1 **Lactase (β -galactosidase) encapsulation in hydrogel beads**
2 **with controlled internal pH microenvironments: Impact of**
3 **bead characteristics on enzyme activity**

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ABSTRACT

Individuals who suffer from lactose intolerance can alleviate many of their symptoms by ingestion of lactase (β -galactosidase) with lactose-containing foods. However, this enzyme is deactivated when exposed to highly acidic gastric fluids, which reduces the amount reaching the small intestine. Oral delivery systems are therefore needed to protect lactase in the stomach, and then release it in an active form in the small intestine. In this study, carrageenan-based hydrogel beads were fabricated that contained lactase and a buffer ($\text{Mg}(\text{OH})_2$). This buffer was selected because it is insoluble under alkaline conditions, but soluble under acidic conditions, and can therefore maintain a neutral pH inside the beads when they are dispersed in a low pH solution (such as acidic gastric fluids). Small ($D = 255 \mu\text{m}$) and large ($D = 2610 \mu\text{m}$) hydrogel beads were prepared using two different injection methods to compare the impact of bead dimensions on the retention of lactase activity. A ratiometric fluorescence method based on confocal laser scanning microscopy was developed to measure changes in the internal pH of the hydrogel beads before and after exposure to simulated gastric conditions. After exposure to stomach conditions, the internal pH of buffer-free beads dropped steeply (from around pH 7.0 to below pH 4.0), but that of buffer-loaded beads remained fairly constant (from around pH 7.2 to pH 6.6). When exposed to small intestine conditions, lactase encapsulated in buffer-loaded beads maintained its enzyme activity, whereas lactase encapsulated in buffer-free beads did not. Smaller beads needed a higher amount of $\text{Mg}(\text{OH})_2$ to maintain a neutral internal pH throughout the gastric phase, which was attributed to faster diffusion of hydrogen ions (H^+) into smaller beads. These results suggest that buffer-loaded hydrogel beads may be useful for encapsulation, protection, and delivery of acid-labile enzymes and other bioactive substances.

Keywords: carrageenan; microgels; polysaccharides; magnesium hydroxide; lactose intolerance

58 1. Introduction

59 Lactose intolerance is a common disease in humans that is associated with the
60 deficiency of a specific enzyme (β -galactosidase) produced within the brush border of
61 the small intestine (Heyman, 2006; Vesa, Marteau, & Korpela, 2000). Individuals
62 with lactose intolerance have a limited ability to digest and absorb lactose in the small
63 intestine. As a result, this disaccharide enters the colon in an undigested form where
64 it can promote health problems, such as gut distension, stomach pain, flatulence,
65 diarrhea, and nausea (Lomer, Parkes, & Sanderson, 2008). Individuals who suffer
66 from lactose intolerance often avoid consuming milk and other dairy products so as to
67 avoid exhibiting these undesirable symptoms. Several other approaches have also
68 been developed to aid individuals with lactose intolerance, including creating
69 lactose-free foods and the co-ingestion of lactase supplements with lactose-containing
70 foods (Montalto, et al., 2006). Enzyme supplement treatments are particularly
71 promising because they do not cause undesirable changes in food quality or
72 nutritional profile. Typically, a tablet or capsule containing lactase is taken at the
73 same time as a lactose-containing food, which promotes lactose hydrolysis within the
74 gastrointestinal tract (GIT). β -galactosidase (β -gal) is the most common form of
75 lactase used in enzyme supplements, but it cannot simply be delivered in its free form
76 because it is highly susceptible to denaturation and deactivation under GIT conditions
77 (Nichele, Signoretto, & Ghedini, 2011). In particular, lactase is usually deactivated
78 when exposed to the highly acidic gastric fluids present within the human stomach
79 (He, Zhang, & Sheng, 2014).

80 The encapsulation of enzymes within porous matrices can improve their stability
81 by creating a protective microenvironment or by acting as a physical barrier to the
82 external environment (Kim, Grate, & Wang, 2006; Xu, et al., 2008). At the same time,
83 the porous matrices can be designed to allow small molecules (such as co-factors,
84 substrates, and products) to easily diffuse into them and reach the catalytic site of the
85 encapsulated enzyme, which enables the enzyme to maintain its activity even after
86 encapsulation (Coviello, Matricardi, Marianecchi, & Alhaique, 2007; Es, Vieira, &
87 Amaral, 2015). Enzymes can be encapsulated within porous matrices using physical
88 entrapment, physical interactions, or chemical bonding (Betancor, Luckarift, Seo,

Brand, & Spain, 2008; Büyükbayram, Kıralp, Toppare, & Yağcı, 2006). The encapsulation of enzymes in hydrogels is particularly attractive for application in the food industry because these semi-solid materials can be fabricated from food-grade ingredients using simple processing operations (Zhang, Zhang, Chen, Tong, & McClements, 2015b). Enzymes are physically entrapped in the hydrogel network during the sol-gel transition, which is a relatively mild process that tends to preserve the structural integrity and activity of the enzymes. After fabrication, the enzymes can be retained within the hydrogel matrix by ensuring that its pore size is appreciably smaller than the enzyme dimensions, or by ensuring that there is a sufficiently strong attraction between the enzymes and the hydrogel matrix.

For many applications it is desirable to utilize hydrogel beads, rather than bulk hydrogels to encapsulate and deliver bioactive substances (Coviello, et al., 2007). Hydrogel beads (also known as microgels) consist of small particles (typically from 100 nm to 1000 μ m) that can easily be dispersed in aqueous solutions. These beads can be fabricated from food-grade biopolymers (typically polysaccharides and/or proteins) using simple processing operations, such as the injection-gelation method (Gombotz & Wee, 2012; Onwulata, 2012; Shewan & Stokes, 2013; Zhang, Zhang, Zou, & McClements, 2016). Typically, the formation of hydrogel beads requires two-steps that may occur consecutively or simultaneously: particle-formation and particle-gelation. For the injection-gelation method, particle-formation is performed by injecting an aqueous solution containing a biopolymer (gelling agent) and a bioactive agent (enzyme) into a gas or immiscible liquid, and then particle-gelation is performed by exposing the particles formed to solution or environmental conditions that promote biopolymer gelation. This process leads to the formation of hydrogel beads with bioactives trapped inside a biopolymer matrix (Li, Hu, Du, Xiao, & McClements, 2011). A serious limitation of this approach for the encapsulation of acid-sensitive enzymes (such as lactase) is that small hydrogen ions (H^+) can easily diffuse through the biopolymer network inside the hydrogel beads, thereby inducing enzyme deactivation. For this reason, a number of research groups have tried to improve the acid-resistant characteristics of hydrogel beads, *e.g.*, by reducing the pore size of the biopolymer network or by coating the beads with biopolymer layers (Srivastava, Brown, Zhu, & McShane, 2005; Taqieddin & Amiji, 2004). However,

these approaches are often unsuccessful because the hydrogen ions are so small that they can still easily penetrate through the biopolymer networks that make up the hydrogel core or shell.

The main objective of the present study was to develop an alternative approach to create hydrogel beads that would protect enzymes from acid-induced deactivation when exposed to gastric conditions. The enzyme (lactase) was co-encapsulated with magnesium hydroxide (a basic buffer) inside the hydrogel beads. $\text{Mg}(\text{OH})_2$ was selected as a buffering agent because it is a widely used food-grade antacid, which is insoluble in water at neutral and basic pH values, but soluble at acidic pH values (Zhu, Mallery, & Schwendeman, 2000). When these buffer-loaded hydrogel beads are dispersed in an acidic solution, the pH inside them remains close to neutral for an extended period. This phenomenon occurs because as hydrogen ions (H^+) from the surrounding aqueous phase diffuse into the hydrogel beads they cause some of the $\text{Mg}(\text{OH})_2$ to dissociate, which generates hydroxyl ions (OH^-) that neutralize any pH changes. Moreover, it has been shown that the presence of Mg^{2+} can increase the activity of lactase (Juers, et al., 2009; Lo, et al., 2010), which may be another advantage of co-encapsulation of this particular buffering agent with lactase. The hydrogel beads were fabricated by an injection-gelation method using an anionic polysaccharide (κ -carrageenan) as the gelling agent and a cationic mineral ion (potassium) as a cross-linking agent. This polysaccharide was selected because it was previously shown that relatively high encapsulation efficiency (around 63%) can be achieved by the carrageenan beads. In addition, β -gal encapsulated within potassium-carrageenan beads had a higher activity than the free enzyme due to the stabilization effects of K^+ ions on enzyme structure (Zhang, Zhang, Chen, & McClements, 2016). The ability of the buffer-loaded hydrogel beads to protect lactase from acid-induced deactivation was demonstrated by exposing them to simulated gastric conditions. Another objective of this study was to show that a quantitative ratiometric fluorescence method based on confocal laser scanning microscopy (CLSM) could be used to map the pH inside the beads before and after exposure to gastric conditions. This study provides information that can be used to facilitate the fabrication of hydrogel beads designed to encapsulate and deliver acid-labile enzymes and other bioactive agents.

2. Materials and methods

2.1. Materials

Fluorescein tetramethylrhodamine dextran (FRD) with an average molecule weight of around 70 kDa was purchased from Molecular Probes (Eugene, OR). Lactase (β -galactosidase) with a specific activity of around 2600 U/g was obtained from Sigma Chemical Co (St. Louis, MO, USA). Carrageenan was kindly donated by FMC Biopolymer (Viscarin SD 389, Philadelphia, PA). The reagents o-nitrophenol (o-NP) and o-nitrophenyl-b-d-galactosidase (o-NPG) were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). All chemicals used were analytical grade. Double distilled water was used to prepare all solutions.

2.2. Hydrogel beads preparation

An aqueous κ -carrageenan (2% w/v) solution was prepared by dissolving the powdered ingredient in distilled water by stirring at 50 °C for an hour, and then reducing the temperature to room temperature with continuous stirring until fully dissolved. The κ -carrageenan solution was then mixed with β -gal solution to obtain a concentration of 1% κ -carrageenan and 130 U β -gal mixture with or without different amount of $\text{Mg}(\text{OH})_2$ co-encapsulation. After continuously stirring, the mixtures were injected into 10% potassium chloride solution using a syringe or a commercial encapsulation unit (Encapsulator B-390, BUCHI, Switzerland) with a 150 μm vibrating nozzle to prepare the hydrogel beads. The encapsulation device was operated under the following conditions: frequency 800 Hz; electrode 750 V; and pressure 450 mbar. The formed beads were held in the K^+ solution for 30 min at ambient temperature to promote bead hardening.

2.3. Simulated gastric conditions

A simulated stomach model was used to investigate the influence of exposure to acidic gastric fluids on the internal pH and lactase activity of the hydrogel beads as described previously (Zhang, Zhang, Zhang, Decker, & McClements, 2015a). Hydrogel beads (with or without $\text{Mg}(\text{OH})_2$) were added to preheated simulated gastric

fluids (pH 2.5, 37 °C) at a ratio of 1:4 (w/w). This mixture was then incubated in an incubated shaker for 2 h at 37 °C to mimic stomach conditions.

2.4. Local pH Determination by Confocal Laser Scanning Microscopy

The fluorescence images were obtained using confocal laser scanning microscopy with a 20 × objective lens (Nikon D-Eclipse C1 80i, Nikon, Melville, NY, USA). The images of the fluorescence probe (FRD) were obtained using emission wavelengths of 543 and 488 nm, and detection wavelengths/bandwidths of 650 nm/LP and 590 nm/50 nm, respectively. All samples were imaged using an exposure time of 0.5 s and a 12.5% excitation power level. The complete images of each sample were typically acquired in less than 2 min with at least eight measurements. The microstructural images for confocal microscopy were acquired using image specialized software (NIS-Elements, Nikon, Melville, NY, USA).

A stock FRD solution (10 mg/mL) was prepared by dissolving powdered FRD dye in phosphate buffer (5mM, pH 7) solution. 5 µL/ml of FRD stock solution was dissolved in a series of phosphate buffer solutions with different pH values and imaged using confocal microscopy to determine the linear range of pH response.

The confocal microscopy images were analyzed using Image J software (1.50I, imagej.nih.gov). The ratio of pixel intensities of images taken using two wavelength channels (488 nm, 543 nm) was calculated and correlated to the pH using the standard curve. Each pH value was estimated from at least eight individual measurements.

2.5. Measurement of lactase activity

The relative activity of β-gal encapsulated in hydrogel beads that was retained after different incubation times in simulated gastric fluids was assayed using a colorimetric test with o-NPG as a substrate. Filtered beads were added to 2 mL of o-NPG in phosphate buffer (0.3 mg/ml) to reach a final alginate to o-NPG ratio of 1:40 (v/v). The formation rate of free o-NP was recorded by measuring the absorbance ($\lambda = 420$ nm) of solutions contained in a 1-cm path length cuvette using a UV-visible spectrophotometer. The relative activity of encapsulated lactase was calculated based on the conversion rate of the substrate (o-NPG) into the reaction

products (o-NP) initially and after stomach phase incubation. At least three freshly prepared samples were used for the activity measurements.

2.6. Measurement of bead dimensions

The particle size distribution of the small beads was measured using laser diffraction (Mastersizer 2000, Malvern Instruments Ltd., Malvern, Worcestershire, UK), which is based on analysis of the angular scattering pattern of particulate suspensions. Samples were diluted in aqueous buffer solutions to avoid multiple scattering effects, and then stirred (1200 rpm) to ensure homogeneity. The diameter of the large beads was measured using a digital micrometer (0–300 mm, EC10, High Precision Digital Caliper, Tresna Instruments, Guilin, China). The bead diameter of at least 5 individual beads was measured, and the mean and standard deviation were calculated from at least three measurements.

2.7. Statistical analysis

All experiments were performed on at least three freshly prepared samples. The results are reported as means and standard deviations calculated using Excel (Microsoft, Redmond, VA, USA). ANOVA analysis was carried out using a statistical software package (SPSS).

3. Results and Discussion

3.1. pH Mapping inside the beads

Knowledge of the local pH inside the hydrogel beads is required to better understand the ability of buffer-loaded beads to protect lactase from acid-induced deactivation in simulated gastric fluids. A pH-sensitive fluorescence probe (FRD) was therefore used to estimate the internal pH of the beads using a ratiometric fluorescence method. FRD has both pH-dependent (FITC) and pH-independent (TMR) fluorescence groups within its molecular structure (Chen & Arriaga, 2006; Deriy, et al., 2009). Consequently, the local pH can be determined by calculating the ratio of fluorescence intensities arising from the FITC and TMR groups. In this study,

the FRD was conjugated to a large molecular weight dextran (70 kDa) to ensure that the fluorescence dye remained trapped inside the hydrogel beads.

The confocal fluorescence microscopy measurements indicated that the emission intensity from the FITC channel (488 nm) decreased when the fluorescent dye was incubated in lower pH buffers, while the emission intensity from the TMR channel (543 nm) remained relatively constant (**Fig. 1**). A standard curve of fluorescence intensity ratio *versus* pH (from pH 4 to 7) was then obtained by taking the ratio of the emission intensities for the FITC/TMR channels (data not shown). The pH value inside the hydrogel beads could then be determined from the confocal fluorescence microscopy images using this calibration curve.

Confocal microscopy images of the hydrogel beads were taken before and after exposure to simulated gastric fluids (**Figs. 2 and 3**). For the buffer-free beads, the fluorescence intensity of the FITC channel (pH-dependent) changed from relatively strong before exposure to very weak after exposure, which is indicative of a substantial reduction in the internal pH of the beads based on the calibration curve (**Fig. 2**). The interior of the buffer-free beads was calculated to be around pH 6.9 before exposure, and below the limit of detection ($\text{pH} < 4$) after exposure. These results suggest that the hydrogen ions (H^+) from the acidic gastric fluids rapidly diffused into the hydrogel beads and decreased their internal pH (see [Section 3.4](#)). Conversely, for the buffer-loaded beads, the fluorescence intensity of the FITC channel only decreased slightly after incubation in simulated gastric fluids (**Fig. 3**). Indeed, the interior of the buffer-loaded beads changed from around pH 7.2 before exposure to around pH 6.6 after exposure. **This slight reduction in pH would not be expected to deactivate the enzyme.** As mentioned earlier, this effect can be attributed to the fact that the encapsulated $\text{Mg}(\text{OH})_2$ is insoluble under neutral conditions, but dissolves under acidic conditions. Consequently, when hydrogen ions from the gastric fluids diffuse into the hydrogel beads, some of the $\text{Mg}(\text{OH})_2$ dissolves, which generates OH^- ions that neutralize the H^+ ions, thereby maintaining a neutral pH inside the beads. Presumably, there was sufficient magnesium hydroxide initially present within the hydrogel beads to counteract all the hydrogen ions arising from the gastric fluids.

Optical microscopy images of the initial hydrogel beads indicated that the magnesium hydroxide particles were evenly dispersed throughout the interior of the hydrogel matrix (data not shown), as has also been reported in a recent study on related systems (Zhang, Zhang, Sun, Park, & McClements, 2017). Moreover, the fact that the fluorescence intensity of the initial hydrogel beads was fairly uniform throughout their interiors (Figure 2), also suggests that the magnesium hydroxide particles were initially fairly evenly distributed throughout the beads.

These experiments demonstrate that the fluorescence dye used in this study is sensitive to pH changes, and that it can be used to measure the local pH inside the hydrogel beads.

3.2. Influence of encapsulation on enzyme activity: Spectrophotometric assay

The local pH that lactase experiences within the stomach phase influences its molecular conformation and enzyme activity. For this reason, the activity of lactase encapsulated in hydrogel beads containing different amounts of co-encapsulated $\text{Mg}(\text{OH})_2$ was measured after incubation in simulated gastric fluids. The impact of bead dimensions on enzyme activity was determined by fabricating large and small hydrogel beads using either a simple syringe or a dedicated encapsulation unit, respectively. The hydrogel beads were then incubated in simulated gastric fluids for different times, and the enzyme activity was measured using a spectrophotometric method.

For both sizes of buffer-free beads, lactase activity was completely lost after 5 min incubation in simulated gastric fluids (Figs. 4 and 5). Presumably, this type of hydrogel bead was unable to prevent acid-induced loss of lactase activity because small H^+ ions easily diffuse through the pores in the biopolymer matrix and rapidly reduced the internal pH (Cook, et al., 2013). The fact that bead dimensions did not have a major impact in this case was probably because the diffusion process occurred so quickly, as highlighted by the mathematical calculations reported in Section 3.4. Conversely, for the buffer-loaded beads, the activity of the encapsulated enzyme depended on both the level of $\text{Mg}(\text{OH})_2$ present and the bead dimensions (Figs. 4 and 5). Lactase activity decreased steeply after 5 minutes incubation for large

buffer-loaded beads containing only 0.06% or 0.1% $\text{Mg}(\text{OH})_2$, with a complete loss of enzyme activity after 25 min incubation (**Fig. 4**). These results suggest that there was insufficient buffer present inside the beads to completely neutralize all the hydrogen ions (H^+) that diffused into them. At 0.15% and 0.3% $\text{Mg}(\text{OH})_2$, the enzyme activity within the large buffer-loaded beads remained > 60% after 120 min incubation (**Fig. 6**), which indicated that these systems were effective at preventing the acid-denaturation of lactase. This effect can be attributed to their ability to maintain a relatively high pH inside the hydrogel beads. For the small buffer-loaded beads, a higher amount (0.6 or 0.8%) of $\text{Mg}(\text{OH})_2$ was needed to maintain a reasonable level of enzyme activity at the end of the gastric phase (26 or 42% respectively) (**Figs. 5 and 6**). The observed impact of hydrogel bead dimensions on the activity of the enzyme can be attributed to the shorter distance that the H^+ ions have to diffuse into smaller beads (**Section 3.4**). In addition, the fraction of lactase molecules in close proximity to the surfaces of the hydrogel beads (and therefore more susceptible to acid denaturation) increases with decreasing bead diameter. This phenomenon may account for the fact that a higher enzyme activity was maintained at the end of the digestion period for the larger beads than the smaller ones (**Fig. 6**).

In the presence of sufficiently high levels of magnesium hydroxide the enzyme activity remained relatively high and constant after the first 5 minutes of incubation (**Figs. 4 and 5**). This suggests that a fraction of the lactase was rapidly inactivated, while the rest remained in an active state. Presumably, the lactase molecules closest to the surface of the hydrogel beads were inactivated when hydrogen ions diffused into them and reduced the local pH before there was time for the magnesium hydroxide particles to dissolve and release neutralizing hydroxyl ions. In addition, some of the lactase molecules at the surfaces of the beads may have diffused into the surrounding gastric fluids and been inactivated.

3.3. Influence of encapsulation on enzyme activity: Visual appearance

Additional insights into the impact of encapsulation on the stability of lactase was obtained by measuring changes in the appearance of hydrogel bead suspensions incubated at pH 2.5 (gastric) and pH 7.0 (intestinal) in the presence of a lactase substrate (o-NPG). Photographic images of the samples were taken periodically to

indicate color changes during incubation: an increase in the intensity of the yellow color of the samples is an indication of a higher conversion of o-NPG to galactose o-NP by lactase.

For the buffer-free beads, the yellow color was evenly distributed within the o-NPG solution during incubation at pH 7 (**Fig. 7a**), which suggests a high enzyme activity under these conditions. Conversely, when buffer-free beads were incubated in the o-NPG solution at pH 2.5, no color change was observed indicating that the enzyme was completely deactivated under these acidic conditions (**Fig. 7b**). For the buffer-loaded beads incubated at pH 2.5, the yellowish color first appeared in the hydrogel beads located at the bottom of the tube, then the external o-NPG solution became yellowish at longer incubation times (**Fig. 7c**). Presumably, the substrate (o-NPG) initially diffused into the hydrogel beads where it was hydrolyzed by the encapsulated lactase. At longer times, some of the reaction products diffused out of the hydrogel beads and into the surrounding aqueous phase, thereby turning the whole solution yellow. A schematic diagram of the proposed reactions taking place in these systems is shown in **Figure 8**.

3.4. Impact of bead dimensions on H^+ ion diffusion

The experimental studies indicated that the hydrogel bead dimensions had a major impact on the stability of the encapsulated lactase to acidic environments, which was attributed to the impact of particle size on the kinetics of H^+ ion diffusion into the beads. In this section, the impact of bead dimensions on hydrogen ion diffusion were therefore investigated using a mathematical model.

The particle size distributions of suspensions of both small and large hydrogel beads contained a single peak (**Fig. 9a**). Digital photographs of the samples showed that they contained fairly uniform size particles that were evenly distributed throughout the system (**Fig. 9b**). The mean particle diameters of the small and large beads were around 255 and 2610 μm , respectively.

The diffusion of hydrogen ions into hydrogel beads with different dimensions can be predicted using the following equation (Crank, 1979):

$$\Phi = \frac{M(t)}{M(\infty)} = 1 - \frac{6}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{n^2} \exp\left(-\frac{D_{gel} n^2 \pi^2 t}{a^2}\right) \quad (1)$$

360

361 Here, Φ refers to the fraction of H^+ ions that have diffused into the hydrogel beads at
 362 time t , $M(t)$ and $M(\infty)$ are the concentrations of H^+ ions inside the hydrogel beads at
 363 time t and at equilibrium, n is an integer, a is the hydrogel bead radius, and D_{gel} is the
 364 diffusion coefficient of the H^+ ions through the hydrogel bead matrix, which can be
 365 estimated based on the following expression (Chan & Neufeld, 2009):

366

$$D_{gel} = D_w \exp\left(-\pi \left(\frac{r_H + r_f}{\xi + 2r_f}\right)^2\right) \quad (2)$$

368 Here, D_w is the diffusion coefficient of the H^+ ions through pure water, r_H is the
 369 hydrodynamic radius of the H^+ ions, r_f is the cross-sectional radius of the biopolymer
 370 chains (κ -carrageenan) in the hydrogel network, and ξ is the mesh pore diameter of
 371 the hydrogel network. The radius (r_f) of the carrageenan chain has been reported to be
 372 around 0.50 nm (Thành, Yasunaga, Takano, Urakawa, & Kajiwara, 2001). Typical
 373 pore sizes of biopolymer hydrogels are reported to be in the range 5 to 500 nm
 374 (Zhang, et al., 2015b), and a value of 15 nm was assumed for the purpose of this
 375 study. This value was selected because it is at the low end of the pore size range, and
 376 therefore provides an estimate of the maximum potential of the beads to limit
 377 diffusion. The hydration (r_H) radius of the H^+ ions (actually H_3O^+ ions) has been
 378 estimated to be around 0.1 nm (Marcus, 2012). The diffusion coefficient of H^+ ions in
 379 water can be obtained from: $D_w = k_B T / 6\pi\eta r_H$, where k_B is Boltzmann's constant, T is
 380 absolute temperature, and η is the viscosity of the solvent. These equations can be
 381 used to estimate the time-dependence of the fraction of H^+ ions diffusing into
 382 hydrogel beads with different sizes after incubation in the stomach phase (**Fig. 10**).

383 The calculations highlight the importance of hydrogel bead dimensions on the
 384 diffusion rate of H^+ ions into the beads. The predictions show that H^+ ions can diffuse
 385 into the small beads much quicker (in a few seconds) than into the large ones, which
 386 would account for the fact that a higher amount of $Mg(OH)_2$ has to be loaded into the
 387 smaller beads to maintain a neutral internal pH. These calculations partly account for

the reason that the smaller beads are less effective at protecting the lactase from acidic conditions than the larger beads

In practice, smaller hydrogel beads are often preferred for applications in the food industry because they give products with more desirable optical, rheological, stability, and mouthfeel characteristics. This study has shown that smaller beads can still be used to protect lactase from acid-induced degradation, but that a higher level of $\text{Mg}(\text{OH})_2$ must be incorporated into the beads. In addition, it may be possible to further enhance enzyme activity by using different types and amounts of biopolymers or cross-linking agents to control the hydrogel pore size and physical interactions.

4. Conclusion

In this study, lactase was encapsulated within potassium carrageenan-based hydrogel beads using the injection-gelation method. The acid-stability of the enzyme could be improved by co-encapsulating it with a basic buffering agent: $\text{Mg}(\text{OH})_2$. This buffer is insoluble under neutral conditions, but dissolves when hydrogen ions diffuse into the hydrogel beads, thereby ensuring a neutral internal pH (as long as some buffer remains). A quantitative ratiometric fluorescence method proved to be highly effective at determining the local pH inside the beads before and after exposure to simulated gastric conditions. The interior of the buffer-free beads rapidly decreased from pH 6.9 to < 4 when exposed to the stomach phase, which led to rapid deactivation of the encapsulated lactase. Conversely, the interior of the buffer-loaded beads remained relatively close to neutral (pH 6.6) even after 2 hours exposure to simulated gastric fluids, which helped to retain lactase activity. A higher amount of $\text{Mg}(\text{OH})_2$ was required to protect lactase encapsulated in small beads than in large beads, which was attributed to faster diffusion of H^+ ions into the smaller bead interiors. This study suggests that buffer-loaded hydrogel beads may be suitable food-grade delivery systems for acid-sensitive enzymes, such as lactase. However, *in vivo* animal and human studies are still needed to confirm that buffer-loaded beads can maintain lactase activity under real life conditions.

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Figure 1. Confocal fluorescent images of a fluorescence probe dispersed within phosphate buffer solutions with different pH values. The images in the top row (TMR channel) and bottom row (FITC channel) represent the fluorescence intensity determined at emission wavelengths of 543 and 488 nm, and detection wavelengths of 650 and 590 nm, respectively.

Figure 2. Fluorescent microscopy images of hydrogel beads without $\text{Mg}(\text{OH})_2$ encapsulation before and after stomach digestion. The images shown in the ratio channel were generated using Image J software (imagej.nih.gov) and the color strip shown in the top right represents the pseudo-color change with pH (intensity of TMR signal was enhanced using Image J to improve contrast).

Figure 3. Fluorescent microscopy images of hydrogel beads containing 0.6% $\text{Mg}(\text{OH})_2$ before and after stomach digestion. The images of the ratio channel were generated using Image J software (imagej.nih.gov) and the color strip shown in the top right represents the pseudocolor change with pH (intensity of TMR signal was enhanced using Image J to improve contrast).

Figure 4. The relative activity of β -galactosidase encapsulated in large hydrogel beads after incubation in simulated gastric fluids for different times. The enzyme was encapsulated in hydrogel beads containing different amounts of $\text{Mg}(\text{OH})_2$ (0 to 0.30%). Samples designated with different letters (a, b) were significantly different (Duncan, $p < 0.05$) when compared between different delivery systems for the final point (120 min).

Figure 5. The relative activity of β -galactosidase encapsulated in small hydrogel beads after incubation in simulated gastric fluids for different times. The enzyme was encapsulated in hydrogel beads combined with different amounts of $\text{Mg}(\text{OH})_2$ (0 to 0.80%). Samples designated with different letters (a, b, c) were significantly different (Duncan, $p < 0.05$) when compared between different delivery systems for the final point (120 min).

Figure 6. The relative activity of β -galactosidase encapsulated in hydrogel beads after 120 minutes of incubation in simulated gastric fluids. The enzyme was encapsulated in hydrogel beads containing different amounts of $\text{Mg}(\text{OH})_2$.

(a)

(b)

(c)

Figure 7. Visual appearance of the color change of lactase-loaded large beads incubated in (a) the o-NPG solution at pH 7 without buffer co-encapsulation, (b) the o-NPG solution at pH 2.5 without buffer co-encapsulation, and (c) the o-NPG solution at pH 2.5 with 0.3% $\text{Mg}(\text{OH})_2$ co-encapsulation.

Figure 8. Schematic representation of physicochemical processes occurring in the lactase-loaded beads incubated in o-NPG solution at pH 2.5 with $Mg(OH)_2$ co-encapsulation.

(a)

(b)

Figure 9. (a) Particle size distributions and (b) visual appearances of carrageenan beads fabricated by syringe (large beads) and Encapsulator (small beads).

Figure 10. Predicted time dependence of the fraction of hydrogen ions (H_3O^+) absorbed by carrageenan beads with a diameter of 255 μm (small size beads) and 2609 μm (larger size beads) with a pore size of 15 nm after submersion in an acidic environment.

“Lactase (β -galactosidase) encapsulation in hydrogel beads with controlled internal pH microenvironments: Impact of bead characteristics on enzyme activity”

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Food Hydrocolloids

Graphical Abstract

Encapsulation of β -galactosidase (β -gal) in the hydrogel beads with self-regulating internal pH property to retain the enzyme activity after exposure to gastric conditions.