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The inhibition of phagocytosis of respirable microspheres by alveolar and peritoneal macrophages

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Abstract

Respirable poly(lactic co-glycolic acid) (PLGA) microspheres (2–3 μm diameter), were fabricated as a model drug delivery system whose uptake by macrophages could be quantified by fluorescent activated cell sorting. The microspheres exhibited minimal release of the entrapped flourophore (rhodamine B) and thus avoided possible fluid phase uptake of the flourophore. Externally bound microspheres were removed from the cell membrane by acid washing. The fluorescent intensity associated with the cells arose, therefore, from the internalised microspheres. NR8383 continuous culture alveolar macrophages were verified against primary cultures as a good model of alveolar phagocytosis. Peritoneal macrophages were also isolated and systemic and alveolar phagocytosis compared. Poloxamer 338 adsorbed at the microsphere surface did not reduce phagocytosis by NR8383 macrophages. It did, however, reduce the number of microspheres contained in primary alveolar macrophages but did not reduce the percentage of phagocytic cells. Poloxamer coatings did not reduce phagocytosis by peritoneal macrophages once the ratio of five microspheres per cell was exceeded. Dipalmitoylphosphatidylcholine (DPPC), the major component of lung surfactant, was added to cultures to model the alveolar environment where it was observed to reduce phagocytosis. In light of this finding, microspheres were coated in DPPC, which reduced their uptake by all cell types at all microsphere to cell ratios. © 2002 Published by Elsevier Science B.V.

Keywords: Alveolar macrophages; Peritoneal macrophages; Phagocytosis inhibition; PLGA microspheres; DPPC; Poloxamer 338

1. Introduction

Administered orally, gene, protein and peptide biotechnology products intended for systemic delivery will be rendered pharmacologically inert by the digestive enzymes of the gastro-intestinal tract. This necessitates their uncomfortable injection at frequent intervals since they are generally rapidly cleared. The lung is a complex organ of over 60 cell types (Stone et al., 1992), well adapted for its primary function of gas exchange

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and provides substantially greater bioavailability for macromolecules than any other port of entry to the body (Byron and Patton 1994; Patton, 1996). The lung has a surface area of approximately 100 m^2 at a total thickness, from air to blood of 0.4 μm. The rate of blood flow through its vasculature is high. The pulmonary artery stems from the left ventricle forcing the whole cardiac output through the lungs via a pulmonary circulation that carries almost the same blood flow as the systemic circulation (Yu and Chien, 1997). These characteristics make it an attractive target for non-invasive administration of aerosolised systemically-active drugs.

The mucociliary escalator does not extend to the peripheral lung. However, particles that do not rapidly dissolve in the alveolar lining fluid will be exposed to the phagocytic pulmonary alveolar macrophage and are likely to be sequestered within this cellular compartment. Either the particulate must avoid such cells, or rapidly release its drug into the alveolar lining fluid to attain maximum bioavailability.

Macrophages are found at all epithelial lung surfaces but are far more frequent in the deeper regions of the lung (Bezdicek and Chrystal, 1997). The number of macrophages in the lung is variable and can be raised on exposure to certain materials, for example cigarette smoke (Crapo, 1993; Crapo et al., 1982; Patton, 1996). In the normal human lung, however, macrophages comprise of over 95% of the mobile cell population and account for 2–5% of the total alveolar cells numbering between 50 and 100 per alveolus (Bezdicek and Chrystal, 1997; Crapo et al., 1982).

Studies of systemic macrophage phagocytosis have shown that coating microspheres with poloxamers prolonged their blood retention times (Gref et al., 1994; Harper et al., 1991; Illum et al., 1986; Illum and Davis, 1982; Moghimi and Davis, 1994; Moghimi and Gray, 1997; Moghimi et al., 1994; Stolnik et al., 1995; Verrecchia et al., 1995). Particles that avoid clearance by the Kupffer cells of the liver are eventually sequestered by the lymphocytes of the spleen (Harper et al., 1991; Illum and Davis, 1982; Illum et al., 1986; Stolnik et al., 1995). Assuming systemic and alveolar macrophages behave similarly and alveolar and systemic environments impact similarly on phagocytosis, poloxamer coatings may also reduce pulmonary phagocytosis.

Poloxamers prolong blood retention times by rendering the particulate more hydrophilic decreasing both opsonisation and the interaction between phagocyte and particulate (Illum et al., 1986; Moghimi and Gray, 1997). Particles opsonised by serum components will be rendered increasingly palatable to macrophages. However, if stabilising agents can withhold the interaction between particle and blood proteins/phagocyte beyond a critical distance then opsonisation and phagocytosis can be prevented. The range of electrostatic interaction between two bodies decreases with increasing ionic strength of the bathing media. In blood, which has an ionic strength of 0.15 moles, electrostatic interactions have a range of less than 1 nm (Stolnik et al., 1995). This concept termed steric hindrance can be applied wherever large molecules preserve a gap, that exceeding the range of van der Waals forces prevents interaction (Moghimi et al., 1994).

Hydrophilic regions of the poloxamer also hydrogen bond with water molecules, forming a protective hydration shell of so called structured water around the particulate. As the molecular weight of these chains and hence their length is increased, the hydration shell of bound water molecules thickens. At some critical value approximating 1500 and 3500 Da the chain length folds in on itself trapping additional molecules between structures. The folding of the hydrophilic chains into a hydrated coil provides a repulsive layer that prevents protein adsorption (Illum et al., 1986; Moghimi et al., 1994; Stolnik et al., 1995). This layer of structured water exhibits liquid properties including a mobility that reduces the contact time of the plasma proteins/phagocyte with the surface and hence lowering adsorption. Particles coated with branching PEGs of lower mobility exhibit higher protein adsorption (Stolnik et al., 1995).

NR8383 alveolar macrophages used in these studies are derived from the BAL of a normal adult male Sprague–Dawley rat. As a continuous cell line they provide more reproducible data than

primary cultures of limited life span. NR8383 displays some properties of freshly isolated alveolar macrophages, as it possesses the FC receptor, produces IL-1 and responds to phagocytic challenge by ingestion and demonstrates a characteristic respiratory burst (Helmke et al., 1987, 1989). It must, however, be verified as an appropriate model of alveolar macrophage phagocytosis, thus the phagocytic response of NR8383 and primary alveolar macrophage cultures have been compared. Peritoneal macrophages were also isolated and the phagocytosis of microspheres determined, allowing comparison between systemic and alveolar macrophage phagocytosis. To deduce whether the alveolar environment affected macrophage phagocytosis DPPC, the major component of pulmonary surfactant was added to in vitro cultures.

2. Materials and methods

2.1. Materials

All solvents were analytical grade and purchased from Fisher Scientific, Loughborough, UK. Water was prepared using a Millipore Milli-Ro system. Poly (lactic co-glycolic acid) 75:25, 90–126 kDa, (PLGA), poly(vinyl alcohol) 13–23 kDa, 87–89% hydrolysed (PVA), and rhodamine B (RB) were obtained from Sigma, Poole, UK. Methylcellulose and poloxamer 338 were obtained from ICI Pharmaceuticals Ltd., Alderley Park, UK. DPPC was purchased from Avanti Polar Lipids Inc., Alabastar, USA.

All cell culture materials including medias, foetal bovine serum (FBS), penicillin/streptomycin antibiotic solution (5000 U ml^{-1}; 5000 μg ml^{-1}, respectively) (P/S) and amino acid supplements were purchased from Gibco Life Technologies, Paisley, UK, dimethylsulphoxide (DMSO), and cytochalasin D were purchased from Sigma chemicals, Poole, UK. Transmission electron microscopy reagents (glutaraldehyde, sodium cacodylate, osmium tetroxide, araldite CY212 resin, uranyl acetate and lead citrate) were purchased from Agar Scientific Ltd., Stansted, UK.

2.2. Methods

2.2.1. Fabrication of poly (lactic co-glycolic) (PLGA) microspheres

PLGA microspheres (MS) were prepared by an O/W solvent evaporation method adapted from Prieto et al. (1994). Twenty milligram of PLGA was dissolved in 1 ml dichloromethane (DCM) containing 0.2 mg of RB. This was dispersed in 4 ml of an aqueous phase of 4% PVA and 0.25% methylcellulose. The resultant emulsion was homogenised for 10 min with an Ultraturax® homogeniser at 13 500 rpm. Subsequent evaporation of the DCM was carried out with mechanical stirring for 6 h at room temperature. MS were collected by centrifugation and washed by dispersion in water (× 3) with subsequent centrifugation. MS were freeze dried to remove remaining water and the yield determined.

2.2.2. Characterisation of PLGA microspheres

MS were sized at room temperature using laser light diffraction (Malvern 2600) in an aqueous media of 0.05% polyoxyethylene-sorbitan monooleate (Tween 80) which ensured that the microspheres were fully dispersed. RIB encapsulation and release rates were evaluated by standard fluorescence methodologies. Microspheres prepared for scanning electron microscopy (SEM) were dried under a nitrogen stream and splutter layered with gold.

Scanning electron micrographs of the microspheres were taken with a Philips XL 20 SEM.

2.2.3. Coating of PLGA microspheres

5 mg of microspheres were incubated in aqueous dispersions of 1% poloxamer or 0.1% DPPC, for 12 h at 37 °C on a plate shaker. Microspheres were washed three times by centrifugation in water to remove excess or unattached substrates. The presence of poloxamer or DPPC was investigated by Fourier transform infrared using a Perkin–Elmer 1625 FTIR. Samples were suspended in a ground matrix of barium chloride and spectra of coated microspheres compared against the adsorbate and uncoated microsphere controls.

2.2.4. Cell isolation

Macrophages were isolated from adult, male wistar rats (150–250 g). Rats were anaesthetised by inhalation of halothane (peritoneal isolation) or by injection of sodium pentabarbitol (60 mg kg^{-1} i.p.) (alveolar isolation) and killed by cervical dislocation.

2.2.4.1. Isolation of peritoneal macrophages. The skin was washed with 70% ethanol and 20 ml of PBS was injected intraperitonealy. The abdomen was massaged for 5 min to free adherent macrophages and the PBS withdrawn by a syringe through a small incision made in the abdominal wall. The lavage fluid was transferred to a centrifuge tube maintained on ice.

2.2.4.2. Isolation of alveolar macrophages. The lungs, bronchi and trachea were dissected out and the connective tissue removed. A cannula was inserted into the trachea and tied firmly into place.

The lungs were filled with 8 ml of PBS, which was collected in a centrifuge tube maintained on ice. This was repeated a further four times producing 40 ml of lavage fluid. To remove surfactant, cells pelleted by centrifugation at $95 \times g$ were washed twice by resuspension in media (minimum Eagles medium supplemented with 10% FBS and 0.5% PIS) recovered each time by centrifugation.

Both peritoneal and alveolar isolates were resuspended in fresh media and 1 ml aliquots of 100 000 cells were incubated in the wells of 24 well plates for 2 h under normal culture conditions (37 °C, 5% CO_2, 95% air, humidified). After gentle agitation the medium was removed and with it non-adherent cells. Following reconstitution in fresh media, some wells were assayed for cell viability by trypan blue exclusion and plates discarded where viabilities were below 95%. Viable cells were cultured for 2 h to allow their attachment to the substratum then used immediately.

2.2.5. NR8383 cell culture

NR8383 cells were cultured in F-12K (Kaighn's modification) nutrient media supplemented with 0.5% P/S and 15% FBS under standard culture conditions. Two phenotypes are expressed, an adherent macrophage population used for experimental purposes and a floating, regenerating phenotype from which subcultures are taken. Both phenotypes exist in equal proportions, approximately 50% of the floating cells becoming attached after reseeding, maintaining this equilibrium. Floating cells were passàged by centrifugation ($95 \times g$ for 8 min) and the cell pellet reconstituted in fresh media. Cells were seeded at 0.4–0.5 million per ml to achieve a floating concentration of approximately 0.25 million per ml. Adherent macrophages were covered by fresh media and removed by light scraping or agitation, before resuspension at 100 000 cells per ml in the wells of 24 well plates.

2.2.6. Transmission electron microscopy (TEM) of NR8383 macrophages

Samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature. The fixed cells were then post-fixed in 1% osmium tetroxide in the same buffer for 1 h then dehydrated in a graded series of ethanol (50, 70, 80, 95 and 3 changes of 100%, each for 10 min at room temperature). Following the dehydration, the samples were incubated overnight in a 1:1 (v/v) mixture of ethanol and araldite CY212 resin. The specimens were embedded in pure resin for 48 h at 60 °C. The polymerised block was then cut into 60 nm thick sections, mounted on pioloform coated grids and counter-stained in 2% uranyl acetate and 5% lead citrate. Ultrastructural examination was performed with a transmission electron microscope (Philips EM 208) operated at an accelerating voltage of 80 kV with magnifications ranging from $\times 1000$ to $\times 5000$.

2.2.7. Fluorescent activated cell sorting (FACS) assay

Cells aliquoted into the wells of 24 well plates were exposed to PLGA microspheres (5, 10 or 100 (excess) per cell) for 45 or 120 min. Cells were then removed and acid washed by centrifugation (2 min at 550 g) in a buffer of 28 mM sodium acetate, 117 mM sodium chloride adjusted to pH 5 with 2 M HCl, to remove microspheres bound

externally to the cell membrane. The inhibitors sodium azide (845 ng ml^{-1}) an inhibitor of the electron transport chain, and cytochalasin D (15 ng ml^{-1}) an actin inhibitor were added to some cultures for the experimental duration and a preincubation stage of 1 h.

Cells and particulates were analysed using a FACScan flow cytometer (Becton Dickinson, Oxford, UK). The fluorescence intensity (FI) of single cells and microspheres was recorded. Cell profiles were constructed according to parameters of side scatter (SSC) a measure of granularity, and forward scatter (FSC) a measure of size. This region was gated thus isolating cellular fluorescence from that of unphagocytosed MS. Fluorescent intensities above autofluorescence (that attributable to native cells) were quantified using WINMDI software, shareware at http://www.uwcm.ac.uk/haem. Results were collected for 5000 cells ($n = 3$) and expressed as the mean of three median fluorescent intensity (MFI) values for each of three experiments. Some remaining cells from FACS samples were mounted on slide and viewed under a fluorescent microscope.

Though microsphere release of RB into the media was minimal it may contribute to cellular MFI. To investigate this possibility, RB was added to the media at concentrations equivalent to the release of 0.5 and 1% of the load encapsulated in 1 mg of PLGA microspheres. These figures were cautionary since RB release over the experimental time period is considerably less and fewer microspheres than those contained in 1 mg were added.

The uptake of poloxamer coated microspheres by peritoneal cells was assayed in three separate media, where FIBS was excluded, included and heat denatured. FIBS was denatured prior to its addition to the media and by retaining the FIBS in a waterbath at 60 °C for 1 h.

2.2.8. Statistics

Paired t-tests were performed on raw data and analysis of variance (ANOVAs; Student–Newman–Keul's) on normalised data expressed as a percentage of control. Significance levels were set at $P < 0.05$.

3. Results

3.1. Characterisation of PLGA microspheres

Fig. 1 shows the size distribution of MS obtained after progressive adaptations to the preparation method of Prieto et al. (1994). After much investigation monodisperse microspheres of a respirable diameter were produced. The MS sample employed in future studies had a $D[v, 0.5]$ of 2.71 ± 0.11 μm with $D[v, 0.9]$ 2.91 ± 0.18 μm and $D[v, 0.1]$ 2.42 ± 0.04 μm. Size measurement was confirmed by S.E.M. (Fig. 2).

Actual yields were always above 90% with at least 18 mg of MS recovered. RB loading efficiency was calculated as $18.0 \pm 2.56\%$ ($n = 10$, mean ± S.D.) producing microspheres containing $0.18 \pm 0.03\%$ w/w RB ($n = 10$ mean ± S.D.).

When microspheres were incubated in water, a minimum burst effect of RB was observed ($\approx$ 0.2% after 1 h), with less than 0.5% being released up to 19 h (Fig. 3). Further incubation produced an increased release of fluorophore amounting to approximately 12% after 1 week.

3.2. Coating of PLGA microspheres

Fig. 4 shows examples of FTIR traces for PLGA and DPPC/PLGA microspheres together with a DPPC spectrum. The presence of phospholipid at the MS surface was identified by reference

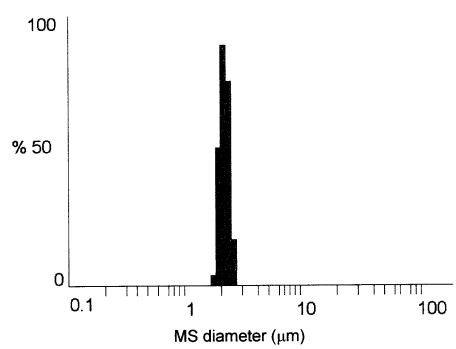

Fig. 1. Representative histogram of a microsphere sample sized by laser diffraction.

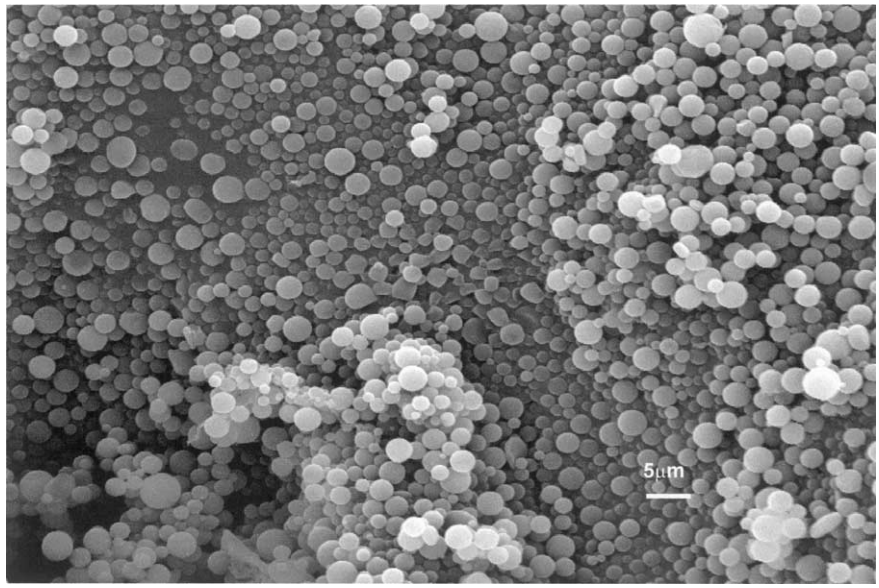

Fig. 2. Scanning electron micrograph of rhodamine B-encapsulated in PLGA microspheres. Magnification $\times 1600$.

to some peaks characteristic of DPPC in the spectrum from DPPC/PLGA microspheres. For example, the CH_2 asymmetric stretch (2920 cm^{-1}) and symmetric stretch (2860 cm^{-1}) are evident. The low intensity doublet on the uncoated MS spectrum (~ 3000 cm) may well relate to PVA^{-1} contamination of the MS surface. The CH_2 symmetrical deformation peak is visible at 1464 cm^{-1} and is absent from the uncoated MS spectrum (no peak between 1453 and 1500 cm^{-1}). The doublet ~ 1236 cm^{-1} on the coated MS is due to the DPPC headgroup PO_2^- antisymmetric stretch: no peaks occur between 1185 and 1276 cm^{-1} on the spectrum of uncoated MS. Also present in the spectrum of the coated MS is the CH_3 symmetrical deformation peak (1380 cm^{-1}). There is, therefore, coverage of the microsphere surface with DPPC. Poloxamer 338 was similarly shown to form an adsorbed film at the MS surface (data not shown) and to adsorb onto polystyrene latex (Jamshaid et al., 1988). Poloxamers have been shown by Cassidy et al. (1999) utilising XPS and Time of Flight Secondary Ion Mass Spectrometry, to adsorb to polymeric particles with the PPO component in contact with the particle surface and the PEO forming the outer surface layer.

3.3. Transmission electron microscopy of the NR8383 alveolar macrophage

TEM of the adherent phenotype revealed extensive pseudopodia, a lobed nucleus and multiple mitochondria and lipid vesicles. Cell counts that included both phenotypes revealed a doubling time of 125 ± 12 h.

3.4. FACS assay

RB was added to the media at concentrations equivalent to 1 mg of PLGA microspheres releasing 0.5 and 1% of their encapsulated rhodamine load. Experiments were conducted at two temper-

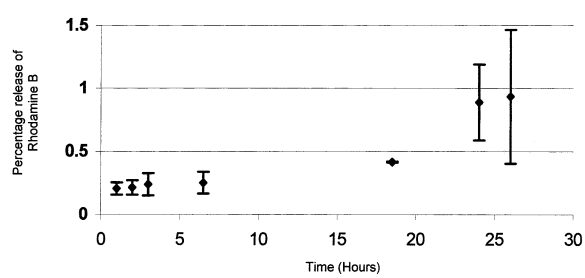

Fig. 3. Percentage release of rhodamine B from PLGA microspheres at 37 °C as a function of incubation time.

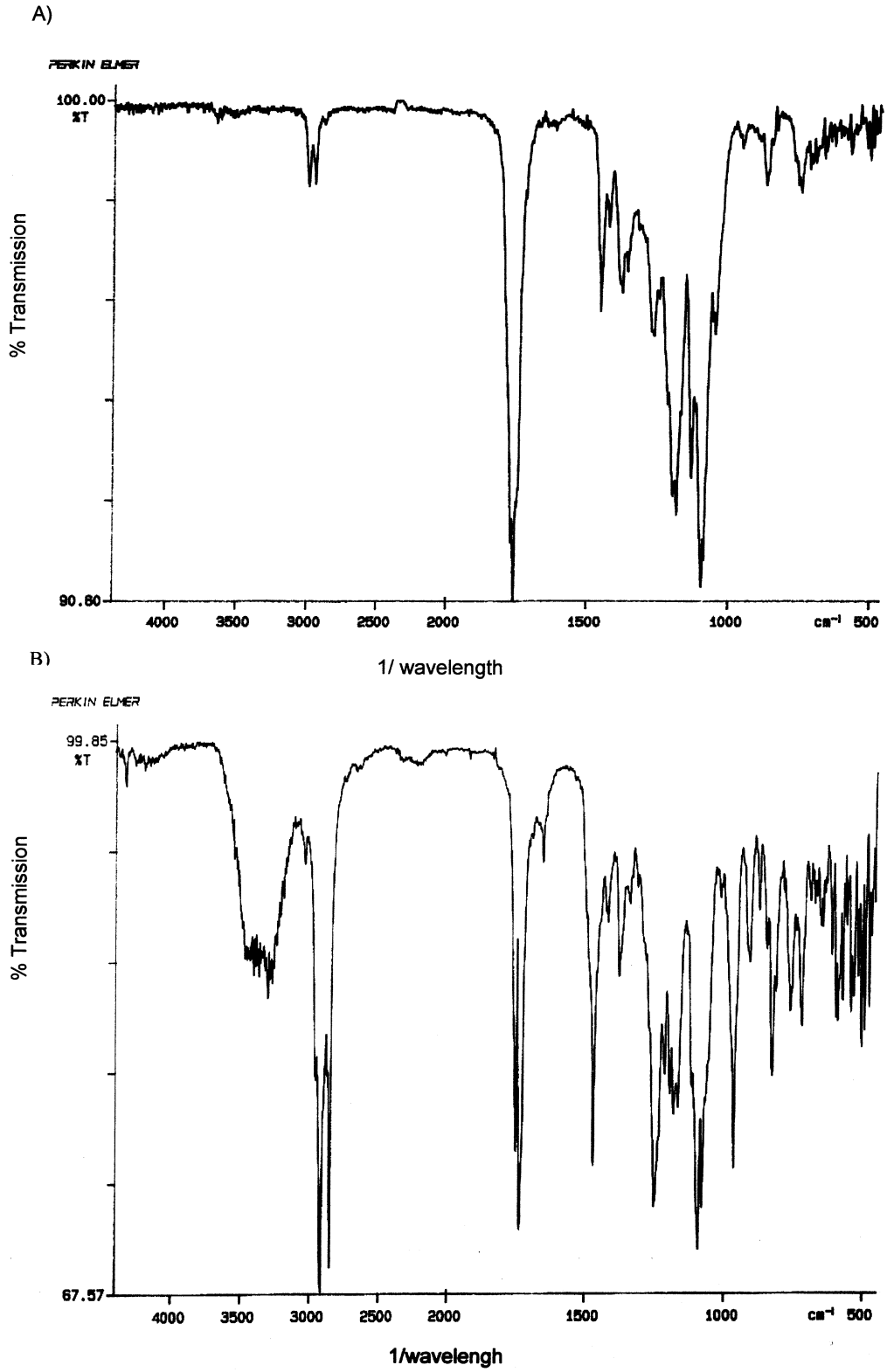

Fig. 4. Representative FTIR spectra of (A) PLGA microspheres, (B) DPPC coated PLGA microspheres and (C) DPPC.

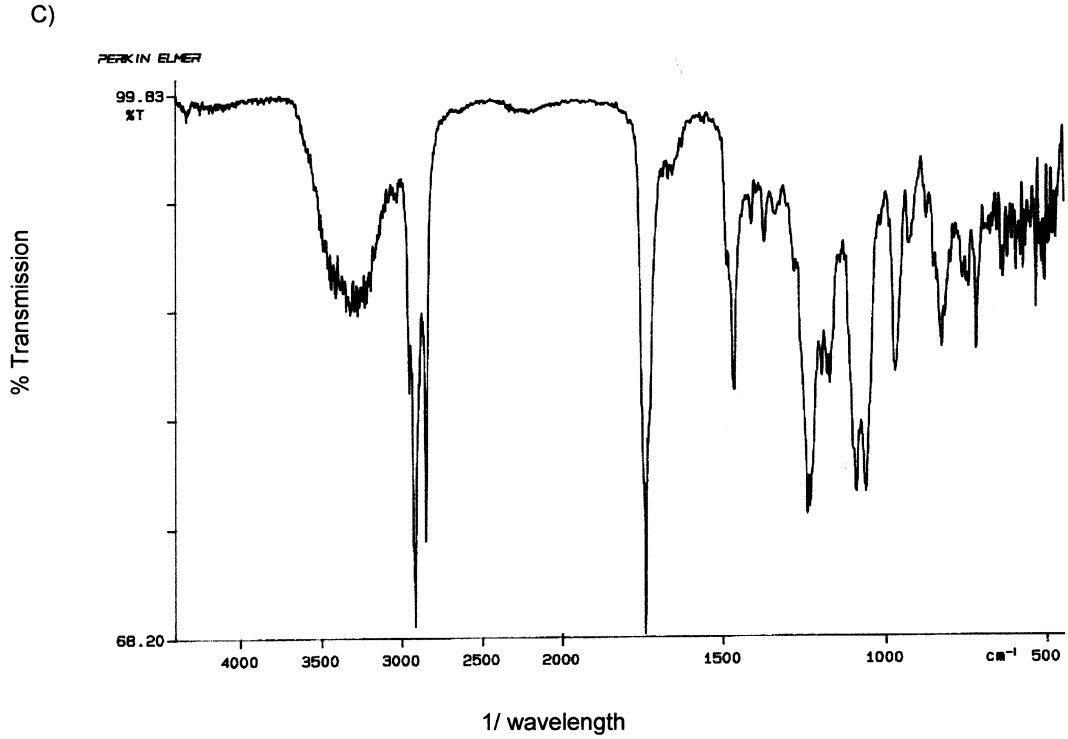

Fig. 4. (*Continued*)

atures 37 and 4 °C (Table 1). Figures represent the percentage of cells expressing RB fluorescence.

RB concentrations equivalent to a 1% release from PLGA-MS caused less than 1% of the NR8383 population to exhibit an increased FI after a 12 h exposure (Table 1). Over an experimental time period of 2 h actual release rates of rhodamine were substantially lower at $0.13 \pm 0.01\%$. Similar results were recorded for cells incubated at 37 °C and controls maintained at 4 °C, indicating the uptake of fluid phase fluorescence to be a passive non-energy dependent process.

Seventy percent of the NR8383 population phagocytosed a mean of 3.24 ± 0.65 microspheres per cell. The use of inhibitors prevented phagocytosis (Table 2) and fluorescent intensities approximated that of autofluorescence subsequent to acid washing ($2.82 \pm 1.30\%$; Fig. 5). Microsphere uptake remained consistent irrespective of the presence, exclusion or integrity of the FBS in the media. Uptake was also unaffected by preincubation of the microspheres in both untreated and heat denatured serum.

Poloxamer 338 coatings (Table 3) significantly reduced microsphere uptake by peritoneal cells at the ratio of five microspheres per cell ($P < 0.05$, paired t-test, poloxamer coated vs. uncoated microspheres). This effect was no longer evident once this ratio was exceeded. This reduced time period of 45 min is that previously used by Illum et al. (1986).

Poloxamer coatings do not reduce microsphere uptake by NR8383 macrophages at ratios of 5, 10 or excess microspheres per cell. Neither the number of microspheres per cell nor the number of phagocytic cells were reduced from control values.

The number of PAMS containing poloxamer-coated microspheres approximates that of control for each microsphere to cell ratio, although the number of microspheres per cell is decreased. This reduction was significant ($P < 0.05$) when compared by paired (poloxamer coated vs. uncoated microspheres) t-test performed on the raw data.

Table 1
Uptake of RB from solution by NR8383 macrophages over a 12 h time period

Time (h)	RB concentration (expressed as % release from PLGA-MS)			
	0.5% (37 °C)	1% (37 °C)	0.5% (4 °C)	1% (4 °C)
2	0.21 ± 0.01	0.18 ± 0.07	0.25 ± 0.14	0.35 ± 0.08
6	0.26 ± 0.19	0.49 ± 0.17	0.07 ± 0.08	0.38 ± 0.5
12	0.16 ± 0.14	0.65 ± 0.31	0.89 ± 0.23	0.67 ± 0.07

DPPC microsphere coatings greatly reduced phagocytosis by all cell types lowering both the number of phagocytic cells and the number of microspheres per cell (Table 4). These reductions were found to be significant by paired t-tests ($P < 0.05$, DPPC coated vs. uncoated microspheres) performed on the raw data for 5, 10 and excess microsphere to cell ratios.

The extent of DPPC induced reduction of phagocytosis is shown in Fig. 6, which compares the response of each cell type to DPPC coated microspheres added to excess. The two alveolar cell types behaved similarly, phagocytosis was inhibited to a larger extent in these cells than in peritoneal macrophages. From a multiple ANOVA (Student–Newman–Keul's test) performed on data encompassing all microsphere to cell ratios, the numbers of primary and NR8383 macrophages phagocytosing DPPC coated microspheres were not significantly different, although there was a significant difference in the numbers of microspheres uptaken per cell ($P < 0.05$). One must be aware, however, that reduction is expressed as a percentage of each cell types respective controls (uptake of uncoated microspheres). In real terms differences in the number of microspheres uptaken by alveolar and NR8383 translate to less than one microsphere per cell.

The same test revealed that DPPC microsphere coatings do not afford the same reduction in phagocytosis for peritoneal as compared with alveolar cell types. A greater number of peritoneal cells were found to uptake more microspheres per cell ($P < 0.05$).

Continuing experiments with NR8383 showed the inclusion of DPPC (0.1 mg ml^{-1}) into the media to be sufficient to inhibit phagocytosis. The number of phagocytic cells was reduced from ~ 70 to $\sim 45\%$ of the population and the number of microspheres per cell from ~ 3.2 to ~ 1.4 (mean, $n = 3$).

Table 5 refers to the phagocytic kinetics of NR8383. Interestingly it can be seen that beyond 0.75 h the number of microspheres per cell does not increase. Further phagocytosis is mediated through the recruitment of more cells as the percentage of the population containing microspheres increases between 0.75 h to a maximum of approximately 70% by 2 h.

4. Discussion

Fluorescent PLGA microspheres have been fabricated with a respirable diameter and low release profiles providing a good model with which to investigate pulmonary phagocytosis. Washing minimised any burst effects and the low release profile minimised fluid phase fluorescence allowing any increase in the fluorescent signal to be attributed to phagocytosis. PLGA is a commer-

Table 2
Percentage of the NR8383 cell population phagocytosing PLGA MS vs. culture conditions ($n = 5$, mean $\pm$ S.D.)

	Percent of cell population containing microspheres
No inhibitors	70.1 ± 1.81
Cytochalasin D (15 μg ml^{-1})	3.00 ± 0.48
Na azide (845 μg ml^{-1}) + cytochalasin D (15 μg ml^{-1})	3.59 ± 0.21
Na azide (845 μg ml^{-1})	5.91 ± 1.45

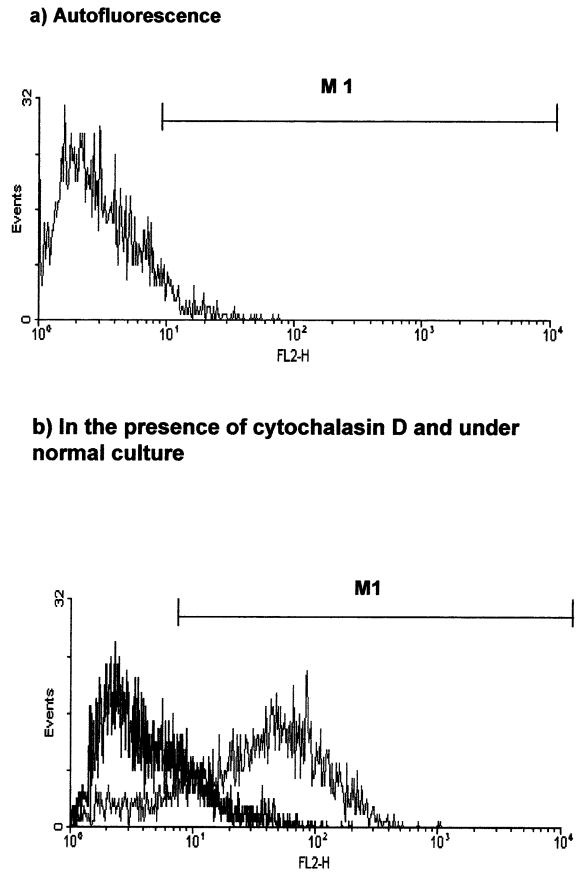

Fig. 5. Representative raw data fluorescence histograms. (a) Autofluorescence and (b) black, incubated with PLGA microspheres in the presence of the inhibitor cytochalasin D; grey, incubated with PLGA microspheres in the absence of inhibitor. The region M1 incorporates less than 1% of the autofluorescent cell population.

cially used polymer for drug delivery and thus comprised microspheres provide a more realistic model than traditionally employed latex.

FTIR demonstrated the presence of poloxamer and DPPC in microsphere samples subsequent to incubations in aqueous solutions of poloxamer and dispersions of DPPC.

A FACS assay has been developed which isolates the fluorescence of internalised microspheres. FACS allows fluorescent measurements to be made on single cells at high speed. Assays utilising this instrumentation are based on the cellular uptake of fluorescent particles. Cells can be discriminated from particulates or indeed other cell types by the unique way in which they scatter a laser beam. Both the fluorescent intensity of each cell and the number of fluorescent cells per sample are recorded.

The incorporation of a fluorescent label into a diversity of substrates has allowed their uptake to be quantified by FACS. The uptake of viruses (Benne et al., 1997; van Iwaarden et al., 1991), bacteria (Busque et al., 1998; Riber and Lind, 1999), fungi and yeasts (Giaimis et al., 1994; Rosseau et al., 1991), latex microspheres (Schurmann et al., 1997; Steinkamp et al., 1982), PLGA microspheres (Torche et al., 1999) and environmental pollutants (Kobzik, 1995) has been investigated in this manner. FACS has had a considerable impact as a clinical tool utilised in leukaemia phenotyping, the CD4 count in HIV and cross match transplantation. It has since found application as a research as well as diagnostic technique used for studies of drug delivery, pharmacodynamics and pharmacokinetics (Ramanathan, 1997).

Uptake by the systemic-reticulo endothelial system has been exhaustively studied and in order to develop pulmonary particulate drug delivery systems the same must be done for the macrophages of the alveoli. It would be unwise to simply extrapolate systemic findings to this differing milieu of surfactant, lower protein concentration and higher oxygen tension. Systemic and alveolar macrophages are heterogeneous and may respond differently to identical stimuli, even when both are in the presence of surfactant (Fels and Cohn, 1986).

Cytochalasin D is a potent inhibitor of phagocytosis preventing the formation of the motile pseudopodia necessary to envelop particulates (Bailly et al., 1991; Brett et al., 1984; Rubtsova et al., 1998; Schliwa, 1982; Urbanik and Ware, 1989, 1988; Verkhovsky et al., 1995; Valentijn et al., 1996; Wakatsuki et al., 1998). When cytochalasin D was included in phagocytic studies, Lacasse et al. (1998) reported microspheres as being in 'association with macrophages regardless of the presence of cytochalasin D' suggesting that co-incubation with cytochalasin D inhibits phagocytosis but particulates still attach to the cell membrane.

Acid washes have been used to dissociate cell membrane receptors from their ligands (Olfesky and Kao, 1982) presumably by protonating regions of the receptor. This approach was investigated as a means of dissociating externally bound, non-phagocytosed microspheres from the cell membrane. Cultures incubated with inhibitors had FIs approximating autofluorescence subsequent to acid washing whilst non-acid washed populations exhibited a degree of increased fluorescence. Microspheres had been removed from the membranes of phagocytically inhibited cells thus acid washing was incorporated into future procedures to isolate the FI of phagocytosed particulates.

FACS samples viewed by fluorescent microscopy also suggested phagocytosis to occur since microspheres and cells could be viewed in association. However, it proved impossible to distinguish between microspheres externally attached to the cell membrane and those actively internalised.

Poloxamer coatings reduced uptake by peritoneal macrophages when microspheres were added at a ratio of 5 per cell confirming the in vitro data of Illum et al. (1986). Here poloxamer 338 coatings reduced latex microsphere phagocytosis to 23% of uncoated controls. Poloxamer coatings in our system reduced phagocytosis (Table 3) to a lesser degree. The number of phagocytic cells were reduced to $62.0 \pm 8.43\%$, and the number of microspheres per cell to $62.5 \pm 6.94\%$, of uncoated controls.

In our in vitro system this reduction was lost when the ratio exceeded 5 microspheres per cell. At low ratios, cells and microspheres may lie in a proximity that allows the coated particulate to avoid an advancing pseudopodium by steric hindrance. At high microsphere to cell ratios a carpet of microspheres overlies the cells that probably results in ingestion as a consequence of an already established contact between cell and particulate. Alternatively, areas of the particulate surface may remain uncovered by their incubation in poloxamer solution. As the number of microspheres is increased so are the chances of contact between cell and such areas thus increasing the likelihood of internalisation.

For NR8383 alveolar macrophages, poloxamer coatings afford no reduction from controls. For PAMS the number of phagocytic cells remains the same, however, the number of microspheres per cell is reduced. Moreover this reduction accounts for less than one microsphere per cell so that phagocytosis between the two cell types can be considered similar.

Incubations with DPPC or indeed microspheres coated with DPPC reduce microsphere uptake by both peritoneal and alveolar macrophages. Evora et al. (1998) included DPPC in their microsphere preparation and concluded DPPC to orientate at the oil/water interface during fabrication. It was demonstrated, by confocal microscopy, that the inclusion of DPPC reduced the number of phagocytic cells from $65.1 \pm 15.87\%$ (control PLGA microspheres) to $26.2 \pm 13.93\%$ (DPPC/PLGA microspheres) of the macrophage population. They also concluded that DPPC improved the flow properties as a dry powder and that 'for all these reasons, DPPC microspheres may be useful to deliver entrapped drugs to the lung, thereby potentially providing a long-acting, inhalable drug delivery system' (Evora et al., 1998).

Table 3
Uptake of poloxamer 338 coated microspheres after a 45 min incubation with each cell type

		5	10	Excess
Peritoneal	%	62.0 ± 8.43*	78.7 ± 19.3	115 ± 11.3
	Number of microspheres	62.5 ± 6.94*	100 ± 17.4	113 ± 21.8
PAMS	%	79.1 ± 13.7	86.6 ± 14.9	91.6 ± 20.1
	Number of microspheres	71.6 ± 3.46*	55.4 ± 17.7*	76.6 ± 10.7*
NR8383	%	83.7 ± 11.3	107 ± 4.95	101 ± 3.27
	Number of microspheres	86.8 ± 18.3	87.0 ± 14.15	99.6 ± 9.39

Results are expressed as a percentage of the uptake of control (uncoated microspheres).

Table 4
Uptake of DPPC coated microspheres after a 45 min incubation with each cell type

		5	10	Excess
Peritoneal	%	43.3 ± 14.8*	40.4 ± 16.9*	53.4 ± 14.8*
	No. MS	46.8 ± 19.4*	42.5 ± 15.4*	57.7 ± 21.6*
PAMS	%	40.1 ± 24.6*	30.8 ± 18.6*	34.8 ± 21.0*
	No. MS	44.1 ± 4.90*	37.6 ± 5.56*	50.1 ± 3.16*
NR8383	%	24.1 ± 7,86*	31.9 ± 3.74*	36.6 ± 3.66*
	No. MS	30.5 ± 4.11*	31.0 ± 1.43*	35.3 ± 1.85*

Results are expressed as a percentage of the uptake of control (uncoated microspheres); %, percentage of the macrophage population containing microspheres; No. MS, mean number of microspheres contained per cell; *, significantly different from control.

DPPC induced inhibition is, however, more pronounced in alveolar cell types than in peritoneal macrophages. Alveolar macrophages are known to actively uptake and process airway surfactant, the efficiency of which is likely mediated through their expression of SP-A receptors (Bates and Fisher, 1996). The conjugation of such receptors and the rapid internalisation of surfactant may amplify the inhibitory stimulus of DPPC in alveolar cell types.

Unlike poloxamers, DPPC does not affect phagocytosis via steric hindrance as its influence is independent of whether it is presented simply in the media or as a coat on the microspheres. Its actual biochemical mechanism is, however, as yet undefined.

Monocyte and macrophage incubation in pulmonary phospholipid surfactant does not affect receptor expression but ingested, it can exert its influence intracellularly interfering in cell signalling pathways beyond ligation (Batenburg, 1992; Geertsma et al., 1997). Two intracellular pathways antagonistically regulate leukocyte antibacterial function. The pathway including Ca^{2+}/calmodulin and phospholipid-dependent activation of protein kinase C (PKC) is generally considered stimulatory and is antagonised by inhibitory pathways including that of cAMP-dependent activation of protein kinase A (PKA) (Geertsma et al., 1997). Alveolar macrophages and those incubated with surfactant have a raised cAMP concentration and it was conjected that the subsequent activation of PKA inhibited the production of reactive oxygen species reducing intracellular killing (Geertsma et al., 1994). However, incubations with PKA inhibitors did not improve intracellular killing (Geertsma et al., 1997). PKC must, therefore, be regulated by another mechanism.

PKC has a peculiar distribution in alveolar macrophages largely localised to the cell membrane as opposed to the cytosol and as a consequence alveolar macrophages fail to respond to other stimuli of the PKC transduction cascade. It was hypothesised that the unusual distribution of PKC caused by surfactant regulated PKC activation inhibited intracellular killing of bacteria by alveolar macrophages. However, further studies reported that the inhibition of PKC did not effect the production of antibacterial oxidants such as NADPH oxidase, thereby disproving a mechanism for surfactant inhibition of intracellular killing. Nevertheless two biochemical consequences of surfactant incubation, an increased cAMP concentration and the peculiar distribution of PKC have been identified, which may provide a mechanism for the impact of surfactant on phagocytosis.

Further studies with NR8383 followed phagocytosis over time. The number of microspheres per cell reached a finite maximum (approximately 3) after 45 min. Further phagocytosis was mediated through the recruitment of more cells until this too reached a maximum (approximately 70% of the population) by 2 h. Beyond this point no further phagocytosis was observed and the macrophage population appears 'saturated'.

Factors that control the extent of systemic phagocytosis cannot be extrapolated to the study of pulmonary phagocytosis. In addition to differ-

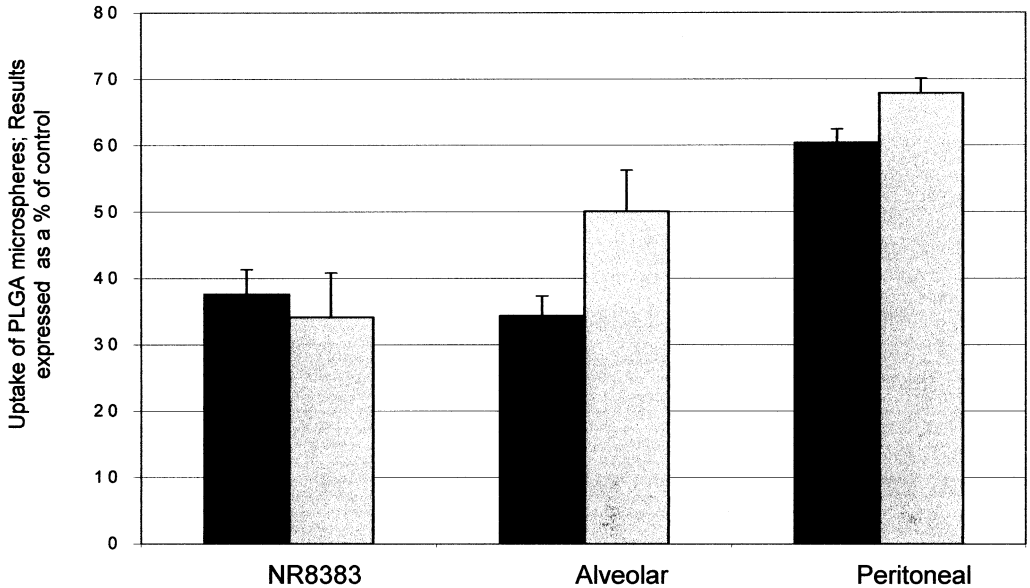

Fig. 6. The phagocytosis of DPPC coated microspheres by primary and continuous culture alveolar and peritoneal macrophages. Data are expressed as a percentage of control and microspheres are present in excess for 2 h. Black bars refer to the number of cells phagocytosing microspheres and the grey bars to the number of microspheres per cell. ($n = 3$ for three experiments, mean $\pm$ S.D., utilising three rats for primary macrophages).

Table 5
Phagocytosis of uncoated PLGA microspheres by NR8383 after increasing incubation periods

Time (h)	% Cell population containing microspheres	Number of microspheres per cell
0.75	48.7 ± 0.32	3.06 ± 0.38
2	71.4 ± 0.14	3.05 ± 0.36
6	70.2 ± 0.36	3.09 ± 0.35
12	72.5 ± 1.88	3.16 ± 0.15

%, Percentage of the macrophage population containing microspheres; No. MS, mean number of microspheres contained per cell; *, significantly different from control.

ences between cells and pulmonary and systemic environments, drug distribution differs. That administered to the bloodstream flows dynamically around the body, that pulmonary delivered resides statically in the alveolar cups until phagocytosed by mobile macrophages.

References

Bailly, E., Celati, C., Bomens, M., 1991. The cortical actomyosin system of cytochalasin D treated lymphoblasts. Exp. Cell Res. 196, 287–293.

Batenburg, J.J., 1992. Surfactant phospholipids—synthesis and storage. Am. J. Physiol. 262, L367–L385.

Bates, R.B., Fisher, A.R., 1996. Surfactant protein A is degraded by alveolar macrophagesm. Am. J. Physiol. 271, L258–L266.

Benne, C.A., Benaissa Trouw, B., van Strijp, J.A.G., Kraaijeveld, C.A., van Iwaarden, J.F.F., 1997. Surfactant protein A, but not surfactant protein D, is an opsonin for influenza A virus phagocytosis by rat alveolar macrophages. Eur. J. Immunol. 27, 886–890.

Bezdicek, P., Chrystal, R.D., 1997. In: Chrystal, R.D., West, J.B., Weiberland, E.R., Bames, P.J. (Eds.), The Lung: Scientific Foundations, vol. 1. Lippincott–Rowan, Philadelphia, pp. 577–588.

Brett, J., Tannenbaum, J., Godman, G., 1984. The actin cytoskeleton of endothelial-cells-response to cytochalasin D. J. Cell Biol. 99, A198.

Busque, P., Higgins, R., Senechal, S., Marchand, R., Quessy, S., 1998. Simultaneous flow cytometric measurement of *Streptococcus suis* phagocytosis by polymorphonuclear and mononuclear blood leukocytes. Vet. Microbiol. 63, 229–238.

Byron, P.R., Patton, J.S., 1994. Drug delivery via the respiratory tract. J. Aerosol. Med. 7, 49–75.

Cassidy, O.E., Rowley, G., Fletcher, I.W., Davies, S.F., Briggs, D., 1999. Surface modification and electrostatic charge of polystyrene particles. Int. J. Pharm. 182, 199–211.

Crapo, J.D., 1993. New concepts in pulmonary edema. Am. Rev. Resp. Dis. 147, 790–792.

Crapo, J.D., Bary, B.E., Gehr, P., Bachofen, M., Weibel, E.R., 1982. Cell numbers and characteristics of the human lung. Am. Rev. Res. Dis. 125, 332–337.

Evora, C., Soriano, L., Rogers, R.A., Shakesheff, K.M., Hanes, J., Langer, R., 1998. Relating the phagocytosis of microparticles by alveolar macrophages to surface chemistry: the effect of 1,2-dipalmitoylphosphatidylcholine. J. Control. Release 51, 143–152.

Fels, A.O.S., Cohn, Z.A., 1986. The alveolar macrophage. J. Appl. Physiol. 60, 353–369.

Geertsma, M.F., Zomerdijk, T.P.L., Nibbering, P.H., van Furth, R., 1994. Pulmonary surfactant inhibits monocyte bacterial functions by altering activation of protein kinase A and C. Immunology 83, 133–139.

Geertsma, M.F., van Furth, R., Nibbering, P.H., 1997. Monocytes incubated with surfactant: a model for human alveolar macrophages. J. Leukocyte Biol. 62, 485492.

Giaimis, L., Lombard, Y., Poindron, P., Muller, C.D., 1994. Flow-cytometry distinction between adherent and phagocytized yeast particles. Cytometry 17, 173–178.

Gref, R., Minamitake, Y., Peracchia, M.T., Trubetskoy, V., Torchilin, V., Langer, R., 1994. Biodegradable long-circulating polymeric nanospheres. Science 263, 1600–1603.

Harper, G.R., Davies, M.C., Davis, S.S., Tadros, T.F., Taylor, D.C., Irving, M.P., Waters, J.A., 1991. Steric stabilisation of microspheres with grafted polyethylene oxide reduced phagocytosis by rat Kupffer cells. In vitro Biomater. 12, 695–704.

Helmke, R.J., Boyd, R.L., German, V.F., Mangos, J.A., 1987. From growth factor dependence to growth factor responsiveness—the genesis of an alveolar macrophage cell line. In vitro Cell Dev. Biol. 23, 567–574.

Helmke, R.J., German, V.F., Mangos, J.A., 1989. A continuous alveolar macrophage cell line—comparisons with freshly derived alveolar macrophages. In vitro Cell Dev. Biol. 25, 44–48.

Illum, L., Davis, S.S., 1982. The targeting of drugs parentally by the use of microspheres. Parental Sci. Tech. 36, 242–248.

Illum, L., Huneyball, I.M., Davis, S.S., 1986. The effect of hydrophilic coatings on the uptake on colloidal particulates by the liver and peritoneal macrophages. Int. J. Pharm. 29, 53–65.

van Iwaarden, J.F., van Strijp, J.A.G., Ebskamp, M.J.M., Welmers, A.C., Verhoeff, J., van Golde, L.M.G., 1991. Surfactant protein A is an opsonin in phagocytosis of herpes virus type I by rat alveolar macrophages. Am. J. Physiol. 261, L204–L209.

Jamshaid, M., Farr, S.J., Kearney, P., Kellaway, I.W., 1988. Poloxamer sorption on liposomes: comparison with polystyrene latex and influence on solute efflux. Int. J. Pharm. 48, 125–131.

Kobzik, L., 1995. Lung macrophage uptake of unopsonized environmental particulates—role of scavenger-type receptors. J. Immunol. 155, 367–376.

Lacasse, F.X., Filion, M.C., Philips, N.C., Escher, E., McMullen, J.N., Hildgen, P., 1998. Influence of surface properties at biodegradable microsphere surfaces: effects on plasma protein adsorption and phagocytosis. Pharm. Res. 15, 312–317.

Moghimi, S.M., Davis, S.S., 1994. Innovations in avoiding particle clearance from the blood by Kupffer cells: cause for reflection. Crit. Rev. Ther. Drug Carrier Syst. 11, 31–59.

Moghimi, S.M., Gray, T., 1997. A single dose of intravenously injected poloxamine-coated long-circulating particles triggers macrophage clearance of subsequent doses in rats. Clin. Sci. 93, 371–379.

Moghimi, S.M., Hawley, A.E., Christy, N.M., Gray, T., Illum, L., Davis, S.S., 1994. Surface engineered nanospheres with enhanced drainage into lymphatics and uptake by macrophages of the regional lymph nodes. FEBS Lett. 344, 25–30.

Olfesky, J.M., Kao, M., 1982. Surface binding and rates of internalisation of ^{125}I-Insulin and IM-9 lymphocytes. J. Biol. Chem. 257, 8667–8672.

Patton, J.S., 1996. Mechanisms of macromolecule absorption by the lungs. Adv. Drug Deliv. Rev. 19, 3–36.

Prieto, M.J.B., Delie, F., Fattal, E., Tartar, A., Puisieux, F., Gulik, A., Couvreur, P., 1994. Characterisation of V3 BRU peptide loaded small PLGA mierospheres prepared by $(W_1/O)W_2$ emulsion solvent evaporation method. Int. J. Pharm. 111, 137–145.

Ramanathan, M., 1997. Flow cytometry applications in pharmacodynamics and drug delivery. Pharm. Res. 14, 1106–1114.

Riber, U., Lind, P., 1999. Interaction between *Salmonella typhimurium* and phagocytic cells in pigs—phagocytosis, oxidative burst and killing in polymorphonuclear leukocytes and monocytes. Vet. Immun. Immunopathol. 67, 259–270.

Rosseau, S., Seeger, W., Pralle, H., Lohmeyer, J., 1991. Phagocytosis of viable *Candida albicans* by alveolar macrophages: flow cytometric quantification. Am. J. Physiol. 267, L211–L217.

Rubtsova, S.N., Kondratov, R.V., Kopnin, P.B., Chumakov, P.M., Kopnin, B.P., Vasiliev, J.M., 1998. Disruption of actin microfilaments by cytochalasin D leads to activation of p53. FEBS Lett. 430, 353–357.

Schliwa, M., 1982. Action of cytochalasin-D on cytoskeletal networks. J. Cell Biol. 92, 79–91.

Schurmann, W.H.T., Frampton, M.W., Schurmann, G., Roberts, N.J., Nichols, J.E., Finkelstein, J.N., Oberdorster, G., Utell, M.J., 1997. A flow cytometric assay of Fc receptor-mediated phagocytosis. J. Aerosol. Med. 10, 1–12.

Steinkamp, J.A., Wilson, J.S., Saunders, G.C., Stewart, C.C., 1982. Phagocytosis-Flow cytometric quantitation with fluorescent microspheres. Science 215, 64–66.

Stolnik, S., Illum, L., Davis, S.S., 1995. Long circulating microparticulate drug carriers. Adv. Drug Deliv. Rev. 16, 195–214.

Stone, K.C., Mercer, R.R., Gehr, P., Stockstill, B., Crapo, J.D., 1992. Allometric relationships of cells numbers and size in the mammalian lung. Am. Resp. Cell. Mol. Biol. 5, 235–243.

Torche, A.M., Albina, E., Le Corre, P., Jestin, A., Le Verge, R., 1999. Flow cytometric and optical microscopic evaluation of poly(D,L-lactide-co-glycolide) microspheres phagocytosis by pig alveolar macrophages. J. Control. Release 58, 289–301.

Urbanik, E., Ware, B.R., 1988. Dose dependence of cytochalasin D on actin assembly. Biophys. J. 53, A572.

Urbanik, E., Ware, B.R., 1989. Actin filament capping and cleaving activity of Cytochalasin-B, D, E, and H. Arch. Biochem. Biophys. 269, 181–187.

Valentijn, K., lacivita, D.Q., Gumkowski, F.D., Jamieson, J.D., 1996. Effects of cytochalasin D on regulated exocytosis and endocytosis in rat pancreatic acinar cells. Mol. Biol. Cell 7 (SS), 3548.

Verkhovsky, A.B., Svitkina, T.M., Borisy, G.G., 1995. Cytochalasin D induces reversal of actin filament polarity at the cell edge. Mol. Biol. Cell 6, 827.

Verrecchia, T., Spenlehauer, G., Bazile, D.V., Murry-Brelier, A., Archimbaud, Y., Veiillard, M., 1995. Non-stealth (poly(lactic acid/albumin) and stealth (poly(lactic acidopolyrthylene glycol) nanoparticles as injectable drug carriers. J. Control. Release 36, 49–61.

Wakatsuki, T., Schwab, B., Elson, E.L., 1998. Cell mechanics response to cytochalasin D. Mol. Biol. Cell 9 (9), 83.

Yu, J., Chien, Y.W., 1997. Pulmonary drug delivery: physiologic and mechanistic aspects. Crit. Rev. Ther. Drug Carrier Syst. 4, 395–493.