

## Maintenance of pDNA Viability Following Aerosolisation

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### Summary

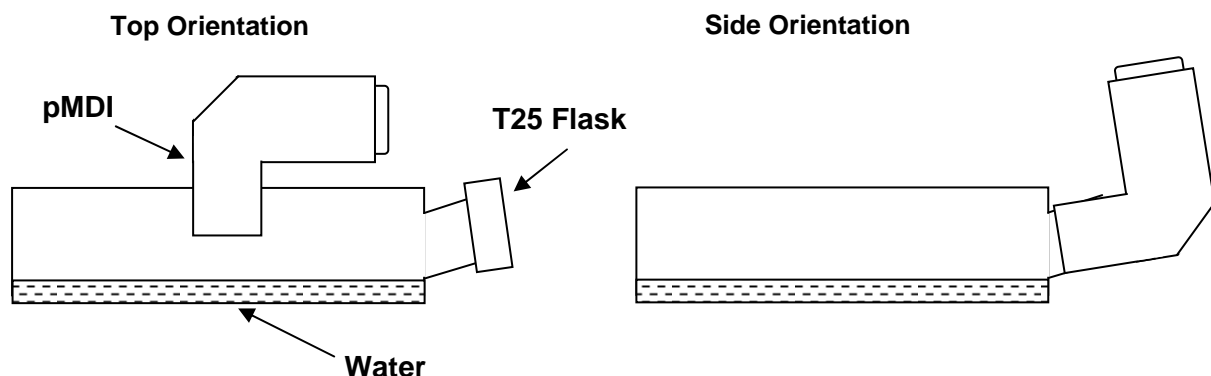
Aerosolised DNA administration could potentially advance the treatment of inheritable lung diseases and lung malignancies and provide immunisation against a range of infectious diseases. We aim to explore whether it is viable to aerosolise and deliver surfactant-coated pEGFP nanoparticles via a pressurised metered dose inhaler (pMDI). Agarose gel electrophoresis, flow cytometry and toxicity assays were used to deduce pDNA integrity, assess biological activity of aerosolised pEGFP and ascertain any formulation toxicity respectively. Results showed pDNA remained functionally active following loading into and actuation from a pMDI system. Aerosolised formulations were found to exert no significant loss in cell viability. The novel low-energy microemulsion process used offers the potential for the incorporation of pDNA nanoparticles into pMDI systems for deep pulmonary delivery of gene vectors.

### Introduction

Aerosolised DNA administration could potentially advance the treatment of inheritable lung diseases and lung malignancies and provide immunisation against a range of infectious diseases. Proof-of-principle studies have shown that gene delivery and expression can correct genetic defects in diseased cells (Ferrari et al 2003). Jet nebulisation, the laboratory and clinical standard for introducing gene therapy formulations into the lung, is inherently inefficient. Shearing forces that may damage labile gene transfer complexes, preferential nebulisation of the solute and adhesion to plastic mean that as little as 10% of material in the nebulisation chamber is emitted through the mouth piece (Birchall et al 2000). While newer nebuliser technologies are under development, pressurised metered dose inhalers (pMDIs) offer an alternative with potential advantages of more efficacious, convenient and rapid administration. We aim to explore whether it is viable to aerosolise and deliver surfactant-coated pDNA nanoparticles using a low-energy nanotechnology process (Dickinson et al 2001), via a pMDI system.

### Methods

Water-in-oil microemulsions containing pEGFP-N1 reporter plasmid were prepared from sucrose solution (lyoprotectant-aqueous phase), lecithin:propan-2-ol (surfactant) and iso-octane (organic phase). Microemulsions were snap-frozen in liquid nitrogen and lyophilised for 24 hours. In some formulations excess surfactant was removed by organic solvent washes and centrifugation. Agarose gel electrophoresis with ethidium bromide staining was used to determine the physical integrity of the lyophilised and solvent washed pEGFP. A semi-quantitative analysis using adjusted volume counts of pDNA signal intensity was used to assess the degree of fluorescence emitted from the supercoiled fraction. Initial pilot studies using pMDIs containing Brilliant Blue dye (supplied by 3M Drug Delivery Systems) were carried out to assess the most quantitatively efficient orientation at which the DNA-loaded pMDIs should be actuated onto the cells (Figure 1). Brilliant Blue pMDIs were actuated a total of 5 times into a T25 flask containing 6 ml of water. The amount of dye deposited into the water was quantified by absorbance using an ultraviolet (UV) spectrophotometer at 629 nm wavelength.



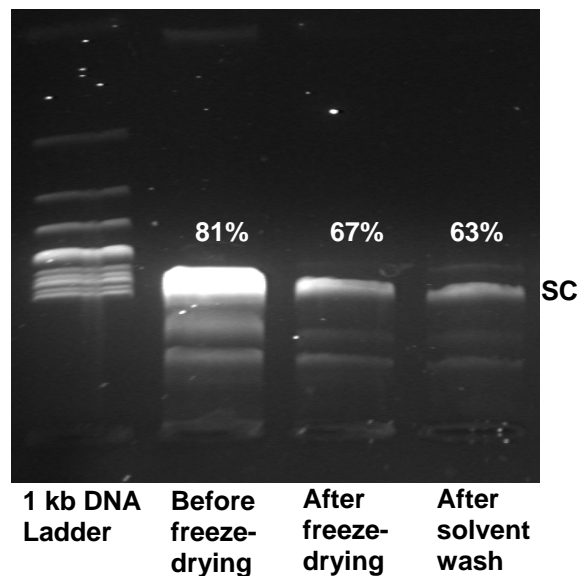
**Figure 1.** Brilliant Blue pMDI actuated from the Top and Side orientation of a T25 flask.

Lyophilised pDNA particulates were incorporated into pMDIs using HFA134a as propellant and ethanol as co-solvent. Formulations were actuated from 50  $\mu$ l Spraymiser retention valves and actuators with an orifice diameter of 0.5 mm (3M drug delivery systems; Loughborough, UK). To assess biological functionality of aerosolised pDNA by transfection, A549 human lung epithelial cells cultured to 50% confluency were subjected to the following treatments: (i) media alone, (ii) unwashed formulation of pDNA particulates, (iii) washed formulation

of pDNA particulates. Formulations (ii) and (iii) were aerosolised from pMDIs directly onto cells covered with media alone or media containing dioleoyl-trimethylammonium propane (DOTAP) cationic lipid to assist cell transfection. An *in vitro* toxicology assay was carried out on treated A549 cells using a colorimetric 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) based toxicity assay. *In vitro* cytotoxicity was determined by spectrophotometrically measuring the activity of living cells post-treatment via their mitochondrial activity.

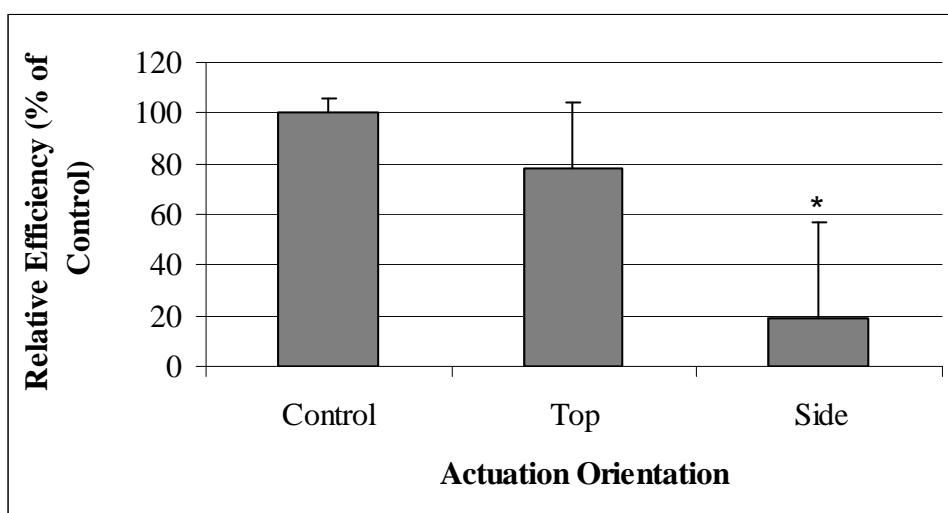
## Results

Controlled lyophilisation of pEGFP incorporated microemulsions enabled the removal of solvent and water to produce surfactant-coated pEGFP particles. Agarose gel electrophoresis with ethidium bromide staining (Figure 2) verified that freeze-dried particles retained pDNA integrity with maintenance of the transfection-competent supercoiled pDNA tertiary structure. A semi-quantitative analysis using adjusted volume counts was used to compare the fluorescence emitted from the supercoiled band with that of the whole pDNA (Figure 2). In respect to the unprocessed pEGFP, the supercoiled band contributed 81% of emitted fluorescence from the total pDNA. Once the pEGFP had been incorporated into a microemulsion and freeze-dried the supercoiled fraction fell to 67% and was reduced further to 63% after the solvent wash. Although a clear reduction in the supercoiled fraction is observed between the unprocessed and freeze-dried pDNA, there is little difference between the freeze-dried and solvent washed pDNA (Figure 2).



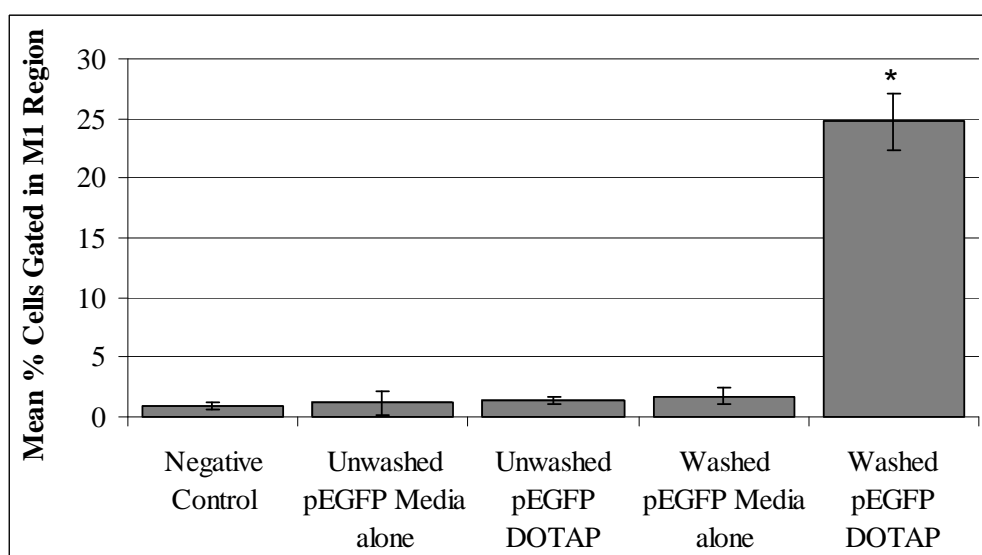
**Figure 2.** Qualitative gel electrophoresis of pEGFP before and after freeze-drying and solvent washes. **SC:** supercoiled fraction. The percentage values represent a semi-quantitative analysis of the ratio of fluorescence emitted from the supercoiled band compared to the total pDNA.

Brilliant Blue dye was used to compare the deposition of MDI output collected in a T25 flask from the top and side orientation (Figure 3). Data was compared to a control, whereby the total emitted dose of Brilliant Blue was collected in a conical flask. The data showed that actuating the Brilliant Blue pMDI from the top orientation gave a greater relative efficiency (78% of the actuated dye was deposited on the bottom of the flask) than actuating onto a flask from the side orientation (19% deposition). Statistical analysis confirmed a significant reduction in the concentration of deposited dye when actuated from a pMDI ( $p \leq 0.05$ ) from the side orientation ( $0.04 \mu\text{g} / 6 \text{ ml} \pm 0.01$ ; mean  $\pm$  SD,  $n = 3$ ) compared to the control ( $0.19 \pm 0.01 \mu\text{g}$ ). Actuating the pMDI from the top orientation demonstrated no significant reduction ( $0.15 \pm 0.04 \mu\text{g}$ ,  $p \geq 0.05$ ) in the concentration of deposited dye relative to the control.



**Figure 3.** Deposition efficiency of Brilliant Blue when actuated from a pMDI in the top and side orientation relative to the control. Data was represented as mean  $\pm$  SD,  $n = 3$ . Statistical analysis by One-Way Analysis of Variance and Dunnett's multiple range test used a significance level of  $p \leq 0.05^*$ .

As a result of the Brilliant Blue pilot study, transfection experiments were conducted by actuating the DNA-loaded pMDIs from the top orientation.

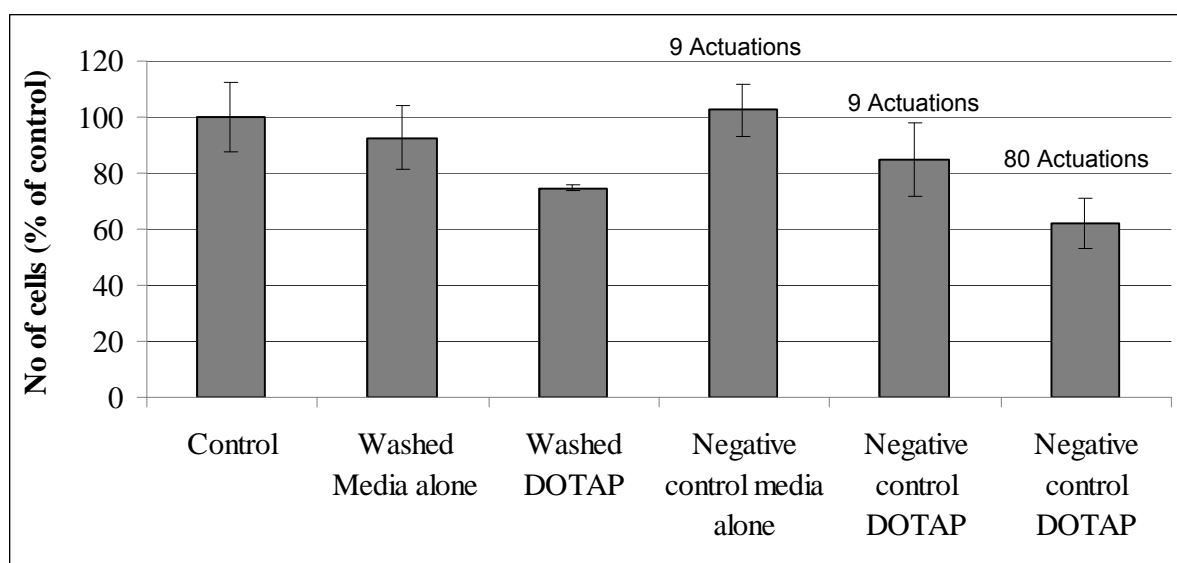


**Figure 4.** Mean percentage of A549 cells emitting fluorescence post pEGFP transfection. Cells were surface-treated with: **Negative Control:** media alone. **Unwashed pEGFP:** pMDI formulation actuated onto media alone. **Unwashed pEGFP DOTAP:** pMDI formulation actuated onto media containing DOTAP. **Washed pEGFP:** pMDI formulation actuated onto media alone. **Washed pEGFP DOTAP:** pMDI formulation actuated onto media containing DOTAP. Data was represented as mean  $\pm$  SD,  $n = 4$ . Statistical analysis by One-Way Analysis of Variance and Duncan's multiple range test used a significance level of  $p \leq 0.001^*$ .

Green Fluorescent Protein (GFP) expressed in cells transfected by pEGFP-N1 provided a marker for retention of biological functionality of the formulated pDNA. Figure 4 revealed washed pDNA remained functionally active following loading into and actuation from a pMDI. Statistical analysis (one-way analysis of variance and Duncan's multiple range test) showed significant difference in the percentage of cells expressing GFP ( $p \leq 0.001$ ) following aerosolisation of the washed pDNA particulates into DOTAP containing media ( $24.75 \pm 2.35\%$ ; mean  $\pm$  SD,  $n = 4$ ) compared with control cells ( $1.23 \pm 0.56\%$ ). Fluorescence microscopy further confirmed gene expression in cells following actuation of the pMDI formulation.

Statistical analysis of the MTT assay (Figure 5) showed no significant loss in cell viability ( $p > 0.05$ ) when comparing UV absorbance values between blank untreated cells ( $1.23 \pm 0.15$ ;  $n = 18$ ) and cells treated with the washed aerosolised pEGFP formulation onto media ( $1.14 \pm 0.13$ ). A significant reduction in cell viability ( $p \leq 0.05$ )

was however observed between cells treated with formulations in the presence of DOTAP ( $0.92 \pm 0.01$ ;  $n = 18$ ) compared to cells treated without DOTAP ( $1.14 \pm 0.13$ ).



**Figure 5.** Percentage cell viability relative to the control. **Control:** untreated cells taken as 100% viable. Cells were surface-treated with: **Washed Media alone:** Washed pEGFP pMDI formulation actuated onto media alone. **Washed DOTAP:** Washed pEGFP pMDI formulation actuated onto media containing DOTAP. **Negative control:** pMDI formulation containing HFA 134a and ethanol actuated onto media alone. **Negative control DOTAP:** pMDI formulation containing HFA 134a and ethanol actuated onto media containing DOTAP. **Negative control DOTAP:** pMDI formulation containing HFA 134a and ethanol actuated onto media containing DOTAP. Data was represented as mean  $\pm$  SD,  $n = 18$ . Statistical analysis by One-Way Analysis of Variance and Duncan's multiple range test used a significance level of  $p \leq 0.05$ .

## Conclusions

Methods have been successfully developed and evaluated to aerosolise surfactant-coated pDNA particles and maintain the integrity of pDNA by processes including the freeze-drying of microemulsions. Furthermore, we have demonstrated that the pDNA particles can be incorporated into an HFA134a pMDI formulation and aerosolised using a standard valve and actuator. The transfection studies demonstrated that the biological functionality of the pDNA after aerosolisation from pMDI systems could be maintained. Toxicity assays show that any loss of cell viability is an effect of DOTAP lipid as opposed to any component of the pDNA particulate formulation. Particles prepared by this novel low-energy microemulsion process may have the potential for stable and efficient delivery of pDNA to cells in the lower respiratory tract via pMDIs. Further studies will focus on the incorporation of a cell transfection agent into the pDNA pMDI formulation.

## References

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