Poster Session 2 – Drug Delivery

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The relative bioavailability of salbutamol to the lungs from a U22 MicroAir nebuliser

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We have shown that the amount of salbutamol excreted in the urine in the first 30 min post inhalation indicates the relative deposition of inhaled salbutamol in the lungs (Hindle & Chrystyn 1992). This index has been used to show that jet nebulisers are inefficient systems to deposit drug into the lungs (Silkstone et al 2002). A U22 MicroAir battery powered hand held nebuliser (Omron, Japan), based on vibrating mesh technology, has recently been introduced. The size of the particles emitted from the mesh is dependent on the mesh size, which also affects the rate of the aerosol output (Dennis et al 2003). We have evaluated the potential of the U22 MicroAir nebuliser to deposit salbutamol into the lungs. U22 nebulisers with a high (U22H) flow rate (emitting 156 mL min^{-1}, MMAD 4.75 μm) and a low (U22L) flow rate (emitting 98 mL min^{-1}, MMAD 3.19 μm) have been compared to a Sidestream jet nebuliser (SIDE; Profile Inhalation Systems, UK) jet nebuliser and to a metered dose inhaler attached to a spacer (M+SP). Ethical Committee approval was obtained and all subjects gave signed informed consent. Each subject inhaled salbutamol respiratory solution (1 mg mL^{-1} – Ventolin Respiratory Solution; GlaxoSmithKline, UK). Randomised study doses (7 days apart) were 1 mg from a U22H and a U22L, 2.5 mg from a SIDE, and finally five 100 μg doses from a M+SP. Inhalation of the doses from the U22 was made using a manual synchronised breathing mode (operator turning it on and off corresponding to their breathing). The maximum nebulisation time was set to 8 min (Silkstone et al 2002). Before each nebulised dose subjects emptied their bladder and then provided a urine sample 30 min after the start of each nebulised dose. The amount of salbutamol excreted (1) (USAL), left in each nebuliser chamber and the spacer (NEB) together with the amounts in the nebuliser mask (MASK) were determined and USAL normalised for the amount available for inhalation (UN) was calculated. Twelve subjects, mean $\pm$ s.d. age and weight of 31.4 $\pm$ 6.8 years and 65.8 $\pm$ 8.9 kg completed the study. Table 1 summarises the fate of the nebulised doses. Statistical analysis (independent *t*-test) revealed that UN for U22L and U22H were more than SIDE ($P < 0.01$) and that M+SP was also greater than SIDE ($P < 0.001$), all other comparisons were not different. The relative bioavailability of salbutamol to the lungs following nebulisation from the MicroAir nebulisers was similar to that of a metered dose inhaler (MDI) attached to a spacer and greater than a Sidestream. The urinary salbutamol excretion suggests that 2.5 mg of salbutamol nebulised from a Sidestream is equivalent to 946 μg nebulised from a U22 MicroAir (High Flow) nebuliser. Also five 100 μg doses inhaled from a MDI attached to a spacer are equivalent to 600 μg inhaled from the U22 MicroAir nebuliser.

Table 1 Amounts of salbutamol from each nebulised method

	USAL (μg)	NEB (μg)	MASK (μg)	UN (%)
U22L	26.2 (13.3)	468 (159)	56 (30)	6.0 (3.7)
U22H	31.5 (10.1)	205 (185)	110 (50)	5.3 (3.1)
SIDE	29.8 (14.3)	1225 (78)	50 (9)	2.4 (1.2)
M+SP	19.1 (7.7)	217 (54)	N/A	7.0 (3.4)

Mean (±s.d.)

Dennis, J. H., et al (2003) *J. Aerosol Med.* **16**: 213
Hindle, M., Chrystyn, H. (1992) *Br. J. Clin. Pharmacol.* **34**: 311–315
Silkstone, V. L., et al (2002) *Br. J. Clin. Pharmacol.* **54**: 115–119

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Pectins as oesophageal protectants; their bioadhesive potential and ability to reduce acid and pepsin diffusion

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Pectins are natural polysaccharides derived from fruits that are structurally similar to alginates. Their structure consists of galacturonic and galacturonic methyl ester units; the degree of methoxylation has effects on the properties of each molecule as it increases the hydrophobicity and affect the hydrogen bonding potential of the molecule. Previous in-vitro work has demonstrated that aqueous solutions of alginate may adhere to oesophageal epithelium for periods of up to 60 min (Batchelor et al 2002). In addition these adhesive layers minimise transfer of acid and pepsin and thus offer protection to the oesophageal epithelium from gastric reflux (Tang et al 2004). This study examines the bioadhesive potential of pectins, as well as their ability to minimise the transport of acid and pectin thus limiting their contact with the oesophageal epithelial surface. The adhesive potential of pectin was assessed using a method that has been described previously (Batchelor et al 2002). Aqueous solutions of pectin (2% m/v) were prepared and the retention of each liquid dose on a substrate that mimics oesophageal mucosa over 30 min was measured. The retention of low, medium and highly methoxylated apple pectins were compared within this study (Table 1). The rate of acid and pepsin transfer through aqueous solutions of pectin was measured using a vertical Franz-diffusion cell. The donor compartment was separated from the receptor compartment by cellulose acetate membrane (12 000 MW cut off). A measured 0.1-mL dose of the pectin was placed onto this membrane before addition of a fixed volume (25 mL) of 0.01 M HCl onto this layer. The receptor compartment contained water with a pH probe inserted into the sample port to allow quantification of acid transfer over time. Pepsin diffusion was measured by addition of 0.3% m/v pepsin within 0.01 M HCl to the donor compartment and using 0.01 M HCl in the receptor with Whatman filter paper separating the two compartments. Quantification of pepsin transfer was via a UV assay; pepsin absorbs at 276 nm, the calibration had an r^2 value of > 0.99. Controls were performed in the absence of a pectin layer; the acid and pepsin diffusion rates were normalised from the control value (Table 2). Linear changes in acid concentration over time were observed thus the rates were calculated as the gradient of these slopes. The pepsin diffusion over time was not linear thus the area under the concentration vs time graph up to 30 min was used as a measure of the rate of diffusion. Low methoxyl pectins demonstrated superior adhesion and protection compared with those with a higher degree of methoxyl esterification; an absence of these methoxyl groups leads to improved flexibility and increased hydrogen bonds sites. These chemical alterations indicate that both adhesion and protection involve hydrogen bonding; further work may be performed to link the methoxyl content and efficacy of adhesion and protection. This study has revealed that low methoxyl pectins have demonstrated in-vitro potential as oesophageal protectants.

Table 1 Retention of pectin at 12 and 30 min

	12 mins	30 mins
LM	40.6 ± 0.2%	32.2 ± 0.2%
MM	8.2 ± 1.0%	0.4 ± 0.2%
HM	0.8 ± 0.2%	0%

Table 2 Normalised rate of diffusion of acid and pepsin

	Acid	Pepsin
Control	100	100
LM	80 ± 11	67 ± 27
MM	72 ± 11	21 ± 48

Batchelor, H. K., et al (2002) *Int. J. Pharm.* **238**: 123–132
Tang, M., et al (2004) *Eur. J. Pharm. Sci.* In press

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Flexibility of phospholipid vesicles containing sodium cholate, Span 80 and Tween 80

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Entrapment of therapeutic agents within ultradeformable vesicles composed of soy bean phosphatidylcholine (PC) with either sodium cholate, Span 80 or Tween 80 can improve drug delivery to and through skin as compared with the use of “traditional” PC vesicles (Cevc et al 1998; El-Maghraby et al 1999). The mechanisms of action of these vesicles in promoting drug delivery remain controversial, and it has been suggested that ultradeformable liposomes may penetrate into or

even permeate across skin membranes intact. Here, the passage of "traditional" PC liposomes (non-deformable) and ultradeformable vesicles (PC:sodium cholate, 86:14% w/w; PC:Span 80, 86:14% w/w; PC:Tween 80, 86:14% w/w) through an artificial barrier with a pore size smaller than the vesicle size has been compared utilizing a novel custom built device. Empty unilamellar PC vesicles (95–110 nm) or of the lipid with the additives were prepared by rotary evaporation, sonication and 10-fold extrusion through 200–100 nm polycarbonate membranes (El-Maghraby et al 1999). For deformability evaluation, 3–4 replicates from each of the 1% w/v vesicle dispersions were passed through 30 nm polycarbonate membranes under a force of 0.6 MPa; compressed air was regulated and the filter was retained using a commercially available filter housing for high pressure studies. The weight of material extruded over 15 min was assessed and vesicle mean diameters (nm) before and after extrusion were measured (Zeta sizer, Malvern Instruments, Malvern, UK). Deformability of vesicles (D) was then calculated using equation 1:

$$D = J^*(r_v/r_p)^2 \quad (1)$$

where J is the amount of vesicle dispersion and the r_v is the vesicle mean diameter after passing the membrane and r_p was the pore size of the membrane. The amounts passing through the artificial membrane were significantly higher (*t*-test, $P < 0.01$) for deformable as compared with pure PC vesicles dispersions (Table 1). Deformable vesicles essentially conserved their size after passing through the 30 nm pore whereas no size could be detected after extrusion of the rigid PC liposomes. Calculated deformability indicated greater flexibility PC-sodium cholate, PC-Span 80 and PC-Tween 80 compared with pure PC vesicles. To conclude, the mean diameter was conserved in ultradeformable vesicles containing Span 80, Tween 80 and sodium cholate whereas pure PC liposomes were disrupted.

Table 1 Vesicle extruded amounts (J), (r_v/r_p) ratios and calculated deformability

Formulation	J (g)	r_v/r_p	Deformability
PC	0.17 ± 0.05	—	—
PC:cholate	2.16 ± 0.12	2.36 ± 0.05	11.97 ± 0.78
PC:Span	1.03 ± 0.15	3.22 ± 0.02	10.69 ± 1.47
PC:Tween	1.10 ± 0.18	2.68 ± 0.06	7.94 ± 1.57

Data are mean ± s.d., n = 3–4.

Cevc, G., et al (1998). *Biochim. Biophys. Acta* **1368**: 226–323
EL-Maghraby, G. M. M., Williams, A. C., Barry, B. W. (1999) *J. Pharm. Pharmacol.* **51**: 1123–1134

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Release study of streptomycin from N,N-bis (2-hydroxyethyl) fatty amide modified polymeric coating

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One of the solutions to overcome medical device-related infection is the use of biodegradable coating incorporated with antimicrobial agent. N, N-bis (2-hydroxyethyl) fatty amide (HAFA) derived from coconut oil has been employed as a curing agent for poly (butyl methacrylate-co-maleic anhydride) based resin (BMA). Coating compositions with varying ratio of BMA/HAFA and streptomycin (1, 2 and 4% w/w) were prepared by dissolving in xylene–acetone (95/5% v/v) mixture with mechanical stirring. The films were prepared by applying the mixture on mild steel panels and then baked in oven at 150°C for 30 min. (Raval et al 1998). Table 1 shows the coating composition and film properties. Streptomycin release was studied by placing 1 square inch of the film in 100 mL of distilled water in stoppered flask in rotary shaker (37°C, 100 oscillations min^{-1}). The amount of streptomycin released as a function of time was determined using spectrophotometer at 540 nm (Raval et al 1997). Table 2 shows the percentage of drug released after 1 week. The ratio of BMA to HAFA has a controlling effect on film properties and drug released. Increasing concentration of streptomycin in the film significantly increased the percentage of drug released. The increasing resistance to water and simultaneous decrease in drug release with increase in HAFA content can be attributed to higher degree cross linking in the film. The scratch hardness of the film decreases with increase in HAFA content. This is attributed to the plasticizing effect of non-polar fatty acid moiety of HAFA in the film. The release of streptomycin coupled with the degradation properties of film indicated its potential as medical device coating.

Table 1 Coating composition and baked film properties

Sr.	BMA:HAFA	Scratch hardness (g) passes			Water resistance		
1	1:1	900	900	800	FB	FB	FB
2	2:1	1500	1400	1100	FC	FC	D
3	3:1	1900	1800	1500	D	D	D

A, Not affected; B, slight swelling and blistering; C, swelling and blistering; D, severe swelling; E, loss of adhesion; F, loss of gloss.

Table 2 Release of streptomycin by 1 week

Sr.	BMA:HAFA	% Release of streptomycin (STR)		
		1% STR	2% STR	4% STR
1	1:1	8.7	9.7	11.8
2	2:1	17.9	28.7	36.2
3	3:1	25.8	37.4	50.8

Raval, D. A., et. al (1997) *Indian J. Pharm. Sci.* **May–June**: 152–157
Raval, D. A., et al (1998) *J. Coatings Technol.* **70**: 85–91

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The development of a novel oral dosage form

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The physico-chemical properties of a range of polymers, including polysaccharides, have been studied in aqueous solution with respect to their viscosity characteristics. The viscosity of a combination of certain polymer blends (e.g. pullulan and carboxymethyl cellulose) is higher than the viscosity of the two polymers measured separately at the same equivalent concentration. A synergistic interaction therefore appears to be taking place between the polymers in solution resulting in greater chain entanglement. The origin of this synergy is unclear, although one possible mechanism may lie in inter-molecular hydrogen bonding between different adjacent polymer chains. The solution properties of these blends have also been studied with respect to the addition of a range of copolymers and cyclic carbohydrates. The measurements were carried out over a range of temperatures and in different solvent environments including various pH and electrolyte concentrations. As a generalisation viscosity typically decreases with increasing temperature and electrolyte concentration. These results suggest that the extent of chain entanglement within the solutions decrease under these conditions. The solutions formed from the polymer blends readily form solid films following evaporation of the solvent. The properties of the resultant film have been found to be highly dependent on such variables as the rate of evaporation and the choice of backing material onto which the film is cast. The films produced have been investigated with respect to their mechanical properties, which include a study of their rigidity and tensile strength. The stability of the films has also been examined in addition to the kinetics of their dissolution and dispersion. Pharmaceutically active compounds having a range of log P values and different molecular masses have been blended with a range of different polymers and polymer blends to produce new film based compounds. These films represent a novel oral dosage form for the delivery of a variety of different drugs. The mechanical and physico-chemical properties of the resultant films have been determined. Dissolution measurements show that the films disperse readily and thereby facilitate a release of the active material. Potential applications for this technology may lie in the development of new oral dosage form for veterinary, geriatric, paediatric and over the counter (OTC) medicines.

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A transdermal delivery system for glipizide

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Glipizide is one of the most commonly prescribed drugs for the treatment of type 2 diabetes. Being a weak acid of poor, pH-dependent water solubility, its release is highly dependent on pH and hydrodynamic conditions of the gastrointestinal tract. Oral therapy with glipizide comprises problems of bioavailability fluctuations and may be associated with severe hypoglycemia; gastric disturbances like nausea, vomiting, heartburn, anorexia and increased appetite have also been observed. On the other hand, since this drug is usually taken for a long period, patient compliance should be taken into consideration. Therefore, as a potential for convenient, safe and effective antidiabetic therapy, the rationale of this study was to develop a transdermal delivery system for glipizide. Inclusion complexes of the drug in β-cyclodextrin (β-CyD), dimethyl-β-cyclodextrin (DM-β-CyD), hydroxypropyl-β-cyclodextrin (HP-β-CyD) and hydroxypropyl-γ-cyclodextrin (HP-γ-CyD) were prepared by the kneading method (Moyano et al 1997). Several percutaneous formulations for the drug and the prepared complexes in different bases (o/w emulsion, PEG, CMC and Carbopol) were developed. Assessment of drug release by the paddle over disk method (Shah et al 1986) revealed improved release from formulations containing glipizide-CyD complexes. Ex-vivo permeation studies through full thickness rat abdominal skin were conducted (Larrucea et al 2002), whereby the effect of several conventional penetration enhancers (propylene glycol, oleic acid, urea, dimethyl formamide, dimethyl sulfoxide, menthol, limonene and cineole) was monitored. Highest flux was found to be that from ointments prepared with Carbopol gel base and containing a combination of propylene glycol and oleic acid. Ointments were applied on the back of male Wistar rats and the plasma glucose level was measured. The results revealed sustained effect of the drug for about 48 h, as well as suppression of hypoglycemia induced in glucose-loaded rats. In this respect, two formulations showed best biological performance. The first was that prepared by incorporating glipizide- DM-β-CyD complex in Carbopol gel base in presence of 15% urea. In the second formulation, the drug was incorporated in the same base in presence of 20% propylene glycol together with 15% oleic acid. The above mentioned results might shed a strong beam of light on the feasibility of transdermal administration of glipizide for treatment of type 2 diabetes with the aim of improving both patient compliance and the pathophysiology of the disease.

Larrucea, E., Arellan, A., Santoyo, S., et al (2002) *Drug Dev. Ind. Pharm.* **28**: 245–248
Moyano, J. R., Arias-Blanco, M. J., Ginés, J. M., et al (1997) *Int. J. Pharm.* **148**: 211–217
Shah, V. P., Tymes, N. W., Yamamoto, L. A., et al (1986) *Int. J. Pharm.* **32**: 243–250

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The stability of liposome entrapped lactate dehydrogenase to nebulisation

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The suitability of nebulisers for delivery of liposomal aerosols is well established, and nebulised liposomes have been employed to alter the pharmacokinetics of entrapped proteins. However, little is known regarding the use of liposomes to enhance the stability of entrapped proteins. In this study, the suitability of liposomes for enhancing the stability of lactate dehydrogenase (LDH) in nebulisers known to degrade that protein (Khatri et al 2001) was investigated. Chloroform was evaporated from a solution of dimyristoylphosphatidylcholine (DMPC) using a rotary evaporator, to form a DMPC film. The film was hydrated at 40°C with a solution of LDH in Sρrensen's modified phosphate buffer and agitated to give liposomes with a lipid concentration of 10 mg mL^{-1}. Liposomes were extruded through polycarbonate membrane filters (pore size 100 nm), and unentrapped LDH was removed by centrifugation. Liposome formulation (5 mL) was placed into Pari LC plus or Pari LC star jet nebulisers used with a Pari Turboby compressor or an Omron U1 ultrasonic nebuliser. Before and after nebulisation, the size distribution of liposomes was measured by photon correlation spectroscopy and LDH activity determined spectroscopically (Wroblewski & LaDue 1955). Data are compared with findings of a previous study of nebulised non-liposomal LDH solutions (Khatri et al 2001). The mean (±s.d.) size of LDH-DMPC liposomes nebulised from the Pari Plus, Pari Star and Omron U1 were, respectively, before nebulisation 175.9 nm (±5.4), 169.6 nm (±5.1) and 154.3 nm (±7.8), and post-nebulisation 173.8 nm (±4.4), 169.8 nm (±2.6) and 151.2 nm (±6.2). The non reduction in mean size ($P < 0.05$) suggests that these small liposomes were not broken up during nebulisation, as occurs for larger vesicles (Bridges & Taylor 2000). LDH is significantly degraded during nebulisation, particularly in the jet nebulisers (Table 1). However, in each nebuliser, entrapment of the protein within the liposomes protects the protein from degradation (Table 1). The protection against protein degradation is probably due to physical protection by the liposomal bilayers, which may be enhanced by a direct interaction of LDH with the bilayers.

Table 1 Activity of LDH in the nebuliser reservoir pre and post nebulisation

	LDH[a]		Liposomal LDH	
	% pre	% post	% pre	% post
Pari LC Plus	100 ± 2.8	24.2 ± 2.7	100 ± 9.6	101.0 ± 7.0
Pari LC Star	100 ± 2.0	61.7 ± 0.4	100 ± 4.9	91.1 ± 7.9
Omron U1	100 ± 3.8	95.2 ± 2.9	100 ± 7.7	101.3 ± 8.0

n = 4 ± s.d. [a]Data from Khatri et al (2001).

Bridges, P. A., Taylor, K. M. G. (2000) *Int. J. Pharm.* **204**: 69–79
Khatri, L., et al (2001) *Int. J. Pharm.* **227**: 121–131
Wroblewski, F., LaDue, J. S. (1955) *Proc. Soc. Exp. Biol. Med.* **90**: 210–214

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Coating of chitosan/DNA particles with hydrophilic polymers improves its stability

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The major obstacle to non viral gene delivery vectors in-vivo is its binding to serum proteins, which restricts its access to target tissues and inhibits its transfection activity in-vitro (Oupicky et al 2002). Covalent attachment of the monovalent hydrophilic polymer, polyethylene glycol (PEG), and the multivalent hydrophilic polymer, poly-[N-(2-hydroxypropyl) methacrylamide] (pHPMA) (Dash et al 2000) was thought to stabilize chitosan/DNA particles, and as a result, to improve the resistance to protein binding and phagocytosis. So, the aim of this study was to investigate the physicochemical changes induced in polymer coated chitosan/DNA particles following incubation with albumin as a model protein. Chitosan/DNA particles were prepared by an ionic gelation procedure (Li et al 2003). Coating of particles was carried out by addition of m-PEG succinimidyl propionate (2 K) and pHPMA with 8 mol% of methacryloylglycineglycine 4 nitrophenyl ester (35 K) (20 mg mL^{-1} in water) to the particles at different molar ratios from 0.25:1 to 30:1 followed by the addition of 1 M HEPES buffer (pH 7.4) to give a final HEPES concentration of 10 mM and 50 mM, respectively. The reaction was allowed to proceed overnight at room temperature and the particles were then purified. The particle physicochemical properties including morphology (transmission electron microscopy), size (photon correlation spectroscopy), and surface charge (zeta potential (ζ) measurement) were characterized. Chitosan particles were also incubated in an albumin (BSA) solution 10% (w/v) for 2 h and 24 h, after which size measurements were made. The degree of coating was determined by measuring the percentage of the free amino groups on chitosan surface using a TNBS (Trinitrobenzene sulphonic acid) assay colorimetrically at 420 nm. The coating of the surface of chitosan/DNA particles with PEG produces almost neutral surface charge, while a negatively charged surface was produced after coating with pHPMA($\zeta = -18.9$ and -21.7 mV for 1:1 and 30:1 coating molar ratios). This can be attributed to the partial hydrolysis of reactive 4-nitrophenyl groups during the coating with the production of negatively charged carboxylate groups. The percentage of free amino groups on particle surface decreases by increasing the amount of the polymer (17.7% and 13.2% for 1:1 and 30:1 coating molar ratios, respectively). It was found that the size of uncoated particles greatly increases after incubation with albumin from 70 nm to more than 1000 nm, whereas coating with either PEG or pHPMA leads to a steric stabilization that is manifested by stable sizes due to increased resistance to albumin-induced aggregation. The key difference between the two

polymers is the ability of pHPMA polymer to bind around the surface of the particles linking together surface amino groups and providing lateral stabilization, which provides the particles with more stability. The findings of this study demonstrate that hydrophilic polymers can be efficiently used to stabilize chitosan particles for improved in-vitro transfection and in-vivo targeting to tissues.

Dash, P. R., et al (2000) J. *Biol. Chem.* **275**: 3793–3802
Li, X. W., et al (2003) *Biochem. Biophys. Acta* **1630**: 7–13
Oupicky, D., et al (2002) *Mol. Ther.* **5**: 463–472

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Fluorescent lipopolyamines: a new class of molecular probes in non-viral gene therapy

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Spermine is a natural polyamine that plays important roles in DNA condensation. We are studying lipospermines, spermine conjugated with lipophilic groups, such as steroids (e.g. cholesterol) or long alkyl or alkenyl chains (e.g. C_{18} fatty acids), as part of a programme to develop more efficient non-viral gene delivery systems for effective and safe non-viral gene therapy (NVGT). The use of a variety of fluorescence techniques enable the intracellular monitoring of NVGT at the molecular level. DNA condensation, the first step in the gene delivery mechanism, was studied by the fluorescence quenching of ethidium bromide (EthBr) to monitor the formation of nanoparticles (Gershon et al 1993; Geall & Blagbrough 2000). The fluorescence yield of EthBr ($\lambda_{ex} = 260$ nm, $\lambda_{em} = 600$ nm) increased on intercalation between adjacent base-pairs, and then gradually decreased when DNA phosphate anions were neutralised by increasing the ammonium/phosphate (N/P) charge ratio. Formation of DNA complexes was confirmed by UV light scattering (LS) at $\lambda = 320$ nm. The plasmid encoding enhanced green fluorescent protein (pEGFP) under the control of CMV promoter (Clontech) was chosen as the DNA to be delivered. Transfected cells with fluorescent imidazolidinone-labelled protein were analysed by fluorescent-activated cell sorting (FACS). Similar experiments were also conducted using the luciferase plasmid (pGL3). N^2, N^3-Dioleoyl spermine (i.e. LipoGen) showed more effective DNA condensation than spermine, at all N/P charge ratios. The in-vitro transfection results using LipoGen were also significantly higher in both primary cell lines (skin fibroblast) and carcinoma, compared with Lipofectin (liposomal cationic lipid). Designed fluorescent-tagged lipopolyamines were prepared by Fmoc chemistry (Blagbrough et al 2003). These probes are designed for studies tracking the major intracellular barriers to efficient NVGT (e.g. cell entry, endosome escape, nuclear localisation). The trafficking of DNA complexes can be monitored by fluorescence microscopy. These conjugate probes can be reporters at different stages of plasmid delivery by the use of Fluorescence Correlation Spectroscopy (FCS) studying the change in fluorophore diffusion behaviour within DNA complexes. Fluorescent tagged plasmid DNA was also prepared by non-enzymatic (random) covalent conjugation and shown to transfect as efficiently. With fluorophores labelling both DNA and vector, the Fluorescence Resonance Energy Transfer (FRET) effect may be observed to offer more insights about NVGT kinetics and barriers, with even the possibility of in-vivo studies. A clearer understanding of the complexity of the barriers to NVGT is crucial in the development of novel efficient vectors.

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Blagbrough, I. S., et al (2003) *Biochem. Soc. Trans.* **31**: 397–406
Geall, A. J., Blagbrough, I. S. (2000) *J. Pharm. Biomed. Anal.* **22**: 849–859
Gershon, H., et al (1993) *Biochemistry* **32**: 7143–7151

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The use of polycarboxylates in local antimicrobial delivery to the buccal mucosa

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The incorporation of bioadhesive polymers into oral healthcare products has been used to increase the retention of antimicrobial agents within the oral cavity, thereby increasing their activity and therapeutic value (Needleman et al 1998). The polycarboxylates investigated in this study exhibit significant bioadhesion to buccal mucosa in-vitro and in-vivo (Kockisch et al 2001) and, due to their low toxicity and biocompatibility, are ideal for inclusion in products intended for everyday use. Zinc salts are bactericidal against oral pathogens and are frequently used in oral healthcare products (Scheie 1989). This project aims to develop zinc/polycarboxylate complexes that are retained within the oral cavity to provide sustained antimicrobial protection against oral pathogens. In this work an equilibrium dialysis technique was developed to study both the ionic association of zinc with the polycarboxylate, and its release in the presence of ions representing those present in human saliva. Zinc/polymer complexes were prepared by adding 1.39 mM zinc sulphate (10 mL) gradually to 0.1% (w/v) aqueous polycarboxylate dispersion (10 mL). This mixture was placed inside pre-treated PVDF dialysis tubing and subsequently into a dialysate solution (480 mL). The release of the zinc from the complex was compared with that from a zinc sulphate solution as the control. Samples removed from the dialysate solution were analysed using Atomic Absorption Spectroscopy with a flame atomiser. The release of zinc from the complex was also investigated in different pHs, chosen to represent the variety of environments within the oral cavity. No zinc was released from the complex after 6 h when dialysing against de-ionised water, compared with > 60% released in the control. After 24 h, zinc released from the complex remained undetectable in the dialysate, compared with > 90% in the control. When the complex was dialysed against an isotonic solution of NaCl and CaCl at pH 5.5, approximately 56% zinc had been released after 6 h; this increased to 77% after 24 h (Table 1). Zinc released from the control was > 70% after just 3 h. When the isotonic solution was adjusted to pH 4.0, the release of zinc from the complex was increased. Conversely, when the isotonic solution was adjusted to pH 7.0, zinc release from the polymer decreased. This study indicates that an association forms between the polycarboxylate and zinc ions. The presence of sodium and calcium ions will allow displacement of the zinc, which is increased as the pH was lowered. Bioadhesive polymers will increase the substantivity of the antimicrobial at a site often affected by poor retention times via mucosal adhesion. These complexes have the potential to form a bio-responsive antimicrobial delivery system that will preferentially release the antimicrobial agent in lower pH environments, such as those generated in the oral cavity by cariogenic microorganisms.

Table 1 Percentage release of zinc from the polycarboxylate complex in the presence of an isotonic solution composed of NaCl and $CaCl_2$ at differing pHs

Time (h)	Control	Complex		
		pH 5.5	pH 4.0	pH 7.0
0	0.00	0.00	0.00	0.00
1	42.87	19.74	28.99	15.53
2	62.53	33.49	51.26	27.04
3	73.51	43.14	65.41	35.53
4	78.22	48.22	71.04	40.67
5	80.88	53.73	75.02	44.13
6	83.70	56.40	77.92	48.38
24	89.22	77.18	85.91	71.93

Release of zinc from the complex was compared to a zinc sulphate control (n = 6).

Kochisch, S., Rees, G. D., Young, S., et al (2001) *J. Controlled Release* **77**: 1–6
Needleman, I. G., Martin, G. P., Smales, F. C. (1998) J. *Clin. Periodontol.* **25**: 74–82
Scheie, A. (1989) *J. Dent. Res.* **68**: 1609–1616

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Spray drying as a method for preparing cationic PLGA microparticles intended for pulmonary delivery

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Polyethylenimines (PEI) as cationic polymers has gained considerable attention as one of the most efficient synthetic DNA carriers (Alpar et al 2002). They offer significantly more efficient protection against nuclease degradation than other polycations (e.g. poly(L-lysine)), possibly due to their higher charge density and more efficient complexation (Benoit et al 2002). Spray drying as a method for preparing dry powders intended for inhalation has many advantages with respect to pulmonary drug delivery as it permits control of particle size, size distribution,

particle shape, density and the possibility to spray dry the heat sensitive macromolecules with negligible degradation (Kristensen et al 2001). The method used was a modified emulsion spray drying method. A volume of PEI solution (internal phase) was added to a volume of the organic polymer (PLGA) solution, homogenized at 20 000 rev min^{-1} for 2 min in ice. This water-in-oil emulsion was immediately transferred into a beaker containing PVA 1% w/w and homogenized at 3000 rev min^{-1} for 5 min (room temperature). The resulting double emulsion was spray dried using a Buchi 191 spray drier. Surface charge density (Zeta potential) was measured using a Malvern Zetamaster 2000. Particles size was determined by laser diffractometry using a Malvern Mastersizer X (Malvern Instruments, UK) and was expressed as weighted mean of the volume distribution. Scanning electron microscopy was used to characterize the particles shape and morphology. DNA (pCMV*lux*) (Clontech, USA) was adsorbed to the spray dried particles and the loading was determined by an indirect (depletion) method using the ultra sensitive fluorescent nucleic acid stain. Adding PEI had a clear impact on the surface charge of the prepared microspheres, as it directly increased by increasing the concentrations of PEI (max 1% w/w PEI). Scanning electron microscopy indicated that the shape and morphology of the microspheres were both affected by PEI. Spray dried particles without PEI had an irregular shape while the particles containing different concentrations of PEI showed a smooth surface and a more distinctive spherical shape. Adding PEI to the spray dried particles increased the loading efficiency (95% loading for 1% w/w PEI) and this may be due to the fact that PEI as a cationic polymer is able to effectively complex DNA molecules possibly due to their higher charge density. The findings of this study demonstrate that spray drying is a suitable technique for the formulation PLGA-PEI microparticles capable of efficiently complexing DNA as a gene delivery system. The effect of PEI content on transfection efficiency is currently under investigation.

Alpar, H. O., et al (2002) *J. Drug Targeting* **10**: 143–151
Benoit, et al (2002) *J. Controlled Release* **81**: 379–388
Kristensen, K. L., et al (2001) *Int. J. Pharm.* **212**: 187–194

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Delivery of liposomes generated from proliposomes using air-jet, ultrasonic, and vibrating-mesh nebulisers

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The suitability of nebulisers for delivering liposomes is well established. Proliposomes (Payne et al 1986) comprise free-flowing particles coated with phospholipids, which produce liposomes on adding aqueous phase. This work investigated whether proliposomes produce potentially inhalable liposomes when hydrated in, and delivered from, nebulisers of different operating principles. A pear-shaped flask containing 2 g sucrose particles (300–500 μm) was attached to a modified rotary evaporator under vacuum, and held at 40°C. A chloroformic solution (60 mg mL^{-1}) of soya phosphatidylcholine (0.264 g) and cholesterol (0.136 g) was added portion-wise, and chloroform removed under vacuum. Proliposomes (0.56 g) were added to 5 mL deionised water in a Pari LC Plus (air-jet) nebuliser attached to a Pari Master compressor, Liberty (ultrasonic) nebuliser or Omron NE-U22 (vibrating-mesh) nebuliser. The Omron nebuliser was shaken manually for 1 min before nebulisation to "dryness" (approx. 30 min). Both Pari and Liberty nebulisers were operated to "dryness" (approx. 25 min and 12 min, respectively) with no prior shaking (these nebulisers' mechanisms of operation provide sufficient agitation). The aerosol was directed into a two-stage impinger, with flow rate of 60 L min^{-1}. Samples were taken from nebuliser reservoirs and impinger stages for transmission electron microscopy (TEM) and size measurement by laser diffraction. In other experiments, aerosol droplet size was measured using laser diffraction, mass output was calculated by weight difference, and phospholipid output and distribution in the impinger was analytically estimated (Stewart 1980). Multilamellar liposomes were observed by TEM. Laser diffraction showed that measured size was greater in the nebulisers than in the impinger, possibly indicating aggregation within the nebuliser and vesicle size reduction of liposomes during nebulisation to the impinger (Table 1). The volume median diameter (VMD ± s.d.) of aerosol droplets was nebuliser dependent, being $2.5 \pm 0.31\,\mu$m, $3.86 \pm 0.27\,\mu$m and $5.54 \pm 1.55\,\mu$m for Pari, Liberty and Omron nebulisers, respectively. Mass outputs (%) were 85.97 ± 1.16, 52.72 ± 6.27 and 90.95 ± 5.13 for the Pari, Liberty and Omron, respectively. These exceeded phospholipid outputs (%), which were respectively 56.53 ± 6.20, 4.57 ± 1.80 and 41.05 ± 8.78, indicating concentration of phospholipids within nebulisers. The delivery of phospholipid (as liposomes) deposited in the 2nd stage of the impinger (representing the fine particle fraction) was in the order Pari > Omron ≫ Liberty nebuliser (Table 2). This study has shown that with air-jet or vibrating-mesh nebulisers, proliposomes provided a liposome formulation with high mass and phospholipid outputs, with a large fraction of phospholipid delivered to the 2nd stage of the impinger. As previously shown for conventional liposomes, a traditional ultrasonic nebuliser was inefficient for liposome delivery (Bridges & Taylor 1998).

Table 1 VMD (μm) of liposomes in the nebuliser and impinger

Nebuliser	Reservoir	1st stage	2nd stage
Pari	6.74 ± 5.45	3.76 ± 0.18	3.18 ± 0.44
Liberty	6.50 ± 0.40	Not detected	Not detected
Omron	15.76 ± 3.52	3.55 ± 0.11	4.00 ± 0.41

Data are means ± s.d., n = 3.

Table 2 Phospholipid distribution between nebuliser and impinger

Nebuliser	Reservoir (%)	1st stage (%)	2nd stage (%)
Pari	43.47 ± 6.20	11.30 ± 0.61	45.23 ± 6.12
Liberty	95.43 ± 1.80	1.56 ± 0.55	3.01 ± 1.26
Omron	58.95 ± 8.78	6.65 ± 4.46	34.41 ± 4.68

Data are means ± s.d., n = 3.

Bridges, P. A., Taylor, K. M. G. (1998) *Int. J. Pharm.* **173**: 117–125
Payne, N. I., et al (1986) *J. Pharm. Sci.* **75**: 325–329
Stewart, J. C. M. (1980) *Anal. Biochem.* **104**: 10–14

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Calorimetric study of conventional liposomes and those formed from ethanol-based proliposomes

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The understanding of liposome phase transitions is important in determining liposome stability in-vitro and in-vivo. Differential scanning calorimetry (DSC) is well established for studying phospholipid phase transitions, with the pretransition reported to be more sensitive than the main (chain-melting) transition to the incorporation of additives such as drugs (Fildes & Oliver 1978). Proliposomes are ethanolic phospholipid solutions which generate liposomes on adding aqueous phase (Perett et al 1991). In this work, the thermal behaviour of conventional multilamellar liposomes (MLVs) and those prepared from proliposomes was studied by high sensitivity DSC (HSDSC). MLVs were made by dissolving dimyristoylphosphatidylcholine (DMPC) in chloroform in a round-bottomed flask. The flask was attached to a rotary evaporator and held at 40°C with vacuum applied for 30 min for solvent evaporation and thin film formation. The film was hydrated at 40°C with deionised water giving a lipid concentration of 62.5 mg mL^{-1} and shaken for 10 min and annealed for 2 h before storage overnight at 6 ± 2°C. Liposomes generated from proliposomes were made by dissolving DMPC (165 mg) in ethanol (132 mg) in a 7-mL glass vial. Proliposomes were hydrated to form liposomes as for MLVs, giving the same phospholipid concentration, then stored or flushed with nitrogen for 5 min before storage. Liposome size was measured using laser diffraction and 0.8-mL samples were studied using HSDSC (Setaram, DSC III) from 6 to 45°C at a scan rate of 1°C min^{-1}. For transitions, the onset (T_o) temperature was determined and enthalpy calculated. The volume median diameter (VMD) of liposomes generated from proliposomes was smaller than for conventional MLVs (Table 1). However, the size of such liposomes increased with nitrogen flushing suggesting that residual ethanol may affect the size and/or aggregation of these liposomes. Conventional MLVs exhibited an endothermic pretransition and a main transition, whereas in both proliposome formulations the pretransition was absent (Table 2). The absence of a pretransition even with flushed samples suggested that residual ethanol remains bound within the liposomes. A previous study indicated that ethanol induced digitations of phospholipid molecules in conventional MLVs, with loss of the pretransition (Verio et al 1987). With proliposome-generated liposomes the main transition enthalpy increased, suggesting ethanol

increased the energy required to effect the transition. This study has shown that liposomes generated from proliposomes exhibit different thermal behaviour compared with conventional MLVs, with no measurable phospholipid pretransition. This suggests such liposomes retain some ethanol within their bilayers, which is not removed by flushing preparations with nitrogen.

Table 1 The effect of formulation on size of liposomes

Formulation	VMD (:m)	Span*
MLVs	7.48 ± 0.18	1.90 ± 0.06
Proliposome	3.88 ± 0.13	1.26 ± 0.02
Proliposome (flushed)	6.87 ± 0.66	1.42 ± 0.00

Data are means ± s.d., n = 3.
*Span = (90% undersize-10% undersize)/VMD.

Table 2 Transition temperature and enthalpy of liposome formulations

Liposome	Pretransition		Main transition	
	Temp. (°C)	ΔH ($kJ\,mol^{-1}$)	Temp. (°C)	ΔH ($kJ\,mol^{-1}$)
MLVs	15.17 ± 0.02	3.33 ± 0.26	24.84 ± 0.05	21.04 ± 0.70
Proliposome	—	—	23.08 ± 0.06	25.03 ± 1.05
Proliposome (flushed)	—	—	24.75 ± 0.03	26.63 ± 0.47

Data are means ± s.d., n = 3.

Fildes, F. J. T., Oliver, J. E. (1978) *J. Pharm. Pharmacol.* **30**: 337–342
Perrett, S., et al (1991) *J. Pharm. Pharmacol.* **43**: 154–161
Verio, J. A., et al (1987) *Biochim. Biophys. Acta* **900**: 230–238

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Stability of spray dried lysozyme with and without additives evaluated by high sensitivity differential scanning calorimetry (HSDSC) and biological activity assay

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One of the best methods to preserve proteins for long periods is to store them in a dry state. There are various methods used to prepare proteins as a solid form (Maa & Prestrelski 2000), including lyophilization and spray drying. The dehydration stress (removal of water) from a protein is one of the stability issues associated with protein manufacturing (Arakawa et al 2001). In this study the effect of gamma-cyclodextrin (γ-CD) and hydroxypropyl-β-cyclodextrin (HP-β-CD) on the stability of lysozyme subjected to thermal and dehydration stress has been investigated. Since the action of a therapeutic protein is dependent on protein molecular conformation, a biological assay for protein activity was employed using *Micrococcus lysodeikticus* as a substrate. An aqueous lysozyme solution (1% w/v) with and without additives was spray dried (BÜCHI B-191 mini spray drier) at a flow of 4–5 $mL\,min^{-1}$ and dried at an inlet temperature of 125 ± 2°C, outlet temperature was 60 ± 1°C. Table 1 represents a comparison between data obtained from HSDSC and activity analysis. The area under the HSDSC trace due to protein unfolding has been used as an estimate of protein integrity. In the case of lysozyme before and after spray drying without additives, it was found that there is a good correlation between data obtained from HSDSC and activity test (91% and 90%, respectively). However, no such correlation was observed in case of γ-CD and HP-β-CD. The %ΔHcal of lysozyme spray dried with HP-β-CD was 92% of the non spray dried one, yet this preparation showed 99% of enzyme activity. The spray dried preparation containing γ-CD gave 96.8% and 91.45% for ΔHcal and activity, respectively, compared with the non spray dried preparation. Similar behaviour was observed for β-galactosidase (Branchu et al 1999). One factor that could explain the effect of HP-β-CD on protein activity is the protective effect imparted by HP-β-CD on the protein upon reconstitution. It is known that reconstitution of dry proteins is a detrimental step for proteins where aggregation has often take place (Branchu et al 1999). Since some cyclodextrins are known to promote unfolding in solution the use of calorimeter data to estimate biological activity is compromised.

Table 1 A correlation between thermodynamic parameters and activity test

Additives	mg ratio of lysozyme: additive	Activity (%)	% ΔHcal from HSDSC (referred to non spray dried)
Without additive	5:0	90.3	91.0
HPCD	5:10	99.0	91.7
γ-CD	5:10	91.5	96.8

Arakawa, T., Prestrelski, S., Kenney, W., et al (2001) *Adv. Drug Deliv. Rev.* **46**: 307–326
Branchu, S., Forbes, R., York, P., et al (1999) *Pharm. Res.* **16**: 702–708
Maa, Y., Prestrelski, S. (2000) *Curr. Pharm. Biotechnol.* **1**: 283–302

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Novel thermal stabilisers of lysozyme evaluated by high sensitivity differential scanning calorimetry

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The advent of genetic engineering has allowed the large-scale production of virtually any protein for therapeutic purposes. However, the native structure and activity of protein are often detrimentally affected during different manufacturing processes (isolation, purification, preparation, storage and delivery) due to the inherent physical and chemical instability of proteins (Branchu et al 1999). Therefore, many attempts have been made to enhance protein stability in solution and in the dried state. They could be summarized into four strategies, which comprise physical modification of protein, chemical modification, using protein engineering and by adding materials, called stabilizers, to proteins (Ó'Fágáin 2003). Since the major instability of large molecules results from the change in molecular conformation, thermodynamic parameters could reflect the changes at molecular level (Leharne & Chowdhry 1998). Using High Sensitivity Differential Scanning Calorimetry, (HSDSC) (Micorocal Inc., MA), the transition temperature, Tm, and folding reversibility of lysozyme have been measured in the presence and absence of additives. The addition (in different concentrations) of the new additive sucrose cocoate-cocoa fatty acid (SCCFA) and other additives, comprising maltohexaose, maltoheptaose, tween 80, gamma-cyclodextrin (γ-CD) and hydroxypropyl-β-cyclodextrin (HP-β-CD), to lysozyme solution (a model protein) have been examined. The results of HSDSC analysis show that the addition of SCCFA, maltohexaose and maltoheptaose lead to an increase in the Tm of lysozyme by 0.3, 0.7 and 0.7°C, respectively, over control. No change in Tm was observed with the addition of cycoldextrins, although a slight decrease in Tm was observed upon addition of tween 80 to lysozyme solution. Regarding folding reversibility, SCCFA showed the highest folding reversibility of lysozyme (84%) compared with control (80.9%), γ-CD (81.6%), HPCD (79.7%) and tween 80 (76.6%). The thermal stability of lysozyme that was obtained in the presence of SCCFA may be attributed to the presence of sucrose in the surfactant composition. Sucrose has been known to stabilize proteins against thermal denaturation Izutsu et al (1991). The enhancement in Tm of lysozyme in the presence of maltohexaose and maltoheptaose was similar to other polyol thermal preservation behaviour with proteins.

Branchu, S., Forbes, R., York, P. et al (1999) *Pharm. Res.* **16**: 702–708
Izutsu, K.-I., Yoshioka, S., Takeda, Y. (1991) *Int. J. Pharm.* **71**: 137–146
Leharne, S., Chowdhry, B. (1998) In: Landbury, J., Chowdhry, B. (eds) *Biocalorimetry: application of calorimetry in the biological sciences*. John Wiley and Sons Ltd, Chichester
Ó'Fágáin, C. (2003) *Enzyme Microb. Technol.* **33**: 137–149

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DNA-coated microcrystals: potential vehicles for pulmonary gene delivery?

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Here we describe a new mode of self-assembly of DNA on crystalline surfaces. This generates micro-crystals of water-soluble compounds such as amino-acids

or sugars, coated with DNA. The formation of DNA-coated microcrystals (DCMC) is straightforward involving a simple coprecipitation from aqueous into a water miscible organic solvent. The self-assembly process utilises crystal lattice energies as the driving force for organising the DNA on the crystal surface: DNA molecules essentially behave like an impurity in a conventional crystallisation but since they are virtually insoluble in the surrounding solvent they remain confined to the crystal surface. This novel process leads to the formation of fine, free-flowing powders of microcrystals coated with DNA. For oligonucleotides coated onto a valine carrier, thin, leaf-like micron sized crystals are typically formed. Confocal laser scanning fluorescence microscopy of DNA-valine microcrystals prepared with a fluorescently-labelled oligonucleotide (DQA-HEX) demonstrated that the oligonucleotide was present on the crystal surface. Tapping mode atomic force microscopy imaging (TM-AFM) showed that the oligonucleotides are located in a fairly uniform layer on the surface of the crystals. Bioactivity of oligonucleotide (DQA-HEX) was retained in dried DNA-coated microcrystals, as demonstrated by PCR: oligonucleotides used as primers for PCR resulted in amplification of the expected gene product from human chromosomal DNA. Sequencing of the gene product showed that the primer sequence remained unchanged during the formation of DNA coated microcrystals and in particular no bases were truncated. We have previously shown that the same principle of crystal lattice mediated self-assembly described above can be exploited to prepare protein-coated microcrystals (PCMC) (Moore et al 1999; Kreiner et al 2001). PCMC have significant potential for the pulmonary delivery of proteins (Moore et al 2003) because they are non-hygroscopic and can be produced with an aerodynamic diameter suitable for efficient delivery to the deep lung. In analogy, it is anticipated that DCMC will have similar useful properties for pulmonary delivery. The lung represents an attractive organ for non-viral gene delivery for the treatment of, for example, cystic fibrosis or lung cancer. As first steps in this direction, HeLa cells were successfully transfected with GFP-plasmid DNA, which was coated onto valine. There was no significant difference to the transfection efficiency between GFP-plasmid-microcrystals and the control (GFP plasmid without any further treatment). Further, particle distribution studies of dried DCMC were carried out using a Multi Stage Liquid Impinger (MSLI). The recovered fine particle fraction (FPF) was approximately 30% of the initial dose delivered to the MSLI. Overall, this indicates that DNA coated microcrystals have a potential as very useful delivery vehicles for pulmonary DNA delivery for gene therapy.

Moore, B. D., et al (1999) Rapid Dehydration of Proteins. PCT/GB00/01854
Moore, B.D., et al (2003) Pharmaceutical Composition. PCT/GB04/000044
Kreiner, M., et al (2001) *Chem. Commun.* **12**:1096–1097

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Engineering the morphology of protein coated amino acid crystals

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An important issue for the delivery of protein therapeutics is the production of free flowing crystalline products with a specific particle size and morphology, together with full retention of bioactivity and high stability. A particle production method that allows a tight control of particle engineering while retaining these characteristics is advantageous. Previously we described a novel method of producing stable protein coated microcrystals (PCMC) with good chemical and physical stability that have a huge potential as therapeutic biomolecule drug delivery vehicles (Moore et al 1999). This method involves the delivery of an aqueous solution containing protein (in this study either bovine serum albumin (BSA) or subtilisin Carlsberg (SC)) and a coprecipitant (here an amino acid) to a water miscible organic solvent (or more precisely antisolvent) under a controlled mixing environment using either a batch or a continuous process. This triggers off the rapid dehydration of the aqueous components and results in the formation of protein coated amino acid crystals. By controlling important process parameters that influence the stability and morphology of the crystals formed an optimum formulation can be produced with desired characteristics, such as particle size or protein-loading, for a particular application. Primarily, the coprecipitating amino acid sets the general shape of the crystals produced, while the organic antisolvent has a more subtle influence on crystal morphology. Taking L-alanine as an example, precipitation in different organic solvents result in needle shaped crystals that vary in the relative rate of growth of the different crystal faces, in particular the horizontal plane. The presence of protein as a coprecipitant inhibits the crystal growth of the amino acid core, resulting in the formation of smaller crystals. The inclusion of additives as coprecipitants can result in some dramatic morphological changes. For example, the precipitation of SC/L-glutamine PCMC in ethanol produces rod shaped crystals upon filtration. However the introduction of NaCitrate and NaCl into the aqueous precipitating solution produces spherical aggregates upon filtration. XRPD of a number of PCMC preparations show the amino acid core to be highly crystalline in all cases. Analysis of the dried formulations by Dynamic Vapour Sorption (DVS) reveal no amorphous-crystalline transitions and show that the amino acid core material exhibits very low hygroscopicity at relative humidities up to 80%. Furthermore DSC and in-situ CD and Raman spectroscopy have highlighted that the protein as presented on the crystal surface, does not undergo secondary structural changes and on reconstitution in aqueous solution adopts its native conformation. Previous Multi Stage Liquid Impinger (MSLI) data suggested that PCMC have the potential to deliver a payload of greater than 40% to the deep lung using basic DPIs (Ross et al 2002). More importantly in this study albumin/L-glutamine PCMC exhibited the same performance in the MSLI before and after exposure to a sorption/desorption cycle set to 80% RH in the DVS. In conclusion these protein-amino acid powder formulations are very promising candidates for pulmonary and parenteral drug delivery. Moreover they offer easy routes to controlled crystal engineering of size and morphology.

Moore, B. D., et al (1999) Patent application number: PCT/GB00/01854
Ross, A. C., et al (2002) Proc. 29th Intern. Symp. Control. Rel.Bioact. Mater. 53

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In-vivo electro-stimulated release of a model drug from chitosan hydrogel

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Interest in responsive hydrogels for utilisation as smart drug delivery system has increased to meet the need for better control of drug delivery. Hydrogels composed of polyelectrolytes respond to the application of an electric current and release drug via changes in gel composition such as gel collapse, syneresis and rheological behaviour. The electro-responsive release of drugs in-vitro from chitosan hydrogels have been previously reported by Ramanathan & Block (2001) and ourselves (Jahan & Mudan 2004). In this abstract, we report on the in-vivo electro-responsive release of a model drug, diclofenac sodium (DFNa) in rats, from chitosan hydrogels. Drug-loaded chitosan hydrogels were prepared at room temperature (~25°C), following a method modified from Ramanathan & Block (2001), by reacting chitosan, ethanol, and acetic anhydride. High molecular weight chitosan (hMr; Mr ~600 000) at 0.5% w/v, was dissolved in 4 mL of 10% v/v acetic acid solution. The viscous chitosan solution was diluted with 6 mL of ethanol and gelation was induced with 0.70 mmol (66 μL) of acetic anhydride. The mixture was then transferred into a Teflon mould and left undisturbed overnight. The gels were homogeneous, transparent, colourless and firm to the touch. DFNa was loaded in the gel as part of the reaction mixture at a concentration of 0.2% (w/v). The drug was dissolved in the ethanol component of the reaction mixture before the addition of acetic anhydride. The DFNa-loaded chitosan hydrogel was hydrated by placing the gel in deionised water for 30 min and then ($\sim 2 \times 2 \times 10$ mm) was surgically implanted subcutaneously under the shaved abdominal skin of anaesthetised male Wister rats (210–230 g). The surgical incision was sealed using cyanoacrylate adhesive. Pulses of electrical current (0.4 mA, 0.5 mA cm^{-2}) were then applied for 10 min at 0, 30, 60 and 90 min using Ag/AgCl resting ECG electrodes placed on the shaved skin of the rat. The anode was placed on top of the implant while the cathode was placed 2 cm away, still on the shaved abdomen. The experiment was followed for 2 h. Blood samples were taken from the tail vein at time zero and after every electrical stimulus and the plasma was analysed for DFNa by High Performance Liquid Chromatography. Passive release experiments (control) were conducted in the same way, except that no electric current was applied. We found that DFNa could be released from chitosan hydrogel in a pulsatile fashion in response to repeated pulses of electrical stimuli. Some release of DFNa during the "off" period was also observed, probably due to drug diffusion along a concentration gradient. The electro-stimulated release of DFNa is attributed to syneresis of the gel, with concomitant drug expulsion and/or due to electrophoresis of the negatively charged drug towards the anode. This reflected passive drug release, again along a concentration gradient, in the control experiments. At the end of the experiment ~70% of DFNa was released from the implant which was electro-stimulated while ~40% was released under passive conditions. In conclusion, we demonstrated the pulsatile drug release from an electro-responsive chitosan hydrogel in-vivo, in rats. Such stimulated release in-vivo has only been reported once before (Kagatani et al 1997).

Jahan, I., Mudan, S. (2004) Proceedings of the Controlled Release Society Annual Meeting, Honolulu, Hawaii.
Kagatani, S., Shinoda, T., Konno, Y., et al (1997) *J. Pharm. Sci.* **86**: 1273–1277
Ramanathan, S., Block, L. H. (2001) *J. Controlled Release* **70**: 109–123

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Electro-stimulated release of a model drug from chitosan microspheres—an in-vivo study

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Many different hydrogels have been investigated as electro-responsive drug delivery systems (DDS). Most of these gels have to be implanted. To make this DDS more patient-friendly, hydrogels can be fabricated into microparticles, which can be injected rather than surgically implanted. Electro-responsive release of model drugs from chitosan hydrogel and microspheres has previously been shown in-vitro (Ramanathan & Block 2001; Jahan & Mudan 2004). In this abstract, we report an investigation in rats to determine whether electro-stimulated release of a model drug can be achieved in-vivo. Microspheres of low molecular weight chitosan (Mr ~150 000) were prepared by a method modified from He et al (1999). Drug-loaded chitosan microspheres were prepared by adding the drug, diclofenac sodium (DFNa) to a chitosan solution before the addition of the cross-linker (2% gluteraldehyde) and spray drying the mixture. The cross-linked particles had volume mean diameter of $3.14 \pm 0.14\,\mu m$, zeta potential of 40.5 ± 0.23 mV and an entrapment efficiency of 55.92 ± 0.83. Unhydrated particles do not conduct electricity, thus the drug loaded microspheres were hydrated in deionised water for 24 h before implantation. Hydrated microspheres suspended in deionised water (1.67 mg mL^{-1}; 2 mL) were injected subcutaneously using a 23 G needle under the shaved abdominal skin of anaesthetised male Wistar rats (210–230 g). Ag/AgCl resting ECG electrodes were placed on the skin, the anode being on the top of the injection site and the cathode being 2 cm apart. Pulses of electrical current (0.4 mA, 0.5 mA cm^{-2}) were then applied for 10 min at 0, 30, 60 and 90 min. Blood samples were collected at time 0 and after each pulse from the tail vein for 2 h and the plasma was analysed for DFNa by High Performance Liquid Chromatography. As control, passive release experiments were conducted in the same way except that no electric current was applied. A burst release of DFNa was observed, after which DFNa levels rose slowly in both electro-stimulated and in control rats. The burst release and the subsequent gradual drug release were greater in rats that had been electro-stimulated; enhanced drug release was probably via ectrophoresis of the drug towards the anode. An "on-off" release pattern was not, however, observed (i.e. drug was still released from the microspheres during the "off" period probably by diffusion along a concentration gradient). This reflects the diffusion of drug from the chitosan microspheres and absorption in blood in control rats. At the end of the experiments which lasted 2 h, ~15% of drug was released from chitosan microspheres under the influence of electric current while ~7% was released in control rats. Despite the absence of an obvious "on-off" pulsatile drug absorption profile, this is the first report of electro-responsive drug release from gel microspheres in-vivo. Further work needs to be conducted on the formulation of chitosan microspheres to improve the "on-off" response in-vivo.

He, P., Davis, S. S., Illum, L. (1999) *Int. J. Pharm.* **187**: 53–65
Jahan, I., Mudan, S. (2004) Proceedings of the Controlled Release Society Annual Meeting, Honolulu, Hawaii
Ramanathan, S., Block, L. H. (2001) *J. Controlled Release* **70**: 109–123

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Mannitol as a carrier for proniosomes

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Proniosomes consist of dry, free flowing powder that on hydration forms a multilamellar niosomal suspension, suitable for administration by oral and other routes. Sorbitol (Hu & Rhodes 1999) and maltodextrin (Welsh & Rhodes 2001) have been used as carriers for proniosomes. In this investigation proniosomes were prepared using mannitol as a carrier and compared with maltodextrin based proniosomes. Ketorolac tromethamine was used as a model drug. Proniosomes of mannitol (MTL) and maltodextrin (MDX) were prepared by slurry method. Maltrodextrin and mannitol were taken in round bottom flask, and varying amount of stock solution containing span 60, cholesterol and dioctylsodiumsulphosuccinate (47.5: 47.5: 5) in diethyl ether was added to it, for different surfactant loading. The flask was attached to rotary evaporator and rotated at 100 rev min^{-1} at 35°C, until the preparation was dry and free flowing. Niosomes were prepared from proniosomes by hydrating the proniosome powder with hot aqueous solution of drug at 80°C and vortexing for 2 min. Niosomes so obtained were characterized for particle size, entrapment efficiency and drug stability. Particle size was determined by optical microscopy. Entrapment efficiency was determined by separating unentrapped drug from niosomal suspension by passing through a 0.2-μm membrane filter. Solution of stability studies and entrapment efficiency were analysed for drug content by injecting 20 μL into HPLC system (Waters), equipped with 600 pump controller, 2847 dual λ absorbance detector, 7725i rhodyne injector, using acetonitrile:water (0.5% o-phosphoric acid) (65:35) in an isocractic run through C8 spherisorb 5 μm (250×4.6 mm i.d.) column. The eluent was monitored spectrophotometrically at 312nm. The results indicate that niosomes prepared from mannitol provided higher entrapment efficiency and stability of Ketorolac than maltodextrin (Table 1). The particle size was found to be in the range 3.06–12.6 μm for MDX and 3.39–4.62 μm for MTL. Thus, mannitol is a suitable carrier for proniosomes.

Table 1 Comparison of mannitol and maltodextrin

Surfactant loading	Entrapment efficiency (%)		Degradation (%)		Particle size (μm)	
	MDX	MTL	MDX	MTL	MDX	MTL
1X	79.0	87.0	48.7	30.3	12.6	4.6
2X	84.0	97.0	49.2	28.7	3.1	4.2
4X	86.0	89.0	30.3	27.1	4.1	3.9
8X	86.4	89.0	58.8	28.2	3.1	3.9
16X	85.6	87.7	17.8	29.0	4.1	3.9
32X	86.0	88.0	24.7	28.5	4.3	3.4
64X	85.6	89.0	25.3	27.9	5.4	3.6

Hu, C., Rhodes, D. G. (1999) *Int. J. Pharm.* **185**: 23–25
Welsh, A. I. B., Rhodes, D. G. (2001) *AAPS Pharm. Sci.* **3**: 1–8

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Chemical modification of the edible film polysaccharide pullulan

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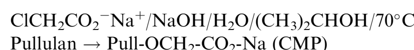
Pullulan is an extracellular water-soluble microbial polysaccharide produced by different strains of *Aureobasidium pullulans*. It is a linear, mixed linkage α-D-glucan consisting of maltotriose units interconnected via α 1–6 linkages. Pullulan has been structurally modified by incorporation of various hydrophobic functionalities resulting in changes in viscosity. The foregoing chemical modifications have been performed in two steps from the parent pullulan. The first step involves carboxymethylation of pullulan with sodium chloroacetate yielding carboxymethyl pullulan (CMP):


$ClCH_2CO_2^-Na^+/NaOH/H_2O/(CH_3)_2CHOH/70°C$
Pullulan $\rightarrow$ Pull-OCH_2-CO_2-Na (CMP)

The second step involves the modification of CMP by coupling alkyl amines of various chain lengths onto the carboxylic groups of CMP, using the coupling agent dicyclohexylcarbodiimide. The alkyl amines used in this modification step were: hexylamine, decylamine, dodecylamine, tetradecylamine and hexadecylamine. When the length of the hydrophobic alkyl tails is increased interchain association becomes more pronounced, resulting in a higher viscosity. Other chemical structural modifications have been performed resulting in different degrees of viscosity change in the resulting polymer compared with the parent pullulan. The hydrophobic modification of pullulan has two purposes: to increase the viscosity of pullulan to enhance formulation characteristics; currently viscosity enhancers or mixtures of polymers are required to improve formulation properties, the provision of hydrophobic domains within the poly-

mer matrix enables the incorporation of small molecular weight material into the polymer. The solution properties of the various pullulan derivatives have been studied over a range of temperatures (30, 40 and 50°C) in 0.05 M sodium sulfate electrolyte solution. Generally pullulan and its derivatives show decreased viscosity with increased temperature. The viscosity of pullulan drastically increases with the addition of long chain alkyl groups and the polymer aggregates intermolecularly. These differing alkyl amine chain lengths show varying viscosity characteristics. The synthesised polymers have been analysed with a number of spectral methods including: NMR, Raman, infra red and GPC. NMR has proved to be useful as an analytical technique; however, its application is limited due to low mobility of the molecules, resulting in poor signal to noise ratios. Raman spectroscopy has shown to be particularly valuable in identifying differing functionalities, and assessing the degrees of polymer modification.