Interaction of α-Gliadin with Polyanions: Design Considerations for Sequestrants Used in Supportive Treatment of Celiac Disease

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ABSTRACT:

Copolymers of sodium 4-styrene sulfonate (SS) and hydroxyethyl methacrylate (HEMA) were investigated as sequestrants of α-gliadin, a gluten protein, for the treatment of gluten intolerance. The interactions of α-gliadin with poly(SS) and poly(HEMA-co-SS) with 9 and 26 mol% SS content were studied at gastric (1.2) and intestinal (6.8) pH using circular dichroism and measurements of turbidity, dynamic light scattering and zeta potential. The interactions and their influence on α-gliadin secondary and aggregated structures depended mainly on the ratio of polymer negative and protein positive charges at pH 1.2, and on polymer SS content at polymer concentrations providing in excess of negative charges at either pH. Poly(SS) could not form complex particles with α-gliadin in a sufficient excess of negative charges. Copolymerization with HEMA enhanced the formation of complex particles. Poly(HEMA-co-SS) with intermediate SS content was found to be the most effective sequestrant for α-gliadin. This study provides insight into design considerations for polymer sequestrants used in the supportive treatment of celiac disease. © 2009 Wiley Periodicals, Inc. Biopolymers 93: 418–428, 2010.

Keywords: poly(HEMA-co-SS); α-gliadin; protein structure; complex particle; polymeric sequestrant

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INTRODUCTION

Celiac disease is a chronic inflammatory disorder characterized by villous atrophy, crypt hyperplasia, and inflammation in the small intestine as well as more general symptoms such as chronic diarrhea, abdominal distension and pain, failure to thrive, weight loss, anemia, fatigue, and depression.[1–3] The disease is triggered by intolerance to gluten from wheat and other grains in genetically susceptible individuals. A gluten subfraction called α-gliadin is known to be the predominant factor in gluten toxicity. This protein is resistant to complete digestion into amino acids by gastric and intestinal proteases, due to the abundance of proline residues in its polypeptide chain. Specific peptide fragments of α-gliadin have been identified as possible triggers of the response to gluten in celiac disease patients.[2,4,5] The expression of celiac disease depends strictly

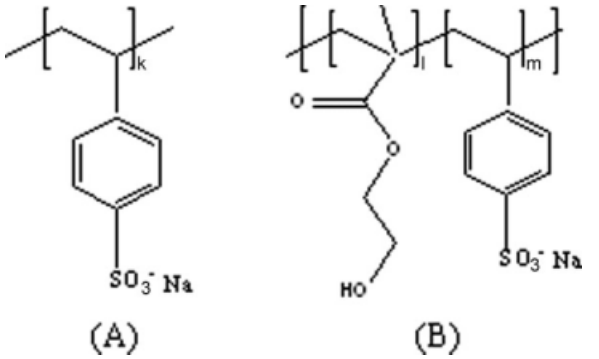

FIGURE 1 Structure of poly(SS) and poly(HEMA-*co*-SS).

on dietary exposure to gluten. The only known effective treatment is a lifelong, strict adherence to a gluten-free diet.[1] However, adherence to such a diet is difficult, since gluten is often used as an additive in processed foods.

High molecular weight polymers have been widely used as sequestrants for lowering the concentration of several endogenous molecules in blood.[6,7] Polyamines can be used to sequester anions such as phosphates and bile acids and eliminate them from the gastrointestinal tract. Sodium 4-styrene sulfonate (SS) homopolymer (Figure 1A) has been reported to sequester toxins A and B of *Clostridium difficile*, a cause of antibiotic-associated diarrhea in hospitals.[8]

We have recently shown that SS homopolymer destroyed intestinal epithelial cell (IEC) morphology when incubating the polymer with IEC-6 cells. The toxic effects could be eliminated by inclusion of hydroxyethyl methacrylate (HEMA) units in the polymer composition (Figure 1B). The resulting copolymer, poly(HEMA-*co*-SS), inhibited α-gliadin-induced alterations in intestinal cell morphology and hindered immunogenic peptide formation and reduced the toxicity of α-gliadin in vivo.[9] Copolymers of this type could therefore be useful in the treatment of gluten intolerance.

We have also shown previously that electrostatic interaction occurs between poly(HEMA-*co*-SS) and α-gliadin at gastric (1.2) and intestinal (6.8) pH.[10] Copolymerization of SS with HEMA may be controlled to obtain variations in the resulting polymer charge density, which has been reported recently to affect polymer interaction with proteins.[11,12] Moreover, the inclusion of HEMA units may affect hydrophobic interaction between SS phenyl and protein hydrophobic residues. In this work, interactions between α-gliadin and polymers with different SS and HEMA contents were studied at gastric and intestinal pH using circular dichroism (CD), turbidity, dynamic light scattering (DLS), and zeta potential measurements. The data gathered from these experiments provide insight into protein/polymer binding mechanisms and could be useful in the design of efficient α-gliadin sequestrants.

EXPERIMENTAL SECTION

Materials

Wheat α-gliadin was kindly supplied by professor Popineau of the Institut National de la Recherche Agronomique (Nantes, France). SS homopolymer (PSS) and its random copolymers with HEMA (PHS1 and PHS2) were synthesized by atom transfer radical polymerization as described previously.[9,13] Their characteristics are summarized in Table I.

Sample Preparation

Stock solutions of α-gliadin were prepared at a protein concentration of 5 g/L in 70% ethanol or 0.06*M* HCl (pH 1.2). Stock solutions of polymers were also prepared at a concentration of 5 g/L, in 0.06*M* HCl (pH 1.2) and 10 m*M* sodium phosphate buffer (pH 6.8). Mixtures of 150 mg/L α-gliadin with polymer at the concentrations varied from 7 to 500 mg/L at pH 1.2 were prepared by adding undiluted acidic protein stock solution to diluted acidic polymer solution. Mixtures with final concentrations of polymers varied from 1 to 500 mg/L at pH 6.8 were prepared by adding undiluted alcoholic protein stock solution to diluted phosphate buffer polymer solution. All mixtures were held for about 2–3 h at room temperature before analysis. All the samples including α-gliadin were prepared using Eppendorf tubes and pipette tips allowing maximum recovery and minimal fluid retention (Axygen Scientific, Union City, CA).

Circular Dichroism

CD spectra were recorded on a Jasco J-710 spectropolarimeter (Jasco, Easton, MD). The path length was 0.1 cm and the 190–250 nm region was scanned. Ellipticity was recorded at a speed of 100 nm/min, 0.2-nm resolution, 10 accumulations, and 1.0-nm bandwidth. Buffer background was subtracted from the raw spectra.

Turbidity Measurement

Turbidity (100 – %T) of α-gliadin/polymer mixtures was determined from the apparent absorbance at 600 nm measured using a HP 8453 UV–Visible spectrophotometer (Agilent Technologies, Santa Clara, CA). The samples were shaken for 1 min to disperse the samples completely before each measurement.

Table I Polymer Characteristics

Sample	Abbreviation	M_w (Da)	SS Content (mol%)
Poly(SS)	PSS	57,500	100
Poly(HEMA-*co*-SS)	PHS1	65,100	26
	PHS2	53,100	9

Dynamic Light Scattering

Particle diameters were measured by quasi-elastic light scattering using a Nicomp 370 Submicron Particle Sizer (Pacific Scientific Division, Hiac-Royce Instruments, Menlo Park, CA). All measurements were done at 23°C and at a scattering angle of 90°. The measured time correlation functions were analyzed in volume-weighted Gaussian analysis mode.

Zeta Potential Measurement

Zeta potentials of α-gliadin/polymer complex particles were examined using a ZetaSizer 2000 (Malvern Instruments, Worcestershire, UK). All measurements were conducted at 25°C. Zeta potentials were calculated automatically from electrophoretic mobility according to the Henry equation and the Smoluchowski approximation.

RESULTS

Influence of Polymers on α-Gliadin Secondary Structure Characterized by CD

At pH 1.2. Gliadin contains a nonrepetitive domain rich in α-helical structure and a heterogeneous repetitive domain rich in β-reverse turns.[14] Figure 2 shows far-UV CD spectra of α-gliadin as a function of the concentration of PSS (A), PHS1 (B), and PHS2 (C), measured at pH 1.2, and at a protein concentration of 150 mg/L. The spectra were collected until a polymer concentration giving a high voltage of up to 600 V at wavelengths below 222 nm was reached. For clarity, only typical far-UV CD spectra are shown. In the absence of polymer, the spectrum exhibited two partly overlapping negative bands with a 222 nm band appearing as a shoulder on a stronger 208 nm band, suggesting that the pure protein adopted an α-helix conformation.[15] At PSS concentrations ranging from 7 to 25 mg/L, both bands disappeared (Figure 2A). As the polymer concentration was increased to 30 mg/L, a new negative band, attributed to β-turn,[10] appeared around 230 nm. With further increases in the PSS concentration, the 230-nm band ellipticity stopped increasing, and the α-helix bands became dominant again. At 73 and 125 mg/L, spectra were almost super-imposable and characterized by a slightly stronger 222-nm band and slightly weaker 208-nm band compared to the pure protein. Above PSS concentrations of 125 mg/L, the spectra became too noisy to analyze α-gliadin structure.

In the presence of PHS1 (26 mol% SS), the α-helix bands gradually flattened and the 208-nm band underwent a 2-nm blue shift as the copolymer concentration increases to 13 mg/L (Figure 2B). From 25 to 125 mg/L, the copolymer induced α-gliadin structural changes somewhat analogous to those obtained with PSS between 7 and 125 mg/L, but appearing at higher concentrations and the longer wavelength. At 125 mg/L, two strong negative bands were present around 211 and 226 nm. The intensity of these bands instead decreased upon further increasing PHS1 concentration to 250 mg/L.

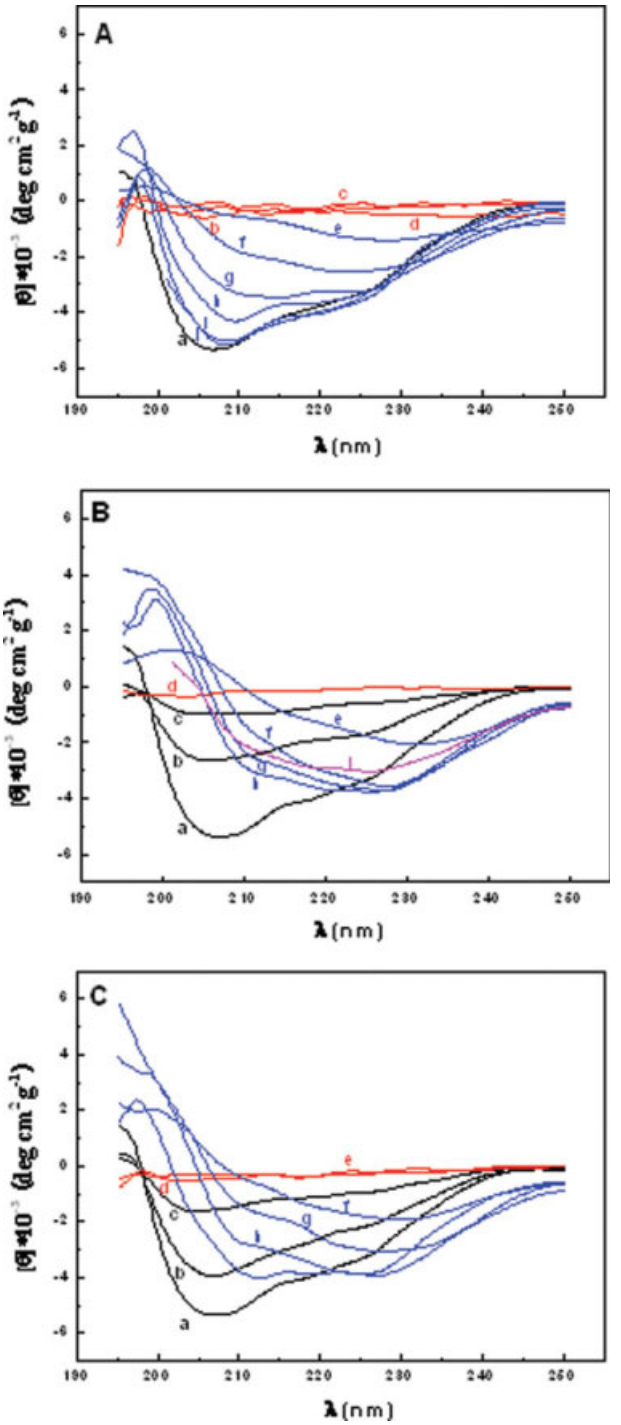

FIGURE 2 Far-UV CD spectra of α-gliadin at 150 mg/L and pH 1.2 in the absence and presence of various concentrations of (A) PSS: (a) 0, (b) 7, (c) 13, (d) 25, (e) 30, (f) 37, (g) 43, (h) 50, (i) 73, (j) 125 mg/L; (B) PHS1: (a) 0, (b) 7, (c) 13, (d) 25, (e) 30, (f) 37, (g) 73, (h) 125, (i) 250 mg/L; and (C) PHS2: (a) 0, (b) 13, (c) 37, (d) 73, (e) 125, (f) 200, (g) 250, (h) 333, (i) 500 mg/L.

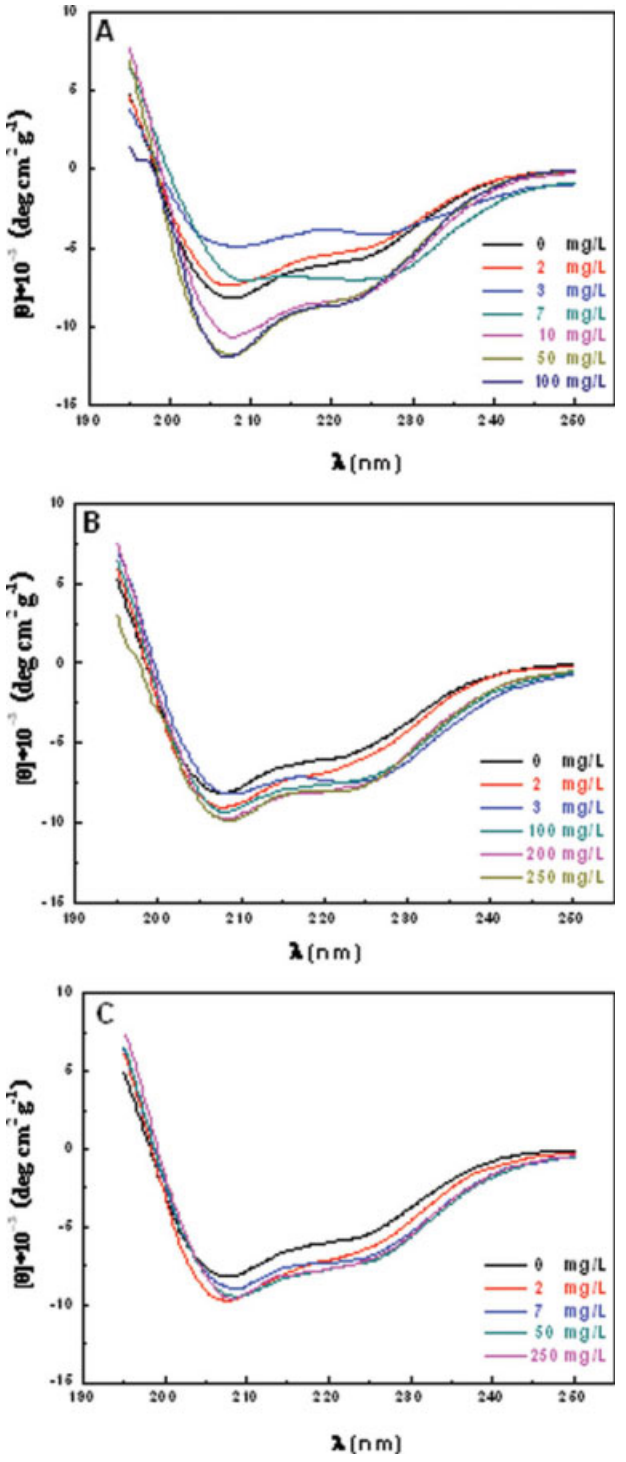

FIGURE 3 Far-UV CD spectra of α-gliadin at 150 mg/L and pH 6.8 in the absence and presence of (A) PSS, (B) PHS1, and (C) PHS2 at various concentrations from 2 to 250 mg/L.

The impact of PHS2 (9 mol% SS) on α-gliadin secondary structure (Figure 2C) was similar to that of PHS1 below 125 mg/L, but occurred at higher concentrations (i.e., disappearance of α-helix bands between 73 and 125 mg/L). At 500 mg/L, the structure of PHS2-bound α-gliadin (ratio of ellipticities $\theta_{208}/\theta_{222}$ of about 1.0) was more similar to that of the pure protein ($\theta_{208}/\theta_{222} = 1.5$) than PHS1-bound protein at 125 mg/L ($\theta_{208}/\theta_{222} = 0.8$). However, PSS-bound protein at 125 mg/L ($\theta_{208}/\theta_{222} = 1.4$) was more similar to that of the pure protein than the PHS2-bound α-gliadin structure.

At pH 6.8. Figure 3 shows far-UV CD spectra of α-gliadin at 150 mg/L and pH 6.8 with increasing amounts of PSS (Figure 3A), PHS1 (Figure 3B), and PHS2 (Figure 3C). For clarity, only typical far-UV CD spectra are shown. In the absence of polymers, the spectral profile characterizing the α-helix was stronger at neutral than acidic pH, due to side-chain repulsion among positively charged basic amino acid residues causing the protein to unfold at acidic pH.[15,16] As PSS concentration increased at pH 6.8, the 208-nm and 222-nm bands first shifted to longer wavelengths, reaching 209 and 226 nm at 7 mg/L, only to return to their original positions at 10 mg/L (Figure 3A). Ellipticity decreased to a minimal value at 3 mg/L and then increased, finally ending up at a value greater than that for the pure protein (Figure 3A). In contrast, PHS1 and PHS2 increased the 208-nm and 222-nm band intensities slightly at 2 mg/L (Figures 3B and 3C). At higher concentrations, both exerted effects similar to those of PSS, but the overall effect was less pronounced for the lower SS content (i.e., the less pronounced intensification of the α-helix profile at the highest concentrations).

As can be seen in Figure 3, the ellipticities around 208 and 222 nm did not change in the same manner. The $\theta_{208}/\theta_{222}$ ratio at pH 6.8 is plotted as a function of polymer concentration in Figure 4. As PSS concentration increased, the ratio first decreased, reaching a minimum of 1.0 at 7 mg/L. It then increased and leveled off near the value obtained with the

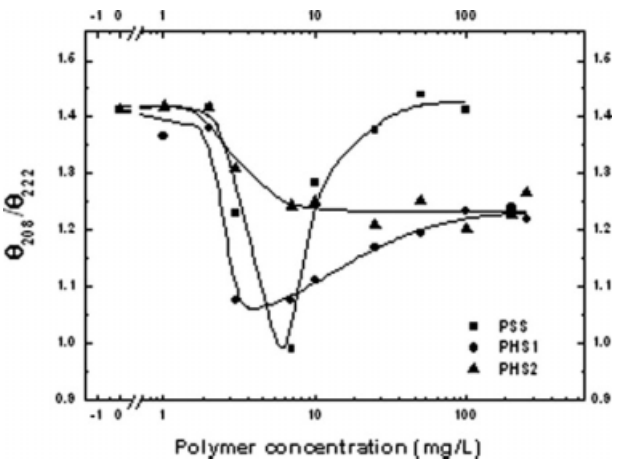

FIGURE 4 Ratio of ellipticities around 208 and 222 nm in far-UV CD spectra of α-gliadin at 150 mg/L and pH 6.8 in the presence of PSS (■), PHS1 (●), and PHS2 (▲) at various concentrations.

pure protein ($\theta_{208}/\theta_{222}$ = 1.4) at PSS concentrations above 50 mg/L. In the case of PHS1, the ratio also followed a profile approaching the inverted bell shape, but reached a plateau at about 1.2 at 100 mg/L. With PHS2, the ratio profile was different, decreasing monotonously and reaching a plateau of about 1.2 at 7 mg/L.

Analysis of α-Gliadin/Polymer Mixture by Turbidity

The turbidity of α-gliadin/polymer mixtures was measured to characterize the formation of protein–polymer complex particles. At pH 1.2, the 150 mg/L α-gliadin pure solution was transparent (Figure 5A). As the concentrations of PSS or poly(HEMA-*co*-SS) were raised, the turbidity first increased and then decreased. The maxima were at polymer concentrations of 10 mg/L for PSS, 25 mg/L for PHS1, and 125 mg/L for PHS2, corresponding to the disappearance of the two negative bands around 208 and 222 nm in Figure 2. The turbidity was zero at PSS concentrations above 50 mg/L, indicating the absence of phase separation and α-gliadin/PSS particle formation. In the case of PHS1 and PHS2, residual turbidities of 18 and 22%, respectively, were measured at the highest copolymer concentrations tested. These data show that either copolymer could form complex particles with the protein over the entire range of concentrations examined at pH 1.2.

At pH 6.8, the solution of pure α-gliadin had a turbidity of about 51% (Figure 5B), indicating that the protein self-aggregated to form particles. The turbidity versus PSS concentration curve peaked at a lower polymer concentration (2 mg/L) at pH 6.8 than at pH 1.2. The turbidity was lower than that of the pure protein when the polymer concentration exceeded 3 mg/L and no turbidity was observed above 10 mg/L. These results indicate that α-gliadin aggregation can be suppressed by interaction with PSS, and PSS/α-gliadin particles form only at the polymer concentrations ≤10 mg/L at pH 6.8. The pattern was noticeably different for poly(HEMA-*co*-SS). In the case of PHS1, turbidity decreased at the polymer concentrations of 1–3 mg/L and then stayed at 18% at higher concentrations. PHS2 did not change the turbidity of the α-gliadin suspension at 1 mg/L, but decreased it at higher concentrations, reaching a plateau of about 18% at 10 mg/L. These data confirm that PHS1 and PHS2 could form complex particles with α-gliadin over the entire range of concentrations examined at pH 6.8.

Analysis of α-Gliadin/Polymer Particle Size by DLS

The size of α-gliadin/polymer complex particles was analyzed by DLS, a technique that has been used widely to investigate the interaction of proteins with polymers.[17–19] At pH 1.2,

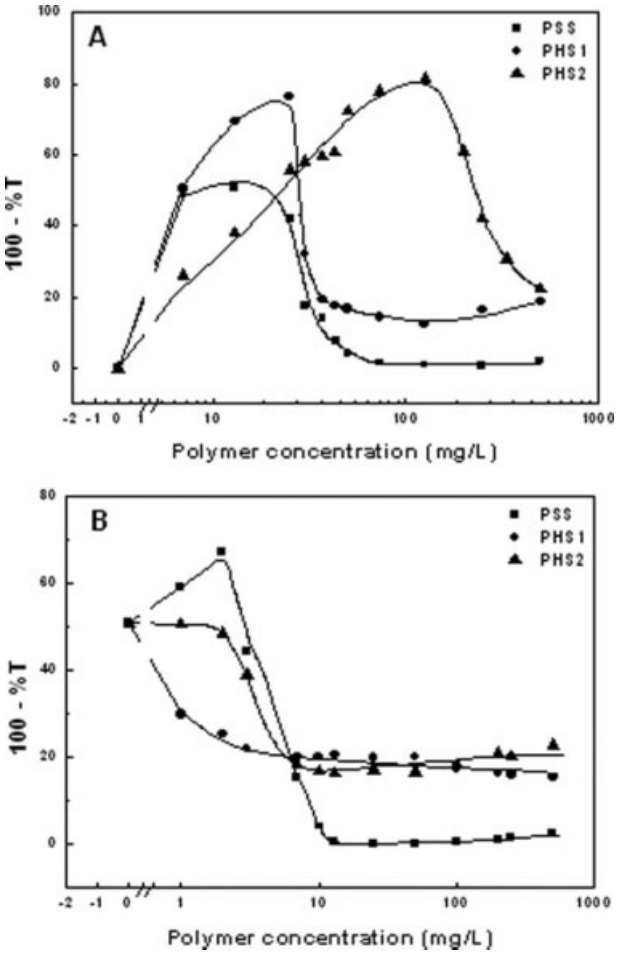

FIGURE 5 Turbidity of 150 mg/L α-gliadin mixtures with increasing amounts of PSS (■), PHS1 (●), and PHS2 (▲) at pH 1.2 (A) and 6.8 (B).

PSS, PHS1, and PHS2 at concentrations, respectively, ≤25, 25, and 125 mg/L produced protein–polymer particles that decreased rapidly in diameter from tens of thousands to a few thousand nanometers over time (data not shown). This may contribute to the initial loss of negative intensity around 208 and 222 nm under such polymer concentration range (see Figure 2).[10] Complex particles were therefore studied only at higher polymer concentrations. Figure 6 shows the distribution of α-gliadin/polymer particle apparent diameter at different polymer concentrations at pH 1.2. The particle diameter gradually decreased from 1260 to 170 nm as PSS concentrations increased from 30 to 50 mg/L (Figure 6A). DLS analysis was not performed at higher PSS concentrations, since no particles formed, as shown earlier by turbidity analysis. A different pattern was obtained with PHS1, the apparent diameter decreasing from 2490 to 290 nm as the copolymer concentrations increased from 30 to 125 mg/L, after which it began to increase, reaching 420 nm at 500 mg/L

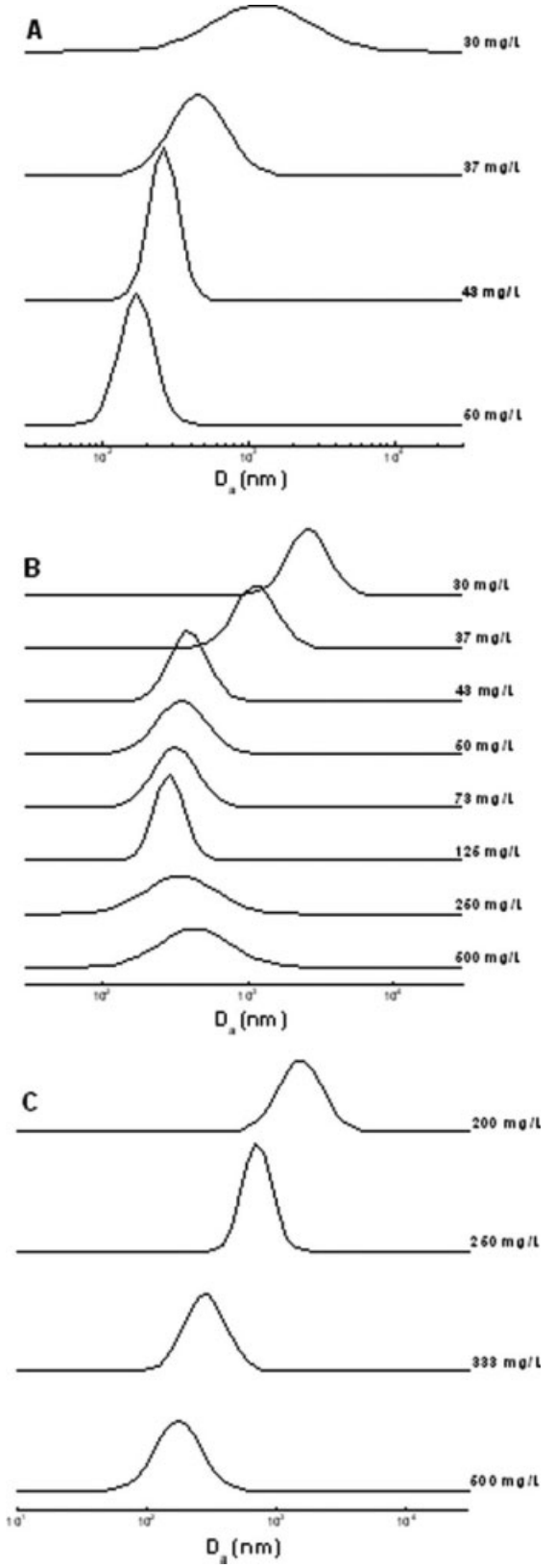

FIGURE 6 Apparent diameter of complex particles of 150 mg/L α-gliadin with (A) PSS, (B) PHS1, and (C) PHS2 at various polymer concentrations and pH 1.2.

(Figure 6B). In the case of PHS2, the diameter of the complex particles decreased from 1650 to 170 nm as the copolymer concentration increased from 200 to 500 mg/L (Figure 6C). The range of effective concentrations for holding α-gliadin in complex particles at pH 1.2 was thus wider for the copolymers than PSS.

Figure 7 shows the distribution of α-gliadin/polymer particle diameter at different polymer concentrations at pH 6.8. At this pH, the diameter of pure α-gliadin particles was about 2600 nm. At 1 mg/L PSS (Figure 7A), the diameter increased slightly to about 2790 nm. As the PSS concentration increased, the particle diameter dropped, reaching 285 nm at 10 mg/L. DLS analysis was not performed above 10 mg/L since no particles formed, as shown earlier by turbidity analysis. In the case of PHS1, the decrease in particle size was already detected at 1 mg/L, the minimum of about 435 nm being reached at 7 mg/L (Figure 7B). With PHS2, the particle diameter decreased almost imperceptibly (to 2590 nm) at 1 mg/L (Figure 7C). The α-gliadin/PHS2 particle diameter then began to decrease as the copolymer concentrations increased, reaching a minimal plateau value close to 435 nm from 7 to 250 mg/L. These data support that copolymerization with HEMA improved the formation of complex particles with α-gliadin, compared to PSS, at concentrations above 10 mg/L at pH 6.8.

Zeta Potential Analysis of α-Gliadin/Polymer Particles

At pH 1.2, the α-gliadin polypeptide carries nine positive charges (one Lys + four His + four Arg),[20] while each PSS, PHS1, and PHS2 chain bears, respectively, 279, 113, and 34 negatively charged sulfonate groups. Electrostatic attraction can therefore occur between the protein and the polymers. Figure 8A shows the change in zeta potential of α-gliadin/polymer complex particles with increasing amounts of polymers at pH 1.2. With each polymer, the zeta potential decreased from a positive to negative value with the surface charge reversal basically consistent with the calculated theoretical stoichiometric equivalence. The polymer concentration, at which surface charge reversal occurred, increased as the SS content decreased (i.e., between 7 and 13 mg/L PSS, 13 and 25 mg/L PHS1, and 50 and 73 mg/L PHS2). The ζ-potential dropped to about −27.8 mV above 30 mg/L PSS, −27.5 mV above 50 mg/L PHS1, and −12.8 mV above 200 mg/L PHS2. At the highest concentration shown in Figure 8A, the negative charges of PSS, PHS1, and PHS2 are about 6, 20, and 7 times of α-gliadin positive charges, respectively, at pH 1.2. In the case of most excess negative charges, zeta potential of −11.6 mV for PHS1 at 500 mg/L in the

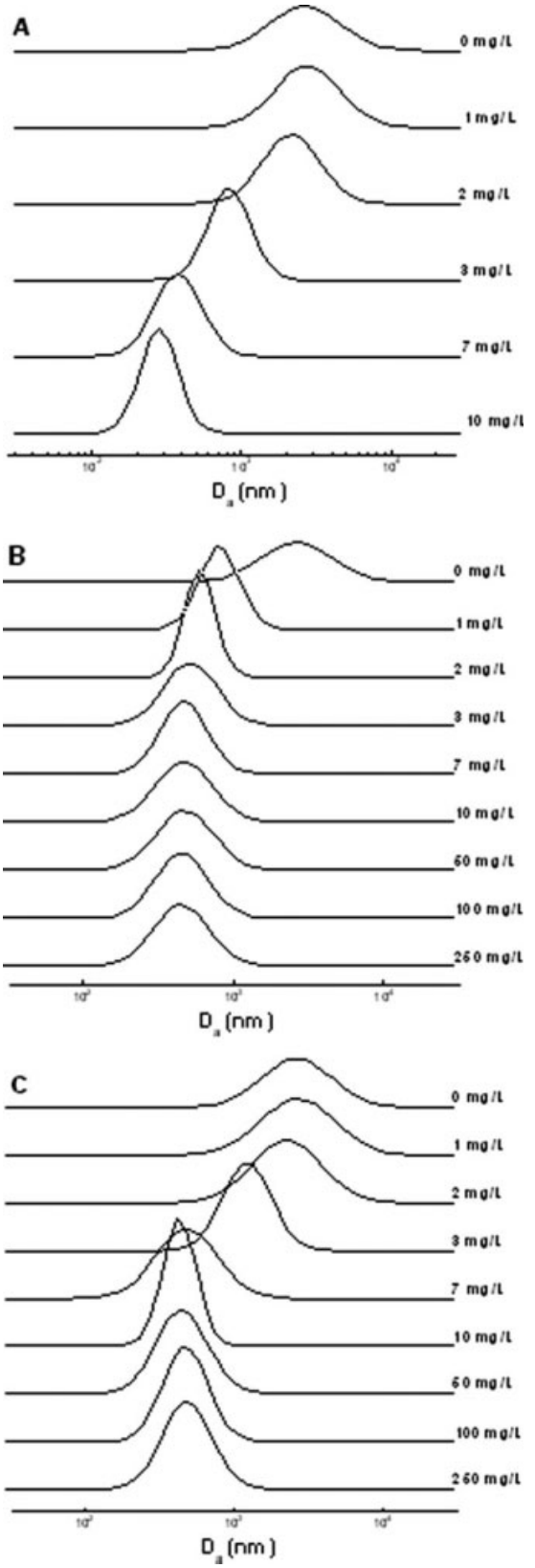

FIGURE 7 Apparent diameter of complex particles of 150 mg/L α-gliadin with (A) PSS, (B) PHS1, and (C) PHS2 at various polymer concentrations and pH 6.8.

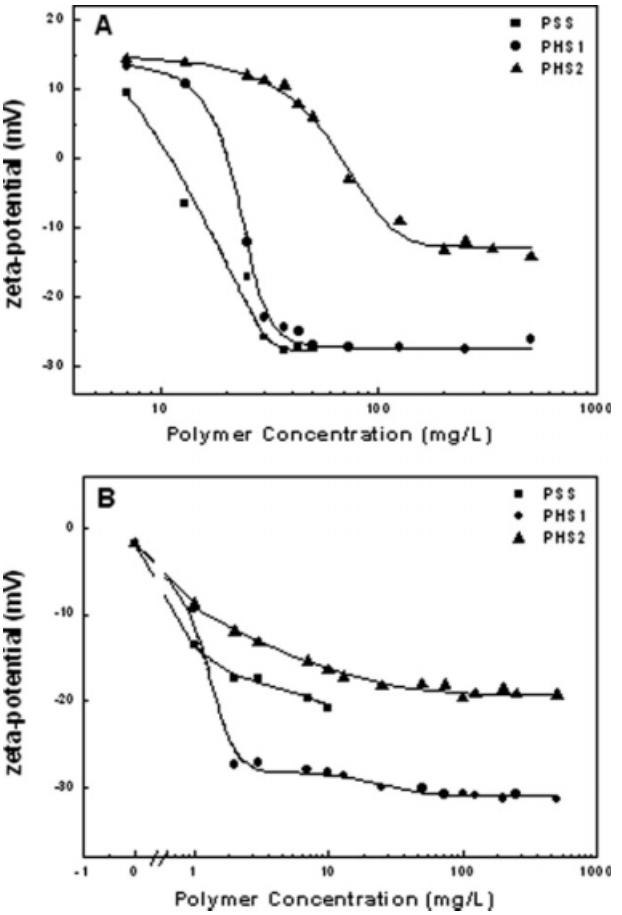

FIGURE 8 Zeta potential of complex particles of 150 mg/L α-gliadin with PSS (■), PHS1 (●), and PHS2 (▲) as a function of polymer concentration at pH 1.2 (A) and 6.8 (B).

absence of α-gliadin is significantly less than that of its complex particles with the protein. This suggests that the complexes should be main component in the solutions and contribute to the zeta potentials obtained in Figure 8A. The lower zeta potential for the complex particles with PHS2 with highest HEMA content (91 mol%) may be due to the enrichment of hydrophilic and electrically neutral HEMA segments on the periphery of the particles.[21]

Figure 8B shows the change in zeta potential of α-gliadin particles as a function of polymer concentration at pH 6.8. The pure protein is characterized by a nearly neutral zeta potential of about −1.8 mV at the pH close to its isoelectric point (Ip 6).[22] Interaction of α-gliadin with PSS and its HEMA copolymers brought drops in particle zeta potential. At 10 mg/L, PSS/α-gliadin particles exhibited a zeta potential of −20.9 mV, substantially different from the value of −47.5 mV for PSS at 10 mg/L in the absence of α-gliadin, indicating that the complexes are main component in the solutions. At the same polymer concentration, the zeta

potential was more negative at neutral than acidic pH. This is likely due to the smaller number of α-gliadin-borne positive charges available at neutral pH to neutralize the polymer negative charges. In the case of PHS1, a more negative zeta potential of −30.9 mV was obtained above 2 mg/L. PHS2 decreased the zeta potential to a lesser extent than PSS and PHS1, with a zeta potential of −19.0 mV at concentrations above 25 mg/L. Zeta potential of −34.3 for pure PHS1 and of −23.2 mV for pure PHS2 at 500 mg/L is not apparently different from that obtained for their complex particles, suggesting free copolymers may also contribute to zeta potentials at the high concentrations in mixed solutions.

DISCUSSION

Mechanism of α-Gliadin/Polymer Interaction at pH 1.2

At pH 1.2, α-gliadin and the binder polymers carry positive and negative charges, respectively. At the stoichiometric equivalence of protein positive and polymer negative charges, charge neutrality occurred (see Figure 8) and electrostatic repulsion between complexes should be minimal. It was under this condition that turbidity was maximal (Figure 5A) and the CD negative bands around 208 and 222 nm disappeared (see Figure 2). Then, the addition of an excess of negatively charged polymer redispersed α-gliadin/polymer complex particles in the solution. Electrostatic attraction with polymers may have weakened electrostatic repulsion among the positive charges of α-gliadin, thus triggering protein self-association. This aggregation increased the content of β-turn, characterized by the negative CD band around 230 nm, and resulted in a decrease of α-helical content near stoichiometric equivalence (see Figure 2).[10]

Electrostatic attraction to positively charged residues on α-gliadin can weaken repulsion forces between negatively charged sulfonate moieties along PSS chain, allowing the formation of complex particles (Figure 6A). However, above 50 mg/L, the polymer negative charges are about five times more abundant than α-gliadin positive charges, and repulsion between excess sulfonate groups appears to be sufficient to prevent the formation of protein–polymer particles,[23] as deduced from the turbidity analysis (Figure 5A). The repulsion may also make α-gliadin self-interaction impossible, thus restoring the α-helix to being the dominant secondary structure at the highest PSS concentrations (Figure 2A).[10]

Copolymerization of SS and HEMA reduces polymer linear charge density and its electrostatic attraction with protein; a higher copolymer concentration was thus needed to achieve stoichiometric charge equivalence (Figure 8A), maximal turbidity (Figure 5A), and similar structural transition (see Figure 2), as SS content decreased at pH 1.2. On the other hand, copolymerization also decreases intra- and/or interchain electrostatic repulsion between sulfonates. This contributed to the formation of α-gliadin/polymer particles at PHS1 or PHS2 concentrations up to 500 mg/L (Figures 5A and 6), at which concentration two copolymer negative charges are about 20 or 7 times of α-gliadin positive charges, respectively. At PHS1 concentrations above 250 mg/L, particle diameter appears to increase (Figure 6B), possibly due to association of polymer/protein complexes.[24] However, such phenomenon was not observed for PHS2 (Figure 6C), possibly due to steric stabilization provided by HEMA segments bound at the surface of complex particles. At 500 mg/L, PHS1 formed larger complex particles with α-gliadin (420 nm) than PHS2 did (170 nm) (Figures 6B and 6C), while PSS was unable to form complex particles with the protein (Figure 5A). This may be the reason why the degree of structural change of α-gliadin induced by polymers ranked as PHS1 > PHS2 > PSS at the highest polymer concentrations examined and at pH 1.2. These findings suggest that SS content when added in great excess is an important factor determining α-gliadin interaction with the three polymers.

Figure 9A summarizes the proposed mechanism of α-gliadin interaction with PSS, PHS1, or PHS2 at pH 1.2. Interaction depends strongly on the ratio of protein positive and polymer negative charges. The protein/polymer particle surface charge changes from positive to negative with an increase in polymer concentrations. The excess negatively charged sulfonates and HEMA segments play a role in the stabilization of complex particles dispersed in the solution at polymer concentrations providing an excess of negative charges. Increasing polymer concentration results in less protein molecules bound per polymer[25] and more hydrophilicity of complex particles that can stabilize a larger surface area,[26] thus forming smaller particles. More negatively charged sulfonates of PSS may be entrapped inside its complex particles with α-gliadin, compared with its HEMA copolymers.[18] The negative charge-induced repulsion inside the particles could make the particles less compact and enlarge the particle size and even produce soluble PSS/complexes in a sufficient excess of negative charges [Figure 9A(a)]. However, introduction of HEMA segments reduces the repulsion between SS groups; two copolymers can thus form the complex particles with α-gliadin over the entire range of concentrations examined at pH 1.2. PHS1 with intermediate SS contents forms larger complex particles [Figure 9A(b)] than PHS2 does [Figure 9A(c)]. In summary, interaction with α-gliadin depends on polymer SS content in a sufficient excess of negative charges at pH 1.2.

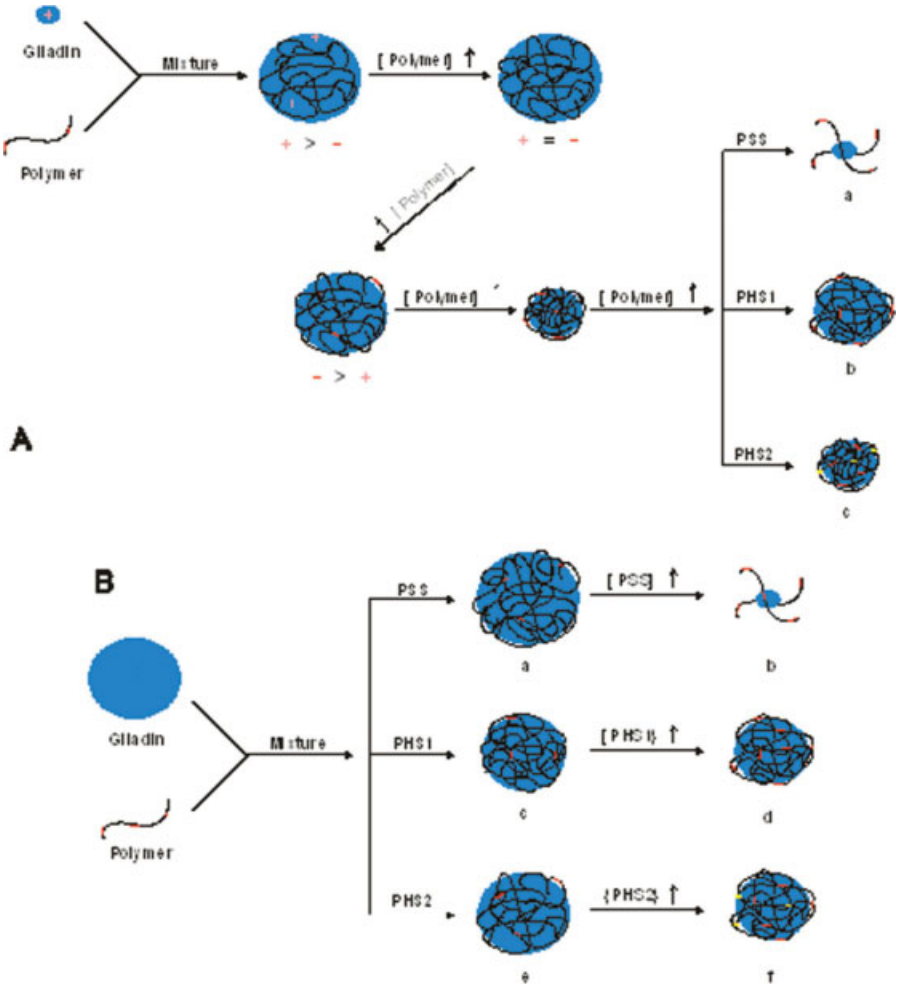

FIGURE 9 Mechanism of α-gliadin interaction with PSS, PHS1, and PHS2 at pH 1.2 (A) and 6.8 (B). Blue = α-gliadin; black = polymer; pink "+" = protein positive charge; red "−" = polymer negative charge; yellow dot = polymer HEMA segment.

Mechanism of α-Gliadin/Polymer Interaction at pH 6.8

At pH 6.8, α-gliadin formed large particles having a nearly neutral zeta potential. Hydrophobic interaction can occur between PSS and proteins[27,28] and should be more important near the Ip of protein.[19] In addition, electrostatic attraction between proteins and polyanions may occur at this pH, due to protein charge patch or heterogeneous distribution.[29,30] However, the charge heterogeneity of protein introduces repulsive forces between polyanion and protein negative domains.[11] No particles formed for PSS above 10 mg/L (Figure 5B), indicating that the repulsion as well as repulsion between excess polymer sulfonate groups appears to be sufficient to inhibit α-gliadin aggregation at pH 6.8.

The optimal polymer composition for interaction with protein would maximize electrostatic and hydrophobic attraction, but minimize repulsion with protein.[11] In copolymers with less SS, the hydrophilic and electrically neutral HEMA segments may sterically hinder the attraction of SS segments with α-gliadin and yet provide stabilization by enrichment on the periphery of complex particles. These factors may explain why PHS1 at 1 mg/L did not, unlike PSS, induce an increase in complex size, but instead suppressed protein aggregation (Figures 7A and 7B). In the case of PHS2, the steric hindrance of the greater HEMA content seemed to apparently decrease the attraction of SS segments to α-gliadin even more. Therefore, no apparent change in protein particle size was observed at 1 mg/L PHS2 and higher concentrations were required to interfere with aggregation (Figure 7C). On the other hand, the introduction of HEMA units should counteract electrostatic repulsions between protein

and polymer as well as along polymer chains. This contributes to the formation of the complex particles with PHS1 and PHS2 at concentrations above 10 mg/L (Figures 5B and 7).

α-Gliadin formed particles with nearly zero net charges at pH 6.8, but was molecularly dissolved carrying nine positive charges at pH 1.2. These different ionization and aggregation states may explain the weaker influence of the polymers on the protein secondary structure at neutral (see Figure 3) than acidic (see Figure 2) pH. At pH 6.8, decreasing the concentration of α-gliadin in the absence of polymer increased α-helix band intensity (data not shown), suggesting that the intensification of α-helix bands is inversely relative to α-gliadin self-aggregation. This is consistent with the greater suppression of α-gliadin aggregation by PSS than PHS1 or PHS2 (see Figure 7), the greater intensification of α-helix bands in the presence of PSS (see Figure 3). The two CD negative bands and their ratio returned to the original position and to the value for pure α-gliadin at the concentrations of PSS that suppressed protein self-aggregation completely (Figures 3 and 4).

Figure 9B summarizes the proposed mechanism of α-gliadin interaction with PSS, PHS1, or PHS2 at pH 6.8. α-Gliadin formed particles with nearly zero net charges at this pH. Its interaction with polymers depended on the polymer concentration and SS content. PSS forms complex particles slightly larger than pure α-gliadin particles at lowest concentration examined [Figure 9B(a)], but suppresses the protein self-aggregation completely at copolymer concentrations producing a sufficient excess of negative charges [Figure 9B(b)]. PHS1 suppresses α-gliadin self-aggregation [Figure 9B(c)], while PHS2 has no apparent effect [Figure 9B(e)] at lowest concentration. Both PHS1 [Figure 9B(d)] and PHS2 [Figure 9B(f)] suppressed protein self-aggregation to a lesser extent than PSS and formed complex particles with protein even in a sufficient excess of negative charges.

Design Considerations for Polymeric Sequestrants of α-Gliadin

Our previous study showed that PSS and its HEMA copolymers could complex with α-gliadin and the copolymer was also found more biocompatible than PSS. The copolymer could hinder immunogenic peptide formation and inhibit the protein-induced alterations in intestinal cell morphology in vitro. Coadministration of SS/HEMA copolymer with gliadin to gluten sensitive mice blocked gliadin-induced intestinal barrier dysfunction and the increase in intraepithelial lymphocyte and macrophage cell counts. Therefore, the copolymer has a potential role in the treatment of patients with gluten-induced disorders.[9] To reduce the toxicity of α-gliadin effectively in vivo, α-gliadin should be entrapped in complex particles. Moreover, the polymers should retain the protein in these particles throughout the gastrointestinal tract including in the presence of gastric acid and under the neutral conditions in the intestine to prevent the formation of the immunogenic digested protein fragments or the possible absorption of α-gliadin through the damaged intestinal mucosa in celiac patients. Like PHS1 and PHS2, PSS was shown to interact with α-gliadin at both gastric and intestinal pH. However, at high concentrations compatible with an oral intake dose (∼0.3–3 g), PSS would not likely form suspended complex particles. α-Gliadin would therefore present a larger specific area for attack by digestive enzymes in addition to remaining at an absorbable size. While both PHS1 and PHS2 were able to form relatively large complex particles with α-gliadin over the entire concentration range examined, PHS1 formed larger complex particles than PHS2 at polymer concentrations above 250 mg/L at pH 1.2. Moreover, at the α-gliadin concentration used in this study (150 mg/L), the amount of PHS1 needed to obtain sufficient negative charges to complex effectively with the protein at pH 1.2 was higher than only 20% (w/w) of the protein concentration, compared to 130% for PHS2. PHS1 therefore appears to be the more efficient sequestrant for α-gliadin.

CONCLUSIONS

This work is a systematic study of the interaction between α-gliadin and PSS or its HEMA copolymers at pH 1.2 and 6.8. The interactions induced changes in α-gliadin secondary structure, which depended on the ratio of negative to positive charges at acidic pH and on polymer SS content at either pH at polymer concentrations resulting in excess of negative charges. Copolymerization with HEMA enhanced the formation of complex particles with α-gliadin, relative to PSS, at polymer concentrations providing sufficient negative charges. Poly(HEMA-*co*-SS) with intermediate SS content (26 mol%) was more effective as an α-gliadin sequestrant than the low-SS (9 mol%) copolymer or PSS. The work has provided not only insight into the mechanism of interaction between proteins and polyanions with different charge densities but also design considerations for polymer sequestrants used in the supportive treatment of celiac disease.

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