The Copolymer P(HEMA-co-SS) Binds Gluten and Reduces Immune Response in Gluten-Sensitized Mice and Human Tissues

MAUD PINIER,* GREGOR FUHRMANN,† HEATHER J. GALIPEAU,§ NATHALIE RIVARD,‖ JOSEPH A. MURRAY,¶ CHELLA S. DAVID,‖ HANA DRASAROVA,‖ LUDMILA TUCKOVA,‖ JEAN–CHRISTOPHE LEROUX,*,‖ and ELENA F. VERDU§

*Faculty of Pharmacy, Université de Montréal, Montréal, Quebec, Canada; †Department of Chemistry and Applied Biosciences, Institute of Pharmaceutical Sciences, ETH Zürich, Zürich, Switzerland; ‡Farncombe Family Digestive Health Research Institute, McMaster University, Hamilton, Ontario, Canada; ‖CIHR Team on Digestive Epithelium, Département d’Anatomie et Biologie Cellulaire, Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, Sherbrooke, Quebec, Canada; §Division of Gastroenterology and Hepatology, Department of Internal Medicine and Department of Immunology, Mayo Clinic, Rochester, Minnesota; and ¶Department of Immunology and Gnotobiology, Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

BACKGROUND & AIMS: Copolymers of hydroxyethyl methacrylate and styrene sulfonate complex with isolated gliadin (the toxic fraction of gluten) and prevent damage to the intestinal barrier in HLA-HCD4/DQ8 mice. We studied the activity toward gluten and hordein digestion and biologic effects of poly(hydroxyethyl methacrylate-co-styrene sulfonate) (P(HEMA-co-SS)). We also investigated the effect of gliadin complex formation in intestinal biopsy specimens from patients with celiac disease.

METHODS: We measured the activity of P(HEMA-co-SS) to reduce digestion of wheat gluten and barley hordein into immunotoxic peptides using liquid chromatography–mass spectrometry. The biodistribution and pharmacokinetic profile of orally administered P(HEMA-co-SS) was established in rodents using tritium-labeled polymer. We assessed the capacity of P(HEMA-co-SS) to prevent the immunologic and intestinal effects induced by a gluten-food mixture in gluten-sensitized HLA-HCD4/DQ8 mice after short-term and long-term administration. We measured the effects of gliadin complex formation on cytokine release ex vivo using intestinal biopsy specimens from patients with celiac disease.

RESULTS: P(HEMA-co-SS) reduced digestion of wheat gluten and barley hordein in vitro, thereby decreasing formation of toxic peptides associated with celiac disease. After oral administration to rodents, P(HEMA-co-SS) was predominantly excreted in feces, even in the presence of low-grade mucosal inflammation and increased intestinal permeability. In gluten-sensitized mice, P(HEMA-co-SS) reduced paracellular permeability, normalized anti-gliadin immunoglobulin A in intestinal washes, and modulated the systemic immune response to gluten in a food mixture. Furthermore, incubation of P(HEMA-co-SS) with mucosal biopsy specimens from patients with celiac disease showed that secretion of tumor necrosis factor-α was reduced in the presence of partially digested gliadin.

CONCLUSIONS: The copolymer P(HEMA-co-SS) reduced digestion of wheat gluten and barley hordein and attenuated the immune response to gluten in a food mixture in rodents. It might be developed to prevent or reduce gluten-induced disorders in humans.

Keywords: Polymeric Binders; Gluten Intolerance; Autoimmune Disorder; Intestine.

Celiac disease is an inflammatory condition of the small intestine triggered by the ingestion of gluten in genetically susceptible individuals. Contrary to previous beliefs, celiac disease is a common disorder with a worldwide prevalence of approximately 1%.1–3 Genetic predisposition is conferred by HLA, since 90%–95% of affected people exhibit HLA-DQ2 molecules and the remainder exhibit HLA-DQ8.4 Recent genome-wide association studies have revealed that non-HLA genetic factors are involved in celiac disease.5,6 During the past 2 decades, tremendous progress has been made toward understanding the pathophysiologic processes in celiac disease and its clinical presentation.7 Some patients without villous atrophy but reporting symptomatic responses to a gluten-free diet (GFD) are labeled “gluten sensitive.”8 Both patients with untreated celiac disease and gluten-sensitive patients with low-grade inflammation have been reported to have increased morbidity and mortality.9,10

No pharmacologic therapies are available to gluten-intolerant patients, and a GFD remains the cornerstone treatment. However, strict gluten restriction for life is far from simple, because it can carry a significant psychosocial and financial burden.11 Nonadherence to a GFD is common because gluten is often used as an additive in processed foods.12 Therefore, complementary therapeutic options are required. Several attractive strategies are currently under investigation.13 Research is being performed to develop celiac-safe wheat by enzymatic treatment14 or by wheat gene modulation15 and bacterial fermentation.16 Another strategy using oral exogenous enzyme intake aims at reducing gluten toxicity by cleaving immunogenic peptide sequences before gluten ingestion or directly in the gut lumen.17–19 Modulators of intestinal permeability have been developed to diminish uptake of gluten peptides into the gastrointestinal mucosa.20,21 Other experimental therapies include restoration of oral tolerance by...
administration of gluten peptides secreted by Lactococcus and immunomodulation by helminths. Finally, tissue transglutaminase inhibitors to prevent peptide selective deamidation, HLA-DQ groove antagonists to block the T-CD4 lymphocyte recognition, and inhibitors of adhesion molecules to hinder inflammatory cell recruitment are also under development.

We have recently explored a novel approach to block gluten toxicity, based on the use of the polymeric binder poly(hydroxyethyl methacrylate-co-styrene sulfonate) (P(HEMA-co-SS)). Such an approach could prove useful as a supportive therapy for celiac disease when the gluten-free quality is not ascertained or when gluten exposure is low (a few milligrams). Polymeric binders are already used in various clinical disorders to sequester compounds in the gastrointestinal tract. Such P(HEMA-co-SS) was shown to complex α-gliadin (the gluten protein fraction predominantly involved in celiac disease) in a relatively selective fashion. The binder counteracted the toxic effects of gliadin on intestinal epithelial cells in vitro. It also reduced the jejunum mucosal toxicity induced by oral gavage of isolated gliadin in mice that had been sensitized with gliadin. Complementary experiments on the ileum mucosa suggested that the deleterious effects of gliadin did not occur farther down in the gastrointestinal tract in the presence of P(HEMA-co-SS). Furthermore, these studies indicated no detectable adverse effects, even at high doses. In this report, we provide evidence that P(HEMA-co-SS) has potential for the treatment of gluten-induced disorders by decreasing the formation of pathogenic peptide sequences from wheat and barley. The efficacy of P(HEMA-co-SS) was tested by measuring jejunum permeability parameters, the mucosal and systemic immune activation after administration of wheat gluten/food mixture to orally gluten-sensitized mice. We further show that the polymer remains mainly in the gastrointestinal tract because it was almost completely recovered in the feces. Lastly, the capacity of P(HEMA-co-SS) to reduce the production of immunogenic gliadin peptides was established by measuring cytokine secretion in mucosal biopsy specimens of patients with celiac disease.

Materials and Methods

Effect of P(HEMA-co-SS) on Wheat Gliadin, Gluten, and Barley Hordein Digestion

All reagents were purchased from Sigma-Aldrich (St Louis, MO) unless otherwise stated. P(HEMA-co-SS) (47:53 ± 2%, 45 and 59 kilodaltons) was synthesized by atom transfer radical polymerization as described elsewhere. After completion of the digestion process, samples were analyzed at least in triplicate by liquid chromatography–mass spectrometry (LC-MS) (Supplementary Materials and Methods).

Absorption Experiments

All animal experiments were approved by the McMaster University and University of Montreal Animal Care Committees, in accordance with the Canadian Council on Animal Care guidelines. [3H]-P(HEMA-co-SS) (1 mCi/mg) was prepared by tritium gas exchange chromatography by American Radiolabeled Chemicals (St Louis, MO). Study design and analytical methods are provided in Supplementary Materials and Methods.

In Vivo Experiments on Transgenic Mice

HLA-HCD4/DQ8 male mice (~26 g), 6 to 8 weeks old, were used. Mice were mucosally sensitized with gluten (500 µg) and cholera toxin (25 µg) as mucosal adjuvant once a week for 3 weeks. One week after the last sensitization, mice were randomly assigned to one of the study groups and orally challenged once. A positive control group received a mixture composed of 2 mg bovine serum albumin (BSA) and 2 mg wheat starch with 2 mg of gluten dissolved in 0.02 mol/L acetic acid. The treatment group with high dose (HD) of P(HEMA-co-SS) consisted of gluten-sensitized mice gavaged with gluten/BSA/starch mixture and P(HEMA-co-SS) (4 mg) (gluten/polymer 1:2 wt/wt). The treatment group with low polymer dose (LD) received 1 mg of polymer (gluten/polymer 2:1 wt/wt). Mice were killed 24 hours after the oral challenge. In another series of experiments, the dose of P(HEMA-co-SS) was kept at 4 mg but the gluten amount increased to 4 mg (higher level of gluten, gluten/polymer 1:1 wt/wt). The negative control group was composed of nonsensitized mice (cholera toxin only) gavaged with BSA/starch mixture but no gluten. P(HEMA-co-SS) was administered 5 minutes before the mixture gavage. Finally, the impact of the treatment on long-term gluten exposure was assessed. Mice (10 male and 10 female) were mucosally sensitized as described previously. One week after the last sensitization, they were orally challenged with gluten twice a day, 3 times a week, for 3 weeks. The positive control group (5 male and 5 female) received the mixture composed of BSA (2 mg) and wheat starch (2 mg) with gluten (2 mg) dissolved in 0.02 mol/L acetic acid. The treatment group (5 male and 5 female) consisted of gluten-sensitized mice gavaged with P(HEMA-co-SS) (4 mg), which was administered 5 minutes before the gluten/BSA/starch mixture intake. The negative control group was composed of nonsensitized mice (5 male and 5 female) gavaged only with BSA/starch mixture. Mice were killed 24 hours after the last oral challenge. Histology, hemogram, and biochemical parameters were assessed as described in Supplementary Materials and Methods.

To verify the impact of sensitization on intestinal permeability, an additional negative control group composed of mucosally sensitized mice gavaged with BSA/starch mixture was used. This control group showed identical immunoglobulin (Ig) A level and 51Cr-EDTA flux as the nonsensitized negative control group. All animal experiments were approved by the McMaster University and University of Montreal Animal Care Committees, in accordance with the Canadian Council on Animal Care guidelines.
centrifuged at 2000g at 4°C for 30 minutes, and the supernatants were collected and stored at −20°C. All samples were processed within 1 week (Supplementary Materials and Methods).

**Effect of P(HEMA-co-SS) on Splenocyte Proliferation in Gluten-Sensitized Mice**

Splenocytes were isolated and a peptic-tryptic digest of gliadin (P-T-gliadin) was prepared as described previously. Detailed methods are provided in Supplementary Materials and Methods.

**Effect of P(HEMA-co-SS) in Biopsy Specimens From Patients With Celiac Disease**

Patients’ biopsy specimens were kindly provided by Dr Pavel Fruhauf (Department of Pediatrics and Adolescent Medicine, 1st Medical Faculty, Charles University, Prague, Czech Republic). Patients were recruited after receiving approval from the local ethics committee. Patients with untreated celiac disease (as diagnosed by serology) attending gastrointestinal endoscopy clinics at various hospitals in the city of Prague were invited to participate in the study. Written informed consent was obtained from all patients. Small intestinal biopsy specimens with confirmed Marsh III lesions were used for cytokine responses to gliadin. In the first set of experiments (5-minute pre-incubation of gliadin with P(HEMA-co-SS) at 37°C before peptic digestion), patients (n = 4) ranging in age from 29 to 49 years (mean, 37 years; female/male, 3/1) were included. In the second set of experiments (pre-incubation of gliadin peptic digest, P-gliadin, with polymer), patients (n = 5) ranging in age from 33 to 49 years (mean, 38 years; female/male, 4/1) were included. Four biopsy specimens (of similar size, 2–3 mm) were obtained from each patient and incubated under 4 different conditions consisting of medium alone, P-gliadin, P(HEMA-co-SS) and gliadin + P(HEMA-co-SS). The gliadin/P(HEMA-co-SS) or P-gliadin/P(HEMA-co-SS) ratios were set at 1:3 (wt/wt). The levels of interleukin (IL)-10, tumor necrosis factor (TNF)-α, IL-15 and IL-21 were determined by enzyme-linked immunosorbent assay as described in Supplementary Materials and Methods.

**Statistical Analysis**

Group values for the comparison of the 2 negative control groups (sensitized vs nonsensitized) were analyzed using a t-test. Group values for in vitro animal experiments were compared using a parametric analysis of variance test, followed by Scheffé, Tukey, or Tukey–Kramer test to determine the significance of all paired combinations (normality was assumed on all experimental data sets). Group values for the biopsy studies were compared using a Kruskal–Wallis test, followed by a Nem- enyi test to determine the significance of the combinations.

**Results**

**P(HEMA-co-SS) Decreases the Formation of Gluten-Derived Toxic Peptides**

Gliadin, wheat gluten, and barley hordein were subjected to in vitro digestion by gastrointestinal enzymes in the presence or absence of P(HEMA-co-SS), and the digestion products were analyzed by LC-MS. This in vitro test was used to provide insight on the mechanisms of action of the polymer. It is semiquantitative and represents a worst-case experimental setup because the cereal proteins are the sole substrates for digestive enzymes, which is not the case in the gastrointestinal tract. As reported in our first study, incubation of gliadin with P(HEMA-co-SS) resulted in a decrease of the overall abundance of generated peptides. P(HEMA-co-SS) greatly reduced (by 70%) the formation, from isolated gliadin, of the 13-mer peptide LGQQPFPFPQPPQFPQ (Supplementary Figure 2), which participates in the innate immune response. Because both gliadin and glutenin are involved in the pathogenesis of celiac disease, in this study further investigations were performed on wheat gluten digests. The analysis of celiac disease.

The production of a gluten sequence, known to bind to the HLA-DQ2 receptor (ie, QLQPFPQPQPLPY), was also reduced by 50%–70% in the presence of P(HEMA-co-SS) (Figure 1A). Besides, the formation of PQQPQPQPQPQPQPQPQ decreased significantly (Figure 1B). When combined, these 2 peptides (QLQPFPQPQPLPY and PQQPQPQPQPQPQPQ) almost completely make up the known 33-mer sequence (QLQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQPQ, one of the identified initiators of the inflammatory reaction in patients with celiac disease. In addition, the gluten sequence SQQQQPFQ was also decreased by 60% (Supplementary Figure 3C).

To test whether the P(HEMA-co-SS) could reduce the production of dominant immunogenic peptides from prolamine of barley, additional analyses with hordein digests were performed. The formation of PQQQPFQPQPQPQPQPQFW, bearing a 9-mer (QQPFPPQQPF) sequence, predicted to be a HLA-DQ2 restricted T-cell epitope was reduced by 40%–45% (Supplementary Figure 4A). More importantly, 2 sequences, PQQQPFQPQPQPQPQPQFRQ and PQQQPFQPQPQPQPQPQPF, reported to stimulate T-cell and T-cell clones from patients with celiac disease, were decreased by 40%–45% and by 60%–65%, respectively (Supplementary Figure 4B and C). Finally, a peptide from γ-hordein (ie, LERPQQLFPQQLQPLPQPQPQ) exhibited a drastic reduction of 85%–90% (Supplementary Figure 4D).

**P(HEMA-co-SS) Is Poorly Absorbed and Mainly Excreted in Feces**

The oral absorption of the polymeric binder and its excretion profiles were first examined in Sprague-Dawley rats after single and long-term dosing. The non-fasted animals were gavaged with [14C]-P(HEMA-co-SS), and blood, feces, and urine were assayed for radioactivity content. After the animals were killed, selected organs were harvested and the radioactivity levels measured. After a single polymer intake (150 mg/kg), the amount of radioactivity found in the blood over 72 hours was very low, reflecting poor absorption of P(HEMA-co-SS) (Supplementary Figure 5A). Indeed, ~98% of the total radioactiv-
ity was recovered in the feces. The total amount excreted in the urine was less than 2% of the total dose. Only residual P(HEMA-co-SS) activity was detected in harvested organs (Supplementary Figure 5B–D). Similar results were obtained after a 3-week administration of P(HEMA-co-SS) at a dosage of 100 mg/kg/day, suggesting no permeabilization of the intestinal mucosa on protracted dosing (Supplementary Figure 6).

To determine whether the absorption of P(HEMA-co-SS) is increased by mucosal inflammation and changes in intestinal permeability triggered by gluten, we administered [3H]-P(HEMA-co-SS) to nonsensitized and gluten-sensitized HLA-HCD4/DQ8 mice. The amount of tritium recovered in the feces exceeded 93% of the total radioactivity and was comparable in gluten-sensitized and nonsensitized mice (Figure 2A). After 72 hours, less than 1.2% of the administered dose was detected in the urine (Figure 2B). Likewise, the radioactivity levels in the blood, liver, and kidney remained similar to background levels (overall amount, 0.1% of given dose) (Figure 2C).

**P(HEMA-co-SS) Reduces Mucosal Dysfunction in Gluten-Sensitized Mice When Administered in a Gluten-Containing Meal**

To evaluate the therapeutic relevance of the polymeric binder toward a wheat gluten containing meal, an in vivo assay was performed on gluten-sensitized HLA-HCD4/DQ8 mice. These mice exhibit increased intestinal permeability and mild mucosal immune activation after gluten or gliadin sensitization. We previously reported that P(HEMA-co-SS) reversed intestinal barrier abnormalities induced by isolated gliadin. However, in the previous study, no other food components were admixed with gliadin, and it was not shown whether the binder was efficient in the presence of whole wheat gluten. Therefore, we measured the activity of P(HEMA-co-SS) in gluten-sensitized mice using a mixture of wheat gluten, BSA, and starch for oral challenge. HLA-HCD4/DQ8 mice gavaged with the food mixture exhibited an increase in 51Cr-EDTA flux, a marker of paracellular permeability (Figure 3A), and in intestinal tissue conductance (Figure 3B) compared with nonsensitized mice receiving the BSA/starch mixture alone. The gluten-induced effects on permeability were normalized in mice treated with P(HEMA-co-SS) under the HD regimen. Thus, P(HEMA-co-SS) decreased gluten-induced effects on barrier function in HLA-HCD4/DQ8 mice even in the presence of additional food components. The same trend was observed at a lower polymer dose (LD), although statistical significance could not be achieved (Figure 3, last bar). An identical pattern was observed when the dose of polymer was unchanged but the amount of wheat gluten was doubled (higher-level regimen) (Supplementary Figure 7A and B), achieving statistical significance in only one permeability parameter (51Cr-EDTA flux).

**P(HEMA-co-SS) Has Immunomodulatory Effects in Gluten-Sensitized Mice**

**Mucosal immunity.** We measured the effect of P(HEMA-co-SS) on anti-gliadin IgA levels in intestinal washes of HLA-HCD4/DQ8 mice. Total anti-gliadin IgAs were increased by 2.4-fold in untreated gluten-sensitized mice (ie, gavaged with gluten-food mixture only) compared with nonsensitized controls (Supplementary Figure 8). In mice receiving P(HEMA-co-SS) (HD and LD regimens) before gluten-food mixture challenge, the IgA levels were comparable to those observed in nonsensitized mice (Supplementary Figure 8). In the higher-level group, the IgA levels exhibited a similar pattern, although statistical significance was not reached (Supplementary Figure 7C). Since statistical significance was not achieved on all parameters (ie, permeability and mucosal immunity) in LD and higher-level groups, further investigations were performed at a gluten-to-polymer ratio of 1:2 wt/wt (HD).
Systemic immunity. The ability of oral P(HEMA-co-SS) to modulate systemic immune responses was examined using a splenocyte proliferation assay. Splenocytes from gluten-sensitized HLA-HCD4/DQ8 mice (subsequently gavaged with gluten-food mixture and/or P(HEMA-co-SS)) and from nonsensitized controls were harvested and incubated with PT-gliadin. Negative controls from all groups were incubated with medium alone and positive controls with concanavalin A (5 μg/mL). Proliferation index of splenocytes incubated with medium alone was low and comparable in all groups. Concanavalin A–stimulated proliferation was robust and similar in all groups (controls, 17.2 ± 3.4 counts per minute; gluten, 16.7 ± 5.3 counts per minute; gluten + P[HEMA-co-SS], 19.4 ± 3 counts per minute). After incubation with PT-gliadin, untreated gluten-sensitized mice had increased thymidine incorporation compared with nonsensitized controls (Figure 4). However, splenocytes from gluten-sensitized mice that were treated with P(HEMA-co-SS) before the gluten-food mixture gavage exhibited a trend of reduced thymidine incorporation versus sensitized mice that had not received P(HEMA-co-SS) (P = .068). To further characterize the immunomodulatory effect of P(HEMA-co-SS), supernatants from splenocyte cultures stimulated with PT-gliadin were assayed for IL-10, TNF-α, and monocyte chemotactic protein 1 (MCP-1). Previous studies in HLA-HCD4/DQ8 mice have shown increases in the immunoregulatory cytokine IL-10.29–31 Our results are in line with these studies showing enhanced IL-10 production after mucosal gluten sensitization (Figure 5A). Administration of P(HEMA-co-SS) before the gluten-food mixture challenge led to a more pronounced IL-10 secretion in vitro (Figure 5A). The proinflammatory cytokine TNF-α was increased in untreated gluten-sensitized mice compared with nonsensitized controls. Administration of P(HEMA-co-SS) before the gluten-food mixture challenge reduced TNF-α secretion by 55% (Figure 5B). In gluten-sensitized mice challenged with the gluten-containing mixture and treated with P(HEMA-co-SS), the TNF-α/IL-10 ratio dropped from 2.3 to 0.5 versus the control group. Nonsensitized mice had low levels of MCP-1, a chemokine that prolongs lymphocyte survival (Figure 5C). Untreated gluten-sensitized mice exhibited higher levels of MCP-1 than controls and than gluten-sensitized mice treated with P(HEMA-co-SS).

P(HEMA-co-SS) Attenuates the Effects of Long-term Administration of Gluten in Gluten-Sensitized Mice

To further evaluate the therapeutic relevance of the polymeric binder, an in vivo assay was performed on gluten-sensitized HLA-HCD4/DQ8 mice after repeated oral challenge with gluten. HLA-HCD4/DQ8 mice gavaged with gluten twice a day, 3 times a week, for 3 weeks exhibited an increase in 51Cr-EDTA flux, intestinal tissue conductance, and total anti-gliadin IgA (Supplementary Figure 9) compared with negative controls. The gluten-induced effects on conductance and anti-gliadin IgA level

---

**Figure 2.** P(HEMA-co-SS) is poorly absorbed and mainly excreted in the feces. Excretion profiles and biodistribution of [H]-P(HEMA-co-SS) in nonsensitized (white bars) and gluten-sensitized (black bars) HCD4/DQ8 mice after single oral dosing (240 mg/kg, 22.4 μCi/kg). (A) Cumulative dose excreted in feces, (B) cumulative dose excreted in urine, and (C) blood, liver, and kidney levels at the time the mice were killed. Mean ± SEM, n = 4/group.
were normalized in mice receiving P(HEMA-co-SS) before the gluten mixture. The same trend was observed for 

\[ {^{51}}\text{Cr-EDTA flux (paracellular permeability)} \]

and (B) intestinal tissue conductance compared with untreated nonsensitized mice. Controls: non-
sensitized mice gavaged with BSA and starch (n = 12–13); gluten mixture group: gluten-sensitized mice gavaged with gluten/BSA/starch (n = 9–10); P(HEMA-co-SS) (HD) + gluten mixture group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) and subsequently with gluten/BSA/starch (gluten/polymer 1:2 wt/wt) (n = 9–10); P(HEMA-co-SS) (LD) + gluten mixture group (second treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) and subsequently with gluten/BSA/starch (gluten/polymer 2:1 wt/wt) (n = 4). Mean ± SEM, \( ^* P < .02 \) for Cr-EDTA and \( P \leq .01 \) for conductance.

Figure 3. Administration of P(HEMA-co-SS) to gluten-sensitized HCD4/DQ8 mice challenged with wheat gluten mixture decreased (A) 

\[ {^{51}}\text{Cr-EDTA flux (paracellular permeability)} \]

and (B) intestinal tissue conductance compared with untreated nonsensitized mice. Controls: non-
sensitized mice gavaged with BSA and starch (n = 12–13); gluten mixture group: gluten-sensitized mice gavaged with gluten/BSA/starch (n = 9–10); P(HEMA-co-SS) (HD) + gluten mixture group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) and subsequently with gluten/BSA/starch (gluten/polymer 1:2 wt/wt) (n = 9–10); P(HEMA-co-SS) (LD) + gluten mixture group (second treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) and subsequently with gluten/BSA/starch (gluten/polymer 2:1 wt/wt) (n = 4). Mean ± SEM, \( ^* P < .02 \) for Cr-EDTA and \( P \leq .01 \) for conductance.

Figure 4. Administration of P(HEMA-co-SS) to gluten-sensitized mice challenged with gluten-food mixture tended to reduce splenocyte prolif-
eration after incubation with PT-gliadin. Data represent the stimulation index from mean [\(^3\)H]thymidine incorporation for triplicate cultures. Mean ± SEM (n = 8–9/group), \( ^* P \leq .001 \). Controls: nonsensitized mice gavaged with BSA and starch; gluten mixture group: gluten-sensitized mice gavaged with gluten/BSA/starch; P(HEMA-co-SS) + gluten mixture group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-
co-SS) and subsequently with gluten/BSA/starch (HD).

The effect of P(HEMA-co-SS) on gliadin digestion was evaluated on mucosal biopsy specimens obtained from patients with celiac disease. Two series of experiments were conducted. First, the gliadin digest was generated in the presence or absence of P(HEMA-co-SS) to mimic a rapid complexation in the stomach (Figure 7). In the second case, P-gliadin was first produced and then admixed to P(HEMA-co-SS) to simulate a situation in which complexation would occur at a later stage when a substantial amount of gluten would have been digested (Supplementary Figure 11). In both cases, the digests were incubated with the isolated mucosa and the secretion of TNF-\( \alpha \) and IL-10 was measured in the culture supernatant. IL-15 and IL-21 were tested, but the levels were below

\[ {^{51}}\text{Cr-EDTA flux (paracellular permeability)} \]

and (B) intestinal tissue conductance compared with untreated nonsensitized mice. Controls: non-
sensitized mice gavaged with BSA and starch (n = 12–13); gluten mixture group: gluten-sensitized mice gavaged with gluten/BSA/starch (n = 9–10); P(HEMA-co-SS) (HD) + gluten mixture group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) and subsequently with gluten/BSA/starch (gluten/polymer 1:2 wt/wt) (n = 9–10); P(HEMA-co-SS) (LD) + gluten mixture group (second treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) and subsequently with gluten/BSA/starch (gluten/polymer 2:1 wt/wt) (n = 4). Mean ± SEM, \( ^* P < .02 \) for Cr-EDTA and \( P \leq .01 \) for conductance.

were normalized in mice receiving P(HEMA-co-SS) before the gluten mixture. The same trend was observed for 

\[ {^{51}}\text{Cr-EDTA flux (paracellular permeability)} \]

and (B) intestinal tissue conductance compared with untreated nonsensitized mice. Controls: non-
sensitized mice gavaged with BSA and starch (n = 12–13); gluten mixture group: gluten-sensitized mice gavaged with gluten/BSA/starch (n = 9–10); P(HEMA-co-SS) (HD) + gluten mixture group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) and subsequently with gluten/BSA/starch (gluten/polymer 1:2 wt/wt) (n = 9–10); P(HEMA-co-SS) (LD) + gluten mixture group (second treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) and subsequently with gluten/BSA/starch (gluten/polymer 2:1 wt/wt) (n = 4). Mean ± SEM, \( ^* P < .02 \) for Cr-EDTA and \( P \leq .01 \) for conductance.

P(HEMA-co-SS) Affects Cytokine Release by

Biopsy Specimens From Patients With Celiac Disease

The effect of P(HEMA-co-SS) on gliadin digestion was evaluated on mucosal biopsy specimens obtained from patients with celiac disease. Two series of experiments were conducted. First, the gliadin digest was generated in the presence or absence of P(HEMA-co-SS) to mimic a rapid complexation in the stomach (Figure 7). In the second case, P-gliadin was first produced and then admixed to P(HEMA-co-SS) to simulate a situation in which complexation would occur at a later stage when a substantial amount of gluten would have been digested (Supplementary Figure 11). In both cases, the digests were incubated with the isolated mucosa and the secretion of TNF-\( \alpha \) and IL-10 was measured in the culture supernatant. IL-15 and IL-21 were tested, but the levels were below

\[ {^{51}}\text{Cr-EDTA flux (paracellular permeability)} \]

and (B) intestinal tissue conductance compared with untreated nonsensitized mice. Controls: non-
sensitized mice gavaged with BSA and starch (n = 12–13); gluten mixture group: gluten-sensitized mice gavaged with gluten/BSA/starch (n = 9–10); P(HEMA-co-SS) (HD) + gluten mixture group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) and subsequently with gluten/BSA/starch (gluten/polymer 1:2 wt/wt) (n = 9–10); P(HEMA-co-SS) (LD) + gluten mixture group (second treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) and subsequently with gluten/BSA/starch (gluten/polymer 2:1 wt/wt) (n = 4). Mean ± SEM, \( ^* P < .02 \) for Cr-EDTA and \( P \leq .01 \) for conductance.
the limit of quantification in all groups. As shown in Figure 7 and Supplementary Figure 11, negative controls (nonstimulated biopsy specimens) released traces of TNF-α (15.6 pg/mL) and IL-10 (31.3 pg/mL). The addition of the P-gliadin in the medium increased the production of TNF-α and tended to increase IL-10. Biopsy specimens incubated with P(HEMA-co-SS) alone secreted traces of TNF-α, suggesting that P(HEMA-co-SS) applied to the intestinal mucosa is not proinflammatory in vitro. The incubation of the biopsy specimens with P-gliadin/P(HEMA-co-SS) mixture (digestion of gliadin before the addition of P(HEMA-co-SS); Supplementary Figure 11) tended to reduce TNF-α, whereas IL-10 concentrations remained unchanged. However, the effect of P(HEMA-co-SS) was more pronounced when intestinal biopsy specimens were incubated with the digested gliadin/P(HEMA-co-SS) complex (Figure 7), showing that TNF-α levels were reduced to very low levels (15.6 pg/mL).

Figure 5. Modulation of cytokine production in splenocyte culture supernatants after PT-gliadin incubation: (A) IL-10, (B) TNF-α, and (C) MCP-1. IL-10 secretion was increased in gluten-sensitized mice receiving P(HEMA-co-SS) compared with controls. Administration of P(HEMA-co-SS) to gluten-sensitized mice led to lower TNF-α and MCP-1 levels in culture supernatants after PT stimulation. Controls: nonsensitized mice gavaged with BSA and starch; gluten mixture group: gluten-sensitized mice gavaged with wheat gluten (2 mg)/BSA (2 mg)/starch (2 mg); P(HEMA-co-SS) + gluten mixture group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) (4 mg) and subsequently with wheat gluten (2 mg)/BSA (2 mg)/starch (2 mg). Challenges were performed twice a day, 3 times a week, for 3 weeks. Representative H&E-stained jejunum section of the various groups observed by optical microscopy.

Figure 6. Administration of P(HEMA-co-SS) reduced the morphologic intestinal abnormalities induced by long-term administration of gluten. (A) Controls: nonsensitized mice gavaged with BSA and starch, (B) gluten mixture group: gluten-sensitized mice gavaged with wheat gluten (2 mg)/BSA (2 mg)/starch (2 mg), (C) P(HEMA-co-SS) + gluten mixture group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) (4 mg) and subsequently with wheat gluten (2 mg)/BSA (2 mg)/starch (2 mg). Challenges were performed twice a day, 3 times a week, for 3 weeks. Representative H&E-stained jejunum section of the various groups observed by optical microscopy.
We have formerly described that administration of P(HEMA-co-SS) with isolated gliadin to HLA-HCD4/DQ8 mice attenuated gliadin-induced changes in the intestinal barrier and reduced intraepithelial lymphocyte and macrophage cell counts.28 Here we show that oral administration of P(HEMA-co-SS) to gluten-sensitized HLA-HCD4/DQ8 mice ameliorated mucosal and systemic consequences of an oral complex gluten-containing food mixture challenge. In mice, P(HEMA-co-SS) restored gluten-induced intestinal barrier dysfunction, reduced the levels of anti-gliadin IgA in intestinal washes, tended to decrease the proliferation of splenocytes incubated with PT-gliadin, and shifted the immune response in vitro toward an anti-inflammatory profile. Our current results also suggest that P(HEMA-co-SS) has the capacity to modulate cytokine production in response to gliadin in mucosal biopsy specimens of patients with celiac disease. Repeated administration of P(HEMA-co-SS) to gluten-sensitized HLA-HCD4/DQ8 mice following long-term challenge with gluten revealed that the polymer exhibited some therapeutic benefits on the intestinal mucosa without affecting weight gain, hemogram, or biochemical parameters.

Innate and adaptive immune responses are involved in the pathogenesis of celiac disease. The intestinal epithelial uptake of gliadin has been extensively studied in the context of celiac disease.41–43 Gliadin has been reported to increase intestinal permeability enabling its paracellular diffusion, thereby subsequently triggering an interaction with the mucosal immune system.41,42 Gliadin peptides can also translocate transcellularly by the transferrin receptor.43 This translocation makes poorly digested gliadin available to the mucosa, leading to antigen presentation of HLA-DQ2/DQ8–bound gliadin peptides to CD4+ T cells and to the generation of effector cells.43,44 Increased availability of gliadin and its by-products in the mucosa and impairment of immunoregulatory mechanisms that control oral tolerance have been proposed as mechanisms that lead to autoimmune enteropathy in celiac disease.46 In the present study, treatment of gluten-sensitized mice with P(HEMA-co-SS) reduced anti-gliadin IgAs in intestinal secretions. Oral P(HEMA-co-SS) also led to increased secretion of IL-10 and to decreased production of MCP-1 and TNF-α by splenocytes after in vitro stimulation with PT-gliadin. The mechanism leading to the increase of IL-10 expression by splenocytes remains to be investigated. It can be hypothesized that the complexation of P(HEMA-co-SS) to gluten, in addition to hindering its degradation, may indirectly influence the immune response to gluten peptides. A recent work reported that the conjugation of mannoside units to BSA could increase IL-10 expression by CD4+ cell type 1 regulatory-like cells and induce oral tolerance in a mouse model of food allergy.47

In human biopsy specimens obtained from patients with celiac disease that were incubated with gliadin, P(HEMA-co-SS) tended to reduce (Supplementary Figure 11) gliadin-induced TNF-α levels when applied with P-gliadin. However, when P(HEMA-co-SS) was incubated with
gliadin before digestion, TNF-α secretion was abolished (Figure 7). In both sets of experiments, IL-10 concentration remained unchanged. However, a trend for increased IL-10 production was observed in human biopsy specimens in the presence of P(HEMA-co-SS), even without gliadin stimulation. This is intriguing in view of previous studies showing that recombinant IL-10 can suppress gliadin-dependent T-cell activation in mucosal T cells obtained from patients with celiac disease. Clinical studies using the polymeric binder will be crucial to determine the therapeutic relevance of such ex vivo observations.

Polymeric binders are commonly used to sequester compounds (e.g., bile salts, phosphate) in the gastrointestinal tract. They are designed not to be absorbed to minimize the risks of systemic toxicity. P(HEMA-co-SS) has a high molecular weight (45–60 kilodaltons) and carries multiple negative charges. Furthermore, on complexation with gliadin, particle sizes in the order of hundreds of nanometers are obtained. Therefore, P(HEMA-co-SS) was not expected to be orally bioavailable. Our present in vivo data in both rats and mice support this hypothesis. Most of the administered P(HEMA-co-SS), whether given as a single dose or in a long-term fashion, was recovered in the feces after 3 days. There was some residual radioactivity found in the blood and in urine, which could be due to tritium exchange or urine contamination by the feces. The lack of absorption of P(HEMA-co-SS) supports the current and previously reported findings of no systemic toxicity as well as no alteration of several biochemical and cellular markers even with long-term administration of high doses. Herein, the fact that P(HEMA-co-SS) absorption was not increased in gluten-sensitized mice, which are known to exhibit changes in intestinal permeability and low-grade inflammation, further supports the safety profile of the binder. Moreover, the direct action in the gastrointestinal tract, bypassing bioavailability issues, might be a tremendous advantage of this strategy.

In conclusion, these data strengthen the evidence in support of luminal gluten-polymeric binder as an effective adjunctive therapy to a GFD. P(HEMA-co-SS) was not systemically absorbed and blocked mucosal toxicity of gluten in mice, likely by decreasing the production of immunogenic peptides in the gastrointestinal tract and by limiting the absorption of gluten by-products. Taken in conjunction with the animal studies, the immunomodulatory effects suggested by our experiments in tissues from patients with celiac disease should encourage future work aiming at assessing the safety of P(HEMA-co-SS) after oral administration to healthy volunteers and progression into phase 1 clinical trials. The ultimate verdict on therapeutic efficacy and utilization guidelines of P(HEMA-co-SS) will only come from clinical studies. Research on numerous adjuvant approaches to a GFD should be encouraged, because a combination of alternative therapies may be beneficial for patients with poorly controlled celiac disease.

**Supplementary Materials**

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.10.038.

**References**


Received May 13, 2011. Accepted October 30, 2011.

Reprint requests

Address requests for reprints to: Jean-Christophe Leroux, BPharm, PhD, Department of Chemistry and Applied Biosciences, Institute of Pharmaceutical Sciences, ETH Zurich, Wolfgang-Pauli-Str 10, HCI H 301, 8093 Zurich, Switzerland; e-mail: jleroux@ethz.ch; fax: (41) (0) 44 633 1314; or Elena F. Verdu, MD, PhD, McMaster University, 1200 Main Street West, Hamilton, Ontario, Canada. e-mail: verdue@mcmaster.ca; fax: (506) 522-3454.

Conflicts of interest

The authors disclose the following: J.-C. Leroux is an inventor of the patent on the use of polymeric binder in treating celiac disease. This patent has been licensed by the University of Montreal to BioLineRx. J.-C. Leroux has a consultancy agreement with BioLineRx. The remaining authors disclose no conflicts.

Funding

Supported by the Canadian Celiac Association, the Fonds Québécois de la Recherche sur la Nature et les Technologies, the Canadian Association of Gastroenterology/Canadian Institute of Health Research, and the Canada Research Chair Program. M. Pinier is a recipient of the Canadian Celiac Association J. A. Campbell Young Investigator Award. E. Verdu holds a McMaster University Department of Medicine Internal Career Research Award and the Canadian Celiac Association J. A. Campbell Research Award. J.-C. Leroux is a recipient of the NSERC Steacie Fellowship. L. Tuckova is supported by Grant Agency of the Czech Republic 310/07/0414. N. Rivard is a recipient of a Canadian Research Chair in Signaling and Digestive Physiopathology.
Supplementary Methods

**Effect of P(HEMA-co-SS) on Wheat Gliadin, Gluten and Barley Hordein Digestion**

Isolation of hordein from barley was performed as previously reported. Briefly, 10 g barley grains were milled and mixed with 32 mL 70% ethanol for 2 h. After centrifugation (30 min at 900 x g) the supernatant was dialyzed against 0.01 M acetic acid and freeze-dried. The lyophilized product was dispersed in 0.01 M acetic acid, centrifuged and the supernatant freeze dried a second time to give the crude hordein.

**Digestion.** Stock solutions of the enzymes were prepared prior to use: i) pepsin (3480 U/mg) in HCl 0.02 M, 6 mg/mL, ii) trypsin (1310 U/mg) in Na2HPO4 buffer (pH 6.8, 20 mmol/L K2HPO4 and 2 mmol/L NaOH), 50 mg/mL, and iii) chymotrypsin (96 U/mg) in Na2HPO4 buffer (pH 6.8, 20 mmol/L K2HPO4 and 2 mmol/L NaOH) 50 mg/mL. \( \alpha \)-Gliadin (supplied by Prof. Poppineau, Institut National de Recherche Agronomique, Nantes; 7.5 mg/mL), gluten (15 mg/mL) or hordein (15 mg/mL) were suspended in HCl 0.02 M for 15 min at 37°C in the presence or absence of P(HEMA-co-SS) at different gliadin (or gluten)/P(HEMA-co-SS) mass ratios. In the calculation of the mass ratios, it was assumed that gluten contained 50% gliadin. Pepsin was added to a final concentration of 0.024 mg/mL and incubated for 2 h, at 37°C under stirring. Thereafter, Na2HPO4 was added (0.12 M), and the pH adjusted to 6.8 by the addition of NaOH 1 M. The suspension was supplemented with trypsin to a final concentration of 0.375 mg/mL and incubated for 2 h, at 37°C under stirring. Subsequently, chymotrypsin was added to the suspension to a final concentration of 0.375 mg/mL and incubated for 2 h, at 37°C under stirring. After completion of the digestion process, samples were heat-inactivated for 5 min at 95°C, freeze-dried and stored at −20°C until analysis. The freeze-dried \( \alpha \)-gliadin and gluten digests were resuspended in ultrapure water and centrifuged (14,000 x g) for 10 min. Supernatants were then further diluted 1 in 10 (v/v) with water containing 0.1% formic acid. Each dilution was injected at least in triplicate.

**Analytical method.** All samples were analyzed using an LC-system composed of a Rheos Allegro quaternary pump (Flux Instruments, Basel, Switzerland), column oven (hot dog 5090), C18-column (Hypersil Gold, 100 x 1 mm, 1.9 \( \mu \)m) and XCalibur control software (all obtained from Thermo Fisher Scientific Inc., San Jose, CA). Samples were injected at a column temperature of 35°C, at a volume of 10 \( \mu \)L and a flow rate of 50 \( \mu \)L/min using water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The gradient for wheat gliadin and gluten analysis consisted of 95% solvent A as initial value, 95–50% A in 1–30 min, 50–5% A in 30–35 min, 5% A in 35–40 min, 5–95% A in 40–41 min and 95% A in 41–50 min. The gradient for analysis of hordein samples was modified as follows: 95% solvent A as initial value, 95–50% A in 1–19 min, 50–5% A in 19–23 min, 5% A in 23–27 min, 5–95% A in 27–28 min, and 95% A in 28–31 min. The LC was directly connected to an LTQ XL linear quadrupole Ion Trap (Thermo Fisher Scientific Inc.). Ionization was conducted via electrospray ion (ESI) source. Data acquisition was carried out by full MS, followed by MS2-fragmentation of the five most intense signals in an automated data dependent scan. Raw data were subjected to the Sequest search algorithm using Proteome Discoverer Software (Thermo Fisher Scientific Inc.). Detected peptides were searched against all sequences from the ExPASy Proteomics database (Swiss Institute of Bioinformatics, Lausanne, Switzerland) that match the following search terms: triticum, aestivum, wheat glutenin and gliadin for gluten and gliadin digests; and hordein, gluten, barley, hordeum vulgare for hordein digests. Exclusively identified peptides with a cross-correlation >1.5 and a peptide probability >30% were considered and reviewed in literature for T-cell stimulation. Quantitative analysis of peptides of interest was performed using LCquan Software (Thermo Fisher Scientific Inc.). The program integrated areas under the peak of individual peptides. The relative abundance (in %) in all samples was calculated setting the respective non-polymer treated sample to 100%. Calibration curve was performed by spiking gluten digest with increasing amounts of LGQQQPFPPQQPY. Linearity of MS quantification was verified by spiking a synthetic gluten peptide (PQPOLYPQOLP) into gluten-digest samples in a dose-dependent manner (\( R^2 = 0.9945 \)).

**Absorption Experiments**

P(HEMA-co-SS) was purified by dialysis against water for 4 days and finally freeze-dried before use.

**Study design.** Male Sprague Dawley rats (250–280 g for single dose study and 410–430 g at gavage time for chronic dose study) were housed individually in metabolic cages. \([3H]-P(HEMA-co-SS)\) was administered to 14 rats by gavage. Seven rats received a single dose of \([3H]-P(HEMA-co-SS)\) (ca. 6.4 \( \mu \)Ci/kg, ca. 150 mg/kg) and seven rats were treated with unlabeled P(HEMA-co-SS) in the drinking water at approximately 100 mg/kg/day for 21 days followed by a single dose of \([3H]-P(HEMA-co-SS)\) (ca. 6.4 \( \mu \)Ci/kg, ca. 150 mg/kg) by gavage on day 22. Total urines and feces were collected from 0 to 12, 12 to 24, 24 to 36, 36 to 48 and 48 to 72 h after administration of \([3H]-P(HEMA-co-SS)\). Aliquots of blood were sampled at 30 min, 1, 2, 4, 8, 12, 24, 48 and 72 h by subclavian vein under isoflurane anesthesia. At sacrifice, exsanguination was performed followed by a saline flush before collecting organs (small intestine, colon, liver, kidneys and spleen).

Transgenic male mice (~25 g), 6 to 8 weeks old and expressing HLA-HCD4/DQ8 (HLA-DQA1*0301; HLA-DQB1*0302) genes in the absence of endogenous mouse
class II genes were used\(^1\). Mice (\(n = 4/\text{group}\)) were orally sensitized by gavaged with gluten (500 \(\mu\)g) and cholera toxin (CT, 25 \(\mu\)g) as mucosal adjuvant, once a week during 3 weeks. Controls (\(n = 4/\text{group}\)) received CT alone. The mice were housed individually in metabolic cages and received a single dose of \([\text{3H}]\)-P(HEMA-co-SS) (ca. 22.4 \(\mu\)Ci/kg, ca. 240 mg/kg) one week after the last sensitization. Urine and feces were collected from 0 to 12, 12 to 24, 24 to 36, 36 to 48 and 48 to 72 h after administration of \([\text{3H}]\)-P(HEMA-co-SS). Aliquots of blood were sampled at 72 h. At sacrifice, exsanguination was performed followed by a saline flush before collecting liver and kidneys.

**Analytical method.** Urines, feces and tissues were weighed and then homogenized. All homogenates were aliquoted (approximately 100–300 mg) and degraded using Solvable (Perkin Elmer, Waltham, MA) at 60°C and then bleached using hydrogen peroxide (30% \(v/v\)). Radioactivity was measured in Hionic Fluor (Perkin Elmer) using a Liquid Scintillation Analyser Tri-Carb 2100TR (Packard, Meriden, CT). Radioactivity in blood, urines, feces and tissues was expressed as a percentage of the administered dose. For blood, the percentage of the administered dose was calculated by multiplying the blood radioactivity per gram by 0.064 \(\times\) body weight in g for rats or 0.072 \(\times\) body weight in g for mice\(^4\).

**In Vivo Experiments on Transgenic Mice**

**Effect of P(HEMA-co-SS) on Intestinal Permeability in Gluten-Sensitized Mice**

Sections of tissues (jejunum) (5 cm) were removed and divided into two segments. Each segment was opened along the mesenteric border, rinsed and mounted in an Ussing chamber (exposed surface area 0.6 cm\(^2\)). Tissues were bathed in oxygenated Krebs buffer containing 10 mM glucose (serosal side) or 10 mM mannitol (luminal side) at 37°C. The net active transport across the epithelium was measured via a short circuit current (Isc) injected through the tissue under voltage clamp conditions. After a 15-min equilibration period, conductance (mS/cm\(^2\)) was recorded. \(^{51}\)Cr-ethylene diamine tetra acetic acid (EDTA) (Perkin Elmer, Waltham, MA) was used to probe paracellular permeability. \(^{51}\)Cr-EDTA (6 \(\mu\)Ci/ml) was added to the luminal buffer once equilibrium was reached. Serosal samples (500 \(\mu\)L) were taken at 30-min intervals for 2 h and replaced with fresh buffer to maintain constant volume. The \(^{51}\)Cr-EDTA was measured by scintillation counter (Beckman Coulter LS6500 Multi Purpose Scintillation Counter, Beckman, CA).

**Effect of P(HEMA-co-SS) on Anti-Gliadin Antibodies in Gluten Sensitized-Mice**

Anti-gliadin-immunoglobulin A (IgA) levels were determined in intestinal contents by ELISA. 96-well ELISA plates (Nunc-Immuno Plates Maxisorp, Roskilde, Denmark) were coated with 50 \(\mu\)L of phosphate buffered saline (PBS) including 5 \(\mu\)g of gliadin dissolved with 70% ethanol. 50 \(\mu\)L of intestinal wash supernatants were added in triplicate to the wells. Goat anti-mouse IgA-horseradish peroxidase (HRP) conjugate (\(\alpha\)-chain specific) was then added as secondary antibodies (50 \(\mu\)L per well). Color was developed with 3,3',5,5'-tetramethylbenzidine substrate (Cedarlane Lab, ON), reaction stopped by HCl 1 N and optical densities (OD) were read at 450 nm using an ELISA plate reader (Bio-TEK Instruments Inc., Winooski, VT). The IgA OD were normalized by total protein concentration (Bio-Rad, Bradford protein assay reagent) per sample.

**Effect of P(HEMA-co-SS) on Splenocyte Proliferation Ratio in Gluten Sensitized-Mice**

Splenocytes were cultured in RPMI 10% fetal bovine serum (FBS) supplemented with either Con A, PT-gliadin (500 \(\mu\)g/mL protein concentrations were measured with bicinchoninic acid assay) or medium alone and incubated 72 h, 37°C, in 5% CO\(_2\) humidified atmosphere. The cells were pulsed with 1 \(\mu\)Ci/well \([\text{3H}]\)-thymidine for additional 18 h. Cells were harvested on an automatic cell harvester and \([\text{3H}]\)-thymidine uptake was measured by counting the radioactivity on filters with Beckman scintillation beta-counter. Results were expressed as stimulation index (SI) (mean cpm of triplicate culture containing Ag)/(mean of cpm of triplicate cells cultured with medium alone). The amounts of IL-10, MCP-1 and TNF-\(\alpha\) in supernatants of cultured splenocytes were determined by ELISA using CBA kit (BD Bioscience ON) according to manufacturer’s instructions.

**Effect of P(HEMA-co-SS) in Gluten-Sensitized Mice After Chronic Administration.**

Mice were weighed daily throughout the study. Twenty-four hours following the final gluten challenge mice were anesthetized and blood was collected via orbital bleed. Plasma samples were analyzed for biochemical parameters (albumin, alkaline phosphatase, alanine amino-transferase, creatinine, Na\(^+\), K\(^+\), Cl\(^-\) and urea). Hemoglobin levels were determined in blood samples using a hemoglobin assay kit (Quantichrom, BioAssay Systems, Hayward, CA). White blood cells, red blood cells, and platelets were counted using a hemocytometer.

**Histology.** Cross-sections of the jejunum were collected, fixed in 10% formalin, embedded in paraffin, and stained with H&E for histological evaluation and measurement of villus-to-crypt ratios using light microscopy. Twenty villus-to-crypt ratios were measured for each mouse in a blinded fashion.

**Effect of P(HEMA-co-SS) in Biopsies from CD Patients**

Endoscopic biopsies obtained from patients with confirmed CD were cultured in RPMI 1640 (BioWhit-
taker, Lonza, Belgium), supplemented with L-glutamine (2 mM), penicillin/streptomycin (100 UI penicillin/mL, 100 μg streptomycin/mL), 10% FBS and 0.001% protease inhibitor cocktail (Cytoskeleton, Denver, CO) and stimulated for 24 h with 100 μg of P-gliadin and/or 300 μg of P(HEMA-co-SS) or P-(gliadin/P(HEMA-co-SS). When applied together, P-gliadin and P(HEMA-co-SS) were pre-incubated for 30 min in 37° C, 5% CO2 and than added to the biopsies. The supernatants were collected and stored in −20°C until use. The levels of IL-10 and TNF-α were determined by ELISA (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions. IL-15 and IL-21 were assayed using ELISA sets (BioLegend and eBioscience, San Diego, CA, respectively). The P-gliadin digestion was performed as previously reported5. Beaded pepsin-agarose gel was tested for lipopolysaccharide contamination and shown to be below the limit of detection (LOD) of the assay. The gliadin/P(HEMA-co-SS) or P-gliadin/P(HEMA-co-SS) ratios were set at 1:3 (w/w).

Supplementary Table 1. Biochemistry and hemogram data. Gluten-sensitized mice (n = 10/group) received gluten mixture (positive control group) or P(HEMA-co-SS) 5 min prior gluten mixture (treatment group) or only starch and albumin (negative control group). Gavages were performed twice a day, 3 times a week for 3 weeks.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Negative control group</th>
<th>Positive control group</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>SD</td>
<td>Average</td>
<td>SD</td>
</tr>
<tr>
<td>Albumin</td>
<td>g/L</td>
<td>36.10 1.29</td>
<td>35.60 1.35</td>
<td>36.80 1.03</td>
</tr>
<tr>
<td>ALP</td>
<td>U/L</td>
<td>129.7 6.07</td>
<td>117.3 29.71</td>
<td>122.7 25.26</td>
</tr>
<tr>
<td>ALT</td>
<td>U/L</td>
<td>14.10 8.61</td>
<td>17.00 6.29</td>
<td>14.20 7.02</td>
</tr>
<tr>
<td>Creatinine</td>
<td>μmol/L</td>
<td>19.20 8.04</td>
<td>16.60 4.03</td>
<td>13.40 5.74</td>
</tr>
<tr>
<td>Na+</td>
<td>mmol/L</td>
<td>146.4 4.00</td>
<td>149.6* 1.35</td>
<td>150.4* 1.78</td>
</tr>
<tr>
<td>K+</td>
<td>mmol/L</td>
<td>4.84 0.39</td>
<td>5.13 0.44</td>
<td>4.95 0.24</td>
</tr>
<tr>
<td>Cl–</td>
<td>mmol/L</td>
<td>104.8 3.40</td>
<td>108.8 3.52</td>
<td>106.9 3.54</td>
</tr>
<tr>
<td>Urea</td>
<td>mmol/L</td>
<td>8.04 1.30</td>
<td>7.76 0.94</td>
<td>7.84 1.21</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>g/100 mL</td>
<td>14.00 0.95</td>
<td>13.70 0.54</td>
<td>14.11 0.83</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>10¹²/L</td>
<td>9.78 0.57</td>
<td>10.16 0.90</td>
<td>10.10 0.67</td>
</tr>
<tr>
<td>White blood cells</td>
<td>10⁹/L</td>
<td>7.13 0.93</td>
<td>6.77 2.01</td>
<td>6.43 1.55</td>
</tr>
<tr>
<td>Platelets</td>
<td>10⁹/L</td>
<td>379.3 48.43</td>
<td>369.4 42.82</td>
<td>361.4 49.75</td>
</tr>
</tbody>
</table>

ALP: alkaline phosphatase, ALT: alanine amino-transferase.
*P < .05 vs. negative control group.

**Supplementary Figure 1.** 
(A) $^{51}$Cr-EDTA flux (paracellular permeability), (B) intestinal tissue conductance and (C) anti-gliadin IgA in intestinal washes in non-sensitized ($n = 12–14$) vs. gliadin sensitized ($n = 8–9$) HCD4/DQ8 mice. Mean ± SEM.

**Supplementary Figure 2.** P(HEMA-co-SS) decreases the formation of the 13-mer peptide, LGQQQPFPPQPY from wheat gliadin. Digested gliadin samples were analyzed by LC-MS. Relative abundance was determined by comparison with the gliadin digest in the absence of P(HEMA-co-SS) (positive control). Mean ± SEM displayed from 3 injections of 3 independent samples, *$P < 0.001$. 

*February 2012 THE COPOLYMER P(HEMA-CO-SS) BINDS GLUTEN 325.e4*
Supplementary Figure 3. P(HEMA-co-SS) decreases the formation of peptides YPTSPQSQGQQL (A), LQPQQPFPQQPQPQFPQ (B) and SQQQQPPF (C) from wheat gluten. Digested gluten samples were analyzed by LC-MS. Relative abundance was determined by comparison with the gluten digest in the absence of P(HEMA-co-SS). Mean ± SEM displayed from 3 injections of 3 independent samples, *P ≤ 0.001.
**Supplementary Figure 4.** P(HEMA-co-SS) decreases the formation of peptides PQQPFPQFPQPQFPFPFW (A), PQPQQPQFPQPQRQ (B), PQPQFPQFPQFPQPQFPF (C), and LERPQQLFPQWLQPPFW (D) from barley hordein. Digested hordein samples were analyzed by LC-MS. Relative abundance was determined by comparison with the hordein digest in the absence of P(HEMA-co-SS) (positive control). Mean ± SEM displayed from 3 injections of 3 independent samples, *P < 0.005.
Supplementary Figure 5. P(HEMA-co-SS) is mainly excreted in feces and poorly absorbed. Pharmacokinetics, excretion profiles and biodistribution of [3H]-P(HEMA-co-SS) in Sprague Dawley rats after single oral dosing (150 mg/kg, 6.4 μCi/kg). (A) Blood concentrations, (B) cumulative dose excreted in the feces, (C) cumulative dose excreted in urine, (D) organ distribution. Mean ± SEM (n = 7).
Supplementary Figure 6. P(HEMA-co-SS) is and mainly excreted in feces and poorly absorbed. Pharmacokinetics, excretion profiles and biodistribution of \[^{3}H\]-P(HEMA-co-SS) in Sprague Dawley rats, after a polymer exposure for 21 days (100 mg/kg/day) and a single dose of \[^{3}H\]-P(HEMA-co-SS) (150 mg/kg, 6.4 μCi/kg) at day 22. (A) Blood concentrations, (B) cumulative dose excreted in the feces, (C) cumulative dose excreted in urine, (D) organ distribution. Mean ± SEM (n = 7).
Supplementary Figure 7. (A) $^{51}$Cr-EDTA flux (paracellular permeability), (B) intestinal tissue conductance, (C) anti-gliadin IgA in intestinal washes. Controls: non-sensitized mice gavaged with BSA and starch (n = 12–14); gluten mixture (HL) (gluten mixture with a higher level of gluten, 4 mg) group: gluten-sensitized mice gavaged with wheat gluten (4 mg)/BSA (2 mg)/starch (2 mg) (n = 8–9); P(HEMA-co-SS) + gluten mixture (HL) group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) (4 mg) and subsequently with wheat gluten (4 mg)/BSA (2 mg)/starch (2 mg) (gluten/polymer 1:1 w/w) (n = 8–9). Mean ± SEM *P ≤ 0.02.
Supplementary Figure 8. Administration of P(HEMA-co-SS) to gluten-sensitized HCD4/DQ8 mice challenged with gluten-food mixture decreased IgA levels. Gluten sensitization and gluten-food mixture challenge to HCD4/DQ8 mice increased OD levels for anti-gliadin IgA in intestinal washes. In gluten-sensitized mice, administration of P(HEMA-co-SS) returned IgA levels to control values. The control group was used as reference (100% optical density at 450 nm, OD₄₅₀). Mean ± SEM, *P ≤ 0.001. Controls: non-sensitized mice gavaged with BSA and starch (n = 14); gluten mixture group: gluten-sensitized mice gavaged with gluten/BSA/starch (n = 10); P(HEMA-co-SS) (HD) + gluten mixture group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) and subsequently with gluten/BSA/starch (gluten/polymer 1:2 w/w) (n = 10); P(HEMA-co-SS) (LD) + gluten mixture group (second treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) and subsequently with gluten/BSA/starch (gluten/polymer 2:1 w/w) (n = 6).
Supplementary Figure 9. Effect of treatment following a chronic exposure to gluten: (A) $^{51}$Cr-EDTA flux (paracellular permeability), (B) intestinal tissue conductance, (C) anti-gliadin IgA in intestinal washes. Controls: non-sensitized mice gavaged with BSA and starch; gluten mixture group: gluten-sensitized mice gavaged with wheat gluten (2 mg)/BSA (2 mg)/starch (2 mg); P(HEMA-co-SS)/H11001 gluten mixture group (treatment group): gluten-sensitized mice gavaged first with P(HEMA-co-SS) (4 mg) and subsequently with wheat gluten (2 mg)/BSA (2 mg)/starch (2 mg). Challenges were performed twice a day, 3 times a week for 3 weeks. Mean ± SEM, (n = 9–10), *$P < 0.02$; #$P = 0.0585$).
Supplementary Figure 10. P(HEMA-co-SS) did not affect the evolution of the weight gain in vivo of HLA-DQ8 transgenic mice after a 3 week challenge. Controls: non-sensitized mice gavaged with BSA and starch; gluten mixture group: gluten-sensitized mice gavaged with wheat gluten (2 mg)/BSA (2 mg)/starch (2 mg); Treatment group: gluten-sensitized mice gavaged first with P(HEMA-co-SS) (4 mg) and subsequently with wheat gluten (2 mg)/BSA (2 mg)/starch (2 mg). Challenges were performed twice a day, 3 times a week for 3 weeks (n = 10). Mean ± SD.

Supplementary Figure 11. Production of TNF-α (white bars) and IL-10 (black bars) in culture supernatants from biopsies of CD patients after incubation with P(HEMA-co-SS) alone or with P- gliadin +/- P(HEMA-co-SS). Negative control corresponds to biopsies incubated with medium only. Mean ± SEM (n = 5). *P ≤ 0.05. BLQ: below limit of quantification (<31.3 pg/mL for IL-10 and <15.6 pg/mL for TNF-α).