Poster Session 2 – Biopharmaceutics

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Concentration-dependent inhibition of pepsin activity by alginates

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Gastro-oesophageal reflux is the entry of gastric contents into the oesophagus without associated belching or vomiting. This is a physiological event but is problematic in a number of the population where reflux leads to symptoms of heartburn, regurgitation and oesophagitis. An estimated 15–44% of adults experience these symptoms on a monthly basis. Persistent reflux damages the delicate squamous oesophageal mucosa resulting in erosive oesophagitis or gastroesophageal reflux disease (GORD). Treatment can be by antacids, H_2 receptor antagonists and proton pump inhibitors. All of these pharmacological interventions act to reduce the amount of acid in the stomach thus increasing the pH so that it is less damaging. However, acid is not the only damaging agent in the gastric refluxate. Other aggressive components include pepsin, bile acids and pancreatic enzymes. Indeed it has been demonstrated that pepsin is far more damaging to the delicate squamous mucosa than acid alone.

Alginates, the active ingredient of the anti-reflux medicine Gaviscon, have previously been shown to have biologically active properties. These include stimulation of fluid phase endocytosis, promotion of cell migration and bioadhesion all of which have potential for use as treatment against gastric reflux and subsequent damage to the oesophageal mucosa. The aim of this project was to test the ability of alginates to inhibit pepsin activity in-vitro.

Pepsin activity was measured using a simple in-vitro colorimetric assay for the generation of new N-terminals during the digestion of a protein substrate, succinyl albumin at pH 2.2 (Hutton *et al* 1986). Eight sodium alginates with contrasting structure and molecular weight were tested at 2, 20, 200, 1000, 2000 and 5000 $\mu g\,mL^{-1}$ (in deionized water) to determine the concentration–response effect on pepsin activity. Alginate solution (100 µL) was added to an equal volume of porcine pepsin in 0.01 M HCl. The weight ratio of alginate:pepsin in the assay ranged from 0.1 to 500 including a 1:1 ratio.

All the alginates tested were able to inhibit the ability of porcine pepsin to degrade the protein substrate to some extent. The degree of inhibition was highly variable with some alginates being able to inhibit pepsin by up to 89%. Pepsin inhibition by alginate showed a concentration-dependent relationship with the greater the concentration of alginate the greater the inhibition. Even at the lowest concentration of alginate (where pepsin was in excess by weight) inhibition ranged from 5 to 20% depending on the type of alginate.

In conclusion, the activity of pepsin can be substantially reduced by aqueous alginate solutions. The extent of inhibition is dependent on the structure of the alginate. There is therapeutic potential for alginates to reduce the damaging capacity of the gastric refluxate in patients with gastro-oesophageal reflux.

Hutton, D. A., *et al.* (1986) *Biochem. Soc. Trans.* **14**: 735

087
An in-vitro study of the effect of heat on the percutaneous absorption of three model penetrants

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It is well established that the stratum corneum invariably provides the principal barrier to the delivery of therapeutic quantities of a medicament either to or through the skin. This has led to the emergence of several techniques designed to reduce the barrier. The aim of this study was to investigate, the use of heat as a means of enhancing the transdermal delivery and skin retention of model penetrants with differing lipophilicity, namely methyl paraben (MP), butyl paraben (BP) and caffeine (CF). Franz cell diffusion experiments were performed at temperatures ranging from 23–45°C, using human epidermis. A saturated system containing undissolved solids (in order to maintain maximal thermodynamic activity) of the model penetrant in deionised water, was introduced into donor compartment of Franz cell. Samples were removed periodically from the receptor compartment over 4 h, after which the skin was removed from the cell, then washed and digested. Quantitative analysis of penetrants was conducted using validated HPLC methods. Transdermal flux and epidermal retention of all penetrants were found to increase with temperature (~ 2-fold in flux for every 7–8°C increment, except in the case of BP where a 3-fold increase was observed from 37°C to 45°C). A significant difference in epidermal localisation was observed for BP at each temperature, whilst MP and CF showed no significant differences in retention at 30°C and 37°C ($P \geqslant 0.05$). The amount of penetrant retained in the epidermis was found to be in the order BP > CF > MP whilst the trend for transdermal flux was MP > BP > CF with increasing temperature. Epidermal diffusivity (E_D), an indirect measure of the penetrant diffusivity in the membrane was estimated from changes in flux/retention (Cross & Roberts 2000). E_D was not affected significantly by the increase in temperature for CF (Table 1). The lower E_D values recorded for BP and CF in comparison with MP at each experimental temperature correlated with the tendency for the two former compounds to be retained within the epidermis. The effect of heat in enhancing the transdermal permeation of the penetrants can be attributed to an increase in diffusive mobility of penetrant from the vehicle or in skin lipid fluidity (Golden *et al* 1986). The findings of this study have clear implications for the percutaneous absorption of active agents or excipients used in barrier semisolids (sunscreens, insect repellents, etc.), when the skin is exposed to elevated temperatures.

Table 1 Estimated epidermal diffusivity (E_D) of penetrants at different temperatures

Receptor temperature (°C)	E_D (10^{-4} cm s^{-1})		
	MP	BP	CF
23	7.14 ± 1.14	0.75 ± 0.21	+
30	11.83 ± 1.54*	0.93 ± 0.22	1.00 ± 0.24
37	26.54 ± 4.17*	1.52 ± 0.22*	1.42 ± 0.54
45	31.05 ± 11.91	2.37 ± 0.49*	1.59 ± 0.42

Data expressed as mean ± s.d. ($n \geqslant 3$). *Significantly higher ($P \leqslant 0.05$) than E_D at preceding lower receptor temperature. +Data not available due to unavailability of human epidermis from same donor

Cross, S. E., Roberts, M. S. (2000) *J. Invest. Dermatol.* **5**: 914–918
Golden, G. M., *et al.* (1986) *J. Invest. Dermatol.* **86**: 307–312

088
Formulation of lipid:DNA complexes for delivery by pressurised metered dose inhaler

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As the discovery of links between genes and disease increases, so does the number of possible therapeutic DNA molecules. Disease states that are particularly benefited by lung delivery are cystic fibrosis and α-1 anti-trypsin deficiency. However, despite a number of clinical trials using virus-based formulations, there have been few successes in the delivery of DNA. Cationic lipids do not have the immunogenicity and carcinogenicity issues of viral vectors and are increasingly finding favour as gene delivery vectors. Cationic liposomes are used to complex plasmid DNA (pDNA) to form a lipoplex, which is the delivery unit for DNA transfection. At present, nebulisation is used to aerosolise lipoplex solutions to the lung. However, nebulisation is inefficient and pressurised metered dose inhalers (pMDIs), which are commonly used for the treatment of asthma, would be better devices for targeting DNA to the lung. The aim of this study was to formulate and characterise lipid:DNA complexes for pMDI delivery to the lung

A model pDNA, pGL3 control vector encoding for luciferase (Promega, UK), was used in the study. The lipid used was a 1:1 w/w mixture of DOTAP and DOPE (Avanti Lipids, USA). The propellant was HFA 134a (DuPont, UK). The pMDI formulations contained Tween 80 (0.5–0.05% w/w) with or without ethanol (1% v/w). Aerosol formulations were produced by complexing lipid with DNA in aqueous solution, usually in presence of surfactant. Lipid:DNA complexation was assessed by gel electrophoresis using ethidium bromide staining and measurement of zeta potential (ZetaPlus, Brookhaven Instruments, UK). The samples were then freeze dried before the addition of ethanol and filling with propellant. PicoGreen Reagent (Cambridge Bioscience, UK) was used to quantify DNA at concentrations between 1 ng mL^{-1} and 1 μg mL^{-1}.

Zeta potential measurements confirmed the complexation of pDNA and lipid in aqueous solution. However, in the presence of higher concentrations of surfactant, complexation was not achieved and at 0.125% w/w surfactant no potential could be detected. Visual assessment of the aerosol formulations showed that in the total absence of surfactant, the lipoplex remained as a freeze-dried mass. With surfactant concentrations up to 0.125% w/w, a clear solution was obtained, while at higher concentrations phase separation was observed. The addition of ethanol resulted in uniform dispersions with a milky appearance, which were stable on standing. Using PicoGreen reagent the emission of aerosolised pDNA was quantified over successive pMDI actuations. Transfection of 16HBE14o- and A549 respiratory cells by the DNA:lipid complex was characterised in preparation for the assessment of the functionality of the DNA emitted from the aerosol formulation. In conclusion, a lipoplex formulation in propellant has been developed, although its nature in propellant requires further investigation, in particular to establish the effects of the surfactant, which may change the solubility of lipid:DNA thereby reducing electrostatic interactions or participate in the formation of reverse micellar/cosolvent systems in propellant. The formulation of pDNA for delivery by pMDI appears to be feasible and the functionality of the aerosolised pDNA is under investigation.

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A tight junction-directed protein delivery system

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Currently, many pharmaceutical targets include proteins whose administration is notoriously problematic. The proposal here is to investigate the potential of the technology of protein encapsulation in biodegradable microspheres by site-directed delivery of the microspheres across the tight junctions of an epithelial cell monolayer. The intention is to utilise zonula occludens toxin (ZOT), an enterotoxic protein secreted by the bacterium *Vibrio cholera*, which reversibly disrupts the tight junctions (tj) between epithelial cells via an unidentified receptor (Fasano 1991). Thus, ZOT may be used in conjugation with the desired target protein or the drug delivery vehicle itself. This study will investigate the potential of ZOT to carry latex microspheres across a confluent Caco-2 cell monolayer.

Genomic DNA was prepared from *V. cholera* TRH7000, an attenuated strain by deletion of the cholera toxin genes, kindly provided by Prof. Hirst, University of Bristol (Hirst *et al* 1984). However, the ZOT gene lies alongside the cholera toxin genes and excision of the cholera toxin genes by *Acc*I digestion of plasmid pJBK16 also removes 280 b from the 3'-end of the ZOT gene. Therefore, this 'missing' 280 b fragment was constructed using recursive PCR (Prodromou & Pearl 1992) and ligated to the truncated ZOT gene amplified from the genomic DNA to provide a full-length ZOT gene, cloned into pBluescript.

The gene encoding for ZOT was subcloned into the expression vector pQE-80L and used to transform *E. coli* DH5α in order to express the polyHis-tagged ZOT protein. Unfortunately, expression of ZOT has proven problematic in our hands, with this construct expressed solely in the cell insoluble fraction and its isolation by denaturing nickle-chelation chromatography being only partially successful. Attempts to overcome this have included subcloning into other expression vectors: pRSET-A, pGEX-4T3 and pMAL-c2x, the latter being the most promising with the fusion protein partially present in the cell soluble fraction.

C- and N-terminal ZOT polypeptides, which span the putative binding site (Di Pierro *et al* 2001), have also been constructed from the wild-type ZOT clone and tested for expression in *E. coli* using the same vectors as above. In particular, the C-terminal ZOT polypeptide expressed as a glutathione-S-transferase (GST) fusion protein can be readily purified by glutathione-affinity chromatography. This fusion protein has been conjugated to carboxyl derivatised fluorescent microspheres (Polysciences), with conjugation of GST as the control. The ability of the C-terminal ZOT polypeptide to 'carry' the microspheres across cell-cell tight junctions will be compared against wild-type ZOT using a confluent Caco-2 cell monolayer grown on 24-well Transwell (Corning) plates.

Di Pierro, M., *et al*. (2001) *J. Biol. Chem*. **276**: 19160–19165
Fasano, A. (1991) *Proc. Natl Acad. Sci. USA* **88**: 5242–5246
Hirst, T., *et al*. (1984) *Proc. Natl Acad. Sci. USA* **81**: 7752–7756
Prodromou, C., Pearl, L. (1992) *Protein Eng*. **5**: 827–829

090
In-vitro membrane transport of captopril carboxylate ester prodrugs

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Delivery of the ACE-inhibitor captopril would benefit from the zero-order kinetics associated with the transdermal route. We have previously described the synthesis and characterisation of a range of carboxyl-ester captopril prodrugs (Moss *et al* 2003). These prodrugs have been designed rationally via a QSAR approach to have range of log P values from 0.84 to 3.66. We measured the membrane transport properties these prodrugs, and thus assessed the success of QSAR methods in designing prodrugs with enhanced percutaneous absorption.

Prodrugs (C_1–C_6 *n*-alkanol esters) were synthesised via a modification of the method of Tai *et al* (1995). Polydimethylsiloxane (Silastic, PDMS) was used as the membrane, and secured in Franz-type cells (n = 3). Each prodrug was at a concentration (0.01–1.0% w/v) that gave consistent thermodynamic activity. The cells were maintained at 37 ± 1°C, sampling the receptor compartment at 0.5, 1, 2, 4, 6, 8, 16 and 24 h. Analysis was by UV spectrophotometry at 206 nm.

Fluxes for prodrugs were calculated from the steady-state (linear) region of the concentration–time profile, and were significantly higher than for the parent drug for C_1–C_3 prodrugs. For example, captopril and its methyl ester prodrug had fluxes of 2.4×10^{-4} and 2.7×10^{-4} mM min^{-1} cm^{-2}, respectively. This gave an enhancement factor of 3.17, when adjusted to consider saturated solubilities. While fluxes decreased due to changes in aqueous solubility for C_4–C_6 prodrugs, larger enhancement factors, relative to the parent drug captopril, were observed when fluxes were corrected for saturated solubility.

These results justified the strategy employed for enhancing percutaneous absorption. However, it must be recognised that use of QSAR models for skin absorption is at present limited to aqueous formulations (Moss *et al* 2002).

Moss, G. P., *et al*. (2002) *Tox. In Vitro* **16**: 299–317
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Tai, D. F., *et al*. (1995) *J. Chin. Chem. Soc*. **42**: 801–807

091
A polyvalent scaffold for use as a non-viral gene delivery vector

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Current work towards non-viral gene delivery systems often involves cationic liposomes or polymers furnished with ligands directed to cell surface receptors in

order to invoke receptor-mediated endocytosis. For example, the 'invasin' protein on the outer surface of *Yersinia psuedotuberculosis* mediates internalisation into mammalian cells via integrin receptors (Isberg & Leong 1990). Unfortunately, linear $[K]_{16}$RGD carriers of DNA have resulted in low transfection rates, despite their specific integrin-targeting domain (Harbottle *et al* 1998). One explanation for this maybe the requirement for polyvalent interaction between integrin receptors and ligand since integrins have been observed to cluster in response to ligand binding (Yauch *et al* 1997). This study describes the development of nanometer-sized agents that have the potential to interact in a polyvalent manner with integrin receptors.

The initial design of a preorganised scaffold has focused on the synthesis of a calixarene-type structure that has been characterised using ^{1}H NMR. This will subsequently be furnished with polycationic 'building blocks' for the complexion of plasmid DNA. Since polylysine or polyarginine building blocks are envisaged, the ability of these cationic polypeptides to complex DNA has been investigated. One microgram of plasmid DNA was complexed with increasing concentrations, 0–2400 pmol, of polylysine (M_r 500–2000) or polyarginine (M_r 5000–15 000) in 40 mM Tris.HCl pH 7.9. Polypeptide–DNA complexes were analysed on 1% agarose gels, stained with ethidium bromide to visualise the ability of the peptides to retard DNA migration (1 retardation unit being defined as the amount of polypeptide just sufficient to prevent the migration of 1 μg DNA by gel electrophoresis; Table 1). The same polypeptide–DNA complexes were then incubated with 1U DNaseI for 30 min at 37°C. Analysis on ethidium bromide stained agarose gel showed that the concentrations of polypeptide equivalent to 1 retardation unit were also just sufficient to prevent the complete digestion of the plasmid DNA by DNaseI.

Table 1 Concentration of polypeptides ≡ 1 retardation unit

Plasmid	Polylysine	Polyarginine
pGL3 (5.3Kb)	2400 pmol	80 pmol
pSV-lac (6.8Kb)	2400 pmol	80 pmol

Given the different M_r between the polylysine and polyarginine it is not surprising that polyarginine has a three-fold lower concentration equivalent to 1 retardation unit. However, the study usefully shows that only a relatively short (5–10 residue) amino acid sequence is sufficient to complex DNA. Our synthetic prototype will therefore be furnished with 5–10 residue polyarginine building blocks and used in mammalian cell transfection studies. The luciferase gene (pGL3) will be used as a reporter assay to measure internalisation of the complex and expression in Caco-2 cells (a β-Galactosidase, pSV-lac, assay will be used as the baseline control). Transfection rates using our target compounds will be compared to lipofectamine transfection.

Harbottle, R. P., *et al*. (1998) *Hum. Gene Ther.* **9**: 1037–1047
Isberg, R. R., Leong, J. M. (1990) *Cell* **60**: 861–871
Yauch, R. L., *et al*. (1997) *J. Exp. Med*. **186**: 1347–1355

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In-vitro evaluation of pH responsive colonic drug delivery dosage forms

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Drug delivery to the colon is considered essential mainly for the local therapy of several colonic disorders such as inflammatory bowel disease, thus reducing the administered dose and associated side effects. Potential pH responsive polymers for colonic drug delivery include Eudragit S as an organic solution or aqueous dispersion, and the recently introduced Eudragit FS (Rohm GmbH). While dosage forms have been developed based on Eudragit S organic solutions, little is known of the drug release characteristics from dosage forms coated with Eudragit S aqueous dispersion and Eudragit FS. We aim therefore to investigate the in-vitro performance of colonic drug delivery dosage forms formulated with these polymer preparations.

Prednisolone 10mg tablets were prepared and film coated with Eudragit S aqueous dispersion (S aqu), Eudragit S ethanolic solution (S org) and Eudragit FS aqueous dispersion (FS), to a 5% weight gain using a fluid bed spray coater. Drug release from the coated tablets was evaluated using the USP paddle apparatus in 900 mL media and 100 rev min^{-1} paddle speed. Tablets were tested first in 0.1 M HCl for 2 h to simulate gastric residence and then in two compendial buffer media: KH_2PO_4/NaOH (B1), Na_2HPO_4/KH_2PO_4 buffer (B2) at pH range 6.8–7.4; and a physiological balanced salt (B3), for 6 h to simulate small intestinal media. B3 test media is similar in ionic composition to intestinal fluid (Banwell *et al* 1971).

There was no drug release from any of the coated tablets in 0.1 M HCl and for up to 6 h in pH 6.8 buffer, thus confirming the resistance of the coating to gastric and upper small intestinal fluid. Drug release occurred at pH 7.0 for the S aqu and FS tablets, and pH 7.2 for the S org tablets. FS tablets, however, showed a quick onset but sustained drug release pattern as evident from a shorter lag time and long $t_{50\%}$, and is thus likely to be more suited to colonic drug delivery.

Table 1 Lag time and $t_{50\%}$ values (min) in the three buffer media

	pH	S aqu		S org		FS	
		lag time	$t_{50\%}$	lag time	$t_{50\%}$	lag time	$t_{50\%}$
B1	6.8	—	—	—	—	—	—
	7.0	160	247	—	—	60	386
	7.2	55	97.5	185	247	50	191
	7.4	35	57.5	65	112	15	77
B2	6.8	—	—	—	—	—	—
	7.0	55	87.5	—	—	55	257
	7.2	25	52.5	120	185	40	145
	7.4	20	47.5	40	70	20	50
B3	7.4	125	227	275	290	120	248

The dissolution rate also varied in the three different media as follows B2 > B1 >>> B3. The considerably slower drug release in B3 (the physiological salt media) suggests that, as well as the pH, the dissolution of these polymers is also dependent on the ionic composition of the test media, and highlights the inadequacy of currently used compendial media which consist of sodium and/or potassium phosphate salts.

Banwell, J. G., *et al*. (1971) *J. Clin. Invest*. **50**: 890–900