Tawfeek, H, Khidr, S, Samy, E, Ahmed, S, Murphy, MF, Mohammed, A, Shabir, A, Hutcheon, GA and Saleem, IY

Poly(Glycerol Adipate-co-ω-Pentadecalactone) Spray-Dried Microparticles as Sustained Release Carriers for Pulmonary Delivery

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9		Organization	Liverpool John Moores University
10		Division	School of Pharmacy & Biomolecular Sciences
11		Address	James Parsons Building, Byrom Street, Liverpool L3 3AF, UK
12		e-mail	I.saleem@ljmu.ac.uk
13	Author	Family Name	**Tawfeek**
14		Particle	
15		Given Name	**Hesham**
16		Suffix	
17		Organization	Liverpool John Moores University
18		Division	School of Pharmacy & Biomolecular Sciences
19		Address	James Parsons Building, Byrom Street, Liverpool L3 3AF, UK
20		Organization	Assiut University
21		Division	Department of Industrial Pharmacy, Faculty of Pharmacy
22		Address	Assiut , Egypt
23		e-mail	
24	Author	Family Name	**Khidr**
25		Particle	
26		Given Name	**Sayed**
27		Suffix	
28		Organization	Assiut University
29		Division	Department of Industrial Pharmacy, Faculty of Pharmacy
30		Address	Assiut , Egypt
31		e-mail	

32		Family Name	**Samy**
33		Particle	
34		Given Name	**Eman**
35		Suffix	
36	Author	Organization	Assiut University
37		Division	Department of Industrial Pharmacy, Faculty of Pharmacy
38		Address	Assiut , Egypt
39		e-mail	
40		Family Name	**Ahmed**
41		Particle	
42		Given Name	**Sayed**
43		Suffix	
44	Author	Organization	Assiut University
45		Division	Department of Industrial Pharmacy, Faculty of Pharmacy
46		Address	Assiut , Egypt
47		e-mail	
48		Family Name	**Murphy**
49		Particle	
50		Given Name	**Mark**
51		Suffix	
52		Organization	Liverpool John Moores University
53	Author	Division	School of Pharmacy & Biomolecular Sciences
54		Address	James Parsons Building, Byrom Street, Liverpool L3 3AF, UK
55		Organization	Liverpool John Moores University
56		Division	General Engineering Research Institute
57		Address	Liverpool , UK
58		e-mail	
59		Family Name	**Mohammed**
60		Particle	
61		Given Name	**Afzaal**
62		Suffix	
63	Author	Organization	Aston University
64		Division	Aston Pharmacy School
65		Address	Birmingham , UK
66		e-mail	
67		Family Name	**Shabir**
68		Particle	
69		Given Name	**Anjum**

70		Suffix	
71		Organization	Aston University
72	Author	Division	Aston Pharmacy School
73		Address	Birmingham , UK
74		e-mail	
75		Family Name	**Hutcheon**
76		Particle	
77		Given Name	**Gillian**
78		Suffix	
79	Author	Organization	Liverpool John Moores University
80		Division	School of Pharmacy & Biomolecular Sciences
81		Address	James Parsons Building, Byrom Street, Liverpool L3 3AF, UK
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86	Abstract		**Purpose:** The aim of this work was to optimize biodegradable polyester poly (glycerol adipate-co-ω-pentadecalactone), PGA-co-PDL, microparticles as sustained release (SR) carriers for pulmonary drug delivery. **Methods:** Microparticles were produced by spray drying directly from double emulsion with and without dispersibility enhancers ($_L$-arginine and $_L$-leucine) (0.5–1.5%w/w) using sodium fluorescein (SF) as a model hydrophilic drug. **Results:** Spray dried microparticles without dispersibility enhancers exhibited aggregated powders leading to low fine particle fraction (%FPF) (28.79 ± 3.24), fine particle dose (FPD) (14.42 ± 1.57 μg), with a mass median aerodynamic diameter (MMAD) 2.86 ± 0.24 μm. However, $_L$-leucine was significantly superior in enhancing the aerosolization performance ($_L$-arginine:% FPF 27.61 ± 4.49–26.57 ± 1.85; FPD 12.40 ± 0.99–19.54 ± 0.16 μg and MMAD 2.18 ± 0.35–2.98 ± 0.25 μm, $_L$-leucine:%FPF 36.90 ± 3.6–43.38 ± 5.6; FPD 18.66 ± 2.90–21.58 ± 2.46 μg and MMAD 2.55 ± 0.03–3.68 ± 0.12 μm). Furthermore, incorporating $_L$-leucine (1.5%w/w) reduced the burst release (24.04 ± 3.87%) of SF compared to unmodified formulations (41.87 ± 2.46%), with both undergoing a square root of time (Higuchi's pattern) dependent release. Comparing the toxicity profiles of PGA-co-PDL with $_L$-leucine (1.5%w/w) (5 mg/ml) and poly(lactide-co-glycolide), (5 mg/ml) spray dried microparticles in human bronchial epithelial 16HBE14o- cell lines, resulted in cell viability of 85.57 ± 5.44 and 60.66 ± 6.75% respectively, after 72 h treatment. **Conclusion:** The above data suggest that PGA-co-PDL may be a useful polymer for preparing SR microparticle carriers, together with dispersibility enhancers, for pulmonary delivery.
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RESEARCH PAPER

Poly(Glycerol Adipate-co-ω-Pentadecalactone) Spray Dried Microparticles as Sustained Release Carriers for Pulmonary Delivery

Hesham Tawfeek • Sayed Khidr • Eman Samy • Sayed Ahmed • Mark Murphy • Afzaal Mohammed • Anjum Shabir • Gillian Hutcheon • Imran Saleem

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ABSTRACT

Purpose The aim of this work was to optimize biodegradable polyester poly(glycerol adipate-co-ω-pentadecalactone), PGA-co-PDL, microparticles as sustained release (SR) carriers for pulmonary drug delivery.

Methods Microparticles were produced by spray drying directly from double emulsion with and without dispersibility enhancers ($_L$-arginine and $_L$-leucine) (0.5–1.5%w/w) using sodium fluorescein (SF) as a model hydrophilic drug.

Results Spray dried microparticles without dispersibility enhancers exhibited aggregated powders leading to low fine particle fraction (%FPF) (28.79 ± 3.24), fine particle dose (FPD) (14.42 ± 1.57 μg), with a mass median aerodynamic diameter (MMAD) 2.86 ± 0.24 μm. However, $_L$-leucine was significantly superior in enhancing the aerosolization performance ($_L$-arginine:%FPF 27.61 ± 4.49–26.57 ± 1.85; FPD 12.40 ± 0.99–19.54 ± 0.16 μg and MMAD 2.18 ± 0.35–2.98 ± 0.25 μm, $_L$-leucine:%FPF 36.90 ± 3.6–43.38 ± 5.6; FPD 18.66 ± 2.90–21.58 ± 2.46 μg and MMAD 2.55 ± 0.03–3.68 ± 0.12 μm). Furthermore, incorporating $_L$-leucine (1.5%w/w) reduced the burst release (24.04 ± 3.87%) of SF compared to unmodified formulations (41.87 ± 2.46%), with both undergoing a square root of time (Higuchi's pattern) dependent release. Comparing the toxicity profiles of PGA-co-PDL with $_L$-leucine (1.5%w/w) (5 mg/ml) and poly(lactide-co-glycolide), (5 mg/ml) spray dried microparticles in human bronchial epithelial 16HBE14o- cell lines, resulted in cell viability of 85.57 ± 5.44 and 60.66 ± 6.75% respectively, after 72 h treatment.

Conclusion The above data suggest that PGA-co-PDL may be a useful polymer for preparing SR microparticle carriers, together with dispersibility enhancers, for pulmonary delivery.

KEY WORDS dry powder inhalation · microparticles · polyester polymers · pulmonary drug delivery · sustained drug release

H. Tawfeek • M. Murphy • G. Hutcheon • I. Saleem (✉)
School of Pharmacy & Biomolecular Sciences
Liverpool John Moores University
James Parsons Building, Byrom Street
Liverpool L3 3AF, UK
e-mail: I.saleem@ljmu.ac.uk

H. Tawfeek • S. Khidr • E. Samy • S. Ahmed
Department of Industrial Pharmacy, Faculty of Pharmacy
Assiut University
Assiut, Egypt

M. Murphy
General Engineering Research Institute
Liverpool John Moores University
Liverpool, UK

A. Mohammed • A. Shabir
Aston Pharmacy School, Aston University
Birmingham, UK

INTRODUCTION

Poly(glycerol adipate-co-ω-pentadecalactone), PGA-co-PDL, is a biodegradable polyester polymer synthesized via lipase enzyme, ~~from *candida albicans*~~, catalyzed ring opening co-polymerization reaction of activated diacid, glycerol and lactone monomers (1). This polymer is synthesized by a one step reaction via a single non-biosynthetic pathway under mild reaction conditions (2), compared to fermentation and other chemical processes that have been extensively studied for the synthesis of biodegradable aliphatic polyesters (3). In addition, these polymers are designed to overcome the lack of chemical functionality associated with poly(lactic acid) (PLA) and its derivatives, due to the presence of pendant hydroxyl groups from the glycerol monomer in the PGA-co-PDL polymer, which permit the attachment of chemical

moieties such as pharmaceutically active drugs. Furthermore, the degree of hydrophilicity can be altered by varying the backbone chemistry (4). Previously PGA-co-PDL has been formulated as microparticles for delivery of dexamethasone phosphate and ibuprofen (5,6), and investigated in our group for delivery of macromolecules using α-chymotrypsin as a model protein (7). In the current investigation, we propose using these polyester polymers as pulmonary carriers for sustained delivery (SR) of therapeutic agents to the lungs.

Pulmonary drug delivery is an attractive, convenient and effective route for the administration of therapeutic drugs, macromolecules (8) proteins and peptides (9), and is an alternative for the treatment of many pulmonary disorders, such as, lung cancer (10) and cystic fibrosis (11) enhancing the pharmacokinetic effect of the therapeutic agent. Dry powder inhalers (DPIs) are commonly used as they are portable and less expensive compared to nebulizers, and are considered to be environmentally friendly due to the absence of propellant, as well as overcoming the synchronization problems associated with pressurized metered dose inhalers (pMDIs) (12,13). Furthermore, there is improved stability in storage for therapeutic agents formulated as dry powders (13).

Lately, research has focused on protecting the therapeutic agent from degradation or premature clearance by a suitable delivery system, and using the lungs as a portal for sustained drug release and absorption over many hours to days. SR therapeutic agents can reduce side effects and the frequency of administration, hence increasing patient acceptability and compliance (14,15). However, the clearance mechanisms of the lung towards foreign particles are likely to jeopardize the potential of a SR formulation to release therapeutic agents over extended periods. Therefore to achieve a SR effect, pulmonary formulations should possess a small mass median aerodynamic diameter (MMAD) and high fine particle fraction (%FPF) in order to minimize central/tracheobronchial deposition and bypass the effects of mucociliary clearance (16). This has generally been achieved using polymeric particles such as, poly(ether-anhydride) and ~~*poly(lactic-co-glycolic acid)*~~, PLGA, as carriers for pulmonary delivery to achieve sustained or controlled release of the intended therapeutic agent (17–21). However, PLGA and PLA have many shortcomings, such as, the polymer backbone cannot be chemically functionalized, stability of macromolecules are affected due to the degradation of PLGA and PLA polymers to its acidic monomers, (22), and are often associated with drug release in a triphasic manner (22,23). This is partly due to the fact that PLGA and PLA were not specifically designed for use in the lungs. Thus, a new polymer which overcomes these problems is imperative in the formulation of carriers for pulmonary delivery.

Previously; we investigated the aerosol performance of PGA-co-PDL microparticles prepared via the emulsion solvent evaporation technique (w/o/w) using sodium fluorescein as a model drug (24). This study emphasized the aggregated properties of the produced microparticles as the %FPF did not exceed 15% (24). Consequently, this investigation aims to enhance the respirable fraction and maximize the drug deposition in the lung, using sodium fluorescein (SF) as a model hydrophilic drug, via spray drying from double emulsion (20,25). Furthermore, the addition of various dispersing agents, such as L-arginine and L-leucine amino acids, as potential dispersibility enhancers (26,27) to improve the aerosol performance was investigated. In addition, to ensure the safety of PGA-co-PDL a toxicity study was also performed in normal human bronchial epithelium cell lines utilizing the MTT assay with comparison to spray dried PLGA microparticles.

MATERIALS AND METHODS

Materials

Novozyme 435 (a lipase from ~~*Candida antartica*~~ immobilized on a microporous acrylic resin) was purchased from Biocatalytics, USA. ω-pentadecalactone, sodium fluorescein (SF), poly(vinyl alcohol) (PVA, 9–10 KMw, 80%), L-leucine and L-arginine, RPMI-1640 medium with L-glutamine and $NaHCO_3$, thiazoly blue tetrazolium bromide (MTT), poly (DL-lactide-co-glycolide) (PLGA) (50:50) inherent viscosity 0.15–0.25, were obtained from Sigma-Aldrich, UK. Dichloromethane (DCM) was purchased from BDH laboratory supplies, UK. Tetrahydrofuran (THF), 75 cm^2/ tissue culture flask with vented cap, 24 well tissue culture plates, 96 well flat bottom plates, Antibiotic/Antimycotic Solution (100X) were purchased from Fisher Scientific, UK. Divinyl adipate was obtained from Fluorochem, UK and Foetal Calf Serum (FCS) heat inactivated was purchased from Biosera UK. 16HBE14o- cells were produced by Dr Dieter Gruenert from the California Pacific Medical Center, University of California San Francisco, USA.

Polymer Synthesis

The co-polymer PGA-co-PDL was synthesized via enzyme catalyzed condensation and ring opening co-polymerization reactions as described by Thompson *et al.* (28). The synthesized polymer was characterized by gel permeation chromatography, GPC (Viscotek TDA Model 300 using OmniSEC3 operating software), calibrated with polystyrene standards (polystyrene standards kit, Supelco, USA), and H^1-NMR spectroscopy (Bruker AVANCE 300, Inverse

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probe with B-ACS 60, Autosampler with gradient chemming) as described by Thompson *et al.* (28).

Microparticles Preparation

PGA-co-PDL microparticles were prepared by spray drying directly from double emulsion (w/o/w). Briefly, 5 mg SF was dissolved in 1.5 ml distilled water and homogenized (IKA yellowline DI 25 basic at 8000 rpm for 3 min) in 13 ml DCM containing 390 mg polymer to form the first w/o emulsion. This was gradually added to the second aqueous phase, 135 ml distilled water containing 1%w/v PVA as an emulsifier, under moderate stirring conditions (Silverson L4RT mixer, 2000 rpm at room temperature, 25°C) to form the w/o/w emulsion (PGA-co-PDL, control). L-arginine (0.5, 1, 1.5%w/w of polymer weight) (Represented in text as: PGA-co-PDL, 0.5% Arg; PGA-co-PDL, 1% Arg and PGA-co-PDL, 1.5% Arg) and L-leucine (0.5, 1, 1.5%w/w of polymer weight)(Represented in text as: PGA-co-PDL, 0.5% Leu; PGA-co-PDL, 1% Leu and PGA-co-PDL, 1.5% Leu) were incorporated into the second aqueous phase in addition to PVA. The produced emulsion was spray dried at room temperature (25°C) utilizing a mini-spray dryer (Büchi, B-290 Flawil, Switzerland) with standard two-fluid nozzle (0.7 mm diameter), inlet and outlet temperature of 100 and 47°C respectively, a pump flow rate of 5–7 ml/min, aspirator at 38 m^3/h and air flow at 600 L/h. Control spray dried PLGA microparticles incorporating L-leucine (1.5%w/w, PLGA, 1.5% Leu), for comparison to optimum PGA-co-PDL microparticles, were produced as above.

Microparticles Characterization

Yield, Encapsulation Efficiency and Drug Loading

10 mg of spray dried microparticle formulations were weighed and solubilized in DCM/water mixture (2:1) to dissolve the polymer and extract SF. The two phases were separated by centrifugation (5 min at 16200 X *g*, accuSpin Micro 17) and the aqueous layer analyzed for SF using spectroscopy at 273 nm. The yield of spray dried microparticles was quantified as a percentage mass of expected total powder yield (n=6). The percentage encapsulation efficiency (EE) and drug loading were determined for all batches using Eqs. 1 and 2 respectively (n=6):

$$\mathrm{EE}(\%) = \left(\frac{\textit{actual weight of SF in sample}}{\textit{theoritical weight of SF}}\right) \times 100 \qquad (1)$$

$$\text{Drug Loading} = \frac{\textit{weight of SF in microparticles}}{\textit{microparticles sample weight}} \qquad (2)$$

Particle Size, Zeta Potential, Powder Density and Primary Aerodynamic Diameter

100 µl microparticle suspension was diluted to 5 ml using double distilled water and the measurements recorded at 25°C ($n=3$) to determine the geometric particle size and zeta potential using a Zetaplus, Brookhaven Instruments, U.K. The poured density of spray dried microparticle powders were determined by adding approximately 0.5 g of powder to a 10 ml graduated cylinder and recording the volume. The tapped density was determined by tapped density measurements on the same samples in a 10 ml graduated measuring cylinder until constant volume was obtained (29) ($n=3$). Carr's Index values for each of the spray dried formulations were calculated according to Eq. 3 (30), and can provide an indication of powder flow. Carr's Index flowability: 5–12%, excellent; 12–18%, good; 18–21%, fair; 21–25%, poor, fluid; 25–32%, poor, cohesive; 32–38%, very poor; >40%, extremely poor. A value less than 25% indicates a fluid powder, whereas a value greater than 25% indicates a cohesive powder (31).

$$\text{Carr's Index}(\%) = \frac{\text{Tapped density} - \text{Poured density}}{\text{Tapped density}} \times 100 \qquad (3)$$

Theoretcial primary aerodynamic diameter (d_{ae}) was calculated using data acquired from geometric particle size (d) and tapped density (p) according to Eq. 4 (32).

$$d_{ae} = d\sqrt{\frac{p}{p_1}} \quad p_1 = 1 \text{ g cm}^{-3} \qquad (4)$$

Amorphous Nature and Water Content

The degree of amorphous material from the spray dried formulations were performed using differential scanning calorimetry (DSC, Perkin Elmer Pyris 1). Briefly, 3–5 mg of sample was placed into a hermetically sealed and crimped pan. The samples were subjected to two scanning programs in the DSC using a heating rate of 20°C/min purged with nitrogen at 20 ml/min as described previously by Thompson *et al.* (6). The weight loss of the powders as a function of temperature was determined using a thermogravimetric analyser (TGA 2050-Thermogravimetric analyzer, UK). Approximately 6–8 mg of each sample was weighed in a platinum pan and heated at the temperature range 25–260°C using a scanning rate of 10°C/min purged under nitrogen at 20 ml/min (n=3).

Particle Morphology

The spray dried microparticles were visualized by scanning electron microscopy (FEI—Inspect S Low VAC Scanning

Electron Microscope). Particles were mounted on aluminium stubs (pin stubs, 13 mm) layered with a sticky conductive carbon tab and coated in gold (10–15 nm) using an EmiTech K 550X Gold Sputter Coater, 25 mA for 3 min. Confocal images were obtained using a Zeiss 510 Meta laser scanning microscope, mounted on a Axiovert 200 M BP computer-controlled inverted microscope. A small amount of spray dried microparticles were placed onto a cover glass chamber slide (Fisher Scientific, UK), and imaged by excitation with an argon ion laser at a wavelength of 488 nm and a Plan Neofluar 63×/0.30 numerical aperture (NA) objective lens. Image analysis was carried out using the Zeiss LSM software.

In-Vitro Aerosolisation Studies

The aerosol performance of spray dried microparticles was determined using a Next Generation Impactor (NGI). Microparticle samples (~20 mg) were manually loaded into hydroxypropyl methylcellulose capsules (HPMC size 2), and placed in a HandiHaler® (Boehringer Ingelheim, Ingelheim, Germany). A pump (Copley Scientific, Nottingham, UK) was operated at a flow rate of 60 L/min for 4 s and the NGI plates were coated with 1%w/w glycerol/methanol solution. Following inhalation all parts of NGI were washed with DCM/water (2:1), and analyzed as above. The fine particle fraction (%FPF) (defined as the mass of drug deposited (d_{ae}<4.6 μm), expressed as a percentage of the emitted dose), mass median aerodynamic diameter (MMAD) (33), and the fine particle dose (FPD), expressed as the mass of drug deposited in the NGI (d_{ae}<4.6 μm), was determined (n=3).

In-Vitro Release Studies

10 mg of spray dried microparticle formulations were added to 1.5 ml microtubes, containing 1 ml phosphate buffer saline pH 7.4 (n=3), and incubated at 37°C on an orbital shaker (IKA KS 130) at 250 rpm. The supernatants were collected to observe the release of SF over 24 h by centrifugation (5 min at 16200 X *g*, accuSpin Micro 17) and analysed using spectroscopy as above. The cumulative drug release was assessed in different release models namely zero order, first order and Higuchi's square root plot, and a correlation coefficient close to unity was used as the mechanism and order of release (34).

Toxicity Study

The toxicity profiles of PGA-co-PDL (control) and PGA-co-PDL, 1.5% Leu were evaluated over 24 h in normal human bronchial epithelial (16HBE14o-) cell line, and compared to spray dried PLGA, 1.5% Leu microparticles. 16HBE14o- cells (passage No. 28) were cultured in 24 well plates with 1 ml RPMI-1640 medium supplemented with 10% FCS/1% Antibiotic/Antimycotic solution for 24 h in a humidified 5% CO_2/95% incubator at 37°C. The wells were replaced with fresh medium (1 ml) containing PGA-co-PDL, PGA-co-PDL, 1.5% Leu and PLGA, 1.5% Leu (0–5 mg/ml) (n=6) and incubated for a further 24 h as above, followed by the addition of 1 ml MTT solution (0.5 mg/ml in PBS, pH 7.4) solution to each well. After further 2 h incubation, the medium was removed and any formazan crystals generated were solubilized with 500 μl of isopropanol. Thereafter, aliquots of the resulting solutions were transferred to 96 well plates and the absorbance was measured using spectroscopy at 570 nm and corrected for background absorbance. The relative cell viability (%) was calculated using Eq. 5 as follows:

$$\text{Viability } (\%) = \frac{A - S}{CM - S} \times 100 \qquad (5)$$

Where A is the absorbance of the test substance concentrations, S is the absorbance obtained for the (isopropanol) and CM is the absorbance obtained for untreated cells incubated with medium (control).

Statistical Analysis

Each formulation was compared with the control formulation 1 by a one-way analysis of variance (ANOVA) with Dunnett multiple comparison test. The formulations were then compared with each other by means of a one-way ANOVA with the Tukey's comparison test. The statistical significance level was set at $p \leq 0.05$.

RESULTS

Polymer Synthesis

The PGA-co-PDL (equimolar monomer ratio, 1:1:1) prepared was a white solid powder, and the nature of the co-polymer was confirmed from the integration pattern of peaks obtained from H^1-NMR spectra (δ_H $CDCl_3$, 300 MHz): 1.34 (s, 22 H, H-g), 1.65 (m, 8 H, H-e, e′, h), 2.32 (m, 6 H, H-d, d′, i), 4.05 (q)-4.18 (m) (6 H, H-a, b, c, f), 5.2 (s, H, H-j) (Fig. 1). The molecular weight of PGA-co-PDL was 23.0 KDa, as determined by GPC.

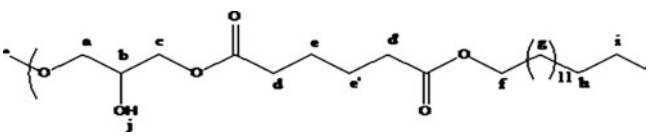

Fig. 1 Chemical structure of PGA-co-PDL polymer (MW 23 KDa).

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Microparticles Characterization

A good yield of over 40% for the different formulations was obtained except for PGA-co-PDL, 1.5% Arg which had the lowest value of 16.4%±1.4 (Table I). Furthermore, an inverse correlation between increasing arginine concentration and yield was observed. There was no significant difference with addition of amino acids in encapsulation efficiency or drug loading when comparing spray dried formulations against control (PGA-co-PDL) ($p > 0.05$, ANOVA/Dunnett). In addition, all formulations had a negative surface charge, with higher values observed in L-leucine modified spray dried microparticles (PGA-co-PDL, 0.5,1, 1.5% Leu and PLGA 1.5% Leu), indicating a greater degree of colloidal stability within the dispersion medium (Table I). It is also worth noting that increasing the L-arginine concentration correlated with increased moisture content (Table I), while an inverse correlation was observed with L-leucine. However, the results for all formulations were within the range of moisture content obtained from spray dried powders (35,36). All formulations had a geometric particle size less than 2 µm (Table I) suitable for targeting the respiratory bronchioles. The tapped densities of all formulations were similar (0.24±0.04–0.31±0.05 g cm^{-3}; Table I), and together with the geometric particle size, were used to calculate the theoretical aerodynamic diameter (d_{ae}). As shown in Table I, the d_{ae} for all formulations was between 0.50±0.13–0.91±0.11. However, the MMAD obtained from cascade impaction studies ranged from 2.18±0.35 to 3.68±0.12 µm, indicating particle aggregation (duplicate or triplicate) compared to geometric particle size. The aggregation was confirmed from Carr's index with values greater than 25 indicating poor and cohesive flowing powders (31).

Figure 2 represents DSC thermograms of PGA-co-PDL polymer, spray dried PGA-co-PDL (control) and PGA-co-PDL, 1.5% Leu formulations respectively. The spray drying process changed the thermal properties of the polymer, resulting in a lower onset of melting, 50.46°C (PGA-co-PDL, control) and 50.35°C (PGA-co-PDL, 1.5% Leu) compared to 55.27°C for the polymer alone. In addition, the endothermic peaks became broader in shape with spray dried formulations coupled with a decrease in area under the endothermic curve and the heat of fusion (ΔH) (Fig. 2). Furthermore, PGA-co-PDL, 1.5% Leu had a broader melting peak and a lower ΔH (2.484 J/g) compared to control formulation (ΔH, 4.621 J/g). Scanning electron microscopy (SEM) confirmed PGA-co-PDL particles had a smooth surface, with no difference between the control (PGA-co-PDL) and amino-acid modified formulations (Fig. 3). However, L-arginine modified microparticles (PGA-co-PDL, 1.5% Arg) were aggregated and appeared to be fused together compared to unmodified control

Table I Physical Characteristics of Spray Dried Microparticles (Values are Means ± SD)

Formu	Yield (%)	EE (%)	Drug loading (µg/mg particle)	Zeta potential (mV)	Particle size (µm)	Water content (%)	Tapped density (g cm^{-3})	Carr's Index		d_{ae} (µm)	MMAD (µm)
								%	Flowability		
PGA-co-PDL	46.2 ± 1.9	20.26 ± 0.01	2.60 ± 0.17	−28.62 ± 1.58	1.76 ± 0.23	1.74 ± 0.0	0.26 ± 0.02	34.48	Very poor	0.90 ± 0.10	2.86 ± 0.24
PGA-co-PDL, 0.5%Arg	49.5 ± 2.4	25.67 ± 0.03	3.29 ± 0.38	−25.92 ± 0.76	1.85 ± 0.13	3.40 ± 0.0	0.24 ± 0.04	30.00	Poor, cohesive	0.91 ± 0.11	2.18 ± 0.35
PGA-co-PDL, 1%Arg	43.9 ± 7.7	21.56 ± 0.01	2.76 ± 0.04	−27.82 ± 1.50	1.17 ± 0.24	4.11 ± 0.0	0.27 ± 0.01	34.48	Very poor	0.61 ± 0.09	2.58 ± 0.19
PGA-co-PDL, 1.5% Arg	16.4 ± 1.4	25.70 ± 0.09	3.29 ± 1.26	−25.39 ± 0.67	0.89 ± 0.17	5.09 ± 0.0	0.31 ± 0.05	30.43	Poor, cohesive	0.50 ± 0.13	2.98 ± 0.25
PGA-co-PDL, 0.5%Leu	41.1 ± 4.9	21.42 ± 0.01	2.74 ± 0.14	−39.58 ± 1.71	1.29 ± 0.20	2.23 ± 0.1	0.25 ± 0.02	33.33	Very poor	0.65 ± 0.10	3.68 ± 0.12
PGA-co-PDL, 1%Leu	52.5 ± 7.2	20.44 ± 0.01	2.62 ± 0.17	−29.95 ± 1.57	1.09 ± 0.20	1.71 ± 0.2	0.28 ± 0.02	33.33	Very poor	0.58 ± 0.08	2.55 ± 0.03
PGA-co-PDL,1.5%Leu	54.7 ± 2.6	18.94 ± 0.01	2.42 ± 0.09	−35.10 ± 0.99	1.49 ± 0.21	1.48 ± 0.1	0.25 ± 0.01	31.03	Poor, cohesive	0.75 ± 0.12	3.43 ± 0.58
PLGA,1.5%Leu	47.8 ± 3.6	22.10 ± 0.09	2.83 ± 0.25	−31.24 ± 1.69	1.08 ± 0.17	1.89 ± 0.23	0.26 ± 0.01	32.12	Very poor	0.56 ± 0.14	2.90 ± 0.41

Yield, encapsulation efficiency (EE), and drug loading (n = 6). Zeta potential, particle size, water content, tapped density and mass median aerodynamic diameter (MMAD) (n = 3)

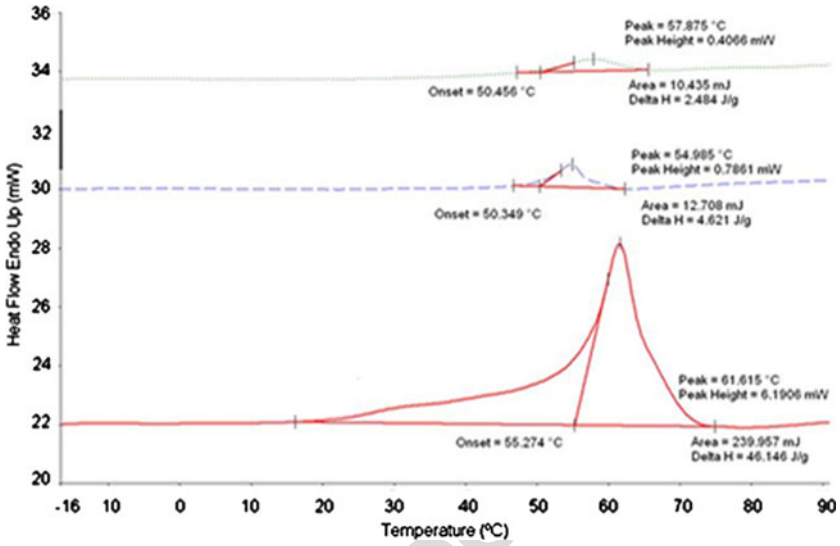

Fig. 2 Comparison of DSC thermograms of blank PGA-co-PDL polymer (*bottom*) and spray dried PGA-co-PDL (*control*) (*middle*) or PGA-co-PDL, 1.5% Leu (*top*).

microparticles (Fig. 3a and b respectively), whereas, L-leucine modified microparticles (PGA-co-PDL, 1.5% Leu) appeared spherical in shape, with no visual evidence of particle fusion (Fig. 3c). Confocal microscopy confirmed SF was homogenously distributed inside the microparticles in control formulation and PGA-co-PDL, 1.5% Leu (Fig. 4) during the emulsion/spray drying process.

In-Vitro Aerosolisation Studies

SF deposition data obtained from spray dried formulations indicated there was a difference in aerosolisation performance between the type and concentration of amino acids used (Fig.5). For example, PGA-co-PDL, 1.5% Arg showed significantly higher powder deposit in the capsule and inhaler compared to the other formulations, including control formulation (PGA-co-PDL) ($p<0.05$, ANOVA/Dunnett) and PGA-co-PDL, 1.5% Leu ($p<0.05$, ANOVA/Tukey's). In addition, L-arginine modified formulations displayed a higher throat deposition in contrast to L-leucine modified microparticles, particularly PGA-co-PDL, 0.5% Arg and PGA-co-PDL, 1.5% Arg formulations, in comparison to control formulation ($p<0.05$, ANOVA/Dunnett) and PGA-co-PDL, 1.5% Leu ($p<0.05$, ANOVA/Tukey's). In addition, PGA-co-PDL, 1.5% Leu resulted in significantly lower powder deposits in capsule and inhaler ($p<0.05$, ANOVA/Tukey's), and throat ($p<0.05$, ANOVA/Tukey's) compared to PLGA, 1.5% Leu. Overall, PGA-co-PDL, 1.5% Leu had the lowest powder deposit in the capsule and inhaler, and throat.

Addition of L-arginine (0.5–1.5%w/w) resulted in no significant change to %FPF ($p<0.05$, ANOVA/Dunnett) compared to control formulation (PGA-co-PDL) (Fig. 6a). In contrast, L-Leucine modified microparticles (PGA-co-PDL, 1% Leu & PGA-co-PDL, 1.5% Leu) produced significantly higher%FPF compared to control formulation

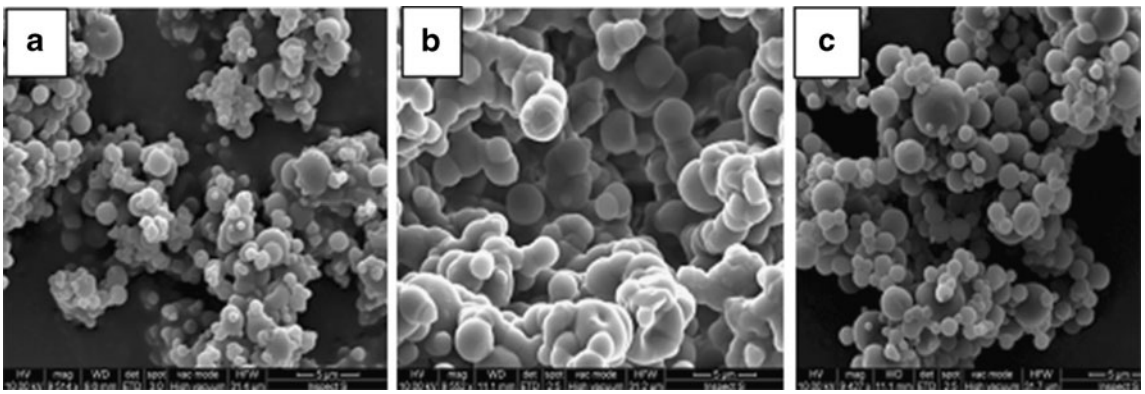

Fig. 3 SEM images comparing PGA-co-PDL (control formulation) (**a**) with PGA-co-PDL, 1.5% Arg (**b**) and PGA-co-PDL, 1.5% Leu (**c**). The scale bar represents 5 μm.

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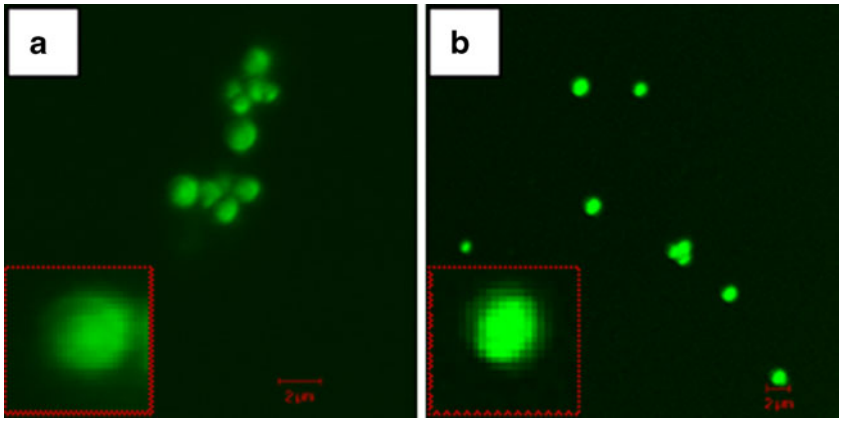

Fig. 4 Confocal laser scanning microscopy images comparing PGA-co-PDL (control formulation) (**a**) and PGA-co-PDL, 1.5% Leu (**b**). The scale bar represents 2 μm.

($p<0.05$, ANOVA/Dunnett) and L-arginine modified formulations (PGA-co-PDL, 1% Arg & PGA-co-PDL, 1.5% Arg) ($p<0.05$, ANOVA,Tukey's). In fact, PGA-co-PDL, 1.5% Leu produced the highest %FPF (43.38±5.61%) which was more than 1.5 times greater than the value obtained with same concentration of L-arginine (26.57±1.85%) ($p<0.05$, ANOVA,Tukey's). However, increasing the L-leucine concentration from 1.0 to 1.5%w/w did not significantly enhance %FPF ($p>0.05$, ANOVA/Tukey's) (Fig. 6a). Addition of amino acids resulted in no significant difference in FPD against control ($p>0.05$, ANOVA/Dunnett) (Fig. 6b). However, incorporating L-leucine, PGA-co-PDL, 1% Leu (21.58±1.21 µg) and PGA-co-PDL, 1.5% Leu (21.42±1.46 µg), resulted in almost double the FPD compared to PGA-co-PDL, 1% Arg (12.40±0.99 µg) ($p<0.05$, ANOVA/Tukey's). Overall PGA-co-PDL, 1.5% Leu had the highest %FPF and FPD, but no significant difference was noted when compared to PLGA, 1.5% Leu ($p>0.05$, ANOVA/Tukey's).

In-vitro Release Studies

It was clear PGA-co-PDL, 1.5% Leu could be considered as an optimum delivery system based on the aerosolisation results (lowest throat deposition, highest FPD and %FPF). Therefore, *in vitro* release studies comparing PGA-co-PDL (control), PGA-co-PDL, 1.5% Leu and PLGA, 1.5% Leu were performed and reported as cumulative percentage SF released over time (Fig. 7). Initially the SF adsorbed on the microparticles surface was removed by washing with 1 ml PBS buffer. A rapid burst release of SF was observed from all three formulations after 30 min, however the release of SF from PGA-co-PDL, 1.5% Leu (24.54%±3.87) and PLGA, 1.5% Leu (24.04%±2.67) was significantly less than PGA-co-PDL (41.87%±2.46) ($p<0.05$, ANOVA/Dunnett). The rapid release continued for all three formulations up to 5 h, where PGA-co-PDL, 1.5% Leu (38.52%±3.27) resulted in significantly less SF released compared to PGA-co-PDL (54.90%±5.76) and PLGA, 1.5% Leu

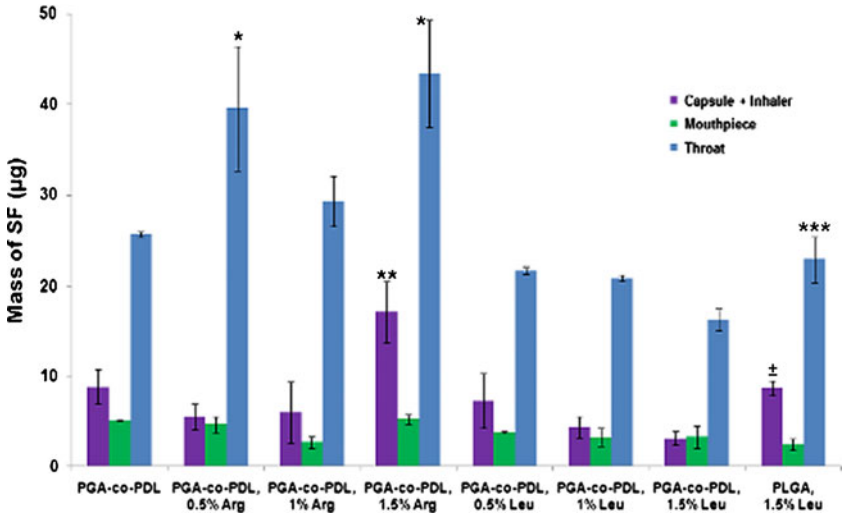

Fig. 5 Comparison of sodium fluorescein deposition in capsule and inhaler, mouthpiece and throat via different formulations. Data represent mean ± S.D., $n=3$. *$p<0.05$ (Throat) PGA-co-PDL, 0.5% & 1.5% Arg *vs* PGA-co-PDL (ANOVA/Dunnett) and PGA-co-PDL, 1.5% Leu (ANOVA/Tukey's), **$p<0.05$ (Capsule & Inhaler) PGA-co-PDL, 1.5% Arg *vs* PGA-co-PDL (ANOVA/Dunnett) and PGA-co-PDL, 1.5% Leu (ANOVA/Tukey's), ***$p<0.05$ (Throat) PLGA, 1.5% Leu *vs* PGA-co-PDL, 1.5% Leu (ANOVA/Tukey's), ±$p<0.05$ (Capsule & Inhaler) PLGA, 1.5% Leu *vs* PGA-co-PDL, 1.5% Leu (ANOVA/Tukey's).

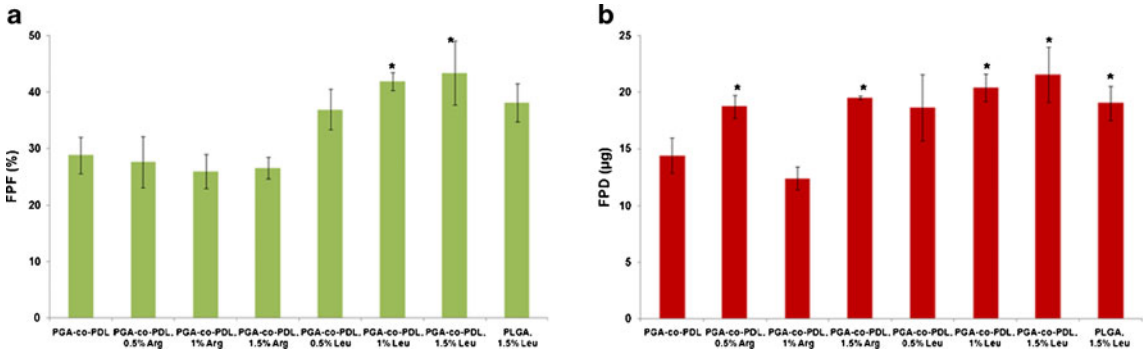

Fig. 6 **a** The percentage fine particle fraction of spray dried microparticles. Data represent mean ± S.D., $n=3$. *p < 0.05 PGA-co-PDL, 1% & 1.5% Leu *vs* PGA-co-PDL (ANOVA/Dunnett) and PGA-co-PDL, 0.5%, 1% & 1.5% Arg (ANOVA/Tukey's). **b** The fine particle dose (µg) of spray dried microparticles. Data represent mean ± S.D., $n=3$. *$p<0.05$ PGA-co-PDL, 0.5 & 1.5% Arg, PGA-co-PDL, 0.5%, 1% & 1.5% Leu and PLGA, 1.5% Leu *vs* PGA-co-PDL (ANOVA/Dunnett).

(54.20%±4.67) ($p<0.05$, ANOVA/Tukey's). After this time period the release of SF reached a plateau providing a slow continuous release phase up to 72 h, with PGA-co-PDL, 1.5% Leu (47.10%±3.78) releasing significantly less SF compared to PGA-co-PDL (61.35%±2.48) and PLGA, 1.5% Leu (63.07%±4.28) ($p<0.05$, ANOVA/Tukey's). In this study SF was released from PGA-co-PDL, PGA-co-PDL, 1.5% Leu and PLGA, 1.5% Leu formulations according to Higuchi diffusion model (R^2 value of 0.890, 0.924 and 0.832 respectively) and the release rate constant (K_h 2.13, 2.68 and 3.95 respectively) (Table II).

Cell Toxicity Study

Unmodified spray dried control formulation, PGA-co-PDL, and L-leucine modified formulation, PGA-co-PDL, 1.5% Leu appear to be well tolerated by normal lung bronchial epithelial cells *in vitro*, compared to PLGA, 1.5% Leu microparticles. Significant reduction in% cell viability was noted between PGA-co-PDL, 1.5% Leu and PLGA, 1.5% Leu microparticles at a concentration of 0.5 mg/ml (91.19±4.32, 82.72±2.58 respectively), 1 mg/ml (87.14±3.40, 74.20±3.13 respectively) and 5 mg/ml (85.57±1.44, 60.66±1.75 respectively) ($p<0.05$, ANOVA/Tukey's). Furthermore, the addition of L-leucine, as a dispersibility enhancer, to the optimum formulation during the emulsion/spray drying process did not alter the% cell viability, with values similar to PGA-co-PDL ($p>0.05$, ANOVA/Dunnett) (Fig. 8).

DISCUSSION

The aim of this study was to investigate the ability of a new family of polyesters, PGA-co-PDL, as SR carriers for pulmonary drug delivery, particularly as it had been investigated and shown promise as a delivery vehicle for both small molecular weight drugs and proteins (6,7). PGA-

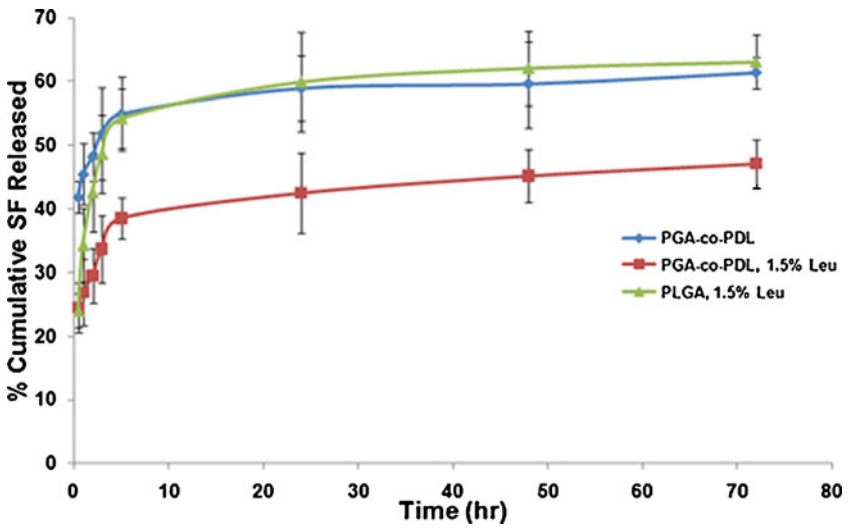

Fig. 7 Cumulative *in-vitro* release of sodium fluorescein from spray dried microparticles in PBS buffer at 37°C. Data represent mean ± S.D., $n=3$.

t2.1 **Table II** Kinetic Analysis of Spray Dried Microparticle Formulations (n = 3)

t2.2 Formulation	Zero Order (R^2)	First Order (R^2)	Higuchi model (R^2)	Mechanism of Release	K_h
t2.3 PGA-co-PDL	0.802	−0.828	0.890	Higuchi	2.13
t2.4 PGA-co-PDL, 1.5% Leu	0.848	−0.869	0.924	Higuchi	2.68
t2.5 PLGA, 1.5% Leu	0.732	−0.786	0.832	Higuchi	3.95

K_h = mg/cm2.min1/2 is the release rate constant for Higuchi diffusion model

co-PDL microparticles were prepared utilizing double emulsion/spray drying technique as our previous investigations indicated preparation of these particles via double emulsion alone were highly aggregated and exhibited poor aerosolisation performance (24).

The spray drying parameters were set to preserve the outlet temperature in the range of 44–47°C, as DSC analysis indicated a low melting point for PGA-co-PDL polymer. Generally, the EE was low in all formulations possibly due to the hydrophilic nature of SF partitioning into the external aqueous phase and a lower concentration remaining in the organic phase of the double emulsion/spray drying process (37). The negative surface charge demonstrated the anionic nature of the produced microparticles, which may be associated with incomplete removal of the PVA emulsifier in the external aqueous phase of the double emulsion. It is accepted that spray drying products are mainly characterized by their amorphous nature or disordered crystalline phase due to rapid drying of droplets (38). This behavior was demonstrated in our study by the broadening of the melting endotherm peaks for spray dried formulations. It is also worth noting that the accumulation of L-leucine at the air-liquid interface and hence the surface of microparticles resulted in physicochemical modifications, such as, surface charge, water content and particle size, which, additionally may have contributed to the enhanced broadening of the endothermic melting peak compared to control formulation (PGA-co-PDL). Furthermore, the shift to a lower temperature and intensity (peak height) indicated distribution of SF inside the PGA-co-PDL microparticles, which was confirmed from confocal microscopy images. Thus L-leucine treated formulations exist in a less crystalline state compared to untreated control formulation, and it is possible that incorporating L-leucine with these polymers may influence the encapsulation efficiency as the drug is mainly encapsulated in the amorphous region (6) and alter the physicochemical properties as noted above, which will inadvertently have an impact on the aerosolisation performance as observed in this study. However, further investigations are required to understand the influence of incorporating amino acids on the crystalline structure and the potential changes this may have on the physicochemical properties of generated spray dried particles.

The geometric particle size, particle shape and morphology are known to affect the aerodynamic properties and pulmonary deposition (39). The theoretical aerodynamic diameters calculated from tapped density indicate the spray dried particles generated are suitable for targeting the alveolar region. However, *in vitro* aerosolisation results from this investigation suggest the formulations did not

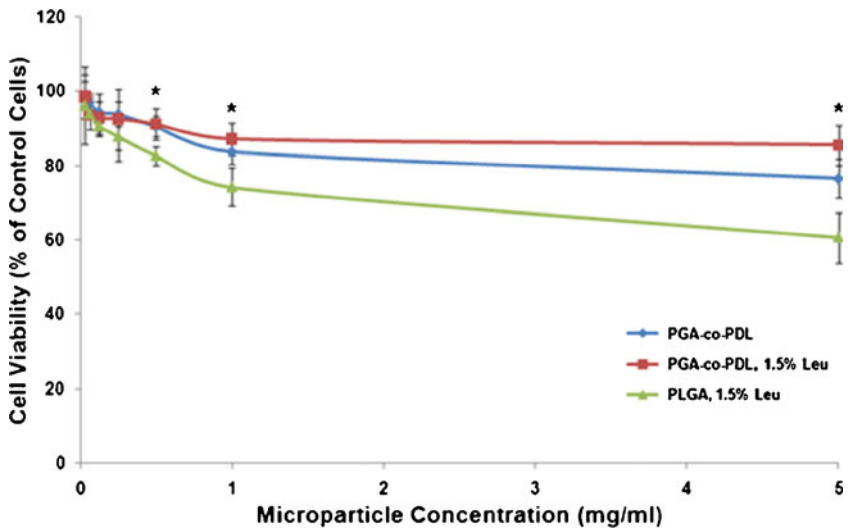

Fig. 8 Cell viability of human bronchial epithelium cell line (16HBE14o) measured by MTT cytotoxicity assay following 24 h exposure to different concentrations of PGA-co-PDL and PLGA microparticles suspension. Data represent mean ± S.D., $n = 6$. *$p < 0.05$ PGA-co-PDL, 1.5% Leu *vs* PGLA, 1.5% Leu (ANOVA/Tukey's).

aerosolize as individual particles, but rather as particle aggregates, as indicated when comparing geometric particle size with MMAD. This most likely occurred due to incomplete powder de-aggregation as van der Waals forces between particles were not completely overcome upon inhalation. In addition, powder aggregation of all spray dried powders generated was confirmed with a Carr's index of ≥30 indicating the flow was very poor and/or cohesive. Moreover, depending upon the addition and concentration of amino acids, different deposition profiles were observed. For example, L-arginine treated microparticles due to their low zeta potential and high percentage of water content were highly aggregated, which affected the deposition pattern by incomplete powder release from the capsule and device, and higher deposition in the throat region, compared to control and L-leucine modified microparticles. Furthermore, increasing the L-arginine concentration resulted in a higher percentage of water content on the surface of microparticles, possibly due to the hydrophilic nature of L-arginine, which increased the tendency of aggregation and consequently affected deposition. Many researchers have indicated the formation of wrinkled surface morphology (40) due to excessive build up of vapor pressure during solvent evaporation in the spray drying process, especially with hydrophobic amino acids, such as L-leucine, for improved aerosolization performance (41). However, this behavior was not observed in particles produced in this investigation, which had a predominantly smooth surface morphology, and may be related to little or no build up of vapor pressure within the particles under spray drying operating conditions used in this study.

The low yield associated with PGA-co-PDL, 1.5% Arg primarily occurred due to production of highly cohesive particles, as indicated from Carr's Index and the high water content, resulting in powder adhesion to the wall of spray drying chamber. Similar results have been reported where enhancing the concentration of L-arginine resulted in decreased spray drying powder yield and aerosol performance, such as %FPF (40). Furthermore, PGA-co-PDL, 1.5% Arg had the lowest zeta potential value, −25.39 ± 0.67, which provided an indication to the instability and cohesiveness as the repulsion force could not exceed the attraction forces between particles. Hence the aggregation, low yield and poor aerosolisation performance (low %FPF, FPD and high powder deposits remaining in the inhaler and capsule, mouthpiece and throat), compared to the other formulations resulted, due to strong van der Waals forces between particles. van der Waals forces are directly proportional to the contact surface area of a particle, and hence an increase in strength is observed with smaller particle sizes due to larger surface area. However, similar zeta potential values were achieved with the other L-arginine modified formulations, but they possessed larger geometric particle sizes resulting in decreased van der Waals forces between particles.

Comparing all formulations, L-Leucine had the highest % FPF and FPD values compared to control formulation (PGA-co-PDL), L-arginine and PLGA modified formulations. The possible mechanisms for the enhanced performance might be related to the surface activity of the relatively strong hydrophobic alkyl side chain of L-leucine accumulating at the particle surface during spray drying (40). Similar reports have also demonstrated the enhanced aerosol performance with L-leucine containing formulations compared to L-arginine and other investigated amino acids (40,42,43). Comparing the three L-leucine formulations, PGA-co-PDL, 1.5% Leu was considered to be the optimum formulation as a carrier for pulmonary drug delivery, as it exhibited the highest %FPF and FPD. Hence, although the powders generated had poor cohesive flow properties, the high zeta potential values indicated good physical stability, which together with the lowest tapped density, water content and relatively large particle size compared to other formulations resulted, in weak van der Waals forces between particles. Consequently, inhalation provided sufficient energy to de-aggregate the particles resulting in an enhanced aerosolisation performance.

The results of this investigation indicate that L-Leucine plays an important role not only in enhancement of the aerosolisation proprieties of the microparticles but also in sustaining drug release over 72 h, as indicated with PGA-co-PDL, 1.5% Leu. Once again this could be attributed to the surface activity of L-leucine coating the microparticles during the spray drying process, resulting in reduced surface adsorption of SF, which can be seen from confocal images, and hence a decreased burst and continuous release (44). Similar results have been reported for other surfactants, such as polysorbate 20 and sodium dodecyl sulphate, which reduced the surface accumulation of certain proteins in a concentration dependant manner (41,45). As a result, it is possible the high burst release associated with PGA-co-PDL may be due to SF particles migrating towards the microparticle surface by residual solvent during spray drying. However; none of the formulations could be considered an optimum SR pulmonary delivery system, as PGA-co-PDL possessed a high burst release and although PGA-co-PDL, 1.5% Leu had a lower burst release, it failed to release its entire pay load during 72 h, with similar results obtained by Thompson *et al.* (6). The incomplete release of SF may be associated with the slow hydrolyzation of the ester linkages in the polymer backbone (46). Data from our laboratory showed approximately 40% loss in polymer molecular weight after 14 days incubation in PBS buffer at 37°C (47) indicating the ester linkages between the monomers were very stable. In this current investigation the

release of SF from control formulation (PGA-co-PDL) and PGA-co-PDL, 1.5% Leu was according to the Higuchi's model, and mediated through the diffusion process with very little contribution from degradation of the polymer. Hence, the controlled release of small molecular weight hydrophilic compounds from modified PGA-co-PDL spray dried particles appears to be a diffusion limited process. The more significant release of SF from PLGA, 1.5% Leu may be associated to the smaller particle size and hence a greater surface area. Future investigations are required to optimize the release profile, and may involve manipulating the polymer characteristics, such as decreasing the molecular weight or increasing its hydrophilic properties by incorporation of poly(ethylene) glycol, PEG, to the polymer backbone.

The results from this investigation indicate that PGA-co-PDL, 1.5% leu was an optimum pulmonary drug delivery carrier. However, the safety of the carrier used for pulmonary drug delivery is an important issue. Normal bronchial epithelial cells (16HBE14o-) were chosen in accordance with the aerosolization and particle size distribution (MMAD) results for the particles generated (48). The cytotoxicity profile data of PGA-co-PDL and PGA-co-PDL, 1.5% Leu was more superior to PLGA, 1.5% Leu spray dried microparticles at 0.5, 1 and 5 mg/ml concentrations. Consequently, this provides an indication about the feasibility of using PGA-co-PDL polymers as alternative safe carriers for pulmonary drug delivery.

CONCLUSIONS

The present investigation suggests that PGA-co-PDL could be considered as an alternative novel biodegradable carrier for pulmonary drug delivery having the ability to control the release of the encapsulated drug. In addition, incorporation of $_{L}$-leucine was found to enhance the aerosolisation performance and decrease both the burst and continues release of encapsulated drug. Toxicity studies revealed the safety of the spray dried PGA-co-PDL modified microparticles compared to PLGA microparticles.

Future studies will be conducted to determine if the polymers elicit an immune response. In addition we will investigate enhancing the aerosolisation performance, encapsulation efficiency and optimizing the release of therapeutic agents from these polymers.

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