Characterization of Methacrylated Inulin Hydrogels Designed for Colon Targeting: *In Vitro* Release of BSA

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Purpose. To characterize methacrylated inulin hydrogels with respect to their release properties.

Methods. Proteins (bovine serum albumin or lysozyme) were used as model drugs and were loaded during or after hydrogel formation. Parameters such as the drug loading method, the molecular weight of the proteins, the initial drug loading concentration, the hydrogel feed composition, degree of substitution, and size of the hydrogel were investigated by determining the release of the model proteins from the hydrogels in a phosphate buffer solution. The biodegradable properties were investigated by studying the release of bovine serum albumin in a solution of inulinase.

Results. *In vitro* protein release from methacrylated hydrogels was influenced by factors such as the drug loading procedure and the molecular weight and loading concentration of the proteins. The feed composition and degree of substitution of inulin seem to be crucial in controlling both the extent and the rate of release. Protein release was clearly enhanced in the presence of inulinase, indicating the biodegradable properties of methacrylated inulin hydrogels.

Conclusions. Several hydrogels show interesting properties with respect to the development of a colon-specific drug delivery system.

KEY WORDS: Hydrogels; colon targeting; protein release; inulin.

INTRODUCTION

Targeting of drugs to the large intestine is therapeutically relevant in the case of treating disorders of the colon such as Crohn's disease, ulcerative colitis, inflammatory bowel disease, and other pathologies that can benefit from local high drug concentration. Although the drug absorption capacity of the colon is less than that of the proximal part of the gastrointestinal tract, the large intestine can be considered an alternative absorption site in the case of drugs that undergo significant degradation in the proximal gut (1). The release of peptides and proteins in the colon is an interesting approach that potentially permits their oral administration (2).

In the past two decades, many systems have been proposed to deliver drugs specifically into the colon (3–4). Among these strategies, systems relying on the metabolic action of the colonic microflora can be considered to be truly site-specific, in contrast with delivery systems exploiting the gastrointestinal pH or transit time. Microflora-triggered biodegradable polymers are therefore interesting excipients. Although significant results with biodegradable azo polymers have been reported during the last decade (4), these materials' potential toxicity might limit their effectiveness. In this respect, naturally occurring polymers are more promising and preferable. Inulin is a naturally occurring polysaccharide (5) that consists of β 2-1 linked D-fructose molecules. Most fructose chains have a glucose unit at the reducing end (6). Inulin was selected as a candidate polymer for the development of colon-specific hydrogels because the β 2-1 osidic bonds are not significantly hydrolyzed by enzymes from the endogenous secretions of the human digestive tract (7). Colonic bacteria, however—and, more specifically, *Bifidobacteria,* which constitute up to 25% of the normal human gut flora (8)—can ferment inulin (9,10).

In a previous study, we reported on the biodegradable properties of hydrogels prepared from methacrylated inulin (11). The objective of the present article is to characterize methacrylated inulin hydrogels by investigating the release of bovine serum albumin (BSA) and lysozyme, two model proteins, from the hydrogels. Several parameters which were thought to influence the release rate were studied.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA) was obtained from Acros Organics (Geel, Belgium) and lysozyme was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bio-Rad Protein Assay Dye Reagent Concentrate was obtained from BIO-RAD Laboratories (Munich, Germany). Novozym 230, an inulinase preparation (1800 U/g) isolated from *Aspergillus ficuum,* was generously donated by Novo Nordisk A/S (Bagsvaerd, Denmark).

All other chemicals were of analytic grade and used as received.

Preparation of MA-IN Hydrogels

The synthesis and characterization of methacrylated inulin (MA-IN) was described previously (12). In brief, 50 g of dried inulin was dissolved in 200 ml of dimethylformamide. After dissolution of 4′-dimethylaminopyridine (DMAP) (10 mol% vs. fructose units), a calculated amount of glycidyl methacrylate was added, depending on the desired degree of substitution of inulin. The reaction mixture was stirred for 72 h at room temperature after which the reaction product was precipitated in isopropanol followed by washing with the same solvent. The precipitate was subsequently dissolved in Milli Q water and dialyzed for ten days. Before isolation/purification, DMAP was neutralized with hydrochloric acid to prevent hydrolysis of the methacrylate ester. After dialysis, MA-IN was recovered by freeze-drying. The hydrogels were prepared as follows: MA-IN solutions (16, 22, and 27% w/w) were prepared in 0.5 M phosphate buffer pH 6.5. After adding 17.5 μmol/ml ammoniumpersulfate and 39.4 μmol/ml of tetramethyl ethylenediamine buffer, the mixture was divided over molds and free radical polymerization took place at room temperature for 2.5 h, resulting in gels with a diameter of 10 mm and a height of 2–3 mm. After polymerization, the hydrogels were removed from the molds and washed in demineralized water to remove unreacted MA-IN and initiating compounds. After washing, the hydrogels were dried at room temperature until weight was constant.

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The degree of substitution (DS, defined as the amount of methacryloyl groups per 100 fructose units) of MA-IN was either 4.4, 8.1, 12.1, or 15.4 (determined with NMR [12]).

Loading of the MA-IN Hydrogels

Loading of the MA-IN hydrogels with proteins was performed according to two frequently used methods (13).

The first method comprises the incorporation of proteins after the formation of the hydrogel network. The obtained hydrogels were allowed to swell until equilibrium was reached at room temperature in 0.5 M phosphate buffer pH 6.5 containing 42 mg protein/ml buffer. After reaching equilibrium swelling, the hydrogels were removed from the protein solution, free surface water was blotted with tissue paper, and the hydrogels were dried at room temperature until weight was constant.

The second method comprises the incorporation of proteins during the formation of the hydrogel network. MA-IN was dissolved in 0.5 M phosphate buffer pH 6.5 containing 42 mg protein/ml buffer (low protein loading concentration) or 80 mg protein/ml buffer (high protein loading concentration). After the two initiators of the free radical solution polymerization were added, the mixture was poured into the molds and allowed to react for 2.5 h at room temperature, yielding protein-loaded MA-IN hydrogels. The hydrogels were removed from the molds, blotted with tissue paper, and dried at room temperature until weight was constant.

In this way, lysozyme and BSA, two model proteins with molecular weights of 14.400 and 65.000, respectively, were loaded.

In Vitro Release Study of Protein from MA-IN Hydrogels

Dehydrated, protein-loaded MA-IN hydrogels were immersed at 37°C in vials containing 10 ml of 0.025 M citrate buffer pH 4.7 with or without prior addition of Novozym 230 (100 U/ml). By adding sodium chloride to the citrate buffer, the ionic strength of the release medium could be adjusted to 0.028 and 0.166, and its effect on BSA release could be determined.

The vials were gently rotated at 20 rpm with a rotary-mixer (Labinco, Breda, The Netherlands). Samples were replaced at regular time intervals with citrate buffer (CB) or inulinase-containing citrate buffer (ICCB). The protein concentration of the collected samples was assayed with the Bio-Rad protein assay (14). Calibration curves were constructed using solutions of known concentration of the appropriate protein.

RESULTS AND DISCUSSION

In Vitro Release of Protein from MA-IN Hydrogels in the Absence of Inulinase

The release of compounds from initially dehydrated hydrogels involves several processes: penetration of release medium into the polymeric network, dissolution of the dispersed drug, effect of swelling on the permeability of the matrix to both penetrant and drug, and release of the drug from the hydrogel under swelling conditions (15).

Hydrogels prepared from 27% w/w solutions of MA-IN DS 12.1 were loaded with BSA (42 mg/ml buffer) both during and after cross-linking. Fig. 1 represents the cumulative percentage release of the protein from the initially dehydrated hydrogels as a function of time. A large and significant difference (*t* test; $p < 0.05$) in release rate can be observed between the applied loading procedures. Loading of BSA into previously prepared hydrogels results in a very fast drug release; the hydrogels appear to be unable to retain the loaded BSA, since all protein is released from the device after 1 h. On the other hand, entrapment of BSA in the hydrogel during cross-linking considerably retards protein release: Only 11.5% of incorporated protein is released after 24 h.

Loading of hydrogels by swelling in a drug-containing solution depends on the size of the hydrogel pores compared with the drug size. Unless the pore size of the hydrogel is substantially larger than the size of the drug, drug diffusion into the hydrogel will be significantly inhibited and most of the drug will be present in the outer region of the hydrogel. The drug molecules will enter only the easily accessible sites from which they will be quickly released. Penetration into regions with a high cross-linking density will be almost impossible. In addition, drying of the hydrogels after loading by equilibrium swelling can produce migration of the drug to the gel surface from which it is quickly released (16,17). Critical reflection suggests that it might well be possible that a large part of the protein was only adsorbed onto the surface of the hydrogel, with only a small part truly entrapped in the hydrogel.

By cross-linking the MA-IN chains in the presence of BSA, the protein will be physically entrapped in the hydrogel network and will be sterically retained. This results in a retarded release. Loading of hydrogels during cross-linking also offers the advantage of allowing easy adjustment of the amount of incorporated drug over a broad range. This does not hold for loading hydrogels by swelling since in this method, the loading percentage depends on the swelling degree of the device, which is often not satisfactory for loading a high drug dose.

Based on these observations, it was decided for further *in vitro* release experiments to load the hydrogels with protein during cross-linking.

Salts and ions may penetrate the hydrogel upon swelling and can interact with network segments. Stretching or con-

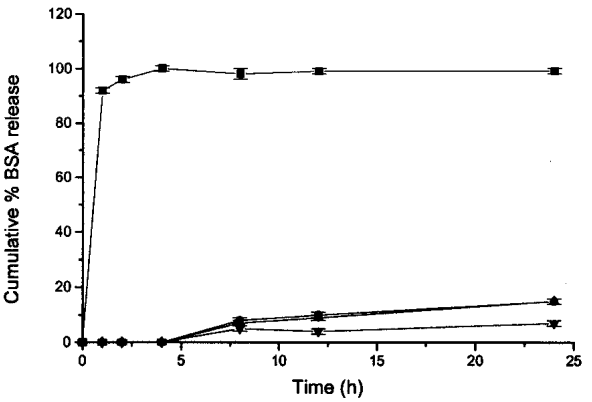

Fig. 1. Cumulative percentage BSA release from hydrogels prepared from 27% w/w solutions of MA-IN DS 12.1 loaded during (▼) and after (■) cross-linking, and BSA release from hydrogels prepared from 22% w/w solutions of MA-IN DS 12.1 in a release medium of ionic strength of 0.028 (▲) and 0.166 (●) (mean ± SD; n = 3).

tracting properties of the network can be altered by this interaction. Solutes have often-unpredictable effects on the swelling of hydrogels because of their interaction with the hydrogel polymer, which can enhance or disrupt specific interactions between swelling solvent and polymer, causing decreased swelling of the hydrogel (18,19). Therefore, the effect of ionic strength of the release medium on BSA release from hydrogels prepared from 22% w/w solutions of MA-IN DS 12.1 and loaded with 42 mg BSA/ml buffer was investigated within the range of ionic strengths encountered in the gastrointestinal tract (20). Figure l shows that the ionic strength has no significant effect (t test; $p > 0.05$) on the release kinetics, which concurs with previously reported data on pure MA-IN hydrogels (21).

The effect of molecular size of the protein, expressed as molecular weight, on the release kinetics was determined by incorporating two model proteins (42 mg/ml buffer) of different molecular weight: lysozyme (mol. wt. = 14.400) and BSA (mol. wt. = 65.000). The cumulative percentage release of these compounds from hydrogels prepared from 27% w/w solutions of MA-IN DS 12.1 is shown in Fig. 2. Release of incorporated protein is considerably reduced with increasing molecular weight. The size of the protein apparently hampers diffusion through and release from the hydrogel network in which the compound is sterically entrapped. However, this observation holds only if the protein is loaded during hydrogel formation, since previously obtained data showed that the release of the two proteins was comparable when they were loaded after hydrogel formation. (Data not shown.)

Figure 2 further illustrates the influence of the hydrogel size (average dry hydrogel weight of 70.1 mg compared with 141.4 mg) on BSA release kinetics: The size appears to have no significant influence on BSA release.

The effect of initial drug loading concentration (42 mg BSAml buffer compared with 80 mg BSAml buffer) on BSA release also is depicted in Fig. 2. Increased drug loading definitely results in a faster initial release, but after 5 h, the release rate is comparable.

The release kinetics of BSA (42 mg/ml buffer) from MA-IN hydrogels of different feed composition with respect to degree of substitution and feed concentration of MA-IN was studied as well. Figure 3 represents the effect of the degree of substitution of MA-IN, whereas Fig. 4 shows the influence of the MA-IN feed concentration. An increasing degree of substitution significantly (t test; $p < 0.05$) delays the release of BSA from the devices. This can be attributed to the relationship between degree of substitution of MA-IN and cross-linking density of the hydrogel networks. Higher amounts of reactive vinyl groups per inulin chain result in the formation of more intermolecular cross-links per volume unit of the network, as concluded from the rheological characterization of the MA-IN hydrogels (22). Release of incorporated protein will be hindered by an increased cross-linking density due to a restricted swelling of the hydrogel networks and thus a lowered permeability.

With respect to the effect of the feed concentration of MA-IN on BSA release, Fig. 4 illustrates the fact that hydrogels prepared from more-diluted solutions release the entrapped protein faster than hydrogels of higher feed concentration. These observations again correlate well with the obtained values of the elastic modulus G' (22) and of the equilibrium swelling ratio q_{eq} (21). Dilution of the hydrogel polymer solution is considered to promote intramolecular cross-linking, resulting in the formation of less-elastic strands per volume unit of the hydrogel network and consequently leading to an increased hydrogel swelling. A hydrogel of lower feed concentration will thus hamper drug release to a lesser extent than a hydrogel prepared from a more-concentrated solution. Moreover, with increasing feed concentration, polymer chain entanglements have to be accounted for (22). These chain entanglements can act as additional cross-links and contribute to the reduced hydrogel permeability. Hennink *et al.* (23) determined the mesh size of methacrylated dextran hydrogels, and Patil *et al.* (24) made the calculations for poly(sucrose acrylate) hydrogels. The mesh size, and thus the porosity of the hydrogels, could indeed be controlled by the feed composition of the hydrogels: With increasing feed concentration and increasing cross-link ratio, the porosity of the hydrogels decreased and the release rate of proteins was consequently reduced.

From Figs. 3 and 4, it can be concluded that hydrogels prepared from 27% w/w solutions of MA-IN DS 8.1,12.1, and 15.4 and hydrogels prepared from 22% w/w solutions of MA-IN DS 12.1 could be considered to be potential candidate

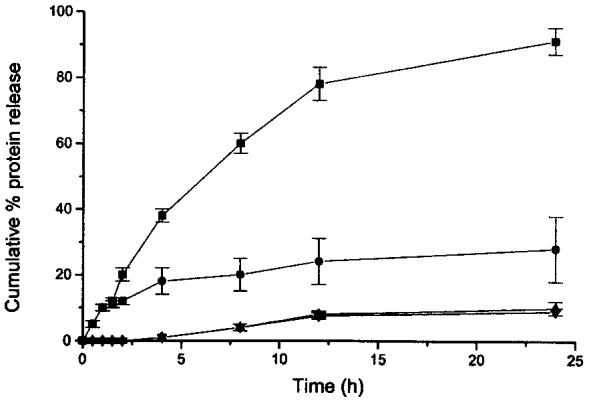

Fig. 2. Cumulative percentage release of lysozyme (■) and BSA (▼) from hydrogels prepared from 27% w/w solutions of MA-IN DS 12.1 (loading concentration = 42 mg protein/ml buffer; average dry hydrogel weight is 70.1 mg), and BSA release from the MA-IN hydrogels with an average dry hydrogel weight of 141.1 mg (▲) or loaded with 80 mg BSA/ml buffer (●) (mean ± SD; n = 3).

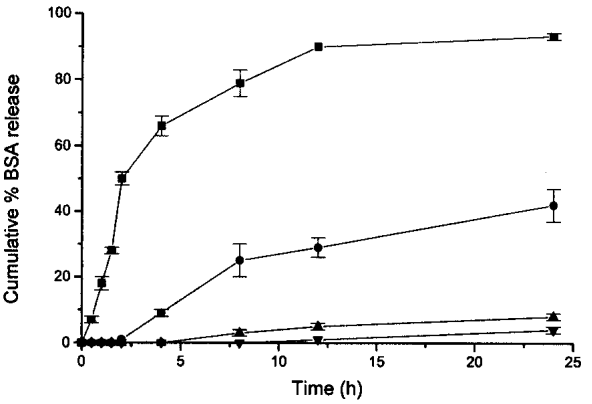

Fig. 3. Cumulative percentage of BSA release from hydrogels prepared from 27% w/w solutions of MA-IN DS 4.4 (■), DS 8.1 (●), DS 12.1 (▲), and DS 15.4 (▼) (mean ± SD; n = 3).

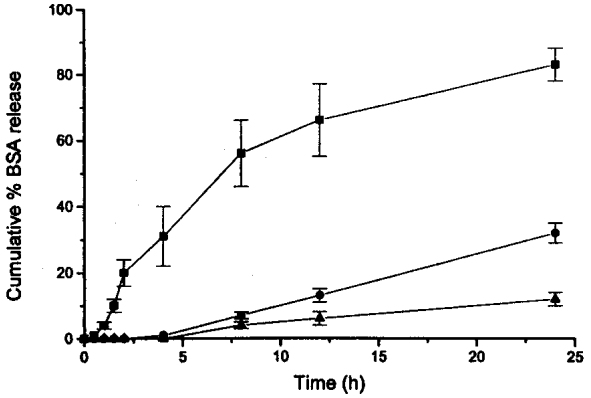

Fig. 4. Cumulative percentage BSA release from hydrogels prepared from MA-IN DS 12.1 solutions with a feed concentration of 16% w/w (■), 22% w/w (●), and 27% w/w (▲) (mean ± SD; n = 3).

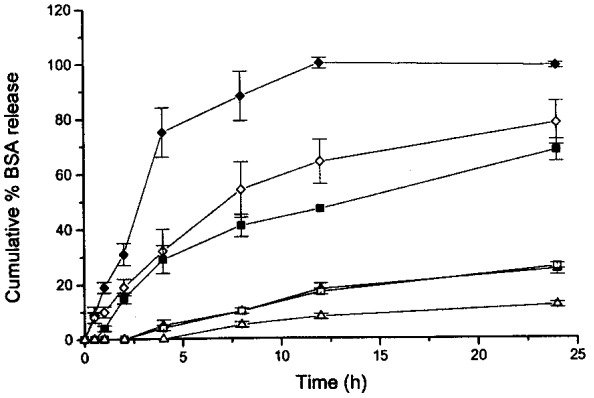

Fig. 6. Cumulative percentage of BSA release from hydrogels prepared from MA-IN DS 12.1 solutions with a feed concentration of 16% w/w (♦), 22% w/w (■), and 27% w/w (▲) in CB (open symbols) and ICCB (closed symbols) (mean ± SD; n = 3).

drug delivery systems for colon-specific protein release. Obviously, the hydrogels have to reach a certain degree of swelling and thus a certain permeability before protein release can start, which might explain, at least partially, the observed lag time. Consequently, these hydrogels will sufficiently delay drug release to prevent excessive premature release during passage of the dosage form through the small intestine.

In Vitro Release of Protein from MA-IN Hydrogels in the Presence of Inulinase

In our next set of experiments, we focused on the effect of inulinase (Novozym 230) on BSA release to study enzymatic hydrogel degradation. Drug release from a hydrogel undergoing a degradation process can be considered as a complex set of interrelated events. Multicomponent diffusion occurs, and the permeability of the hydrogel network changes continuously because of the combination of swelling and degradation (16).

Figures 5 and 6 represent BSA release plots as a function of time for MA-IN hydrogels incubated in an ICCB of pH 4.7 at 37°C. This pH is not typical for the colon but was necessary to perform the test at the optimal activity of the fungal inulinase. Previous studies have shown that pH does not influence the swelling properties of the (no-ionic) hydrogels under investigation (21). The hydrogels are prepared from MA-IN with different degree of substitution (Fig. 5) or different feed concentration (Fig. 6). To differentiate more easily between the effect of hydrogel degradation and simple leaching of the protein from the swelling device, the release plots of BSA in CB without inulinase also are included in the two figures. From 2 hours on, the release in ICCB was always significantly higher (t test; $p < 0.05$) than that in CB. An overview of the percentage BSA released in ICCB and CB after 24 h, which falls within the range of average colonic transit time, is represented as a function of the degree of substitution of MA-IN in Fig. 7, and as a function of the feed concentration of MA-IN in Fig. 8. The release of BSA after 24 h was significantly higher in all cases except for DS 4.4 (t test). The loose structure of this network apparently permits release by simple diffusion of the protein. The release of BSA from the devices is enhanced in the presence of inulinase. This can be explained by the enzymatic degradation of the hydrogel sugar backbone, which loosens the hydrogel network and results in an increased permeability of the hydrogels. Hydrogels prepared from 27% w/w solutions of MA-IN DS 4.4 even dissolved completely in the inulinase solution within the 24-h period of the release experiment.

From this *in vitro* release study, two hydrogels seemed to be promising candidate carriers to be further developed for colon-specific delivery of proteins since they provide a sufficient lag time for drug release to prevent premature release in

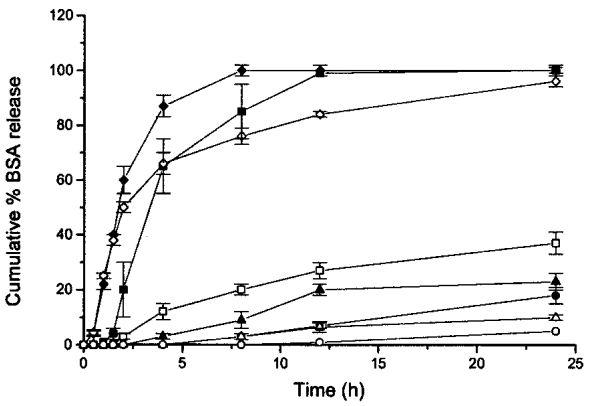

Fig. 5. Cumulative percentage of BSA release from hydrogels prepared from 27% w/w solutions of MA-IN DS 4.4 (♦), DS 8.1 (■), DS 12.1 (▲), and DS 15.4 (●) in CB (open symbols) and ICCB (closed symbols) (mean ± SD; n = 3).

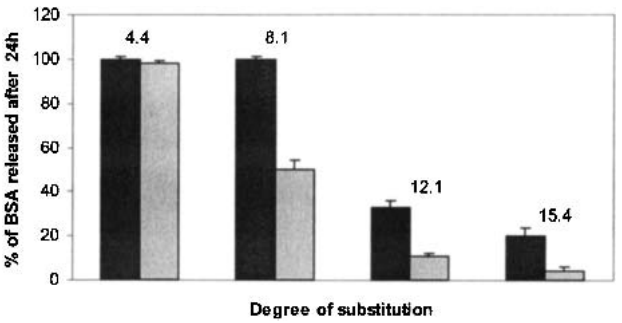

Fig. 7. Percentage of BSA released after 24 h in CB (gray) and ICCB (black) from hydrogels prepared from 27% w/w solutions of MA-IN as a function of the degree of substitution of MA-IN (mean ± SD; n = 3).

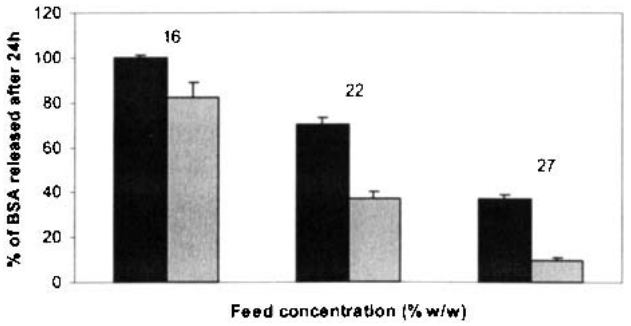

Fig. 8. Percentage of BSA released after 24 h in CB (gray) and ICCB (black) from hydrogels prepared from MA-IN DS 12.1 as a function of the feed concentration of MA-IN (mean ± SD; n = 3).

the small intestine and 100% of drug can be released within the period of colonic residence. The two selected devices comprise hydrogels prepared from 27% w/w solutions of MA-IN DS 8.1, with only 12.6% BSA release after 4 h in CB and 100% BSA release after 24 h in ICCB, and hydrogels prepared from 22% w/w solutions of MA-IN DS 12.1, with only 1.1% BSA release after 4 h in CB and 68.1% BSA release after 24 h in ICCB.

CONCLUSIONS

In vitro protein release from MA-IN hydrogels is affected by the procedure used to load the hydrogels and by the molecular weight and loading concentration of the protein. Ionic strength of the release medium and size of the hydrogels appear to be of minor importance, at least in the range used in this study. The feed composition of the MA-IN hydrogels, including both the degree of substitution and feed concentration of MA-IN, is an important parameter that has to be considered in controlling protein release.

By adding inulinase to the release medium, protein release can be enhanced. However, the extent of this enhancing effect depends on the feed composition of the hydrogels.

From this screening study, two hydrogels can be selected that open perspectives for further research in view of the development of a colon-specific drug delivery system: MA-IN hydrogels prepared from 27% w/w solutions of MA-IN DS 8.1 and MA-IN hydrogels prepared from 22% w/w solutions of MA-IN DS 12.1. The selection of these hydrogels is based on the lag time for drug release, which will prevent premature protein release in the small intestine, and on the enhancement of protein release by inulinase within average colonic residence time, which depends on the extent of degradation of the hydrogels. These two parameters define the colon specificity of the MA-IN hydrogels.

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