ELSEVIER

International Journal of Pharmaceutics 122 (1995) 121–128

international journal of pharmaceutics

Alginates as drug carriers: covalent attachment of alginates to therapeutic agents containing primary amine groups

Suzanne M. Morgan [a], Aymen Al-Shamkhani [b], Dominique Callant [c], Etienne Schacht [c], John F. Woodley [d], Ruth Duncan [e,*]

[a] *Institute for Cancer Studies, The Medical School, The University of Birmingham, Edgbaston, Birmingham B15 2TJ, UK*
[b] *Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, UK*
[c] *Biomaterial and Polymer Research Group, Department of Organic Chemistry, University of Ghent, Krijgslaan 281, B-9000 Ghent, Belgium*
[d] *Drug Delivery Group, Department of Biological Sciences, Keele University, Keele ST5 5BG, UK*
[e] *The School of Pharmacy, University of London, 29 / 39 Brunswick Square, London WC1N 1AX, UK*

Received 28 November 1994; accepted 25 January 1995

Abstract

We have been developing a new generation of alginate drug delivery systems based on covalent attachment of therapeutic agents to this macromolecular carrier. This approach involves covalent coupling of the therapeutic agent either directly through its primary amine group, or via a linker terminating in a primary amine group, to propylene glycol alginate. A model immunogenic peptide was synthesised and bound directly to alginate, and the effect of conjugation on its immunogenicity was assessed. We found that conjugation to alginate resulted in a 4-fold decrease in the humoral antibody response to the peptide following repeated systemic administration to mice. A conjugate of alginate and 5-aminosalicylic acid (5ASA) was also prepared. 5ASA was first coupled to a linker (6-aminohexanamide-L-phenylalanine) specifically designed to be cleaved by the enzyme α-chymotrypsin, and subsequently bound to alginate. In vitro release studies showed that 5ASA was gradually released from this conjugate in the presence of α-chymotrypsin (48% released/24 h), and that the initial rate of drug release was approx. 6-fold slower than that from the low molecular weight analogue, 6-aminohexanamide-L-phenylalanyl-5ASA.

Keywords: Alginate; Polymeric drug carrier; Peptide delivery system; 5-Aminosalicylic acid; α-Chymotrypsin-sensitive linker; Inflammatory bowel disease

1. Introduction

Alginates are natural polymers composed of (1–4)-linked β-D-mannuronic acid and α-L-guluronic acid residues (Gacesa, 1988). Although alginates have been developed as drug delivery systems, most studies have used methods involving non-covalent entrapment of drugs within the alginate matrix or within calcium alginate gels (Bodmeier and Paeratakul, 1991; Kim and Lee, 1992; Lin and Ayres, 1992). Covalent coupling of

* Corresponding author. Tel. 44 171 753 5932; Fax 44 171 753 5931.

0378-5173/95/$09.50
SSDI 0378-5173(95)00059-3

proteins and drugs to macromolecular carriers on the other hand, offers the possibility of tailoring more sophisticated drug delivery systems, having improved drug pharmacokinetics and bioavailability and this approach has already begun to realise clinical potential (reviewed in Duncan, 1992).

In this study, we have developed a general method for the covalent coupling of alginates to therapeutic agents containing primary amine groups. We used a peptide antigen (peptide VP2) composed of a B cell-T helper cell epitope representing two sites on the structural protein VP2 of the human rhinovirus type 2 (Francis et al., 1989) to study the effect of conjugation to alginate on its immunogenicity. Conjugation of proteins to polymers such as polyethylene glycol (Tsuji and Hirose, 1985) and *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer (Flanagan et al., 1990) have been shown to result in a reduction in the immunogenicity of the coupled proteins. However, alginates are known to have immunostimulatory activity (Otterlei et al., 1991; Espevik et al., 1993) and therefore may exhibit adjuvant properties on coupling to antigens.

Here we also describe a method which can be used for the coupling of drugs such as 5-aminosalicylic acid (5ASA) indirectly to alginate by the incorporation of a linker terminating in a primary amine group. We used a linker which is specifically cleaved by the enzyme α-chymotrypsin, hence tailoring this conjugate to enable drug release in the gastrointestinal tract.

2. Materials and methods

2.1. Synthesis of peptide VP2

Peptide VP2 (VKAETRLNPDLQPTETS QDVANAIVC) was chemically synthesised using the solid-phase process according to the procedure of Merrifield (1963), with the B cell epitope at the amino terminus and an non-natural cysteine residue at the carboxyl terminus. The peptide was purified by reverse-phase HPLC and characterised by fast atom bombardment mass spectrophotometry.

2.2. Preparation of alginate-peptide conjugate

Propylene glycol alginate (a commercial product supplied by Kelco Division of Merck and Co. Inc.) was reacted with peptide VP2 in 0.2 M bicarbonate buffer (pH 9) using a 3:1 molar ex-

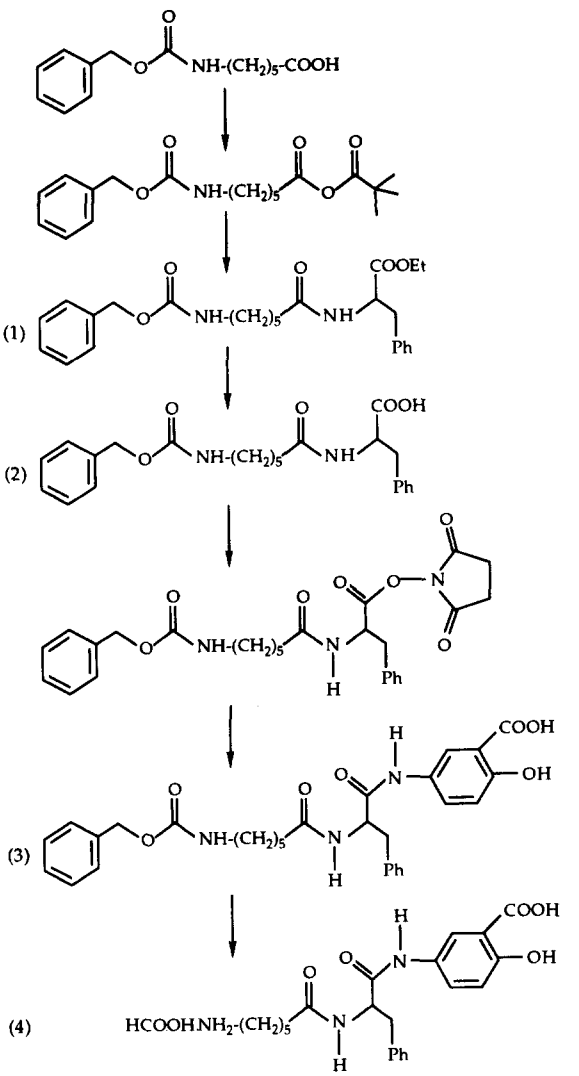

Fig. 1. Synthetic pathway of 6-aminohexanamide-L-phenylalanyl-5ASA. The major intermediate compounds (1–4) are referred to in section 2: (1) 6-(*N*-benzyloxycarbonylamino) hexanamide-L-phenylalanyl ethyl ester, (2) 6-(*N*-benzyloxycarbonylamino) hexanamide-L-phenylalanine, (3) 6-(*N*-benzyloxycarbonylamino) hexanamide-L-phenylalanylamido-5ASA, (4) 6-(*N*-benzyloxycarbonylamino) hexanamide-L-phenylalanyl-5ASA formate.

cess of esterified carboxyl groups to free amine groups, and a 5 mg/ml concentration of peptide. The reaction was maintained at pH 9 for 72 h at 4° C. Uncoupled peptide was removed by dialysis against sodium bicarbonate buffer (pH 10) and then against distilled water. The resulting product was analysed by size-exclusion HPLC using a TSK G3000 PW analytical column (7.5 mm × 300 mm), and eluted with phosphate-buffered saline containing 0.4 M NaCl at a flow rate of 0.75 ml/min. A variable-wavelength UV detector (280 nm) was used for detection. The column was calibrated using pullulan molecular weight standards. The amount of peptide bound was determined by the protein assay of Lowry et al. (1951).

2.3. Preparation of alginate-6-aminohexanamide-L-phenylalanyl-5ASA conjugate

5ASA was derivatised to 6-aminohexanamide-L-phenylalnyl-5ASA and subsequently conjugated to alginate. The derivatisation procedure is shown in Fig. 1.

2.3.1. Synthesis of 6-(N-benzyloxycarbonylamino) hexanamide-L-phenylalanyl ethyl ester (1)

To a cooled solution (−5° C) of 6-(*N*-benzyloxycarbonylamino) hexanoic acid (20 g, 0.075 mol) and *N*-methylmorpholine (16.6 ml, 0.151 mol) in dry tetrahydrofuran (200 ml), trimethylacetyl chloride (9.3 ml, 0.075 mol) was added while stirring. The solution was stirred for 3 h at −5° C and then phenylalanyl ethyl ester hydrochloride (18.2 g, 0.074 mol) and *N*-methylmorpholine (8.7 ml, 0.079 mol) dissolved in acetonitrile (200 ml) were added. The reaction mixture was left to stir overnight at room temperature. The precipitate was removed by filtration and the filtrate was concentrated under reduced pressure. The oily residue was dissolved in 400 ml of ethyl acetate, washed three times with 150 ml of a 10% aqueous solution of citric acid, and finally three times with a saturated solution of sodium bicarbonate. The organic layer was dried over magnesium sulphate. Concentration of the organic solution yielded an oil which crystallised on standing. The solid was recrystallised by dissolving in 150 ml of a 2/1 (v/v) mixture of chloroform and ether, and the addition of excess hexane.

2.3.2. Synthesis of 6-(N-benzyloxycarbonylamino) hexanamido-L-phenylalanine (2)

Sodium hydroxide solution (3%, 100 ml) was carefully added to a solution of derivative (1) in 400 ml methanol (30 g, 0.068 mol) at 0° C. The solution was stirred for 6 h at room temperature, and the methanol was then evaporated under reduced pressure. After adding water (100 ml) to the residue, the solution was acidified with concentrated hydrochloric acid to pH 2 at 0° C. The precipitate was removed by filtration and dissolved in ethyl acetate (600 ml). The organic phase was washed first with a saturated solution of sodium chloride (150 ml) and then with water (150 ml). The ethyl acetate phase was dried over magnesium sulphate. Concentration of the organic solution yielded the product (derivative 2).

2.3.3. Synthesis of 6-(N-benzyloxycarbonylamino) hexanamido-L-phenylalanylamido-5ASA (3)

Derivative (2) (25 g, 0.061 mol) and *N*-hydroxysuccinimide (7 g, 0.061 mol) were dissolved in dry tetrahydrofuran (400 ml) and cooled to −20° C. Solid *N,N'*-dicyclohexylcarbodiimide (12.5 g, 0.0607 mol) was added and the solution was stirred overnight at 0° C. The precipitate was removed by filtration and the solution of the succinimide ester was added to a solution of 5ASA (10.7 g, 0.069 mol) and *N*-methylmorpholine (15.3 ml, 0.14 mol) in dry dimethylformide (250 ml) at 5° C, and the reaction mixture was stirred at room temperature. After 24 h the solvent was evaporated under reduced pressure, the oily residue was dissolved in 800 ml of ethyl acetate and washed with saturated sodium chloride (250 ml) and water (250 ml). The product was dried over magnesium sulphate and the organic phase was removed under reduced pressure. The product was recrystallised by dissolving in methanol.

2.3.4. Synthesis of 6-aminohexanamide-L-phenylalanyl-5ASA formate (4)

Derivative (3) (3 g, 5.5 mmol) was dissolved in a methanol-formic acid mixture (300 ml, 10:1,

v/v), Pd/C (10%, 0.3 g) was added as a catalyst and the solution was placed under a hydrogen atmosphere (35 bar). After 12 h the catalyst was removed by filtration and the filtrate was concentrated under reduced pressure to 50 ml. Diethyl ether was added to the residue until a white precipitate appeared. After filtration the product was dried.

All of the derivatives synthesized were characterised by IR and ^{1}H-NMR spectroscopy.

2.3.5. *Binding of 6-aminohexanamide-L-phenylalanyl-5ASA formate to alginate*

Derivative (4) (80 mg, 0.17 mmol), dissolved in a mixture (2:5) of dimethyl sulphoxide and 0.5 M NaOH (7 ml), was added to a solution of propylene glycol alginate (80 mg in 10 ml of distilled water). The resulting solution was adjusted to pH 10 with NaOH (1 M) while stirring thoroughly at room temperature. The solution was then left reacting at 4° C. After 72 h, the alginate-drug conjugate was precipitated with excess acetone, filtered and redissolved in a small amount of distilled water adjusted to pH 10 with NaOH (1 M). In order to remove unbound drug, the solution was first dialysed against bicarbonate buffer (pH 10.5) for 24 h and then against distilled water for 48 h, prior to lyophilisation. The conjugate was characterised by size-exclusion HPLC using a TSK G4000 PW column (7.5 mm × 300 mm) connected in series with a TSK G2000 PW column (7.5 mm × 300 mm), and eluted with phosphate-buffered saline containing 0.4 M NaCl at a flow rate of 1 ml/min. Detection was performed with a refractive index detector maintained at 25° C. The columns were calibrated using pullulan molecular weight standards. The amount of bound drug was determined spectrophotometrically at 306 nm using derivative (4) as standards.

2.4. *Evaluation of the immunogenicity of the alginate-peptide VP2 conjugate*

An equivalent dose of peptide VP2 (200 μg) in its free and conjugate form was administered intraperitoneally in 0.5 ml PBS/Freund's complete adjuvant mixture (1:1) to groups of five inbred Balb/c mice (8–12 weeks old). Control animals included a naive group and a group administered 0.5 ml PBS/Freund's complete adjuvant mixture (1:1) only. The mice were boosted on day 28, with Freund's complete adjuvant being replaced with Freund's incomplete adjuvant. The animals were killed 1 week after boosting. The blood was obtained by cardiac puncture and then left to clot at 4° C. The serum was collected, centrifuged to remove erythrocytes and stored at −20° C. The anti-peptide VP2 IgG response in the serum sample of each animal was tested using an indirect ELISA, following the method of Francis et al. (1990).

2.5. *In vitro release of 5ASA*

To 0.9 ml of alginate-6-aminohexanamide-L-phenylalanyl-5ASA conjugate, or 6-aminohexanamide-L-phenylalanyl-5ASA in Tris buffer, pH 7.8 (equivalent to ~ 1.2 mM 5ASA), 100 μl of α-chymotrypsin (10 mg/ml, EC. 2. 4. 21. 1., Sigma Chemical Co. Ltd) were added. Following incubation at 37° C, samples (100 μl) were removed at various times up to 24 h, and analysed by reverse-phase HPLC (as described below). As a control, incubations were also carried out in the absence of α-chymotrypsin to assess hydrolytic stability. The controls consisted of 0.9 ml of the appropriate substrate solutions and 100 μl of buffer. Determination of 5ASA was carried out by reverse-phase HPLC using a C18 (Kromasil) analytical column (4.6 × 150 mm) and a variable-wavelength detector (λ = 328 nm). Samples (100 μl) containing 5ASA were made up to 700 μl with Tris buffer, pH 7.8 (0.1 M adjusted with HCl) and perchloric acid (50 μl) was added. After 10 min on ice, the samples were centrifuged (4000 × *g*, 4° C) and aliquots (600 μl) were removed. The solution was adjusted to pH 2 by the addition of NaOH (35 μl, 4 M) before a sample (20 μl) was injected onto the HPLC column. Elution was isocratic using water/trifluoroacetic acid (0.1% v/v), and the flow rate was set at 1 ml/min. A calibration curve (peak area) was constructed using a range of 5ASA concentrations (4.5–225 μg/ml).

3. Results

3.1. Preparation of alginate-peptide VP2 and alginate-6-aminohexanamide-L-phenylalanyl-5ASA conjugates

The alginate-peptide VP2 and alginate-6-aminohexanamide-L-phenylalanyl-5ASA conjugates were prepared according to the general synthetic scheme shown in Fig. 2. Analysis of the alginate-peptide VP2 conjugate by size-exclusion

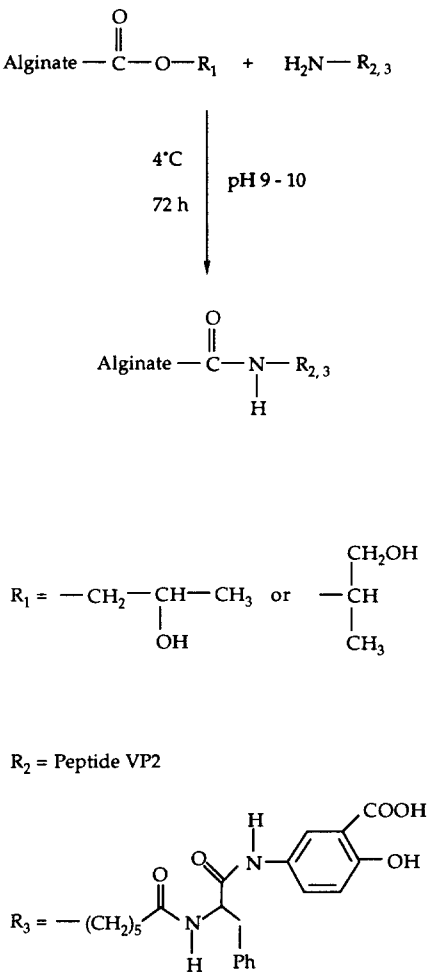

Fig. 2. Synthetic pathway of alginate-peptide VP2/5ASA conjugates. R_1 represent the two types of ester groups; primary 2-hydroxyprop-1-yl and secondary 1-hydroxypropyl-2-yl which exist in equilibrium, R_2 represents peptide VP2 and R_3 denotes 6-aminohexanamide-L-phenylalanyl-5ASA.

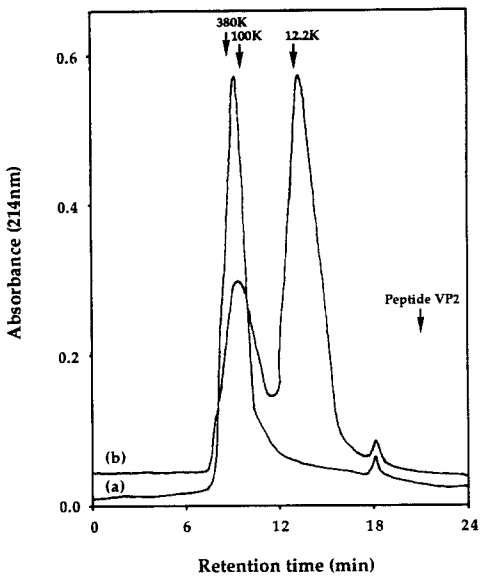

Fig. 3. Size-exclusion HPLC profiles of propylene glycol alginate (a) and the alginate-peptide VP2 conjugate (b). Analysis was performed using a TSK G3000 PW column as described in section 2. Arrows indicate the retention time of pullulan molecular weight standards and free peptide VP2.

HPLC showed that the dialysed preparation was composed of two components with different molecular weights, but did not contain free peptide (Fig. 3). Both components of the alginate-peptide VP2 conjugate preparation were of lower molecular weight (retention times of 9.4 and 13.2 min) than the alginate precursor (9.0 min). Using pullulan as molecular weight standards, the data obtained from size-exclusion HPLC showed that the alginate-peptide VP2 conjugate had an apparent molecular weight within the range of 100 000–11 000. The alginate-peptide VP2 conjugate contained 11.5% (w/w) peptide VP2, as determined by the Lowry protein assay (Lowry et al., 1951). 5ASA was bound to alginate via the peptidyl spacer 6-aminohexanamide-L-phenylalanine. The alginate-5ASA conjugate contained 1.7% (w/w) 5ASA. On analysis by size-exclusion HPLC (Fig. 4), this conjugate appeared to have a lower weight average molecular weight (M_w = 66 000) and a broader molecular weight distribution (M_w/M_n = 3.9) than the starting propylene glycol alginate (M_w = 689 000, M_w/M_n = 2.7).

3.2. Evaluation of the immunogenicity of the alginate-peptide VP2 conjugate

The anti-peptide VP2 serum IgG response following the intraperitoneal administration of free peptide VP2 and alginate-peptide VP2 was analysed using an indirect ELISA (Francis et al., 1990). An anti-peptide VP2 IgG response was not detected in either of the control groups. Administration of both free peptide VP2 and alginate-peptide VP2 in adjuvant elicited a significant anti-peptide VP2 serum IgG response when compared with the control group, administered PBS/adjuvant mixture only (Fig. 5). However, the IgG response to peptide VP2 when administered in the conjugate form was significantly lower than the IgG response to the free peptide, with $P < 0.05$ and < 0.006 within the 4–8 and 16–512 reciprocal antibody dilution ranges, respectively (Fig. 5). Based on the titre values, the alginate-peptide VP2 conjugate was approx. 4-fold less immunogenic than the free peptide; the titre values for alginate-peptide VP2 conjugate and free peptide VP2 were 32 and 120, respectively. By

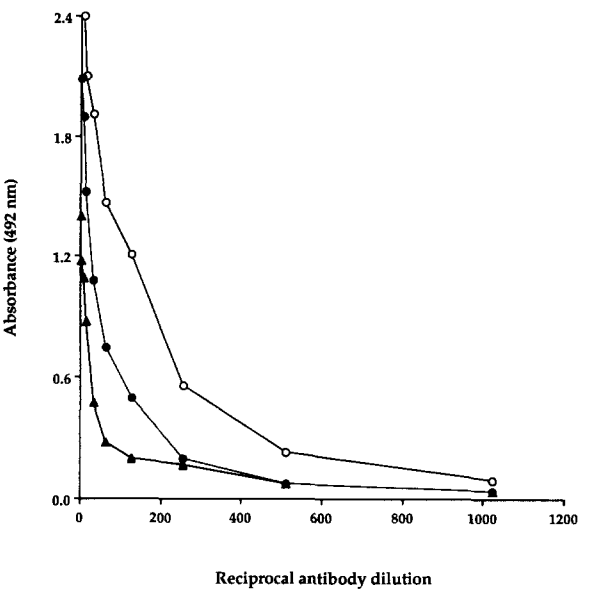

Fig. 5. Immunogenicity of peptide VP2 and alginate-peptide VP2 in mice following parenteral administration. Groups of mice ($n = 5$) were immunised with peptide VP2 (○) and alginate-peptide VP2 conjugate (●) as described in section 2, and the response of these two test groups was compared with that of a naive group (▲). Detection of anti-peptide VP2 IgG antibody in serum was performed using an indirect ELISA.

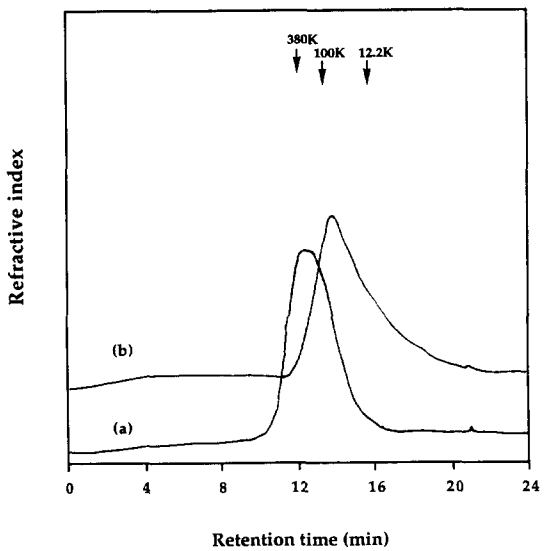

Fig. 4. Size-exclusion HPLC profiles of propylene glycol alginate (a) and alginate-5ASA conjugate (b). Analysis was performed using TSK G4000 PW and TSK G2000 PW columns connected in series as described in section 2. Arrows indicate the retention time of pullulan molecular weight standards.

reference to the respective response curves, the titre value was taken as the reciprocal antibody dilution at 50% of the average maximum absorbance (492 nm).

3.3. In vitro release of 5ASA from alginate-6-aminohexanamide-L-phenylalanyl-5ASA

5ASA was released from alginate-6-aminohexanamide-L-phenylalanyl-5ASA in the presence of α-chymotrypsin. The release of 5ASA from the alginate conjugate was linear for the first 4 h of the incubation period and then began to decline gradually (Fig. 6). The total amount of 5ASA released during the 24 h incubation period was 48%. For comparison, the release of 5ASA from the low molecular weight analogue, 6-aminohexanamide-L-phenylalanyl-5ASA, was also examined in vitro. It was shown that in the presence of α-chymotrypsin the initial rate of drug release from the alginate-6-aminohexanamide-L-phenyl-

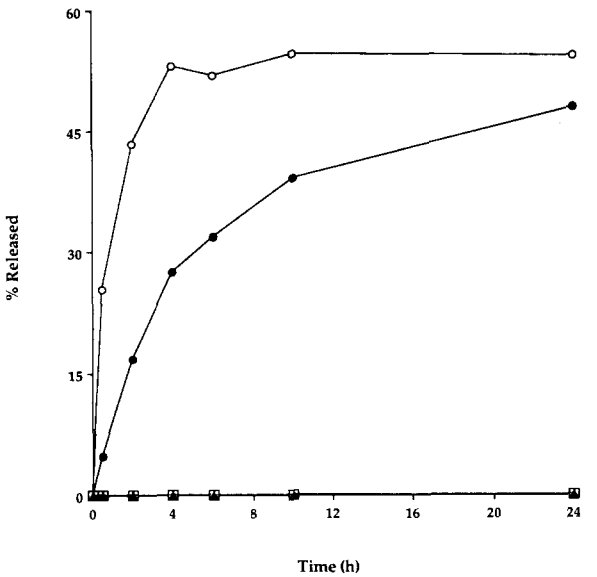

Fig. 6. Percentage of 5ASA released from alginate-5ASA conjugate (●) and the low molecular weight analogue, 6-aminohexanamide-L-phenylalanyl-5ASA (○) when incubated in the presence of α-chymotrypsin. Incubation in the absence of the enzyme is also shown (alginate-5ASA conjugate, ▲; 6-aminohexanamide-L-phenylalanyl-5ASA, □).

alanyl-5ASA conjugate was approx. 6-fold slower than that from 6-aminohexanamide-L-phenylalanyl-5ASA (Fig. 6). No 5ASA release was observed from either the alginate-6-aminohexanamide-L-phenylalanyl-5ASA or the low molecular weight analogue in the absence of α-chymotrypsin.

4. Discussion

Coupling of peptide VP2 and 6-aminohexanamide-L-phenylalanyl-5ASA to alginate was carried out by aminolysis of the ester groups of propylene glycol alginate under alkaline conditions. Under these conditions, the remaining unreacted ester groups are hydrolysed to the free carboxylate groups. Some degradation of propylene glycol alginate is known to occur under alkaline conditions (BeMiller and Kumari, 1972; Gray et al., 1990), and as predicted the alginate-peptide VP2 and the alginate-5ASA conjugates were shown by size-exclusion HPLC to have lower molecular weights than the alginate precursor.

It has been shown that polypeptides exhibit decreased antigenicity following modification by synthetic polymers, such as polyethylene glycol (Tsuji and Hirose, 1985) and HPMA copolymer (Flanagan et al., 1990). Our data show that covalent coupling to the natural polymer alginate also results in a reduction in the immunogenicity of peptide VP2. This reduction in immunogenicity is likely to be caused by steric hinderance of the antigenic determinants from immune surveillance. More specifically, we anticipate that the covalently attached alginate polymer would decrease the rate of interaction between the peptide epitopes and their respective immune receptors. Clearly, while alginates have previously been shown to have immunopotentiating properties (Otterlei et al., 1991; Espevik et al., 1993), our results indicate that alginates do not exhibit adjuvant properties, but rather decrease the antigenicity of a covalently coupled peptide. This property could be useful for the parenteral delivery of therapeutic peptides/proteins which are inherently immunogenic.

It has been suggested that peptidase enzymes of the gastrointestinal tract can be utilised for the controlled release of drugs from specifically designed peptidyl side chains of polymeric drug carriers (Woodley, 1992). Here we show that in the presence of α-chymotrypsin, 5ASA was gradually released from the alginate conjugate, and the initial rate of release was slower than that from the low molecular weight analogue. The slower rate of drug release from the alginate conjugate is probably due to a decrease in the rate of enzyme-substrate complex formation as a result of steric hindrance by the polymer. The decline in the rate of 5ASA release, observed in vitro, with the alginate conjugate and the low molecular weight analogue is likely to be caused by enzyme inactivation due to auto-hydrolysis, and is unlikely to be a limiting factor in vivo. As chymotrypsin, or chymotrypsin-like activity is present in the colon as well as the small intestine (Woodley, 1991), continuous release of 5ASA from alginate-6-aminohexanamide-L-phenylalanyl-5ASA conjugate would be expected to take

place throughout the whole length of the bowel. This type of release should be suitable for the treatment of both small intestine and colon inflammatory lesions. Alginate-5ASA conjugate could also be incorporated within calcium-alginate gel beads (Kim and Lee, 1992) thus further modifying the rate of drug release. The swelling of the beads would be accompanied by gradual release of the drug conjugate and/or the diffusion of the enzyme into the beads with the subsequent hydrolysis of the spacer and release of the drug.

Previously, we have shown that alginates are poorly immunogenic and are well tolerated following systemic administration (Al-Shamkhani et al., 1991; Al-Shamkhani and Duncan, 1995). These properties of alginates together with the data obtained in this study suggest that alginates should be useful as drug delivery systems for therapeutic peptides/proteins and drugs, with the possibility of designing alginate-drug conjugates with specific controlled release properties.

Acknowledgements

This work was supported by Kelco Division of Merck and Co. Inc. (San Diego, USA) and The Wellcome Foundation Ltd (Beckenham, UK).

References

Al-Shamkhani, A. and Duncan, R., Radioiodination of alginate via covalently-bound tyrosine amide allows monitoring of its in vivo. *J. Bioact. Compat. Polym.*, 10 (1995) 4–13.

Al-Shamkhani, A., Bhakoo, M., Tuboku-Metzger, A. and Duncan, R., Evaluation of the biological properties of alginates and gellan and xanthan gums. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 18 (1991) 213–214.

BeMiller, J.J. and Kumari, G.V., beta-Elimination in uronic acids: evidence for an E1cB mechanism. *Carbohydr. Res.*, 25 (1972) 419–428.

Bodmeier, R. and Paeratakul, O., A novel multiple-unit sustained release indomethacin-hydroxypropyl methylcellulose delivery system prepared by ionotrophic gelation of sodium alginate at elevated temperatures. *Carbohydr. Polym.*, 16 (1991) 399–404.

Duncan, R., Drug-polymer conjugates: potential for improved chemotherapy. *Anti-Cancer Drugs*, 3 (1992) 175–210.

Espevik, T., Otterlei, M., Skjak-Braek, G., Ryan, L., Wright, S.D. and Sundan, A., The involvement of CD14 in stimulation of cytokine production by uronic acid polymers. *Eur. J. Immunol.*, 23 (1993) 255–261.

Flanagan, P.A., Duncan, R., Rihova, B., Subr, V. and Kopecek, J., Immunogenicity of protein-*N*-(2-hydroxypropyl)-methacrylamide copolymer conjugates in A/J and B10 mice. *J. Bioact. Compat. Polym.*, 5 (1990) 151–166.

Francis, M.J., Hastings, G.Z., Brown, A.L., Grace, K.G., Rowlands, D.J., Brown, F. and Clarke, B.E., Immunological properties of hepatitis B core antigen fusion proteins. *Proc. Natl. Acad. Sci. USA*, 87 (1990) 2545–2549.

Francis, M.J., Hastings, G.Z., Brown, A.L., Grace, K.G., Rowlands, D.J., Brown, F. and Peat, N., T-cell help for β-cell antibody production to rhinovirus peptides. In Lerner, R.A., Ginsberg, H., Chanock, R.M. and Brown, F. (Eds), *Vaccines 89*, Cold Spring Laboratories, NY, 1989, pp. 437–444.

Gacesa, P., Alginates. *Carbohydr. Polym.*, 8 (1988) 161–181.

Gray, C.J., Griffiths, A.J., Stevenson, D.L. and Kennedy, J.F., Studies on the chemical stability of propylene glycol alginate esters. *Carbohydr. Polym.*, 12 (1990) 419–430.

Kim, C.-K and Lee, E.-J., The controlled release of blue dextran from alginate beads. *Int. J. Pharm.*, 79 (1992) 11–19.

Lin, S.Y. and Ayres, J.W., Calcium alginate beads as core carriers of 5-aminosalicylic acid. *Pharm Res.*, 9 (1992) 1128–1131.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193 (1951) 265–275.

Merrifield, R.B., Solid phase peptide synthesis. 1. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.*, 85 (1963) 2149–2154.

Otterlei, M., Ostgaard, K., Skjak-Braek, G., Smidsrod, O., Soon-Shiong, P. and Espevik, T., Induction of cytokine production from human monocytes stimulated with alginate. *J. Immunother.*, 10 (1991) 286–291.

Tsuji, J.I. and Hirose, K., Studies on antigenicity of the polyethylene glycol(PEG)-modified uricase. *Int. J. Immunopharmacol.*, 7 (1985) 725–z730.

Woodley, J.F., Peptidase activity in the GI tract: distribution between luminal contents and mucosal tissue. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 18 (1991) 337–338.

Woodley, J.F., Peptidase enzymes of the GI tract: barriers to peptide delivery, but potential for controlled release. *Proc. Int. Symp. Controlled Release Bioact. Mater.*, 19 (1992) 2–3.