Sustained gastrointestinal activity of dendronized polymer–enzyme conjugates

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Abstract

Methods for stabilizing and retaining enzyme activity in the gastrointestinal tract are rarely investigated due to the difficulty of protecting proteins from an environment which nature has evolved to promote their digestion. This study showcases the first spatiotemporally resolved *in vivo* assessment of covalent conjugation to polymers as a means of stabilizing therapeutic orally administered enzymes at different locations in the gastrointestinal tract. Architecturally and functionally diverse polymers were evaluated to sterically protect enzymes from inactivation and to promote interactions with stomach mucin. Findings suggest that an exceptional enhancement of the *in vivo* performance of enzymes is achievable, both in the stomach and/or in the small intestine, and can be sustained (even in the stomach) for several hours. These findings provide new insight and a firm basis for the development of new therapeutic and imaging strategies based on orally administered proteins using a simple and accessible technology.

Main text

Proteins, in particular enzymes, are structurally and functionally complex entities that are pharmaceutically and chemically relevant due to their specificity and high activity[1-4]. Over the past 30 years, substantial effort has been placed on stabilizing[5-8] and prolonging the circulation lifetime[9,10] of these sensitive biomacromolecules after systemic administration. In contrast, the stabilization and retention of orally administered enzymes (*e.g.*, for celiac disease[11], phenylketonuria[12], lactose intolerance[13] or exocrine pancreatic disease[14]) in the gastrointestinal (GI) tract have received little attention. In fact, methods for stabilizing enzymes in the upper GI tract and in particular in the stomach, are only seldom investigated *in vivo*. This is likely due to the paramount challenge in protecting a protein from conditions which nature has evolved to promote its denaturation. The discovery of new approaches for preserving the activity of proteins in this environment should stimulate the development of new classes of protein-based oral therapies for a variety of diseases.

The stomach represents a harsh milieu in which proteins are subjected to inactivation by acid and peptidases. Recently, the activity of exogenous proline-specific endopeptidases (PEPs) has been monitored during their transit through the GI tract[15]. PEPs are of considerable interest as potential adjuvant therapy for celiac disease[16-18], an autoimmune disease triggered by the ingestion of dietary proteins (gluten) that provokes inflammation in the upper small intestine[19-21]. Our previous study suggested that some PEPs undergo denaturation with concomitant loss of activity in the stomach[15]. Efficient stabilization of orally administered enzymes, especially in the upmost parts of the GI tract, could greatly improve the treatment of celiac disease[22,23] (and several other

illnesses) given that immunogenic peptides (or other toxic compounds) could be efficiently degraded enzymatically prior to reaching the small intestine. Based on these findings, new methods to stabilize proteins in the stomach must therefore not only address digestion by peptidases (*e.g.*, pepsin), but also unfolding and aggregation.

This work reports the first straightforward, yet highly effective, approach for stabilizing orally administered enzymes in the stomach through covalent conjugation to a polycationic dendronized polymer (poly-(3,5-bis(3-aminopropoxy)benzyl)methacrylate) PG1 (Fig. 1a)[24-26]. A dendronized polymer possesses a linear main chain and dendron-type side-chains[24]. A bacterial PEP from *Myxococcus xanthus* (MX) was selected as a model enzyme because it is easily deactivated in the stomach, and has promiscuous activity towards gluten proteins[15,27]. MX was modified with PG1, which promoted *in vivo* adhesion to the stomach mucosa (Fig. 1b). Retention of MX and unprecedented stabilization of activity for over 3 h was observed in the stomach. Comparable experiments with other polyionic or neutral polymers, including poly(acrylic acid) (PAA), α-poly(D-lysine) (PDL) or methoxy poly(ethylene glycol) (mPEG, the gold standard for stabilizing proteins for the systemic route[10]), offered little or no retention/protection of enzymes in the stomach. However, enhanced activity in the small intestine was observed for enzyme conjugates of mPEG and PAA (Fig. 1c). Our findings are discussed in terms of their benefits for therapies requiring activity at different locations in the GI tract.

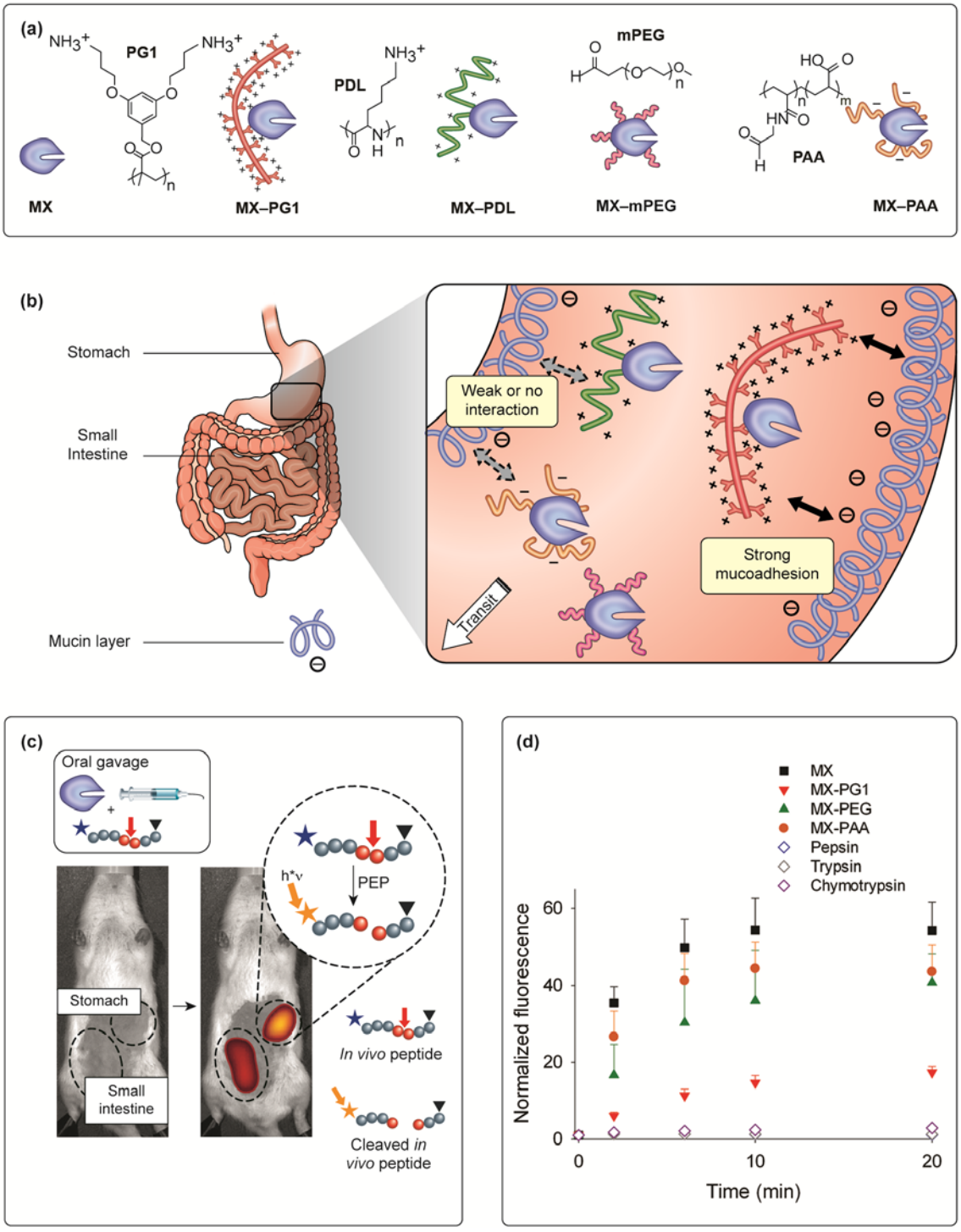

Figure 1. Gastric stabilization and retention of exogenous enzymes by polymer modification. (a) Chemical structures of the four polymers examined: a cationic dendronized polymer PG1, cationic PDL, neutral mPEG and anionic PAA. **(b)** Schematic depiction of behavior of MX–polymer conjugates in the GI tract. After oral

administration, MX–PG1 interacts with the negatively charged gastric mucin layer leading to strong mucoadhesion. MX–PDL, MX–PAA and MX–mPEG do not (or weakly) interact with the mucin layer. Please note that proportions of MX and polymers are not to scale. **(c)** The activity of the individual MX–polymer conjugates was measured using an *in vivo* fluorescence assay. A peptide substrate (HiLyte Fluor™647)-LPYPQPK-(QXL™670) was administered orally to rats followed by MX or MX–polymer conjugates. Fluorescence emission after peptide cleavage (indicated by arrow) was detected by non-invasive imaging in the GI tract of rats (stomach or small intestine). **(d)** Prior to animal experiments, the uncleaved peptide substrate (3 µM) was incubated *in vitro* with MX, MX–polymer conjugates or with endogenous enzymes (1 $\mu g_{(protein)} \cdot mL^{-1}$). All MX–polymer conjugates induced a significant increase in fluorescence intensity after peptide cleavage. Endogenous GI enzymes could not hydrolyze the peptide. Mean + SD, $n = 3$.

Results

Mucoadhesion *in vivo*. PG1 and PDL were selected on the basis that their electrostatic interactions with mucin should induce complexation and mucoadhesion. Below pH 9, PG1 (M_n 80.5 kDa, free of counter-salt, DP_n ~176) possesses a high charge density (two positive charges per repeating unit), branched structure and non-biodegradability. PDL (of comparable number of repeating units to PG1) is a poorly biodegradable, but bioadhesive polymer[28]. PAA is a weakly mucoadhesive polymer[29] and mPEG represents an important control for evaluating steric stabilization imparted by the polymer modification. The structure of all polymers and their expected behavior are summarized in Fig. 1.

The ability of the selected polymers to complex porcine stomach mucin was screened *in vitro* through turbidity experiments (Fig. 2a and b). PG1 and PDL induced

enhanced optical density, indicating complexation with the mucin suspension. Complexation was most efficient at pH 4.5 (fed stomach pH) and less pronounced at pH 1.8 (fasted stomach pH)[30,31] due to partial neutralization of mucin ($pK_{a,COOH}$ ~1–2). This underlines the importance of electrostatic interactions for mucoadhesion. Hydrophobic interactions and hydrogen bonding may also be involved because complexation at pH 1.8 was partially disrupted by salts or alcohol (Fig. 2a). In contrast to the cationic polymers, neither PAA nor mPEG efficiently complexed mucin.

To evaluate mucoadhesion in rats, fluorescent MX–polymer conjugates were tracked by *in vivo* imaging[15]. As outlined in the Supplementary Methods, PG1 and PDL were labeled with (on average) 20 molecules of DyLight™800 (DL800, $\lambda_{ex/em}$ = 745/800 nm). The labeled polymers were then conjugated to MX *via bis*-aryl hydrazone (BAH) bond formation to yield MX–PG1–DL800 and MX–PDL–DL800, respectively (Supplementary Table S1 and Figs. S1 and S2). After purification, each enzyme bore ca. 1 polymer chain, each chain possessing a comparable number of repeating units (176 for PG1 and 208 for PDL). Note that the BAH bond is stable at pH 2 for over 24 h at 37 ºC (Supplementary Fig. S3). The fluorescent conjugates were administered to rats fasted for 12 h prior to gavage. Transit of the conjugates was monitored under discontinuous anesthesia (*i.e.,* short periods of anesthesia) to avoid reduced stomach motility due to the anesthetic gas[15]. Retention in the stomach was evaluated as an indicator of *in vivo* interaction of the conjugates with gastric mucin. Fluorescence of MX–PG1–DL800 in the stomach was strong and constant for ~6 h and then decreased with time (Figs. 2c and d). In comparison, MX–PDL–DL800 eluted from the stomach at a rate which was similar to that of a simple fluorescently labeled mPEG (Fig. 2d).

Analysis of the dissected, opened and emptied stomach confirmed that the fluorescence was localized to the epithelial mucin layer and to a lesser extent to residual chyme (Fig. 2e). Although GI mucus turnover times can fluctuate, renewal rates of 4–6 h have been reported for rats[32,33], consistent with the profile observed for MX–PG1–DL800. This suggests that this conjugate interacted mainly with the loosely adherent mucosal layer and less with the firmly adherent glycocalyx layer[34]. To further examine this, the opened stomach was rinsed with neutral phosphate buffer leading to a significant decrease of the fluorescent signal (Fig. 2e), likely due to disruption of the loosely adherent mucin layer and elution of conjugate–mucin complex. Histology of stomach tissue indicated that MX–PG1–DL800 did not penetrate beyond the mucosal layer (Supplementary Fig. S4). No difference between the PDL conjugate and the labeled mPEG was observed (Fig. 2d), indicating poor interaction with stomach mucin *in vivo*. No residual fluorescent signal was observed after 20 h for any conjugate. The mucoadhesive properties of MX–PG1–DL800 are presumably related to electrostatic interactions with the gastric mucosal layer, which may be enhanced due to high accessibility of the positive charges along the expected extended main chain of PG1, as observed by atomic force microscopy for its Boc-protected precursor (Supplementary Figure S5)[25]. This contrasts to PDL, which has a lower charge density and is expected to behave as a random coil at acidic pH[35], with some charges being inaccessible for interaction. Moreover, the dendron-type side chains may provide additional interactions through aromatic residues (*e.g.,* π-stacking) and H-bonds.

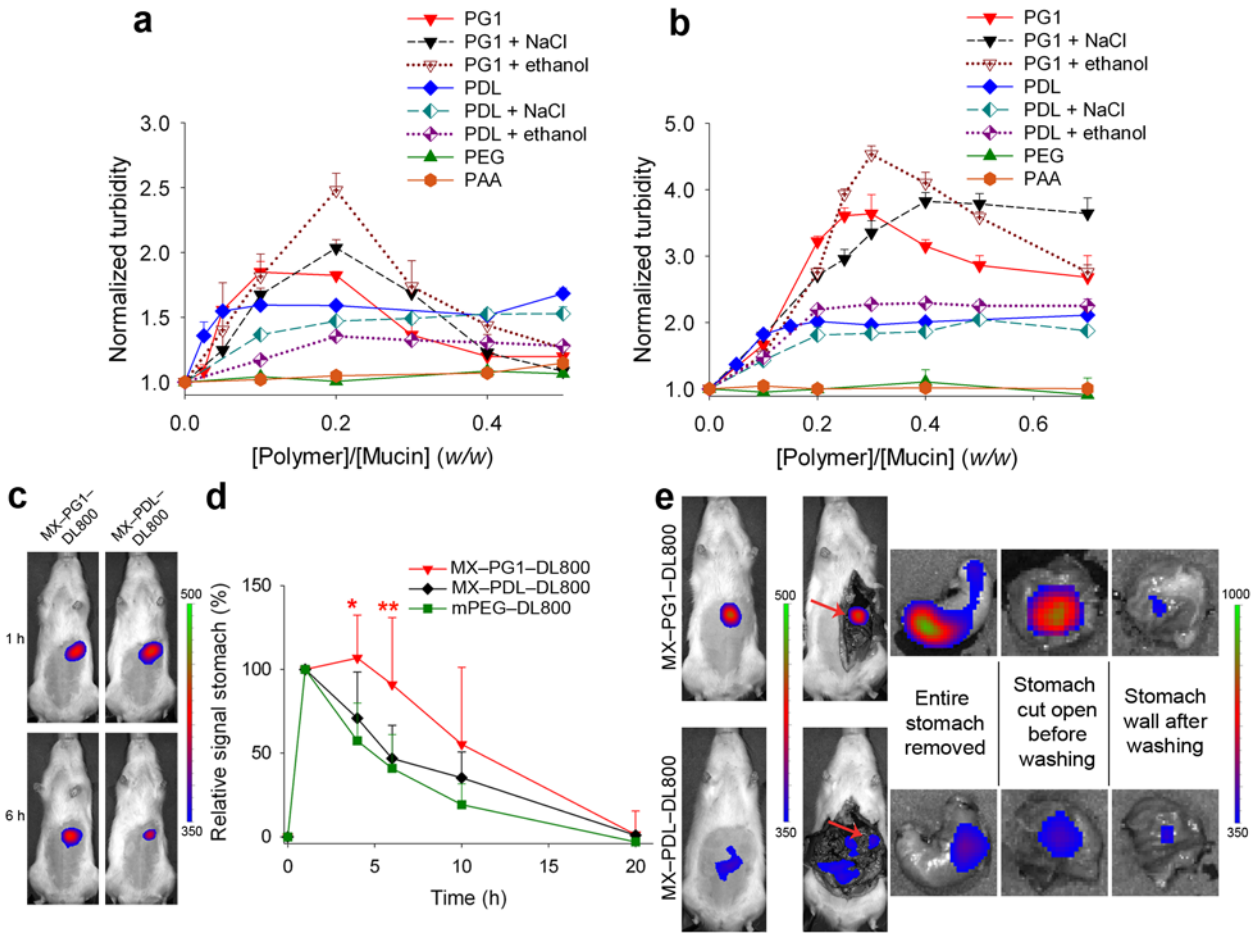

Figure 2. *In vitro* and *in vivo* characterization of mucoadhesion. (a,b) Turbidity was measured at 400 nm after incubation of mucin with polymers at **(a)** pH 1.8 and **(b)** 4.5. Mean ± SD, *n* = 3. **(c–e)** The fluorescence of MX–PG1–DL800, MX–PDL–DL800 and the control mPEG–DL800 was tracked over time *in vivo* after oral administration. **(c,d)** Evolution of fluorescence in the stomach. Mean + SD, *n* = 8–12. One star (*) or two stars (**) in the same color as the corresponding plot denotes significant difference from mPEG–DL800, or all other conjugates, respectively ($p < 0.05$). **(e)** After 6 h, rats were sacrificed and their abdominal wall opened. Fluorescence of MX–PG1–DL800 in the stomach (arrow) could be clearly detected, and was much stronger than that of MX–PDL–DL800. After opening the stomach and removing residual contents, MX–PG1–DL800 was found to be localized to the inner stomach wall (before washing) but was removed by a single washing with neutral buffer. Note that little or no signal was observed in the small intestine due to slow elution of the fluorescent entity, whose concentration was then below the detection limit of the imaging system.

Activity in model fluids. As it was *a priori* unclear which polymer would provide the best stabilization of MX in the GI tract, independently of complexation to mucin, MX was modified at its surface-exposed lysine residues with PG1, PDL, PAA or mPEG (MX–PG1, MX–PDL, MX–mPEG and MX–PAA; Supplementary Figs. S2, S6–8). The degree of MX modification was ca. 1 polymer chain for PG1 or PDL, and 14 or 15 chains for mPEG and PAA, respectively. Although the DP of the mPEG chains was lower than for the other polymers, the overall mass ratio of protein-to-polymer was comparable for all conjugates. The MX–mPEG conjugate, which bore multiple mPEG chains, was designed to favor stabilization in the GI tract owing to the expected poor performance of MX modified with a single chain of high molecular weight mPEG. Conjugate activity was first evaluated in fluids simulating the stomach and the small intestine. The degree of modification corresponded to the maximal extent of conjugation achievable before a dramatic decrease of activity was observed *in vitro*. In a simulated fed stomach (pH 4.5 with pepsin), the activity of MX and MX–polymer conjugates (2 $\mu g_{(protein)} \cdot mL^{-1}$) towards the model substrate *Z*-Gly-Pro-*p*NA was lost within 5 min (Fig. 3a). However, in simulated intestinal fluid (USP pH 6.8 with pancreatin), all polymers except PDL provided strong stabilization in comparison to native MX up to ~30 min. PG1 was the most effective polymer, despite the fact that only a single polymer chain was conjugated to MX, consistent with stabilization to unfolding evaluated by a tryptophan fluorescence (Supplementary Fig. S9). Interestingly, unconjugated PG1 also stabilized MX at pH 6.8 (Fig. 3b), suggesting a protecting role of polyion complexation between PG1 and MX (pI_{MX} ~7, Supplementary Fig. S2).

PAA and mPEG showed similar stabilizing effects, expected to originate from steric shielding from proteases (Fig. 3a). Comparable results were obtained for two other PEPs derived from *Flavobacterium meningosepticum* (FM) and *Sphingomonas capsulate* (SC; Supplementary Figs. S3, S10 and S11) pointing to a more general nature of these findings. Moreover, polymer modification had little effect on Michaelis-Menten parameters (Supplementary Fig. S12; three different PEPs conjugated to three different polymers each), suggesting that these findings are not specifically related to the active site of PEPs, and therefore applicable to a broader range of enzymes (*i.e.*, non-PEP).

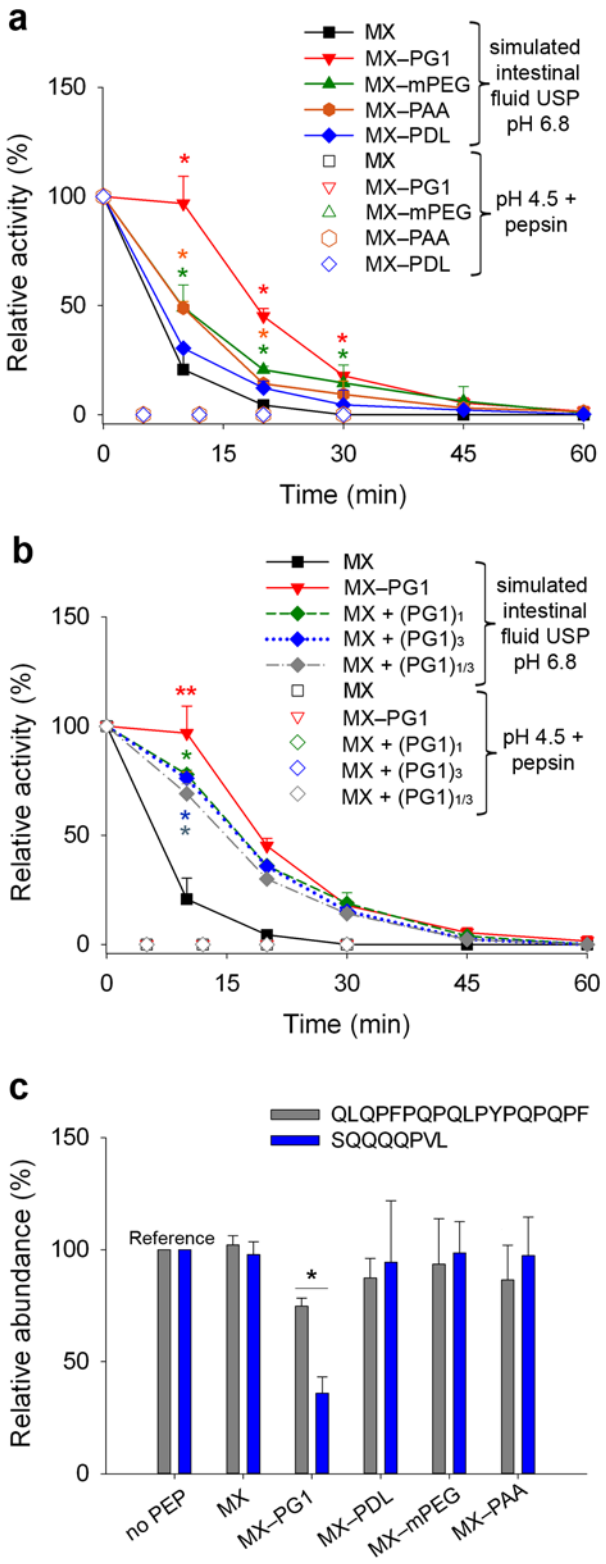

Figure 3. Stability and activity of MX and MX–polymer conjugates in simulated GI tract conditions. (a) Activity of MX or MX–polymer conjugates (2 $\mu g_{(protein)} \cdot mL^{-1}$) or **(b)**

MX with unconjugated PG1 (1, 3 and 1/3-fold molar excess) at pH 4.5 with pepsin (0.6 mg·mL^{-1}) or in intestinal fluid USP (pH 6.8 with 10 mg·mL^{-1} pancreatin) towards *Z*-Gly-Pro-*p*NA. Under acidic conditions all enzymes are inactivated in <5 min. Note that MX and MX–PG1 are identical in **(a)** and **(b)** and are presented for comparison purposes. **(c)** Abundance of immunogenic peptides after digestion of gluten (1:100, *w/w*) in simulated gastric environment (pH 4.5 with pepsin for 1 h) followed by intestinal conditions (pH 6.8 with trypsin and chymotrypsin). All plots shown as mean + SD, n = 3–7. One star (*) or two stars (**) in the same color as the corresponding plot denotes that activity was statistically different from MX, or all other conjugates, respectively (p < 0.05).

Macromolecular substrates. The covalent modification of enzymes with polymers typically hinders the processing of macromolecular substrates. To examine this, the proteolysis of whole wheat gluten, the trigger of celiac disease, by MX and MX–polymer conjugates was examined *in vitro*. Conjugates were incubated in acidic conditions mimicking a fed stomach along with whole wheat gluten for 1 h at 37 °C (average transit time from stomach to small intestine[15]). The solution was then neutralized to pH 7, trypsin and chymotrypsin added to simulate transition to the small intestine and incubation pursued for 2 h at 37 °C. After heat inactivation, the reaction mixture was analyzed by liquid chromatography-mass spectrometry for the abundance of gluten peptides (QLQPFPQPQLPYPQPQPF from α-gliadin[15] and SQQQQPVL from glutenin) (Fig. 3c). MX–PG1 significantly decreased the abundance of these peptides in comparison to controls with and without native MX. Conversely, MX–PDL, MX–PAA and MX–mPEG had no effect, indicating that steric stabilization would be inefficient in this model therapy involving a macromolecular substrate. These combined results show that the selective modification of MX with a single chain of PG1 may permit more facile

access to the larger gluten-derived substrates than for the MX–polymer conjugates of PAA and mPEG bearing multiple copies of polymer chains. This result shows that the electrostatic interactions between PG1 and MX (which were found above to stabilize MX–PG1 in neutral media) may permit a certain extent of dynamic reorganization to allow gluten peptides to access the active cleavage site of MX.

***In vivo* activity in the stomach and small intestine.** Based on the strong complexation of PG1 with mucin and its stabilizing effect on MX in model fluids, MX–PG1 was evaluated *in vivo* in comparison to MX–PAA and MX–mPEG, which served as anionic and neutral polymer controls. MX–PDL was not assessed due to the poor stabilizing effect observed using PDL *in vitro* and the lack on mucoadhesion *in vivo*. To monitor activity, a fluorescence-quenched peptide probe bearing both a fluorophore (HiLyte Fluor™647) and corresponding quencher (QXL™670) at each extremity was used as enzymatic substrate ((HiLyte Fluor™647)–LPYPQPK(QXL™670)) (Fig. 1c). Proteolysis of this probe led to a quantifiable 20–50-fold increase in fluorescence signal *in vitro* for all MX–polymer conjugates (Fig. 1d). The probe was gavaged 5 min after MX (or MX–polymer conjugates) to rats fasted for 4 h. Recovery of fluorescence was quantified to assess residual activity and location of proteolysis (stomach or small intestine) by *in vivo* imaging (Fig. 4a). A pre-cleaved peptide was used as positive control to normalize fluorescence intensity, and the probe alone was used as negative control. The dose of native MX (0.5 µg) was selected so that no *in vivo* activity was observed (stomach or small intestine) to highlight the stabilizing effect of polymer conjugation. MX–polymer conjugates were administered on an equimolar basis *versus*

the native enzyme. Rats did not show any signs of discomfort and their weight gain was normal (average weight increase >200% during 3 months). MX–PAA and MX–mPEG were not active in the stomach but regained activity in the small intestine (Fig. 4b), indicating that both polymers exerted a protecting effect, likely steric, during transit through the stomach. Furthermore, the comparable evolution of signal for rats treated with MX–PAA and MX–mPEG in the small intestine suggest that, in our *in vivo* model, these polymers were weakly or not mucoadhesive. Of particular interest, MX–PG1 was the only conjugate to be active in the stomach, leading to recovery of 41% of the signal of the positive control 1 h after intake (Fig. 4c). To put this finding in a more general context, SC, a PEP that differs from MX from the point of view of origin and sequence homology (<25%), was also examined *in vivo*. SC, SC–mPEG and SC–PG1 showed comparable results to their MX counterparts (Supplementary Fig. S13). These findings are somewhat surprising on the basis of a comparison with *in vitro* results (Fig. 3). These observations warranted further examination of the influence of the polymer on the *in vivo* stability of MX–PG1 conjugates in the stomach.

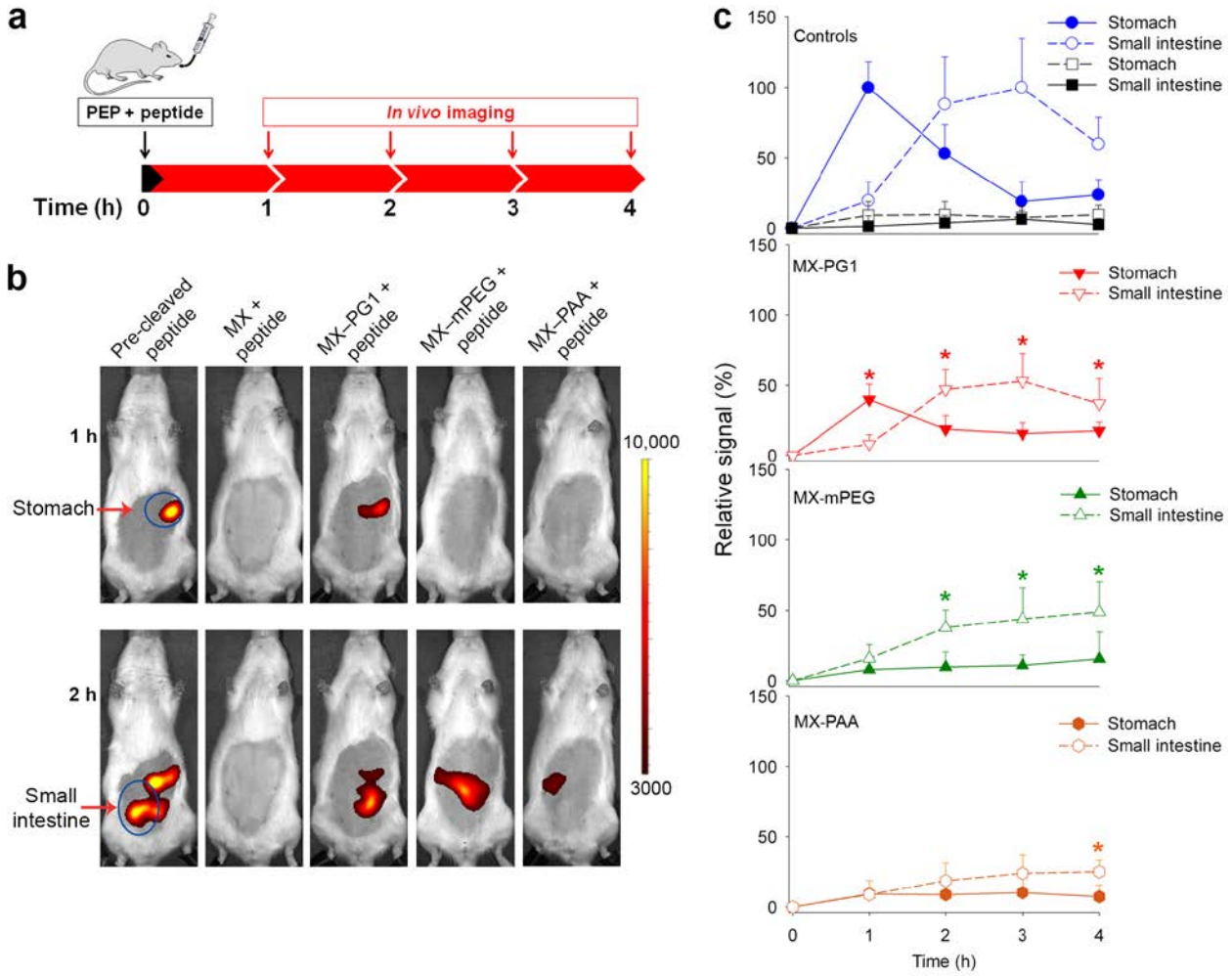

Figure 4. ***In vivo*** **activity of MX and MX–polymer conjugates. (a)** Schematic representation of experimental timeline used to analyze *in vivo* activity. MX or MX–polymer conjugates were administered by oral gavage 5 min prior to application of the probe substrate. **(b)** Images show the evolution of the fluorescent signal throughout the GI tract. MX–PG1 was the only conjugate to be active in the stomach. For each group a representative animal is shown. **(c)** Relative fluorescence signal in the region-of-interest of the stomach and the small intestine with time. Control samples were the pre-cleaved peptide (blue curves) and MX + peptide (black curves). MX–mPEG was active in the small intestine, while the signal produced by MX–PAA was only significantly different from native MX at 4 h. Mean + SD, n = 6–9. One star (*) indicates that fluorescence intensity was statistically different from that produced by native MX ($p < 0.05$).

Stability of MX–PG1 in the stomach. In light of its strong retention in the stomach, the stability of MX–PG1 was assessed over a 6 h period. MX–PG1 was administered to rats and then the probe substrate was given either 2 or 4 h afterwards (Fig. 5a). After 3 h residence in the stomach, this conjugate displayed comparable activity (32%) to that observed in Fig. 4c indicating little or no deactivation of the enzyme over this period (Figs. 5b and c). Moreover, fluorescence due to probe proteolysis coincided with the fluorescent signal for the labeled MX–PG1–DL800 (Fig. 5c). The activity then vanished after 5 h residence in the stomach (Figs. 5c and d). These unprecedented results point to PG1 providing a strong stabilizing effect in the harsh environment of the stomach in addition to mucoadhesion. In rats, the gastric emptying has been reported to be around 15 min for liquids[36] and 60–120 min for solid dosage forms, even after brief isoflurane anesthesia[37]. Our findings show that PG1 is not easily washed away with the normal gastric peristalsis.

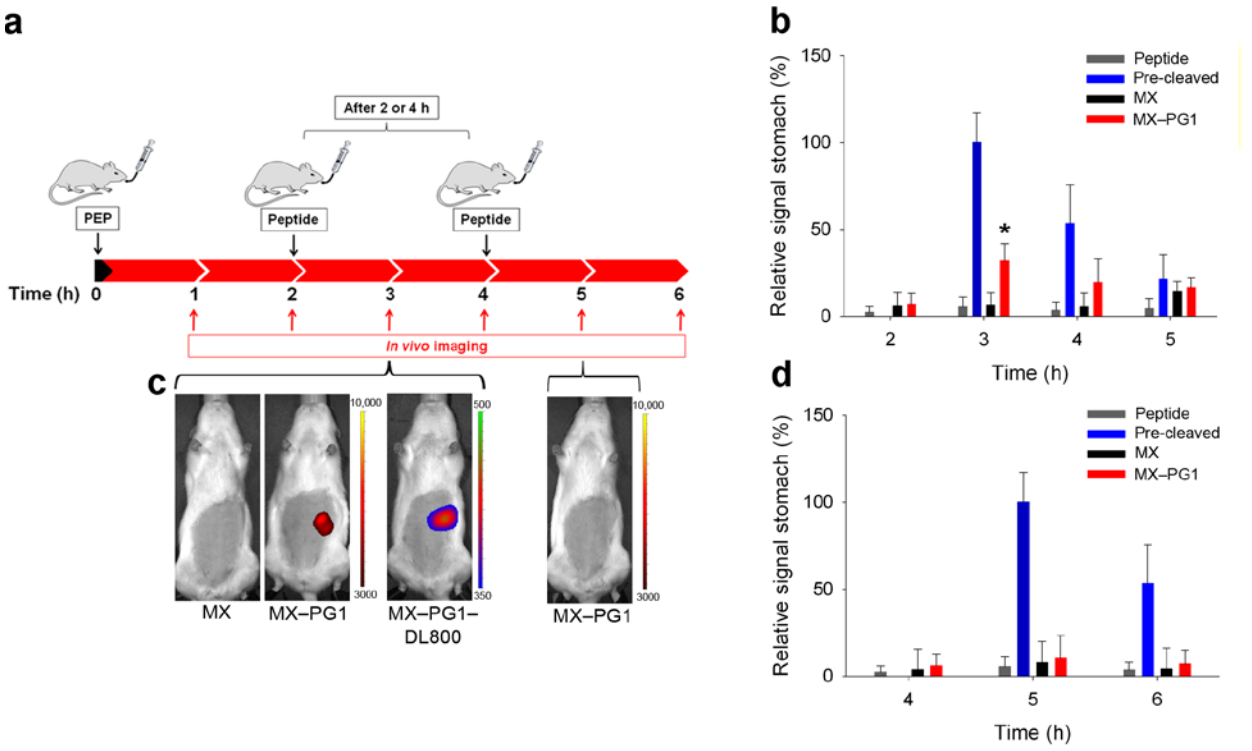

Figure 5. Activity of MX–PG1 in the stomach. (a) Schematic depiction of experimental timeline used to analyze the gastric stability of MX–PG1. MX or MX–PG1 were gavaged orally at 0 h. The probe substrate was administered either **(b)** 2 h or **(d)** 4 h after the PEP. The evolution of fluorescence was then followed by *in vivo* imaging between 1 and 6 h to measure residual enzyme activity. **(b)** At 3 h in the stomach, MX–PG1 was still active. In **(c)** representative animals are shown at 3 h and 5 h, after having received the probe at 2 h (MX or MX–PG1) or 4 h (MX–PG1). Co-localisation of the cleaved peptide (λ_{em} = 700 nm) and MX–PG1–DL800 (λ_{em} = 800 nm) was observed in a control experiment performed under identical conditions (middle). **(d)** After 5 h in the stomach, MX–PG1 was no longer active. One star (*) denotes that fluorescence intensity was statistically different from that produced by native MX ($p < 0.001$). Data plotted as mean + SD, n = 5–8.

Discussion

For some diseases, processing of potentially deleterious agents is desirable in the upper part of the GI tract to prevent any harm or absorption in the small intestine or colon (gluten in celiac disease[38,39], phenylalanine in phenylketonuria[40] or lactose in hypolactasia[13]). Recently, a PEG-modified enzyme evaluated as a therapeutic option for phenylketonuria was able to reduce phenylalanine levels by 37% (*vs.* 27% for the native enzyme)[41]. In comparison, PG1 stabilized MX considerably more than mPEG (stomachal activity persisting >3 h), while concomitantly promoting retention in the stomach and preserving the ability to process macromolecular substrates. The mucoadhesion observed for PG1 is also reported for other cationic polymers[42,43]. However, the comparably poor mucoadhesive properties of PDL indicate that the density of electrostatic interactions as well as polymer architecture, conformation and functionality play an important role for this phenomenon in the stomach.

The activity of exogenous enzymes in the small intestine is desired for conditions such as exocrine pancreatic insufficiency[14,44]. Currently, enteric coated formulations, typically as microparticles[45], prevent the gastric degradation of enzymes until they can be released in the intestine. However, variability of size, which affects migration into the duodenum, and fluctuations of stomach pH affect their dissolution profile. Consequently, GI tract bioavailability can be erratic[46]. The results above indicate that MX can be strongly stabilized by conjugation with several chains of the neutral polymer mPEG, in agreement with other *in vivo* findings[41]. It should be noted, however, that MX–mPEG was unable to hydrolyze whole wheat gluten derived peptides under simulated GI conditions. Thus, steric stabilization may be limited to therapeutic enzymes whose

activity involves small molecules. The lack of activity of MX–mPEG in the stomach, however, suggests that while (at least) partial unfolding of MX in acidic medium may be occurring, the polymer chains prevent permanent inactivation. Interestingly, PG1 had the greatest stabilizing effect of all polymers in neutral conditions *in vitro*, possibly due to electrostatic interaction between the positively charged polymer and MX, which becomes slightly negatively charged at neutral pH (pI_{MX} ~7). A limitation of PG1 for applications where activity is desired in the small intestine is its high mucoadhesion in the stomach. A future challenge to be addressed would be to tailor PG1 to display lesser mucoadhesion while nevertheless preserving its stabilizing effect[47,48].

In summary, a straightforward and easily applicable approach for stabilizing therapeutic proteins at different locations in the GI tract is presented. Polymers typically used for the *in vivo* stabilization of proteins after systemic administration are generally neutral and designed to poorly interact with cells and biomacromolecules[49,50]. Conversely, in the GI tract, strong mucoadhesive interactions between the polymer and GI tract components prolonged retention and protected the former towards denaturation and proteolysis. This study portrays the first real time *in vivo* examination of the usefulness of modifying oral therapeutic proteins with polymers, and showcases significant enhancement of the *in vivo* performance of orally administered enzymes, both in the stomach or in the small intestine. Based on the fact that the observed results did not appear to arise from a specific interaction with the catalytic site of the PEPs, our findings should be applicable to other proteins. A better understanding of how polymer interactions *in vivo* can influence the performance of proteins in the GI tract will

undoubtedly contribute to improve therapeutic strategies based on orally administered enzymes designed to be active in different locations in the GI tract.

Methods

Preparation of MX–polymer conjugates. The synthesis, purification and characterization of labeled and unlabeled conjugates of MX with PG1, PDL, mPEG or PAA is described in detail in the Supplementary Methods section.

Analysis of mucoadhesion. *In vitro* mucoadhesion was analyzed using type II stomach porcine mucin. An aqueous stock suspension of mucin (3 $mg \cdot mL^{-1}$ water) was prepared in an ultrasonic bath (15 min) and was used for all experiments. This stock suspension was diluted with either PG1, PDL, mPEG or PAA in 50 mM citric acid buffer (pH 1.8) or 50 mM acetate buffer (pH 4.5). A fixed concentration of mucin (0.375 $mg \cdot mL^{-1}$) was used and the polymer–mucin ratio (*w/w*) was varied between 0–0.7. Turbidity was measured at 400 nm after incubation at 37 °C for 30 min. To analyze the mechanism of mucoadhesion, NaCl (0.2 M) or ethanol (10 vol %) were added to the polymer–mucin mixtures.

Animal experiments were approved by the Cantonal Veterinary Office Zurich and were conducted as previously described[15]. Female Sprague-Dawley rats (5–18 weeks old, 100–300 g) were shaved in their abdominal region and were fasted for 12 h prior to each experiment. For *in vivo* mucoadhesion MX–PG1–DL800 and MX–PDL–DL800 (corresponding to 9.5 $\mu g_{(protein)}$ and 0.4 $\mu M_{(polymer)}$), or mPEG–DL800 (0.4 $\mu M_{(polymer)}$) in

300 µL sodium cholate (0.5 wt %; to ensure proper solubilization) was administered orally to rats by gavage. The fluorescence signal from the animals was measured at specific time points using an *in vivo* imaging system (IVIS® Spectrum, Caliper, Mainz, Germany) equipped with a heated platform (37 °C) and the following settings: $\lambda_{ex/em}$ = 745/800 nm, binning 8, f/stop 2, exposure time 2 s. For each animal, the intensity in the stomach before any application was used for normalization purposes.

For analysis of mucoadhesion to stomach mucin, rats were administered orally MX–PG1–DL800 or MX–PDL–DL800 (as above). After 6 h, rats were sacrificed, their abdominal wall opened and their stomach removed. The stomach was cut open in a line from the oesophagus to the duodenum and residual food was removed carefully using forceps. A fluorescence picture of the inner stomach wall recorded with the IVIS® instrument (settings as above). Subsequently, the empty stomach was washed for 30 s with 10 mM phosphate buffer (pH 7) and an additional image recorded.

Enzymatic activity in model fluids. *In vitro* stability and activity of MX and MX–polymer conjugates were evaluated as described in the Supplementary Methods section.

***In vivo* imaging of enzyme activity.** Prior to *in vivo* analysis, the ability of MX and MX–polymer conjugates (1 $\mu g_{(protein)} \cdot mL^{-1}$) to hydrolyze the probe (HiLyte Fluor™647)–LPYPQPK(QXL™670) (2.5 µM) in 50 mM phosphate buffer (pH 7) was verified by monitoring fluorescence over time ($\lambda_{ex/em}$ = 640/700 nm). In addition, the inability of

pepsin (0.6 mg·mL^{-1}, in 50 mM acetate buffer pH 4.5), trypsin and chymotrypsin (0.4 mg·mL^{-1} in 50 mM phosphate buffer pH 7) to hydrolyse the probe was also verified.

For *in vivo* imaging, MX or MX–polymer conjugates (0.5 µg protein in 250 µL phosphate buffer (10 mM, pH 7)) were mixed with gliadin (2 mg·mL^{-1}) and gavaged orally to rats pre-fasted for 4 h. The fluorescence-quenched probe (2.5 µM in 200 µL sodium cholate 0.5 wt %) was administered orally 5 min, 2 or 4 h after the enzyme, as displayed in the timelines shown in Figs. 4a and 5a. For imaging, rats were anesthetized (1.5–2% isoflurane, 0.5 mL·min^{-1} oxygen) and placed in an IVIS® equipped with a heated platform (37 °C). White-light images were taken, and the fluorescence signal coming from the animal was recorded ($\lambda_{ex/em}$ = 640/700 nm, binning 8, f/stop 2, exposure time 2 s), which was normalized to the pre-gavage intensity. A positive (*ex vivo* pre-cleaved probe) and a negative control (peptide alone) were administered separately and set as 100% and 0% of relative signal, respectively. Pictures were smoothed by 7 × 7 pixels to reduce background noise. Auto-fluorescence not related to the peptide cleavage and outside the abdominal area was removed in all displayed pictures.

Statistical analysis. One-way analysis of variance (ANOVA) followed by a Tukey *post-hoc* test was used for pairwise comparisons. Differences were considered significant at $p < 0.05$.

References

1 Bulow, L. & Mosbach, K. Multienzyme systems obtained by gene fusion. *Trends Biotechnol.* **9**, 226-231 (1991).

2 Zaks, A. & Klibanov, A. M. Enzymatic catalysis in organic media at 100 degree C. *Science* **224**, 1249-1251 (1984).

3 Klibanov, A. M. Improving enzymes by using them in organic solvents. *Nature* **409**, 241-246 (2001).

4 Swartz, M. A., Hirosue, S. & Hubbell, J. A. Engineering approaches to immunotherapy. *Sci. Transl. Med.* **4**, 148rv149 (2012).

5 Lele, B. S., Murata, H., Matyjaszewski, K. & Russell, A. J. Synthesis of uniform protein-polymer conjugates. *Biomacromolecules* **6**, 3380-3387 (2005).

6 Mahmoud, E. A., Sankaranarayanan, J., Morachis, J. M., Kim, G. & Almutairi, A. Inflammation responsive logic gate nanoparticles for the delivery of proteins. *Bioconj. Chem.* **22**, 1416-1421 (2011).

7 Zhu, G. Z., Mallery, S. R. & Schwendeman, S. P. Stabilization of proteins encapsulated in injectable poly (lactide-co-glycolide). *Nat. Biotechnol.* **18**, 52-57 (2000).

8 Keefe, A. J. & Jiang, S. Poly(zwitterionic)protein conjugates offer increased stability without sacrificing binding affinity or bioactivity. *Nat. Chem.* **4**, 59-63 (2012).

9 Frokjaer, S. & Otzen, D. E. Protein drug stability: a formulation challenge. *Nat. Rev. Drug Discov.* **4**, 298-306 (2005).

10 Harris, J. M. & Chess, R. B. Effect of pegylation on pharmaceuticals. *Nat. Rev. Drug Discov.* **2**, 214-221 (2003).

11 Pinier, M., Fuhrmann, G., Verdu, E. & Leroux, J.-C. Prevention measures and exploratory pharmacological treatments of celiac disease. *Am. J. Gastroenterol.* **105**, 2551-2561 (2010).

12 Sarkissian, C. N. *et al.* A different approach to treatment of phenylketonuria: Phenylalanine degradation with recombinant phenylalanine ammonia lyase. *Proc. Natl. Acad. Sci. USA* **96**, 2339-2344 (1999).

13 Enattah, N. S. *et al.* Identification of a variant associated with adult-type hypolactasia. *Nat. Genet.* **30**, 233-237 (2002).

14 Leeds, J. S., Oppong, K. & Sanders, D. S. The role of fecal elastase-1 in detecting exocrine pancreatic disease. *Nat. Rev. Gastroenterol. Hepatol.* **8**, 405-415 (2011).

15 Fuhrmann, G. & Leroux, J.-C. In vivo fluorescence imaging of exogenous enzyme activity in the gastrointestinal tract. *Proc. Natl. Acad. Sci. USA* **108**, 9032-9037 (2011).

16 Shan, L. *et al.* Structural basis for gluten intolerance in celiac sprue. *Science* **297**, 2275-2279 (2002).

17 Mitea, C. *et al.* Efficient degradation of gluten by a prolyl endoprotease in a gastrointestinal model: implications for coeliac disease. *Gut* **57**, 25-32 (2008).

18 Di Sabatino, A. & Corazza, G. R. Coeliac disease. *The Lancet* **373**, 1480-1493 (2009).

19 Jabri, B. & Sollid, L. M. Tissue-mediated control of immunopathology in coeliac disease. *Nat. Rev. Immunol.* **9**, 858-870 (2009).
20 Tack, G. J., Verbeek, W. H. M., Schreurs, M. W. J. & Mulder, C. J. J. The spectrum of celiac disease: epidemiology, clinical aspects and treatment. *Nat. Rev. Gastroenterol. Hepatol.* **7**, 204-213 (2010).
21 Sollid, L. M. Coeliac disease: dissecting a complex inflammatory disorder. *Nat. Rev. Immunol.* **2**, 647-655 (2002).
22 Husby, S. *et al.* European Society for Pediatric Gastroenterology, Hepatology, and Nutrition guidelines for the diagnosis of coeliac disease. *J. Pediatr. Gastroenterol. Nutr.* **54**, 136-160 (2012).
23 Cerf-Bensussan, N., Matysiak-Budnik, T., Cellier, C. & Heyman, M. Oral proteases: a new approach to managing coeliac disease. *Gut* **56**, 157-160 (2007).
24 Guo, Y. *et al.* Tuning polymer thickness: synthesis and scaling theory of homologous series of dendronized polymers. *J. Am. Chem. Soc.* **131**, 11841-11854 (2009).
25 Grotzky, A., Nauser, T., Erdogan, H., Schlüter, A. D. & Walde, P. A fluorescently-labeled dendronized polymer-enzyme conjugate carrying multiple copies of two different types of active enzymes. *J. Am. Chem. Soc.* **134**, 11392–11395 (2012).
26 Grotzky, A., Manaka, Y., Kojima, T. & Walde, P. Preparation of catalytically active, covalent alpha-polylysine-enzyme conjugates via UV/Vis-quantifiable bis-aryl hydrazone bond formation. *Biomacromolecules* **12**, 134-144 (2011).
27 Shan, L., Mathews, II & Khosla, C. Structural and mechanistic analysis of two prolyl endopeptidases: role of interdomain dynamics in catalysis and specificity. *Proc. Natl. Acad. Sci. USA* **102**, 3599-3604 (2005).
28 Liu, P. & Krishnan, T. R. Alginate-pectin-poly-L-lysine particulate as a potential controlled release formulation. *J. Pharm. Pharmacol.* **51**, 141-149 (1999).
29 Patel, M. M. *et al.* Mucin/poly(acrylic acid) interactions: a spectroscopic investigation of mucoadhesion. *Biomacromolecules* **4**, 1184-1190 (2003).
30 Ward, F. W. & Coates, M. E. Gastrointestinal pH measurement in rats: influence of the microbial flora, diet and fasting. *Lab. Anim.* **21**, 216-222 (1987).
31 Cook, M. T., Tzortzis, G., Charalampopoulos, D. & Khutoryanskiy, V. V. Microencapsulation of probiotics for gastrointestinal delivery. *J. Control. Release* **162**, 56-67 (2012).
32 Lehr, C.-M., Poelma, F. G. J., Junginger, H. E. & Tukker, J. J. An estimate of turnover time of intestinal mucus gel layer in the rat in situ loop. *Int. J. Pharm.* **70**, 235-240 (1991).
33 Lai, S. K., Wang, Y.-Y. & Hanes, J. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. *Adv. Drug Deliv. Rev.* **61**, 158-171 (2009).
34 Ensign, L. M., Cone, R. & Hanes, J. Oral drug delivery with polymeric nanoparticles: The gastrointestinal mucus barriers. *Adv. Drug Deliv. Rev.* **64**, 557-570 (2012).
35 Orts Gil, G., Łosik, M., Schlaad, H., Drechsler, M. & Hellweg, T. Properties of pH-responsive mixed aggregates of polystyrene-block-poly(l-lysine) and nonionic surfactant in solution and adsorbed at a solid surface. *Langmuir* **24**, 12823-12828 (2008).

36 Nilsson, F. & Johansson, H. A double isotope technique for the evaluation of drug action on gastric evacuation and small bowel propulsion studied in the rat. *Gut* **14**, 475-477 (1973).
37 Torjman, M. C., Joseph, J. I., Munsick, C., Morishita, M. & Grunwald, Z. Effects of isoflurane on gastrointestinal motility after brief exposure in rats. *Int. J. Pharm.* **294**, 65-71 (2005).
38 Green, P. H. R. & Cellier, C. Celiac disease. *New Engl. J. Med.* **357**, 1731-1743 (2007).
39 Turner, J. R. Intestinal mucosal barrier function in health and disease. *Nat. Rev. Immunol.* **9**, 799-809 (2009).
40 Blau, N., van Spronsen, F. J. & Levy, H. L. Phenylketonuria. *The Lancet* **376**, 1417-1427 (2010).
41 Sarkissian, C. N., Kang, T. S., Gamez, A., Scriver, C. R. & Stevens, R. C. Evaluation of orally administered PEGylated phenylalanine ammonia lyase in mice for the treatment of phenylketonuria. *Mol. Genet. Metab.* **104**, 249-254 (2011).
42 Griffiths, P. C. *et al.* PGSE-NMR and SANS studies of the interaction of model polymer therapeutics with mucin. *Biomacromolecules* **11**, 120-125 (2009).
43 Vandamme, T. F. & Brobeck, L. Poly(amidoamine) dendrimers as ophthalmic vehicles for ocular delivery of pilocarpine nitrate and tropicamide. *J. Control. Release* **102**, 23-38 (2005).
44 DiMagno, E. P., Go, V. L. W. & Summerskill, W. H. J. Relations between pancreatic enzyme outputs and malabsorption in severe pancreatic insufficiency. *New Engl. J. Med.* **288**, 813-815 (1973).
45 Domínguez–Muñoz, J. E. Chronic pancreatitis and persistent steatorrhea: what is the correct dose of enzymes? *Clin. Gastroenterol. Hepatol.* **9**, 541-546 (2011).
46 Fieker, A., Philpott, J. & Armand, M. Enzyme replacement therapy for pancreatic insufficiency: present and future. *Clin. Exp. Gastroenterol.* **4**, 55-73 (2011).
47 Hawker, C. J. & Wooley, K. L. The convergence of synthetic organic and polymer chemistries. *Science* **309**, 1200-1205 (2005).
48 Matyjaszewski, K. & Tsarevsky, N. V. Nanostructured functional materials prepared by atom transfer radical polymerization. *Nat. Chem.* **1**, 276-288 (2009).
49 Gauthier, M. A. & Klok, H.-A. Polymer-protein conjugates: an enzymatic activity perspective. *Polym. Chem.* **1**, 1352-1373 (2010).
50 Bertrand, N. & Leroux, J.-C. The journey of a drug-carrier in the body: An anatomo-physiological perspective. *J. Control. Release* **161**, 152-163 (2012).

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Author Contributions

G.F., M.A.G. and J-C.L. designed and conceived the study; G.F. and A.G. prepared and characterized conjugates *in vitro* with the help of R.L. and S.M.; G.F. conducted and analyzed all *in vivo* experiments; P.L., B.Z. and H.Y. synthesized and contributed compounds; G.F., M.A.G., A.G., P.W., A.D.S and J.-C.L. wrote the paper. All authors discussed the results and implications and commented on the manuscript at all stages.

Competing Financial Interests statement

None to declare.