Influence of Surface Properties at Biodegradable Microsphere Surfaces: Effects on Plasma Protein Adsorption and Phagocytosis

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Objective. The objective of this work was to determine plasma protein adsorption and macrophage phagocytosis of biodegradable polyanhydride, polylactic acid and polylactic-co-glycolic acid microspheres prepared by both spray-drying and solvent evaporation techniques.
Methods. Microspheres were characterized by scanning electron microscopy (SEM), confocal laser microscopy, particle size distribution and zeta (ζ) potential determination. Plasma protein adsorption onto the microspheres was determined using a fluoroaldehyde reagent. Phagocytosis was evaluated by incubating microspheres containing the angiotensin II antagonist, L-158,809, with the macrophages in the presence or absence of the phagocytosis inhibitor cythochalasin D. The extent of phagocytosis was established by fluorescence determination of L-158,809 and by optical microscopy. The effect of amphiphilic poly(ethylene glycol) (PEG) derivatives on phagocytosis was determined using PEG-distearate incorporated into the microspheres.
Results. The average diameter of the microspheres, which depended on the polymer and the initial formulation, ranged from 0.9 to 3.2 micrometers. ζ potential studies showed strong negative values irrespective of the polymer used for the spray-dried formulations. The ζ potential was masked by the incorporation of PEG 400- or PEG 1,400-distearate in the formulation. Confocal laser microscopy showed a homogenous dispersion of PEG (measured as PEG-fluorescein) in the microspheres. Protein adsorption was not observed for any of the microsphere formulations following incubation with bovine serum. Incubation of microspheres with murine macrophages showed that PEG-distearate inhibited phagocytosis at appropriate levels (0,1% w/w). Higher levels >1% w/w of PEG-distearate) resulted in enhanced association with macrophages, despite the presence of the phagocytosis inhibitor cytochalasin D, indicating fusion between the microspheres and the plasma membrane.
Conclusions. These results demonstrate that spray-dried PEG-containing microspheres can be manufactured and that an appropriate concentration of this excipient in microspheres results in decreased phagocytosis.

KEY WORDS: microspheres; plasma protein adsorption; phagocytosis; PEG.

During the last decade, many classes of polymers as homopolymers of lactic acid (PLA), glycolic acid and their polylactic-co-glycolic copolymers (PLGA), have been studied for controlled-delivery applications (1). Due to the biocompatible and biodegradable nature of these polymers, controlled-release systems have been developed (2). More recently, the use of polyanhydrides as matrices have been evaluated for drug delivery systems (3,4). Polymeric microspheres for drug delivery have been prepared using two principal methods: solvent evaporation (5,6) and spray-drying (7–11). The solvent evaporation method requires the use of an emulsifying surfactant such as polyvinyl alcohol (PVA) which has been reported to be carcinogenic (12,13). Methylene chloride, the common solvent used in the spray-drying method, is very easy to remove leaving residual solvent concentrations lower than the required 50 p.p.m. (11).

One of the most important problems for particulate carriers that are to be injected parenterally is their recognition by the cells of the reticuloendothelial system. Intravenous administration results in an immediate interaction of microspheres with plasma and blood components. The interaction can result in opsonization due to adsorption of proteins on the surface of the particles, followed by macrophage recognition and uptake (14). This phenomenon may be explained by several parameters such as serum opsonic factors (15), the size and the surface potential of the particles (16), and the composition of the particles (17). More recently, a number of studies have demonstrated the "stealth" behaviour of decreased uptake by the reticuloendothelial system following the coating of particles with poly(ethylene glycol) (18,19), poloxamer, poloxamine and poly(ethylene oxide) (20). This phenomenon can be observed by a decreased surface charge and zeta (ζ) potential (21,22).

In this study, the preparation of biodegradable polyanhydride, PLA and PLGA microspheres by both spray-drying and solvent evaporation techniques is reported. The spray-dried formulations were made with and without varying concentrations (0.1, 1 and 10% w/w) of poly(ethylene glycol)(PEG) 400- and 1,400-distearate. The microspheres were characterized by scanning electron microscopy (SEM), confocal laser microscopy, photon correlation spectroscopy and ζ potential. Plasma protein adsorption and phagocytosis have been correlated with the presence of amphiphilic PEG derivatives in the formulation.

MATERIALS AND METHODS

Materials

The synthesis of D,L-PLA was carried out as previously described (23) with slight modification. Briefly, D,L-dilactide from Aldrich, (Milwaukee, U.S.A.) was synthesized by a ring opening method using tetraphenyltin as a catalyst under anhydrous and high vacuum conditions. The crude reaction product was purified by precipitation of PLA in water after dissolution in acetone. The PLA was then dried under vacuum over phosphorous pentoxide for 2–3 days. Poly(carboxyphenoxy-propane-*co*-sebacic) acid (CPP-SA)(20/80) (polyanhydride) was synthesized following the method described by Langer *et al.* (6).

PLGA (RG503) was obtained from Boreinger (Ingelheim, Germany). PEG 400-distearate and fluorescein isothiocyanate (FITC) were obtained from Aldrich (Milwaukee, U.S.A.). PEG 1,400-distearate was purchased from Calgene Chemicals (Skokie, Il., U.S.A.). Methoxy-PEG-5,000-NH_2 was obtained from Shearwaters Polymers Inc. (Huntsville, Al. U.S.A.).

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Mouse IgG2a(UPC 10) and IgG2b(MOPC 141) were purified immunoglobulins (solution in 0.02 M Tris buffered saline, pH 8.0 containing 0.02% sodium azide). Poly-L-arginine hydrochloride and thin layer chromatography plates (polyester silica gel) were obtained from Sigma (St-Louis, MO., U.S.A.). L-158,809 was obtained from Merck Frosst (Kirkland, Canada).

The Bio-Rad protein assay was purchased from Bio-Rad (Richmond, CA., U.S.A.). The O-phtalaldehyde reagent solution was obtained from Pierce (Rockford, Il., U.S.A.). Bovine serum albumin (fraction V) was obtained from Boehringer (Mannheim, Germany).

Characterization of PLA

The molecular weights of the PLA samples were determined by gel permeation chromatography (GPC) using a Waters 717 Autosampler coupled to a Waters™ 600E System Controller and a Waters 410 Differential Refractometer. The columns used were Ultrastyragel 10^3 Å and 10^4 Å. Chloroform from Fischer (Neapan, Ontario, Can.) was used as the mobile phase at a flow rate of 1 ml/min.

Preparation of Microspheres

Microspheres of polyanhydride (MW 24,000), PLA (MW 82,000), and PLGA (MW 70,000) were prepared using the following procedures:

Spray-dried microspheres: a methylene chloride solution containing 1% w/v of polymer was prepared. For the samples containing PEG-distearate, 0.1%, 1% and 10% w/w of PEG 400- or PEG 1,400-distearate were added to the methylene chloride solution. For the samples containing L-158,809, 10% w/w of the compound was added to the methylene chloride solution. The sample was then spray-dried with a Büchi Mini Spray Dryer-Model 190 (Büchi, Flawil, Switzerland) using a 0.5 mm nozzle. The process parameters were as follows: 58–60°C inlet air temperature; 36–38°C outlet air temperature; aspirator control 10; pump control 10 (245 ml/h); 600Nl/h air flow.

Microspheres made by solvent evaporation: a methylene chloride solution containing 5% (w/v) of polymer was emulsified with an aqueous phase containing 0.27% (w/v) of polyvinyl alcohol. The emulsion was stirred overnight and then washed 3 times with 250 ml of distilled water. The microspheres were collected following centrifugation, 3,000 rpm for 15 minutes, and lyophilized.

Particle Morphology

Morphological evaluation of the microspheres were made by scanning electron microscopy (JSM 820 JEOL). The microspheres were attached to the specimen holder with a double-coated adhesive tape and coated for 3 minutes at 40 mA with a layer of gold (Coating unit: Polaron E5100).

Particle Size Measurement and ζ Potential

The mean particle size of the samples was determined using photon correlation spectroscopy (N4 Plus, Coulter Electronics Inc, Hialeah, FL). Microspheres were ultrasonicated (Branson 3210 Ultrasonic bath) for 30 seconds, and were diluted with phosphate buffer (pH 7.4) to give a particle count rate between 5×10^4 and 1×10^6 counts per seconds. The mean particle diameter was calculated, in size distribution processor mode (SDP), using the following conditions: 1.33 fluid refractive index; 20°C temperature; 0.93 centipoise viscosity; 90.0° angle of measurement; 10.5 ms sample time, and sample run time of 60 seconds.

The particle ζ potential was measured using a Delsa 440SX (Coulter Electronics Inc, Hialeah, FL) at the following conditions: 0.7 mA current; 500 Hz frequency range; 20°C temperature; 1.33 fluid refractive index; 0.93 centipoise viscosity; 78.3 dielectric constant; 16.7 ms/cm conductivity; on time of 2.5 s, off time of 0.5 s, and a sample run time of 60 seconds.

PEG-Fluorescein Synthesis

PEG-Fluorescein was synthesized by adding a tetrahydrofurane solution containing 0.8% (w/v) of fluorescein isothiocyanate to a tetrahydrofurane solution containing 1.7% (w/v) methoxypoly(ethylene glycol)-amine (MeO-PEG-5,000-NH_2). The solution was stirred for 1 hour at 25°C. Confirmation of PEG-fluorescein synthesis was carried-out using thin layer chromatography (TLC). The TLC plates were developed using chloroform/methanol (9:1) and the presence of only one spot indicated that all the FITC was conjugated with the PEG-5,000-NH_2.

Incubation with Plasma Protein

Free L-158,809 microspheres (10% w/v) were incubated with 2 ml bovine serum for 2 hours at 37°C. The preparation was then centrifuged for 10 min at 3,000 r.p.m.. The supernatant was removed and the microspheres resuspended in 2 ml of phosphate buffer pH 7.4. The centrifugation washing step was repeated 10 times. The final suspension was analyzed spectrophotometrically at 595 nm with a Hewlett-Packard 8452A diode array spectrophotometer for adsorbed proteins using the Bio-Rad protein assay (minimum sensivity = 1 μg/ml). The sensitivity of detection was increased by using the O-Phtalaldehyde (fluoroaldehyde) reagent solution, which has a minimum sensitivity lower than 50 ng/ml. Bovine serum albumin (fraction V) was used as a standard for the two tests.

Incubation of Microspheres with Cationic Poly-L-arginine Hydrochloride and IgG

Free L-158,809 PLA and polyanhydride microspheres 1% (w/v) were incubated for 2 hours in 5 ml of two cationic solutions. The first solution consisted of 10% (w/v) poly-L-arginine hydrochloride pH 7.4, and the second consisted of 10% (w/v) IgG pH 5.2. The same procedure as that used for the adsorption of plasma proteins was followed, except that the Bio-Rad reagent was sufficient to evaluate the level of poly-L-arginine hydrochloride and IgG adsorption.

Phagocytosis Assay

Macrophages were obtained by intraperitoneal (I.P.) injection of female CD1 mice (Charles River, St-Constant, Canada) with 1.5 ml sterile Brewer's thioglycollate broth (Difco, Illinois, U. S. A.). The peritoneal exudate (>85%

macrophages) was harvested 4 days later and washed by centrifugation in Hank's balanced salt solution (HBSS). It was then seeded in 6-well flat-bottom micro plates at 1.0×10^6 macrophages/well in RPMI-1640 containing 10% heat-inactivated fetal calf serum (FCS), 50 mg/ml gentamycin sulfate and 20 mM of HEPES (all from Gibco Life Science, Burlington, Canada). The cells were allowed to adhere for 18 hours at 4°C (no phagocytosis at this temperature) and at 37°C in an atmosphere of 5% CO_2 after which non-adherent cells were removed by gentle washing with warm medium. The macrophage monolayer was incubated in the presence or absence of the phagocytosis inhibitor cytochalasin D (Sigma, St-Louis, U.S.A.) with: 1) 1,2-dioleylphosphatidylethanolamine(DOPE) and 1,2-dioleyl-3-trimethylammonium propane(DOTAP) liposomes (DOPE-DOTAP as a positive standard) containing the fluorescence phospholipid derivative NBD-PE (Avanti Polar Lipids, Alabasser,Al.); 2) with free L-158,809 (as a negative standard); and 3) PLA microspheres containing 0.1 or 1%(w/w) PEG 400-distearate, and 10% (w/w) L-158,809. The monolayer was washed extensively with warm medium. Total phagocytosis was determined by incubating the cells with 1.0% v/v Triton X-100 in water to induce lysis, followed by fluorescence (excitation :460 nm, emission :535 nm) determination of L-158,809.

In Vitro Cytotoxicity Assay

Macrophage (1.0×10^5 cells/well in a volume of 200 µl medium) were placed in 96-well tissue culture plates and incubated with; 1) PLA microspheres; 2) free L-158,809 (0.01 mg/ml 0.2 mg/ml and 10 mg/ml); and 3) PLA microspheres containing both 10% (w/w) PEG 400-distearate and L-158,809 at 37°C, 5% CO_2 for 24 hours. Lactate dehydrogenase (LDH) activity in the medium, used as an indicator of cell death, was determined by means of a commercial kit (Sigma Chemical Co.). Total LDH activity was determined by incubating the cells with 1.0% v/v Triton X-100 in water to induce lysis, followed by vigorous agitation.

RESULTS AND DISCUSSION

Microspheres made with polyanhydride, PLA, and PLGA were analyzed by photocorrelation spectroscopy for their size distribution. Table I shows the size distribution of these microspheres. The largest average diameter observed was 3.3 µm for the spray-dried PLGA microspheres containing 10% PEG 400-distearate. This indicates that all of these microsphere formulations may be used for an intravenous injection. Furthermore, it was shown that the mean diameters were smaller for the polyanhydride microspheres irrespective of the method of preparation used. This could be attributed to the microsphere's low molecular weight and lower viscosity, which results in fine dispersions. In addition, the small standard deviations observed for all of the microsphere formulations confirm the presence of only one population size. Scanning electron microphotographs of polyanhydride and PLA microspheres, Figure 1a and 1b respectively, showed very spherical particles with a smooth surface as no visible macropore could be observed. The composition of the microspheres did not affect their morphology.

The ζ potential of the different microsphere formulations is reported in Table II. The ζ potential was found to be highly negative for microspheres made with pure polymers by the spray-drying process. The significant variation in ζ potential (−13 to −46 mV) can be explained partly by the different particle sizes and the polymer polydispersity which affects the number of end carboxylate groups. The highly negative ζ potential could be due to the polymer composition and more specifically to the bond implicated in the backbone of the polyanhydride, polylactic-co-glycolic acid and poly(D,L)-lactic acid monomers. The free electron doublets, especially in the anhydride bond, are not alone in contributing to a negative ζ potential for these polymers as there are large regions of backbone that contain phenyl groups, ether bonds and anhydride bonds. As a consequence, all the π bonds and oxygen doublets give this region a high polarity. The acetyl group is not implicated as a charge. The charge in PLA and PLGA was observed to be less negative. This could be due to both the ester bonds and the free terminal carboxylic acid groups. It should be kept in mind that the ζ potential is not necessarily due to an ionic charge but can also be due to a charge generated by brownian motion of the particle. Moreover, it is interesting to note in Table II that the ζ potential decreases for spray-dried microspheres containing any PEG distearate and for the samples made by solvent evaporation. In a previous work (24), we had assumed that during the spray-drying process, the PEG-distearate was well dispersed on and in the microspheres. The confocal microphotographs shown in Figure 2 now confirm this

Table I. Size Distribution of Biodegradable Microspheres

Polymer	Microspheres	Size (nm) ± S.D.
Polyanhydride	Spray-dried	580.9 ± 96.7
	Spray-dried + 10% PEG 400(C_{18})$_2$	1051.1 ± 231.4
	Spray-dried + 10% PEG 1,400(C_{18})$_2$	1288 ± 451.1
	Solvent evaporation	2910.1 ± 1036.0
PLGA	Spray-dried	899.6 ± 201.6
	Spray-dried + 10% PEG 400(C_{18})$_2$	3230.1 ± 1512.1
	Spray-dried + 10% PEG 1,400(C_{18})$_2$	892.9 ± 231.5
	Solvent evaporation	1104.2 ± 318.0
PLA	Spray-dried	1809.8 ± 259.2
	Spray-dried + 10% PEG 400(C_{18})$_2$	894.3 ± 224.8
	Spray-dried + 10% PEG 1,400(C_{18})$_2$	1481.7 ± 459.7
	Solvent evaporation	1464.2 ± 458.2

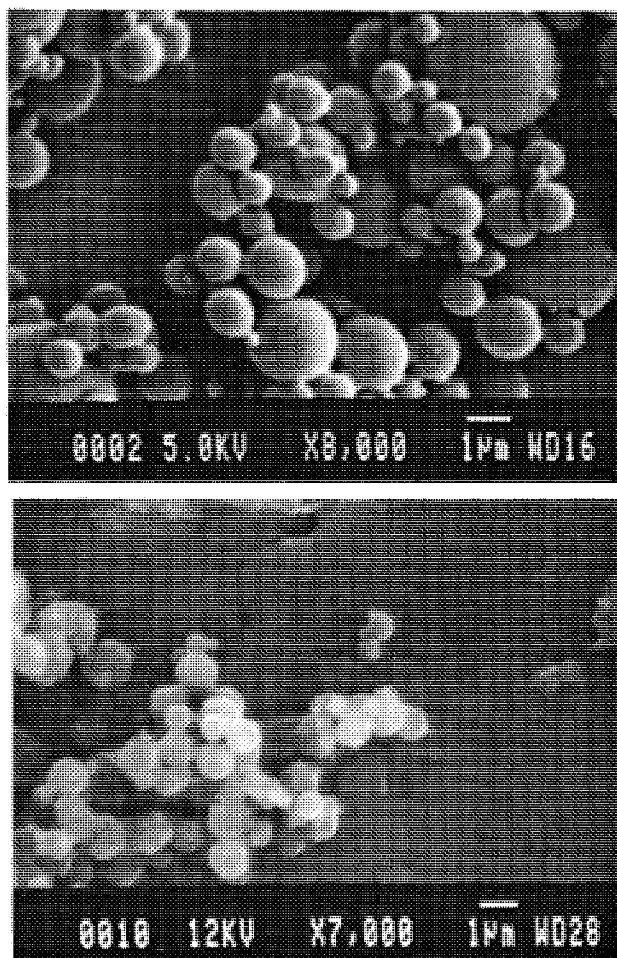

Fig. 1. (a) Scanning electron micrograph of spray-dried PLA microspheres. (b) Scanning electron micrograph of spray-dried polyanhydride microspheres.

assumption. Indeed, with 0.5% and 10% (w/w) PEG in the initial formulation, it is shown that the PEG (measured as PEG-fluorescein) is homogeneously dispersed in the microsphere. Due to its molecular conformation, the PEG-distearate can occupy the region of the electrical double layer at the surface of the micro-

Table II. Zeta Potentials (mV) of Biodegradable Microspheres

Microspheres	Zeta (mV) ± S.D.
PLA spray-dried	−21.25 ± 4.16
PLGA spray-dried	−13.34 ± 3.18
Polyanhydride spray-dried	−46.47 ± 1.36
PLA spray-dried + 10% PEG 400(C_{18})$_2$	−0.37 ± 0.02
PLGA spray-dried + 10% PEG 400(C_{18})$_2$	−0.44 ± 0.03
Polyanhydride spray-dried + 10% PEG 400(C_{18})$_2$	−1.01 ± 0.20
PLA spray-dried + 10% PEG 1,400(C_{18})$_2$	0.07 ± 0.57
PLGA spray-dried + 10% PEG 1,400(C_{18})$_2$	0.25 ± 0.19
Polyanhydride spray-dried + 10% PEG 1,400(C_{18})$_2$	−0.01 ± 0.04
PLA solvent evaporation.	−0.27 ± 0.05
PLGA solvent evaporation	−0.37 ± 0.01
Polyanhydride solvent evaporation	−0.33 ± 0.01

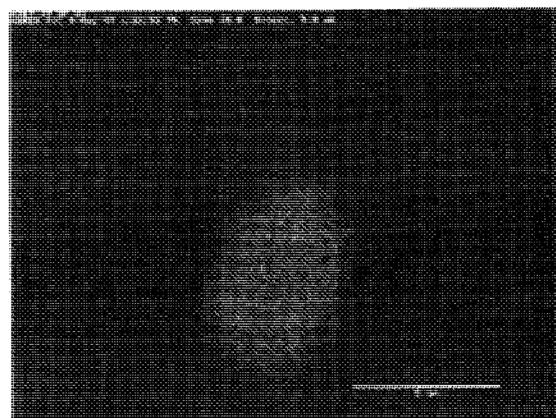

Fig. 2. Confocal laser-scanning micrograph of PEG-Fluorescein microsphere.

sphere, and as a result, a neutral ζ potential is observed. The same phenomenon is displayed with microspheres made by the solvent evaporation technique as residual polivinyl alcohol (PVA) is still present at the surface even after 3 washes. This last hypothesis was demonstrated by using the X-Ray-Photoelectron Spectroscopy XPS technique (25).

Taking into account the sensitivity (50 ng/ml) of the assay, no trace of protein adsorption was found for any of the microspheres incubated in plasma using the Bio-Rad reagent or the O-phtalaldehyde reagent. Furthermore, the ζ potential of spray-dried microspheres without PEG-distearate after incubation with plasma was unchanged (data not shown) supporting the hypothesis that no protein or peptides were adsorbed. Due to the strong negative ζ potential of the spray-dried microspheres, the stealth role of PEG on the surface of particulate carriers, and the electronegativity of serum proteins, a positive control was made with poly-L-arginine hydrochloride. This linear polypeptide was chosen as a control because of its strong positive charge and its particular secondary structure (26). As soon as the spray-dried microspheres, without PEG-distearate, were exposed to the polypeptide solution, there was an instantaneous precipitation. Table III shows the amount of poly-L-arginine

Table III. Concentration of Poly-L-arginine Adsorbed on Biodegradable Microspheres

Microspheres	μg poly-L-arginine/mg microspheres ± S.D.
Polyanhydride spray-dried	166.4 ± 9.8
Polyanhydride spray-dried + PEG 400 (C_{18})$_2$	41.1 ± 1.3
Polyanhydride + PEG 1,400 (C_{18})$_2$	39.0 ± 1.3
Polyanhydride solvent evaporation	120.2 ± 9.1
PLA spray-dried	70.2 ± 5.2
PLA spray-dried + PEG 400 (C_{18})$_2$	18.4 ± 0.7
PLA spray-dried + PEG 1,400 (C_{18})$_2$	0
PLA solvent evaporation	51.3 ± 1.5
PLGA spray-dried	91.6 ± 1.4
PLGA spray-dried + PEG 400 (C_{18})$_2$	10.4 ± 0.4
PLGA spray-dried + PEG 1,400(C_{18})$_2$	0
PLGA solvent evaporation	71.6 ± 8.1

hydrochloride adsorbed on the microspheres. These concentrations were similar after one, three and ten washes which confirms the fact that poly-L-arginine is well adsorbed on microspheres made by spray-drying or solvent evaporation. Indeed, the Bio-Rad assay demonstrated the presence of adsorbed poly-L-arginine on all three types of microspheres.

The sixteen-fold higher concentration of adsorbed cationic protein found on the polyanhydride microspheres compared to PLA and PLGA microspheres may be due to several factors. The first factor involves the potentially strong interaction between poly-L-arginine and the polyanhydride microspheres due to their distinct chemical properties. Polyanhydride microspheres have the strongest negative zeta potential (before and after incubation) because of the negative surface charge and the high number of anhydride radicals that contain a significant number of delocalized electrons. On the other hand, poly-L-arginine is positively charged as arginine is a positive component of the histonic nucleoprotein on which negatively charged DNA is coiled. Furthermore, polyanhydride is made of sebacic acid ($C_{10}H_{18}O_4$) which is more hydrophobic than lactic acid ($C_3H_6O_3$) and glycolic acid ($C_2H_4O_3$). Poly-L-arginine is also a homopolymer of arginine ($C_6H_{14}N_4O_2$). A second factor explaining adsorption is the possible hydrophobic interaction of the aliphatic carbon skeleton of the polyanhydride with the poly-L-arginine. Thirdly, the specific area of the polyanhydride microspheres is larger than PLA or PLGA microspheres because of the smaller microsphere size. The high specific area of these microspheres could also explain the higher level of poly-L-arginine adsorption. Finally, ζ potential measurements taken after incubation with polyarginine showed neutral values (data not shown) for the three kinds of microspheres, thus confirming adsorption.

The amount of poly-L-arginine adsorbed on spray-dried PEG-coated microspheres or made by solvent evaporation is lower because of the presence of PVA and PEG 400 or PEG 1,400-distearate which, in principle, may form a hydrophilic, hydrated steric barrier on the particle surface. Because the PEG is conjugated with distearate, PEG on the surface may be represented like hair-pins. In addition, there would be a better steric protection with PEG 1,400 than with the PEG 400 because of a greater swelling of the poly(ethylene glycol). This hypothesis is supported by data shown in Table III where it is evident that there is less poly-L-arginine adsorbed on microspheres made with PEG 1,400-distearate in comparison with those made with PEG 400-distearate. The linear nature of the poly-L-arginine molecule may result in its interaction with the microsphere surface by interdigitating between the PEG 400 arms, but not between the longer PEG 1,400 arms. To validate this assumption, microspheres made of PEG 400 and 1,400-distearate were incubated with IgG, a globular protein with a pI of 6.8 that is positively charged at pH 5.2. No IgG was found adsorbed on the surface of PEG-containing microspheres (data not shown), even with the PEG 400-distearate, indicating that the steric barrier conferred by PEG 400-distearate is sufficient to provide protection against the globular conformation of the IgG. These results are in contrast to previous work. Privitera *et al.* (21), and Norman *et al.* (27) found significant plasma protein adsorption on the surface of polystyrene microspheres that had a strong negative ζ potential. There are however, two major differences in their protocol in comparison with ours. The first is that their ζ potential was evaluated using electrophoretic mobilities that give empirical results. The second is in their evaluation of plasma protein adsorption. After incubation at an unspecified temperature, their microspheres were washed with water. Since the study was not conducted in optimal conditions, proper pH and ionic strength, serum protein precipitation may have occurred due to protein denaturation or conformational change, consequently affecting hydrophobic interactions. Chonn *et al.* (28), and Devine *et al.* (29) found plasma proteins adsorbed on liposomes, particularly complement factor C3. This interaction required the inclusion of phospholipids bearing a net charge, and was estimated in a semi-qualitative method using ELISA.

No LDH activity was observed at any of the doses tested, indicating a lack of *in vitro* cytotoxicity of the drug, the carrier, and the polymer (data not shown). Table 4 shows the results of phagocytosis, evaluated by fluorescent determination of L-158,809. Fluorescence evaluation of free macrophages showed that no fluorescence could be attributed to any macrophage component. The fluorescence evaluation with free L-158,809, as a negative control, showed that there was no cell-association, thus confirming the fact that the L-158,809 is a non-permeable compound. The DOPE/DOTAP liposomes were used as a positive standard and results in Table 4 show that there was an association between the positively charged carrier and the macrophages. In contrast, the addition of PEG 2,000 in the liposome formulation decreased this association. Figure 3a, 3b, and Table IV illustrate that in the absence of PEG, there was an association between the microspheres and the macrophages. This association was inhibited by cytochalasin D. The presence of PEG-distearate at 0.1% (w/w) in the microspheres inhibited this association. At a PEG concentration of 1% (w/w), there was an association with macrophages regardless of the presence of cytochalasin D. These

Table IV. Fluorescent Evaluation of Phagocytosis Level

Samples	Microspheres phagocytosis rate (nmol + S.D./5.10^5 cell/4h.)	
	with cytochalasin D	without cytochalasin D
Free L-158,809	0	0
PLA + 0.1% PEG 400 $(C_{18})_2$	2.2 ± 0.9	3.1 ± 1.1
PLA + 0.1% PEG 1,400 $(C_{18})_2$	1.9 ± 1.0	2.1 ± 1.3
PLA + 1% PEG 400 $(C_{18})_2$	53.7 ± 10.2	60.2 ± 11.3
PLA + 1% PEG 1,400 $(C_{18})_2$	55.9 ± 13.5	57.9 ± 15.2
DOPE/DOTAP	—	28.1 ± 6.1
DOPE/DOTAP + DPPE-PEG_{2000}	—	1.2 ± 0.6

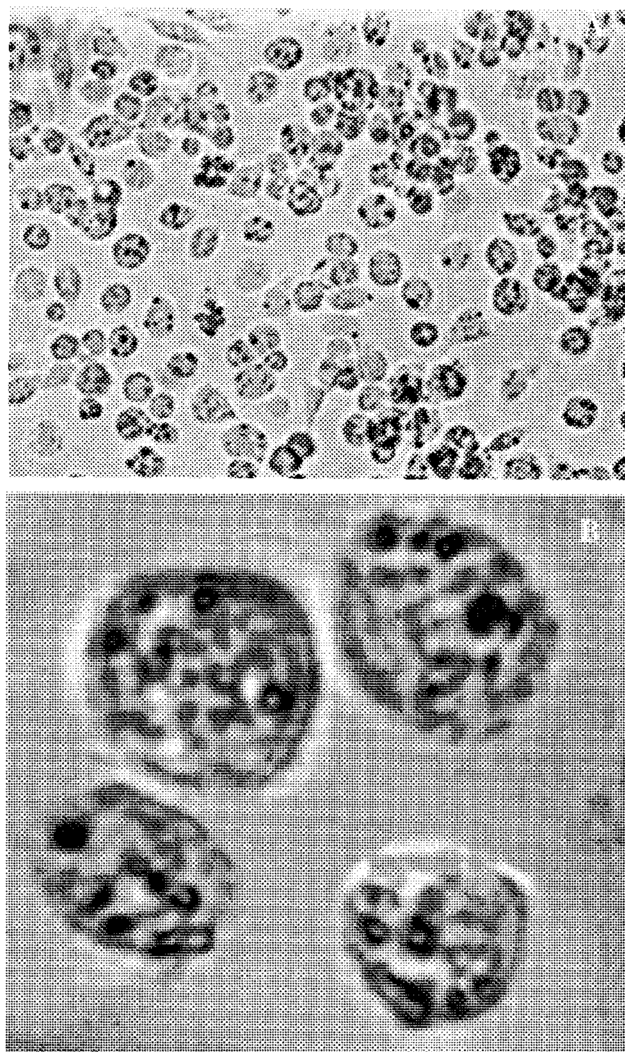

Fig. 3a,b. Optical micrograph of phagocytosed microspheres.

observations indicate that high levels of PEG-distearate at the surface of the microspheres may induce fusion with the macrophage plasma membrane. Hence, in view of the above findings, it may be concluded that the concentration of PEG-distearate in the microspheres appears to be critical in governing the association of these microspheres with the macrophages. At levels of less than 1% (w/w), PEG-distearate is effective in inhibiting phagocytosis. Despite the presence of cytochalasin D however, greater levels (1% w/w) of PEG-distearate induce fusion of the microspheres with plasma membranes.

CONCLUSIONS

In this study, we have evaluated the correlation between microsphere ζ potential, plasma protein adsorption and macrophage phagocytosis after manufacturing spray-dried microspheres with appropriate concentrations of PEG-distearate in their formulations. PEG-conjugates have been used in liposomes to decrease liposome-protein and liposome-macrophage interaction (19,22). The results of the present studies demonstrate that the expression of PEG-distearate at the surface of the microspheres masks their negative ζ potential and also decreases their interaction with phagocytic macrophages.

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