Optical Meets Mechanical: Use of Luminescence Spectroscopy To Assess Ageing in Biodegradable Films

Louis Colaruotolo

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OPTICAL MEETS MECHANICAL: USE OF LUMINESCENCE SPECTROSCOPY TO ASSESS AGEING IN BIODEGRADABLE FILMS

A Thesis Presented

by

LOUIS A. COLARUOTOLO

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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Food Science
OPTICAL MEETS MECHANICAL: USE OF LUMINESCENCE SPECTROSCOPY TO ASSESS AGEING IN BIODEGRADABLE FILMS

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With the growing concern of the accumulation of plastic-based food packaging waste, the search for bio-based biodegradable packages is on the rise. These materials differ from their petro-based counterparts in their degradation rates, which are much higher in the former. Not only do bio-based biodegradable materials degrade faster during post-usage processes but also they age faster during usage and storage, which affects their performance and functionality. The application of noninvasive testing methods with the capability to report on the matrix’s state could assist in the development of a more ubiquitous way to assess ageing in food packaging, particularly in biodegradable ones. To this end, the performance of a luminescence spectroscopy technique based on three luminescent probes, one intrinsic to the matrix and two added, was monitored and the sensitivity of the probes to report on ageing was analyzed.

Biodegradable films were made of 2% gelatin (type A) and 0.5% glycerol (plasticizer). Gelatin contains an intrinsic fluorophore, the aromatic amino acid tyrosine (Tyr), which can report on the molecular mobility of a matrix. Additionally, the films were doped with two extrinsic fluorophores, Fast Green FCF (FG) at 0.124 mM and
pyranine (Pyr) at 0.05 mM, which can report on the physical state and available free water within a matrix, respectively. Films were casted onto plastic Petri dishes and stored at five relative humidities (RHs), namely 2.5, 25, 33, 53, and 75%, for five weeks with measurement collection every week. Films were tested using fluorescence spectroscopy at excitation and emission range wavelengths optimized depending on the assessed probe. Additional measurements to determine moisture content, changes in secondary protein structure using FTIR spectroscopy, and mechanical properties using a Universal Testing Machine in tensile mode aided in the evaluation of the sensitivity of the luminescent probes in sensing ageing.

Luminescent probes, intrinsic or added, have the capability to assess the physical state of the films in situ and can provide molecular level sensing of their local environment. Tyr emission showed a sharp increase in fluorescence intensity in films stored at low RH as a function of time. FG showed a similar pattern to that of Tyr but higher sensitivity to changes along the observed period. The two characteristics emission bands of Pyr provide information on the state of water within the matrix. Although the results on this probe hinted microstructural rearrangements within the films as a function of time, the sensitivity of this probe was not high enough at the conditions evaluated and provided limited information on films’ solvation. The sensitivity of the luminescent probes to changes during ageing were revealed through correlation of the photophysical properties of the two effective probes, Tyr and FG, and the mechanical properties of the films at different RH through storage. Both methods, mechanical and optical, were similarly sensitive to changes during ageing particularly after 3-week storage. However, it can be speculated that because of the different scales at which optical and mechanical
measurements report (local vs. bulk), the methods, they could also complement each other. These findings suggest that, in principle, a luminescence spectroscopy technique using intrinsic and extrinsic probes can replace mechanical testing to noninvasively monitor structural changes and stability of biodegradable packaging as a function of time.
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CHAPTER 1
INTRODUCTION

Food packaging significantly contributes to the buildup of waste in the environment. Of particular concern is petro-based plastic packaging due to its persistence in landfills and water streams when not properly recycled\(^1\). Convenience, freshness, and safety of food products have extensively benefited from the utilization of petro-based plastic packaging\(^2\). However, environmental concerns and sustainability have fueled the search for bio-based biodegradable alternatives.

The development of biodegradable food films and edible coatings has been an area of interest dating back to the 1800s\(^3\). These films and coatings are mainly composed of renewable bio-based materials, and even organic waste or by-products have been incorporated in their composition as additives or main structural components\(^4-11\). Their purpose is to extend the shelf life of foods and provide protection from environmental factors\(^12, 13\). These films and coatings can be either consumed with the products or are biodegradable. Thus, their impact on the environment post-usage is minimized\(^9\).

The interest in biodegradable films for food applications has continuously increased since the 1960s\(^14\), becoming an extremely active area of research in the last two decades\(^6, 7, 9, 15\). Although these alternative materials have been studied and optimized for many years, they are seldom seen in grocery store shelves, due to some challenges presented in their usage. Among their shortcomings, their inability to fully match petro-based plastics’ functionality and their relatively short shelf life have hindered their widespread distribution in the food supply chain\(^6\). Although both petro- and bio-based food films exhibit changes in physical properties, structure, and functionality with time
due to a phenomenon called “ageing”\textsuperscript{16}, the deterioration rate of the latter is normally higher. The comparatively fast deterioration of the alternative materials also can impact the overall shelf life of the food product that they contain, normally reducing its stability. Therefore, before a biodegradable food film is deemed acceptable for use in retail products, the kinetics of the film’s deterioration processes during storage should be fully mapped to evaluate and/or predict its potential effects on the safety and shelf life of a food product\textsuperscript{12,17}.

Understanding ageing in food films requires assessing changes in their most relevant attributes through time. Typically, the changes in the mechanical properties of the films, e.g., tensile strength, elongation, Young's modulus, are used as indicators of a film’s stability. These properties are commonly evaluated using instruments such as Universal Testing Machines (UTM)\textsuperscript{18}. There are several drawbacks associated with this approach; a) it requires the acquisition of specific equipment (UTMs), which can be expensive and in some case prohibited depending on the capabilities of a film’s manufacturer, b) the complex geometry of the equipment’s attachments sometimes does not provide a proper characterization of the mechanical properties and produces empirical results that can only be used for comparison\textsuperscript{19}, c) the variability among measurements can be high, e.g., commonly > 20-30\textsuperscript{o}\textsuperscript{20}, d) the tests are destructive, and in some cases the methodology is material-specific, and e) the measurements can seldom be performed \textit{in situ}\textsuperscript{21}. Therefore, identifying novel, simple, and more sensitive methods to assess ageing in packaging materials could help overcome the challenges presented, while also accelerating and facilitating the development of new bio-based biodegradable films.
Luminescence spectroscopy has been identified as a useful technique to assess changes in the physical properties in a variety of food matrices. The single molecule sensitivity of this technique combined with environmentally responsive probes can detect small structural changes in films as a function of storage time. In principle, luminescent probes, intrinsic (i.e., native to the film matrix) or extrinsic (i.e., purposely added) can report on the local environment of the films. Thus, luminescence spectroscopy can provide an effective way to monitor changes as a function of time, such as those observed during ageing.

These techniques can become feasible, in situ, cost-effective tools to assess the mechanical properties of bio-based food films, and even petro-based ones. Moreover, they can be applied to intact films allowing for continuous measurements in real-time of structural properties during storage. New tools and procedures for monitoring the structural integrity of food films could contribute to make bio-based biodegradable films a more common type of food packaging. Not only can optical monitoring tools aid in the development and continuous assessment of ageing but they can also be operationalized as real-time sensors embedded in packaging to monitor integrity from production to post handling steps (controlled decomposition).

In the next chapters, this document briefly summarizes the state of the art on bio-based biodegradable food films, highlighting the ageing process and how it can be monitored and quantified (Chapter two). The third chapter details the procedures, including materials and methodology, used to assess the challenges, opportunities, and limitations of using optical tools as potential replacements for mechanical methods. In particular, luminescence spectroscopy is evaluated as an alternative to assess structural
changes in bio-based films over time and the effect of relative humidity on the ageing process. The fourth chapter discusses the results obtained and provides a comparison of optical and mechanical methods as tools to monitor the film’s ageing. The fifth chapter concludes this thesis with a summary of findings presented and discusses future work.

1.1 Objectives

This study aims to evaluate the feasibility of using optical techniques, particularly luminescence spectroscopy, to monitor changes in biodegradable films during storage. To this end, the following specific objectives have been set forward as milestones in this study:

1) To evaluate and compare the efficacy of environmentally sensitive intrinsic and extrinsic luminescence probes as potential sensors of ageing in gelatin-based biodegradable films. Completion of this objective will establish the potential applicability of luminescence-based techniques in sensing structural changes in films as a function of storage.

2) To gain insights on the ageing processes that the luminescent probes report upon. This will help to establish: a) to what extent the luminescent probes are sensitive to changes during aging, and b) if the changes in luminescent properties of the films could be related to specific changes in the matrix, e.g., the secondary structure of the gelatin within the films.

3) To establish potential correlations between optical and mechanical measurements and reveal if the techniques could be interchangeable or complementary.
CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Packaging has evolved through time to better protect and preserve food during storage. The use of petro-based packaging materials has indeed increased food quality, safety, variety, and convenience. However, its widespread presence throughout the food supply and poorly planned disposal have extensively contributed to environmental pollution. Petro-based plastics can be reprocessed into new products through recycling, but the amount reprocessed is not nearly as high as the plastics consumed. The 2015 report from the Environmental Protection Agency (EPA) claims that only 9.1% of the total amount plastics generated are effectively recycled and repurposed\textsuperscript{30}, this proportion is even lower for selected materials such as polyisoprene. Also, it is estimated that two-thirds of all packaging waste is food-related\textsuperscript{31}. The use of plastics for food applications has taken a toll in the environment and has become a topic of constant concern in recent news\textsuperscript{32}. One potential solution to this problem is the development of alternatives, i.e., biodegradable films, that are just as functional as conventional petro-based packaging while having minimal impact on the environment.

Bio-based biodegradable films are composed of one or several biomaterials typically obtained from agricultural products or produced through bacterial fermentation. These films are naturally biodegradable, so with time, they break down to simple carbohydrates and proteins that can be repurposed, for example by composting, with limited detriment to the environment. Their limited stability is beneficial from an
environmental standpoint, but potentially problematic from a food safety and food quality perspective, since the fast degradation of these materials can result in packaging that provides less protection towards re-contamination and environmental factors. Throughout their usage and shelf life, these films exhibit structural changes due to ageing. It should be noted, however, that petro-based plastics also undergo deterioration throughout their life span, however, their deterioration rate is orders of magnitude slower (years vs. months) than that of bio-based materials. The shorter shelf life and stability of bio-based films limit their functionality, usage, and applications in food protection.

Assessing temporal changes in films, petro- or bio-based, is not a trivial task. First one must consider the material of which the films are made of. Different sources have been utilized to produce biodegradable films and can be broadly categorized into carbohydrate, protein, and synthetic-based (e.g., poly(ethylene-co-methyl-acrylate-co-glycidyl-methacrylate), each of them with distinct characteristics, deterioration and break down mechanisms. Since the type of material affects their properties and functionality, the need for adequate tests that can provide insights on a film’s structure, mechanical and barrier properties, among others, is evident.

This chapter will briefly summarize the materials used to produce biodegradable films, their main properties, and functionality. It should be noted, however, that the ageing process and the most frequent techniques used to monitor the deterioration in biodegradable food films along their shelf life will be the main focus of this chapter.
2.2 Biodegradable products: from food films to utensils

Traditional petro-based plastics and bio-based food films share, to some extent, a similar makeup; their matrix is formed by the polymerization of monomers from a variety of sources. Films made from biomaterials that have a faster degradability than petro-based plastics are certainly not a recent scientific discovery. Biodegradability, the feasibility of a material to break down (decomposed) by microorganisms into its basic monomers is a process that can range greatly in time and is highly dependent on environmental conditions like temperature, relative humidity, pH, and the bacterial strain used35.

Bio-based food films can be separated into two main categories: a) films to replace petro-based packaging, and b) films to be applied directly onto food surfaces as edible coatings. Both applications play a role in food safety and extension of shelf life by providing protection and preventing postproduction contamination of food products. Biodegradable films will differ from edible food coatings in their application and preparation techniques. Edible coatings are typically manufactured using similar materials as films but are applied directly to food surfaces to act as a protective barrier between the food and the environment36. Both bio-based films and edible coatings shelter the food from environmental factors like moisture and oxygen, and act as a barrier from oils, gases, and vapors, while optionally carrying active substances like antioxidants, antimicrobials, coloring, and flavorings37. The thickness of these thin films is often within 10-100 μm38, and both their height and width are determined by the product needs or preparation methods.
Edible and biodegradable food films origins date back to the 1800s with the application of waxes to fruit surfaces to extend their shelf life\textsuperscript{39, 40}. In these early years, the films were typically edible coatings applied directly to foods and only in the 1950s corn-based films were introduced to contain and preserve meat and fish products\textsuperscript{14}. Since then, films made from numerous biomaterials have been developed and tailored to specific purposes.

Although not novel, the development of biodegradable films for food applications has received increasing attention lately due to pressure from consumers, nonprofit organizations, and governmental offices for environmentally conscious and sustainable options to conventional food packaging. The push towards eco-friendly packaging has been acknowledged by a few companies that have implemented innovations to address their packaging-related waste \textsuperscript{41, 42}. For example, PepsiCo and Coca-Cola have been developing biodegradable bottles made from natural resources, and Frito Lays unveiled a noisy but 100\% compostable chip bag in 2009\textsuperscript{43}. Additionally, the expansion of novel biodegradable items for food service has increased in recent years. Alternatives to disposable utensils, plates, cups, and straws for food service are constantly being sought\textsuperscript{44}. For example, companies like Bioenvelop, EarthShell Corp., and Metabolix have been forerunners in the production and distribution of biodegradable products for food service\textsuperscript{45}. Films in the food service industry are also widespread due to their convenience and disposability and have been proven more difficult to replace than other items.

2.2.1: Biodegradability of bio-based films: standards, regulations and trends

A major advantage of using biomaterials for the production of food packaging is their potential low impact on the environment. The ambiguity of the terms biodegradable,
bioplastic, and compostable, usually leads to “green-washing.” Green-washing refers to the misleading use of vague terms or labels (e.g., biodegradable, compostable, etc.) often by manufacturers to advertise the potential environmental impact of their products. To avoid misleading and confusion, several standards and regulations have been put in place to ensure that biodegradable materials launch into the market can be disintegrated or decomposed effectively.

Several countries use commonly established rates of disintegration and biodegradation. For example, the European Parliament requires that biodegradable plastics achieved “at least 90% disintegration after 84 days in composting facilities” and “at least 90% biodegradation in 180 days in composting facilities leaving no visible or toxic residues.” These guidelines inform the standard for biodegradability of food films. Regulations for plastic-based packaging on specifications, disposal, and even sustainability are covered in several documents from the International Organization for Standardization (ISO). Guidelines by ISO provide information on conducting Life Cycle Assessment (LCA) tests on packaging material from production to disposal. The LCA system makes it possible to identify which stages in the life cycle contribute the most to environmental pollution. It is suggested that these ISO’s could be applied to all types of new packaging when conducting LCA’s on biodegradable food packaging before their adoption by the food industry. In addition to LCA guidelines, standards for testing new packaging, including mechanical and physical properties to degradation rates, are available. Standard methodologies published by the American Society for Testing Materials (ASTM) provide film manufacturers with appropriate methods to assess the biodegradability of new films with testing parameters and conditions, reducing the
burden of method development from a manufacturing aspect. Currently, the United States of America uses ASTM D5338-92 and ASTM D6868-17 to determine proper disintegration and biodegradation of materials intended for aerobic composting. Specifically ASTM D6868-17 states proper disintegration (90% of the original weight of the waste) must occur within 84 days, and Biodegradation within 180 days, with the clause that no negative impacts are observed on the resulting compost. A summary of the main guidelines that are normally applied to guide the development and manufacturing of biodegradable packaging materials are summarized in Table 2.4.

<table>
<thead>
<tr>
<th>Standard (Ref. Number)</th>
<th>Topic</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISO 14041</td>
<td>Life Cycle Inventory of BD films</td>
<td>Catalog the resources inputs and outputs during BD films production</td>
</tr>
<tr>
<td>ISO 14042</td>
<td>Life Cycle Impact Assessment of BD films</td>
<td>Classify and evaluate the impact of the resources identified using ISO 14041</td>
</tr>
<tr>
<td>ISO 14043</td>
<td>Life Cycle Interpretation of BD films</td>
<td>Provide value assessments of ISO 14042</td>
</tr>
<tr>
<td>ASTM D882-18</td>
<td>Tensile Properties of Thin Plastic Sheets</td>
<td>Provide guidelines to test the mechanical properties of thin film</td>
</tr>
<tr>
<td>ASTM D5247-92</td>
<td>Aerobic Biodegradability of Plastics</td>
<td>Suitability and requirements for microbial decomposition of degradable plastics</td>
</tr>
<tr>
<td>ASTM 5338-92</td>
<td>Aerobic Degradation of Plastics during Composting</td>
<td>Suitability and requirements for composting degradable plastics</td>
</tr>
<tr>
<td>ASTM D5272-92</td>
<td>Outdoor exposure testing of photodegradable plastics</td>
<td>Assessment of degradation of light-sensitive plastics</td>
</tr>
<tr>
<td>ASTM D6868-17</td>
<td>Labeling of end items designed to be aerobically composted</td>
<td>Provide standards of disintegration and biodegradation</td>
</tr>
</tbody>
</table>

Several trends and priorities have informed the development of novel packaging during the last decades, as outlined by Han and others (2014) and summarized in Table 2.2. The current decade has been characterized by an increasing interest in reducing
carbon footprint. However, the amount of biodegradable food films found on retailer’s shelves is limited. The lack of biodegradable packaging used is deterred by the lower price and greater stability of non-biodegradable petro-based polymers, and the high cost of infrastructure needed to effectively collect and compost biodegradable packaging\textsuperscript{48}. But as public pressure moves towards decreasing single-use plastics, the replacement of current packaging by environmentally friendly alternatives is expected.

Table 2.2: Priorities that informed the development of food packaging in the last 60 years

<table>
<thead>
<tr>
<th>Period</th>
<th>Function and Issues</th>
</tr>
</thead>
<tbody>
<tr>
<td>1960's</td>
<td>Convenience, point-of-purchase marketing</td>
</tr>
<tr>
<td>1970's</td>
<td>Lightweight, source reduction, energy saving</td>
</tr>
<tr>
<td>1980's</td>
<td>Safety, evidence of tampering</td>
</tr>
<tr>
<td>1990's</td>
<td>Environmental impact (solid waste)</td>
</tr>
<tr>
<td>2000's</td>
<td>Safety and security</td>
</tr>
<tr>
<td>2010's</td>
<td>Carbon footprint reduction</td>
</tr>
</tbody>
</table>

Source: Innovations in Food Packaging, 2\textsuperscript{nd} edition\textsuperscript{9}.

As in the case of traditional plastic-based films, biodegradable food film’s composition and mechanical properties must be appropriate for their intended use and application. Food packaging serves many purposes. The most important ones are protection, communication, convenience, and containment\textsuperscript{49, 50, 51}. In 1985, Yokoyama and others outlined the most relevant requirements of effective food packaging; ability to be mass produced, suitability in structure and form, convenience, and low environmental impact of its disposal\textsuperscript{9}. Regardless of the type of packaging used, these requirements apply for all. The utilization of bio-based materials as packaging poses extra challenges, such as their inherent lower stability. Additionally, when transitioning from petro-based packaging to bio-based films, it is critical to assess how a product’s properties and functionality will be affected by compositional and structural changes.
Maintaining the quality and safety of food is the main priority in the development of bio-based films. Therefore, it is important to hold up novel food films to, at least, the standards of traditional packaging. Developing food films with good physical properties, i.e., mechanical and barrier, thermal and chemical attributes is critical to prevent spoilage of foods, regardless of the deterioration being caused by chemical, biochemical, or biological agents.

The introduction of novel functionality to food films gave rise to the production of intelligent coatings and packaging. These films can further improve quality, extend shelf-life, and even enhance safety by including, for example, antimicrobial agents. Smart films contain in their matrices, embedded or attached, sensors able to report on quality and safety attributes of the contained food. In addition to acting as barriers, smart packaging can provide the consumer with dynamic information about the food or the packaging itself, e.g., characteristics, stability, and integrity of packaging films (breakage, shrinkage, etc.). Therefore, the incorporation of sensors for integrity in bio-based packaging could elevate its functionality by actively reporting on its state.

2.2.2 Biomaterials and additives used in food packaging

Biomaterials used in food films are generally categorized as carbohydrate, protein, and copolymer, i.e., a single polymer made from two unique monomers,–based. Lipid-based bilayers and emulsions are excluded from this section as they are most typically applied as edible coatings rather than films. Biodegradable films and coatings are typically formed from several components: a primary polymer, plasticizer(s), and solvent(s). Protein-based films can be produced using animal (casein, whey, collagen, gelatin, albumen) or vegetal (soy globulin, wheat glutenin, and gliadin, corn zein)
sources. Carbohydrate-based films can be classified into gum-based (agar, alginate, carrageenan, gellan, pectins, chitosan) and starch-based (cross-linked, substituted such as methylcellulose, carboxymethylcellulose, partially hydrolyzed). Plasticizers are low molecular weight compounds that increase a film’s flexibility by decreasing the glass transition temperature ($T_g$) of the matrix\textsuperscript{53}. These small molecules confer plasticity to food films, which can significantly affect their mechanical and barrier properties.

The variety of biomaterials used in food packaging is constantly expanding as researchers search for alternatives to petro-based options. Table 2.2 provides examples of biodegradable food films within all the above-cited categories. Besides their composition, the added functionality provided by one or many of its components is reported in Table 2.3.
<table>
<thead>
<tr>
<th>Material Class</th>
<th>Main Component</th>
<th>Composition</th>
<th>Additives</th>
<th>Purpose of the study and potential added functionality</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>Starch</td>
<td>Pea/Potato/Cassava</td>
<td>Glycerol (% N/A)</td>
<td>Effect of composition on structural characteristics</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Corn HMC (hydroxymethylcellulose)</td>
<td>Glycerol (0.25% polymer-based)</td>
<td>Retarding of ageing</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Corn epsilon-poly-lysine</td>
<td>Glycerol (3% polymer based)</td>
<td>Antimicrobial activity</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wheat Lignin</td>
<td>Glycerol (1.2% polymer based)</td>
<td>Antioxidant activity</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cassava (77%) Chitosan (shellfish – 5%)</td>
<td>Glycerol (18% polymer based)</td>
<td>Antimicrobial activity</td>
<td>58</td>
</tr>
<tr>
<td>Gums</td>
<td>Xanthan gum (<em>Xanthomonas campestris</em>)</td>
<td>Glycerol (% N/A)</td>
<td>Effect of composition on structural characteristics</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Locust bean gum</td>
<td>Glycerol (% N/A)</td>
<td>Effect of composition on structural characteristics</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>Chitosan</td>
<td>Shellfish</td>
<td>Glycerol (0.3% total weight)</td>
<td>Antimicrobial activity</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shellfish</td>
<td>-</td>
<td>Effect of RH on stability</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Shellfish</td>
<td>Potato starch</td>
<td>Glycerol (25% polymer based)</td>
<td>Effect of composition on structural characteristics</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Shellfish</td>
<td>Poly Lactic Acid (Synthesis)</td>
<td>-</td>
<td>Effect of composition on structural characteristics</td>
<td>63</td>
</tr>
<tr>
<td>Material Class</td>
<td>Main Component</td>
<td>Composition Details &amp; Secondary Components</td>
<td>Additives</td>
<td>Purpose of the study and potential added functionality</td>
<td>Ref.</td>
</tr>
<tr>
<td>----------------</td>
<td>----------------</td>
<td>------------------------------------------</td>
<td>-----------</td>
<td>-----------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Proteins</td>
<td>Vegetal</td>
<td>Soy protein isolate, Corn zein</td>
<td>Glycerol (30%)</td>
<td>Antimicrobial activity</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soy protein isolate</td>
<td>Glycerol (30, 40, 50% polymer based)</td>
<td>Effect of plasticizers on stability during storage</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soy protein isolate</td>
<td>-</td>
<td>Effect of fabrication method (spinning) mechanical properties</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pea protein</td>
<td>Various glycols</td>
<td>Effect of composition on structural characteristics</td>
<td>67</td>
</tr>
<tr>
<td>Animal</td>
<td>β-casein (milk)</td>
<td>Glycerol (2.5% polymer based)</td>
<td></td>
<td>Effect of temperature on structural characteristics</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gelatin type A</td>
<td>Rutin, Glycerol (3% polymer based)</td>
<td>Antioxidant Activity</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myofibrillar fish protein (sardines)</td>
<td>Glycerol (35% polymer based)</td>
<td>Effect of film thickness on performance</td>
<td>38</td>
</tr>
<tr>
<td>Co-Polymers</td>
<td></td>
<td>Poly(ethylene-co-methyl-acrylate-cp-glycidyl-methacrylate) (PEMAGMA)</td>
<td>-</td>
<td>Ageing and biodegradability</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polyurethane</td>
<td>Catechin</td>
<td>Antioxidant Activity</td>
<td>70</td>
</tr>
<tr>
<td>Composites</td>
<td>Gelatin/Flour</td>
<td>Gelatin (Fish), Flour (Rice)</td>
<td>Glycerol (30% polymer based)</td>
<td>Effect of composition on stability during casting</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Gelatin/Pea</td>
<td>Gelatin (cod), Pea protein isolate</td>
<td>Glycerol (0.25%), Sorbitol (0.75%)</td>
<td>Effect of pea protein inclusion on structural characteristics</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>Polymers from organic acids</td>
<td>Poly(lactic acid), poly(3-hydroxybutyrate)</td>
<td>Oligomers of lactic acid (0-15%)</td>
<td>Antimicrobial Activity</td>
<td>73</td>
</tr>
</tbody>
</table>
Table 2.2 provides a glimpse into the variety of materials employed in the production of bio-based films to highlight the multifunctionality of some biodegradable food films. This table only shows some of the traits often incorporated into these materials, which range from antimicrobial and antioxidant activities to structural improvement.

Due to the increasing demand for active and smart packaging, biodegradable food films with added functionality are often developed. The incorporation of additives that can act as antioxidants or that exhibit antimicrobial activity enhance the performance and contribute to expanding the shelf life of foods. Studies that focus on the added functionality of biodegradable films help build a foundation for food films that go beyond the requirements of containment and protection. Increasing the functionality of food films through additives can increase the economic viability of biodegradable food films from a food manufacturer standpoint and may encourage the switch from traditional petro-plastics to bio-based ones.

2.2.3 Biodegradable films with antimicrobial activity

Packaging can actively prevent food spoilage through the addition of antimicrobial agents to the films to delay or inhibit microbial growth. In 1998, Padgett and others explored the use of antimicrobial agents, i.e., lysozyme and nisin, to hinder the growth of gram-positive bacteria\textsuperscript{64}. Since then, numerous antimicrobial agents, synthetic and natural, have been incorporated into film matrices and their antimicrobial activity, stability, and release kinetics have been monitored\textsuperscript{74-76}. The incorporation of essential oils or their active components has gained popularity to confer antimicrobial activity to
biodegradable films. For example, compounds like carvacrol, the predominant
antimicrobial agent in oregano essential oil, has been incorporated in several
biodegradable films\textsuperscript{73, 75}. Tampau and others (2018) introduced carvacrol in electro-spun
corn starch-sodium caseinate and poly-epsilon-caprolactone films. In this study, poly-
epsilon-caprolactone films exhibited a higher carvacrol encapsulation efficiency than
corn starch-sodium caseinate ones\textsuperscript{77}. Due to the higher carvacrol carrying capacity of the
poly-epsilon-caprolactone films, their antimicrobial activity also increased. The
antimicrobial activity of eugenol in whey protein/soy lecithin/ maltodextrin films with the
addition of oleic acid and/or chitosan was tested by performing an inoculation study with
\textit{L. innocua} and \textit{E. coli} \textsuperscript{74}. The addition of oleic acid and chitosan decreased the
antimicrobial activity as these components decreased the encapsulation efficiency of the
antimicrobial agent, i.e., eugenol.

Although antimicrobial compounds provide additional functionality to films, it is
critical to determine how the addition of these compounds may change the films’
structure. Zhang and others (2015) studied the incorporation of epsilon-poly-lysine, a
known antimicrobial agent, in starch-based films. This group reported no significant
impact on the mechanical properties of the film due to the incorporation of the poly-
lysine\textsuperscript{56}. Similarly, Pranoto and others (2004) monitored the mechanical properties of
chitosan films with the addition of garlic oil. Garlic oil did not significantly affect the
tensile strength at concentrations below 400 μL/gram of chitosan. Additionally, no
significant changes in film elongation for any of the concentrations tested was observed\textsuperscript{78}.
Cargi and others (2001) studied the mechanical properties of whey protein films with the
addition of sorbic acid at increasing concentrations. In contrast to the reports on garlic oil
and poly-lysine, as the sorbic acid concentration progressively increased from 0 to 1.0 (w/v %), the tensile strength of the films decreased\textsuperscript{79}. Therefore, when including additives with selected functionality in films, their effect on their structure and stability should be carefully monitored to avoid interactions among components that may weaken the performance of the film.

Antimicrobial activity can be attained not only by the addition of specific additives to a film matrix but also, by using biopolymers naturally bestowed of antimicrobial activity as main or secondary structural components. Particularly, the use of chitosan, derived from shellfish, as both a structural and antimicrobial component has received extensive attention during the last decade\textsuperscript{58, 60, 80, 81}. Chitosan’s structure provides the film with antimicrobial activity since the positively charged chitosan molecules interact with negatively charged bacteria disrupting the cell membrane and leading to the death of the microorganism\textsuperscript{82}. Aligned with the efforts of the multi-hurdle approach to food safety, researchers have combined chitosan-based films with active antimicrobial agents, like curcumin, and reported a synergistic antimicrobial effect\textsuperscript{83}. Biomaterials that simultaneously provide structure and antimicrobial or bacteriostatic activity produce multifunctional films, which in turn increases the value and viability of these films.

2.2.4 Biodegradable films with antioxidant activity

Packaging that can hinder deteriorative processes in foods such as oxidation has also become more prevalent through recent years\textsuperscript{10, 84}. The addition of antioxidants, particularly encapsulated or partially protected within the matrix consistently results in sustained and high antioxidant activity.
Lipid oxidation can be retarded by embedding antioxidants, e.g., metal chelating agents to the film matrix or crosslinking them to their internal surface\textsuperscript{85}. The addition of microparticles loaded with rutin, a plant-based flavonoid with antioxidant activity, to gelatin films was studied by Dammak and others (2017)\textsuperscript{69}. Rutin loaded microparticle films exhibited greater antioxidant activity than films doped with non-encapsulated rutin\textsuperscript{69}. Antioxidant-loaded electrospun chitosan/ poly(vinyl alcohol) films optimized for water vapor barrier and film thickness were studied to determine their release kinetics and antioxidant activity during storage. The films that had higher encapsulation efficiency, exhibited more antioxidant activity\textsuperscript{86}.

As in the case of chitosan, lignin has been explored as a multifunctional component. Acosta and others (2015) have reported that lignin improves the structural properties of films while also exhibiting antimicrobial activity. The antioxidant activity, and not surprisingly, the mechanical properties of the films depended on the lignin molecular weight, extraction procedure, and botanical sourcing. This study observed that as lignin concentration increased in the final film also did its antioxidant activity\textsuperscript{57}. Panzella and others (2019) developed a pre-processing method to increase the antioxidant activity of spent coffee grounds, which were used as a filler to stabilize films. Besides improving the mechanical properties of the film, the spent coffee grounds provided the films with antioxidant activity\textsuperscript{87}.

Studies not purposely designed to improve or confer antioxidant activity to films have revealed interesting findings. For example, catechin was added to polyurethane-based films to increase the film’s thermal stability. Not only did catechin significantly increase thermal stability, but it also improved the elongation without rupture during film
production\textsuperscript{70}, and provided antioxidant activity to the film. Also, the addition of antioxidants has often resulted in an increase in tensile strength. Green tea extract was added in squid gelatin films to increase their antioxidant activity. At concentrations above 10\% (polymer based), the green tea extracts unintendedly increased the tensile strength and percent elongation of the films\textsuperscript{88}.

As it has been presented in previous sections, the biomaterials and additives used in the production of a food film confer distinct properties (structural, mechanical, barrier) to the final product even among materials within a single category, e.g., carbohydrate, protein, or copolymer. To provide adequate background to this thesis, the mechanical properties of biodegradable materials from different sources are discussed in the following section.

2.3 Mechanical properties of biodegradable films

Biodegradable food films, much like traditional plastics, must be able to contain food without losing their integrity or falling apart throughout the different stages of the supply chain. Biodegradable films produced with biopolymers tend, in general, to exhibit weaker mechanical properties than conventional materials, particularly tensile strength and elongation (\%)\textsuperscript{5,9,39}. A comparison of the mechanical properties (tensile strength in MPa and elongation in \%) of biodegradable food films and popular petro-based films are presented in Table 2.4.
Table 2.4: Comparison of mechanical properties among and between film categories

<table>
<thead>
<tr>
<th>Class</th>
<th>Material</th>
<th>Additives</th>
<th>Tensile strength (MPa)</th>
<th>Elongation (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal</td>
<td>Calcium caseinate</td>
<td>Glycerol</td>
<td>1-10</td>
<td>&lt;1</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Whey protein isolate</td>
<td>Sorbitol</td>
<td>1-10</td>
<td>10-100</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Whey protein isolate</td>
<td>Glycerol</td>
<td>10-100</td>
<td>10-100</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Fish myofibrillar protein</td>
<td>Glycerol</td>
<td>10-100</td>
<td>10-100</td>
<td>91</td>
</tr>
<tr>
<td>Vegetable</td>
<td>Pea protein</td>
<td>Glycerol</td>
<td>1-10</td>
<td>10-100</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>Wheat gluten</td>
<td>Glycerol</td>
<td>1-10</td>
<td>&gt;100</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>Banana flour</td>
<td>Glycerol</td>
<td>10-100</td>
<td>1-10</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>Glycerol</td>
<td>10-100</td>
<td>1-10</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Cassava starch</td>
<td>Glycerol</td>
<td>10-100</td>
<td>1-10</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Rice starch</td>
<td>Glycerol</td>
<td>10-100</td>
<td>1-10</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Methylcellulose</td>
<td>n/a</td>
<td>&gt;100</td>
<td>1-10</td>
<td>95</td>
</tr>
<tr>
<td>Composite</td>
<td>Collagen/Cellulose</td>
<td>Glycerol</td>
<td>1-10</td>
<td>10-100</td>
<td>38</td>
</tr>
<tr>
<td>Synthetic</td>
<td>Polystyrene</td>
<td>n/a</td>
<td>&lt;1</td>
<td>10-100</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>LDPE (low density polyethylene)</td>
<td>n/a</td>
<td>10-100</td>
<td>&gt;100</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>HDPE (high density polyethylene)</td>
<td>n/a</td>
<td>10-100</td>
<td>&gt;100</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 2.4 highlights the ability of biodegradable food films to achieve mechanical properties like those of synthetic petro-based polymers. For example, the tensile strength of fish and wheat proteins plasticized with glycerol compares to that of LDPE and HDPE. It is also notable that plasticizers can have significant effects on the films’ mechanical properties. Whey protein isolate films plasticized with either sorbitol and glycerol have elongation values within the same range, but the addition of glycerol instead of sorbitol results in an order of magnitude greater tensile strength. Consequently, many studies have focused on the optimization of plasticizer type and concentration to provide films with different mechanical properties. Sanyang and others (2015) prepared palm starch films keeping constant the concentration of polymer but independently changing the type and concentration of plasticizers (glycerol, sorbitol, or a combination of thereof) in the films. They determined that higher plasticizer concentrations, regardless of the type, lowered the density and increased both the thickness and moisture content of the films. In a
follow-up study, the same group assessed the mechanical properties of films made with different plasticizer contents. They observed that the addition of plasticizers increased the tensile strength of the films in the following order: sorbitol > sorbitol/glycerol > glycerol. All polyols affected the tensile strength in a dose-response way, with the highest tensile strength consistently at 15% w/w plasticizer. Martino and others (2009) studied amorphous poly(lactic acid) films plasticized with commercial polymeric adipates with different molecular weight. The addition of polyadipates caused a decrease in the glass transition temperature ($T_g$) of the matrix, and significantly increased the deformation at the break at plasticizer concentrations $\geq 20\%$ w/w. No significant differences were observed between the two molecular weight adipates. Many plasticizers are used in biodegradable films, and without them, these films do not possess the mechanical properties required to be sufficiently flexible for their use in food packaging.

The macromolecular structural differences between polysaccharides and proteins provide their corresponding films with different mechanical and barrier properties. While polysaccharides films tend to have, in general, high tensile strength and elongation, their water vapor barrier properties are normally poor. A comprehensive and recent review by Cazon (2017) on polysaccharide-based films, particularly on starch-based materials, thoroughly explores recent advances in these type of films. Protein films are further categorized based on the type of proteins that are used in their composition as fibrous or globular, which also affects their function and properties. A fibrous protein film tends to be insoluble in water and provides excellent water vapor barrier properties. Globular protein films normally are water soluble and are structured preferentially by covalent and H-bonds. The prevalence of covalent over H-bond, normally results in stiffer matrices,
and higher mechanical properties\textsuperscript{100}. A comprehensive review of protein films by Zhang
and Mittal (2010), which focuses on plant proteins used for film production, provides
extensive information on these materials \textsuperscript{101}.

When comparing polysaccharide and protein films, it is generally agreed that
polysaccharide films have lower tensile strength and elongation (%) than protein films,
with the exception of few materials, namely chitosan, and methylcellulose (see Table
2.4)\textsuperscript{15}. Conversely, protein films made from zein and soy protein isolates have
significantly lower extensibility than chitosan films\textsuperscript{102}.

The development of blended films, i.e., films that combine carbohydrates and
proteins, has received additional attention in recent years. The rationale behind the
development of composite films is to reinforce structural features and improve
mechanical and barrier properties by leveraging from the unique and sometimes
complementary properties of both types of polymers. For example, the addition of
sodium caseinate to starch films was studied by Jimenez and others (2012). These
carbohydrate-protein films were less stiff, hence more flexible, deformable and more
resistant to fracture than pure starch films. Additionally, their oxygen permeability
increased as the concentration of protein increased\textsuperscript{103}. Tripathi and others (2016) looked
into the use of lactic acid-grafted-gum arabic as a filler to poly(lactic acid) films to
improve their gas barrier properties. A ten-fold decrease in oxygen permeability was
observed when the filler was used. Also, water permeability diminished, although to a
lesser extent (only 27\%), when 5\% of filler was added\textsuperscript{104}. Li and others (2015) developed
pea starch films reinforced with maize starch nanocrystals. The addition of the crystals
increased the tensile strength while decreasing water permeability. However, once the
nanocrystal content was above 7%, the crystals began to aggregate, and the structure changed to a longitudinal fibrous form which resulted in lower tensile strength and elongation than compositions with nanocrystal contents < 7%\textsuperscript{105}. The incorporation of chitosan nanoparticles to high- and low-density methyl pectin films was tested by Lorevice and others (2016). The addition of the nanoparticles significantly affected the tensile strength of the pectin films, providing a 1.5 and 2.25 fold increase in this property when added to high- and low-methyl pectin films, respectively\textsuperscript{106}. Material combinations and addition of fillers are often aimed at increasing the tensile strength while decreasing vapor permeability in films. This strategy to improve upon existing biodegradable films aims to bringing the mechanical and barrier properties of biodegradable films closer to those of traditional petro-based packaging and, consequently expanding the application of the bio-based films.

### 2.4 Ageing processes in biodegradable films

As it has been mentioned in previous sections, during their usage and storage, biodegradable films undergo appreciable changes in their structure and functionality\textsuperscript{107, 108}. This process is referred to as ageing. Ageing poses an important challenge in the development and utilization of bio-based biodegradable films as it reduces a film’s shelf life, and that of the concomitant food product. To some extent, faster ageing in biodegradable films can be considered desirable, since it signals their transient nature that is linked to their capacity to decompose. Unlike a traditional petro-based polymer, in which biodegradability is not a desirable property, biodegradable films should be programmed to breakdown easily during, for example, composting processes. It should
also be noted that in the case of composted biodegradable films, their components will return to the environment.

Regardless of their main structural material being a carbohydrate, protein, or a composite, films undergo ageing as a consequence of the rearrangement of the polymers within the matrix that significantly affects their mechanical\(^{109}\), optical\(^{52}\), and barrier properties\(^{18}\). These physical changes can extensively affect the functionality of the film and reduce its stability. Therefore, identifying aging mechanisms and quantifying the extent of aging constitute an active area of research in biodegradable films to expand their use and applicability.

### 2.4.1 Ageing mechanisms in biodegradable films

The wide variety of components that can provide structure to a biodegradable film results in multiple mechanisms that are, individually or in combination, responsible for ageing. Carbohydrate, protein, or composite biodegradable films undergo distinct ageing processes as time elapses. Biodegradable films consist of a network of interweaving polymer chains with water (or an appropriate solvent) trapped throughout the film. During wet casting of films, a common method used in films’ production, a solution is prepared, poured, and left to dry until the appropriate amount of solvent has evaporated. The remaining solvent acts as a plasticizer giving the film flexibility by providing a hydration shell between polymer chains\(^{110-112}\). The amount of solvent in the film can vary greatly depending on the matrix of the film, which in turn affects its mechanical and barrier properties. In general, ageing can be attributed to an increase in polymer-polymer interactions over polymer-solvent ones, decreasing flexibility and increasing tensile strength\(^{16, 18, 99, 109}\).
In carbohydrate films where the composition is mainly starch, the main ageing mechanism is retrogradation, i.e., the recrystallization of the starch material\textsuperscript{113}. Hydrated or gelatinized starch is used in the preparation of starch-based films. During storage, the starch in biodegradable films undergoes physical changes from a solvated polymer to a crystallized amorphous structure (See Figure 2.1). This re-crystallization causes a rearrangement of the polymers forcing the water and the plasticizer (if other than water) out of the polymer network. This re-arrangement results in increasing polymer-polymer interactions, which in turn causes an increase in the film’s brittleness\textsuperscript{7, 114}. The crystalline structure of a retrograded film is evidenced by changes in the optical properties of the film, e.g., more opaqueness, and water/plasticizer exudation, which can cause total loss of functionality in extreme cases\textsuperscript{115}. Kim and others (2009) hypothesized that the molecular weight of the plasticizers used could influence the extent of retrogradation by hindering plasticizer migration in starch-based films. This group studied poly(ethylene glycol) plasticizers with varying molecular weights from 300-100000 g/mol. Poly(ethylene glycols) with a molecular weight above 8000 g/mol exhibit low molecular mobility when the starch began to retrodegrade\textsuperscript{116}. Gutierrez and others (2015) compared films made from yam and cassava starch for their ability to retain plasticizer within their matrix during storage. They determined that cassava-based films remained more flexible than yam films due to glycerol binding more tightly to the cassava starch and ultimately inhibiting the recrystallization of the starch in the films. Since cassava starch bound to glycerol better than yam, the resulting film had more extensibility\textsuperscript{117}. 
Figure 2.1: Gelatinization and subsequent recrystallization (retrogradation) of starch as a function of storage time. Reprinted from Shujun and others (2015)\textsuperscript{118}

Protein-based biodegradable films, made from animal or vegetable proteins, undergo ageing in a similar way as carbohydrate-based films, albeit differences that arise from their distinct molecular structures, e.g., folded structure of proteins. The increase of polymer-polymer interactions over polymer-solvent interaction augments the brittleness of the film and decreases its extensibility. Olabarrieta (2005) used SEM to visualize films’ structure during storage. In Figure 2.2 B and D tighter, highly cross-linked structures are noticeable, which were correlated to increased tensile strength and decreased extensibility\textsuperscript{119}.

Protein film ageing mechanisms are not nearly as studied as those in carbohydrate-based films, so alternative and complementing theories are proposed in the literature. Ciannamea and others (2018) studied films made from pea protein and glycerol as a plasticizer. They suggested that the loss of plasticizer and water in the film increased brittleness; this effect was more pronounced in films with low glycerol content (e.g., < 30% w/w of the polymer). The progressive transition from unordered structures to tighter and more organized structures of intermolecular hydrogen bonded in films was
speculated to be responsible for the loss of plasticizer and water in those films\textsuperscript{120}.

Duconseille and others (2017) attributed the ageing of gelatin films to the formation of inter-molecular cross-links and changes in the protein’s secondary structure from triple helix, an extremely ordered and restricted structured, to random coil, more opened and with higher water interactions to $\beta$-sheets, tightly aligned again\textsuperscript{121}. In this study, ageing was reported to depend on biological and regional variations in the gelatin source. Additionally, the changes in the protein secondary structure increased intermolecular cross-links making more predominant polymer-polymer over polymer-solvent interactions\textsuperscript{121}.

![Figure 2.2: Cross sectional scanning electron micrographs of wheat gluten films prepared in acidic conditions (A, B) and basic conditions (C, D), before ageing (A, C) and after ageing (B, D) at 50% RH and room temperature for 120 days. Reprinted from Olabarrieta (2005)\textsuperscript{119}](image)

As it was mentioned earlier, composite films made from both carbohydrate and protein sources are extensively being studied based on the possibility of modulating functionality through changes in composition, e.g., the proportion of each component. Their ageing mechanisms are suspected to be a combination of both their carbohydrate
and protein contributions. Jimenez and others (2012), when studying ageing of starch (carbohydrate) films reinforced with sodium caseinate (protein), observed more uniform films with fewer defects in the composites than in their non-blended controls – see Figure 2.3103. However, after a five-week storage at 25°C and 53% RH, all films became significantly more brittle due to an increase in polymer-polymer interaction, but the blended films were less brittle than the single component films. The ageing process was attributed to the rearrangement of the polymer chains through intermolecular attraction forces such as van der Waals interactions and H-bonding between amino groups of amino acids and starch hydroxyl groups. Multiple ageing mechanisms operating in concert due to the combination of materials within a film complicate the interpretation of results during storage, which justifies the use of single-sourced materials when assessing ageing mechanisms.
Figure 2.3: Cross-sectional SEM micrographs of a starch/sodium caseinate (pure and combination) films. Pure starch (A), 75:25 starch:sodium caseinate (B), 50:50 starch:sodium caseinate (C), pure sodium caseinate (D). Reprinted from Jimenez and others (2012)\textsuperscript{103}

2.4.2: Effects of environmental factors on ageing

The ageing process is influenced to different extent by environmental factors such as the relative humidity (RH) and the temperature during storage. Due to the important effect of solvent-polymer interactions, RH is one of the main factors reported as an external parameter in film stability studies because it modulates the moisture content of the films. However, since biodegradable film studies usually focus on the performance of novel biomaterials or their combinations as structuring agents, most film ageing is conducted at a single RH. Although testing at a single condition provides insights on the potential effect of environmental factors on the deterioration kinetics of a material, it
seldom allows for estimation of alternative scenarios since information is missing, e.g.,
the dependence of the deterioration rate on RH. It should be noted, however, that food
films may be exposed to a variety of dynamic conditions along the supply chain.

Numerous studies have reported changes in the mechanical and barrier properties
of their films due to storage RH. Relative humidity has been deemed integral to the
ageing of soybean protein films, as a reorganization of protein secondary structure was
observed at different RHs. Ciannamea and others (2015) studied changes in protein
secondary structure within films and reported the inability of a film to retain a plasticizer
with ageing. The delocalization of the plasticizer resulted in increased tensile strength
and stiffness in the films. Eventually, it also leads to the formation of small cracks in the
matrix during storage, which also increased its oxygen permeability.65

Aguirre and others (2016) draw attention to the limitations of conducting ageing
studies at a single environmental condition, e.g., storage at just one RH. In this study,
chitosan films stored at high RHs (75, 84, 90, 100%) had high film elongation and water
vapor permeability, and low tensile strength61 at the end of the storage. Conversely, these
changes were not observed at RHs below 50%. Basiak and others’ study on wheat starch
films reported that the films’ storage RH had a larger impact on water vapor permeability
than changes in polymer concentration122. In general, these studies correlated high water
vapor permeability and low tensile strength with storage at high RHs, e.g., >70%. This
has been systematically verified for a variety of film types, demonstrating the importance
of considering storage RH in stability tests.

Another factor that effectively affects the rate and extent of ageing is storage
temperature. The temperature dependence of the rates of many foods and food-related
processes, e.g., oxidation, compounds degradation, etc., are widely reported and often characterized using the Arrhenius equation\textsuperscript{123,124}. Therefore, storage temperature is usually taken into consideration when performing biodegradable films’ stability studies. Food films can be exposed to a variety of temperatures from freezing to refrigeration and room temperature. At each of these conditions, the rates of ageing will be different, which justifies the selection of a material based on its storage requirements. As in the case of RH, ageing studies are often conducted at a constant single temperature, for the sake of practicality. Studies at multiple temperatures could allow characterizing the temperature dependence of the ageing rates and the potential estimation of ageing under fluctuating temperatures, often observed along the food supply chain.

2.5 Assessing ageing in biodegradable films

Mechanical testing has been extensively used for studying ageing in films. Since mechanical properties like tensile strength and percent elongation are crucial to film performance, it is not surprising that changes during ageing are assessed by these means. Several other techniques have been used to study ageing in films or bio-based biodegradable matrices, examples of these techniques and their uses are presented in Table 2.5.
Table 2.5: Examples of commonly used techniques to assess structural and physical properties in films and biodegradable matrices

<table>
<thead>
<tr>
<th>Technique</th>
<th>Targeted Property</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scanning Electron Microscopy (SEM)</td>
<td>Structural features and phase separations</td>
<td>125</td>
</tr>
<tr>
<td>Confocal laser scanning microscopy (CLSM)</td>
<td>Distribution of polymer on the surface</td>
<td>126</td>
</tr>
<tr>
<td>Thermal Gravimetric Analysis (TGA)</td>
<td>Thermal stability of films</td>
<td>125</td>
</tr>
<tr>
<td>Dynamic Scanning Calorimetry (DSC)</td>
<td>Glass transition temperature of films</td>
<td>125</td>
</tr>
<tr>
<td>Dynamic mechanical analysis</td>
<td>Viscoelastic behavior of polymers</td>
<td>127</td>
</tr>
<tr>
<td>Dynamic mechanical thermal analysis</td>
<td>Glass transition temperature of films</td>
<td>128</td>
</tr>
<tr>
<td>Fourier Transform Infrared Spectroscopy (FTIRS)</td>
<td>Protein structure, miscibility of polymers</td>
<td>59</td>
</tr>
<tr>
<td>Luminescence spectroscopy (phosphorescence)</td>
<td>Molecular mobility in amorphous solids</td>
<td>129</td>
</tr>
<tr>
<td>Luminescence spectroscopy (fluorescence)</td>
<td>Protein conformation within films</td>
<td>66</td>
</tr>
<tr>
<td>Wide Angle X-ray Diffraction</td>
<td>Crystal structure retrograded carbohydrates</td>
<td>130</td>
</tr>
<tr>
<td>Zeta potential</td>
<td>Potential compatibility of blended polymers in film dispersions before casting based on electrostatic repulsion</td>
<td>131</td>
</tr>
</tbody>
</table>

Despite the plethora of techniques available to monitor ageing in biodegradable food films, few of these can be applied *in situ* and in real time, which makes difficult the continuous assessment of ageing during, usage, and storage. Embedding optical sensors within films or, even better, using natural present ones, could simplify and facilitate monitoring structural properties within biodegradable films.

### 2.5.1. Optical techniques to assess ageing in biodegradable films

A variety of optical methods to survey ageing in bio-based (and petro-based) films has been proposed as possible alternatives to mechanical ones. IR, luminescence, and Raman spectroscopy approaches have high sensitivity to changes in molecular structures and local physical properties. Thus, they have the potential to accurately sense
these structural changes in flexible biodegradable films. The uses of luminescence spectroscopy and Fourier Transform Infrared Spectroscopy (FTIRS) techniques are continuously expanding in chemical, biological, and engineering applications\textsuperscript{22, 23, 132, 133}. In principle, luminescence spectroscopy could detect changes in the photophysical properties, e.g., intensity, energy, polarization of lumiphores, i.e., probes that fluoresce or phosphoresce upon photoexcitation, within a matrix as it ages. Complementary to luminescence spectroscopy, FTIRS can report on changes in the molecular arrangements within a film. These highly sensitive methods have been replacing conventional methods of observing structural changes in films with the added benefit of molecular level sensitivity, particularly in biodegradable food films as a function of time\textsuperscript{29, 132}.

2.5.1.1. Luminescence spectroscopy: techniques and applications

Luminescence spectroscopy is a versatile tool to assess the molecular environment of many matrices, potentially including biodegradable food films. Luminescence spectroscopy is hailed as an incredibly sensitive method of analysis, although it is important to note the equipment’s detection limit ultimately determine the sensitivity of these methods\textsuperscript{134, 135}.

Luminescence is the emission of light from a lumiphore not induced by heat but by a photon. The radiative decay of an excited lumiphore from a singlet to its ground state is referred to as fluorescence, which is characterized by a short lifetime (1ps – 10ns)\textsuperscript{136}. Conversely, the radiative decay of an excited lumiphore from a triplet state to its ground state is referred to as phosphorescence, which has characteristically longer decay times potentially orders of magnitude greater than fluorescence emission. Since the
current study utilizes primarily fluorescence spectroscopy in the following sections this methodology will be predominately discussed.

Wolfbeis (2008) has summarized the photophysical properties of fluorescent natural organic compounds, including those present in foods\textsuperscript{137}. More recently, Christensen and others made available a web-based food fluorescence library (www.models.kvl.dk). Not only the identification of the numerous fluorescent compounds in foods has been explored in recent years but also reports on the environmental sensitivity of several fluorophores has been reported. \textsuperscript{23, 138-142}. This supports the potential use of endogenous fluorophores (individually or in combination) as intrinsic reporters of the state of their surrounding media, i.e., a food matrix. Additionally, probes from food origin could be directly incorporated into the foods or their packaging as intrinsic sensors of food quality and consumed without any safety or regulatory concerns due to their generally recognized as safe (GRAS) or permitted status.

The photophysical properties, environmental sensitivity and current and potential uses of selected luminescent probes are presented in the following sections.

Aromatic amino acids, namely phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp), are among the most common intrinsic fluorophores in foods. All these amino acids are present to different extent in most proteins. Their molecular structures and main photophysical properties, e.g., optimal excitation and emission wavelengths, are presented in Figure 2.4 and Table 2.6, respectively.
Phenylalanine, Phe, due to its relatively low quantum yield quantum yield is seldom used in protein fluorescence studies. Additionally, as it can be observed in Table 2.6, the emission energy, i.e., wavelength, of the Phe overlaps with the excitation wavelength of tyrosine, Tyr. Therefore, if both amino acids are present in a protein and are close to each other, resonance energy transfer (RET) from Phe and Tyr occurs and detection of Phe emission is virtually impossible. Similarly, and due to the overlapping of the emission wavelength of Tyr and the excitation wavelength of Trp (see Table 2.6), the emission of the Tyr is absorbed by Trp, i.e., the energy is transferred, provided that both moieties are within a characteristic distance commonly referred to as Förster distance. These energy transfer phenomena determines that Trp is normally the dominant emitting species in proteins. It should be noted that if the residues are not within the Föster distance, individual peaks pertaining to each amino acid can be identified. Native folded conformations in proteins usually favored RET

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Code</th>
<th>Excitation wavelength $\lambda_{ex}$ (nm)</th>
<th>Emission wavelength $\lambda_{em}$ (nm)</th>
<th>Quantum yield $\phi_F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>Phe</td>
<td>260</td>
<td>282</td>
<td>0.02</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr</td>
<td>275</td>
<td>303</td>
<td>0.14</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Trp</td>
<td>295</td>
<td>350</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Source: Lakowicz (2006)
The emission of Tyr and Trp are highly sensitive to the characteristics of their surrounding environment, for example polarity. Their environmental sensitivity makes them important beacons of the state of a protein with a matrix. Solvent polarity causes differences in the emission spectrum of Trp because of the ability of the imino group on the amino acid to form H-bonds. Tyr sensitivity to polarity is based on the state of hydroxyl group off the benzene ring, the functional group that distinguishes Tyr from Trp. Deprotonation of this group by a base normally causes a solvatochromic (red) shift. Red shifts in the emission spectra of Trp are often observed in polar solvents, and hypsochromic (blue) shifts are reported in non-polar solvents. These observations have been broadly extended to changes in the aromatic amino acids environment due to conformational changes in proteins. A simplistic understanding of spectral shifts during protein folding and unfolding equates a red shift to the expose of Tyr or Trp to polar solvents and a blue shift due to the inclusion or burring of the amino acid within the hydrophobic core of a protein. Although applicable to certain cases, spectral shifts in amino acids are more commonly the net result of a sum of phenomena operating in concert as extensively explained by Lackowicz. Most proteins contain multiple Tyr and/or Trp residues, therefore moieties that are affected by different environments collectively contribute to the total fluorescence emission.

In foods, the environmental sensitivity of the photophysical properties of aromatic amino acids have been used to evaluate thermal effects on protein stability and conformational changes at interfaces in food systems. For example, an 8-nm red shift in Trp emission, along with a slight but non-significant decrease in emission intensity was observed in samples of β-conglycinin during heat treatments. The authors
attributed these changes to losses of the tertiary structure in this protein, exposing more Trp residues causing a peak shift and lower emission intensities, i.e., quenching\textsuperscript{148} This type of studies support the potential used of the photophysical properties of amino acids to understand protein rearrangements within a matrix.

A luminescent molecular rotor (MR) is a molecule that can undergo partial or full internal rotation (See Figure 2.5) and its internal rotation affects its photophysical properties. Their ability to rotate in these molecules is affected by the physical properties of the environment in which they are embedded. These probes are sensitive to molecular crowding in their surrounding environment, which makes them good reporters of conditions or processes that restrict their internal movement\textsuperscript{29}. Internal rotation of a MR has been attributed to cis-trans isomerization\textsuperscript{149} or the formation of a Twisted Intermolecular Charge Transfer (TICT) state\textsuperscript{150} upon photoexcitation.

\begin{figure}[h]
\centering
\includegraphics[width=0.7\textwidth]{figure25.png}
\caption{Schematic view of the hypothetical twisting in an azo dye (Citrus Red 2) MR upon photoexcitation. Reprinted from Du and others (2014)\textsuperscript{151}}
\end{figure}

Regardless of the mechanism of rotation, a high molecularly crowded environment will restrict rotation and will favor deactivation from the excited state through emission of a photon. Therefore, higher emission intensity, quantum yields, and lifetimes have been associated to more rigid or more viscous environments around a MR. Loutfy and Arnold (1982) studied the relationship between molecular crowding,
evidenced as viscosity, and fluorescence quantum yield in several solvents\textsuperscript{152}. They observed an increase in lifetime of a MR as viscosity of its solvent medium increased. As it is shown in Figure 2.6 (top), the emission intensity of the molecular rotor Sunset yellow (a synthetic food dye) increases with the viscosity of the medium.

Forster and Hoffman (1971) established a proportional relationship between solvent viscosity and fluorescent quantum yield. The Förster-Hoffmann equation describes this relationship between the of the surrounding solution and can be expressed as follows\textsuperscript{153}:

$$\log \varphi_F = c + x \log \eta$$

\textbf{Figure 2.6: Fluorescence spectra of Sunset Yellow FCF in solution with increasing viscosity solutions (top), and normalized fluorescence intensity versus viscosity (bottom) plotted in linear and logarithmic coordinates and fitted with Eq. 2. Reprinted from Corradini and others (2016)\textsuperscript{23}}
where \( \Phi_F \) is the fluorescence quantum yield of a molecular rotor, \( \eta \) is the viscosity of the surrounding medium, \( c \) is a MR-specific constant and \( x \) is a constant related to MR-solvent interactions. Since fluorescence emission intensity and quantum yield are proportional; a MR fluorescence intensity, as well as its lifetime, exhibits a power law relationship to a medium’s molecular crowding as it can be seen in Figure 2.6 (bottom). This relationship is often expressed as (Eq. 2)\(^{154}\):

\[ I_F = \alpha \eta^x \]  

(2)

where \( \alpha \) is usually considered a measure of the probe’s brightness and \( x \) a measure of its sensitivity to local viscosity. Many synthetic and natural food dyes, such as Sunset Yellow, have been recently listed among the newly identified MRs. Table 2.7 presents examples of food colorants with presumptive MR behavior, their structures and relevant photophysical properties.

**Table 2.7: Luminescent food probes with presumptive molecular rotor behavior**

<table>
<thead>
<tr>
<th>Type</th>
<th>Compounds</th>
<th>Structure</th>
<th>( \lambda_{Ex} ) (nm)</th>
<th>( \lambda_{Em} ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azo Dyes</td>
<td>Allura Red, Sunset Yellow, Tartrazine, Citrus Red</td>
<td><img src="structure.png" alt="Structure" /></td>
<td>450-540</td>
<td>540-610</td>
</tr>
<tr>
<td>Triarylmethane Dyes</td>
<td>Fast Green, Brilliant Blue</td>
<td><img src="structure.png" alt="Structure" /></td>
<td>580-600</td>
<td>650-700</td>
</tr>
<tr>
<td>Natural colors</td>
<td>Betaxanthines, Conjugated chlorophylls*</td>
<td><img src="structure.png" alt="Structure" /></td>
<td>470, 440</td>
<td>500, 685</td>
</tr>
</tbody>
</table>

The structure is of the first molecule listed. *Derived from chlorophylls. Adapted from Corradini and others (2016)\(^{147}\)

The sensitivity of the above-listed MRs to local and bulk viscosity has been validated and quantified by Corradini and Ludescher’s groups. Based on these studies, these permitted-in-foods MR can be used in food systems to noninvasively and
nondestructively assess food matrices, potentially including biodegradable food films during ageing, since this process normally results in a progressive stiffening of the matrix.

Luminescence probes sensitive to solvation dynamics can be used to assess the extent and state of hydration shells in different matrices (surrounding a probe). Spectral shifts in the absorption or emission bands of chromophores in solvents with different polarity have been extensively observed. These shifts have served to build scales for solvent classifications. For example, the Reichardt’s $E_{T}(30)$ parameter and scale are based on the exceptional absorbance hypsochromism of the Dimroth–Reichardt's betaine dye (4-(2,4,6-triphenylpyridinio)phenolate) in different solvents. The spectral shifts of the chromophores are due to solvation free energy differences between their ground and excited states.

Among the probes sensitive to the solvation dynamics of the environment, pyranine (Sodium 8-hydroxypyrene-1,3,6-trisulfonate) has received additional attention lately. Roche and others (2006) used pyranine to assess molecular level changes in its hydration shell of pyranine embedded in protein solutions. The addition of glycerol to a protein/pyranine solution reduced the water available and consequently the H-bond stabilization of the protein structure, which allow the authors to infer the role and state of water within these systems. Similarly, Kashi and others used pyranine to demonstrate how the state of water within a matrix, free to bound, could affect the ability of a MR to report on rheological bulk properties of a matrix. Pyranine sensitivity to these changes arises from the dissociation of the ionizable hydroxyl on the molecule, marked in Figure 2.7.
Figure 2.7: Ionizable hydroxyl in pyranine responsible for the formation of two tautomers with distinct emission bands

When pyranine is solvated, the hydroxyl group is predominantly deprotonated. The deprotonated tautomer exhibits a peak at 513 nm, whose intensity is higher if an extensive solvation shell surrounds the pyranine. If the hydration is disturbed, for example the addition of glycerol, and less free water is available, the pyranine is predominantly protonated and the peak corresponding to this tautomer at about 435 nm has a higher intensity (see Figure 2.8). Pyranine’s dual peak emission allow characterizing the environment using a concentration independent index derived from the ratio of the two peaks 2.9155
This makes pyranine a suitable probe for sensing the local solvation in a matrix, its sensitivity to free water and dual emission make it a cogent reporter on the solvation of a material at the molecular level.

2.5.2 Fourier Transform Infrared Spectroscopy (FTIRS)

Infrared (IR) spectroscopy has been a long-trusted analytical tool as a clean, simple, and non-destructive technology to assess the unique vibrational relaxation of compounds and structures within a sample. IR spectroscopy is often divided into three categories based on energy of the light source used. Far IR (1000-15000 cm\(^{-1}\) (FIR)), Mid IR (700-1000 cm\(^{-1}\) (MIR)) and Near IR (750- 2500 cm\(^{-1}\) (NIR)), are subdivisions with wavelength ranges that increase in energy, respectively.

IR spectroscopy is based on the unique vibrational relaxation of specific chemical moieties within a sample when subjected to a given radiation. NIR spectroscopy (NIRS) can report on overtones and combinations of fundamental vibrations that allow assessing
sample composition. NIRS is currently used in food science to detect transgenic foods\textsuperscript{156}, assess the lipid content in grains\textsuperscript{157}, and lipid oxidation in ground beef\textsuperscript{158}, among others. Comprehensive reviews by Osborne\textsuperscript{159, 160} outline the uses of NIRS in food applications. In contrast to NIRS, MIR spectroscopy (MIRS) is used to monitor fundamental vibrational modes of molecules, that produce a chemical profile or fingerprint of a sample and report on its structure. Vibrational relaxation is not a high energy phenomenon and this is taken advantage of because there is no need for sample dilution to obtain a result within a detectable limit\textsuperscript{161}. This is advantageous because solid intact samples can be analyzed without altering their composition or structure. The vibrational relaxation spectra for each molecule is different due to bond relaxation mechanics like stretching and bending, which causes characteristic peaks in the fingerprint region (1500-500 cm\textsuperscript{-1}). Due to the complexity of the IR spectra, the use of Fourier Transform Infrared Spectroscopy (FTIRS) has been developed. In this technique, the signal is tackled using a Fourier transform, from which the technique derives its name. This mathematical function converts the IR spectrum into a more readable output through the decomposition of the signal into constituent frequencies.

FTIR has the ability to test intact samples, however, the presence of chemical species such as water with intense infrared absorption complicates the use of MIRS in food systems. The H-O-H bending vibration is significantly strong within the amide I band region, an important region used to elucidate secondary structures of proteins. To overcome this challenge, working with deuterated water (D\textsubscript{2}O) instead of H\textsubscript{2}O has been proposed. Although D\textsubscript{2}O does not absorb in the amide I region, the reagent’s cost and the potential different effect of D\textsubscript{2}O in the molecular dynamics of the sample had advanced
the search for alternative solutions. Performing the measurements in the attenuated total reflectance (ATR) mode allows avoiding or reducing the attenuation of the signal by high absorbing materials, e.g., systems with high water content, due to its short path length. The traditional light path of FTIR splits the IR light source using a beam splitter, sending the light to one stationary mirror and one moving mirror. Each mirror reflects the light back and through the sample, and the light absorbed or transmitted from the sample is reported on by a detector\textsuperscript{162}. In the ATR mode, the light passes through a crystal that has a higher reflective index than the sample (Figure 2.9). Passing light through the crystal causes the formation of an evanescent wave which bounces off the sample and allows for a small wave penetration depth of 0.2 to 5 μm\textsuperscript{132}. This attenuates the reflectance because the evanescent wave decays exponentially with distance from the crystal, making previously undetectable peaks, due to their high absorbance intensity, now detectable. ATR determinations can typically be performed using a FTIR setup by adding an accessory that houses the crystal. The attenuated beam decreases the depth of penetration of the light, which causes the absorption or transmission spectra to arise from the chemical nature of the material that comes in direct contact with the ATR crystal. This is beneficial for thin films and other food applications because no additional sample preparations are needed, and the film can be measured intact and nondestructively\textsuperscript{163, 164}. 
Figure 2.9: Schematics of the light path in conventional FTIR spectroscopy (top), and the modified light path of ATR-FTIR. Reproduced from Blum and John (2011)\textsuperscript{162}

FTIRS in food films has been predominately used to determine interactions of polymers in composite films. This application allows monitoring polymer compatibility and polymer ratios that result in effective interactions. Kurt and others (2017) used FTIR to determine the extent of chemical interactions between the two polymers, namely xanthan and locust bean gum, in blended films. The shift in peak positions from 1150 to 1147 cm\textsuperscript{-1} was an indicator of chemical interaction between blended polymers at the molecular level. Peak shifts signaled increasing chemical interactions between the different blend ratios of the two polymers\textsuperscript{59}. Sindhu and others (2006) studied starch-chitosan films using FTIR to determine to which extent interactions between the hydroxyl...
groups of starch and amino groups of chitosan were present. These data informed the selection of compositions that promoted compatibility between the two polymers and resulted in films with higher tensile strength than those made solely from each component\textsuperscript{62}.

FTIRS has also been used to assess the secondary protein structure of a sample nondestructively. This application began with the groundbreaking work by Elliott and Ambrose in 1950\textsuperscript{165}. They determined that absorbance at the amide I band (1700-1600 cm\textsuperscript{-1}) and correlated their peaks to specific secondary structures in proteins. IR absorption in this region is dictated by the C=O double bond in amino acid residues\textsuperscript{166}. The absorption of IR in this region can be broken down into subsections which correspond to various protein secondary structures, including but not limited to: β-angle, β-fold, α-helix, and random coil. Although absorbance at specific frequencies that correspond to different secondary structures differ slightly depending on the reference material, Table 2.8 reports the generally agreed upon frequency assignments of secondary structures within the amide I region\textsuperscript{167}.

Table 2.8: Frequency assignments of protein secondary structure of the FTIR spectrum within amide I region

<table>
<thead>
<tr>
<th>Secondary structure assignments</th>
<th>Frequency Ranges (cm\textsuperscript{-1}) in solution</th>
<th>Frequency Ranges (cm\textsuperscript{-1}) in hydrated films</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-sheet</td>
<td>1610-1640</td>
<td>1613-1637</td>
</tr>
<tr>
<td>β-turns</td>
<td>1691-1699</td>
<td>1662-1682</td>
</tr>
<tr>
<td>α-helix</td>
<td>1650-1659</td>
<td>1645-1662</td>
</tr>
<tr>
<td>Unordered Structures (Random coil)</td>
<td>1640-1650</td>
<td>1637-1645</td>
</tr>
</tbody>
</table>

Sources: Byler (1986)\textsuperscript{167,168}, Goormaghtigh (1990)\textsuperscript{168}

Multiple peaks normally overlap since they correspond to a mixture of protein conformations within a sample. Thus, calculating the second derivative of the signal and plotting it against the signal’s frequency “discovers” the hidden peaks\textsuperscript{169}. Figure 2.10
shows the ability to find hidden peaks in an FTIRS absorbance of wheat gluten when the second derivative method is used in the amide I region.

![Figure 2.10: FTIRS absorption spectra of wheat gluten (including deconvoluted peaks) (top). Second derivative of same region showing more inflections that provide better identification of the peak for each structure (bottom). Reproduced from: Nawrocka (2014)170](image)

The use of FTIRS to identify and quantify protein conformation gained popularity when Goormaghtigh in 1990 studied thin films made from animal protein, he used ATR-FTIR when trying to develop a novel method as an alternative to x-ray crystallography. The results from ATR-FTIR were not significantly different from data collected using x-ray crystallography, and required less sample preparation168. Gueguen and others (1998) studied the influence of plasticizers on pea protein films and determined that the addition of plasticizers can shift the wavenumber of the β-sheet conformation peaks when using IR spectroscopy. In this study, the use of plasticizers was intended to decrease polymer-polymer interactions in the films as the chain length of the plasticizer increased,
significant reductions in tensile strength and percent elongation were observed as plasticizer chain length increased. The authors suggested that the decrease in tensile strength (of shorter chain lengths) was due to the increase of bonds between the plasticizer and the β-sheet structures. When studying the effects of increasing soybean protein isolate in cod gelatin blended films, it was discovered that soy protein isolate at concentrations equal or above 25% in the films caused gelatin conformational changes that could be detected using FTIR.

FTIR has proven to be a useful tool to assess polymer compatibility and identify and quantifying changes in protein secondary structure.

Efforts to reduce dependence on petro-based materials have made the study and development of biodegradable food films extremely relevant in recent years. Ageing of these films results in changes in functionality during storage which limits their applicability, deterring their use in the food industry. These changes and their kinetics need to be characterized, potentially using the above described techniques, to better understand the ageing process. Although mechanical tests have been so far the gold standard to monitor ageing, due to their high variability and lack of versatility, optical methods could provide an alternative methodology to monitor ageing in real time in biodegradable food films. Development of new tools to assess film ageing could help to better understand ageing mechanisms, which could facilitate the optimization of biodegradable films’ production and storage processes and the ultimate displacement of petro based films by this more sustainable option.
CHAPTER 3
MATERIALS AND METHODS

The use of luminescence spectroscopy techniques to assess physical changes in bio-based films during storage at different relative humidity (RH) conditions can provide a promising alternative to the traditional monitoring of aging in films using mechanical properties, such as tensile strength. By employing a gelatin-based film as a model system, a storage study was conducted to monitor structural changes as the films’ ageing progressed. Two optical methods (fluorescence and Fourier Transform Infrared spectroscopy (FTIRS)) were used to assess structural changes in the films at the molecular level. Mechanical testing of the films was conducted in tandem and compared to the results of the optical methods. This comparison aimed at determining if, and to what extent, optical measurements were complementary or could replace mechanical measurements. Findings from this study could advance the development of a more sensitive method to sense structural changes in biodegradable food films, and even petro-based ones, during ageing. Film production, testing parameters, and the methodology used to determine moisture content, optical and mechanical properties, are described below.

3.1 Bio-based biodegradable food films

3.1.1 Preparation and storage

Gelatin type A (acid hydrolysis from collagen, porcine skin, food grade powder with a bloom number of 225) at 2% w/v (MP Biomedicals LL, Solon, OH) was dispersed in double distilled ultrapure water (Thermo Scientific Barnstead GenPure, Waltham,
MA). The dispersion was heated to 40°C until the gelatin was completely dissolved. Pure glycerol at 0.5% w/v (Sigma, St. Louis MO) was added to the solution to act as a plasticizer. Two luminescent probes were separately added to the solution. Fast Green FCF (disodium; 2-[[4-ethyl-[(3-sulfonatophenyl)methyl]amino]phenyl]-[4-ethyl-[(3-sulfonatophenyl)methyl]azaniumylidene]cyclohexa-2,5-dien-1-ylidene]methyl]-5-hydroxybenzenesulfonate, >85% purity, Sigma, St. Louis MO), a luminescent triarylmethane dye with molecular crowding sensitivity, was incorporated into the solution at a concentration of 0.124 mM. Pyranine (sodium 8-hydroxypyrene-1,3,6-trisulfonate, >97% purity, Sigma, St. Louis MO), a ratiometric probe sensitive to local solvation, was added at a concentration of 0.05 mM. Optimal concentration of both probes in the films was determined by a fluorescence intensity versus concentration study (see Section 3.1.2., for details). Films were casted on 60 or 150 mm diameter dishes (Thermo Scientific, Waltham, MA) for spectroscopic and mechanical testing, respectively. The casting procedure was adapted and modified from Aguirre-Loredo and others (2016)61, and was conducted as follows. The deposited amount of solution in the dishes was measured gravimetrically to achieve a final solids density of 84 g/m³. All films were dried at 20°C and 23% RH for 72 hours. Sample water loss was determined gravimetrically to corroborate if the target solids density was achieved before removal of the films from the casting surface. The films were peeled intact from the surface and cut to different dimensions based on the specific needs of various tests. Films were stored in chambers at 20°C equilibrated at five specific RHs for periods up to 6 weeks. The RHs within the chambers were modulated using phosphorus pentoxide (RH = 2.5%)171, the commercial desiccant Drierite (RH = 25%), and supersaturated solutions of magnesium
nitrate (RH =33%)\textsuperscript{172}, magnesium chloride (RH =53%)\textsuperscript{172} and sodium chloride (RH =75%)\textsuperscript{172}. All reagents were procured from ThermoFisher Scientific (Waltham, MA). Continuous relative humidity and temperature measurements were performed using data loggers (EL-USB-2-LCD, Lascar Electronics Inc. US, Erie, PA) to confirm that the storage chambers maintained their set RHs throughout the length of the experiments.

3.1.2 Optimization of the luminescent probes concentration

The concentration of the added luminescent probes, i.e., Fast Green FCF (FG) and pyranine (Pyr), was optimized by performing a concentration study. Films were doped with aliquots of the probe stock solution to attain increasing concentrations of the probes in the films. Then, their fluorescence emission intensity was measured at their corresponding excitation and emission wavelengths. The optimal probe concentration to be used in the experiments was selected so that the FG and Pyr exhibit a detectable signal in recently prepared films and also allowed for the fluorescence intensity to increase or decrease multi-fold without surpassing either the inferior or superior limit of detection throughout the storage experiments\textsuperscript{173}.

3.1.3 Moisture content determination

The moisture content of the films was recorded during storage to verify if the samples have attained equilibrium. Additionally, this measurement allows discarding changes in the fluorophores’ content within the films, e.g., dilution or concentration, during storage. A significant change in fluorophore content would compromise the use of fluorescence intensity, a concentration-dependent photophysical property, as a reliable measurement. The procedure for this analysis was adapted and slightly modified from
Cano and others (2017)\textsuperscript{174}. Five samples per storage week and RH were tested per replicate, and the experiment was performed in triplicate. In brief, films (1cm x 1cm) were stored in the previously reported RH environments, i.e. 2.5, 25, 33, 53, 75\%. The samples were weighed before drying using a vacuum oven (Lindeberg Blue M, ThermoFisher Scientific, Waltham, MA) for 48 hours at 60\(^\circ\)C with a chamber vacuum pressure of 25inHg. Once removed from the oven, the samples were placed in a chamber for twelve days at 2.5\% RH, maintained using phosphorous pentoxide (P\(_2\)O\(_5\)), until a constant weight was achieved. Moisture content (MC) was recorded on the day the films were peeled from the casting surface and after 7, 21, and 35 days of storage at each chosen relative humidity. Moisture content was expressed as percentage and calculated using the following equation:

\[
MC \ (\%) = \left( \frac{P_1 - P_2}{P_1} \right) \times 100 \
\]

(3)

where \(P_1\) is the weight of the film before drying, and \(P_2\) is the weight of the film after drying and stabilization. MC (\%) corresponds to the grams of water per 100g of film.

3.2 Optical measurements

Optical measurements were used as non-invasive and non-destructive tools to monitor the structural properties of the films at the molecular level and their changes as a function of time. Two main optical methods were utilized for this study, namely fluorescence and Fourier Transform Infrared Spectroscopy (FTIRS). The procedures utilized for both techniques are detailed in the following sections.
3.2.1 Steady-state fluorescence spectroscopy

Fluorescence spectroscopy was used to assess the local characteristics of the matrix using three probes, one intrinsic and two extrinsic ones, with reported environmental sensitivity. Because of this technique’s high sensitivity\textsuperscript{134}, fluorescence-based tests have gained popularity to monitor changes in the local physical properties of thin solid matrices, such as viscosity or rigidity. Steady-state fluorescence spectra of the intrinsic and added probes were recorded using a Fluoromax 4 spectrophotometer (Horiba Scientific Inc., Edison, NJ) (Figure 3.1).

![Fluoromax 4 spectrophotometer equipped with thermostatic chamber](image)

**Figure 3.1: Fluoromax 4 spectrophotometer equipped with thermostatic chamber**

Films were cut and affixed to quartz slides (Technical Glass Products, Snoqualmie, WA) 13.5mm wide x 30mm long x 0.6 mm thick (Figure 3.2) and placed in 1 cm light path quartz cuvettes (FireflySci, Staten Island, NY) at a 45-degree angle. The cuvettes were inserted in the thermostatized testing chamber at 23°C and kept undisturbed for 5 min before testing. Samples exposure to the environment was reduced to an absolute minimum to prevent changes in the moisture content of the films before testing. The samples were placed in holding chambers to prevent their de-equilibration.
while they awaited testing. Long pass filters (ThorLab, Newton, NJ) that attenuate shorter wavelengths and predominately transmit longer wavelengths than their cut off values were used to reduce the contribution of the excitation source to the recorded signal.

Figure 3.2: Films mounted on a quartz slide. Top: control with no added probe but intrinsic one (Tyr), Bottom: film doped with FG

Tyrosine (Tyr), a luminescent aromatic amino acid, is native to porcine gelatin, the kind of structural material used in this study. Its fluorescence and phosphorescence emission has been correlated to the stiffness, solvation, and rigidity of its surroundings, among other characteristics. The fluorescence spectra of Tyr in all films were recorded using an excitation wavelength of 280 nm within an emission wavelength range from 290 to 350 nm. The excitation and emission slits were set at 1 and 2 nm, respectively. A 280 nm long pass filter was utilized. Control spectra were obtained by recording the signal of the quartz slide and the empty cuvette at the same excitation and emission settings as the films. Each sample’s spectrum was corrected by subtracting the spectrum of the control to eliminate background noise (Figure 3.3).
Fast Green (FCF), a luminescent molecular rotor, has the ability to report on molecular crowding in a matrix upon photo excitation\textsuperscript{23, 29}. Tyr and Fast Green (FG) photophysical properties can report on similar characteristics of the films, giving rise to a multiple approach technique to assess the changing molecular environment of a biodegradable food film. However, it is hypothesized that the molecular rotor behavior of FG might confer it higher sensitivity to physical properties such as an increase or decrease in viscosity/rigidity of liquid or solid matrices than that of an intrinsic fluorophore, i.e., Tyr. The fluorescence spectra of FG in the films were recorded using an excitation wavelength of 600 nm over an emission wavelength range from 620 to 700 nm. The excitation and emission slits were set at 1 and 2 nm, respectively. No filter was used in this acquisition as this probe emits in the far red (~655 nm), therefore its emission band is easy to differentiate from background\textsuperscript{29}. Each sample’s spectrum was corrected by subtracting that of the control, i.e., gelatin films without added FG probe measured at the same excitation and emission settings (Figure 3.4).
Pyranine (Pyr) is a solvation probe with dual peak emission. As mentioned before, the sensitivity of this probe to bound and free water in a matrix has been reported. In this study, its addition to the matrix was expected to help monitor changes in water affinity throughout the matrix and overall solvation. The fluorescence spectra of Pyr in the films were recorded using an excitation wavelength of 350 nm over an emission wavelength range from 375 to 575 nm. The excitation and emission slits were set at 1 and 1 nm, respectively. A 400 nm long pass filter was utilized. The sample spectra were corrected by subtracting those of the controls, i.e., films without the addition of pyranine, measured with the same excitation and emission settings (Figure 3.5).
**Figure 3.5: Typical fluorescence emission spectrum of 50μM pyranine in a solid biodegradable gelatin film (λ_{ex}= 350 nm, long pass filter 400 nm)**

Samples were tested every seven days of storage for six consecutive weeks.

Emission spectra were collected using optimized conditions for each probe and analyzed for maximum intensity in counts per second, and energy of the maximum intensity (i.e., location of the peak in nm). Maximum peak intensities during storage were normalized to the same sample’s original intensity on day 0. The spectral data were managed using FluorEssence™ software (Horiba Scientific Inc., Edison, NJ), and later processed using OriginPro (Origin Labs, Northampton, MA).

### 3.2.2 Fourier Transform Infrared spectroscopy

Fourier Transform Infrared Spectroscopy (FTIRS) was used to analyze the secondary structure of the proteins that formed the films. These measurements can also provide unique peaks for the added luminescent probes, FG and Pyr. Changes in the secondary structure of the protein may unveil information about the mechanisms of ageing in the films. Changes in the peaks of FG and Pyr may indicate interactions between the probe and the protein matrix that may influence their corresponding photophysical properties\(^{176, 177}\).
An IRTracer100 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) equipped with an Attenuated Total Reflectance (ATR) MIRacle attachment (Pike, Madison, WI) (Figure 3.6) was utilized to collect the reflectance spectra of the films at frequencies from 400-4000 cm\(^{-1}\). Happ-Genzel apodization functions were calculated using a resolution of 4 and mirror speed of 2.8 averaging a composite of 25 scans. Measurements were taken after removing the films from the casting surface and every 14 days for six consecutive weeks. Films were prepared using double distilled ultrapure water. To evaluate the noise provided by the water in the samples, films with the same composition, prepared using deuterated water (99.9% purity, Sigma, St. Louis MO) as a solvent, were also tested.

![Figure 3.6: Attenuated Total Reflectance MIRacle attachment for FTIR](image)

The spectra were collected using the Lab solutions software (Shimadzu Corporation, Kyoto, Japan) and interpreted in conjunction with KnowItAll spectral database (Bio-Rad, Hercules, CA). Amide I peaks were analyzed to determine protein secondary structure, second derivative plot peaks were deconvoluted\(^{169}\) and quantified using a Lorentzian peak fit function in OriginPro. Each experiment was conducted with a
sample size of two and repeated in triplicate. The Lorentzian equation used to fit the peaks can be expressed as follows:

\[
y = y_0 + \frac{2A}{\pi} \frac{w}{4(x-x_c)^2 + w^2}
\]  

(4)

where \(A\) is the area of the peak, \(x_c\) corresponds to the wavenumber (cm\(^{-1}\)) at the maximum height of the peak, and \(w\) is the width of the peak at half height. Properties of the fitted amide I peaks such as center gravity (cm\(^{-1}\)), full width at half height (cm\(^{-1}\)), and area of the peak (AUC) were used to calculate changes seen in peaks during storage as they correspond to secondary structures of the protein in the films.

3.3 Mechanical properties of the biodegradable films

The films’ mechanical properties were used to assess changes in the structure during the ageing process. As mentioned before, changes in tensile strength of the films impact the functionality of the material and have been extensively observed during ageing processes.

A Universal Testing Machine (Model 5542, Instron Corporation, Norwood, MA) equipped with a 500N load cell and tensile grip attachments was used to determine the mechanical properties of the films in a tension mode (Figure 3.7). Tests were performed on the day of peeling from the casting surface and after 7, 21, and 35 days of storage for all relative humidity environments. Film strips (1.25 cm wide x 10 cm long) were mounted between tensile grips (Instron Corporation, Norwood, MA). The specimens were deformed at a constant speed of 10 mm/min. Acquisition time (s), extension (mm), and load (N) were used to calculate the Engineering stress, \(\sigma_E\), and Hencky strain, \(\varepsilon_H\), as follows:
\[ \text{Engineering Stress} = \sigma_E = \frac{F}{A} \quad (5) \]
\[ \text{Hencky Strain} = \varepsilon_H = \ln \left( \frac{L}{L_0} \right) \quad (6) \]

where \( F \) is the force in Newtons (N), \( A \) corresponds to the cross-sectional area of the film strips in \( \text{mm}^2 \), \( L \) is the momentary length of material in mm at time \( t \), in s, and \( L_0 \) is the initial length of the film strips at time zero.

![Figure 3.7: Instron Universal Testing Machine model 5542 with tensile grip attachments (left), film strip loaded in tensile grips prior to deformation (right)](image)

Maximum engineering stress before break (maximum force exerted on films before failure), toughness (area underneath the curve of stress v. strain), and Young’s modulus (slope of the linear portion of stress v. strain curve) were calculated\(^{178} \). All mechanical tests were conducted with a sample size of six specimens, and the experiments were performed in triplicate.
The methodologies for this study were carefully selected based on the available literature on the characterization of biodegradable food films. Using these methodologies, the characteristics of 2% gelatin films doped with FG or Pyr were tested to assess changes in their structural integrity as a function of time and storage RH. These methodologies seek to unveil the ageing mechanisms of biodegradable food films and help further understand the dynamics that contribute to the loss of functionality of biodegradable food films during usage and storage.
CHAPTER 4
RESULTS AND DISCUSSION

The sensitivity of the selected intrinsic and extrinsic fluorescent probes to report on changes in the local environments of the films during ageing in controlled environments (constant relative humidities and temperature) are discussed in this section. The mechanical properties and secondary structures of the film main component as a function of storage time and conditions are also reported.

4.1 Moisture content in biodegradable films

Moisture content is commonly monitored during film production and storage because water acts as a plasticizer in films and can affect their performance and mechanical properties. Assessing moisture content during storage in this study is particularly important since it will contribute to verify that changes in fluorescence intensity throughout storage are not driven by a significant concentration or dilution of the luminescent probes in the samples.

As shown in Figure 4.1 and Table 4.1, and as expected, the films stored at different relative humidities (RHs) exhibit moisture contents consistent with the ambient RH in which they were stored. For most storage conditions, the moisture content of the specimens did not change during the studied storage period. This observation suggests that in all cases the concentration of the probes did not significantly change during storage and that the samples were appropriately equilibrated prior to testing. The samples stored at 2.5% RH were the exception. At this RH, a significant reduction in moisture content was progressively observed during the whole storage period. The films stored at 2.5% RH continued to dry as their MC reached equilibrium with the environment (Table
This caused the films to experience a significant, though small, decrease, e.g. no larger than 2%, in moisture content at each storage interval. The equilibration rate in the films stored at 2.5% RH was slower due to the state of the water in highly dehydrated films (e.g., bound) and the small driving force due to the moisture content differences between the almost equilibrated films and their environment. The slow equilibration rates in gelatin films was noted in Yakimets and others (2005).

Table 4.1: Moisture content of films stored for 5 weeks at different RHs

<table>
<thead>
<tr>
<th>Storage (weeks)</th>
<th>Moisture content (%) of samples stored at RHs (%) of</th>
<th>2.5</th>
<th>25</th>
<th>33</th>
<th>53</th>
<th>75</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>2.01 ± 0.44&lt;sup&gt;A&lt;/sup&gt;</td>
<td>3.88 ± 0.87&lt;sup&gt;B&lt;/sup&gt;</td>
<td>7.43 ± 1.9&lt;sup&gt;A&lt;/sup&gt;</td>
<td>10.5 ± 2.3&lt;sup&gt;A&lt;/sup&gt;</td>
<td>19.5 ± 1.8&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1.40 ± 0.29&lt;sup&gt;B&lt;/sup&gt;</td>
<td>6.52 ± 0.98&lt;sup&gt;A&lt;/sup&gt;</td>
<td>7.53 ± 2.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>11.5 ± 3.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>20.2 ± 4.1&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.40 ± 0.2&lt;sup&gt;C&lt;/sup&gt;</td>
<td>4.06 ± 1.3&lt;sup&gt;B&lt;/sup&gt;</td>
<td>6.53 ± 1.9&lt;sup&gt;A&lt;/sup&gt;</td>
<td>11.1 ± 2.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>20.2 ± 2&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Significance determined at p<0.05 (triplicate, n=3).

Figure 4.1: Moisture content of gelatin films stored at five RHs (%) reported at 1, 3, and 5 weeks of storage (triplicates, n=5, significance determined at p<0.05)
The results from this study correlate well with those obtained by Acosta and others (2015), 2% gelatin cassava starch composite blend films plasticized with glycerol (25% of polymer w/w) with moisture contents ranging from 7-11% when stored at 53% RH for 5 weeks\(^{180}\). Although the films studied by Acosta and others was a composite of gelatin and cassava starch, the moisture contents are within the same range and indicate similarities between the values obtained from both studies, although the materials of the films must be considered when comparing the two systems.

When stored at 25% RH, the films in this study underwent changes in the moisture content that were not permanent. At 25% RH, a significant increase in the moisture content of the sample was observed after three-week storage, followed by a subsequent decrease in week five. Oses and others (2009) studied the ageing of whey protein isolate films during storage at different RHs. Small changes in moisture content were observed during the 180-day study but remained insignificant from the beginning to the end of the trial\(^{181}\). Temporary changes in moisture content may be attributed to rearrangement of the structure in the proteins and moisture migration within the film\(^{103}\). Georget and others (2006) used FTIRS to monitor wheat protein’s secondary structure as a function of moisture content during heating. Changes in the secondary β-sheet structures were observed at low moisture content (13%) after heating. Upon cooling the secondary structures returned to their initial state\(^{182}\). This shows that systems (proteins) with low moisture content can experience changes in structure and consequently moisture affinity that are reversible. Proteins-solvent interactions confer flexibility to a protein through hydrogen bonding of water to the protein. The amount of H-bonds formed is dependent on molecular arrangement of the protein promoting increased flexibility per
secondary structures hydrogen bonding decreases. The level of hydration of the protein is influenced by its structure, which has been established as subject to change depending on humidity. Since protein hydration is structure-dependent and secondary structure of proteins can be altered during ageing, it can be hypothesized that the slight change in moisture seen at 25% RH, and to some extent those at 2.5%, could be attributed to these effects. The temporal change of MC in the 25% appears to be a short-phenomenon due to structural changes and relocation of water within the films. Studying these films at different but constant MCs allows modulating the films mechanical properties for further testing and comparison using and optical tools and evaluating the effect of RHs on ageing.

4.2 Optical measurements

Luminescence and FTIR spectroscopy can provide insights into structural changes in the films during storage. In the following sections, the photophysical properties of tyrosine (Tyr), Fast Green (FG), and pyranine (Pyr) embedded in gelatin-based films are followed using luminescence spectroscopy. The observed changes in these properties are examined based on potential changes in molecular mobility, molecular crowding, and solvation, respectively. FTIRS (section 4.2.2) was used as a complementary measure to provide insights into processes that drive ageing in films and the sensitivity of the luminescence spectroscopy technique to assess them.

4.2.1 Steady-state fluorescence spectroscopy measurements

Fluorescence spectroscopy techniques allow testing a solid sample non-invasively and in real time. These techniques rely on the presence of environmentally sensitive
fluorophores within the sample that can report on attributes of the local environment, in this particular case, the physical properties and molecular mobility of the matrix. Not only does the fluorescence intensity change with the surrounding medium, but also other photophysical properties, such as energy (emission wavelength), are different in diverse environments. All probes showed a progressive increase in intensity as their surrounding media became more rigid, i.e., water < 2% gelatin gel < 2% gelatin film (data not shown). Besides, these expected changes, spectral shifts were observed in the three systems. The effect of the changing environment on the emission wavelength is presented for Tyr, FG, and Pyr in Figure 4.2.

**Figure 4.2: Emission spectra of Tyr, FG, and Pyr in three solutions: water (orange), 2% gelatin gel (yellow), 2% gelatin film (green)**

Red or solvatochromic shifts have been usually associated with hydrophilic environments around luminescent probes, particularly of aromatic amino acids. However, hybrid quantum mechanical-molecular dynamics simulations have demonstrated that this could be an oversimplification. Although, the exposure of a fluorophore to a more hydrophilic environment often results in a red shift, in water/protein environments any field that channel electrons in the direction of electron transfer will result in a solvatochromic or red shift. The fields in these systems will arise from the dipole moments of all intervening polarizable molecules, and the net effect will determine the direction of the shift. Regardless of the underlying mechanisms of the shift, its presence...
signals orientation pockets in the enclosed water and/or configuration changes in the intervening proteins\textsuperscript{187}. Spectral shifts for a wide range of fluorophores, other than aromatic amino acids, embedded in a “host” medium have extensively been observed due to, for example, probe aggregation or depolarization\textsuperscript{188, 189}. A red shift in structured environments has been reported for other water/protein systems such as whey proteins during liquid antisolvent precipitation\textsuperscript{147}.

In Figure 4.2, tyrosine (Tyr) emission exhibits a modest peak shift as the medium changes from water to gel and to film (see Table 4.2. FG fluorescence emission, on the other hand, underwent a comparatively larger peak shift from 645 nm in water to 651 nm in gel and an additional 4nm shift to 655 nm when in a film. As the rigidity of the matrix increases from gel to film the MR experiences more molecular crowding, which makes the MR favor emission rather than internal rotation as a decay pathway.

A more pronounced, but expected change is seen in pyranine (Pyr), since as it was mentioned in section 3.2.1 Pyr is a probe sensitive to free and bound water. A high intensity peak located at 511nm is seen in the pyranine aqueous solution, and dual emission is observed in the Pyr film. The predominant peak of pyranine in pure water suggests that the majority of pyranine is surrounded by a hydration shell and consequently it is unprotonated. As the free water available decreases when the matrix is replaced by a film, the peak emission attributed to bound water (435m) emerges due to an increase proportion of protonated Pyr.
Table 4.2: Spectral shifts of the used fluorophores in three distinct media relevant to the study

<table>
<thead>
<tr>
<th>Media</th>
<th>Peak location (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Water</td>
<td>307</td>
</tr>
<tr>
<td>2% Gelatin Gel</td>
<td>308</td>
</tr>
<tr>
<td>2 % Gelatin Film</td>
<td>309</td>
</tr>
</tbody>
</table>

The shifts in fluorescence emission of the three probes are reported to exemplify how the characteristics of different media, particularly the water distribution and affinity towards the matrix can affect the photophysical properties of the probes, besides their intensity.

4.2.1.1 Photophysical properties of tyrosine in a biodegradable film during storage

Tyrosine (Tyr), the luminescent aromatic amino acid naturally present in gelatin, was used as an intrinsic probe to explore changes in molecular mobility within the films during storage. The ability of Tyr to report on a matrix’s molecular mobility in solid protein matrices\(^{129,190}\) arises from the changes in its photophysical properties due to modifications in its local environment. For example, if surrounded by an extensive hydration shell, Tyr intensity and lifetime decrease. Conversely, if buried within a protein, its intensity and lifetime increase\(^{143}\).

Figure 4.3 shows the extent of the increase in Tyr fluorescence intensity as a function of time at different RHs. The data in Figure 4.3 were collected in films stored at 2.5, 33, and 75% RH after equilibration (before storage – 0 week), and after 3 and 5 weeks of storage. The data were normalized to the initial intensity for each relative humidity to facilitate comparison. The relative increase in fluorescence intensity for Tyr in the films stored at 2.5% RH is the highest during the studied period, followed by 33%
RH and 75% RH, suggesting that the changes observed during storage were more predominant when the films were kept at lower RHs.

![Figure 4.3: Emission spectra of Tyr in gelatin films stored at 2.5 (left), 33 (middle), 75 (right)% RH for zero (blue), 3 (orange), and 5 (gray) weeks](image)

As the RH increases, the differences between 0, 3, and five weeks of storage becomes less predominant. This effect is further displayed in Figure 4.4, in which the emission intensity is normalized to the initial sample emission. This figure shows that, at the beginning of the study, the spectra for all films follow a similar pattern, albeit a lower intensity for films stored at higher RH (75%). After five-week storage, the Tyr maximum intensity in samples kept at 2.5% RH increased about 2-fold compared to that of the samples stored at 75% RH.

![Figure 4.4: Emission spectra of Tyr in gelatin films stored at 2.5 (green), 33 (blue), 75 (yellow)% RH after initial equilibration (left) and five weeks of storage (right)](image)

The relative fluorescence intensity of Tyr in films as a function of time for all RHs studied is reported in Figure 4.5. Changes in Tyr maximum fluorescence intensity
during the entire storage study are more prominent at lower RHs, e.g., 2.5 and 25%. Not only are significant differences observed at low RHs, but there is a notable increase in intensity in all samples near the end of their storage. Additionally, the onset of perceivable changes differs among samples, the lower the RH, the sooner the changes appear. These results suggest that potential changes in the conformation of the proteins during storage restrict the mobility of the probe, resulting in an extensive increase in intensity. Additionally, it can be inferred that these conformational changes initiate earlier and are more extensive in samples stored at lower RHs. This hypothesis is further discussed in section 4.2.2.
Figure 4.5: Maximum normalized fluorescence intensity of tyrosine in gelatin films stored at different RHs for five weeks (Triplicate, n=3, significance at p<0.05)

The mean of the maximum emission intensity of Tyr during storage and their standard deviations are summarized in Table 4.3. The results show a 2.8-fold increase in Tyr emission after five weeks of storage at 2.5% RH, but only a 0.3-fold increase when stored at 75% RH. It should be noted that the extent of changes might be underestimated due to the normalization procedure selected, i.e., normalization to the initial intensity. It should be explored if revisiting the normalization procedure to better account for initial fluorescence intensity at t=0 is necessary.
Table 4.3: Normalized maximum intensity of Tyr as a function of storage at different RHs

<table>
<thead>
<tr>
<th>Storage (weeks)</th>
<th>Normalized fluorescence intensity (-) of Tyr in samples stored at the following RHs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>0</td>
<td>1.00 ± 0.00^A</td>
</tr>
<tr>
<td>1</td>
<td>1.01 ± 0.05^A</td>
</tr>
<tr>
<td>2</td>
<td>0.96 ± 0.06^A</td>
</tr>
<tr>
<td>3</td>
<td>1.53 ± 0.20^A</td>
</tr>
<tr>
<td>4</td>
<td>2.56 ± 0.78^B</td>
</tr>
<tr>
<td>5</td>
<td>2.79 ± 0.63^B</td>
</tr>
</tbody>
</table>

Significance determined at p≤0.05 (triplicate, n=3)

As it was mentioned before, spectral shifts provide information on conformational changes. In these samples, non significant (p≤0.05) but moderate red shifts, ≤6nm, were observed in films stored at 2.5 % RH decreasing in magnitude at subsequent RHs (see Table 4.4). This might suggest a more extensive conformational change in samples stored at low RHs.

Table 4.4: Peak location of the maximum intensity of Tyr in gelatin films stored at different RHs for five weeks

<table>
<thead>
<tr>
<th>Storage (weeks)</th>
<th>Peak location of the maximum intensity of Tyr in samples stored at the following RHs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>0</td>
<td>302 ± 2.4</td>
</tr>
<tr>
<td>1</td>
<td>305 ± 1.9</td>
</tr>
<tr>
<td>2</td>
<td>306 ± 1.8</td>
</tr>
<tr>
<td>3</td>
<td>305 ± 3.0</td>
</tr>
<tr>
<td>4</td>
<td>308 ± 0.9</td>
</tr>
<tr>
<td>5</td>
<td>308 ± 0.9</td>
</tr>
</tbody>
</table>

Triplicate, n=3

Changes in polymer-polymer and water/plasticizer- polymer interactions, increased rigidity, and protein conformational changes may act in concert in a biodegradable film as it ages. All these processes may affect fluorescence intensity during storage, since they contribute to rearrangements in the protein structure\textsuperscript{143}. The
results reported in Tables 4.3 and 4.4 suggest that during storage the local environment around the Tyr moieties is losing flexibility and becoming more rigid. Conformational changes that could result in the amino acids being buried within the protein matrix would restrict the solvation and movement of amino acids. This scenario is supported by the data since buried/sheltered amino acids have higher fluorescence intensities than exposed or solvated ones. It is critical to note that gelatin from porcine skin consists 21 tyrosine amino acids, which due to structural arrangements may be exposed to different environments within the same film. The results observed correspond to the net emission of all tyrosines in their potentially distinct local pockets.

Anker and others (2001) have attributed ageing to changes in moisture distribution within a matrix during storage. Changes in solvation have been linked to the migration of hygroscopic plasticizers to the surface of films due to low binding constants of proteins and the plasticizer. Eventual migration of the plasticizer during aging is inevitable and has been reported in many studies. Anker and others (2001) showed the migration of plasticizer (glycerol) in whey protein isolate films stored at 50% RH for 45 days. Park and others (1994) also reported glycerol migration in corn-zein protein films after 20 days of storage at 50% RH and correlated this to increases in tensile strength, but decreases in film elongation (%), which suggest that the material was becoming more brittle. Not surprisingly, Georget and others (2006) reported that plasticizer migration is higher in films stored at low RHs and that moisture content within the film and plasticizer are the driving forces in the restructuring of the protein matrix during ageing. The above studies provide evidence of a change in the matrix to a stiffer, less flexible
structure consistent with the increase in Tyr fluorescence emission during storage, particularly at low RHs.

4.2.1.2 Photophysical properties of Fast Green in a biodegradable film during storage

Fast Green FCF (FG) exhibits molecular rotor behavior and has been previously utilized to report on the physical properties of food model systems and products\textsuperscript{22, 23}. FG was used as an extrinsic probe to monitor the matrix’s molecular crowding in the biodegradable gelatin films during storage. Due to its segmental mobility, FG can relax from the excited stated predominately with no emission in fluid environments and radiatively when the probe is immersed in a more rigid environment\textsuperscript{150}. Increased fluorescence emission is indicative of movement restriction within the matrix. For this reason, FG is a probe sensitive to changes in physical properties of several matrices.

Before including the probe within the films, it is important to identify an adequate concentration of the fluorophore to avoid inner filter effects during testing. This requires the probe concentration to be high enough to provide a signal within limits of detection and sensitivity of the equipment, yet low enough to avoid reduction in emission intensity due to re-absorbance of the emitted fluorescence\textsuperscript{29}. Figure 4.5 depicts the results of the FG concentration study conducted in gelatin films, the change of pattern in the relationship at high concentrations is evidence of inner filter effects. The selection of the concentration of FG in the final films was determined by selecting a molarity within the linear region of the relationship that also allows detecting a signal when the intensity increased and decreased multi-fold without reaching either inferior or superior limit of detection on the equipment.
Significant changes in FG emission intensity during storage time were observed at all the RHs studied. Figure 4.6 shows the emission spectra at three RHs, i.e., 2.5, 33, 75%, and storage periods, i.e., after equilibration (no storage), 3 and five weeks. The maximum normalized intensity of FG in samples stored in 2.5% RH increase from 1 to ~35 after three weeks of storage and ~45 after five weeks. These large differences might be attributed to FG being in a more crowded or rigid environment, which restricts the movement of the probe and results in higher emissions throughout. This trend is observed during ageing at all the RHs studied, however, the effect is less pronounced when the samples were stored at higher RHs.

Figure 4.7 shows that all films started with a similar FG fluorescence emission intensity, when normalized to their initial values. However, after five weeks of storage,
the samples stored at 2.5% RH exhibited a ~15-fold increase in FG emission intensity, while the increase of the samples at 75% RH after the same storage period was only 2-fold. Based on these results, it can be inferred that the environment around FG in the 2.5% RH films might be more rigid due to a lack of hydration than that of 75% RH.

Figure 4.8: Emission spectra of FG in gelatin films stored at 2.5 (green), 33 (blue), 75 (yellow) % RH after initial equilibration (left) and 5 weeks of storage (right)

Figure 4.8 compares the maximum fluorescence intensity of FG during the entire storage study at all RHs. Larger differences in intensity were observed in the samples stored under drier environments, i.e., 2.5 and 25% RH. It should be noted that significant increases in intensity were observed in all samples near the end of the storage period. As for tyrosine, the onset of significant differences in intensity is later in samples stored at high RHs.
Table 4.5 summarizes the changes in maximum fluorescence intensities of FG during film storage. A ~11-fold increase in FG intensity is observed at the end of the storage period when the samples were kept at 2.5% RH. However, only a 0.2-fold increase was detected in samples equilibrated at 75% RH. The drastic relative change, in comparison to the values observed for Tyr, reinforces the notion that molecular rotors, due to their emission mechanism, have higher sensitivity to matrix rigidity than other type of fluorescence probes. Consequently, in principle, they could be deemed better.
reporters of ageing in biodegradable packaging. Like the trends seen in the tyrosine emission results, the onset of significant changes occurred sooner at 2.5% RH than at any other RH.

**Table 4.5: Normalized maximum intensity of FG as a function of storage relative humidity and time**

<table>
<thead>
<tr>
<th>Storage (weeks)</th>
<th>Normalized Fluorescence Intensity of FG in samples stored at the following RHs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>0</td>
<td>1.00 ± 0.00&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>1.18 ± 0.08&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>1.14 ± 0.13&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>2.84 ± 2.21&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>7.68 ± 5.86&lt;sup&gt;BC&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>11.1 ± 6.46&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Significance determined at p≤0.05(triplicate, n=3)

The large increase in fluorescence intensity in the films stored at 2.5% RH, could have been driven by an increase in concentration due to further equilibration of the samples moisture content during storage (See Section 4.1). However, it should be noted that the further loss of moisture in the films from 2.01 to 0.40%, could result only in a change in concentration from 0.124 mM to 0.136 mM. Based on the results in the concentration study (Figure 4.6), this change in concentration accounts for an increase in intensity of less than 7.5%. Therefore, this increase in concentration could not be responsible for the observed changes in the films stored at 2.5% RH.

No significant in peak shifts were observed in any of the films during storage. Non significant (p≤ 0.05) but moderate blue shifts, approximately <5nm were observed in films stored at all RHs (Table 4.6). Since these shifts were consistent in the two most extreme storage conditions, it would be extreme to consider the information on peak shift to be indicative of changing dynamics in the protein, making this property an inadequate measure of the changing structure of the films.
Table 4.6: Maximum peak intensity location statistics of FG emissions in gelatin films stored at different RHs for five weeks

<table>
<thead>
<tr>
<th>Storage (weeks)</th>
<th>Peak location of the maximum intensity of FG in samples stored at the following RHs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>0</td>
<td>657 ± 3.0</td>
</tr>
<tr>
<td>1</td>
<td>655 ± 0.9</td>
</tr>
<tr>
<td>2</td>
<td>655 ± 0.7</td>
</tr>
<tr>
<td>3</td>
<td>657 ± 1.0</td>
</tr>
<tr>
<td>4</td>
<td>655 ± 1.4</td>
</tr>
<tr>
<td>5</td>
<td>655 ± 1.2</td>
</tr>
</tbody>
</table>

Experiments conducted in triplicate (n=3)

4.2.1.3 Photophysical properties of pyranine in biodegradable films during storage

As it was previously mentioned, pyranine, upon photoexcitation at 350nm exhibits dual emission bands at 420-445 nm and near 500-515 nm. This probe was used to potentially monitor the solvation status of the gelatin films during storage.

The results of the concentration study to optimize the amount of Pyr in the films are shown in Figure 4.9. The Pyr concentration was selected based on the final concentration that would provide a large enough signal to noise ratio, but not so high that would ensue inner filter effects. A 0.05 mM concentration was selected to ensure that the intensity could increase or decrease without surpassing the inferior or superior limit of detection of the equipment.
Figure 4.10: Emission spectra of Pyr at different concentrations in gelatin films (left). Normalized fluorescence intensity as a function of Pyr concentration (right)

Figure 4.10 shows the relationship of spectra of Pyr in films stored at 2.5, 33, and 75% RH after equilibration (before storage), and after five weeks of storage. It can be seen the relative increase in Pyr fluorescence intensity at 2.5% RH is the largest during the storage period, following a smaller change at 33% RH and the smallest amount of change at 75% RH.

Figure 4.11: Emission spectra of Pyr in gelatin films stored at 2.5 (left), 33 (middle), 75 (right)% RH for zero weeks (blue), 5 weeks (orange)

The ratio of fluorescence intensity of the protonated and deprotonated Pyr in films during storage as a function of RH is shown in Figure 4.12. A paired significance test shows no significant difference in the ratios during storage for any of the RH environments. However, a progressive reduction in the ratio for all samples was observed as the samples aged (See Table 4.7). These observations suggest that there might be some
water migration or changes in the hydration shells around the probe along storage. A smaller ratio ensues due to the increase in intensity of the unprotonated tautomer band, a reduction in intensity of the protonated tautomer band or both, which equates to less free water available inside the matrix.

Figure 4.12: Ratio of pyranine fluorescence intensity (FI) at 430 nm and 510 nm in gelatin films stored at different RHs for five weeks (triplicate, n=3)

Table 4.7: Ratios of Pyr intensity at 430 and 510 nm in gelatin films stored at different RHs for five weeks

<table>
<thead>
<tr>
<th>Storage (weeks)</th>
<th>Ratio of intensities at 430 and 510 nm in samples stored at the following RHs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>0</td>
<td>2.66 ± 0.27</td>
</tr>
<tr>
<td>1</td>
<td>2.64 ± 0.16</td>
</tr>
<tr>
<td>2</td>
<td>2.47 ± 0.20</td>
</tr>
<tr>
<td>3</td>
<td>2.55 ± 0.14</td>
</tr>
<tr>
<td>4</td>
<td>2.59 ± 0.32</td>
</tr>
<tr>
<td>5</td>
<td>1.73 ± 1.08</td>
</tr>
</tbody>
</table>

Triplicate, n=3
It was hypothesized that a decrease in the ratio would be seen in films during storage. Statistically no significant changes (p ≤ 0.05) in the Pyr emission ratio were observed. However, redistribution of the water within the films cannot be ruled out based on the trends observed.

A significant change (p ≤ 0.05) in moisture content in the films stored at ~2.5% RH was observed during storage (Figure 4.1). Although a change is observed in the ratio after 5-week storage at that RH, the scatter of the measurements does not render that difference significant.

Kashi et al (2015) reported Pyr intensity ratios of 2.95 for hydrocolloids solutions at 2% and 1.32 in 70% sucrose solution. In this study the ratios for all films were about 2.5 ± 0.5. These values indicate that the amount of free water in the films was less than in hydrocolloids solutions, however extensive hydration shells existed around the probes and that the water has a higher molecular mobility than in highly molecular crowded systems such as concentrated sugars or polyol solutions.

4.2.2 Fourier transform infrared spectroscopy

The use of Fourier Transform Infrared Spectroscopy (FTIRS) to assess the types of secondary structure most prevalent in protein films as a function of storage time and RH is not a novel. It has been used to assess miscibility of antimicrobial agents in film matrices, and changes in structural properties in protein-based films. FTIRS in this study was used to obtain supplemental information on the kind of changes observed in the films and to facilitate explaining changes in the luminescent properties of Tyr and FG embedded in films during aging.
A comparison of the absorption spectra of purified gelatin from a database and the 2% gelatin films used in this study is shown in Figure 4.13. The characteristics peaks in the fingerprint region (1500-500 cm⁻¹) of the two spectra line up well with each other.

![Figure 4.13: Comparison of the FTIR spectrum of the 2% gelatin film without plasticizer and the reference spectrum of purified bovine gelatin from KnowItAll spectral database. Fingerprint region bound by dashed line](image1)

FTIRS was carried out on a sample of FG solubilized in deuterated water D₂O to determine the characteristic bands of the probe (See Figure 4.14). The tested FG sample had a low relative absorbance that never reached above 0.1 AU. The use of D₂O was effective at reducing the background noise since there was no contribution from the solvent due to H-O-H bending²⁴, 192, 193. This rationalized the use of D₂O in sample preparation of films as a comparison to samples prepared with water.

![Figure 4.14: Comparison of the FTIR spectra of Fast Green from a deuterated water sample and reference from KnowItAll spectral database](image2)
The spectrum of 2% gelatin films doped with FG was compared against the spectrum of purified gelatin from the spectral database (Figure 4.15). This was done to assess if any interactions between the FG and gelatin or alterations within the amide I region could be seen. No spectral shifts were observed in the spectra upon the addition of FG.

![Figure 4.15: Comparison of the FTIR spectrum of 2% gelatin-glycerol-FG film and the reference spectrum of purified bovine gelatin from KnowItAll spectral database](image)

The films were subjected to storage at 5 RHs, and their FTIRS spectra were collected every other week. Figure 4.16 shows the spectra of the films during storage at 2.5% RH at the beginning and end of this study. A decrease in the absorbance intensity and minor spectral shifts were observed. Films were only analyzed after equilibration (no storage) and at the end of 5 weeks of storage.
Based on a literature review which indicated that most prevalent changes in biomaterials films were observed in the amide I region of the spectrum, the absorption bands within the amide I region (1600-1700 cm\(^{-1}\)) were analyzed. Fourier deconvolution was needed to obtain distinct peaks within the amide I region. A Lorentzian peak fitting protocol was applied to the amide I regions and a sample of this fit is presented in Figure 4.17.
Figure 4.17: Example of the deconvolution of the amide I peak. Two peaks (green) were fit using Lorentzian peak fit equation (Eq. 2) (green), combined spectra from peak fits (red) were calculated and compared to original second derivative of spectra (black).

As it can be seen in Figure 4.17, two major peaks have been identified. The parameters of the fits corresponding to both peaks, and their respective areas including their changes during storage at all the RHs studies are summarized in Tables 4.8 and 4.9, respectively.

Table 4.8: Parameters of the Lorentzian fit of the first peak in amide I region of the films' spectra

<table>
<thead>
<tr>
<th>Relative Humidity (%)</th>
<th>Storage (weeks)</th>
<th>Fit area (%)</th>
<th>Center of Gravity (cm⁻¹)</th>
<th>FWHM (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>equilibrated</td>
<td>0</td>
<td>47 ± 1.5</td>
<td>1656 ± 0.3</td>
<td>18.0 ± 0.6</td>
</tr>
<tr>
<td>2.5</td>
<td></td>
<td>64 ± 0.8</td>
<td>1658 ± 0.4</td>
<td>17.0 ± 0.7</td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>74 ± 2.5</td>
<td>1655 ± 0.4</td>
<td>17 ± 0.4</td>
</tr>
<tr>
<td>33</td>
<td></td>
<td>71 ± 3.1</td>
<td>1655 ± 0.2</td>
<td>17 ± 0.4</td>
</tr>
<tr>
<td>53</td>
<td></td>
<td>70 ± 4.9</td>
<td>1656 ± 0.8</td>
<td>17 ± 1.1</td>
</tr>
<tr>
<td>75</td>
<td></td>
<td>72 ± 4.3</td>
<td>1655 ± 0.9</td>
<td>18 ± 0.4</td>
</tr>
</tbody>
</table>

Triplicate, n=2
Two main structures could be predominantly identified in the films based on the analysis of the amide I region. Based on typical band assignments, the bands could be attributed to $\alpha$-helix (~1655 cm$^{-1}$) and $\beta$-pleated (~1627 cm$^{-1}$). The relative proportions of these two structures changed as a function of RH and storage time. During storage, regardless of the RH, the area of the band attributed to $\alpha$-helix increase (Table 4.8) and that attributed to $\beta$-pleated (Table 4.9) decreased. According to Zhu and Bundle (1996), $\alpha$-helix secondary structures present lower water accessibility than $\beta$-pleated ones, which corresponds well with an increase in rigidity of the biodegradable films and increases in the fluorescence emission intensity of the studied probes due to water delocalization and reduce plasticization at local points throughout the matrix.

Table 4.9: Parameters of the Lorentzian fit of the second peak in amide I region of the films’ spectra

<table>
<thead>
<tr>
<th>Relative Humidity (%)</th>
<th>Storage (weeks)</th>
<th>Fit area (%)</th>
<th>Center of Gravity (cm$^{-1}$)</th>
<th>FWHM (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>equilibrated</td>
<td>0</td>
<td>53 ± 1.5</td>
<td>1631 ± 0.2</td>
<td>14 ± 0.6</td>
</tr>
<tr>
<td>2.5</td>
<td>0</td>
<td>36 ± 0.8</td>
<td>1629 ± 0.3</td>
<td>11 ± 0.5</td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>27 ± 2.5</td>
<td>1627 ± 0.5</td>
<td>8 ± 0.8</td>
</tr>
<tr>
<td>33</td>
<td>5</td>
<td>30 ± 3.1</td>
<td>1627 ± 0.3</td>
<td>9 ± 0.4</td>
</tr>
<tr>
<td>53</td>
<td>5</td>
<td>30 ± 4.9</td>
<td>1627 ± 0.5</td>
<td>9 ± 0.8</td>
</tr>
<tr>
<td>75</td>
<td>2</td>
<td>28 ± 4.3</td>
<td>1628 ± 0.5</td>
<td>9 ± 0.9</td>
</tr>
</tbody>
</table>

Triplicate, n=2

As it was mentioned in previous sections, Anker (2001) reported that ageing was accelerated by migration of plasticizers\textsuperscript{18}. Similarly, Duconseille and others (2017) attributed ageing to the increase of intermolecular bonds decreasing protein-solvent interactions and increasing protein-protein ones\textsuperscript{121}. Although this will be aligned with the observations in these experiments, it should be noted that Duconseille and others reported changes from predominantly triple helix to random coil and $\beta$-turn in their samples. The
differences could be related with a different protein source or the lack of identification of 
a peak for unordered structures in the biodegradable films studied. The relatively large 
full width at half maximum (FMWD) values could support the latter. Additionally, it 
should be noted that frequency ranges and their correspondence to specific secondary 
structures have been reported to be to some extent sample specific and exhibit shifts 
depending if the protein is tested in solution or a film (See Table 2.8).

To better understand how the secondary structure of proteins play a role in film 
ageing and how it correlates to the fluorescence spectroscopic results, the peaks in amide 
I could be corroborated with amide III. The use of the III region is currently common to 
corroborate the results of amide I peaks. A further investigation of the mechanisms 
of protein ageing as a function of secondary structure would be beneficial in identifying 
the intermolecular interactions that increase rigidity in protein films.

4.3 Mechanical measurements

Maximum engineering stress, toughness, and Young’s modulus were estimated 
from stress strain curves of the films during storage. These relationships were recorded in 
tension mode using a UTM. Typical engineering stress v. Hencky strain of the films 
stored at different RHs are presented in Figure 4.18.
Figure 4.18: Engineering Stress (σE) versus Hencky’s strain (εH) relationship of gelatin films stored at 2.5% (yellow), 33% (blue), and 75% (green) RH (%) after one week of storage (left) and five weeks of storage (right)

The highest engineered stress, σE, after both 1 and five weeks corresponded to the samples stored at 2.5% RH. The εH of the films before break increased after 5 weeks at 2.5% RH but decreased in 33 and 75% RH. This increase in strain before the break is typical of a more rigid matrix with increased tensile strength. The gelatin films can be classified as a ductile material based on the shape of the curve and the characteristic linear region of stress and strain relationship, and during ageing the same shape was maintained with increases in tensile strength in samples stored at drier RHs.

Table 4.10: Maximum Engineering Stress (σE) of samples stored at different RHs for five weeks

<table>
<thead>
<tr>
<th>Storage (weeks)</th>
<th>Engineering Stress (σE) in MPa of samples stored at the following RHs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>25</td>
</tr>
<tr>
<td>1</td>
<td>4.7 ± 0.4^A</td>
</tr>
<tr>
<td>3</td>
<td>5.1 ± 0.2^A</td>
</tr>
<tr>
<td>5</td>
<td>6.0 ± 0.2^B</td>
</tr>
</tbody>
</table>

Triplicate, n=6, significance at p<0.05

Figure 4.19 shows the changes in maximum engineering stress, σE, as a function of storage time and RH (figure 4.19). The differences in engineering stress at different RHs are significant, but due to the data scatter changes during storage within the same
RH are not as apparent. Significant changes were just observed in the samples during the last week of storage at 2.5% RH.

Figure 4.19: Maximum $\sigma_E$ of gelatin films stored at 5 RHs (%) for five weeks. (triplicates, $n=6$, significance determined at $p<0.5$)

Toughness was recorded and plotted as a function of storage time and RH in Figure 4.20, the values are also shown in Table 4.11. High variability without significant change is seen during storage. It should be noted that in all the tests, errors above 20-30% were recorded. This is one of the issues highlighted in the drawbacks of mechanical testing of these types of materials. Probably due to the high scatter, the only significant changes in toughness was seen in 2.5% and 25% RH after five weeks of storage, which matched the changes observed in $\sigma_E$. 
Table 4.11: Toughness (AUC) of samples stored at different RHs for five weeks

<table>
<thead>
<tr>
<th>Storage (weeks)</th>
<th>2.5</th>
<th>25</th>
<th>33</th>
<th>53</th>
<th>75</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.31 ± 0.05&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.09 ± 0.04&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.34 ± 0.09&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.06 ± 0.01&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.02 ± 0.02&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>0.34 ± 0.05&lt;sup&gt;AB&lt;/sup&gt;</td>
<td>0.26 ± 0.11&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.28 ± 0.15&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.04 ± 0.02&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.03 ± 0.02&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>0.51 ± 0.22&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.34 ± 0.09&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.20 ± 0.09&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.03 ± 0.21&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.01 ± 0.01&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Triplicate, n=6, significance at p<0.05

Figure 4.20: Toughness of gelatin films stored at 5 RHs (%) for five weeks (triplicates. n=6, significance determined at p<0.05)

Lastly, Young’s moduli were calculated from the linear slope of the stress v. strain curve and plotted as a function of storage time and RH in Figure 4.21. Less variability in these measurements are seen when compared to toughness, but the data is still fairly spread. Trends are similar to that of maximum engineering stress.

Table 4.12: Young’s modulus of samples stored at different RHs for five weeks

<table>
<thead>
<tr>
<th>Storage (weeks)</th>
<th>Young’s modulus (MPa) of samples stored at the following RHs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>1</td>
<td>94.5 ± 8.5&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>94.8 ± 8.5&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>94.8 ± 5.7&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Triplicate, n=6, significance at p<0.05
The differences in the mechanical properties of the films stored at different RHs are presented in Figures 4.19–4.21. The reported mechanical properties of the films in this study were in some cases an order of magnitude less than published work. These differences in the measurements might have been caused by differences in the procedure, specifically the size of the sample, between this study and other published work. This discrepancy shows that the geometry of film strips tested highly contributes to the observed values and variability of measurements, another disadvantage of mechanical tests. It should be noted that in the case of optical based measurements, only the sample thickness not the overall, size of the tested surface will affect the measurement, reducing its variability.
Nonetheless, as expected, storage RH and water absorption play a significant role in these properties, due to water’s role as a plasticizer that modulates the stiffness of the films. Consequently, the re-location of water with a biodegradable film matrix, as suggested by the results, possibly drives the increase in maximum engineering stress and toughness after five weeks of storage at 2.5% RH.

In overall, the studied films showed a comparable mechanical behavior during storage to those previously published. For example, Anker and others (2001) studied changes in the mechanical properties of whey protein films during storage. These authors observed an increase in tensile strength and a decrease in the strain at break after storage for 120 days. Although the trends in the gelatin films studied here are in line with Anker and others’, the gelatin samples were only tested for a shorter time, i.e., less than a third of the duration. It could be hypothesized that the gelatin films in the current studied will perform similarly after storage during longer periods as it was observed for glutenin films plasticized with glycerol for 16 weeks, soy protein isolate films after 14 weeks, and after 13 weeks. Several studies use the standard of a five-week storage test, but increasing the length of storage could be beneficial to further evaluate and verify the trends observed in terms of mechanical properties.

Biodegradable films require a thorough characterization of their mechanical properties for application purposes, and traditional tensile tests are the standard methodology to achieve these measurements. Mechanical testing of the gelatin samples in this study showed minor changes in engineering stress, toughness, and Young’s modulus as a function of storage time. On the contrary, changes in fluorescence intensity of the probes embedded in the films suggested that structural changes in the films start earlier.
than detected in the matrices studied. Therefore, the last section of this chapter is devoted to assessing correlation between mechanical and optical parameters to understand their respective applicability.

4.4 Correlation between optical and mechanical properties

Both mechanical and luminescent techniques have provided valuable information about the studied matrices and their changes during storage. To further compare mechanical and fluorescent properties of the films, the normalized fluorescence intensity of the embedded probes, Tyr or FG, was plotted vs, the maximum engineering stress for all samples, storage times and RHs (See Figure 4.22). The results show that the optical measurements are more effective at detecting changes in relatively rigid matrices. The changes in the optical measurements were more noticeable once a certain level of rigidity in the matrix was attained.

![Figure 4.22: Normalized fluorescence intensity of Tyr (left) and FG (right) versus engineering stress, $\sigma_E$, of all films during storage at all RHs fitted with Eq. 6](image)

Similar results, in terms of the optical probe’s sensitivity to report physical properties, were observed when using a molecular rotor, namely Citrus Red 2, to measure the microviscosity of an oil within a fat crystal network. The relationship between the optical and the physical properties of the fat crystal networks could be appropriately
characterized using a logarithmic exponential model. This model was slightly reworked for this study and it can expressed as:

\[ F(I, \sigma_E) = a + \ln\left[ 1 + e^{k(\sigma_E - \sigma_c)} \right] \]  

(7)

where \( a \) is the baseline intensity of equilibrated films, \( k \) is the extent of the change of the normalized fluorescence intensity as a function of the engineering stress, and \( \sigma_c \) is the critical at which a pronounced increased in fluorescence intensity is observed.

The \( \sigma_c \) parameter in this model provides information on the sensitivity of the probes, by providing a cut off value of engineering stress at which the optical parameters show higher responses. For Tyr and FG, the \( \sigma_c \) value was about 5 MPa, which indicates that the sensitivity of the probes is enhanced in the same type of matrices. Although \( \sigma_c \) were similar for the tested probes, the extent of the increase denoted by the parameter \( k \) was significantly higher for FG than Tyr, 9.3 vs 1.7 MPa\(^{-1}\), respectively.

To better understand the correlations presented in this section, two factors pertaining to the involved methods should be addressed. First, it is important to recognized that optical and mechanical test involved measurements at two different scales. Mechanical tests report on the bulk physical properties of the film, while optical techniques based in lumiphores with environmental sensitivity report on local molecular environments. Because bulk and local properties of a film matrix may be different, therefore the sensitivity and physical meaning of both measurements may or may not overlap. Secondly, the relationship is affected by the normalization procedure employed for fluorescence emission intensity. To best show the differences in fluorescence intensity of Tyr and FG, each sample emission through storage was normalized to the original intensity of that film after equilibration (before storage). Therefore, this normalization
procedure reflects the differences in the films stored at a RH from the beginning to the end of the study rather than the differences among the films at different RHs.

The correlation of mechanical and photophysical properties of Tyr and FG emphasizes the different principles behind the techniques that can mainly report on changes at the bulk (mechanical) and local (optical) level and how they relate to structural changes in the films during ageing.
CHAPTER 5

CONCLUSIONS AND FUTURE WORK

Biodegradable food packaging could reduce the amount of petro-based waste in the environment. The inherent biodegradability of these materials, although desirable for disposal purposes, make bio-based food films less stable during storage. This could also decrease the shelf life of the food product they contain. Being able to easily evaluate the stability of the extant and novel packaging material in a more convenient rapid and sensitive manner can contribute to extend shelf life, ensure food safety, and increase the penetration of more sustainable materials into the market to replace petro-based ones.

This study aimed to determine the feasibility of using a luminescent technique to evaluate the physical state of biodegradable food films during ageing. To this end, two fluorescent probes Tyr and FG were used for their high environmental sensitivity to sense structural changes in their surrounding matrices.

Significant increases in fluorescence intensity during storage at low RHs was observed using both probes. Tyr emission intensity is normally higher in rigid matrices, for example when buried in a protein matrix not exposed to solvent or molecular oxygen that can quench its fluorescence emission. FG, a luminescent molecular rotor, is highly sensitive to molecular crowding through a few potential mechanisms like the formation of the TICT state or cis-trans tautomerization. Although both Tyr and FG could report on changes within their local environment, that correlated to other signs of ageing in the films, FG sensitivity was proven to be higher based on the extent of the response to the same stimuli and the equal critical mechanical parameters above which both probes were sensitive. For samples stored at the same amount of time at the same RH, the increase in
fluorescence emission of FG in that matrix could be up to 4 times higher than that of Tyr. The restriction of internal rotation in FG in the films seemed to confer it higher sensitivity than Tyr, i.e., smaller changes in structure resulted in higher changes in intensity. Besides its presumed sensitivity to ageing, the status of FG as an approved color in foods could facilitate its operationalization as a sensor of ageing in biodegradable films without raising regulatory or toxicological concerns.

Although the results in this study suggest that MRs such as FG could be adequate sensors of ageing, before their potential implementation in food films, a thorough characterization of their advantages and limitations should be performed. The selection of a MR that best suits the needs of a specific product must be identified. This study selected a single MR that was applied to a single protein matrix as a proof of concept. Biodegradable films are made from a plethora of biomaterials, therefore the performance of Tyr or FG within different matrices may significantly differed from the one reported here, i.e., it could be better, worse, or even nonexistent. Thus, the efficacy of this approach with other MRs or intrinsic probes such as Tyr and other matrices should be explored.

The use of the optical probes applied in this study, might not be sufficient to scout and characterize the physical properties of a films as it ages. In films stored at high RHs, limited changes were seen in fluorescence intensity of either fluorophore during storage. Although no changes were observed in the mechanical properties of these films using a tensile test, their FTIR spectra indicated that protein conformation had changed at the end of the study. These changes were not detected by either the mechanical or optical approach.
The luminescent probe Pyr was used to monitor changes in the state of the water within the films, however it showed limited sensitivity in the studied matrices potentially due to insignificant changes in moisture content through storage. In contrast, Tyr and FG could report on changes in the local environment and provided quantifiable differences in structure of a matrix at the beginning and end of the study.

This study used a complimentary optical technique, FTIRS, to provide insights on the film matrix as it aged. FTIRS was used to determine the secondary structure of proteins within the films as a function of RH and storage time. Shifts in the protein structure from 50:50 $\alpha$-helix and $\beta$-sheet at the beginning of the study to a predominantly $\alpha$-helix structure at the end were observed. Both secondary structures are more structured than random coil, however further analysis of the data should be pursued to establish the effect of these changes on the physical properties of the films or its ageing.

This study is the first to report a luminescence spectroscopy technique based on lumiphores with environmental sensitivity to assess ageing in biodegradable food films. In principle, this technique was able to detect potential changes in a matrix as it ages earlier and more accurately than conventional mechanical tests. Beyond the use in biodegradable food films, these methods could be applied to traditional petro-based materials. Advancements in assessing ageing via luminescent spectroscopy techniques would help make more biodegradable packages with better mechanical properties faster by simplifying the testing procedures. By speeding the development of this type of packaging, more options will be available to food manufacturers and industry as alternatives to petro-based plastics.
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