Basic Strategies and Testing Methods to Develop Effective Edible Antimicrobial and Antioxidant Coating

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1. Introduction

Due to the continuous increase in microbial outbreaks originated form minimally processed and ready-to-use food [1,2], the active food packaging has become one of the top research topics in food science. A properly designed active packaging might increase the microbial safety or delay the spoilage of food (antimicrobial packaging), it might delay oxidative changes in food (antioxidant packaging), or it might help increasing functional properties of food by delivery of nutrients or bioactive substances to food surface in bioavailable form (bioactive packaging) [3–5].

The antimicrobial packaging is the most popular active packaging method and materials used in this method could be developed by (1) addition of antimicrobial containing sachets or pads into food packages; (2) coating, immobilization, or direct incorporation of antimicrobials into food packaging materials; or (3) use of packaging materials that are inherently antimicrobial [3]. However, the direct incorporation of antimicrobials into packaging materials is the most promising and effective method since films produced by this method deliver the desired amounts of antimicrobial directly at the food surface and they enable use of controlled release technologies. The antimicrobial packaging is effective mainly at the food surface, the most susceptible part of food against microbial contamination and development [6]. However, its effectiveness in depths of food is controlled mainly by diffusivity of delivered antimicrobials into food, interactions between food components and antimicrobials, and susceptibility of food against microbial spoilage. Thus, the future application of antimicrobial packaging is considered mainly as a leg of hurdle technology applied in combination with chilling, vacuum packaging, modified atmosphere packaging, and nonthermal processing methods [7,8]. The current trend in antimicrobial packaging also indicates that this technology will be applied mainly by use of natural antimicrobials (or antioxidants) with edible packaging materials [3,9,10]. This is due to the increased health concerns of consumers originated from chemical preservatives and environmental problems originated from plastic packaging materials. It is also important to note that the technological problems such as denaturing effects of thermal

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polymer-processing methods, extrusion, and injection molding are also a great disadvantage to use heat-labile natural antimicrobial in plastic films [3,11,12].

The application of antimicrobial packaging might be conducted by wrapping of food by precast active self-standing films or by placing precast self-standing films at food surface or between layers of food. However, a more practical and economically feasible alternative active packaging method is application of the film-forming solution at food surface by coating. The foods might be coated by (1) dipping of food directly into film-forming solution, (2) by spraying of the film-forming solution on food surface, or (3) by brushing of film-forming solution on food surface [13]. The film formation occurs mostly by applying a cross-linking solution by dipping, spraying, or brushing. Alternatively, some film-forming strategies might depend on drying, cooling, or heating of the coated food for fixation of the film layer.

Although there are different methods to apply the films on food surface, during the film development studies conducted in the laboratory, the performances and properties of the films (self-standing film or coating film) are optimized and tested by using films obtained by the standard casting method. This chapter focuses on basic strategies and testing methods of developing effective antimicrobial and antioxidant films and coatings using edible materials and natural active agents.

2. Basic Strategies to Develop Effective Edible Antimicrobial and Antioxidant Coatings

The studies to develop active edible films or coatings might be "Film Material Dependent" (FMD) or "Food System Dependent" (FSD) [10]. The FMD-based strategies aim mainly at the evaluation of a certain problematic waste material or a low value by-product rich in a film-making biopolymer. The discovery of novel film-making biopolymers or improvement of the film-making properties of known biopolymers is also FMD-type studies. On the other hand, FSD-type studies aim primarily to improve the safety and quality of a specific food product by use of active packaging. The film material selection in FSD-type studies is based solely on its high compatibility with food and selected antimicrobial or antioxidant compound that will be employed as active agent.

The FMD studies frequently start with extraction and characterization of the molecular and film-making properties of the film-forming biopolymer [14]. The formulation of the film-forming solutions to improve target physicochemical properties (such as mechanical, textural, morphological, hydrophilic/hydrophobic, barrier, or permeability characteristics) of films, and to evaluate the compatibility of major antimicrobial and antioxidants with the film and target food systems. It is generally desired that the antimicrobial and antioxidant activity in the food system should be obtained by use of minimum amount of active agents. This is important to avoid potential allergenic effects of natural active agents and minimize potential effects of natural active agents on taste, flavor, and color of food. Thus, release tests to determine amount of soluble and bound active compound per gram (or cm^2) of film are conducted and active

agent concentration is optimized based on its soluble fraction's concentration in the film. An active agent having high soluble/bound ratio in the film system might be compatible if it got sufficient stability in the film system and got a proper release profile (release kinetics) from the film. Further in vitro tests are then continued to characterize antimicrobial and antioxidant activity of films with laboratory tests in proper media. Then, the coating performance on target food is tested, and antimicrobial and antioxidant activities of films in the food system are evaluated. The FMD-type studies have some great limitations since the developed film system might be incompatible with some major natural active agents and food systems. Moreover, the physical and mechanical properties of the biopolymer might be problematic and it could be challenging to overcome these problems by classical edible film−making ingredients like plasticizers, cross-linkers, emulsifiers, antisticking agents, or thickening agents [10].

On the other hand, the FSD-type studies aim at improving the safety of a specific risky food product (e.g., beef burger, hamburger, smoked salmon, fresh cheese, poultry) against one or several major pathogenic bacteria such as *Listeria monocytogenes*, *Escherichia coli O157:H7*, *Salmonella typhimurium,* and *Campylobacter jejuni* [6,15,16]. In some cases, specific spoilage fungi such as *Botrytis cinerea* observed on table grapes [17], *Penicillium commune* observed on cheese [18], or a species of fungi like *Penicillium* species in bread [19] might also be targeted to prevent microbial origin economic losses. Thus, it is important to report that the selection of the effective antimicrobial against the target microorganism in FSD type studies is quite critical. The second step is the selection of a suitable biopolymer compatible with the target food and the selected active agent. The formulation of film-making solutions, physicochemical tests, release tests, test of antimicrobial, and antioxidant performance in laboratory media and in target food systems are then conducted to evaluate and optimize the film formulation and application methods.

3. Formulation of Edible Antimicrobial and Antioxidant Coatings

The formulation of the edible coating material is one of the important steps that determine the success of the application in the final food product. This step consists of selection of suitable film-forming material and active agent as well as choosing of suitable aiding agents to improve film physicochemical properties.

3.1 Selection of Edible Coating Material

The edible coating materials include polysaccharides such as chitosan, cellulose and derivatives, pullulan, carrageenan, pectin and alginate; proteins such as gelatin, collagen, gluten, zein, whey, casein and soy proteins [11,20−22], and lipids such as waxes, fats, and oils [23,24] (Table 4.1). However, it is quite important to use suitable edible coating materials for each food product. For example, materials from cellulose, casein, zein, soy protein, and chitosan could be employed for coating of whole intact

Table 4.1 Examples of Active Agent–Free Edible Coating Materials Applied on Different Food Products

Edible Coating	Major Target	Product Coated	References
Zein	To delay ripening and to reduce moisture loss	Apple	[25]
		Pear	[26]
		Mango	[27]
		Tomato	[28]
Alginate	To reduce microbial growth and moisture loss	Meat, poultry, fish	[29]
Chitosan	To control trans–shell penetration of *Salmonella Enteritidis*	Egg	[30]
Whey protein	To reduce oxidative changes	Low-fat sausage	[31]
Caseinate–Whey protein composite	To reduce molding	Strawberry	[32]
Whey protein	To delay ripening	Apple	[33]
Hydroxypropyl methylcellulose	To delay ripening	Apple	[34]
Galactomannan	To control gas exchange and water loss	Cheese	[35]

fruits and vegetables to suppress their respiration rates, since these materials could form films having desired gas barrier/permeation properties and give odorless, tasteless, and transparent coatings [36]. For a successful fruit and vegetable coating application, the gas permeability characteristics of the coating material and the product respiration rate should be compatible. This helps to achieve the "modified atmosphere effect" that is formed by the reduction of fruit or vegetable respiration rate under reduced O_2 and elevated CO_2 atmospheres [36,37]. Such films could also be incorporated by natural antimicrobials to inhibit the growth of spoilage fungi such as *B. cinerea* and spoilage bacteria such as *Erwinia amylovora, Erwinia carotovora*, *Xanthomonas vesicatoria,* and *Pseudomonas syringae* [13]. On the other hand, materials such as alginate, gellan, whey protein concentrate, pectin, and chitosan are suitable for coating of minimally processed fruits and vegetables [37]. The materials applied for minimally processed fruits and vegetables might contain not only antimicrobial agents, but also antioxidants (such as ascorbic acid and derivatives) and enzyme inhibitors (chelating agents such as malic and citric acids) that control enzymatic browning caused by polyphenoloxidase, and firming agents (such as $CaCl_2$) to improve textural properties [38]. The edible coating agents in the minimally processed fruits and

vegetables could retain at wet cut vegetable surfaces and act as a reservoir for the active agents. For example, the whey protein and chitosan films could be applied on product surface simply by dipping or spraying. A draining to remove excess film solution and a following mild drying step might be applied to prevent leaking of the film and fixing it on product surface. However, in some cases the film-forming solution could be fixed to food surface simply by chemical cross-linking (e.g., cross-linking of alginate and low methoxy pectin coating with $CaCl_2$). The use of such films is quite advantageous since they enable increasing film thickness at the product surface. This is important when one needs to use coating layer not only as a reservoir for active agents, but also as a reservoir for water to prevent drying of product surface by evaporation. In fact, this explains why the alginate films are used frequently for coating of meat, poultry, and fish [29].

The application of zein as a coating material attracts a growing interest since this edible material is an important coproduct of the oil and bioethanol industries. The zein, a rare hydrophobic protein that is soluble in ethanol, also gives excellent coatings with good gas- and moisture-barrier properties [39]. Moreover, the zein does not create many interactions with the incorporated natural active compounds since it contains only residual amounts of charged hydrophilic groups. This ensures solubility of the incorporated active agents within the films and provides an effective delivery system for different natural active compounds including phenolic compounds [39,40]. Moreover, the hydrophobic zein films show a limited swelling in the food system, and this help controlling release rates of antimicrobial or antioxidants from the films [6,16].

3.2 Selection of Antimicrobial and Antioxidant Compounds

Different natural compounds suitable for antimicrobial coating include antimicrobial peptides such as polylysine, bacteriocins such as nisin, pediocin, and lacticin, and antimicrobial enzymes such as lysozyme, lactoperoxidase, and glucose oxidase [10]. The phenolic rich plant extracts and essential oils could serve both as antimicrobial and antioxidant agents for the active coating [3,12,16,41,42]; while natural compounds such as ascorbic acid and derivatives, tocopherols, and protein isolates could serve mainly as antioxidants [38,43].

The lysozyme produced from hen egg white is one of the most popular generally recognized as safe (GRAS) status antimicrobial that is used in active packaging (Table 4.2). The enzyme is effective mainly on gram-positive bacteria by splitting the bonds between the N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan (PG) layer in their bacterial cell wall. The effectiveness of the lysozyme on the critical pathogen *L. monocytogenes* is the primary reason of employing this enzyme in antimicrobial packaging [16,46,47]. However, it is important to note that some gram-positive bacteria such as *Staphylococcus aureus* could show a great resistance against lysozyme. The *S. aureus* not only shows an extreme resistance against lysozyme, but also it is capable to boost its capacity to produce protective biofilm formation in presence of this enzyme [54]. On the other hand, due to the protective lipopolysaccharide (LPS) layer around their PG layer lysozyme is not directly effective on gram-negative bacteria. However, the combination of lysozyme with chelating agents like EDTA in

Table 4.2 Examples of Antimicrobial and Antioxidant Packaging and Coating With Different Edible Films Incorporated With Active Agents

Film	Active Agent(s)	Major Effect Achieved	Method of Application	Food Applied	References
Zein	Lysozyme, catechin, gallic acid	Inhibition of *Listeria monocytogenes* and lipid oxidation	Films were placed on both surface	Fresh cheese	[6]
Gelidium corneum	Catechin	Inhibition of *Escherichia coli* O157:H7 and *L. monocytogenes*, and lipid oxidation	Film was wrapped on the surface	Sausage	[44]
Whey protein	Lysozyme	Inhibition of *L. monocytogenes*	Film placed at the surface was activated by acidification	Smoked salmon	[45]
Whey protein	Lysozyme	Inhibition of *L. monocytogenes*	Acidified film was placed at the surface	Smoked salmon	[46]
Chitosan	Lysozyme	Inhibition of *L. monocytogenes, E. coli, Pseudomonas fluorescens*	Film was applied by dip coating	Mozzarella cheese	[47]
Zein	Lysozyme and EDTA	Inhibition of total coliforms	Films were placed on both surface	Beef patty	[16]
Na-Caseinate	Nisin	Inhibition of *Listeria innocua* (as indicator of *L. monocytogenes*)	Film was placed at the surface	Cheese	[48]

Bacterial cellulose	Nisin	Inhibition of *L. monocytogenes*	Film was wrapped on the surface	Sausage	[49]
Chitosan	Natamycin or pomegranate extract	Inhibition of total yeast and mold	Film was applied by dip coating	Strawberry	[50]
Starch	Nisin and natamycin	Inhibition of total yeast and mold	Film was placed at the surface	Cheese	[51]
Fish gelatin	α-Tocopherol	Inhibition of lipid oxidation	Film were placed at the surface	Lard	[52]
Whey protein	Ascorbic acid	Inhibition of lipid oxidation	Film was applied by dip coating	Peanut	[53]

edible films causes the destabilization of LPS layer of gram-negative bacteria and enables use of lysozyme against critical pathogens such as *S. typhimurium* and *E. coli O157:H7* [16].

The nisin, a GRAS-status bacteriocin obtained from lactic acid bacteria, is also an alternative natural active compound suitable for use in antimicrobial packaging [16,55]. The nisin is a cationic peptide that owes its antimicrobial activity to its ability to interact with the anionic phospholipids at the bacterial surfaces to form pores and to dissipate proton motive forces at the bacterial membrane [54]. It is not effective on gram-negative bacteria, but it shows potent antimicrobial activity on many gram-positive bacteria including pathogenic ones like *L. monocytogenes* and *S. aureus* [54]. Thus, active packaging conducted by nisin-incorporated coatings could be suggested particularly for fresh cheeses since their microbial risks are associated mainly from contamination of *L. monocytogenes* and *S. aureus* [54,56,57].

ϵ-Polylysine, a polypeptide formed by 25−35 L-lysine residues, produced commercially from aerobic fermentation by *Streptomyces albulus*, is another alternative active agent suitable for antimicrobial packaging [58−61]. The major advantage of using ϵ-polylysine is that it might show antimicrobial effect on both gram-positive and gram-negative food pathogenic bacteria including *L. monocytogenes*, *E. coli* O157:H7, and *S. typhimurium* [59,62]. The antibacterial action of ϵ-polylysine is attributed to its polycationic and surface active nature that enable its interaction with bacterial membrane and with bacterial DNA when it is penetrated into cells [63,64]. In Japan, ϵ-polylysine has been approved for use in sliced fish and fish surimi, boiled rice, noodle soup stocks, noodles, and cooked vegetables [60] while the FDA in the United States recognized ϵ-polylysine as safe (GRAS) for use in cooked or sushi rice [58]. Ünalan et al. [16] tested ϵ-polylysine with chitosan, whey, alginate, and zein films. These authors reported that the most compatible film with this agent is zein since it did not cause complex formation and immobilization of this antimicrobial within the film matrix by charge−charge interactions [16].

Phenolic compounds are other potential active compounds suitable for edible coatings. The use of phenolic compounds obtained from plant-based agro-industrial wastes and by-products in active packaging is very attractive not only to evaluate waste material, but also to employ an invaluable active agent that shows antimicrobial and antioxidant activities in the food system [44], and improves the health benefits of food products [5,65]. The phenolic compounds could show their antimicrobial activity with multiple mechanisms including complex formation with cell walls, membrane disruption, inhibition of bacterial adhesion, or inactivation of bacterial enzyme systems [66]. On the other hand, the antioxidant activity of phenolic compounds originates mainly from their free radical scavenging and iron binding capacities [67]. An edible coating incorporated with proper phenolic compounds could effectively prevent lipid oxidation in food [6]. However, it is important to note that the undesired taste and flavor changes in food caused by phenolic compounds could limit their use at elevated concentration that is necessary for microbial inhibition. Thus, designing a multifunctional film having both antioxidant and antimicrobial properties needs the use of phenolic compounds highly compatible with the aroma and flavor characteristics of food system. Otherwise, it is essential to combine phenolic compounds with

alternative antimicrobial agents. The enzyme lysozyme is a very suitable natural antimicrobial to be combined with phenolic compounds. It was reported that the lysozyme combined with mixture of catechin and gallic acid in zein-based films is highly stable and it could be employed successfully to suppress the growth and to inhibit *L. monocytogenes* in actively packed cheeses [6]. Kaewprachu et al. [68] also successfully controlled microbial load of actively packed minced pork by combining lysozyme with catechin in gelatin films.

The most critical factors for using phenolic compounds in edible materials is that both carbohydrate- and protein-based edible film−forming materials could bind phenolics strongly. The polar groups of carbohydrates could bind phenolic hydroxyl groups with noncovalent interactions such as hydrogen bonds and van der Waals forces [69−71]. On the other hand, the protein−phenolic interaction occurs by hydrogen bonds formed between phenolic hydroxyl and protein -NH and -CO groups, and hydrophobic interactions formed between nonpolar phenolic groups and nonpolar protein domains [15,72]. Thus, it is quite important to conduct detailed release tests of developed edible films and ensure the presence of sufficient amounts of soluble phenolic compounds in the films.

Due to their effectiveness to prevent microbial and oxidative changes in the food systems, well-characterized health benefits, and abundance in human diet the green tea catechins (e.g., catechin, epicatechin, epicatechin gallate, and epigallocatechin gallate) are among good candidates for use in active packaging [6,39,40,44,73,74]. The use of grape seed extract in edible films also attracts a particular interest, but these extracts contain mainly the oligomeric proanthocyanidins that are red colored and have an astringent taste that might me incompatible with the sensory properties of most food [75]. The rosemary extract containing diterpene phenolics, carnisol, and carnosic acid is one of the rare odorless and tasteless phenolic extracts [76,77] that could be used in antioxidant packaging. The essential oils such as eugenol and citral that have a GRAS status are also suitable phenolic-based antioxidant and antimicrobial agents for the edible films [12]. Khalil and Deraz [78] employed eugenol while [13] employed both eugenol and citral to develop antimicrobial zein films. However, it is important to note that the use of essential oils is possible only when their distinctive odor and taste are compatible with the food system. Gutierrez et al. [79] suggested the combination of essential oils with aroma compounds to obtain an acceptable organoleptic profile. This strategy might be a feasible solution to increase use of phenolic compounds in antimicrobial and antioxidant coating, but further studies are needed to determine compatible phenolic compound−aroma compound−food system trios and to obtain detailed consumer feedbacks for acceptability of these formulations.

3.3 Selection of Other Film Ingredients

Besides the film-making polymer and active agents, proper use of other film ingredients including plasticizing agents, emulsifying agents, and cross-linkers is also essential to obtain edible coatings with desired physicochemical and active properties. The alcohol glycerol is one of the most frequently employed GRAS agents for plasticization of edible films. The major objective of using glycerol is to reduce the brittleness of

dried coatings. The glycerol content of whey protein films might be as high as 60% of whey protein by weight [45], while zein films might contain glycerol at 35% of zein by weight [43]. However, the necessary amount of glycerol should be determined by preliminaries for each specific edible coating formulation. Other agents that could be used for plasticizing edible films include sugar alcohols such as sorbitol [80], sugars such as glucose, galactose, and fructose [81], and fatty acids such as oleic and linoleic acids [82]. The surface-active agents such as lecithin might be used to mix hydrophilic and hydrophobic components in edible film−making solutions [39]. On the other hand, cross-linking of films might be applied by different agents to improve mechanical properties, and gas and moisture barrier properties of films, or to turn soluble films into insoluble form by gelation. For example, the enzymes transglutaminase [83] and tyrosinase [84] or oxidized phenolic compounds [85] might be applied for cross-linking of protein-based films. On the other hand, divalent ions like Ca^{++} and Mg^{++} might be employed for cross-linking of some carbohydrate-based films from low methoxyl pectin, alginate, and carrageenan [86,87].

4. Preparation of Film-Forming Solutions

A film-forming solution containing the biopolymer and active agents (antimicrobial and/or antioxidant) should be mixed at proper concentrations and ratios in true sequence together with different ingredients. The solubilization, heating, and pH setting are the critical factors during preparation of film-forming solutions. The solubilization of film-forming material and active agents and ensuring their presence in soluble form during processes such as heating, cooling, pH setting, homogenization, and drying is extremely important to obtain a homogenous and effective coating at the final food product. Some carbohydrate-based materials like pectin and lipid-based materials should be solubilized by means of heating applied during homogenization. In such cases, the mixing of other heat-labile ingredients should be conducted following cooling of the heated film-forming solution. In particularly protein-based coatings, the proper heating of film-forming solution is essential to achieve sufficient protein denaturation that affects film-forming properties of the coating. The heating affects the hydrophilic/hydrophobic balance of the protein since it modifies the protein conformation, and this is highly effective on affinity of coating on food surface. Therefore, the heating conditions of each film-forming solution should be optimized considering the film properties and affinity of final coating on targeted food surface. The setting of the pH of coating is another important factor since it affects not only film-forming properties, but also solubility of the active agents within the film. For example, the films of whey protein (isoelectric point (pI) between 4.4 and 5.4) have mainly a negative charge close to neutrality. Thus, incorporation of a positively charged natural agent like lysozyme (pI: 11.4) into these films close to neutral pH resulted with binding of lysozyme by negatively charged whey protein film matrix. This causes immobilization of the enzyme and prevents its release from the films if coated food pH is also close to neutrality [45]. Thus, the pH of whey protein films should be set at least to pH 4.0 to solubilize majority of immobilized lysozyme in the films (Table 4.3).

Table 4.3 Lysozyme Released From Whey Protein Films Prepared Without Acidification

pH of Buffer Used in Release Tests	Activity Released (U/cm^2)	(Activity Released/Activity Incorporated) × 100
3.0	69,138	102
4.0	47,019	70
4.5	35,021	52
5.0	14,776	22
5.5	No considerable activity	0
6.0	No considerable activity	0

Data obtained from D. Boyacı, F. Korel, A. Yemenicioglu, Development of activate-at-home-type edible antimicrobial films: an example pH-triggering mechanism formed for smoked salmon slices using lysozyme in whey protein films, Food Hydrocoll. 60 (2016), 170−178.

5. Basic Testing Methods of Antimicrobial and Antioxidant Coatings

Development of edible antimicrobial and antioxidant coatings could be a challenging process depending on susceptibility of food against microbial and oxidative spoilage. The use of controlled release technology might also be needed depending on the rate of spoilage reactions that determines the shelf life, diffusion rate of antimicrobial compound from food surface to interior parts of food, and rate of neutralizing reactions between active agent and food components. Thus, different traditional and custom tests including release tests, antimicrobial tests, antioxidant tests, etc. could be needed to design the specific properties of active coating. This section discusses the major tests necessary to measure the performances of edible films.

5.1 Release Tests

The release tests are conducted mainly to determine the amount of soluble antimicrobial or antioxidant agent in the films. Moreover, the release tests also provide data about the release kinetics of active agent from the films. In most studies, the release tests were conducted in distilled water, but suitable buffers at a specific pH, model gels, and the target food itself might also be used to determine the release profiles of an active agent.

5.1.1 Release Tests in Liquid Media

The release tests conducted in a suitable buffer system set at target food pH gives realistic results to calculate the soluble active agent content of films. However, the distilled water is also frequently employed in some film-development studies when the target

food system is uncertain. In general, the tests were conducted by placing films at a certain size in suitable glass or stainless steel containers containing buffer or distilled water. The test should be conducted at a suitable temperature considering the storage and marketing temperatures of target food to be coated. For example, a release test at 4°C is essential if the target food will be stored and marketed under refrigeration. Moreover, the container should be shaken (not mixed by stirrer since this could disturb film) horizontally at a slow speed to enable continuous diffusion of active agent from films to release medium. The release test is conducted until reaching of the equilibrium for the released active agent or until an insignificant increase occurred in active agent concentration by time. The monitoring of the release of active agent is conducted by taking samples at different time intervals and quantifying concentration (or activity) of agent in the release medium. However, at this point the amount of active agent removed during sampling should be considered during calculation of total released agent at each time point. At this point, the most accurate results are obtained by taking minimum amounts of liquid in sampling to minimize the change of volume in release test medium. The results for each time point were expressed as amount released per cm^2 of films (mg/cm^2) or as amount released per gram of films (mg/g). The release curve is formed by plotting concentration (or activity) of active agent versus release period (h or min). Fig. 4.1 shows the release of water soluble phenolic compounds in zein films incorporated with different amounts of clove phenolic extract. These curves clearly showed that the amounts of soluble phenolic compounds in the films correspond to almost one-fifth of the incorporated clove extract by weight. This result

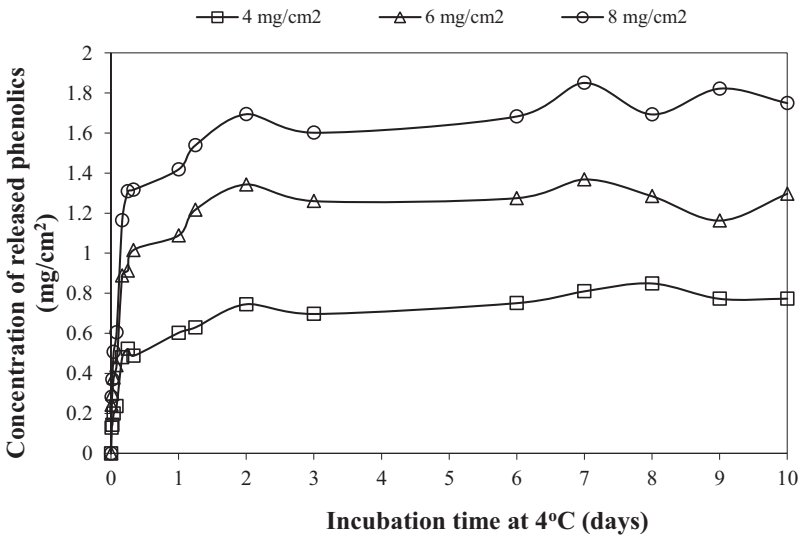

Figure 4.1 Release profiles of phenolic compounds from zein films incorporated with different concentrations of clove extract (Notes: 4 × 4 cm films were incubated in distilled water at +4°C. The phenolic concentration was determined by the Folin−Ciocalteu method) [88].

provides essential information to understand the effective concentration of the clove extract in films and in the target food system.

One of the most critical factors during release tests conducted in distilled water and buffer is related with the density of films. The films having a low density might float in the water or buffer and one side of the film remains in the air. This causes release from only one side of the film and might affect the time necessary to reach equilibrium. Such film-floating problems occur frequently when composite films are formed by mixing protein or carbohydrate-based film-forming materials with lipid-based materials in presence of emulsifiers. To prevent this problem, a custom-made simple apparatus that keeps film immersed into release medium might be employed (Fig. 4.2A). This apparatus could simply be formed by placing films between two glass frames, fixation of frame by inert elastic material, and placing it into sufficient volume of buffer or water to cover the apparatus. Some glass pieces at proper dimensions (e.g., 1 cm^3) should be placed at the bottom of the apparatus to allow circulation of water or buffer around apparatus during shaking. The same apparatus should also be employed for release tests of high-density films that sink into release medium and stitch to the bottom surface of container used in the release test. A particular type of apparatus is also needed when films are asymmetric and researchers are specifically interested in release from one side (dense or porous side) of the films. The apparatus needed for release tests of asymmetric films is similar with that described in Fig. 4.2A. However, the bottom frame should be changed with a glass plate and two plates should be fixed after placing a waterproof material (e.g., properly cut frame from parafilm or inert rubber) between them to cut the contact of one side of the film with water or buffer (Fig. 4.2B).

5.1.2 Release Tests in Gel Media

The release tests in model solid gel medium are conducted to simulate and better understand the swelling and release properties of films in a potential food application. It is sometimes a really problematic process to conduct release tests in a food system since effective extraction and quantification of some released natural active agents from food is quite challenging. In contrast, a model solid media designed properly could be extracted more easily to quantify the released agent. For example, the agar, a basic gelling agent used in microbial media, might be a good alternative to conduct

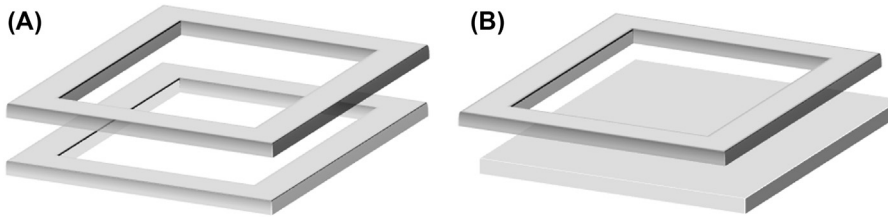

Figure 4.2 Release test apparatus suitable for low-density films that have floating problem (A) and for asymmetric films that should be tested separately for dense and porous surfaces (B).

release tests [15,89]. The hardness of the agar could be modified by changing its concentration, and different food components such as sugars, acids, salts could be added into this medium to simulate the food system.

5.1.3 Release Tests in Target Food

The release tests in target food system are possible when the active agent released from films to food could be extracted and quantified with sufficient efficiency. The active agent is first released from films onto food surface. Then, it diffuses into depths of food form surface and might form insoluble or inactive complexes with the food components. For example, phenolic active agents released from films might form complex with food hydrocolloids [90]. The nisin is neutralized in fresh meat since it forms inactive complexes with glutathione by catalysis of glutathione S-transferase [91]. Lysozyme forms strong complexes with milk caseins [92]. Thus, monitoring of lysozyme diffusion in actively packed cheeses might be challenging. Boyacı et al. [45] extracted and measured lysozyme activity released form whey-based protein films to smoked salmon with 50%−76% recovery in enzyme activity after 72 h at +4°C. On the other hand [93], successfully monitored antimicrobial products (SCN- and OSCN-) in smoked salmon slices coated with lactoperoxidase incorporated whey protein films. Thus, it is clear that the release test in food is beneficial only if active agent is extracted and measured effectively. Otherwise, the release tests with target food should be supported by model systems (gel or buffer) to understand the diffusion kinetics and exact concentrations and activity of released active agents in the food system.

5.2 Test of Antimicrobial Properties

The testing of antimicrobial properties is the most important and challenging step in development of active coating materials. There is no single method with laboratory media to simulate the real antimicrobial performance of active coatings. However, there are reports that a film performed well in properly designed laboratory media might show antimicrobial activity in the target food system [41]. The films succeeding in antimicrobial tests in laboratory media generally contain maximum amounts of soluble, but minimum amounts of bound antimicrobial compounds, and they show a sufficient release rate for these antimicrobial compounds [16,45]. In contrast, films containing bound (immobilized) antimicrobials or soluble antimicrobials with relatively slow release rates generally perform bad in laboratory tests [45].

5.2.1 Classical Zone Inhibition Tests on Agar Media

The classical zone inhibition test is the most frequently applied method used for antimicrobial testing of active films. This method includes placing discs (rarely square shaped) of edible film on suitable agar medium previously inoculated with target microorganism. The best method to prepare discs of edible film aseptically is to use a sterile cork borer at a suitable diameter. The inoculation level of the target

microorganism is mostly set significantly higher than that of possible contamination levels in the target food. It is essential to obtain a dense microbial growth at agar surface since insufficient number of colony formation at the agar surface could cause zone boarders hardly identifiable. However, the use of high levels of inoculum in antifungal tests might cause no zone formation even for the most effective antimicrobial coatings. Thus, it is best to conduct an optimization study to determine the level of proper inoculum in the antifungal tests. During comparison of antimicrobial performances of films having different release profiles for the active compounds, it is best to keep the Petri dishes for several hours at +4°C before transferring them to microbial incubation temperatures. This helps better seeing the performances of films with slow release rates before initiation of microbial growth at optimal growth temperature. In general, the microbial incubation conditions are 3−5 days at 25−30°C for most fungi and 1−2 days at 37°C for most bacteria. However, the Petri dishes might sometimes be incubated between 7 and 10°C for 7−10 days when target microorganism is psychrotrophic. After the incubation period, films that have antimicrobial activity form zones around their discs placed on the agar surface. The clear zones (Fig. 4.3) are accepted as an indication of good antimicrobial activity and they are measured for their diameter to calculate the zone area (mostly reported as mm^2). The partially clarified hazy (turbid) zones are generally not measured for their zone areas, but this might be noted as a limited antimicrobial activity after ensuring that they are formed by reduced microbial growth at the zone area. In some cases, the measurement of the area of clear zones could be challenging since the shape of clear zones might be distorted or clear zones are formed only

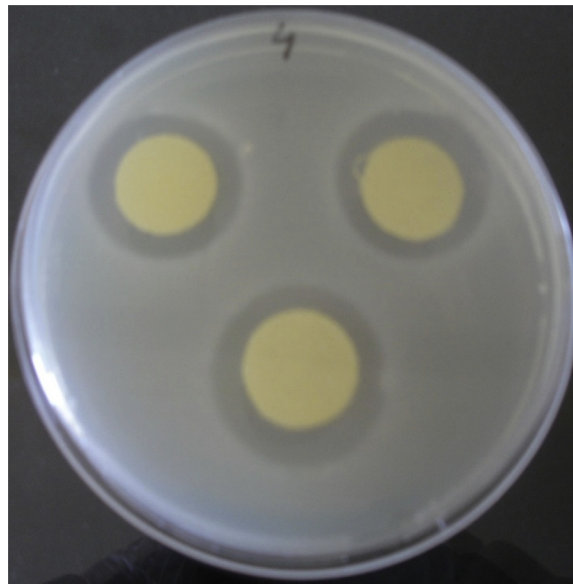

Figure 4.3 Clear zones formed on nutrient agar against the plant pathogenic bacteria *Erwinia amylovora* by edible zein films incorporated with 4 mg/cm^2 vanillic acid as an antimicrobial agent [88].

at one side of the films. These problems occur mostly due to heterogeneities in distribution of antimicrobial within the films or poor film casting conditions that cause differences in film thickness. The testing of films that contain immobilized antimicrobial agent is also problematic. The films containing immobilized antimicrobials did not form zones, but it might be possible to check their antimicrobial activity qualitatively by removing films carefully from the agar surface and observing the clarity of film contact surface. A clear spot at the same size with the removed discs of film might be considered for antimicrobial activity only if microbial development is observed in similarly treated control film locations. The zone inhibition method could provide useful data to optimize the film-making method and set the inhibitory concentration for a given antimicrobial. However, the results obtained might sometimes be quite tricky. For example, a film that contains a high molecular weight antimicrobial could show smaller zones than a similar film that contains a low molecular weight antimicrobial at the same concentration and antimicrobial potency. This is due to the differences in diffusion rates of high and low molecular weight agents from films to agar and within the agar. Thus, the evaluation of zone inhibition tests together with the release profiles of antimicrobials always provides a more realistic approach.

5.2.2 Inhibition Tests in Broth Medium

The antimicrobial packaging is developed mainly to inhibit microbial growth at the solid or semisolid food's surface, the most susceptible area of food for microbial contamination and development. However, the active packaging is an innovative area and future applications of antimicrobial packaging in liquid food might need test of antimicrobial properties of films in broth media. Moreover, it should also be reported that the antimicrobial tests in broth medium might be very useful when it is aimed to compare the differences in antimicrobial performances of films having fast and sustained release properties for the same antimicrobial agent. The films capable of releasing antimicrobials slowly at a certain release rate could fail to show any antimicrobial properties in the classical zone inhibition test that last mostly 24 or 48 h depending on growth rate of target microorganism. Moreover, it is also important to note that the antimicrobial tests in the classical zone inhibition test should be conducted at the specific growth temperature (mostly at 37°C for most bacteria) of target microorganism. Otherwise, it is not possible to obtain a dense growth at the agar surface and observe the clear zone areas. In contrast, the tests in broth medium might be conducted even at refrigeration temperatures that last for several weeks and simulate the performance of films against a specific pathogen during a targeted shelf life. The antimicrobial tests in broth medium are also useful to evaluate the minimum inhibitory concentrations (MIC) of different candidate antimicrobials compatible with the edible films. The MIC tests against the target microorganism are conducted by testing a series of concentrations (prepared by two-fold increases) for the antimicrobial against the target microorganism. In such a test, the MIC value is mostly determined by monitoring the microbial growth based on turbidity of the broth media. The lack of turbidity might indicate lack of microbial growth. However, it is

necessary to conduct microbial counts in the broth media when microbial growth does not cause changes in turbidity.

5.2.3 Antimicrobial Tests on Specific Food Samples

The antimicrobial tests on specific food sample are essential to ensure the proper design and effectiveness of films. At this step, the antimicrobial effectiveness of the coating is tested against the most critical microbial treat(s) causing poisoning or spoilage. For this purpose, the food should be inoculated with target microorganisms and the effect of antimicrobial packaging on initial counts and later counts during storage should be monitored with proper intervals. The application of proper selective media during enumeration is critical and should be decided and performed in collaboration with an experienced microbiologist. It is worth noting that the main objective of a food application might be to increase safety of food within the normal shelf life solely against the most critical pathogenic bacterium. For example, the smoked salmon is a typical *L. monocytogenes* risk food that causes deadly infections [94–96]. Thus, inhibition of this bacterium and reduction of deaths from smoked salmon–originated listeriosis might be the sole objective for the designed antimicrobial packaging. Alternatively, the main objective of active packaging might be much more challenging and it might target increased safety against majority of critical pathogenic bacteria that might present in the food within the normal shelf life. A good example for this challenging strategy was applied by Leleu et al. [30] who developed chitosan coatings to inhibit *Salmonella enteritidis* together with *S. typhimurium, L. monocytogenes, E. coli*, and several other pathogenic bacterial species. On the other hand, the main objective of antimicrobial coating might be the inhibition of spoilage microorganism (such as fungi) when the food product does not contain any major risk from pathogenic bacteria due to its low pH and/or a_w (some dried fruits and bakery products). At all cases, it is very important to inoculate and spread known numbers of a suitable strain of the target microorganism homogenously at the food surface. The target strain could be the most pathogenic or resistant one if study was conducted by an experienced microbiologist in a risk laboratory. However, the use of nonpathogenic strains that show similar resistances with the pathogenic ones (such as use of *Listeria innocua* in place of *L. monocytogenes*) is preferred when possible.

5.3 Test of Antioxidant Properties

The process of designing and testing an antioxidant film should be closely based on the nature of major oxidative reaction in the target food system. Different performance tests are needed to develop films capable of neutralizing free radicals, to chelate metal atoms or to inhibit oxidative enzyme systems. Moreover, extensive microbial tests might also be needed if antioxidant films should possess also antimicrobial potential. This section focuses on basic tests necessary to obtain an antioxidant edible film or coating. The antioxidant tests in laboratory are initiated mostly by measurement of soluble and bound antioxidant activity originated intrinsically from biopolymer and incorporated active agent. The bound antioxidant properties of biopolymers might

be related with the active properties of their monomers that act as building blocks. For example, the proteins owe their antioxidant activity to their constituent amino acids that could donate protons to free radicals (such as aromatic, sulfur containing, and basic amino acids) or chelate metal ions (such as basic and acidic amino acids) [97–99]. Moreover, the presence of a bound antioxidant (such as bound phenolic compounds in hydrocolloids) in a biopolymer might also contribute significantly in bound antioxidant capacity. On the other hand, the soluble antioxidant capacity of an edible film is related mainly with the antioxidant compound incorporated into the films. The incorporated active compound might also form a significant portion of bound antioxidant activity if it is trapped within the film matrix, or if it forms covalent or noncovalent bonds and interactions with the film matrix. For example, Arcan and Yemenicioğlu [100] reported the binding of 30%–40% of incorporated catechin in zein films. The binding of antioxidant by the film matrix could reduce the effectiveness of antioxidant coating if oxidative changes in the food develop very rapidly. A strategy to increase the soluble antioxidant content of a film might be the addition of an inert (neutral) agent (such as a wax) within the films to form a composite structure [6,100]. The increase of the amount of inert component's fraction within the composite structure could reduce the amount of bound active agent (antimicrobial or antioxidant) while it increase the amount of soluble active agent [45].

5.3.1 Soluble Antioxidant Properties

The determination of the soluble antioxidant properties of a film could be done simply by monitoring concentration and antioxidant activity (with a proper method) of active agent during a release test conducted at a specific temperature (e.g., +4°C or 25°C). It is very critical to conduct the test in sufficient volumes of release test medium (buffer at target pH or water) and to consider the activity of total solubilized active agent at the equilibrium. The monitoring of antioxidant properties with a suitable method and expression of results with a meaningful unit are also essential. Many different methods exist to determine the antioxidant capacities of active agents [101]. However, the 2,2-azinobis(3-ethylbenozthiazoline-6-sulfonate) (ABTS) test conducted by radical of chemically oxidized ABTS and 2,2-diphenyl-1-picrylhydrazyl (DPPH) test conducted by radical of DPPH are the most popular spectrophotometric methods to determine free radical scavenging based antioxidant capacity of H-atom donors such as phenolics, proteins, and amino acids [97–99,101]. The iron chelating capacities (ICC) of active agents, on the other hand, might be determined by the classical spectrophotometric method that employs ferrozine reagent for detection of iron [102]. The results of measurements could be reported as μmol equivalents of a known antioxidant (Vitamin C or Trolox for ABTS and DPPH and disodium EDTA for ICC) released per cm^2 (or g) of an edible film.

5.3.2 Bound Antioxidant Properties

The bound antioxidant capacities of films could also be determined by using the ABTS, DPPH, and ICC methods specified in Section 5.3.1. However, this method is applied by

mixing a solution of reactants with a certain piece of film (not a certain volume of release test medium as in Section 5.3.1) [43]. The most critical part of this test is the use of film free from soluble antioxidants. This is achieved by using a film previously exposed to a release test that reached the equilibrium for the released antioxidant. The films exposed to release test could be employed in bound antioxidant activity measurements after they are further washed with suitable amounts of water or buffer to ensure removal of residual soluble antioxidants. The tests are generally conducted by mixing a weight piece of film (e.g., 1−6 cm^2) with a certain volume (e.g., 10−50 mL) of reactant (ABTS or DPPH radical solution, or iron solution for ICC) and determining antioxidant capacity formed (or iron bind) within a certain time period [21]. The selection of time period for the assay could be compatible with that employed for soluble antioxidant activity measurements. However, for both soluble and bound antioxidant activity tests, it is best to monitor the progress curves of ABTS and DPPH tests and use suitable incubation periods covering the activity region. The incubation period of films or solution of active agents in iron (for ICC test) should also be optimized by monitoring iron-binding capacity for different incubation periods. The results of measurements could be reported as μmol equivalents of a known antioxidant (Vitamin C or Trolox for ABTS and DPPH, and disodium-EDTA for ICC) per cm^2 (or g) of an edible film.

5.3.3 Antioxidant Tests in Specific Food Samples

The antioxidant tests conducted in laboratory media are good to optimize film composition and film-making processes, to determine antioxidant release rates and release profiles of films, and to compare soluble/bound fractions and antioxidant performances of different active compounds incorporated into films. However, a food application is the only realistic test to measure the true antioxidant potential of films. A food application is conducted mostly by active coating (or packaging) of a food with the developed coating (or film) and monitoring of lipid oxidation products during refrigerated and/or room temperature storage. Different methods exist for the measurement of primary and secondary lipid oxidation products in food [103,104]. However, the most popular method to monitor lipid oxidation products in food extracts is the spectrophotometric measurement of thiobarbituric acid reactive substances (TBARS). This method measures malondialdehyde (MDA) that is a dialdehyde produced by a two-step oxidative degradation of fatty acids with three or more double bonds, and aldehydic lipid oxidation products [104]. The TBARS method is preferred since it is simple and shows reasonable correlations with sensory analysis of food suffering from lipid oxidation [105,106]. However, if there are limitations (e.g., presence of interfering agents) to apply TBARS method for the selected food sample, monitoring of specific carbonyl compounds (such as hexanal, 2, 4-decadienal, etc.) by gas chromatographic methods might be considered to support the obtained sensory analysis [103].

6. Conclusions

This chapter emphasizes the importance of applying a smart strategy and a proper planning of testing methods to obtain effective antimicrobial and antioxidant edible films

and coatings. The formulation studies, physicochemical tests, release tests, and antimicrobial and antioxidant tests are all very important to develop effective active films and coatings. However, performing a detailed microbial risk analysis in the target food to select true natural antimicrobial agent(s) against a true target pathogenic or spoilage microorganism is the most critical step in antimicrobial film and coating development. On the other hand, the choosing of an effective antioxidant compound suitable for the type of oxidation reactions as well as compatible with the flavor and aroma characteristics of food system is the most critical step in development of antioxidant films and coatings. The selection of suitable coating or film-forming material compatible with the selected antimicrobial and antioxidant agent and applicable to the target food system is the second most critical step in active film and coating development. All other formulation studies and testing procedures are then applied to optimize the physicochemical properties, to improve and to prove effectiveness, and to optimize coating process for the selected target food system. The active edible coating is one of the most promising emerging technologies that could help increase safety and quality of ready-to-eat and minimally processed food. The current developments in this technology suggest that the active edible coating incorporated with natural active agents will be an important leg of hurdle concept applied on chilled foods in future. Further studies are also needed to understand exact benefits and feasibility of combining active coating with emerging nonthermal processes.

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Further Reading

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