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Interactions of a Cationic Antimicrobial (ε-Polylysine) with an Anionic Biopolymer (Pectin): An Isothermal Titration Calorimetry, Microelectrophoresis, and Turbidity Study

Yuhua Chang,[†,‡] Lynne McLandsborough,[†] and David Julian McClements*[,†]

[†]Department of Food Science, University of Massachusetts, Amherst, Massachusetts 01003, United States

[‡]College of Food Engineering and Nutritional Science, Shaanxi Normal University, Xi'an, Shaanxi 710062, P.R. China

ABSTRACT: ε-Polylysine (ε-PL) is a food-grade cationic antimicrobial that is highly effective against a range of food pathogens and spoilage organisms. In compositionally complex environments, like those found in most foods and beverages, the antimicrobial activity of cationic ε-PL is likely to be impacted by its interactions with anionic components. The purpose of this study was to characterize the interactions between cationic ε-polylysine and an anionic biopolymer (high methoxyl pectin, HMP) using isothermal titration calorimetry (ITC), microelectrophoresis (ME), and turbidity measurements. ITC and ME measurements indicated that ε-PL bound to pectin, while turbidity measurements indicated that the complexes formed could be either soluble or insoluble depending on solution composition. Ionic strength and pH were also shown to affect the interactions significantly, highlighting their electrostatic origin. This study demonstrates that ε-PL can form either soluble or insoluble complexes with anionic biopolymers depending on the composition of the system. Our study provides basic knowledge that will facilitate the more rational application of ε-PL in complex food systems.

KEYWORDS: ε-Polylysine, pectin, interaction, isothermal titration calorimetry, ITC, microelectrophoresis

INTRODUCTION

There is a growing need for effective antimicrobials that can be used to inhibit microbial growth in food and beverage applications. A number of studies have shown that ε-polylysine (ε-PL) is a highly effective antimicrobial that has potential for utilization in food products.[1−3] ε-PL is a homopolymer consisting of L-lysine monomers (typically between 25 and 35) linked together by isopeptide bonds between ε-amino and α-carboxyl groups.[4−6] ε-PL was discovered more than 30 years ago in Japan, in culture filtrates of *Streptomyces albulus* ssp. *lysinopolymerus* strain 346.[4] The compound is now industrially produced by aerobic fermentation, using a mutant derived from strain 346 which exhibits increased production of ε-PL.[6−8] Because of the presence of primary amine groups along its backbone, ε-PL is cationic when the environmental pH is lower than its isoelectric point, which is around 9.0.[6] The proposed antimicrobial mechanism is that ε-PL is absorbed onto negatively charged cell surfaces of microorganisms through electrostatic interactions, leading to stripping of the outer membrane, abnormal distribution of the cytoplasm, and finally cell death.[9] ε-PL is reported to have a wide antimicrobial spectrum, against both Gram-negative and Gram-positive bacteria, yeasts, and molds.[2,6,9,10] On the basis of absorption, distribution, metabolism, excretion, and toxicity (ADMET) studies, ε-PL has been proven to be safe for consumption.[11] For this reason, it has been approved as generally recognized as safe (GRAS) within the United States for certain food applications (GRAS No. 000336 and GRAS No. 000135).[12,13]

ε-Polylysine has the potential to be a highly effective ingredient for controlling or preventing microbial growth in food products because of its high antimicrobial activity, nontoxicity, water-solubility, and good thermal stability. However, its application may be limited for a number of reasons: (i) it may form undesirable precipitates if it interacts with anionic components within the food matrix; (ii) it may bind to anionic biopolymers in the mouth, leading to perceived astringency or bitterness; and (iii) its antimicrobial activity may be weakened if it interacts with anionic ingredients. Therefore, both the beneficial attributes (antimicrobial) and detrimental attributes (precipitate formation and perceived bitterness) of ε-PL are likely to depend on its electrostatic interactions with other molecules in the food systems in which it will be utilized. It is therefore important to systematically characterize the interactions between ε-PL and anionic food ingredients so as to gain a better understanding of how to control the functionality of ε-PL in compositionally complex food products.

In this study, we used a variety of analytical methods to characterize the interactions between cationic ε-PL and anionic pectin. Pectin is an anionic heteropolysaccharide of partially esterified α-1,4 linked D-galacturonides, containing varying amounts of covalently attached rhamnose and branches of L-arabinose, D-galactose, D-xylose, and L-rhamnose. Pectin functions as a gelling, thickening, and stabilizing agent in foods. This functionality is related to the molecular weight, degree of esterification (% DE), distribution of ester groups on the backbone, presence of nonuronide components, and other structural characteristics.[14,15] Pectin has carboxylic acid side groups that are

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negatively charged across a wide range of pH values ($pK_a \approx 3.5$). The charge density and charge distribution along the backbone determine the functionality of pectin in foods. Previous studies have shown that anionic pectin molecules can interact with cationic groups on other food ingredients, thereby altering their functionality.[16,17] Studies have also shown that cationic polylysine can interact with various anionic polysaccharides. Studies with carrageenan,[18] pectate,[19,20] alginate,[21] chondroitin-6-sulfate,[22] and heparin[23] have shown that they form electrostatic complexes with L-polylysine and that the interaction may promote a coil-to-helix transition in the polylysine depending on the charge characteristics of the anionic polysaccharide involved. A major driving force for this research has been the development of delivery systems for biologically active components in the pharmaceutical industry.[24,25]

Ultimately, we believe that a better understanding of the interactions between cationic ε-PL and anionic pectin molecules in aqueous solutions will facilitate the rational design of ε-PL delivery systems with improved functionality in foods and other applications, for example, enhanced antimicrobial activity and/or reduced bitterness and undesirable precipitation reactions.

MATERIALS AND METHODS

Materials. The cationic antimicrobial ε-polylysine (50:50 mixture with maltodextrin) was provided by Purac (Puraq Xtend FX50P; lot#: 1091102). High methoxyl pectin (HMP) with a degree of esterification (DE) of approximately 71% (Pectin 1400) was donated by TIC Gums (Belcamp, MD). The mineral content of the pectin was reported by the manufacturer to be 451 mg of sodium, 152 mg of potassium, and 148 mg of calcium per 100 g. Maltodextrin (dextrose equivalent 4.0−7.0; catalog no. 419672-100G) was purchased from Sigma-Aldrich (St Louis, MO). Doubly distilled water was used for the preparation of all solutions.

Solution Preparation. An aqueous ε-PL solution was prepared by dispersing powdered ε-polylysine ingredient into doubly distilled water to a final concentration of 1.0 w/v % ε-PL (and therefore 1.0 w/v % maltodextrin). The solution was then adjusted to pH 3.5 using either HCl or NaOH. Pectin solutions (0.3 w/v %) were prepared by dispersing powdered pectin into doubly distilled water under stirring, followed by heating until boiled, and then continued stirring for at least 2 h at room temperature to ensure complete dispersion and dissolution. The pectin solution was then adjusted to pH 3.5 using HCl or NaOH.

For the variation of pH and salt concentration experiments, ε-PL and pectin solutions were also prepared at pH 2, 3, 4, 5, 6, and 7, and with 0, 50, 100, and 150 mM NaCl at pH 3.5. It should be noted that there would be a slight difference in the ionic strength of solutions at different pH values due to the different amounts of acid or base they contained.

Isothermal Titration Calorimetry (ITC). An isothermal titration calorimeter (VP-ITC, Microcal Inc., Northampton, MA) was used to measure enthalpies of mixing at 30.0 °C. Fifty-eight 5 μL aliquots of ε-PL solution (1.0 w/v% or 0.25 w/v%, pH 3.5) were injected sequentially into a 1480 μL titration cell initially containing either water (adjusted to pH 3.5) or 0.3 w/v % pectin in water (adjusted to pH 3.5). Each injection lasted 12 s, and there was an interval of 360 s between successive injections. The solution in the titration cell was stirred at a constant speed of 315 rpm throughout the experiments.

Microelectrophoresis (ME) and Turbidity Measurements. Microelectrophoresis and turbidity measurements were used to provide information about the electrical charge and aggregation state of ε-PL−pectin complexes, respectively. The experiments were designed to mimic the ITC experiments, that is, using a similar total ε-PL concentration range and ε-PL-to-pectin ratio. Aliquots of surfactant solution (0−1500 μL, 1.0 w/v % ε-PL, pH 3.5) were injected into glass test tubes initially containing 7.5 mL of 0.3 w/v % pectin solution (pH 3.5). The resulting solutions were then mixed thoroughly and stored overnight prior to analysis.

The electrical charge (ζ-potential) of the particles in the solutions were then measured using a particle electrophoresis instrument (Zetasizer Nano-ZS, model ZEN3600, Malvern Instruments, Worchester, U.K.). The ζ-potential was calculated from the measurement of the electrophoretic mobility of particles in an applied oscillating electric field using laser Doppler velocimetry. All measurements were conducted on at least two freshly prepared samples and repeated three times per sample. We observed that the light scattering intensity (counts rates) was much greater for pectin solutions than for polylysine solutions. For example, the count rates were 12, 53, and 62 kcps for aqueous solutions containing 1% polylysine, 0.3% pectin, and 0.3% pectin/0.162% polylysine (pH 3.5), respectively. This meant that in systems containing any excess polylysine the overall signal would be dominated by the presence of the complexes.

The optical turbidity (at 600 nm) of the pectin and pectin−polylysine solutions was measured using a UV−visible spectrophotometer (Ultraspec 2000, Pharmacia Biotech) at room temperature. The samples were contained within 1 cm path length optical cells, and distilled water was used as a control. Turbidity measurements were carried out on at least two freshly prepared samples.

RESULTS AND DISCUSSIONS

The purpose of our experiments was to obtain a better understanding of the interactions and physicochemical properties of mixed polylysine−pectin systems. The interactions between ε-PL and pectin were characterized using ITC, electrical charge, turbidity, and visual appearance measurements. These experiments were carried out by titrating ε-PL solutions into pectin solutions (pH 3.5) and recording the resulting changes in enthalpy, ζ-potential, turbidity (τ_{600}), and visual appearance.

Enthalpy Changes Associated with ε-Polylysine−Pectin Interactions. The enthalpy changes associated with polylysine−pectin interactions were quantified by isothermal titration calorimetry (ITC) at 30 °C. Heat flow versus time profiles resulting from sequential injections of 5 μL aliquots of ε-PL solution (0.25 or 1.0 w/v %) into a 1480 μL titration cell initially containing either water (pH 3.5) or pectin (0.3 w/v %, pH 3.5) were measured (data not shown). The dependence of the enthalpy change per unit amount of polylysine titrated into the reaction cell on the ε-PL concentration in the reaction cell was calculated by integration of the heat flow versus time profiles (Figure 1). The major differences between the ε-PL titrated into water and the ε-PL titrated into pectin solution profiles indicated that there were strong interactions between the cationic ε-PL and anionic pectin.

In the absence of pectin, enthalpy changes resulting from titration of polylysine into water were initially slightly exothermic and became progressively less exothermic with increasing ε-PL concentrations (Figure 1). The observed decrease in enthalpy can be attributed to solute dilution effects: when polylysine is titrated into the reaction cell, the ε-PL molecules move further apart, which alters the overall magnitude of the molecular interactions in the system, leading to an enthalpy change. The enthalpy change decreased in magnitude with increasing ε-PL concentration because the concentration difference between the polylysine in the injector and that in the reaction cell got progressively less.

The enthalpy changes resulting from an injection of ε-PL solution (1.0%, w/v) into 0.3 w/v % pectin solutions were also measured using ITC (Figure 1). The presence of pectin in the

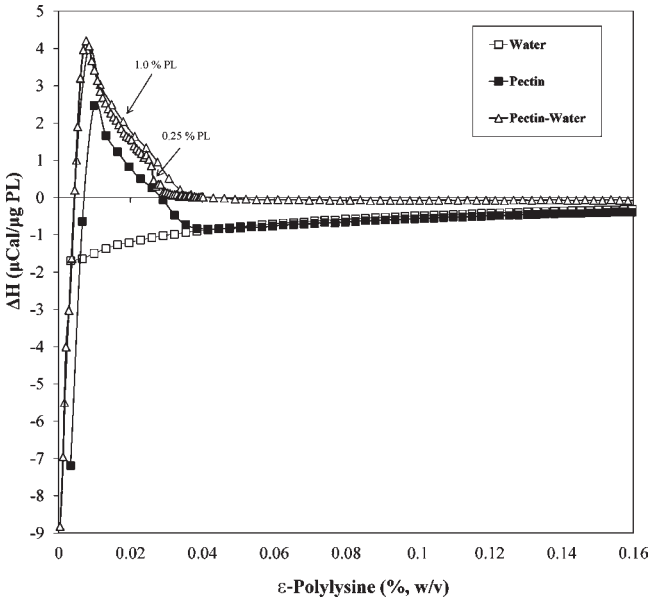

Figure 1. Dependence of enthalpy change (ΔH) per unit mass (μg) of polylysine titrated into the reaction cell on the average ε-PL concentration in the reaction cell after the injection when either 0.25 or 1.0 wt % ε-PL was injected into a reaction cell containing either 0 or 0.3 wt % pectin (pH 3.5, 30 °C). The overall enthalpy change associated with the interaction was calculated as the difference between titration into pectin solution and titration into water.

reaction cell caused an appreciable alteration in the enthalpy change versus ε-PL concentration profiles, indicating that there was some form of interaction between the cationic ε-PL and anionic pectin. The enthalpy changes associated with the interaction were highlighted by plotting the difference in enthalpy changes in the presence and absence of pectin: $\Delta H_{int} = \Delta H_{pectin} - \Delta H_{water}$ (Figure 1). The above experiment was repeated using a more dilute ε-PL solution (0.25%, w/v) to inject into the reaction cell, so as to highlight the concentration range where the major enthalpy changes occurred. There was excellent agreement between the measurements made using 0.25 and 1.0 w/v ε-PL solutions (Figure 1).

The interactions between polylysine and pectin molecules could be conveniently divided into a number of different concentration regimes (Figure 1). At relatively low ε-PL concentrations (0−0.01 w/v %), the interaction enthalpy went from highly exothermic to highly endothermic with increasing ε-PL concentration. At intermediate ε-PL concentrations (0.01−0.03 w/v %), the interaction enthalpy decreased from highly endothermic to close to zero with increasing ε-PL concentration. At relatively high ε-PL concentrations (>0.03 w/v %), the interaction enthalpy was close to zero, suggesting that little further interactions occurred.

Presumably, the cationic ε-PL molecules titrated into the reaction cell interacted with the anionic pectin molecules, which led to changes in the organization and interactions of the various molecules involved: ε-PL, pectin, small ions, and water. For example, when two or more oppositely charged biopolymers come into contact with each other, there may be a number of alterations in the system: there will be an increase in the number of attractive intermolecular electrostatic interactions between anionic groups on pectin and cationic groups on polylysine; there may be a change in the number of repulsive intramolecular electrostatic interactions between anionic groups on pectin and between cationic groups on polylysine; there will be a change in the distribution of counterions around the charged groups on the two biopolymers; the molecular conformations of the two biopolymers may change (e.g., coil−helix); and, the degree of hydration of the two biopolymers may have altered.[18,26,27] There will be enthalpy changes associated with each of these molecular events, which contribute to the overall enthalpy change measured by ITC. Indeed, one of the major limitations of ITC is that it measures the net enthalpy changes associated with an interaction, and it is often difficult to determine the precise molecular origin of the observed changes. Recently, molecular dynamics simulations of the interactions between oppositely charged polyelectrolytes have been carried out in an attempt to relate enthalpy changes to specific molecular events.[26] These simulations have shown that the overall enthalpy change may be negative or positive depending on the strength of the electrostatic interactions between the two polyelectrolytes. In future studies, it would be useful to use ITC in combination with a variety of other analytical methods to provide more detailed insights into the molecular changes involved.

Despite limitations in relating the enthalpy changes measured by ITC to precise molecular events, the technique still provides some valuable information about the interactions involved. First, they suggest that there are at least two different physicochemical phenomena occurring: one at low ε-PL levels (<0.01% w/v) and one at intermediate ε-PL levels (0.01−0.03% w/v). Second, they suggest that the pectin molecules became saturated with ε-PL molecules above 0.03% w/v ε-PL since no further enthalpy changes were observed (Figure 1). This corresponds to a mass ratio of ε-PL-to-pectin of ≈0.1-to-1, or a molar ratio of ≈0.11-to-1, when expressed on a monomer-basis assuming a molecular weight of ≈180 g for the monomers in pectin and ≈120 g for the monomers in polylysine.

It should be noted that the ε-PL sample used in this study contained 50% maltodextrin; consequently, there is a concern of its potential interaction with pectin and contribution to the measured enthalpy changes. We therefore measured the enthalpy changes resulting from an injection of maltodextrin solution (1.0 wt/v%, pH 3.5) into 0.3 wt % pectin solutions (pH 3.5) and found that they were negligible (data not shown). The most likely reason for this observation is that maltodextrin is electrostatically neutral, and therefore, it would not be expected to strongly interact with pectin.

Electrical Characteristics of ε-Polylysine−Pectin Complexes. The electrical characteristics (ζ-potential) of polylysine−pectin complexes were measured by microelectrophoresis in order to obtain more information about the nature of the interactions involved (Figure 2). A titration experiment was carried out that mimicked the ITC experiment: the ζ-potential was measured when increasing amounts of 1.0 wt % ε-PL solution were titrated into the 0.3% w/v pectin solution (pH 3.5).

In the absence of polylysine, the ζ-potential was highly negative ($\zeta \approx -22$ mV), which can be attributed to the presence of partially charged carboxylic acid groups along the pectin chain at pH 3.5.[28] As the ε-PL concentration was increased, the ζ-potential went from negative to positive, with a point of zero charge around 0.03% ε-PL, indicating that the cationic polylysine formed complexes with the anionic pectin. These measurements show that the polylysine−pectin complexes were negatively charged at low ε-PL-to-pectin mass ratios, but positively charged at high mass ratios. Charge neutralization occurred at a mass ratio

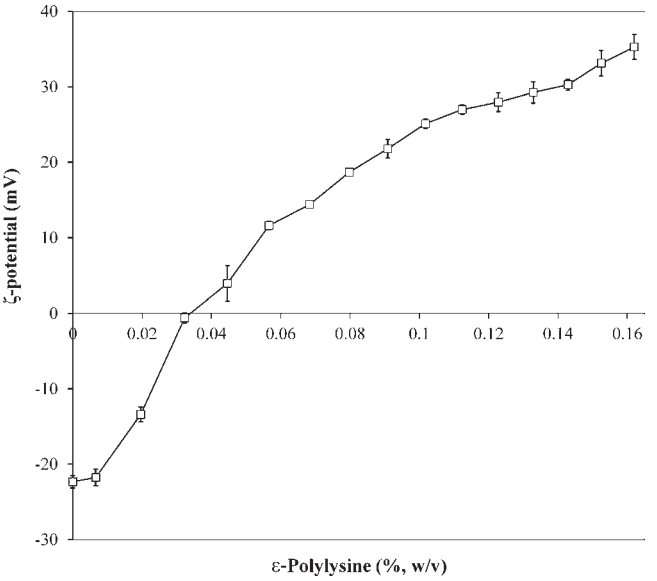

Figure 2. Dependence of ζ-potential of aqueous polylysine−pectin solutions on the ε-PL concentration in the reaction cell. An ε-PL−pectin solution (1.0 wt %, pH 3.5) was titrated into a pectin solution (0.3 wt %, pH 3.5).

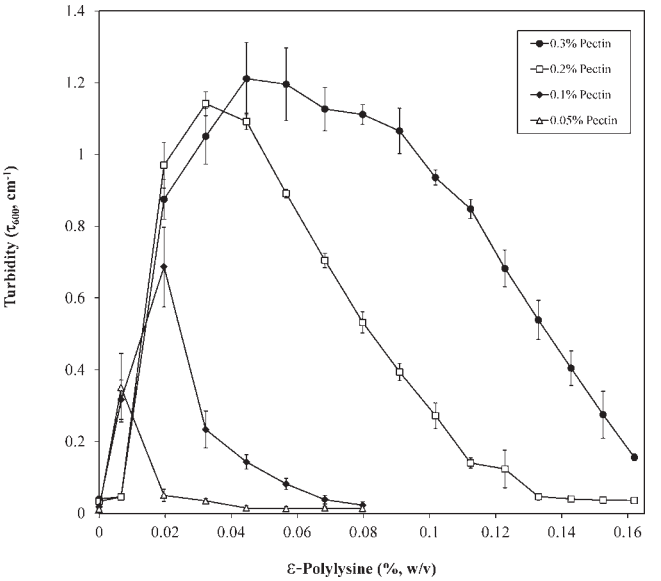

Figure 3. Dependence of solution turbidity (at 600 nm) on ε-PL concentration for mixed polylysine−pectin solutions. An ε-PL solution (1.0 wt %) was titrated into aqueous solutions containing various pectin concentrations (0.05−0.3 wt % pectin) at pH 3.5.

of PL-to-pectin of ≈0.1-to-1 or a molar ratio of 0.11-to-1 (see previous section). The fact that the complexes contained much less polylysine than pectin at charge neutralization can be attributed to differences in the charge densities of the two biopolymers. In polylysine, all of the monomers have an amino group ($pK_a \approx 9$) that is fully positively charged at pH 3.5, leading to a high positive linear charge density. However, in high methoxy pectin, only a fraction of the monomers have a carboxyl group ($pK_a \approx 3.5$), and only about 50% of these would be ionized at pH 3.5.

Knowledge of the electrical characteristic of the complexes has important consequences for understanding the physicochemical properties and functional behavior of polylysine−pectin complexes in food applications. One would expect negatively charged complexes to bind to positively charged ingredients and surfaces, and vice versa. The formation of anionic complexes may alter the tendency for polylysine to bind to the negative surfaces of bacteria, which could reduce its antimicrobial efficacy. However, the formation of anionic complexes might reduce the undesirable tendency for polylysine to bind to anionic biopolymers in human saliva (causing astringency) or to bind to anionic ingredients in foods (causing precipitation).

Aggregation Characteristics of ε-Polylysine−Pectin Complexes. The aggregation characteristics of polylysine−pectin complexes were determined by turbidity and visual observation. Small soluble complexes do not scatter light strongly, leading to the formation of clear solutions with low turbidity. However, large insoluble complexes scatter light strongly, leading to the formation of cloudy colloidal suspensions with high turbidity. A titration experiment was carried out that mimicked the ITC and microelectrophoresis experiments: the turbidity and visual appearance of the samples were measured when increasing amounts of 1.0 wt % ε-PL solution were titrated into 0.3% w/v pectin solution (pH 3.5). We also carried out a number of additional experiments using a series of different pectin concentrations (0.05, 0.1, and 0.2%) in the reaction cell. For the turbidity measurements, the samples were vortexed prior to analysis to ensure that any complexes present were evenly distributed throughout the whole sample. For visual observation, the samples were stored at ambient temperature for 24 h to determine the stability of any complexes formed due to gravitational separation.

For the solutions initially containing 0.3% w/v pectin in the reaction cell, the turbidity (τ_{600}) increased gradually when the polylysine concentration was increased from 0 to 0.01% w/v (Figure 3), but the values remained relatively low (<0.06 cm^{-1}). This suggested that the size of the polylysine−pectin complexes formed was relatively small since they did not scatter light strongly. The turbidity then increased steeply when the ε-PL concentration was increased from 0.01 to 0.03% w/v, suggesting that large insoluble complexes were formed that scattered light strongly. Finally, the turbidity progressively decreased as the ε-PL concentration was increased further, suggesting that the size of the complexes decreased. Visual observation of the solutions (Figure 4) indicated that they appeared only slightly turbid at low polylysine concentrations (<0.01% w/v), formed turbid colloidal suspensions or white sediments at intermediate polylysine concentrations (0.01 to 0.14% w/v), and formed relatively stable turbid colloidal dispersions at high polylysine concentrations (>0.14 wt %).

A similar type of general behavior was observed when lower levels of pectin were initially present in the reaction cell: with increasing polylysine concentration, the turbidity initially increased until it reached a maximum value, and then decreased (Figure 3). Nevertheless, the height and position of the turbidity maximum depended on pectin concentration. As the initial pectin concentration was decreased, the height of the maximum and the polylysine concentration where the maximum occurred both decreased. In addition, the range of concentrations where sedimentation occurred decreased. This effect can be attributed to the fact that less cationic groups on polylysine are required to saturate the anionic groups on the pectin molecules at lower pectin concentrations. These results are in good agreement with previous studies of the interactions

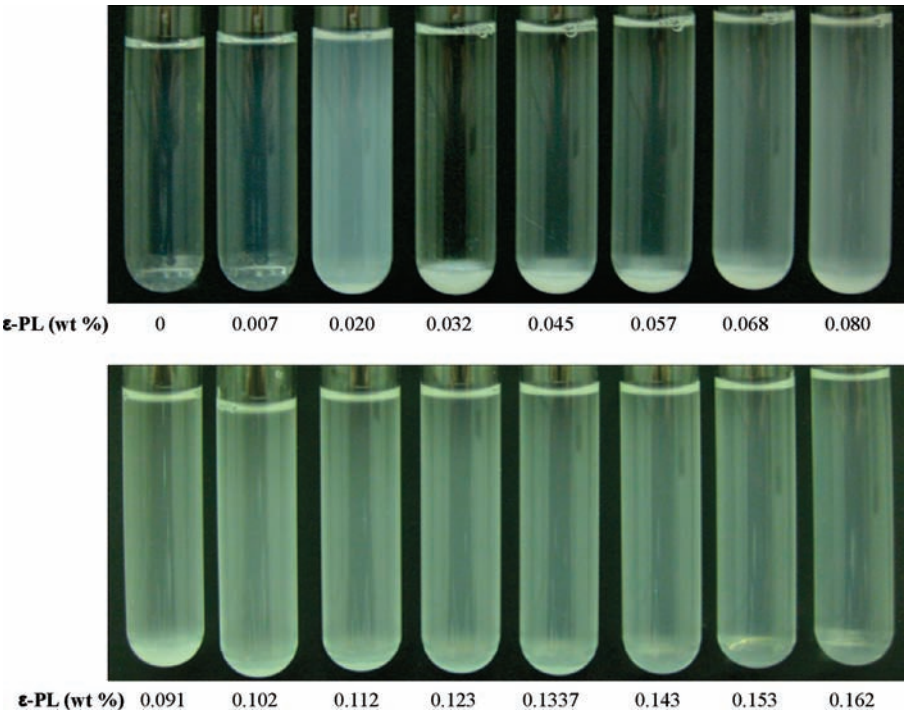

Figure 4. Visual appearance of aqueous solutions containing 0.3% pectin (pH 3.5) and different levels of ε-polylysine.

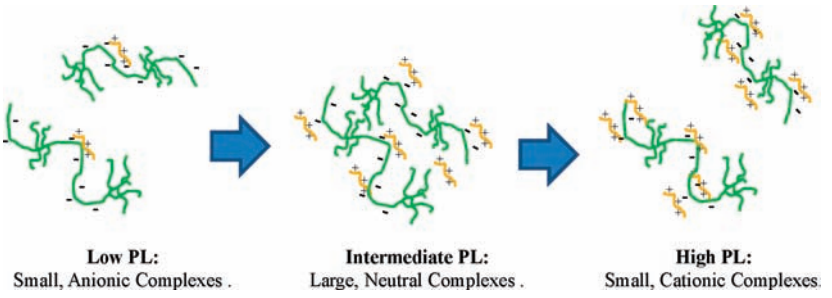

Figure 5. Schematic diagram of the complexes formed between anionic pectin (green) and cationic polylysine (orange) as the polylysine-to-pectin ratio is increased.

between oppositely charged biopolymers, which have been reviewed in detail elsewhere.[29−31]

Classification of ε-Polylysine−Pectin Interactions. On the basis of previous studies and our experimental data, it was possible to divide the interactions between polylysine and pectin into three different regions. Previous computer simulations and experimental measurements suggest that oppositely charged polyelectrolytes can form a variety of structures when the ratio of the two polyelectrolytes is varied.[30−32] Consider the case of a positive polyelectrolyte ($P+$) being titrated into a solution of negative polyelectrolyte ($P-$). Initially, when a small amount of $P+$ is added, the two types of polyelectrolytes associate with each other to form relatively small "primary complexes" with a high negative charge that inhibits further aggregation. As the $P+$ concentration is increased further, the charge on the primary complexes tends toward zero due to charge neutralization, and therefore, the primary complexes associate with each other to form large "secondary complexes", which may further associate into a coacervate phase. When the $P+$ concentration is further increased, the net charge on the primary complexes becomes highly positive, and therefore, the large secondary complexes dissociate. In the system used in our study (0.3% w/v pectin, pH 3.5), the interaction between polylysine and pectin can be divided into three ε-PL concentration regions (Figure 5):

Region I: 0−0.01 w/v % ε-PL. When the ε-PL concentration was increased in this region, (i) the enthalpy change went from highly exothermic to highly endothermic (Figure 1); the electrical charge was highly negative (Figure 2); and (iii) the turbidity remained relatively low (Figures 3 and 4). We postulate that the cationic ε-PL molecules bound to the anionic pectin molecules and formed relatively small soluble primary complexes that did not scatter light strongly. These primary complexes contained considerably more negative charges from pectin than positive charges from polylysine, and therefore, their net charge was highly negative. The formation of larger secondary complexes was inhibited due to the relatively high negative charge on the primary complexes, which generated a strong electrostatic repulsion between them.

Region II: 0.01−0.03 w/v % ε-PL. When the ε-PL concentration was increased in this region, (i) the enthalpy change became increasingly less exothermic (Figure 1); the electrical charge on the complexes became relatively small and close to zero

(Figure 2); and (iii) the turbidity was relatively high (Figures 3 and 4). In this case, the primary complexes contained approximately equal amounts of negative charges from pectin and positive charges from polylysine. We postulate that many ε-PL−pectin primary complexes associated together to form large secondary complexes that scattered light strongly. These large complexes were able to form because of the relatively low net charge on the primary complexes, which meant that the electrostatic repulsion between them was weak. The resulting complexes were large enough to sediment to the bottom of the test tubes and form a coacervate phase because they were denser than the surrounding water.

Region III: >0.03 w/v % ε-PL. When the ε-PL concentration was increased in this region, (i) the enthalpy change remained close to zero (Figure 1); the electrical charge on the complexes became increasingly positive (Figure 2); and (iii) the turbidity decreased (Figures 3 and 4). In this case, the primary complexes contained considerably more positive charges from polylysine than negative charges from pectin, and therefore, their net charge was highly positive. We postulate that large ε-PL−pectin complexes dissociated at higher polylysine concentrations because the positive charge on the primary complexes became increasingly large, which would have generated a strong electrostatic repulsion between them. Presumably, the large secondary complexes would completely dissociate at sufficiently high polylysine concentrations (as observed when the experiments were carried out with lower pectin concentrations in the reaction cell (Figure 3)).

In summary, the complexes contain an excess of anionic groups from pectin in Region I, a fairly even number of anionic groups from pectin and cationic groups from polylysine in Region II, and an excess of cationic groups from polylysine in Region III. The analytical methods used in this study were not able to provide detailed information about the conformation or structural organization of the two different kinds of biopolymer within the complexes, but this would certainly be a useful area of research in future studies.

Influence of Ionic Strength on ε-PL−Pectin Interactions. Salts are known to screen electrostatic interactions in aqueous solutions and would therefore be expected to influence the formation and properties of electrostatic complexes.[29−31,33] Practically, it is important to understand how ionic strength influences the functional properties of ε-PL−pectin complexes since different food products contain different levels of salt. For this reason, we examined the influence of NaCl on the formation and properties ε-PL−pectin complexes (pH 3.5, 30 °C).

In the absence of ε-PL, the ζ-potential of pectin (0.3% w/v) was highly negative at 0 mM NaCl but became increasingly less negative as more salt was added (Figure 6), which can be attributed to electrostatic screening effects, i.e., accumulation of sodium ions around the negative groups on the pectin molecules. In the presence of ε-PL, the ζ-potential of the polylysine (0.0162% w/v)−pectin (0.3% w/v) complexes was highly positive at 0 mM NaCl but became less positive and then negative as more salt was added (Figure 6). Measurements of the effects of salt on the ζ-potential of 0.0162% w/v polylysine could not be made since the signal was too small to measure. The charge reduction and reversal observed in the mixed system can be attributed to a combination of electrostatic screening effects (charge reduction) and complex dissociation (charge reversal).

The ζ-potential versus polylysine concentration profile was measured at different salt concentrations (Figure 7a), and the

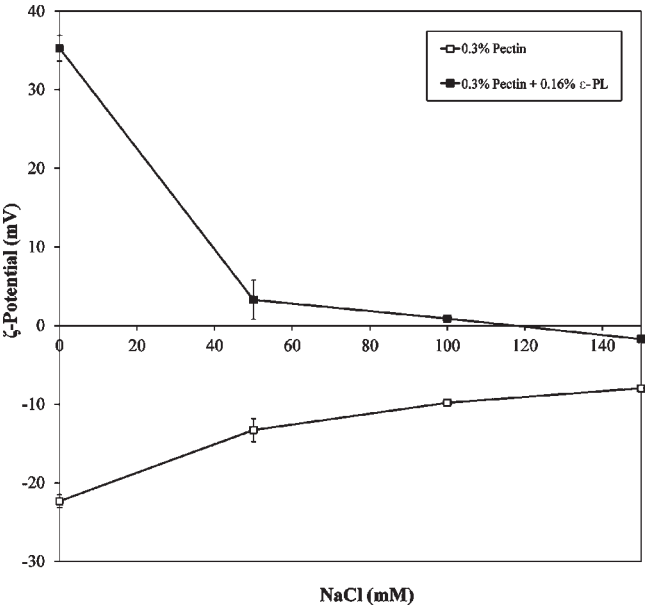

Figure 6. Influence of ionic strength on the ζ-potential of pectin (0.3% w/v) and ε-PL−pectin (0.0162% w/v−0.3% w/v) mixture at pH 3.5.

amount of ε-PL required to neutralize the negative charge on pectin (0.3% w/v) was determined from these profiles (Figure 7b). In the absence of added NaCl, the ζ-potential went from highly negative to highly positive as increasing amounts of ε-PL were titrated into the pectin solution, which was attributed to complex formation as discussed earlier. At higher NaCl concentrations, the ζ-potential still became less negative as increasing amounts of polylysine were added to the system, but the magnitude of the change was much less than in the absence of salt. This effect can be partly attributed to the ability of salts to reduce the ζ-potential of particles through electrostatic screening, but it may also be because fewer polylysine molecules bound to the pectin molecules due to the reduction in electrostatic attraction in the presence of salt. The ε-PL concentration needed to neutralize 0.3% w/v pectin increased with increasing ionic strength (Figure 7b). We postulate that the electrostatic attraction between the pectin and polylysine was weakened in the presence of NaCl, which meant that more ε-PL had to be added to form a complex with pectin with a specific stoichiometry, thereby a higher concentration was required to reach charge neutralization.

The change in turbidity was measured when ε-PL solution (1 w/v %, pH 3.5) was titrated into pectin solutions (0.3 w/v%, pH 3.5) containing different salt levels, i.e., 0 to 150 mM NaCl (Figure 8). Our measurements clearly show that ionic strength appreciably affected the formation and properties of ε-PL−pectin complexes. At low ionic strength (0 mM NaCl), there was a large maximum in the turbidity versus ε-PL profile corresponding to the formation of large complexes. At intermediate ionic strengths (50 or 100 mM NaCl), the mixed solutions were still turbid, but the height and position of the maximum turbidities were different than in the absence of salt. The reduction in the height of the turbidity maximum suggests that smaller complexes were formed. At high ionic strength (150 mM NaCl), all the ε-PL−pectin solutions were either transparent or only slightly turbid, which suggested that the formation of large complexes had been completely suppressed.

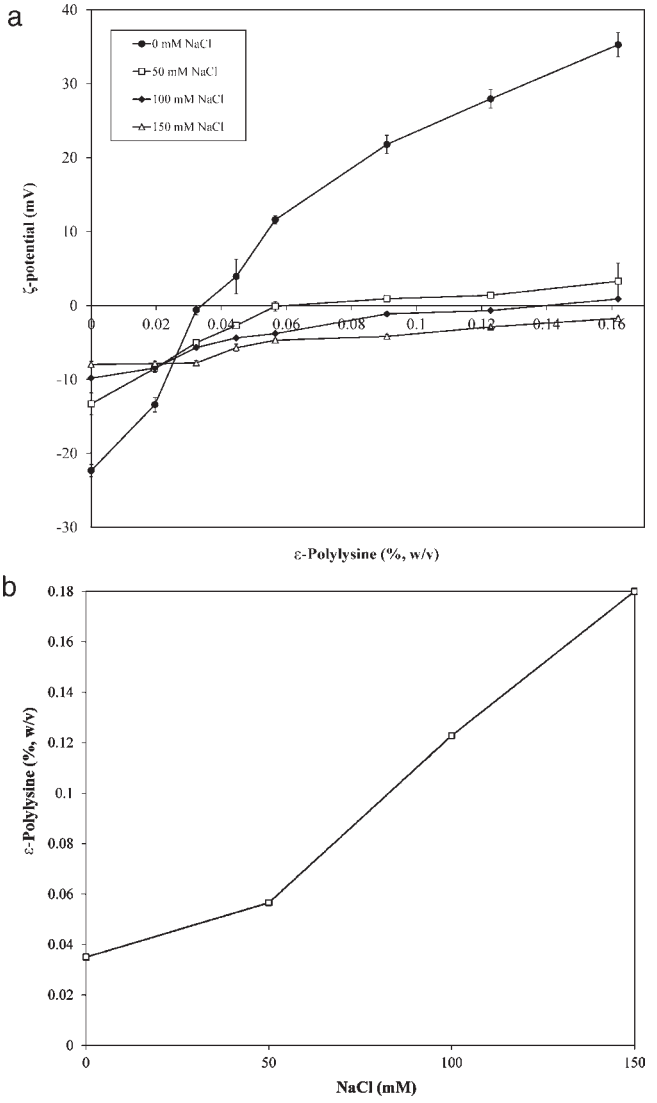

Figure 7. (a) Influence of ionic strength on the dependence of the ζ-potential of aqueous polylysine−pectin solutions on the ε-PL concentration in the reaction cell. An ε-PL−pectin solution (1.0 wt %, pH 3.5) was titrated into a pectin solution (0.3 wt %, pH 3.5) containing different amounts of NaCl. (b) Critical concentration of ε-PL needed to neutralize pectin (0.3 wt %) at pH 3.5 under different ionic strengths (0 to 150 mM NaCl).

The visual appearance of the samples after 1 day of storage also depended on the salt and ε-PL levels present (data not shown). Transparent or slightly turbid solutions were formed at low ε-PL concentrations, opaque colloidal dispersions or sediments were formed at intermediate ε-PL concentrations, and transparent solutions or slightly turbid dispersions were formed when the ε-PL concentrations were sufficiently high. The precise ε-PL concentrations demarcating the low, intermediate, and high regions depended on salt concentration.

These results indicate that the addition of salts altered the formation and properties of the electrostatic complexes, presumably because the attractive interactions between the cationic ε-PL and anionic pectin molecules were weakened through electrostatic screening effects. Our observations are in agreement with previous studies of the impact of salts on the interactions of oppositely charged biopolymers.[29−31]

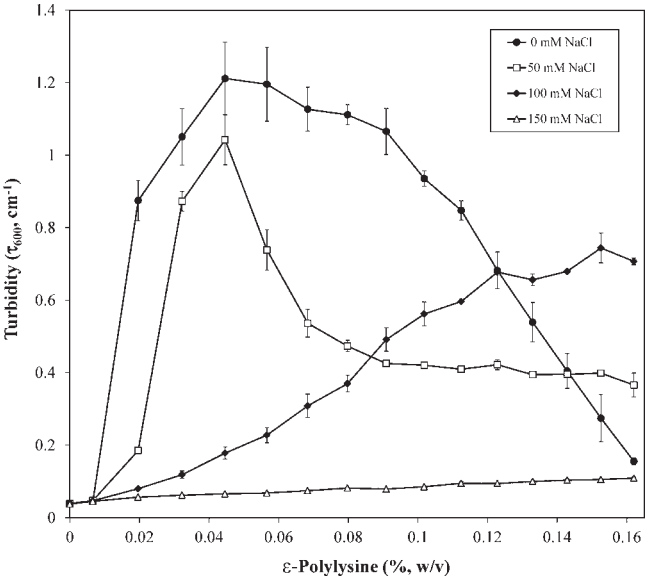

Figure 8. Influence of ionic strength (0−150 mM NaCl) on the turbidity of ε-PL−pectin solutions. An ε-PL solution (1.0 wt %) was titrated into aqueous pectin solutions (0.3 wt % pectin) at pH 3.5, containing 0−150 mM NaCl.

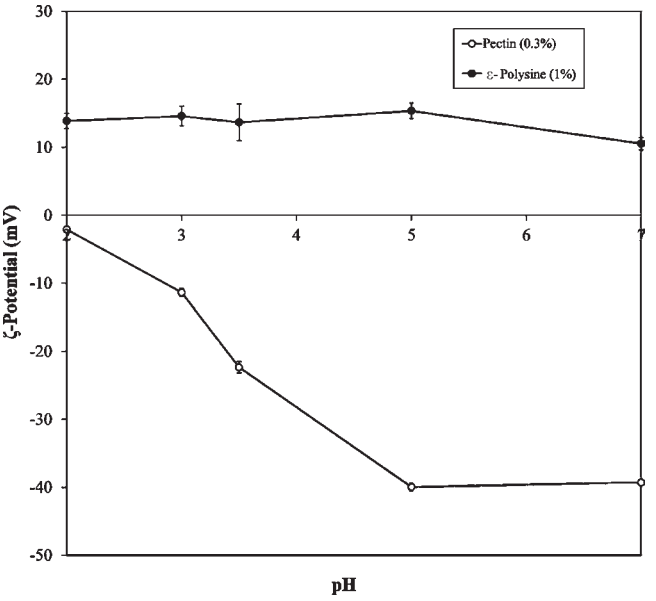

Figure 9. Influence of pH on the ζ-potential of pectin (0.3 wt %) and ε-PL (1 wt %). A relatively high concentration of polylysine was needed for these measurements since it did not scatter light strongly.

Influence of pH on ε-PL−Pectin Interactions. The electrical characteristics of biopolymers are often affected by pH, which may influence the formation and properties of electrostatic complexes. We therefore studied the influence of pH on the electrical properties and aggregation behavior of individual biopolymers and ε-PL−pectin mixtures (0 mM NaCl, 30 °C).

Measurements of the pH-dependence of the ζ-potential of the individual biopolymers were initially carried out. Polylysine remained highly positive from pH 7 to 2 (Figure 9), which can be attributed to the fact that the amino groups have a $pK_a \approx 9$ so that they kept their full positive charge across the entire pH range

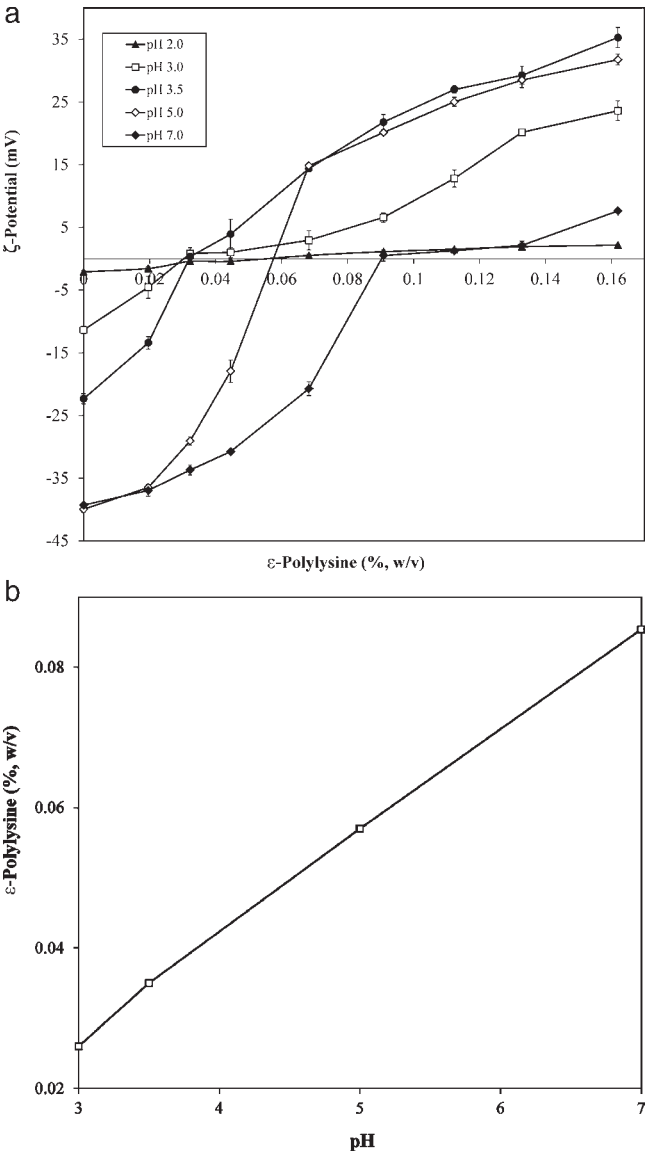

Figure 10. (a) Dependence of the ζ-potential of aqueous polylysine−pectin solutions on the ε-PL concentration in the reaction cell. An ε-PL−pectin solution (1.0 wt %) was titrated into pectin solutions (0.3 wt %) at different pH values. (b) Concentration of ε-PL needed to neutralize pectin (0.3 wt %, 0 mM NaCl) at varying pH (3−7).

studied. However, pectin was highly negative from pH 7 to 5 but became increasingly less negative when the pH was reduced further, until at pH 2 it had a charge close to zero (Figure 9). This effect can be attributed to the fact that the carboxyl groups on pectin have a $pK_a \approx 3.5$, and therefore, they lose most of their negative charge when the pH falls below this value.

The ζ-potential versus polylysine concentration was measured at a number of different pH values (Figure 10a). The concentration of ε-PL required to neutralize the negative charge on 0.3% w/v pectin was determined from these curves (Figure 10b). The ζ-potential went from negative to positive with increasing polylysine concentration at most pH values, but the shape of the curves depended on pH. At pH 2, there was little change in ζ-potential with polylysine concentration (Figure 10a), which can be attributed to the fact that the pectin had little negative charge at this pH so that it did not interact with the cationic polylysine.

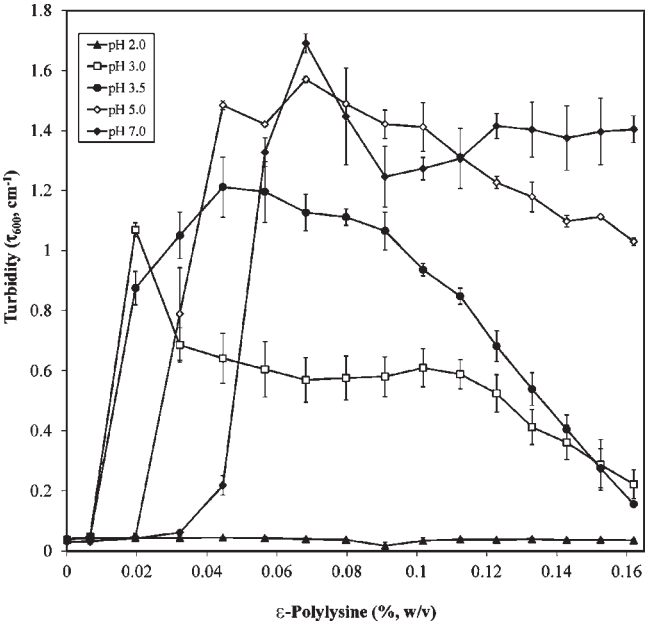

Figure 11. Influence of pH on the turbidity of ε-PL−pectin solutions. An ε-PL solution (1.0 wt %) was titrated into aqueous pectin solutions (0.3 wt % pectin) with pH values from 2 to 7.

At higher pH values, the negative charge on the pectin molecules was appreciable, which promoted complex formation, as seen by the ζ-potential becoming less negative and then positive (Figure 10b). Interestingly, there were some significant differences in the electrical properties of the complexes depending on pH. At pH 5 and 7, the pectin molecules had fairly similar negative charges in the absence of polylysine, but the ζ-potential of the complexes at high ε-PL concentrations was strongly negative at pH 5, but only slightly negative at pH 7 (Figure 10a). This suggests that there was some alteration in the nature of the complexes formed at the higher pH values. The ε-PL concentration required to neutralize the pectin increased with increasing pH (Figure 10b), which can also be attributed to changes in the electrical characteristics of pectin. The negative charge on the pectin molecules was greater at high than at low pH (Figure 9), and therefore, more cationic ε-PL molecules would be required to neutralize the charge on the pectin at higher pH values. Almost all the carboxylic groups ($pK_a \approx 3.5$) on pectin would be fully dissociated (negative) at pH 5 and higher, which accounts for the fact that the measured electrical charge on the pectin molecules did not change much from pH 5 to 7 (Figure 10a). However, the electrical charge on the cationic ε-PL molecules decreased slightly when the pH was increased from 5 to 7 (Figure 9), which may account for the fact that more ε-PL was needed to neutralize pectin at pH 7, and the difference in the charge characteristics of the complexes at pH 5 and 7 at high ε-PL concentrations.

The changes in turbidity when increasing amounts of polylysine were titrated into pectin solutions were also measured at different pH values (Figure 11). At pH 2, the solutions remained transparent at all ε-PL levels added, which suggested that no large complexes were formed. This phenomenon can be attributed to the fact that the pectin molecules lost most of their negative charge at pH 2 (Figure 9) and hence could not form electrostatic complexes with cationic polylysine. At higher pH values, there was evidence of complex formation at certain polylysine

concentrations, as indicated by an increase in turbidity (Figure 11). In general, the turbidity versus polylysine−pectin interactions occurred: (i) at low ε-PL, the solutions were transparent or only slightly turbid; (ii) at intermediate ε-PL, the solutions were highly turbid; (iii) at high ε-PL, the turbidity of the solutions decreased somewhat. As discussed earlier, large complex formation is inhibited in Regions I and III because the ε-PL−pectin primary complexes have either a high negative or a high positive charge (respectively) so that there is a strong electrostatic repulsion between them. However, large complexes are formed in Region II because the ε-PL−pectin primary complexes only have a low net charge, and therefore, they can further associate with each other. As the pH was increased, the concentration range where the mixed solutions remained transparent increased (Region I), and the ε-PL concentration where the solutions had a maximum turbidity increased (Region II). These effects can be attributed to the fact that the negative charge on the pectin molecules increased with increasing pH, and therefore, more polylysine molecules were needed to reach charge neutralization. At pH 3 and 3.5, the turbidity decreased appreciably with increasing polylysine concentration at the higher ε-PL concentrations (Region III), which can be attributed to some dissociation of the complexes due to the relatively high positive charge on the polylysine−pectin primary complexes. At higher pH values, the turbidity only decreased slightly with increasing ε-PL concentrations in Region III, presumably because more polylysine molecules were needed to bind to the pectin molecules and form strongly positively charged complexes. These observations are in agreement with previous studies of the impact of pH on the formation of electrostatic complexes from other types of oppositely charged biopolymers.[29−31]

In summary, this study used isothermal titration calorimetry (ITC), microelectrophoresis (ME), turbidity measurements, and visual observations to characterize the interactions between cationic ε-PL molecules and anionic pectin molecules. The main results of this study are the following:

(1) ITC and ME measurements indicated that ε-PL bound to pectin, while turbidity measurements and visual observation indicated that the complexes formed could be either soluble or insoluble depending on solution composition. The cationic ε-PL bound to the anionic pectin and formed a molecular complex, with charge neutralization occurring at a ε-PL-to-pectin mass ratio of ≈0.1:1.

(2) The nature of the complexes formed depended on the ratio of ε-PL-to-pectin molecules since this determined the overall electrical characteristics of the system. When pectin was present in high excess, small anionic electrostatic complexes were formed that led to transparent solutions. At intermediate polylysine-to-pectin ratios, large complexes were formed with a low net charge, which led to the formation of turbid or opaque systems that were often prone to sedimentation. When polylysine was in high excess, small cationic electrostatic complexes were formed that led to transparent solutions.

(3) The formation and properties of ε-PL−pectin complexes were highly dependent on solution ionic strength and pH due to the impact of these factors on electrostatic interactions. The electrostatic interactions in the mixed systems were screened by adding salt (0 to 150 mM NaCl). Adding low levels of salt (50 and 100 mM), partially suppressed the formation of ε-PL−pectin complexes, as seen by changes in the height and position of the maximum turbidity in turbidity versus ε-PL profiles. The addition of high levels of salt (150 mM NaCl), almost completely suppressed the formation of electrostatic complexes, presumably by its ability to weaken electrostatic attractive forces. Solution pH also affected the formation of the electrostatic complexes by altering the electrically properties of the individual biopolymers. In general, the ε-PL−pectin interactions became stronger at higher pH values because the negative charge on the pectin molecules increased.

The results of this study have provided an improved understanding of the interactions between cationic ε-PL and anionic pectin in aqueous solutions, which will prove useful in the rational application of ε-polylysine as a functional ingredient in food systems. In particular, this study has shown that polylysine will interact with oppositely charged polysaccharides in aqueous food products. These interactions may cause the product to become turbid or opaque and may even lead to sedimentation of the electrostatic complex formed. These changes in appearance and physical stability would have an adverse impact on the quality attributes of many products, e.g., beverages that should remain homogeneous and transparent (or only slightly cloudy) during storage. In addition, the formation of these complexes may reduce the antimicrobial efficacy of polylysine so that it becomes less effective at preventing microbial growth. We intend to examine the impact of electrostatic complex formation in antimicrobial activity of cationic antimicrobials in future studies. This study shows that the potential for complex formation should be taken into account when designing food systems containing cationic antimicrobials.

AUTHOR INFORMATION

Corresponding Author

*Phone: 413 545 1019. Fax: 413 545 1262. E-mail: mcclements@foodsci.umass.edu.

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