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# Physicochemical And Toxicological Assessment Of Antimicrobial ε-Polylysine-Pectin Complexes

Cynthia L. Lopez Pena
*University of Massachusetts - Amherst*

# PHYSICOCHEMICAL AND TOXICOLOGICAL ASSESSMENT OF ANTIMICROBIAL ε-POLYLYSINE-PECTIN COMPLEXES

A Dissertation Presented

By

CYNTHIA LYLIAM LOPEZ PENA

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Approved as to style and content by:

_____
D. Julian McClements, Chair

_____
Hang Xiao, Member

_____
Lynne McLandsborough, Member

_____
Richard Wood, Member

_____
Eric A. Decker, Department Head
Department of Food Science

**DEDICATION**

To my parents, who encouraged my scientific curiosity from a young age (and put up with many messes in the kitchen during my scientific quests) and inspired me to follow my dreams.

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My greatest gratitude and appreciation is to my parents. Not only have they provided me with unconditional love, support, encouragement, and words of wisdom throughout my life, but they are also the inspiration and driving force behind everything I do.

## ABSTRACT

## PHYSICOCHEMICAL AND TOXICOLOGICAL ASSESSMENT OF ANTIMICROBIAL ε-POLYLYSINE-PECTIN COMPLEXES

MAY 2015

CYNTHIA LYLIAM LOPEZ PENA, B.S., CORNELL UNIVERSITY

PhD., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor D. Julian McClements

ε-Polylysine is an appealing FDA-approved, all natural antimicrobial biopolymer effective against a wide range of microorganisms. Its implementation is greatly limited by its strong cationic charge, which has been linked to instability in food systems, perceived astringency and bitterness, and the ability to inhibit lipid digestion. Previous studies have shown that controlled complexation of ε-polylysine with anionic pectin is able to prevent instability and astringency in simplified model food systems, while maintaining the antimicrobial character of polylysine. Isothermal titration calorimetry, micro-electrophoresis, microscopy, and turbidity analyses of the stability of electrostatic pectin-polylysine complexes in the presence of strongly anionic κ-carrageenan, and carrageenan-polylysine complexes in the presence of pectin at different mass ratios (pH 3.5) suggested that although polylysine-carrageenan interactions were much stronger, polylysine-pectin complexes maintained their stability in the presence of carrageenan.

*In vitro* digestion models showed that electrostatic interactions between bile salts and polylysine, which have been suggested as the mechanism for lipase inhibition by polylysine (2ppm), were affected by components in the sample's matrix. The implementation of an anionic (quillaja saponin) versus a non-ionic surfactant (Tween 20) in corn oil emulsions (2.5%w/w) showed a marked decrease of lipase inhibition, suggesting that electrostatic complexes formed by polylysine with other components prior to its exposure to bile salts in the small intestine may prevent the lipase-inhibiting polylysine-bile salts complex from occurring.

Corn oil emulsions (2%w/w) stabilized by Tween 20 subjected to oral, gastric, and intestinal digestion in the presence and absence of mucin and polylysine (200ppm) demonstrated that polylysine forms electrostatic complexes with bile salt-stabilized mixed micelles, potentially decreasing lipid absorption and altering its metabolism. Complexes formed between polylysine and mucin prior to addition of bile salts showed a decrease in insolubilized oil after digestion, suggesting that interactions between polylysine and bile salts were somewhat inhibited.

The influence of polylysine and pectin on the *in vitro* digestibility of animal feed either as individual components or as an electrostatic complex was assessed as part of a subchronic toxicity study. While pectin appeared to increase the rate and extent of lipid digestion, there did not seem to be any inhibition generated by polylysine.

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# CHAPTER 1

# INTRODUCTION

## 1.1 Background

Foodborne illnesses are estimated to affect 48 million Americans each year, cause 128,000 hospitalizations and 3,000 deaths (Centers for Disease Control and Prevention, 2014), and have an annual economic impact between $51.0 and $77.7 billion (Scharff, 2012). The control of pathogenic and spoilage organisms, commonly referred to as food protection (Jay, 2005), has been one of the main interests of the Food Industry throughout its history. In order to achieve this goal, the Food Industry has conceived, implemented, and combined numerous techniques including thermal and non-thermal processing of food, smart packaging, fermentation with specific microorganisms, and decreasing water content, to name but a few (Jay, 2005). The aforementioned techniques are habitually combined with the addition of antimicrobial compounds (or preservatives) in order to further decrease the likelihood of spoilage and pathogenic microorganisms proliferating.

The appeal of using preservatives is their low minimum inhibitory concentrations and high affectivity against microorganisms. Although the preservatives presently utilized by the Food Industry are effective and have undergone extensive examination to be deemed Generally Regarded As Safe (GRAS) by the Federal Drug Administration (FDA), consumers' growing disdain for synthetic/non-natural food ingredients and demands for cleaner labels in recent

years have incited the search for effective, safe, all-natural alternatives (Gyawali & Ibrahim, 2014; Tajkarimi, Ibrahim, & Cliver, 2010).

A promising alternative is ε-polylysine (ε-PL), an all-natural potent antimicrobial effective against various Gram (+) and (-) bacteria, yeasts, molds, and even some bacteriophages (S. S. Chang, Lu, Park, & Kang, 2010; Geornaras, Yoon, Belk, Smith, & Sofos, 2007; Shima, Matsuoka, Iwamoto, & Sakai, 1984; Yoshida & Nagasawa, 2003). Despite having a prolific antimicrobial range that surpasses that of more popular preservatives (Jay, 2005), ε-PL's highly cationic nature has prevented its widespread implementation in food products. Regarding incorporation in food matrices, ε-PL has a high propensity to interact with anionic components and may potentially generate instability in food systems (Y. H. Chang, L. McLandsborough, & D. J. McClements, 2011). Additionally, it is bitter, astringent, and has been documented to interfere with lipid digestion (Kido, Hiramoto, Murao, Horio, Miyazaki, Kodama, et al., 2003; Tsujita, Takaichi, Takaku, Aoyama, & Hiraki, 2006; Tsujita & Takaku, 2009).

The formation of anionic electrostatic complexes between cationic ε-PL and anionic high methoxyl pectin have been shown to minimize any destabilization in model food systems caused by the highly cationic charge of ε-PL, while maintaining ε-PL's antimicrobial properties (Y. Chang, L. McLandsborough, & D. J. McClements, 2011a; Y. H. Chang, L. McLandsborough, & D. J. McClements, 2011).

## 1.2 Objectives

The overall goal of this project is to conduct an in-depth investigation regarding the feasibility of implementing antimicrobial ε-PL-pectin complexes,

focusing on both their stability in the presence of food components that may disrupt them, as well as any toxicological hazards that may arise from their regular consumption. Due to the resistance of both ε-PL and pectin to digestive enzymes, as well as ε-PL's ability to inhibit lipid digestion, it is especially important to scrutinize any undesirable changes in the composition of the gut microbiota, as well as any impediment to the absorption of lipophilic nutrients at the estimated consumption levels.

**Specific Project Objectives:**

**A. Stability of Antimicrobial ε-PL-Pectin Complexes:** ε-PL-pectin complexes will be exposed to different concentrations of extremely anionic food ingredients in order to determine if there is a propensity for the more anionic polymers to sequester ε-PL from the complex. If so, this could jeopardize the antimicrobial functionality of ε-PL, as well as possibly causing instability and undesirable changes in sensory attributes.

**B. Impact of ε-PL On Digestibility of Fat and Bioaccessibility of Lipophilic Nutrients:** The inhibitory effect of extremely high concentrations of ε-PL on lipid digestibility has been documented; ε-PL-pectin complexes could pose a serious concern for the absorption of lipophilic nutrients and nutraceuticals typically found in the diet. Therefore, careful analysis testing whether the implementation of ε-PL-pectin complexes at the suggested usage levels may decrease the digestion and bioaccessibility of dietary lipophilic compounds is imperative.

### C. Toxicological Assessment of ε-PL-Based Antimicrobial Delivery System:

**System:** One of the primordial characteristics that any food additive must have is a nonexistent toxicity. Because ε-PL and pectin are both unaffected by digestive enzymes, it is possible that this complex may disrupt the regular digestion and absorption of lipids and affect lipid metabolism in the colon. Furthermore, the arrival of this antimicrobial complex to the large intestine may pose a threat to the bacteria that inhabit the colon. Therefore, thorough toxicological analyses implementing both *in vivo* and *in vitro* models must be conducted, focusing on blood markers, abnormalities in organs or body weight, and any changes in the composition of the gut microbiome.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Antimicrobial Agents in Food Systems

Processed foods made up approximate 70% of the average food consumed per capita in the United States in 2013 (Warner, 2013). Frequently, processed foods have to travel long distances from manufacturing plants to grocery stores where consumers can purchase them, continue their voyage from grocery stores to consumers' households, and often times extend their mileage to schools and offices where they are finally enjoyed. Ensuring that food reaches the final stage of its journey in good condition and maintaining its innocuousness requires the design and implementation of a strategy focused primarily on preventing contamination and spoilage (Becerril, Manso, Nerin, & Gomez-Lus, 2013; Cheng, Friis, & Leth, 2010). These strategies include (i) utilizing physical barriers to prevent contamination and physical damage; (ii) maintaining environmental conditions that will minimize microbial growth and undesirable chemical and physical changes; and (iii) incorporating additives to extend the shelf life of a product (Carocho, Barreiro, Morales, & Ferreira, 2014).

Food additives have been defined by the U.S. Congress and the Codex Alimentarius as:

> "Any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including

organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport, or holding of such food results, may be reasonably expected to result (directly or indirectly) in it or its by-products becoming a component or otherwise affecting the characteristics of such foods (Codex Alimentarius, 2014; Gaynor, 2006)."

However, "the term does not include contaminants or substances added to food for maintaining or improving nutritional qualities (Codex Alimentarius, 2014)." Their use in food products is closely regulated, and will be discussed in *Section* 2.2 Regulation of Preservatives by the U.S. Food and Drug Administration. There are currently over 2,500 additives approved for use in food systems (Branen AL, 2001; Carocho, Barreiro, Morales, & Ferreira, 2014), out of which antimicrobial agents are one of the substances most frequently incorporated into formulations precisely to control or retard the natural spoilage of food and/or to reduce contamination by both spoilage and pathogenic microorganisms (Tajkarimi, Ibrahim, & Cliver, 2010).

Many of the most widely employed preservatives are not naturally derived, and despite their safety certifications and approval for use from the FDA have become progressively unpopular. This growing mistrust stems from consumers' concerns regarding the possible toxicology of synthetic or overly-processed ingredients, which have led them to seek more natural food ingredients and labels (Tajkarimi, Ibrahim, & Cliver, 2010; Topper, 2014). This has prompted food companies and researchers alike to search for all natural, label-friendly alternatives to replace preservatives that are currently ill received by consumers.

### 2.1.1 Natural Antimicrobials

Although natural antimicrobial agents abound in nature and can be readily extracted from different sources (**Figure 1**), finding feasible label-friendly options to incorporate in food systems is rather challenging. There are a myriad of considerations to take into account when selecting preservatives, natural or not. These considerations, depicted in **Figure 2**, tend to focus on the antimicrobial affectivity of the compound of interest, any impact on sensory attributes that may arise upon its incorporation in a food product, its compatibility with food matrices

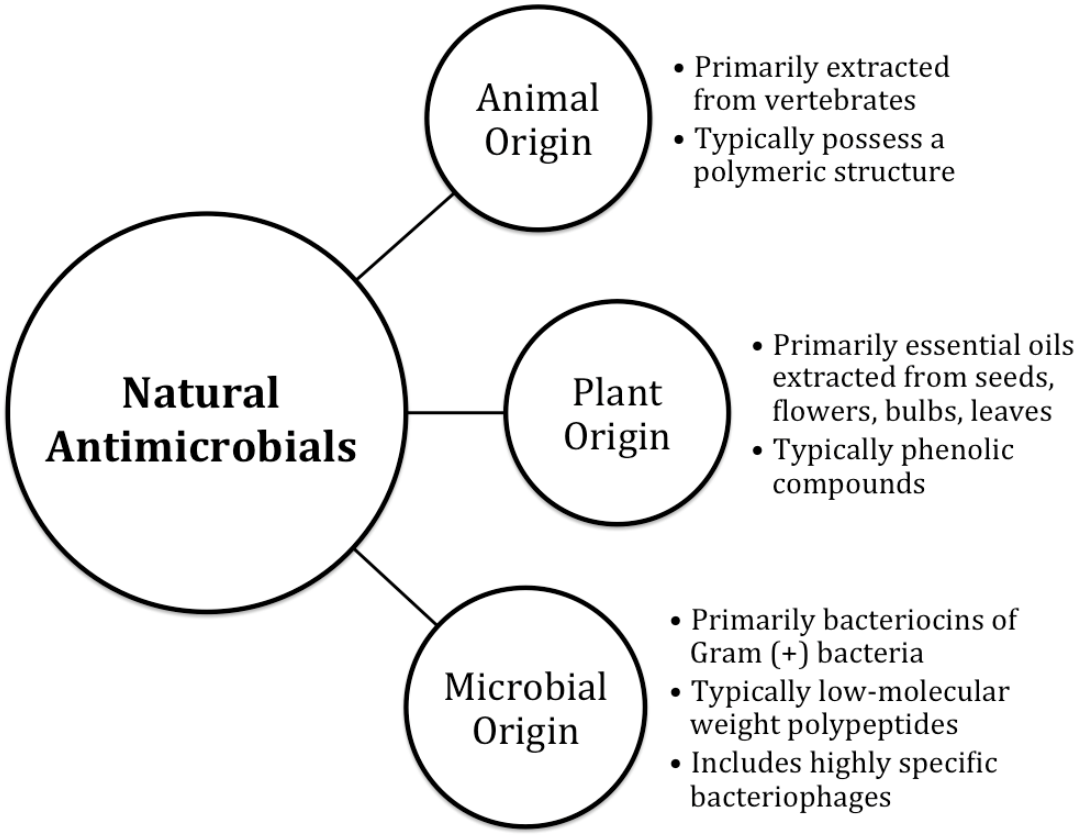



**Figure 1. Sources of natural antimicrobial agents[1].**

[1] Adapted from Davidson, Critzer, and Taylor (2013)

and processing, and any possible toxicological hazards that it may impose to those who may consume it (Davidson, Critzer, & Taylor, 2013).

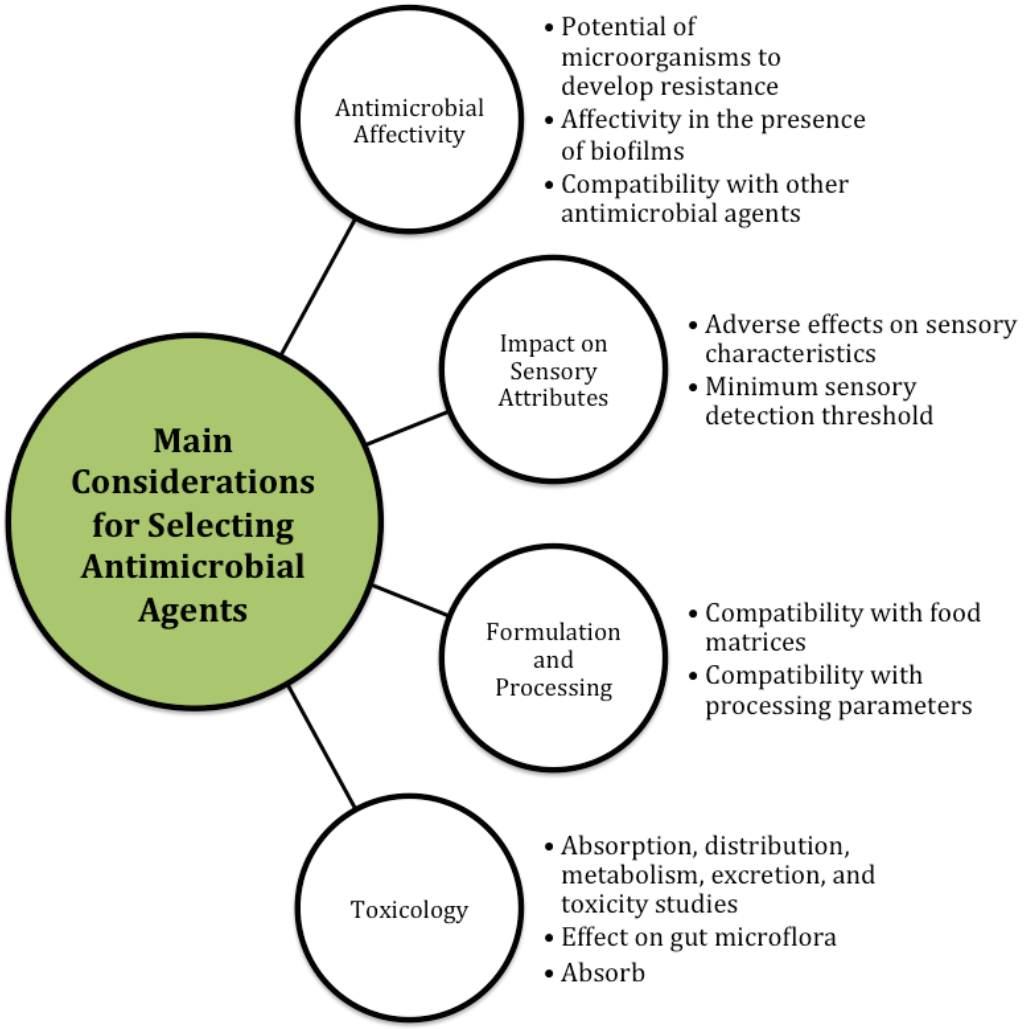



**Figure 2. Considerations for selecting an antimicrobial agent.**[2]

[2] Adapted from Davidson, Critzer, and Taylor (2013)

Based on the aforementioned considerations, an ideal antimicrobial agent should have a high affectivity at low concentration, be effective against a wide range of spoilage and pathogenic microorganisms, not cause undesirable organoleptic changes, pose no toxicological danger to consumers, and have a low cost of production and implementation (Davidson, Critzer, & Taylor, 2013). These characteristics are presented in **Figure 3**.

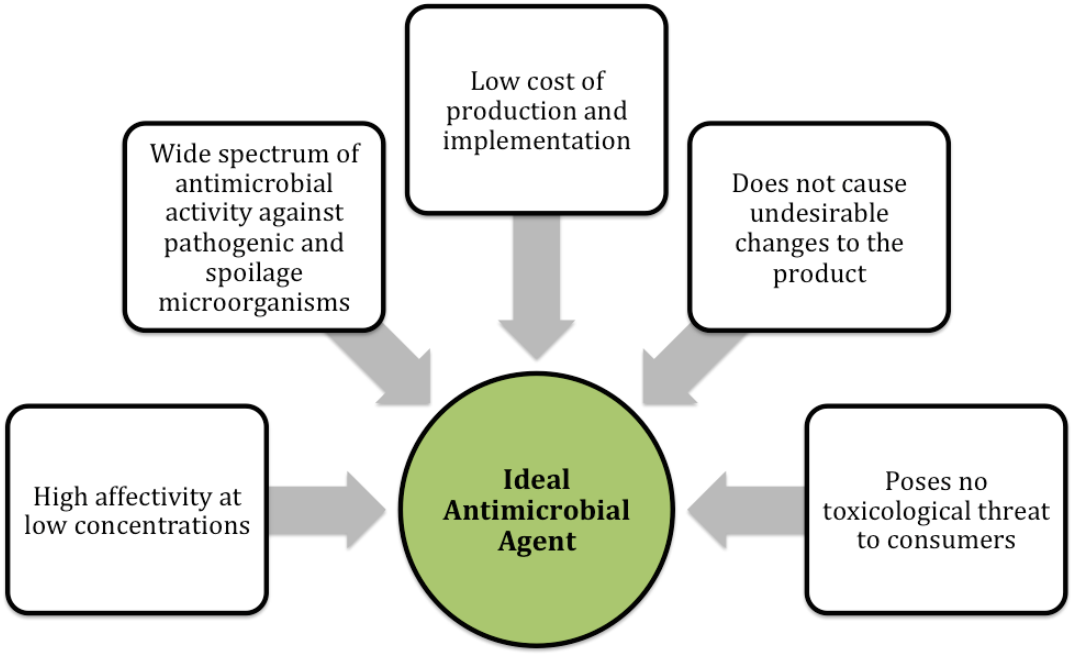



**Figure 3. Ideal characteristics of antimicrobial agents**[3]

One of the main and unexpected difficulties that arise from using natural antimicrobials stems precisely from their natural origin. Due to the complexity of the systems from which natural preservatives are obtained, those that possess a sufficiently strong antimicrobial potency that allows them to be added (and labeled)

[3] Adapted from Davidson, Critzer, and Taylor (2013)

as a whole food or extract are rare; most require undergoing a purification or refining process to be successfully implemented in foods. This processing, however, transforms the preservative's label nomenclature into something much less appealing to consumers (Davidson, Critzer, & Taylor, 2013).

Furthermore, many sources have reported that the incorporation of natural antimicrobial agents in complex food matrices commonly results in undesirable organoleptic changes – particularly flavors, odors, and the formation of aggregates that may impact the appearance and acceptability of the product. These complexes are often a product of the natural preservative interacting and even reacting with proteins, lipids, sugars, and cationic or anionic compounds, commonly resulting not only in the destabilization of the food matrix, but also a diminished antimicrobial activity (Chobpattana, Jeon, Smith, & Loughin, 2002; Davidson, Critzer, & Taylor, 2013; Devlieghere, Vermeulen, & Debevere, 2004; Gaysinsky, Taylor, Davidson, Bruce, & Weiss, 2007; Tserennadmid, Tako, Galgoczy, Papp, Vagvolgyi, Gero, et al., 2010; von Staszewski, Pilosof, & Jagus, 2011). The mechanism behind the formation of such complexes and many of their consequences in food products will be described in *Section* 2.1.2.1.1.1 Electrostatic Interactions.

Due to the frequency with which the aforementioned problems have been observed, researchers have devoted time and resources to identifying suitable natural antimicrobial agents and developing technologies that conserve – and in some cases enhance – their antimicrobial power while simultaneously preventing them from interacting with components of the food matrix.

### 2.1.2 ε-Polylysine: A Potent Antimicrobial

One of the most promising groups of all-natural preservatives are antimicrobial peptides. These compounds are secreted by some insects, reptiles, mammals, and plants as part of their natural immune system to ward off infection (Blin, Purohit, Leprince, Jouenne, & Glinel, 2011; Brogden, 2005; Soares & Mello, 2004; Zasloff, 2002) and by some microorganisms as part of their competitive advantage to obtain dominance over other microorganisms in complex ecologies (Davidson, Critzer, & Taylor, 2013). Their wide-spectrum antimicrobial efficacy, minimum inhibitory concentrations (MIC) of a few parts per million (ppm), and low propensity to generate resistance in microorganisms have led them to be proposed as an all-natural alternative superior to conventional preservatives in food systems (Blin, Purohit, Leprince, Jouenne, & Glinel, 2011; R. E. W. Hancock & H. G. Sahl, 2006; Zasloff, 2002).

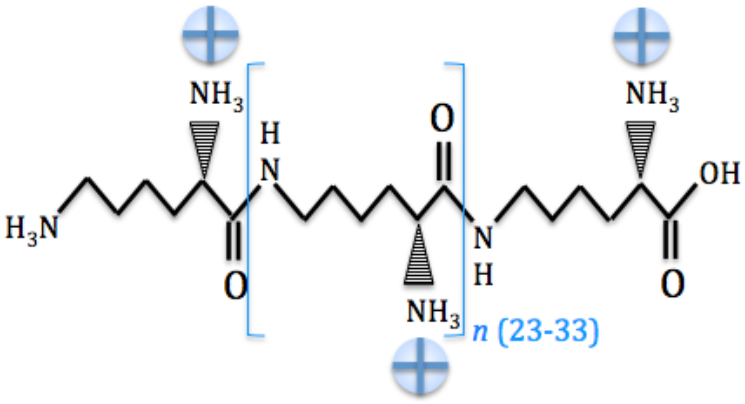



**Figure 4. Representation of the structure of ε-PL at pH < pI[4].**

[4] Modified from (Food and Drug Administration, 2011)

A member of the antimicrobial peptide family that has received much attention from academia as a promising antimicrobial agent in food systems (Y. Chang, L. McLandsborough, & D. J. McClements, 2011a; Y. Chang, McLandsborough, & McClements, 2012; Islam, Oishi, Machida, Ogura, Kin, Honjoh, et al., 2014; Liu, Pei, Han, Feng, & Li, 2015; Muriel-Galet, Lopez-Carballo, Gavara, & Hernandez-Munoz, 2014) is ε-polylysine (ε-PL). This compound is a homopolymer of 25 to 35 L-lysine residues (**Figure 4**) naturally secreted by *Streptomyces albulus* ssp. *Lysinopolymerus* strain 346 (J. Hiraki, Ichikawa, Ninomiya, Seki, Uohama, Seki, et al., 2003; Kahar, Iwata, Hiraki, Park, & Okabe, 2001; Shima & Sakai, 1977). It has shown to surpass the antimicrobial spectrum of widely implemented preservatives (**Figure 5**) such as

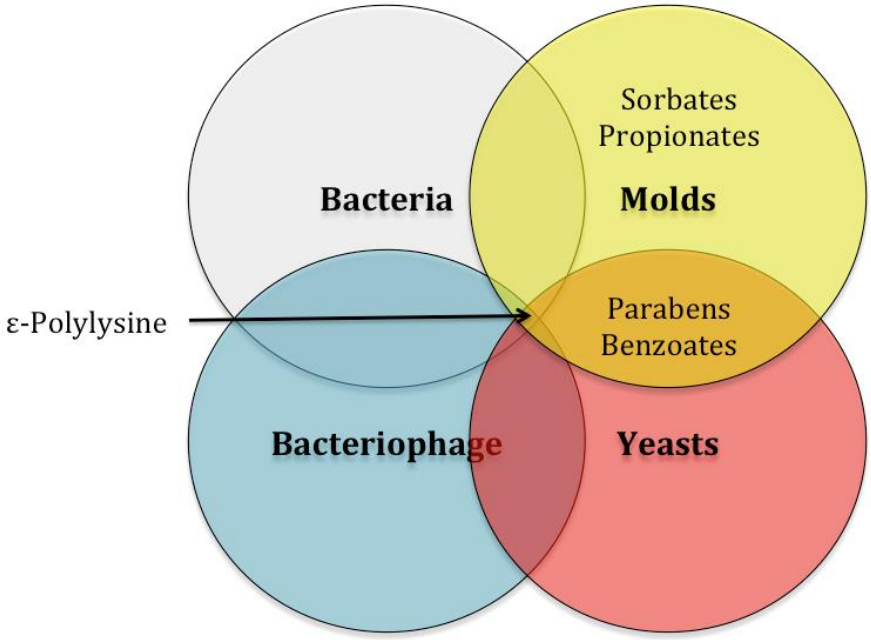



**Figure 5. Diagram comparing the antimicrobial spectrum of commonly utilized preservatives with ε-PL[5].**

[5] Adapted from (Jay, 2005).

propionates, sorbates, benzoates, and parabens (Jay, 2005), as it is highly effective against various Gram (+) and (-) bacteria, yeasts and molds, and even some bacteriophages (S. S. Chang, Lu, Park, & Kang, 2010; Geornaras, Yoon, Belk, Smith, & Sofos, 2007; Shima, Matsuoka, Iwamoto, & Sakai, 1984; Yoshida & Nagasawa, 2003). The list of microorganisms susceptible to ε-PL includes many of the pathogenic microorganisms responsible for major foodborne diseases outbreaks (**Table 1**), making it an even more interesting alternative.

The concentration of primary amine groups ($NH_3^+$) along ε-PL's backbone provide the polymer with a high isoelectric point (pI~9), making it positively charged at pH values commonly found in foods (Yoshida & Nagasawa, 2003), as shown in Figure 4. This positive charge is what makes ε-PL a potent antimicrobial; ε-PL interacts electrostatically with the negatively charged surface of microorganisms, becoming adsorbed. Once adsorbed, ε-PL permeabilizes the membrane by forming pores or structural defects, eventually stripping the outer membrane and causing cell death through the abnormal distribution of the cytoplasm (Blin, Purohit, Leprince, Jouenne, & Glinel, 2011; Brogden, 2005; Shima, Matsuoka, Iwamoto, & Sakai, 1984; Zasloff, 2002). Studies on *E. coli* O157:H7 have shown that ε-PL may also affect gene expression and cause oxidative stress by reactive oxygen species (Ye, Xu, Wan, Peng, Wang, Xu, et al., 2013).

**Table 1. Examples of pathogenic microorganisms susceptible to ε-polylysine, and their impact on the annual number of reported foodborne illness cases and deaths[6].**

| Pathogenic Microorganism | Number of Cases | Number of Deaths |
|---|---|---|
| *Bacillus cereus*[7] | 63,400 | 0 |
| *Campylobacter* spp.[8] | 845,024 | 76 |
| *Escherichia coli* (Shiga toxin-producing O157:H7 and non-O157:H7, enterotoxigenic, and other diarrheic strains)[7] | 205,781 | 20 |
| *Listeria monocytogenes*[7] | 1,591 | 255 |
| *Mycobacterium* spp.[7] | 60 | 3 |
| *Salmonella enterica* Typhi[7] | 1,821 | 0 |
| *Staphylococcus aureus*[7] | 241,148 | 6 |

### 2.1.2.1 Applications of ε-polylysine in the Food Industry

ε-PL has undergone the rigorous process required by the FDA to assess its safety (*Section* 2.2 Regulation of Preservatives by the U.S. Food and Drug

[6] Modified from (Scallan, Griffin, Angulo, Tauxe, & Hoekstra, 2011; Scallan, Hoekstra, Angulo, Tauxe, Widdowson, Roy, et al., 2011; Scharff, 2012)
[7] Shima, Matsuoka, Iwamoto, and Sakai (1984)
[8] Food and Drug Administration (2011)

Administration), including extensive toxicology studies (J. Hiraki, et al., 2003) and studies focusing on effects on reproductive, neurological, and immunological functions for two generations in rats (J. Hiraki, 1995; Neda, 1999). With additional documentation submitted by the Chisso Corporation and Purac Biochem b.v., the FDA has deemed ε-PL to be a Generally Regarded As Safe (GRAS) food ingredient (Food and Drug Administration, 2004, 2011), and has approved its use in a wide number of food systems at concentrations up to 0.025% by weight (**Figure 6**).

Despite ε-PL being a potent all-natural antimicrobial with GRAS status and an industrial scale production (J Hiraki, 1999; Shih, Shen, & Van, 2006; Yoshida & Nagasawa, 2003), it is not widely used by the Food Industry in the United States. This is largely due to the high positive charge of the primary amine groups, which presents significant hurdles to the implementation of this natural preservative: ε-PL may (i) interact electrostatically with anionic components present in the food matrix, generating complexes that could increase turbidity, cause sedimentation, and (ii) reduce its antimicrobial efficacy; (iii) confer astringency by interacting

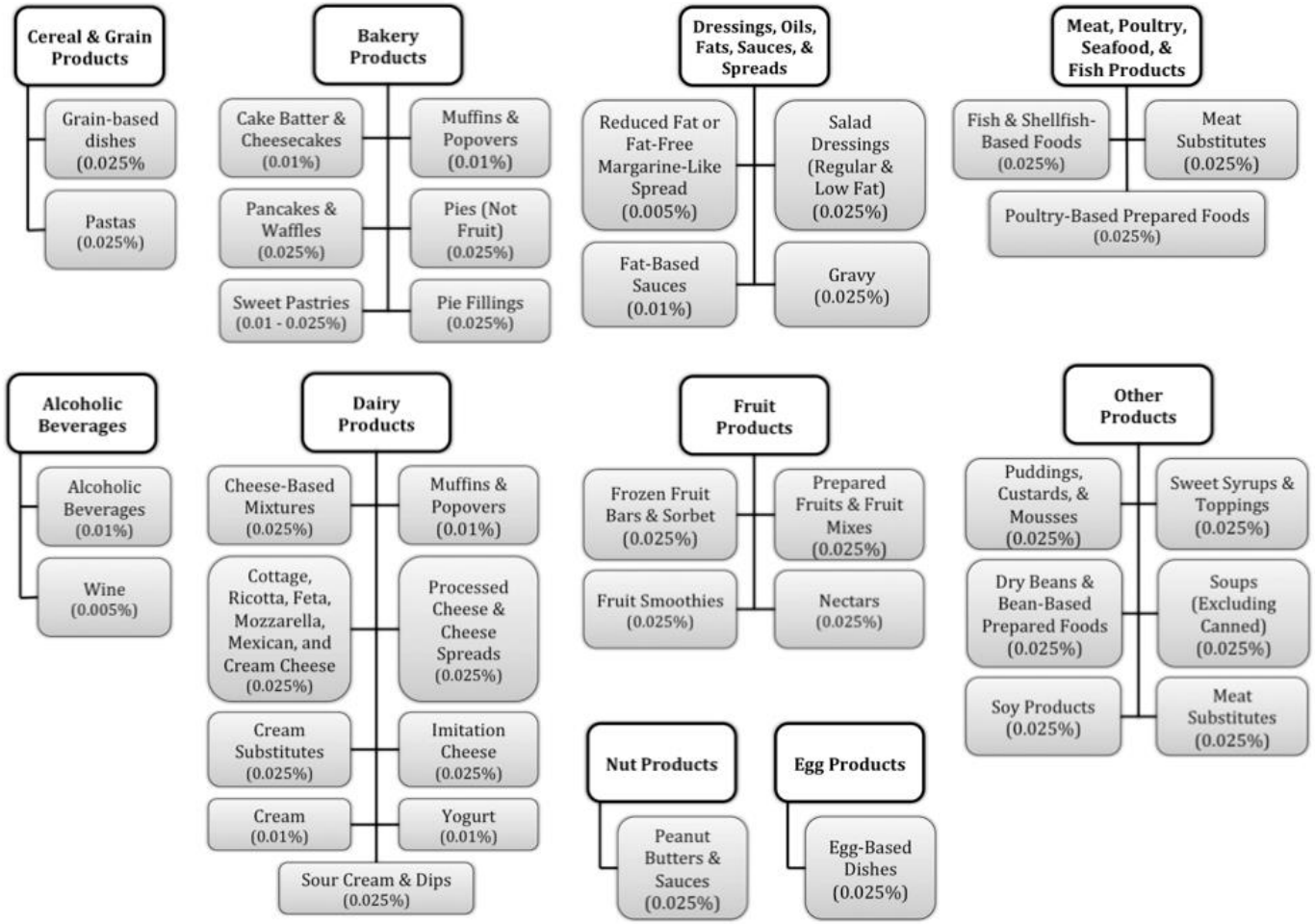



**Figure 6. Intended uses of polylysine in food, as declared by the Food and Drug Administration (2011)**

electrostatically with anionic mucin in human saliva; or (iv) be detected due to its bitter taste (Y. Chang, L. McLandsborough, & D. J. McClements, 2011a; Y. H. Chang, L. McLandsborough, & D. J. McClements, 2011)

#### 2.1.2.1.1 Complexation of ε-PL With Pectin

As mentioned in the previous section, the high cationic charge of ε-PL makes it extremely capable of electrostatic interactions with numerous anionic biopolymers, often with undesirable results. In order to provide an effective solution to this problem, it is important to first have a more thorough understanding about electrostatic interactions.

##### 2.1.2.1.1.1 Electrostatic Interactions

Electrostatic interactions refer to interactions that occur between molecules or complexes with a permanent electrical charge (Israelachvili, 1992; JN Murrell, 1982; Norde, 2003; Reichardt, 1988; Rogers, 1989); these charges can emanate from ions or an uneven distribution of electrons in an otherwise neutral molecule, i.e., its polarity (D. McClements, 2005c). As a rule of thumb, molecular moieties will attract each other if they possess opposite electrical charges, and will repel each other if their electrical charges are the same. The strength of the electrostatic interaction between the molecular systems depends on the magnitude of their charges, the distance between their centers, and the orientation of any dipoles present. The interactions are most intense between systems with strong electrical charges, whose dipoles are oriented towards each other, and whose charges have little physical distance between them (D. McClements, 2005c).

When adequate conditions such as the pH and salt concentration in a system are met, biopolymers can present electrical charges in their structure. These charges may lead to electrostatic interactions with oppositely charged biopolymer molecules, forming electrostatic complexes. These electrostatic interactions have been shown to cause significant changes in the functional properties of the biopolymers involved – particularly changes in solubility, which may have detrimental effects on the overall stability and sensory properties of food systems (ZE Sirorski, 2008)

#### 2.1.2.1.1.2 Complexation of ε-PL with Pectin

The formation of ε-PL-pectin electrostatic complexes has been suggested as a promising solution to the aforementioned issues related to the implementation of ε-PL in food systems (Y. Chang, L. McLandsborough, & D. J. McClements, 2011a; Y. Chang, McLandsborough, & McClements, 2012; Y. H. Chang, L. McLandsborough, & D. J. McClements, 2011; Y. H. Chang, McLandsborough, & McClements, 2014).

Pectin is a popular food ingredient used as a gelling, thickening, and stabilizing agent, primarily extracted from citrus peel and apple pomace (Thakur, Singh, & Handa, 1997). The polymeric structure of pectin is composed of a linear chain of (1→4)-linked α-D galactopyranosyluronic acid units, some periodic L-rhamnose units, and, in some instances, covalently-bound branched arabinogalactan chains and/or shorter D-xylosyl and/or L-rhamnosyl chains (James N. BeMiller, 2008; Thakur, Singh, & Handa, 1997). Additional variations of the pectin building blocks, and perhaps the most important ones in terms of particle charge and functionality, are the different forms in which the carboxylic acids may be present:

the methyl ester form (-$COOCH_3$), free acid form (-COOH) or salt form (-$COO\text{-}Na^+$). Pectins with 50% or more of their carboxylic groups present in the methyl ester form, i.e. a 50% or higher degree of esterification (DE) or degree of methylation (DM), are referred to as high-methoxyl (HM) pectin. Conversely, those with less than a 50% DE are referred to as low-methoxyl (LM) pectin (James N. BeMiller, 2008). The differences between HM and LM pectin can be observed in **Figure 7**. Regardless of the DE, the carboxylic acid side groups give pectin a $pK_a$ of approximately 3.5, making the polymer negative at pH values around and above a pH of 3.5 (Asker, Weiss, & McClements, 2011).

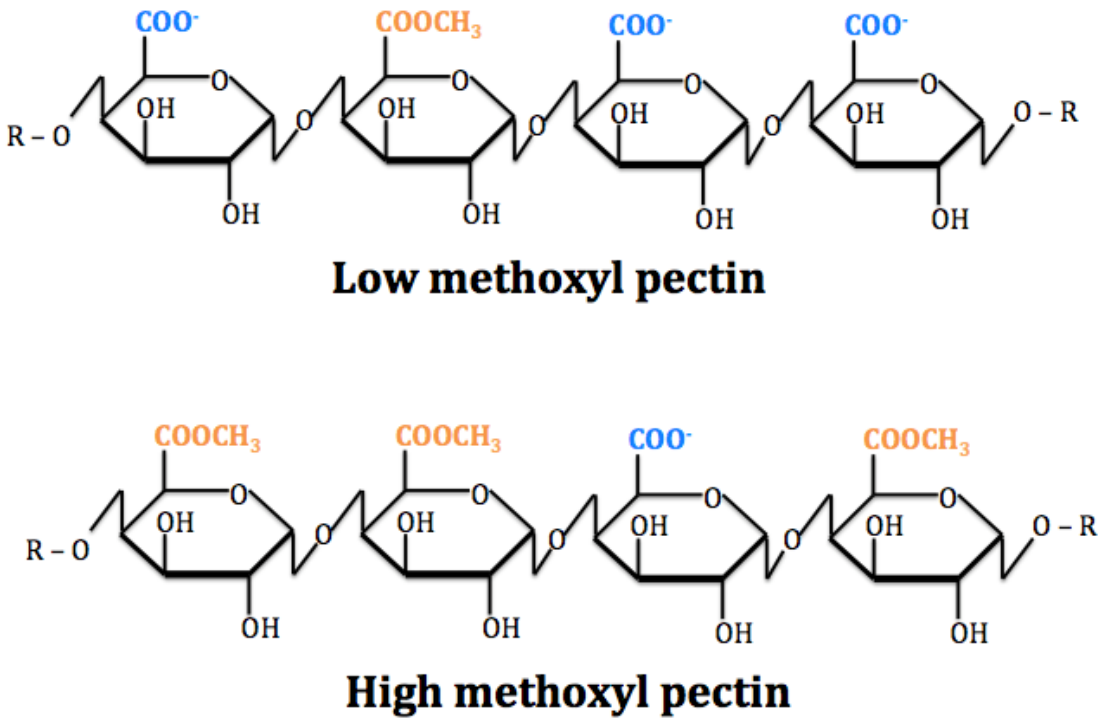



**Figure 7. Representation of the structure of low and high methoxyl pectin at pH ≥ $pK_a$**[9]

[9] Adapted from (Kabir, Wang, Lau, & Cardona, 2012; Tharanathan, 2003)

Electrostatic complexes between pectin and ε-PL can be formed easily by dispersing both polymers in a solution at a pH equal to or above the $pK_a$ of pectin and below the pI of ε-PL. Under these conditions, they possess opposite charges and are able to interact electrostatically with each other (Y. Chang, McLandsborough, & McClements, 2012). The interaction between the cationic primary amine groups of ε-PL and anionic polymers has been shown to form electrostatic complexes that promote the transition from ε-PL's coil structure to a helical structure (Sedlmeyer, Brack, Rademacher, & Kulozik, 2004; Tholstrup Sejersen, Salomonsen, Ipsen, Clark, Rolin, & Balling Engelsen, 2007). An important consideration when forming electrostatic complexes between pectin and ε-PL is that the DE may have important implications, as it could result in radically different negative charge intensity between HM and LM pectins. The use of lower DE pectin (which have a lower number of the carboxyl groups' charge neutralized by the esterification of methoxyl groups) results in a stronger, more densely packed complex that may cause considerable turbidity and even sedimentation (Y. Chang, McLandsborough, & McClements, 2012), as well as potentially quenching the antimicrobial efficacy of ε-PL.

This complexation strategy has been shown to be successful in decreasing the overall charge of ε-PL at mass ratios of 1:1 – 1:20 ε-PL : HM pectin (pH 3.5), conferring pectin's anionic charge to ε-PL while still maintaining ε-PL's antimicrobial efficacy against the acid-resistant yeasts *Zygosaccharomyces bailii* and *Saccharomyces cerevisia*. Furthermore, this successful negatively charged antimicrobial complex possesses a low turbidity and is stable in a model green tea

beverage system (Y. Chang, L. McLandsborough, & D. J. McClements, 2011a; Y. Chang, McLandsborough, & McClements, 2012; Y. H. Chang, L. McLandsborough, & D. J. McClements, 2011).

While these are promising results, additional testing assessing the stability of these complexes in more multifarious food models, their digestibility, toxicology, and overall impact on consumer health are required.

## 2.2 Regulation of Preservatives by the U.S. Food and Drug Administration

In the United States, the FDA plays a crucial role in monitoring the plethora of current and potential food additives. The Center for Food Safety and Applied Nutrition's (CFSAN) Office of Food Additive Safety monitors circa 80% of the food supply, employing a science-based system to scrutinize the safety of food additives, while communicating and exchanging scientific information with the interested parties (Alger, Maffini, Kulkarni, Bongard, & Neltner, 2013). The system relies heavily on science, defining safety as

> "[Having] a reasonable certainty in the minds of competent scientists that [a] substance is not harmful under the intended conditions of use" (§170.3(i)).

In order to allow the utilization of additives in food systems, the FDA requires that the interested party (the sponsor) submit a detailed report with scientific evidence that attests to the substance's safety (Gaynor, 2006). These reports must answer the three mandated queries established by Congress in order to determine whether or not the substance of interest is safe:

> "(i) An estimate of the amount of the substance itself and any other substances that may be ingested due to the incorporation of the substance of interest; (ii) the amassed effect of ingesting the aforementioned amount of the substance of interest and any other substance ingested due to the incorporation of the substance of interest; and (iii) the safety factors that, in the opinion of experts, are generally recognized as appropriate" (§170.3(i)).

These reports include detailed information about the nomenclature, formulation, purity, stability, intended technical effects and uses, intake estimates, a detailed methodology to analyze and detect the additive in food systems, and a thorough report of the extensive toxicology analysis, along with a rationale from the sponsor summarizing why the substance of interest should be considered GRAS for its intended use (United States Food and Drug Administration 2009). The submitted information is carefully reviewed and evaluated by a group of qualified experts, who must reach a consensus regarding the safety of the substance of interest under the conditions of its intended use. After the consensus is reached and the final decision is made, the FDA gives one of three answers: (i) "FDA does not question the basis for the notifier's GRAS determination;" (ii) "The notice does not provide a sufficient basis for GRAS determination;" or (iii) "The agency has, at the notifier's request, ceased to evaluate the GRAS notice" (Gaynor, 2006).

True to their openness with information, the FDA's website publishes their regulation guidelines for the Food Industry in the FDA Redbook, an online database with all the additives and ingredients permitted to be incorporated into food

systems ("Everything Added to Food In the United States"), and a database with all the GRAS notices that the FDA has ever received (Branen AL, 2001; Food and Drug Administration, 2007, 2013; Gaynor, 2006).

### 2.2.1 Toxicology Analysis

The toxicological analyses are the most crucial section of the GRAS notification process, as they will provide the solid scientific evidence for the approval or rejection of the food additive from the FDA. Due to their importance, the FDA has included a set of guidelines with examples of suggested studies that could be included in the documentation (Food and Drug Administration 2014).

The proposed studies include genetic toxicity tests, short-term toxicity tests with rodents, subchronic toxicity studies with rodents, subchronic toxicity studies with non-rodents, one-year toxicity studies with non-rodents, chronic toxicity, carcinogenicity studies with rodents, reproduction studies, developmental toxicity studies, metabolism and pharmacokinetic studies, and human studies (Food and Drug Administration 2014). One of the main considerations when conducting toxicological studies is to first determine the estimated daily intake (EDI); that is, the average daily intake of the substance of interest over a lifetime based on the average consumer's diet (Food and Drug Administration 2009). The EDI is then used as a base, changing the amount that the test subjects will be exposed to accordingly to their body weight, desired exposure level, length of the study, etcetera. After the experiment has been conducted, the highest dosage (mg/kg body weight) at which no negative effects were observed in the most susceptible test subject is identified. This value, the "No Observed Adverse Effect Level" or "NOAEL"

is multiplied by case-specific conversion factors to establish the maximum usage levels for the substance of interest, also known as "Acceptable Daily Intake" or ADI (Carocho, Barreiro, Morales, & Ferreira, 2014).

## 2.3 Gastrointestinal Tract and Experimental Models

### 2.3.1 Human Digestive Tract

The gastrointestinal (GI) tract is composed a flexible muscular tube that encompasses the mouth, pharynx, esophagus, stomach, and small and large intestines (**Figure 8**), whose joint main function is to efficiently extract and absorb nutrients from ingested food. The epithelial cells of these organs secrete a protective mucus layer that contains mucin, a group of glycosylated proteins with a large molecular weight. The high level of glycosylation of these proteins provides them with an extremely high water-holding capacity (Kufe, 2009; Perez-Vilar & Hill, 2004), which lubricates the epithelial surfaces to decrease friction with the passage of food, traps and immobilizes pathogens, and is permeable enough to allow nutrients to diffuse through it to be absorbed by the epithelial cells in certain sections of the GI tract (Mackie, 2012). Each of the sections or organs that make up the GI tract – particularly the mouth, stomach, and intestines – are equipped with specific muscular activity, enzymes, and other characteristics that make their internal conditions vastly dissimilar from one another, and consequently provide them with an essential role in the digestion and absorption of nutrients and excretion of waste (Basit, 2005). Additional glands and organs such as the salivary

glands, liver, pancreas, and gallbladder provide the enzymes and digestive juices that are crucial

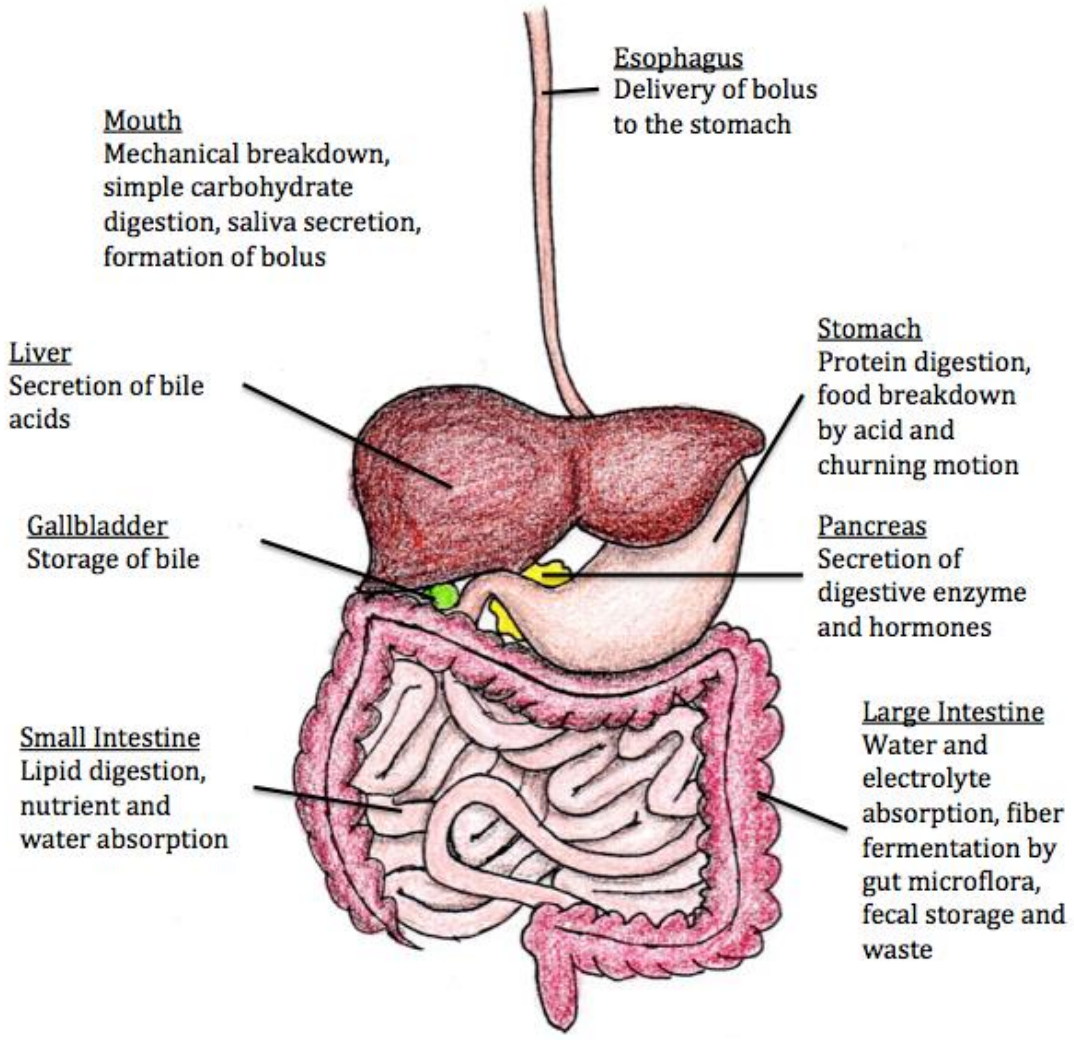



**Figure 8. Diagram of the Digestive Tract[10].**

to the digestion of foods (National Institute of Diabetes and Digestive and Kidney Diseases, 2013). The muscles that line the GI tract contract continuously, moving food from organ to organ. This action – called peristalsis – occurs at varying rates

[10] Adapted from National Institute of Diabetes and Digestive and Kidney Diseases (2013)

and intensities in different parts of the GI tract, providing additional turbulence and even a grinding motion in some organs (Whitney, 2005).

#### 2.3.1.1 Mouth

The digestive process begins in the mouth, where food is introduced into the body and undergoes physical and chemical transformations into a moist, soft, paste-like substance called the bolus. Within seconds, chewing breaks down large fragments of food into small particles – simultaneously relating sensory information such as aromas, flavors, and texture – in order to prevent physical damage to the digestive organs and increase the surface area of the ingested food. This larger surface area promotes interaction of food with digestive enzymes and juices, increasing their efficiency and favoring the absorption of nutrients in other digestive organs. The small amount of saliva injected into the mouth by the salivary glands – which contains water, salts, mucus, and enzymes such as amylase – moistens the food and degrades the majority of carbohydrates, making the ingested food more manageable in the stomach. The resulting ground and partially digested food is swallowed and passes through the esophagus into the stomach (Mackie, 2012; Whitney, 2005).

#### 2.3.1.2 Stomach

The bolus enters the stomach through the esophageal sphincter, which seals itself to prevent the undesirable re-entry of the bolus into the esophagus. The bolus is kept in the upper portion of the stomach and is gradually transferred to the lower

section, where it undergoes gastric digestion. The conditions in the lower stomach are vastly different from those in other parts of the digestive system: gastric glands release juices that contain the proteolytic enzyme pepsin and the hydrolytic enzyme gastric lipase, water, and hydrochloric acid; the pH drops to 1.5-3 depending on the ingested food and whether the subject has been fasting; and strong muscle contractions provide a grinding action. The low pH renders the salivary enzymes inactive, and it is the proteolytic activity of lipase that dominates in the stomach. As the acid and enzymes act on the bolus, it is slowly transformed into a semiliquid mass called chyme. As the chyme is produced, sensors in pyloric sphincter detect certain nutrients and regulate gastric emptying based on the stimuli they receive (Mackie, 2012; Whitney, 2005).

#### 2.3.1.3 Small Intestine

Chyme is slowly released from the stomach through the pyloric sphincter into the upper portion of the small intestine (the duodenum), where it interacts with intestinal juices secreted by the gallbladder through the bile duct. These juices contain sodium bicarbonate, digestive enzymes, and the surfactants bile salts and phospholipids; as the juices come in contact with chyme, the pH rises to approximately 6.5, and the enzymes degrade any remaining lipids, carbohydrates, and peptides (Whitney, 2005).

The intestinal walls are especially designed to absorb nutrients: the mucous layer that protects them contains digestive enzymes that act on any undigested nutrients and is permeable enough to allow their passage, while the epithelial cells that make up the wall have an extremely large surface area. As chyme travels

through the three sections of the small intestine (duodenum, jejunum, and ileum) and is in constant contact with the intestinal walls, the enzymatically broken down nutrients are absorbed by the body (Mackie, 2012). The remaining chyme, at this time primarily water and electrolytes, leaves the small intestine through the ileocecal valve and enters the large intestine (Whitney, 2005).

#### 2.3.1.4 Large Intestine

Unlike the other organs of the digestive tract, the colon does not have strong or rapid peristaltic movements, high concentrations of acid or bile, or significant enzymatic activity, and maintains a near neutral pH (Evans, Pye, Bramley, Clark, Dyson, & Hardcastle, 1988; Gibson, McFarlan, Hay, & Macfarlane, 1989; Van den Mooter, Stas, Damian, Naesens, Balzarini, Kinget, et al., 1998; Yang, Chu, & Fix, 2002). Rather, the role of the large intestine is to absorb any remaining water or electrolytes present in the remaining chyme as it travels through the large intestine, act as a reservoir of fecal material until the latter is excreted (Whitney, 2005), and act as a biofermentor. Biofermentation is achieved through the large number of microorganisms that reside in the different areas of this organ, which degrade compounds that were not digested and/or absorbed in the upper digestive tract (Edwards, 1997). These microorganisms, also known as the gut microflora, play a crucial role on the host's health, and will be discussed in *Section* 2.4 Gut Microbiota.

### 2.3.2 Digestion, Absorption, and Transport of Lipids

The average person consumes anywhere between 20 to 160 grams of fat every day, out of which approximately 97% are triglycerides (TAG), 1% are cholesterol, less than 1% are other lipophilic compounds, and 2% are phospholipids. All these compounds undergo digestion and absorption in the body, and do so with an efficiency of approximately 98% (Lairon, 2009).

Ingested dietary fat begins the digestive process in the mouth, where it is subjected to a small degree of degradation by lingual lipase. Despite the presence of gastric lipase in the stomach, no significant degradation of TAG occurs in this organ. This is primarily due to the low pH, which prevents the fatty acids (FAs) from disassociating from the glycerol molecule, possibly deactivating the enzyme (Mackie, 2012). However, the violent grinding motion of the gastric muscles disperses any fat present into smaller droplets. This increased surface area makes the fat droplets more susceptible to the enzymatic activity of lipase in the small intestine (Lairon, 2009).

As chyme enters the small intestine, the fat droplets contained in it trigger the secretion of the hormone cholecystokinin (CCK), which prompts the gallbladder to inject bile and pancreatic bicarbonate into the small intestine (Lairon, 2009; Mackie, 2012). The secretion of bicarbonate increases the pH to 6.5-7, permitting the ionization of the acid groups present in the fat (Lairon, 2009). Bile acids or salts, the primary component of bile, are natural surfactants produced in the liver. Their chemical formula consists of a hydrophobic molecule of cholesterol complexed with a hydrophilic amino acid. The primary structure formed in the liver is conjugated

with taurine and glycine (ROMAŃSKI, 2007), and numerous variations occur naturally (Meredith, Caprio, & Kajiura, 2012; Mukhopadhyay & Maitra, 2004). An example of a bile salt is presented in **Figure 9**. Bile salts and phospholipids present

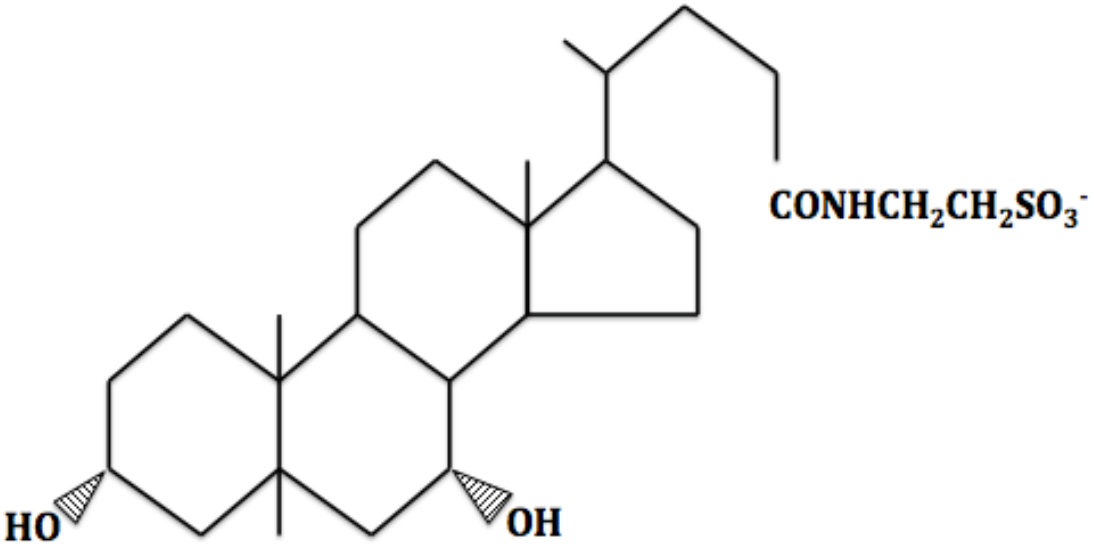



**Figure 9. Chemical structure of taurochenodeoxycholic acid, an example of a bile salt.**[11]

in bile – or any other surfactant – stabilize the dispersed fat droplets by placing themselves on the water-lipid interface, with the hydrophobic sections of the molecules interacting with the fat droplets whereas the hydrophilic sections interact with the aqueous phase of chyme (Maldonado-Valderrama, Wilde, Macierzanka, &

[11] Adapted from (Meredith, Caprio, & Kajiura, 2012)

Mackie, 2011). This facilitates the dispersion and solubilization of lipids including cholesterol in the aqueous phase within the intestine (ROMAŃSKI, 2007)

In order to cleave fatty acids present in phospholipids, TAGs, or complex sterols, lipase becomes adsorbed onto the surface of the bile acid- or phospholipid-stabilized fat droplets and hydrolyzes the FAs. For TAGs, this enzymatic activity typically results in two free fatty acids chains and one monoglyceride (MAG) molecule (Lairon, 2009). Glycerol and short- and medium-chain FAs can diffuse easily into the intestinal cells, where they rearranged into TAG, incorporated into transport vessels called chylomicrons, and released into the lymphatic system (Mackie, 2012). After serving their purpose in emulsifying fat, bile salts are either reabsorbed from the intestine and reused, or they can be trapped by fibers excreted with feces (Lairon, 2009).

#### 2.3.2.1 Emulsion-Based Delivery Systems

Emulsions consist of the combination of two immiscible phases (typically an aqueous phase and an oil phase), where one is dispersed in another. Oil-in-water emulsions, such as milk, are composed of small oil droplets dispersed in an aqueous continuous phase. Water-in-oil emulsions, such as butter, are the inverse; small water droplets dispersed in a continuous oil phase (D. McClements, 2005a). These systems are generally stabilized though the incorporation of emulsifiers, which are surface-active molecules that can become adsorbed on the surface of the emulsion droplets. Once adsorbed, they are able to decrease the surface tension between the water-oil interphase and provide a physical barrier that prevents emulsion droplets from interacting and aggregating, thus increasing their stability (D. McClements,

2005a). The production of emulsions is commonly achieved through homogenization, which consists of subjecting the aqueous phase, oil phase, and emulsifier to a high degree of mechanical action. This causes small, spherical, and mostly homogeneous droplets of the discontinuous phase to be dispersed throughout the continuous phase. As the new droplets are formed, the emulsifier becomes adsorbed to their surface and exerts its aforementioned stabilizing and protective activity (D. McClements, 2005a). Emulsifiers are selected based on the type of oil used, desired particle sizes and/or charge of the emulsion system, compatibility with the matrix in which it will be implemented, and any other preferred additional functional characteristics (Acosta, 2009; Gutierrez, Gonzalez, Maestro, Sole, Pey, & Nolla, 2008; S. J. Lee, Choi, Li, Decker, & McClements, 2011; Qian & McClements, 2011).

Emulsions are a versatile system, and have nearly endless applications in food systems. In recent years, their functionality has included controlling the release of certain ingredients in a food matrix, targeting the release of bioactive components to their absorption site in the digestive tract (Salvia-Trujillo, Qian, Martin-Belloso, & McClements, 2013b), protecting sensitive ingredients from degradation or undesirable interactions with specific components (Zhang, Decker, & McClements, 2014), and modifying texture in food products (Paradiso, Giarnetti, Summo, Pasqualone, Minervini, & Caponio, 2015), to name but a few.

When forming successful emulsion-based delivery systems for bioactive components – which are of particular interest to the research presented here, it is imperative to have a thorough understanding of the physicochemical and

physiological processes that food – and the intended delivery system – undergoes in the digestive tract, as well as the factors that hinder the rate and extent to which the compound of interest is absorbed from a food matrix and becomes available at the physiological site of action, i.e. its bioavailability (Lesmes & McClements, 2009).

#### 2.3.2.1.1 Emulsion Instability

There are four main mechanisms by which an emulsion system may forego its original properties over time: creaming, sedimentation, flocculation, coalescence, and phase inversion (**Figure 10**). Changes in particle density triggers gravitational separation, which in turn causes either creaming or sedimentation. When the emulsion droplets' density becomes lower than that of the continuous phase, the particles rise to the surface of the continuous phase and "creaming" is observed, whereas the opposite is true for sedimentation. Flocculation and coalescence both refer to the aggregation of emulsion droplets; however, flocculation is observed in a system when the aggregated droplets retain their individual characteristics, whereas coalescence encompasses the merger of two or more droplets to form a single, considerably larger, droplet (D. McClements, 2005a). Phase inversion, on the other hand, refers to the phenomenon observed when system-specific conditions cause an oil-in-water emulsion to become a water-in-oil system, and vice versa (D. McClements, 2005a).

There are many techniques to prolong the stability of emulsion systems, including controlling droplet size, manipulating the viscosity of the continuous phase to decrease the rate of collision between droplets, and decreasing the physical

interaction between droplets through electrostatic or steric repulsion, to name but a few (D. McClements, 2005b).

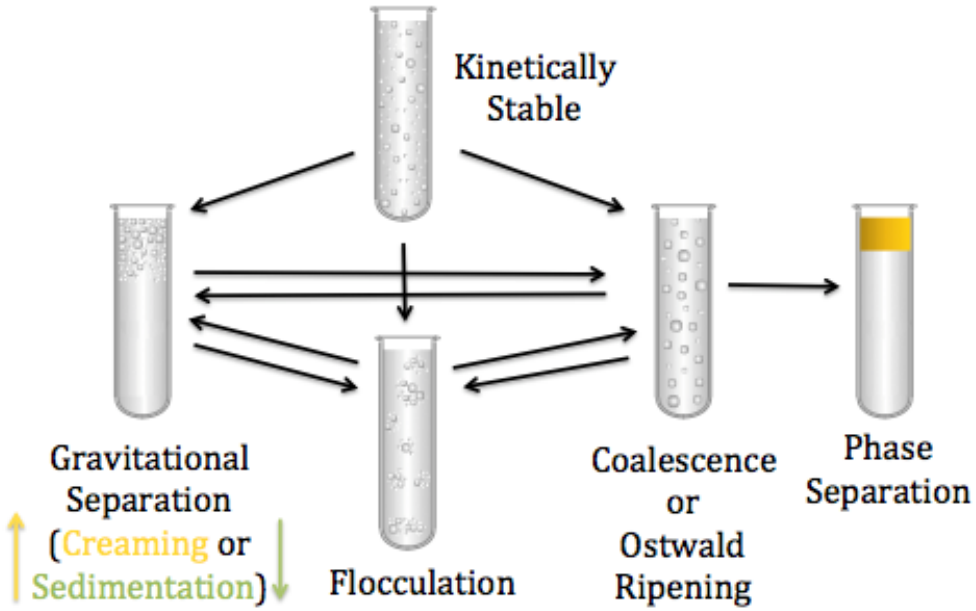



**Figure 10. Representative diagram of the main mechanisms of emulsion instability[12].**

### 2.3.2.2 Polylysine-Induced Hindrance of Fat Absorption

ε-PL has been shown to inhibit lipid digestion *in vivo* and *in vitro* (Kido, et al., 2003; Takahiro Tsujita, 2006; Tsujita, Takaichi, Takaku, Aoyama, & Hiraki, 2006; Tsujita & Takaku, 2009). Due to the negative charge of bile salts and the positive charge of ε-PL, it has been hypothesized that the observed phenomenon originates

[12] Adapted from D. McClements (2005b)

from a strong electrostatic interaction between these two compounds. However, the inhibition mechanisms are not as clear.

It has been proposed that ε-PL inhibits lipid digestion through interacting with the mixed micelles stabilized by bile salts. According to Kido, et al. (2003), ε-PL is attracted to the bile salts on the lipid-water interface and forms an electrostatic complex. The formation of this complex can either (i) generate a steric hindrance to the adsorption of lipase onto the droplet surface or (ii) destabilize and effectively destroy the emulsion droplet, efficaciously inhibiting the enzymatic activity of lipase. However, given that bile salts are a fundamental part of lipid digestion and absorption (Macierzanka, Rigby, Corfield, Wellner, Boettger, Mills, et al., 2011; Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011), it is possible that even electrostatic interaction on the aqueous phase between available bile salts and ε-PL may also generate some degree of inhibition.

It is extremely important to understand the possible effect that the incorporation of ε-PL into food products formulations may have on the overall digestion of lipids, as a decrease in lipid digestion may also impact the success of delivery systems specifically designed to deliver lipophilic nutrients to consumers (Y. Li & McClements, 2011; Porter, Trevaskis, & Charman, 2007), and decrease the bioaccessibility and bioavailability of ingested lipophilic bioactive compounds.

### 2.3.3 *In Vitro* Simulations of the Digestive Tract

Due to the complexity and difficulty of acquiring and analyzing data directly from the human digestive tract – as well as high costs, ethical implications, and concern for the test subjects, researchers often turn to simplified models that

simulate the human digestive process. The typical recreations employed are *in vitro* simulations, cell cultures, and animal models, each presenting their own advantages and disadvantages (Mackie, 2012).

*In vitro* digestion models are, as their name implies, designed specifically to mimic conditions of the GI tract. Although they're not as biologically accurate as other models, their numerous advantages make them the most widely utilized model of the digestive tract: they are relatively inexpensive, ideal for rapid, more direct tests, perfectly suited for determining physicochemical mechanisms, specific interactions or reactions, and have a wide range of applications (Lesmes & McClements, 2009; Mackie, 2012). Some of the most popular applications include assessing organoleptic properties, fat perception (Mackie, 2012), and monitoring the disintegration and release of delivery systems at different locations within the GI tract (Lesmes & McClements, 2009).

#### 2.3.3.1 Lipid Digestion Models

There are numerous methodologies implemented to assess the degree of lipid digestion *in vitro*, and although the instruments and specific assays may vary between research groups, the principle behind the simulation of the digestive process is the same: a sample is exposed to the physiological conditions (digestive juices, enzymes, and pH) that a food would encounter in each digestive organ, holding those simulated conditions for a period of time that would most closely simulate the degree of degradation that the sample would undergo in each organ (Mackie, 2012).

The *in vitro* digestion model presented in the experimental section of this document consists of subjecting the sample of interest to three digestion stages, simulating oral, gastric, and intestinal duodenal digestion conditions (**Figure 11**). Each phase has its own simulated digestive juices: artificial saliva (AS), simulated gastric juices (SGJ), and simulated intestinal juices (SIJ), their composition simplified from the digestive juices and secretions mentioned in *Section* 2.3.1 Human Digestive Tract (Yan Li & McClements, 2010; Mao & McClements, 2012; Salvia-Trujillo, Qian, Martin-Belloso, & McClements, 2013b). It is important to consider that because this model does not simulate the chewing motion or the peristaltic movements in the GI

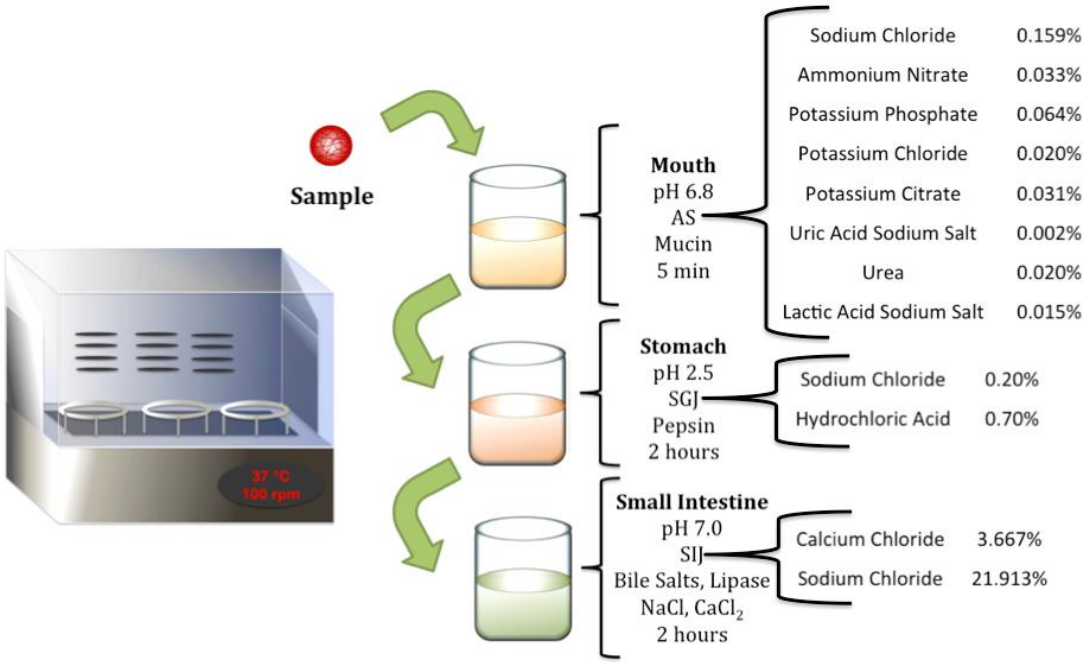
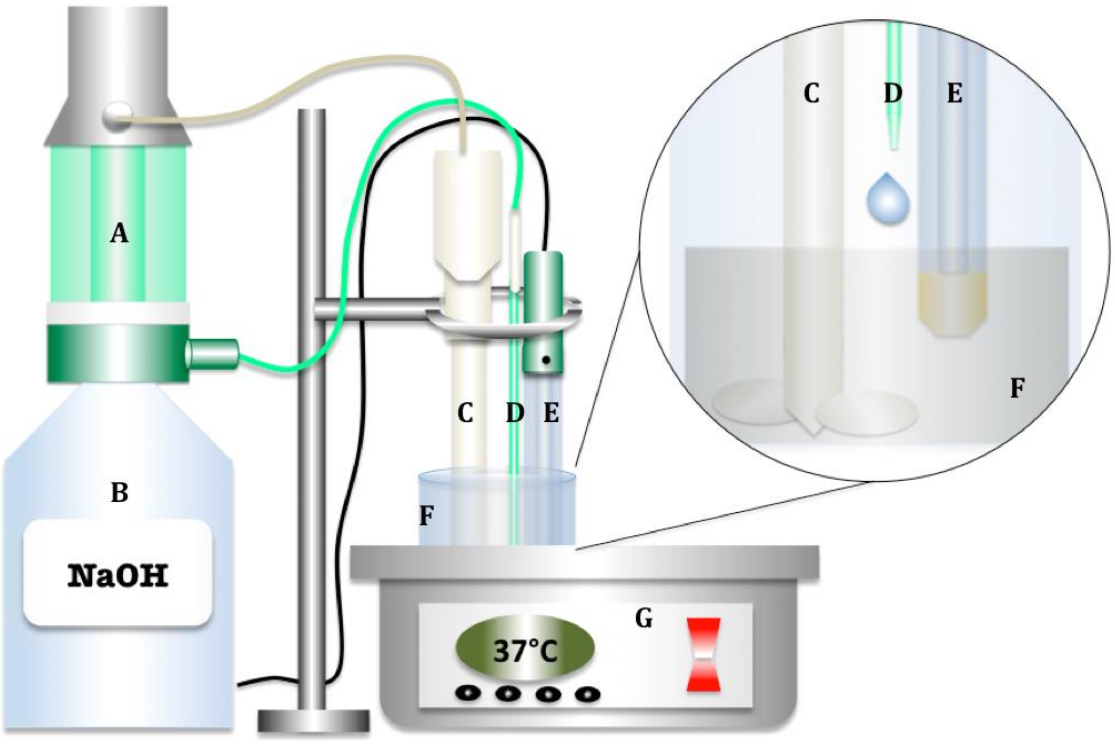


**Figure 11. Representative diagram of the conditions and duration of the mouth, stomach, and small intestine stages during *in vitro* digestion[13].**

tract, the duration of certain organ stages – particularly the mouth – have been modified in order to ensure that the interaction with the digestive juices render equivalent degradation results. The mouth and stomach phases consist of adding the corresponding juices and adjusting the system's pH to the 6.8 and 2.5, respectively, and letting the digestion simulation take place at 37 °C without further modification under gentle agitation. It is the small intestine phase of this model that sets it apart.




[13] Based on the method described by Yan Li and McClements (2010), Mao and McClements (2012), and Salvia-Trujillo, Qian, Martin-Belloso, and McClements (2013b)

**Figure 12. Representative diagram of a sample undergoing intestinal-duodenal *in vitro* digestion in a pH Stat.**[14]

The *in vitro* simulation of the small intestine presented in the experimental portion of the present work relies on the utilization of a pH Stat (857 Titrando, Metrohm USA, Riverview, FL). The principle behind this method is to quantify the amount of free fatty acids (FFAs) released from TAGs during the intestinal digestion through changes in pH. After the sample has undergone oral and gastric digestion, it is placed in a reaction chamber (**Figure 12F**) with the pertinent intestinal juices, bile salts, and lipase concentrations under stirred conditions (**Figure 12C**). The pH is adjusted to 7, and continuously monitored by the instrument (**Figure 12E**); as the bile salts and lipase act in conjunction to digest TAGs, the hydrolysis and subsequent release of free fatty acids (FFA) decreases the pH of the system. As the pH decreases, a solution with a predetermined concentration of sodium hydroxide (**Figure 12B**) is automatically titrated into the reaction chamber (**Figure 12D**) to maintain the pH at 7 throughout the experiment. The volume of the alkaline solution that was titrated throughout the digestion (**Figure 12A**) is then utilized to determine the amount of TAGs digested through the following equation (Y. Li & McClements, 2011):

$$\%FFA = 100 \times \frac{V_{NaOH} \times m_{NaOH} \times m_{lipid}}{w_{lipid} \times 2}$$

[14] Based on the method described by Yan Li and McClements (2010), Mao and McClements (2012), and Salvia-Trujillo, Qian, Martin-Belloso, and McClements (2013b)

Where %FFA is the percentage of released FFAs; $V_{NaOH}$ is the volume of titrant in liters; $m_{NaOH}$ is the molarity of the sodium hydroxide solution used; $M_{lipid}$ is the molecular weight of the oil used (grams per mol); and $w_{lipid}$ is the weight of the oil in the digestion system (grams).

## 2.4 Gut Microbiota

The term "gut microbiota" refers to the 10-100 trillion Gram (+) and Gram (-) bacteria, viruses, and fungi that permanently inhabit the mucous layers and luminal area of the large intestine, particularly the colon (Faith, Guruge, Charbonneau, Subramanian, Seedorf, Goodman, et al., 2013; Hooper & Gordon, 2001). They are also known as "commensal microbiota" (Reid, Howard, & Gan, 2001) and they exert a "co-metabolic" activity: they increase the energy extracted from foods (Tremaroli & Bäckhed, 2012; Turnbaugh, Ley, Mahowald, Magrini, Mardis, & Gordon, 2006) and have a strong effect on the bioavailability, functionality, and synthesis of nutrients that were not absorbed in the small intestine (Krajmalnik-Brown, Ilhan, Kang, & DiBaise, 2012), vitamins (Arumugam, Raes, Pelletier, Le Paslier, Yamada, Mende, et al., 2011; Kau, Ahern, Griffin, Goodman, & Gordon, 2011), and drugs (Sousa, Paterson, Moore, Carlsson, Abrahamsson, & Basit, 2008; Wallace & Redinbo, 2013). Their functionality also includes fecal bulking, increasing transit of colonic contents, increasing nitrogen utilization in the gut, and forming short chain fatty acids (Cummings & Macfarlane, 1991). Such is the health impact of the gut microbiota that research has linked it to numerous health issues, including both relatively innocuous and severe gastrointestinal infections, autoimmune disorders (Crohn's disease), inflammatory maladies (irritable bowel syndrome), chronic disorders

(type II diabetes, rheumatoid arthritis, and food allergies), several types of cancer, and even obesity (Cani, Amar, Iglesias, Poggi, Knauf, Bastelica, et al., 2007; Kalliomaki, Salminen, Poussa, Arvilommi, & Isolauri, 2003; Y. K. Lee, Puong, Ouwehand, & Salminen, 2003; Natividad & Verdu, 2013; Rinkinen, Jalava, Westermarck, Salminen, & Ouwehand, 2003; Rowland, 2000; Salminen, von Wright, Morelli, Marteau, Brassart, de Vos, et al., 1998; Wellen & Hotamisligil, 2005; Yudkin, Juhan-Vague, Hawe, Humphries, di Minno, Margaglione, et al., 2004).

### 2.4.1 Gut Health

The composition of a healthy gut microbiota has been demonstrated to vary considerably from one individual to another – primarily due to dietary differences, yet it is relatively stable within an individual (Delgado, Suárez, Otero, & Mayo, 2004; Donaldson, 1968; S. L. Gorbach, Nahas, Lerner, & Weinstein, 1967; Jalanka-Tuovinen, Salonen, Nikkilä, Immonen, Kekkonen, Lahti, et al., 2011; Mitreva, 2012). Despite the person-to-person changeability, studies have shown that bacteria from the genera *Bacteroides*, *Bifidobacterium*, *Eubacterium*, and *Lactobacillus* predominate over the more than 400 species of bacteria in a healthy gut microbiota (S. Gorbach, 1971). These commensal bacteria have been suggested to have key roles in the immune system (Hooper, Wong, Thelin, Hansson, Falk, & Gordon, 2001; Smith, McCoy, & Macpherson, 2007; Zhao & Shen, 2010), particularly as modulators of the intestinal barrier function (Hooper & Gordon, 2001; Smith, McCoy, & Macpherson, 2007), i.e., "the regulation of transport and host defense mechanisms at the mucosal interface with the outside world" (Baumgart & Dignass, 2002). The commensal bacteria exert their synergistic relationship with the colonic immune

system in three main ways: (i) saturation of colonization sites and competition for nutrients; (ii) decreasing luminal pH by the reduction of short chain fatty acids; (iii) communicating with the epithelial cells to promote the secretion of mucin, antibodies, and antimicrobial peptides; and, lastly, by (iv) triggering an immune and inflammatory response (Kailasapathy & Chin, 2000; Khosravi & Mazmanian, 2013; Reid, Howard, & Gan, 2001). When the healthy composition of the gut microbiota is compromised (usually through antibiotic treatments or drastic dietary changes), pathogenic microorganisms have fewer hurdles to overcome in order to cause an infection.

Although it might be assumed that the biggest pathogenic threat comes from ingesting pathogens, this is not always the case. Chow and Mazmanian (Chow & Mazmanian, 2010) coined the term "pathobiont" to describe a typically small number of bacteria that exist with the commensal bacteria in a healthy individual, which can bloom and exhibit pathogenic behavior if given the opportunity or provided with favorable conditions. This pathogenic behavior is accompanied with the secretion of toxic compounds typical of pathogenic bacteria, which will usually trigger immune activation and inflammation. It is these toxins that have been linked to many of the disorders previously mentioned (Zhao & Shen, 2010). It is important to note that factors that will favor pathobionts will typically cause a disruption in the overall composition of the gut microbiota, as they are usually detrimental to beneficial and innocuous bacteria. Consequently, they will likely decrease the population of the latter whilst increasing the population of pathogenic strains. Something as simple as an imbalanced diet could trigger the growth of pathobionts.

Research has shown that ε-PL is resistant to the action of digestive enzymes in the human gastrointestinal tract (J. Hiraki, 1995), potentially maintaining its antimicrobial attributes and posing a threat to the composition of the gut microbiome. Additionally, the "anti-obesity" effect of ε-PL may generate a difference in the normal lipid profile that reaches the gut microbiota (Kido, et al., 2003) – particularly an greater amount of potentially toxic bile salts (ROMAŃSKI, 2007), possibly causing changes in microbial signaling and, in turn, differences in the gut microbiota's composition.

# CHAPTER 3

# OPTIMIZING DELIVERY SYSTEMS FOR CATIONIC BIOPOLYMERS: COMPETITIVE INTERACTIONS OF CATIONIC POLYLYSINE WITH ANIONIC κ-CARRAGEENAN AND PECTIN

## 3.1 Abstract

Polylysine is a cationic biopolymer with a strong antimicrobial activity against a wide range of microorganisms, however, its functional performance is influenced by its interactions with anionic biopolymers. We examined the stability of polylysine–pectin complexes in the presence of carrageenan, and vice versa. Polylysine–pectin or polylysine–carrageenan complexes were formed at mass ratios of 1:0 to 1:32 (pH 3.5), and then micro-electrophoresis, turbidity, microscopy, and isothermal titration calorimetry (ITC) were used to characterize them. Solutions containing polylysine–pectin complexes were slightly turbid and relatively stable to aggregation at high mass ratios, whereas those containing polylysine– carrageenan complexes were turbid and unstable to aggregation and precipitation. Pectin did not strongly interact with polylysine–carrageenan complexes, whereas carrageenan displaced pectin from polylysine–pectin complexes, which was attributed to differences in electrostatic attraction between polylysine, carrageenan, and pectin. These results have important implications for the design of effective antimicrobial delivery systems for foods and beverages.

## 3.2 Introduction

Polylysine (PL) is a natural cationic polymer that has proved to have

antimicrobial activity against a wide range of Gram (+) and Gram (-□) bacteria, as well as some yeasts, molds, and bacteriophages (N. A. El-Sersy, A. E. Abdelwahab, S. S. Abouelkhiir, D.-M. Abou-Zeid, & S. A. Sabry, 2012; Moschonas, Georgnaras, Stopforth, Wach, Woerner, Belk, et al., 2012; Shima, Matsuoka, Iwamoto, & Sakai, 1984; H. Yu, Y. Huang, & Q. Huang, 2010; C. Zhou, P. Li, X. Qi, A. R. M. Sharif, Y. F. Poon, Y. Cao, et al., 2011). It is currently used in Japan as an antimicrobial agent in foods, such as fish surimi, boiled rice, noodle soup stocks, noodles, and vegetables. It has also been used in potato salads, steamed cakes, and custard creams. The FDA has designated polylysine as Generally Recognized As Safe (GRAS) for use in cooked or sushi rice (FDA, 2004; J Hiraki, 2000; Unalan, Ucar, Arcan, Korel, & Yemenicioglu, 2011).

Recent research has shown its efficacy in reducing *Salmonella* in chicken products (Moschonas, et al., 2012), *Escherichia coli*, *Listeria innocua*, *Salmonella typhimurium* and *Staphylococcus aureus* in edible films (Unalan, Ucar, Arcan, Korel, & Yemenicioglu, 2011), and *S. typhimurium* and *Listeria monocytogenes* in roast beef (Chang, Lu, Park, & Kang, 2010). Polylysine has a number of potential advantages for application in food products: it has a relatively low minimum inhibitory concentration (MIC) against common food pathogens, i.e., 5–100 lg/ml; it has high selectivity; it has low toxicity to mammalian cells: it is biodegradable and it has a low propensity to promote pathogen resistance (Blin, Purohit, Leprince, Jouenne, & Glinel, 2011; N. A. El-Sersy, A. E. Abdelwahab, S. S. Abouelkhiir, D.-M. Abou-Zeid, & S. A. Sabry, 2012; Georgnaras & Sofos, 2005; R. E. W. Hancock & H.-G. Sahl, 2006; P. Li, Zhou, Rayatpisheh, Ye, Poon, Hammond, et al., 2012; Shima, Matsuoka, Iwamoto, &

Sakai, 1984; H. Yu, Y. Huang, & Q. Huang, 2010; Zasloff, 2002).

Similarly to other cationic antimicrobial compounds, such as lauric arginate and chitosan, the widespread application of polylysine in foods is currently limited since: its potency as an antimicrobial agent may be reduced if it interacts with anionic components within the food matrix; undesirable precipitation or turbidity may occur if it binds to anionic components in food matrices and it may be perceived as bitter or astringent if it interacts with anionic biopolymers in the mouth, such as mucin (Asker, Weiss, & McClements, 2011; Y. Chang, L. McLandsborough, & D. J. McClements, 2011b).

Previous work has shown that anionic pectin will interact electrostatically with cationic ε-polylysine, leading to the formation of soluble complexes (Y. Chang, L. McLandsborough, & D. J. McClements, 2011b). These complexes retain the antimicrobial properties of e-polylysine and are anionic (rather than cationic), potentially minimizing the interaction of ε-polylysine with other anionic compounds present in food systems and decreasing its perceived bitterness. Y. Chang, L. McLandsborough, and D. J. McClements (2011b) also demonstrated that these complexes could be added to green tea beverage systems without adversely affecting their appearance or physical stability. Although this is a promising result, the stability of this complex in the presence of highly anionic compounds that could potentially be found in more complex food systems, has not been assessed. It is possible that ε-polylysine, being a cationic polymer, may favor interactions with compounds that are more strongly anionic than pectin. These competitive

ingredient interactions could alter the antimicrobial efficacy and stability of polylysine–pectin complexes in foods. In this study, we therefore examined the influence of a strongly charged anionic food polymer (carrageenan) on the stability of ε-polylysine–pectin complexes.

Carrageenans are natural anionic compounds that are normally extracted from red seaweeds (Cui, 2005). These polymers are linear chains of D-galactopyranosyl units joined with alternating (1→3)- a-D- and (1 → 4)-b-D-glycosidic linkages, with most sugar units having one or two sulphate half-ester groups. These sulphate groups are responsible for the negative charge of the polymer, as they are always ionized at the pH values present in foods. κ-Carrageenan is one of the most common forms of carrageenans used in foods, and it is characterized by having D-galactose-4-sulphate, 3,6-anhydro-D-galactose-2-sulphate as a building block, and has a double-helix conformation (Cui, 2005). The popularity of this ingredient in the food industry is due to the ability of its linear helical portions to associate to form a three-dimensional gel in the presence of appropriate cations. In addition, it may interact with various food proteins through electrostatic interactions and increase their aggregation stability (Belitz, Grosch, & Schieberle, 2009; Damodaran, Parkin, & Fennema, 2008).

The overall objective of this study is to test the stability of ε-polylysine–pectin complexes when exposed to κ-carrageenan. We hypothesize that the more highly charged carrageenan molecule may be able to displace the pectin from the ε-polylysine–pectin complexes, thereby altering their functional properties.

## 3.3 Materials

ε-Polylysine (50:50 mixture with dextrin) was provided by Purac (Lincolnshire, IL) (Puraq Xtend FX50P; lot 112022). High-methoxyl (DE 69–77%) pectin (TIC Pretested Pectin 1400; lot 512401) and κ-carrageenan (Ticaloid 710 H Powder; lot 21311) were provided by TIC Gums (Belcamp, MD).

## 3.4 Methods

### 3.4.1 Solution Preparation

All solutions were prepared in double-distilled water. Stock solutions of e-polylysine (0.1% w/v), pectin (0.5% w/v), and κ-carrageenan (0.5% w/v) were prepared by dissolving the corresponding amounts of powdered reagents into double-distilled water. The solutions were left to stir overnight, and were then adjusted to the appropriate volume and to pH 3.5, the latter by addition of diluted solutions for either HCl or NaOH. A pH of 3.5 was used to simulate the acidic conditions found in food products where polylysine might be used (such as beverages and dressings).

### 3.4.2 Micro-electrophoresis (ME) and turbidity measurements

The electrical charge on the complexes was assessed through micro-electrophoresis (Malvern Zetasizer ZS, Malvern Instruments, Worcestershire, United Kingdom), while the aggregation of complexes was studied using turbidity measurements (Ultrospec 3000, Biochrom Ltd., Cambridge, United Kingdom). Four sets of experiments were devised to assess the competitive interactions between the polylysine and different anionic polymers (pH 3.5). In the first experiment, a 1 ml

aliquot of 0.1% w/v e-polylysine solution was titrated into a series of glass tubes containing increasing amounts of 0.5% w/v pectin solution, to achieve mass ratios ranging between 1:1 and 1:32 ε-polylysine:pectin (PL-P). In the second experiment, the pectin solution was replaced with 0.5% w/v κ-carrageenan. In the third experiment, a 1 ml aliquot of 0.5% w/v pectin solution was titrated into a series of tubes containing 0.5% w/v of ε-polylysine-κ-carrageenan (1:24 PL-KC) complex. In the fourth experiment, a 0.5% w/v pectin solution was injected into a solution containing 0.5% w/v of ε-polylysine:pectin (1:24 PL-P). This polylysine:pectin ratio was selected since it has previously been shown to form stable electrostatic complexes that maintain their antimicrobial activity (Y. Chang, McLandsborough, & McClements, 2012). All samples were supplemented with double-distilled water to reach a final volume of 10 ml. The turbidity was measured at k = 600 nm using a UV–visible spectrophotometer immediately after mixing, and again after 24 h.

### 3.4.3 Isothermal Titration Calorimetry Measurements

Enthalpies of mixing (at 30 °C) were measured using an isothermal titration calorimeter (VP-ITC, General Electric, Fairfield, CT), modified from the methodology presented by Y. Chang, McLandsborough, and McClements (2012). Fifty-eight □ 5 μl aliquots of e-polylysine solution (0.1% w/v, pH 3.5) were injected sequentially into a 1480 μl titration cell initially containing either 0.5% w/v pectin (pH 3.5) or 0.5% w/v κ-carrageenan (pH 3.5). The competitive interaction was assessed by titrating either a pectin solution (0.5%, pH 3.5) or a κ-carrageenan solution (0.5%, pH 3.5) into a PL-KC complex solution (1:24 mass ratio, pH 3.5) or a PL-P solution (1:24 mass ratio, pH 3.5), respectively. Each injection lasted 12 s, and there was an

interval of 240 s between successive injections. The solution in the titration cell was stirred at a constant speed of 315 rpm throughout the experiments. The resulting heat flow-time curves were integrated using the instrument's software to generate interaction enthalpy versus ε-polylysine concentration profiles.

### 3.4.4 Microscopy

Selected samples were observed at 200X or 400X magnification using an optical microscope (Nikon D-Eclipse C1 80i, Nikon, Melville, NY). The polymer mass ratios selected were 1:8, 1:16, 1:12, and 1:16 for the (PL-P), (PL-KC), (PL-P)-KC, and (PL-KC)-P systems, respectively. The selections of the mass ratios to be observed under the microscope were chosen based on the visualization of the samples with the naked eye. Those containing visibly discernible particles were selected for microscopy.

## 3.5 Results and Discussion

### 3.5.1 Aggregation stability: turbidity and microscopy measurements

#### 3.5.1.1 General

Initially we examined the stability of the electrostatic complexes to aggregation using turbidity measurements. An increase in turbidity is indicative of the formation of particles sufficiently large enough to scatter light strongly. Measurements were made after 0 and 24 h and found to be similar (data not shown), suggesting that complex formation occurred rapidly and that complex structure did not change during storage. For the sake of clarity, we only show the

data after 0 h storage (**Figure 13**). The change in turbidity with polymer mass ratio (R) depended strongly on the nature of the biopolymers initially in the reaction cell and in the injector. Square brackets are used to designate the biopolymers that were initially present in the reaction cell.

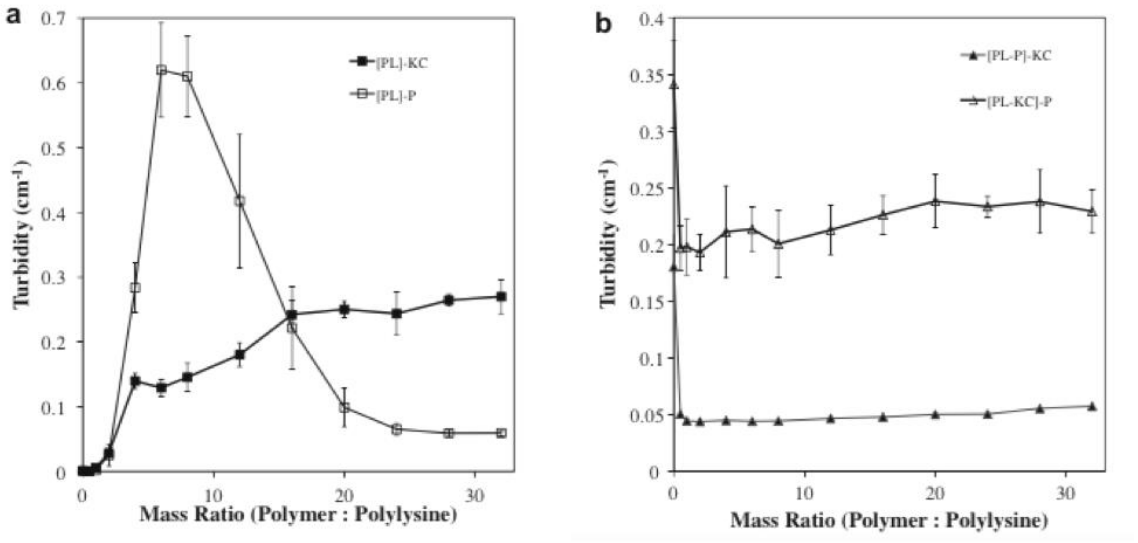



**Figure 13. (a) Influence of polymer:polylysine ratio on the turbidity (at k = 600 nm) for mixed binary biopolymer solutions. (b) Influence of polymer:polylysine ratio on the turbidity (at λ = 600 nm) of mixed ternary biopolymer solutions: PL = polylysine; P = pectin; KC = κ-carrageenan (pH 3.5)**[15]**.**

### 3.5.1.2 Binary systems

When pectin was injected into a polylysine solution ([PL]-P), the turbidity remained relatively low (R = 0–1), increased steeply (R = 1–6), had a maximum value at R (6–8), decreased steeply (R = 8–20), and then reached a relatively

[15] C. L. Lopez-Pena and McClements (2014)

constant low value at higher polymer ratios (**Figure 13a**). The observed increase in turbidity can be attributed to the formation of electrostatic polylysine– pectin complexes that were large enough to scatter light. A white sediment was observed at the bottom of these samples after 24 h storage (data not shown), which could be re-suspended into a cloudy suspension by mild agitation. Thus, some of the electrostatic complexes formed were large enough to undergo gravitational separation. Indeed, optical microscopy images of samples at high polymer ratios indicated the presence of large aggregates (**Figure 13a**). The turbidity maximum observed at intermediate pectin concentrations may have been due to charge neutralization of the complexes (see next section), since this would have reduced the electrostatic repulsion between them leading to more extensive aggregation. The fact that the turbidity decreased at higher mass ratios suggests that the complexes may have partially dissociated, presumably because of the increase in electrostatic repulsion between them (see next section).

When κ-carrageenan was injected into a polylysine solution ([PL]-KC), the turbidity remained relatively low (R = 0–1), increased gradually (R = 1–20), and then reached a relatively constant high value at higher polymer ratios (**Figure 13a**). Visually, the solutions containing PL-KC mixtures appeared to consist of a few hair-like structures floating in a clear aqueous solution. These hair-like structures sedimented to the bottom of the samples after 24 h storage, and could not be readily re-dispersed by simply shaking. The presence of these hair-like structures was confirmed by optical microscopy (**Figure 14b**). These structures may have formed by assembly of linear carrageenan and polylysine molecules into fibers. Our results

show that the polylysine–carrageenan complexes had different aggregation

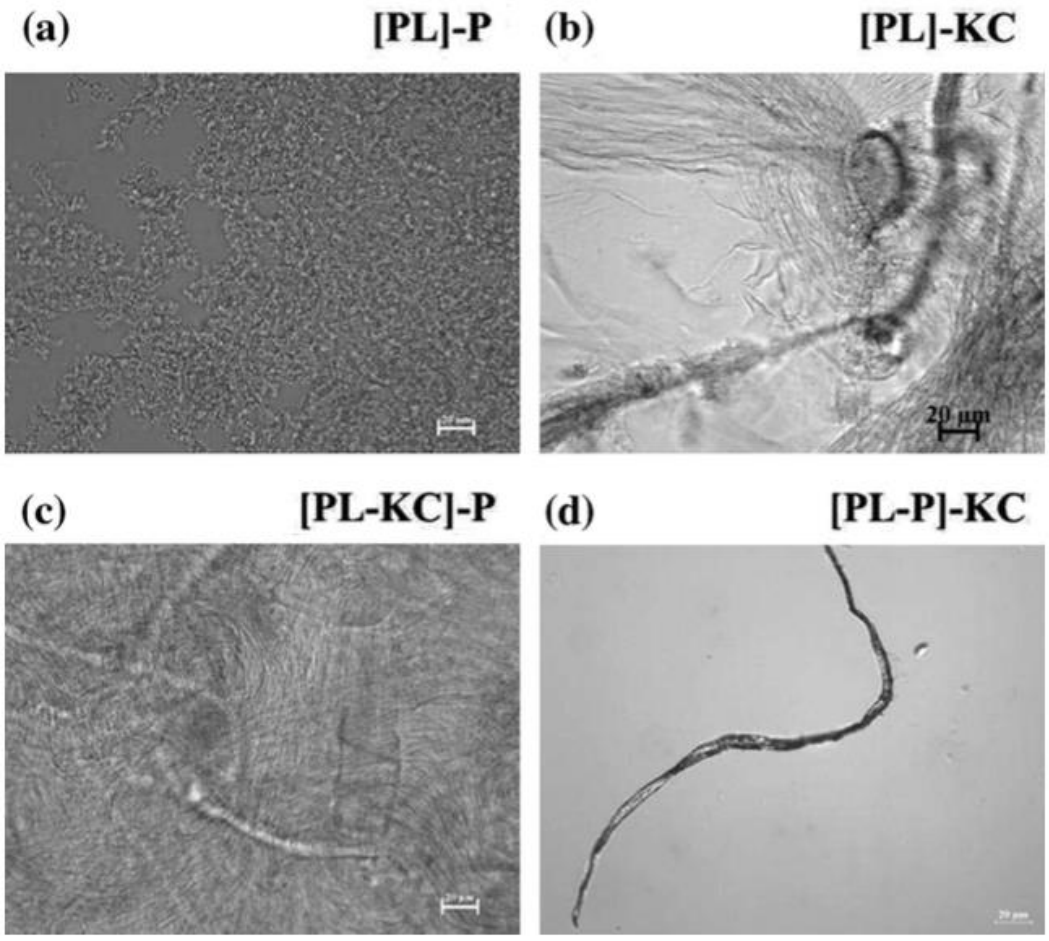



**Figure 14. Optical microscopy images of representative structures observed in biopolymer mixtures.**

characteristics than the polylysine–pectin ones. We attribute this effect to differences in the electrical charge and structure of the two anionic biopolymers. Carrageenan has a fairly rigid linear anionic backbone with a high charge density, whereas pectin has a more flexible anionic back-bone with neutral side chains attached with a lower overall charge density (Cui, 2005). Consequently, linear polylysine molecules would be expected to form stronger electrostatic complexes

with carrageenan molecules than with pectin molecules. Based on the differences in molecular structure we also hypothesize that the [PL]-KC complexes have a rod-like structure (which would account for the formation of the hair-like structures), whereas the [PL]-P complexes have a more disordered structure (**Figure 15**).

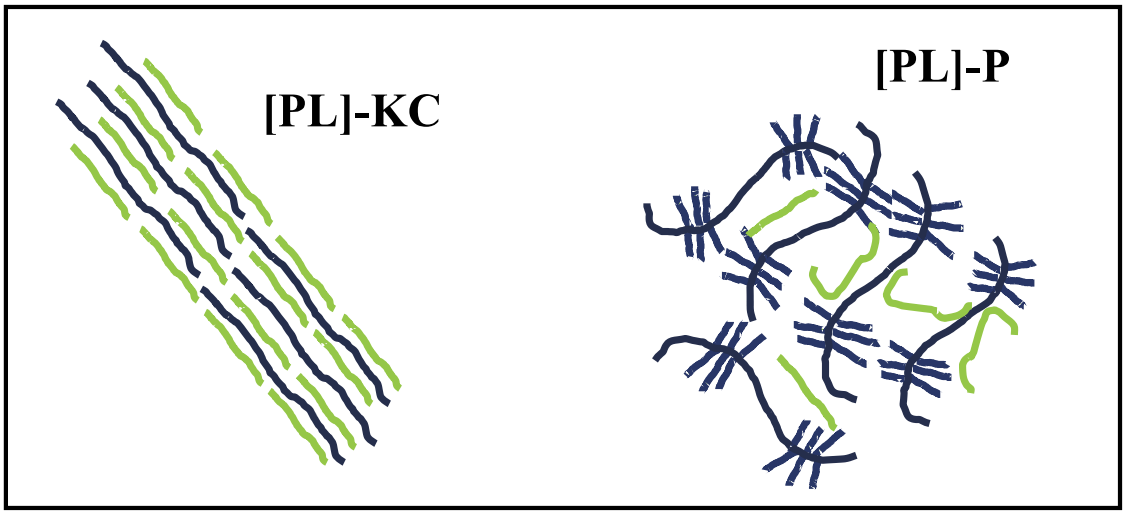



**Figure 15. Highly schematic diagram of possible structures formed in polylysine– carrageenan and polylysine–pectin electrostatic complexes. PL is the short green linear molecule, KC is the long blue linear molecule, and P is the blue branched molecule**[16]**.**

### 3.5.1.3 Ternary systems

Qualitatively, the samples that initially contained electrostatic complexes in the reaction cell ([PL-P]-KC and [PL-KC]-P), rather than single biopolymers, behaved fairly similarly (**Figure 13b**). There was initially a sharp decrease in turbidity after the first injection of the anionic biopolymer (KC or P), and then the turbidity

[16] (C. L. Lopez-Pena & McClements, 2014)

remained relatively constant at higher polymer mass ratios. However, the final turbidity of the [PL-KC]-P mixtures (around 0.23 $cm^{-1}$) was appreciably higher than that of the [PL-P]-KC mixtures (around 0.06 $cm^{-1}$). This suggested that the amount and/or size of the electrostatic complexes formed in the [PL-KC]-P system was higher than those formed in the (PL-P)-KC system at high R. The structure of the aggregates formed within the ternary biopolymer mixtures was observed by optical microscopy. The [PL-KC]-P system (**Figure 14c**) contained some hair-like structures similar to those observed in the [PL]-KC system (**Figure 14b**), which suggests that addition of pectin molecules could not dissociate the strong complexes which form between polylysine and carrageenan. Some hair-like structures were also observed in the [PL-P]-KC system (**Figure 14d**), but these were much thinner and difficult to see with the naked eye compared to those observed in the [PL]-KC system. This result suggests that carrageenan may have displaced some of the pectin molecules from the [PL-P] complexes, leading to the formation of hair-like structures (since these were not observed in the [PL]-P systems).

### 3.5.2 Micro-electrophoresis measurements

#### 3.5.2.1 General

In this series of experiments, micro-electrophoresis was used to determine the electrical charge (ζ-potential) of the electrostatic complexes. The electrical characteristics of the complexes depended strongly on the nature of the biopolymers in the reaction cell and injector (**Figure 16**).

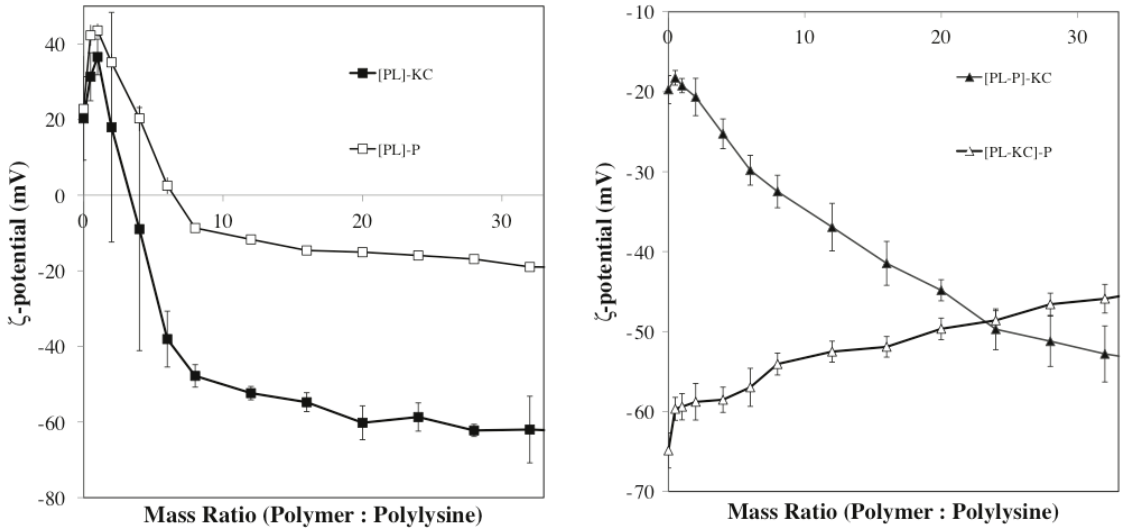



**Figure 16. (a) Influence of polymer:polylysine ratio on the ζ-potential of binary complexes formed when a polylysine solution is titrated into an anionic biopolymer solution. (b) Influence of polymer:polylysine ratio on the ζ-potential of ternary complexes formed when a polylysine solution is titrated into a binary biopolymer complex solution: PL = polylysine; P = pectin; KC = κ-carrageenan (pH 3.5)[17].**

### 3.5.2.2 Binary systems

In the absence of anionic biopolymers, the electrical charge on the polylysine in the reaction cell was around +20 mv (**Figure 16a**), which can be attributed to ionization of the amino groups on the polypeptide chains ($-NH^{+}_{3}$). Qualitatively similar behavior was observed when either pectin or carrageenan was titrated into the polylysine solution: there was a slight increase in positive charge from R = 0 to 1, then a sharp decrease in positive charge/increase in negative charge from R = 1 to 8, and then a more gradual increase in negative charge at higher polymer ratios.

[17] C. L. Lopez-Pena and McClements (2014)

These results indicate that both types of anionic biopolymer bind to the cationic polylysine molecules and formed electrostatic complexes. Nevertheless, the final negative charge on the complexes at high R values was much greater for the [PL]-KC system ($\zeta$ = -62 mv) than for the [PL]-P system ($\zeta$= -19 mv), which may be attributed to the higher charge density of carrageenan compared to pectin.

A comparison of the turbidity and $\zeta$-potential measurements also indicated some interesting differences in the behavior of the [PL]-P and [PL]-KC systems. The maximum in turbidity observed for the [PL]-P system (**Figure 13a**) occurred at a polymer ratio ($R \approx 7$), which is fairly similar to the polymer ratio where charge neutralization occurred (**Figure 14a**). The decrease in turbidity observed at higher polymer ratios for this system may therefore be due to an increase in the electrostatic repulsion between [PL-P] complexes. On the other hand, there was no maximum observed in the turbidity of the [PL]-KC systems, despite the fact that charge neutralization occurred around $R \approx 4$. One explanation for this difference is that the interaction between polylysine and pectin molecules was relatively weak, therefore PL could easily move from one pectin molecule to another. Thus, as more pectin molecules were added to the system, some of the PL molecules dissociated from the original complexes and bound to new pectin molecules. On the other hand, the interaction between polylysine and carrageenan was relatively strong, and so PL could not easily move from one carrageenan molecule to another. In this case, once the initial polylysine–carrageenan complexes have formed they could no longer dissociate.

The origin of the initial increase in the positive charge on the complexes at low R values is currently unknown. A number of possible explanations may account for this phenomenon: changes in biopolymer conformation; alterations in the spatial arrangement of charge groups or release of counter-ions after binding. Further experiments using additional analytical tools are clearly needed to elucidate the origin of this interesting effect.

#### 3.5.2.3 Ternary systems

We also measured the change in electrical characteristics in ternary systems when one type of anionic biopolymer (KC or P) was titrated into a reaction cell containing an electrostatic complex made up from the other type of biopolymer ([PL-P] or [PL-KC]). Initially, the [PL-KC] complexes had a higher negative charge than had the [PL-P] complexes, which can be attributed to the larger charge density of KC. When increasing amounts of pectin were added to the reaction cell initially containing [PL-KC] complexes, the ζ-potential became slightly less negative, which may have been due to some replacement of the KC molecules in the [PL-KC] complexes with pectin, or due to the contribution of free pectin molecules to the micro-electrophoresis signal. Conversely, when increasing amounts of carrageenan were added to the reaction cell initially containing [PL-P] complexes, the ζ-potential became considerably more negative, which may have been due to some replacement of pectin molecules in the [PL-P] complexes with carrageenan, or due to the contribution of free carrageenan molecules to the micro-electrophoresis signal. In the [PL-P]-KC system, the final ζ-potential was fairly similar to that of the [PL-KC] system at high polymer ratios, which suggested that carrageenan might have

replaced much of the pectin in these complexes.

### 3.5.3 Isothermal Titration Calorimetry

Finally, isothermal titration calorimetry experiments were carried out to provide additional information on the molecular interactions associated with complex formation.

#### 3.5.3.1 Binary systems

Major differences were observed in the interaction enthalpy versus polymer ratio profiles when the two different anionic biopolymers (P or KC) were titrated into the reaction chamber containing cationic polylysine (**Figure 17a**). There was a relatively small mainly endothermic (positive DH) interaction enthalpy when pectin was titrated into the polylysine solution, with the interaction occurring from about R = 0 to 7. The polymer ratio where the interaction appeared to be complete (R = 7), corresponded to the point where the turbidity reached a maximum value (**Figure 13a**), and the f-potential reached a relatively constant value (**Figure 16a**). This suggests that all of the cationic groups on the polylysine molecules had interacted with anionic groups on the pectin molecules at this polymer ratio. The [PL]-KC system exhibited quite different behavior. There was a large exothermic (negative DH) interaction enthalpy when carrageenan was titrated into the polylysine solution, with the interaction occurring from about R = 0 to 4 (**Figure 17a**). In this case, the polymer ratio where the interaction appeared to be complete (R = 4), corresponded to the point where the turbidity (**Figure 13a**) and ζ-potential (**Figure 16a**) reached relatively constant values. As discussed earlier, the fact that the

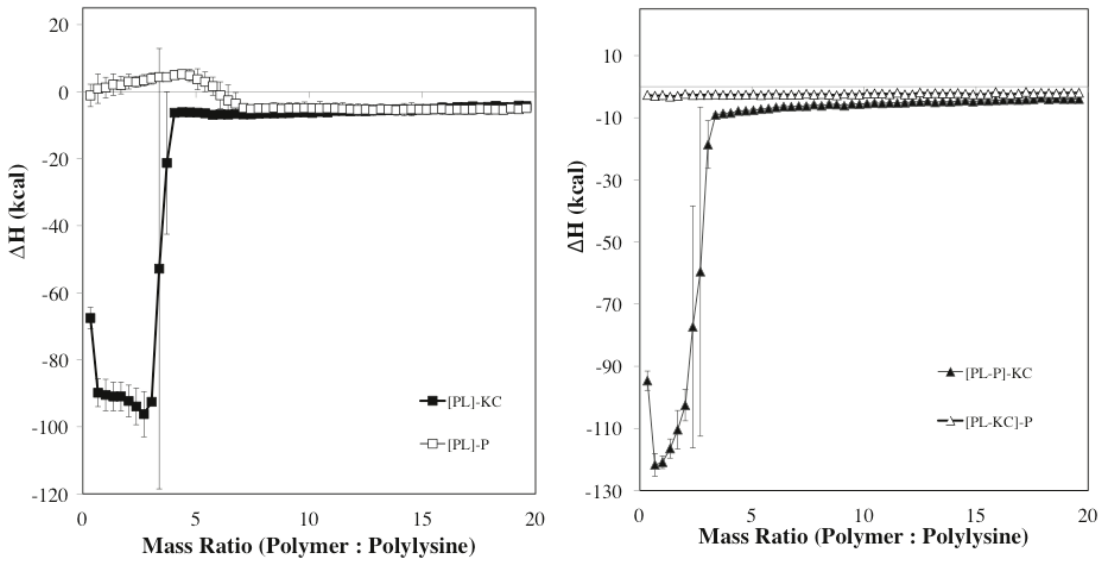



**Figure 17. (a) Change in interaction enthalpy (DH) when polylysine was titrated into a reaction vessel containing anionic biopolymer solution, either pectin (P) or j- carrageenan (KP), at pH 3.5 and 30 °C. (b) Change in interaction enthalpy (DH) when polylysine was titrated into a reaction vessel containing electrostatic complexes in solution, either PL-P or PL-K), at pH 3.5 and 30 °C[18].**

electrostatic complexes dissociated at high polymer ratios for pectin but not for carrageenan can be attributed to the weaker electrostatic attraction between PL and pectin compared to PL and carrageenan.

### 3.5.2.3 Ternary systems

We also measured the interaction enthalpies in ternary systems when one type of anionic biopolymer (KC or P) was titrated into a reaction cell containing an electrostatic complex made up from the other type of biopolymer ([PL-P] or [PL-

[18] C. L. Lopez-Pena and McClements (2014)

KC]). Interestingly, when pectin was titrated into a reaction cell containing [PL-KC] we observed no change in the interaction enthalpy across the entire polymer ratio studied (**Figure 17b**), which suggests that pectin was unable to displace carrageenan from the [PL-KC] complexes. On the other hand, when carrageenan was titrated into a reaction cell containing [PL-P] we observed a large exothermic interaction enthalpy from R = 0 to 4 (**Figure 17b**), fairly similar to the one observed in the absence of pectin (**Figure 17a**). This result strongly suggests that the carrageenan was able to displace the pectin molecules from the PL-P complexes leading to the formation of PL-KC complexes. This would explain the occurrence of hair-like structures in the [PL-P] systems after carrageenan was added (**Figure 14**).

In future studies, it may be useful to determine the composition of the complexes formed in mixed polymer systems. For example, insoluble complexes could be separated from the surrounding solution by centrifugation or filtration, and then the concentration of polymers remaining in the soluble fraction could be determined using suitable analytical tools.

### 3.5.4 Proposed Interaction Mechanism

Overall, our results suggest that the complexes formed through electrostatic attraction between ε-polylysine and κ-carrageenan were considerably stronger than those formed between ε-polylysine and pectin. In particular, measurement of the interaction enthalpies using ITC showed that carrageenan could displace pectin from [PL-P] complexes, but pectin could not displace carrageenan from [PL-KC] complexes. Nevertheless, there were still some differences in the nature of the

aggregates formed in binary and ternary systems. In binary systems, [PL]-KC complexes tended to form hair-like structures that rapidly sediment to the bottom of the samples. However in ternary systems ([PL-P]-KC), which would be expected to contain [PL]-KC complexes at high polymer ratios, much fewer hair-like structures were formed (**Figure 14d**) and the overall turbidity was much lower (**Figure 16b**). This suggests that the pectin molecules did have some impact on the structure of the electrostatic complexes formed in the ternary systems.

## 3.6 Conclusions

Our results have important implications for the utilization of cationic biopolymers (such as polylysine) as functional ingredients in foods and beverages. Previous studies have shown that problems associated with the utilization of cationic polymers in food products (such as precipitation and astringency) can be overcome by forming electrostatic complexes with pectin (Y. H. Chang, L. McLandsborough, & D. J. McClements, 2011). However, if these complexes are introduced into food or beverage matrices that contain other anionic biopolymers that can compete with the pectin, the functionality of the cationic polymers might be altered in an undesirable way. It is therefore essential for food manufacturers to carefully formulate their products taking into account the various kinds of molecular interactions that can occur in complex multicomponent food systems.

## 3.7 Acknowledgments

This material is based upon work supported by the Cooperative State Research, Extension, Education Service, United States Department of Agriculture,

Massachusetts Agricultural Experiment Station and a United States Department of Agriculture, CREES, NRI and AFRI Grants. We greatly thank Purac and TIC-GUMs for donating the polysaccharides used in this study.

# CHAPTER 4

# EFFECT OF CATIONIC BIOPOLYMER (ε-POLYLYSINE) ON DIGESTION OF EMULSIFIED LIPIDS

## 4.1 Abstract

ε-Polylysine (ε-PL) is a cationic biopolymer that may be used as a food ingredient because of its strong antimicrobial activity and potential to inhibit pancreatic lipase. We examined the effect of polylysine on the digestion of corn oil-in-water emulsions stabilized by either a natural anionic surfactant (quillaja saponin) or a synthetic non-ionic surfactant (Tween 20). Emulsions were prepared using high pressure homogenization (microfluidization) and then subjected to *in vitro* digestion in the absence or presence of polylysine at the maximum level allowed in foods by the FDA. Samples were characterized before and after *in vitro* digestion using electrophoresis, confocal microscopy, and static light scattering. The presence of polylysine decreased the hydrolytic activity of pancreatic lipase by around 53% and 28% in the Tween 20- and saponin-stabilized emulsions, respectively. The lipase-inhibiting properties of polylysine were attributed to its electrostatic interaction with bile salts, which may have inhibited bile salt and/or lipase adsorption to lipid droplet surfaces, as well as interfere with fatty acid solubilization in mixed micelles. These results have important implications for the incorporation of polylysine into food systems, particularly those containing lipophilic nutrients.

*Keywords*: polylysine; lipids; digestion; emulsions; nanoemulsions; lipase inhibition

## 4.2 Introduction

ε-Polylysine (ε-PL) is an appealing antimicrobial agent for utilization within the food industry due to its natural origin and its strong antimicrobial activity against a wide range of Gram (+) and Gram (-) bacteria, yeasts, molds, and bacteriophages (N. A. El-Sersy, A. E. Abdelwahab, S. S. Abouelkhiir, D. M. Abou-Zeid, & S. A. Sabry, 2012; Moschonas, et al., 2012; Shima, Matsuoka, Iwamoto, & Sakai, 1984; H. L. Yu, Y. P. Huang, & Q. R. Huang, 2010; C. C. Zhou, P. Li, X. B. Qi, A. R. M. Sharif, Y. F. Poon, Y. Cao, et al., 2011). The Food and Drug Administration (FDA) has deemed it as a Generally Regarded As Safe (GRAS) food ingredient, and has approved its use in a variety of food systems including alcoholic and non-alcoholic beverages, pastries, meats, soup, dairy products, fruit and vegetables products, and pasta (Food and Drug Administration, 2011).

Despite its approval by the FDA, the high cationic charge density of ε-PL – which is responsible for its antimicrobial properties (Blin, Purohit, Leprince, Jouenne, & Glinel, 2011; Brogden, 2005; Shima, Matsuoka, Iwamoto, & Sakai, 1984) – has limited its widespread use as an ingredient in food systems. This is due to the cationic biopolymer's potential to interact with anionic components in food matrices, which can decrease its antimicrobial efficacy and cause undesirable turbidity and precipitation in products. In addition, cationic polylysine may interact with negatively charged biopolymers in the saliva and mucus coating the mouth and sensory receptors on the tongue, which may lead to bitterness or astringency (Asker, Weiss, & McClements, 2011; Y. H. Chang, L. McLandsborough, & D. J. McClements, 2011; C. L. Lopez-Pena & McClements, 2014). A successful strategy

was developed to overcome the adverse effects associated with these undesirable electrostatic interactions, which was based on forming weak electrostatic complexes between polylysine and anionic polysaccharides (Y. Chang, L. McLandsborough, & D. J. McClements, 2011a; Y. Chang, McLandsborough, & McClements, 2012; Y. H. Chang, L. McLandsborough, & D. J. McClements, 2011; Y. H. Chang, McLandsborough, & McClements, 2014; C. L. Lopez-Pena & McClements, 2014). These electrostatic complexes maintained the antimicrobial activity of polylysine, without causing extensive precipitation or sediment formation. However, another potential challenge that might limit the widespread application of ε-PL as a food ingredient is associated with its ability to participate in electrostatic interactions with bile salts in the small intestine that may inhibit the enzymatic activity of lipase (Kido, et al., 2003; Tsujita, Sumiyoshi, Takaku, Momsen, Lowe, & Brockman, 2003; Tsujita, Takaichi, Takaku, Aoyama, & Hiraki, 2006). These interactions could lead to the malabsorption of both dietary fat and lipophilic nutrients from food.

After ingestion, foods pass through the different regions of the gastrointestinal tract (GIT) where they are exposed to stresses, flow profiles, enzyme activities, salts, surface active substances, and pH changes, which aid in the digestion and absorption of nutrients (Basit, 2005). The three main intestinal components that facilitate lipid digestion and absorption are pancreatic lipase, bile salts, and phospholipids (Duan, 2000). Lipases in the mouth, stomach, and small intestine convert ingested triacylglycerols into free fatty acids and monoglycerides, with most of the digestion and absorption occurring in the small intestine (Lairon, 2009; Mackie, 2012; D. J. McClements & Li, 2010; D. J. D. McClements, Eric A.; Park,Y.,

2007; Singh, Ye, & Horne, 2009). Bile salts and phospholipids also play a number of key roles: (i) they help emulsify lipids by adsorbing to lipid droplet surfaces and forming a protective layer; (ii) they displace some of the original emulsifiers from the lipid droplet surfaces thereby altering interfacial composition in a manner that favors lipase adsorption; and (iii) they participate in the formation of mixed micelles that can solubilize free fatty acids and transport them to the epithelium cells (Hismiogullari, Bozdayi, & Rahman, 2007). After adsorption to lipid droplet surfaces, lipase breaks down triacylglycerols into fatty acids and 2-monoacylglycerols. The resulting compounds are incorporated into the mixed micelles, transported across the mucus layer covering the intestinal wall, absorbed by intestinal microvilli, and then eventually enter the bloodstream (Kido, et al., 2003).

As previously mentioned, research has shown that polylysine possesses the ability to inhibit lipid digestion. This phenomenon is proposed to be due to the electrostatic interaction between cationic ε-PL and anionic bile salts and phospholipids through three main mechanisms: (i) ε-PL binding to these digestive components, thereby preventing them from adsorbing to lipid droplet surfaces; (ii) ε-PL forming a cationic coat around anionic lipid droplets, thus preventing lipase from coming into close proximity to the lipids; (iii) ε-PL binding to bile salts and phospholipids, consequently retarding the formation of mixed micelles capable of solubilizing and transporting digested lipids (Kido, et al., 2003).

Because a decrease in lipid digestion may adversely affect the absorption of essential lipophilic nutrients and nutraceuticals (Y. Li & McClements, 2011; Porter,

Trevaskis, & Charman, 2007), it is important to understand the possible impact of the incorporation of ε-PL into food products on lipid digestion. Previous studies that have shown the lipase-inhibiting properties of ε-PL have typically been carried out using ε-PL concentrations that far exceed the maximum levels approved by the FDA for food use. Additionally, these studies have used homogenization methods and emulsifiers that are not widely used in the food industry (Kido, et al., 2003; Tsujita, Sumiyoshi, Takaku, Momsen, Lowe, & Brockman, 2003; Tsujita, Takaichi, Takaku, Aoyama, & Hiraki, 2006). The objective of the current study was therefore to test whether ε-PL at the highest permitted concentration allowed by the FDA (Food and Drug Administration, 2011) has an impact on lipid digestion in emulsion-based delivery systems stabilized by food-grade ionic or non-ionic surfactants.

## 4.3 Materials And Methods

### 4.3.1 Emulsion Preparation And Optimization

Emulsions were prepared by combining an oil phase (4% w/w) with an aqueous phase (96% w/w). The oil phase consisted of a commercial food-grade corn oil (Mazola, ACH Food Companies, London, England). The aqueous phase was composed of 5 mM phosphate buffer (pH 7) and either non-ionic surfactant (0.03-1.5% w/w Tween 20) or anionic surfactant (0.05-0.95% quillaja saponin). Tween 20 was acquired from Acros Organics (Hampton, New Hampshire, lot 091M1417V), while the quillaja saponin was donated in the form of Q-Naturale by Ingredion (Westchester, IL, lot QEU-151112-01, 14% purity). ε-Polylysine was purchased from Wilshire Technologies (Princeton, NJ). The 5 mM phosphate buffer was prepared by

dissolving 1.1676 g of sodium phosphate monobasic (Sigma Aldrich, St. Louis, Missouri, lot BCBB2118) and 3.0932 g of sodium phosphate monobasic (Sigma Aldrich, St. Louis Missouri, lot 129K0053) to a final volume of 4 l. The pH was adjusted to a final value of 7.0 by adding either hydrochloric acid or sodium hydroxide at varying concentrations.

A coarse emulsion was first prepared by mixing the oil and the aqueous phases with a high-sheer blender (Bamix® Basic, Mettlen, Switzerland) at 7,000 rpm for 2 minutes. The resulting course emulsion was then passed five times through a microfluidizer (M-110P, Microfluidics, Westwood, MA) at 9,000 psi to produce a fine emulsion.

### 4.3.2 *In Vitro* Digestion

*In vitro* digestions simulating only the small intestine stage were carried out by modifying the protocol established by Y. Li, Hu, and McClements (2011). Solutions for this experiment were prepared as follows. Bile salts (Sigma Aldrich, St. Louis, Missouri, lot 031M0106V) were prepared by dissolving 0.1875 g in 3.5 ml of phosphate buffer pH 7.0 and stirring overnight. A salt stock solution was produced by diluting 16.44 g of sodium chloride (Fisher Scientific, Hampton, New Hampshire, lot 111354) and 2.57 g of calcium chloride (Sigma Aldrich, St. Louis, Missouri, lot 39H0085) in double-distilled water to a final volume of 75 ml.

Lipase was prepared by dissolving 0.06 g of the powdered enzyme (Sigma Aldrich, St. Louis, Missouri, lot SLBC9250V) in 2.5 ml of 5 mM phosphate buffer, followed by stirring for 30 minutes. The enzyme was used immediately after its preparation.

For the systems without polylysine, 18.75 ml of the prepared emulsions were diluted with 11.25 ml of 5 mM phosphate buffer. For systems containing polylysine, 18.75 ml of sample were diluted with 11.25 ml of a solution containing 0.16% (w/w) polylysine (Willshire Technologies, Princeton, New Jersey, lot 20130228) in 5 mM phosphate buffer, pH 7. When the emulsion was combined with the polylysine solutions, the final oil concentration for each system was 2.5% (w/w) corn oil, and 2 ppm polylysine.

The diluted emulsion sample was combined with the bile salts, stock salt solution, and lipase to simulate the digestion process in the small intestine. The digestion simulation took place using an automated pH Stat titration method (857 Titrando, Metrohm USA, Riverview, Florida). The principle behind this intestinal digestion simulation is quantifying the lipase-mediated release of free fatty acids (FFA) from the triacylglycerols that make up the oil system. The desired sample – in this case a corn oil emulsion – is dispersed in a solution containing specific amounts of digestive components including bile salts, lipase, sodium chloride, and calcium chloride. As the lipase exerts its enzymatic activity over the oil present in the sample, FFAs are released. This production of FFAs causes a decrease in pH, which is monitored by the automated titration unit; as the pH changes, the instrument automatically titrates sodium hydroxide solution to maintain it at pH 7.0. The amount of sodium hydroxide that was utilized throughout the digestion simulation is recorded *versus* time, and the percentage of FFA released can be calculated utilizing the equation below (Y. Li, Hu, & McClements, 2011):

$$\%FFA = 100 \times \frac{V_{NaOH} \times m_{NaOH} \times m_{lipid}}{w_{lipid} \times 2}$$

Where %FFA is the percentage of released FFAs; $V_{NaOH}$ is the volume of titrant in liters; $m_{NaOH}$ is the molarity of the sodium hydroxide solution used; $M_{lipid}$ is the molecular weight of the oil used; and $w_{lipid}$ is the weight of the oil in the digestion system in grams.

It should be noted that a highly simplified *in vitro* digestion method was utilized in this study that only focused on the events occurring within the small intestine region. This method was used as an initial screening tool to establish whether polylysine would interfere with the processes occurring the small intestine where the majority of lipid digestion and absorption normally occurs. In future studies, more sophisticated *in vitro* digestion models that include mouth, stomach and small intestine stages should be used, as well as *in vivo* studies using animals (Minekus, Alminger, Alvito, Ballance, Bohn, Bourlieu, et al., 2014).

### 4.3.3 Particle Characterization

The resulting emulsions and digestion products were characterized by measuring particle size (Malvern Mastersizer 2000, Malvern Instrumentst, Worcestershier, United Kingdom), zeta potential (Malvern Zetasizer ZS, Malvern Instruments, Worcestershire, United Kingdom), and confocal microscopy (Nikon D-Eclipse C1 80i, Nikon, Melville, NY) immediately after the emulsions were produced and after the conclusion of the digestion process. The experimental protocols used for each of these methods have been described in detail elsewhere (Salvia-Trujillo, Qian, Martin-Belloso, & McClements, 2013a).

### 4.3.4 Data Analysis

All experiments were performed in triplicate. The results were then reported as averages and standard deviations of these measurements.

## 4.4 Results And Discussion

### 4.4.1 Initial Emulsion Properties

Initially, we aimed to produce oil-in-water emulsions that had relatively low concentrations of free surfactant in the aqueous phase, so as to prevent any interactions between ε-polylysine and excess surfactant that might interfere with data interpretation. Emulsions were therefore prepared using fixed homogenization conditions (oil content, homogenization pressure, and number of passes), but increasing concentrations of surfactants in the aqueous phase. At low surfactant concentrations, there is insufficient surfactant to coat all of the small droplets formed within the homogenizer, and so the droplet size is mainly determined by the total amount of surfactant present. In this "surfactant-limited" regime, the mean particle diameter decreases with increasing surfactant concentration, and there is a relatively low concentration of free surfactant in the aqueous phase. At higher surfactant concentrations, the mean particle diameter remains relatively constant as the surfactant level is increased because the particle size is limited by the homogenization device, rather than the amount of surfactant present. In this "homogenizer-limited" regime, the amount of free surfactant in the aqueous phase increases as the total amount of surfactant in the system increases.

Consequently, it is important to be within the surfactant-limited regime so as to reduce the amount of free surfactant present.

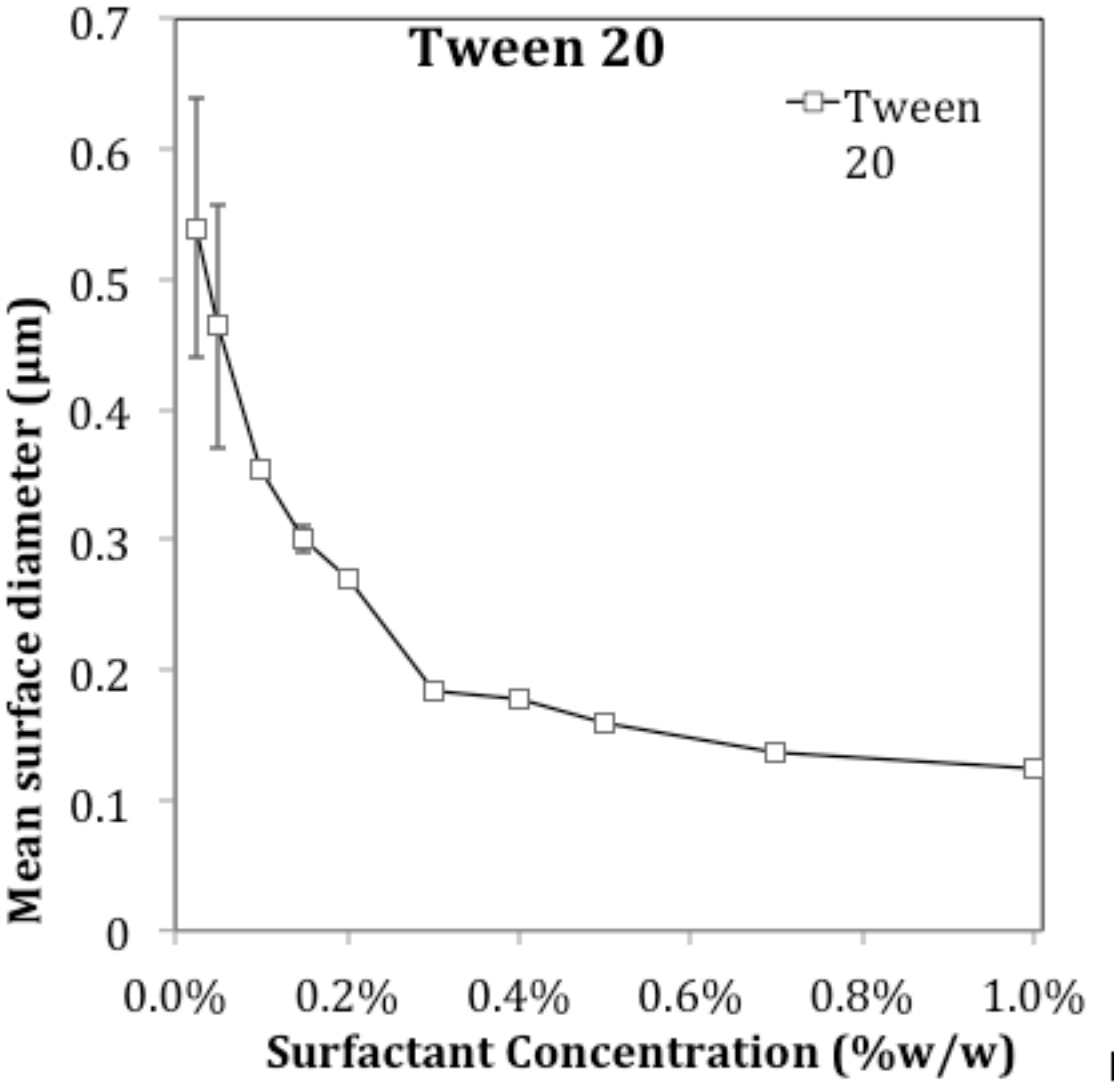



**Figure 18. Influence of surfactant concentration on particle size of T20-stabilized nanoemulsion droplets[19].**

[19] Lopez-Pena and McClements, 2015)

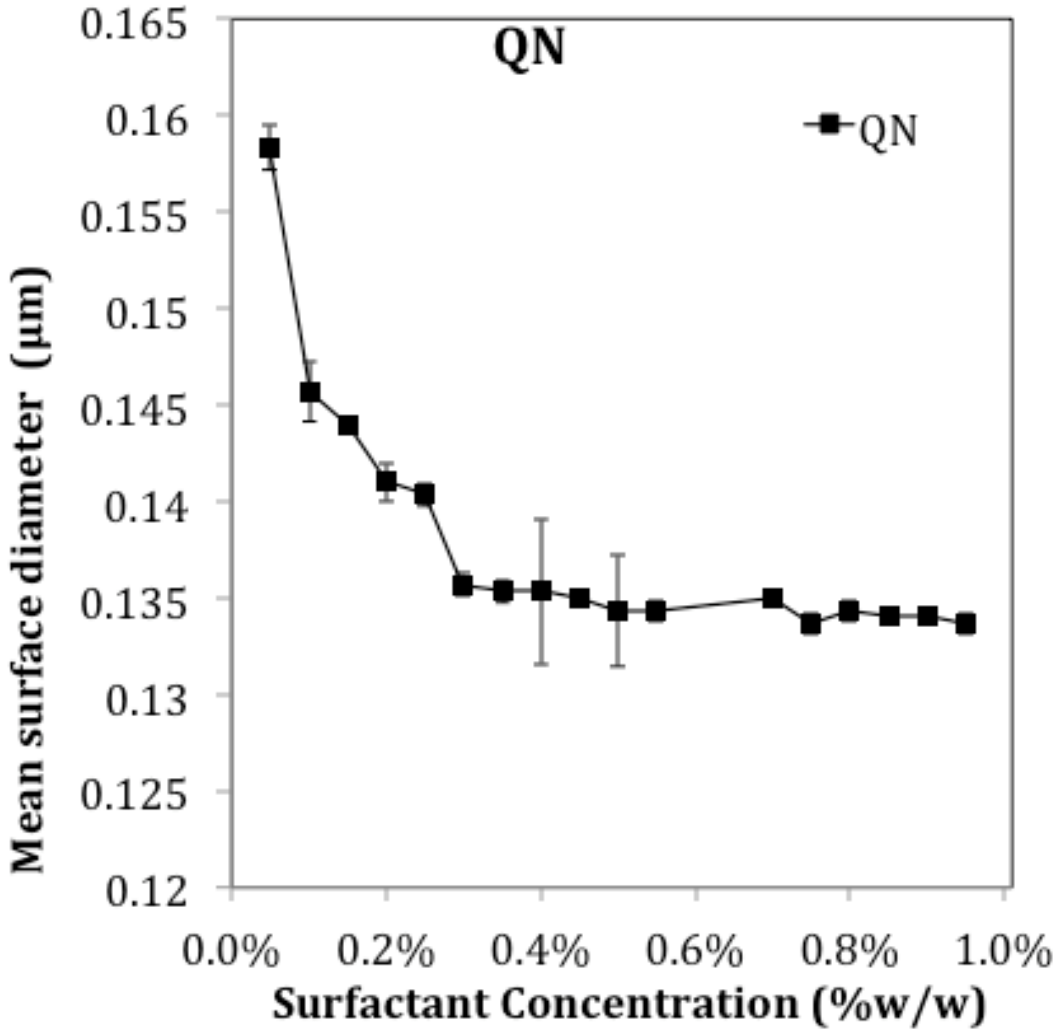



**Figure 19. Influence of surfactant concentration on the mean surface weighted diameter particle size of QN-stabilized nanoemulsion droplets [20].**

The influence of surfactant concentration on the mean particle diameter for emulsions prepared using either Tween 20 (T20) or quillaja saponin (QN) were therefore measured (**Figure 18** and **Figure 19**). In general, the dependence of the particle size on surfactant concentration was similar for both surfactants: initially

[20] Lopez-Pena and McClements, 2015)

there was a decrease in mean droplet diameter with increasing surfactant, followed by a leveling off. The optimal surfactant concentration was taken to be the value at which there was little further decrease in droplet size with increasing surfactant concentration, and the emulsion remained stable for at least 24 hours after production. For T20 this value was 0.33%, whereas it was 0.35% surfactant for QN. Consequently, these surfactant concentrations were used in the subsequent experiments.

### 4.4.2 Particle Size Analysis And Confocal Microscopy

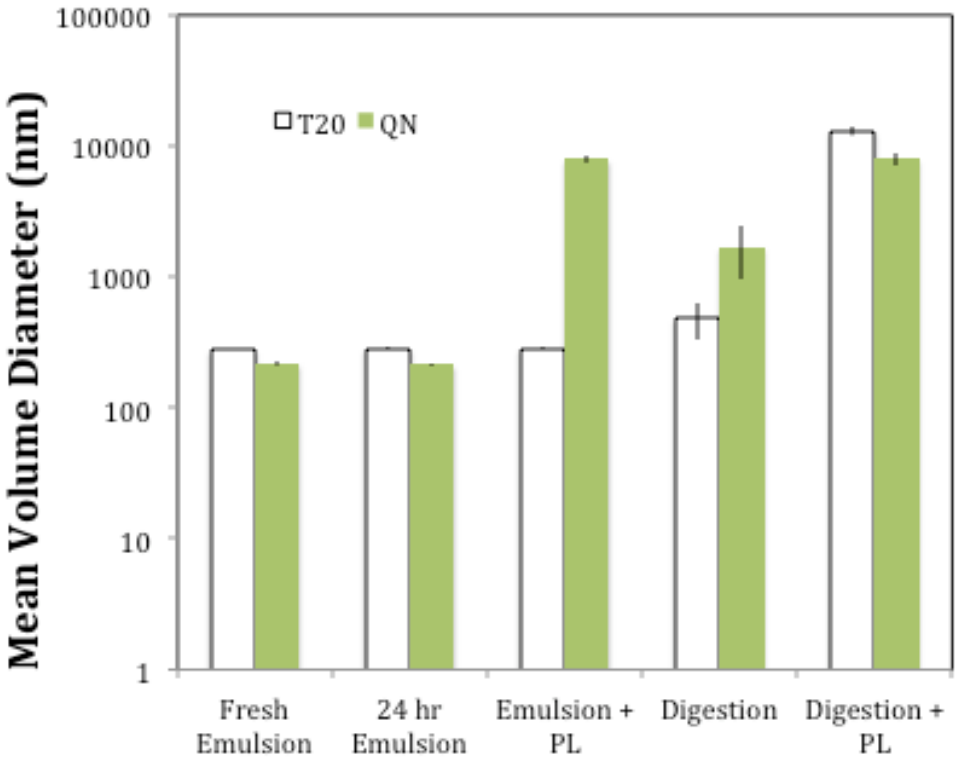



**Figure 20. Particle size (nm) of the original and digested emulsion systems with and without ε-PL for both T20 and QN systems[21].**

[21] Lopez-Pena and McClements, 2015)

Initially, we measured the influence of emulsifier type and ε-PL addition on changes in the particle size and microstructure of the emulsions after digestion. In the absence of ε-PL, the mean diameter of the particles in the T20-emulsions increased appreciably after being subjected to *in vitro* digestion, changing from around 276 to 484 nm (**Figure 20**). Conversely, there was a decrease in mean particle diameter after digestion for the QN-emulsions, changing from around 191 to 123 nm (**Figure 20**). In general, a change in particle size distribution of emulsions after digestion can be attributed to a number of phenomena, including droplet hydrolysis, coalescence, flocculation, formation of mixed micelles (micelles and vesicles), and formation of insoluble precipitates (such as calcium soaps). The difference in particle size measured after digestion for the two surfactants suggests that there were some differences in the structural properties of the undigested emulsion droplets or lipid digestion products produced. These results were supported by the microstructure images obtained through confocal microscopy, which showed that both emulsions contained relatively small evenly dispersed lipid droplets before digestion, but that the T20 system contained some relatively large particles after digestion (**Figure 21**).

The addition of polylysine to the T20-emulsions prior to digestion did not alter the particle size of the droplets, which can be attributed to the fact that cationic ε-PL did not interact strongly with the non-ionic surfactant coated lipid droplets. Presumably, there was no strong electrostatic attraction between the polylysine and the droplet surfaces, but there was a strong steric repulsion between the polylysine and the neutral polymeric head groups of this surfactant. On the other hand, there

was a large increase in the mean particle diameter in the T20-emulsions containing ε-PL after digestion (**Figure 20**), which suggested that the cationic biopolymer promoted extensive aggregation of the anionic undigested lipid droplets and/or mixed micelles.

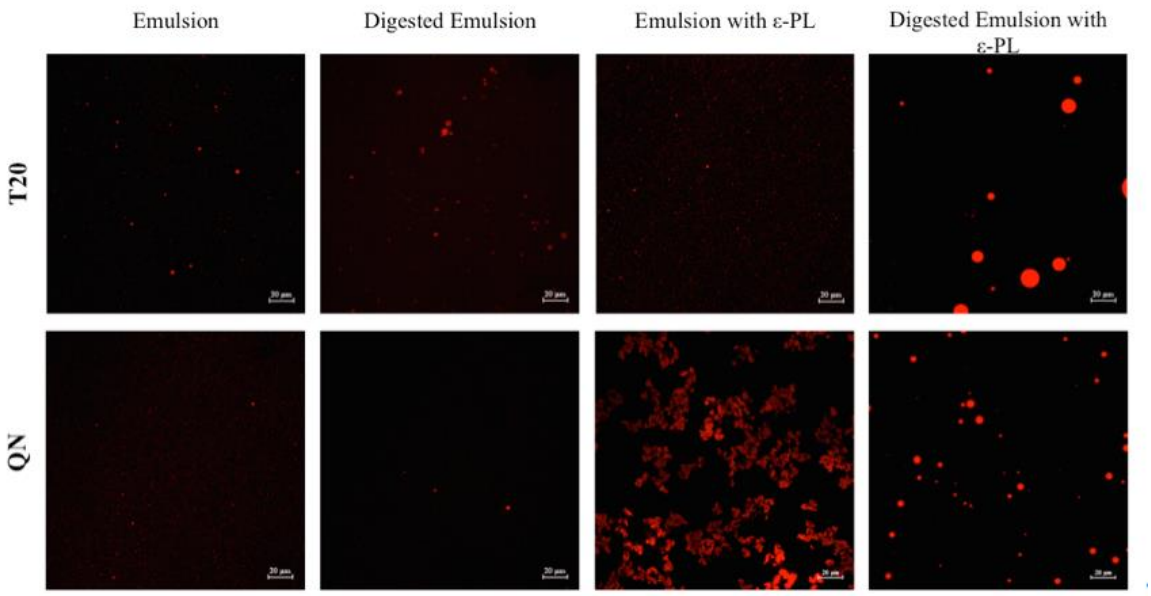



**Figure 21. Images obtained via confocal microscopy of the T20- and QN-stabilized nanoemulsion systems before and after digestion, with and without the addition of ε-PL[22].**

Prior to digestion, the addition of polylysine to the QN emulsions caused a large increase in their mean particle diameter, which can be attributed to bridging flocculation of the anionic droplets by the cationic biopolymer. After digestion, the QN emulsions containing polylysine also had very large particle sizes, indicating that they were highly flocculated. This effect can be attributed to the formation of electrostatic complexes between cationic ε-PL molecules and anionic species in the

[22] Lopez-Pena and McClements, 2015)

digesta, such as bile salts, phospholipids, free fatty acids, and undigested fat droplets.

Confocal microscopy images of the emulsions containing polylysine supported the light scattering results (**Figure 21**). Prior to digestion, the addition of polylysine to the T20-emulsions had little influence on their microstructure, but its addition to the QN-emulsions promoted extensive droplet aggregation. After digestion, the emulsions contained spherical lipid particles, which may have been undigested fat droplets or vesicles formed by bile salts, phospholipids and fatty acids.

### 4.4.3 Micro-Electrophoresis Measurements

In this section, we characterized changes in the surface charge of the two emulsions with and without ε-PL, as well as before and after digestion. As mentioned previously, these two surfactants were partly selected for this study because of their different surface characteristics, which would have been expected impact their interactions with ε-PL (**Figure 22**). Prior to digestion, the T20-emulsion containing no polylysine had a relatively low negative charge (-5.8 mV), whereas the QN-emulsion has a relatively high negative charge (-63.2 mV). After digestion, the negative charge on the particles present in the T20-emulsions increased (-74.5 mV) appreciably, which can be attributed to the presence of the anionic bile salts, phospholipids, and free fatty acids. The particles in the QN-emulsions was also highly negative after digestion (-42.1 mV), but it was less negative that the emulsions prior to digestion. These results suggest that there were

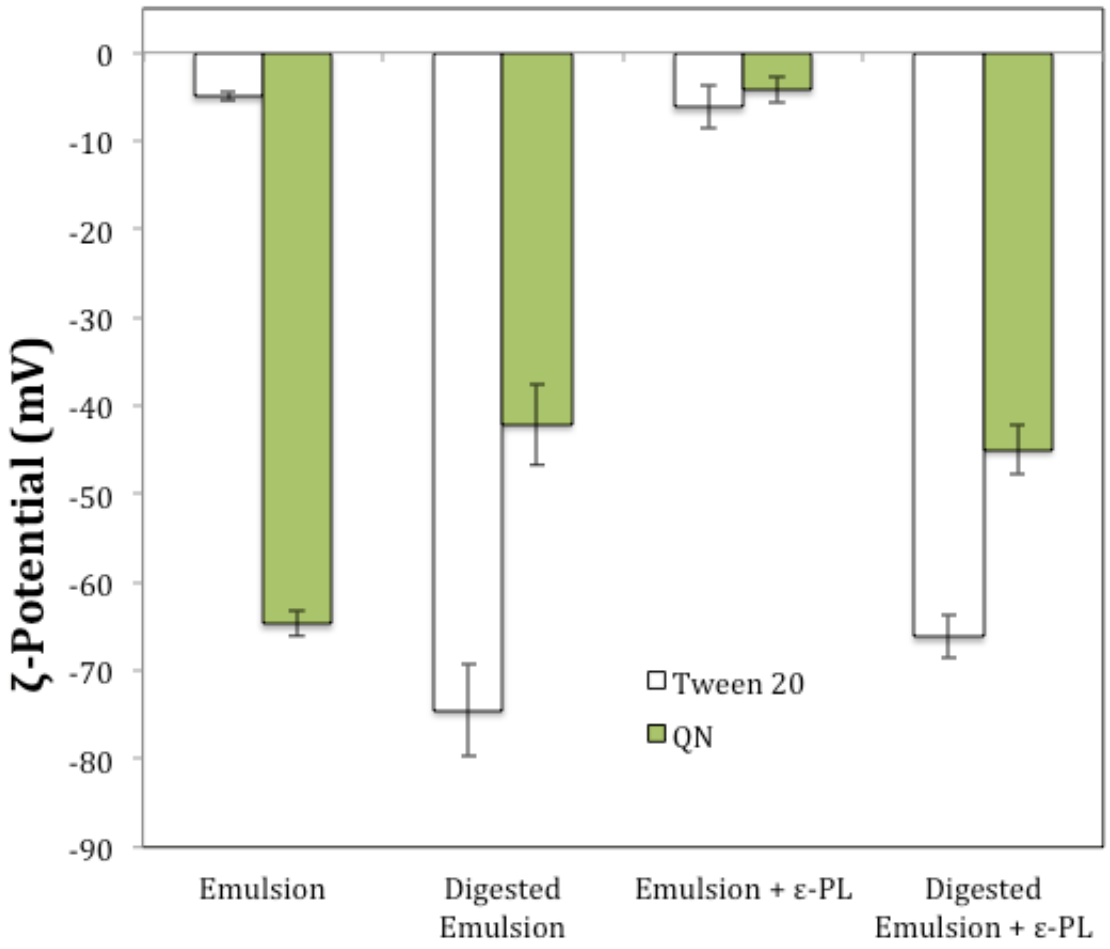



**Figure 22. ζ-Potential (mV) of the original and digested nanoemulsions with and without ε-PL for both T20 and QN systems[23].**

different kinds of structures in the digesta for the quillaja saponins than for the Tween 20.

Prior to digestion, the addition of polylysine to the T20-emulsions caused little change in the electrical characteristics of the droplets (-6.2 mV) compared to the samples with no polylysine (- 5.8 mV). This suggests that polylysine did not

[23] Lopez-Pena and McClements, 2015)

interact strongly with the surfaces of the lipid droplets coated by the non-ionic surfactant. Conversely, the incorporation of polylysine into the QN-emulsions prior to digestion caused an appreciable change in their surface charge characteristics, with the □-potential going from -63.2 to -4.2 mV upon addition of polylysine. This suggests that the cationic polylysine molecules adsorbed to the surfaces of the anionic QN-coated lipid droplets through electrostatic attraction, thereby partially neutralizing their charge.

### 4.4.4 *In Vitro* Digestion

The main objective of this study was to assess the potential inhibitory action of polylysine on lipid digestion in the small intestine, and to determine the influence of initial surfactant type on this effect. A simulated small intestine (pH stat) method was therefore used to focus on the events occurring within this region of the GIT where the majority of lipid digestion normally occurs. In reality, an emulsion passes through the mouth and stomach before reaching the small intestine, which may cause alterations in the size and surface characteristics of the lipid droplets. Nevertheless, previous studies have shown that ε-PL is resistant to degradation by digestive enzymes in the upper gastrointestinal tract (Tsujita, Takaichi, Takaku, Aoyama, & Hiraki, 2006), and that lipid droplets coated by some small molecule surfactants do not undergo appreciable changes in their properties in the mouth and stomach regions (D. J. McClements, Decker, & Park, 2009; D. J. McClements & Li, 2010; D. J. D. McClements, Eric A.; Park,Y., 2007; Singh, Ye, & Horne, 2009). Hence, the results obtained in this study should provide some valuable insights into the potential role of polylysine on lipid digestion in the small intestine.

In general, the free fatty acid release profiles of the different systems exhibited a similar behavior: initially there was a rapid increase in FFAs released followed by a more gradual increase at longer times (**Figure 23**). However, the precise shape of the FFA release profiles depended on emulsifier type and ε-PL addition. In the

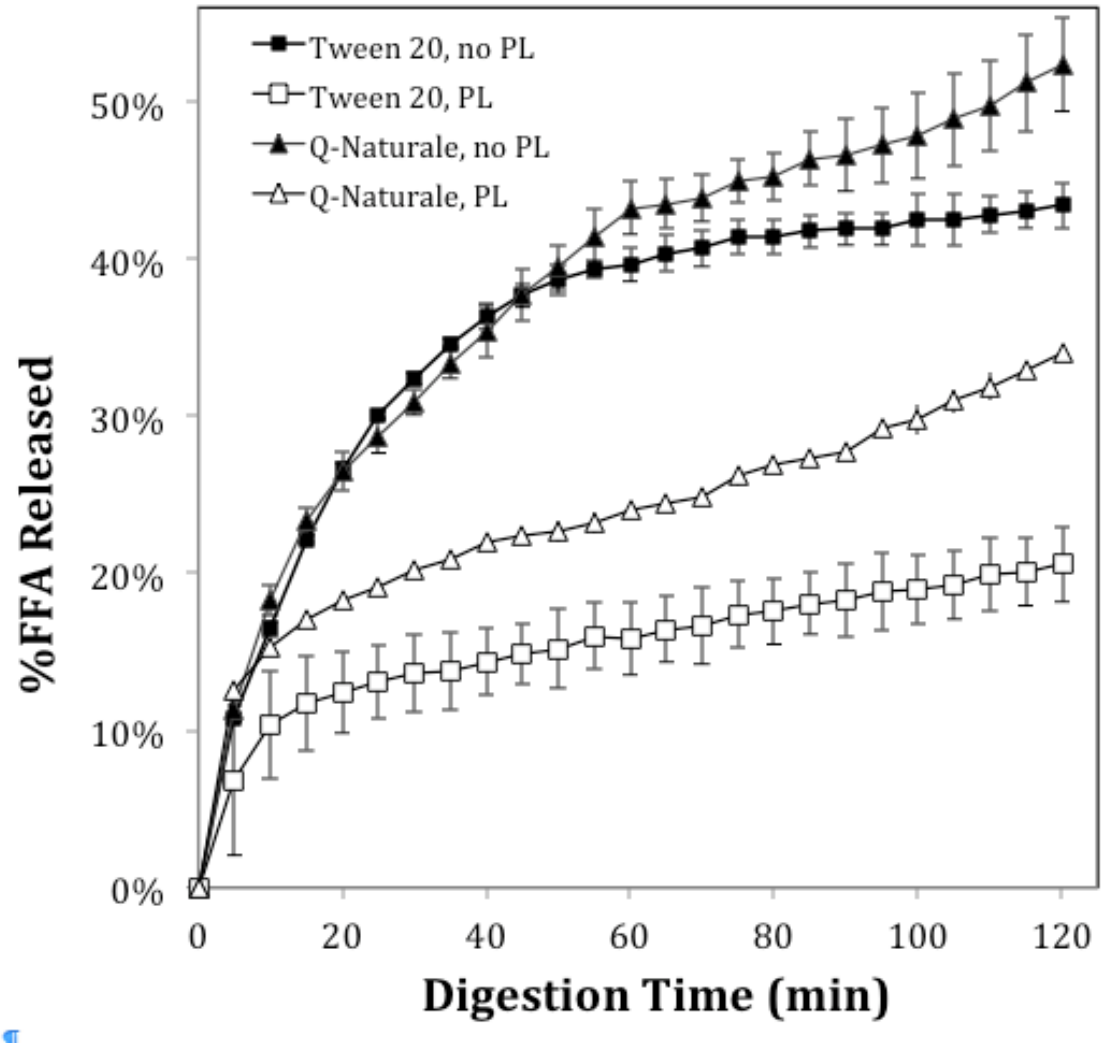



**Figure 23. Influence of surfactant charge and addition of ε-PL on the percentage of free fatty acids (FFA%) released from nanoemulsions formulated with anionic (QN) and neutral (T20) surfactants. The final lipid content in the digestion medium was 2.5%[24].**

[24] Lopez-Pena and McClements, 2015)

absence of polylysine, the fraction of the lipid phase digested after 2 hours incubation was about 43% and 52% for the T20 and QN systems, respectively (**Figure 23**). The relatively low amount of FFAs released from the emulsions can be attributed to the relatively high level of lipids present in the initial samples, *i.e.,* there were too many triacyglycerol molecules for the lipase to completely digest and/or for the mixed micelles to completely solubilize (Y. Li, Hu, & McClements, 2011). These experiments may therefore be more representative of conditions where a high fat load is ingested as part of a meal. The addition of ε-PL had a major impact on lipid digestion for both surfactant types leading to a decrease in the rate and extent of FFA production. For example, at the end of the 2 hour incubation period, the amount of FFAs released was around 21% and 34% for T20 and QN systems, respectively. These values correspond to a decrease of 53% and 35% in FFA production compared to the samples with no polylysine. The suppression of lipid digestion by ε-PL observed in this study is in agreement with that reported in earlier studies using different types of emulsions, where an inhibitory effect of up to 50% was also reported (Kido, et al., 2003; Tsujita, Sumiyoshi, Takaku, Momsen, Lowe, & Brockman, 2003; Tsujita, Takaichi, Takaku, Aoyama, & Hiraki, 2006; Tsujita & Takaku, 2009).

There are a number of potential physicochemical mechanisms that may account for the observed suppression of lipid digestion in the presence of polylysine. Cationic polylysine may adsorb to the surface of anionic lipid droplets and form a protective coating that inhibits the ability of the lipase to interact with

the lipids. Cationic polylysine may interact with anionic bile salts and phospholipids through electrostatic interactions thereby changing their functional properties. Bile salts and phospholipids play a number of important roles in the lipid digestion process: they aid in lipid emulsification within the GIT; they alter lipid droplet surfaces in a manner that promotes lipase adsorption and function; and, they aid in the solubilization and transport of lipid digestion products by forming mixed micelles (Kido, et al., 2003). In this study, the lipid droplets were homogenized prior to entering the small intestine, and therefore the role of bile salts and phospholipids on emulsification is less important. However, the ability of ε-PL to induce droplet flocculation may alter the rate and extent of lipid digestion. Previous studies have shown that highly flocculated lipid droplets are digested more slowly than non-flocculated ones because of a reduction in the surface area of lipid phase directly exposed to the lipase molecules (Day, Golding, Xu, Keogh, Clifton, & Wooster, 2014). The adsorption of bile salts to oil droplet surfaces (and displacement of the original surfactants) is often critical to lipid digestion, as the formation of a bile-salt coated interface promotes lipase adsorption and activation (Aloulou, Rodriguez, Fernandez, van Oosterhout, Puccinelli, & Carriere, 2006; Borgström & Brockman, 1984; Tsujita, Takaichi, Takaku, Sawai, Yoshida, & Hiraki, 2007). Moreover, the activity of lipase is determined by the amount that adsorbs to the lipid droplet surfaces, rather than the total amount present in the system (Moreau, Moulin, Gargouri, Noel, & Verger, 1991; Ransac, Gargouri, Marguet, Buono, Beglinger, Hildebrand, et al., 1997; Tsujita, Takaichi, Takaku, Sawai, Yoshida, & Hiraki, 2007). When no ε-PL is present, bile salts are free to interact with the oil

droplets, establishing suitable conditions on the droplet surfaces for the subsequent adsorption and activation of lipase. However, when cationic polylysine is present, it interacts with the anionic bile salts and prevents them from coating the oil droplet surfaces, thereby altering the adsorption and activity of lipase (Tsujita, Sumiyoshi, Takaku, Momsen, Lowe, & Brockman, 2003; Tsujita, Takaichi, Takaku, Sawai, Yoshida, & Hiraki, 2007).

When anionic surfactants (such as saponin) are present in an emulsion, the polylysine may interact with them as well as the bile salts. Consequently, there may be more free bile salts to interact with the lipid droplet surfaces and form mixed micelles, thereby promoting digestion. The changes in ζ-potential observed for both systems upon the introduction of ε-PL further explains the difference in the degree of lipase inhibition exhibited. Tsujita, Sumiyoshi, Takaku, Momsen, Lowe, and Brockman (2003) have shown that for ε-PL to exhibit lipase-inhibiting behavior in an emulsion system with a fixed amount of bile salts, the positive charges contributed by ε-PL must surpass the negative charges in the system. The inclusion of a negative surfactant and subsequent increase in negative charge means that the system no longer complies with this premise, limiting the inhibitory effect of ε-PL.

## 4.5 Conclusions

There is interest in utilizing polylysine as a functional ingredient in foods and beverages because it is a natural antimicrobial agent. However, our results and those of other groups working with phospholipid-stabilized emulsions, suggest that this cationic biopolymer may interfere with the normal lipid digestion process due to its ability to interact with anionic components in the gastrointestinal tract, such

as lipid droplets, bile salts, and phospholipids. These interactions depend on surfactant type, and may have an impact on the absorption of lipophilic bioactive agents, such as oil-soluble vitamins or nutraceuticals. In future studies, we intend to use a more comprehensive *in vitro* digestion model and animal feeding studies to assess the potential influence of polylysine on the bioaccessibility of lipophilic nutrients.

## 4.6 Acknowledgments

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# CHAPTER 5

# EFFECT OF MUCIN ON INHIBITION OF LIPASE ACTIVITY BY E-POLYLYSINE

## 5.1 Abstract

The cationic biopolymer, ε-polylysine (ε-PL), is a natural generally regarded as safe (GRAS) wide spectrum antimicrobial agent suitable for utilization in the food industry. This biopolymer has also been shown to possess the ability to inhibit lipid digestion both *in vivo* and *in vitro*. However, many of the previous studies in this area have utilized polylysine concentrations that are substantially higher than those stipulated by the FDA, or have not accounted for the interactions of polylysine with other components in the mouth or stomach. We therefore examined the effect of polylysine on the digestion of corn oil-in-water emulsions stabilized by Tween 20 in the presence and absence of mucin. Emulsions were passed through a simulated gastrointestinal tract that included oral, gastric, intestinal duodenal phases. Samples were characterized before and after each stage using electrophoresis, optical microscopy, and static light scattering. Our results showed that there was no statistically significant difference between emulsions with and without ε-polylysine and with and without mucin. However, our results suggest that ε-polylysine does form strong electrostatic complexes with mixed micelles, potentially decreasing the absorption of lipids in the small intestine. Samples containing mucin had a lower amount of insoluble sediment formed after digestion and a higher free fatty acid content, suggesting that it may somewhat prevent the interaction between cationic polylysine and anionic bile salts. These results have important implications for the

incorporation of polylysine into food systems, particularly those containing lipophilic nutrients.

*Keywords*: polylysine; lipids; digestion; emulsions; nanoemulsions; lipase inhibition; antiobesity

## 5.2 Introduction

ε-Polylysine is an FDA-approved (Food and Drug Administration, 2004, 2011) natural antimicrobial agent that is effective against a wide range of microorganisms including Gram (+) and Gram (-) bacteria, bacteriophages, yeasts, and fungi (N. A. El-Sersy, A. E. Abdelwahab, S. S. Abouelkhiir, D. M. Abou-Zeid, & S. A. Sabry, 2012; Moschonas, et al., 2012; Shima, Matsuoka, Iwamoto, & Sakai, 1984; H. L. Yu, Y. P. Huang, & Q. R. Huang, 2010; C. C. Zhou, et al., 2011). The high antimicrobial activity of this cationic biopolymer is mainly attributed to its ability to interact electrostatically with anionic microbial cell membranes and disrupt their integrity (Blin, Purohit, Leprince, Jouenne, & Glinel, 2011; Brogden, 2005; Shima, Matsuoka, Iwamoto, & Sakai, 1984; Zasloff, 2002).

The cationic nature of this antimicrobial agent has also been linked with inhibition of lipid digestion both *in vitro* and *in vivo* (Kido, et al., 2003; Cynthia Lyliam Lopez-Pena & David Julian McClements, 2015; Tsujita, Sumiyoshi, Takaku, Momsen, Lowe, & Brockman, 2003; Tsujita, Takaichi, Takaku, Aoyama, & Hiraki, 2006). The inhibitory mechanism has been proposed to be due to the electrostatic interaction between cationic ε-polylysine and anionic bile salts within the small intestine (Kido, et al., 2003). Bile salts, phospholipids, and lipase act in conjunction within the small intestine to hydrolyze triacylglycerols and other lipids (Duan, 2000). Bile salts adsorb to the surface of the fat droplets entering the small intestine from the stomach, which facilitates the adsorption of lipase and triggers its enzymatic activity. As lipase exerts its enzymatic activity, triacylglycerols are cleaved to fatty acids and 2-monoacylglycerols. Bile salts and phospholipids – both

amphiphilic molecules – act as surfactants for the fat droplets entering from the stomach, as well as solubilizing the lipid digestion products in mixed micelles. These mixed micelles are absorbed by the microvilli lining the intestinal walls, and eventually enter the bloodstream (Kido, et al., 2003). The electrostatic interaction between ε-polylysine and bile salts can result in a number of effects that may alter lipid digestion: (i) formation of a coating around the surfaces of bile- or phospholipid-emulsified fat droplets entering the small intestine, thereby inhibiting lipase adsorption; (ii) flocculation of the fat droplets thereby reducing the available surface area for lipase adsorption; or, (iii) decreasing the formation of free fatty acid micelles after digestion, limiting the activity of lipase (Kido, et al., 2003). In addition, the binding of polylysine to mixed micelles may lead to insoluble complexes that reduce the bioaccessibility of any encapsulated lipophilic agents.

Although there have been numerous studies documenting the strong inhibitory effect of ε-polylysine, the methodologies followed for the *in vivo* and *in vitro* systems have focused on the interactions between ε-polylysine and bile salts under intestinal conditions (Kido, et al., 2003; Cynthia Lyliam Lopez-Pena & David Julian McClements, 2015; Takahiro Tsujita, 2006; Tsujita, Sumiyoshi, Takaku, Momsen, Lowe, & Brockman, 2003; Tsujita, Takaichi, Takaku, Aoyama, & Hiraki, 2006), but have not taken into account interactions that ε-polylysine might have had with other anionic components in the mouth (such as mucin), and in many instances have utilized polylysine concentrations that exceed the concentration currently permitted by the FDA (Food and Drug Administration, 2011) by several orders of magnitude. We hypothesized that cationic ε-polylysine interacts with anionic mucin

in the mouth, thereby altering its subsequent gastrointestinal fate. Mucin is a family of heavily glycosylated negatively charged proteins with a high proportion of the sulfide-containing amino acid cysteine, naturally secreted by epithelial cells to form a protective mucus layer. Its high glycosylation provides it with a high water-holding capacity and immunity to proteases, making them an extremely effective protective barrier in the digestive tract (Kufe, 2009; Perez-Vilar & Hill, 2004). Due to the negative charge of this biopolymer, it is possible that it may interact electrostatically and form an electrostatic complex with ε-polylysine prior to the latter's exposure to other anionic gastrointestinal constituents (such as bile salts).

ε-Polylysine has been shown to interact quite strongly with negatively-charged compounds, and the electrostatic complexes formed with one anionic biopolymer may remain intact even after addition of another anionic biopolymer (C. L. Lopez-Pena & McClements, 2014). Given the strong negative charge of mucin, it is possible that electrostatic complexes formed between ε-polylysine and mucin in the mouth or stomach would prevent it from interacting electrostatically with bile salts and other anionic components in the small intestine, thereby reducing its lipase-inhibiting activity.

The objective of this work is therefore to assess whether the formation of electrostatic complexes formed between ε-polylysine and mucin in the oral stage of digestion have an effect on the lipase-inhibiting activity of ε-polylysine. This objective was achieved by passing corn oil-in-water emulsions through a simulated gastrointestinal tract in the absence and presence of both ε-polylysine and mucin.

## 5.3 Materials And Methods

### 5.3.1 Emulsion Preparation

Emulsions were prepared by combining an oil phase (4% w/w) composed of a commercial food-grade corn oil (Mazola, ACH Food Companies plf, London, England) with an aqueous phase (96%) containing 5 mM phosphate buffer (pH 7) and 0.33% Tween 20 purchased from Acros Organics (Hampton, New Hampshire). The phosphate buffer was produced by combining sodium phosphate monobasic (Sigma Aldrich, St. Louis, Missouri) and sodium phosphate dibasic (Thermo Fisher Scientific, Waltham, MA) and adjusting the final pH through addition of hydrochloric acid or sodium hydroxide at the appropriate concentrations. The oil and aqueous phases were first converted into a coarse emulsion utilizing a high-shear blender (bamix® Basic, bamix of Switzerland, Mettlen, Switzerland) at 7,000 rpm for 2 minutes. The coarse emulsion was then homogenized by passing it five times through a microfluidizer (M-110P, Microfluidics, Westwood, MA) operating at a pressure of 10,000 psi.

### 5.3.2 *In Vitro* Digestion

To analyze the effect of mucin on the lipase-inhibiting activity of ε-polylysine, a simulated gastrointestinal tract was used that mimicked the mouth, stomach, and small intestine stages. A 4 %w/w ε-polylysine (Wilshire Technologies, Princeton, NJ) in 5 mM phosphate buffer solution was prepared. *In vitro* digestions were carried out based on the protocol established by Y. Li, Hu, and McClements (2011), with some modifications.

*Simulated Oral Fluids:* A stock solution of artificial saliva was prepared by mixing sodium chloride (Fisher Scientific, Hampton, New Hampshire, lot 138498), ammonium nitrate (Sigma Aldrich, St. Louis, Missouri), potassium phosphate monobasic (Fisher Scientific, Hampton, New Hampshire), potassium chloride (Fisher Scientific, Hampton, New Hampshire), potassium citrate (Sigma Aldrich, St. Louis Missouri), uric acid sodium salt (Sigma Aldrich, St. Louis, Missouri), urea (Fluka, St. Louis, Missouri), and lactic acid sodium salt (Sigma Aldrich, St. Louis, Missouri) in double distilled water, as described by Y. Li, Hu, and McClements (2011). For each sample, 0.6 g mucin Type II (Sigma Aldrich, St. Louis, Missouri) was dispersed in 20 ml of the artificial saliva stock solution and allowed to stir overnight.

*Simulated Gastric Fluids:* A stock solution of simulated gastric fluids was prepared by dissolving 2 g sodium chloride (Fisher Scientific, Hampton, New Hampshire) and 7 ml hydrochloric acid (Sigma Aldrich, St. Louis, Missouri) in a final volume of 1 liter of double distilled water. 30 minutes prior to use, 0.064 g of pepsin (Sigma Aldrich, St. Louis, Missouri) were dissolved in 20 ml of the gastric solution.

*Simulated Small Intestinal Fluids:* For the small intestine phase, 5.5 g calcium chloride (Fisher Scientific, Hampton, New Hampshire) and 32.85g sodium chloride (Sigma Aldrich, St. Louis, Missouri) were dissolved in 1 liter of double distilled water to form a stock solution of simulated small intestinal fluids. For each sample, 0.1875 g bile salts (Sigma Aldrich, St. Louis, Missouri) were dispersed in 4 ml phosphate buffer (5 mM, pH 7.0) and allowed to stir overnight. 30 minutes prior to use, 0.06 g lipase (Sigma Aldrich, St. Louis, Missouri) were dispersed in 2.5 ml phosphate buffer

(5 mM, pH 7), as previously described by Cynthia Lyliam Lopez-Pena and David Julian McClements (2015) and by Y. Li, Hu, and McClements (2011).

*Passage through simulated GIT*: Prior to digestion, concentration adjustments were made to the emulsions: 10 ml 4% w/w corn oil emulsion was diluted with either 10 ml of phosphate buffer (5 mM, pH 7.0) or combined with 10 ml of 0.4 %w/w ε-polylysine to obtain a final ε-polylysine concentration of 200 ppm, which is one hundred times higher than the dosage approved by the FDA for use in food systems. Increasing the concentration one hundred times is a common practice when assessing the toxicology of a compound *in vivo* and *in vitro*. The samples then underwent the *in vitro* digestion process, as described below.

For the mouth phase, 20 ml diluted emulsion was combined with either 20 ml mucin solution or 20 ml artificial saliva stock solution. The pH was adjusted to 6.8 utilizing sodium hydroxide and/or hydrochloric acid at varying concentrations, and was then placed in a shaking incubator (Excella E24 Incubator Shaker Series, New Brunswick Scientific, Enfield, CT) at 37 °C and 100 rpm for 10 minutes. At the end of the mouth phase, 20 ml of the resulting digesta was titrated into 20 ml simulated gastric fluids with freshly prepared pepsin. The pH was adjusted to 2.5, and reintroduced into the shaking incubator for 2 hours. Upon completion of the gastric stage of the digestion, 30 ml was taken from the stomach digesta and transferred to a different vessel and connected to a pH stat (857 Titrando, Metrohm USA, Riverview, FL). Here, it was combined with the simulated intestinal juices and bile salts, the pH adjusted to 7.0, and freshly prepared lipase was added. Lipid digestion was quantified through the aforementioned pH Stat, which monitors changes in pH

and titrates a sodium hydroxide solution at a specific concentration to maintain the pH at 7.0.

The principle of this method is based on lipase cleaving free fatty acids (FFA) from triacylglycerol molecules, effectively decreasing the pH of the system due to the release of protons ($H^+$). The volume of sodium hydroxide (0.1 N) required to maintain the system at a constant pH (7.0) was recorded throughout the experiment. The amount of FFA released by the action of lipase from the oil are calculated utilizing the equation reported by Y. Li, Hu, and McClements (2011):

$$\%FFA = 100 \times \frac{V_{NaOH} \times m_{NaOH} \times m_{lipid}}{w_{lipid} \times 2}$$

Where %FFA is the percentage of released FFAs; $V_{NaOH}$ is the volume of titrant in liters; $m_{NaOH}$ is the molarity of the sodium hydroxide solution used; $M_{lipid}$ is the molecular weight of the oil used (grams per mol); and $w_{lipid}$ is the weight of the oil in the digestion system (grams).

### 5.3.3 Particle Characterization

The emulsion with and without ε-polylysine and the digestion products with and without mucin were characterized by measuring their particle size (Malvern Mastersizer 2000, Malvern Instrumentst, Worcestershier, United Kingdom) and □-potential (Malvern Zetasizer ZS, Malvern Instruments, Worcestershire, United Kingdom), as well as observing all systems under optical and confocal microscopy (Nikon D-Eclipse C1 80i, Nikon, Melville, NY) immediately after the emulsions were produced and after the conclusion of the digestion process.

### 5.3.4 Data Analysis

Each experiment was performed at least two or three times and the mean and standard deviations were calculated. Statistical significance between means were determined through analysis of variance (ANOVA) following Tukey pairwise comparisons with a confidence interval of 95% with Minitab® 17.1.0 (Minitab Inc., State College, Pennsylvania).

## 5.4 Results And Discussion

### 5.4.1 Particle Size Analysis And Microscopy

The addition of ε-polylysine to the initial oil-in-water emulsion had no discernable effect on the particle size, with both samples having a mean diameter of about 0.26 μm (**Figure 24**) and no visible changes in appearance were observed (**Figure 25a** and **e**). The most likely reason for this observation is that the cationic biopolymer did not promote bridging flocculation of the fat droplets because they were coated by a non-ionic surfactant. In addition, the polylysine concentration used was not sufficient to generate a strong osmotic attraction that would have promoted depletion flocculation.

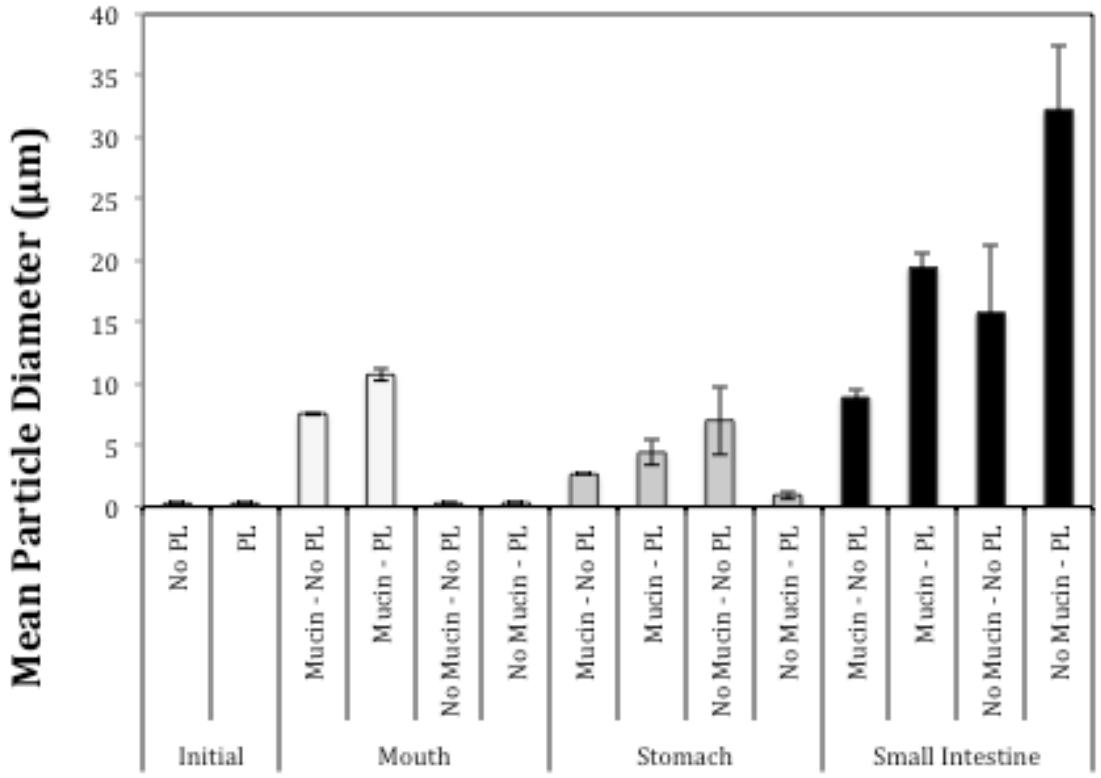


**Figure 24. Volume-weighted mean particle diameter (μm) of the nanoemulsion with and without ε-polylysine prior to digestion and after each stage of digestion, with and without mucin[25].**

[25] (Lopez-Pena, Zheng, Sela, Xiao, Decker, and McClements, 2015)

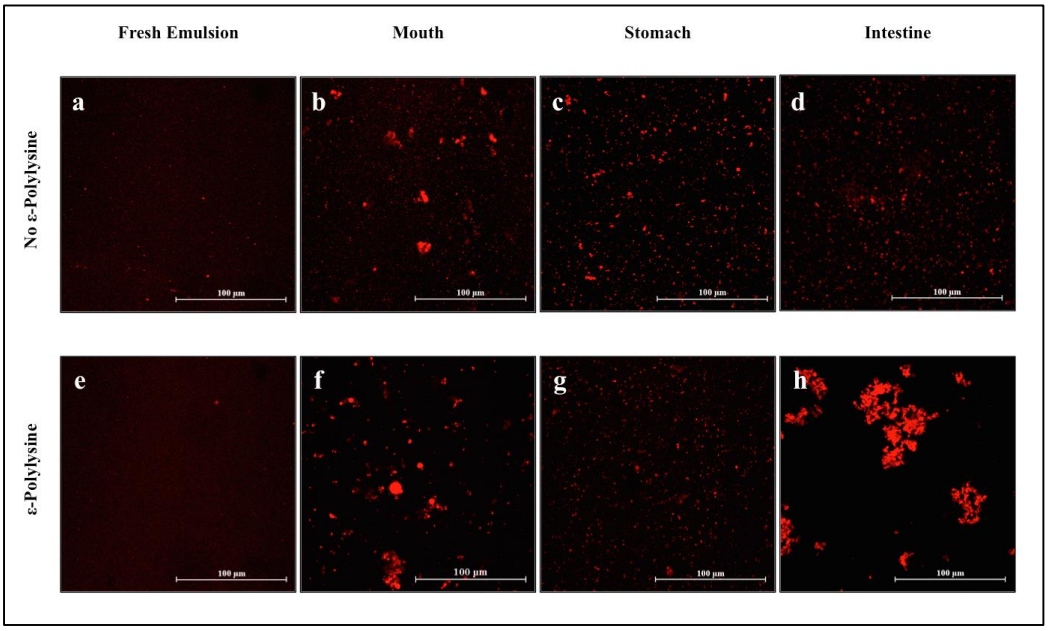


**Figure 25. Images obtained via confocal microscopy of the emulsion with and without ε-polylysine prior to digestion and after each stage of digestion. These samples contained mucin added in the mouth phase[26].**

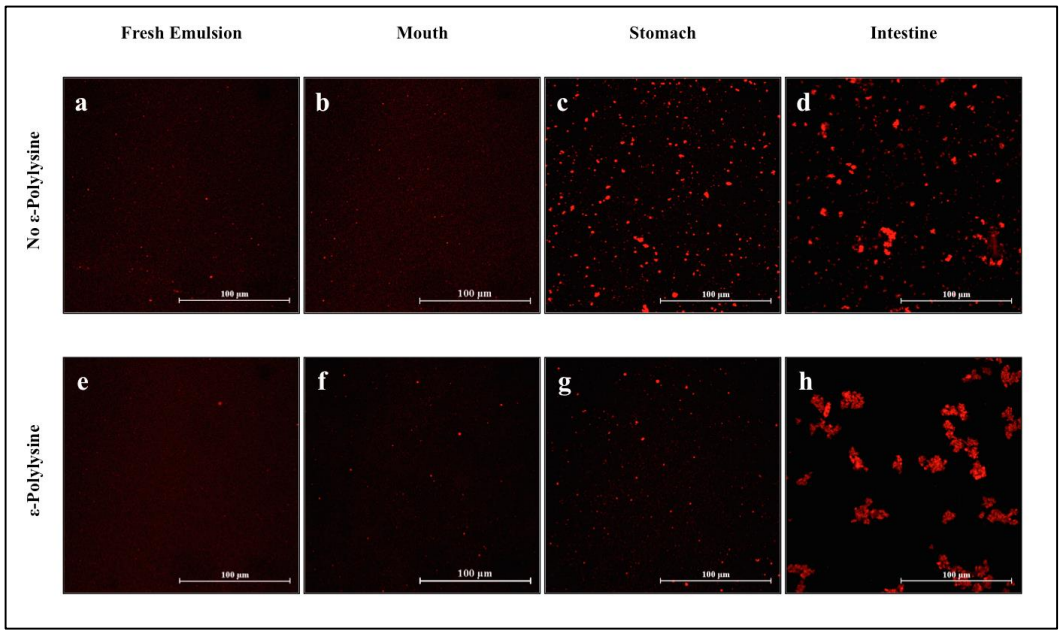


**Figure 26. Images obtained via confocal microscopy of the emulsions with and without ε-polylysine added prior to digestion and after each stage of digestion. These emulsions contained no mucin[27].**

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[26] (Lopez-Pena, Zheng, Sela, Xiao, Decker, and McClements, 2015)

#### 5.4.1.1 Oral Phase

The first difference in the behavior of the emulsions became apparent in the oral phase when mucin was added to only one set of samples. In the absence of mucin, there was little change in particle size (**Figure 24**) or appearance (**Figure 26b** and **f**) of the samples after exposure to the simulated oral fluids. Indeed there was no change in the mean particle diameter of the emulsions containing no ε-polylysine (0.26 μm), and only a slight increase in the mean particle diameter of the emulsions containing ε-polylysine (0.31 μm). On the other hand, there was a pronounced increase in the mean particle diameter for the emulsions containing mucin: ≈ 8 and 11 μm in the absence and presence of ε-polylysine, respectively (**Figure 24**). This change in particle size was clearly evident when the emulsions were observed by confocal microscopy: extensive flocculation was observed in both systems, with larger clusters occurring in the sample containing ε-polylysine (**Figure 25b** and **f**). Presumably, the mucin molecules promoted flocculation of the oil droplets in the emulsion containing no ε-polylysine through either a depletion or a bridging mechanism. The increased aggregation in the system containing cationic ε-polylysine was probably due to its ability to form electrostatic complexes with anionic mucin that trapped some of the fat droplets.

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[27] (Lopez-Pena, Zheng, Sela, Xiao, Decker, and McClements, 2015)

#### 5.4.1.2 Gastric Phase

After exposure to simulated gastric fluids there was an appreciable change in the particle size and microstructure of the emulsions, which depended on the presence of both ε-polylysine and mucin.

*Without mucin:* The emulsions without mucin (which were stable under oral conditions) exhibited an appreciable increase in particle size after exposure to the gastric phase (**Figure 24**). The mean particle diameter of the samples containing ε-polylysine increased to around 0.94 μm, while the samples without ε-polylysine increased to around 7.0 μm. The particle size measurements therefore suggested that appreciable droplet aggregation had occurred in these emulsions after exposure to gastric conditions, with the extent of aggregation depending on the presence of the cationic biopolymer. Confocal microscopy of these samples supported the light scattering measurements (**Figure 26c** and **g**): the emulsion containing ε-polylysine exhibited some limited aggregation (slight increase in particle size), while the emulsion without ε-polylysine exhibited much more extensive aggregation. The presence of the cationic polypeptide therefore appeared to inhibit droplet aggregation under gastric conditions. The physicochemical origin of this effect is currently unknown. The ε-polylysine may have adsorbed to the lipid droplet surfaces and formed a protective coating, or it may have interacted with the pepsin thereby limiting its ability to interact with the oil droplets. Although a physical interaction between ε-polylysine and pepsin is likely, it is unlikely that pepsin would exert any enzymatic activity, as it has been reported that the peptide

bonds in ε-polylysine make are resistant to hydrolysis by gastric enzymes (Tsujita, Takaichi, Takaku, Aoyama, & Hiraki, 2006).

*With mucin*: The samples containing mucin exhibited a decrease in particle size when they moved from the oral phase (where they were highly aggregated) to the gastric phase, although the mean particle diameters were still larger than those in the original emulsions (**Figure 24**). For example, the mean particle diameter of the emulsions without ε-polylysine decreased to around 2.6 μm after exposure to the simulated gastric fluids, while that of the emulsion containing ε-polylysine decreased to around 4.4 μm. Confocal microscopy also showed that the large aggregates formed in the mouth phase due to the interaction of the oil droplets with mucin were dissociated somewhat after exposure to the gastric phase (**Figure 25c** and **Figure 26g**). A number of possible physicochemical factors may contribute to the dissociation of these aggregates. First, the samples were diluted and stirred during the simulated gastric phase, which may have promoted some dissociation. Second, there was a change in pH and ionic strength when the oil droplets moved from the mouth to the stomach, which may have altered any electrostatic interactions in the emulsions. Third, there may have been some proteolysis of the mucin molecules by pepsin in the stomach, however, this effect is likely to be small since the high glycosylation of mucin confers some resistance to protease activity (Perez-Vilar & Hill, 2004).

#### 5.4.1.3 Small Intestine Phase

After exposure to the simulated intestinal phase, all the samples exhibited an appreciable increase in mean particle size, with those containing ε-polylysine exhibiting the largest increase (**Figure 24**).

*Without Mucin:* In the absence of mucin, the emulsions without ε-polylysine increased to around 16 μm after exposure to simulated intestinal conditions, whereas the ones with ε-polylysine increased to around 32 μm. The impact of the cationic biopolymer on the microstructure of the emulsions was confirmed by confocal microscopy: larger aggregates were present in the samples containing ε-polylysine (**Figure 26h**) than in those without ε-polylysine (**Figure 26g**). Optical microscopy images also showed that large aggregates were formed in the presence of ε-polylysine (**Figure 27**). These results suggest that the ε-polylysine promoted the formation of large aggregates within the digesta formed due to lipid digestion. A number of different types of particles may have been present in this digesta, including undigested oil droplets, micelles, vesicles, calcium salts of fatty acids, and insoluble biopolymer complexes. Presumably, the cationic polypeptide was able to electrostatically interact with anionic molecular species in the digesta, such as bile salts, phospholipids, free fatty acids, and proteins, thereby promoting their aggregation.

*With Mucin*: The presence of mucin in the emulsions had an appreciable impact on the microstructure of the digested emulsions. The light scattering measurements indicated that the mean particle diameter of the digesta was around 9 μm without ε-polylysine and around 32 μm with it. These results were supported

by the confocal microscopy measurements, which also showed that much larger aggregates were observed when ε-polylysine was present (**Figure 25h**) than when it was absent (**Figure 25d**). Optical microscopy images also confirmed that large aggregates were formed in the presence of ε-polylysine (**Figure 27**). Thus, the presence of the cationic polypeptide appeared to promote aggregation in both the presence and absence of mucin.

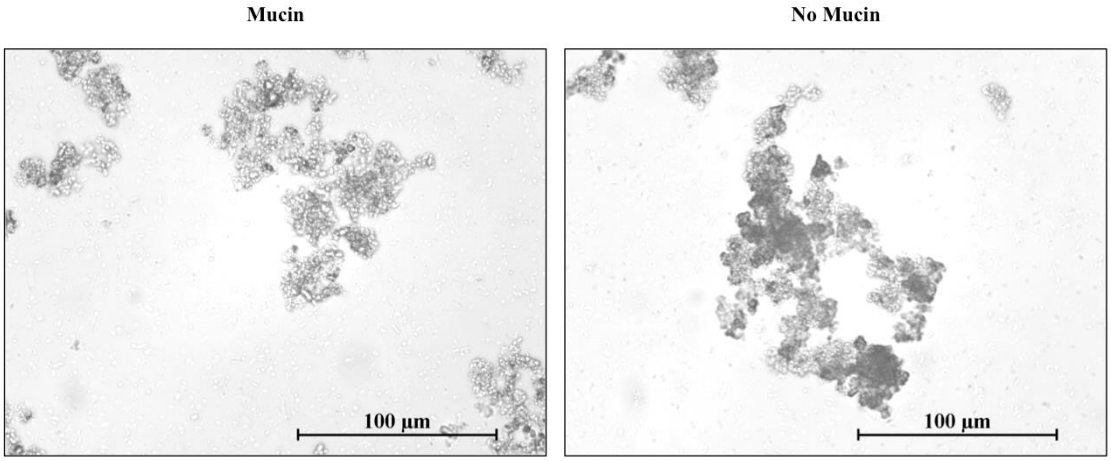


**Figure 27. Optical microscopy of the intestinal phase of the *in vitro* digestion samples containing ε-polylysine, with and without the addition of mucin in the mouth phase[28].**

Comparison of the digested samples in the absence and presence of mucin (**Figure 24**, **Figure 25**, and **Figure 26**), suggest that mucin was able to partially suppress the formation of large aggregates in the small intestine phase. The precise physicochemical origin of this effect is unknown, but it may be due to the interactions of the mucin with various components involved in the lipid digestion

[28] (Lopez-Pena, Zheng, Sela, Xiao, Decker, and McClements, 2015)

process, including lipase, bile salts, phospholipids, free fatty acids, calcium ions, and polylysine. Further studies are required to elucidate the potential role of mucin within the small intestine.

### 5.4.2 Micro-Electrophoresis Measurements

In this section, we characterized changes in the electrical characteristics of the emulsions with and without ε-polylysine as they passed through the various stages of the simulated gastrointestinal tract (**Figure 28**). The initial control emulsion had a slightly negative charge (≈ -9.1 mV), which can be attributed to anionic impurities in the surfactant or oil phase, since the droplets were stabilized by a non-ionic surfactant. The addition of ε-polylysine to the control emulsion caused the charge to change from around -9.1 mV to around +1.3 mV. This change in electrical characteristics could have been caused by two phenomenon: (i) adsorption of cationic ε-polylysine molecules to the surfaces of the anionic oil droplets: (ii) contribution of the cationic ε-polylysine molecules to the light scattering signal detected by the particle electrophoresis instrument. The particle size measurements indicated that the emulsions were stable to aggregation after addition of ε-polylysine (**Figure 24**), which suggests that if any biopolymer adsorption did occur, then it did not promote bridging flocculation.

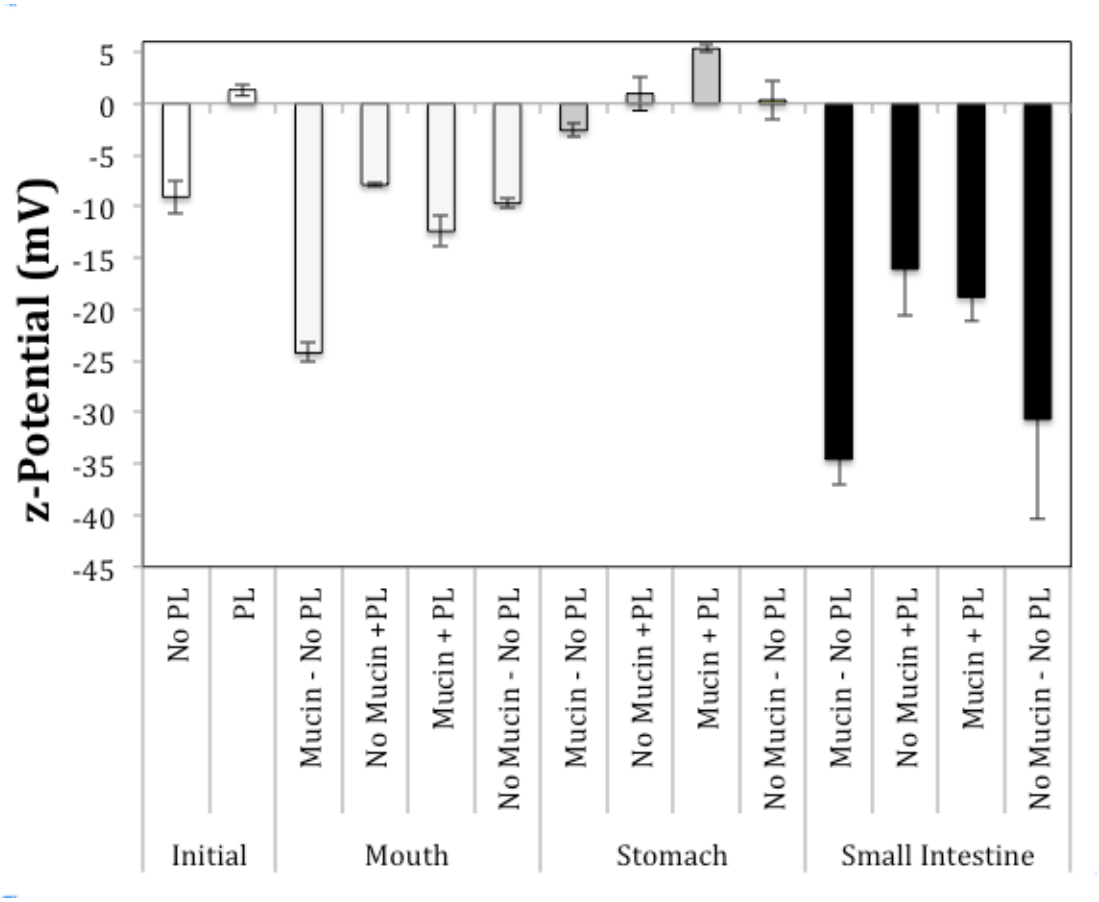


**Figure 28. ζ-Potential (mV) of the original nanoemulsions with and without ε-polylysine prior to digestion and after each stage of digestion, with and without mucin[29].**

### 5.4.2.1 Oral Phase

The presence of mucin and ε-polylysine influenced the electrical characteristics of the oil droplets after exposure to the simulated oral fluids (**Figure 28**). The control emulsions (no mucin or ε-polylysine) had fairly similar charges to the original emulsions because the pH was similar (around neutral). In the absence of mucin, the emulsions containing ε-polylysine had a slightly lower negative charge

[29] (Lopez-Pena, Zheng, Sela, Xiao, Decker, and McClements, 2015)

than those without ε-polylysine. However, the net charge was no longer positive (as in the initial emulsions containing ε-polylysine), which suggests that the higher ionic strength of the simulated oral fluids may have promoted desorption of the cationic biopolymer from the droplet surfaces. The incorporation of mucin led to a considerable decrease in the particle charge as compared to the samples with no mucin. For example, in the absence of ε-polylysine the □-potentials were -24.0 and -9.7 mV with and without mucin, respectively, while in the presence of ε-polylysine they were -12.4 and -7.9 mV, respectively. The negatively charged mucin molecules may have contributed to the overall charge characteristics of the systems due to their ability to adsorb to droplet surfaces or form colloidal particles in the surrounding aqueous phase.

#### 5.4.2.2 Gastric Phase

After exposure to the simulated gastric fluids, there was an appreciable change in the electrical characteristics of all the samples (**Figure 28**). In the absence of mucin the □-potential became +0.96 and +0.30 mV for the systems with and without ε-polylysine respectively, whereas in the presence of mucin the equivalent values were +5.3 and -2.6 mV. The change in the particle charge of the samples when they moved from the mouth to the stomach phase is likely due to the low pH conditions causing protonation of certain ionizable groups (*e.g.,* -COOH or $-NH_3^+$). The systems containing ε-polylysine had a higher surface charge (more positive or less negative) than their counterparts containing no ε-polylysine, illustrating the strong effect that this cationic biopolymer contributes.

#### 5.4.2.3 Small Intestine Phase

The electrical charge on all of the samples became strongly negative after exposure to the small intestine conditions, but the magnitude of the negative charge depended on system composition (**Figure 28**). In the absence of mucin, the ζ-potential was -16.1 and -30. 7 mV with and without ε-polylysine, respectively, while in the presence of mucin it was -18.8 and -34.6 mV, respectively. The overall negative charge on the particles in the different samples after exposure to small intestine conditions can be attributed to a number of factors. First, the neutral pH conditions will promote deprotonation of many kinds of charged groups (*e.g.,* $-COO^-$ and $-NH_2$). Second, the addition of anionic bile salts and phospholipids will contribute to the overall negative charge. Third, the formation of anionic free fatty acids due to the digestion of the triacylglycerols will also contribute to the negative charge. The difference in the charges between samples can be attributed to the influence of the cationic ε-polylysine and anionic mucin on the electrical characteristics of the particles present. Samples containing ε-polylysine had a lower negative charge than the ones without ε-polylysine, which can be attributed to the positive charge associated with this biopolymer. Interestingly, the samples containing mucin had fairly similar charges to the ones containing no mucin, which suggests that the mucin did not make a major contribution to the overall charge characteristics.

### 5.4.3 *In Vitro* Digestion

The main objective of this section was to assess the impact of mucin and ε-polylysine on lipid digestion using an *in vitro* digestion method (pH stat).

Surprisingly, the free fatty acid (FFA) release profiles during lipid digestion very fairly similar for all of the systems studied (**Figure 29** and **Figure 30**). The presence of mucin and ε-polylysine in the emulsions therefore did not appear to have a major

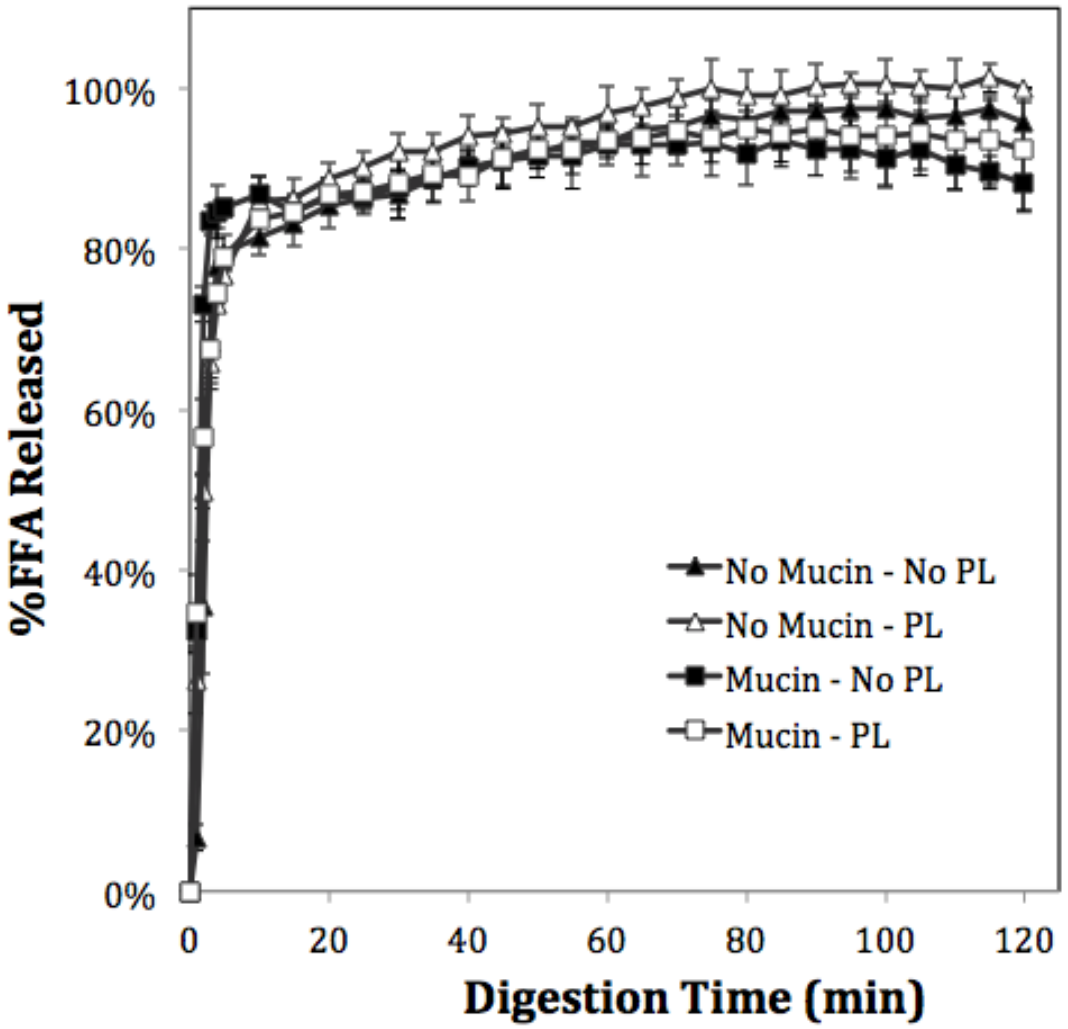


**Figure 29. Influence of the presence of mucin and addition of ε-PL on the percentage of free fatty acids (FFA%) released from nanoemulsions formulated with corn oil and Tween 20. The final lipid content in the digestion medium was 0.5%[30].**

[30] (Lopez-Pena, Zheng, Sela, Xiao, Decker, and McClements, 2015)

influence on their lipid digestion, even though they did influence their microstructure and electrical properties during passage through the simulated GIT. These results are different from those previously reported in the literature, where it was shown that polylysine may inhibit lipid digestion or absorption (Kido, et al., 2003; Cynthia Lyliam Lopez-Pena & David Julian McClements, 2015; Tsujita, Sumiyoshi, Takaku, Momsen, Lowe, & Brockman, 2003; Tsujita, Takaichi, Takaku, Aoyama, & Hiraki, 2006). There may be a number of reasons for this difference. First, considerably higher ε-polylysine concentrations were utilized in many of the previous studies that have reported that this cationic biopolymer can inhibit digestion. Second, different types of lipid-based delivery samples were tested (*e.g.,* oil and emulsifier types), which may have impacted the ability of the ε-polylysine to interact with the lipid droplets. Third, different analytical methods were used in different studies to monitor the effects of polylysine on lipid digestion. Fourth, ε-polylysine may demonstrate an anti-obesity effect due to its ability to inhibit fatty acid absorption, rather than lipid digestion.

Despite the fact that we did not observe a significant difference ($p < 0.05$) in lipid digestion amongst the samples, we did notice some interesting differences in their physical appearance after digestion (**Figure 30**). The oil phase of these emulsions initially contained a highly lipophilic red dye (Nile Red), which therefore enables one to determine the location of the lipids after digestion. Visual observation of the samples after digestion indicated that there were appreciable differences in their color, turbidity, and sedimentation. The samples without ε-polylysine (**Figure 30B** and **C**) had an aqueous phase that was a brighter shade of


**Figure 30. Appearance of emulsion samples with and without ε-polylysine prior to digestion (A and F, respectively) and samples after undergoing full digestion with mucin and no ε-polylysine (B), no mucin and no ε-polylysine (C), mucin and ε-polylysine (D), and no mucin with ε-polylysine (E)[31].**

pink (corresponding to a higher lipid content), were more turbid, and formed a white sediment at the bottom of the tubes. Conversely, the samples with ε-polylysine (**Figure 30D** and **E**) had a more translucent aqueous phase, had a thin ring of oil on their surface, and formed a bright pink sediment. The difference between the samples with and without mucin was mainly associated with the amount of precipitate formed. Samples containing mucin (**Figure 30B** and **D**) had a smaller amount of sediment than those without mucin (**Figure 30C** and **E**). These results suggest that the inclusion of ε-polylysine might not prevent lipid digestion by lipase, but that it may inhibit lipid absorption in the small intestine by interacting electrostatically with bile salts and forming insoluble aggregates that sediment. The

[31] (Lopez-Pena, Zheng, Sela, Xiao, Decker, and McClements, 2015)

thin layer of oil on the surface of samples containing ε-polylysine suggests that it may have promoted droplet coalescence, while the bright pink sediment in these samples suggests that the anionic mixed micelles may have formed electrostatic complexes with the cationic ε-polylysine, making them unavailable for absorption.

## 5.5 Conclusions

Overall, our results suggest that both mucin and ε-polylysine alter the microstructure and electrical characteristics of emulsified lipids under simulated gastrointestinal conditions. However, they do not appear to have a major influence on the rate and extent of lipid digestion by lipase. Visual observation of the distribution of an oil-soluble dye in the samples after digestion suggests that the bioavailability of any encapsulated lipophilic agents may be altered by the presence of polylysine. Nevertheless, further work is required to determine if this effect is important for food-grade bioactive components, such as oil-soluble vitamins and nutraceuticals.

## 5.6 Acknowledgments

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# CHAPTER 6

# POTENTIAL IMPACT OF BIOPOLYMERS (ε–POLYLYSINE AND/OR PECTIN) ON GASTROINTESTINAL FATE OF FOODS: *IN VITRO* STUDY

## 6.1 Abstract

Food-grade biopolymers, such as proteins and polysaccharides, may impact the gastrointestinal fate of foods through various mechanisms. In this study, we examined the influence of ε-polylysine (an antimicrobial) and pectin (a thickening agent) on the behavior of animal feed (full-fat and fat-free) in a simulated gastrointestinal tract that included mouth, stomach, and small intestine phases. Powdered biopolymers were incorporated into the animal feed in either individual or complexed form. The presence of the biopolymers altered the microstructure and charge characteristics of the gastrointestinal contents. In particular, the presence of pectin appeared to increase the rate and extent of lipid digestion, which may have been due to its ability to inhibit extensive protein aggregation. Our results do not support the hypothesis that polylysine inhibits lipid digestion, as has been reported previously. Overall, the results of this study may be useful for interpreting animal feeding studies of the influence of biopolymers on the gastrointestinal fate of foods.

*Keywords*: Gastrointestinal; Polylysine; Pectin; In vitro study; Digestion

## 6.2 Introduction

Foodborne diseases are a major cause of illness and death in the USA, and lead to substantial economic losses (Scharff, 2012). Consequently, the development of effective antimicrobial treatments to control pathogenic and spoilage organisms is a major concern of the food industry (Jay, 2005). These antimicrobials undergo extensive examination by the Food and Drug Administration and must be approved as Generally Regarded As Safe (GRAS) before they can be utilized by the food industry (FDA, 2009). The requirements for this status rely on a science-based system (Alger, Maffini, Kulkarni, Bongard, & Neltner, 2013), which defines safety as "[having] a reasonable certainty in the minds of competent scientists that [a] substance is not harmful under the intended conditions of use" (FDA, 2015). The most critical documentation presented to the FDA to assess the safety of food additives are toxicology studies, such as short-term toxicity tests, genetic toxicity tests, subchronic toxicity studies, chronic toxicity, carcinogenicity studies, reproduction studies, developmental toxicity studies, and metabolism and pharmacokinetic studies often conducted on rodents (FDA, 2014).

Many consumers are concerned about the utilization of synthetic preservatives in food products, which has stimulated research into the development of effective, safe, all-natural antimicrobials (Gyawali & Ibrahim, 2014; Tajkarimi, Ibrahim, & Cliver, 2010). ε-Polylysine (ε-PL) is a GRAS natural antimicrobial that is effective against various Gram (+) and Gram (-) bacteria, yeasts, molds, and even some bacteriophages (S. S. Chang, Lu, Park, & Kang, 2010; FDA, 2011; Geornaras, Yoon, Belk, Smith, & Sofos, 2007; Shima, Matsuoka, Iwamoto, & Sakai, 1984; Yoshida

& Nagasawa, 2003), surpassing the antimicrobial range of more popular preservatives (FDA, 2011; Jay, 2005). However, this biopolymer's highly cationic nature limits its widespread implementation in food products, as it has a high propensity to interact with anionic components and promote precipitation and sedimentation in food systems (Y. H. Chang, L. McLandsborough, & D. J. McClements, 2011). Additionally, polylysine has been reported to have a bitter and astringent mouthfeel, which limits its application in many foods (Kido, et al., 2003).

Previous work has shown that electrostatic interactions between cationic ε-PL and anionic pectin generates a negatively-charged complex that maintains the antimicrobial properties of ε-PL whilst minimizing any undesirable interactions with other anionic components that might be present in food matrices (Y. Chang, L. McLandsborough, & D. J. McClements, 2011a; Y. H. Chang, L. McLandsborough, & D. J. McClements, 2011; C. L. Lopez-Pena & McClements, 2014). These antimicrobial complexes have been shown to perform successfully in model food systems (Y. Chang, McLandsborough, & McClements, 2012), and are therefore promising all-natural alternatives to synthetic antimicrobials currently used. Nevertheless, it is important that toxicological studies are conducted to assess the safety of these complexes prior to their incorporation in food systems. For example, it is possible that these antimicrobial complexes may alter the normal digestion of macronutrients, or that they may reach the colon and alter the microbial microflora.

The objective of this study was therefore to determine the potential influence of ε-PL-pectin complexes on the gastrointestinal fate of foods, and on the composition of the colonic microflora. This aim will be achieved through the

implementation of a combination of *in vitro* and *in in vivo* studies, focusing on macronutrient digestion, metabolic markers in blood, abnormalities in body and organ weight, and impact on the gut microbiome. In the current portion of the study, our objective was to obtain an understanding of the potential influence of the ε-PL-pectin complexes on the gastrointestinal fate of foods using an in vitro digestion model. This work was carried out because previous studies have shown that polylysine may interfere with lipid digestion (Kido, et al., 2003). Previous studies have usually used simple model systems to pass through simulated gastrointestinal tracts. In this study, we utilized the same powdered samples that were used in the animal feeding studies so that the results of this *in vitro* study could be directly compared with the results of *in vivo* feeding studies.

## 6.3 Materials And Methods

Maltodextrin DE 18 (Maltrin® M180) was provided by Grain Processing Corporation (Muscatine, IA); ε-polylysine was obtained from Wilshire Technologies, Inc.; high-methoxyl pectin was donated by TIC Gums (White Marsh, MD); high-fat mixed lipid diet (Dyet #180605) and its fat-free equivalent were acquired from Dyets, Inc. (Bethlehem, PA).

### 6.3.1 Powder Production And Characterization

In this section, the methods used to convert biopolymer solutions into powders that could be incorporated into animal feed are described.

#### 6.3.1.1 Liquid Feed Preparation

Four different solutions were prepared to be subjected to spray drying: 20% w/w maltodextrin; 0.1% w/w maltodextrin - 1% w/w pectin (MR 1:10); 0.1% w/w maltodextrin – 1% w/w pectin – 0.05% w/w ε-polylysine (MR 10:20:1); and 20% w/w maltodextrin – 2% w/w ε-polylysine (MR 10:1). The maltodextrin and ε-polylysine solutions were prepared by dispersing the corresponding amounts of reagents in double distilled water. Solutions of HCl and NaOH at varying concentrations were used to adjust the pH to a final value of 3.5. Pectin stock solutions (2% w/w) were prepared by dispersing powdered high methoxyl pectin into hot double-distilled water, and then stirring at 550 rpm under heated conditions (37 °C) for at least 3 hours. The solution was then allowed to stir overnight at room temperature to ensure full dispersion, adjusted to pH 3.5, and brought to the adequate volume the next day. Corresponding volumes of the maltodextrin or maltodextrin-ε-polylysine solutions were combined with the pectin stock solution, and thoroughly stirred to ensure homogeneity.

#### 6.3.1.2 Spray Drying Conditions

The liquid feeds were subjected to spray drying using two different spray dryers. Maltodextrin (MD), maltodextrin-ε-polylysine (MD+PL), and some of the maltodextrin-pectin (MD+P) and maltodextrin-pectin-ε-polylysine (MD+P+PL) were processed in a Büchi Mini Spray Dryer B-290 (Büchi Laboratorium-Tecnik, Flawil, Switzerland) under the following experimental conditions: inlet temperature 120 °C, outlet temperature 67-72 °C, Q-flow 40, pump flow 30% (spray flow feed rate 9 ml/min), aspirator 100%. Due to the large quantities of feed that needed to be

processed, large volumes of the solutions containing pectin were processed using a Niro Atomizer Versatile Utility Spray Dryer (NGEA Process Engineering A/S, Søborg, Denmark). The inlet temperature was 120 °C, and the flow rate 5.55 l/hr. Samples were stored in a desiccator after production.

#### 6.3.1.3 Powders Characterization

Prior to use, the moisture content of the powders was determined following the standard gravimetric method described by the International Dairy Federation (1993) and the (FAO, 1997). The procedure consisted of weighing 1 to 3 g of the sample into previously dried aluminum capsules, and storing the sample in an oven at 102 ± 2 °C for 2 hours. The samples were stored in a desiccator for 30 min to allow them to cool, and were then weighed. The samples were then returned to the oven for 1 hour for further drying, and weighed after spending 30 min in the desiccator. This process was repeated until the difference between measurements was 0.5 mg or less. The moisture content (% w/w) was obtained through the following formula:

$$\%Moisture = \frac{100 \times (M_1 - M_2)}{M_1 - M}$$

where:

$M$ is the mass of the empty capsule (g); $M_1$ is the initial mass of the capsule with the sample (g); $M_2$ is the final mass of the capsule with sample after drying (g).

### 6.3.2 *In Vitro* Digestions

*In vitro* digestions including oral, gastric, and intestinal simulations were adapted from the method described by Y. Li, Hu, and McClements (2011) and C.L.

Lopez-Pena and D.J. McClements (2015), with some modifications. Stock solutions for all phases three were prepared with the specifications described by C.L. Lopez-Pena and D.J. McClements (2015): (i) artificial saliva containing sodium chloride (Fisher Scientific, Hampton, New Hampshire), ammonium nitrate (Sigma Aldrich, St. Louis, Missouri), potassium phosphate monobasic (Fisher Scientific, Hampton, New Hampshire), potassium chloride (Fisher Scientific, Hampton, New Hampshire), potassium citrate (Sigma Aldrich, St. Louis Missouri), uric acid sodium salt (Sigma Aldrich, St. Louis, Missouri), Urea (Fluka, St. Louis, Missouri), and lactic acid sodium salt (Sigma Aldrich, St. Louis, Missouri) dissolved in double distilled water; (ii) simulated gastric fluids composed of sodium chloride (Fisher Scientific, Hampton, New Hampshire) and hydrochloric acid (Sigma Aldrich, St. Louis, Missouri) dissolved in distilled water; and (iii) simulated intestinal juices comprised of calcium chloride (Fisher Scientific, Hampton, New Hampshire) and sodium chloride (Sigma Aldrich, St. Louis, Missouri) dissolved in double distilled water. The mouth phase required that a mucin solution be prepared by dispersing 0.6 g mucin Type II (Sigma Aldrich, St. Louis, Missouri) in 20 ml artificial saliva stock solution, stirred overnight. For the gastric phase, 0.064 g pepsin (Sigma Aldrich, St. Louis, Missouri) were dissolved in 20 ml simulated gastric fluids, stirred for 30 minutes and used immediately after production. For the intestinal phase, 0.1875 g bile salts (Sigma Aldrich, St. Louis, Missouri) was stirred overnight in 4 ml 5mM phosphate buffer [sodium phosphate monobasic (Sigma Aldrich, St. Louis, Missouri) and sodium phosphate dibasic (Thermo Fisher Scientific, Waltham, MA)] pH 7; and 0.06 g lipase

(Sigma Aldrich, St. Louis, Missouri) was dispersed in 5 mM phosphate buffer pH 7, stirring for 30 minutes and using immediately after production.

Prior to conducting *in vitro* digestions, adequate amounts of spray-dried powders (0.052 g MD, 0.061 g MD+PL, 0.170 g MD+P, or 0.176 g MD+P+PL) were combined with 4.04 g full fat animal feed or 3.24 g fat-free animal feed and 20 ml 5 mM phosphate buffer (pH 7), and stirred in a water bath at 37 °C for at least 1 hour. The powders containing pectin (MD+P and MD+P+PL) were allowed to stir on a hot plate with the surface heated to 37 °C overnight prior to mixing with the animal feed. **Table 2** presents the composition of the animal feed utilized, as reported by Dyets, Inc.

As described by C.L. Lopez-Pena and D.J. McClements (2015), the mouth phase of the *in vitro* digestion was initiated by mixing 20 ml of the prepared powder/feed solution sample with 20 ml mucin solution, adjusting the pH was adjusted to 6.8 utilizing solutions of hydrochloric acid and/or sodium hydroxide at different concentrations. The resulting solution was placed in a shaking incubator (Excella E24 Incubator Shaker Series, New Brunswick Scientific, Enfield, CT) at 37 °C, 100 rpm for 10 minutes to simulate oral digestion. Upon completion of the first stage, 20 ml of the oral digesta was combined with 20 ml fresh pepsin solution and the pH was adjusted to 2.5. The solution was reintroduced in the shaking incubator for 2 hours.

The small intestine phase focuses on quantifying the digestion of any triacylglycerols present in the sample by the action of lipase. In order to achieve

**Table 2. Composition of animal feed Dyet #180605 provided by Dyets, Inc. (Bethlehem, PA)**

| Component | %w/w | Kcal/kg |
|---|---|---|
| Casein | 23.5 | 841.3 |
| Cornstarch | 35.7 | 1285.2 |
| Dextrose | 9.02 | 328.33 |
| DL-Methionine | 0.35 | 14 |
| Cellulose | 5.9 | 0 |
| Lipids[32] | 20 | 1800 |
| Beef tallow | 3.2 | |
| Lard | 2 | |
| Anhydrous Milkfat | 2.4 | |
| Soybean Oil | 6 | |
| Peanut Oil | 1 | |
| Corn Oil | 5.4 | |
| Mineral Mix #200000 | 4.11 | 19.32 |
| Vitamin Mix $300050 | 1.18 | 46.26 |
| Choline Bitartrate | 0.24 | 0 |
| | | |
| TOTAL | 100 | 4334.40 |

[32] (Reddy, 2006)

this, an automated pH Stat titration method (857 Titrando, Metrohm USA, Riverview, Florida)was utilized: as the sample is exposed to bile salts, simulated intestinal juices, and lipase, free fatty acids (FFA) are released from triacylglycerols contained in the lipidic portion of the sample. Whilst FFAs are released and generate a decrease in pH, the instrument identifies any drops in pH and automatically titrates sodium hydroxide at a specific concentration into the vessel containing the sample, maintaining the pH at 7. The instrument records the amount of sodium hydroxide titrated throughout the duration of the digestion, and the percentage of FFA released can be calculated utilizing the equation below (Y. Li, Hu, & McClements, 2011):

$$\%FFA = 100 \times \frac{V_{NaOH} \times m_{NaOH} \times m_{lipid}}{w_{lipid} \times 2}$$

Where %FFA is the percentage of released FFAs; $V_{NaOH}$ is the volume of titrant in liters; $m_{NaOH}$ is the molarity of the sodium hydroxide solution used; $M_{lipid}$ is the molecular weight of the oil used; and $w_{lipid}$ is the weight of the oil in the digestion system in grams. Due to the complexity of the lipid phase, we estimated the %FFA released based on the molecular weight of corn oil.

#### 6.3.2.1 Particle Characterization

All samples before and after each stage of digestion were characterized by measuring particle size (Malvern Mastersizer 2000, Malvern Instruments, Worcestershier, United Kingdom) and □-potential (Malvern Zetasizer ZS, Malvern Instruments, Worcestershire, United Kingdom), as well as by conducting optical and confocal microscopy (Nikon D-Eclipse C1 80i, Nikon, Melville, NY). Samples were

maintained at approximately 37 °C between measurements to avoid any changes in structure due to the potential solidification of the fat phase, and measurements were conducted at approximately 37 °C. The refractive index of the liquid fat was determined to be 1.471 utilizing a refractometer (Abbe 3L, Bausch & Lomb, Rochester, NY). This refractive index was used to determine particle size and ⍰-potential.

### 6.3.3 Data Analysis

All experiments were performed at least in triplicate. Statistical significance between means were determined through analysis of variance (ANOVA) following Tukey pairwise comparisons with a confidence interval of 95% with Minitab® 17.1.0 (Minitab Inc., State College, Pennsylvania).

## 6.4 Results And Discussion

The main purpose of this study was to understand the potential gastrointestinal fate of antimicrobial biopolymer complexes. We therefore tested the behavior of four samples: Control (maltodextrin only); Electrostatic complex (polylysine, pectin, and maltodextrin); Polylysine (polylysine and maltodextrin); Pectin (pectin and maltodextrin). Experiments were carried out using a regular animal feed (full fat), and the same system without fat, so as to determine the role of the biopolymer complexes on lipid digestion.

### 6.4.1 Sample Preparation And Characterization

The amount of each biopolymer powder to be added to the animal feed was calculated based on the estimated annual average consumption of soft drinks in the

United States per capita (Mintel, 2014), considering that carbonated beverages would contain 0.025 %w/w ε-polylysine (FDA, 2011), and therefore 0.5 %w/w high methoxyl pectin (Y. Chang, L. McLandsborough, & D. J. McClements, 2011a; Y. Chang, McLandsborough, & McClements, 2012; Y. H. Chang, L. McLandsborough, & D. J. McClements, 2011; C. L. Lopez-Pena & McClements, 2014). Soft drinks were selected because this is one of the applications where polylysine may find utilization as an antimicrobial agent. The daily dosage (g/kg body weight) of the powdered samples fed to the mice was based on the average body weight of Americans (U.S. Department of Health and Human Services, 2008), which was in turn used to calculate the exposure levels for the mice used in a subchronic toxicology analysis for 13 weeks. The amounts utilized for this experiment correspond to the amount of each treatment powder added during the last week of the *in vivo* portion of the toxicology analysis.

Due to possible differences in water content generated by variations during processing as well as due to the diverse water-retention capabilities of the components of the powders, it was necessary to analyze the powders' moisture content prior to use. The amounts to be incorporated were then determined on a dry weight basis of the powders. The samples containing pectin had a slightly higher moisture content (4.0%) than those without pectin (3.6%) (**Table 3**). This information was taken into account when calculating the amount of biopolymers added to each diet.

**Table 3. Moisture content of the produced powders for week 13.**

| Powder Composition | Moisture Content (w/w%) |
|---|---|
| MD | 3.61 ± 1.12% |
| MD+PL | 3.60 ± 0.28% |
| MD+P | 4.12 ±0.16% |
| MD+P+PL | 3.97 ±0.16% |

### 6.4.2 Particle Size Analysis, Micro-Electrophoresis, And Confocal Microscopy

The particle size and surface charge of samples containing different types of biopolymers (MD, MD+PL, MD+P, and MD+P+PL) were analyzed before and after each stage of the *in vitro* digestion. Experiments were carried out using animal feed that contained fat ("full-fat") and no-fat ("fat-free) to determine the influence of the biopolymers on lipid digestion and gastrointestinal fate.

#### 6.4.2.1 Initial Conditions

Initially, both the full-fat ($d_{43}$ = 35 to 50 μm) and fat-free ($d_{43}$ = 88 to 152 μm) animal feed contained relatively large particles for all samples (**Figure 31** and **Figure 32**). The optical and confocal fluorescence microscopy images also indicated that these samples contained large particles of various sizes and shapes (**Figure** 33 and **Figure 34**). Based on the known compositions of the animal feeds, these particles were probably cellulose fibers, starch granules, protein aggregates, and fat globules. It is possible that cationic polylysine promoted aggregation of anionic components within the system (C. L. Lopez-Pena & McClements, 2014), while

anionic pectin promoted aggregation of cationic components (Matalanis & McClements, 2012). Nevertheless, it is difficult to unambiguously determine the role of different components in the system from the light scattering measurements or microscopy images. The lower mean particle size in the full-fat systems suggest that the fat may have been able to inhibit some particle aggregation. The full-fat systems all had relatively high anionic surface charges (-21 to -28 mV) prior to

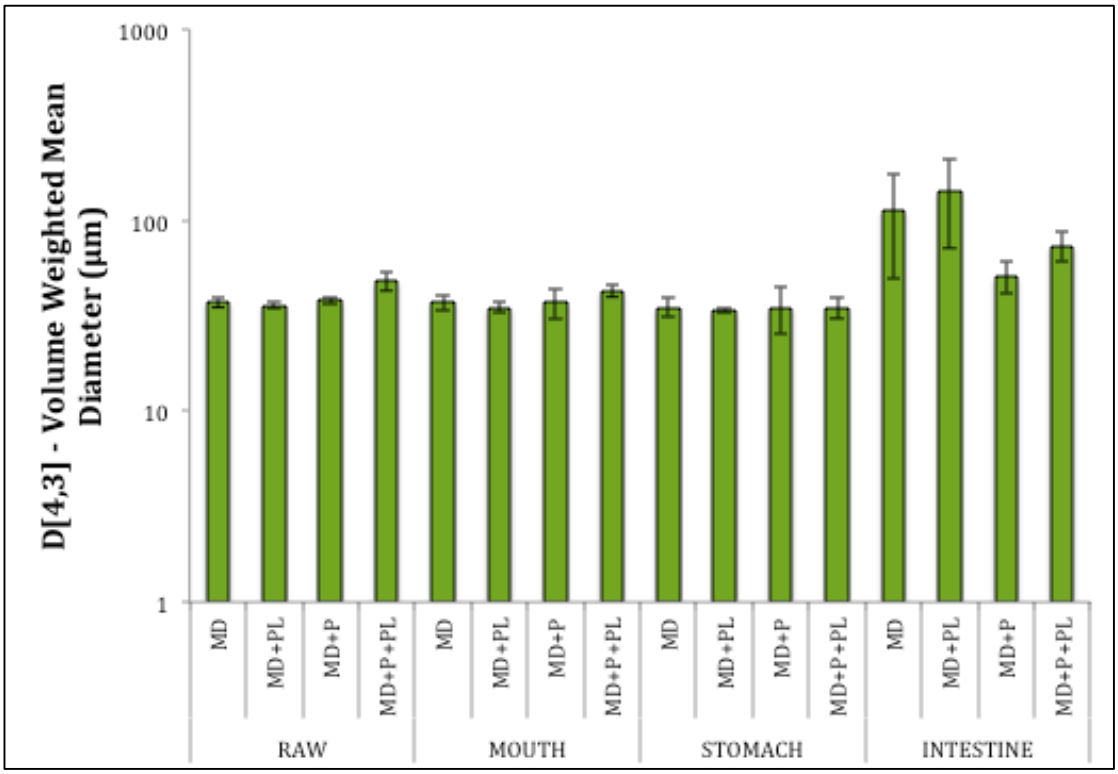


**Figure 31. Influence of addition of pectin (P) and/or ε-polylysine (PL) on particle size of fat-free animal feed containing casein, maltodextrin, cornstarch, dextrose, DL-methionine, cellulose, vitamins, minerals, choline bitartrate, beef tallow, lard, anhydrous milk fat, soybean oil, peanut oil, and corn oil during *in vitro* digestion[33].**

[33] (Lopez-Pena, Song, Xiao, and McClements, 2015)

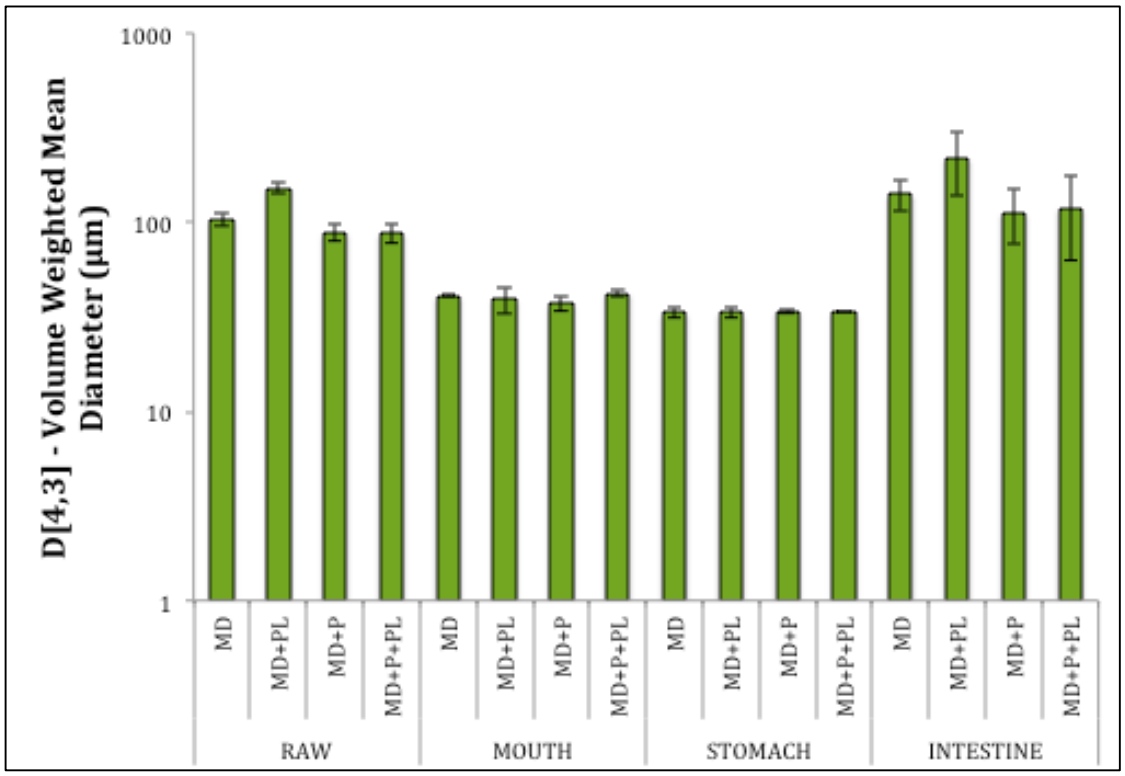


**Figure 32. Influence of addition of pectin (P) and/or ε-polylysine (PL) on particle size of fat-free animal feed containing casein, maltodextrin, cornstarch, dextrose, DL-methionine, cellulose, vitamins, minerals, and choline bitartrate during *in vitro* digestion[33].**

digestion (**Figure 36**). The fat-free systems (**Figure 37**) were also negatively charged, but the magnitude of the charge was smaller than the full-fat systems (-16 to -19 mV). This may have been because protein-coated fat droplets in the full-fat systems made a large contribution to the overall light scattering signal used to measure the ζ-potential. The addition of polylysine alone (which should be positively charged) did not have a major impact on the overall charge characteristics of the samples, which may have been because of the relatively high concentration of anionic species in the animal feed (*e.g.,* protein).

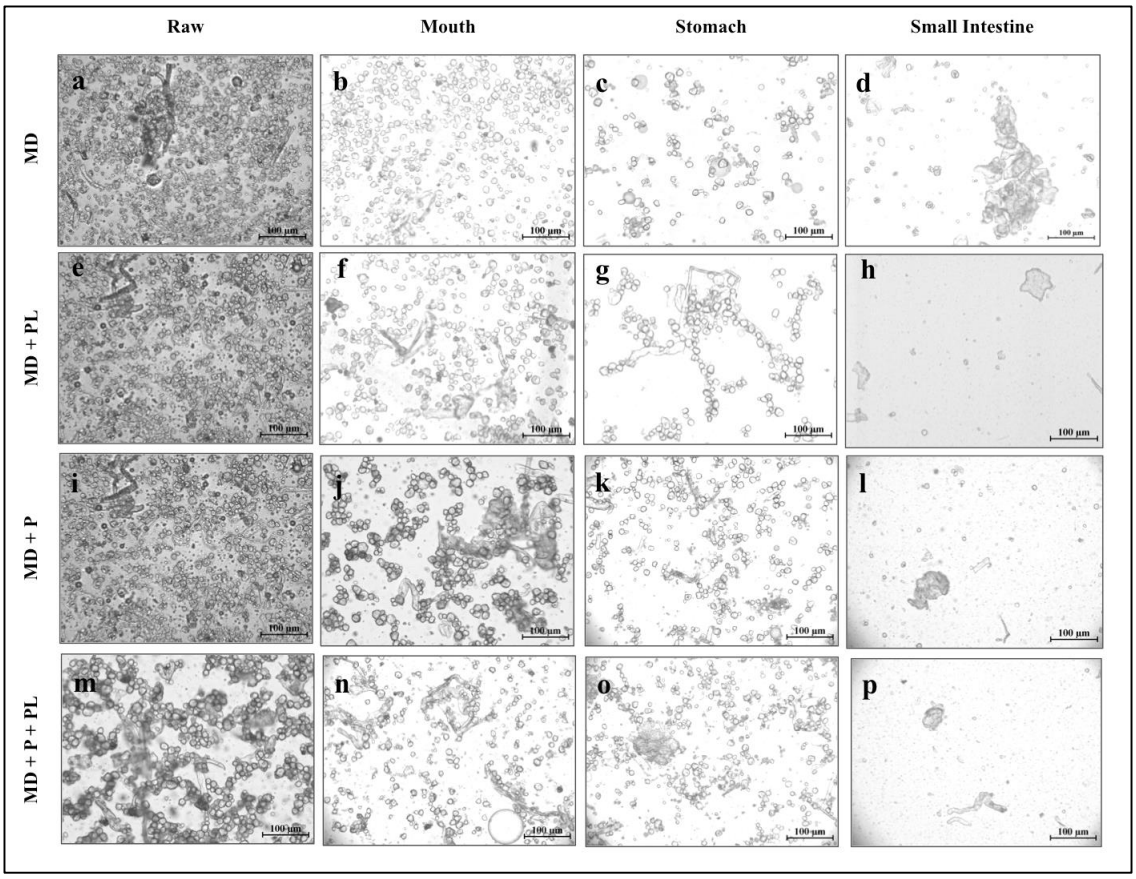


**Figure 33. Optical microscopy demonstrating the influence of addition of pectin (P) and/or ε-polylysine (PL) on particle structure of full-fat animal feed containing casein, maltodextrin, cornstarch, dextrose, DL-methionine, cellulose, vitamins, minerals, choline bitartrate, beef tallow, lard, anhydrous milk fat, soybean oil, peanut oil, and corn oil during *in vitro* digestion[34].**

[34] (Lopez-Pena, Song, Xiao, and McClements, 2015)

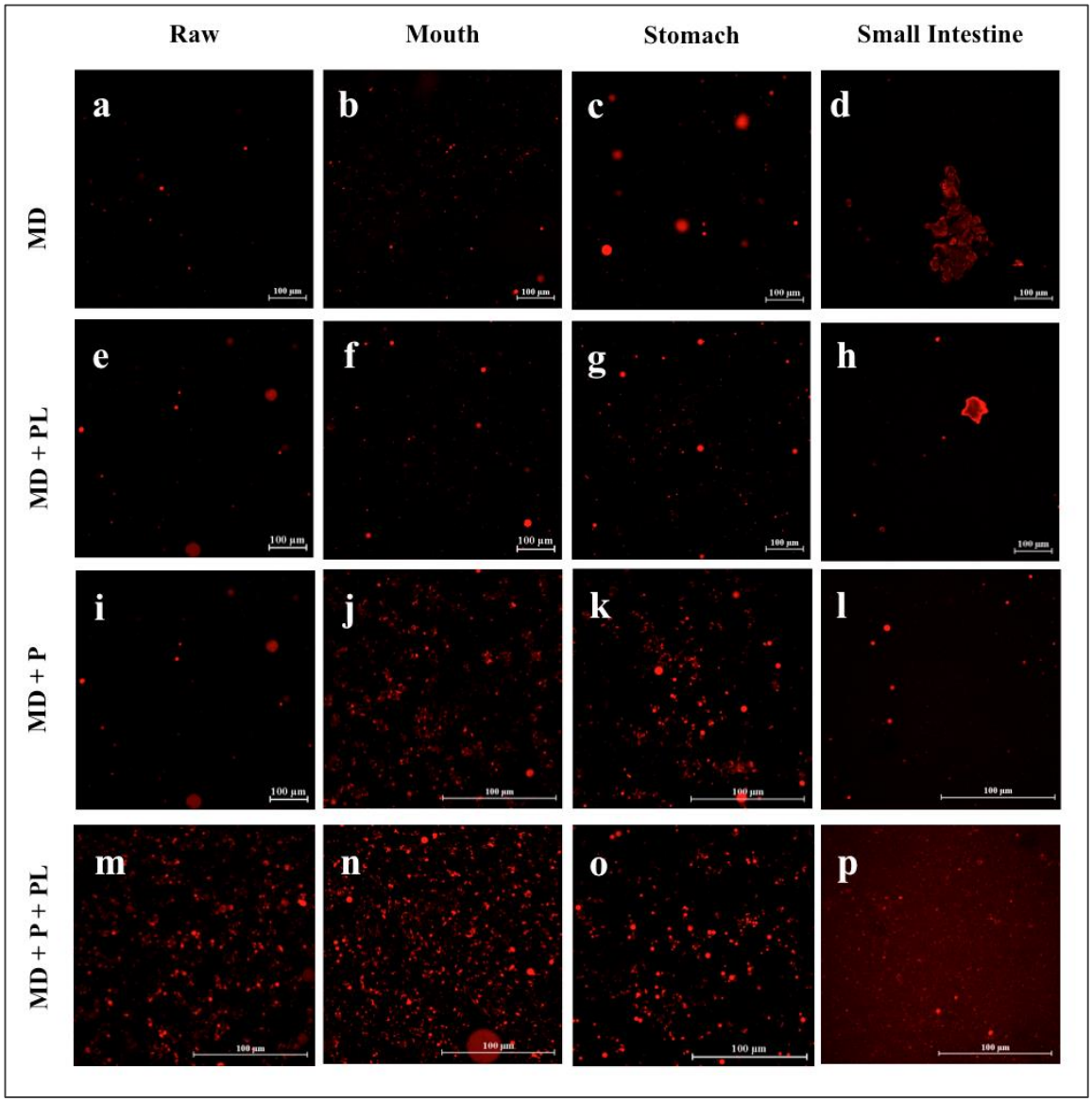


**Figure 34. Confocal microscopy demonstrating the influence of addition of pectin (P) and/or ε-polylysine (PL) on particle structure of full fat animal feed containing casein, maltodextrin, cornstarch, dextrose, DL-methionine, cellulose, vitamins, minerals, choline bitartrate, beef tallow, lard, anhydrous milk fat, soybean oil, peanut oil, and corn oil during *in vitro* digestion[35].**

[35] (Lopez-Pena, Song, Xiao, and McClements, 2015)

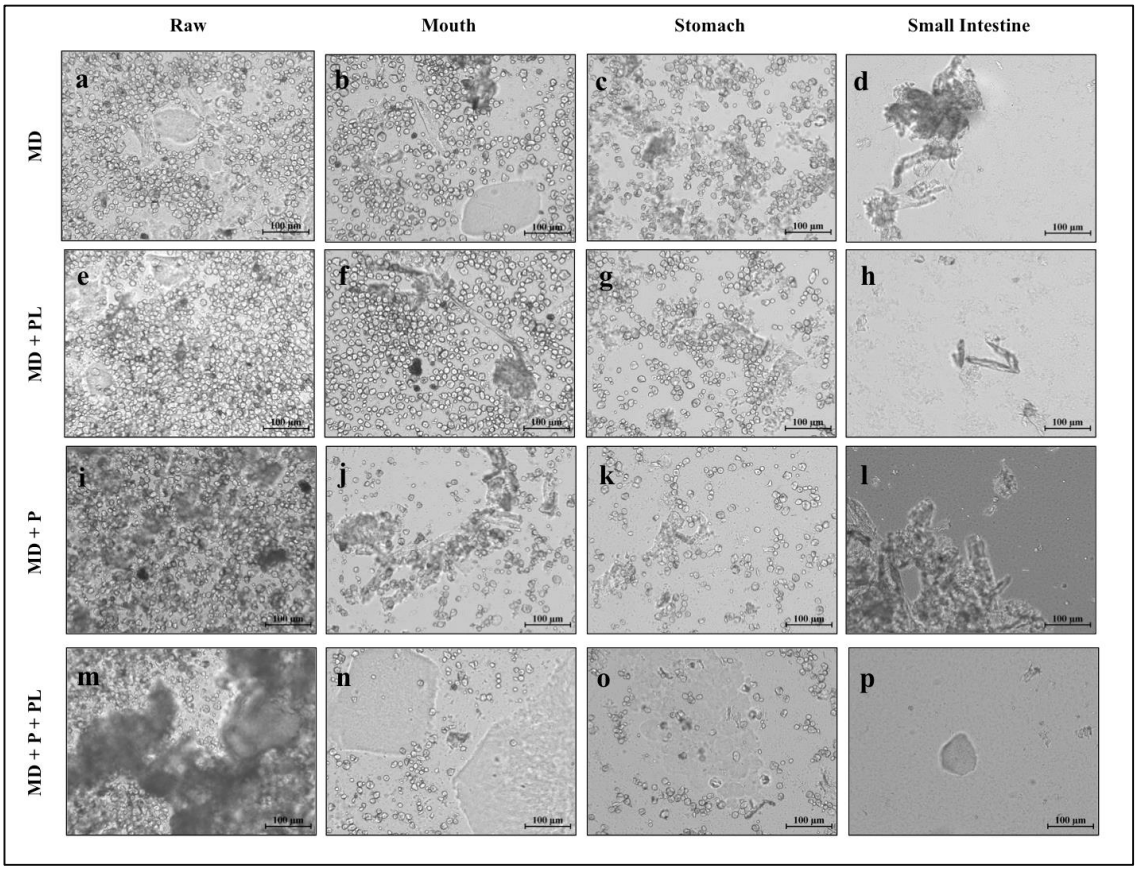


**Figure 35. Optical microscopy demonstrating the influence of addition of pectin (P) and/or ε-polylysine (PL) on particle structure of fat-free animal feed containing casein, maltodextrin, cornstarch, dextrose, DL-methionine, cellulose, vitamins, minerals, and choline bitartrate during *in vitro* digestion[36].**

[36] (Lopez-Pena, Song, Xiao, and McClements, 2015)

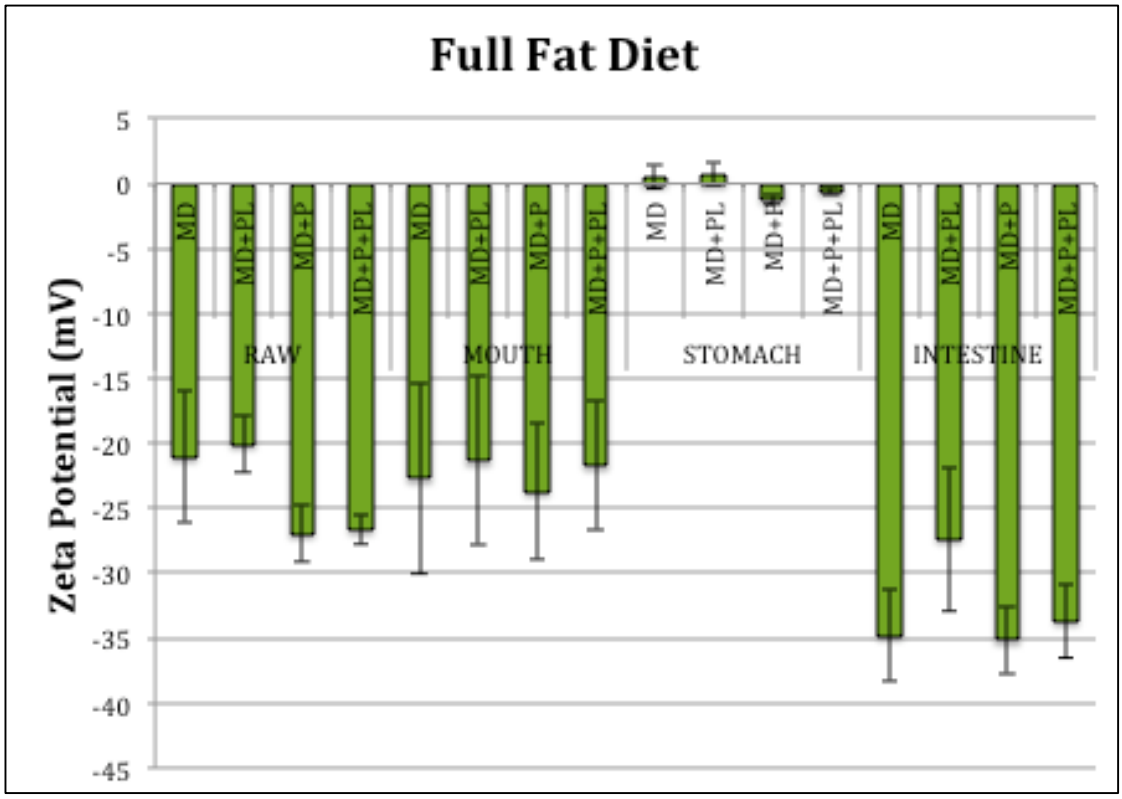


**Figure 36. Influence of addition of pectin (P) and/or ε-polylysine (PL) on particle charge of fat-free animal feed containing casein, maltodextrin, cornstarch, dextrose, DL-methionine, cellulose, vitamins, minerals, choline bitartrate, beef tallow, lard, anhydrous milk fat, soybean oil, peanut oil, and corn oil during *in vitro* digestion[37].**

[37] (Lopez-Pena, Song, Xiao, and McClements, 2015)

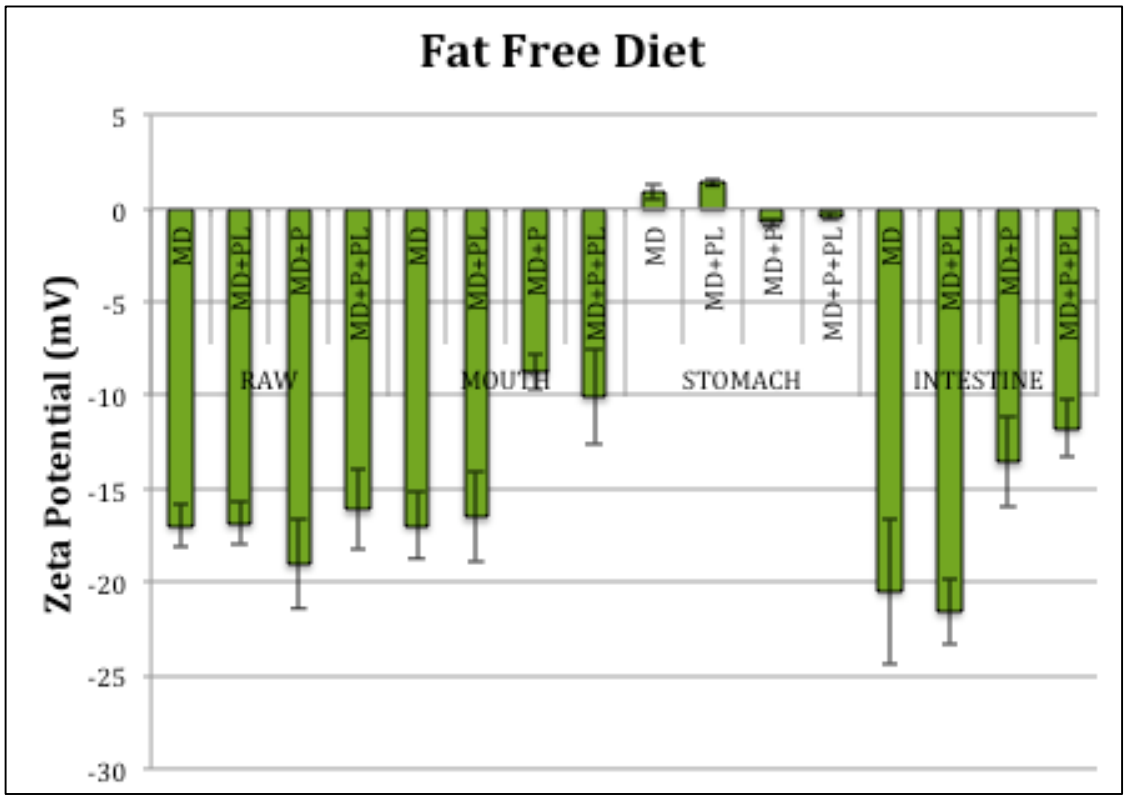


**Figure 37. Influence of addition of pectin (P) and/or ε-polylysine (PL) on particle charge of fat-free animal feed containing casein, maltodextrin, cornstarch, dextrose, DL-methionine, cellulose, vitamins, minerals, and choline bitartrate during *in vitro* digestion[37].**

### 6.4.2.2 Oral Digestion

There were appreciable changes in the mean particle sizes and microstructures of all of the samples after exposure to the oral phase of the *in vitro* gastrointestinal model (Figures 1 to 4). These effects can be attributed to dilution of the initial samples with simulated saliva fluids. The mean particle size of the full fat samples did not change appreciably after dilution, which suggested that the large particles present in the initial sample remained intact (**Figure 31**). On the other hand, there was an appreciable decrease in the mean particle size of the fat-free

samples, which suggested that simulated oral conditions promoted some breakdown of aggregates in these samples (**Figure 32**). For example, the change in pH or ionic strength, the presence of mucin, or the application of shear forces may have led to some aggregate disruption.

Optical and confocal microscopy of the full fat systems (**Figure 33** and **Figure 34**) showed that there was an increase in the space between particles, which can be at least partially attributed to simple dilution effects. In addition, there was evidence of extensive particle flocculation and phase separation in some of the systems. These effects may have been due to bridging or depletion flocculation by the biopolymers, but again it is difficult to unambiguously identify the origin of these effects from microscopy images. Again, this highlights one of the challenges of studying complex food matrices using simulated GIT methods.

The electrical charge on the full-fat systems remained highly negative after exposure of the samples to oral conditions, which can be attributed to the fact that the pH was still around neutral and so the proteins will be negatively charged. In addition, the mucin molecules in the simulated saliva are also negatively charged. The electrical charge on the fat-free systems was again less negative that that on the full-fat systems, which suggests that the fat droplets played a role in determining the overall electrical characteristics of the systems.

#### 6.4.2.3 Gastric Conditions

The mean particle diameter remained relatively constant when both the full-fat and fat-free samples moved from the oral to the gastric phase (**Figure 31**and **Figure 32**), which suggested that at least a population of the particles remained

intact. The gastric phase contained pepsin, and therefore one would have expected some degradation of the proteins in the animal feed. This result suggests that indigestible particles (probably cellulose fibers) mainly contributed to the light scattering signal used to determine the particle size. The observed decrease in particle concentration may also be attributed to the simple fact that the samples were diluted (1:1) when they moved from the mouth to stomach phases.

In all the samples, the ζ-potential was close to zero after exposure to gastric conditions, which can be attributed to the change in the electrical characteristics of the proteins and peptides at the low pH of the gastric environment. The electrical charge on proteins (such as the caseinate in the animal feed) moves from negative at high pH to positive at low pH with a point of zero charge near the isoelectric point (pI ≈ 5). Presumably, the electrical charge did not become strongly positive in our samples due to the presence of the anionic mucin molecules that would form electrostatic complexes with any cationic proteins, thereby reducing the net charge of the complexes.

#### 6.4.2.4 Intestinal Conditions

After exposure to the intestinal phase of the GIT model, there was an appreciable increase in the mean particle size measured by light scattering (**Figure 31** and **Figure 32**) and evidence of large aggregates in the microscopy images (**Figure 33, Figure 34**, and **Figure 35**) in all of the samples. This result suggests that some components in the simulated intestinal fluids promoted particle growth. In the full-fat samples, one would expect the lipase to interact with the lipid phase and promote the formation of lipid digestion products, such as free fatty acids and

monoacylglycerols. These lipid digestion products form micelles and vesicles (collectively known as "mixed micelles") in the intestinal fluids, which may account for the large lipid-rich objects observed in some of the confocal fluorescent microscopy images (**Figure 34**). In addition, the bile salts in the simulated intestinal fluids may have interacted with some of the components in the animal feed and caused particle aggregation. The confocal microscopy images also suggested that there were some differences in the distribution of the lipids in the samples after exposure to simulated intestinal conditions (**Figure 34**). There appeared to be relatively large domains that contained all of the lipids in the samples containing no pectin (MD and MD+PL), but the lipids appeared to be more evenly distributed throughout the samples containing pectin (MD+P and MD+P+PL). This result suggests that the pectin may have altered the ability of the lipase to interact with the lipids in the animal feed.

It is interesting to note that the vast majority of starch granules in all the full-fat and fat-free systems were digested (**Figure 33** and **Figure 35**), which is likely due to the presence of amylase activity in the enzyme extracts used.

The particle charge for all systems became markedly more negative after exposure to intestinal conditions, which can be attributed to the presence of various anionic species after digestion, such as bile salts, free fatty acids, and peptides. The negative charge was higher in the full-fat samples than in the fat-free samples, which may have been due to the generation of free fatty acids due to lipid digestion.

### 6.3.3 *In Vitro* Digestion

Previous studies suggest that incorporation of dietary fibers (such as cellulose and pectin) into animal feed may cause incomplete digestion of macronutrients in the stomach, thereby causing an increase in the amount of non-digested protein or fat in the small intestine (Bergner, Simon, Partridge, & Bergner, 1985; Howard & Mahoney, 1989; Shah, Atallah, Mahoney, & Pellett, 1982; Southgat.Da & Durnin, 1970). We therefore used pH-stat measurements on full-fat and fat-free animal feeds to determine the influence of digestive enzymes on their hydrolysis (**Figure 28**). Measurements were run on the MD, MD+P, MD+PL, and MD+P+PL samples to establish the influence of biopolymer type and complexation on digestion.

For all fat-free samples, there was an appreciable increase in the amount of alkali that had to be titrated into the reaction vessel to maintain a constant neutral pH, which can be attributed to the breakdown of peptide bonds by the proteases in the simulated GIT fluids. The amount of alkali required to neutralize the samples was higher for all the full-fat samples, which is due to the conversion of the triacylglycerol molecules into free fatty acids by lipase.

The potential influence of the biopolymers on lipid digestion was compared by calculating the amount of free fatty acids released after the pH-stat profile for the fat-free animal feed was subtracted from the full-fat one (**Figure 39**). Interestingly, the rate and extent of lipid digestion appeared to be higher in the samples containing pectin (MD+P and MD+P+PL) than the ones containing no pectin (MD

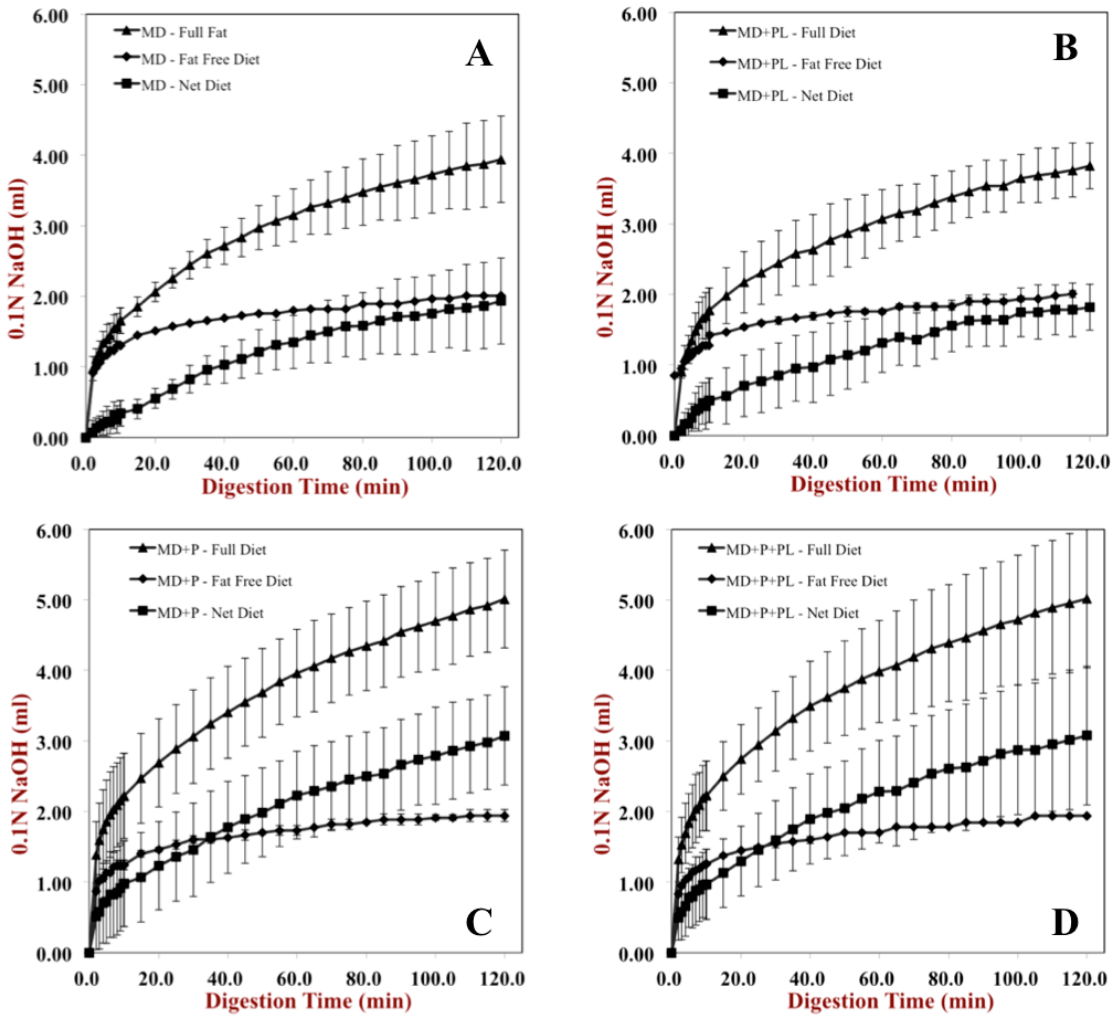


**Figure 38. Influence of the presence (full fat) or absence of fat (fat-free) on the amount (ml) of 0.1N NaOH solution titrated into the (A) maltodextrin (MD), (B) maltodextrin and ε-polylysine (MD+PL), (C) maltodextrin and pectin (MD+P), and (D) maltodextrin, pectin, and ε-polylysine samples during simulated intestinal digestion[38].**

[38] (Lopez-Pena, Song, Xiao, and McClements, 2015)

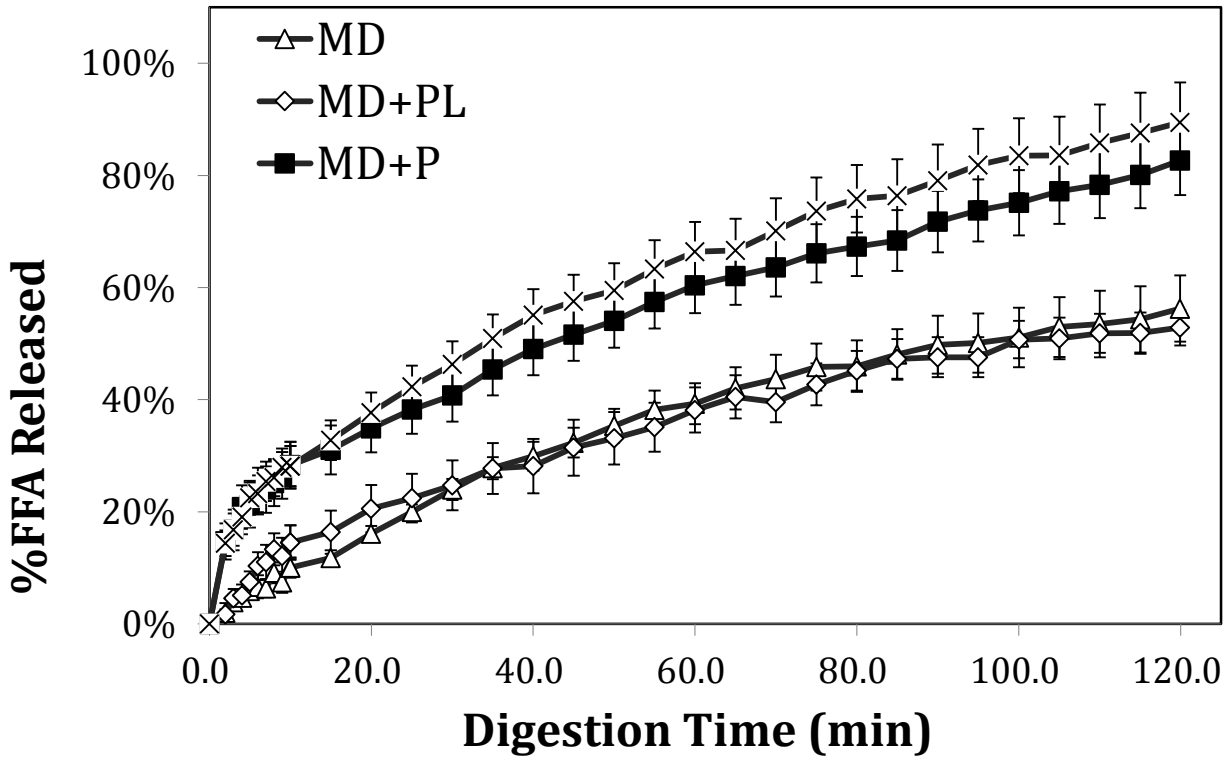




**Figure 39. Influence of addition of pectin (P) and/or ε-polylysine (PL) on the percentage if free fatty acids (%FFA) released from animal feed containing casein, maltodextrin, cornstarch, dextrose, DL-methionine, cellulose, vitamins, minerals, choline bitartrate, beef tallow, lard, anhydrous milk fat, soybean oil, peanut oil, and corn oil during simulated intestinal digestion. The final lipid content in the digestion medium was 0.5%[39].**

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[39] (Lopez-Pena, Song, Xiao, and McClements, 2015)

and MD+PL). These results suggest that the presence of pectin may have facilitated lipid digestion. One potential mechanism is that the pectin inhibited extensive aggregation of the protein in the stomach and small intestine, thereby allowing the lipase to access the surfaces of the lipid phase more easily, as has been reported in previous studies (Espinal-Ruiz, Parada-Alfonso, Restrepo-Sanchez, Narvaez-Cuenca, & McClements, 2014). Indeed, the confocal fluorescence microscopy images suggest that the pectin may have led to a more even distribution of lipids in the samples after exposure to the stomach and intestinal phases (**Figure 34**).

## 6.4 Conclusion

The *in vitro* portion of this study described here suggests that the presence of ε-polylysine did not have a significant effect on the rate or extent of lipid digestion when compared to a control. On the other hand, the presence of pectin, delivered either alone or as a complex with polylysine, appeared to promote lipid digestion. The origin of this effect was attributed to the ability of pectin to prevent extensive protein aggregation in the stomach and small intestine, thereby allowing the lipase to more easily access the lipid phase. In order to more fully assess the potential gastrointestinal fate of these biopolymers, *in vivo* models focusing on the absorption and metabolism of fat have been conducted (reported elsewhere).

## 6.5 Acknowledgments

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Massachusetts Agricultural Experiment Station and a United States Department of Agriculture, CREES, NRI and AFRI Grants.

# CHAPTER 7

# CONCLUSION

The overall goal of this work was to first assess the stability of antimicrobial electrostatic polylysine-pectin complexes (Y. H. Chang, L. McLandsborough, & D. J. McClements, 2011) in the presence of more anionic biopolymers, to then identify any potential toxicology stemming from its habitual consumption via *in vitro* and *in vivo* analyses. Special attention was given to the lipase-inhibiting properties of polylysine.

Results showed that although the interactions between polylysine and carrageenan were stronger than those between pectin and polylysine, it was the order in which the anionic biopolymers interacted with polylysine that would determine which complexes were formed, more so than the strength of their electrostatic interaction. That is, pectin-polylysine complexes formed prior to exposure to carrageenan were relatively stable, with seemingly only a small amount of polylysine being displaced to interact with carrageenan. These results suggest that the antimicrobial polylysine-pectin complexes may be successfully incorporated into food systems with strongly anionic food components. It is important to note, however, that interactions may occur with other components present in a food matrix. Thus, its is essential for food manufacturers to carefully formulate their products taking into account the various kinds of molecular interactions that can occur in complex multicomponent food systems when considering the implementation of this antimicrobial complex.

In order to fully understand and assess any possible toxicology stemming from the incorporation of polylysine-pectin complexes in food, it was essential to first study the behavior of polylysine in the digestive tract, focusing on its ability to interact electrostatically with bile salts and inhibit lipase. From this work, a few observations were reached:

a. When only focusing on simulations in the small intestine *in vitro*, the surfactant type utilized to stabilize corn oil emulsion systems had a strong impact on polylysine's "anti-obesity" properties in conditions with excess oil (2.5%w/w), when 2ppm polylysine was utilized. Negatively charged surfactants (quillaja saponins) seemed to compete with bile salts to interact with polylysine, thus limiting the inhibition on lipase's enzymatic activity as compared to a non-ionic Tween 20-stabilized corn oil emulsions when there.
b. When no excess oil (0.5%w/w) was present in the intestinal tract, there did not seem to be an inhibitory effect of polylysine (200 ppm) on lipase activity. However, interaction of polylysine with mixed micelles containing bile salts and free fatty acids promoted their sedimentation, and seemed to decrease the amount of solubilized free fatty acids in the aqueous phase. This phenomenon may have important implications on the absorption and metabolism of lipids, particularly on the bioaccessibility of lipophilic nutrients and nutraceuticals.
c. Both polylysine and the anionic glycoproteins contained in mucin seemed to alter the microstructure and electrical characteristics of emulsified lipids *in vitro.*

Based on the observations mentioned above, further investigation is required to determine whether incorporating polylysine in food system systems may indeed decrease the bioaccessibility of lipophilic compounds. Additional work is currently underway to assess the impact of polylysine on the bioaccessibility of fat-soluble vitamins.

Subchronic toxicological studies performed on mice were designed to test any toxicological effect of the habitual consumption of pectin-polylysine complexes in dosages 100 times higher than their recommended use. Animal feed containing cellulose, different types of fat, vitamins, minerals, starch, and casein were supplemented with body weight-based amounts of spray dried pectin-polylysine, pectin, polylysine, or only maltodextrin powders. The *in vitro* portion of the study suggested that pectin had a significant effect on lipid digestion as compared to the control, but not polylysine. However, it is possible that polylysine may still exert an impact on the solubilization of mixed micelles containing free fatty acids regardless of not inhibiting lipid digestion, as mentioned in previous sections. Additional information from the *in vivo* study regarding body weight, organ size and weight, blood markers, fecal analysis, and changes in the gut microbiome are required to fully assess any toxicological and metabolic effects stemming from the habitual consumption of pectin-polylysine complexes. These studies are currently underway.

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