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Relating the phagocytosis of microparticles by alveolar macrophages to surface chemistry: the effect of 1,2-dipalmitoylphosphatidylcholine

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Abstract

This study examines the potential of 1,2-dipalmitoylphosphatidylcholine (DPPC), a major component of lung surfactant, to reduce the phagocytosis of microspheres by altering the cellular interactions occurring in the alveoli. These microspheres could be designed to act as a controlled delivery system for small molecules, peptides or proteins for pulmonary administration. Microspheres were prepared using poly(lactic-co-glycolic acid) (PLGA, 50/50) and encapsulated peroxidase as a model protein. DPPC was included in some formulations. The interaction of PLGA and DPPC-PLGA microspheres with phagocytic cells was evaluated using lung macrophages in culture. X-ray Photoelectron Spectra (XPS) results indicate that the inclusion of DPPC in the microspheres alters the microsphere surface chemistry, with the DPPC covering a large portion of the microsphere surface. The dominance of DPPC on the microsphere surface is highly beneficial in moderating the interactions occurring between the microspheres and phagocytic cells in the lung. Fluorescent confocal microscopy indicates that only 25% of cells internalized DPPC-coated particles, whereas 70% of those cells exposed to particles without the DPPC coating internalized particles after one hour of incubation. © 1998 Elsevier Science B.V.

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1. Introduction

The low bioavailability of peptides and proteins given via the oral route has motivated the investigation of other non-parenteral routes of administration, including the pulmonary route, for these molecules [1]. The lung presents a number of potential

advantages over the gastrointestinal tract (GIT) for systemic protein delivery. The gas exchange function of the lung demands that the organ possesses a very high mucosal surface area with an extensive blood supply and these attributes can also potentially assist protein absorption [2]. In addition, absorption from the lung reduces first pass hepatic metabolism. Drug metabolism is further minimized by lower concentrations of extracellular enzymes in the lung as

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compared to the GIT [2]. Evidence of acceptable bioavailability following pulmonary administration has been reported for a number of proteins, including human γ -interferon, parathyroid hormone (PTH), salmon and human calcitonin [3], insulin [4], growth hormone [5], recombinant human granulocyte colony stimulating factor (rhG-CSF) [6,7] and leuprolide [8].

The advantages of pulmonary administration of drugs, including proteins, could be enhanced by their formulation into controlled release systems. Such systems may alleviate the need for multiple daily administrations over a long treatment period. Biodegradable microparticles may be a suitable delivery vehicle to achieve this goal [9–16]. However, a number of problems must be addressed to formulate successful microparticulate delivery systems. These problems include the high loss of inhaled aerosol in non-absorptive areas of the lung (i.e. the upper airways) and removal of microparticles due to phagocytosis by lung macrophages. In previous studies it has been shown that the inclusion of DPPC, a phospholipid endogenous to the lung, in microparticle formulations results in a significant improvement in the transport of the particles to absorptive regions of the lungs. This improvement due, in part, to a decrease in particle density and improved flow properties [16].

Mechanisms of reducing phagocytosis of microparticles administered intravenously have been actively investigated. Particular success has been demonstrated by coating the surfaces of microparticles with poly(ethylene glycol) (PEG) [17–20]. For example, it has been demonstrated that phagocytic cells have a much reduced ability to phagocytose particles sterically stabilized by pendant PEG chains, compared with those stabilized by ionic charge. Promising results have also been obtained by Gref et al. [21] who compared the potential of biodegradable nanospheres composed of copolymers of PEG and poly(lactic acid) (PLA). For particles with a non-ionic surface coating of PEG, the number of cells with engulfed particles decreased with increasing surface hydrophilicity [22].

Because phospholipids such as DPPC constitute the primary component of the absorptive surface of the lung, we have chosen to study the potential of this surfactant to reduce phagocytosis of controlled

release microsphere aerosols. On a molecular level, phospholipids represent the major constituents of the surfaces relevant to polypeptide absorption: alveolar cell membranes (type I and type II pneumocytes, macrophages), endothelial cell membranes, and pulmonary surfactant. Lung surfactant is composed of 80–90% lipid, of which phosphatidylcholines (PC) and phosphatidylglycerols (PG) constitute 70–80 and 5–10% of the total lipid by weight, respectively [23]. Approximately 60% of the PC in the surfactant is disaturated and in the form of DPPC. Given the abundance of DPPC in the lung, we hypothesized that the presence of this phospholipid at the surfaces of microparticles would reduce the adsorption of opsonic proteins, thereby reducing microsphere phagocytosis occurring in the alveoli. In this paper, we test this hypothesis by assessing the effect of microsphere surface chemistry on the extent of microsphere phagocytosis.

2. Materials and methods

Poly(vinyl alcohol) (PVA, Mw 25 kDa, 88 mole % hydrolyzed) was obtained from Polysciences, Inc. (Warrington, PA). Poly(D,L-lactic-co-glycolic acid) with a molar ratio of 50:50 (PLGA 50:50, Resomer RG503) was obtained from Boehringer Ingelheim (distributed by B.I. Chemicals, Montvale, NJ). 1,2-dipalmitoylphosphatidylcholine (DPPC), and isothiocyanate-labeled peroxidase-rhodamine (RITC-Peroxidase), were obtained from Sigma Chemical Co., Inc. (St. Louis, MO).

2.1. Microsphere preparation and characterization

Microspheres were prepared by a modified double-emulsion solvent-evaporation method [24]. In brief, the inner emulsion was formed by homogenization of 1 ml RITC-peroxidase aqueous solution (2 mg/ml) into 4 ml polymer solution in methylene chloride (100 mg polymer). Homogenization was performed by sonication at output 4 (Model VC-250, Sonics and Materials Inc., Danbury, CT) for 15 s in an ice bath. This emulsion was added to 4 ml of a 2.5% aqueous PVA solution and sonicated at output 4 for 20 s. This emulsion was then poured into 100 ml of 1% aqueous PVA solution and homogenized (L4R

Homogenizer, Silverson Machines, Inc. East Longmeadow, MA) at 8500 rpm using a 5/8" tip for 2 min in an ice bath. Removal of methylene chloride occurred by solvent evaporation while the second emulsion was magnetically stirred for 2 h. Microspheres were then collected by centrifugation, washed three times with double-distilled water and freeze-dried. Microspheres containing DPPC were prepared by an identical method with DPPC dissolved in the polymer solution at 3 mg/ml prior to the initial emulsification.

Drug entrapment efficiency was determined by digesting 10.0 mg microspheres in 3.0 ml 0.8 N NaOH overnight at 37°C, filtering with a 0.45 µm Millex HA filter (Millipore, Bedford, MA), and measuring the fluorescence relative to a standard curve (554 nm excitation and 574 nm emission) using a fluorimeter (PTI Photon Technology International, Canada). Drug loading was determined by dividing the amount of the drug encapsulated by the theoretical amount assuming all was encapsulated. Microsphere size distributions were determined using a Coulter Multisizer II (Coulter Electronics Limited, Luton, UK).

2.2. Surface chemistry analysis

X-ray photoelectron spectra were obtained using a Surface Science Laboratories X-100 spectrometer. An electron flood gun (energy 5 eV) was used to compensate for charging during XPS data acquisition. For all spectra the X-ray spot size was 600 µm. For all samples, a survey spectrum was recorded over a binding energy range of 0 to 1000 eV using a pass energy of 300 eV. The survey spectra recorded the presence of only oxygen (O1s 533 eV) and carbon (C1s 285 eV) at the surface. C1s region data was recorded with a pass energy of 150 eV. Curve fitting was performed using the manufacturer supplied software. A Gaussian–Lorentzian function with an 80% Gaussian component was applied to all data.

Microsphere samples were prepared on glass substrates by drop casting a 10 µl aliquot of a 100 mg/ml aqueous suspension of the microspheres. After approximately 12 h, the suspension had dried leaving a compact bed of particles that completely covered the substrate. Prior to polymer coating, the glass substrates were cleaned for 24 h in a 1%

solution of RBS 35 Detergent Concentrate (Pierce, Rockford, Illinois) and rinsed with deionized water.

2.3. Phagocytosis assay

2.3.1. Cell isolation

Alveolar macrophages (AM) were recovered from male Wistar rats (200–250 g) by intratracheal lavage. Rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (658 mg/kg body weight). Subsequently, a cross-sectional cut exposing the underlying intestines was made, and the rat was completely exsanguinated by severing the abdominal aorta. The trachea was exposed and a PE-190 polyethylene tubing (Becton Dickinson, Parsippany, NJ) was used for tracheal cannulation. The lung was lavaged using intratracheal instillation of 10 ml calcium and magnesium free Dulbecco's balanced salt solution (DBSS) (Gibco, Grand Island, NY) with 0.5 mM Na₂EDTA (Fisher, Fairlawn, NJ). The lavage procedure was repeated until a total volume of 30 ml was collected. Macrophages were isolated from the lung lavage fluid, pelleted by centrifugation at 400×g for 10 min, resuspended in 500 µl of 0.1% w/v BSA in Hanks' balanced salt solution (phenol red-free HBSS, Gibco, Grand Island, NY) at pH 7.4, and counted using a hemocytometer. The total cell volume was diluted to obtain 1×10⁶ cells/ml. Aliquots of 1 ml of 0.1% w/v BSA in HBSS containing 4×10⁵ cells were added on glass coverslips (12 mm diameter) placed in the wells of 24-cell well plates (Costar, Cambridge, MA) and incubated in a humidified chamber at 37°C with 5% CO₂ for 1 h.

2.3.2. Particle loading

After 1 h incubation, the media was withdrawn and the nonadherent cells removed by washing with PBS. Subsequently, 1 ml of media containing microspheres in a concentration of 0.7 mg/ml (100 particles/cell) was added into each well and allowed to co-incubate with the cells in a humidified chamber at 37°C in 5% CO₂ for 60 min. The 100 particles/cell ratio is higher than the typical ratio of 10 particles per cell such as routinely used in macrophage phagocytosis assays using heat-killed yeast or bacteria. However, pushing the system makes it easier to identify differences in particle uptake

among the various treatments because the particles were often smaller than 0.5 microns in size. The choice of incubation of 1 h was determined because of the known time course of phagocytic uptake by alveolar macrophages. Surface binding of particles by macrophages occurs rapidly, usually within 5 min, but in a previous assay it was found that 1 h is the time required for particle internalization.

2.4. Microscopic analysis

2.4.1. Cell fixation and staining

Cells were briefly washed with cytoskeleton buffer, pH 6.2, and fixed with the same buffer containing 0.5% Triton[®] X-100 (Sigma Chemical Co., Inc.). The fixative was removed and 2% *p*-formaldehyde solution was added for 15 min. To identify internalized particles and distinguish them from surface-bound particles, the microfilament network of the cytoskeleton cell was stained for 45 min at room temperature in the dark using 0.2 units FITC-Phalloidin/ml (Sigma Chemical Co., Inc.) dissolved in PBS. Cell staining in this fashion revealed the actin-rich cortical margin and gave suitable contrast to the cells and particles. Coverslips containing stained cells were sealed in microwell chambers containing 3 μ l antifade agent prior to microscopic examination.

2.4.2. Anti-peroxidase staining

Peroxidase-containing particles were incubated in 25 μ g/ml FITC-conjugated goat anti-horseradish peroxidase antibody solution (Jackson Immuno Research, West Grove, PA) for 1 h at room temperature, washed in PBS and mounted in microwell chambers for microscopic analysis.

2.4.3. Fluorescent confocal microscopy

Adherent alveolar macrophages were viewed using a Sarastro 2000 confocal laser scanning microscope (CLSM) (Molecular Dynamics, Sunnyvale, CA) fitted with a 25 mW argon-ion laser. The microscope was configured for dual channel fluorescent imaging with simultaneous 488 and 514 nm excitation, 10% laser transmission and 18 mW laser power. Emission spectra from the rhodamine-labeled peroxidase was collected by a photomultiplier tube (channel 1) operated at a voltage of 600–650 V. Another optical

path was selected for emission spectra emitted from the FITC-labeled actin filaments and was collected through a filter of 540 nm with a 15 nm bandwidth. Fields of cells were randomly selected and images were recorded in a 512 pixel image size format using a 60 \times 1.4 numeric aperture objective. At least 100 macrophages were examined in each test.

3. Results and discussion

3.1. Preparation of microspheres

The entrapment efficiency of RITC-peroxidase into DPPC containing particles was $95 \pm 1.35\%$ ($n=6$ individual batches) and into non-DPPC containing particles was $88 \pm 0.18\%$ ($n=6$ individual batches). In this study, the microspheres were completely spherical and the diameter for PLGA and DPPC-PLGA microspheres were 3.3 ± 1.00 and 3.5 ± 1.72 μ m ($n=6$ measurements per batch), respectively. In both cases the sizes were in the range applicable to inhalation administration [25].

3.2. Microsphere surface chemistry

During microsphere fabrication by the double emulsion method a large surface area interface is created between the methylene chloride/polymer phase and the aqueous/drug phase. As methylene chloride is removed by evaporation this interface converts to a polymer-water interface. The method of stabilization of these interfaces determines the final microparticle surface chemistry. For example, if surfactant adsorption stabilizes the oil/water interface, the surface chemistry of the resulting microspheres will be dominated by the surfactant. Therefore, the chemical nature of the surfactant used is likely to have an impact on the *in vivo* behavior of the microparticles and, in particular, on the response of the alveoli macrophages to the presence of the particles.

The XPS data displayed in Fig. 1 shows the C1s (atomic orbital 1s of carbon) regions of the RITC-peroxidase containing PLGA particles prepared without DPPC. We have previously shown that this C1s region data is indicative of the presence of poly(vinyl alcohol) on the microparticle surface [26]. Peaks 1 to

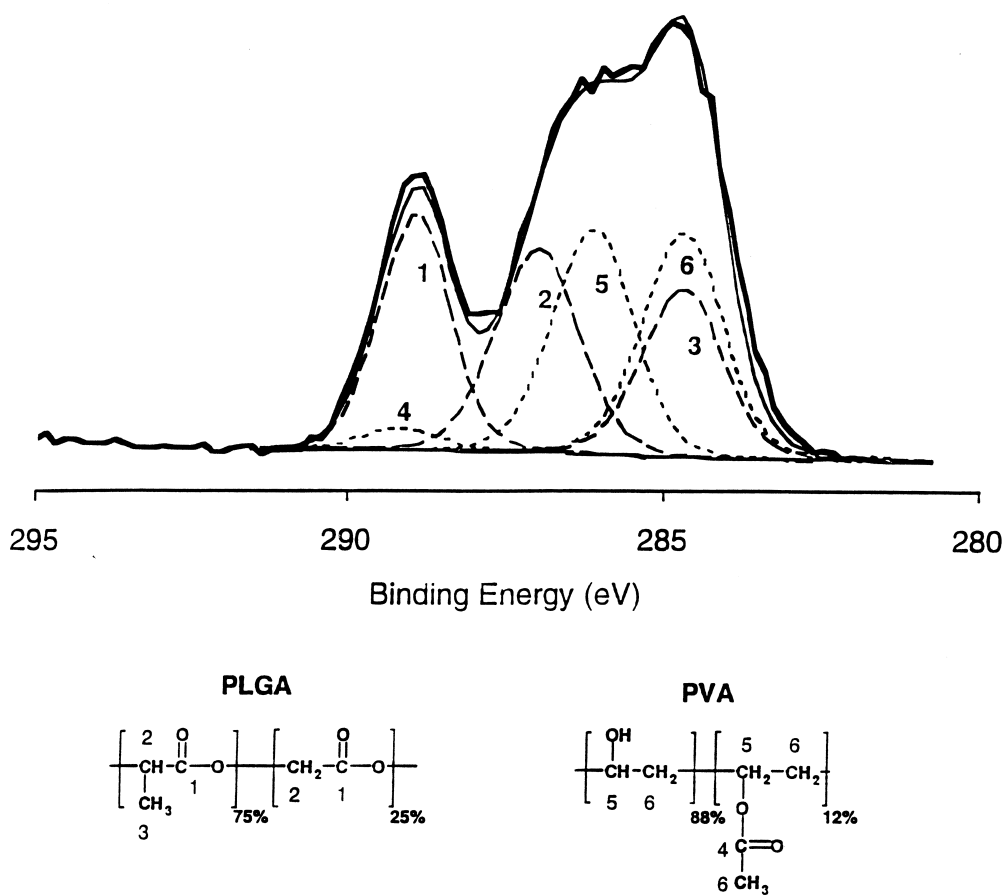


Fig. 1. Surface chemistry analysis of PLGA microparticles by XPS.

3 are contributed by the three carbon environments present in the PLGA monomer units. Peaks 4 to 6 are contributed by the 88% hydrolyzed PVA. The presence of PVA is expected due to the adsorption of this surfactant at the methylene chloride/water interface during microsphere preparation.

The inclusion of DPPC in the microparticle formulation caused a significant change in surface chemistry. The C1s region of the DPPC-containing PLGA microparticles incorporating RITC-peroxidase is shown in Fig. 2a along with a C1s region recorded from a DPPC film (Fig. 2b). If we first consider the DPPC film (Fig. 2b) the largest contribution to the C1s region is at a binding energy of 285.0 eV (72.8%). This peak is generated by the methylene groups of aliphatic chains. There are also minor contributions at binding energies of 286.4 eV (ether

carbon) (14.6%), 287.0 eV (ether carbon alpha to carboxyl) (8.6%) and 289.0 (carboxyl carbon) (4%). Comparison of the DPPC film C1s regions to the C1s region of the DPPC-containing microparticles reveals that the microparticle surface chemistry is dominated by DPPC. This can be inferred from the large area peak at binding energy 285.0 eV compared to the small peak area at binding energy 289.0 eV. However, there is a greater contribution from the carboxyl carbon (binding energy 289.0) to the microparticle C1s region as compared to the DPPC film C1s region. This indicates that the microparticle surface has a minor contribution from the biodegradable polymer and therefore, the DPPC is dominating the surface chemistry but does not entirely mask the PLGA.

This XPS data demonstrates that the low surface

energy of DPPC surfactant ensures that the methylene chloride-water, and the final polymer-water interfaces are stabilized by DPPC and not PVA. The presence of PVA at the microparticle surface would cause a high intensity to be observed over the range 285.0 to 286.5 eV, not just at 285.0 eV. Therefore, DPPC containing microparticles are stabilized by the phospholipid component, not the surfactant of the final aqueous phase.

3.3. *In vitro* phagocytosis

For a controlled release aerosol to be effective, it must be retained in the lung for a prolonged period so that the therapeutic agent can be released and absorbed over time. When foreign particulate material reaches the alveoli, where no ciliated epithelium is present, deposited particles may stay for longer times. However, in the alveolar region of the lung, microparticles come into contact with another lung defense mechanism, the alveolar macrophages. Therefore, the controlled delivery of drugs to or via the lung requires, in many cases, the ability of biodegradable particles to escape detection and uptake by alveolar macrophages. To enable the success

of controlled drug delivery systemically, it is necessary to minimize the probability of particle phagocytosis.

The use of a macrophage culture system has proved to be an effective means of studying particle phagocytosis and it can therefore be adopted for determining whether or not selected materials can alter phagocytic functions [27].

In this study, lung macrophages adhering to glass coverslips were used to investigate the interaction of microspheres and cells. Confocal microscopy was used to quantitate the uptake of microparticles. Fig. 3 shows fluorescent confocal micrographs of rat alveolar macrophages exposed to the same numbers of particles with and without the DPPC coating for 1 h. Uptake was influenced by microsphere surface characteristics. PLGA particles showed evidence of a significant number of particles within the cell cytoplasm (Fig. 3a), whereas the DPPC coating reduces the phagocytic uptake of fluorescent peroxidase-containing PLGA microspheres (Fig. 3b). Fig. 4 illustrates the percentage of phagocytic cells containing microspheres following incubation of the cells with the particles for 60 min. Only $26.2 \pm 13.93\%$ ($n=6$ experiments) of cells internalized DPPC-coated par-

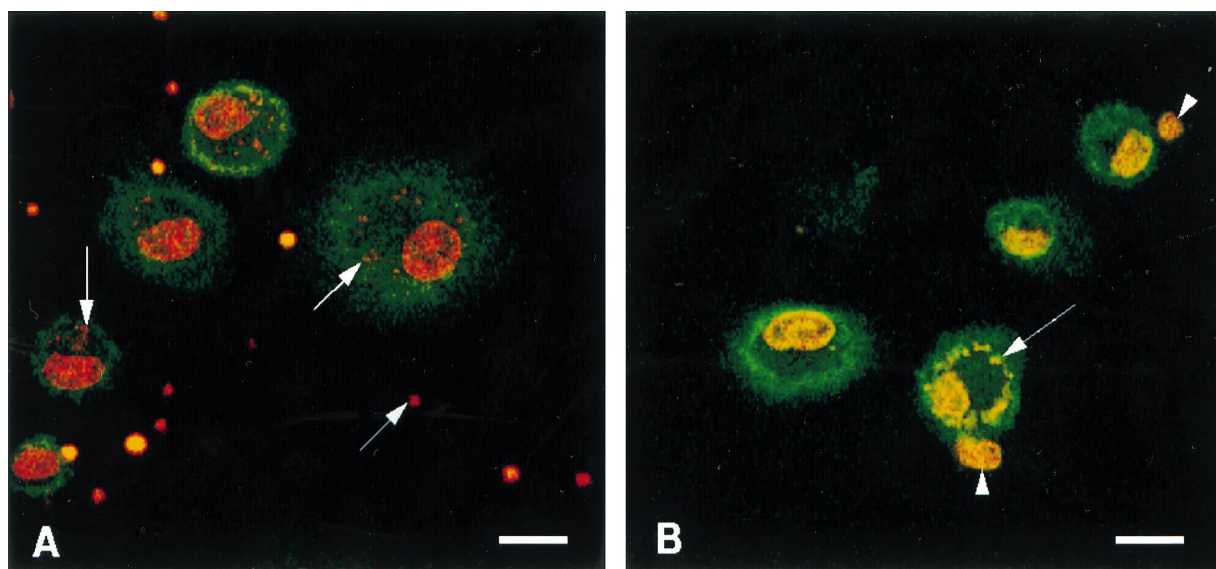


Fig. 3. (A) Fluorescent confocal micrographs of rat alveolar macrophages (green) stained with FITC-phalloidin after a one hour exposure to peroxidase-containing PLGA particles (arrows). (B) Rat alveolar macrophages exposed to peroxidase-containing PLGA particles (arrow) prepared with DPPC. Large particles are occasionally seen bound to cell surfaces. Bar represents 10 microns.

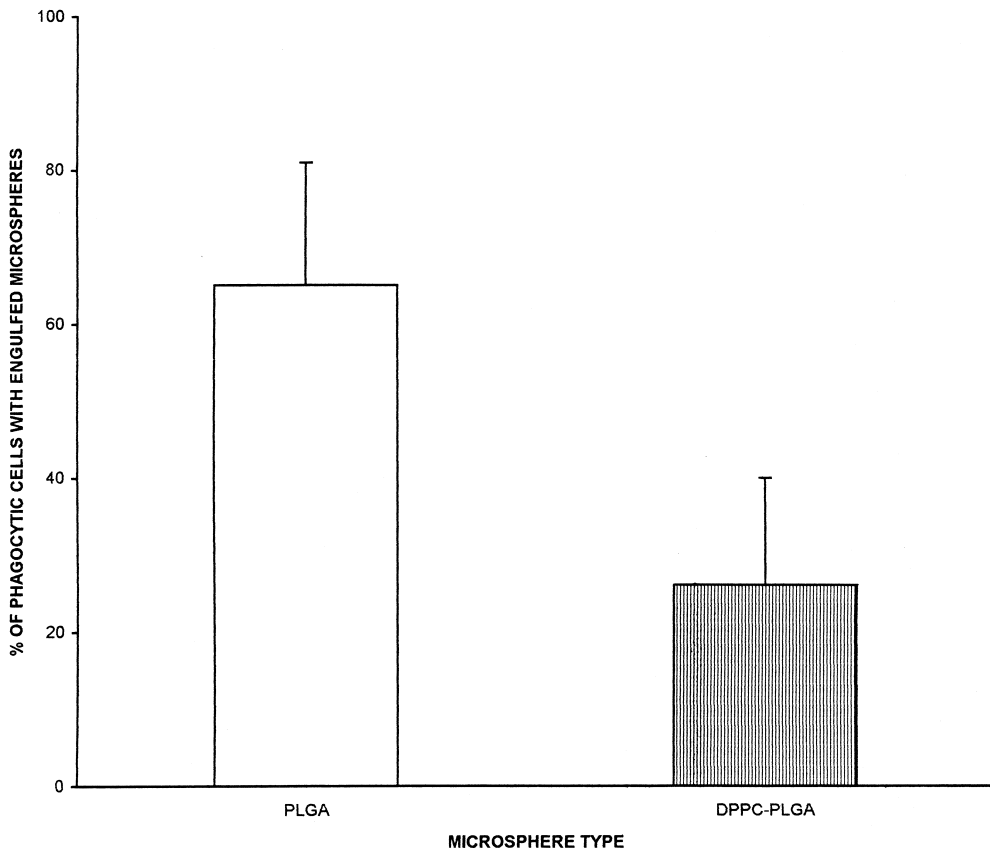


Fig. 4. The % cells engaged in phagocytic uptake of peroxidase-containing PLGA particles without and with surface modifications by DPPC.

ticles, whereas $65.1 \pm 15.87\%$ ($n=6$ experiments) of cells exposed to particles without DPPC coating internalized particles. These results were analyzed by Student's *t*-test ($P=0.0018 < 0.05$). Therefore, particle phagocytosis was dramatically reduced when DPPC-PLGA particles, with a high surface density of DPPC, were used.

Tabata et al. [22] have shown that macrophage phagocytosis of polymer microspheres is enhanced by surface precoating with some opsonic proteins such as immunoglobulin, fibronectin, and gelatin. For this reason, the presence of protein on the surface of the formulation was checked by a surface antigen assay for peroxidase. Particles were immunostained using anti-horseradish peroxidase antibody (Fig. 5) and only those particles without the DPPC coating (Fig. 5a) appeared to exhibit the green surface stain from the anti-peroxidase staining pro-

cedure. The presence of protein on the surface of this formulation, and the hydrophobic surface of PLGA microspheres, could explain the higher uptake of these particles by lung macrophages.

4. Conclusions

DPPC oriented to, and dominated the chemistry of, the surface of microspheres prepared by the double emulsion solvent evaporation process, as shown by XPS. If DPPC was excluded from the formulation, the surface chemistry was a mixture of PLGA and PVA [26]. The DPPC coating significantly reduces phagocytic uptake of the microspheres. Previously, it has been shown that DPPC improves the performance of PLGA microsphere formulations with respect to flow properties as a dry

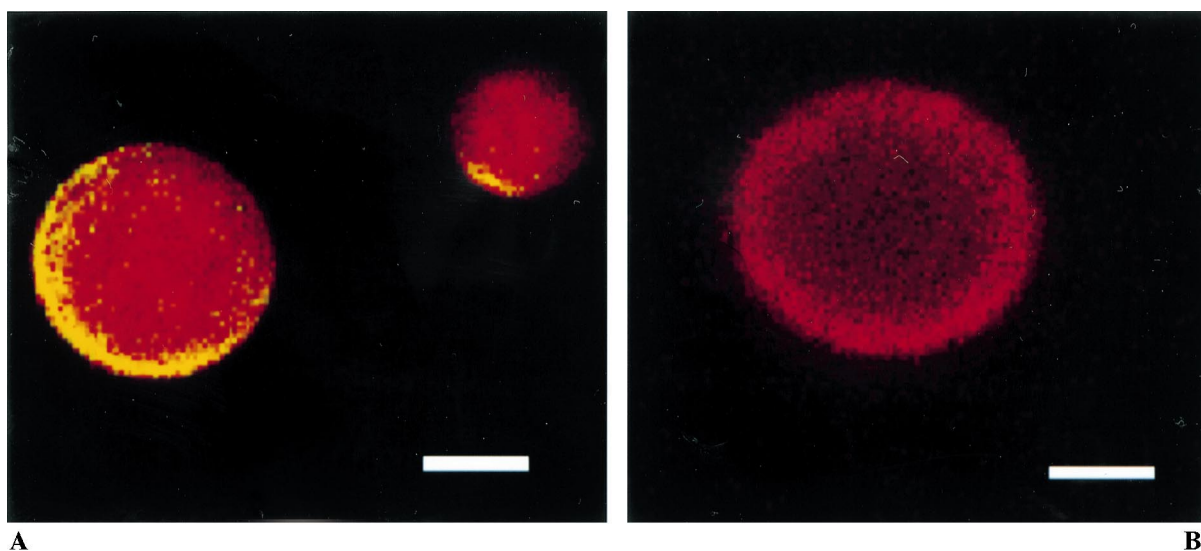


Fig. 5. Fluorescent confocal micrographs of peroxidase-containing PLGA particles (red) without (A) or with DPPC coating (B). Bar represents 2 microns.

powder [16] and it has also been suggested that it enhances pulmonary absorption of peptides and proteins [28]. 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.

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