NON-MIGRATORY BIOACTIVE PACKAGING: COVALENT ATTACHMENT OF BIOACTIVE PEPTIDES TO POLY(ETHYLENE) FOOD PACKAGING FILMS

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Matthew David Steven

May 2004

NON-MIGRATORY BIOACTIVE PACKAGING: COVALENT ATTACHMENT OF BIOACTIVE PEPTIDES TO POLY(ETHYLENE) FOOD PACKAGING FILMS

Matthew David Steven, Ph.D.

Cornell University 2004

Non-migratory bioactive packaging is a novel form of active packaging. It is packaging which elicits a desirable biological response from food systems without the active component migrating from the packaging into the food. Possible applications for this include in-package enzymatic processing and non-migratory antimicrobial packaging. Poly(ethylene glycol) (PEG) oligomers were covalently attached by one end to surface-oxidized poly(ethylene) (PE) films using carbodiimide coupling; bioactive peptides were covalently linked to the free terminus of the PEG chains using the same reaction. Reactions were confirmed and monitored through contact angle measurements, dye adsorption assays, x-ray photoelectron spectroscopy and atomic force microscopy.

Bioactivity of the modified films depended on the peptide attached: antibacterial activity was found for films treated with antimicrobial peptide E14LKK; no activity was found for lactase attached to PE films. Antibacterial activity was demonstrated against *E.coli* for films to which side-chain-protected E14LKK was attached and then deprotected.

Further investigation of the lactase coupling indicated difficulties with amino-carboxy-PEG attachment, despite prior successes with diamino-PEG; amino-

carboxy-PEG coupling requires further optimization. Lactase attached directly to PE was inactive, regardless of lactose inclusion in the coupling buffer, however PEGylated lactase is active: the inclusion of a PEG spacer should improve immobilized lactase activity.

The effect of the modifications on PE properties important in packaging was assessed through physical, mechanical and optical testing. Properties primarily dependent on the bulk characteristics of the material were generally unaffected by our film modifications. Properties sensitive to film surface characteristics (permeability, optical properties, friction) were affected by the modifications; the changes in these properties will need to be allowed for when designing processes and packages involving the modified films, but should not affect the utility of the film for the standard applications of PE.

BIOGRAPHICAL SKETCH

Matthew was born, raised and educated in New Zealand prior to arriving at Cornell. He received a Bachelor of Technology (Food Technology) with First Class Honours from Massey University in May 1997, graduating top of his class. Moving to Ithaca in August 1997 after serving three months as an Assistant Lecturer at Massey, Matthew entered Cornell and commenced the studies leading, eventually, to this document. Returning to New Zealand in January 2001 to marry Joanne (née Cuthbert), the pair returned to Ithaca together. An avid telemark skiier, whitewater canoeist and general outdoors freak, Matthew is well known for his enthusiasm and involvement in the outdoors community at Cornell and is looking forward to finding some real mountains again in the near future.

To Jill and David Steven, for a lifetime's worth of guidance and support.

To Jo, for being there, keeping me going and getting me out.

ACKNOWLEDGEMENTS

In any advanced degree there are numerous people who assist in various ways; it is never possible to list them all. I would like to thank all those of you who have assisted in my research. I truly appreciate the time and effort you have put into assisting me. I would also like to thank all those who have made my time here in Ithaca enjoyable: friends, colleagues and fellow outdoor nuts. You have been a huge part of my life and have helped me stay sane when the going got tough and the tough wanted to run like hell. There's not much to say, but thanks.

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Introduction: Non-migratory Bioactive Packaging

Food is often prepared a significant time before, and distance away from, where it is consumed (Branen, 1993). The basic function of food processing and packaging is to preserve the food between preparation (production) and consumption, maintaining a safe, nutritious food supply. Antimicrobial agents (preservatives) are an important part of this preservation process and have long been paired with packaging systems to extend the shelf life of foods. Preservatives and packaging systems have become more intertwined as technology has progressed; similarly, the processing and packaging of foods has become more intertwined. Non-migratory bioactive packaging can be viewed as the end point of this evolution, the combination of processing functions (*in-package* enzymatic processing) or preservatives (non-migratory antimicrobial packaging) with the packaging function in a single material.

Non-migratory bioactive polymers (NMBP) are a class of polymers that possess biological activity without the active components migrating from the polymer to the substrate. This concept has primarily been applied to immobilized enzyme processing to date (Bachler et al., 1970; Brody and Budny, 1995; Katchalski-Katzir, 1993; Mosbach, 1980; Ikada, 1994) but is now becoming of interest in packaging applications (Appendini and Hotchkiss, 1997; Soares, 1998).

Bioactive materials are based on molecules that elicit a response from living systems. The goal of NMBP is to create bioactive packaging for which the re-

sponse is desirable for either the package or the product, for example inhibition of microbial growth or flavor improvement. Enzymes are classic examples of bioactive substances, as are many peptides, proteins, and other organic compounds. A functional definition of bioactive has been adopted, based upon the way the substance interacts with living systems. Purely physical processes, for example adsorption or diffusion, are excluded. Bioactive polymers can be formed by attachment of bioactive molecules to synthetic polymers, as in the case of enzyme immobilization (Appendini and Hotchkiss, 1997; Soares, 1998), or may result from an inherent bioactive effect of the polymer structure, as with chitosan (Collins-Thompson and Cheng-An, 2000; Tanabe et al., 2002). They have potential applications in the packaging of food and other biological materials, in food processing equipment, on biomedical devices (Sodhi et al., 2001; Sun and Sun, 2002) and in textiles (Edwards and Vigo, 2001; Sun and Sun, 2002).

Non-migratory polymers are defined as those for which the bioactive component does not migrate out of the polymer system into the surrounding medium (see Figure 1.1). Migration, at its most basic level, is mass transfer of the bioactive compound from the packaging material into the packaged product. Migration can be prevented by covalent attachment of the active component to the polymer backbone, by entrapment of the active component within the polymer matrix, or by using inherently bioactive polymers.

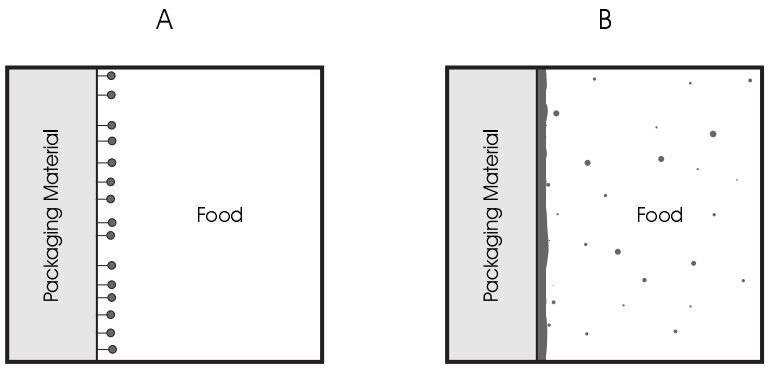


Figure 1.1: Non-migratory (A) vs. migratory (B) bioactive packaging. Adapted from Han (2000).

1.1 Non-migratory bioactive polymers: benefits and limitations

In order for a new technology to be considered, it needs to have advantages over the *status quo*. Typically, these advantages come with limitations, in application or utility, and frequently with an increase in cost. Benefits and limitations may be generic to all NMBP or specific to one type of NMBP.

1.1.1 Benefits

The benefits of NMBP can be divided into four main areas: technical benefits, regulatory advantages, marketing aspects and the food processor's perspective. This list is not exhaustive: particular applications will involve some, or all, of these plus other considerations specific to the application.

Technical benefits

The technical benefits of NMBP include improved stability of the bioactive substance and concentration of the activity at a specific locus. Improved stability is a

consideration for covalently immobilized bioactive substances; biological molecules, e.g. enzymes, are typically very sensitive to environmental conditions. They are readily denatured by some solvents, by high, and in some cases low, temperatures; by high pressures, high shear or ionizing radiation; by certain pHs or in the presence of high electrolyte concentrations (Richardson and Hyslop, 1985). Conjugation to polymer supports has been shown to dramatically enhance the stability of these molecules. Topchieva and colleagues (Topchieva et al., 1995) demonstrated improved thermal stability of chymotrypsin when conjugated to poly(ethylene glycol) (PEG) (Figure 1.2). Appendini and Hotchkiss (2001) similarly demonstrated improved thermal stability of a small antimicrobial peptide when covalently attached to a PEG-grafted poly(styrene) (PS) support. The immobilized peptide remained active when dry-heated to 200℃ for 30 minutes and when autoclaved at 121℃ for 15 minutes. Polymers are often processed at temperatures that would denature native proteins; improving the thermal stability of peptides is important for their inclusion in packaging materials.

Appendini (1999) also demonstrated the improved activity of the conjugated peptide over a range of pHs (Figure 1.3). Other authors have also reported improved stability of polymer-conjugated enzymes to pH and temperature (Gaertner and Puigserver, 1992; Yang et al., 1996, 1995a,b; Zaks and Klibanov, 1984; Chen et al., 2000). The extended range of pH stability may provide activity in a broader range of food products than would be the case for the native compound.

The stability of proteins to inimical media, such as organic solvents, supercritical fluids and gases, may also be improved by polymer conjugation, and applications have developed to exploit this in non-aqueous enzymology (Mabrouk, 1997; Panza et al., 1997; Veronese, 2001; Yang et al., 1995a,b; Zaks and Klibanov, 1984).

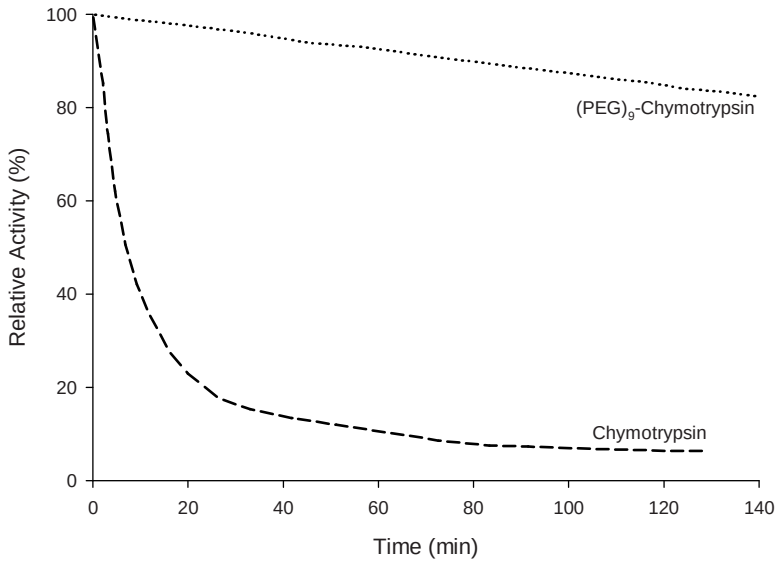


Figure 1.2: Activity of PEG-conjugated chymotrypsin and native chymotrypsin held at 45℃. Activity is expressed in percent relative to the initial activity of each enzyme preparation. Adapted from Topchieva et al. (1995).

This enhanced stability to organic solvents is potentially useful in extending the range of solvents and chemicals that can be used in casting, cleaning/sterilising or treating polymer films prior to package filling without damaging the functional characteristics of immobilized bioactive constituents.

The long-term stability of immobilized peptides and proteins is generally enhanced compared to native compounds (Katchalski-Katzir, 1993; Panza et al., 1997), which will help ensure the activity of bioactive packaging is retained for the shelf life of the packaged food product. Long-term stability is also important in ensuring adequate storage life of the active packages prior to filling; packaging materials are often warehoused for extended periods prior to use.

Concentration of biological activity at a specific locus within the package and/or food allows the activity to be focussed where it will be most effective. For many minimally processed food products, such as fresh meat and fresh-cut fruit and veg-

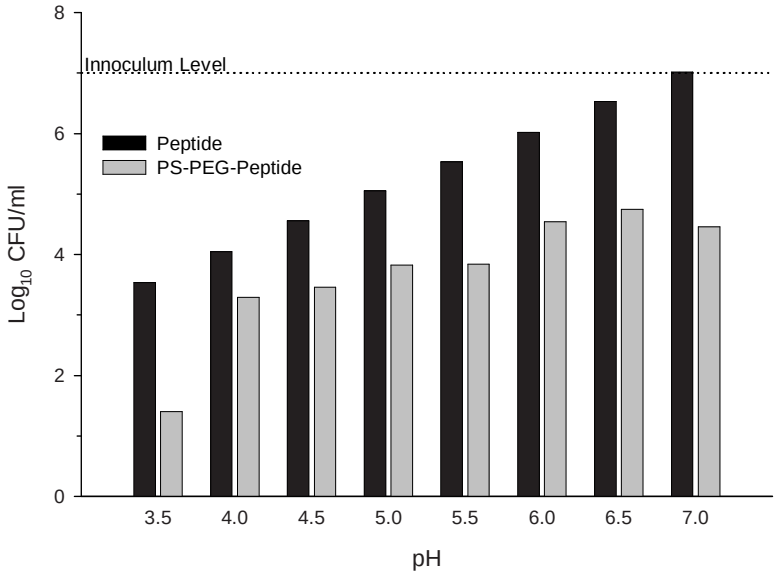


Figure 1.3: *E.coli* 0157:H7 survival in the presence of a small synthetic peptide in 0.1M citrate buffer across a range of pHs. Activity is shown for the native peptide (■) and peptide attached to a PS surface through a PEG spacer (■). Equivalent peptide concentrations were used in each determination. Adapted from Appendini (1999).

etables, the majority of microbial spoilage occurs on the product surface (Collins-Thompson and Cheng-An, 2000; Hotchkiss, 1995). Concentrating antimicrobials on the surface of the product, as with antimicrobial packaging, allows minimal amounts of the active compounds to be used to maximal effect.

Regulatory advantages

Regulations relating to active food packaging are still evolving. As new technologies develop, regulations must be modified to encompass them.

As noted by various authors (de Kruijf et al., 2002; de Kruijf and Rijk, 2003; Meroni, 2000; Vermeiren et al., 2002, 1999), there are currently no specific EU regulations for active or intelligent packaging. Rather, these packaging systems are subject to the same regulations as traditional packaging. These regulations require

that all components used to manufacture food contact materials be on "positive lists," set down migration limits for both overall migration and migration of specific components, and require that the packaging does not affect the composition of the packaged food. The migration requirements should not be problematic for NMBP, although a lack of migration will need to be established as detailed in the appropriate regulations. The compounds used to manufacture NMBP will need to be included on the relevant positive lists, however, which may be an issue for some systems. Additionally, the entire purpose of some active packaging is to modify the composition of the food, which directly contravenes the legislation. The key Directive (regulation) of concern is 89/109/EEC.

An amendment was proposed to 89/109/EEC on November 17, 2003 specifically to deal with active and intelligent packaging (Anon, 2003a). This amendment allows for the use of packaging materials that change the composition or organoleptic properties of food contained within them, provided the changes comply with other EU regulations applicable to food (Anon, 2003b); the regulation clearly states that these changes must in no way mislead consumers. 2003/0272(COD) allows the specification of both overall and specific migration limits for active and intelligent packaging separate from those set for traditional packaging. It is expected that this regulation will be voted into effect during 2004.

In the United States, regulations relating to food contact materials can be found in the Code of Federal Regulations (CFR) Title 21 Parts 170 through 190 (Anon, 2002a). The regulations revolve around determining whether the compounds in the packaging materials may be considered food additives. Food additives are defined as substances "the intended use of which results or may reasonably be expected to result, directly or indirectly, either in their becoming a direct component of

food or otherwise affecting the characteristics of food". Further, "If there is no migration of a packaging component from the package to the food, it does not become a component of the food and thus is not a food additive" unless it is used "to give a different flavor, texture of other characteristic in the food", in which case it "may" be a food additive (21 CFR §170.3 (e) (1)). The regulations also establish guidelines for determining limits below which migration can be considered negligible, negating food additive classification of that substance in that specific application.

These regulations can be interpreted as saying that any substance for which it can be shown that there is negligible migration into a food product is not a food additive (21 CFR §170.39). This would imply that NMBP needs to meet the regulations required of items for food contact use, but do not need to meet the more stringent food additive regulations, provided a lack of migration is proven. However, additive classification may also depend on the intended function of the component. If it is intended that a packaging component be active in the food product, as with an immobilized antimicrobial intended to extend the shelf life of a packaged food, then that component might be classified as a Direct Food Additive and would be required to comply with the food additive regulations (Brackett, 2002). There is, therefore, some ambiguity as to the status of NMBP materials. If the active components of NMBP are not considered food additives, then it could be a significant advantage, allowing the use of substances not currently permitted as food additives in packaging systems, provided that there is negligible migration. The process of obtaining food contact approval for packaging materials has been recently reviewed (Heckman and Ziffer, 2001).

The above discussion on US regulations focuses on the issues of covalently im-

mobilized or entrapped bioactive compounds in packaging. For inherently bioactive polymers, a different situation exists. Assuming a lack of migration, if the intended function of the bioactive agent is in the packaging film, not in the food, then it will not be considered a food additive (Brackett, 2002). A structural component of the packaging film is unlikely to be classified as a direct food additive, e.g. antibacterial UV irradiated nylon (Shearer et al., 2000), even if it has an effect in the food. For edible films, e.g. antibacterial applications of chitosan (Coma et al., 2002), food additive legislation would apply.

Marketing aspects

In recent years, consumers have become more aware and concerned about the composition and safety of their food. There have been increasing demands for safe, but minimally processed and preservative-free products (Appendini and Hotchkiss, 2002; Collins-Thompson and Cheng-An, 2000; Vermeiren et al., 1999; Jay, 1992). This is against a background of recent food-borne microbial disease outbreaks (Appendini and Hotchkiss, 2002; Mead et al., 1999). NMBP may have a key role to play in this area. Incorporating non-migratory antimicrobials in packaging materials may significantly reduce microbial contamination and extend shelf-life, while providing minimally processed, preservative-free food products. Similarly, immobilized enzyme packaging (Soares, 1998) may provide *in-package* processing opportunities which would not otherwise be possible for "fresh" products, enhancing the acceptability and shelf life of minimally processed foods.

The food processor's perspective

From the perspective of the food processor, NMBP could have several advantages. A general benefit would be increased shelf life, but certain NMBP technologies and applications may offer specific benefits. As an example, consider the production of lactose free milk: the demand for this product is not high, although there is a definite place for it in the market; it sells at a high price due to the high cost of production and the low sales volume. Using lactase-active packaging, however, milk could be packed off a normal production run and a lactose reduced product obtained after a short period of storage. A migratory enzyme, or the direct addition of lactase to milk, cannot be used in this application due to post-pasteurization restrictions in the pasteurized milk ordinance (Anon, 1999) and the heat lability of lactase. Similarly, for other products some of the processing may be accomplished in package, instead of in the processing plant, reducing processing costs and increasing flexibility for the food processor.

1.1.2 Limitations

As noted above, any new technology must have benefits over current technologies in order to be successful, but these benefits typically come with limitations. The limitations of NMBP may include: a limited locus of activity, specific requirements on the mechanism of activity of the active agent, reduced activity, availability of appropriate technology and an increase in packaging cost.

Limited locus of activity

One possible limitation of NMBP is the need for the reaction constituents to be transported to the package-product interface. This limits the function to areas in

intimate contact with the packaging material for solid and viscous liquid foods. For low viscosity foods agitation during distribution will mix the product, so this less problematic. For viscous liquids the high viscosity limits fluid mixing during distribution; diffusive mixing is also limited. For solid products, diffusive migration will be inadequate to provide the desired effect throughout the product. Even when the product surface is the target, the need for intimate contact with the packaging material may prevent action of the active agent within crevices and folds on the item surface, although this can be alleviated somewhat by careful package design and vacuum packaging. It should be noted that migratory active packaging technologies are often similarly limited in their diffusion and mixing requirements.

Mechanisms of action

In order for a bioactive agent to be active when covalently anchored to a packaging material, the conformation of the immobilized active component (compared to the free form), the location of the covalent link to the polymer, and the mechanism by which the agent interacts with the environment must all be considered. If, for example, an antimicrobial agent must enter the microbial cell to be effective, then it is unlikely to be active in a tethered state, whereas an antimicrobial agent active at the cell surface may remain active when tethered. If attachment causes conformational changes in the bioactive compound, or an active site is altered, then activity will be disrupted. Consider also the attachment of an enzyme that requires a co-enzyme for activity. If this coenzyme is not present in the food or otherwise attached along with the primary enzyme, then the primary enzyme will be inactive. Understanding the mechanism of the active agent is key in developing

NMBP.

Reduced activity

One concern in immobilizing bioactive compounds is the potential for loss of activity. In many cases, activity is reduced compared to the native compound (Katchalski-Katzir, 1993) and may be lost completely. With appropriate coupling methodology, however, activity can normally be retained, albeit at a lower level than for the free compound. Appendini (1999) compared the activity of a small antimicrobial peptide in solution and immobilized to PEG grafted PS beads and found that it was 200–7000 times less active when covalently immobilized. It still possessed significant antimicrobial activity, however, and was effective against *E.coli* 0157:H7 at immobilized peptide concentrations of 4 µmol/ml in growth media.

Soares (1998) found that naringinase retained 23% of its free activity when immobilized. Soares also found that at pHs less than 3.1, the immobilized naringinase was more active than free naringinase. This often occurs with immobilized enzymes — their increased stability leads to higher activity than the free enzyme under extreme conditions, even when activity is reduced under optimum conditions. Mosbach (1980) also suggested that for sequential enzyme pathways, the activity of the immobilized enzymes could be higher than that of the enzymes in free solution if the enzymes were immobilized in close proximity.

Technology availability

The commercial availability of the technology required to produce NMBP could limit applications. Technologies for basic polymer surface functionalisation are

readily available, but newer technologies allowing controlled surface functionalisation still require development, particularly with regards the high throughput required for packaging production. Surface functionalisation is discussed in more detail in §1.2.3.

Other than very basic surface functionalisation, surface modification of packaging films is not practised commercially. Production of most NMBP will require significant surface modification, probably involving wet chemical treatments for immobilization of active agents; adaptation of existing technologies will be required to implement these treatments. Over time the technology will become more readily available and this limitation will disappear; these issues regarding commercial availability of required technology are true for most novel technologies.

Cost

Intensive modifications, such as the attachment of proteins, will incur significant cost increases because of the additional processing steps required, the chemicals used in processing, and the high cost of the active agent itself. The peptides, proteins and enzymes involved are typically very expensive, although increased demand should result in cost reductions in the long term. The need to recover research and development expenses and capital equipment costs will further increase finished product costs. Over time, reduced equipment costs and increased availability, combined with reduced costs for the raw materials, should lead to the overall cost of the films decreasing. Again, this is the typical cycle for the introduction of new technologies.

1.2 Types of Non-Migratory Bioactive Polymers

1.2.1 Inherently bioactive synthetic polymers

As previously mentioned, there are three main types of NMBP: inherently bioactive polymers, polymers with entrapped bioactives and polymers with covalently immobilized bioactives. For inherently bioactive polymers, the structural polymer itself is bioactive. This includes structural polymers with modified backbones, for example UV irradiated nylon and naturally bioactive materials such as chitosan. These polymers differ from those with immobilized or entrapped bioactives in that no previously synthesized bioactive compound is incorporated into the polymer. Several materials have been found to have inherent bioactivity (Oh et al., 2001; Ozdemir and Sadikoglu, 1998; Shearer et al., 2000; Vigo, 1999; Vigo and Leonas, 1999) and new ones are underdevelopment (Tew et al., 2002). All the examples of inherently bioactive polymers to date involve antimicrobial activity.

Chitosan

Chitosan is the possibly the most studied inherently bioactive NMBP (Coma et al., 2002; Oh et al., 2001; Tanabe et al., 2002), possesses broad spectrum antimicrobial activity in simple media, and is commercially available as an antifungal coating for fresh fruit (Appendini and Hotchkiss, 2002; Padgett et al., 1998). Chitosan is the deacetylated form of chitin (poly-/*beta*-(1,4)-N-acetyl-D-glucosamine), a common natural biopolymer extracted from the shells of crustaceans. Production of chitosan from chitin involves demineralization, deproteinization, and deacetylation (Oh et al., 2001). The properties of chitosan films, including antimicrobial efficacy, mechanical and barrier properties, are significantly affected by the degree of

deacetylation (Oh et al., 2001; Paulk et al., 2002). Recent research suggests that the polycationic chitosan molecule interacts with, and disrupts, the negatively charged outer membrane of bacteria, leading to increased membrane permeability (Helander et al., 2001; Tsai and Su, 1999). The inhibitory activity is dependant on the media composition and the organism. Due to its membrane effect, chitosan can act synergistically with other compounds, for example bile acids and dyes.

Chitosan activity has been tested against a broad range of microorganisms by researchers in many different fields, including dentistry and pharmaceuticals (Ikinci et al., 2002), textiles (Takai et al., 2002) and food packaging (Oh et al., 2001; Paulk et al., 2002; Tanabe et al., 2002). It has been found effective against gram-positive and gram-negative bacteria, along with some yeasts and molds. In food applications, chitosan has been tested with some success in mayonnaise (Oh et al., 2001) and milk (Tsai et al., 2000).

Most research on chitosan activity has been conducted in solution, not with chitosan films, so extrapolation to packaging applications is difficult. The high solubility of chitosan in aqueous systems is an issue, as it results in migration into aqueous products, which would include many foods, violating the non-migratory principle. Chitosan is Generally Recognized as Safe (GRAS), however, so migration and consumption is not overly problematic; it has been investigated as an edible antimicrobial film and shows significant promise in this application (Coma et al., 2002, 2003; Tsai et al., 2002).

UV irradiated nylon

Physico-chemical surface modification of polymers can lead to antimicrobial activity, for example treatment of nylon with an excimer laser at UV frequencies

(193 nm) (Kelley et al., 1995; Ozdemir and Sadikoglu, 1998; Paik et al., 1998; Shearer et al., 2000). The UV treatment converts amides on the nylon surface to amines, which remain part of the polymer chains. The antimicrobial effect is strongly dependent on the UV wavelength used, the temperature and the composition of the test media. A side effect is etching (roughening and ablation) of the film surface. The mechanism of antimicrobial activity is assumed to be similar to that of chitosan, poly-L-lysine and other cationic polymers: interaction with negatively charged microbial membranes leading to membrane disruption. The results of antimicrobial assays of UV irradiated nylons have not been definitive; more investigation is needed to determine whether the decrease in bacterial counts is due to bacterial adsorption to the film surface or bacterial inactivation.

Hydantoins

Recent work in textiles shows significant promise for the production of regenerable antimicrobial polymers (Sun, 2001; Sun and Sun, 2002). Although this work was conducted on fibrous substrates, similar technology could provide antimicrobial food packaging films. The regenerable nature of the polymers also makes them ideal for use on food processing equipment. Hydantoin groups are introduced to the polymer chain by chemical modification, are activated by a dilute chlorine bleach solution and inactivate microorganisms by oxidative processes associated with the chloramine function. Bacterial inactivation results in loss of the chlorine atom, which can subsequently be regenerated by another treatment with dilute chlorine bleach (Figure 1.4).

At this point it is unclear whether this is truly a non-migratory technology, as there are two proposed mechanisms of action for the hydantoin group: (i) direct

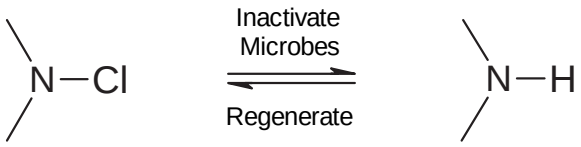


Figure 1.4: Bacterial inactivation and regeneration of the chloramine group of hydantoins

interaction of the microbes with the chloramine group resulting in inactivation of the microbes, or (ii) release of active chlorine from the chloramine groups by interaction with water and inactivation of microbes by the free active chlorine. It is highly probable that the chlorine migrates out of the polymer.

Others

Many other polymers have been investigated for potential bioactivity. An interesting possibility is the design of biomimetic antimicrobial polymers. Tew et al. (2002) have designed a series of amphiphilic acrylic polymers that possess very similar structure and activity to the magainin class of amphiphilic antimicrobial peptides. These peptides are active against a broad range of microorganisms by disrupting cell membranes.

Poly(ethylene glycol) (PEG) has been reported to possess antimicrobial activity (Jinkins and Leonas, 1994; Vigo, 1999; Vigo and Bruno, 1993; Vigo and Leonas, 1999). Our work with PEG has indicated that it does possess antimicrobial activity, but only at high concentrations (80mg/ml in liquid media). The water soluble nature of this polymer suggests it might be best applied as tethered graft chains on a structural polymer backbone, if it remains active in this state, rather than as an independent polymer layer. PEG is known to reduce the adhesion of proteins and cells to polymers (Sofia and Merrill, 1998; Zalipsky and Harris, 1997).

Poly-l-lysine and poly(lactic acid) have both been reported to possess limited antimicrobial activity (Appendini and Hotchkiss, 2002; Ariyapitipun et al., 2000; Mustapha et al., 2002). Poly-l-lysine is a cationic polymer that promotes cell adhesion and is thought to be active by a similar mechanism to chitosan (Appendini and Hotchkiss, 2002). Low molecular weight poly(lactic acid) has been found to be active against several organisms, although the mechanism of antimicrobial activity is not known (Ariyapitipun et al., 2000; Mustapha et al., 2002). It has been suggested that it releases lactic acid and the activity derives from this. Polymer modification with quaternary ammonium and phosphonium groups to produce polycationic biocides has also been reported (Kenawy and Mahmoud, 2003).

An important note: many of the aforementioned compounds are water-soluble and initial trials have been conducted on the soluble form of the polymer. Activity of the soluble form does not necessarily carry over to activity of the polymer in a film, either as an independent layer or as a graft layer on another backbone polymer. It is important to test film activity as well as solution activity if there is to be any application of these new materials in food packaging.

1.2.2 Polymers containing entrapped bioactive compounds

In some cases large compounds can be immobilized by entrapment in the polymer matrix such that they do not migrate out of the polymer under conditions of use. This requires an active agent larger than the channels present in the molecular structure of the polymer and minimal polymer chain mobility, for example a polymer with a high glass transition temperature or one that is highly cross-linked. This strategy is difficult to control and more likely to result in migration of the

active compound than covalent immobilization or inherently bioactive polymers. Elevated polymer processing temperatures may also result in significant migration and loss of the active agent. This strategy was not considered in this work.

1.2.3 Polymers with covalently immobilized bioactive compounds

The third major type of NMBP is an inactive polymer backbone to which an active agent is covalently attached. The active agent may be a peptide, protein or enzyme; it can be synthesized on the surface, or it can be synthesized or extracted separately and then covalently linked to the polymer. To date there has been more research conducted in this area than in that of inherently bioactive polymers, and a number of examples have been commercialized, although little of this work, and none of the commercial examples, has focussed on food packaging applications.

Enzymes or peptides that are adsorbed on polymers are often described as being immobilized (Scannell et al., 2000), however, these compounds are not truly immobilized as they can readily migrate out of the polymer in suitable non-reactive solvents. The term immobilized, as used here, implies covalent attachment of bioactive molecules to the polymer backbone. Covalent attachment of graft chains avoids their delamination and ensures long-term chemical stability, in contrast to physically coated or adsorbed systems (Kato et al., 2003).

Developing NMBP by immobilization

The polymer backbone is of prime concern in designing attachment schemes: if the polymer is essentially inert, such as poly(ethylene) (PE), then reactive functional groups need to be created on the polymer backbone to provide sites for attachment.

This step is termed functionalisation, and needs to be optimized to develop the maximum number of target functional groups for the desired coupling (immobilization) chemistry while minimizing polymer degradation and side reactions. For polymers which already possess suitable functional groups in the polymer backbone, for example poly(acrylic acid), the coupling chemistry needs to be chosen to target the available functional groups.

Most functionalisation and coupling chemistries are surface-centric, but modification of the bulk of the polymer can be achieved by treating the polymer as a fine powder, then thermally processing the powder, e.g. extrusion or molding, or by treating the polymer in solution. This results in distribution of the bioactive component throughout the bulk of the polymer. Polymers can be modified in solution using appropriate solvents, but these solvents often denature bioactive proteins; most reactions used for surface coupling are also applicable to solution coupling. Some proteins can be protected from solvent denaturation by conjugation with suitable polymers, e.g. PEG oligomers. Solution modification is applicable for water-soluble biopolymers such as chitosan, zein and poly(lactic acid). With covalent immobilization of an active agent throughout the bulk of the polymer, the active agent will be unable to migrate from the bulk to the polymer surface, so food packaging applications are limited. Bulk covalent immobilization may be of interest for constructing food-processing equipment where surface abrasion and wear is an issue.

For surface modification, the polymer should be preformed into the final package form prior to modification, if possible, to prevent surface rearrangement during later thermal processing/forming. Surface modification of a polymer film is the simplest situation; more complex shapes may require different modification

strategies.

Solvents may swell polymers without dissolving them, allowing reagents to penetrate the polymer matrix. Polymer swelling allows increased modification of the polymer surface, but may render some of the active agent inaccessible if the packaged product does not similarly swell the polymer. Careful selection of the solvents used for the reactions can control swelling.

Polymer functionalisation For inert polymers, such as PE, the polymer backbone requires functionalisation prior to attaching or generating the bioactive agent of interest. The literature contains many examples of this. The simplest methods for laboratory use involve wet chemical oxidations of the polymer surface with, for example, chromium trioxide, potassium hypochlorite or potassium permanganate in concentrated sulphuric acid (Eriksson et al., 1984; Larsson et al., 1979). Although these methods are simple, the hazardous nature of the reagents makes them undesirable in commercial applications. A recent development reduces the problems of wet chemical methods by utilizing a microwave-catalyzed reaction between solid potassium permanganate and powdered polyolefins (Mallakpour et al., 2001a,b), but this process still produces wastewater containing high concentrations of $KMnO_4$.

Wet chemical oxidations introduce various carbonyl groups, predominantly carboxylic acids, aldehydes and ketones, to polymer surfaces. The reaction can be optimized to produce the maximum concentration of the desired carbonyl function (Eriksson et al., 1984; Holmes-Farley et al., 1985; Rasmussen et al., 1977). Side reactions include incorporation of sulphate groups and surface etching/ablation. Sulphate groups can be removed by nitric acid treatment post-oxidation; surface

etching can be controlled to an extent by optimizing reaction conditions. Two recent reviews (Garbassi et al., 1994; Bergbreiter, 1994) of polymer surface modification are recommended.

The commercial application of wet chemical modifications is limited by numerous safety and environmental concerns. More common commercially are "physical" surface treatments such as flame treatment and corona discharge (Shi et al., 1998). Corona discharge involves applying a high voltage (10–40 kV) at a high frequency (1–4 kHz) between a discharge electrode and an earthed roller carrying the film (Robertson, 1993). This oxidizes the surface of the film, introducing a range of oxygen and nitrogen functional groups to the polymer backbone. Careful control is required to prevent excessive etching. Flame treatment also produces an oxidized film surface and introduces a range of oxygen and nitrogen functions, but is more difficult to control than corona treatment. Both treatments require specialized equipment, but this equipment is common in polymer processing and converting operations. The disadvantage of both these methods is that it is very difficult to control the exact nature of the functional groups created on the surface of the film. It may be possible to control the surface functionalisation in corona discharge treatment by varying the gas composition of the treatment atmosphere, although the typical installation does not have this capability.

Control of the treatment atmosphere has allowed controlled surface functionalisation in plasma treatment of polymer surfaces (Groning et al., 2001; Klapperich et al., 2001; Schroder et al., 2001; Terlingen et al., 1995). The disadvantage of classic plasma processing is that it requires a high vacuum to generate a stable plasma and, as such, is a batch process not suited to high throughput polymer converting operations. A new development in plasma processing is the Atmospheric Pressure

Non-Equilibrium Plasma (APNEP) system (Shenton and Stevens, 1999). This has been tested with a range of common polymers and various atmospheric compositions (Shenton et al., 2001; Shenton and Stevens, 1999, 2001; Shenton et al., 2002); controlled surface functionalisation appears to be possible, similar to that obtainable through vacuum plasmas. A disadvantage of the APNEP system is that the plasma is at a very high temperature, so care is needed to prevent thermal degradation of polymers during treatment. This is achieved by placing the films in the downstream afterglow region of the plasma rather than in the plasma itself; the distance from the plasma source is an important variable for this system. The APNEP system typically causes more surface etching than vacuum plasmas.

Plasma treatment technologies, or possibly controlled atmosphere corona discharge treatments, are likely to be the most useful commercial techniques for controlled surface functionalisation of a broad range of polymers, although the use of inherently functional polymers, e.g. poly(lactic acid), poly(acrylic acid), poly(methacrylate) or derivatives, may be more feasible. Reviews of physical methods for polymer surface modification/functionalisation are available (Lane and Hourston, 1993; Ozdemir et al., 1999b,a), although these do not include the novel APNEP technology. The review by Ozdemir et al. (1999b) is particularly useful as it approaches surface functionalisation from the food packaging standpoint, albeit with different intended applications.

Polymeric spacers One of the major difficulties in attaching bioactive agents to polymeric systems is the necessity of maintaining the native conformation and structure of the attached compound; the activity of most bioactive compounds is closely related to their structure and may be lost if this structure is significantly

disturbed. The hydrophobic nature of many common polymers may disrupt the structure of a hydrophilic bioactive compound if they are directly coupled. To prevent this, a hydrophilic spacer molecule may be used between the bioactive compound and the polymer backbone (Ikada, 1994). A spacer also helps reduce steric hindrances to activity (Weetall, 1993) and allows increased mobility of the bioactive compound. Some peptides, for example small amphiphilic antimicrobials (e.g. magainins), require association of multiple units for activity to be evident; if the peptides are not mobile, then they are unlikely to be active in the bound state. A bioactive attached directly to the polymer backbone is unlikely to be mobile, while one attached via a flexible (low T_g) spacer will be mobile and is more likely to retain its activity.

Many different oligomers can be used as spacers, although the most common is PEG. The main considerations in selecting a spacer for a food packaging application are: it does not disrupt the structure of the bioactive compound; it is approved for food contact use; and suitable chemistry exists for coupling it to both the polymer backbone and the bioactive compound. PEG is safe, well characterized (Zalipsky and Harris, 1997) and approved for food use (Anon, 2002a). It has been extensively used for conjugation with peptides and proteins (Zalipsky and Harris, 1997; Yang et al., 1995a; Herrwerth et al., 2003), and a large range of derivatives are available for reaction with different functional groups (Shearwater Corporation, 2001). Methods are well established for grafting it to polymer backbones, typically by attaching preformed PEG chains of defined molecular weight using various coupling chemistries (Bae et al., 1999; Emoto et al., 1998; Kang et al., 2001; Malmsten et al., 1998; Sofia and Merrill, 1998; Herrwerth et al., 2003). PEG is water soluble, allowing coupling in aqueous media and increasing the probability

that liquid food products will swell the polymer surface, increasing the interactions between the attached active agent and target constituents in the food.

Coupling chemistries There are many coupling chemistries available for covalently linking bioactive compounds to polymers and many different types of linkage that can be formed. Amide bonds can be formed between an amino group (on either the bioactive agent or the polymer) and a carboxylic acid group. Other common linkages are esters and thioesters, formed by interactions between carboxylic acids and alcohols or thiols, respectively. All these groups are common constituents of bioactive compounds. The coupling chemistries which have been explored for polymer conjugation are often the same as those used for peptide synthesis; texts on peptide synthesis are good sources of information on coupling techniques (Bodanszky, 1993a,b; Bodanszky and Bodanszky, 1994).

The carbodiimide method is a well-established and apparently simple coupling technique that forms amide bonds. It can be used in organic solvents or aqueous systems, depending on the carbodiimide used. 1,3-dicyclohexyl carbodiimide (DCC) is typically used in organic solvents (Bodanszky and Bodanszky, 1994), whereas 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC or WSC, water soluble carbodiimide), is the most common carbodiimide for aqueous coupling (Bae et al., 1999; Carraway and Koshland, 1972; Herrwerth et al., 2003; Hinder et al., 2002; Kang et al., 2001; Nakajima and Ikada, 1995; Plummer and Bohn, 2002; Valuev et al., 1998). The ideal situation for carbodiimide coupling is to have carboxylic acid groups on the film surface react with amino functions from the bioactive compound. The preference is for the carboxyl functions to be on the film surface because it is the carboxylic acid that is activated by the carbodi-

imide; if amines are present within the structure of the activated compound, as would be the case if activating carboxylic acids within a bioactive peptide, then the activated carboxylic acids can spontaneously couple with them leading to inter- and intra-molecular cross-linking and inactivation of the peptide. A similar effect can occur if a single step coupling reaction is used (Valuev et al., 1998); a two step procedure, activation followed by coupling (Kato et al., 2003), has been used here to avoid these problems: the film is immersed in a buffered solution of carbodiimide to activate the carboxyl functions, removed and gently rinsed, then immersed in a buffered solution containing the bioactive compound to be attached. The exact conditions, e.g. time, temperature and pH, require fine-tuning for each polymer-peptide combination. Peptides have previously been coupled to carboxy-PEG coated surfaces using WSC coupling (Herrwerth et al., 2003).

A second common method for coupling bioactive agents to polymers uses glutaraldehyde. Glutaraldehyde has long been recognized as an efficient cross-linking agent for use with proteins and other biological molecules (Bigi et al., 2001; Kikuchi et al., 2002; Weissman, 1979) and has also been used to immobilize bioactive materials onto polymeric backbones (Appendini and Hotchkiss, 1997; Molday et al., 1975; Soares, 1998). Glutaraldehyde (OHC-CH2-CH2-CH2-CHO) is a bifunctional short-chain aldehyde that reacts with amines. It requires amines on both the support and the bioactive molecule, and forms three-dimensional, cross-linked aggregates.

A third possibility for coupling reagents are succinimidyl succinate active esters and their derivatives. These are commercially available (Shearwater Corporation, 2001) and have been extensively employed for PEG conjugation to peptides and proteins. Succinimide esters react with free amine groups to form stable amide

linkages. The most commonly used derivative is the succinimidyl propionic acid ester of PEG (PEG-SPA), which has been used to conjugate PEG with insulin (Caliceti and Veronese, 1999) and human growth hormone antagonist (Olson et al., 1997). The reaction conditions can be modified to suit the system of interest.

A succinimidyl ester, N-hydroxy succinimide (NHS), can also be used in an extension of carbodiimide coupling (Hinder et al., 2002; Plummer and Bohn, 2002). Carbodiimide activated carboxylic acids are relatively unstable and may not be ideal for two step coupling processes, particularly if there is a significant delay between the activation and coupling steps. Instead, the activation is performed in the presence of NHS, creating a more stable, but still reactive, NHS ester of the carboxylic acid. The NHS-activated film is then immersed in a solution of the amine-containing compound to be coupled and coupling proceeds with the formation of amide linkages. NHS active esters of PEG can also be obtained commercially (Shearwater Corporation, 2001).

1.3 Applications of NMBP with immobilized bioactive agents

Although there are many possible applications of immobilized bioactives in food packaging, only a few have been explored to date. These can be broken into three main areas: *in-package* processing, antimicrobial packaging/shelf life extension and intelligent packaging.

1.3.1 *In-package* processing

In-package processing involves immobilizing an enzyme on the surface of the packaging material to perform in the package what would otherwise be a processing step in the plant prior to packaging. An example is the hydrolysis of naringin, one of the bitter compounds in citrus juices, with immobilized naringinase (Soares and Hotchkiss, 1998; Soares, 1998). This packaging material was able to significantly reduce the bitter naringin content of grapefruit juice during storage, providing what was perceived as a sweeter product as storage progressed. Another potential application exists for immobilized lactase (β-galactosidase) packaging to produce lactose-reduced milk products at a reduced cost compared to current methods. Reducaed-lactose milk products are required for consumers who are lactose-intolerant and unable to consume regular milk products. Lactase immobilization has been one focus of this research.

Only the number of enzymes with potential processing applications limits the range of potential applications for *in-package* processing. Other examples include hydrolases for non-thermal inactivation of shelf-life limiting enzymes; glucose isomerase to convert glucose to fructose, increasing the sweetness of products; and cholesterol reductase to reduce the cholesterol content of products. The concepts of lactase and cholesterol reductase immobilized on packaging materials were explored by Pharmacal Biotechnologies in the early 1990s (Brody and Budny, 1995), although no commercialization is apparent.

The basic principle of in-package processing with immobilized enzymes is shown using the example of cholesterol reductase in Figure 1.5. The interior surface of the packaging contains immobilized cholesterol reductase in contact with the food. The enzyme substrate, in this case cholesterol, contacts the immobilized enzyme

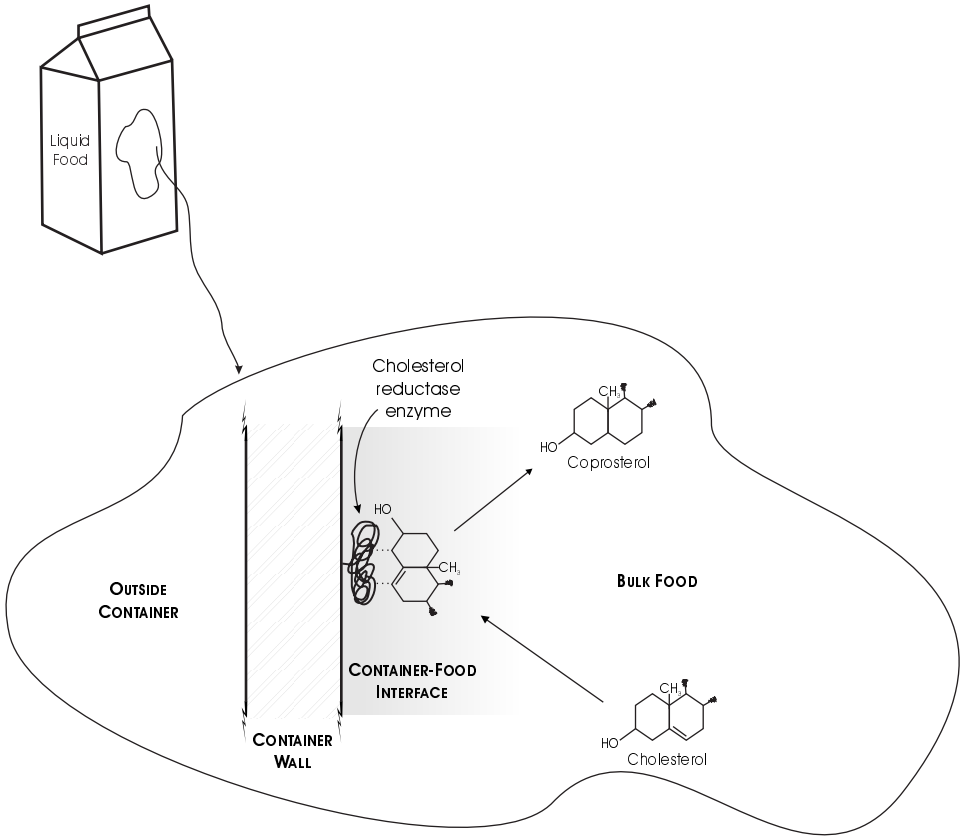


Figure 1.5: The principle of *in-package* processing utilizing NMBP: cholesterol reduction of milk with covalently immobilized cholesterol reductase enzyme. Adapted from Brody and Budny (1995).

as a result of natural convective currents or diffusion within the product or mixing caused by product agitation during handling and distribution. The substrate is acted on by the enzyme, producing the desired effect, and the reaction products are released back into the food system. Over storage, the composition of the packaged food changes to that desired by the food processor for optimum consumer acceptability, nutritional content, or shelf life.

1.3.2 Antimicrobial packaging and shelf life extension

The primary concern of all food processors should be the safety of the food they produce. A recent review estimated that there were 76 million illnesses, 325,000 hospitalizations and 5000 deaths per year due to food-related illness in the United States (Mead et al., 1999). The role of antimicrobial packaging in reducing food-borne illness has two major aspects: sanitation of packaging materials and reduction of pathogen growth in packaged foods. Although less dire than food poisoning, food spoilage due to microbial action is also a problem, resulting in the loss of large quantities of food. Retarding microbial growth and extending product shelf-life may allow food to be transported to places where it is needed. Several reviews of antimicrobial food packaging have recently been published (Appendini and Hotchkiss, 2002; Collins-Thompson and Cheng-An, 2000; Han, 2000; Vermeiren et al., 2002).

Shelf life extension and reduction of food-borne disease are the main goals of antimicrobial packaging. Considering the later, it is clearly important to maintain pathogen levels in food below the level that will cause illness, be it by toxin production or food-borne infection; it is important to prevent pathogen growth and to inactivate those present in the food. For shelf life extension, however, complete

growth inhibition and microbe elimination is not necessary. To extend the shelf life of a product where microbial growth is limiting, it is only necessary to reduce the growth rate or extend the lag phase of the organisms, i.e. to retard microbial growth. Even where microbial growth is not the limiting factor for shelf life, immobilized enzymes may have a role in extending shelf life if they can target shelf life limiting reactions, e.g. oxidation, by removing catalysts or reactants.

Both synthetic and natural compounds have been investigated for attachment to polymers to create non-migratory antimicrobial packaging. Appendini (1996); Appendini and Hotchkiss (1997) investigated the attachment of lysozyme, derived from hen egg white, to poly(vinyl alcohol), nylon and cellulose triacetate (CTA). Although lysozyme was successfully immobilized on all materials, it's activity on nylon and poly(vinyl alcohol) was insufficient for commercial use. Greater activity was retained on CTA films and significant bacterial retardation was observed in trypticase soy broth, although there was still a significant reduction in activity compared to free lysozyme. Activity of the CTA-lysozyme film also decreased with repeated usage, indicating that the lysozyme was either inactivated over time/with use or migrated out of the film.

Another natural antimicrobial system that could be used for antimicrobial packaging is the lactoperoxidase system of milk. The immobilization of lactase and glucose oxidase enzymes on nylon pellets has been investigated with the goal of producing hydrogen peroxide to activate the lactoperoxidase system naturally present in milk (Garcia-Garibay et al., 1995). The system investigated was designed for use in a bioreactor, rather than in packaging, but the principle is equally applicable to packaging. The advantage of this system is that milk regulations in many countries prevent the addition of preservatives to milk; activating a natural antimi-

crobial system already present in the milk provides antimicrobial activity without contravening regulations. Lactase and glucose oxidase were immobilized onto nylon pellets using glutaraldehyde coupling with a poly(ethyleneimine) spacer. The system resulted in reductions of 0.5–2 log cycles in the natural microflora of raw milk. Milk samples were only exposed to the enzymes for 3 minutes, and microbial counts were taken 24 hours after exposure (storage at 8°C). Modifying this system for use in a package, with prolonged exposure of the milk to the hydroperoxide, would probably result in greater bacterial inhibition, but might also result in oxidation of milk components by hydroperoxides leading to undesirable sensory characteristics.

A small synthetic antimicrobial peptide, E14LKK, has been investigated for non-migratory antimicrobial packaging applications (Appendini, 1999; Appendini and Hotchkiss, 2001; Haynie et al., 1995; Haynie, 1998). E14LKK is an amphipathic, α-helical derivative of magainin II (Haynie et al., 1995). It has the sequence HOOC-L-K-L-L-K-K-L-L-K-L-L-K-K-L-NH_2, where L is Leucine and K is Lysine. In the work of Appendini (1999), the peptide was synthesized attached to a PEG-grafted PS support, but this support could not be reprocessed into useful packaging. A high activity was observed for the immobilized peptide, with significant antimicrobial activity against a broad range of microorganisms: gram positive and gram negative bacteria, yeasts and moulds. Activity is presumed to be a result of pore formation in the cell membrane via the barrel-stave mechanism proposed by Ojcius and Young (1991). There is some doubt whether the peptide remained bound to the support in the work of Appendini (1999); some evidence suggested that the PEG spacer was hydrolyzing, releasing PEG-peptide conjugates into solution. The conditions that caused the hydrolysis were not investigated. The degree

of activity was found to be dependent on the test media; positive activity was found in buffer, trypticase soy broth, apple juice and meat exudate. The original objective of the research reported here was to extend this work to create a PE film with immobilized, active E14LKK, although a broader outcome is now envisaged: the system developed may potentially be used to attach any peptide to modified PE film. Bacteriocins have also been investigated for incorporation into antimicrobial packaging (Scannell et al., 2000; Padgett et al., 1998; Cha et al., 2003; Cooksey and Wu, 1998; Cooksey, 2000).

Other enzymes with indirect antimicrobial activity, typically by modifying package atmospheres, have also been immobilized onto food packaging polymers. Glucose oxidase was immobilized in conjunction with catalase for use as an oxygen scavenger (de Kruijf et al., 2002; Labuza and Breene, 1989). The glucose oxidase oxidizes glucose to produce glucono-delta-lactone and hydrogen peroxide. Hydrogen peroxide could lead to potential undesirable oxidations of food components, so is degraded to water and oxygen by catalase. The net reduction in oxygen content is half a mole per mole of reactions. A review of the mechanism and kinetics of glucose oxidase can be found in the literature (Labuza and Breene, 1989). Alcohol dehydrogenase has also been immobilized to polymer films and can also be used as an oxygen scavenger (Labuza and Breene, 1989). Reduced oxygen concentration inhibits the growth of aerobic microbes, especially yeasts and molds, however care must be taken that anaerobic, low acid, high moisture conditions do not result, as these may favor the growth of certain pathogens, e.g. *Clostridium botulinum*.

Immobilized enzymes could also be used to control carbon dioxide concentration. Non-enzymatic carbon dioxide emitters and absorbers have been developed and commercialized (Labuza and Breene, 1989), although there has been no com-

mercialization of enzymatic carbon dioxide control systems to date. Other immobilized enzymes could be used to produce ethanol, which has well known antimicrobial activity. Commercial ethanol emitters are not typically enzymatic (Labuza and Breene, 1989), but use slow-release encapsulated ethanol. The ethanol vapors released inhibit microbial growth, particularly that of fungi.

In addition to their indirect antimicrobial effect, many enzymes that modify the gaseous atmosphere of packaged products can also extend product shelf life by inhibiting non-microbial degradative mechanisms. The main application of this is reduced-oxygen packaging that inhibits the oxidation of food components and prevents the resulting negative sensory and nutritional effects. Catechin has recently been immobilized on acrylic polymer beads as an antioxidant system (Ihara et al., 2003). Carbon dioxide control can also be important in extending shelf life, as in the case of packaged coffee. Enzyme systems may be used to prevent taints and off-flavors by metabolizing compounds of concern. Care needs to be taken, however, that the taints inhibited are not indicative of microbial spoilage. Off-odors and off-flavors can be key indicators to consumers that food is spoiled and unfit for consumption; removing these indicators may result in consumption of spoiled, possibly pathogenic, foods.

In all cases where indirect enzyme action is used to control microbial growth, and in fact for all immobilized enzyme reactions in food packaging, the by-products of the reaction must be carefully considered. As noted above, glucose oxidase produces hydrogen peroxide, which could cause potentially detrimental oxidations of food components. Other enzymes also produce by-products that may have detrimental effects on the sensory characteristics or shelf life of the packaged food. Complete understanding of the catalyzed reactions is required to ensure undesirable

by-products are minimized.

1.3.3 Intelligent Packaging

Intelligent Packaging[1] has recently received a lot of attention (Ahvenainen, 2003; Taoukis and Labuza, 2003; Jarvi-Kaariainen, 2003; Smolander, 2003). Immobilized enzymes and antibodies are common components of intelligent packaging systems. A range of different indicators involving immobilized bioactive compounds have been developed, including time-temperature integrators (de Kruijf et al., 2002; Labuza and Breene, 1989), spoilage indicators (Anon, 2001) and indicators of chemical or other contamination (Woodaman, 2001). Time-temperature integrators (TTIs) based on enzyme-catalyzed reactions are available commercially (de Kruijf et al., 2002; Labuza and Breene, 1989). Although the commercial versions do not include NMBP, this is an area in which NMBP could be effective. For microbial spoilage, enzymatic TTIs may be particularly well suited for accurately modelling microbial growth since microbial growth depends on enzyme-catalyzed reactions.

For indicators of microbial or toxicant contamination, there are two main methods by which immobilized bioactive compounds can be used: (i) enzyme-catalyzed reactions requiring microbial metabolites or contaminant chemicals as substrates, or (ii) immobilized antibodies specific to bacterial metabolites and toxins, or contaminating chemicals. Both the enzymes in (i) and the antibodies in (ii) could be used to develop NMBP contamination indicators.

A final paradigm for the use of immobilized bioactive compounds in intelligent

[1]defined as packaging systems that monitor the condition of packaged food and communicate information on food quality during transport and storage (de Kruijf et al., 2002)

packaging is as detection units on biosensors. The incorporation of biosensor systems in packaging films is an area for future research. Biosensors may allow remote monitoring of package conditions or point of sale testing of product condition by interfacing with appropriate electronic devices. These may be useful for TTIs, for the detection of contaminating bacteria or toxicants, or in other areas yet to be explored.

Chapter 2

Research Objectives

The initial goal of this project was to develop a non-migratory antimicrobial packaging film by covalently attaching antimicrobial peptide E14LKK to the surface of poly(ethylene). During the course of the work, however, it became apparent that this system has a broader potential, so the objectives were modified to reflect this:

1. Develop a method for covalently immobilizing peptides and proteins on the surface of poly(ethylene) film.

2. Investigate the possibilities for producing lactose-reducing films by lactase immobilization.

3. Attach peptide E14LKK to the surface of poly(ethylene) and investigate the antimicrobial properties of the film.

4. Determine the effects of these surface modifications on the packaging-relevant properties of poly(ethylene).

Chapter 3

Materials and Methods

3.1 Materials

Low density poly(ethylene) (LDPE) pellets (∼0.03g) with a specific gravity of 0.92 and an approximate molecular weight of 50,000 were purchased from Scientific Polymer Products (Ontario, New York) and used as purchased. High density poly(ethylene) (HDPE) film (3mil, Lot#SC00030053) blown from Sclair 19A low-additive resin (NOVA Chemicals, Calgary, Alberta, Canada) was kindly donated by Dupont (Wilmington, DE). HDPE film had a mean thickness (n=10) of 79.5μm (3.13mil). Additive-free blown LDPE film was kindly donated by the Dow Chemical Company (Midland, MI). LDPE film had a mean thickness (n=10) of 100.6μm (3.96mil).

Chromium trioxide (99.75% purity, Mallinckrodt, St Louis, MO), sulphuric and nitric acids (AR grade, Mallinckrodt, Paris, KY), dichloromethane (AR grade, Fischer Scientific, USA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma Chemicals, St Louis, MO), diamino-poly(ethylene glycol) 3400MW (PEG-NH_2) and ω-amino-α-carboxyl-poly(ethylene glycol) 3400MW (NH_2-PEG-COOH) (Shearwater Polymers, Hunstville, AL), toluidine blue O (Fisher Chemicals, Fairlawn, NJ), orange II sodium salt (Sigma-Aldrich, St Louis, MO), bromophenol blue (BioRad, Hercules, CA), acid blue 45 (ICN Biomedicals Inc. Aurora, OH) were used as purchased.

Antimicrobial peptide E14LKK, having the sequence LKKLLKLLKKLLKL, was synthesized at the Sheldon Biotechnology Center (McGill University, Mon-

treal, Quebec). The peptide was received both as a free peptide and with side chain (lysine) amines protected with the *tert*-butyloxycarbonyl (Boc) group. Deprotection of the protected peptide was carried out post coupling, as described below (§3.3.5).

Lactase (GODO YNL-2, Valley Research Inc., South Bend, IN) was filtered through a 0.45μm syringe filter, then purified by centrifugal separation using a 30,000 dalton cut off membrane (Centricon YM-30, Millipore Corporation, Bedford, MA). 2ml of lactase solution was centrifuged for 2 hours at approximately 4500×g (4000RPM) using an IEC B-20A Centrifuge and a #872 Rotor (International Equipment Company, Needham Heights, MA). The tube was topped with distilled water and centrifuged for a further 2 hours; more water was added and the centrifugation repeated. This process was repeated two further times. The filtrate was discarded, the filter inverted and centrifuged for 2 hours. The retentate was then removed and placed in a clean test tube. 1ml of distilled water was added to the filtrate container, the filter returned (inverted) to the centrifuge and centrifuged a final 45mins to extract enzyme retained in the filter. The retentate and the wash water were combined and the resulting solution used for lactase coupling.

Films were cut into 25×50 mm pieces using a precision strip cutter (Testing Machines Incorporated, Amityville, NY), then stored in clean glass containers at ambient temperature until further treatment. Film Thickness was assessed with a Scherr-Tumico, St James, MN Dial Thickness Gauge.

3.2 Film Preparation

3.2.1 Heat-Pressing LDPE Films

Heat-pressed LDPE films were prepared from LDPE pellets using the method of Al-Ati (2002). Approximately 0.3g of pellets were placed between two pieces of mylar film and these placed between two preheated parallel metal plates in a preheated film press (Model 341-20, Loomis Engineering and Manufacturing Company, Caldwell, NJ). The press was closed and the pellets tempered at ∼140℃ for 15–20 minutes. 30,000(±2000) pounds of force (133±8 kN) was then applied to the plates with the press. The area of the smaller plate was 0.0283 m^2 giving a pressure between the plates of 4.71 MPa. The pressure was maintained for 15–20 minutes, after which the mylar films were removed and immediately immersed in distilled ice water. The PE film was removed from the mylar and dried under ambient conditions.

The thickness of pressed films ranged from 1.5–4 mils (38-102 μm). Thickness variation across a single 25×50 mm piece of film was typically less than 0.5 mil (13 μm).

3.2.2 Film Cleaning

Prior to surface treatment, all PE films were first cleaned by refluxing 12–24 hours in dichloromethane (DCM) and vacuum drying overnight at 60℃, ∼27 kPa, abs. (∼22 inches Hg vac). After cleaning, films were stored in clean glass containers at ambient temperature until further analysis or treatment.

3.3 Film Modification

Figure 3.1 shows the film modification process; the detailed methodology follows.

3.3.1 Surface Oxidation of PE film using Chromic Acid

Surface oxidation was performed using methodology described by the Whitesides group (Rasmussen et al., 1977; Holmes-Farley et al., 1985). Cleaned PE films were immersed in chromic acid solution (CrO_3:H_2O:H_2SO_4 = 29:42:29 by weight) at 70±2°C for 1 minute, with agitation, followed by three successive washes in distilled water at ambient temperature. Film samples were then transferred to 70% nitric acid at 50℃ for 15 minutes followed by three further distilled water washes. Surface oxidized films were typically analyzed, or further treated, immediately. If storage was required, films were stored in distilled water under refrigeration to minimize rearrangement of the film surface and loss of surface carboxyl functions (Holmes-Farley et al., 1985).

3.3.2 PEG attachment to PE-COOH using WSC Coupling

Oxidized poly(ethylene) films were grafted with NH_2-PEG-NH_2 or NH_2-PEG-COOH using 1-ethyl-3-(3-aminopropyl)-carbodiimide (Water Soluble Carbodiimide, WSC) coupling. Three films were immersed in 30 ml of 0.35 mM WSC in degassed distilled water[1] (adjusted to ∼pH 4.5 with HCl/NaOH) at room temperature and incubated for 1 hour with agitation (Lin and Tseng, 2001; Bae et al., 1999; Anon, 2002b,c). After incubation, the films were gently washed three

[1] in addition to obvious problems with buffers containing amino or carboxy groups, it has been suggested that phosphate buffers might also cause problems with WSC coupling (Anon, 2002c). Distilled water was recommended.

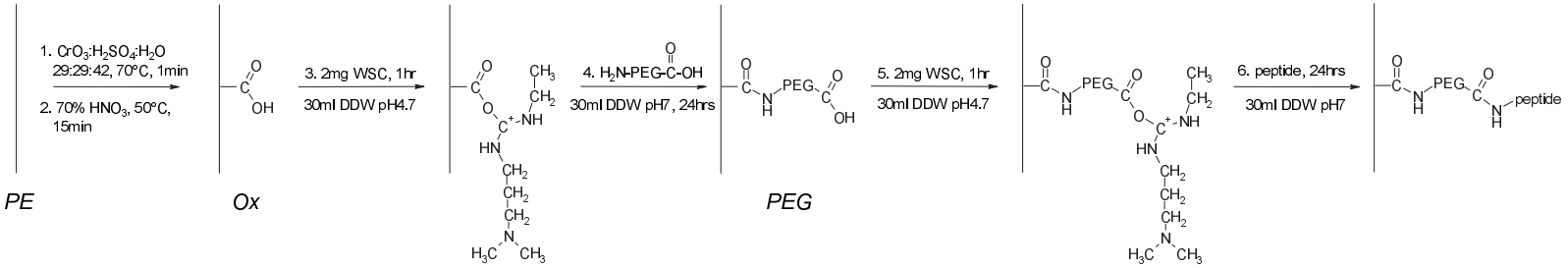


Figure 3.1: Film modification process. *PE*, *Ox* and *PEG* designate stages of the reaction scheme. Intermediate structure based on Nakajima and Ikada (1995)

times with distilled water and placed in 30 ml of 0.1 mM PEG in degassed distilled water (adjusted to ∼pH 7), incubated for ∼24 hours at 4℃ with agitation, then removed and gently rinsed in distilled water (×3). The films were washed by shaking in 40 ml (per 3 films) of 10% aqueous ethanol for 1 hour, then gently rinsed in distilled water (×3). Films were stored under moist conditions at 4℃ until further analysis or modification.

3.3.3 Lactase attachment to PE-PEG-COOH and PE-COOH

As for PEG attachment, three films were immersed in 30 ml of 0.35 mM WSC in degassed distilled water (pH 4.5) and incubated at room temperature for 1 hour with agitation. The films were rinsed (×3) in distilled water and added to 30 ml of degassed distilled water (pH 7). Half the retentate from 2 ml of lactase solution, purified as described above (§3.1), was added to each 30ml of coupling solution and the films incubated in this at 4℃ for 24 hours with agitation. Films were removed, rinsed three times in distilled water and stored under moist conditions at 4℃ until further analysis or modification.

Before and after coupling, the lactase activity of the coupling solution was tested by mixing a few drops of coupling solution with a few drops of 8 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG; described in §3.6.1). The rapid development of a yellow color indicated a positive test for lactase activity.

3.3.4 E14LKK attachment to PE-PEG-COOH and PE-COOH

Coupling of E14LKK was conducted similarly to PEG and Lactase coupling. The carboxy groups on the film were activated by agitation in 0.35 mM WSC solution (pH 4.5) for 1 hour at room temperature. The films were then placed in degassed distilled water (pH 7, 3 films/30ml) to which peptide solutions had been or were subsequently added: for E14LKK with unprotected side chain amines (*unE14LKK*), 5 mg of peptide was dissolved in the distilled water prior to adding the films; for E14LKK with Boc protected side chains (*proE14LKK*) approximately 5–10 mg of peptide previously suspended in distilled water was added after the films. The film/peptide solutions were incubated at 4℃ for 24 hours with agitation, then rinsed briefly in distilled water (×3). Films were stored at 4℃ under moist conditions until further testing.

The bactericidal activity of the coupling solutions was assessed post-coupling by adding 1 ml of coupling solution to 9 ml of Butterfields Buffer containing 0.1ml of an overnight culture (10^8 cfu/ml) of *Escherichia coli* ATCC 25922. Samples (triplicate) were incubated for 24hours at 37℃, after which 0.1ml was spread onto the surface of Standard Methods Agar plates. The plates were incubated at 37℃ and visually assessed at 12 and 24hours. The *unE14LKK* coupling solution significantly reduced the growth of the bacteria compared to the control, with the difference most apparent at 12hours. The *proE14LKK* was not notably different from the control.

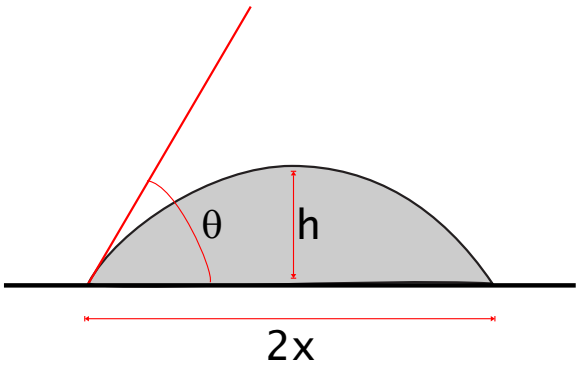


Figure 3.2: Determining contact angle of droplet

3.3.5 Deprotection of Immobilized Peptide E14LKK

The peptide was supplied with side-chain amines protected by Boc groups to prevent attachment via the side-chain amines. For the peptide to be active once attached to a film, however, the Boc groups must first be removed. The films with immobilized, protected peptide were immersed in a solution of 1:1 trifluoroacetic acid : dichloromethane for 30 minutes with agitation (Pennington, 1994; Calbiochem-NovaBiochem, 1999). They were removed, rinsed thoroughly (×3) in distilled water, then in 0.1N NaOH (×3), and again in distilled water (×3).

3.4 Film Surface Analyses

3.4.1 Contact Angle Measurement

With a suitable measurement system, contact angles can be one of the most sensitive methods of obtaining information on the outermost layers of a polymer surface (Kato et al., 2003). 5×50 mm pieces of film were attached to glass microscope slides with double sided tape at each end. Care was taken to only touch the ends of a film when attaching it to the slide and only untouched sections of film were used

for the contact angle measurements as skin oils alter the wetting characteristics of the film (Holmes-Farley et al., 1985). The film was attached flat as surface irregularities or slope alter the shape of water drops.

A 0.5 μl drop was delivered to the film surface using a 2.5 μl hand-held syringe (Hamilton, #87942) with a flat-tipped, Teflon-coated needle (Hamilton, #80471). The drop was viewed through the microscope eyepiece, with the magnification set such that the drop size was comparable to the linear scale in the eyepiece. The relative height and width of the drop was measured using this scale and the contact angle calculated from the drop dimensions (Bartell and Zuidema, 1936; Mack, 1935):

$$\tan\frac{\theta}{2} = \frac{h}{x} \tag{3.1}$$

where θ is the contact angle, h is the height and x the half-width of the drop (Figure 3.2). The height was measured first, then the width. Drop height is more sensitive to evaporation than is drop width. To reduce evaporation, drafts around the apparatus were minimized and drop measurement was completed within $\sim$30 seconds of applying the drop. 5–7 drops were measured for each film sample and averaged to determine the contact angle for that sample; the results from at least three, and generally six, film samples were averaged to determine the contact angle for a treatment.

3.4.2 Dye Adsorption Assays for Surface Chemistry

These dye tests are quantitative analyses of the acid or base functions present on the surface of a film. They were adapted from published methods (Hu et al., 2002; Kato and Ikada, 1996; Sano et al., 1993).

Toluidine Blue O Assay

Film samples were soaked in 0.1mg/ml aqueous Toluidine Blue O solution (adjusted to pH 11 with NaOH) at room temperature for 6 hours, removed, rinsed in distilled water, then in 0.1M NaOH and again in distilled water, then dried hanging in a desiccator[2]. The dye was desorbed by sonicating each film twice for 20minutes in separate 5ml aliquots of 1:1 Hexane : Trifluoroacetic acid (TFA)[3]. The aliquots were combined and the absorbance measured at either 605 or 677nm[4]. The dye concentration was calculated by comparison to a standard solution using Beer's Law:

$$A = a \cdot b \cdot C \tag{3.2}$$

where A is the absorbance, a the absorbtivity, b the path length (constant at 10mm) and C the concentration.

Standard solutions were prepared by dissolving a known amount ($\sim$2mg) of Toluidine Blue in 10ml of Hexane : TFA to give a stock solution ($\sim$0.2mg/ml), then diluting 0.1ml of this stock solution in 9.9ml of Hexane:TFA to obtain a standard solution ($\sim 2\mu$g/ml). This solution was sonicated for 20minutes prior to absorbance measurement to duplicate sample treatment. Three separate standard

[2]It is important to dry the films completely prior to placing in the Hexane : TFA solution. The presence of even minute amounts of water will cause phase separation.

[3]Previous researchers (Hu et al., 2002; Kato and Ikada, 1996; Sano et al., 1993) used 50% aqueous Acetic Acid to desorb the toluidine blue from the film, however, this did not fully extract the dye from the film, even with sonication. Maximum dye extraction was obtained with 1:1 Hexane : Trifluoroacetic Acid, although even after this treatment the films retained significant color.

[4]The absorption maximum of toluidine blue is 677nm. Issues with one spectrophotometer prevented use of wavelengths greater than 605nm; sufficient absorption remains at this wavelength to obtain accurate results. When using an alternative spectrophotometer, the absorbance was measured at the optimal 677nm. Samples were compared to standards measured at the same wavelength.

solutions were prepared on two separate occasions, with concentrations of 2.2, 2.3 and 3.3μg/ml; the absorbtivities of these solutions were averaged to calculate dye concentrations in the desorbing solutions.

The concentration of acid groups on the film surface was calculated based on the desorbed dye concentration, the solution volume and the film area. The concentration was adjusted for the dye absorption found for unmodified PE film, which was assumed to have no surface acid groups.

Acid Orange 7 Assay

Films were soaked in 1.0mg/ml aqueous Acid Orange 7 (Orange II Sodium Salt, Sigma) solution (adjusted to pH 3 with HCl) at room temperature for 6 hours. They were removed and rinsed in distilled water, then in 0.1M HCl and again in distilled water. The dye was extracted by sonicating the film in 5ml of 6N NaOH for 20 minutes. The absorbance of the extraction solution was measured at 485nm and the dye concentration calculated, in comparison to the absorbance of a standard, using Beer's Law (equation 3.2). Standard solutions were prepared with dye concentrations of 1.0, 2.0 (twice) and 3.5μg/ml; the average absorbtivity of these four standards was used to calculate the concentration of basic groups on film surfaces, as per the Toluidine Blue assay.

3.4.3 Instrumental Analysis of Film Surfaces

Atomic Force Microscopy (AFM)

AFM scans were conducted using a Digital Instruments Dimension 3100 Scanning Probe Microscope (Veeco Instruments, Inc., Woodbury, NY) in tapping mode with Ultrasharp Silicon Cantilevers (MikroMasch, Germany, part number NSC14/W2C).

The scan size was 10 μm, with 128 lines per scan, 512 sampling points per line, a tip velocity of 12 μm/s and scan rate of 0.598 Hz.

X-Ray Photoemission Spectroscopy (XPS)

XPS analysis was conducted by Dy Michael Kelley and colleagues at Jefferson Laboratory (Newport News, VA) using a Vacuum Generators ESCALAB Mk II (VG Scientific, West Sussex, England) with a Mg anode (1253.6eV) operated at 15kV and 200W.

3.5 Functional Testing of Modified Films

3.5.1 Water Vapor Transmission Rate

WVTR was measured using a modification of ASTM Method E96–00 (American Society of Testing and Materials, 2000). Film samples measuring 25×50 mm were prepared and mounted on metal can lids using silicon sealant (732 Multi-purpose Sealant, Dow Corning, Midland, MI), covering two 16 mm diameter holes previously cut in the lids. The lids were sealed over aluminum dishes containing Drierite Indicating Desiccant (8 Mesh, W.A.Hammond Drierite Co., Xenia, OH) using melted Bowax paraffin wax (Strohmeyer & Arpe, Millburn, NJ). Sample dishes were incubated at 35℃ in a desiccator containing a saturated aqueous solution of $ZnSO_4.7H_2O$ in the base cavity, providing a relative humidity (RH) of approximately 90%[5], and weighed every 12–48 hours for 2–3 weeks (n=3).

[5] a saturated solution of $ZnSO_4.7H_2O$ provides a RH of 90% at 20℃ (Anon, 2004a); the RH is expected to decrease slightly, probably by ∼2%, as temperature increases (based upon data presented by Labuza (2004))

3.5.2 Tensile Testing

Tensile testing methodology was based on ASTM Method D882–97 (American Society of Testing and Materials, 2000) and conducted on a Texture Technologies (Scarsdale, NY) TA–XT2 Texture Analyzer. The method was modified for use with 50 mm long strips of film by using an initial grip spacing of 25 mm; 5 mm wide strips of film were tested for HDPE (n=12) and 10 mm wide strips were tested for LDPE (n=10); sample thickness was measured at three locations on each test film prior to testing. The initial strain rate was set at 4.2 mm/s (∼250 mm/min). Tensile Strength, Percent Elongation at Break and Yield Strength were calculated per the ASTM standard.

3.5.3 Tear Propagation Resistance

Tear propagation testing was conducted using a modification of ASTM Method D1938–94 (American Society of Testing and Materials, 2000) on a Texture Technologies (Scarsdale, NY) TA–XT2 Texture Analyzer. The method was adapted for samples measuring 25×50mm. Samples were slit lengthwise using a Precision Strip Cutter (Testing Machines Inc, Amityville, NY) leaving 25mm of uncut material between the tip of the slit and the end of the sample. The initial spacing of the tensile tester grips was approximately 25 mm and the initial separation rate was 4.2 mm/s (n=6). The normalized tear propagation force was calculated by dividing the area under the tear propagation force-distance curve (the work to tear the sample) by the length of the tear and the thickness of the sample. This calculation was used because the samples showed various tear patterns (see Figure 3.3 for examples), so the length of the tear varied.

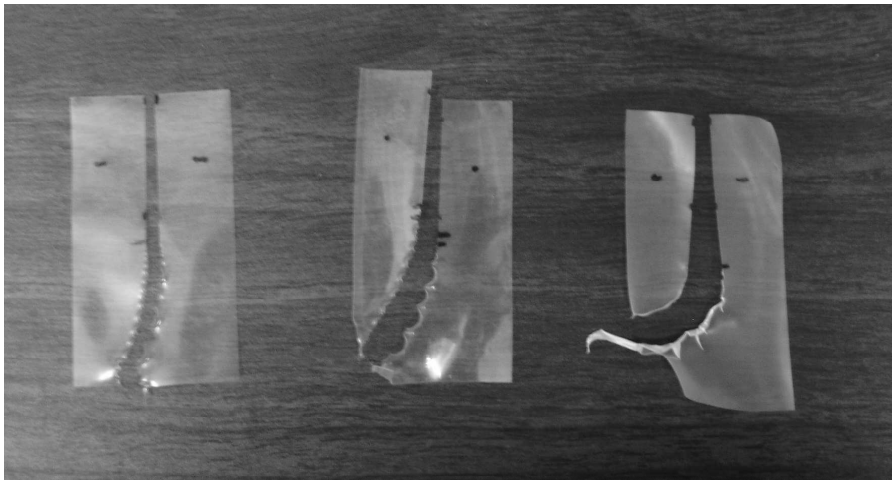


Figure 3.3: The range of tear patterns evidenced in the tear propagation experiment. From left to right: a relatively straight tear (LDPE); a diagonal tear (LDPE); a sideways tear (HDPE).

3.5.4 Evaluation of Film Friction Coefficients

Static and Kinetic Friction coefficients were determined for LDPE films according to ASTM Method D1894–99 (American Society of Testing and Materials, 2000). Testing was conducted by Advanced Plastic and Material Testing, Inc. (Ithaca, NY). Film samples measured 65mm square (n=5).

3.5.5 Transition Temperature Determination

Melting and glass transition temperatures were determined for LDPE films using ASTM Standard D3418–99 (American Society of Testing and Materials, 2000). Testing was conducted on a TA Instruments (New Castle, DE) DSC 2920 Modulated DSC operated with a refrigerated cooling system. Data acquisition was performed with TA Instruments Thermal Analysts 2100 system (v8.10b) software. The purge gas was Nitrogen ("High Purity", Airgas Inc., Radnor, PA) with a flowrate of 100 ml/min. Samples consisted of 5–6 discs of LDPE, each measuring

```
1. Equilibrate sample to 30°C.
2. Turn Data Storage On.
3. Ramp temperature at 10°C/min to a final temperature of 170°C.
4. Hold sample isothermally for 10 minutes.
5. Ramp temperature at 10°C/min to a final temperature of 20°C.
6. Hold sample isothermally for 5 minutes.
7. Ramp temperature at 10°C/min to a final temperature of 170°C.
8. Turn Data Storage Off.
9. Jump temperature to 30°C.
```

Figure 3.4: DSC Temperature Program for Melting Point Determination

∼3 mm in diameter, cut from a single 25 mm square piece of film, giving a total sample weight of approximately 6 mg. Samples were weighed to the nearest 0.1mg and hermetically sealed into aluminum sample pans (TA Instruments part numbers (base) 900793 and (lid) 900794). The temperature program used to determine melting temperatures of the film samples (n=3) is shown in Figure 3.4.

3.5.6 Surface Gloss Evaluation

Surface gloss of LDPE and HDPE films was assessed according to ASTM Methods D523–89(99) and D2457–97 (American Society of Testing and Materials, 2000). Testing was conducted by Advanced Plastic and Material Testing, Inc. (Ithaca, NY) using an angle of 60°and samples mounted on a matte black fabric backing. Samples prepared for these tests measured 25×70mm (n=3).

3.5.7 Film Transmittance and Haze

The effect of the film modifications on light transmittance was assessed for both LDPE and HDPE films according to ASTM Method D1003–00 Procedure B (American Society of Testing and Materials, 2003). Testing was conducted by Advanced Plastic and Material Testing, Inc. (Ithaca, NY) on samples measuring 30×50 mm,

using CIE Illuminant C with the observer at 2°(n=3).

3.6 Bioactivity Evaluations of Modified Films

3.6.1 Lactase activity assay

Lactase activity was measured using a synthetic lactose substrate, o-nitrophenyl-β-D-galatopyranoside (ONPG). 8 mM of ONPG solution was prepared as follows: 0.24 g of ONPG was added to 100 mL of 0.1 M potassium phosphate buffer (adjusted to pH 6.7 with HCl and NaOH) containing 2 mM $MgCl_2$ and 0.06 mM EDTA. Two 25×25 mm pieces of film were sterilized by dipping in 95% ethanol, dried over gentle heat, then submerged in 10 ml of filter-sterilized 8 mM ONPG solution and incubated at for ∼3 weeks and 35℃ with agitation. After storage, the absorbance of the solutions was measured at 420 nM against an 8 mM ONPG standard (stored at 4℃). PE-PEG-Lactase and PE-Lactase films were compared to three controls: no film, oxidized PE Film and PE-PEG-NH_2 film (n=3). Attempts at recording time series data (daily measurements) was unsuccessful due to problems with microbial contamination leading to ONPG hydrolysis.

3.6.2 Antimicrobial Efficacy Evaluations

Film Overlay Method

To evaluate the antimicrobial efficacy of E14LKK modified films, a simple spread-plate assay was designed. The assay is based on the method used to count bacterial plates that contain ≥250 colonies; in this situation, squares measuring 10 mm^2 are selected at random from the plate and the number of colonies in each square counted. These square counts are then averaged and multiplied by 57 to determine

the total count for the plate (Speck, 1984). The pieces of film used in the antimicrobial assays measured approximately 25 mm square, so cover just over four 10 mm squares on the surface of the petri dish; when the count per square is between 10 and 100 colonies, four random squares are averaged to determine the plate count. A target of 20 colonies per square was selected so plate counts could be determined from just four squares; this requires a total plate count of approximately 1.1×10^3 colonies. The effect of the film could then be evaluated by comparing the count of colonies underneath the film (in contact with the antimicrobial surface) to the colony count on uncovered areas of the plate. Three films could be tested per plate, allowing control and E14LKK samples to be run side-by-side on a single culture plate.

0.1 ml of a $\sim 10^4$ cfu/ml culture of *Escherichia coli* (ATCC 25922) was spread on the surface of pre-poured standard methods agar plates using a sterile plastic 'hockey stick'. Test films were overlaid on the surface using sterile forceps and pressed gently to ensure complete contact with the agar surface; three films were placed on each plate. The agar plates were incubated for 24 hours at 35℃ and four uncovered 10 mm squares were counted to determine the initial inoculum concentration for the plate. Plates were then incubated a further 48 hours and four 10 mm squares were counted under each piece of film. The extra time was allowed because it was observed that colony development was slowed under a PE film. For each plate, the counts per square were averaged to determine the 'plate count' for the uncovered and covered areas of the plate. The differences were evaluated statistically using an Analysis of Variance (ANOVA) model. Antimicrobial efficacy evaluations were only conducted for LDPE (n=3).

Solution Method

The results from the Film Overlay assay were not conclusive. To further investigate the antimicrobial activity of the films, the films used for the overlay assay were removed from the plates, soaked in 95% aqueous ethanol for two minutes, then placed in sterile sealed glass tubes for 3 hours until used in the solution assay.

Films were dipped in 95% ethanol, then dried over low heat and placed in 10 ml of trypticase soy broth to which 0.1 ml of a 10^{-4} dilution of an overnight culture (10^{8} cfu/ml) of *E.coli* ATCC 25922 had previously been added. Films were shaken to fully immerse in the growth broth, then incubated 37℃ with gentle agitation (90 RPM). After 24 hours, serial dilution were prepared from the test cultures (n=3) and counted using duplicate pour plates (Speck, 1984) of the 10^{-5}, 10^{-6} and 10^{-7} dilutions. Plates were incubated 24 hours at 37℃prior to counting. Culture tubes with no film and with neither film nor inoculum served as controls, in addition to a range of control films.

3.7 Statistical Analysis

Statistical evaluations were conducted using Minitab® (v13.1 or v14.0, Minitab Inc., State College, PA). Mathematical manipulations of data, prior to and following statistical analysis, were conducted in either Minitab or Microsoft Excel 2002 (Microsoft Corporation, Seattle, WA). Graphical analysis was conducted with Sigmaplot® (v8.02, SPSS Inc., Chicago, IL). Error bars on all graphs are the standard deviations. Error values given in the text are 95% confidence intervals calculated using the student's t-distribution. Specific statistical methods used for each experiment are detailed in the text.

Chapter 4

Film Modification

The first stage in polymer film modification is to develop and test a modification protocol. Rather than reinvent new coupling chemistry, the protocol developed here has been assembled from complementary existing chemistries. A similar coupling protocol was recently described for a poly(acrylic acid) grafted poly(ethylene terephthalate) surface (Kim et al., 2000), whereby PEG was attached to surface carboxy groups using WSC coupling, and a peptide was then attached to the PEG, again using WSC coupling.

The detailed chemistries for each step of the modification process are outlined in Chapter 3; an overall schematic of the protocol is shown in Figure 3.1. This discussion focusses on the changes observed in the film at each step of the modification process. These changes allow a better understanding the effect of the modifications, allow predictions of the effect of the modifications on film properties and provide evidence that the desired chemical changes are occurring on the surface of the film.

4.1 Film Preparation

The surface of the film as received or as pressed is fouled by environmental contaminants and processing aids (e.g. oils); these need to be removed prior to working with the film. To this end the films were refluxed in dichloromethane (DCM), then heated to 60℃ under vacuum to remove the DCM. This caused a slight increase in contact angle (CA) and a reduction in CA variability (Figure 4.1). The treatment

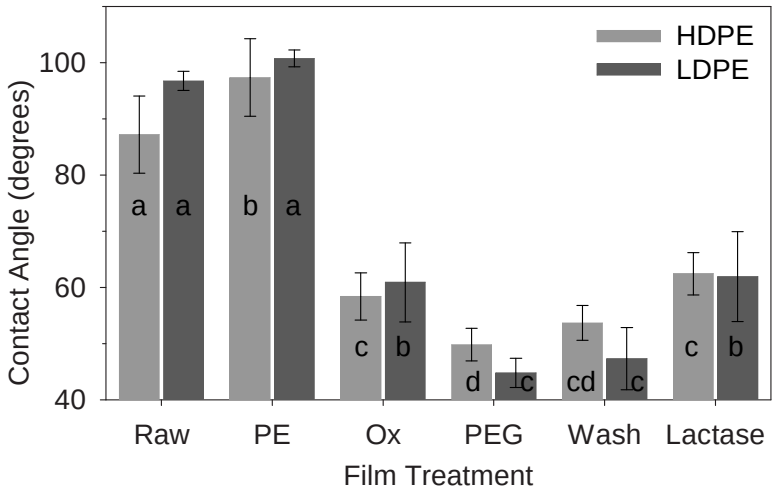


Figure 4.1: Water contact angles of LDPE and HDPE films at various stages of the modification process (n≥6). Letters represent significant differences within LDPE or HDPE samples. ■ HDPE, ■ LDPE

may have the additional benefit of extracting any additives from the films that might otherwise interfere with later reactions.

4.2 Oxidation

Oxidation of the PE surface caused a significant decrease in CA (Figure 4.1) from 101° to 61° for LDPE and from 97° to 58° for HDPE; these CAs agree with the reference data (Table 4.1).

A change in film behavior post-oxidation was discovered when first drying the oxidized films: when dried on a clean glass surface, the dried oxidized films adhered strongly ot the glass. No adhesion was observed for unmodified films when they were similarly dried. A similar effect was noted by Kato et al. (2003). In subsequent work, films were dried hanging in a desiccator.

An interesting variant of CA analysis was used by both Holmes-Farley et al. (1985) and Sarkar et al. (1997) where contact angles were measured across a range

Table 4.1: Reference contact angle data for various films. All CAs were determined using the sessile drop method, unless otherwise noted. Oxidized films (PE-COOH) were oxidized using chromic acid.

Film	Contact Angle	Comments	Reference
LDPE	103	aqueous buffers 1≤pH≤14	Holmes-Farley et al. (1985)
LDPE	95	water	Kong et al. (2001)
LDPE	95	water	Kato (1976)
LDPE	102	water	Schonherr and Vancso (1998)
LDPE-COOH	60	unbuffered solutions pH≤9, identical oxidation	Holmes-Farley et al. (1985)
LDPE-COOH	65	water, identical oxidation	Schonherr and Vancso (1998)
LDPE-COOH	66	water, weaker oxidation	Kong et al. (2001)
LDPE-COOH	55	water	Kato (1976)
LDPE-COOH	72	water	Kato (1977)
LDPE(COOH)PEG-NH_2	70	water, carboxy groups and amino-PEG chains	Sarkar et al. (1997)
LDPE-PEG-NH_2	40	water	Sarkar et al. (1997)
LDPE-PEG-OH	38	water, 4000MW PEG	Kiss et al. (1996)
PET-PEG	34	water, 3500MW PEG	Gombotz et al. (1989) cited in Kiss et al. (1996)
PU-PEG	48	water, 3300MW PEG	Bae et al. (1999)
HDPE	95	water	Kato (1976)
HDPE-COOH	43	water	Kato (1976)
HDPE-COOH	53	water	Kato (1977)

of solution pHs. At pHs corresponding to acid-base transitions (pKa values) of acid/base groups on the surface, the CA changes. For buffered solutions, a broad transition is observed, whereas unbuffered solutions show distinct, well-defined transitions. A similar analysis was applied in this work, using buffered solutions, with the results shown in Figure 4.2. For the oxidized films the CA decreases as the pH increases, while no change is evident for the unmodified films. The decrease in CA results from increasing ionization of surface carboxylic acid groups with increasing pH; an ionized surface is more hydrophilic than a non-ionized surface so has a lower contact angle. This confirms the presence of acid groups on the oxidized surfaces.

Difficulties with the high variability inherent in these contact angle measurements and a need for more information on the chemical changes occurring at the film surface required the use of additional techniques. Dye adsorption assays (Table 4.2) indicated a significant increase in surface acid groups after oxidation, as was expected.The surface carboxylic acid concentration created with this oxidation has previously been estimated to be 2.7 nmol/cm^2 (Holmes-Farley et al., 1985; Rasmussen et al., 1977), slightly higher than estimated here. The difference can be attributed to the differing methods of estimation and to incomplete dye extraction in this work: even after extraction, some color (and therefore dye) remained in the films. Holmes-Farley *et al.* further determined that the oxygen functions introduced by the oxidation procedure consisted predominantly of ketones (40%) and carboxylic acids (60%).

A side reaction of the oxidation process is etching or ablation of the film surface. This can be measured both gravimetrically and by analyzing the surface topography (discussed in §4.6). Film samples prepared from heat pressed LDPE varied

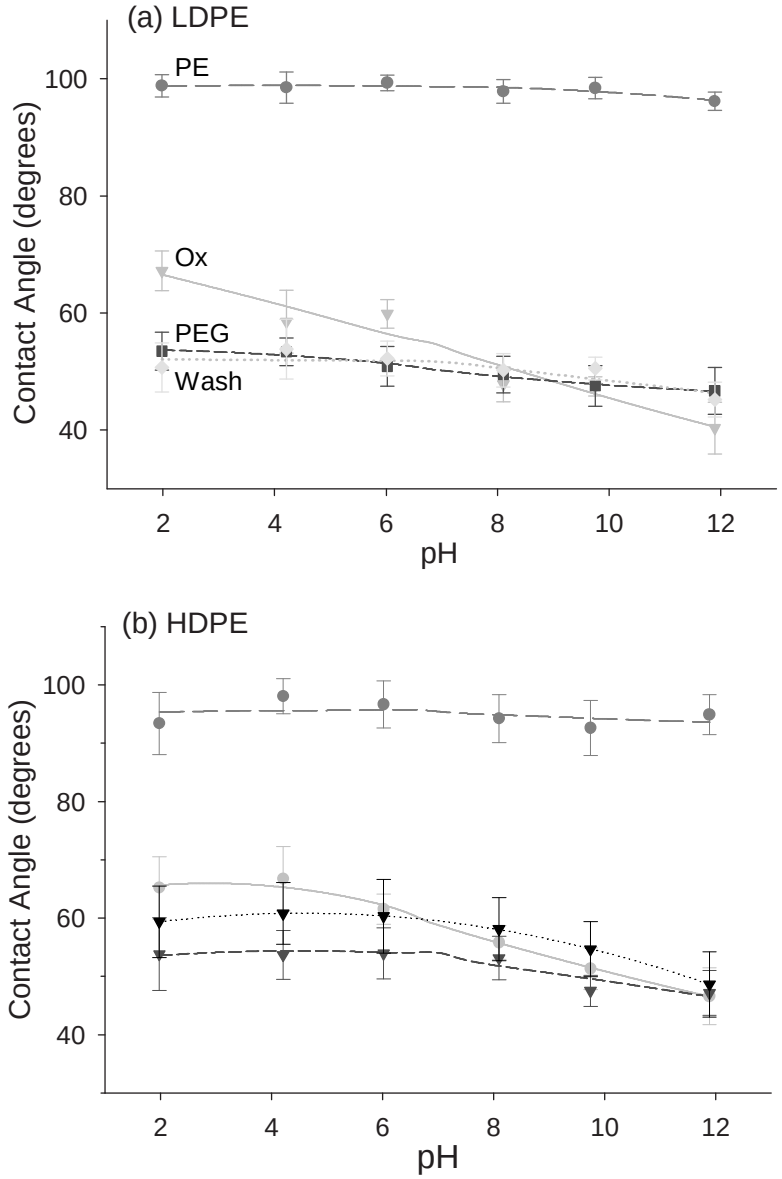


Figure 4.2: Contact angle variation with pH; measured with pH adjusted 50mM phosphate buffers. The lines plotted are smoothed, second-order polynomial Loess curves fitted to the data (n≥3).

Table 4.2: Absorbance of dyed film extraction solutions and estimates of surface acid and base concentrations (from Beers Law) for LDPE (n≥3). Letter superscripts denote significant differences within a column. Adjusted absorbance values account for differences in sample area and extract volume. *PEG* and *Wash* samples utilized diamino-PEG, whereas the *Lact* sample utilized amino-carboxy-PEG. — indicates not determined.

Sample	Toluidine Blue Extract A_{677}	Adjusted Absorbance ($A \cdot ml/cm^2$)	Acid Groups ($nmol/cm^2$)	Acid Orange 7 Extract A_{485}	Adjusted Absorbance ($A \cdot ml/cm^2$)	Basic Groups ($nmol/cm^2$)
PE	0.031^{a}	0.009^{a}	0	0.006^{a}	0.003^{a}	0
Ox	0.235^{b}	0.066^{b}	1.8	0.009^{a}	0.002^{a}	0.1
PEG	0.164^{c}	0.046^{c}	1.2	0.044^{b}	0.018^{b}	1.3
Wash	—	—	—	0.014^{ac}	0.008^{a}	0.4
Lact	0.119^{c}	0.100^{d}	2.5	0.033^{bc}	0.018^{b}	1.3

Table 4.3: Elemental composition of LDPE film surfaces from XPS analyses. The base LDPE film was heat pressed in the laboratory, as detailed in §3.2.1. *PEG* data is for films treated with diamino-PEG.

Sample	%Carbon	%Oxygen	%Nitrogen
PE	93.3	6.7	nd[a]
Ox	86.7	13.3	nd
PEG	77.9	19.4	2.6

[a]not detected

in weight from 50–80 mg depending on sample thickness. LDPE films typically decreased in weight by ∼0.6 mg during oxidation, indicating that some etching occurs. Increasing the duration of the oxidation does not increase the concentration of surface oxygen functions above a saturation level, but does continuously increase weight loss due to surface etching (Kato, 1977). It has previously been noted that LDPE etches faster than HDPE (Garbassi et al., 1994); oxidation weight loss was not measured here for HDPE films. No difference was found in the thickness of films before and after oxidation.

The effect of oxidation on the elemental composition of the film surface was measured by X-ray Photoelectron Spectroscopy (XPS, Table 4.3). As expected, the oxidation increased the oxygen content of the surface. The high initial oxygen content of the surface (6.7%) is thought to relate to oxygen incorporation in the film during heat pressing; commercial extruded PE films have surface oxygen contents of ∼1% (Kelley, 2004).

4.3 PEGylation

Key changes observed after PEGylation were further increased hydrophilicity, as shown by the decreased contact angle (Figure 4.1); further increase in the surface oxygen content (Table 4.3); the presence of basic groups on the film surface, as shown by dye adsorption assays (Table 4.2); and the presence of nitrogen on the film surface (Table 4.3). Neither surface nitrogen nor basic groups had previously been detected, but both are consistent with the presence of primary amines at the free terminus of tethered diamino-PEG chains. The combination of this evidence confirms the PEG coupling reaction was successful.

Considering the effect of pH on contact angle, the shape of the curve differs for the PEGylated film compared to the oxidized film. Although there is still an overall decreasing trend for contact angle as pH increases, suggesting the presence of unreacted acid groups, the difference between contact angles at low and high pH is reduced for PEGylated films. The presence of unreacted acid groups on the PEGylated film surface is confirmed by the dye adsorption results (Table 4.2). There is the suggestion of a maximum on the contact angle curve for PEGylated films, indicating increasing hydrophilicity (decreasing CA) at low pH, consistent with the ionization of primary amine groups under acid conditions; this is most apparent on the HDPE curve. The presence of unreacted acid groups and the greater initial hydrophilicity of the PEGylated film may obscure the effect of amine ionization on contact angle.

4.4 Washing

In early experiments investigating the stability of the PEGylated film surfaces, it quickly became apparent that washing the PEGylated film caused increases in CA, indicating some loss of the hydrophilic PEG coating. Even after vigorous washing, however, the CA did not increase back to the level of the oxidized films. It was hypothesized that some PEG was adsorbed to the surface, while the remainder was covalently immobilized. This was confirmed by repeating the coupling procedures without the activating agent (WSC) and with non-reactive PEG in the presence of WSC and observing a decrease in contact angle both times (Figure 4.3). The presence of adsorbed PEG is problematic as any active agent attached to adsorbed PEG could readily migrate from the packaging material, violating non-migratory principle. To prevent this, the adsorbed PEG must be removed prior to attaching the active agent.

Three different wash solutions were tested: water (data not shown), pH 5 phosphate buffer and 10% aqueous ethanol. The washing protocol involved shaking three test films for 1 hour in 30 ml of test solution at room temperature (Figure 4.4). Water washing resulted in a change similar to washing with pH 5 buffer. Ethanol (10%, aq.) was found to be the most effective wash solution. More severe treatments were not tested as it was considered they might adversely effect the retention of the covalently immobilized PEG. Future testing with a pH 3 solution would be advisable if the final film is to be used with acidic food products.

Figure 4.4 also allows the comparison of three different coupling methods based on contact angle differences before and after washing. Unfortunately, a large variability was associated with these results, so statistically valid comparisons were not possible. These results do indicate that higher levels of carbodiimide are not

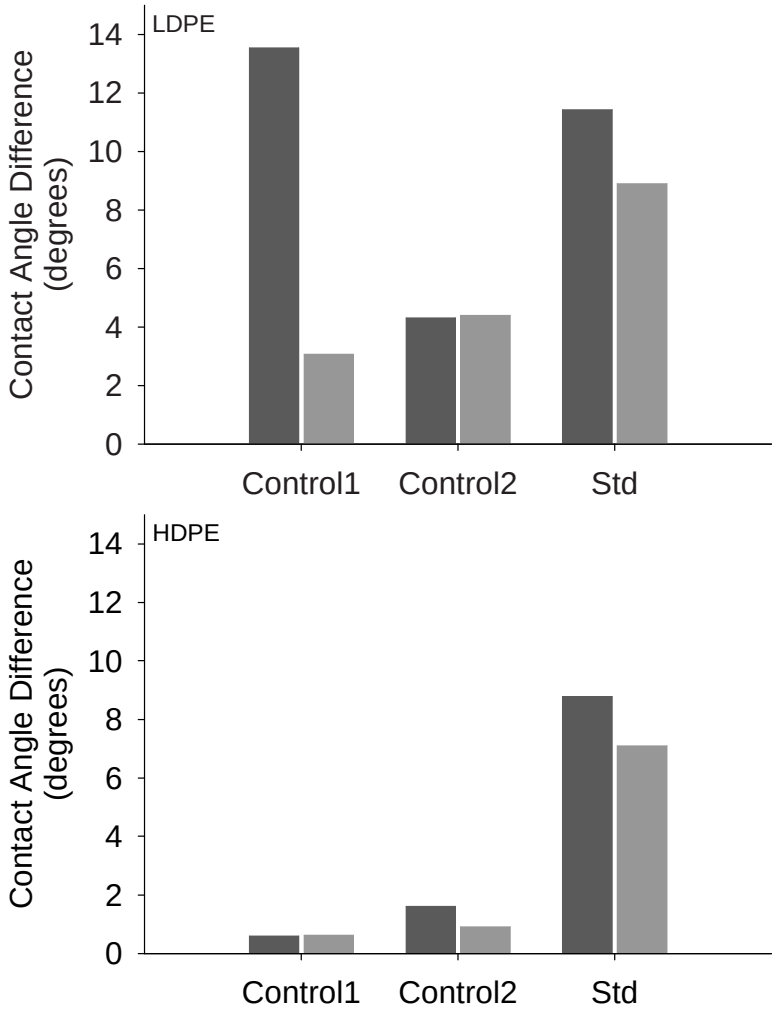


Figure 4.3: Adsorption of PEG by HDPE and LDPE. Data plotted is the difference between the oxidized and PEGylated film contact angles. Std: standard coupling protocol; Control 1: standard coupling omitting the carbodiimide (WSC); Control 2: regular coupling substituting dihydroxy-PEG for diamino-PEG. (n=6) ■ No wash step; ■ 10% aqueous ethanol wash.

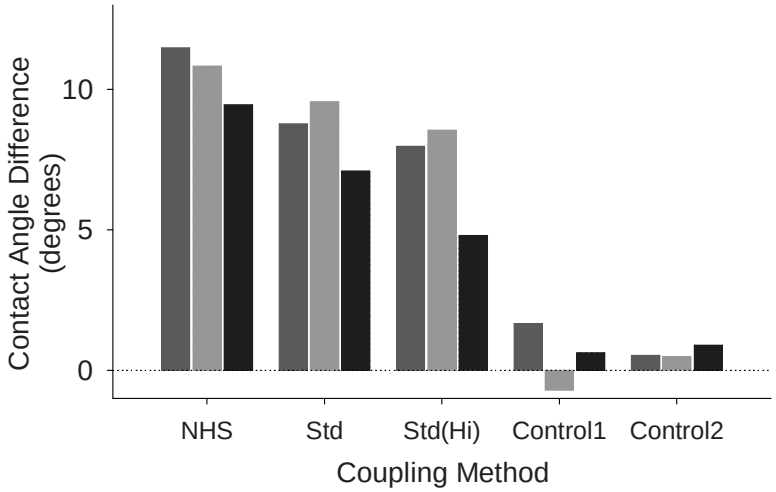


Figure 4.4: Comparison of five different coupling methods and two wash methods for HDPE films. The difference between the oxidized and PEGylated film contact angles is plotted. Std: standard coupling protocol; Std(Hi) standard coupling using an increased level of WSC; NHS: addition of N-hydroxy succinimide to the activation (carbodiimide) solution; Control 1: standard coupling omitting the carbodiimide (WSC); Control 2: regular coupling substituting dihydroxy-PEG for diamino-PEG. (n=3) ■ No wash step; ■ pH5 phosphate buffer wash; ■ 10% aqueous ethanol wash.

advantageous and may actually reduce immobilization; this was also described by Nakajima and Ikada (1995). Addition of N-hydroxy succinimide to the activation (WSC) solution may be advantageous, which has also been suggested by other authors (Kato et al., 2003), and should be explored further. Optimization of the coupling reaction needs to be conducted using more precise analysis systems than the current CA methodology.

Briefly considering pH–CA trends (Figure 4.2), the washed PEGylated films show nearly identical trends to the unwashed PEGylated films. For LDPE films, no significant difference was observed between unwashed and washed PEGylated films in this experiment, whereas for HDPE films the expected increase in contact angle was observed after washing.

4.5 Lactase Attachment

After attachment of lactase to the washed, PEGylated PE film, the CA (Figure 4.1) increased to 62° for both LDPE and HDPE. These films were indistinguishable from oxidized films on the basis of CAs. The dye adsorption assays, however, allowed a distinction to be made (Table 4.2); lactase coupling significantly increased the number of both acidic and basic groups present on the film surface. The basic groups present on the lactase-modified film do not originate from the PEG, as it may initially appear. The PEGylated film analyzed for the dye-adsorption assays was treated with diamino-PEG, providing the (free amine) basic groups found in the dye adsorption assays; the PEGylated film used to create the lactase-modified films, however, utilized amino-carboxy-PEG with a carboxylic acid free terminus. Hence, the basic groups found on the lactase modified film can be assigned to the lactase and not the underlying PEG.

Lactase attachment is discussed further in Chapter 5.

4.6 Surface Topography

Surface topography was investigated using Atomic Force Microscopy; it is of interest both because it provides further evidence to the progress of the reactions and because changes in film surface structure may have direct or indirect effects on other analytical results, on film properties and on the activity of an immobilized compound. Representative AFM images are shown in Figures 4.5 (LDPE) and 4.6 (HDPE).

It is immediately clear that there are significant differences between LDPE and HDPE. The differences relate to the microstructure of the poly(ethylene) in each of

Figure 4.5: Surface topography of modified and unmodified LDPE films shown using Atomic Force Microscopy. A: *PE*; B: *Ox*; C: *PEG*; D: *Wash*.

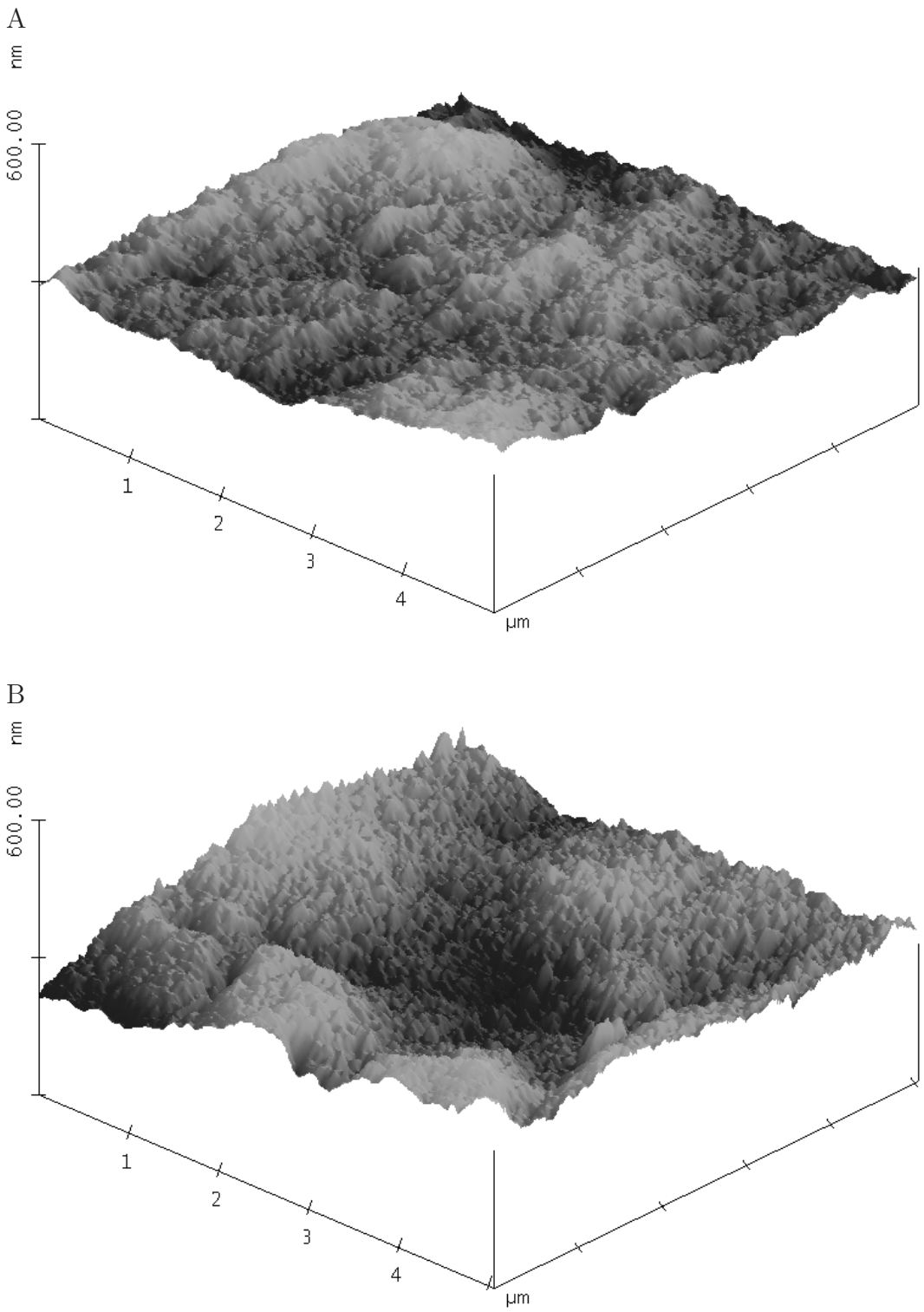

A
nm
600.00
1
2
3
4
µm
B
nm
600.00
1
2
3
4
µm

Figure 4.5 (continued)

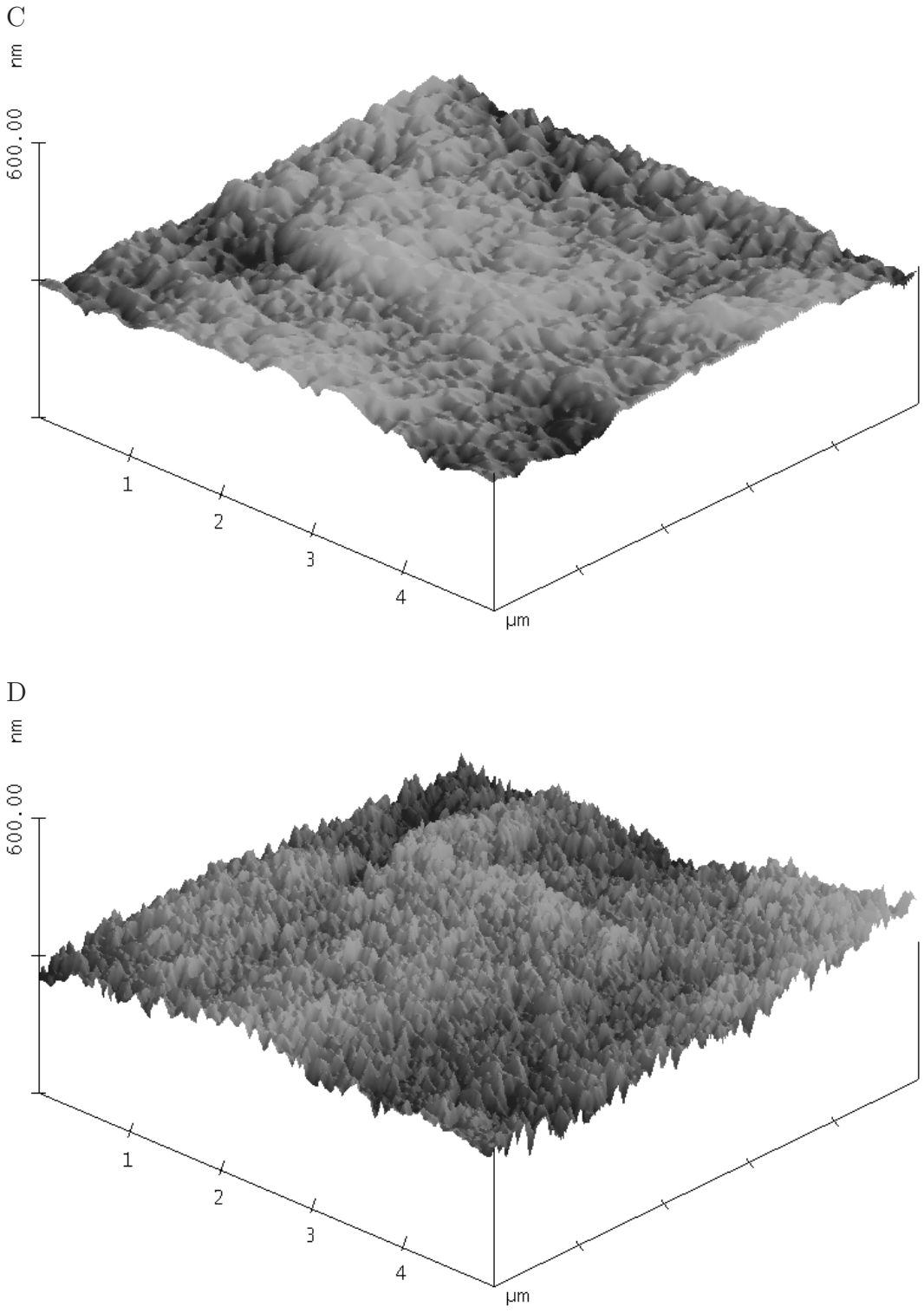
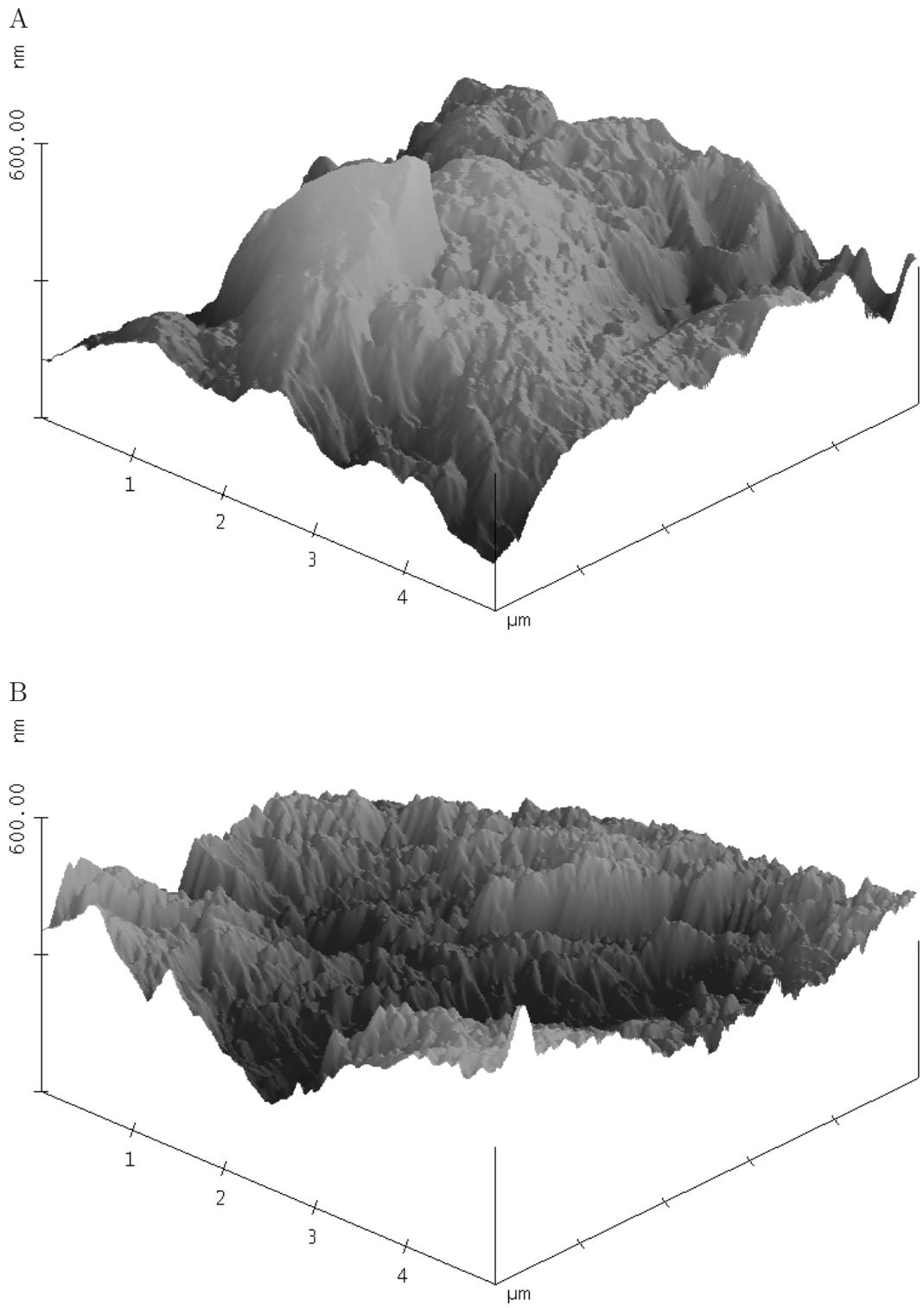


Figure 4.6: Surface topography of modified and unmodified HDPE films shown using Atomic Force Microscopy. A: *PE*; B: *Ox*; C: *PEG*; D: *Wash*.


A
nm
600.00
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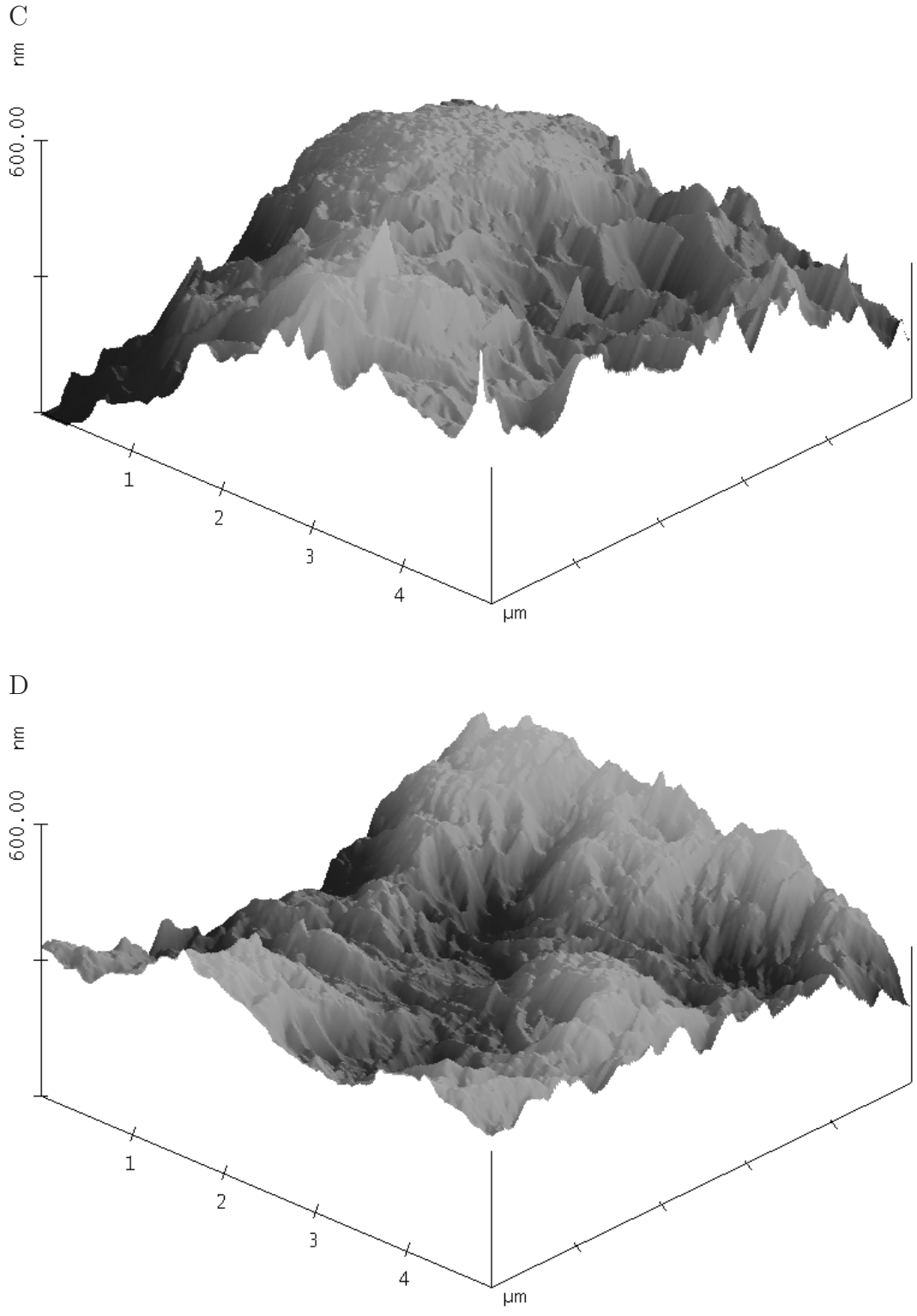
2
3
4
µm
B
nm
600.00
1
2
3
4
µm

Figure 4.6 (continued)



these materials. LDPE is predominantly an amorphous material, whereas HDPE has a high crystalline content — as can be seen from the large obtrusions and the general angular nature of the surfaces visible in Figure 4.6.

The effects of the surface modifications are less obvious than the contrast between LDPE and HDPE, but are still distinct. These differences will first be discussed for LDPE, for which they are most apparent. The unmodified film surface is made up of broad, fairly low-angle peaks. After oxidation, however, the surface is very different: it has a more regular pattern, with an increased number of taller, steeper peaks. The change is due to etching and ablation of the film surface by the chromic acid.

Most previously published images of chromic-acid etched poly(ethylene) films are from Scanning Electron Microscopy (SEM) and do not directly correspond to AFM images due to the differing aspects and sample preparation. Based on the SEM images, however, the etching and surface structuring found here appears to be similar to what has previously been observed (Kato, 1977; Schonherr and Vancso, 1998; Lin and Tseng, 2001).

The unwashed PEGylated film surface is virtually flat, consisting of broad, low-angle peaks. The troughs present on the oxidized film appear to have been filled in by the PEG.

The washed PEGylated film is a different picture again, showing a similar number of peaks to the oxidized film, but the peaks appear to be taller (greater vertical scale) and steeper. The PEG which had previously filled in the troughs between the peaks was, apparently, adsorbed to the surface, not covalently attached and was removed by washing. To account for the increased height and steepness of the peaks, it is hypothesized that the PEG chains preferentially attach to the tips

of the peaks on the oxidized film; the tips would be more accessible to PEG chains in solution than the troughs (steric hindrance). PEG attachment at the tips of the peaks would result in them becoming taller and steeper, as observed.

It has previously been noted (Kato et al., 2003) that polymer chains grafted to an otherwise smooth surface tend to attach in clusters, leading to a surface apparently coated with freestanding pillars. This is favorable from a thermodynamic viewpoint[1] and a similar process occurring at the most accessible points (the tips of the peaks) on the oxidized LDPE film provides an additional reason for preferential PEG attachment to the tips of the peaks present on the oxidized film.

Similar differences to those just discussed for LDPE are somewhat apparent for HDPE, but are largely obscured by the facetted, crystalline nature of the surface; for HDPE it is more difficult to distinguish differences between the images.

4.7 Summary of Findings and Recommendations

Overall, the reaction sequence appears to be successful for covalent immobilization of peptides on poly(ethylene) film, although it does require further optimization. Optimization will require ready access to better analytical facilities than have been available to date. Such facilities could consist of a more precise means for measuring contact angles or reliable access to either XPS or ATR-FTIR. It is possible that addition of NHS to the carbodiimide activation solution will improve the coupling yield; this needs further investigation.

[1] reduction in the surface area created, compared to independent attachment of graft chains

Chapter 5

Bioactivity of Modified Films

The objective of this research was to attach bioactive peptides to poly(ethylene) film to create non-migratory bioactive food packaging films. It has already been shown (Chapter 4) that the coupling protocol appears to be effective at attaching first PEG and then the peptide to PE film. In the following discussion, the bioactivity of E14LKK and lactase modified films is investigated and the film modification reactions are examined further.

5.1 Antimicrobial Peptide Modified Films

The goal of antimicrobial films should be shelf-life extension. In this function they must inhibit the growth of microbes, but are not required to totally eliminate them. Antimicrobial films are less suited to food-safety applications requiring complete pathogen elimination.

Shelf-life extension of microbially-limited products can occur in two ways: extension of the microbial lag period or reduction of the maximal microbial growth rate. Antimicrobial films should be considered a hurdle technology in the food processing and packaging mix; they are not a solve-all "silver bullet". An advantage of antimicrobial packaging over current technologies could be in reducing the required severity of processing conditions and/or reducing use of "artificial" preservatives in the product formulation. There are also possible regulatory advantages and technical benefits from isolating the antimicrobial effect at the packaging surface. These advantages were discussed in more detail in Chapter 1.

An antimicrobial film was successfully developed (Table 5.1). Clear antibacterial activity was evidenced in the solution assay for the deprotected film to which side-chain-protected E14LKK had been attached.

5.1.1 Antimicrobial Assays

The method used to assess antimicrobial activity always needs to be carefully considered when testing potentially antimicrobial films. Key considerations include migration of the antimicrobial, mechanism of antimicrobial activity and similarity of the assay to the target application of the films.

In this work, three separate assays for film antimicrobial activity were performed using two different methods: a agar film-overlay method and a solution method. The second overlay assay and the solution assay were performed using the same set of films (Table 5.1). The first assay, a film overlay assay (data not shown), was unsuccessful as the plate inoculum was too low — there were insufficient colonies per square centimeter, and the colonies were unevenly ditributed. This demonstrated two difficulties with this method — the need for a very accurate count of the inoculum and the need for uniform colony distribution across the plates. These factors were understood prior to conducting the assay, but, despite significant care, problems still arose.

In the second film overlay assay (Table 5.1) the inoculum was ideal for the method. The uncovered colonies on the film plates could not be counted, however, due to colony overgrowth and merging; a shorter incubation time prior to counting the uncovered colonies or a lower incubation temperature would have prevented this. Fortunately, a control plate was produced using a reduced inoculum from the same culture, so an accurate "uncovered" count was available.

Table 5.1: Results from the antimicrobial assays of the treated films. Mean values are followed (in brackets) by standard deviations (n=3). Significant differences, determined from a one-way ANOVA with Tukey pairwise comparisons ($\alpha = 0.05$), within an experiment are indicated by the letters. — indicates not determined

Film	Film Overlay Method colonies/cm^2	Solution Assay log(cfu/ml)
Control	50 (—)a[a]	9.1 (0.1)a
PE	38 (11)a	8.8 (0.1)a
Ox	59 (21)a	8.6 (0.2)a
PEG	40 (21)a	9.0 (0.1)a
Wash	43 (8)a	8.1 (0.2)a
unE14LKK	34 (8)a	8.1 (0.2)a
proE14LKK	55 (—)a[b]	—
deE14LKK	29 (3)a	5.9 (0.3)b[c]

[a]Colonies on uncovered areas had spread too much for accurate counting on the film plates; this value is from 10 squares on a single (n=1) spread plate control (no films) prepared with a 0.1ml inoculum; the lower inoculum (0.1ml c.f. 0.2ml) allowed for accurate counting. The result has been doubled to allow for easy comparison to the film results.

[b]n=1

[c]estimated count, all plates had less than 30 colonies

Despite a relatively even colony distribution on the plates and an ideal colony count, the film-overlay assay failed to show statistical significance between controls and test samples with an ANOVA. The high variability of the control samples suggests that, despite the appearance of uniformity, the bacterial distribution on the test plates was inadequately uniform, suggesting a major shortcoming of this method. By grouping the results from the control films (*PE*, *Ox*, *PEG*, *Wash*) together and comparing the mean of the controls to the mean for each of the test films (*unE14LKK* and *deE14LKK*) with one-way t-tests ($\alpha = 0.05$), the colony count for *deE14LKK* was found to be significantly lower than the control counts. The difference between *unE14LKK* and the control films was not significant at the 95% confidence level, but was at the 90% confidence level. This statistical analysis, however, is not as valid as an ANOVA test.

These differences prompted the final solution-based antimicrobial assay, which showed definitive differences between *deE14LKK* and the control films using an ANOVA (Table 5.1). This suggests that the film overlay assay developed here is insufficiently sensitive to adequately evaluate the antibacterial action of the modified films. It does show some promise, but a completely uniform bacterial distribution would be required for it to be useful. A uniform distribution may be possible using an automated plate seeding technique as opposed to the manual inoculation and spreading conducted here. The film-overlay assay was designed to mimic the antibacterial effect of films used for bacterial inhibition on the surface of solid foods. The solution assay was effective and is most suited to evaluating films for use packaging liquid food products.

5.2 Lactase Modified Films

Lactase active films could produce reduced-lactose fluid milk products without requiring special milk processing, potentially leading to significant cost savings and simplified production for these products. A lower price for reduced-lactose milk could significantly expand this market: according to the National Institutes of Health (2003), 30–50 million Americans (10–18%) suffer from lactose intolerance, with the rate significantly higher for certain ethnic groups (African-Americans, up to 75%; Asian-Americans, up to 90%). Internationally, caucasians have the lowest rate of lactose intolerance (between 2% and 25% depending on country), with rates generally above 50% for Indians, South Americans, Africans and Asians; incidences as high as 100% have been reported for some Asian populations (Sahi, 1994; Vesa et al., 2000). Worldwide it is estimated that two thirds of the adult human population has some degree of lactose intolerance. Clearly the potential market for reduced-lactose dairy products is significant, yet consumption of such remains low. It may be postulated that this is in part due to the higher price and lower availability of these products. Reduced-lactose milk is often 2–3 times more expensive than regular milk and is frequently unavailable in smaller markets; very few reduced-lactose processed dairy products are available. Lowering production hurdles and decreasing production costs could significantly improve the marketability of these products and potentially enrich the lives of many people.

The bioactivity of lactase modified films was measured by storage with a synthetic lactose substrate, *o*-nitrophenyl-β-D-galactopyranoside (ONPG). Lactase hydrolyses ONPG, releasing galactose and yellow o-nitrophenol; activity can be monitored by measuring the color change of the solution with a visible spectrophotometer at 420nm. For free lactase the color change occurs extremely quickly,

however to allow for possible reduced activity of immobilized lactase, a three week storage trial was conducted. Daily measurements could not be conducted without compromising the sterility of the sample solutions — various bacteria are also able to hydrolyze ONPG so their growth will also cause the solution to turn yellow — so a single point measurement was used to investigate the possibility of activity. Unfortunately, no activity was found for lactase-modified films, even after three weeks of storage, regardless of PEG pre-treatment or the inclusion of lactose in the coupling solution (Table 5.2).

To verify the coupling reactions and confirm the presence of lactase on the films, XPS surface analysis was conducted on samples from the same reaction batch as those used for the lactase activity assay, with some interesting results (Table 5.3).The presence of lactase on the films was confirmed (distinct increase in %N and %O), however no PEG appears to have been immobilized during the previous modification step; there was no significant increase in oxygen content[1] on PEG treatment. Although a small amount of nitrogen was observed, this was significantly less than observed previously when PEG attachment was confirmed (Table 4.3). The previous analysis was conducted on *PEG* films treated with diamino-PEG, whereas the coupling progression for peptide attachment uses amino-carboxy-PEG. Diamino-PEG was used in early work because it was available and was significantly less expensive than amino-carboxy-PEG. It had been assumed that the reactions would proceed equivalently for the two compounds, but this was clearly not the case. The differences between the two PEGs prevented the

[1]in the first XPS analyses, heat pressed films with a high initial %O were used. In the later analysis shown in Table 5.3 commercially extruded LDPE films were used; a typical surface oxygen content of commercial LDPE films is 1% (Kelley, 2004) so an oxidized value of 7.6% confirms the oxidation. Untreated samples were not analyzed.

Table 5.2: Lactase activity of modified commercial LDPE films. Increasing absorbance indicates increased lactase activity (n=3); data from two separate activity experiments is shown. Standard deviations are given in brackets; no significant differences were found. *Lact*: Lactase attached to PEGylated PE; *PELact*: Lactase attached directly to oxidized PE; *B*: coupling conducted in presence of lactose.

Film	Storage Time (days)	Absorbance 420nm
Control	23	0.17 (0.02)
PE	23	0.15 (0.03)[a]
Ox	23	0.17 (0.01)
PEG	23	0.20 (0.02)[a]
Lact	23	0.14 (0.04)
PELact	23	0.16 (0.02)
Control	25	0.10 (0.01)
PE	25	0.10 (0.01)
Lact B	25	0.12 (0.02)
PELact B	25	0.11 (—)[b]

[a] n=2
[b] n=1

Table 5.3: Surface atomic composition of Lactase-track modified commercial LDPE films determined from XPS analyses. nd: not detected.

Film	%C	%O	%N
Ox	92.4	7.6	nd
PEG	90.9	8.4	0.7
Wash	92.1	7.6	0.3
Lact	82.1	12.3	5.6
PELact	81.3	13.2	5.5

amine group on the amino-carboxy-PEG from coupling with the activated carboxyl groups on the film surface.

Revisiting the exact chemical make-up of the amino-carboxy-PEG, it was noted that the terminal amine function was complexed with HCl. The reaction is conducted in unbuffered distilled water adjusted to pH 7; on dissolution of the PEG the HCl may reduce the pH of the reaction solution, hindering the reaction. Alternatively, if the amino-carboxy-PEG exists as a zwitterion, which is probable at roughly neutral pHs, then inter- and intra-molecular ionic bonds may form between the charged amino and carboxyl groups. These ionic bonds could interfere with the reactions.

If pH alteration by the HCl is the cause of coupling failure, then one solution could be to conduct the coupling in a buffered solution. Unfortunately, most common buffers (e.g. citric, phosphate) are unsuitable as they interfere with carbodiimide coupling (Anon, 2002c); MES buffer (2-[N-morpholino]ethane sulfonic acid buffer) has been successfully used for WSC coupling (Biltresse et al., 2000), so should be investigated as an alternative to inorganic buffers.

If zwitterion formation is interfering with the coupling, a solution could be to adjust the pH of the coupling solution; an optimal pH may exist where the zwitterion form of the molecule is not predominant, but the coupling reaction still proceeds. The use of buffered solutions is recommended if pH modification is investigated. As well as pH, other reaction conditions can also be varied to enhance PEG attachment. Kingshott et al. (2002, 2003) conducted PEG immobilization at elevated temperatures and salt concentrations in what they term "cloud-point" grafting. The temperature selected was the lower critical solution temperature of the PEG (60℃); under these conditions PEG solubility is reduced and surface coupling is enhanced. This should also be investigated. Before any further reaction optimization can be conducted, however, ready access to appropriate equipment needs to be arranged to monitor the reaction outcomes; contact angles have proven insufficient for this task.

In summary, lactase was successfully attached directly to PE film, but no activity was found in this state. Activity was not improved by including lactose in the coupling buffer. As previously mentioned, the purpose of hydrophilic spacers is to separate the hydrophilic peptides from the hydrophobic PE to prevent disruptions to the peptide structure (§1.2.3). It is assumed that such disruptions are the reason for the lack of lactase activity. The effectiveness of the spacer at ameliorating this problem, however, cannot be experimentally evaluated at this time due to the difficulties with amino-carboxy-PEG attachment. It is likely that inclusion of a PEG spacer will improve lactase activity, as other research has shown excellent activity for PEGylated-lactase (Talbert, 2004).

5.3 Summary of Findings and Recommendations

Lactase immobilization without PEGylation did not produce a lactase-active LDPE film, regardless of lactose inclusion in the coupling buffer. Amino-carboxy-PEG was not successfully attached to the oxidized LDPE surface, despite success with diamino-PEG attachment. Further investigation of the coupling reactions is required to optimize coupling of amino-carboxy-PEG. It is unknown if lactase immobilized on a PEGylated LDPE film will be active, but this is considered likely based the confirmed activity of PEGylated lactase (Talbert, 2004).

An antibacterial LDPE film was successfully produced. The film was prepared by immobilizing antimicrobial peptide E14LKK (with Boc-protected side-chain amines) onto PEG-treated LDPE film, then deprotecting the peptide. Antibacterial activity was demonstrated against *E.coli* in a solution assay.

Chapter 6

Effect of Modifications on Film Properties

When modifying an existing material for enhanced performance, e.g. by providing targeted bioactivity, it is necessary to consider the original properties and characteristics of the material and how they relate to its production, use and disposal. Typically, the intended applications of the new material are similar to current applications of the base material; for the modified material to be suitable for existing applications, key characteristics of the original material must not be adversely affected by the modifications, or, if they are affected, the changes must be understood and adjusted for during process or package design. This chapter discusses the affect of these film surface modifications on characteristics important to the production, use and disposal of PE packaging films.

Each of the tests conducted is related to important properties of PE film in real packages: transition (melting) temperatures are important in many processing operations and for heat sealing; tensile properties are important when a film is a structural component of a package and also in many processing operations; coefficients of friction are important in film processing and also in the transport, display and handling of packages; optical properties are important for the communication (marketing) function of the package; and moisture permeability is important for the preservation of many food products.

Material properties can be classified as either surface-sensitive or surface-insensitive. Surface-sensitive properties are ones significantly affected by nanoscale

Table 6.1: Classification of film properties as 'Surface Sensitive' or 'Surface Insensitive'.

Surface Sensitive Properties	**Surface Insensitive Properties**
Coefficients of Friction	Melting Temperature
Specular Gloss	Tear Propagation Force
Haze & Luminous Transmittance	Yield & Tensile Strengths
Moisture Permeability	Elongation at Break

physical and chemical changes to the surface layers of the film. Surface-insensitive properties, on the other hand, are relatively unaffected by physical or chemical changes to the surface layers of the film, but are primarily governed by the bulk characteristics of the material. The division of the properties examined here into surface-sensitive and surface-insensitive is shown in Table 6.1. It is important to realize that these classifications are not absolutes; surface sensitivity is really a spectrum, varying linearly from completely unaffected by material surface characteristics to completely dominated by surface characteristics. This scale is the inverse of the influence of the material bulk characteristics on the property under consideration.

For the majority of these analyses both LDPE and HDPE films were tested. In three assays (melting point, friction and moisture permeability) only LDPE was tested; analogous results are expected for HDPE. The films tested were either unmodified (PE), oxidized (Ox) or diamino-PEG grafted (PEG). It was expected that any changes caused by the peptide immobilization scheme would be evident after the PEGylation of the film. These results can be extrapolated for peptide-modified films or additional testing can be designed if deemed necessary based on

these results.

Results were analyzed using General Linear Models with the film (LDPE or HDPE), the treatment (*PE*, *Ox* or *PEG*) and the interaction term as predictor variables. Significant differences were evaluated using Tukey pairwise comparisons with an overall confidence level of 95% ($\alpha = 0.05$). In all bar charts, letters within the bars indicate significant differences within each group of bars: the same letter denotes no significant difference; different letters denote significant differences. For groups of treatments with no significant differences, the values given in the text for that group are the mean across all treatments within the group.

6.1 Physical and Mechanical Properties

Most of the physical and mechanical properties of polymer films are dependent predominantly on the bulk properties of the material; these properties can therefore be considered surface insensitive. In other words, they are relatively independent of the state of the film surface, so it was hypothesized that they would be minimally affected by these surface modifications. The exception to this, amongst the physical and mechanical properties examined, are the coefficients of friction, which are inherently surface sensitive; it was hypothesized that the modifications would affect the coefficients of friction.

6.1.1 Transition Temperatures

For a mixed polymer system with distinct, homogenous regions, as in the case of a brush-grafted polymer such as PEG-grafted PE, multiple transition temperatures may be observed; the individual transition temperatures depend on the bulk properties of the different homogenous regions. It would not, therefore, be unex-

Table 6.2: Reference transition temperature data for LDPE, HDPE and PEG.

Material	T_{m} (℃)	T_{g} (℃)	$T_{\mathrm{softening}}$(℃)	Notes	Reference
	137	−120	—	—	Rodriguez (1996)
HDPE	134–137	−110	—	—	Bakker (1986)
	130–137	−140–−100	72–133	—	Ellis (2000)
	110	−120	—	—	Rodriguez (1996)
LDPE	112	−110	—	—	Bakker (1986)
	80–115	−125–−120	86–102	—	Ellis (2000)
	66	−67	—	—	Rodriguez (1996)
PEG	53–56	—	—	4000 MW	Ellis (2000)
	—	−17	—	6000 MW	Ellis (2000)

pected to observe two distinct transitions — one for PEG, one for PE — for each of the major thermo-physical transitions, for example melting and the glass transition. The detection of these transitions, however, depends on the quantities of each homogenous region present in the polymer system and the sensitivity of the measurement system. The DSC method employed to determine transition temperatures in this work uses samples of approximately 5mg, with the PEG comprising less than 1%, by weight, of the film sample. It was considered highly unlikely that the PEG transition would be observed, and this was indeed the case; no transition was observed corresponding to the melting point of PEG for the PEG-modified film samples.

In some polymer systems, transition temperatures vary depending on the presence of other polymers in the system due to intermixing and interaction of the polymers involved. This is not normally the case for polymer systems with dis-

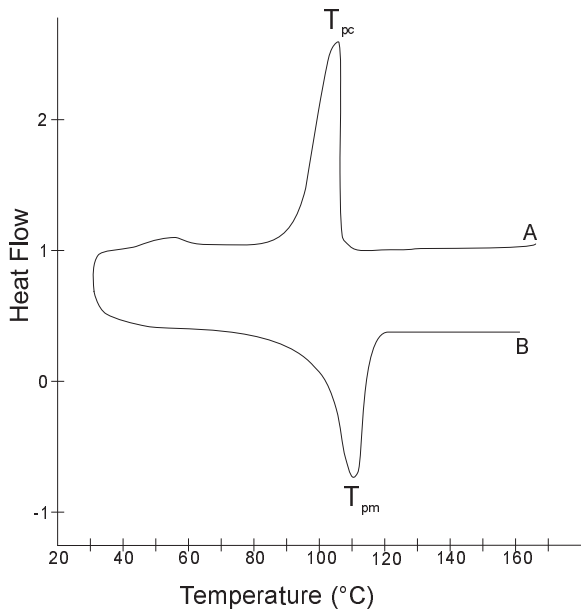


Figure 6.1: Example of typical DSC melting-point determination curve for LDPE, showing the melting (T_{m}) and crystallization (T_{pc} temperatures and the shoulder-peak on the crystallization curve. The sample starts at A, is cooled and then reheated to finally reach B. Cooling and heating rates are constant at 10℃/minute.

tinct, homogenous zones. As was expected, there was no change in the melting temperature associated with the LDPE between the modified and unmodified samples. In all cases, the melting point (T_{pm}) of the samples was found to be 110.9($\pm$0.4)℃ and the crystallization point (T_{pc}) 93.9($\pm$0.4)℃. These results are comparable with reference values from the literature (Table 6.2). A shoulder-peak was also observed on the crystallization curve at a temperature of 57.8($\pm$0.4)℃ (see Figure 6.1). This is within the range of T_{m} values found for PEG in the literature, however this shoulder peak is present on all the curves, not just those of the PEG-modified PE, so it is not associated with PEG. This shoulder-peak may be related to a molecular rearrangement within or phase change of the PE lattice.

Regarding glass transition temperature determination, the equipment was not well suited to the low temperatures required (see Table 6.2). An attempt was made

to determine glass transitions using manual liquid nitrogen cooling of the sample cell, however the sample could not be cooled sufficiently and the cooling rate was too slow and too variable for an accurate determination to be made. An estimated glass transition (n=1) was found at -26.9℃ (T_{mg}) for unmodified LDPE film. This does not approximate the reference values and may be a 'phantom peak'; it may have resulted from the inability to adequately control sample temperature. It was considered very unlikely that a T_{g} would be observed for the PEG based on the T_{m} results and given that a secondary transition (T_{g}) is typically harder to detect than a primary transition (T_{m}). As such, no further attempts were made to measure T_{g} values.

6.1.2 Tensile Properties

Tensile properties of a material, and structural properties in general, are dominated by the bulk characteristics of the material. Surface specific changes typically have negligible effect, although surface roughness can affect stress concentration and, correspondingly, mechanical properties; significant differences were not expected between the properties of the modified and unmodified films. Significant differences were expected between LDPE and HDPE films, and this was indeed the case for all the tensile properties examined.

For LDPE, no significant differences were found between modified and unmodified films for Tensile Strength (12.6 ± 0.4 MPa, Figure 6.2), Yield Strength (10.2 ± 0.2 MPa), Elongation at Break (530 ± 50 %) or Normalized Tear Propagation Force (32.7 ± 0.9 N/mm, Figure 6.3), as was expected. All properties were measured in the machine direction. The measured Tensile and Yield Strengths are within the ranges presented by the reference values (Table 6.3). The Elongation at Break

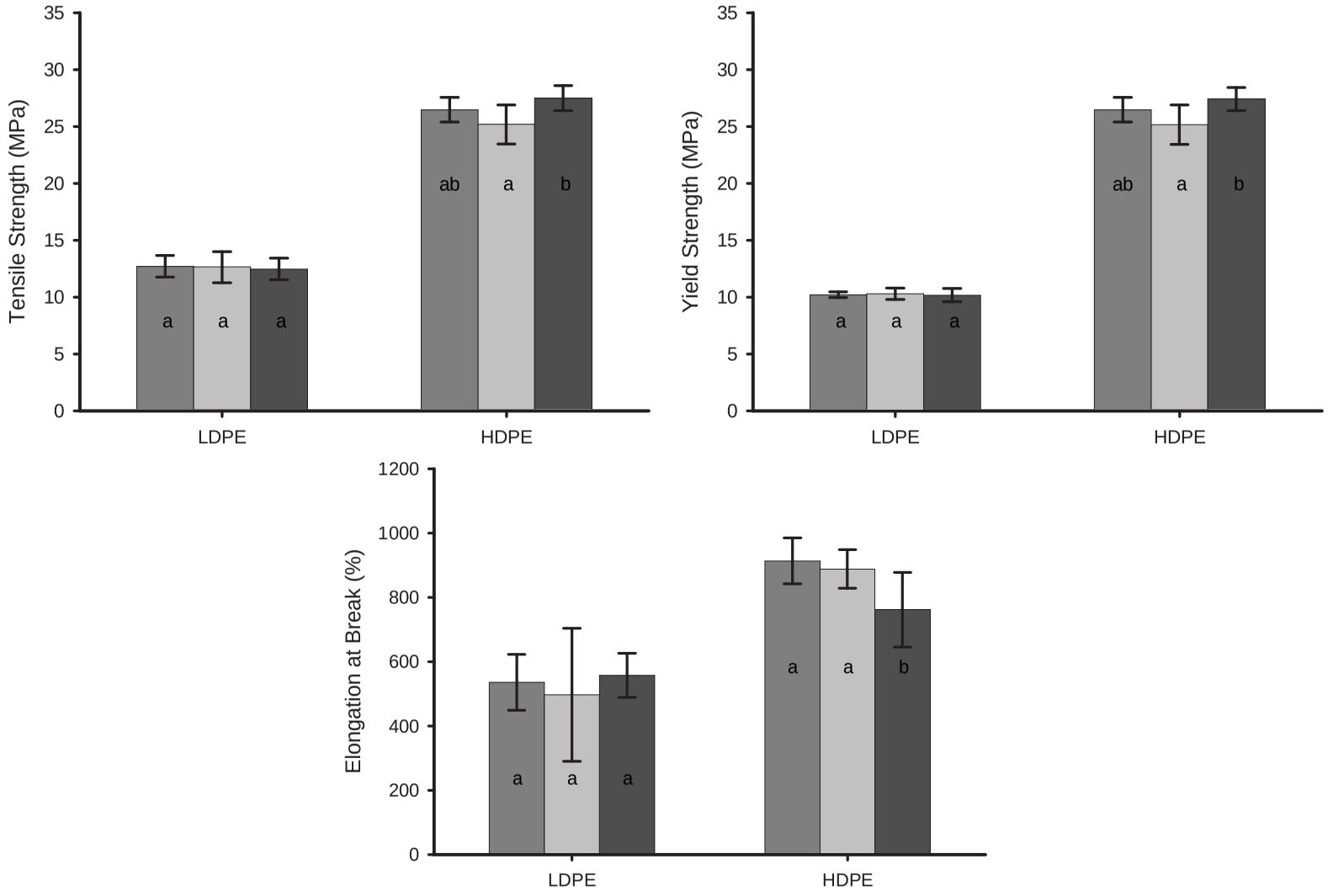


Figure 6.2: Tensile properties of modified and unmodified LDPE (n=10) and HDPE (n=12) films, as per ASTM method D882–97. Significant differences within each group bars are indicated by the letters within the bars ($\alpha = 0.05$). *PE*, *Ox*, *PEG*.

Table 6.3: Reference data on the physical, mechanical and optical properties of LDPE, HDPE and PEG films. Footnotes indicate the standards or calculations used to determine a value if these are known to be different from those used here.

Material	Sample Info	Tensile Strength (MPa)		Yield Strength (MPa)		Elongation at Break (%)		Tear Propagation Force (N/mm)		Haze (%)	Gloss (%)	Coefficient of Friction	Reference
		MD[a]	TD[b]	MD	TD	MD	TD	MD	TD				
	Sclair 19A, 38µm	39	22	27	22	—		—		78	5	—	NOVA (2002)
	Sclair 19A, 25.4µm	50	36	—	28	700	400	—		—	—	—	Anon (1995)
	ρ=0.952–0.965	22–31		26–33		10–1200		—		—	—	—	Callister (1996)
HDPE	(no information)	17–35		—		300		—		—	—	—	Bakker (1986)
	High MW[c]	36	35	—		450	500	8[d]	58[d]	78	—	0.3[e]	Bakker (1986)
	Medium MW	32	30	—		350	550	6[d]	39[d]	78	—	0.3[e]	Bakker (1986)
	(no information)	22–31		26–33		—		—		—	—	—	Ellis (2000)
	70µm film	26–28[f]	17–21[f]	—		200–250	600	—		<8–<15	<10–<35[g]	—	Anon (1995)
	50µm film	16–24	13–20	—	9–12	250–450	540–600	—		<7–14	15 >30–>40[g] 52–60[h]	0.2–0.7	Anon (1995)
	40µm film	21–31	15–22	—	9–15	190–425	510–600	—		4–11	60–75[h]	<0.1–1.0	Anon (1995)
LDPE	30µm film	23–24	17–20	—	9–12	260–310	590–600	—		3.8–5	60–77[h]	0.2–0.6	Anon (1995)
	ρ = 0.917–0.932	8–32		9–15		100–650		—		—	—	—	Callister (1996)
	38µm ρ=0.921–0.923	19–20	18–21	—		—		34–62[d]	38–43[d]	—	—	—	Bakker (1986)
	(no information)	10–34	14–21	—	9–13	—		—		15	45-75[g] 10–100[h]	—	Ellis (2000)
PEG	(no information)	16	13	11.7		—		—		—	—	—	Ellis (2000)

[a] Machine Direction
[b] Transverse Direction
[c] molecular weight
[d] ASTM D1922: pendulum method for tear propagation
[e] face-to-face friction
[f] Ultimate Tensile Strength obtained using standard ISO 527.
[g] angle of measurement = 20°
[h] angle of measurement = 45°

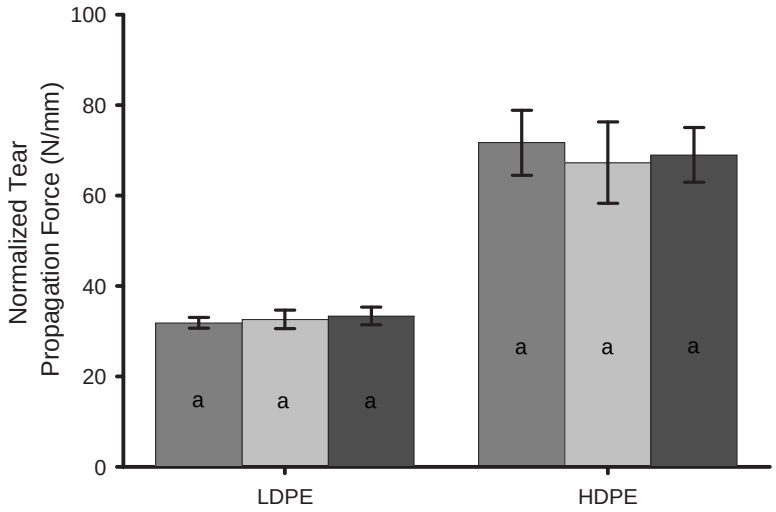


Figure 6.3: Normalized tear propagation force for modified and unmodified LDPE and HDPE films, as per ASTM method D1938–94 (n=6). Significant differences within each group of bars are indicated by the letters within the bars ($\alpha = 0.05$). ■ *PE*, ■ *Ox*, ■ *PEG*.

is slightly greater than normally found for LDPE films, but significant variability was found in these results, which may account for the difference. The low tensile strength of the films may also account for some of this difference, as films with lower tensile strengths often exhibit greater extensibility. For Tear Propagation, the measured value is less than the reference range, however different methodologies were used in the reference tests to that used here, so the difference is not surprising. The reference data were measured using a Pendulum tear test (ASTM D1922), whereas a "Trouser" tear test (ASTM D1938) was used here; when films are aligned in the Machine Direction, the Trouser Tear test measures tear propagation in the machine direction, whereas the Pendulum test actually measures tear propagation in the Transverse direction. This needs to be accounted for in comparing the results to the reference data.

For HDPE, no significant differences were found between the modified and unmodified films for Normalized Tear Propagation Force (70±4 N/mm). Some

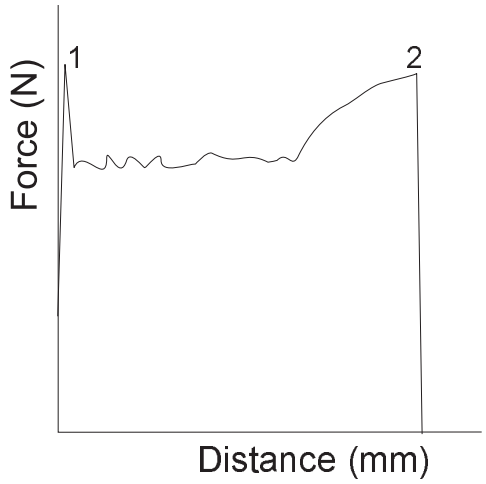


Figure 6.4: A typical tensile test force-distance curve for HDPE. 1: yield load; 2: failure load. Maximum tensile load is the absolute maximum of the curve, which may be either 1, or 2, or a point in between.

statistically significant differences were found in the other tensile properties, however. For Tensile Strength and Yield Strength, *Ox* (25.2±1.7 MPa for both) was significantly different to *PEG* (27.5±1.1 MPa and 27.4±1.0, respectively), although neither were significantly different from the unmodified HDPE (26.5±1.1 MPa for both). It needs to be noted that, for many of the individual films tested, the maximum tensile strength and the yield strength were one and the same: the maximum tensile strength was taken from the absolute maximum of the force-distance plot, which for HDPE often corresponded to the yield point, the initial maximum on the plot (see Figure 6.4). It is, therefore, to be expected that if either of these properties shows significant differences, then the other will as well.

The pattern of significant differences for Elongation is similar to that of Yield and Tensile strengths, with *PEG* significantly different to both *PE* and *Ox* and these last not significantly different from each other. The direction of change of the values of Elongation is the inverse of Tensile and Yield Strengths, as expected, with *PEG* showing less Elongation than *Ox* or *PE*; for Tensile and Yield Strengths,

PEG had higher strength values than *Ox*.

The slight increase in the tensile and yield strengths between the oxidized and PEGylated films may be explained from the surface topographies and layered structures of these films. First, consider the surface topography: a rough surface could lead to a reduction in film strength by providing more sites for tear initiation (sharp concavities where the stress will concentrate) than would a smooth surface. The greater the number of initiation sites, the greater the probability that the film will tear at a lower load. Additionally, the sharper the concavities, the greater the stress concentration and the increased probability of tear initiation. Considering the films examined here and comparing the tensile data to the surface topography (AFM analysis, §4.6), it is clear that the oxidized surface is both weaker and significantly rougher, with an increased number of sharper peaks, and assumably sharper valleys, than the PEGylated film (pre-washing). From the tensile data, it appears that the oxidized film is slightly weaker (lower tensile and yield strengths) than the untreated film, and that the untreated film is slightly weaker than the PEGylated film, although these differences are not statistically significant. These trends match the topographical trends shown in the AFM analysis of the film surfaces.

Although surface roughness is one plausible explanation of the differences in film strengths, the microstructure of the films should also be considered. The important concept here is effect of layering on film strength and the layering present in the PEGylated films, which have a PE core coated on both sides with a thin layer of PEG. If a film is coated with complete layers of a material with differing tensile properties, then the overall properties of the film may be effected, even if the coating is thin. The important considerations are the strength of the coating

layers and the ease of tear initiation in these layers compared to the bulk of the film. Given that PEG films are typically weaker[1] than PE films, PEG coating will not increase the strength of PE films by tensile reinforcement. If tear initiation is more difficult in PEG than in PE, however, then the PEG coating could lead to stronger films. No data on this was available.

Comparing the measured HDPE data to reference values, for all treatments we see that the measured values for Tensile and Yield strengths are similar to the reference data, regardless of significant differences between treatments. The measured Elongation at Break is slightly higher than the reference range, however the differences are not considered significant; as for LDPE these differences are most likely related to the high variability and relatively low tensile strength of the test films. Even when there are differences between the modified and unmodified films, these differences are not significant compared to the normal range of HDPE properties and should not affect the application of the modified films. The differences may, however, need to be accounted for during package and process design. As for LDPE, the measured Tear Propagation Force does not fall within the range of the reference data; again, this is most likely related to the differing measurement techniques used.

6.1.3 Coefficients of Friction

Coefficients of friction are important in processing situations when webs of film are being run through and over equipment. The static coefficient of friction is a measure of the force that must be overcome to start a stationary film moving over a surface, so is important for process start-up. The kinetic coefficient of

[1] personal observation

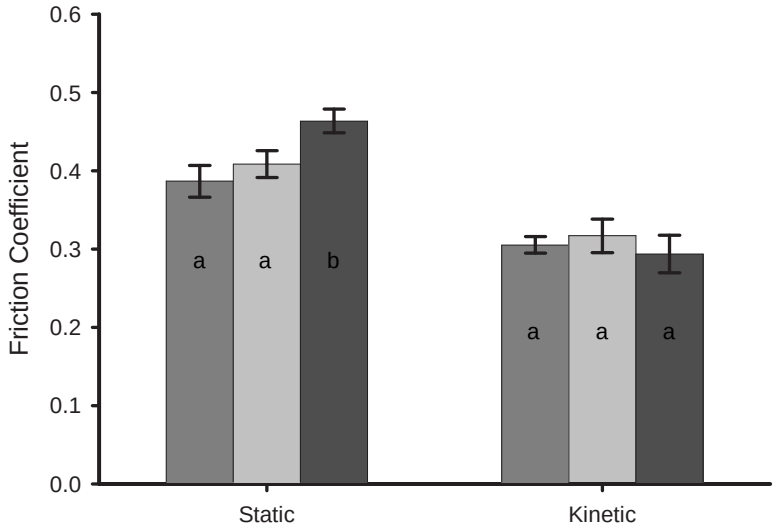


Figure 6.5: Static and kinetic coefficients of friction for modified and unmodified LDPE films, as per ASTM method D1894–01 (n=5). Significant differences within each group of bars are indicated by the letters within the bars ($\alpha = 0.05$). ■ *PE*, ■ *Ox*, ■ *PEG*.

friction is a measure of the force that must be overcome to keep a film moving over surface once it is already moving, so is important while running film processing plants. Friction properties are also important in finished packages when stacking or handling the packages, although this is only important for the outer layer of the packaging film. Given that the target of the bioactive films is the food contained in the package and the additional cost of these materials, they are unlikely to be used as an external layer on a food package, so friction considerations relating to finished packages are unlikely to be of concern. Coefficients of friction were only measured for LDPE films and are considered surface sensitive; it was hypothesized that the film treatments would effect the coefficients of friction.

There was no significant difference between treated and untreated films for the kinetic coefficient of friction (Figure 6.5), and these values also agree with the reference data (Table 6.3). The static coefficients of friction, however, were sig-

nificantly different for the different film treatments, although the measured values were all within the range of the reference data[2].

Coefficients of friction are affected by surface roughness and the material (e.g. PE or PEG) of the surface layer; environmental variables are controlled in the test protocol. There are significant differences in the surface roughness of the treated films (see §4.6) and the PEGylated film has an external PEG layer[3], so there was expected to be differences in the coefficients of friction for the variously treated films.

The lack of difference in the kinetic coefficient of friction between the native and PEG treated films is of some concern, as this could be explained by abrasion or deformation (Kato et al., 2003) of the PEG layer due to the shear forces developed in sliding the film over the test surface. Any loss of or damage to the PEG layer could also lead to loss of an attached bioactive compound and a reduction in film bioactivity. This needs to be further investigated before commercialization of the modified films.

6.2 Optical Properties

Optical properties are affected by interfaces between materials with differing refractive indices, variations in surface roughness and the existence of irregularities or inhomogeneities in the film greater in size than the wavelength of visible light (Robertson, 1993; Jenkins and Harrington, 1991; Briston, 1983). PEG has a different refractive index to PE, so the PEG layer is likely to alter the optical properties

[2]no distinction was made between static and kinetic coefficients in the reference literature

[3]although no data was found for the friction properties of PEG, it is anticipated that these are different to those of PE.

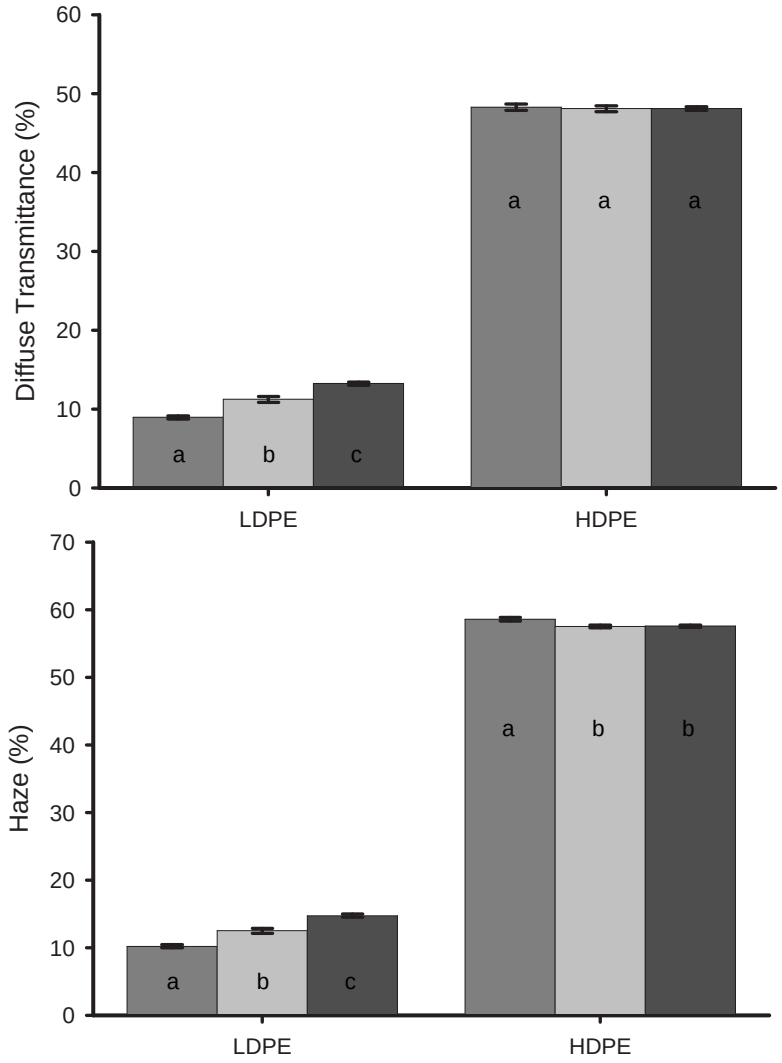


Figure 6.6: Diffuse Transmittance and Haze of modified and unmodified LDPE and HDPE films, as per ASTM method D1003–00 (n=3). Significant differences within each group of bars are indicated by the letters within the bars ($\alpha = 0.05$). ■ *PE*, ■ *Ox*, ■ *PEG*.

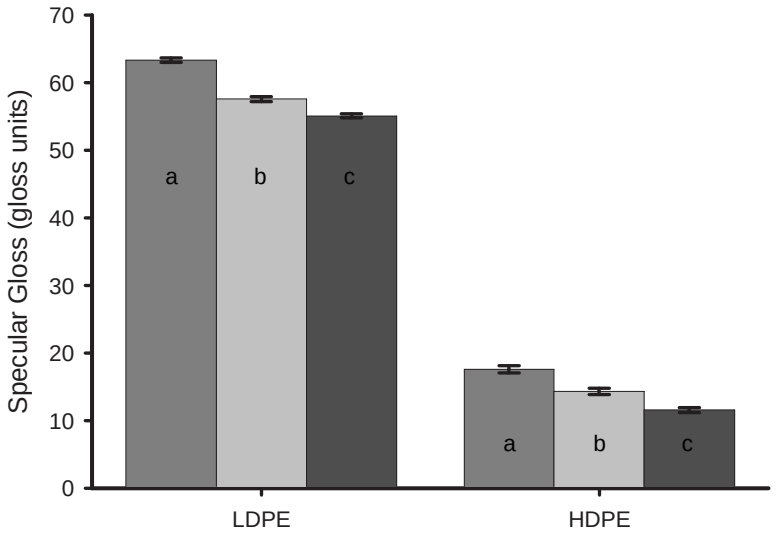


Figure 6.7: Specular gloss of modified and unmodified LDPE and HDPE films, as per ASTM methods D523–89(99) and D2457–97 (n=3). Significant differences within each group of bars are indicated by the letters within the bars ($\alpha = 0.05$). ■ *PE*, ■ *Ox*, ■ *PEG*.

of the film; surface modifications also had a significant effect on surface roughness (§4.6), so it was expected that film optical properties would be affected by the modifications. This was indeed the case (Figures 6.6 and 6.7).

Haze is defined (American Society of Testing and Materials, 2000) as the percentage of transmitted light scattered by more than 2.5° from the path of the incident beam and can be thought of as the degree of milkiness or cloudiness in a film (Briston, 1983). Diffuse transmittance is the ratio of the incident and diffusely transmitted fluxes (Anon, 2004b). Gloss is defined as "*the degree to which a surface simulates a perfect mirror in its capacity to reflect incident light*" (Robertson, 1993) and is the ratio of amount of light incident to the surface at a particular angle to that which is reflected at the same angle.

For LDPE there were significant differences between treatments for all these properties. This was not, however the case for HDPE: there were no significant

differences found in Diffuse Transmittance between the different film treatments and there was no significant difference in Haze between oxidized and PEGylated films. The lack of differences for HDPE, while the expected differences were found for LDPE, can be related to the initial states of the two materials. Unmodified HDPE is a hazy, somewhat opaque film, whereas LDPE is relatively clear. For HDPE, the slight change caused by the modifications is insignificant compared to the initial Haze of the films and, as such is undetectable.

Comparing the measured data to reference values (Table 6.3), it can be seen that the measured Haze is slightly below and the Gloss slightly above the reference values for HDPE; no reference values were found for Diffuse Transmittance. These HDPE reference values, however, are point estimates and do not provide ranges, yet these properties will vary depending on the molecular characteristics of the polymer and the method used to produce the films. The reference values are sufficiently similar to the measured values that the difference is not significant. The differences in Gloss and Haze found between the modified HDPE films are not apparent to the human eye.

For LDPE, the measured Haze values for both modified and unmodified films are within the range provided by the reference values, so are within the normal spectrum for LDPE films. As such, the increased Haze of the modified films is unlikely to be a problem in most applications, although it may be in issue in applications requiring exceptional clarity. It should be noted that the difference in Haze between the unmodified and oxidized films is apparent to the human eye, albeit barely.

The Gloss values are more difficult to evaluate as Gloss values depend on the angle at which they are measured, the value typically increasing as the angle in-

creases. Most of the literature values were measured at lower angles than used here. Extrapolating from these lower angle measurements, it appears that the measured values are comparable to reference values. The differences in Gloss between the modified and unmodified LDPE films was not visually apparent.

6.3 Moisture Permeability

Moisture permeation through a polymer film consists of first sorption of the permeant on one side of the polymer film, then diffusion through the polymer and finally desorption of the permeant from the opposite side of the film (Krochta, 2003). Diffusion is a bulk property and will not be affected by surface modifications. Sorption is defined as the initial penetration and dispersal of the permeant into the polymer matrix (Robertson, 1993) and is surface-sensitive, so will be affected by chemical changes at the film surface. As seen previously from contact angle analysis (Table 4.1), the surface hydrophobicity is altered by the film treatments; as surface hydrophobicity decreases, moisture sorption to the surface will increase, leading to increases in moisture permeability.

As expected, the hydrophilic modified films showed statistically significant increases in WVTR compared to the hydrophobic unmodified LDPE film (Table 6.4). Similarly, the PEGylated film (pre-washing) had a higher WVTR and lower CA than the washed PEGylated films, although these differences were not statistically significant for either CA or WVTR. The unexpected result was that the WVTR of the oxidized film was higher than the WVTRs of the PEGylated films, even though the contact angles of the PEGylated films was lower than that of the oxidized film.

The difference in transmission rates between PEGylated films and oxidized films may relate to moisture diffusion through the PEG layer. No data was found

Table 6.4: Comparison of contact angles and measured water vapor transmission rates for modified and unmodified LDPE films. Standard errors are given in parentheses following the measured values. Significant differences ($\alpha = 0.05$) are indicated by the letters following the data. WVTR test conditions were 35℃ and 90% relative humidity (RH); contact angles were measured at room temperature using 0.5µl drops of distilled water. N/A = not applicable.

Film	Contact Angle	WVTR (mg/hr)	WVTR (g·mm/m^2·d)
LDPE	101 (3)a	0.22 (0.03)a	1.3 (0.2)
Ox	61 (2)b	0.45 (0.03)b	2.7 (0.2)
PEG	45 (1)c	0.39 (0.03)c	2.4 (0.2)
Wash	47 (1)c	0.29 (0.03)c	1.8 (0.1)
Foil	N/A	0.23 (0.03)a	0.38 (0.05)
Control	N/A	−0.03 (0.02)d	N/A

Table 6.5: Reference WVTR data for LDPE.

Reference WVTR (g·mm/m^2·day)	Test Conditions	Reference
0.26–0.59	unknown	Anon (1995)
0.37–0.5	38℃, 90%RH	Bakker (1986)

relating to the permeability of water through PEG films, however, PEG is a polar, water soluble polymer; the affinity of water molecules for the polar PEG chains may reduce the diffusivity of water through an immobilized PEG matrix. If the PEG coating has a lower water diffusivity than the bulk LDPE, then the presence of PEG coatings on both sides of the PE in PEGylated films could reduce the moisture permeability compared to the (pure PE) oxidized films. The higher permeability of the PEGylated films compared to the unmodified PE does not preclude this; it would indicate that the increased sorption of the PEGylated films dominates over the decreased diffusion through the PEG coating.

The measured WVTR of the unmodified film was significantly higher than the reference data for LDPE (Table 6.5). This is likely related to differences in the methods used: a modified method was used in this work to allow for smaller film samples. In the modified method, the sample was sealed across two 5/8 inch (15.9 mm) diameter holes in a metal can lid using silicon sealant. The metal can lid was then sealed to the test dish (containing desiccant) using paraffin wax. The *Control* sample was an intact metal lid similarly sealed to a sample dish. The rate of weight change of the *Control* sample[4] indicates that there was negligible moisture permeation through the wax.

A second control (*Foil*)was used where a sample of aluminum foil was prepared identically to the polymer films. The significant WVTR recorded for *Foil* (theoretically the value for foil should approach be zero) indicates that there is moisture permeation through the silicon sealant sealing the films to the lid[5]. The measured WVTR of the foil could also be due to pinholes present in the test foil — pinholes are common in foils thinner than 25.4 µm (Robertson, 1993) — however, given a

[4]not significantly different from zero

[5]early trials found problems using silicon sealant to seal the lid to the test dish

foil thickness of 27.9 µm, pinholes are unlikely to be present. Assuming that the moisture permeation measured for foil is due to permeation through the silicon, the WVTR for the unmodified LDPE film can be adjusted by this amount to compensate, yet this still yields a WVTR significantly greater than the reference data.

Permeation through the silicon from the edges of the films inwards to the holes in the can lid, as would be the case for the impermeable foil, is unlikely to be the sole cause of the difference. If moisture is assumed to permeate through the silicon from the edge of the film, then it can also permeate through the film into the silicon and from there into the test dish. The effective area of permeation for the film would then be significantly greater than the area of the holes (assumed as the area for permeation in the WVTR calculations). Moisture could permeate through the entire area of the film, although the rate of permeation would decrease as the distance from the holes in the lid increased. This is the most probable explanation of the differences between the measured WVTRs and the reference data. To avoid these uncertainties in future work, it is recommended that wax be used to seal the films to the metal lid. For improved precision, the film thickness of each sample should also be measured prior to testing.

The uncertainties just described prevent ready comparisons of the modified film WVTRs to the reference data. The numerical range of the measured data can also not be compared directly to the range of the reference data as the effect of the silicon permeability will vary depending on the WVTR of the film. A rough comparison can be made, however, using the ratios of the highest and lowest measured WVTRs ($2.7/1.3 = 2.1$) and the highest and lowest reference values ($0.59/0.26 = 2.3$)). Given that the ratio for the measured data is less than the

ratio for the reference data, it is thought unlikely that the higher WVTR induced by these surface modifications will be problematic in PE packaging applications of the modified films. The difference in WVTR of the modified films compared to unmodified films will, however, need to be accounted for during package design, particularly if PE is the main water barrier in the package.

Finally, a note on the statistical analysis. Using the SAS statistical package (SAS Institute Inc., Cary, NC) to properly control for covariates (Minitab was not able to do this), a statistically significant effect was found for the effect of time on the rate of the transmission; there was a slight reduction in the rate measured over the course of the experiment. This effect, however, was three orders of magnitude less than the effect of the different treatments, so will not have affected WVTR comparison between the different treatments. The most probable cause of the time effect was incomplete equilibration of the samples to a steady state before measuring the data used in the rate calculations.

6.4 Summary of Findings and Recommendations

The measured properties of the modified and unmodified LDPE and HDPE films are summarized in Table 6.6. For many properties, no changes were caused by the modification. Even when differences were observed, the properties remain within (or very close to) the normal range for PE film, as defined by the literature. Some of the altered properties of modified films will need to be accounted for in the package design process, but the changes should not affect the suitability of the modified films for the normal applications of PE.

Table 6.6: Summary of Film Property Data of the Modified Films. Values are followed (in brackets) by their standard deviations. Where significant differences exist for a property, between treatments within a material, they are indicated by letters after the values. A dash (—) indicates the property was not measured for that film/treatment combination.

Material & Treatment		T_m (°C)	Tensile Strength (MPa)	Yield Strength (MPa)	Elongation at Break (%)	Tear Propagation Force (N/mm)	Haze (%)	Diffuse Transmittance (%)	Specular Gloss (%)	Coefficients of Friction		WVTR ($\frac{g \cdot mm}{day \cdot m^2}$)
										Static	Kinetic	
	PE	—	26.5 (1.1)ab	26.5 (1.1)ab	910 (70)a	71.8 (7.2)	58.6 (0.3)a	48.3 (0.4)	17.6 (0.5)a	—	—	—
HDPE	Ox	—	25.2 (1.7)a	25.2 (1.7)a	890 (60)a	67.3 (9.0)	57.6 (0.3)b	48.1 (0.4)	14.3 (0.5)b	—	—	—
	PEG	—	27.5 (1.1)b	27.4 (1.0)b	760 (120)b	69.1 (6.1)	57.6 (0.2)b	48.1 (0.2)	11.6 (0.4)c	—	—	—
	PE	110.6 (0.2)	12.7 (0.9)	10.2 (0.3)	540 (90)	31.9 (1.2)	10.2 (0.2)a	9.0 (0.2)a	63.4 (0.3)a	0.39 (0.02)a	0.31 (0.01)	1.3 (0.2)a
LDPE	Ox	110.8 (0.5)	12.7 (1.4)	10.3 (0.5)	500 (210)	32.7 (2.0)	12.5 (0.4)b	11.2 (0.4)b	57.6 (0.4)b	0.41 (0.02)a	0.32 (0.02)	2.7 (0.2)b
	PEG	111.1 (0.7)	12.5 (1.0)	10.2 (0.6)	560 (70)	33.4 (2.0)	14.8 (0.2)c	13.2 (0.2)c	55.1 (0.3)c	0.46 (0.02)b	0.29 (0.02)	2.4 (0.2)c

Chapter 7

Conclusions and Recommendations

- An antibacterial LDPE film was produced. Antibacterial activity was achieved by immobilization of side-chain protected peptide E14LKK on the surface of a PEG-treated, oxidized LDPE film, followed by peptide deprotection.

- For many film properties, no differences were observed between the modified and unmodified films. Where differences were observed, the properties remain within (or very close to) the normal range associated with PE film.

- Where properties were changed by the modification, the changes will need to be accounted for in the package design process, but are unlikely to affect the suitability of the modified films for the normal applications of PE.

- Lactase immobilized directly to LDPE, without a PEG spacer, was not active.

- Although oxidized-poly(ethylene) was successfully modified, the use of a functional graft polymer, such as poly(acrylic acid) or poly(lactic acid), attached to the poly(ethylene) surface should be explored as a method of increasing the number of available grafting sites on the film surface. The use of these functional polymers as independent films should also be investigated.

- Attachment of amino-carboxy-PEG was not successful, despite the success with diamino-PEG. Further optimization of the coupling parameters for the heterobifunctional PEG is necessary. Initial avenues for investigation would be the use of buffered coupling solutions and variation of pH, temperature and salt levels. This optimization requires ready access to suitable analytical

equipment.

- The use of N-hydroxy succinimide in the WSC activation solution should be explored as it shows potential to improve the coupling yield.
- A solution-based antimicrobial assay was effective at demonstrating antimicrobial activity; it models use of the modified films with liquid food products.

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