Poster Session 1 – Drug Delivery

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Screening of inhalation formulations: quantification of individual particulate interactions

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Drugs delivered via the respiratory tract are frequently micronised creating microparticles in the respirable range. Such powders possess high surface areas/energies, and form agglomerates or adhere to packaging surfaces, leading to problems with dosage control, dispersion and delivery in inhalers. Factors contributing to adhesion include capillary, electrostatic forces, particle surface free energy, etc. Atomic Force Microscopy (AFM) can measure forces between particulates adhered to AFM tips and a sample surface (Ducker & Senden 1992). The use of AFM for screening inhalation components by measurement/mapping of adhesive interaction forces, preliminary electrostatic forces and work of adhesion for single particles, is reported. AFM measures the degree, locality and nature of interactions between inhaler components (i.e. characterising forces between an active drug, micronised carrier particles and polymer canister coating). These measurements are obtained by attaching the particle of interest to an AFM cantilever and obtaining force-distance curves using a DI Multimode IIIa (Eve et al 2002) or Enviroscope. Such data calculates adhesion forces between the immobilised drug microparticle and substrates of interest, at $< 5\%$ RH to reduce capillary interaction forces. Force measurements were made against HOPG and contact area assessed, allowing the work of adhesion to be quantified for single particles (Hooton et al 2003). Particle tips were imaged using tip characterisation gratings. Electric Force Microscopy (EFM) allows mapping of electrostatic charges using a biased probe in controlled humidity environments. In particle-particle measurements, if the spring constant of the cantilever is determined, using the force-distance curves obtained ($\geq$100 for each substrate), the magnitude of the tip/substrate adhesion is calculated. Measurements are typically recorded for three different tips. A consistent adhesion ranking to different components is obtained, with variations in magnitude of adhesion between each tip, due to differences in geometry/chemistry of the attached particles. Compensation for particle roughness and contact area is achieved through estimation of contact radii using specially designed grids. The work of adhesion for single particles is also determined. A force of adhesion map between a functionalised tip and a large carrier particle can be obtained. Spatial variations in adhesion are observed, which may correlate to active sites for active particle absorption. Repeated contact of the functionalised probe to the polymer substrate revealed a large increase in the force of adhesion, attributed to tribo-charging of the polymer, which was investigated using EFM. Mapping of polarity/magnitude of charge across the polymer shows an overall negative charge on the surface, with some spatial heterogeneity. AFM allows ranking of interactions between inhalation components, at a single particle level. It is possible to obtain spatial maps of variations of adhesion of an active particle across a carrier. These measurements can be performed with controlled humidity, or a variety of solvent environments. A 3-D representation of the contact area involved in the interaction was produced, and allows both qualitative and quantitative comparisons between different samples. This provides new methods of early screening of small quantities of novel active ingredients for suitability of aerosol delivery. The ability to image/measure the magnitude of the electrostatic interaction at varying humidity provides a novel method to screen potential mechanisms for irreproducibility of administered dosage.

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018
Challenges in the development of drug eluting stents: swelling behaviour characterisation

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Second generation coronary stents encompass drug loaded with polymer coatings on the surface. Successful development of these stents requires knowledge of the property of the polymer coatings after being immersed in relevant environments to understand and control the integrity and durability of the coating, drug release behaviour and potential bulk biological interaction of polymer with tissue. This study highlights the possibility of monitoring the swelling behaviour of polymer systems using atomic force microscopy (AFM). To study the capability of AFM to quantify the swelling behaviour of a polymer coating on stents, a hard gelatin film was first used as a model sample. The sample was prepared by partially immersing the film into cold ultra pure water for a short period of time. AFM was then used to locate, and image the interfacial region between dry and wet parts of the sample. It has been identified that the swollen region appeared as a high area and the non-immersed as a low area in the AFM height image. Furthermore, the use of cross-section analysis allows the height difference between the swollen and the dry gelatin to be measured. In this test, the film had swollen by about 1.29 μm in the z axis. This experiment hence proves that by systematically imaging along a stent section in the region of the interface it is possible to measure changes in the z-direction caused by exposure to solution. A polymer coating system on a stent was studied following the same method. A sequence of images were recorded for the partially wetted stent along a straight dry strut, moving towards the region observed to have been immersed in buffer solution. The results show that the surface morphology of the coating in the non-immersed part was consistent with that observed for the completely dry stent. However when the sampling point moved to the wet part, a change in topographical and phase images was clearly seen. The height image indicates that large domains appeared on the sample surface. Bright phase contrast corresponding to swollen domains in the height image was also observed. Furthermore, the appearance of unavoidable noise in the phase images suggests a change in material properties, possibly due to neck formation between a very tacky material and the AFM tip. Cross-sectional analysis reveals the maximum height of the swollen domains to be 520 nm above the surrounding material. A method has been developed to reveal the changes occurring at a device surface during exposure to a simulated in vivo environment. The current method utilises a partial exposure of the device to allow the extent to which the coating swells to be quantified. Both hydrophilic and hydrophobic polymers used for drug eluting stents designs have been characterized using this method. Extensions to this method may also allow in-situ monitoring in the simulated environment.

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Investigation of extruded nalidixic acid-poly(ε-caprolactone) blends for medical device applications

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The bacterial colonization of implanted medical devices is responsible for a great number of rejections and failures presenting increased risks of patient morbidity and mortality and consequently, major attention has been focused on the treatment of these polymeric surfaces to reduce the levels of adhering organisms. The use of biodegradable coatings on urological device surfaces is just one potential method of overcoming this problem (Jones et al 2002) whereby the coating functions to provide a renewable surface through degradation resulting in the removal of adherent organisms and encrustation deposits. The inclusion of a suitable active agent within the matrix may also serve to provide a means of combating infection before the onset of degradation. This study investigates the release of nalidixic acid (NA), an antimicrobial agent used in the treatment of acute or chronic urinary tract infections, from extruded poly(ε-caprolactone) (PCL), a biodegradable linear polyester. NA was blended with PCL (Capa 6506) in powder form in a high speed mixer at 1500 rev min^{-1} (1, 2.5, 5, 10 and 25% w/w). The prepared mixtures were then injection moulded into plaques and circular discs were stamped out for drug release studies. Individual drug release samples were placed in a beaker containing 50 mL of pH 9 Tris buffer which had been preheated to 37°C. Samples (5 mL) of the release medium were removed at selected time intervals and replaced with fresh buffer solution (5 mL). The mass of NA released was quantified by analysis of the solutions via HPLC using a 0.1% phosphoric acid:methanol (55:45) mobile phase. The results below show the percentage of NA released from each blend after 1-, 24-, and 168-h periods (Table 1) and the change in the rate of NA released after 1 h, day 2 and day 6 (Table 2). The fraction of drug released was shown to decrease as drug loading increased. The rate of NA release was also shown to decline over time for each of the formulations investigated. These materials may therefore be of potential use as urological device coatings, the indwelling time of which is dependent upon the medical condition, whereby the release of the antimicrobial agent can be maintained over a prolonged period and the desired rate of release achieved through appropriate formulation.

Table 1 Effect of drug loading on the percentage of NA released

Fraction of NA in blend	NA released (%)		
	after 1 h	after 24 h	after 168 h
1%	10.97 ± 0.69	31.46 ± 0.76	69.8 ± 1.47
2.5%	6.07 ± 0.43	17.15 ± 1.17	39.29 ± 2.11
5%	2.72 ± 0.17	8.70 ± 0.31	21.19 ± 4.76
10%	1.79 ± 0.10	5.37 ± 0.23	13.06 ± 0.78
25%	0.79 ± 0.04	2.4 ± 0.07	5.67 ± 1.70

Table 2 Rate of NA release (dQ/dt) from NA:PCL blends

Fraction of NA in blend	dQ/dt (mg h^{-1})		
	after 1 h	after day 2	after day 6
1%	117.76 ± 10.22	4.75 ± 0.21	1.84 ± 0.13
2.5%	168.27 ± 13.17	6.89 ± 0.60	2.9 ± 0.28
5%	147.21 ± 11.76	7.2 ± 0.28	3.41 ± 0.2
10%	190.28 ± 12.34	8.83 ± 0.42	4.27 ± 0.27
25%	213.84 ± 13.93	9.45 ± 0.48	4.56 ± 0.23

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020
Evaluation of a novel, anti-infective and biomimetic silicone for urological use

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Biomaterials are employed in the manufacture of medical devices, such as urinary catheters, endotracheal tubes and ureteral stents. Unfortunately, despite their widespread use, one complication that has been reported for all medical devices is device-related infection, leading to increased morbidity and mortality (Dickinson & Bisno 1989). Bacteria adhere to the device, reproduce and, after secretion of exopolymeric material, establish a biofilm. While suspended populations of bacteria (planktonic) readily respond to antimicrobial treatment, the bacteria within biofilms are protected from even the most aggressive of treatment regimes (Costerton et al 1993). Biomaterials are currently being developed that may reduce microbial biofilm formation and hence lower the incidence of medical device related infection. In a recent study we have described the formulation of novel silicone biomaterials that exude a biocompatible organic liquid and, in so doing, enhance the lubricity of the biomaterial (Woolfson et al 2003). In this study, the antimicrobial properties of a related novel silicone biomaterial that has been formulated containing a series of antimicrobial agents are described. The novel lubricious silicone elastomer was prepared in a similar way to conventional two-part, condensation type (tin-catalysed), room temperature vulcanised silicones. The poly (dimethylsiloxane) molecules are crosslinked with a novel crosslinking agent, teraoctyldodecoxysilane (TODDOS). The material is cured at 90°C for 3 min. Crosslinking with TODDOS produces a silicone elastomer, which exudes a lubricious alcohol, octyldodecanol (ODD), that is distributed homogeneously throughout the matrix. This ODD is released over time, resulting in a renewable surface. Release of ODD was analysed over time through weight loss studies and expressed as a percentage of the total ODD present. The mechanical properties of the TODDOS were determined by tensile strength testing using a TA-XT2 Texture Analyser. This apparatus was modified to perform coefficient of friction testing on all the various TODDOS silicones. A CAHN dynamic contact angle analyser was used to determine surface characteristics of the materials, indicating their hydrophobicity or hydrophobicity. Various antimicrobials and antifungals were incorporated directly into the TODDOS matrix and the suitability of the TODDOS for use as a urinary catheter biomaterial was determined by comparing its lubricity and ability to resist bacterial adherence to that of a conventional silicone. The adherence and persistence of activity of the antimicrobial-incorporated TODDOS silicones were tested against both *E. coli* and *E. faecalis*. Adherence was tested at 4 and 24 h, with the persistence of activity determined through zone of inhibition testing. The antimicrobial-incorporated TODDOS elastomers showed significantly lower adherence and a significantly longer persistence of activity, compared with the control silicone. The incorporation of the antimicrobials did not significantly affect the tensile strength or the contact angle of the TODDOS silicone. The coefficient of friction of the novel TODDOS was significantly lower than the control silicone, due to the production and release of the oily alcohol, ODD. The results demonstrate the potential benefits of the antimicrobial-incorporated TODDOS over existing silicones through the production of this renewable lubricious surface and may also provide a means of managing bacterial biofilm formation and ultimately urinary tract infection.

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021
Protein encapsulated microparticles produced by supercritical fluid mixing

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Controlled protein delivery from biodegradable polymers is making a clinical impact in a range of therapeutic areas (Orive et al 2003). However, a number of difficulties are still faced when attempting to formulate proteins in a microparticle system. The method of manufacture of polymer microparticles remains an area of difficulty due to the need to expose the polymer phase to heat or organic solvent to mobilise it. Many therapeutic proteins are denatured by temperature changes or exposure to organic solvent. Therefore, a number of approaches have been developed to protect protein function throughout a manufacturing route (Cook et al 2002). In this paper we use supercritical carbon dioxide ($scCO_2$) as a processing medium for the fabrication of poly(DL-lactic acid) $P(_{DL}LA)$ microparticles that encapsulate a protein material. The attraction of using $scCO_2$ is that the processing can be performed at 35°C and in the complete absence of conventional organic solvents. Particles of $P(_{DL}LA)$ containing 10% (w/w) ribonuclease A were produced at 35°C and with a $scCO_2$ pressure of 320 bar. Microparticle size and morphology were controlled by manipulation of the processing conditions. A comparison of particle morphology, by scanning electron microscopy and size analysis by Coulter LS230, with and without protein encapsulation, shows that particle morphology and size was not influenced dramatically by the encapsulation of protein. Under the stated processing conditions the particles produced were irregularly shaped and porous, probably caused by rapid loss of CO_2 upon depressurisation. The enzymatic activity of ribonuclease was found to be 92.7% ± 4.7 after processing. The $scCO_2$ processing was repeated using 5% (w/w) lysozyme. Particle morphology and size distribution data were similar to data for ribonuclease encapsulation. Enzyme activity was recorded at 92.4% ± 5.0 compared with control samples that were not exposed to $scCO_2$. Building on the foundation of the model protein work, we prepared microparticles containing either 10% (w/w) insulin or 0.00025% (w/w) calcitonin. Microparticles containing encapsulated insulin were tested for protein activity using an ELISA assay. The assay was performed immediately after microparticle fabrication and then after microparticle storage at 25°C for one week and one month. Activity of insulin, as measured by this ELISA assay was identical to the control on day 1. On storage at 25°C, both the microparticles and control insulin powder showed a decrease in activity for both batches. However, the fall in activity was less pronounced for insulin encapsulated in $P(_{DL}LA)$ than for the control insulin powder. The calcitonin assay is based on the reduction of motility of rat osteoclasts. The concentration of calcitonin released on day 1 was too low to assay by a method independent of protein activity. However, this assay did confirm that the calcitonin released on day 1 generated a 100% inhibition of osteoclasts. This equates to a released concentration of fully active calcitonin of above 6 pg mL^{-1} (Chambers & Moore 1983). Therefore, this $scCO_2$ method provides an alternative route to encapsulated polymeric microparticles that avoids high processing temperatures and processing in organic solvent. Thus the $scCO_2$ method has the potential to improve the manufacture of protein encapsulated products.

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022
DNA loaded DRV niosomes: stabilisation of surfactant bilayer using disaccharides

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Previous studies have shown that non-ionic surfactant vesicles (niosomes) prepared by dehydration-rehydration method, are effective in DNA delivery and vaccination (Perrie et al 2002). These dehydration-rehydration vesicles (DRV) are prone to membrane destabilization during freeze-drying process leading to vesicle fusion. It has been established that sugars, due to their non-eutectic nature, can be used to stabilize bilayers by interacting with polar headgroups and also forming a stable glassy state preventing fusion and vesicle aggregation. Here, we evaluated the physicochemical characteristics of DNA loaded DRV niosomes in presence of sucrose and trehalose as cryoprotectants. PRc/CMV HBs (100 μg) encoding the S region of HbsAg (subtype ayw) was mixed with preformed SUV composed of 1-monopalmitoyl-rac-glycerol, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, cholesterol and cholesteryl 3β-N-(dimethyl amino ethyl) carbamate (16:8:4:4 μmol, respectively) incorporating variable quantities of sucrose or trehalose to provide sugar to surfactant/lipid mass ratios of 0, 1, 3, 5, 10, 15, 20, were freeze-dried below glass transition temperature of sugar, followed by controlled rehydration with ddH_2O. Niosome z-average diameter and zeta potential were measured on Zeta plus (Brookhaven Instruments) in ddH_2O and 0.001 M PBS, respectively. Percentage of DNA encapsulation was determined fluorimetrically using PicoGreen reagent. Release of surface bound DNA was studied performing agarose gel electrophoresis with 1% SDS. Results showed that DRV niosomes exhibited vesicle sizes over a micron in the absence of sugars (1511 nm) and also when sucrose to surfactant/lipid mass ratios were 1 & 3, (1724 & 1594 nm, respectively), suggesting a pronounced fusion or aggregation of the bilayers. Increasing the mass ratio (to 5, 10, 15, 20) led to formation of submicron sized vesicles (871, 925, 892, 664 nm, respectively) apparently due to the protection offered by excess sugar by replacing hydrated water molecules on surfactant head groups with saccharide molecules (Crowe & Crowe 1993). Vesicle size with trehalose at mass ratio of 20 was relatively higher (944 nm), as trehalose unlike sucrose is more hydrophobic and is likely to penetrate the polar head groups reaching the hydrophobic regions during dehydration step. Trehalose tends to promote bilayer expansion by remaining there even after rehydration (Viera et al 1993) thus increasing vesicle size. Zeta potential in all preparations remained fairly constant (~40 mV) and no significant effect was seen on entrapment efficiency, which was around 98% both in presence and absence of sugars tested, since it is proposed that DNA is predominantly entrapped within the bilayers of DRV, presumably bound to the inner cationic charges with only a minor portion interacting with surface cationic charges. Gel electrophoresis revealed that the release of surface-bound DNA decreased with increase in sucrose presumably due to either high internalisation of DNA or due to the carbohydrate having a stabilising effect on the bilayer. In conclusion, these results demonstrate that disaccharides, such as sucrose and trehalose, could efficiently be employed in DNA encapsulated DRV niosomes, without significantly influencing encapsulation efficiency; a pronounced effect was observed on vesicle size yielding submicron sizes, which could further be lowered by increasing surfactant hydrophobicity and thereby reducing the overall surface free energy.

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023
Molecular simulation of the mechanism of action of oleic acid as a drug penetration enhancer

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The promise of delivery of drugs by the transdermal route remains largely unrealised because for many drug molecules the skin constitutes a significant barrier. One approach is to use penetration enhancer molecules that interact with the skin lipids to facilitate the transport of the drug through skin. While there is considerable interest in identifying penetration enhancers, the mechanisms of action at the molecular level are not fully understood. A greater understanding of the mechanism of action of these molecules could assist in their rational design. Oleic Acid (OA) is a fatty acid that has been shown to enhance the transdermal delivery of drugs. It is clear that OA interacts with and modifies the lipid domains of the stratum corneum. However, the details of the interactions and a molecular explanation for the concentration dependent effects are not yet clear (Williams & Barry 2004). In this study we have investigated the possible mechanisms of action of OA in a lipid system by way of molecular simulation. We have carried out molecular dynamics (MD) simulations of OA in a dipalmitoylphosphatidylcholine (DPPC) bilayer patch comprising about 500 molecules. The simulations were carried out at a range of concentrations of OA (0–60% by number). In a MD simulation one follows the trajectory of interacting particles in a system with time. A coarse-grained model that represents small groups of atoms by single interaction sites has been used. By reducing the complexity of the problem, the model endeavours to include all the essential physics, while having the advantage that longer time scale events such as the transport of a molecule through a membrane become accessible. The results of the simulations confirm that the OA molecules do indeed interact with the hydrocarbon chains of the lipid bilayer. We have observed that OA disperses throughout the bilayer and does not tend to form separate lipid phases as proposed by Ongpipattanakul et al (1991). Analysis of the trajectories shows that the DPPC chains distort from their equilibrium conformation as they interact with the unsaturated hydrocarbon chain of OA. The lateral diffusion constants of the lipids increase with increasing concentration of OA, indicating that OA increases the fluidity of the membrane in a concentration dependent manner. In conclusion, it has been found that at the concentrations studied OA disperses heterogeneously into the bilayer without forming pools and disrupts the molecular packing in the bilayer. The fact that we have not observed pooling of the oleic acid is probably due to the selected phase of the lipid, namely the liquid-crystalline phase rather than the solid-crystalline phase or possibly due to the phase separation process occurring over a longer time scale than the simulation times. These investigations are currently being extended to include permeation studies of simple drug molecules in the presence or absence of penetration enhancers using free energy calculations.

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024
An in-vitro examination of solutions of sodium alginate as oesophageal protectants against damage caused by gastric reflux

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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 uses microscopy as a tool to evaluate the protection offered by solutions of sodium alginate to oesophageal tissue sections exposed to acid and acidified pepsin solutions (two aggressors within gastric refluxate). Porcine oesophagi were collected from the abattoir on the day of sacrifice; the outer muscle layers were removed and the inner epithelium was stored at −70°C until use. Immediately before the experiment, the oesophagus was thawed at room temperature. The upper epithelial layer of the central portion of the oesophagus was separated from the entire epithelium via immersion in saline solution for 1 min at 60°C (Diaz del Consuelo et al 2003). This upper epithelial layer was kept in saline solution at room temperature until required. The isolated epithelium was placed on a Transwell insert (diameter 1.5 cm) and 2.5 mL acid or acidified pepsin solution was dispersed on the surface. Paired epithelium sections were used with a control or 0.04 mL aqueous alginate applied to the surface before dispensing the acid or acidic pepsin solutions. Acid solutions of pH 1, 2 and 3 were investigated, as well as solutions of 0.1, 0.2 and 0.3% m/v pepsin within an acidic solution at pH 1. After 1 h, the epithelium was removed, sectioned and stained (haemotoxilin and eosin stain). A ZEISS, Axioskop microscope was used to view the surface damage and images were captured. Assessment criteria were set up (8 score system: score of 0 = no damage, 2 = slight damage, 4 = moderate damage, 6 = significant damage, 8 = serious damage). The images were randomly numbered. Ten subjects were asked to assess the score for each photo according to the criteria. The score results were analysed statistically using analysis of variance. As expected the damage observed was greater at low pH where the acid concentration is greatest (Table 1). In addition, as the concentration of pepsin increased the damage to the epithelial surface also increased (Table 2). The presence of alginate layer on the tissue surface demonstrated lower damage score in all cases and significantly reduced damage compared with the control ($P < 0.05$). Table data show mean ± standard deviation (n > 10). A new technique using microscopy

and an independent scoring system was set up to assess the level of oesophageal tissue damage. This technique was successfully used in this study to assess the extent of the oesophageal epithelium damage caused by acid and acidified pepsin solutions and the effect of aqueous alginate layers in protecting the oesophageal epithelium from this damage.

Table 1 Score of epithelium damaged by acid

	pH 1	pH 2	pH 3
Control	5.51 ± 0.91	2.67 ± 1.17	1.53 ± 0.88
Alginate	2.71 ± 1.29	0.60 ± 0.86	N/A

Table 2 Score of epithelium damaged by acidic pepsin

	0.1% w/v	0.2% w/v	0.3% w/v
Control	3.70 ± 0.75	5.74 ± 0.95	7.73 ± 0.46
Alginate	1.25 ± 0.80	2.81 ± 0.88	0.50 ± 0.67

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025
Dispersibility of spray-dried formulations for pulmonary drug delivery

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Considerable research efforts have been directed towards the development of dry powder inhaler systems due to their lack of propellant, ease of portability, low cost and ease of administration, for the delivery of therapeutic agents for both local and systemic effect. However, the flow and aerosolisation properties of the dry powder determine the site of deposition within the lung. A number of methods have been developed to improve the deposition pattern, including the use of modified carrier particles (Steckel & Müller 1997) and particle surfaces (Larhrib et al 2003). Spray-drying is an alternative effective and convenient method of producing appropriately sized respirable particles, in a one-step process. We have previously shown that inclusion of leucine in spray-dried lactose-based powders enhances the in-vitro deposition pattern (Li et al 2003). In this study, we investigate the in-vitro deposition of lactose-, trehalose- and mannitol-based spray-dried powders incorporating leucine as a dispersibility enhancer and salbutamol sulphate as a model drug. Aqueous solutions of salbutamol sulphate and lactose, trehalose or mannitol, in the presence or absence of leucine, were spray-dried (Büchi 191 mini spray-dryer) using standard operating conditions of inlet temperature, spray flow rate and pump rate to generate dry powders. Laser diffraction was used to determine the particle size of the dry powders, and their apparent morphology was assessed using scanning electron microscopy (SEM). Powders were aerosolised using a Spinhaler dry powder inhaler into a Multistage Liquid Impinger (MSLI), and the mass of salbutamol sulphate deposited at each stage quantified using HPLC. The fine particle fraction (FPF) was calculated as the proportion of the powder deposited in the lower stages of the MSLI (i.e. aerodynamic diameter $\leq 3.1\ \mu$m). SEM indicated that, for the leucine-free formulations, trehalose- and lactose-based powders consisted of spherical particles, whereas the particles in the mannitol-based powders were more irregular in morphology. Inclusion of leucine in the solution before spray-drying increased the surface roughness of the particles, and altered the surface morphology. Particle size analysis revealed a unimodal distribution for powders containing leucine, whereas those powders without leucine displayed a multimodal size distribution. The emitted dose of the leucine-free lactose and mannitol formulations ($< 55\%$) was significantly lower than that of the leucine-containing powders ($> 90\%$), while the spray-dried trehalose formulations with or without leucine had a similar emitted dose of about 94%. All the formulations containing leucine produced greater deposition in the lower stages of the MSLI, and hence had an FPF at 40–50%, which was significant higher than that of the leucine-free powders with maximum FPF of approximately 12%. Lactose is almost exclusively employed when formulating dry powder inhaler systems. We have shown that trehalose-based spray-dried powders may offer an improvement over lactose-based powders, and that the inclusion of leucine into the formulation before spray-drying can enhance the emitted dose and respirable fraction of the spray-dried powder.

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026
Site-specific drug delivery to the oesophagus using alginate micro-particles

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Site-specific drug delivery to the oesophagus is difficult and remains a relatively unstudied area. However, oesophageal disease is extremely debilitating, can result in severe patient morbidity and therefore presents a challenge to the formulation scientist. In this study, we have investigated the use of alginate microparticles as a sustained delivery system for oesophageal delivery. Alginate microparticles were successfully manufactured using a water-in-oil emulsion preparation technique based on that of Wan et al (1992). The effect of manufacturing conditions on the quality of the microparticles was examined, with these being optimised to give a free-flowing powder with a mean particle diameter of 200 μm. Scanning Electron Microscopy indicated that these particles were roughly spherical. Sudan Red and sodium fluorescein were used as model hydrophobic and hydrophilic compounds, respectively, to assess incorporation of active material into the microparticles, and also to enable visualisation during the retention experiments. The sodium fluorescein was found to be incorporated into the interior of the micro-particles, whereas the Sudan Red was found to be loosely adhered to their external surface, indicating the likely incorporation of drugs of differing physico-chemical natures. The retention of the sodium fluorescein-labelled microparticles was examined using an ex-vivo model as previously described by Batchelor et al (2002). Excised porcine oesophagus was mounted on an inclined platform, the alginate microparticles applied and the surface of the oesophagus washed to simulate salivary flow. At intervals post-application, the oesophageal tissue was examined using confocal laser scanning microscopy (CLSM) to assess the percentage retention. At all times up to 60 min (end of the experiment) the retention of alginate microparticles on the oesophageal tissue was significantly higher than that of an equivalent alginate solution, with approximately 53% retention being observed for the microparticles after 1 h compared with approximately 20% retention for the alginate solution. For both formulations, the greatest loss was observed in the first 20 min (i.e. approximately 34% loss for the microparticles and 53% loss for the solution), although the solution continued to show greater loss thereafter. The implications of this work are that it may be possible to develop an alginate microparticulate formulation that is retained on the surface of the oesophagus for prolonged periods of time, thus enabling the incorporated or attached drug to be adsorbed onto or absorbed into the oesophageal tissue, hence maximising the therapeutic potential of the drug. The use of the ex-vivo model and CLSM in conjunction enabled retention of micro-particles on the oesophagus to be assessed in a simple, rapid and direct manner.

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027
Microneedle facilitated cutaneous delivery of a non-viral gene complex to the viable epidermis

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The efficient localised expression of gene therapy formulations within the skin will enable potential therapeutic applications, including the correction of heritable skin disorders and genetic vaccination, where a gene codes for a specific antigen to which a rapid systemic immune response is initiated. However, the delivery of macromolecular therapies to cells of the epidermis and dermis is complicated by the significant barrier properties of the outermost skin layer,

the stratum corneum (SC). To date, the most successful methods of facilitating epidermal gene expression include direct intradermal injection of plasmid DNA (Hengee et al 1995) and particle mediated gene delivery using a biolistic device (Lin et al 2000). Microneedle arrays are a novel intra/transdermal delivery device, spawned by recent advances in microfabrication technologies, that provide access to the viable cells of the epidermis through the creation of microchannels within the SC. Investigations within our laboratory aim to combine the cutaneous delivery potential of a microneedle device with the rational design of non-viral gene therapy formulations to create a controllable therapeutic gene delivery system. Zeta potential measurements and Photon Correlation Spectroscopy have been used to develop a representative fluorescent colloidal model of the gene delivery vector, a lipid:polycation:pDNA LPD complex. Franz cell diffusion studies using this model nanoparticulate formulation predict the influence of a vectors physicochemical characteristics on its movement through the conduits created by the microneedle array. Subsequent microscopy investigations confirm the capability of a silicon microneedle device to create micron-sized channels within the human SC. In-vitro cell culture and ex-vivo human skin organ culture experiments are used to study the expression of reporter plasmid DNA within the human keratinocytes of the viable epidermis. To predict the microneedle influenced delivery of a non-viral gene complex through the stratum corneum, a synthetic diffusion model has been developed. A lipid-coated fluorescent latex bead has been created to provide an LPD mimic that can be easily visualised and quantified (Table 1). Current studies use this mimic to determine the influence of zeta potential on the diffusion of a colloidal particle through the uniform circular pores of a polycarbonate track-etched membrane.

To predict the microneedle influenced delivery of a non-viral gene complex through the stratum corneum, a synthetic diffusion model has been developed. A lipid-coated fluorescent latex bead has been created to provide an LPD mimic that can be easily visualised and quantified. Current studies use this mimic to determine the influence of zeta potential on the diffusion of a colloidal particle through the uniform circular pores of a polycarbonate track-etched membrane. The visualisation of microconduits within the SC, approximately 15 μm in diameter, was succeeded by the microneedle mediated delivery of fluorescent colloidal nanoparticles to the viable cells within the human epidermis. These studies confirm the capability of a microneedle array to create a route of delivery for macromolecular gene therapy formulations. Franz cell diffusion studies suggest that the size (~80 nm) and surface charge (zeta potential +33.9 mV at pH 7.4) of an LPD complex and the diameter of the created microchannel are important factors governing the efficiency of microneedle mediated gene delivery. To promote successful gene delivery to the epidermis it will therefore be important to optimise the formulation characteristics and the microneedle device structure. Cell culture studies have demonstrated the ability of an LPD vector containing β-galactosidase and green fluorescent protein reporter plasmids to successfully transfect keratinocyte (HaCaT) cells. Investigations within our laboratory have proven the concept of microneedle mediated delivery of macromolecules to the epidermal and subepidermal skin layers. To optimise this method of cutaneous gene delivery it is important to consider physicochemical factors such as the surface charge of particulate formulation and the diameter of the channels created by the microneedles. In-vitro keratinocyte transfection has proven to be an efficient, reproducible process; however, the delivery and expression of non-viral gene therapy vectors within ex-vivo skin presents a much greater challenge.

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028
The design and activity of small interfering RNA (siRNA) as a potential therapeutic agent for the down-regulation of the epidermal growth factor receptor (EGFR)

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Molecules that inhibit epidermal growth factor receptor (EGFR) signalling have been shown to prevent tumour growth and viability in-vitro and in-vivo; this makes EFGR a promising therapeutic target for the development of novel agents. Small interfering RNA (siRNA) molecules comprise of short duplexes, 19–23 RNA nucleotides, which can cause effective gene silencing in cell culture and have recently also been shown to be potent in-vivo (Song et al 2003). The development of an active siRNA molecule targeting EGFR would be a useful biological tool and may a represent a potential therapeutic agent. For siRNA to be successful in gene silencing several factors should be addressed: siRNA molecules must be designed to target specific mRNAs, be biologically stable, delivered efficiently to cells and be potently efficacious in target cells. Since not all siRNA designs are active, it is important to understand the rules governing the design of efficacious siRNA molecules. In this study we firstly investigated whether targeting siRNA to hybridization accessible regions of EGFR mRNA was important for siRNA-mediated gene silencing activity. The hybridization accessibility of EGFR mRNA was determined using scanning oligonucleotide microarrays, a technique known to be effective in designing both efficacious antisense and siRNA sequences (Beale et al 2003). Three regions of 120–150 nucleotides within the first 600 nt downstream of 5′ AUG initiation codon of EGFR mRNA were analysed for accessibility. siRNA sequences were then designed to target regions of differing hybridization accessibility. In addition, and as control sequences, siRNAs targeting EGFR were designed from a knowledge of published rules that do not account for mRNA target structure (Elbashir et al 2002). Array-designed siRNA sequences delivered to A431 cells using Oligofectamine and 25 kDa branched polyethylenimine dendrimer (PEI) were effective in inhibiting A431 cell growth and EGFR protein expression. The level of siRNA activity generally correlated well with the degree of hybridization accessibility in EGFR mRNA as indicated by intensity values on scanning arrays. The siRNA targeted against accessible sites significantly inhibited growth greater than 50% compared with controls ($P < 0.01$) while siRNAs designed to non-accessible regions were largely ineffective ($P > 0.05$%). In addition siRNA sequences derived from published design rules and on-line algorithms were not effective if the targeted site had poor accessibility ($P > 0.05$). These results suggest that effective siRNA design may require a knowledge of both the target mRNA structure and the thermodynamic parameters relating to the siRNA duplex structure. Further work is planned to produce improved targeting of EGFR mRNA in cancer cells and to refine the rules of siRNA design.

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029
Unravelling the spatial distribution of daunorubicin within human leukaemic cells displaying differential drug distribution and sensitivity

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Multi-drug resistance (MDR) presents a major problem in therapeutic approaches to combating a variety of diseases, including cancer (Gottesman & Pastan 1993). MDR is a highly complex process as individual cells may exhibit several defence mechanisms including cellular drug sequestration and an inability to overcome biological barriers mediated by drug transporters such as P-glycoprotein (P-gp). Treatment of haematological malignancies such as acute myeloid leukaemia is also hampered by MDR, but the exact mechanism still awaits clarification (Kappelmayer et al 2004). There is therefore a need to gain a further understanding of the resistance mechanisms in leukaemia cells, ultimately leading to the design of more effective MDR modulators. We have studied the resistance mechanisms in two human leukaemia cell lines: chronic myeloid leukaemia-K562, and the highly resistant acute myeloid leukaemia- KG1a. A novel immunofluorescence microscopy technique was initially developed for assessing the intracellular distribution of P-gp relative to cellular organelles, early endosomes, recycling endosomes, Golgi, lysosomes and mitochondria. Live cell fluorescence imaging was used to consolidate the data in fixed cells and also to visualise the intracellular distribution of the cytotoxic agent daunorubicin, in comparison with internalised ligands that label specific compartments of the endocytic pathway. Daunorubicin distribution differed greatly in KG1a and K562 cells, being located in diffuse cytoplasmic vesicles in the former, compared with a dense perinuclear region in the latter. Daunorubicin in both cell lines co-localised with endocytosed dextran, a marker for the lysosomes. In view of this data, the daunorubicin sequestering compartments were, as expected, sensitive to treatment with lysosomal pH disrupting agents, bafilomycin A1 and nigericin. These both resulted in a dramatic increase in the nuclear localisation of the drug in both cell lines. In agreement with previous studies, we find that KG1a cells supported the majority of their P-gp intracellularly within small cytoplasmic vesicles (Ferrao et al 2001). We find that the morphology of these vesicles is sensitive to treatment with wortmannin, suggesting that they are of endosomal origin. P-gp inhibitors verapamil and cyclosporin A resulted in nuclear localisation of daunorubicin in KG1a but not K562 cells, suggesting that intracellular P-gp in KG1a cells actively reduces the nuclear localisation of the drug. In conclusion, we identified major differences regarding the possible resistance mechanisms in KG1a and K562 cells. We propose that there are two resistance mechanisms in KG1a, a P-gp dependent efflux critical for drug exclusion from the nucleus and also a P-gp independent mechanism of drug sequestration into lysosomes. However, in K562 cells the daunorubicin was extensively sequestered within

lysosomes. Targeting lysosomal sequestration of daunorubicin and other weak base drugs, as well as direct inhibition of P-gp, may provide more effective modulators of MDR in acute myeloid leukaemia cells.

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030
Phage Display library identification of peptides binding to brain microvascular endothelial cells in-vitro

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With an ageing population disorders of the brain are becoming more prevalent, however, the blood-brain barrier (BBB) is, and will remain, a significant obstacle to the delivery of biologics to the central nervous system (CNS). The BBB is comprised of microvascular endothelial cells that line the capillaries traversing the brain. The existence of highly restrictive tight junctions and the relatively low abundance of morphologically evident endocytic vesicles restricts both paracellular and transcellular access to the brain of therapeutic proteins and peptides (Gumbleton & Audus 2001). Nevertheless, highly regulated endocytotic and transcytotic events across the BBB occur and are an essential feature of normal BBB physiology. As part of an ongoing programme to identify novel ligands that mediate endocytotic events within the BBB we report here the use of a Phage Display library to identify peptides that bind to in-vitro brain microvascular endothelial cell BBB models. Such binding experiments will identify peptides that can be further studied for endocytic potential. Use was made of a Phage Display Library (Ph.D.-C7C, New England Biolabs) representing 3×10^9 unique genotypes encoding random -7mer disulphide constrained peptides genomically fused to the pIII coat protein of the filamentous phage M13. This library was used in subtractive biopanning procedures (4°C) over the luminal surface of confluent b.End3 (murine), RBE4 (rat) or primary brain capillary endothelial (PBCE; porcine) (Gumbleton & Audus 2001; Omidi et al 2003) cell monolayers. Following biopanning, non-binding phage were removed with detergent washes and the remaining binding phage eluted with a low pH wash. These binding phage were then taken through further iterative biopanning rounds (four in total) with each round representing an increase in binding selectivity. Following the fourth round of biopanning the phage binders underwent DNA sequencing to determine the corresponding peptide library sequences. The peptide sequences were entered into BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to search for "short, nearly exact" protein matches to examine for motif homologies. Peptides identified as binding moieties are shown in Table 1. BLAST results highlighted a number of similarities between the binding peptides and known pathogens that enter the CNS including *Plasmodium falciparum* and *Bacillus anthracis*. Of note, PCB-PEP-7C-1 (Table 1) shares a significant homology with the V3 region of the human immunodeficiency virus 1 (HIV-1) gp120 envelope glycoprotein (amino acid sequence NISVSKW). Studies suggest that gp120 plays a key role in the uptake of HIV-1 into the brain inducing both adsorptive endocytosis and transcytosis across the BBB(Banks et al 2001). In summary, this current work has utilised a phage display library to identify short peptide sequences that can bind to in-vitro blood BBB endothelial cell models. This technology will be further employed to identify peptides that mediate brain capillary endothelial cell entry via endocytotic and transcytotic pathways, with the ultimate goal for incorporation of such peptides into vectors that specifically target and cross the BBB.

Table 1 b.End3, RBE4 and PBCE binding peptide sequences

Peptide	Cell line	Amino acid sequence
PCB-PEP-7C-1	b.End3	NTSVSKW
PCB-PEP-7C-2	b.End3	NSPRHWT
PCB-PEP-7C-3	RBE4	NELGHYQ
PCB-PEP-7C-4	RBE4	KANNHYH
PCB-PEP-7C-5	RBE4	SIHSPRV
PCB-PEP-7C-6	RBE4	NLTLKNL
PCB-PEP-7C-7	RBE4	SPGHGRL
PCB-PEP-7C-8	RBE4	LDTSPRL
PCB-PEP-7C-9	PBCE	NDPLHTT

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031
In-situ monitoring of solution crystallisation using energy dispersive X-ray diffraction

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In-situ monitoring of the crystallisation process from solution is of both academic and commercial interest, particularly with regards determining growth kinetics, characterizing polymorphic transformation and rationalizing the effects of crystal growth conditions on the overall process. Progress in applying X-rays to this area has been hampered by the quality of diffractograms obtainable from a low solute weight fraction typical of solution crystallization, as the solution background scatter dominates any diffraction from the solute. To avoid this problem and hence improve the sensitivity of X-ray diffraction in studying liquid phase crystallisation processes, a novel batch crystalliser has been designed, which allows the classification of crystals into a well-defined zone through which the X-ray beam passes. The work presented here describes our initial findings during the commissioning of the cell using the dispersive instrument on Station 16.4 at Daresbury Laboratory, UK. Data on three systems — urea, citric acid and glutamic acid — are reported (the initial solutions used were: urea, 144 g per 100 cm^3 water, citric acid 180 g per 100 cm^3 water, glutamic acid 6.5 g dissolved in 100 cm^3, with added 10 weight % slurry of α crystals). Data collection was undertaken using fixed detector angles of (top) 7.796°, (middle) 4.996° and (bottom) 2.196°. The white light x-ray source used was in the range 20–70 Kev in energy. Agitation was at 200 rev min^{-1} unless otherwise stated. Typically, diffraction patterns were recorded every 30 s. The resulting diffraction ratio profile (I{2-12}/I{background}; peaks calibrated using silicon dioxide and benzamide samples (the former is well-defined standard, and the latter has a number of significant reflections over the d-spacing range of interest) for citiric acid clearly showed the induction time for the onset of nucleation, to be 21 s, followed by the overall increase in diffraction intensity as crystallisation progressed. Examination of other significant reflections (viz (202), (002) and (201)) showed similar trends. It is noted that all these data show maxima, (between 24 to 40 s), during the course of the crystallisation. A maxima in diffraction intensity as crystallisation proceeds may be attributed to preferred orientation effects because of the evolving crystal habit as the crystallisation process proceeds. Future work would be needed to explore this area further. The initial traces for glutamic acid clearly result from crystals of the α form. Over the 110-min period, the peaks due to the α form at 3.34 Å and 3.75 Å gradually decrease while those due to the β form at 4.02 Å and 4.14 Å increase in intensity. In this case the transformation may be tracked using the time dependence of ratio of a significant peak (3.75 Å for α, and 4.14 Å for β) for each form. The onset of the nucleation of the β form occurred after 40 min and the transformation due to Ostwalds rule stages occurring between 45 and 56 min. The effect of agitation on the orientation of the suspended urea crystals (once crystallisation has gone to completion) was observed in the diffractograms. At 0 up to 80 rev min^{-1} only the {111} reflections are observed. As the agitator speed increases through 100–200 rev min^{-1}, the {110} reflection becomes dominant with the {002} just appearing. Above 300 rev min^{-1} only the {002} reflection is seen. This data indicates that without stirring the crystals appear to settle under gravity with their long axis inclined slightly off from the vertical. As the agitator speed rises, the rotation of the fluid lifts the crystals up such that the needle axes lie with increasing incline to the horizontal. At higher agitator speeds where vertical and horizontal movement of the liquid occurs the crystals re-align with their c-axes horizontal. Our current work shows how a novel approach to cell design and the utilization of the intensity X-rays from a synchrotron source overcomes this problem and allows the crystallization process to be clearly followed.

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The relative incorporation of cholesterol and beclomethasone diproprionate into dipalmitoylphosphatidylcholine monolayer films as assessed by neutron reflectance

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Liposomes have been widely studied as potential drug delivery systems, with applications in a number of therapeutic states. However, liposome research has tended to focus on the more clinical aspects, with relatively little work being conducted on the physico-chemical aspects of liposome formulation. In

particular, the determination of the amount of drug incorporated into liposomes, either in the aqueous pores or within the lipid bilayer remains difficult to predict. In addition, there is a paucity of suitable techniques for such examination, most requiring sample preparation, which may in itself affect the determination of percentage incorporation. In this work, we have used two-dimensional phospholipid monolayers as models for the three-dimensional liposomes, and have used the technique of neutron reflectance to examine the incorporation of cholesterol and beclomethasone diproprionate (BDP) into monolayers composed of dipalmitoylphosphatidylcholine (DPPC). Neutron reflectivity measurements were performed using the SURF reflectometer at the Rutherford Appleton Laboratory (Didcot, UK). Using a Langmuir trough, phospholipid/drug monolayers were spread onto sub-phases of H_2O or D_2O by careful dropwise addition of the appropriate chloroform solution. Subsequently, the monolayer was compressed to a surface pressure of $25\,mNm^{-1}$ before the neutron reflectivity experiments being conducted. Scattering data were analysed using proprietary software. Reflectivity profiles of DPPC monolayers containing 0, 10 and 50 mole% cholesterol were significantly different, indicating that the cholesterol is predominantly or totally associated with the phospholipid monolayer. In contrast, the reflectivity profiles of DPPC monolayers containing 0, 10 and 50 mole% BDP were identical, suggesting that the BDP was predominantly dissolving into the aqueous subphase, despite its low aqueous solubility. Presaturation of the sub-phase with BDP, by shaking for several hours, decreases the dissolution effect markedly, and moves the reflectivity profiles for the BDP-phospholipid system towards that obtained for the cholesterol-phospholipid system. This study demonstrates that subtle differences in steroid structure (in this case probably the slightly enhanced hydrophilicity of the BDP because of the oxygenated functional groups arranged around the steroid backbone) can have profound effects on incorporation of the molecules into simple membrane models. This has implications not only for the in-vitro study of such systems, but also the in-vivo behaviour of these systems once administered to a patient, where the interaction with an aqueous environment could be expected have significant impact on the integrity of any drug-phospholipid system. Additionally, the study has shown that neutron reflectance is a useful technique in the examination of such systems.

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Liposomal solutions to problematic drugs

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Due to their biphasic character, the liposomes can act as carriers for both lipophilic and hydrophilic drugs. The solubility and partitioning behaviour of a drug molecule governs its location in the liposome structure; highly hydrophilic drugs (logP < 1.7; e.g. arabinosylcytosine) are located exclusively in the aqueous compartment of the liposomes while lipophilic drugs with logP > 5 (e.g. cyclosporine) are entrapped in the lipid bilayer. Drugs with intermediate partition coefficients with logP between 1.7 and 4.0 pose a problem because they partition easily between the lipid and aqueous phases and are lost very easily from liposomes (Gulati et al 1998). The aim of this work was to enhance the solubility of such problematic drugs (logP between 2 and 4) by exploiting the highly adaptable physicochemical properties of liposomal systems. Liposomes were prepared by the hand shaking method (Bangham et al 1965) using phosphatidylcholine (PC)/dimyristoyl PC (DMPC)/distearoyl PC (DSPC) and cholesterol (Chol) (16:4 μmol) with 1.25 mg of a range of poorly soluble drugs. Liposome drug loading was assessed by spectrophotometric analysis of the supernatant after liposome separation by centrifugation. Liposomes were sized on a Malvern Mastersizer X in ddH_2O and the zeta potential measured on a zetaPlus (Brookhaven Instruments) in 0.001 M PBS. Our investigations show that both drug log P and liposomal hydrophobic volume influence bilayer drug loading: incorporation studies demonstrated drug loading could be enhanced by increasing the liposomal hydrophobic volume although the extent of this enhanced drug loading was strongly dictated by the log P of the drug. For instance, liposomal loading of both sulindac and ibuprofen (which have log P values around 3.5) increased by only 10% (to 59 and 43%, respectively) when phosphatidylcholine (PC) was replaced by the longer alkyl chain length lipid distearoyl phosphatidylcholine (DSPC) within the liposomal composition. In contrast, incorporation of higher log P drugs, such as flurbiprofen (log P = 4.2) increased by 40% when PC was replaced by DSPC and resulted in incorporation values of 93%. In terms of phyiscochemical characteristics there was no significant difference in zeta potential values of the different formulations (around −6 mV with or without loaded drug) indicating that drug incorporation into the bilayer had no influence on the charge. Drug loading into liposomal systems was shown to have a significant effect on the mean volume diameter of the liposomal systems: for example incorporation of ibuprofen into vesicles resulted in a 23% increase in vesicle diameter (from 4.3 to 5.3 μm, respectively). These results indicate that both the physicochemical properties of the drug molecules and the lipid bilayer composition dictate liposomal drug loading and liposome characteristics therefore such factors must be considered collectively when designing liposome-based solubilisation systems.

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034
Effect of molecular weight and concentration hyaluronan on the in-vitro release characteristics of ibuprofen and diclofenac sodium across cellulose membrane

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Hyaluronan (HA) is a naturally occurring polyanionic glycosaminoglycan. Drugs formulated in HA and applied topically are retained in the skin with little systemic absorption (Brown et al 2000, 2001). However, the precise mechanism of action of HA that facilitates drug delivery and promotes drug localization remain unclear. In this study we investigated the in-vitro release characteristics of two NSAID drugs, ibuprofen (IBF) and diclofenac sodium (DFS), incorporated into vehicles containing four different molecular weight (MW) grades and different concentrations of HA across cellulose membrane. Briefly, infinite dose of formulations were applied to cellulose membrane mounted on calibrated Franz cells kept at 32°C. At predetermined time intervals, samples of the receptor fluid were taken and analysed for drug content using a validated HPLC method. Low MW HA (MW = 137.72 kDa) was found to have no significantly effect ($P > 0.05$) on the release of IBF, whereas, the release of IBF was significantly enhanced ($P < 0.05$) from the vehicles containing HA with MW greater than 524.25 kDa in comparison with that obtained from deionized water (control). Release of IBF decreased as a function of concentration of HA in the vehicles from 0.1% to 2.5% w/w when the vehicles contained HA with MW greater than 524.25 kDa. The release rate of DFS decreased as a function of concentration of HA from 0.0% to 2.5% w/w. However, the MW grade of HA did not appear to affect the release of DFS ($P > 0.05$). This work suggests that HA, when included as an excipient in topical formulations, might affect drug release from the vehicle due to the altered solubility or diffusivity of active drug in the HA vehicles in a drug-dependant manner.

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035
A continuous production system for dry protein formulations

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The ability to stabilize a protein structure for an indefinite time still largely eludes formulation scientists. The inherent sensitivity of protein structures often result in structural perturbations during formulation, that invariably lead to loss of protein activity. Freeze-drying and spray-drying are the current methods of protein stabilization, however these processes are not without significant drawbacks. Both methods are expensive, technically demanding, and often not all protein activity is retained. A method that offers a rapid cost effective route to dry, active protein would be advantageous to a range of applications, including the pharmaceutical formulation of therapeutic proteins. Protein coated micro-crystals (PCMCs) offer a novel, versatile approach to protein stabilization. Via a rapid co-precipitation process, active protein is uniformly deposited on a water-soluble coprecipitant, which is then isolated as a dry, active protein formulation. After drying, the non-hygroscopic micron-sized particles can be stored at ambient temperature until reconstitution whereupon they rapidly redissolve in aqueous to provide free active protein. Kreiner et al (2001) discovered and pioneered the early development of PCMCs. Investigating a variety of different coprecipitants, they utilised salts, sugars

and amino acids to successfully stabilize a range of proteins, for applications in both therapeutic formulation and biocatalysis. Proteins were shown to retain their enzymatic activity, even after storage at increased humidity for prolonged periods. The procedure for the preparation of PCMCs is essentially a very simple co-precipitation crystallization procedure. An aqueous protein solution is mixed with a concentrated solution of excipient. This combined protein aqueous mixture is then dispensed drop-wise into a highly mixed water-soluble anti-solvent, whereupon the protein and excipient instantly co-precipitate. Depending on the protein payload, the co-precipitated PCMC particle is typically micron-sized, with the protein molecules located on the surface of the excipient crystal. Producing milligrams quantities of PCMCs can easily be performed with basic laboratory apparatus although a larger scale production system was required to provide pre-clinical quantities of material. Simply increasing the size of the batch apparatus proved unfruitful because the mixing rheology differs on larger scale apparatus. Consequently a continuous flow co-precipitation was envisaged. Using two pumping units and a specially designed mixing flow cell a continuous flow co-precipitation system was constructed. This allowed protein/excipient solution and anti-solvent to be continuously co-precipitated, producing an outflow of PCMC suspension. The PCMCs could be harvested directly from this suspension. Using this strategy, grams of PCMCs could be produced per hour of production. Initial studies using a continuous flow strategy have produced PCMCs with good retention of protein activity, plus excellent particle characteristics. Typically particles are $< 5\,\mu$m, which makes them especially amenable to the pulmonary delivery of therapeutic peptides, proteins, nucleic acids and other water-soluble bioactive molecules.

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036
Use of small-angle neutron scattering (SANS) and surface tension to better understand the mechanism of membrane and surfactant micelle interaction of endosomolytic polyamidoamines

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Polyamidoamines (PAAs) are a family of synthetic, water-soluble, linear polymers, prepared by hydrogen-transfer polyaddition of aliphatic amines or *bis*-secondary amines to *bis*-acrylamides (Ferruti et al 2000). They display pH-dependent breakage of model membranes (e.g. red blood cells (RBCs)) and can disrupt the intracellular endosomal (endosomolytic) and lysosomal membrane to mediate intracellular delivery of genes and proteins (Richardson et al 2001). However, their mechanism of membrane perturbation is still poorly understood. Recent studies using SANS and pulsed-gradient spin-echo NMR have shown that the size of the PAA ISA23Cl polymer coil changes with pH (Griffiths et al 2004). With decreasing pH the ISA23 radius of gyration increases to a maximum ($R_g \sim 8$ nm) at ~pH 3, before subsequently decreasing, until at very low pH, the coil collapses ($R_g < 2$ nm). The aim of this study was to investigate the interaction between PAA and surfaces providing representative model membranes using techniques commonly employed in synthetic surfactant research. To investigate the polymer-membrane interaction, RBCs, surfactant micelles and liposomes were chosen as models and ISA23Cl as the PAA. Combinations of anionic (SDS and phosphatidylcholine) and non-ionic surfactants (C_{14}BNMG and $C_{12}E_4$), were used to prepare the 'surfaces'. Surface tension technique was used to study the onset of the interaction between these model surfactant micelles (10 mM to 0.008 mM) and ISA23Cl (0.2 wt%) as a function of pH. Maximum bubble pressure was used to measure the surface tension. Scattering and spectroscopy techniques probe the structure and dynamics of the complex directly. SANS has been used to quantify the dimensions of the surfactant micelles, polymer-surfactant and polymer-membrane complexes. The neutron scattering of the liposome (1 mM) in the absence and presence of ISA23Cl (1 wt%) at pH 7.4 and 5.5 was measured. The SANS measurements were performed on LOQ diffractometers at the ISIS Spallation Neutron Source, Oxfordshire (UK). The liposomes (phosphatidylcholine: phosphatidylethanolamine, ratio 5:2) were prepared using freeze/thaw extrusion method. ISA23Cl demonstrated pH-dependent haemolytic activity. At pH 7.4 and 6.5 no haemolytic activity was seen (0.7 and 1.1%, haemoglobin release, respectively), but at pH 5.5 a significant haemolysis was observed (18% haemoglobin release). The surface tension ($mN\,m^{-1}$) versus log[total concentration of surfactant] curve does not change in shape following the addition of ISA23Cl, at pH 8, but a clear change is observed at pH 4, which is due to the interaction occurring between ISA23Cl and the surfactant micelle. And similar conclusions can be gained from electron paramagnetic resonance (EPR) studies. The scattering of the liposome at pH 7.4 is not affected by the addition of ISA23Cl, but at pH 5.5 a clear change in scattering is observed, the size of the liposome seems to increase in size at this pH. From the surface tension studies it is clear that ISA23Cl was found to interact with the surface at low pH when the polymer and the surface have opposite charge but not when both bear similar charge. And this is an agreement with SANS data, which demonstrates polymer-liposome interaction at a low pH, again when ISA23Cl and liposome are oppositely charged.

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037
Amphiphilic doxorubicin conjugates alter the intracellular distribution and activity of the drug

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Efficacy of chemotherapy is often limited by the narrow therapeutic index of anti-cancer drugs. Hence, various strategies are being employed to alleviate the side effects of these drugs, by modifying drug pharmacokinetics and biodistribution. Amongst the various strategies employed, the exploitation of the EPR effects has been reported, which takes advantage of the leaky tumour vessel endothelium. To potentially utilize the EPR effect for targeting and simultaneously explore the potential of modified intracellular trafficking to overcome P-glycoprotein (P-gp) related multi-drug resistance, we have synthesised amphiphilic conjugates of doxorubicin. Novel amphiphilic SDox and UDox conjugates were synthesised by the covalent attachment of DSPE-PEG to doxorubicin and DSPE-PEG-NH_2 to Cis Aconitic-doxorubicin. These novel polymers were able to form particulates. The acid liability of the unstable conjugate was determined. In-vitro investigations involved determining the potential modulation of the P-gp pump by employing the parental cell line, which does not express P-gp and is sensitive to doxorubicin and the resistant cell line (A2780AD) which exhibits P-gp mediated doxorubicin resistance. This involved cytotoxicity studies, uptake and intracellular trafficking of SDox and UDox conjugate for the A2780 and A2780AD cells. Tumourocidal efficacy of UDox and doxorubicin was determined in nude mice bearing A2780 tumour. Biodistribution studies of UDox and doxorubicin were attempted. HPLC method was developed to analyse in-vivo doxorubicin metabolites. SDox and UDox both had an affinity for cellular membranes, for the A2780 and the A2780 AD cell line. The conjugates appear to initially accumulate in the cell membrane and within small spherical compartments within the cell indicating endocytosis uptake, potentially by passing the P-gp pump. The conjugates were similar in cytotoxicity for the parental and resistant cell line. The UDox showed and increase in dose tolerance in comparison with doxorubicin and similar in tumour growth delay to doxorubicin.

038
The effect of NaCl on the formation of theophyline microballoons in an o/w emulsion

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Emulsion solvent diffusion is a method to prepare microballoons as floating controlled-release systems in the stomach (Kawashima et al 1992). The major problem of o/w emulsification technique is the low encapsulation efficiency of moderately water-soluble drugs such as theophylline, caffeine and salicylic acid. The drug can diffuse from the organic dispersed phase into the aqueous continuous phase, which results in poor entrapping (Watts & Davies 1990). In this study, the influence of variation in continuous phase composition of emulsion on the physical characteristics of resultant theophylline microspheres was investigated. Theophylline, ethyl cellulose and butylphthalate were dissolved in dichloromethane/alcohol mixture, added to 0.1 N HCl containing different amount of polysorbate 80, polyvinyl alcohol, NaCl (20%) or theophylline (saturated). The mixture was stirred at 600 rev min^{-1} for 3 h. Resultant microspheres were separated from solution by filtration. Floating behaviour of microspheres was studied in the solution of HCl 0.1 N containing 0.02% polysorbate 80. The solution was stirred at 100 rev min^{-1} for 12 h and the buoyant beads were counted every hour. Size distribution of prepared microspheres was measured by the sieve analysis method. To assess the drug loading, microballoons were dissolved in ethanol and

added to a large amount of water. Precipitated ethyl cellulose was filtered and remaining solution was analysed specterophotometrically at 271 nm. Resultant microspheres tended to float over the simulated gastric medium for more than 12 h. Addition of NaCl (20%) and polysobate 80 (0.2%) to aqueous phase increased the drug loading and size uniformity of microballoons, respectively. Rapid diffusion of alcohol into aqueous medium and evaporation of dichloromethane resulted in the formation of pours in microspheres and decreased their densities. The presence of a salt in the microencapsulation medium decreased the solubility of theophyline and increased encapsulation efficiency. Polysorbate 80 as an emulsifier accelerated the formation of drops and stabilized them in external phase.

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039
Multiparticulate systems based on enteric polymers

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This research is concerned with studies on polymer matrices in the form of multiparticulate systems based on enteric polymers for the prolonged delivery of drug to the stomach and as conventional enteric systems. Enteric and floating beads have been prepared from enteric polymer solutions containing sodium diclofenac as a representative of insoluble drug and paracetamol as a representative of soluble drug. The beads were prepared from polymer solutions containing either dissolved or suspended drug (1% drug was dissolved and 10% was suspended). The method relies on the precipitation of the enteric polymers, when their solutions in an aqueous alkaline media are dropped into an acidic environment. Three enteric polymers have been used for current studies: hydroxypropyl methylcellulose phathalate (HPMCP), hydroxypropyl methyl cellulose acetate succinate (HPMCAS) and Eudragit L100-55 (enteric form). Sodium diclofenac representing a poorly soluble, has been successfully incorporated in the HPMCP beads. Only the floating beads were produced due to using freeze-drying as a drying method. Paracetamol, representing a soluble drug, was incorporated in the HPMCAS and Eudragit beads. Both floating and enteric beads were produced using two different drying methods. Conventional drying of the resultant beads leads to the formation of a solid system; freeze drying forms a porous floating system and air-drying forms an enteric system. Drug release studies show that the relatively insoluble compound (sodium diclofenac) is well protected in an acidic environment but rapid release occurs when the pH is raised. The drug release studies of a soluble compound (paracetamol) show that 40% and 20% of drug were released from the HPMCAS and Eudragit beads, respectively, in 2 h, which is not acceptable for enteric systems. Solute migration during drying forms a high surface concentration with the soluble material allowing the release in acid. The best formulation incorporating paracetamol (soluble drug) was 15% Eudragit containing 10% paracetamol. SEM studies of internal and external structure of the beads showed that the drug was distributed in both the surface and matrices but most was in the matrices. DSC curves of the pure paracetamol, pure Eudragit and the mixtures of the different concentrations of them show there is no physical interaction between paracetamol and eudragit. It was observed that most of the beads prepared by freeze-drying were light beads, which would float in media solutions. Whitehead et al (1996) showed that alginate beads prepared by dropping an alginate solution into calcium chloride solution and dried by freeze-drying were capable of floating. Zaniboni et al (1995) also showed that freeze-drying produced a porous system, which caused the beads to float. Conventional drying, on the other hand, produces solid beads which sink in aqueous media.

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040
Indomethacin release from pellets coated with Eudragit S100 and Eudragit L100 combinations in media with different pH: an approach to colon delivery

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In recent years, colon targeted delivery has been the focus point of investigations not just for delivery of drugs for treatment of local diseases of colon, but also for its potential for the delivery of labile molecules such as proteins. To achieve successful colonic delivery, a drug needs to be protected from absorption or the environment of the upper gastrointestinal tract and then be abruptly released into the proximal colon. Various approaches have been used for oral delivery of drugs to colon, including covalent linkage of a drug with a carrier, coating with pH-sensitive polymers, formulation of timed released systems and exploitation of carriers that are degraded specifically by colonic bacteria and bioadhesive systems. But so far, the pH-dependent systems have found practical application. In these systems the cores containing the active drug should be coated with pH-dependent material that remains intact at pH of stomach (pH 1–2, residence time 2 h) and of the proximal part of small intestine (pH 6.1–6.5, residence time 1 h), start to slowly dissolve at the pH of lower part of small intestine (pH 6.5–7, residence time 2 h) and rapidly release at the terminal ileum and preferably at the ileocecal junction (pH 7.2–7.5) (Evans et al 1998). The main objective of this study was to assess the suitability of the coating systems of Eudragit S100 and Eudragit L100 combinations at different levels of coating for colon delivery of indomethacin pellets. Eudragit L100 and Eudragit S100 dissolve at pH 6 and 7, respectively, and hence none of these polymers are suitable to be used alone. Indomethacin suspension in aqueous solution containing 7% polyvinylpyrrolidone K30 was sprayed on the nonpareils (850–1180 μm), using a fluidized bed coating apparatus (Glatt). The drug load was 20% w/w. These pellets were coated with formulations prepared based on the 3^2 factorial design. The independent variables are the ratio of Eudragit S100/Eudragit L100, and the level of coating. Coating solutions consisted of 10% w/v polymer, 1% w/v triethylcitrate as plasticizer and 0.5% w/v talc as anti adherent, in isopropanol–water (9:1). The coated pellets were assessed by scanning electron microscopy for uniformity of the coat. Dissolution test was carried out for coated pellets in media with different pH (1.2, 6.5, 6.8 and 7.2) using Pharmatest dissolution tester. The dissolution data revealed that the ratio of polymers used and also the level of coating is very important to achieve our main objectives. It was shown that when the polymer combination contained 80–85% Eudragit S100, the pellets were intact at pH 1.2, and no indomethacin was released at pH 6.5, but it was slowly released at pH 6.8 (about 20% in 3 h). These pellets exhibited a fast release of indomethacin at pH 7.2. The results of this study revealed that a coating formulation of Eudragit S100 and Eudragit L100 combination containing 80–85% Eudragit S100 is suitable for colon targeted delivery systems.

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041
In-vitro adhesion and release from drug delivery formulations targeted to the oesophagus

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The delivery of drugs to the oesophagus has implications in a number of disease states including those relating to oesophageal motility and cancers. Previous work has demonstrated that polyacrylic acids including Noveon AA1 demonstrate good adhesion to the oesophagus in-vitro (Russell et al 2003). In addition emulsions that adhere to the oesophagus for extended periods of time have also been reported (Cuca et al 1999). This study compares the in-vitro oesophageal retention of Noveon AA1 and simple emulsions; in addition the rate of drug release from these formulations was measured. Isosorbide dinitrate (ISDN) and nifedipine (NIF) were used as model drugs. Noveon AA1 (0.1% w/v) formulations contained the drugs suspended or dissolved in water (1.0 or 2.5 mg mL^{-1}) and the pH was adjusted using NaOH solution to pH 4.7 or 6.0, as these values have previously demonstrated enhanced retention (Russell et al 2003). Emulsions were prepared using either heavy mineral oil (HMO) or light mineral oil (LMO) in water, with Tween 85 as the surfactant. Drug-containing formulations were made by first dispersing the drug in the lipid phase at a concentration of 1.0 or 2.5 mg mL^{-1} before addition of the aqueous phase. Bioadhesion was tested using an in-vitro model described previously (Batchelor et al 2002). All formulations were better retained than the water control. At 10 min the HMO and LMO emulsions were 1.3% and 2.5% retained, respectively. Noveon AA1 showed better retention at pH 6.0 than pH 4.7 (0.7% retained after 10 min for pH 6.0). Drug release was determined using a Franz-style diffusion cell at 37°C, with acetonitrile: water (60:40) as a common receiver fluid, and dialysis membrane (MW cut-off 12–14 kDa) between the chambers. One microlitre of drug-containing formulation was placed in the donor chamber, the receiver fluid was sampled at regular intervals and drug content determined by HPLC. The results are shown in Tables 1 and 2. Drug release for ISDN was significantly greater than NIF from all formulations (analysis of variance $P < 0.05$). For 1.0 mg mL^{-1} NIF and 2.5 mg mL^{-1} ISDN, drug release from Noveon AA1 was related to

viscosity, with significantly improved release from the lower viscosity pH 4.7 solution. At high concentrations, ISDN was released faster from LMO emulsion compared with HMO emulsion, yet the reverse was true for NIF. In conclusion, both Noveon AA1 and the emulsions tested demonstrated bioadhesive potential to the oesophagus in-vitro. Drugs were successfully released within a short time and the systems have potential for oesophageal delivery.

Table 1 ISDN released after 10 min ($\mu g\,cm^{-2}$)

ISDN concn ($mg\,mL^{-1}$)	Noveon AA1 0.1% w/v		Emulsion	
	pH 4.7	pH 6.0	HMO	LMO
1.0	1.94	1.51	0.82	1.40
2.5	2.91	1.96	1.37	2.53

Table 2 NIF released after 10 min ($\mu g\,cm^{-2}$)

NIF concn ($mg\,mL^{-1}$)	Noveon AA1 0.1% w/v		Emulsion	
	pH 4.7	pH 6.0	HMO	LMO
1.0	0.05	0.02	0.09	0.05
2.5	0.13	0.13	0.12	0.80

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042
A modified dehydration-rehydration procedure for the production of small liposome-entrapped DNA

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Liposome-entrapped DNA vesicles prepared by the dehydration-rehydration procedure (DRV) (Gregoriadis et al 1999) produce liposomes of relatively large size (> 200 nm). In-vivo, these liposomes can be rapidly cleared from blood circulation and as a result may not reach the desired site to elicit appropriate immunity to the encoded antigen (Zadi & Gregoriadis 2000). In such instances, the production of small liposomes (< 200 nm) may be advantageous. Here, we assess a modification of the DRV procedure, to produce small liposomes, which maintain high entrapment of plasmid DNA. Small unilamellar liposomes (SUV), composed of 16 μmol egg phosphatidylcholine, 8 μmol 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine and 4 μmol of the cationic lipid cholesterol 3β-*N*-(dimethyl-aminoethyl)carbamate were prepared by the DRV procedure. Total lipid mass of each SUV sample was 10.2 mg, in which increasing amounts of sucrose was added to provide sucrose/lipid mass ratios of 0, 1, 3 and 5. Plasmid antigen encoding the hepatitis B surface antigen (pRc/CMV HBS; 50 μg) was added to each sample and subsequently freeze-dried for 72 h, at a shelf temperature of −40°C. After freeze-drying, the lipid films were rehydrated with 100 μL ddH_2O and 0.5 mL PBS. The liposome z-average diameter and zeta potential were measured on a ZetaPlus (Brookhaven Instruments) in ddH_2O and 0.001 M PBS, respectively. The percentage of plasmid DNA encapsulation was determined by PicoGreen reagent. In the absence of sucrose, the size of liposome-entrapped DNA vesicles after freeze-drying were larger than those vesicles freeze-dried and rehydrated in the presence of sucrose (757 nm vs 198 nm). Before freeze-drying, the size of SUV-DNA liposome complexes is approximately 200–250 nm. In the absence of sucrose, liposomes experience a considerable amount of fusion during rehydration, as the lipid bilayers undergo a phase transition from gel to liquid crystalline phase, resulting in destabilisation of the liposome membrane and reformation to larger vesicles (Gregoriadis et al 1999). When a disaccharide (e.g. sucrose) is present during the DRV procedure, vesicle size is reduced significantly to 198 nm. The sucrose molecules infuse between and interact with the head groups of the phospholipids (Oliver et al 1998), enabling the lipid bilayer to maintain within its liquid crystalline state during freeze-drying, avoiding the phase transition upon rehydration and preventing aggregation of the liposome membranes. Due to destabilisation of the lipid membrane in the absence of sucrose, a high percentage (98%) of plasmid DNA becomes entrapped within lipid bilayers upon rehydration when the liposomes aggregate and reform into larger vesicles. At a low sucrose to lipid ratio, the percentage of DNA entrapped within the liposomes remains high at 95%, indicating sufficient bilayer destabilisation to enable the DNA to become encapsulated. Gel electrophoresis analysis suggested that using a low sucrose/lipid ratio continued to promote entrapment rather than surface complexation of DNA. As the ratio increases, the percentage DNA entrapment decreases very gradually due to the increasing concentrations of disaccharide enhancing membrane stability. In conclusion, addition of a disaccharide to the DRV procedure produces small liposomes, which maintain a high level of DNA entrapment and may offer the potential for parenteral gene delivery.

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043
Aerosolisation properties of disodium cromoglycate microparticles spray dried from different water to ethanol ratio

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Disodium cromoglycate (DSCG) has emerged as one of the first-line agents in the treatment of mild to moderate asthma (Gilman 2001). Spray drying of DSCG from absolute ethanol results in the production of a crystalline sample with improved aerosolisation capability (Najafabadi et al 2003). The aim of this study was to investigate the effects of water to ethanol ratio on the physical properties and aerosolisation behaviour of spray dried DSCG. The ratio of water to ethanol in the feed varied between 50:50 and 12.5:87.5. The absolute ethanol was used as the control. All DSCG suspensions were spray dried at the same conditions. The powder properties of the samples were examined by laser diffraction, scanning electron microscopy, helium densitometry and X-ray diffraction. In-vitro deposition analysis was done using Andersen Cascade Impactor operated at 60 L min^{-1} (Eur. P. 2000). The aerosolisation properties of pure DSCG were evaluated, without carrier particles. An amorphous/crystalline nature was found for all materials spray dried in the presence of water. The X-ray pattern of DSCG spray dried from absolute ethanol was different from the other samples. The volume median diameter of the samples varied between 2.41 μm and 5.07 μm. All spray dried samples had similar density values in the range 1.60-1.62 g cm^{-3}. The particle shape of DSCG varied according to the water to ethanol ratio in the feed. Fine particle fractions (FPF), percent of emitted doses (ED%) and dispersibility percents (D%) for all samples are shown in Table 1. The physical properties of DSCG samples were different. This study showed that the water to ethanol ratio can influence significantly ($P < 0.01$) the deposition profile of spray dried DSCG.

Table 1 Deposition data for DSCG spray dried from different water to ethanol ratio

Water/ethanol ratio	FPF	ED%	D%
50:50	4.2	24.0	17.5
32.5:67.5	6.8	30.3	21.7
25:75	18.1	36.5	45.2
12.5:87.5	22.6	58.6	34.2
0:100	21.4	59.7	33.9

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044
A bioadhesive formulation for the delivery of antifungal agents to the oesophagus

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Oesophageal candidiasis is an increasingly common clinical condition associated mainly with immuno-compromised patients; an effective topical therapy

would minimise the amount of drug used and reduce the many unwanted side effects associated with systemic therapy. This study will investigate the in-vitro potential of a drug delivery formulation designed to target the oesophagus for the delivery of antifungal agents. Poor blood supply to the oesophagus and difficulties associated with swallowing are two features that complicate the treatment of oesophageal candidiasis; a bioadhesive liquid formulation that coats the infected area and acts directly from the luminal surface can overcome these inherent difficulties. Xanthan and chitosan were used as potential bioadhesive carrier polymers for the model drug miconazole. Previous work has demonstrated that these polymers are suitable for delivery of antifungal agents (Ruissen et al 1999; Senel et al 2000). Aqueous solutions of these polymers were prepared at a concentration of 2% m/v within McIlvaine's buffer at pH 4. The retention of these polymers on oesophageal tissue was measured in-vitro according to a technique that has been described previously by Batchelor et al (2002). Miconazole was incorporated into these carriers at a concentration of either $12.5\,mg\,mL^{-1}$ or $25\,mg\,mL^{-1}$ using either 10 or 25% m/v PEG to aid solubility. Franz diffusion cells were used to measure the release rate of drug from the formulation over time; dialysis membrane was used to separate the upper drug containing formulation from the receptor phase. The receptor phase consisted of pH 4 McIlvaine's buffer with either 10 or 25% m/v PEG incorporated to match the formulation within the donor compartment. The porthole diameter was 17 mm meaning that the effective release area was $2.52\,cm^2$. Retention studies indicated that at 12 min 62% of the original dose of xanthan was retained on the tissue surface whereas 40% of chitosan was retained. At 30 min the retention was 22% for chitosan and 34% for xanthan. Tables 1 and 2 show the results for the release studies of $12.5\,mg\,mL^{-1}$ and $25\,mg\,mL^{-1}$ miconazole incorporated into 2% m/v formulations of polymers with PEG added at either 10 or 25% m/v to aid solubility. The results are shown as the percentage of the drug applied that was released after 15 min ($n = 4 \pm$ standard deviation). These results demonstrate that the release of miconazole incorporated at $12.5\,mg\,mL^{-1}$ was significantly reduced as the percentage of PEG within the formulation increased from 10 to 25% m/v, whereas no significant differences were observed with the higher concentration of miconazole. Release from chitosan was greater than from xanthan with the high dose of drug, yet no significant differences were observed fro the low concentration. This study has demonstrated that liquid adhesive formulations have potential in the treatment of oesophageal candidiasis.

Table 1 % of miconazole released from chitosan

	10% PEG	25% PEG
$12.5\,mg\,mL^{-1}$	48 ± 2	32 ± 2
$25\,mg\,mL^{-1}$	44 ± 3	43 ± 5

Table 2 % of miconazole released from xanthan

	10% PEG	25% PEG
$12.5\,mg\,mL^{-1}$	43 ± 3	28 ± 3
$25\,mg\,mL^{-1}$	24 ± 7	28 ± 5

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045
Permeation of curcumin (turmeric pigment) through enhancer-treated rat skin

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Curcumin is the principle pigment and active component of *Curcuma* species including *C. longa* (turmeric). It has several pharmacological effects including anticancer, anti-hepatotoxicity and the well known anti-inflammatory effect; which seems to be stronger than that of hydrocortisone (Ammon & Wahl 1991). Curcumin has poor oral bioavailability (Ammon & Whal 1991) and its topical application could be of choice, particularly for local effects. *Curcuma* species have been used in Asian traditional medicine as topical anti-inflammatory drugs (Mujumdar et al 2000). In Iranian traditional medicine, it is used as home-made egg yolk-containing cataplasm. Curcumin is a promising drug and lots of studies are in progress on this molecule (e.g. Lin et al 2000). However, its percutaneous absorption and the effects of enhancers on this phenomenon, which are the subject of this investigation, are not well studied yet. In this investigation, permeation of curcumin through excised rat skin was studied at 25°C using static diffusion cells. As curcumin is not soluble in water, an aqueous solution of $Tween_{20}$ (0.5%, w/v) was used as the receptor phase. Dried curcumin (deposited on the skin after evaporation of solvent), and its saturated solutions in 50:50 and 75:25 ethanol:water systems were used as donor phases. Ethyl acetate:n-hexane (1:1, v/v), aqueous solutions of lecithin ($1\,mg\,mL^{-1}$) and sodium lauryl sulphate (SLS, 6%, w/v) were used as penetration enhancers. Skin samples were treated with enhancers for 18 h before permeation studies. Drug determination was by spectrophotometry at 424 nm. Results showed that permeation flux of curcumin from 75% ethanolic solution was about 3 times more than that of dried powder ($P < 0.05$). As both systems are in their maximum thermodynamic activities, this could be due to effects of ethanol and/or water on the barrier performance of skin. Decreasing the ethanol concentration to 50% reduced the enhancement effect to 1.9 times, but still significant ($P < 0.05$). Saturated solution of curcumin in 50% ethanolic solution was used as the donor phase for the rest of experiments. Permeation flux of curcumin from this system and in the absence of enhancers was $1.38 \pm 0.25\,\mu g\,cm^{-2}\,h^{-1}$. Flux increased significantly ($P = 0.035$) by about 2.2 times to $3.08 \pm 0.96\,\mu g\,cm^{-2}\,h^{-1}$ (mean ± s.d., s.d., $n = 3$) in lecithin-treated samples. This might show the reason behind the inclusion of egg yolk in the traditional turmeric cataplasm. SLS increased the flux significantly ($P = 0.001$) to $3.19 \pm 0.42\,\mu g\,cm^{-2}\,h^{-1}$ ($n = 4$); comparable with that of lecithin. Ethyl acetate:n-hexane system showed the highest enhancement effect by increasing the flux by about 3.5 times to $4.90 \pm 1.89\,\mu g\,cm^{-2}\,h^{-1}$ (mean ± s.d., $n = 4$, $P = 0.026$). These results show that permeation of curcumin can be significantly increased by different types of penetration enhancers. Further investigations including the effects of vehicle on in-vitro and in-vivo permeation of curcumin and *Curcuma* extract are in progress in our laboratories.

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046
Design of novel, bioresponsive polymer-protein conjugates

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Although protein-based therapeutics are finding increased use, proteins as drugs have a number of disadvantages including short circulation time, antigenicity and rapid metabolism (Veronese & Morpurgo 1999). PEGylation of proteins has been developed to address these problems, and several PEG conjugates have already entered routine clinical use (Harris & Chess 2003). None-the-less PEG does have limitations. The polymer is not degradable. A PEG molecular weight must be used that allows renal elimination, and moreover, chronic, parenteral administration is still not ideal. Consequently, the aim of this study was to develop a novel approach for polymer-protein conjugation. A biodegradable polymer is used to create protein conjugates that are inactivated in transit, but, by triggered degradation of the polymer at the appropriate site of action, unmasking of protein activity can be localised to the target. To evaluate the feasibility of this concept, trypsin was chosen as a model protein and dextrin selected as the polymer for conjugation. Dextrin-trypsin conjugates were synthesised in two stages. First, dextrin was functionalised, by succinoylation (16–32 mol%). This intermediate was subsequently conjugated to trypsin using EDC and S-NHS as coupling agents. The conjugate was purified by dialysis. Succinoylated dextrin and the trypsin conjugates were characterised by titration, FTIR, GPC, FPLC and SDS-PAGE. The total trypsin content was determined by BCA assay. Enzyme activity of free and conjugated trypsin was measured using N-benzoyl L-arginine *p*-nitroanilide (L-BAPNA) as a substrate. To examine the potential effect of triggered degradation of the polymeric carrier, the activity of the dextrin-trypsin conjugate was also assessed after incubation with α-amylase for 16 h at 37°C. Dextrin-trypsin conjugates were synthesised using 16, 24 and 32 mol% modified dextrin. In the succinoylated intermediate, characteristic peaks of the succinic ester and acid could be seen by FT-IR and ^1H and ^{13}C NMR. No distinct band of free trypsin in the conjugates was seen by SDS-PAGE, or using GPC and FPLC. The trypsin content of the conjugates was 6–7 wt% and this value was used to calculate trypsin equivalents for

enzyme activity studies. The polymer-bound trypsin showed reduced activity compared to free trypsin. As anticipated this was 40–80%, dependent on the degree of dextrin succinoylation. Increased polymer modification leading to the greatest reduction in activity. Following incubation of the conjugate with α-amylase, degradation of the polymer occurred leading to a significant reappearance of trypsin activity (unpaired t-test, $P < 0.05$). Those conjugates with the highest degree of polymer modification showed the most pronounced "unmasking" effect. In conclusion, these results support the concept of masking the activity of the protein payload by conjugation to a biodegradable polymer whose degradation can be triggered. Reinstatement of the trypsin activity was significant and could be modulated by the extent of polymer conjugation.

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047
Evaluation of HPMA copolymer-Dox-AGM conjugates in-vitro: a novel polymer combination for the treatment of breast cancer

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Despite the emergence of new, more selective drugs, the high incidence of breast cancer (1 in 8 women) and the low survival in the metastatic disease (less than 20%) show that current therapy is not fully effective. Polymer-drug conjugation has been shown to decrease drug toxicity, increase tumour targeting by the enhanced permeability and retention (EPR) effect and has the ability to bypass some resistance mechanisms (Duncan 2003). In addition, N-(2-hydroxypropyl)-methacrylamide (HPMA) copolymer-doxorubicin (Dox) has already shown activity in breast cancer patients in Phase I/II clinical trials (Vasey et al 1999). The aim of our work is to design a polymer-drug combination therapy where the chemotherapy and the endocrine therapy are delivered by attachment to the same polymer chain. For these studies, the cytotoxic agent Dox and the aromatase inhibitor AGM were chosen as model drugs, and the HPMA copolymer was chosen as the carrier due to its favourable chemical properties and proven clinical safety. The synthesis and characterisation of this novel class of polymer-drug conjugates has been described elsewhere (Vicent et al 2003). HPMA-copolymer GFLG (10 mol%)-Dox-AGM, HPMA-copolymer GFLG (5 mol%)-AGM and HPMA-copolymer GFLG (5 mol%) Dox were evaluated in-vitro in this study. We used the estrogen-dependent cell-line MCF-7 and its sub-line MCF-7ca (transfected with the aromatase gene) as a model to study conjugate cytotoxicity and ability to inhibit aromatase. Cytotoxicity was evaluated using MTT assay (72 h incubation). To study aromatase inhibition it was first necessary to establish a model system. Therefore, the two cell lines were grown in medium deprived of steroids but supplemented with different concentrations of androstenedione. The HPMA-copolymer-AGM-Dox conjugate showed higher cytotoxicity than HPMA-copolymer-Dox in both the cell lines used ($IC50 = 51.7 \pm 24.8$ to 5.9 ± 3.5 in $\mu g\,mL^{-1}$; $IC50 > 130\,\mu g\,mL^{-1}$, respectively). Furthermore, it showed higher activity even when compared with a mixture of the HPMA copolymer conjugates carrying these drugs individually ($IC50 > 130\,\mu g\,mL^{-1}$). Androstenedione increased growth of MCF-7ca cells, but not in MCF-7 cells, due to its mitogenic activity mediated by aromatase. Androstenedione had the highest mitogenic activity at concentrations of 10^{-7} to 10^{-9} M. When AGM was added, at a concentration of $0.2\,mg\,mL^{-1}$, to MCF-7ca cells incubated with androstenedione (5×10^{-8} M) it completely blocked the growth stimulatory effect of androstenedione. It appears that HPMA copolymer-AGM conjugates also demonstrate aromatase inhibitory activity. In conclusion, the HPMA copolymer-AGM-Dox combination therapy showed marked superiority in-vitro compared with the individual polymer conjugates. These results prompt us to carry out further studies to understand better the intracellular mechanisms responsible for these observations, and to assess whether these conjugates potential to inhibit tumour growth in-vivo.

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048
Preparation and characterisation of ibuprofen pellets based on Eudragit RS PO and Eudragit RL PO or their combination

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Among the various types of sustained release (SR) dosage forms, pellets have attracted much attention. Extrusion-spheronization technology is a widely accepted procedure for production of pellets in the pharmaceutical industry. Matrix SR pellets are much preferred over coated SR pellets due to ease of manufacture. Attempts have been made to produce SR matrix pellets by this technology with limited success. Series of materials were incorporated into pellet formulations to slow drug release (Goskonda et al 1994; Kojima & Nakagami 2002). Despite the large volume of work performed on production of pellets, the effect of incorporation of some release-retarding materials into pellet formulation requires further investigation. In this work we study the application of Eudragit RS PO, Eudragit RL PO and their 1:1 combination for production of matrix SR pellets. The effect of three formulation parameters, namely drug loading, binder concentration and Eudragits ratio, were evaluated on pellet characteristics. Ibuprofen pellets were prepared by extrusion-spheronization method. Pellet formulation consisted of ibuprofen (40, 60 or 80%), Avicel PH 101 (10%), PVP as a binder (1, 3 or 5%) and Eudragit RS, Eudragit RL or their 1:1 combination. The amounts of Eudragit(s) added were varied based on the drug load and binder concentration to make 100% w/w. The drug, Avicel, Eudragit(s) and binder were dry blended and then kneaded with addition of water to make a proper wet mass. The wet mass was passed through an extruder with 1-mm screen. The extrudates were processed in a spheronizer fitted with a cross-hatched plate rotated at $1000\,rev\,min^{-1}$ for 2 min and dried at 40°C for 8 h. The pellets were characterized by determining the percentage of pellets obtained in the range of 0.8–1.2 mm (sieve analysis), sphericity (image analysis), elastic modulus of pellets (material testing machine) and drug release profiles (USP dissolution apparatus I). The results showed that all formulations produced an acceptable yield at the desired range. More than 70% of the pellets were in the size range of 0.8–1.2 mm. Drug load, binder concentration and type of Eudragit did not affect the percentage yield of pellets. The sphericity of pellets were in the range of 0.81–0.88, indicating that near spherical pellets were obtained by these formulations. Sphericity of pellets was not dependent on drug load, binder concentration and also the type of Eudragit used. Elastic modulus of pellets was considerably affected by drug loading. Increase in drug load decreased the elastic modulus of pellets. At low drug loading increase in binder concentration decreased the elastic modulus of the pellets but at higher drug loading no clear relationship was observed between binder concentration and elastic modulus of pellets. Overall mean dissolution times (MDT) calculated for pellets with 60% drug loading were shorter than other pellets. Higher amounts of Eudragit present in pellets with 40% drug load and more hydrophobic nature of the pellets with 80% drug loading were possible explanations for higher MDT of these pellets. However, neither binder concentration nor Eudragit type affected MDT considerably.

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049
Calcium-mediated interaction of DNA with zwitterionic phospholipid monolayers

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In the search for suitable non-viral vehicles for gene delivery, the enthusiasm for using vesicles formed from synthetic cationic lipids has been severely dampened by reports of their cytotoxicity and immunogenicity (Filion & Philips 1997). Vesicles formed from non-cytotoxic natural phospholipids have previously been overlooked as DNA vectors because of the lower affinity shown by the zwitterionic lipids for DNA. Recent x-ray diffraction studies by McManus et al (2003), however, have suggested that the interaction of DNA with zwitterionic lipids might be enhanced by addition of calcium. In the work reported here, our aim was to establish the existence and extent of such interactions using neutron reflectivity, examining the effects of DNA, in the presence and absence of calcium, on a monolayer formed at the air-water interface by the zwitterionic phospholipid 1,2,-distearoyl-sn-glycero-3-phosphocholine (DSPC). Neutron reflection experiments were carried out on the SURF reflectometer at the Rutherford Appleton Laboratory (Didcot, UK).

The DSPC monolayers were achieved by dropwise addition of chloroform solutions of the alkyl chain deuterated form of the surfactant (d_{70}-DSPC; Avanti Polar Lipids) onto an aqueous subphase in a Nima Langmuir trough. Neutron reflectivity using a pulsed white neutron source, with the incident angle fixed at 1.5°, was measured with the adsorbed layer maintained at a surface pressure of 40 mN m^{-1} at 298 K. The intensities were calibrated with reference to D_2O (MSD Isotopes). Neutron reflectivity was measured from d_{70}-DSPC on a subphase of neutron-transparent water (8% D_2O:92% H_2O) for 1 h. Calf thymus DNA (Sigma) was then injected beneath the layer and measurements were continued in 30-min time slices for 3 h. Analyses of the reflectivity data were carried out using optical matrix methods (Penfold 1990), treating the adsorbed layer as a single slab of uniform scattering length density. Structural changes to the DSPC monolayer were observed in the presence of calcium that are a direct result of interaction with DNA. Although the thickness of the lipid hydrocarbon layer remained constant (at ~25 Å) after the addition of DNA, there was a marked decrease in the scattering length density of the adsorbed layer (from 6.4 Å^{-2} to 4.4 Å^{-2} after 3 h). This indicates insertion of hydrogenous material into the layer (i.e., DNA), and/or a structural rearrangement of the molecules within the layer to occupy a greater interfacial area. Changes of a similar nature were observed in the absence of calcium but occurred much more slowly and were of a much smaller magnitude. On the basis of these findings, we concur with McManus et al (2003) that calcium mediates the interaction with DNA by bridging the gaps between DNA phosphate groups and the lipid head groups or, alternatively, that the calcium interposes between adjacent lipid molecules, causing conformational changes in the trimethylamine portions of their head groups so that they are more favourably oriented for interaction with DNA. In either event, it is clear that calcium promotes interactions between zwitterionic phospholipids and DNA, and that these systems might thus make suitable (non-toxic) vectors for gene delivery.

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050
Formulation of lipid-based carrier for TB vaccines

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Globally, tuberculsosis kills two million people a year, with one-third of the world's population currently infected with the bacillus, *Mycobacterium tuberculosis* (Mtb). While most cases and deaths occur in developing countries, resistant Mtb strains are emerging. The only way to eradicate TB is to find an effective vaccine. The only current vaccine against TB is the *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine. However, BCG only protects infants from severe TB, its efficacy is highly variable, and it fails to protect the adult population against contagious TB. It is now thought that the primary reason for the lack of efficacy of the BCG vaccine is that it gives protection for only a limited period of time (Doherty et al 2002). However, repeated booster vaccination does not offer a solution since, to induce a significant degree of protection, BCG seems to require a period of multiplication in the host that is blocked in individuals with residual immunity, resulting from prior BCG vaccination or exposure to environmental mycobacteria (Brandt et al 2002). Thus, new improved vaccines are consequently needed to circumvent this problem. The aim of this new research programme is to develop and rigorously characterize effective lipid-based adjuvant delivery systems for sub-unit TB vaccines able to promote a specific immune response and applicable for large-scale manufacture. Initial systems will be based on previously developed adjuvants (e.g. the dimethyl dioctadecylammonium bromide, DDA) (Brandt et al 2000). Our initial work focuses on the physico-chemical characteristics of the cationic DDA adjuvant in combination with monophosphoryl lipid A (MPL). DDA vesicles were prepared in two different media: 0.9% sodium chloride (NaCl) and 5% dextrose. Using photon correlation spectroscopy results reveal that preparation of the DDA/MPL vesicles in 0.9% NaCl produced large vesicle structures (2702 ± 36 nm) while, in contrast, systems prepared in 5% dextrose resulted in significantly smaller vesicles (903 ± 52 nm). Immediately after preparation, both formulations had positive zeta potentials (~80 mV) due to the cationic DDA component of the formulation. However, storage of DDA/MPL vesicles in 0.9% NaCl for up to 10 days at 20°C resulted in dramatic reduction in zeta potential to below 30 mV. Similarly, over this time DDA/MPL vesicle size significantly increased (to over 5 microns) when stored in NaCl. In contrast, the 5% dextrose formulation showed no significant change in vesicle size or zeta potential over this time period. Investigations continue to identify both the structural and chemical outcome of such changes, with the aim of avoiding such problems to ensure systems may be sufficiently formulated for clinical trials.

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051
Investigation into the dominant role of swollen lamellar α-crystalline gel phase on the microstructure and stability of fatty alcohol-Tween 60-water ternary systems

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Pharmaceutical and cosmetic creams are semisolid oil-in-water emulsions prepared with a mixture of sparingly soluble fatty alcohol and non-ionic/ionic surfactant. It has been shown that emulsions are stable on the addition of mixed homologue (e.g. cetostearyl alcohol, CSA) and unstable when pure fatty alcohols (e.g. C16 or C18) are used (Eccleston 1997). To gain an insight into the gel phase responsible for the microstructure and stability of these creams, the microstructure, rheology and thermal properties of fatty alcohol-Tween 60-water ternary systems, prepared with various C16-C18 fatty alcohol combinations (C16:C18 100:0, 90:10, 70:30, 50:50, 30:70, 20:80, 15:85, 10:90, 0:100), were investigated. Such systems are models to represent the continuous phase of multiphase oil-in-water emulsions. The ternary systems (fatty alcohol 16 g, Tween 4 g, water 180 g) were prepared and stored for 3 months at 25°C. Rheological tests (continuous shear) were performed at 25°C using CSL 100 Rheometer (TA Instrument, UK) with cone and plate geometry (4 cm, 2°). In addition, differential scanning calorimetry (DSC) experiments (Mettler, Switzerland) and microscopy (Polyvar, UK) were also used to characterize the systems. All the systems were white semisolids immediately after preparation. Rheological results were classified into 3 groups according to the homologue admixtures used. The 1st group containing 30–70% C18 alcohol remained semisolid throughout storage. The structure of these systems built up significantly over approximately the first week of storage and followed by slight reductions. The initial increase and subsequent minor decrease in consistencies in these systems are possibly due to further hydration of polyoxyethylene groups of Tween 60 to form additional swollen α-crystalline gel phase and re-organization of Tween 60 within the gel network on storage, respectively. Although systems in the 2nd group containing 80–85% C18 or 90% C16 alcohols also showed the initial increase in consistency, the apparent viscosities of this group rapidly diminished on further storage as these systems became mobile with some crystallization visible microscopically. In contrast, the consistencies of the 3rd group containing pure alcohols or 10:90 C16:C18 alcohols declined dramatically from semisolids to mobile liquids within days. The DSC data for stable systems showed a broad endotherm starting at 53°C due to melting of α crystals and α-crystalline gel phase, which was stable throughout storage at 25°C. In contrast, an additional low temperature transition of β,γ to α crystals was observed with unstable systems, indicating the stability of β,γ forms at storage temperature. Because the β, γ crystals did not swell to form lamellar gel phase, the α-crystalline gel phase formed during the cooling of preparation gradually crystallized out (to β,γ crystals) on storage and systems thinned. In conclusion, the lamellar swollen α-crystalline gel phase that remains stable on storage is mainly responsible for the stability of semisolid oil in water emulsion. The deterioration of pharmaceutical and cosmetic creams from semisolids to milky emulsions is observed when the α-crystalline gel phase converts into non-swollen β,γ crystals on storage.

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052
Dendrimeric delivery systems for siRNA and gene therapy intrinsically alter gene expression in human epithelial cells

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Viral and non-viral vectors are two paradigms for gene therapy, however safety concerns such as immunogenicity has narrowed the use of viral vectors (Ferber

2001). Non-viral vectors such as polycationic dendrimers appear promising candidates for oligonucleotide, small interfering RNA (siRNA), DNA and gene delivery (Li & Ma 2001; Merdan et al 2002). However, these delivery systems should possess the highest genocompatibility, ideally with no induction of intrinsic gene expression. We have previously reported gene expression changes induced by cationic lipid delivery systems (Lipofectin and Oligofectamine) using a cDNA microarray approach (Omidi et al 2003). In this current work, the toxicogenomics of starburst polyamidoamine dendrimers (Superfect, SF, and Polyfect, PF) and two lower generations (G2 and G3) of polypropylenimine diaminobutane dendrimers (DAB8 and DAB16) have been investigated in human epithelial A431cells by comparative transcriptome studies using cDNA microarray-based gene expression profiling. Cultured cells (40–50% confluency) were exposed to polymers for 4 h then to normal media for 24 h. Total RNA was then extracted and subjected to gene expression profiling using a cDNA microarray methodology described previously (Omidi et al 2003). Briefly, 10 μg total RNA (from treated and untreated cells) was used to prepare aminoallyl-cDNA, which was post-labelled with cyanine dyes (either Cy3 or Cy5) and hybridized on glass microarrays housing 200 or 20 000 genes. The microarray expression data were validated using a semi-quantitative RT-PCR analysis of multiple genes. Intact (PF) and fractured (SF) starburst dendrimers, at manufacturer's recommended concentrations, induced significant (> 2-fold) gene expression changes (e.g., 1488 genes exhibited altered expression for PF treatment). Such gene changes were sufficient to induce apoptosis (programmed cell death) in A431 cells. Complexation of PF with DNA also induced gene expression changes (457 altered genes), but this signature of gene changes was significantly different from that induced by the PF dendrimer alone. Gene expression changes were also observed upon treatment of A431 cells with DAB8 and DAB16. The nature of the genes whose expression was induced by these dendrimeric nanostructures was diverse and included some common genes including those related to apoptosis (bcl2, caspase 9, cytochrome C and TGFb1) and stress (heat shock proteins) as well as membrane receptors and transcription factors. Our data suggest that dendrimers, and other non-viral vectors can induce diverse gene expression changes in cells and cannot truly be considered to be genocompatible. Thus, their gene expression signature should be determined in target cells before use in gene therapy or gene silencing experiments. There appears to be considerable potential for delivery system-induced genes changes to compete or enhance the desired effects of gene-based therapies. Hence, such gene changes should be taken into consideration when using dendrimeric delivery systems for gene-based therapies.

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053
Polyethylenimine-mediated delivery of small interfering RNA targeting the epidermal growth factor receptor: a comparison of linear and branched polymer architecture

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Small interfering RNA (siRNA) are short RNA duplexes (usually 19–23 nucleotides) that can induce sequence-specific gene silencing via RNA induced silencing complex (RISC) that is located in the cytosol of cells. Targeted gene silencing by siRNAs has been extensively demonstrated in cell culture and more recently in-vivo (Song et al 2003). The epidermal growth factor receptor (EGFR) is known to be involved in the initiation, progression and pathology of several diseases including cancers of the breast and brain and its selective inhibition is likely to have significant therapeutic value in such conditions. Indeed, we have recently shown that siRNA-mediated inhibition of EGFR, through lipoplex-mediated delivery, produced a more potent anticancer effect in A431 epidermoid cancer cells than antisense oligonucelotides, ribozymes or DNAzymes (Beale et al 2003). However, the efficient delivery of siRNAs to cells in-vitro and in-vivo needs further improvement. In this study, we have examined the use of linear and branched architectures of polyethylenimine (PEI) of the same molecular weight (25 kDa) as potential delivery vectors for siRNAs targeting the EGFR. Cellular uptake and biological activity of anti-EGFR siRNA was examined in A431 cancer cells following dual transfections with PEI similar to that described previously (Beale et al 2003). Polymer toxicity was determined at a cellular level using MTT cytotoxicity assay as well as at genomic level using microarrays (and validated by RT-PCR analysis) as described by Omidi et al (2003). Determination of the optimal ratio of polymer:siRNA for uptake of fluorescently labelled siRNA was determined through flow cytometry, while the subcellular distribution was investigated using live cell fluorescent microscopic analysis as described by Hollins et al (2004). The biological activity of the delivered siRNA was determined using a cell proliferation assay and quantification of EGFR protein expression by Western blotting (Beale et al 2003; Hollins et al 2004). Consistent with previous studies on polymer toxicity in human cells, the linear PEI architecture exhibited a higher IC50 ($\sim$321 μg mL^{-1}) compared than the branched ($\sim$159 μg mL^{-1}). A toxicogenomic evaluation of these polymer architectures using microarray-based transcriptomics confirmed that branched PEI induced a greater number of gene expression changes relative to the linear PEI. At predetermined sub-toxic concentrations, branched PEI enhanced siRNA uptake by 6-fold. In contrast, linear PEI showed little or no improvement in siRNA delivery compared with naked siRNA alone. Fluorescent microscopy demonstrated that PEI-siRNA complexes were largely distributed within the cytosol of the cell- the site also for where RISC is thought to reside (Dorsett & Tuschl 2004). Anti-EGFR siRNA delivered by branched PEI inhibited A431 cell growth and produced a > 60% reduction in total EGFR expression (normalised against the housekeeping protein β-actin). Our studies suggest that efficient siRNA uptake and activity is dependent on the PEI polymer architecture. Further studies are ongoing to understand the underlying mechanism of this phenomenon.

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054
An investigation into the mechanisms of the drug release from the fatty acid-based oral formulations

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Stearic acid and palmitic acid have been used in paediatric oral particulate formulations for taste masking purposes. The drug release from the matrix is highly dependent on the pH of the dissolution medium (Robson et al 1999) and the composition of the formulation, with faster release from matrices composed of stearic acid/palmitic acid (1:1 w/w) in alkaline conditions. In previous studies we have obtained evidence of sodium acid-soap formation during the dissolution. In this investigation, freeze-fracture scanning electron microscopy (FFSEM), high sensitivity differential scanning calorimetry (HSDSC) and neutron reflection were applied for studying the further potential mechanisms on the drug release from this fatty acid-based formulation. The drug-free microspheres (50SA50PAM) were prepared using customized spray-chilling apparatus using 50:50 (w/w) 99% purity stearic acid and palmitic acid. The freeze-fractured microspheres were prepared by grinding the particles with liquid nitrogen for 5 min. The SEM results of freeze-fractured 50SA50PAM showed significantly mottled texture on the surface with a multi-layered internal structure. After 4 h exposed to pH 8.0 alkaline buffer at room temperature, followed by washing and dry, the 50SA50PAM showed large amount of flakes build up gradually from the surface to the inside, which may relate to the new formation of acid-soaps and this instable structure eventually led to the collapse of the microspheres. The HSDSC was preformed from 20°C to 80°C using a heating rate of 1°C min^{-1} and the mixture of microspheres with alkaline buffer in sample vessel and the same buffer solution as reference. The HSDSC results of the 50SA50PAM in buffer exhibited multiple transitions. Two of these transitions, at 55–57°C and 66–68°C, are similar to the previous DSC studies of the washed and dried buffer treated 50SA50PAM, which has been confirmed the new formation is its acid-soap by using XRPD. The same transitions were found in the HSDSC results of the buffer treated 50SA50PAM re-dispersed into water and using water as reference. Neutron reflection was used to measure the average scattering length density and thickness of a monolayer consisting of deuterated and hydrogenous stearic and palmitic acids. The 1.0 mM chloroform solution of hydrogenous (*h*-) and deuterated (*d*-) stearic and palmitic acid cross combination mixture of 50:50 were spread on pH 8.0 alkaline buffer. The results of the *d*-stearic acid/*h*-palmitic acid showed the scattering length density increases with the gradual increase of the proportion of stearic acid, which corresponded to the surface loss of palmitic acid over time. It was supported by the result of the *h*-stearic acid/*d*-palmitic acid (50/50) where its scattering length density gradually decreases as the palmitic acid fractional coverage reduced from 50% to 37% in 60 min. It indicates the dissolution of palmitic acid into the alkaline solution. In conclusion, this study provides more evidence of the formation of its acid-soaps

play an important role in the drug release from the 50SA50PAM in alkaline buffer.

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Investigating the mechanism of formation of protein coated microcrystals

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Formulation of therapeutic biomolecules as dry powders is an area of increasing interest because it provides for stabilisation of bioactivity and serves as route to new drug delivery technologies. Powders made by conventional routes such as spray-drying generally contain amorphous excipients and this can limit the range of humidity and temperature over which stable powder performance may be obtained. In contrast, protein coated microcrystals (PCMC; Kreiner et al (2001)) with a crystalline core made from amino-acids such as valine or glutamine are found to exhibit unchanged powder characteristics even following exposure to high humidity and elevated temperature. Importantly this feature extends to aerodynamic properties. Protein coated microcrystals are prepared by a coprecipitation process. This involves addition of an aqueous mixture containing a bioactive molecule and a concentrate of carrier material to a water miscible solvent such as isopropanol. The PCMC precipitate out directly as a suspension within the solvent and may be isolated to yield a fine free-flowing dry powder. The coprecipitation process may be carried out either batch-wise or continuously with the latter favoured for scale-up. It is clear that formation of PCMC involves a self-assembly process but detailed investigations are required to pin down the mechanism more precisely so that the process can be optimised. This contribution will describe Zeta potential measurements and dynamic light scattering studies aimed at more precisely characterising the coating process. Zeta potential measurements were aimed at determining whether electrostatic binding plays an important role in binding the bimolecule to the crystal surface. Model proteins adenosine deaminase, trypsin and lysozyme with pI of 4.85, 9.3 and 11.0, respectively, were coated onto amino-acid crystals at different pH and the Zeta potential of the resultant PCMC measured using a Malvern Zetasizer. The values obtained were compared with those of 'naked' microcrystals prepared under identical conditions. The results clearly show that the Zeta potentials of the microcrystals were altered by the presence of the protein and the size of the particles was reduced, consistent with coating of protein on the surface. At pH 7, trypsin and lysozyme coated microcrystals gave rise to more positive Zeta potentials while those coated with adenosine deaminase were more negative. This accords with the expected charge on the proteins at this pH. The fact that both positive and negative proteins coat microcrystals in a similar way suggest electrostatic binding is not a key driver in the formation mechanism of protein coated microcrystals.

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Alveolar epithelium is able to internalise macromolecules via the caveolar membrane system

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It is now widely recognized that proteins and peptides display relatively good systemic bioavailability when administered by inhalational aerosol to the lung. In particular, for systemic delivery of such biologics it is the lung alveolar epithelium that is considered the target absorption barrier. As a corollary there has been significant improvements in aerosol device technology to afford reproducible aerosol deposition patterns that encompass the deeper peripheral lung regions. Nevertheless, it is evident that significant loss of inhaled therapeutic protein and peptides still occurs within the lung airspaces primarily due to clearance mechanisms involving degradation and/or the mucociliary escalator. Understanding the absorption process for macromolecules across lung alveolar epithelium may provide a means for its modulation, and hence a shift in the competing kinetic forces of airway clearance and systemic absorption. Lung alveolar epithelium itself is comprised of squamous alveolar type I epithelium (95% surface area), which represents the limiting barrier in transport between alveolar airspace and capillary blood. These are very thin ($\sim$0.3 μm) cells possessing a high density of vesicular structures termed caveolae, whose main structural protein is caveolin-1. It has been proposed that caveolae may mediate the transport of inhaled proteins from alveolar airspace to blood. Previous work by our group has exploited live cell imaging of alveolar epithelial cells expressing Green Fluorescent Protein tagged Caveolin-1 (GFP-Cav-1) chimeras (Hollins et al 2002), and shown that caveolae structures in the alveolar cells are poorly mobile. However, given the thin attenuated nature of the in-vivo cell type and the high density of caveolae that they possess, then even limited vesicular mobility may allow for macromolecular transport. Here we sought to use fixed cell immunofluorescence microscopy to examine if macromolecular probes could be colocalised within caveolae structures in in-vitro alveolar epithelial cell models. Type II human lung adenocarcinoma (A549) cells were cultured on glass coverslips in the presence of 0.1 μM dexamethasone, which promotes their transformation from a type II to type I phenotype. The endocytic pathway in these cells was investigated using fluorescent conjugates of cholera toxin B subunit (CtxB), bovine serum albumin (BSA) and transferrin (Tf) in conjunction with antibodies against early endosomes (EEA-1), lysomomes (LAMP-1), *trans*-Golgi network (TGN-46) and caveolin-1 (Cav). Images were acquired in X-Y-Z mode using a Leica TCS SP2 RS confocal microscope. As a positive control, transferrin and EEA-1 were shown to colocalise in these cells. No significant colocalisation between caveolin-1 and EEA-1, LAMP-1 and TGN-46 was evident, indicating caveolae to represent distinct vesicular compartments. Substantial intracellular accumulation of CtxB was evident within 10 min of probe endocytosis, the majority of the CtxB label present immediately beneath the plasmalemma. CtxB vesicles showed considerable colocalisation with Cav and to a lesser extent with EEA-1. Longer incubation of 20–40 min resulted in movement of CtxB labeled vesicles to a perinuclear/Golgi location of the cell with retention of some co-localisation with Cav still evident. Colocalisation between BSA and Cav was also demonstrated in these cells. These studies have shown for the first time that caveolae within alveolar epithelium are able to internalize macromolecular probes and therefore provide in-vitro evidence that the caveolae membrane system are likely to be important in the pulmonary alveolar absorption of therapeutic proteins.

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Effect of HLB of emulsifier on the encapsulation efficiency of PLA microspheres containing naltrexone

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Various techniques have been used for fabricating microparticulate drug delivery systems, among which water in oil in water double emulsification solvent evaporation may be the most widely used method for encapsulation of water-soluble drug molecules such as peptides and proteins. In general, stability of the primary emulsion is a prerequisite for the successful encapsulation of drug molecules into microparticles. Addition of emulsifiers to the internal emulsion (O/W_1) could have a dramatic effect on emulsion stability, and thus on microsphere properties. In this study, poly (d, l-lactide) microspheres containing naltrexone hydrochloride were prepared using W/O/W double emulsion solvent evaporation technique. Effect of addition of one of the five different emulsifiers, tween 20 (HLB 16.7), tween 80 (HLB 15), tween 85 (HLB 11), span 20 (HLB 8.6) and span 80 (HLB 4.3), to the first aqueous phase (W_1) on the encapsulation efficiency was investigated. Briefly, PLA was dissolved in methylene chloride to form a 3% w/v solution. An aqueous solution containing 10% w/w naltrexone hydrochloride (with respect to PLA) was prepared separately (W_1). This water phase was emulsified into the polymer phase at 18 000 rev min^{-1} for 2 min. One percent (w/v) of one of the above-mentioned emulsifiers was added to the system. Afterwards, the primary emulsion was added to a 0.5% w/v poly vinyl alcohol solution (W_2) at 600 rev min^{-1} to form the double emulsion ($W_1/O/W_2$). Mixing continued for 4 h at room temperature until evaporation of methylene chloride completed and hence microspheres were formed. The experiments were repeated at least three times. The encapsulation efficiency of different formulations is presented in Table 1. In general, incorporation of surfactant led to higher drug encapsulation efficiency by improving the W_1/O emulsion stability. Span 80 (HLB 4.3) led to the highest encapsulation efficiency among the different emulsifiers. This may be due to the lower HLB of span80. Therefore it is most suitable for stabilizing the O/W emulsions and this stability led to better naltrexone loading. By increasing the HLB value of emulsifiers (span20 and tween85) encapsulation efficiency decreased. Contrary to expectation, encapsulation efficiency increased in high HLB values (HLB > 15). Tween20 and tween80, due to their high HLB values,

have strong tendency to stabilize W/O emulsions. Their noticeable effect on improving the encapsulation efficiency could be attributed to the fact that during the process of microsphere preparation, a part of such hydrophilic surfactants could migrate to the interface between oil and the outer aqueous phase (W_2) modifying the surface properties of the microspheres. This migration may reduce the amount of drug adsorbed onto the microsphere surface and hence reduce the number of pores and channels (Rojas et al 1999) and consequently decrease leaching out of drug to the W_2. In conclusion, this study demonstrated that HLB of emulsifier could have a significant effect on the encapsulation efficiency of poly lactide microspheres prepared by double emulsion technique.

Table 1 Effect of different emulsifiers on the encapsulation efficiency of PLA microspheres (n = 3)

Type of emulsifier	HLB	Mean encapsulation efficiency	± s.d.
None	—	11.9	1.54
Tween 20	16.7	34.7	3.46
Tween 80	15.0	33.1	1.56
Tween 85	11.0	26.8	3.61
Span 20	8.6	25.4	2.30
Span 80	4.3	37.2	2.78

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In-vivo behaviour of pectinate-TMC beads intended for colon-specific drug delivery

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The oral route of administration of drugs is the most convenient route. Peptide drugs, however, should be protected from the harsh gastric environment and peptidase enzymes of the small intestine so as to reach intact the relatively mild environment of colon and at last their absorption should be enhanced at this site, if they are to be administered orally. We developed a pectinate bead system and incorporated trimethyl chitosan chloride (TMC) as an absorption enhancer into the system and evaluated it in-vitro. Zinc pectinate beads were prepared based on 2^3 full factorial design by ionotropic gelation of low-methoxy amidated pectin (P) gel in zinc acetate (El-Gibaly 2002), by a Nisco encapsulator. Coomassie Brilliant Blue G (CB) and TMC were incorporated into the pectin gel. TMC was synthesized in a two step method (Sieval et al 1998). Some factors were kept constant due to the results of some preliminary screening experiments: crosslinking time was 2 h; TMC concentration was 1/6 of pectin's; CB concentration was 0.3%. The evaluated factors, pectin concentration, zinc acetate concentration and the encapsulator voltage (V) are summarized in Table 1. The loading efficiency (LE) of the beads was determined by assessing the amount of CB lost in the crosslinking solution by the equation LE = [(Theoretical amount of CB − Amount lost in the crosslinking solution)/Theoretical amount of CB] × 100. A pre-weighed amount of beads were evaluated for their swelling ratio (SR) and in-vitro release of CB in test tubes containing 10 mL of phosphate buffer (pH = 7.4), in a shaker water bath at 37°C. The SR was determined by the following equation: SR = [(Weight of beads at time t − Dry weight)/Dry weight] × 100. The loading efficiency was 100% in formulation containing TMC, but in formulation without TMC it was approximately 95%. Drug release results are shown in a Table 1. The TMC concentration was fixed at 1/6 of pectin concentration, since increasing the ratio would dramatically cause early disintegration of the beads. Another interesting observation was that although we expected the inclusion of TMC as a soluble ingredient to cause CB to release faster, at the mentioned concentration it delayed the release of CB compared with the formulation without TMC. Therefore it can be suggested that a complex is formed between TMC^+ and CB^-. This was partly proved by titration of a fixed amount of CB with TMC and centrifuging the samples and reading the UV absorption of the supernatant. It appears that an insoluble complex is formed at a certain ratio of CB:TMC. From the release and SR data one concludes that P5% is better than P3.5%. All the formulations released less than 40% of their drug contents in 5 h. However, comparing with SR studies, some beads partially disintegrated within 5 h but retained more than 60% of their drug content.

Table 1 Swelling ratio (SR) and release of CB from pectinate–TMC beads

	P(%)	ZnAc (M)	V (KV)	% Release after 5 h	S.R. during 5 h		
					1 h	3 h	5 h
F1	3.5	0.15	2	34 ± 7.7	220 ± 33.8	183 ± 5.7	−100 ± 0
F2	5	0.15	2	14.6 ± 4	300 ± 14	315 ± 70	150 ± 28
F3	3.5	0.6	2	30 ± 6.5	153 ± 5.7	−60 ± 30	−100 ± 0
F4	5	0.6	2	28.1 ± 7	240 ± 0	85 ± 21	−80 ± 28
F5	3.5	0.15	0	34.4 ± 4.9	286 ± 32	186 ± 23	−76 ± 25
F6	5	0.15	0	18.3 ± 1	275 ± 21	265 ± 7	205 ± 49
F7	3.5	0.6	0	32.1 ± 7.7	200 ± 10	−50 ± 15	−100 ± 0
F8	5	0.6	0	36.5 ± 1.8	290 ± 0	220 ± 28	−35 ± 10

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Skin permeation studies of transdermal formulations of fentanyl through hairless mouse stratum corneum

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Different permeation parameters such as lag time (T_{lag}) and flux (J) were calculated to explain the mechanism of fentanyl penetration through stratum corneum. These investigations represent the effects of various polymeric vehicles on percutaneous absorption of fentanyl in solution formulation and in pressure sensitive adhesive (PSA) matrix across the hairless mouse skin. In-vitro permeation studies were carried out at 32°C, using stratum corneum obtained from hairless mouse skin in a modified Franz diffusion cell with 1.2 cm diameter and 5.6 mL volume. Full-thickness hairless mouse skin was excised from fresh carcasses. Subcutaneous fat was removed carefully with scissors and scalpel. Phosphate buffer solution (PBS) pH 7.2 was used as receiver medium. The hairless mouse skin was mounted onto each receiver cell, and O-ring and donor cells were placed on the top of each skin, and then any air bubbles that remained in the receiver cells and below the skin were removed. When hydro-alcoholic solution formulations were tested, 2 mL of fentanyl solution in a hydroxyl ethyl cellulose gel vehicle was placed in the donor compartment. When matrix formulations were tested, a PSA matrix disc with the surface area of 1.2 cm^2 was applied to the epidermal side of the mouse skin with slight pressure before mounting on the receiver cell. At each sampling time interval, a 100-μL specimen was withdrawn and replaced with fresh PBS. The cumulative amount of fentanyl was determined by HPLC. The analysis was carried out by a full-validated method on a Capital C18 (250 × 4.6 mm) column using a mobile phase consisting 4 volumes of ammonium acetate solution (1 in 100) and 6 volumes of a mixture of methanol, acetonitrile and glacial acetic acid (400:200:0.6) with a pH of 6.6 ± 0.1 at a flow rate of 2 mL min^{-1}. The retention time and plate numbers were 4.1 min and 2050, respectively. The permeation profiles of fentanyl from solutions were different depending on vehicles used. The amount of alcohol up to 15% increased the J and decreased the T_{lag}. Those vehicles may act as permeation enhancers by increasing the thermodynamic activity of the drug and/or changing the barrier property of the skin (Møllgaard & Hoelgaard 1983). These results might also be explained by skin hydration and possible shunt route penetration provided by hydro- alcoholic solution (El Maghraby et al 2001). The flux of fentanyl also increased significantly as its concentration in the solutions increased. Incorporation of vehicles into the acrylic adhesive matrix significantly enhanced the permeation rate and shortened the T_{lag} of fentanyl. The maximum J obtained from pressure sensitive adhesive matrix seemed to be high enough to obtain therapeutic effect. As a conclusion, the maximum J obtained from solution and matrix formulation was 6.4 and 4.2 $\mu g\,cm^{-2}\,h^{-1}$, respectively. The human skin is generally less permeable than the hairless mouse skin (Kim et al 2000), so the actual size of the system must be determined after evaluating the permeation rate across the human skin.

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