Poster Session 3 – Drug Delivery

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Penetration characteristics of 5-aminolevulinic acid through keratinized structures; potential of photodynamic therapy for the treatment onychomycosis

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Onychomycosis is a fungal infection of the keratinized tissue of the nail plate, which has been traditionally and notoriously difficult to diagnose and treat (Stier *et al* 2001). It accounts for approximately one-third of all fungal infections of the skin and an increase in prevalence has recently been experienced. A recent survey revealed that 2.7% of the population of the UK (1.2 million people) and 2–18% of the world's population are now affected (Einarson *et al* 1996). Available treatment modalities for consideration are topical and oral therapies or surgical intervention. The poor success rates of topical treatments are attributed to the impenetrability of the compact keratinized structure of the nail.

There is growing interest in photodynamic therapy (PDT), primarily as a novel treatment for eradication of neoplasias, but also in the treatment of infectious diseases. PDT comprises administration of a photosensitizer, such as aminolevulinic acid (ALA), followed by exposure to light, which induces a cascade of photochemical and photobiological events causing irreversible damage to cellular structures. The aim of this work was to evaluate the delivery and penetration profile of ALA through avulsed human nail and pig hoof membrane as a first step in PDT of onychomycosis.

ALA was formulated into a bioadhesive patch along with a known amount of ^{14}C-ALA. Penetration into hoof was evaluated using a microtome sectioning technique. Penetration through nail was evaluated using a modified Franz diffusion cell. Results for nail penetration parameters from three experiments are shown in Table 1.

Table 1 Penetration characteristics of ALA through human nail

Replicate	Depth of nail (cm)	Flux (mg cm^{-2} s^{-1})
1	7.82×10^{-2}	9.39×10^{-5}
2	7.77×10^{-2}	2.24×10^{-4}
3	7.66×10^{-2}	5.55×10^{-7}

Results indicate that ALA penetrates nail structures, with a flux that compares favourably to literature values of conventional antimicrobial agents, such as chloramphenicol (8.02×10^{-8} mg cm^{-2} s^{-1}) and nystatin (4.02×10^{-9} mg cm^{-2} s^{-1}). Variation in flux (Table 1) was attributed to interpatient differences, where some harvested nails appeared more dense than others. PDT may, thus, be a potential means to eradicate fungal spores that harbour within the nail matrix.

Einarson, T. R., *et al.* (1996) *Pharmacoeconomics* **9**: 307–320
Stier, D. M., *et al.* (2001) *J. Am. Podiatr. Med. Assoc.* **91**: 521–527

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Evaluation of ALA-induced cell death rates during photodynamic therapy using the HeLa cell line

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Treatment of neoplastic superficial lesions in the lower female reproductive tract can be accomplished using a wide range of options, such as cryotherapy, laser ablation and radiotherapy. These, along with surgical intervention, all carry some degree of morbidity and poor cosmetic outcome, all of which make treatment options less attractive to younger women. Photodynamic therapy (PDT) is emerging as an alternative treatment procedure that has distinct advantages over convention therapies, not least of which is selective cell death and healing without scarring (Morton *et al* 2002). PDT comprises the administration of a photosensitizer, such as aminolevulinic acid (ALA), followed by exposure to light, which induces a cascade of photochemical and photobiological events causing irreversible damage to cellular structures.

At present, drug delivery to superficial lesions in the lower female reproductive tract is hampered by poor dosage form design. No clear information on the dosage of ALA required or the light dosimetry exists. The aim of this work was to use a cervical cell line, HeLa, as a model to evaluate the optimum dose of ALA required, the length of exposure to drug and the dose of light needed. Cultured HeLa cells were exposed to 0.01, 0.1, 1.0, 10.0 and 100 mM ALA and irradiated at 100 J cm^{-2} (635 nm) for different time periods. Viable cell numbers were evaluated afterwards using the MTT assay. Results showed that exposure for 5 minutes and concentrations at 1.0 mM were sufficient to achieve substantial levels of cell death (Table 1).

Table 1 Effect of ALA concentration on HeLa survival after 5 min irradiation

Concn of ALA (mM)	Mean viable cell count
0.01	46210 ± 760
0.1	41691 ± 2327
1.0	5247 ± 2327
10.0	8025 ± 2327
100.0	5528 ± 2327

Data are means ± s.d., n = 5 replicates

Results from this work show that a tissue concentration of 1.0 mM must be achieved within the lesion if a drop in viable cell count is to be expected. This information is currently being used to optimize the design of topical bioadhesive systems for ALA delivery to gynaecological neoplasias.

Morton, C. A., *et al.* (2002) *Br. J. Dermatol.* **146**: 552–567

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Delivery of 5-aminolevulinic acid using a novel bioadhesive patch for the photodynamic therapy of vulval Paget's disease

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Paget's disease is a rare, neoplastic condition occurring predominantly on the nipple and areolar region, with close associations with underlying invasive ductal carcinoma. The extra-mammary variant of Paget's disease (EMPD) develops around the anogenital areas, with the most prevalent form affecting the vulval area of white, postmenopausal women. Treatment of EMPD is difficult, but recent published work has shown efficacy of photodynamic therapy (PDT) in the treatment of vulval Paget's disease (Shieh *et al* 2002). Although still relatively new, the technique has been applied to the clinical management of a wide range of neoplastic conditions (Hopper 2000). It involves the interaction between a photosensitizer, light at a specific wavelength and molecular oxygen. The administration of photosensitizers, such as aminolevulinic acid (ALA), is followed by exposure to a light, which induces the photosensitizer to trigger a cascade of photochemical and photo-biological events causing irreversible damage to tumour tissue.A bioadhesive patch is described that overcomes the challenge facing delivery of ALA to both intact skin and superficial lesions (i.e., the need for prolonged ALA application, often exceeding 4 h). This difficulty is compounded by the geography of vulval lesions, which exist between skin folds that are close to the urethra and anus and subjected to substantial frictional stresses. To overcome these problems that are specific to the shape of the vulva, a water-soluble bioadhesive patch has been developed that delivers a defined dose of ALA to

vulval lesions. The patch is comfortable and does not require occlusion. Importantly, it remains in place in the ambulant patient, who is free to return home during the drug administration phase.

The patch was applied to a 66-year-old Caucasian female who had been referred for treatment of vulval Paget's disease. She had had an 8-year history of persistent vulval itch, which had been treated unsuccessfully with antifungal, antibiotic and steroid creams. Upon patch removal, the target area was exposed to red light at 630 nm generated by incoherent lamp source, which delivered a light dose of 100 J cm^{-2}. Subsequent clinical and histological evaluation revealed complete clearance of Paget's cells. Patient evaluation reported disappearance of all symptoms. This case report demonstrated the clinical applicability of the bioadhesive patch for PDT of vulval Paget's disease.

Hopper, C. (2000) *Lancet Oncology* **1**: 212–219
Shieh, S., *et al*. (2002) *Br. J. Dermatol*. **146**: 1000–1005

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Genistein — a pharmaceutical formulation

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Postmenopausal women are amongst the largest consumers of food supplements (Albertazzi *et al* 2002). Soy and soy-derived supplements are widely consumed in this age group as a "natural" form of hormone replacement therapy. Genistein is the most estrogenic of the isoflavones present in soy. We describe the development of our formulation of a genistein capsule to use in a study in osteoporosis.

A source of food grade material genistein was located and its identity verified by high-pressure liquid chromatography (HPLC) retention time and ultraviolet spectroscopy peak maxima. Its purity was determined by comparison with an authentic standard using HPLC. The microbial count was determined using Total Viable Count. A limit test for heavy metals was determined (10 ppm). A capsule containing 90 mg genistein was hand-packed using an appropriate powder vehicle, by St Mary's Pharmaceutical Unit in Cardiff. The capsule sample was then subjected to quality control testing in an accredited pharmaceutical laboratory. Capsule fill, genistein content, and capsule dissolution were verified. Capsule dissolution was compared by United States Pharmacopoeia (2000) method with estradiol 2 mg tablet (Trisequens).

A mean capsule content of 95.3% of the nominal value was achieved, though a number of capsules in the sample varied from the mean causing the sample to fail the uniformity test (mean 86 mg, range 77–103 mg, limit 81–99 mg per capsule, coefficient of variability CV 8.1%). Genistein was found to have very low water solubility both when tested for dissolution from the capsule (mean 9%, range 8–10%), and when compared with estradiol (100%).

There is no monograph in a pharmacopoeia for genistein or genistein capsules. The capsules were tested according to the general BP monograph for hard gelatine capsules. Total viable count indicated the raw material was suitable for oral use. The assay requirement was set at 90–110% of stated. The nominal content has been verified to the set standard although the capsule to capsule variation just failed to meet BP uniformity standard. We accept that factors of scale and automation could deliver a product which would meet the BP specification but feel that the observed results are what one could expect for a handmade product. The poor dissolution of genistein was expected due to low water solubility. Disintegration (at mean 5.6 min, range 5.5–6.1 min, CV 4.3%) was within BP limits. Preliminary tests indicated that both genistein and estradiol did not dissolve in aqueous buffer or dilute acid solutions. The USP method using sodium lauryl sulphate 0.3%w/v indicated a difference between the solubility of the genistein product and Trisequens 2 mg tablet. Sodium lauryl sulphate is a novel dissolution medium and the pharmacokinetic significance is unknown. The dissolution results will be considered when analysing the trial results.

A 90 mg genistein capsule has been prepared to an acceptable standard for our pilot study. The trial has commenced recruitment and we look forward to reporting the results in due course.

Albertazzi, P., *et al*. (2002) *Climacteric* **5**: 374–382
United States Pharmacopoeia (2000) 678

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Vaccination of BALB/c mice against cutaneous leishmaniasis using fusogenic liposomes incorporated with recombinant major surface glycoprotein of leishmania (rGP63)

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Vaccination could be one of the answers to the control of cutaneous leishmaniasis. The protective immunity against the leishmaniasis is the cell-mediated immunity (CMI). Recombinant major surface metalloproteinase glycoprotein of Leishmania (rGP63) is one of the promising candidates for subunit vaccine against leishmaniasis (Handman 2001). Liposomes are microscopic vesicles consisting of phospholipid bilayers which enclose aqueous compartments and utilized as a delivery systems for drugs, proteins and DNA. Liposomes can also function as an efficient immunoadjuvant in inducing immune responses to protein antigens (Gregoridis 1990). The objective of this study was to formulate liposome preparations loaded with rGP63 to selectively induce CMI in susceptible BALB/c mice and protect them against cutaneous leishmaniasis.

Liposomes containing rGP63 was prepared as dehydration-rehydration vesicles (DRV) and composed of DPPC (Dipalmitoylphosphatidylcholin) and cholesterol (Chol) (DRV-DPPC/Chol-rGP63 7:2 molar ratio) (Gregoridis *et al* 1987). To this formulation, DOPE (dioleoylphosphatidyl-ethanolamine) (DRV-DPPC/DOPE/Chol-rGP63 7:1:2 molar ratio) was added to produce fusogenic liposomes (Kono *et al* 2000). Liposome preparations containing rGP63 (2 μg), rGP63 (2 μg) by itself (PBS-rGP63), PBS (Phosphate buffer saline) and a control empty liposome (DRV-DPPC/Chol 7:2 molar ratio) were injected subcutaneously (SC) three times in groups of 10 female BALB/c mice with three weeks interval. Three weeks later the mice were tested for delayed type hypersensitivity (DTH) by injecting 2 μg of rGP63 SC to the left footpads; and for comparison the right footpads were injected with PBS. After 24, 48 and 72 h the footpad thickness was measured on both foot pads. One week after the DTH test, the mice were challenged with virulent L. major promastigotes (1×10^6/50 μL) SC to the left footpads and the footpad thickness was measured for 10 weeks (Russell *et al* 1988).

All the liposome formulations were heterogeneous in size, ranging from 0.1 to 1.5 μm, but their encapsulation rate was high (46–52%). The results of DTH test indicated that among different groups, the DRV-DPPC/DOPE/Chol-rGP63 had the greatest positive DTH response compared with the control groups ($P < 0.01$). The DTH responses of DRV-DPPC/Chol-rGP63 and PBS-rGP63 groups were also positive ($P < 0.05$); however, they were less. In the challenge test, the DRV-DPPC/DOPE/Chol-rGP63 showed a very good protective effect (83%) ($P < 0.001$) compared with the controls. The DRV-DPPC/Chol-rGP63 had also a suitable protective effect (75%) ($P < 0.001$) compared with the controls but it was less than the DRV-DPPC/DOPE/Chol-rGP63. The PBS-rGP63 showed only partial protection (55%) ($P < 0.05$). The protective effects of the DRV-DPPC/DOPE/Chol-rGP63 and DRV-DPPC/Chol-rGP63 were significantly ($P < 0.05$) more than PBS-rGP63. These results indicate that DRV-DPPC/DOPE/Chol-rGP63, which is fusogenic liposomes, could be a suitable immunoadjuvant for rGP63 to induce selective CMI in BALB/c mice and protect the mice against cutaneous leishmaniasis.

Gregoridis, G. (1990) *Immun. Today* **11**: 89–97
Gregoriadis, G., *et al*. (1987) *Vaccine* **5**: 145–151
Handman, E. (2001) *Clin. Microbiol. Rev.* **14**: 229–243
Kono, K., *et al*. (2000) *J. Controlled Release* **68**: 225–235
Russell, D. G., *et al*. (1988) *J. Immunol*. **140**: 1274–1279

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Effect of internal aqueous phase volume on evaluation parameters of PDLLA microparticles loaded with insulin

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Insulin is presently the most effective drug in treatment of insulin dependent (Type I) diabetes mellitus. Current dosage regimens of insulin comprise up to four subcutaneous injections per day. Compliance with such demanding dosage regimens is difficult, hence the development of a delivery system that is effective for long term delivery is the need of the hour. Insulin loaded microparticles of PDLLA (Poly-dl-lactic acid) polymer were made using multiple emulsion (w/o/w) technique and the effect of internal aqueous phase volume on release profile, morphology, particle size and encapsulation efficiency of insulin was evaluated. An insulin aqueous solution (400 IU mL^{-1}) was emulsified (by sonication) with 2 mL 10% w/v polymer in dichloromethane (DCM). The primary emulsion thus formed was then sonicated with 2% PVA (polyvinyl alcohol). The resulting w/o/w emulsion was stirred overnight under ambient conditions to evaporate DCM. The microparticles were centrifuged, freeze dried and stored at $-20^{\circ}C$ under desiccant. Table 1 indicates the resulting encapsulation efficiencies and mean particle size of microparticles. Encapsulation efficiency was determined using Micro BCA protein reagent kit. Particle size was determined by laser diffractometry using Malvern Mastersizer. Surface topography was assessed using scanning electron microscopy.

Table1 Encapsulation efficiency and mean particle size

Batch no.	Int. Aq. phase vol. (μL)	Encapsulation efficiency (%)	Mean particle size (μm)
A	100	42.0	31.0
B	200	65.6	63.4
C	800	31.0	28.3

Increase in internal aqueous phase volume from 100 μL to 200 μL resulted in two fold increase in particle size from 31 μm to 63 μm and hence encapsulation efficiency is increased from 42% to 66%. Increase in size due to increase in w/o ratio is related to reduction in agitation, which occurred because of increase in mixing efficiency associated with larger volume. A reduction in mixing efficiency produced an increase in size of emulsion droplets formed during process. As a result of decreased size there is increase in particle volume which leads to more encapsulation efficiency. These results are supported by Sturesson *et al* (1999) who have reported doubling of particle size of PLGA microspheres encapsulating, rotavirus when aqueous phase volume was increased from 1 mL to 5 mL. Decrease in encapsulation efficiency using higher volumes shows the requirement of an optimum phase volume for the particles of desired size and encapsulation efficiency. Lamprecht *et al* (1999) have reported similar results with BSA entrapped in PLGA nanoparticles. Burst release was observed to some extent with all PDLLA microparticles. This fact is similar to reported by Uchida *et al* (1997). Less burst effect was observed in case of batch A. This is due to the fact that smaller internal aqueous phase volumes seems to be advantageous for controlling the internal burst release. Hence an optimum phase volume is required to obtain the particles of desired size with high encapsulation efficiencies and sustained release with minimum burst effect.

Lamprecht, A., *et al*. (1999) *Int. J. Pharm*., 184: 97–105
Sturesson, C., *et al*. (1999) *J. Controlled Release* **59**: 377–389
Uchida, T., *et al*. (1997) *Chem. Pharm. Bull*. **45**: 1539–1543

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Conjugation of biomolecules to medical device materials for improved haemo- and biocompatibility

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Upon implantation in the body many biomaterials in contact with blood or living tissue may trigger adverse responses such as thrombosis, inflammation and device-associated infections. As a result of this various techniques have been involved in the surface modification of polymeric implants including the immobilization of biomolecules, the incorporation of hydrophilic grafts to minimise protein absorption, and the creation of microdomains to regulate cellular and protein adhesion (Schierholz *et al* 2002).

The use of pure polydimethylsiloxane for manufacture of medical devices is not possible due to its poor mechanical strength, however this material possesses many desirable properties including low-surface tension, extreme hydrophobicity, good oxidative, thermal and UV stability in addition to having high haemo- and bio-compatibility (Tang *et al* 1999). Similar suppressed protein adsorption has also been ascertained with the use of poly(ethylene oxide) (Kim & Kim 2002). Due to their incompatibility, upon blending of these materials the air-polymer interface is generally dominated by the low-surface energy silicone providing a more biocompatible surface than that of the bulk polymer. Covalent linkage via suitable functionalities provides a means of permanently anchoring biomolecules preventing diffusion and subsequent loss of the surface properties.

The conjugation of an amine-functionalised silicone and poly ethylene glycol methyl ether to commercially available polyethylene-graft-maleic anhydride (PE-g-MA) copolymers has been carried out. Polydimethylsiloxane-co-3-aminopropyl-methylsiloxane was added to a solution of PE-g-MA in hexane and the mixture refluxed. Polymeric conjugates were synthesised with an approximate silicone content of 15%. Conjugation of the ether was carried out in a similar fashion with an equimolar amount of PE-g-MA and characterisation of these materials, including the study of protein adsorption with the use of a Quartz Crystal Microbalance, has been undertaken. Incorporation of silicone into the PE-g-MA materials showed a reduction of 32% (15 Hz) in the frequency of the quartz crystal in comparison to a quartz crystal coated with PE-g-MA upon exposure to a fibrinogen solution (100 μg mL^{-1}), demonstrating the lower capacity for protein adsorption, and hence higher haemocompatibility of silicone-incorporated material.

Kim, J. H., Kim, S. C. (2002) *Biomaterials* **22**: 2015–2025
Schierholz, J. M., *et al*. (2002) *Int. J. Microbial Agents* **19**: 511–516
Tang, L., *et al*. (1999) *Biomaterials* **20**: 1365–1370

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Rapidly dissolving and dispersing films

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Dry thin film technologies are making an impact as novel dosage forms in both the healthcare and pharmaceutical markets. Pullulan, a bioadhesive polysaccharide of low viscosity, has particular advantages for making rapidly dissolving films. The intrinsic low viscosity, however is a disadvantage in manufacture but handling can be improved by the addition of viscosity modifiers. Whereas an increased viscosity can have additional benefits in an oral healthcare product, it is not desirable in a rapidly dispersing oral pharmaceutical dosage form.

We have developed the use of a variable viscosity modifier to make a dosage form that has high viscosity during manufacture but low viscosity in use.

Sodium alginate is a naturally occurring co-polymer of mannuronic and guluronic acid salts. It is water soluble above pH 4, but under more acidic conditions it is

converted to the insoluble but water swellable (gelling) alginic acid. We have prepared viscous polymer mixtures of pullulan and sodium alginate at around pH 3.5, which can be readily cast. Included in these mixtures is a volatile acid that is removed during subsequent film drying. When rehydrated at a pH of 6.4–8 (i.e. the pH of the mouth), the resultant solution has low viscosity and rapidly disperses. Therefore we have developed a rapidly dissolving and dispersing oral dosage form that has good handling properties during manufacture.

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Transport of modified PAMAM dendrimers across Caco-2 monolayers

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Polyamidoamine (PAMAM) dendrimers are a class of highly branched polymers, the surface of which possess either positively charged primary amine groups (full generation) or negatively charged carboxyl groups (half generation) (Tomalia 1995). Dendrimers have been shown to cross cell barriers at sufficient rates to act as potential delivery systems (D'Emanuele *et al* 2003). The objectives of this study were to investigate the cytotoxicity and transport of PAMAM dendrimers (G2, G2.5, G3, G3.5 and G4) using monolayers of the human intestinal adenocarcinoma cell line (Caco-2). The influence of dendrimer surface modification by conjugation with the absorption enhancer lauroyl chloride on dendrimer cytotoxicity and transepithelial transport was evaluated. Transport mechanisms were investigated by examining the influence of transport modifiers, namely, the Ca^{2+} chelator ethylenediamine tetraacetic acid (EDTA, which is known to cause disruption of tight junctions), and of colchicine (endocytosis inhibitor) on transepithelial electrical resistance (TEER) and permeation of dendrimers. The effect of dendrimers on the permeability of monolayers to the paracellular marker [^{14}C]mannitol was also examined.
Dendrimer conjugates were synthesised and characterised by ^{1}H NMR. Cytotoxicity was determined using the 3-(4, 5-dimethythiazole-2-yl)-2, 5-diphenyl tetrazolium bromide assay. The effects of dendrimers on monolayer integrity was measured from TEER and [^{14}C]mannitol apparent permeability coefficient (P_{app}). The P_{app} of dendrimers and dendrimer conjugates labelled with fluorescein isothiocyanate through monolayers was measured in both the apical (A) to basolateral (B) and B to A directions in the presence and absence of transport modifiers and at 37°C and 4°C. The cytotoxicity of PAMAM dendrimers increased with both concentration and generation. Anionic dendrimers showed lower toxicity than cationic dendrimers. The conjugation of lauroyl chains onto cationic dendrimers caused a decrease in toxicity. The P_{app} of cationic PAMAM dendrimers was higher than that of anionic. The P_{app} of lauroyl-conjugated cationic dendrimers increased with the number of attached lipid chains. P_{app} values of dendrimers and lauroyl-conjugated cationic dendrimers were lower at 4°C than at 37°C. Incubation with cationic PAMAM dendrimers resulted in a pronounced decrease in TEER, whereas no change was observed with incubation of anionic PAMAM dendrimers and paracellular markers. Within 24 h of dendrimer removal TEER indicated recovery of tight junction integrity. The decrease in TEER of cells exposed to lauroyl-dendrimer conjugates was less than that observed with unmodified dendrimers. The P_{app} values of dendrimers, dendrimer conjugates and mannitol were higher in the presence of EDTA indicating permeation via the paracellular pathway. Colchicine caused the A to B P_{app} values to decrease, which, combined with the observation of lower P_{app} values at 4°C suggests that the transport mechanism of dendrimers and conjugates across Caco-2 cell monolayers may also involve a transcellular route. Conjugation of cationic PAMAM dendrimers with lauroyl chloride decreased their cytotoxicity to Caco-2 cell monolayers and increased their permeation. The route of transepithelial transport, for both dendrimers and dendrimer conjugates is via both paracellular and transcellular pathways.

D'Emanuele, A., Attwood, D., Abu-Rmaileh, R. (2003) Dendrimers. In: *Encyclopaedia of pharmaceutical technology*. 2nd Edition (3), pp 1–21
Tomalia, D. A. (1995) *Sci. Am.* **272**: 62–66

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Microencapsulation of diphtheria toxoid in polycaprolactone and vitamin E TPGS blends using spray drying

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It is well known that association or encapsulation of antigens with polymeric microparticles acts as an adjuvant. Earlier studies used non biodegradable particles for delivery of vaccines. However, biocompatible microparticles prepared with PLGA or PLA have attracted much attention in recent years. One major drawback of these polymers is the loss of vaccine antigenicity due to the extreme acid environment produced by their degradation. Polycaprolactone (PCL) is a biocompatible and biodegradable polyester polymer, which has been studied as a carrier for oral vaccines and delivery of drug moieties. Its biodegradability has been exploited by blending with aliphatic polyesters (Blanco *et al* 2003). Moreover, it has a very low degradation rate, and therefore, does not produce an acid environment (Baras *et al* 2000)
The aim of the present work is to prepare microspheres of diphtheria toxoid in PCL and Vitamin E TPGS 1000 blends using spray-drying technology. Conventional microencapsulation techniques like solvent evaporation/extraction poses a critical problem of degradation, as these methods include the use of high shear forces and a long exposure time to organic solvents. Spray-drying with its mild processing conditions gives an attractive alternative for protein entrapped microparticles (Baras *et al* 2000). We have employed the double emulsion technique using polyvinyl alcohol (PVA) as a stabiliser wherein the corresponding emulsion was subjected to spray-drying using a Buchi Mini Spray Dryer, model 190 (Buchi Laboratoriums-Techniik AG, Switzerland). Vitamin E TPGS 1000 in different concentrations was incorporated in the organic phase (dichloromethane). The size and zeta potential of the microspheres was measured using a Malvern Mastersizer and ZetasizerMicrosphere loading was calculated by micro BCA assay. The parameters for spray drying were: inlet temp 40°C ± 2°C, outlet temp 26°C ± 2°C, aspirator setting 50, pump rate 5 mL min^{-1}, airflow 600 L h^{-1}.
Microspheres obtained were in the size range of 2–5 μm. The concentration of Vitamin E TPGS was shown to influence microsphere characteristics. From these blends, 20% Vitamin E TPGS 1000 gave a very narrow size distribution. The zeta potential of the particles decreased with the increase in Vitamin E TPGS concentration. The loading was 1.6 times more for the 20% Vitamin E TPGS blend ($P = 0.007$). The product yield with 20% and 40% Vitamin E TPGS was 54.5% and 66%, respectively, whereas, without Vitamin E TPGS the yield was 10–20%. Further studies are ongoing for microspheres prepared using PCL and Vitamin E TPGS blends with reference to antigen release, stability of antigen and degradation in comparison with PCL microspheres alone.

Baras, B., *et al.* (2000) *J. Microencaps.* **17**: 485–498
Blanco, M. D., *et al.* (2003) *Eur. J. Pharm. Biopharm.* **55**: 229–236

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Vaccine-coated microcrystals: enhanced thermal stability of diphtheria toxoid

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According to the World Bank, immunisation is the most cost-effective intervention in medicine. Unfortunately, it has been estimated that approximately half of all supplied vaccines is wasted. In most cases, the thermal instability of the vaccine seems to be the main reason for this wastage (Brandau *et al* 2003). Increasing vaccine stability to thermal stress is thus very important to ensure resistance to degradation during shipping and storage.

Here, we report some preliminary data on the formulation of vaccine-coated microcrystals, with the aim of providing stress resistant dry powder vaccine. Protein-coated microcrystals (and in this case, vaccines) represent a novel particle engineering approach for the formulation of a wide range of biomolecules, including proteins; peptides and DNA/RNA. Protein-coated microcrystals (PCMC) are water-soluble micron-sized particles that typically consist of a core crystalline material, such as an amino-acid, sugar or salt on which is coated the therapeutic biomolecule. PCMC are prepared in a one-step process that simultaneously dehydrates these two components and results in immobilisation of the protein on the surface of the crystalline core carrier.

In this study, diphtheria toxoid (DT) was used as a model antigen. DT-coated microcrystals were prepared as follows: appropriate volumes of the stock DT solution and the crystalline-forming material (in this case L-glutamine) were added to each. This combined solution was dispensed into 2-propanol to produce the DT-coated microcrystals. The PCMC were then dried to produce a free-flowing dry powder. Scanning electron microscopy of the crystals obtained revealed flat plates, with an average size of 5–10 mm. The actual loading of DT were calculated to be 3.95% wt/wt as determined by Bradford assay.

Vaccine stability to stress conditions was tested by incubating the DT-coated crystals and the controls (free DT solution) at room temperature for 2 weeks; at 37°C for 2 weeks or at 45°C for 2 days, prior to suspension in a phosphate buffered saline and then intramuscular administration to groups of female Balb/c mice (n = 5). Each mouse received 5 μg of DT in 50 μL of suspension. Booster doses were administered 4 weeks later. The mice were bled from the tail vein on days 21 and 42 and the levels of antigen-specific antibody in the serum were determined by ELISA. Administration of DT solution which had been incubated at 45°C for 2 days produced no antibody response. However, it was found that the thermal stability of DT was improved when the antigen was coated onto the microcrystals and a strong IgG response was achieved when DT-microcrystal (treated in the same way) were administered. Therefore, from this preliminary data, vaccine-coated microcrystals appear to afford protection against heat-induced vaccine inactivation. This study suggests the potential application of vaccine-coated micro-crystals as a means of formulating thermally stable vaccines.

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Enhanced immune response to purified bluetongue virus (BTV-1) particles in guinea-pig model with chitosan-aluminium phosphate adjuvant

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Bluetongue virus (BTV) is a dsRNA virus that causes a non-contagious, infectious, arthropod borne disease of ruminants. To date 24 serologically distinct serotypes have been identified and recognised internationally. Vaccination against one serotype does not usually confer protection against any of the other serotypes. Bluetongue virus is the type species of the genus *Orbivirus* within the family *Reoviridae* (Roy *et al* 1994).

Development of effective vaccines has greatly decreased the incidence of many infectious diseases worldwide in man and livestock. The addition of an effective adjuvant to a vaccine is often critical to the generation of the most beneficial immune responses. Adjuvants can enhance these responses in various ways (O'Hagan *et al* 2001). The adjuvanticity of alum (aluminium hydroxide) has been attributed partly to its ability to act as a short-term antigen depot so that it can continually present an antigen to the immune system over a defined period of time. The objective of this study is to evaluate the immune response in guinea-pigs following vaccination with purified BTV-1 in the absence of adjuvant and in the presence of chitosan glutamate and alum phosphate either separately or in combination.

Five groups of three guinea-pigs were immunized subcutaneously with 10 μg of purified BTV-1 particles either in 1 mL of sterile PBS, 0.2% w/v chitosan glutamate, 0.2% w/v chitosan plus 300 μg aluminium phosphate or PBS containing 300 μg aluminium phosphate. Blood samples were collected on day 22 and assayed for BTV antibody and competitive ELISA. The immune responses recorded in guinea-pigs that were given purified BTV-1 with adjuvant were markedly greater than those given purified BTV-1 alone. Antibody titres were highest in the guinea-pig group that was given purified BTV-1 in combination with the two adjuvants, chitosan and aluminium phosphate. The groups of guinea-pigs that received purified BTV-1 with chitosan and aluminium phosphate separately recorded similar antibody titres.

This study indicates that a combination of chitosan and aluminium phosphate as adjuvants enhances the immune response to BTV-1 particles. Further investigations are ongoing to help elucidate the mechanism for the cumulative effect of the aluminium phosphate and chitosan glutamate.

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Encapsulation of purified bluetongue virus 1(BTV-1) particles into chitosan nanoparticles

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Chitosan is a cationic, bioabsorbable, non-toxic polysaccharide. This polymer has been studied previously as a carrier for a variety of bacterial and viral proteins and DNA. This is the first report exploring the use of chitosan nanoparticles carrying bluetongue virus. These studies are designed to investigate the value of virus/antigen encapsulation in the preparation of safe and efficacious, inactivated and recombinant vaccines against bluetongue.

Chitosan nanoparticles were prepared according to the method of Calvo *et al* (1997). Nanoparticles were obtained by the addition of a tri-polyphosphate (TPP) in phosphate buffer solution (0.5 mL, 0.2% w/v) to an aqueous solution of chitosan glutamate (4 mg, 0.2 or 0.5% w/v) under magnetic stirring at room temperature for 30 min. Purified BTV-1 particles were incorporated into the TPP solution, prior to the formation of the nanoparticles. The nanoparticles were separated from the aqueous suspension by centrifugation at 14 000 *g* for 10 min. The amount of purified BTV-1 particles (Mertens *et al* 1996) loaded into the nanoparticles was calculated to be the difference between the total amount of antigen used and the amount remaining in the supernatant after encapsulation as determined by titration in the BTV indirect sandwich ELISA.

The morphology of the chitosan nanoparticles after loading with purified BTV-1 was revealed by transmission electron microscopy. Dilutions of purified BTV-1, purified BTV-1 in chitosan nanoparticles and purified BTV-1 with various concentration of chitosan solution (0.5%, 0.25%, 0.125% w/v) were inoculated intravenously into 11-day-old embryonated chicken eggs (ECE). Briefly, 0.1 mL of each preparation was inoculated into each of 3 ECE. The eggs were incubated at 33.5°C and examined daily for 7 days. Virus end-point titres were calculated according to the number of embryos that died between days 2 and 7. Electron microscopical examination of the chitosan nanoparticles loaded with purified virus BTV-1 showed a characteristic dense spherical structure. The efficiency of BTV-1 encapsulation into chitosan particles, determined by ELISA, was around 82.5%. The mechanism by which the BTV is associated with the chitosan nanoparticles is mediated by electrostatic interaction. Preliminary studies in eggs showed that chitosan particles alone were not toxic. The infectivity of the BTV-1 encapsulated in chitosan nanoparticles was comparable with that of pure BTV-1 particles, whereas BTV-1 particles mixed with various concentrations of chitosan solution influenced on infectivity of virus. For example, at higher concentrations of chitosan solution the virus infectivity was reduced while at lower concentrations a measurable increase in virus infectivity was observed.

These studies show that purified BTV particles can be encapsulated into chitosan nanoparticles without any apparent loss in virus infectivity. Further studies are continuing to explore the in-vivo immunopotentiating activity of BTV-1/chitosan nanoparticles and the potential value of these particles as a vaccine carrier.

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Spray-dried rifampicin loaded PCL microspheres

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The oral bioavailability of rifampicin is high which in turn makes oral administration likely to cause systemic side effects. If rifampicin is targeted to the lung it may increase the local therapeutic effect and reduce systemic exposure. It has been proposed that biodegradable aerosol microparticles containing anti-tubercular agents may be delivered to the lungs to improve the treatment of tuberculosis (Patrik *et al* 2000). For this, the particles must have a aerodynamic mean diameter of 2–5 μm. We have used a biocompatible and biodegradable polyester polymer, polycaprolactone (PCL), to load rifampicin in conjunction with Vitamin E TPGS 1000 (Mu & Feng 2003). This water-soluble derivative of Vitamin E is formed by esterification of Vitamin E succinate with polyethylene glycol 1000 (Eastman chemical company, USA) and can be used for reversing or preventing Vitamin E deficiency and a safe excipient.

Microparticles were characterised according to size, zeta potential, loading and release. The concentration of rifampicin in microspheres was detected by UV spectrophotometery. Absorbance was observed at 262 nm and a standard curve was plotted using rifampicin in chloroform. We have employed two techniques for spray-drying. The first method consist of dissolving 10% (w/w) of rifampicin and PCL in dichloromethane and subsequently spray drying using a Buchi Mini Spray Dryer, model 190 (Buchi Laboratoriums-Techniik AG, Switzerland). The second method was exactly the same as outlined above, except prior to spray drying, a double emulsion was prepared employing polyvinyl alcohol (PVA) as an emulsifying agent. Additionally, in the second method, Vitamin E TPGS was incorporated into the organic phase at various concentrations. The parameters for spray drying were: Inlet temp. 40 $\pm$ 2°C, outlet temp. 33 $\pm$ 2°C, aspirator setting 50, pump rate 5 mL min^{-1}, airflow 600 L h^{-1}.

The direct spray drying method gave almost 100% loading of rifampicin but the morphology of the particles formed was flake-like with sizes ranging up to 50 μm. Using the second method the particles formed were around 1–5 μm and the loading was in the range of 42–56%. It was found that Vitamin E TPGS decreased the zeta potential of the particles. The product yield with 20% and 30% Vitamin E TPGS was 47% and 56%, respectively, which is comparatively much higher when compared with the product yield without Vitamin E TPGS (less than 10%). Rifampicin loaded microparticles, formulated with PCL and Vitamin E TPGS using the methodology outlined above, have thus been shown to have potential for further development for the application of the local administration of rifampicin to the lung, possessing desirable characteristics in respect of aerodynamic mean diameter, yield and loading.

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Chitosan-Quil-A enhances the immune response to rectal and vaginal delivery of diphtheria toxoid

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The potential to generate both a local and systemic immune response makes the mucosal immune system an attractive site for immunization. However, mucosal administration of protein and peptide antigens generally results in a poor immune response. Successful mucosal vaccination is therefore largely dependent on the development of effective mucosal adjuvants. In recent years, many adjuvants and vaccine delivery systems have shown an ability to enhance immune responses to mucosally administered antigens. These include bacterially-derived products such as monophosphoryl lipid A, toxins and immunostimulatory DNA sequences.

Cholera toxin consists of a pentameric B oligomer that binds to GM-1 receptors and an enzymatically active A subunit that is responsible for the toxicity of this agent. The toxicity of the CT holotoxin limits its usefulness as an adjuvant; therefore the cholera toxin B-subunit (CTB) which consists only of the non-toxic B-subunit of the cholera enterotoxin is more widely used in candidate mucosal vaccines (Goto *et al* 2000). Fractions prepared from *Quillaja saponaria* (Quil-A) can also be used as adjuvants. Quillaja saponins are known to increase the effectiveness of both injected and oral vaccines (Sjolander & Cox 1998). In previous studies we have shown that chitosan is able to enhance the effects of other adjuvants when administered intranasally (Bramwell *et al* 1999). In this study we have examined the effect of vaginal and rectal administration of diphtheria toxoid in the presence of the experimental combination of chitosan plus Quil-A or CTB as a known mucosal adjuvant on the systemic and mucosal immune response.

Three groups of five BALB/c mice were immunized via either the rectal or vaginal route (six groups in total) with 20 μg of diphtheria toxoid in either 30 μL of sterile PBS, 0.2% w/v chitosan glutamate plus 15 μg of Quil-A or 10 μg of CTB on day 1, day 7 and day 21. Blood samples, vaginal washes and faecal matter were collected on day 14 and 27 and assayed for anti-diphtheria specific antibody by indirect ELISA.

The results showed that CTB produced the highest serum IgG response compared with the group of mice that received free toxoid or the group of mice that received free toxoid with chitosan glutamate plus Quil-A. The group of mice that received diphtheria toxoid with chitosan plus Quil-A also showed enhanced systemic IgG responses compared with the group of mice that received free toxoid. This trend was the same for either rectal or vaginal administration. We are in the process of evaluating the mucosal and cellular responses of these groups, but the preliminary results presented in this study clearly show that chitosan plus Quil-A can act as an adjuvant for rectally and vaginally delivered antigens.

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Evaluation of new polymeric binders with potential for use in extended release tablets

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The oral route is the most convenient for drug administration and extended release tablets provide the opportunity to increase patient compliance. Most commercial tablets are based on starch-based excipients, but often a large percentage of the drug is released quickly so frequent dosing is still required. This has led to the search for new polymeric excipients that might help in designing improved extended release tablets. The aim of this study was to synthesise and characterise acid functional co-polymers of vinyl acetate-maleic anhydride and methylmethacrylate-maleic anhydride (Raval *et al* 1998). The co-polymers were characterised according to their solubility, softening point, molecular weight and antibacterial activity (as a measure of general toxicity). To evaluate their potential as binders for extended release tablets, paracetamol tablets (at different ratios of binder to drug) were prepared using an automatic compression and ejection device (Kharis 1997). The hardness and friability values of the tablets were measured and drug release investigated at different pH values using an in-vitro dissolution method (Sugiyanto 1998). A mathematical model was established to allow prediction the drug release at different concentrations and time intervals.

The vinyl acetate-maleic anhydride copolymer was soluble in water, acetone and methanol whereas the methylmethacrylate-maleic anhydride copolymer was insoluble in water and soluble in acetone. The characteristics of the co-polymers are shown in Table 1. The softening point was 180°C and the molecular weight was 1200 Da. From titration, the maleic anhydride content of the copolymer was 26%. Antibacterial activity was determined to assess general toxicity using *Escherichia coli*. The copolymer showed no inhibition of growth indicating low toxicity.

Table 1 Characteristics of the co-polymers and the paracetamol tablets made using them

Co-polymer	Melting point (°C)	MW (Da)	Time for 100% release of paracetamol (min)
Poly (vinyl acetate-co-maleic anhydride)	160	1200	90
Poly (methylmethacrylate-co-maleic anhydride)	180	1300	360

The time period for 100 % release of paracetamol from a commercial starch-based tablet was 10 min. In contrast tablets prepared from the new co-polymers gave an extended release profile (Table 1). The release profile was found to be directly proportional to binder concentration and inversely proportional to pH. The established mathematical model showed an 85% fit with respect to predicted and experimental values.

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