MICROBIOLOGICAL ANALYSIS OF MILK LP SYSTEM ACTIVATED BY GOX-IN-PLA MICROFIBERS

The antimicrobial efficacy of GOX-in-PLA fibers was tested for bovine milk stored at 4 and 30°C. Three different raw milk samples (100 mL) were prepared: (a) raw milk alone; (b) milk fortified with 2.7 mL 50 mM glucose, 0.51 mL 30 mM SCN⁻ and GOX-in-PLA fibrous mat (5 cm × 5 cm; 0.0025 and 0.00367 g); (c) milk fortified with 0.51 mL 30 mM SCN⁻ and GOX-in-PLA fibrous mat (5 cm × 5 cm; 0.00272 and 0.0044g). Accumulated OSCN⁻ and microbial growth were monitored for up to 8 days at predetermined time intervals. The amount of SCN⁻

used here was in accordance with the guideline suggested by the International Dairy Federation (IDF 1988).

The concentration of OSCN⁻ in milk was determined based on the procedure described in section 7.2.2. To elucidate the efficacy of the GOX-immobilized electrospun fibers, viable plate counts were performed in duplicate using 0.1% peptone water as a diluent in the following media and conditions: (1) total bacteria were enumerated on PCA plates incubated at $32 \pm 1^{\circ}\text{C}$ for 24 ± 1 h; (2) pseudomonads were determined on Pseudomonas agar at $21 \pm 1^{\circ}\text{C}$ for 25 ± 1 h; (3) lactobacilli were counted on lactobacilli MRS Agar in a reduced-oxygen atmosphere generated using a GasPakTM EZ anaerobe patch at $32 \pm 1^{\circ}\text{C}$ for 48 ± 2 h (Frank *et al.* 1992).

To obtain the spiral plate count (SPC), 50 μL of each sample was spirally plated on a 100 mm plate of medium using an Autoplate 4000[®] from Spiral Biotech, Inc. (Norwood, MA). The actual number of microorganisms was calculated from the colony count on plates using a Spiral Plater cfu calculator (described in Appendix). The automated spiral plater has a detection range from $20 - 4 \times 10^5$ cfu/mL on one plate of 100 mm diameter prior to dilution.

7.3. RESULTS AND DISCUSSIONS

7.3.1. LP ACTIVITY OF MILK

The LP activities obtained for raw milk, skim milk, and heated milk were 2.85 ± 0.039 , 2.80 ± 0.020 , and 1.80 ± 0.086 U_{LP}/mL, respectively, which are of the same order of magnitude as those cited by Kamau *et al.* (1990) and within the activity range reported by Fonteh *et al.* (2002). The fact that raw and skim

milk had comparable LP activity values ($P > 0.05$) indicated that the necessary components for the LP system existed mainly in the non-lipid phase. This implies that skimming of milk will not affect the LP activity. By contrast, heating of milk resulted in an LP activity that was 36.8% lower than in the unheated control ($P < 0.05$), indicating that the LP system will have a less effect in pasteurized milk. Nevertheless, others have reported that under the typical pasteurization condition of 72°C for 15 s, adequate LP activity should remain in the milk for GOX-in-PLA microfibers to activate the LP system (Griffiths 1986).

7.3.2. PRELIMINARY STUDY ON ACTIVATION OF LP SYSTEM IN MILK BY GOX-IN-PLA MICROFIBERS

The addition of GOX-in-PLA fibers to unfortified skim milk (non-pasteurized) caused a progressive increase in OSCN⁻ concentration for up to about 9 h (Figure 15A). This showed that the naturally occurring SCN⁻ and glucose in milk were adequate to activate the GOX and LP catalyzed reactions. Compared with the unfortified milk, fibers added to the milk fortified with glucose resulted in a higher OSCN⁻ concentration (Figure 15B), whereas fibers added to milk fortified with SCN⁻ resulted in a similar OSCN⁻ profile (Figure 15C). These results seem to suggest that glucose was a limiting factor in the GOX reaction. A lack of glucose may have prevented the formation of the H₂O₂ needed in the subsequent LP-catalyzed reactions (oxidation of SCN⁻). This hypothesis is supported by the further observation that fibers added to the milk fortified with both glucose and SCN⁻ resulted in a substantially higher OSCN⁻ concentration (Figure 15D). Here, the added glucose allowed the GOX-catalyzed limiting reaction to proceed, thereby generating additional H₂O₂ to oxidize the fortified

SCN⁻. The OSCN⁻ data agreed well with the quality attributes of the test samples (photos inserted in Figure 15). At the end of the 24 h test period at 22°C, off-odor, coagulation and discoloration were observed in the control milk (not treated with GOX-in-PLA fibers). For milk samples treated with GOX-in-PLA fibers, the extent of deterioration was less severe. In contrast, samples fortified with glucose and SCN⁻ showed no visible deterioration or detectable off-odor. Thus, the efficacy of GOX-in-PLA fibers should be enhanced in lactose-free or lactose-reduced milks, in which the glucose content is higher than in regular milk.

Figure 15. Activation of LP system at $21 \pm 2^\circ\text{C}$ using GOX-in-PLA microfibers in skim milk samples without fortification or fortified with glucose and/or SCN^- . (A) Without fortification; (B) 1.46 mM glucose; (C) 0.49 mM SCN^- ; (D) 1.42 mM glucose + 0.47 mM SCN^-

7.3.3. MICROBIOLOGICAL ANALYSIS MONITORED WITH OSCN- ACCUMULATION

Figure 16 shows the OSCN^- content of all milk samples stored at 4°C over an eight day period. The OSCN^- concentration for all samples increased initially and reached the maximal level on the 3rd day. Compared to sample C fortified with SCN^- alone, the OSCN^- concentration in sample B maintained a higher level due to the sustained generation of H_2O_2 caused by the fortified glucose. Since both sample B and C were treated with GOX-in-PLA microfibers, therefore their OSCN^- concentrations were higher than the control (sample A). Data from Figure 16 shows that the LP system can be activated in natural milk during storage at 4°C (sample A), although higher OSCN^- concentration can be achieved by treating the milk with SCN^- and GOX-immobilized fibers (samples C).

Figure 16. Monitoring of OSCN^- in skim raw milk stored at 4°C : (A) without fortification; (B) GOX-in-PLA microfibers + 1.31 mM glucose + 0.15 mM SCN^- ; (C) GOX-in-PLA microfibers + 0.15 mM SCN^- .

Figure 17. Growth of total bacteria in skim raw milk at 4°C: (A) without fortification; (B) GOX-in-PLA microfibers + 1.31 mM glucose + 0.15 mM SCN⁻; (C) GOX-in-PLA microfibers + 0.15 mM SCN⁻.

Figure 18. Growth of pseudomonads in skim raw milk at 4°C: (A) without fortification; (B) GOX-in-PLA microfibers + 1.31 mM glucose + 0.15 mM SCN⁻; (C) GOX-in-PLA microfibers + 0.15 mM SCN⁻.

In terms of the microbial growth, at 4°C no significant difference between the control and GOX-in-PLA microfibers treated milk samples was found for total

plate count (Figure 17). Due to the glucose and immobilized GOX in milk sample B, a different inhibitory effect on pseudomonads between sample B and control was observed from day 1 through day 8 (Figure 18). However, after 8 days, the plate counts for total bacteria and pseudomonads reached ~ 7 Log cfu/mL, at which time the product is deemed to be unacceptable due to flavor defects and microbial proliferation (Murphy 2007). However, no lactobacillus was detected in any of the milk samples throughout the 8-day storage (graph not shown). Visually, no deterioration was observed in the samples.

Figure 19. Monitoring of OSCN⁻ in skim raw milk at 30°C: (A) without fortification; (B) GOX-in-PLA microfibers + 1.31 mM glucose + 0.15 mM SCN⁻; (C) GOX-in-PLA microfibers + 0.15 mM SCN⁻.

The microbial plate counts and OSCN⁻ concentrations for samples stored at 30°C were monitored only for 24 h due to the rapid deterioration rate. In the first 3 hours, the growth of total bacteria (Figure 20) and pseudomonas (Figure

21) in all milk samples was static, during which OSCN^- accumulated to maximum (Figure 19).

Figure 20. Growth of total bacteria in skim raw milk at 30°C: (A) without fortification; (B) GOX-in-PLA microfibers + 1.31 mM glucose + 0.15 mM SCN^- ; (C) GOX-in-PLA microfibers + 0.15 mM SCN^-

Figure 21. Growth of pseudomonads in skim raw milk at 30°C: (A) without fortification; (B) GOX-in-PLA microfibers + 1.31 mM glucose + 0.15 mM SCN⁻; (C) GOX-in-PLA microfibers + 0.15 mM SCN⁻

Figure 22. Growth of lactobacillus in skim raw milk at 30°C: (A) without fortification; (B) GOX-in-PLA microfibers + 1.31 mM glucose + 0.15 mM SCN⁻; (C) GOX-in-PLA microfibers + 0.15 mM SCN⁻

The effects of GOX-fibers against lactobacillus in milk are shown in Figure 22. Growth of lactobacillus was detected for samples A and C after 3 hours, while no colony was detected up to 6 hours in sample B. Considering the detection limitation of the spiral plate method, a lower limit 20 cfu/mL was used for data analysis. Overall, sample B (fortified with glucose) exhibited a greater growth inhibitory effect than samples A and C for up to 20 hours. The main antibacterial effects of LP system occurred between 6 to 20 h when a maximum ~2-3 log cfu/mL reduction in count was observed. The greatest growth inhibitory effect on total bacteria, pseudomonas and lactobacillus occurred at 10, 6, and 6

hour, respectively, which might be due to the different mechanisms of OSCN⁻ with each type of microorganism.

From Figure 19, it is worth noting that during the OSCN⁻ ramp up phase (the first hour), no detectable microbial growth was observed for all treatments (Figure 20 to Figure 22). The growth inhibitory effect on total bacteria and pseudomonads became evident only several hours later after maximal OSCN⁻ concentration has been established. Here, the greater the maximal OSCN⁻ concentration attained during the first hour, the greater were the bactericidal/bacteriostatic effects observed. This observation suggests that the peak content of OSCN⁻ is critical for the efficacy of the LP system, and that the accumulated OSCN⁻ is capable of exerting its antimicrobial function throughout storage. The rapid decrease of OSCN⁻ after the first hour observed in Figure 19 might be due to the spontaneous degradation of OSCN⁻ as it is being formed (Aune and Thomas 1977; Korhonen 1980).

When exposed to the LP system, the bacterial cytoplasmic membrane of both streptococci and coliforms are damaged or changed because of the leakage of potassium ions, amino acids and polypeptides, resulting in bacteriostatic and bactericidal effects on these microorganisms, respectively. The difference is due to their cell morphology. As gram-positive organisms, streptococci may be less susceptible to the oxidation of OSCN⁻ because of their cell wall (Reiter and Harnulv 1984). In a similar way, our current study showed that the bactericidal

effect of LP system was more obvious on gram-negative pseudomonads than gram-positive lactobacillus, which agreed the previous conclusion drawn from structure difference of microorganisms.

In the study of Bjorck *et al.* (1975), raw milk samples were fortified with SCN⁻, glucose and free GOX at 30°C. *Pseudomonas fluorescens* in the treated milk sample declined rapidly in the first 6 hours of incubation, after which the bacteria grew at the same growth rate as the control sample. The observed higher inhibitory effect against *Pseudomonas fluorescens* compared to our study might be due to: 1) LP system is more effective against *fluorescens* species; 2) free GOX is more available for generating H₂O₂; 3) glucose content is less in our study.

A few recent studies utilized electrospinning to immobilize GOX (Ren *et al.* 2006; Wang *et al.* 2009), but none described the activation of LP system from milk. Nevertheless, other carriers have been reported for immobilization of this enzyme to extend the shelf-life of milk. Garcia-Garibay *et al.* (1995) treated raw milk with lactase and GOX immobilized in nylon pellets. The counts of *Pseudomonas* spp., the predominant psychrotrophs in milk, were reduced by 91% compared to the control raw milk at 8°C during the 24 hour test period. By contrast, in our study at 4°C, the GOX-in-PLA microfibers reduced the *Pseudomonas* spp. by ~50% on day 3, ~89% on day 6 and ~73% on day 8. The higher temperature will enhance the activity for both LP and GOX. Also, in Garcia-

Garibay's study, their GOX was immobilized through covalent bonding to the carrier surface, providing more active sites to react. Bjorck also immobilized GOX and lactase on glass beads (Bjorck and Rosen 1976). After treating with the immobilized enzyme system, the whey from fresh pooled milk enhanced with 0.26 mM SCN⁻ became bactericidal against *P. fluorescens*. The bacterial numbers decreased by 99% within 2 hours. The differences in antimicrobial efficacy between our studies and those reported in the literature may be due to the different test conditions used, immobilization method employed, and the composition (e.g., [glucose], [SCN⁻] and LP activity) of the milk tested. As pointed out by Fonteh *et al.* (2002), LP activity in milk had a huge variation throughout lactation of cows, especially during the early stage of lactation. In our studies, the amounts of SCN⁻ and H₂O₂ are the limiting factors in LP system. Thus, the LP activity is expected not to be a contributing factor that affects the efficacy of the LP system.

For total bacteria and pseudomonads, the LP system at 30°C was more bactericidal than at 4°C, which might due to the higher peak content of OSCN⁻ (Figure 19) observed at the higher temperature (Figure 16). It is reported that the maximum growth rate of lactobacilli occurs at ~35°C (Collins and Hartlein 1982), which meant they were more susceptible to the effects of LP at higher temperatures. This can explain that why lactobacillus was not detectable at 4°C in our study.

Zajac *et al.* (1983) reported that the LP system, which was activated by direct addition of NaSCN and sodium percarbonate, can retard bacterial multiplication (standard plate count, coliform, and psychrotroph) when the temperature increased from 10 to 17°C. Harnulv and Kandasamy (1982) recommended 15°C as the maximum temperature for overnight storage of raw milk due to the activity of the LP system. These studies also indicated a strong dependence of the antibacterial effect of the LP system on temperature. Obviously, 4°C is not the optimum working temperature for the LP system. The combination of the LP system and other storage temperatures, such as 10°C and 17°C, might be a potential milk preserving method. Storage at these higher temperatures will reduce energy required for cooling. According to our findings and the information reported in the literature, the LP system appears to be most effective during the first 72 hours. Since the activated LP system is more effective at higher temperatures than at refrigeration temperatures, the active electrospun fibers developed in the current study may be useful to reduce the impact of thermal abuse during transportation or storage of raw milk before further processing.

The LP system is not only effective against typical microorganisms in milk, but also other microflora such as *Streptococcus* spp. (Marshall *et al.* 1986), *Escherichia coli* (Dionysius *et al.* 1992; Bosch *et al.* 2000), fungi (Garcia-Garibay *et al.* 1995), *Listeria* spp. (Earnshaw and Banks 1989; Kamau *et al.* 1990) and *Staphylococcus* spp. (Kamau *et al.* 1990; Bosch *et al.* 2000).

7.4. CONCLUSION

At 30°C, the maximal OSCN⁻ concentration occurred in the first few hours upon the additional of GOX-immobilized fibers. However, at 4°C, the maximal OSCN⁻ concentration was not established until the 3rd day. These results indicate the crucial role of temperature in the accumulation and degradation rate of OSCN⁻. Storage at 4°C and activation of the LP system with GOX-immobilized fibers both contributed to the extended shelf-life of milk. Keeping milk at refrigerated temperatures appeared to be more important than OSCN⁻ concentration for maintaining the microbial keeping quality of milk. However, the antimicrobial effect exerted by the LP system in milk became more prominent when the sample was stored at higher temperature. At 30°C, the LP system activated by GOX-in-PLA microfibers can limit the growth of spoilage microorganisms in an acceptable range within the first 10 hours, while the LP system has a minimal impact at 4°C,

This study elucidated that the electrospun GOX-in-PLA fibers may be useful for activating the LP system for the preservation of raw milk. They may be prepared as a non-woven reusable sachet, which would be added to the milk barrel/bucket for stabilization of raw milk during temporary storage and transportation prior to further processing. This application may be useful for milk preservation in underdeveloped regions of the world where refrigeration infrastructure is lacking.

8 CONCLUSIONS AND FUTURE WORKS

Overall, we have achieved the main goal of this research, which is to develop an enzyme immobilization technique for forming bioactive electrospun fibers to activate the LP system in milk.

In this study, we avoided the direct addition of H_2O_2 to milk by using the GOX/glucose enzymatic system to achieve controlled release of H_2O_2 . Here the GOX is immobilized through entrapment in PLA polymer, which maintains an acceptable enzymatic activity. This study shows that electrospinning is a simple and productive technique for immobilizing enzyme in ultrafine fiber carriers. We elucidated the activity and stability of immobilized GOX, The antimicrobial activities of the bioactive fibrous membrane was studied in a model LP system as well as in raw milk to test its inhibitory effect on total bacteria, pseudomonads and lactobacillus at 4 and 30°C.

In order to prepare a homogeneous fiber-forming emulsion for electrospinning, we employed SMP (Span 40) to emulsify the hydrophilic GOX aqueous solution and the apolar solvent chloroform for PLA. The optimal process parameters for electrospinning, namely infusion rate, applied voltage, and distance between spinneret and target were identified as 1.0 mL/hr, ~10 kV, and ~16 cm, respectively. By using an environmental chamber, elevated RH was found to result in a more focused fibrous mat distribution on the target PE film. By contrast, temperature did not influence the distribution of the fibrous mat. Electrospinning was conducted at 20°C and 30% RH because these conditions

provided the most stable process and consistent fiber morphology. While SMP facilitated the formation of a uniform emulsion, an excessive amount of SMP inhibited the evaporation of chloroform during spinning, causing the formation of droplets and fusing of fibers when they landed on the target. Therefore, the use of SMP should be minimized. The final optimized formulation used for preparing the bioactive PLA fiber was 1% w/w SMP and 0.2% w/w GOX aqueous solution.

Chapter 5 reported the enzymatic activity of GOX-in-PLA fibers. Thicker GOX-in-PLA membranes had less enzyme activity per weight of polymer because of their limited mass transfer. In contrast, the fibrous membrane formed from electrospun GOX-in-PLA fibers had as much as ~20 times enzyme activity as compared to the cast films. This might be due to the large surface area of electrospun fibers, which allowed greater diffusion of reactive molecules across the polymer carrier. Also two key important characteristics of immobilized enzyme, storage stability and reusability were investigated in the current study. During 26 days of storage at 4°C in a jar with desiccant, GOX-in-PLA fibers exhibited lower enzyme activity initially than the free enzyme. However, the free enzyme degraded faster and had a significantly lower activity at the end of the stability study. The dipping test of the enzymatic fibers shows the enzyme might not be all encapsulated within the fibers. This speculation is based on the fact that the activity decreased by ~36% after the first immersion test in water. Nonetheless, the enzyme activity reduced only by ~24% during the subsequent six immersion/reuse cycles, resulting in ~40% of the initial activity being retained

by the fibers. The bioactive fibrous membrane shows potential for commercial use as an enzyme immobilization carrier.

The OSCN⁻ production profiles for the LP system, when activated by H₂O₂, free GOX and glucose, and GOX immobilized in PLA fibers and glucose are reported in Chapter 6. Compared to direct activation of LP using H₂O₂, free GOX, and glucose, the GOX-in-PLA microfibers provided a more sustained production of OSCN⁻, presumably due to the oxidative of OSCN⁻ and deactivation of LP by the excess H₂O₂ present. The more sustainable formation of OSCN⁻ is expected to be more effective for inhibiting microorganisms over a longer period.

Chapter 7 discussed the application of GOX-in-PLA fibers in raw bovine milk. Microbiological analysis of activated LP systems in bovine milk was conducted at 4°C and 30°C. At 30°C, the bioactive fibers exerted a remarkable antimicrobial effect by activating the LP system, although the milk at 4°C had a much longer shelf-life due to the preservation effect of low temperature storage. The efficacy of the LP system is temperature dependent and we concluded that its efficacy at elevated temperature was most effective during the first 72 h following activation. Compared with the data reported in the literature, our study showed less reduction of some microorganisms, possibly due to the different experimental conditions used and diffusion-limited reaction in our encapsulated enzyme system.

In North America, we have well-established transportation systems and methods for processing raw bovine milk. This is not achievable in many

developing and underdeveloped countries, where much of the product is wasted due to the lack of robust refrigeration control during transportation and distribution. The natural antimicrobial LP system may be used to stabilize raw milk to offset the lack of refrigerated facilities. The fibrous PLA membrane immobilized may find applications in active packaging by attaching the enzyme-immobilized carrier (in a form of sachet, patch, film, etc.) onto the interior surface of packaging materials, so that the shelf-life of milk potentially can be extended.

While significant progress has been made by this research, there still exist knowledge gaps in several areas. Some of these that need to be considered and investigated in future works are outlined below:

- Since safety is a key concern for food, the residual chloroform concentration in the electrospun fibers after electrospinning needs to be determined. The search for other non-toxic solvents for electrospinning of enzyme immobilized fiber is recommended;
- In order to improve the reusability of immobilized GOX, the distribution of enzyme in fibers should be determined, using , for example, transmission electron microscopy (TEM) or field emission scanning electron microscopy (FESEM);
- The robustness of PLA fibrous membrane on PE film needs to be evaluated when the active sachet is attached to packaging materials to ensure that the fibrous mat remains attached to the PE backing material;

- The stability of the immobilized GOX needs to be evaluated under different storage conditions to ensure that the enzymatic activity remains acceptable;
- For more practical usage, minimum inhibitory concentration (MIC) of OSCN⁻ for each targeted microorganism needs to be measured;
- Other quality indices should be monitored during the activation of the LP system, such as protein hydrolysis, lipolysis, and vitamin A;
- Besides PLA, other non-water soluble fiber carriers should be investigated, especially those such as zein and other prolamins that can be dissolved in non-toxic solvents;
- Since glucose can be generated from hydrolysis of lactose (rich in milk) through enzymatic action of lactase, the incorporation of GOX along with lactase in electrospun PLA fiber may potentially be beneficial for overcoming the limited glucose problem for the LP system; also the application of GOX-in-PLA fibers in lactose-reduced or -free milks could help to mitigate sweetness of those milks as well;
- Microbiological analysis should be carried out with different levels of initial counts since the antimicrobial efficacy of the LP system may be affected by different growth phases of microorganisms.

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APPENDICES

Method for colony counting through spiral plating (Spiral Biotech 1999; Scher 2000)

1. Instructions:

1.1. Count two opposite quadrants (A & A) or (B & B) (Figure 23) a minimum of 20 colonies on each.

1.2. Report the counts on each quadrant.

1.3. Report which was the highest segment that you count the colonies on (8 to 13 in Table 4)

Figure 23. Spiral counting grid (Spiral Biotech 1999).

1.4. Report the dilution that you plated

1.5. The real colony counts can be calculated following equation

Table 4. Deposition volumes in each segment.

Segment number Number	μL per segment
8	1.214
9	2.968
10	5.500
11	9.157
12	14.482
13	25.015
All	50.030

$$C_R = (C_1 + C_2)/V_S \times 1000 \times 10^D$$

Where C_R is the real colony count, C_1 and C_2 are the counts in quadrant 1 and 2 respectively, V_S is the deposition volume of each segment, and D is the dilution times.