ELSEVIER

International Journal of Pharmaceutics 195 (2000) 147–158

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Anti-mucus polyclonal antibody production, purification and linkage to the surface of albumin microspheres

A.B. MacAdam [a,*], Z.B. Shafi [a,1], C. Marriott [b], G.P. Martin [b], S.L. James [a]

[a] *School of Pharmacy and Biomolecular Science, University of Brighton, Moulsecoomb, Brighton, UK*
[b] *Department of Pharmacy, King's College London, Franklin-Wilkins Building, 150 Stanford Street, London, UK*

Received 2 August 1999; received in revised form 20 October 1999; accepted 1 November 1999

Abstract

This aim of this study was to develop a microparticulate based oral drug delivery system, which could prolong gut transit time by binding via specific interactions to the gut mucus layer. Porcine gastric mucus was semi-purified and used as an antigen to raise a polyclonal antiserum in rabbits. The immunoglobulin fraction of this serum was isolated, purified and tested for homogeneity and cross reactivity. High cross-reactivity was displayed when the antiserum was challenged against types of mucus other than that used as an antigen, but no significant cross-reactivity occurred when challenged against some other common macromolecules. The antibody fraction of this serum was covalently linked to three types of albumin microspheres (MS) using 1-ethyl-3(3-dimethyl aminopropyl) carbodiimide. The MS employed had either a hydrophobic, a hydrophilic or a carboxymethylated surface, and were prepared and characterised as described earlier (MacAdam, A.B., Shafi, Z.B., Martin, G.P. and James, S.L. 1997. Preparation of hydrophobic and hydrophilic MS and determination of surface carboxylic acid and amino residues. Int. J. Pharm. 151, 47–55). Binding of these MS to both radioiodinated mucin in suspension and to isolated gut segments was measured. Hydrophilic and carboxymethylated MS with surface-associated antibody bound significantly more mucin from a suspension than did uncoated controls. Similarly, anti-mucus antibody-coated hydrophilic and carboxymethylated MS bound more strongly to an isolated gut segment than did uncoated controls or controls coated in an antibody specific for albumin. These results suggest anti-mucus antibody coated albumin MS may be a useful model to act as comparators in studies aimed at developing drug delivery systems with delayed gastrointestinal transit.

Keywords: Polyclonal antibodies; Bovine serum albumin; Albumin microspheres; Ligand binding; Antibody production; Antibody isolation; ^{35}S anti-rabbit IgG; Antibody linkage; ELISA; PAGE; Carboxymethylated MS; Bioadhesion; Mucoadhesive strength; Radio-iodinated mucus; Perfusion of rat jejunum segments; Carboxymethylated mucus

* Corresponding author. Tel.: +44-273-642097; fax: +44-273-679333.
E-mail address: a.macadam@brighton.ac.uk (A.B. MacAdam)
[1] Present address: Wyeth Ayerst Research, Pharmaceutical Sciences, Fareham Road, Gosport, Hants, UK.

0378-5173/00/$ - see front matter
PII: S0378-5173(99)00390-7

1. Introduction

The use of mucoadhesive formulations for the delivery of drugs to the gastrointestinal tract (GIT) is an attractive approach for two major reasons. Firstly, since the transit time in humans for material to pass from mouth to anus is variable, and may be as little as 5 h, drugs which are only slowly absorbed from the gut will display a variable absorption profile, and rarely be totally taken up. A marked increase in gut transit time would overcome this effect, as absorption would be complete before the distal parts of the gut are reached. Secondly, and related to this, dosing intervals could be extended, hopefully leading to better patient compliance, especially in the elderly.

The relatively hostile environment in the gut may lead to significant degradation of drug formulations during transit, particularly if those drugs are peptide or oligosaccharide in nature. A popular approach to solving this problem is to entrap such drugs in microparticulates, such as liposomes, microspheres (MS) or nanoparticles. Association of mucoadhesive technologies with such a delivery device has the benefit of developing a widely applicable formulation, as well as avoiding any necessity to structurally modify the drug itself.

Mucoadhesion may be either specific or non-specific. Non-specific mucoadhesion is usually mediated via the bulk material of the microparticulate displaying adhesive qualities with respect to the mucosa, for instance, by a dehydration process (Smart et al., 1991). However, the use of polyacrylate derivatives to retard gastrointestinal transit has met with limited success (Lehr et al., 1992; Tirosh and Rubinstein, 1998). Specific mucoadhesion is usually accomplished using biological ligands specific for binding sites on the mucosal surface. Such ligands include antibodies and lectins. This latter approach is often technologically more difficult to employ than the former, but allows greater discrimination in targeting.

The mucosa of the GIT is covered in a layer of mucus, reportedly ranging in depth from as much as 500 μm in the stomach, to supposedly only a few μm in the colon (Bickel and Kauffman, 1981; Matsuo et al., 1997). This protects the underlying epithelium from noxious agents, but is just as likely to prevent targeting devices aimed at epithelial cellular ligands from reaching their goal. However, the mucus itself can act as a target, being composed of distinctive *O*-glycosidically linked mucins forming a highly hydrated gel. Thus, both non-specific and specific mucoadhesive formulations may be targeted to the mucus.

Mucus is known to be continually produced from the epithelial surface in the gut, and lost to the lumen. This turnover may be expected to displace microparticles specifically bound to the mucus surface. Furthermore, soluble mucins in the gut lumen may be expected to block ligand binding sites. Both antibody, and lectin binding to their respective ligands are equilibrium processes, and may be expected to detach from, and rebind mucin epitopes in the environment of high mucin concentration at the mucus surface. Lehr et al. (1991) have shown mucus turnover rate in the rat intestine is comparable with mean residence time for mucoadhesive microparticles in an intestinal loop model, but the relatively short loop employed in that study may not have been sufficiently long to allow rebinding of particles.

Linkage of antibodies to MS surfaces may be achieved by adsorption via second antibody attachment, or by a specific covalent linkage. Adsorption of antibodies has been successful using a variety of microparticulates such as nitrocellulose (Steinez and Tamir, 1995), polystyrene (Galisteogonzalez et al., 1994; Tadikonda and Davis, 1994) and magnetic MS (Fe_3O_4) (Shen et al., 1996). The use of second antibody techniques is widespread in immunology, where magnetic immunobeads, for example Dynabeads®, are used extensively for cell separation protocols (Grimsley et al., 1993). Both of these binding techniques rely on secondary bonding, and may lose integrity in the gut where wide variations in pH and tonicity are experienced. We thus favoured the use of a covalent linkage between antibody and MS, although care must be taken not to compromise the antibody affinity (Pimm et al., 1982).

The mucus layer in the GIT will display numerous diverse epitopes, and so a polyclonal antiserum raised against partially purified mucins is

likely to be a more successful targeting ligand, in this case, than a monospecific antibody produced by monoclonal technologies. Indeed, a number of workers have now produced monoclonal antibodies specific to individual products of the various members of the *MUC* gene family in humans (Porchet et al., 1991; Ho et al., 1995). While these would be inappropriate for general targeting to mucus, they may, of course, be useful in targeting if a product of a particular *MUC* gene were found to be associated with a particular disease state.

It was the aim of this work to produce unmodified and surface modified biodegradable albumin MS, and link polyclonal anti-mucus antibodies to these microparticulates, using the water soluble carbodiimide linking agent EDAC (Ezpeleta et al., 1996). The binding capacity of these products was to be tested by measuring the binding of radioiodinated mucins to the MS at saturation, and the retention in an isolated rat ileum segment.

2. Materials

Sodium chloride, sodium sulphate, sodium carbonate, Tween 20, sodium hydroxide (pellets), cocktail T 'scintran', dichloromethane, gelatin powder from pig skin (150 bloom) and urea were supplied by BDH, Poole, Dorset, UK.

Anti-rabbit immunoglobulin (whole molecule) conjugated with alkaline phosphatase, phenyl methyl sulphonyl fluoride (PMSF), immunoglobulin A (human), immunoglobulin G (rabbit), bovine serum albumin (fraction V powder, 96-99% albumin), 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide (EDAC), *p*-nitro phenyl phosphate-disodium hexahydrate (phosphatase substrate), deoxyribonucleic acid (DNA) (single stranded, from calf thymus, lyophilised powder containing approximately 65% DNA), anti-human albumin immunoglobulin G (raised in rabbits), iodoacetamide and 1,3,4,6-tetra-3α, 6α diphenyl glycouril (Iodogen) were supplied by Sigma Chemical Company, Poole, Dorset, UK.

Sepharose CL4B, DEAE cellulose and Sephadex G20 in prepacked columns were supplied by Pharmacia, Milton Keynes, Bucks, UK.

Bacillus calmette-guerin (BCG) vaccine was supplied by Evans Medical, Houghton Regis, Dorset, UK.

^{14}C methylated bovine serum albumin (0.19 MBq, 129.5 MBq/mMol) and sodium ^{125}I iodide, (37 MBq, 3700 MBq/ml in sodium hydroxide) were supplied by Amersham International, Little Chalfont, Bucks, UK.

3. Methods

3.1. Partial purification of porcine gastric mucus (PGM)

Stomachs from fifty pigs within 15 min of slaughter were collected onto ice and subsequently rinsed with normal saline (0.9% w/v). Mucus was collected by gentle scraping of the entire mucosa with a wooden spatula avoiding bile contamination, giving a total volume of approximately 250 ml of mucus. This was transferred to an equal volume of protease inhibiting saline solution (Normal phosphate buffered saline plus 0.1 mM PMSF). This crude mucus was solubilised by homogenising the mixture in a domestic blender for 1 min, and this was then centrifuged at $17\,000 \times g$ for 1 h to separate the food and cellular debris (which formed the pellet) from the solubilised mucus (in the supernatant). The supernatant was filtered twice through glass wool, and 50 ml of the resultant solution was diluted to 180 ml with protease inhibiting saline solution, which was subsequently applied to a Sepharose CL4B exclusion chromatography column (30 cm in length and 9.5 cm in diameter). The sample was eluted with protease inhibiting saline solution at a rate of 300 ml h^{-1} at 4°C, the eluate being monitored spectrophotometrically at 280 nm.

The volume of the excluded fraction (peak A), which contains the glycoprotein, was reduced to approximately 50 ml, using an ultrafiltration cell equipped with 100 000 Dalton cut-off ultrafiltration plates (Millipore minitan ultrafiltration system, Millipore Corporation, Bedford, MA, USA). The resultant solution was transferred to a dialysis tubing sack with a 12 000 Dalton cut-off and dialysed exhaustively over a 36 h period

against distilled water (10 l) at 4°C, changing the dialysis solution every 12 h. The dialysed mucus was frozen, freeze dried for 24 h and stored at −20°C until required. This procedure (Lethem et al., 1990) fields a partially pure mucin preparation, with some contamination by high molecular weight proteins and nucleic acids.

3.2. Antibody production

Three, male, New Zealand white rabbits were used for antibody production. Partially purified PGM (10 mg/ml) was dissolved in distilled water and 1 ml was emulsified with 1 ml non-ulcerative Freunds adjuvant (Morris), and 0.2 ml BCG vaccine for subcutaneous use. Each rabbit was injected with two 0.5 ml intramuscular injections into each back leg and two 0.5 ml subcutaneous injections into the neck.

After 14 days, the rabbits were bled from the marginal ear vein. The rabbits were then boosted by injection in the same sites with the same volume of the above materials except that the BCG vaccine was omitted and again bled for antibodies after 14 days. The rabbits were then boosted and bled as required, and the serum anti-mucin antibody titre was measured by an enzyme linked immunosorbent assay (ELISA).

3.3. Isolation of antibody from serum

Serum (50 ml) was warmed to 25°C and 9 g sodium sulphate was added and dissolved with stirring to precipitate the globulin fraction. After centrifugation at $3000 \times g$ for 30 min at 25°C, the supernatant was discarded and the pellet was redissolved in distilled water to give a 25 ml volume. The process was repeated and the pellet was redissolved in 15 ml of distilled water. The 15 ml volume was transferred to a dialysis tubing sack with a 12 000 Dalton cut-off and dialysed exhaustively over a 36 h period against 10 l phosphate buffer (0.07M, pH 6.3) at 25°C, changing the dialysis solution every 12 h.

The 15 ml dialysed sample was applied to a 30 cm DEAE cellulose column, pre-equilibrated with phosphate buffer (0.07M, pH 6.3) at room temperature. Elution was carried out at a rate of 300 ml/h, using phosphate buffer as the mobile phase. Fractions (10 ml) were collected and monitored spectrophotometrically at 280 nm. The first peak to be eluted in the mobile phase was collected and dialysed as before. The dialysed sample was frozen, freeze dried and stored at −20°C until required. The product was then tested for purity using a standard polyacrylamide gel electrophoresis (PAGE) technique, running the gel at 200 V for 6 h and then staining with Coomassie brilliant blue R (0.025% w/v) and destaining in ethanol/acetic acid.

3.4. Enzyme linked immunosorbent assay

The mucus antigen (10 μg/ml) was dissolved in sodium carbonate solution (0.05 M; pH 9.6) and 200 μl was pipetted into each well of a microELISA plate. The plate was incubated at 4°C overnight, after which the solution remaining in the wells was aspirated with a pasteur pipette. The wells were washed twice with 200 μl of phosphate buffered saline (PBS) containing BSA (1%w/v) and Tween 20 (0.5% v/v) leaving the washing solution in contact with the well for 3 min intervals at room temperature. PBS plus BSA (1% w/v; 200 μl) was added to the wells and incubated for 1 h at room temperature, to block vacant adsorption sites, after which the wells were washed as before. The serum isolated previously was diluted with PBS plus BSA (1% w/v) and Tween 20 (0.5% v/v) in a ratio of 1 to 50 and 200 μl was added to the wells.

This serum was further diluted with the same diluent to produce a final dilution of 1:100, and 200 μl of this dilution was added to further wells. Control wells were set up by adding the diluent solution (200 μl) to 18 wells which had not been exposed to antigen. The plate was incubated for 2 h at room temperature. The wells were washed three times employing the method described previously. A dilution (1:500) of alkaline phosphatase conjugated anti-rabbit IgG in PBS with Tween 20 (0.05%w/v) was prepared, 200 μl added to each well and the plate was incubated for a further 2 h at room temperature. The wells were washed three times employing the method described previously. Two-hundred microlitres of a freshly made solu-

tion of *p*-nitrophenylphosphate (1 mg/ml) in glycine buffer was added to each well and the plate was incubated for 30 min at room temperature. Sodium hydroxide (50 μl, 3M) was added to each well and the absorbance in each well was measured using a micro-ELISA reader at 405 nm.

This ELISA was employed to measure the antibody titre in the serum obtained following the bleeds described above. It was also used to test different cross reactivity with different mucins viz bovine cervical mucus (BCM), murine jejunal mucus (MJM) and human sputum (HS), all purified as described above, as well as to test that the antibodies produced did not cross-react with other components found in serum, such as DNA, BSA, and IgA.

3.5. Linking radioactive antibody to the MS surface

Hydrophobic, hydrophilic and carboxymethylated BSA MS were manufactured using a high shear technique described previously (Townes et al., 1989; Shafi et al., 1995; MacAdam et al., 1997). Briefly, hydrophobic MS have a predominantly non-polar surface and thus there are few reactive surface residues to which ligands can be bound. Hydrophilic MS (Longo et al., 1982) are produced after dispersion in a relatively polar disperse phase, although the polarity may be introduced as a result of incorporation of polymethylmethacrylate into the MS (MacAdam et al., 1997). Carboxymethylated MS are treated with iodoacetic acid to yield increased numbers of surface carboxylic acid residues (MacAdam et al., 1997).

Five batches of 5 mg of each type of MS were separately suspended in 1.8 ml sodium chloride solution (0.1 M, pH 7.0) and dispersed by sonication for 10 min. Five lots of EDAC (0, 0.5, 1, 2, 4 and 8 mg) were dissolved in 0.1 ml sodium chloride solution (0.1 M, pH 7.0), added to the MS suspensions and mixed at 4°C. ^{35}S anti-rabbit IgG (5.032×10^{-3} MBq, 0.1 ml) was added to the MS suspensions and mixed at 4°C for 2 h. The MS were recovered from the mixtures by centrifugation at $1000 \times g$ for 3 min, after which a washing procedure was carried out employing 5 ml urea (8M) twice and 5 ml saturated sodium chloride solution once to remove antibody bound to the MS by secondary bonds. The MS were re-suspended in 5 ml cocktail-T scintillation fluid and counted in a liquid scintillation counter for 1 min.

3.6. Carboxymethylation of mucus

PGM (100 mg) purified as discussed above was dissolved in 100 ml iodoacetamide (250 mM) and stirred for 24 h in the dark under an atmosphere of nitrogen. The carboxymethylated mucus was dialysed exhaustively over a 36 h period at 4°C against 10 l of distilled water which was changed approximately every 12 h. The dialysed mucus was frozen (-20 °C) until required.

3.7. Radio-iodination of mucus and carboxymethylated mucus

Dichloromethane (20 μl) containing 20 mg iodogen was pipetted into the bottom of a polypropylene tube (14 ml volume) and allowed to stand at 20°C until the dichloromethane had evaporated leaving a fine coating of the iodogen in the tube. Mucus (0.5 mg) or carboxymethylated mucus (0.5 mg) was dissolved in 50 μl borate buffer (0.2M, pH 8.2) and added to the tube containing the iodogen. Sodium ^{125}I iodide (18.5 MBq) was added to the tube, which was capped and gently shaken for 2 min. Phosphate buffer (500 μl, 0.05M, pH 7.4) was added to the tube and the contents were eluted from a Sephadex G-20 column (1×10 cm) under gravity with phosphate buffer (0.05M, pH 7.4) containing gelatin (1 g/l). Fractions (1.5 ml) were collected and counted for to determine the amount of associated radioactivity.

3.8. Binding assay of mucus to antibody-linked MS

Hydrophobic, hydrophilic and carboxymethylated (both coated with anti-PGM antibodies and uncoated) MS (5 mg) were suspended in 1 ml PBS. Samples (100 μl) of radio-iodinated mucus were added to the MS suspension and stirred for 2 h at 20°C. Following centrifugation at $1000 \times g$

for 3 min, the MS were resuspended in 1 ml distilled water and centrifuged under the same conditions to obtain a pellet. Following two further repetitions of this washing procedure, the MS were transferred to a gamma counter and counted for 60 s.

3.9. Adhesion of radiolabelled antibody coated MS to rat jejunum

Carboxymethylated MS were produced as described previously (Shafi et al., 1995; MacAdam et al., 1997) except that the BSA employed to produce the MS had ^{14}C methylated BSA (0.037 MBq) added. The radiolabelled carboxymethylated MS obtained were linked either to anti-PGM antibodies or to anti-albumin antibodies (as a negative control) using the following procedure. The radiolabelled carboxymethylated MS (50 mg) were suspended in 19 ml sodium chloride solution (0.1 M, pH 7.0) and dispersed by sonication for 10 min. EDAC (40 mg) dissolved in 0.5 ml sodium chloride solution was added to the MS suspension and mixed at 4°C. Anti-PGM antibody or anti-BSA antibody (1 mg) was dissolved in 0.5 ml sodium chloride solution and then added to the MS suspension and mixed at 4°C for 2 h. The MS were recovered from the mixture by centrifugation at $1000 \times g$ for 3 min, after which they were resuspended in 50 ml urea (8 M) and centrifuged under the same conditions to obtain a pellet. Following a further two repetitions of this washing procedure, employing 50 ml urea (8 M) for one wash and 50 ml saturated sodium chloride for the other wash, the resultant pellet was resuspended in 20 ml distilled water and dialysed exhaustively at 4°C over a 36 h period against 10 l distilled water, changing the solution every 12 h. The dialysed antibody-linked MS were frozen, freeze dried and stored at -20 °C until required. Male Wistar rats (250–300 g) were starved overnight and sacrificed by cervical dislocation and the abdominal cavity exposed. The entire length of the small intestine (pylorus to caecum) was removed and the section of jejunum (3 cm) immediately proximal to the duodenum was mounted in the apparatus shown in Fig. 1 by securing it tightly to two glass nipples employing cotton sutures. Aerated Ringers solution at 37°C was continually pumped by a peristaltic pump at a rate of 0.5 ml/min through the lumen of the rat jejunum for 5 min before the start of each experiment. The inner compartment was also filled with Ringers so the outside of the jejunum was surrounded by fluid. Uncoated radiolabelled carboxymethylated MS or the same MS coated either with anti-PGM antibodies or with anti-albumin antibodies (5 mg) were suspended in 0.5 ml Ringers by stirring with a magnetic stirrer. The perfusion of Ringers through the lumen of the jejunal section was stopped and a 1 ml plastic syringe containing the MS suspension was connected to the perfusion line behind the pump but before the tissue. The MS suspension was then

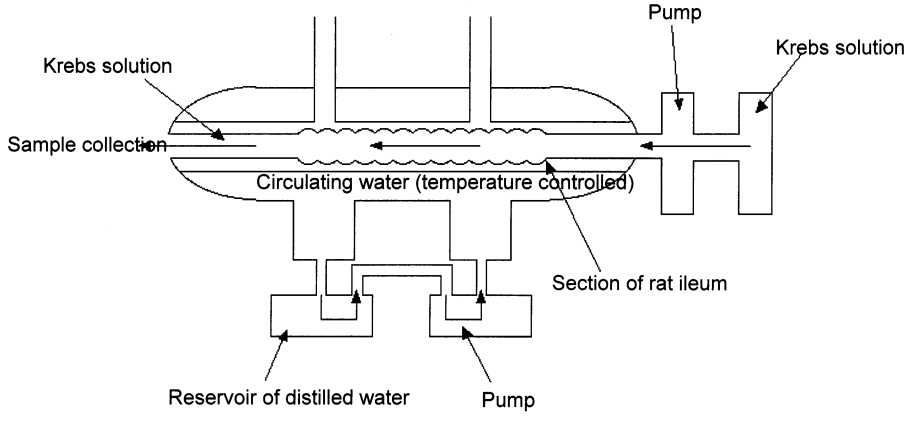

Fig. 1. Line Diagram of apparatus employed in the perfusion study

Table 1
IgG content fo serum from rabbits 1, 2, and 3 during antibody production as determined by an IgG specific ELISA[a]

Rabbit number	Bleed number	Mean absorbance at 405 nm ± SD
1	1	0.567 ± 0.100
1	2	1.088 ± 0.183
1	3	>3.0
2	1	1.165 ± 0.186
2	2	1.602 ± 0.292
2	3	>3.0
3*	1	0.785 ± 0.104
3*	2	0.912 ± 0.398
Blank	1	0.131 ± 0.033
Blank	2	0.091 ± 0.026
Blank	3	0.063 ± 0.016

[a] Results at 1/50 are shown and are expressed as raw absorbance values since a full titre curve was not carried out for each bleed.

* Excessive venoconstriction in rabbit 3 prevented successful collection of bleed 3.

introduced into the lumen of the jejunum and allowed to interact for 10 min. Perfusion of the lumen of the segment was recommenced (flow rate 0.5 ml/min) and continued for 5 min during which time the perfusate was collected. The rate of flow was increased to 1 ml/min for 5 min and the perfusate collected separately. The tissue was removed from the apparatus and the contents of the lumen were removed, together with adherent mucus. The samples collected were frozen at −20°C and freeze dried. The residues were re-dissolved in 5 ml scintillation fluid and the amount of ^{14}C in each sample determined by liquid scintillation counting.

4. Results

4.1. Mucus purification

The eluant from the excluded volume of the Sepharose CL4B was analysed for protein and hexose, and was shown to have a profile characteristic of mucin glycoproteins as reported previously (Hughes, 1988; Lethem et al., 1990).

4.2. Polyacrylamide gel electrophoresis

The results for PAGE are described as follows: Track 1, molecular weight ladder (29 000, 45 000, 66 000, 97 000, 116 000 and 205 000 Daltons). Tracks 2 and 3 were 5 and 10 μl loadings, respectively, of Ig obtained from the final bleed of one rabbit. Likewise tracks 4 and 5 were 5 and 10 μl loadings of Ig obtained from the final bleed of a second rabbit. These four tracks had the same two bands of protein with molecular weights 22 000 and 53 000 Daltons. Tracks 6 and 7 were 5 and 10 μl loadings of IgG obtained from a commercial supplier. These two tracks also had same two bands of protein with molecular weights 22 000 and 53 000 Daltons. No other bands were found on tracks 4–7. Tracks 8 and 9 were 5 and 10 ml loadings, respectively, of BSA and these tracks also gave two bands corresponding to molecular weights 66 000 and 132 000 Daltons.

4.3. Enzyme linked immunosorbent assay

The results of ELISA showed all three rabbits had an increasing amount of IgG in their serum between the bleed following the initial inoculation and the booster inoculation (Table 1). Since the second antibody is specific for IgG isotype, this figure represents the IgG fraction obtained. Rabbits 1 and 2 were found to have the highest levels of IgG. For this reason they were further boosted and bled and these bleeds gave IgG levels which yielded further elevated titres.

When the same technique was employed to assess the cross-reactivity of the anti-PGM IgG antibodies with possible common PGM components, consisting of DNA, BSA and IgA, the absorbance values obtained for the three potentially crossreacting macromolecules were less than 3% of that obtained for PGM (Table 2). This indicated that there was negligible cross-reactivity of anti-PGM antibodies with DNA, BSA and IgA whereas there was marked binding to the PGM.

When different mucus types at various concentrations (from 0.001 to 10 μg/l) were challenged with serum from rabbit 2 at 1/100 dilution, significant cross-reactivity was displayed for all mucus types at all concentrations (Table 3). Indeed, the

Table 2
ELISA results of serum samples (from rabbit 2: 1/100 dilution) challenged with possible different antigens found in PGM[a]

Antigen	Mean absorbance at 405 nm ± SD ($n = 18$)
PGM	1.807 ± 0.056
DNA	0.056 ± 0.006
IgA	0.015 ± 0.004
BSA	0.000 ± 0.000

[a] Results expressed as raw absorbances for comparative purposes.

antibody was more avid for MJM than PGM at all but the lowest concentration. At 10 μg antigen concentration, all mucins cross-reacted at a level above the saturation point of the assay.

This result suggests that this antibody has a broad enough specificity to be useful in targeting to all mucin types.

4.4. Linking radioactive antibody to MS

Fig. 2 a–c shows considerable variation between replicates, in the amount of antibody bound to the MS with increasing EDAC concentration. Generally antibody was found to bind in greater quantities to the hydrophilic and carboxymethylated albumin MS with saturation indicated at EDAC concentrations of approximately 1 mg/ml and 2 mg/ml, respectively. Furthermore, at zero EDAC concentration, residual and unwashable binding to all types of MS was seen. This suggests that although a majority of the binding to the MS may be covalent, a non-specific adsorption also occurs.

4.5. Radioiodination of mucus

The amount of radioactivity associated with each fraction of eluate was monitored. The radioactivity was highest in the excluded volume of the column for both types of mucus. The activity associated with the mucus fraction after the carboxymethylation process was higher than when iodination of non-carboxymethylated mucus was attempted. Specific activities were calculated and found to be 3.5×10^8 and 4.9×10^8 cpm/mg for the most active fraction obtained from the non-carboxymethylated and carboxymethylated mucus, respectively.

4.6. Binding assay of mucus to antibody-linked MS

The amount of carboxymethylated and non-carboxymethylated mucus bound to the various MS preparations when incubated with 100 μl samples of radio-iodinated mucin are shown in Table 4.

These results indicate that after accounting for non-specific binding to the reaction tube, antibody coated and uncoated hydrophobic MS bound the same quantity of non-carboxymethylated mucus, whereas there was slightly less carboxymethylated mucus bound to the uncoated hydrophobic MS compared with antibody-coated hydrophobic MS.

Hydrophilic MS coated with anti-PGM antibodies bound over forty times more carboxymethylated mucus and more than five times more non-carboxymethylated mucus compared

Table 3
ELISA results for serum samples (from Rabbit 2: 1/100 dilution) challenged with different mucus types[a]

Antigen	Mean absorbance at 405 nm ± SD ($n = 18$)				
	0.001 (μg)	0.01 (μg)	0.1 (μg)	1.0 (μg)	10 (μg)
PGM	0.580 ± 0.05	0.590 ± 0.02	1.030 ± 0.05	>3	>3
MJM	0.381 ± 0.04	0.802 ± 0.02	>3	>3	>3
BCM	0.434 ± 0.03	0.510 ± 0.02	0.800 ± 0.05	1.810 ± 0.12	>3
HS	0.422 ± 0.03	0.504 ± 0.02	1.060 ± 0.16	2.900 ± 0.16	>3

[a] Mucus types where: PGM is pig gastric mucus; MJM is murine jejunal mucus; BCM is bovine cervical mucus; HS is human spetum.

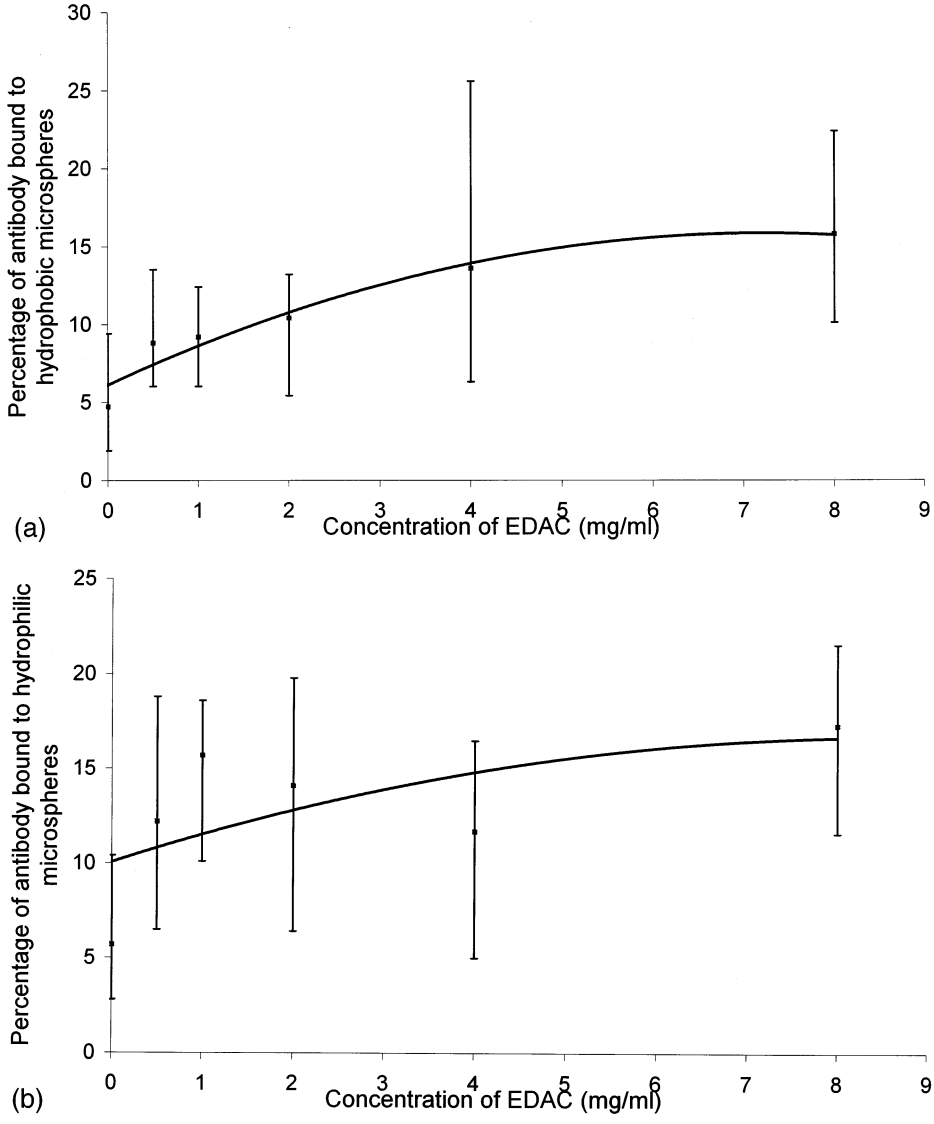

Fig. 2. (a): Radioactive antibody bound to hydrophobic microspheres as a function of EDAC concentration (mean $\pm$SD, $n=3$). (b): Radioactive antibody bound to hydrophilic microspheres as a function of EDAC concentration (mean $\pm$SD, $n=3$). (c): Radioactive antibody bound to carboxymethylated microspheres as a function of EDAC concentration (mean $\pm$SD, $n=3$)

with the respective quantity of mucus bound by uncoated hydrophilic MS.

Carboxymethylated MS coated with anti-PGM antibodies bound about five times more carboxymethylated mucus and about twice the amount of non-carboxymethylated mucus compared with the respective quantity of mucus bound by the uncoated carboxymethylated MS.

4.7. Adhesion of radiolabelled antibody coated MS to rat jejunum

The results in Table 5 show that in terms of the counts measured in the sample as a percentage of the total counts recovered, approximately 75% of uncoated carboxymethylated MS and anti-albumin antibody coated (i.e. negative control) carboxymethylated MS were removed following a

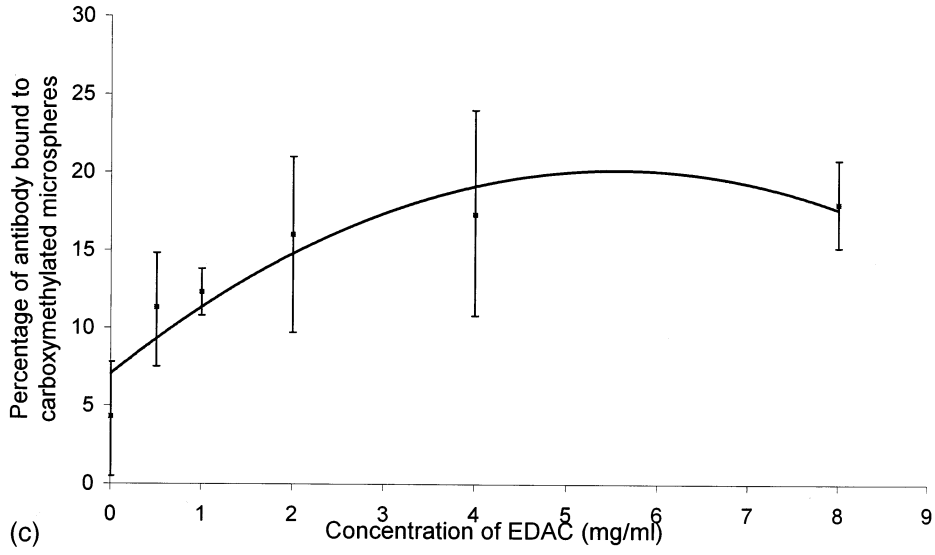

Fig. 2. (*Continued*)

flow rate of 0.5 ml/min, whereas only 33% of anti-PGM antibody coated carboxymethylated MS were removed at this flow rate. At the higher flow rate of 1 ml/min, less than 7% of all three types MS were detached from the jejunal wall. When the contents of the piece of tissue were counted for the radioactive tracer, only $18.9 \pm 18.5\%$ of the uncoated carboxymethylated MS and $22.9 \pm 11.8\%$ of the anti-BSA antibody coated carboxymethylated MS had remained attached to the jejunal wall whereas $62.6 \pm 6.7\%$ of the anti-PGM antibody linked carboxymethylated MS were retained by the jejunal tissue.

4.8. Discussion

Antibodies were successfully produced in a polyclonal antiserum with a specificity towards epitopes on partially purified porcine mucus. Such an ill-defined antigen would be expected to yield a widely cross-reacting antiserum, binding not only mucin epitopes, but determinants on many other constituents of the mucus preparation. The antiserum was shown to have very low cross reactivity with IgA and DNA, and no detectable cross reactivity with serum albumin. However, a significant cross reactivity with mucus from other sources was seen. This suggests the presence of antibodies in the serum specific for ubiquitous mucin epitopes, possibly in the naked peptide region of the glycoprotein, or common hexose residues such as *N*-acetylglucoasmine (GlucNac) and *N*-acetylgalactosamine (GalNac). This is unsurprising considering the considerable homology

Table 4
Amount of radio-iodinated non-carboxymethylated and carboxymethylated mucus bound to samples (5 mg) of anti-PGM antibody coated MS and to uncoated MS

	Amount of radio-iodinated mucus bound ((g) (corrected for non-specific binding)	
MS ($n = 3$)	Non-carboxylated	Carboxylated
Hydrophobic	0.25	0.11
Anti-PGM antibody coated hydrophobic	0.25	0.19
Hydrophilic	0.16	0.03
Anti-PGM antibody coated hydrophilic	0.89	1.28
Carboxymethylated	0.37	0.18
Anti-PGM antibody coated carboxymethylated	0.83	0.88
Total count (10μl)	3.36	3.35

Table 5
Percentage of the total number of three types of radioactive carboxymethylated MS collected following perfusion through a section of murine jejunum by PBS at different flow rates[a]

	Percentage of the total number of radioactive MS collected in eluate (±SD)		
	Flow rates (ml/min)		
	0.5	1.0	Remaining in jejunal tissue
Uncoated carboxymethylated MS	74.3 ± 21.1	6.8 ± 4.4	18.9 ± 18.5
Anti-PGM antibody coated carboxymethylated MS	72.2 ± 10.3	4.9 ± 3.1	22.9 ± 11.8
Anti-PGM antibody coated carboxymethylated MS	33.3 ± 6.8*	4.1 ± 3.2	62.6 ± 6.7*

[a] 0.5 followed by 1 ml/min and remaining in the tissue following inter-luminal incubation of the MS (5 mg) with the tissue for 10 min ($n = 3$).

* Denotes significant differences between Anti-PGM antibody coated and both Anti-BSA antibody coated and uncoated MS ($P < 0.02$) employing a two tailed Mann–Whitney U-test.

between the various members of the *MUC* gene family in humans, and furthermore, significant homology (74-87%) between human *MUC* 1 and rodent *Muc*-1, (Spicer et al., 1991.) suggesting some *MUC* sequences to be common to other species. We therefore suggest that this antiserum is suitable for targeting to intestinal mucus in a variety of model species.

A water soluble carbodiimide based coupling reaction was used to link the antibodies to the MS. A maximum of 20% of the antibody added was bound irreversibly to the hydrophilic MS, and a little less to the carboxymethylated spheres. The optimum EDAC concentration for binding was different in these two cases, being 1 and 2 mg/ml, respectively. This is unsurprising since both these surfaces may be expected to possess both free amino and free carboxylic acid residues available for conjugation to the antibodies. The considerable variability in results is likely to be as a result of polymerisation of the antibodies prior to the attachment to the MS, since these proteins display both amino and carboxylic acid residues. This may result in random chain lengths of immunoglobulin polymers, either left in suspension, or indeed, attached to the MS surface. This phenomenon may also explain the relatively low maximum binding of the antibody. An alternative explanation is that the available binding sites on the MS surface are simply saturated. The post-reaction integrity of the antibodies is, as evidenced by their affinity, demonstrated in Table 4. Substantial increases in the amount of mucus bound to both carboxymethylated and hydrophilic MS was seen when surface antibodies were present. The lack of an increase in mucus binding to antibody coated hydrophobic MS may indicate a loss of antibody affinity, possibly because of the proximity of the hydrophobic MS surface. Alternatively, the antibody may be associated with the hydrophobic surface in a different orientation to the way it was bound to the other two types of MS, such that the variable region of the antibody is less or more available to complex with the mucus.

Table 5 clearly demonstrates a significantly ($P < 0.02$) increased retention of anti-PGM coated MS in a rat gut segment when compared with control MS coated in an unrelated antibody, or no antibody. Whilst the time of perfusion at a rate of 0.5 ml/min is relatively short at 5 min, the results demonstrate that a further 5 min perfusion at double that flow rate failed to remove any further significant numbers of spheres, suggesting those remaining to be firmly attached to the gut mucosa. Whether these MS are embedded in the mucus layer, or simply attached to the superficial surface is not clear, but the total binding of the MS must be specific, since anti-BSA antibody coated microparticles do not mimic this effect. It must also be strong enough to resist simple shear stress induced removal.

The potential chemical and physical liability of antibodies within the gastrointestinal tract is an issue not addressed in these studies. In addition the issue of mucus turnover in vivo and the effect on retention of particles within the gastrointestinal tract remains to be investigated. Nevertheless these results suggest that such a system provides a useful model for the study of specifically targeted MS aimed at prolonging gut transit time, and may form the basis of a drug delivery system which can improve the bioavailability of poorly absorbed drugs.

References

Bickel, M., Kauffman, G.L., 1981. Gastric gel mucus thickness: 16,16-dimethyl prostaglandin E_2 and carbenoxolone. Gastroenterology 80, 770–775.

Ezpeleta, I., Irache, J.M., Stainmesse, S., Chabenat, C., Gueguen, J., Orecchioni, A.M., 1996. Preparation of lectin-vicilin nanoparticle conjugates using the carbodiimide coupling technique. Int. J. Pharm. 142, 227–233.

Galisteogonzalez, F., Martinrodriguez, A., Hidalgoalvarez, R., 1994. Adsorption of monoclonal IgG on polystyrene microspheres. Colloid Polym. Sci. 272 (3), 352–358.

Grimsley, P.G., Amos, T.A., Gordon, M.Y., Greaves, M.F., 1993. Rapid positive selection of CD34 + cells using magnetic microspheres coated with monoclonal antibody QBEND/10 linked via a cleavable disulphide bond. Leukemia 7 (6), 898–908.

Ho, S.B., Robertson, A.M., Shekels, L.L., Lyftogt, C.T., Niehans, G.A., Toribara, N.W., 1995. Expression cloning of gastric mucin complementary DNA and localization of mucin gene expression. Gastroenterology 109, 735–747.

Hughes, D.R.L., 1988. PhD thesis, Brighton Polytechnic.

Lehr, C.M., Poelma, F.G.J., Junginger, H.E., Tukker, J.J., 1991. An estimation of the turnover time of intestinal mucus. Int. J. Pharm. 70 (3), 235–240.

Lehr, C.M., Bouwstra, J.A., Kok, W., Deboer, A.G., Tukker, J.J., Verhoef, J.C., Breimer, D.D., Junginger, H.E., 1992. Effects of the mucoadhesive polymer polycarbophil on the intestinal absorption of a peptide drug in the rat. J. Pharm. Pharmacol. 44 (5), 402–407.

Lethem, M.I., James, S.L., Marriott, C., 1990. The role of mucus glycoprotein in the rheologic properties of cystic fibrosis sputum. Am. Rev. Resp. Dis. 142, 1053–1058.

Longo, W.E., Iwata, H., Lindheimer, T.A., Goldberg, E.P., 1982. Preparation of hydrophilic microspheres using polymeric dispersing agents. J. Pharm. Sci. 71, 1323–1328.

MacAdam, A.B., Shafi, Z.B., Martin, G.P., James, S.L., 1997. Preparation of hydrophobic and hydrophilic MS and determination of surface carboxylic acid and amino residues. Int. J. Pharm. 151, 47–55.

Matsuo, K., Ota, H., Atamatsu, T., Sugiyama, A., Katsuyama, T., 1997. Histochemistry of the surface mucous layer of the human colon. Gut 40 (6), 782–789.

Pimm, M.V., Jones, J.A., Price, M.R., Middle, J.G., Embleton, M.J., Baldwin, R.W., 1982. Tumour localisation of monoclonal antibodies against a rat mammary carcinoma and suppression of tumour growth with adriamycin-antibody conjugate. Cancer Immunol. Immunotherap. 12, 125–134.

Porchet, N., Nguyen, V.C., Dufosse, J., Audie, J.P., Guyonnet-Duperat, V., Gross, M.S., Dennis, C., Degand, P., Bernheim, A., Aubert, J.P., 1991. Molecular cloning and chromosomal localization of a novel human tracheobronchial mucin cDNA containing tandemly repeated sequences of 48 base pairs. Biochem. Biophys. Res. Comm. 175, 414–422.

Shafi, Z.B., Martin, G.P., Olliff, C.J., James, S.L., 1995. Surface modification of albumin microspheres. J. Drug Target. 3, 53–56.

Shen, R.S., Wang, R.Z., Xing, R.Y., Li, Y.Q., Zhou, F.Q., Jiang, S.H., Lin, Z.H., Xu, B.L., 1996. Magnetic microparticle antibodies and their application to RIAs. J. Radioanal. Nuc. Chem. 206 (2), 205–218.

Spicer, A.P., Parry, G., Patton, S., Gendler, S.J., 1991. Molecular-cloning and analysis of the mouse homolog of the tumor–associated mucin, MUC1, reveals conservation of potential *o*–glycosylation sites, transmembrane and cytoplasmic domains and a loss of minisatellite-like polymorphism. J. Biol. Chem. 266 (23), 15099–15109.

Steinez, M., Tamir, S., 1995. An improved method to create nitrocellulose particles suitable for the immobilization of antigen and antibody. J. Immunol. Methods 197 (1), 171–177.

Smart, J.D., Carpenter, B., Mortazavi, S.A., 1991. Is mucus dehydration an important factor in mucoadhesion. Proc. Int. Sympt. Release Bioact. Mater. 18, 629–630.

Tadikonda, K.R., Davis, R.H., 1994. Cell separations using targeted monoclonal antibodies against overproduced surface proteins. Appl. Biochem. Biotech. 45–6, 233–244.

Tirosh, B., Rubinstein, A., 1998. Migration of adhesive and nonadhesive particles in the rat intestine under altered mucus secretion conditions. J. Pharm. Sci. 87 (4), 453–456.

Townes, A.B., Martin, G.P., James, S.L., Marriott, C., 1989. Microparticulate surface groups. J. Pharm. Pharmacol. (Suppl.) 41, 140P.