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Colon-Specific Delivery of IgG from Highly Swellable Dextran Hydrogels: In-Vitro Study

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Abstract

Colonic drug delivery has gained increased importance for the delivery of peptide and protein drugs. In this study, the strategy is to investigate release of high molecular weight (MW) protein drugs from dextran-based hydrogels. The hydrogels were synthesized in the presence of crosslinking agents, N,N'-methylenebisacrylamide (MBAm) and epichlorohydrin (ECH). While dextran-MBAm hydrogels are having average equilibrium swelling ratios between 12.0 (at pH 2) and 14.0 (at pH 7), dextran-ECH hydrogels have values between 9.0 (at pH 2) and 12.5 (at pH 7). The model high molecular weight protein, immunoglobulin G (IgG), was loaded into the 50% (by weight) crosslinker-containing dextran hydrogels by two ways, i.e. during the crosslinking reaction or by soaking method. The loading capacity was varied between 3.0 and 4.0 mg per g of dry gel by depending upon the loading procedure. In-vitro release experiments were performed in the simulated gastrointestinal system in the presence and absence of dextranase. The diffusion exponents were calculated by means of the semi-empirical power law equation indicated that IgG was mainly released by non-Fickian diffusion. The release of IgG from both hydrogels was substantially higher than that of similar systems especially in the presence of dextranase, which was attributed to the high swellability of the dextran hydrogels synthesized here.

Key Words: Dextran; protein delivery; hydrogels; IgG; colon-specific delivery.

INTRODUCTION

Colon-specific drug delivery has a number of important implications in the field of pharmacotherapy. Applications of colonic drug delivery include the local treatment of large intestine disorders such as Crohn's disease, ulcerative colitis, and colorectal cancer, and the oral administration of protein and peptide drugs. To achieve successful colonic delivery, a drug needs to be protected from absorption and/or the environment of the upper gastrointestinal tract (GIT) and then be abruptly released into the proximal colon, which i considered the optimum site for colon-specific delivery of drugs because of its high retention time for drugs and low digestive enzyme activity [1].

Targeting drugs to the colon can be achieved by different routes: coating drugs with pH-sensitive [2] or bacterially degradable polymers [3], covalent linking of drug with a carrier [4], and using osmotic controlled drug delivery systems [5]. Recently, polymeric matrices have been designed based on enzyme degradable polymer backbones [6] or crosslinks [7]. A special kind of microbial enzymes,

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dextranases, which are able to degrade the polysaccharide dextran, were found to be present in the colon [8]. Taking advantage of these enzymes, dextran hydrogels have been under investigation for many years as colon-specific delivery system for especially protein drugs. It has been demonstrated by a proper selection of the hydrogel matrix, proteins can be released for prolonged periods of time [9]. Stenekes and co-vorkers reported the use of biodegradable dextran-based hydrogels as protein releasing matrices [10]. Polymeric prodrugs were designed by Hovgaard and Brondsted [11]. They have proposed to crosslink dextran with diisocynate for colon-specific drug delivery. Degradation of the hydrogels has been studied in vitro using dextranase. It was found that by changing the chemical composition of the hydrogels it is possible to control the equilbrium degree of swelling, drug release profile and degradability. Güner et al. studied crosslinking reactions of dextran with epichlorohydrin (ECH), phosphorus oxychloride ($POCl_3$), N,N'-methylenebisacrylamide (MBAm) and ^{60}Co- -irradiation [12]. Previously, we prepared dextran hydrogels by crosslinking dextran with ECH, glutaraldehyde (GA) and MBAm and we performed in vitro release studies of Bovine Serum Albumin (BSA) from these dextran hydrogels [13]. Doğan et al. investigated controlled release of epidermal growth factor (EGF) and basic-fibroblast growth factor (bFGF) from dextran hydrogels crosslinked with ECH [14]. In this study, we synthesized highly swellable dextran hydrogels crosslinked with MBAm and ECH. Then, the parameters affecting the colon-specific delivery of model protein, immunoglobulin-G (IgG), were studied in-vitro.

MATERIALS AND METHODS

Materials

Dextran (T-70; the molecular weight characteristics are determined by the manufacturer as the weight average and number average molecular weights of 70.000 and 46.800 g/mol, respectively) and crosslinking agents N,N'-methylenebisacrylamide (MBAm) and epichlorohydrin (ECH) were purchased from Sigma. Sodium hydroxide was used in the preparation of alkaline medium for the crosslinking reactions and it was supplied by Sigma. Hydrochloride acid (37%) and 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) buffer used in the protein release medium were obtained from Merck. Dextranase D-1508 from *Penicillium ssp.*(EC 3.2.1.11, specific activity 500 U/mg protein) and sodium azide (NaN_3) were obtained from Sigma. All other chemicals were analytical grade and were used as received.

Synthesis and characterization of the dextran hydrogels

The synthesis of dextran hydrogels was described previously by us [15]. In brief, intermolecular side-chain reaction of dextran hydroxyl groups with monomeric crosslinking agents such as MBAm and ECH in alkaline leads to the formation of dextran network. In a typical experiment, dextran was dissolved in distilled water containing 2.8 M NaOH at a concentration of 20% (w/v). 50% (w/w) of MBAm or ECH was added to this solution and stirred magnetically for 10 min. The polymer mixture was then poured into glass test tubes of 6 mm in diameter. In the case of MBAm, the crosslinking reaction was completed in 24 h at 25±0.5°C. For ECH-containing gels, crosslinking was completed in 2 h at 25°C then 60 h at 37.5°C. The hydrogels were removed from the tubes and cut into discs (diameter: 6 mm, thickness: 3 mm). Then they were washed thoroughly in distilled water under stirring for 7 days to remove the impurities sourced from uncrosslinked dextran and excess of crosslinkers. Washed hydrogel discs were dried in vacuum (EV 018, Nüve) at 25°C until no weight loss could be detected. The dry discs were kept in hermetically until further use.

The time-dependent swelling ratios of hydrogels were determined at pHs 2.0 and 7.0. The preweighed dry hydrogel discs were immersed in 25 mL Tris buffer solutions at 37°C. Changes in weight of the hydrogel discs were recorded at time intervals until equilibrium swelling was reached. The swelling of the hydrogels was expressed as swelling ratio (S) by using the following equation:

$$S = ((M_t - M_o)/M_o) \quad (1)$$

Where M_o is the dry weight of hydrogel (initial weight), M_t is the weight of swollen gel at a given time (t). In addition, initial swelling rate $(dS/dt)_o$ of hydrogels was determined by means of second-order kinetics proposed by Schott [16].

A scanning electron microscope (SEM, JEOL JSM-840 A) was used to analyze the surfaces and cross-sections of the dextran hydrogels. The hydrogel disc samples were prepared for microscopy by drying at vacuum from the swollen state. The dry disc samples were mounted onto stubs and sputter with gold.

The molecular weight between the crosslinks (M_c) was determined by density measurements according to the well known equation of Flory-Rehner [17], modified by Peppas and Merrill [18] for gels in which the crosslinks are introduced in solution. Details of the synthesis method and the characterization of dextran hydrogels was described in our previous publication [15].

Model protein:IgG

IgG (MW=165 000 Da, technical grade, Sigma) was used as model protein. The hydrodynamic diameter of IgG was calculated using the Einstein-Stokes equation:

$$D_o = kT/3\pi\eta d \quad (2)$$

Where η is the viscosity of the solvent, k is the Boltzman constant, T is the absolute temperature, d and D_o are the hydrodynamic diameter and the diffusion coefficient of the protein, respectively. The reported D_o value is: 4.0 x 10^{-7} cm^2/s for IgG [19]. Using the Einstein-Stokes equation, the hydrodynamic diameter of IgG is calculated as 11.3 nm.

IgG assay was performed using fluorescence spectrophotometer (Shimadzu RF 1501, Japan). All measurements were made with the freshly prepared solutions of IgG in PBS, using 1 cm^2 quartz cells. The intrinsic tryptophan fluorescence observed from protein solutions at 280 nm excitation (λ_{ex}) and 342 nm (λ_{em}) emission wavelengths. The calibration results showed that, concentrations up to 3 μg/mL are linear curve fit and minimum detectable concentration is 0.1 μg/mL.

Preparation of IgG loaded dextran hydrogel discs

IgG loading was realized according to two frequently used methods [20]. In the first method, briefly soaking method, the hydrogel discs were immersed at 37°C in Tris buffer (pH 7.0) containing 0.5 mg IgG/mL buffer. Then discs were allowed to swell until equilibrium. Incorporation of IgG was accomplished by soaking 1 g of each set of the dried discs in 10 mL of the IgG solution. Experiments in which the loading time was varied showed that 48 h was sufficient for complete loading. Then the hydrogel discs were dip-rinsed in buffer in order to wash off any protein solution on the gel surface. Then, free surface water was blotted with filter paper. The IgG loaded gels were dried under vacuum at room temperature until weight was constant and then stored in an evacuated dessicator. The concentrations of loading solutions were determined fluorimetrically. The amount of loaded protein to each hydrogel disc was calculated from the

difference between original and final reservoir concentrations and it was represented as “milligram protein per gram of dry hydrogel”.

The second method comprises the incorporation of proteins during the formation of the hydrogel network (during crosslinking reaction). Dextran (50 mg/mL buffer) was first dissolved in Tris buffer (pH 7.0) containing 0.5 mg IgG/mL buffer and then crosslinker (50 mg/mL buffer) was added to this solution. The mixture was poured in a glass tube and allowed to react at incubator with appropriate temperature program described before, yielding IgG-loaded dextran hydrogels. The hydrogel was removed from glass test tube, cut into discs (diameter 6 mm, thickness 3 mm) and dried in vacuum at room temperature until no weight loss could be detected. The dry discs were kept in hermetically until further use.

In vitro release study of IgG from dextran hydrogel discs

The release of IgG from hydrogels was investigated in the model gastrointestinal (GI) system containing 0.02% sodium azide at perfect sink conditions. This is why, dry protein loaded hydrogel discs were immersed in Tris buffer at pH 2.0 for 2 h (simulating gastric medium). Then samples were transferred to Tris buffer at pH 7.0 (simulating intestinal medium). Incubation was conducted by rotating at 50 rpm on a shaker (Nüve S 400, Turkey) thermostated at 37°C. Samples were withdrawn with 15 min intervals initially and 1 h intervals later and replaced with fresh release medium. The released amount of IgG from each hydrogel disc was determined by fluorescence spectrophotometer in the same way as loading process.

In order to mimic in-vivo release after oral administration of the hydrogel disc, the release studies were also realized in the presence of dextranase at the same conditions described above. This is why, 4th h of the release experiment dextranase solution in the range of 0.01-0.5 U/mL was added to then release medium. The cumulative amount of released IgG from each hydrogel disc was determined by fluorimetric measurements.

All experiments (loading and release) were repeated at least 3 times and the results were reported as average values.

RESULTS AND DISCUSSION

Synthesis and characterization of dextran hydrogels

The colon is largely being investigated as a site for administration of protein and peptides, which are degraded by digestive enzymes in the upper gastrointestinal tract. In addition, drugn administration to the site of action for local diseases of the colon does not only reduce the dose to be administered but also decreases the side effects [21]. The polysaccharide hydrogels have been extensively used as a colon-targeted delivery system because of the fact that they degrade at the targeted sites [21]. They are also highly stable, safe, nontoxic and hydrophilic. The polysaccharide used as protein (IgG) delivery device is dextran and it is widely used in pharmaceutical field since it is water soluble and inert in biological systems. In addition, it is hydrolyzed by dextranases present in the colon.

Dextran hydrogels that were used in this study were prepared with MBAm and ECH acting as the crosslinker. Since MBAm is a polyfunctional monomeric crosslinker, dextran is crosslinked with MBAm through side-chain reaction of dextran hydroxyl groups with amine groups of MBAm in the presence of NaOH [12]. In the case of ECH, the opening of epoxy groups with formation of free chlorohydrin fragments in the side chain of linear

macromolecules is proceeded. The chlorohydrin fragments formed can be easily transformed to epoxy functionality by dehydrochlorination in the presence of NaOH. Dehydrochlorination reaction between two macromolecules containing OH and Cl^- substitute, respectively, is realized providing formation of crosslinking structure [12,15]. In our previous study, we prepared a number of dextran hydrogels by varying the amounts of crosslinker in the polymer mixture [15]. By taking into account the swelling and mechanical properties of all that hydrogels, this study was carried out only the gels prepared with 50% (wt) crosslinkers.

The synthesized dextran hydrogels demonstrated typical characteristic properties (Table 1). The value of M_c for dextran hydrogels was estimated according to the Flory-Rehner model. M_c values for both hydrogels are higher than 5000 g/mole, this is why, the hydrogels synthesized here can be considered as loosely crosslinked networks. Hydrogel mesh size, ξ, is an important parameter in order to predict the release behavior of macromolecule, e.g. protein release from hydrogels. The calculated mesh sizes of hydrogels are approximately 19 nm which means that it will not likely result in a screening effect for most protein drugs. Because the hydrodynamic diameters of most proteins are smaller than 19 nm, such as for IgG it was calculated as 11.3 nm. The equilibrium swelling ratios of both hydrogels, dextran-MBAm and dextran-ECH, are higher at basic medium than acidic medium, however, dextran-MBAm hydrogels have higher swelling ratios and higher initial swelling rates $(dS/dt)_o$ than that of dextran-ECH hydrogels (Table 1). The state of equilibrium was reached within 4 h. While dextran-MBAm hydrogels are having average equilibrium swelling ratios between 12.0 (at pH 2) and 14.0 (at pH 7), dextran-ECH hydrogels have values between 9.0 (at pH 2) and 12.5 (at pH 7). These values are significantly greater than those observed by Chiu et al. [22] from the acrylic acid containing dextran hydrogels and about 10 times higher than those observed by Hennink et al. [23] from the glycidyl methacrylate-containing dextran hydrogels.

Table 1. Structural characteristics and swelling properties of dextran hydrogels synthesized in this study.

Hydrogel	M_c (g/mol)	ξ (nm)	$(dS/dt)_o$ (g water/g gel)		S_{eq} (g water/g gel)	
			pH 2.0	pH 7.0	pH 2.0	pH 7.0
Dextran-MBAm	7385	19.23	0.120	0.161	12.00	14.17
Dextran-ECH	7128	19.02	0.088	0.133	9.20	12.55

Loading of the dextran hydrogels with IgG as model protein drug

Dextran-ECH and dextran-MBAm hydrogels were loaded with 3.00 and 4.00 mg IgG/g dry disc, respectively, during crosslinking. They are the highest amounts of loadable protein which do not cause the disintegration of hydrogel structures. FTIR spectra of loaded gels showed that IgG was physically entrapped within the dextran hydrogel. This method is often satisfactory for loading a high protein dose.

Dextran-MBAm hydrogels were also loaded 3.60±0.25 mg IgG/g dry hydrogel disc by soaking method. Loading of hydrogels by swelling in IgG-containing buffer solution depends on the size of the hydrogel pores (19.23 nm) compared with IgG size (the hydrodynamic diameter of IgG is 11.3 nm) and the swelling properties of the hydrogel. IgG diffusion into dextran-MBAm hydrogel is significantly realized since the pore size of the hydrogel is larger than the size the protein. However, dextran-ECH hydrogels lost their stability in the loading solution containing IgG. This is why, only dextran-MBAm

hydrogels were loaded with IgG by applying the soaking procedure. Increased solute loading concentrations above 0.5 mg/mL did not change the amount of loaded drug to each hydrogel. By taking into account the pH-dependent water sorption property of the hydrogels, IgG was loaded at pH=7, which allows high swelling and thus high loading capacity without causing denaturation of IgG molecules.

Characterization of IgG loaded dextran hydrogel discs

The swelling ratios of each protein-loaded samples at two pHs (2.0 and 7.0) were determined by swelling studies at 37°C in tris buffer (Table 2). It was shown that there was a little detectable difference in the S values of protein loaded and unloaded hydrogels. This indicates that in the protein loaded hydrogels no osmotic pressure due to the dissolved proteins was present. However, it appeared that the initial swelling rates of the gels were decreased in the presence of protein. All samples were stable in swollen media up to a 1 month period.

The SEM pictures of protein loaded hydrogel discs are presented in Figure 1 a-d. Figure 1a shows a surface view of IgG dextran-ECH hydrogel loaded with IgG during crosslinking reaction. The material is porous in its native state. Figure 1b represents a cross-sectional view at higher magnification (x 655).

The surface and cross-sectional micrograph of dextran-MBAm hydrogel loaded with IgG during crosslinking reaction are seen in Figures 1c and d, respectively. Dispersed IgG molecules are seen in Figure 1d. In the case of dextran-MBAm hydrogels the network becomes less ordered and more porous (Figure 1 d).

Table 2. Loading capacities and swelling properties of IgG loaded dextran hydrogels.

Hydrogel	Amount of IgG (mg/g dry hydrogel)	S_{eq} (g water/g gel)		$(dS/dt)_o$	
		pH 2.0	pH 7.0	pH 2.0	pH 7.0
Dextran-MBAm/crosslinking-IgG	4.00	12.11	14.37	0.082	0.094
Dextran-ECH/crosslinking-IgG	3.00	9.26	12.62	0.046	0.087
Dextran-MBAm/soaking-IgG	3.60	12.12	14.28	0.087	0.103

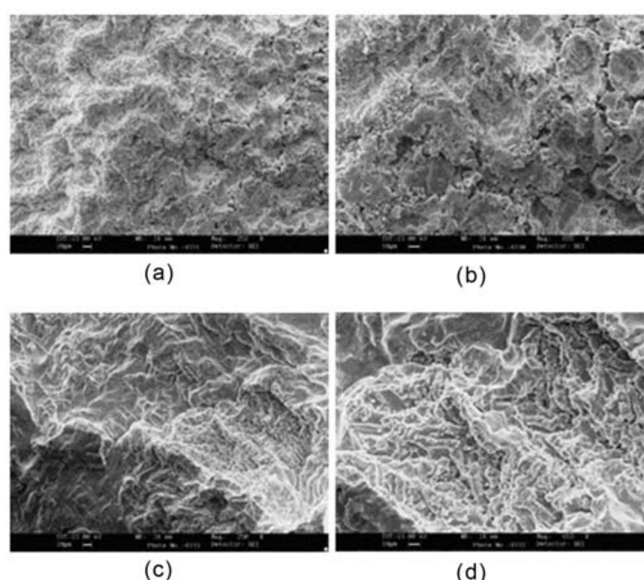

Figure 1. Scanning electron micrographs of IgG loaded dextran hydrogel discs: (a) Surface structure of IgG loaded dextran-ECH hydrogel during crosslinking reaction (X252) (b) cross-section of the same hydrogel (X655) (c) surface structure of IgG loaded dextran-MBAm hydrogel during crosslinking reaction (X250) (d) cross-section of IgG loaded dextran-MBAm hydrogel during crosslinking reaction (X653).

In vitro release of IgG from dextran hydrogels in the model GIT

<u>In the absence of dextranase:</u>

Figure 2 represents the cumulative release of IgG from dextran hydrogels as a function of time in

model GIT in the absence of dextranase. A significant difference in release rate can not be observed between the applied loading procedures. It was seen that IgG release rate from dextran-MBAm hydrogels are almost same with the dextran-ECH's although the swelling ratio of dextran-MBAm is higher than that of dextran-ECH's.

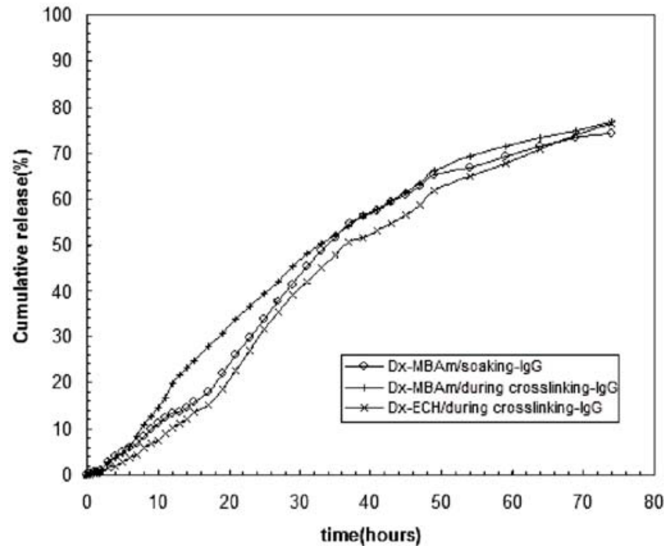

Figure 2. Cumulative release of IgG from dextran hydrogels in model GIT in the absence of dextranase.

It is the most important point in Figure 2 that the cumulative release of IgG is minimum level (0.5%) in pH 2.0 medium (stomach) for all hydrogel samples. In addition, 40% of IgG is released during 30 h (minimum space time in intestinal tract) and the 76% of IgG is released during 74 h (maximum space time in intestinal tract).

The diffusion exponents (n) were calculated with the semi-empirical power law equation (Eq.3), as suggested by Ritger and Peppas [24]. This equation is appropriate for the release of drugs from swellable matrices and it has been frequently used in the literature to describe the drug transport mechanism.

$$M_t/M_\infty = kt^n \qquad (3)$$

where M_t is the cumulative amount of released protein at time t, M_∞ is the amount of loaded protein present in the hydrogel network, M_t/M_∞ (F) represents the fractional release of the protein at time t, k is a constant characteristic for the protein loaded matrix system, and n, the diffusion exponent, is a characteristic for the release mechanism.

The n and k values determined from the initial portion of ($F \leq 0.6$) log-log plots of F vs time are presented in Table 3 together with the regression coefficients. According to the model suggested by Peppas for swellable cylindirical devices [24]. Fickian diffusion corresponds to a diffusion exponent n = 0.45, whereas polymer relaxation occurs at higher values (n= 0.89) (Case-II diffusion). Non-Fickian diffusion also corresponds to the diffusion exponents $0.45 < n < 0.89$. Table 3 shows that n values vary between 0.65-0.69 for IgG release at pH=7.0 from all hydrogels. In Non-Fickian diffusion, the Fick diffusion rates and polymer relaxation rates are competition with each other.

Table 3.The difussion parameters which are related to IgG release from dextran hydrogels at pH: 7.0.

Hydrogel	n	k	R^2
Dextran-MBAm/ crosslinking-IgG	0.66 ± 0.002	0.003	0.990
Dextran-ECH/ crosslinking-IgG	0.69 ± 0.003	0.001	0.992
Dextran-MBAm/ soaking-IgG	0.65 ± 0.004	0.002	0.991

In the presence of dextranase

The release of BSA from dextran hydrogels in the model GIT containing in the range of 0.01-0.5 U/mL varying concentrations of dextranase was studied. The results are shown in Figures 3a-c together with release data in the absence of dextranase.

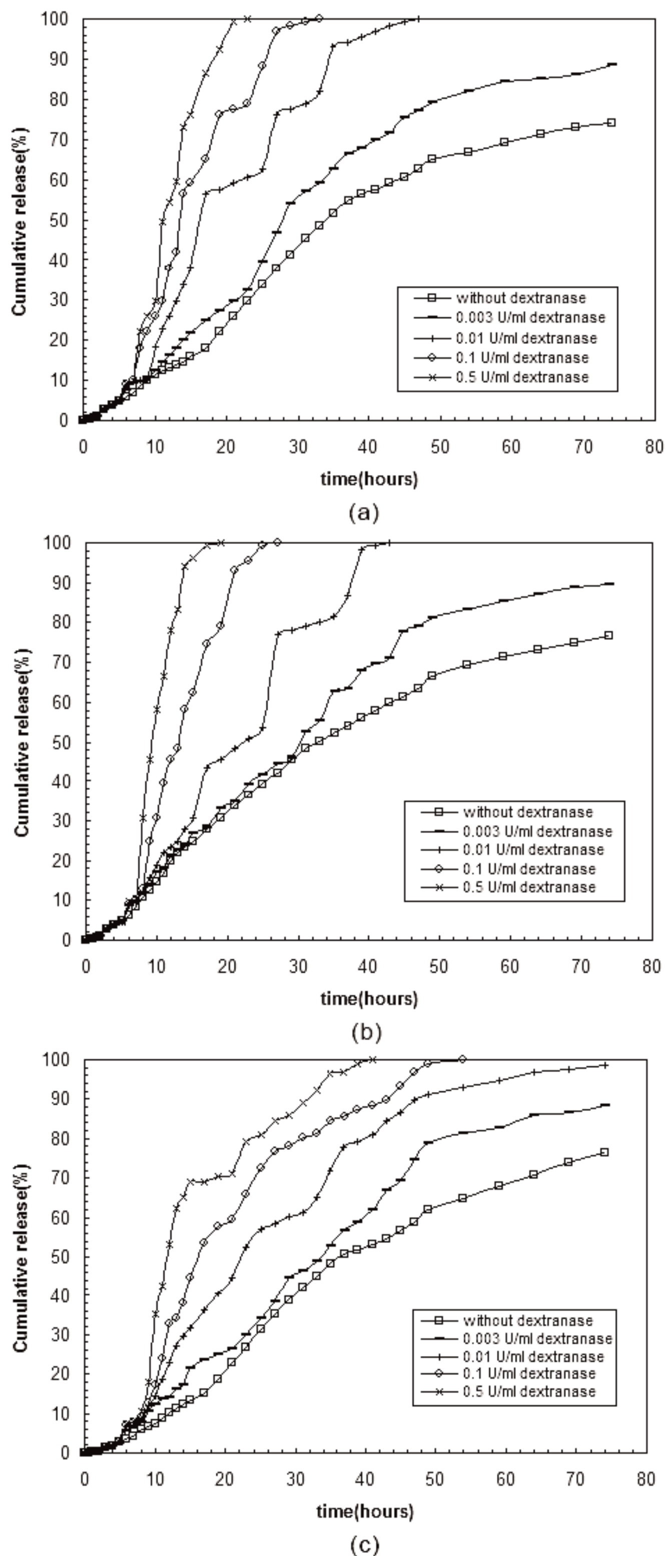

Figure 3. Comparison of release profiles of IgG from dextran hydrogels in the presence and absence of the dextranase in model GIT. (a) For dextran-MBAm hydrogels loaded by soaking; (b) For dextran-MBAm hydrogels loaded during crosslinking; (c) For dextran-ECH hydrogels loaded during crosslinking.

When no dextranase is present, the cumulative IgG release is about 75% from all hydrogels at the end of 70 h incubation period and controlled by simple diffusion. In the presence of high concentrations (0.5 U/mL) of dextranase, a fast release of IgG was observed from all hydrogels and 100% of the BSA was released in 19 h, 23 h and 39 h from dextran-MBAm (crosslinking), dextran-MBAm (soaking) and dextran-ECH (crosslinking), respectively. The release of IgG from dextran hydrogel discs was enhanced in the presence of dextranase. This can be explained by the enzymatic degradation of the dextran backbone, and results in an increased permeability of the hydrogels. Furthermore, it was observed that the release rate is independent of the crosslinker type and the loading method. In addition, it was seen that the release rate is changing with respect to the enzyme concentration. Hydrogels prepared from 50% w/w solutions of dextran and crosslinker even dissolved completely in the dextranase concentration of 0.5 U/mL between 75-80 h period of the release experiment.

Both hydrogels provide a sufficient lag time for protein release to prevent premature release in the small intestine. Furthermore, protein can be released completely within the period of colonic residence.

CONCLUSION

The results presented in this study demonstrate that highly swellable dextran hydrogels synthesized in the presence of MBAm and ECH can be used for the colon-specific release of high molecular weights proteins such as IgG. Nearly equal amounts of IgG were loaded into the hydrogels by crosslinking and soaking procedures. It was indicated that the loading procedure does not affect the release profile of IgG. The release rate depends on and can be manipulated by the type of crosslinker, release

medium pH and the presence of dextranase in the release medium.

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