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Dextran and 5-aminosalicylic acid (5-ASA) conjugates: synthesis, characterisation and enzymic hydrolysis

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Abstract—Mesalamine (5-aminosalicylic acid) is the drug of choice for the treatment of Crohn's disease. A scheme for the synthesis of 5-aminosalicylic acid (5-ASA) conjugates of dextrans was developed with a focus on Crohn's disease applications. Dextrans were oxidised using sodium periodate ($NaIO_4$), where the aldehyde groups formed were coupled with the α-amino ($-NH_2$) group of 5-ASA. The resulting imine bonds were unstable in water and were consequently reduced to secondary amine groups. The effects of different aspects of the conjugation reaction were studied. These included the following: the molecular weight of the dextrans, the molar proportion of $NaIO_4$ to the dextrans (for periodate oxidation), the pH of the conjugation solutions, the ratio 5-ASA to oxidised polysaccharide and the relationship between the degree of conjugation and the amount of enzyme hydrolysis. Conjugates incubated in HCl were stable in 0.5 and 1.0 M HCl, but they underwent degradation in 2.0 and 4.0 M HCl. Dextrans (MW 20,000) with various degrees of oxidation (12%, 26%, 46%, 65%, 90% and 93%) were also prepared. Each oxidised dextran sample was conjugated with 5-ASA, and the product was quantified by high-performance liquid chromatography (HPLC). Dextrans with a maximum degree of oxidation (93%) unsurprisingly gave maximum conjugation of 5-ASA (49.1 mg per 100 mg of product) but were resistant to dextranase hydrolysis. Less oxidised dextrans (12%) conjugated proportionally less 5-ASA (15.1 mg per 100 mg of product) but were successfully hydrolysed by dextranase, suggesting their potential applications for the treatment of Crohn's disease in the distal ileum and proximal colon.

Keywords: Dextran; 5-ASA; Drug conjugates; Periodate oxidation; Schiff's base

1. Introduction

Polymer–drug conjugation is a relatively novel technique for drug delivery with a history of around 25 years. Polymer–drug conjugates (PDCs) act as carriers for drugs and target them to the desired site in the body. The carriers then undergo enzymic hydrolysis, and the active drug is released, sometimes with sustained release profiles. The major attributes of PDCs include the following: capacity to be stored in depots, unique pharmacokinetic profiles, potential body distribution and pharmacological efficacy.[1] Commercially available PDC products include polyethylene glycol (PEG) with somavastatin (Somavert), asparginase (Oncasper) and interferon-α-2 (Pegasys). PEGylation of therapeutic proteins increases: (a) in vivo half life (due to decreased enzymatic degradation and reduced kidney excretion rates); (b) performance (due to reduced immunogenicity, antigenicity and toxicity); (c) physicochemical properties (e.g., water solubility) and (d) reduced frequency of administration with increased patient compliance.[2] To date there are no commercially available polysaccharide–drug conjugate delivery systems prescribed for any specific therapies.

Dextrans may be used as model polysaccharides for conjugation. They have excellent water solubility, low toxicity and immunogenicity and are available in a wide molecular-weight range with low polydispersity. Dextrans contain primarily glucose α-(1→6) linkages with side chains of additional α-(1→4) and α-(1→3) bonds. Dextrans have been successfully conjugated in the laboratory with low-molecular-weight drugs (indomethacin, 5-aminosalicylic acid and doxorubicin)[3] and therapeutic

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proteins (insulin, immunoglobulin and human growth hormone).[4–6] These polysaccharides are not readily degraded in the blood and for molecular weights above 70,000 D are not filtered through the kidneys (consequently the dextrans have also been used as plasma expanders). Dextran-hydrolysing enzymes (dextranases) are produced by mammalian cells and colonic bacteria and are hence found in the liver, spleen, kidney and in the lumen of the large intestine.[7]

The aspirin derivative 5-ASA is a very effective form of treatment of inflammatory bowel disease (IBD) with minimum side effects. It is used alone or in combination with other drugs in chronic IBD disease conditions.[8] The 5-ASA molecule is amphoteric, and its solubility and ionisation characteristics are dependent upon pH and the pK_a values of the carboxylic and amino groups.[9] It is very soluble above pH 5.5 and thus it is readily absorbed from the gastrointestinal tract as soon as it passes through the stomach. Systemic absorption of 5-ASA creates various physiological side effects. Hence research has focused on local (topical) delivery of 5-ASA at the diseased site (distal ileum and proximal colon) with intentionally minimised systemic absorption. The mechanism of action of 5-ASA is not fully understood, but it is suggested that it reduces inflammation by blocking cyclooxygenase and lipoxygenase in the arachidonic acid pathway and inhibits the production of prostaglandins and other inflammatory mediators in the intestine[10] (Fig. 1). Patients prescribed conjugates of 5-ASA (non-carbohydrate) have shown a better response compared to 5-ASA therapy alone.[8] The commercially available 5-ASA conjugates include sulfasalazine (5-ASA-sulfapyridine), olsalazine (5-ASA dimer) and balsalazide (5-ASA–4-aminobenzoyl-β-alanine). Although 5-ASA is chemically bonded in these systems, a small portion of intact prodrug gets absorbed from the upper GI tract, which may be avoided by using polymer conjugates.

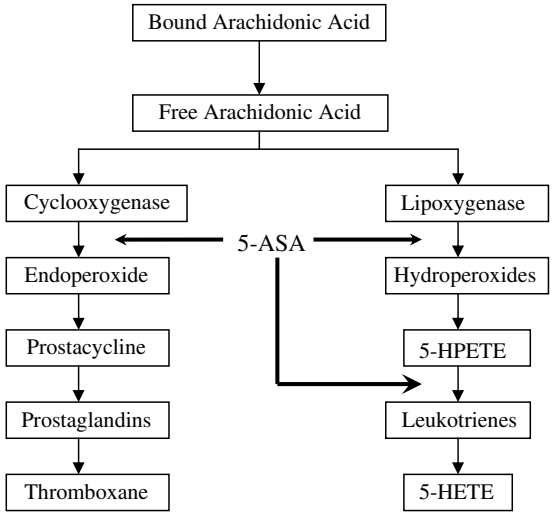

Figure 1. Mechanism of action of 5-aminosalicylic acid (5-ASA).[10]

The objective of this work was to prepare 5-ASA conjugates of dextran, appropriate for sustained and targeted delivery of 5-ASA to the distal ileum and proximal colon. Covalent conjugation of 5-ASA with dextran has the potential to reduce or eliminate the problems associated with rapid blood clearance and drug-related side effects and provides new formulations with a low dose and targeted delivery to the inflamed intestine. Dextran–5-ASA conjugates are potentially safer than sulfapyridine in sulfasalazine formulations, with improved release profiles due to colonic microflora fermentation.

2. Experimental

2.1. Materials and methods

Glucose (16,325), fructose (15,760) and dextrans of different molecular weights (6000, $M_r/M_n = 1.7$, 31,388; 20,000, $M_r/M_n = 1.1$, 31,387; 40,000, $M_r/M_n = 2.3$, 31,389; 70,000, $M_r/M_n = 1.7$, 31,390; 100,000, $M_r/M_n = 1.5$, 31,391 and; 500,000, $M_r/M_n = 1.4$, 31,392), 5-ASA (09410) and borane–pyridine complex (15,583) were all obtained from Fluka Biochimika, Gillingham, UK. All the other chemicals were of analytical reagent grade. Vivaspin-20, 5000 molecular-weight cut-off membranes (MWCO) (VS2012) and 0.2 μm syringe filters (Ministart 16532K) were obtained from Vivascience AG, Hanover, Germany. Dialysis membrane (DTV 12000.10.000) of 12–14,000 MWCO was obtained from Medicell International Ltd, London, UK. Dextranase (EC 3.2.2.1.11, D5884 from *Penicillium* sp.; 12.9 U mg^{-1}) and pancreatin (76,190, from hog pancreas, 140 U mg^{-1}) were obtained from Sigma and Fluka (both Gillingham, UK), respectively. Dextran standards (5000, 31,417; 12,000, 31,418; 50,000, 31,420 and 80,000, 31,421), maltohexaose (63416), maltose (63,418) and maltotriose (63,430) were also obtained from Fluka and were used for calibration of the GPC column as 1% solutions (below).

2.2. Periodate oxidation of polysaccharides

Different methods were evaluated to optimise conjugation. Glucose solutions (20 mg mL^{-1}) were prepared in either water[4] or phosphate buffer[11] (0.2 M; pH 6.0). To 25 mL of these glucose solutions (contained in 100-mL dark bottles), 25 mL of 0.1 M $NaIO_4$ was added to initiate the reaction. The contents of the sealed bottles were mixed and stored at room temperature. Solutions (1 mmol per 5 mL) of glucose, fructose and different dextrans were also prepared together with 2.5, 2.0, 1.5, 1.0, 0.75 and 0.50 mM $NaIO_4$. To 5 mL of these

carbohydrate solutions (also contained in dark bottles), 10 mL of $NaIO_4$ was added by a pipette. The contents were mixed, and the sealed bottles were stored at room temperature. The amount of $NaIO_4$ consumed by the oxidation reactions was determined by back titration of unreacted reagent by measuring the free iodine released from KI with 0.05 M sodium thiosulfate using 1% soluble starch as an indicator. Formic acid generated (assumed to be the only acid product) by the reaction was titrated with 0.05 M sodium hydroxide using 1% phenolphthalein as indicator. For bulk synthesis, polysaccharides were oxidised in the same ratio, and at the end point the reaction was stopped by adding an excess of ethylene glycol. The solution was dialysed for 24 h to remove the by-products and then freeze dried.

2.3. Schiff's base conjugation of 5-ASA

The 5-ASA was conjugated with dialdehyde dextran according to the scheme presented in Figure 2. To understand the effect of reaction pH and time, samples of 5-ASA (408.46 mg, equivalent to 2 mM) were accurately weighed into 100-mL volumetric flasks to which 50 mL distilled water, acetate buffer (0.1 M; pH 5.0), phosphate buffer (0.1 M; pH 6.0, 7.0, 8.0 and 9.0) or carbonate buffer (0.1 M; pH 10.0) was added. Dialdehyde dextran (162 mg, equivalent to 1 mM oxidised glucose unit) was accurately weighed into small weighing boats, then quantitatively washed into the flasks containing the 5-ASA solutions (with buffer or water), and the volumes were adjusted to 100 mL. These were thoroughly mixed. After 12, 24, 48 or 78 h incubation at room temperature, the solutions were centrifuged at 4800 rpm (1800*g*) for 40 min using Vivaspin-20 tubes to remove unconjugated drug.

For quantitative analysis of 5-ASA, a five-point standard calibration curve (0.4, 0.8, 1.0, 1.2 and 1.5 mg mL^{-1} in distilled water) was determined spectrophotometrically at 335 nm. Stock solutions of 5-ASA conjugates of dextran were also prepared quantitatively in distilled water and similarly characterised. The conjugated products were profiled by high-performance liquid chromatography (HPLC). The HPLC system consisted of a degasser (model DG2, Waters), isocratic pump (model 1515, Waters), RI detector (model 2414, Waters) and dual-wavelength UV absorbance detector (model 2487, Waters). A gel-permeation chromatography (GPC) Waters Ultrahydrogel 250 (7.8 mm ID × 30 cm) column was used and eluted with an aqueous mobile phase (containing 0.02 M sodium azide filtered through a 0.45 μm membrane) at a flow rate of 0.8 mL min^{-1} and at a pressure of 380 psi. The column effluent was monitored by both the UV (at 335 nm) and RI detectors. Waters Breeze software was used for data analysis.

To understand the relationship between the concentration of 5-ASA and degree of conjugation, different solutions (100 mL) of 5-ASA (0.1, 0.5, 1.0, 1.5, 2.0 and 2.5 mM) were prepared in 0.1 M (pH 10.0) sodium carbonate buffer containing dialdehyde dextran (162 mg, equivalent to 1 mM oxidised glucose unit). After 24 h incubation, the solutions were centrifuged at 4800 rpm (1800*g*) for 40 min using Vivaspin-20 tubes and were characterised by HPLC (as above). For bulk synthesis of conjugates, the reaction was performed in the same ratio of the reactants: after 24 h pyridine–borane (equivalent to moles of 5-ASA) was added to the solution, and after 8 h the product was dialysed against distilled water (5 L, 6 changes) for 24 h.

2.4. Enzyme study of native, oxidised and conjugated polysaccharide

Solutions of native, oxidised and conjugated polysaccharides (5 mg mL^{-1}), dextranase (13, 65 or 130 U mL^{-1}) and pancreatin (15, 50 or 150 U mL^{-1}) were prepared in tris buffer (0.1 M, pH 6.7). To 25 mL

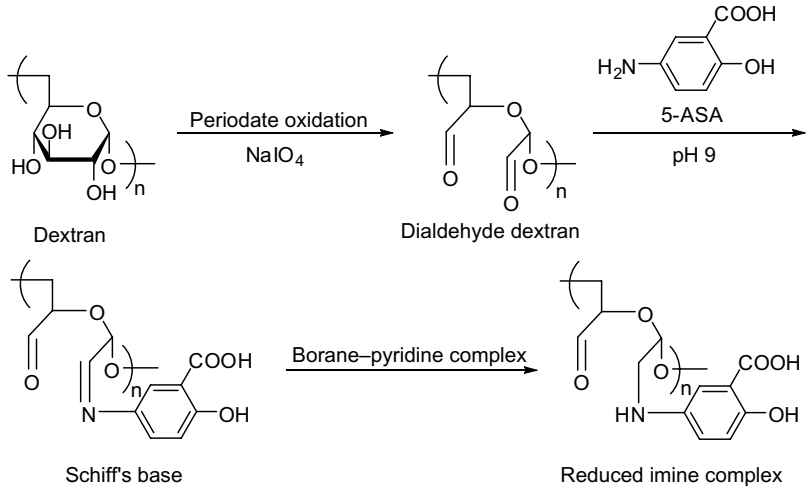

Figure 2. Schematic representation of the synthesis of dextran–5-ASA conjugates.

Table 1. Periodate oxidation of 1 mmol of carbohydrates using different methods

Carbohydrates (1 mmol)	Theoretical value (mmol)		Experimental value (mmol)	
	$NaIO_4$ consumed	Formic acid produced	$NaIO_4$ consumed	Formic acid produced
Glucose[4]	5	5	3.9 ± 0.1	3.9 ± 0.1
Glucose[11]	5	5	3.9 ± 0.2	3.9 ± 0.3
Glucose[a]	5	5	4.9 ± 0.1	4.9 ± 0.1
Fructose[a]	5	3	4.8 ± 0.1	2.8 ± 0.1
Dextran 6000[a]	2	1	1.9 ± 0.1	1.0 ± 0.1

[a] Modified method[4,11] where volume was reduced from 50 to 15 mL.

aliquots of polysaccharide solutions in 50 mL screw-capped flasks, 1 mL of either pancreatin or dextranase solution was added by pipette, and the sealed and mixed flasks were incubated at 37 °C. Sampling was undertaken at different time intervals up to 8 h. For analysis, 2-mL aliquots of the reaction mixture were transferred by a pipette to clean and dry 10-mL screw-capped tubes. The sealed tubes were placed in boiling water for 5 min, cooled, filtered through 0.2 μm syringe filters, then characterised by HPLC (as above). To study the chemical stability of 5-ASA–dextran conjugates in acid, they were incubated in 0.5, 1.0, 2.0 and 4.0 M HCl. After 2 h the HCl was neutralised with equimolar NaOH and analysed by HPLC (as above). Data were expressed as the mean of triplicate determinations with standard deviation from the mean.

3. Results

Periodate oxidation of glucose as a pre-screen for studies including dextran in water[4] or buffer[11] provided only partial oxidation with similar results by either method (Table 1). The methods were consequently developed to optimise the effects of time, temperature and molar strength of $NaIO_4$ on the extent of oxidation. Using this approach, it was found that extending the temperature and time of the reaction did not have any significant influence on the degree of oxidation (data not shown). In terms of the effect of molar strength on periodate oxidation, glucose was oxidised with 0.05, 0.1, 0.2, 0.3 or 0.4 mM $NaIO_4$. As the molar strength of $NaIO_4$ increased, the degree of oxidation also (unsurprisingly) increased. For $NaIO_4$ solutions with a molar strength from 0.05 to 0.2 mM, it was found that the number of moles of $NaIO_4$ consumed were equal to the number of moles of formic acid produced (Fig. 3). However, at higher concentrations (0.3 and 0.4 mM $NaIO_4$), there was an increase in the consumption of $NaIO_4$, but a decrease in the production of formic acid. Presumably this was because as the concentration of $NaIO_4$ increases, it starts oxidising formic acid to carbon dioxide and water.

When the reaction volume used for periodate oxidation as per the published methods[4,11] (50 mL) was reduced to 15 mL, it produced stoichiometric results for glucose, fructose and dextran 6000 (Table 1). This procedure was thus adopted for the oxidation of

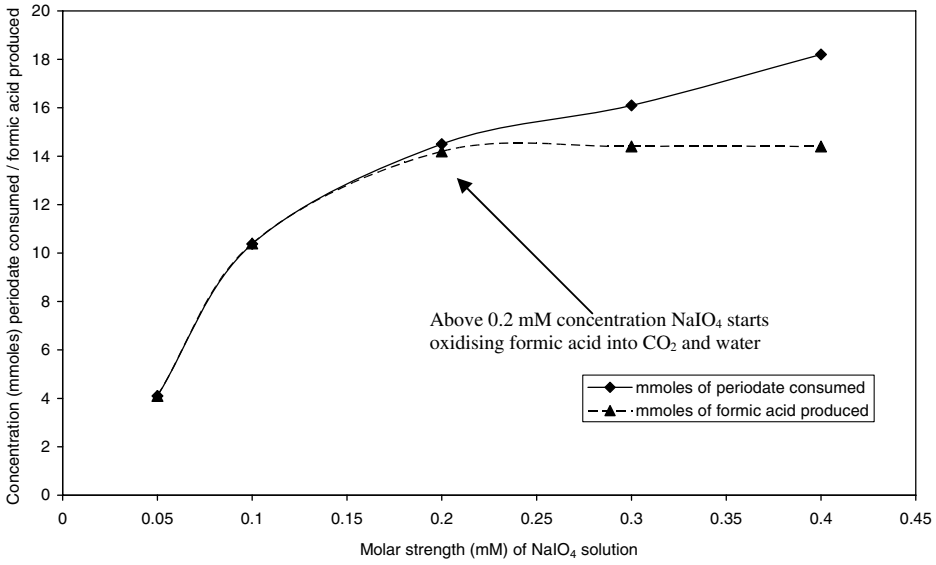

Figure 3. Effect of molar strength of $NaIO_4$ on the oxidation of a 1 mmol solution of glucose after 24 h at 25 °C in water.

dextrans. When 1 mmol of dextrans was treated with 2 mM $NaIO_4$, it was observed that as the molecular weight of dextran increased the degree of oxidation decreased but the stoichiometric ratio of the reaction was maintained, that is, 2 mmol of $NaIO_4$ was consumed and 1 mmol of formic acid was produced (Fig. 4). Dextran 20,000 was treated with different solution concentrations of $NaIO_4$ (0.5, 0.75, 1.0, 1.5, 2.0 and 2.5 mM) where it was found that as the molar strength increased the degree of oxidation also increased (Table 2). At low concentrations, 0.5 and 0.75 mM $NaIO_4$, the ratio of periodate consumed and formic acid formed was higher than 2:1, that is, individual glucose units were not oxidised completely. At higher molar strengths of $NaIO_4$ (1.0 mM and above), the ratio was 2:1. From these data, it is apparent that the extent of C–C oxidation can be selected by the choice of $NaIO_4$ concentration.

Dextran was activated to dialdehyde dextran to facilitate the conjugation of newly formed aldehyde groups with amino groups of the 5-ASA (Fig. 2). The influence of pH on the conjugation reactions was studied at different pHs (4.6, 5.0, 7.0, 9.0 and 10.6), and it was observed that the efficiency of the 5-ASA–dextran conjugation was highly dependent on the pH. Maximum conjugation occurred at pH 7.0 (Fig. 5a). The time required for the completion of the reaction at pH 7.0 was also assessed. It was found that the conjugation reaction was complete within 24 h (Fig. 5b). When dextran 20,000 (with various degrees of oxidation) was conjugated with 5-ASA, it was found that the degree of conjugation increased linearly with the degree of oxidation (Table 3) due to the greater number of available aldehyde groups. As expected, the conjugation reaction was dependent on the degree of oxidation, not on the molecular weight of the dextrans. The Schiff's base was unstable; hence, it was reduced to amine using pyridine–borane, a specific reducing agent for Schiff's bases.[12]

For sustained release of drug from a polymer, it is important that the drug–carrier bonds are hydrolysed slowly with possible facilitation of this process by hydrolysis of the polymer itself. In this study, when different molecular-weight dextrans were incubated with dextranase, within 8 h all the dextrans were hydrolysed to the same extent, but at a different rate (Fig. 6). Hence the molecular weight of dextran played a part in the overall release process of the 5-ASA. A typical hydrolytic pattern of dextran by dextranase is provided in Figure 7. The effects of the degree of oxidation on hydrolysis of dextrans were also studied. Dextran 20,000 was oxidised to a different extent (12%, 26%, 45%, 65%, 90% and 93%) where it was found that as

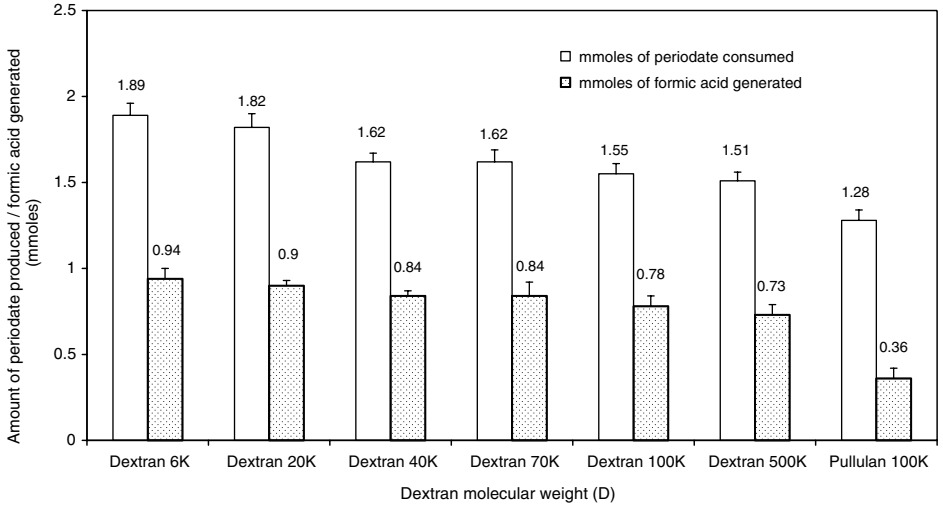

Figure 4. Oxidation of 1 mmol solution of dextrans of different molecular weights with 2 mM $NaIO_4$ using modified method at 25 °C.

Table 2. Effect of molar strength of $NaIO_4$ on periodate oxidation of dextran 20,000

Molar strength of $NaIO_4$ (M)	Periodate oxidation of dextran 20,000	
	$NaIO_4$ consumed (mmol)	Formic acid produced (mmol)
2.00	1.87 ± 0.1	0.93 ± 0.1
1.50	1.81 ± 0.1	0.90 ± 0.2
1.00	1.34 ± 0.2	0.65 ± 0.1
0.75	1.05 ± 0.1	0.45 ± 0.1
0.50	0.75 ± 0.2	0.26 ± 0.2
0.10	0.53 ± 0.3	0.12 ± 0.2

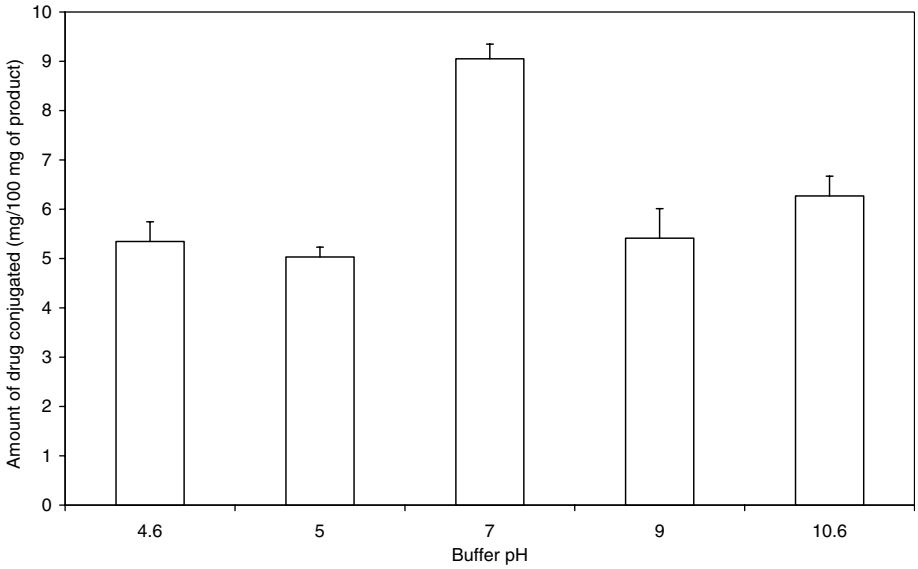

Figure 5a. Effect of buffer pH (0.1 M acetate, phosphate and carbonate buffer) on the extent of 5-ASA–dextran conjugation at 25 °C.

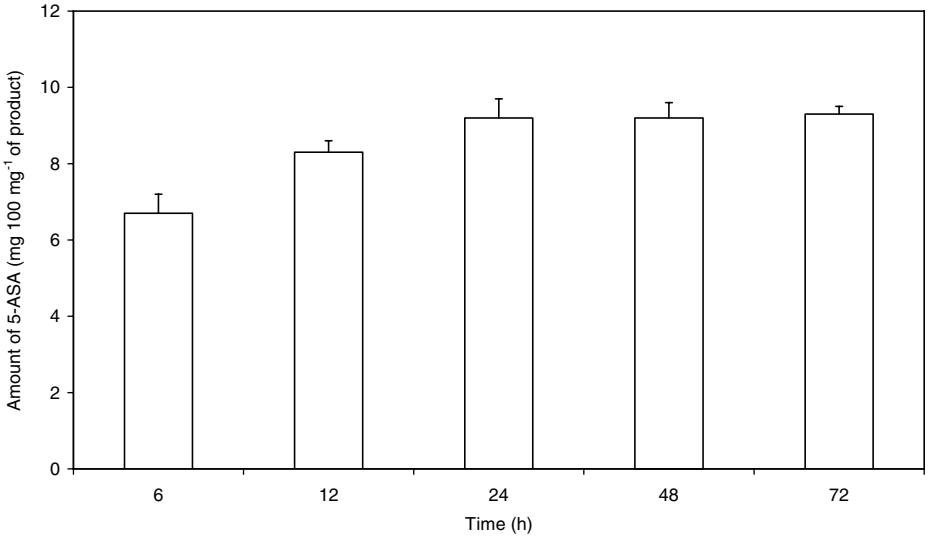

Figure 5b. Effect of time on the extent of 5-ASA–dextran conjugation at 25 °C.

the degree of oxidation increased, the rate and extent of hydrolysis decreased (Table 3). A '12% oxidised' dextran underwent hydrolysis to the same extent as the native dextran but with a slower rate. In contrast '93% oxidised' dextran underwent only partial hydrolysis under these conditions. Thus the extent of oxidation of dextran also played a role in the overall hydrolysis profile.

To predict the stability of dextran–5-ASA conjugates in the stomach, the conjugates were incubated in HCl (0.5, 1.0, 2.0 and 4.0 M) at 37 °C, where it was observed that they remained stable in 0.5 or 1.0 M HCl but were partially hydrolysed in 2.0 or 4.0 M HCl (Table 3). Taken with the enzymatic data discussed above, these data suggest that the dextran–5-ASA conjugates should be able to bypass the stomach without modification.

When 5-ASA–dextran conjugates were incubated with dextranase at 37 °C, it was observed that the products with greatest degrees of conjugation were most resistant to enzymatic hydrolysis (Table 3). Hence, the degree of conjugation was also a controlling factor for dextranase hydrolysis of the polysaccharide. The effects of pancreatin on dextran–5-ASA conjugate hydrolysis were also studied. The native, oxidised and conjugated dextrans were found to be unhydrolysed by the pancreatin enzymes, which was not surprising, as the organ synthesises neither dextranases nor deaminases (which could hydrolyse the conjugate bonds to the polysaccharides) (Table 3). This confirms that dextran–5-ASA conjugates would potentially be stable in the duodenum and jejunum.

Table 3. Stability of oxidised and conjugated dextran in dextranases, pancreatin and HCl solutions

Oxidation of dextran (%)	Amount of 5-ASA conjugated with ox. dextran as mg per 100 mg of product (DS)[a]	Molecular weight					Reduction in the amount of conjugate after 2 h incubation in HCl (%)	
		Oxidised dextran	Oxidised dextran hydrolysis with dextranase[b]	5-ASA–dextran conjugate	5-ASA–dextran conjugate hydrolysis with dextranase[a]	5-ASA–dextran conjugate hydrolysis with pancreatin[a]	0.5/1.0 M	2.0/4.0 M
93.0	49.1 ± 0.2 (1.0)	10,600	5900	98,000	86,000	98,000	0.8 ± 1.0	82 ± 1.6
90.0	41.0 ± 0.2 (0.9)	11,400	5600	85,000	77,000	85,000	—	—
65.0	35.2 ± 0.2 (0.6)	12,600	3800	78,000	52,000	78,000	1.0 ± 0.9	79 ± 1.4
45.0	25.1 ± 0.3 (0.4)	14,400	2300	53,000	50,000	53,000	—	—
26.0	19.8 ± 0.1 (0.3)	14,600	1000	47,000	33,000	47,000	—	—
12.0	15.1 ± 0.7 (0.2)	17,200	600	37,000	16,000	37,000	0.6 ± 1.3	85 ± 1.5

[a] Degree of substitution.
[b] Incubation time = 24 h.

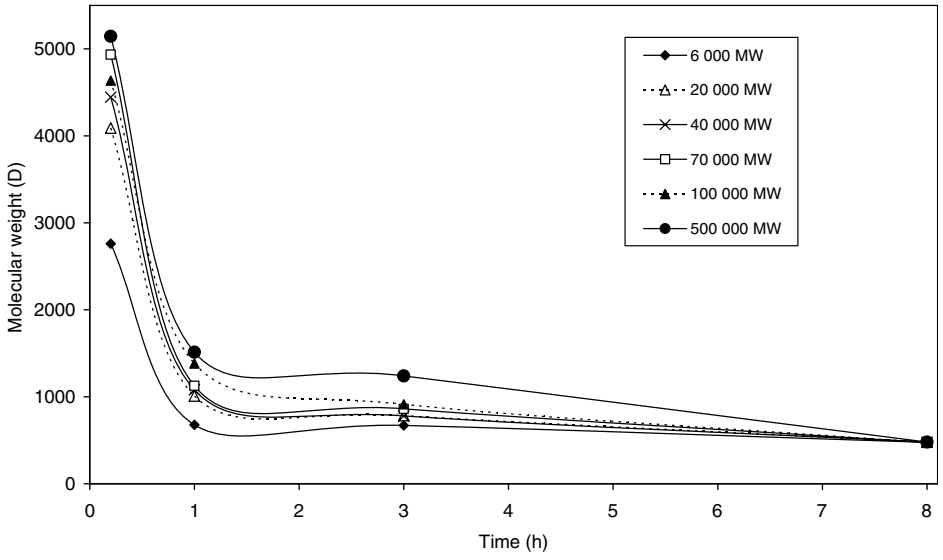

Figure 6. Decrease in the molecular weight of dextrans by the action of dextranase as a function of the original molecular weight of the polysaccharides in tris buffer at 37 °C (0.1 M, pH 6.7) over 8 h duration.

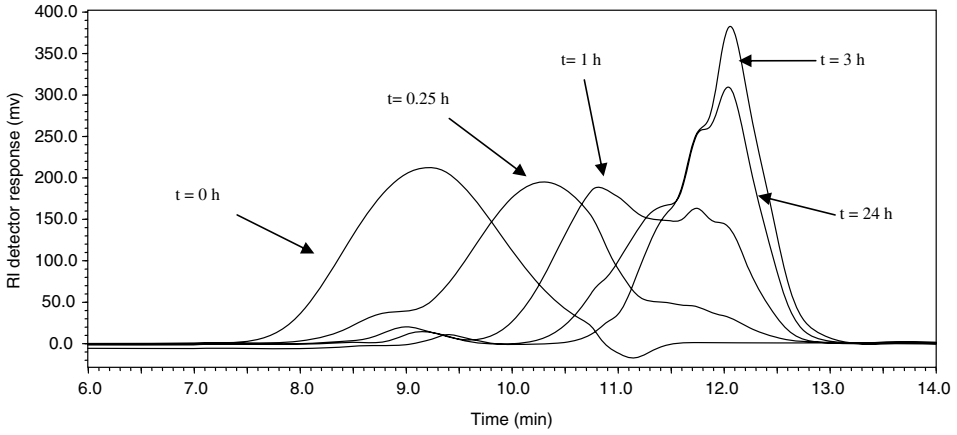

Figure 7. A typical hydrolytic pattern of dextran (20 K) by dextranase in tris buffer (0.1 M, pH 6.7) at 37 °C over 24 h duration using HPLC.

Table 4. Sources of dextranase and deaminase in humans

Enzyme	Source (tissue/bacteria)
Dextranase[15]	Liver, spleen, kidney, mucosa of distal part of jejunum, Bacteroides
Deaminase[16]	*E. coli*, Bacteroides, Clostridium, *Aspergillus melleus*

4. Discussion

The data presented above suggest that the 5-ASA conjugates with dextran may bypass the stomach and small intestine intact. The products formed from classical periodate oxidation[13] and Schiff's base formation[14] contain relatively acid and enzyme (as found in the body) resistant structures. However, they have the potential to be hydrolysed in the distal ileum and proximal colon by gut microflora and hence release 5-ASA to reduce inflammation at the diseased site. The human gut microflora (Table 4) apparently produce both dextranases and deaminases, which could hydrolyse dextran and primary amine bonds, respectively. The 5-ASA conjugates produced in this work comprise secondary amine groups, and it is recognised that certain deaminase enzymes (focused towards primary amines) probably cannot hydrolyse these bonds. However, there are probably other relevant enzyme systems (specific secondary amine deaminases) in the gut, which are effective in this respect. This approach may, therefore, be used as a potential therapeutic tool for Crohn's disease for targeted sustained delivery of 5-ASA. In subsequent paper, physicochemical properties and microbial fermentation of 5-ASA–dextran conjugates will be discussed.

5. Conclusions

The extent of hydrolysis of 5-ASA–dextran conjugates (secondary amines) by dextranase is in part controlled by the degree of oxidation and by the amount of conjugated 5-ASA. This is not surprising as the 5-ASA molecules affect dextranase interaction with the substrate to cleave the constituent bonds. The bonds between the 5-ASA and the dextran are resistant to acid hydrolysis, and in view of the apparent lack of dextranases or deaminases in the small intestine, it is anticipated that the 5-ASA–dextran conjugates would be able to reach the distal ileum without modification. This provides a potential topical treatment at the interface between the distal ileum and proximal colon, where 5-ASA release from the dextran molecules could be achieved due to the production of secondary amine deaminases by the colonic microflora.

References

1. Ouchi, T.; Ohya, Y. *Prog. Polym. Sci.* **1995**, *20*, 211–257.
2. Veronese, F. M.; Morpurgo, M. *Il Farmaco* **1999**, *54*, 497–516.
3. Mehvar, R. *J. Controlled Release* **2000**, *69*, 1–25.
4. Battersby, J.; Clark, R.; Hancock, W.; Campanella, E. P.; Haggarty, N.; Poll, D.; Harding, D. *J. Controlled Release* **1996**, *42*, 143–156.
5. Fagnani, R.; Hagan, M. S.; Bartholomew, R. *Cancer Res.* **1990**, *50*, 3638–3645.
6. Baudys, M.; Letourneur, D.; Liu, F.; Mix, D.; Jozefonvicz, J.; Kim, S. W. *Bioconjugate Chem.* **1998**, *9*, 176–183.
7. Shingel, K. I. *Carbohydr. Res.* **2004**, *339*, 447–460.
8. Evans, B. K. Inflammatory bowel disease. In *Clinical Pharmacy and Therapeutics*; Walker, P., Edward, C., Eds.; Churchill Livingstone: London, 2002; pp 163–178.
9. French, D. L.; Mauger, J. W. *Pharm. Res.* **1993**, *10*, 1285–1290.
10. Tornhamre, S.; Edenius, C.; Smedegard, G. *Eur. J. Pharmacol.* **1989**, *169*, 225–234.
11. Domb, J. A.; Linden, G.; Polaxheck, I.; Benita, S. *J. Polym. Sci., Part A: Polym. Chem.* **1996**, *34*, 1229–1236.
12. Cabacungan, J. C.; Ahmed, I. A.; Feeney, R. E. *Anal. Biochem.* **1982**, *124*, 272–278.
13. Percival, E. G. V.; Percival, E. Periodate oxidation of polysaccharides. In *Structural Carbohydrate Chemistry*; Percival, E. G. V., Ed.; J. Garner Miller Ltd.: London, 1962; pp 197–235.
14. Wong, S. S. Reactive groups of proteins and their modifying agents. In *Chemistry of Protein Conjugation and Cross-Linking*; Wong, S. S., Ed.; CRC Press: London, 1991; pp 7–48.
15. Larsen, C. *Adv. Drug Delivery Rev.* **1989**, *3*, 103–154.
16. Ilett, K. F.; Tee, L. B. G.; Reeves, P. T.; Minchin, R. F. *Pharmacol. Ther.* **1990**, *46*, 67–93.