



A novel enzymatic technique for limiting drug mobility in a hydrogel matrix

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Abstract

An oral colon specific drug delivery platform has been developed to facilitate targeted release of therapeutic proteins as well as small molecule drugs. A simple enzymatic procedure is used to modify the molecular architecture of a lightly chemically crosslinked galactomannan hydrogel as well as a model drug–galactomannan oligomer conjugate, fluorescein isothiocyanate (FITC) tagged guar oligomer, to entrap the model drug. The enzyme-modified hydrogel retains the drug until it reaches the colonic environment where bacteria secrete enzymes (namely β -mannanase) to degrade the gel and release the drug molecule. Laser scanning confocal microscopy combined with fluorescence recovery after photobleaching is used to quantify the diffusion of the drug conjugate. The diffusion coefficient of solutes in the lightly crosslinked galactomannan hydrogel is approximately equal to the diffusion coefficient in the guar solution for simple diffusional drug loading. After drug loading, α -galactosidase treatment generates additional physical crosslinks in the hydrogel matrix as well as between the drug–oligomer conjugate and the hydrogel, which reduces diffusion of the drug–oligomer conjugate significantly. Degradation of the hydrogel by β -mannanase results in a slow and controlled rate of FITC–guar oligomer diffusion, which generates an extended release profile for the model drug.

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1. Introduction

Hydrogels have been gaining acceptance in a wide variety of biological applications such as drug carriers

and artificial organs [1]. Through selective chemical crosslinking, the hydrogels can be tailored to obtain specific water uptake, physical strength, and/or other physio-chemical properties. However, chemical crosslinking also has its limitations making it highly desirable to be able to create crosslinks without resorting to chemical methods. A prime example in this regard is guar galactomannan that has been used

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as the base material for oral colon specific drug delivery vehicles [2]. In this application, effective encapsulation and delivery of solutes requires a combination of strength and limited solute diffusion. Therefore, chemical crosslinkers are often added to provide the necessary mechanical stability, reduce hydrogel solubility and limit solute diffusion. However, initial crosslinking of the hydrogel to impart proper mechanical stability can make subsequent drug loading very restrictive, if not impossible. On the other hand, addition of drug prior to guar crosslinking, may cause the chemical crosslinker to react with the drug as well as the guar, creating drug variants which may become inactive or worst yet, toxic. The promiscuous behavior of chemical crosslinkers can therefore be a difficult problem to overcome. The use of enzymes, which have very high specificity, provides a powerful alternative to circumvent this issue. In particular, this approach can be exploited with guar galactomannan, which is susceptible to enzymatic hydrolysis [3].

Guar galactomannan (Guar) is a plant polysaccharide composed of a main chain of β -(1 \rightarrow 4) linked mannose sugars with single sugar α -(1 \rightarrow 6) linked galactose branches [4]. There are a variety of galactomannan polysaccharides with the only structural difference being the number of galactose branches [4]. Typically, the number of galactose branches in a galactomannan is quantified by the ratio of mannose to galactose (M:G), with guar having an M:G ratio of \sim 2. The M:G ratio plays a key role in determining the rheological and microstructural characteristics [5]. Without any galactose branches, mannan (linear linked mannose) will aggregate due to intermolecular hydrogen bonding of *cis*-hydroxyls on mannose and be completely insoluble in water [6]. These intermolecular associations are referred to as “hyper-entanglements”. As galactose branches are added, steric hindrance prevents intermolecular hydrogen bonding and aggregation. Guar gum has the most steric hindrance and hydrates in water almost instantaneously.

The varied characteristics of galactomannans with different M:G ratios are a result of the specific structure of the linear backbone of β -(1 \rightarrow 4) diequatorially linked mannose sugars, which allows the formation of hyper-entanglements in portions of the backbone devoid of galactose branches. These hyper-

entanglements were first observed when studying viscosity versus concentration of galactomannan [7]. A higher dependence on concentration was observed over other random coil biopolymers at concentrations above the entanglement concentration (c^*). This behavior has recently been synthetically produced by adding ethyl (hydroxyethyl) functional groups onto cellulose [8]. This addition enables the ethyl (hydroxyethyl) cellulose to self-aggregate through hydrogen bonding in segments of the polymer backbone that are unbranched [9,10]. While in the case of cellulose, the ability to self-aggregate was modulated by chemical synthesis of ethyl (hydroxyethyl) branches, the amount of hyper-entanglements in galactomannan polysaccharides can be increased and tailored by the use of non-chemical means such as enzyme modification.

An enzyme that can be used to modify the amount of galactose branches on guar is α -galactosidase. The α -galactosidase enzyme is commercially available and cleaves the α -(1 \rightarrow 6) glycosidic bond between the galactose branches and the mannan backbone [11]. By using the enzyme to remove galactose branches from guar, the molecular architecture of the guar can be changed from that of a homogeneous polymer to a “block” copolymer (regions with galactose and regions without galactose branches). The advantage of changing the structure to a block copolymer is that the new sections of the guar without galactose branches are able to self-aggregate, thus allowing the guar to form strong intermolecular physical crosslinks. (Fig. 1) The reduced number of galactose units on the guar backbone also enables it to form synergistic gels with polysaccharides such as xanthan [12]. The physical bonds created (following α -galactosidase action) in these synergistic gels, as well as homogeneous gels of guar mimic the effects of chemical crosslinks [13].

The purpose of this study is to investigate a potential platform for oral colon specific drug delivery based on the use of α -galactosidase to cleave off the galactose side chains from guar galactomannan hydrogels that have been formed through limited amount of chemical crosslinks. Enzyme modification after the drug has been loaded would enable the hydrogel to self-aggregate through hyper-entanglements of the galactose-depleted regions. As self-aggregation occurs, the model drug will be trapped

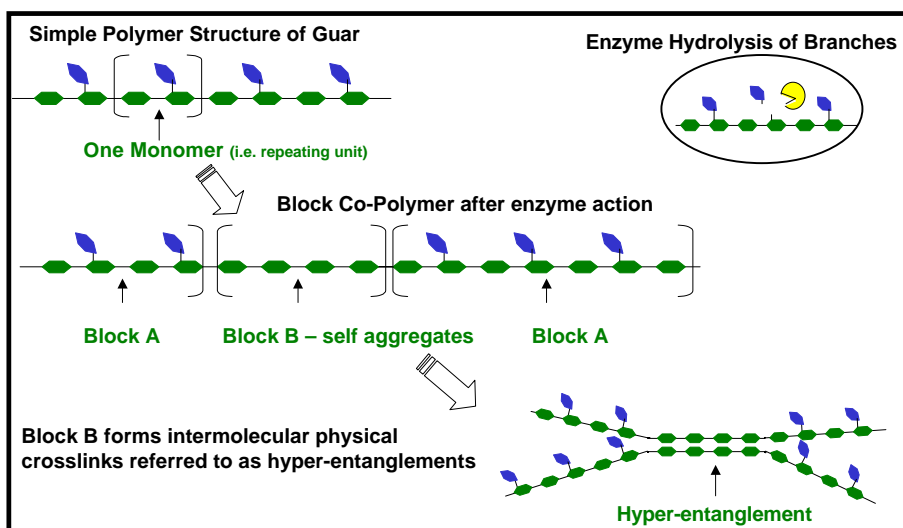


Fig. 1. Pictorial representation of the formation of hyper-entanglements in guar galactomannan due to enzymatic cleavage of the galactose side sugar by α -galactosidase.

in the hydrogel matrix by a decrease in the effective pore size of the hydrogel and limited mobility of the drug due to physical constraints. In order to prevent premature drug release, the model drug will be conjugated with a short guar oligomer, which will also be modified by α -galactosidase. Therefore, hyper-entanglements will also form between the hydrogel matrix and the drug–oligomer resulting in a further decrease in drug mobility. Drug release will occur when the hydrogel arrives in the lower GI tract and enzymes (i.e., β -mannanase) secreted from the microflora degrade the hydrogel and the oligomer releasing the drug. In this study, the effectiveness of the drug delivery platform in vitro is analyzed using the laser scanning confocal microscope, which allows the quantification of the mobility of the drug–oligomer conjugate. In particular, a partially hydrolyzed guar with a fluorescein isothiocyanate (FITC) attachment is used as a model for the drug–oligomer conjugate. The diffusion of this probe through the hydrogel is compared with the diffusion of a non-interacting probe (fluorescent tagged dextran) of similar molecular weight to obtain insights on the degree of hyper-entanglement of the guar oligomer with the hydrogel. The effect of enzymatic modification by both α -galactosidase and β -mannanase on probe diffusion is examined to obtain insights on solute (drug) entrapment and release under different

conditions, mimicking the enzyme activity in the colonic environment.

2. Materials and methods

2.1. Guar solution preparation

Guar gum, purchased from Aldrich, was sprinkled slowly into the vortex of water to a concentration of 7 mg/mL. This solution was vigorously mixed for 1 h followed by low shear mixing for 24 h. The solution was then centrifuged at 7000 rpm for 30 min. The supernatant was collected and 2 volumes of ethanol were added. The precipitate was collected and lyophilized for 48 h. The purified guar was ground to a fine powder with a mortar and pestle and redissolved in DIH₂O to the appropriate concentration. Sodium azide at 0.2 mg/mL was added as a biocide, 20 mM glycine was added and the pH was adjusted to 9. Both sodium azide and glycine were used as received from Sigma.

2.2. Preparation of titanium–guar hydrogels

Hydrogels were formed by mixing a purified guar solution (prepared as mentioned above) and Tyzor 131 (titanium crosslinker) to yield a guar hydrogel. The

final concentration of the guar and titanium were varied and the specific concentrations are mentioned for each experimental result. Tyzor 131 was a gift from Dupont Performance Chemicals and is a chelate of titanium used to crosslink aqueous polymer solutions.

2.3. Fluorescent probes of dextran and guar

The diffusion of two different probe molecules, a fluorescein isothiocyanate dextran (FITC–dextran) molecule and a FITC–guar conjugate, in the hydrogels were examined. Fluorescein isothiocyanate dextran (FITC–dextran) probes of molecular weight 31 kDa were purchased from Sigma. The wavelength of absorption maxima (λ_{abs}) and emission maxima (λ_{emis}) for the FITC–dextran probes were 488 and 514 nm, respectively. The concentration of the FITC–dextran probe in the hydrogel was kept constant at 0.4 mg/mL.

To prepare the fluorescently tagged guar oligomer, Benefiber, a partially hydrolyzed guar gum product from Novartis Nutrition (Minneapolis, MN), was used as the starting material. The Benefiber product was further purified by dialysis with a 10,000 MWCO Membrane 12 mL capacity Slide-A-Lyzer Cassette (Pierce). Dialysis was performed for one week in 1 L of water, with 0.2 mg/mL sodium azide, which was exchanged daily. The purified Benefiber product is hereon referred to as guar oligomer. Analysis of dialysis products was performed using size exclusion chromatography (SEC). FITC was attached to the guar oligomer following the protocol developed by de Belder and Granath (1973) and previously used by Khan and co-workers [14–16]. In summary, Benefiber (1 g) was dissolved in methyl sulphoxide (10 mL) containing a few drops of pyridine. Isothiocyanate fluorescein (0.05 g) was added, followed by dibutyltin dilaurate (20 mg), and the mixture was heated for 2 h at 95 °C. The solution was then dialyzed for 1 week against deionized water, which was exchanged daily. The resulting FITC–guar oligomer solution was lyophilized and dissolved in pH 9 20 mM glycine at 6 mg/mL. The degree of substitution was approximately 0.007 mol of sugar per mol of FITC. This was determined spectrophotometrically by heating the solution to 90 °C for 20 min and comparing its absorbance to an FITC standard curve. The FITC–guar

oligomer concentration used for diffusion coefficient measurement was 0.4 mg/mL.

2.4. Size exclusion chromatography (SEC)

Size exclusion analysis was performed with a Waters 2690 Separation Module and a Wyatt Optilab DSP Interferometric refractometer. TriSEC Data Acquisition System 1 software (Version 3.0, Viscotek) was used to collect the data, TriSEC Conventional GPC Module software (Version 3.0, Viscotek) was used to analyze the data and Millennium³² (Version 3.05, Waters Corp.) was used to control the equipment. The solvent used was 0.2 M NaNO₃ and was prepared by dissolving 34 g of NaNO₃ into 2 L of DIH₂O, filtered with a 0.2 µm membrane filter at a flowrate of 0.8 mL/min with a continuous online degasser and a column temp of 25 °C. Three Waters Ultrahydrogel columns 2000, 500 and 120 were used in series as well as a Waters Ultrahydrogel guard column. Pullulan polysaccharide standards were used: $dn/dc = 0.1460 \pm 0.0015$ @633 nm in 0.2 M NaNO₃ (determined by offline refractometer measurements in the laboratory) and the dn/dc of guar = $0.1505 + 0.0017$ @633 nm in the same solvent. These results are consistent with previous literature, which reports pullulan in 0.2 M NaNO₃ at 514 nm to have a dn/dc of 0.147 and guar in water at 633 nm to have a dn/dc of 0.153 [17,18]. Samples were prepared at 0.5 mg/mL and filtered by a 0.45 µm autovial filters (Fischer). The molecular weights of the pullulan standards used were: 1600, 380, 212, 100, 48, 22, 11 and 5 kDa. The SEC procedures used were based on previous work [17,19].

2.5. Diffusion coefficient measurements

The diffusion coefficient of FITC–dextran probes was quantified using laser scanning confocal microscopy (LSCM) and fluorescence recovery after photobleaching (FRAP) technique as previously mentioned [13,20]. An alternative approach to FRAP would have been to use fringe pattern bleaching and recovery (FPBR); however, this would not have affected the observed trends and outcomes of this study. Guar–titanium hydrogels were synthesized in a Grace Bio-Labs incubation chamber (PC200), placed on an ESCO frosted microscope slide and a 31 kDa FITC–

dextran probe was added after 24 h. To create a hydrogel with a homogeneous FITC–dextran concentration, the sample was then placed on a Thermolyne RotaMixer (Type 48200) for 24 h at 100 rpm. Aluminum foil was placed over the sample to prevent stray light from bleaching the FITC–dextran probes [13]. A Leica TCS NT laser scanning confocal microscope with a 10×0.3 NA dry PL Fluotar Objective and an argon ion laser was used to perform the FRAP experiments. The LSCM–FRAP experiment was summarized in a previous paper Burke et al. [13]. However, a few modifications of the previous procedure were performed during data analysis. A data fit program was used to predict the initial intensity of the bleached spot as well as a non-linear least square regression data fit program was used to extract the diffusion coefficient from the experimental data based on the theoretical equation developed by Blonk et al. [21]. These modifications yielded more accurate diffusion coefficient calculations.

2.6. Enzyme modification

Guar seed α -galactosidase (Megazyme, Ireland, Lot 50701, 150 U/mL) and *Aspergillus niger* β -mannanase (Megazyme, Ireland, Lot 50401, 41 U/mg, 297 U/mL) enzymes were used without further purification. Enzyme stock solutions were prepared at 3.32 U/mL for β -mannanase and 33.2 U/mL for α -galactosidase. Enzyme modifications were performed as explained in a previous paper [13], except for enzyme deactivation when using FITC–guar as a probe. When using FITC–guar, the probe was added to the hydrogel prior to enzyme addition and the reaction was stopped by adding 1-deoxygalactonojirimycin hydrochloride (DGJ) (Industrial Research Limited, New Zealand). DGJ was added at a concentration of 12.5 μ M and the hydrogels were immediately placed at 4 °C for five days, following which the diffusion coefficient was measured. During the β -mannanase incubation, the diffusion measurements were performed in situ to avoid enzyme deactivation by heat treatment at 90 °C for 10 min, which unfortunately also results in the hydrolysis of the thiocarbamoyl linkage releasing the FITC from the guar oligomer.

The concentration of β -mannanase used to mimic the in vivo concentration was adapted from the work

of Wong et al. who determined that 0.1 mg/mL of Gamanase best predicted in vivo performance [22]. Based on previous data from Tayal and Khan on viscosity and molecular weight reduction of guar solutions with Gamanase and purified β -mannanase (the main component of Gamanase), it was determined that 0.166 U/mL of purified β -mannanase best matched that of 0.1 mg/mL Gamanase [19,23].

2.7. Relative activity and enzyme stability measurements

The kinetics of α -galactosidase hydrolysis was measured using *p*-nitrophenol- α -D-galactopyranoside (PNPG, Sigma) as a substrate. The reaction was quantified by the in vitro hydrolysis of *p*-nitrophenol from PNPG and the subsequent increase in absorbance at 405 nm in a Perkin-Elmer Spectrophotometer. Data was collected and analyzed using UV KinLab software. For determination of the relative activity of α -galactosidase in the presence of DGJ, the same buffer conditions were used as mentioned above for the hydrogel preparation, with 50 mM PNPG at 22 °C. Relative activity was defined as the percent activity compared to the solution without DGJ. For the determination of enzyme stability, a 50 mM sodium phosphate buffer at pH 6 with 5 mM PNPG at 30 °C was used. In this case, the relative activity was defined as the percent activity compared to the enzyme solution immediately prepared and measured at pH 6. Enzyme kinetics were performed at an enzyme concentration of 1.66 U/mL.

3. Results and discussion

The strategy employed in this study has been to conjugate a model drug, FITC, with a guar oligomer, embed it in a guar hydrogel, and enzymatically modify both using α -galactosidase. The molecular weight of FITC (389 Da) is similar to many small molecule drugs and allows the use of established methods to determine diffusivity using confocal laser scanning microscopy. The conjugation of FITC to a short guar oligomer will enable the formation of hyper-entanglements between the drug–oligomer and the hydrogel matrix, thereby reducing drug mobility. Yet, when exposed to colonic enzymes, the guar oligomer will

degrade and release the FITC in a fashion similar to an enzymatically cleaved prodrug. In addition, the molecular weight of the drug–guar oligomer conjugate is within the limits of renal excretion of polymeric prodrugs [24,25]. Fig. 2 presents an overview of the strategy through a pictorial representation of the crosslinking/drug loading procedure. In the first step of the procedure, the guar galactomannan solution is lightly crosslinked chemically to provide some initial mechanical stability during drug loading, but not enough to reduce the solute diffusion coefficient from that in the guar solution. In this paper, the hydrogel was crosslinked with titanium but a variety of crosslinkers can be used for this purpose. Next, the drug, which is conjugated to a guar oligomer, is loaded into the hydrogel by diffusion, taking advantage of the fact that the drug–oligomer mobility in the hydrogel is similar to that in solution. Finally, α -galactosidase is added to the hydrogel to create physical crosslinks within the hydrogel matrix as well as between the guar oligomer and the hydrogel.

3.1. Isolation, characterization and FITC conjugation of the guar oligomer

To implement this strategy, a guar oligomer was attached to a model drug. A specific size oligomer was first isolated by dialysis with a 10,000 molecular

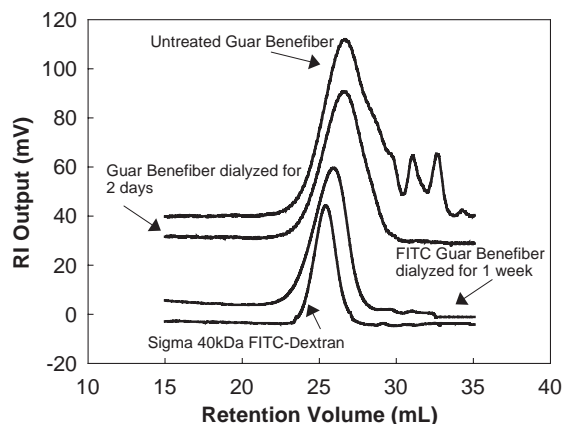


Fig. 3. Refractometer chromatograms from SEC experiments for the analysis of the guar oligomer purification and molecular weight determination. The untreated Benefiber had many small molecular weight contaminants, which were removed by a 10 kDa MW cut-off slide-a-lyzer (Pierce). After two days of dialysis, the molecular weight was 20.6 kDa but the polydispersity index (PDI) was 2.37, higher than preferred. Dialysis for 1 week yielded a molecular weight of 34.1 kDa and a good polydispersity of 1.80. The HPLC chromatogram of a Sigma 40 kDa FITC–dextran (MW=31 kDa and PDI=1.3) is also shown for comparison.

weight cut-off membrane from Benefiber, a mixture of hydrolyzed guar oligomers [26]. Fig. 3 shows chromatograms of the untreated Benefiber and the dialyzed sample, which has a molecular weight of ~20 kDa. The isolated guar oligomer was then conjugated

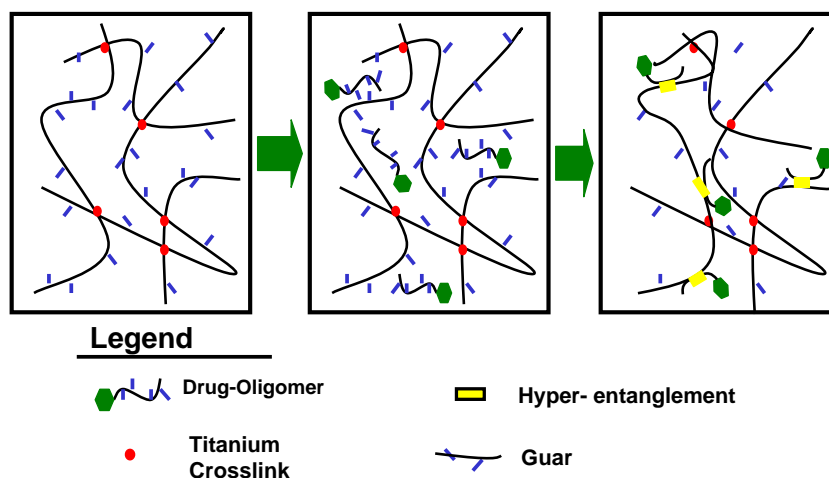


Fig. 2. Pictorial representation of drug (FITC)–guar oligomer entrapment in the hydrogel by enzyme modification of the guar galactomannan with α -galactosidase. The removal of galactose branches from the backbone of guar by the enzyme creates exposed regions of the mannan backbone, where hyper-entanglements between the FITC–guar oligomer and the hydrogel matrix can occur, i.e., physically crosslinking of the drug to the hydrogel.

to the model drug, which in this case was fluorescein isothiocyanate (FITC). Conjugation with FITC allows quantitative mobility measurements of the drug–oligomer conjugate to be performed in vitro using confocal microscopy combined with FRAP. The FITC labeled guar oligomer was dialyzed again for one week to isolate a 34.1 kDa fraction with a polydispersity of 1.8 that was commensurate in size with a commercially available FITC–dextran probe having a molecular weight of 31 kDa and a polydispersity of 1.3 (Fig. 3).

Diffusion analysis in aqueous solutions of the FITC–dextran and FITC–guar oligomer reveals that the diffusion of the two probes are virtually identical (Fig. 4). When the FITC–guar oligomer is placed in a guar solution, the diffusion coefficient is only slightly lower than FITC–dextran due to the natural entanglements between the FITC–guar oligomer and the high molecular weight guar through the latter's exposed portion of the mannan backbone. After α -galactosidase modification the FITC–guar oligomer will act as an interacting/active probe through formation of strong hyper-entanglements with the guar while the dextran probe will act as a non-interacting/passive probe. A comparison of the diffusion of the two probes could therefore be used to determine the extent of hyper-entanglement formation.

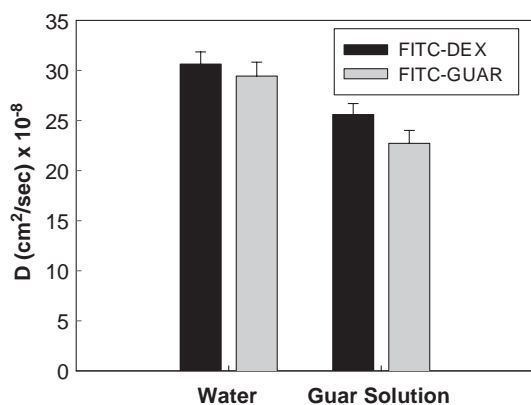


Fig. 4. Diffusion coefficient of the Sigma 31 kDa FITC–dextran probe and the FITC–guar oligomer probe in an aqueous solution and a 5 mg/mL guar solution. The guar oligomer and the dextran probe have essentially identical diffusion coefficients in water, within error bars. The natural amount of hyper-entanglement present in the solution due to exposed portions of the mannan backbone only slightly reduces the diffusion of the guar oligomer compared to the dextran probe.

Although a simple model drug is used for the present research, the conjugation of actual drug products may reveal some limitations to this drug delivery vehicle. However, if a protein therapeutic is used, the conjugation may be easier and more reproducible than expected. Recent advances in enzymology through site-directed mutagenesis have been able to transform those enzymes, which hydrolyze polysaccharides, into enzymes that are able to perform transglycosylation reactions effectively [27,28]. Using these advances in combination with nature's protein glycosylation pathways would provide a simple and very specific addition of sugars and polysaccharide oligomers to protein therapeutics. In addition, adding the guar oligomer by an enzymatic route versus a chemical technique may allow the oligomer to be completely hydrolyzed from the protein by enzymes such as β -mannanase and β -mannosidase.

While the use of FITC as a model drug provides many advantages such as simple conjugation methods and accurate mobility measurements, one disadvantage is the heat labile nature of the thiocarboamoyl linkage [14]. In previous work on enzyme incubation of guar hydrogels, the enzyme was deactivated by heating to 80 °C for 10 min, followed by FITC–dextran probe addition after the hydrogel was cooled to room temperature [13]. However, this heat procedure cannot be used with the FITC–guar oligomer because enzyme modification of the guar oligomer is also needed. If the FITC–guar oligomer is added to the hydrogel before enzyme modification, the FITC would be hydrolyzed from the guar oligomer during the enzyme deactivation step. When the FITC–guar oligomer was modified separately from the hydrogel and then added to it, a heterogeneous distribution of the probe in the hydrogel was found. It is believed that the probe formed hyper-entanglements on the surface of the hydrogel, rapidly reducing probe mobility and preventing homogeneous dispersion in the hydrogel.

3.2. 1-Deoxygalactonojirimycin hydrochloride α -galactosidase inhibition

To circumvent these problems, an alternative technique to deactivate the enzyme by use of the α -galactosidase inhibitor, 1-deoxygalactonojirimycin hydrochloride (DGJ), was chosen. DGJ is known to

be a strong, reversible inhibitor for α -galactosidase from a variety of sources but has not been used with α -galactosidase isolated from guar seed [29,30]. The relative activity of the enzyme in the presence of DGJ at various concentrations is examined in Fig. 5. The enzyme rapidly loses activity at very low concentrations of DGJ; however, a basal level of enzyme activity (approx. 5%) is found at all DGJ concentrations due to the reversible nature of the inhibitor. Therefore, in addition to adding the DGJ inhibitor to the hydrogel to stop the α -galactosidase activity, the hydrogel was placed at 4 °C to stop the enzyme reaction at the appropriate time interval.

The synthesis of the hydrogel is typically conducted at pH 9 to generate effective titanium cross-linking [31]. At this pH, the enzyme might lose additional activity, even at 4 °C. So the experimental conditions were simulated and the enzyme activity determined after a 20 h α -galactosidase modification (Fig. 6). The enzyme loses only ~7% of its activity during the 20 h incubation at room temperature at pH 9. In contrast, addition of the DGJ results in a significant drop in relative activity to ~1%. After adding the DGJ inhibitor, the enzyme was immediately placed in a cold room at 4 °C. Portions of the enzyme sample were removed periodically and its

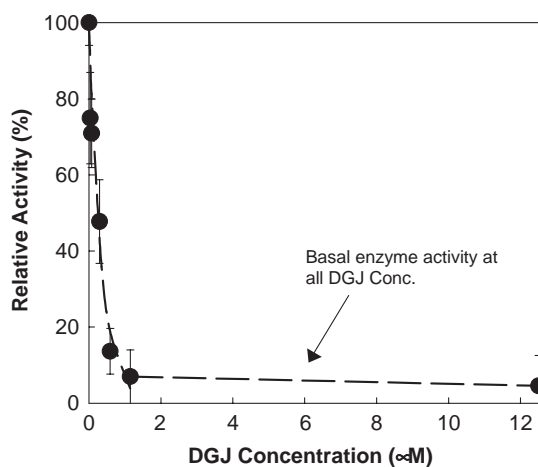


Fig. 5. Relative activity of α -galactosidase in the presence of deoxygalactonojirimycin hydrochloride (DGJ) at 22 °C and pH 9 in 20 mM glycine. The enzyme concentration was 1.66 U/mL. The enzyme activity decreased significantly upon addition of the DGJ; however, the enzyme continued to display approximately 5% relative activity even at high DGJ concentrations due to reversible rather than irreversible inhibition.

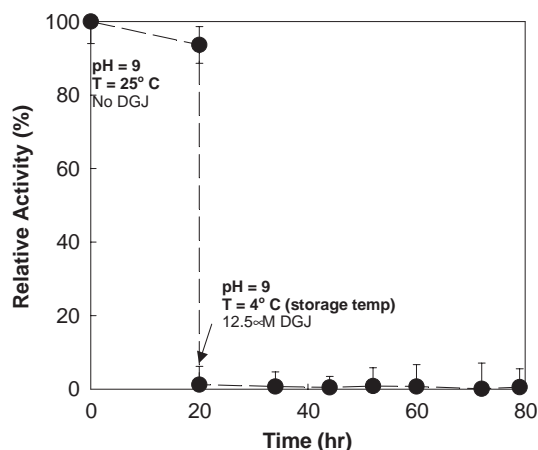


Fig. 6. Enzyme stability during simulated experimental conditions at pH 9 and in the presence of 12.5 μ M deoxygalactonojirimycin hydrochloride (DGJ). The enzyme was kept at pH 9 for 20 h at 25 °C, during which the enzyme lost only ~7% of its activity. However, addition of the inhibitor immediately dropped the enzyme activity to ~1%. After inhibitor addition, the enzyme was immediately placed in a cold room at 4 °C. Samples were removed at various time intervals and the activity of the enzyme with the DGJ was measured and remained below 1% for the extent of the experiment. Note that the activity of the enzyme was always measured at pH 6 and 30 °C during this experiment.

activity measured. Fig. 6 shows that the enzyme activity remains at a level of 1% throughout the rest of the experiment. It is important to note that the activity of the enzyme was measured at pH 6 and 30 °C versus pH 9 and 22 °C (in Fig. 5) because the substrate used, *p*-nitrophenol- α -D-galactopyranoside (PNPG), has significant auto hydrolysis at pH 9 and adjusting the pH to a lower value allowed more accurate measurements at the low levels of enzyme activity. However, in the previous analysis, it was essential to establish that the inhibitor was effective at the experimental conditions (pH 9) and maintained its inhibition ability at high pH (Fig. 5). Based on these results, one can conclude that the activity of the enzyme is significantly reduced to levels virtually below detection. In addition, storage of the enzyme-modified samples at 4 °C until the time of diffusion coefficient measurement further insured the inactivity of the enzyme.

3.3. Diffusion in α -galactosidase modified hydrogels

An important issue to consider in studying hydrogels is the effect of time on gel microstructure.

Previous work in the laboratory using rheology and confocal microscopy has revealed that guar solutions could take as much as 20 days to reach an equilibrium state after being treated with the α -galactosidase enzyme [12]. This issue is examined in Fig. 7, which shows the diffusion coefficient of both probe molecules, in an enzyme-modified hydrogel, as a function of the time interval between enzyme treatment and diffusion measurements. One finds that the diffusion coefficient of both the FITC–dextran probe and the FITC–guar oligomer probe decreases initially with increasing waiting time and then reaches a plateau value. The decrease in the diffusion coefficient is related to the time required for hyper-entanglements to form in the hydrogels. However, this time is found to be considerably shorter than what was observed in the earlier study from the group [12]. The FITC–dextran samples reach an equilibrium value after approximately 4 days, while the FITC–guar oligomer takes 3 days to reach equilibrium. The slightly expedited equilibrium in the latter case could be attributed to guar oligomer serving as a limited catalyst to promote the formation of hyper-entanglements.

The diffusion coefficient of FITC–dextran and the FITC–guar oligomer in hydrogels modified to different extents using α -galactosidase is shown in Fig. 8. These measurements were taken 3–4 days after enzyme modification to allow the samples to reach

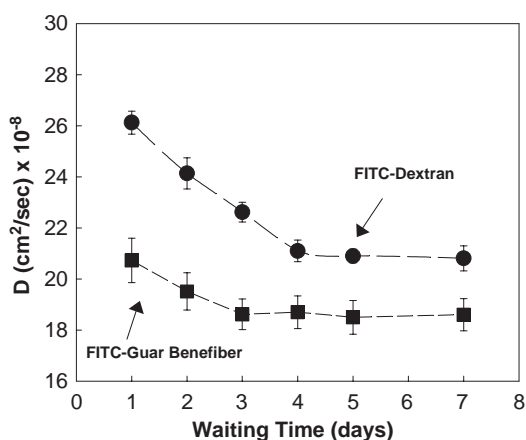


Fig. 7. The diffusion coefficient as a function of waiting time of a 31 kDa FITC–dextran (●) and an FITC–guar oligomer (■) probe in an enzyme-modified guar hydrogel incubated for 20 h with 1.66 U/mL α -galactosidase. Waiting time represents the time interval from the point at which the enzyme reaction is stopped to the point at which the diffusion coefficient is measured.

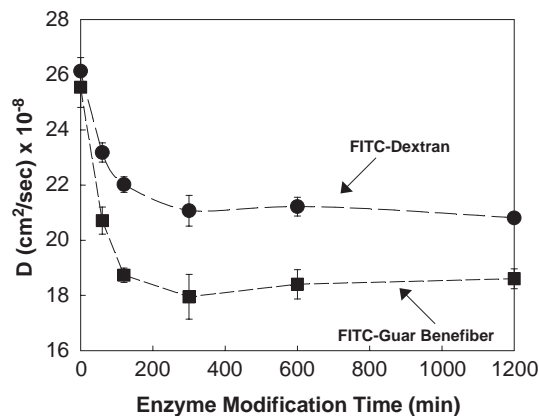


Fig. 8. Diffusion coefficient of a 31 kDa FITC–dextran (●) and an FITC–guar oligomer (■) probe in an enzyme-modified guar hydrogel (5 mg/mL guar–0.2 mg/mL titanium) plotted as a function of enzyme incubation time. 1.66 U/mL α -galactosidase was used in each experiment.

equilibrium. Both the FITC–dextran and the FITC–guar probes exhibit similar behavior — a decrease in diffusion coefficient followed by a plateau. However, the diffusion coefficient of the guar oligomer probe is found to be lower than the dextran probe at all enzyme modification times. Since the molecular size of both probes are similar based on SEC analysis, the additional reduction in the FITC–guar oligomer probe could be attributed to the formation of hyper-entanglements/associations between the guar hydrogel matrix and FITC–guar as depicted earlier in Fig. 2.

The less-than expected reduction in diffusion coefficient of the FITC–guar oligomer may be a consequence of the pH of the gel and/or the presence of limited absorption sites for hyper-entanglements. In particular, hyper-entanglements are known to be alkali-labile in solutions such as 1 M NaOH. At high pH, the hydroxyl groups of the sugars are ionized and the electrostatic repulsion destabilizes the intermolecular associations between constituent chains [6]. At pH 9, it is anticipated that a portion of the hydroxyl groups on the sugar backbone of the samples would be ionized; these ionized groups would limit the strength of the hyper-entanglements formed. If this hypothesis is accurate, a reduction in the pH to neutral conditions would reduce the amount of ionized hydroxyl groups and increase the strength of the hyper-entanglements. The effect of pH on hyper-entanglement was tested by examining the micro-

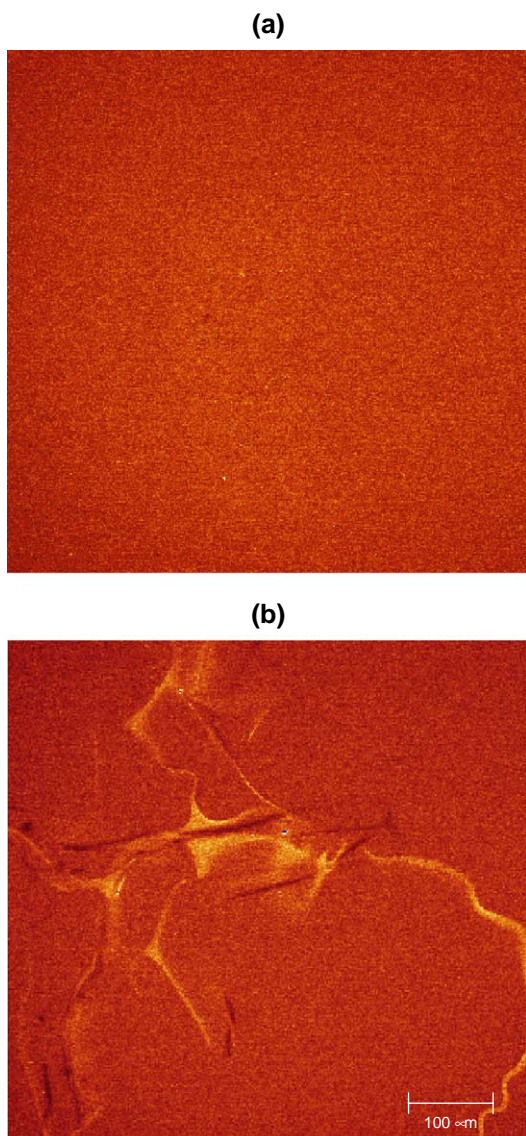


Fig. 9. Laser scanning confocal microscope images of a 20 h α -galactosidase treated guar hydrogel (5 mg/mL guar–0.2 mg/mL titanium) at pH 9 (a) and pH 7 (b) five days after enzyme treatment.

structure of an enzyme-modified hydrogel containing FITC–guar oligomer, at two different pH. Fig. 9a shows a confocal image of the hydrogel at pH 9. A uniform image within the limits of the magnification of the confocal microscope is observed. At pH 7, on the other hand, the same gel (Fig. 9b) shows presence of considerable microstructure. Particularly, bright, fluorescent-rich striations are observed that may be

caused by the FITC–guar aggregating with itself or on the crosslinked guar network. Although preliminary in nature, the presence of such structure seems to suggest that the hyper-entanglements are enhanced at neutral pH. These observations are also consistent with results that show galactomannans with an M:G ratio of 4 to have an intrinsic viscosity at neutral pH that is more than double the intrinsic viscosity in 1 M NaOH [6]. The increase in hyper-entanglement with a reduction in pH will be beneficial to colonic drug delivery by preventing premature release of the drug in the stomach or small intestine.

Since the strength of the hyper-entanglements is reduced at pH 9 compared to neutral conditions, the physical crosslinks in the system can be considered to be acting as reversibly absorbed intermolecular interactions. One can therefore apply the simple absorption equilibrium equation to the data and calculate the absorption equilibrium constant using the relation:

$$D_{\text{eff}} = D / (1 + K_{\text{eq}})$$

where, $K_{\text{eq}} = [\text{hyper-entangled probe}] / [\text{freely diffusing probe}]$ is the absorption equilibrium constant [32,33]. The FITC–dextran probe diffusion coefficient corresponds to D , diffusion without absorption, whereas the FITC–guar oligomer probe diffusion coefficient corresponds to D_{eff} , diffusion with absorption. Fig. 10 displays the K_{eq} as a function of α -galactosidase modification time. The K_{eq} rapidly increases during the first 2 h of enzyme modification

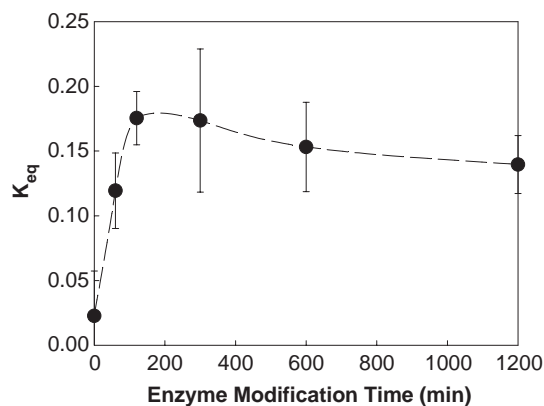


Fig. 10. The absorption equilibrium constant K_{eq} of the FITC–guar oligomer probe during incubation with 1.66 U/mL of α -galactosidase, plotted for various enzyme modification times.

by α -galactosidase, then levels off quickly at approximately $K_{eq}=0.2$, indicating that approximately 20% of the probes are hyper-entangled at any given time. This low value of K_{eq} is consistent with the fact that the diffusion coefficient of FITC–guar oligomer is not substantially lower than that of FITC–dextran. Interestingly, K_{eq} could be an interesting tool to use to predict and correlate the maximum drug that can be effectively loaded using this technique.

3.4. Degradation of α -galactosidase modified hydrogels

A key factor to consider when synthesizing a hydrogel based on this technique is the ability of the hydrogel to be degraded by the enzymes secreted by the microflora of the colon. Specifically, β -mannanase, which is responsible for the cleavage of the guar galactomannan backbone, is the most significant enzyme to consider for degradation leading to drug release. When guar galactomannan hydrogels are crosslinked by chemical methods, the crosslinking sites may not be susceptible to β -mannanase hydrolysis. Further the hyper-entanglements produced by α -galactosidase modification may not be susceptible to hydrolysis. To examine this question, 20 h α -galactosidase modified hydrogels were further incubated with β -mannanase and the diffusion coefficient was measured at various time intervals (Fig. 11). The first point at “zero” time corresponds to diffusion

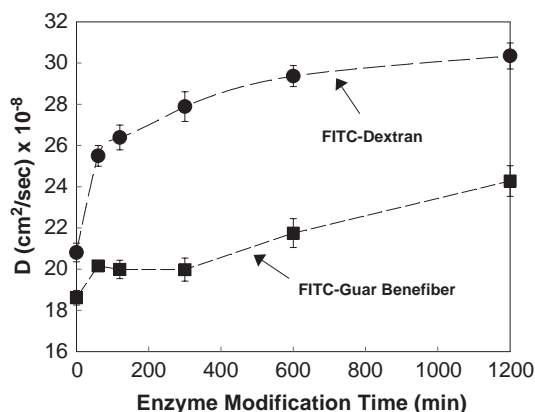


Fig. 11. Diffusion coefficient of a 31 kDa FITC–dextran (●) and an FITC–guar oligomer (■) probe in an enzyme-modified guar hydrogel (5 mg/mL guar–0.2 mg/mL titanium) as a function of time incubated with 0.166 U/mL β -mannanase.

without enzyme. The degradation of the hydrogel by β -mannanase results in a rapid increase in the diffusion of the FITC–dextran probe, with the diffusion coefficient rapidly increasing to the value of the diffusion coefficient found in water. The diffusion coefficient of the FITC–guar oligomer increases after 1 h of β -mannanase treatment but remain constant for approximately 4 more hours before increasing slowly at longer times. This suggests that the β -mannanase degradation of the hyper-entanglement occurs at a much slower rate. The binding of *A. niger* β -mannanase for effective cleavage is known to require only four mannose residue with only one galactose branch allowed near the midpoint of the mannose residue [34]. Additional galactose branches can prevent effective hydrolysis [34]. Therefore, the α -galactosidase modified guar hydrogels should have very rapid degradation. However, it appears that hyper-entanglements slow the hydrolysis rate of β -mannanase. This fact may prove beneficial in regard to drug release, which is preferred to occur at a constant rate for many controlled release drug delivery vehicles. In addition, the rate of enzymatic degradation by β -mannanase can be further controlled by incorporation of a newly discovered β -mannanase inhibitor [3].

It should be pointed out that previous work with crosslinked guar gels have examined release of drug from the gel to the surrounding medium by placing the sample in a large quantity of aqueous medium [35–37]. The current work with enzyme degradation is entirely different as the focus is on intra-gel (i.e., not surface or diffusion/release in the surrounding media) diffusion in which the diffusion coefficient is measured within the gel. A release (burst or otherwise) of surface bound probes observed in the previous studies is not possible in this case, with enzymatic breakdown of the gel being the only plausible change occurring in the system. As such, the results presented here prove the in vitro feasibility of the hydrogel system to release the drug substance, more specifically that the hyper-entanglements can be degraded by β -mannanase. However, future work will have to be performed to develop the hydrogel system into a pharmaceutical product including evaluation of the in vitro dissolution rate of various drug molecules similar to that performed by Wong et al. [22]. In addition, pre-clinical pharmacokinetic and gamma-

scintigraphic evaluations will have to be performed in the beagle dog model or rat model to verify in vivo performance [35–37].

4. Conclusions

This study shows that it is possible to tailor the molecular architecture of galactomannan hydrogels as well as guar oligomer–drug conjugate to entrap and limit the diffusion of model drugs using a simple enzymatic modification technique. The diffusion coefficient of solutes in the lightly crosslinked galactomannan hydrogel is approximately equal to the diffusion coefficient in a guar solution for simple diffusional drug loading; however, α -galactosidase treatment can effectively reduce solute diffusion after drug loading without affecting the drug or therapeutic protein. The diffusion coefficient of a model drug (FITC) guar oligomer conjugate probe decreased more than that of a non-interacting probe of the same molecular weight. Furthermore, the physical crosslinks generated by α -galactosidase treatment slowed the rate of hydrogel degradation by β -mannanase, providing a constant rate of solute diffusion from the 1 to 4 h mark followed by an increase in the diffusion coefficient thereafter. These results taken together suggest that the basis for a platform, which could potentially entrap a wide variety of molecules and target the delivery to the colon at a controlled rate, has been established.

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References

- [1] S.A. Hoffman, Hydrogels for biomedical applications, *Advanced Drug Delivery Reviews* 43 (2002) 3–12.
- [2] K.S. Soppimath, A.R. Kulkarni, T.M. Aminabhavi, Chemically modified polyacrylamide-g-guar gum-based crosslinked anionic microgels as pH-sensitive drug delivery systems: preparation and characterization, *Journal of Controlled Release* 75 (2001) 331–345.
- [3] M.D. Burke, S.A. Khan, Triggered enzymatic degradation of a water-soluble polymer solution using a novel inhibitor, *Biomacromolecules* 1 (2000) 688–695.
- [4] V.D. Scherbukhin, O.V. Anulov, Legume seed galactomannans (Review), *Applied Biochemistry and Microbiology* 35 (1999) 229–244.
- [5] F. Bayerlein, Technical applications of galactomannans, in: F. Meuser, D.J. Manners, W. Seibel (Eds.), *Plant Polymeric Carbohydrates*, 1993.
- [6] F.M. Goycoolea, E.R. Morris, M.J. Gidley, Viscosity of galactomannans at alkaline and neutral pH: evidence of “hyperentanglement” in solution, *Carbohydrate Polymers* 27 (1995) 69–71.
- [7] E.R. Morris, A.N. Cutler, S.B. Ross-Murphy, D.A. Rees, Concentration and shear rate dependence of viscosity in random coil polysaccharide solutions, *Carbohydrate Polymers* 1 (1981) 5–21.
- [8] M. Nydén, O. Söderman, An NMR self-diffusion investigation of aggregation phenomena in solutions of ethyl (hydroxyethyl) cellulose, *Macromolecules* 31 (1998) 4990–5002.
- [9] M. Nydén, O. Söderman, G. Karlström, A PFG NMR self-diffusion investigation of probe diffusion in an ethyl (hydroxyethyl) cellulose matrix, *Macromolecules* 32 (1999) 127–135.
- [10] G. Robinson, S.B. Ross-Murphy, E.R. Morris, Viscosity–molecular weight relationships, intrinsic chain flexibility, and dynamic solution properties of guar galactomannan, *Carbohydrate Research* 107 (1982) 17–32.
- [11] A.J. Fellingner, J.M.A. Verbakel, R.A. Veale, P.E. Sudbery, I.J. Bom, N. Overbeeke, C.T. Verrips, Expression of the α -galactosidase from *Cyamopsis tetragonoloba* (Guar) by *Hansenula polymorpha*, *Yeast* 7 (1991) 463–473.
- [12] V. Pai, S.A. Khan, Gelation and rheology of enzyme-modified guar/xanthan blends, *Carbohydrate Polymers* 49 (2002) 207–216.
- [13] M.D. Burke, J.O. Park, M. Srinivasarao, S.A. Khan, Diffusion of macromolecules in polymer solutions and gels: a laser scanning confocal microscopy study, *Macromolecules* 33 (2000) 7500–7507.
- [14] A.N. de Belder, K. Granath, Preparation and properties of fluorescein-labelled dextrans, *Carbohydrate Research* 30 (1973) 375–378.
- [15] V. Pai, Ph.D. thesis, North Carolina State University, 2001.
- [16] V. Pai, M. Srinivasarao, S.A. Khan, Evolution of microstructure and rheology in mixed polysaccharide systems, *Macromolecules* 35 (2002) 1699–1707.
- [17] W.S. Bahary, M.P. Hogan, M. Jilani, M.P. Aronson, Size-exclusion chromatography of dextrans and pullulans with light scattering, viscosity, and refractive index detection, in: T. Provder, H.G. Barth, M.W. Urban (Eds.), *Chromatographic Characterization of Polymers*, vol. 247, American Chemical Society, Washington, DC, 1995.
- [18] B.R. Vijayendran, T. Bone, Absolute molecular weight and molecular weight distribution of guar by size exclusion chromatography and low-angle laser light scattering, *Carbohydrate Polymers* 4 (1984) 299–313.

- [19] A. Tayal, R.M. Kelly, S.A. Khan, Rheology and molecular weight changes during enzymatic degradation of a water-soluble polymer, *Macromolecules* 32 (1999) 294–300.
- [20] S.C. De Smedt, T.K.L. Meyvis, J. Demeester, Diffusion of macromolecules in dextran methacrylate solutions and gels as studied by confocal scanning laser microscopy, *Macromolecules* 30 (1997) 4863–4870.
- [21] J.C.G. Blonk, A. Don, H. Van Aalst, J.J. Birmingham, Fluorescence photobleaching recovery in the confocal scanning light microscope, *Journal of Microscopy* 169 (1993) 363–374.
- [22] D. Wong, K. Larrabee, K. Clifford, J. Tremblay, D.R. Friend, USP dissolution apparatus III (reciprocating cylinder) for screening of guar-based colonic delivery formulations, *Journal of Controlled Release* 47 (1997) 173–179.
- [23] A. Tayal, S.A. Khan, Degradation of a water-soluble polymer: molecular weight changes and chain scission characteristics, *Macromolecules* 33 (2000) 9488–9493.
- [24] K. Hoste, K. De Winne, E. Schacht, Polymeric prodrugs, *International Journal of Pharmaceutics* 277 (2004) 119–131.
- [25] A.J.M. D'Souza, E.M. Topp, Release from polymeric prodrugs: linkages and their degradation, *Journal of Pharmaceutical Sciences* 93 (2004) 1962–1979.
- [26] N.H. Alam, R. Meier, H. Schneider, S.A. Sarkar, P.K. Bardhan, D. Mahalanabis, G.J. Fuchs, N. Gyr, Partially hydrolyzed guar gum-supplemented oral rehydration solution in the treatment of acute diarrhea in children, *Journal of Pediatric Gastroenterology and Nutrition* 31 (2000) 503–507.
- [27] C. Mayer, D.L. Zechel, S.P. Reid, A.J. Warren, S.G. Withers, The E358S mutant of *Agrobacterium* sp. β -glucosidase is a greatly improved glycosynthase, *FEBS Letters* 466 (2000) 40–44.
- [28] L.F. Mackenzie, Q. Wang, R.A.J. Warren, S.G. Withers, Glycosynthases: mutant glycosidases for oligosaccharide synthesis, *Journal of the American Chemical Society* 120 (1998) 5583–5584.
- [29] J.-Q. Fan, S. Ishii, N. Asano, Y. Suzuki, Accelerated transport and maturation of lysosomal α -galactosidase A in Fabry lymphoblasts by an enzyme inhibitor, *Nature Medicine* 5 (1999) 112–115.
- [30] N. Asano, S. Ishii, H. Kizu, K. Ikeda, K. Yasuda, A. Kato, O.R. Martin, J.-Q. Fan, In-vitro inhibition and intracellular enhancement of lysosomal α -galactosidase A activity in Fabry lymphoblasts by 1-deoxygalactonojirimycin and its derivatives, *European Journal of Biochemistry* 267 (2000) 4179–4186.
- [31] M.D. Burke, Dissertation, Dept. of Chemical Engineering, North Carolina State University, 2001.
- [32] Y. Cheng, Dissertation, Dept. of Chemical Engineering, Princeton University, 2002.
- [33] Y. Cheng, R.K. Prud'homme, Reaction–diffusion of enzyme molecules in biopolymer matrices, in: R.A. Gross, H.N. Cheng (Eds.), *Biocatalysis in Polymer Science*, American Chemical Society Symposium Series, vol. 240, Oxford University Press, Washington, DC, 2003.
- [34] B.V. McCleary, β -D-mannanase, *Methods in Enzymology* 160 (1988) 596–610.
- [35] D.A. Adkin, C.J. Kenyon, E.I. Lerner, I. Landau, E. Strauss, D. Caron, A. Penhasi, A. Rubinstein, I.R. Wilding, The use of scintigraphy to provide “proof of concept” for novel polysaccharide preparations designed for colonic drug delivery, *Pharmaceutical Research* 14 (1997) 103–107.
- [36] I. Gliko-Kabir, B. Yagen, A. Penhasi, A. Rubinstein, Low swelling, crosslinked guar and its potential use as colon-specific drug carrier, *Pharmaceutical Research* 15 (1998) 1019–1025.
- [37] I. Gliko-Kabir, B. Yagen, M. Bauluom, A. Rubinstein, Phosphated crosslinked guar for colon-specific drug delivery: II. In vitro and in vivo evaluation in the rat, *Journal of Controlled Release* 63 (2000) 129–134.