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Poly-(D,L-Lactic Acid) Microspheres Incorporating Histological Dyes for Intra-pulmonary Histopathological Investigations

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Abstract

Polylactic acid (PLA) microspheres incorporating fluorescein as a histological marker have been prepared and used as a model for the testing of inhaled PLA microspheres (2–5 μm) in the lung. PLA microspheres (20 mg) were delivered to rabbits in the form of a saline nebulization.

The distribution pattern within the pulmonary system showed that the fluorescein-labelled microspheres were distributed about the four lobes in discrete groups. The comparative numbers of these groups showed a trend towards a reduced concentration in the lower lobes. Subsequent histological examination revealed that the microsphere-treated lungs had been significantly damaged after 24 h.

Histological damage was assessed in terms of pulmonary haemorrhage, eosinophilia and neutrophil infiltration.

Drug delivery via the respiratory tract presents a viable alternative to parenteral and oral administration. Problems of poor absorption through the intestinal wall may be overcome by the intra-pulmonary route as the alveoli are in close contact with the blood capillaries. Additionally, a drug may be absorbed via aqueous pores, carrier-mediated transport or phagocytosis (Shanker 1978; Snipes et al 1989). The problem of hepatic first-pass metabolism is also avoided by the pulmonary route of administration.

The site in the lungs where the drug is deposited and the efficiency with which it is delivered depend upon the size of the particles (Gouda 1981) and their shape (Wong et al 1989). Larger particles do not pass beyond the upper airways, but particles of 3 μm or less are deposited in the lower airways by sedimentation. However, previous studies (Newman et al 1982; Zainudin et al 1989; Philips et al 1990) have reported that only 10% of an actual dose reaches the lung, and some of this is inevitably lost on exhalation. Moreover, the retention of the particles in the airways will be determined by the efficiency of the host-defence mechanism in clearing them (Hallworth & Malton 1984). Nevertheless, aerosols have been used in the successful treatment of inflammatory disorders such as asthma.

It is our intention to treat inflammatory lung disorders using drug-loaded poly-(lactic acid) (PLA) microspheres which can be given by nebulization. The microspheres will biodegrade and release the drug in a controlled manner. However, such a system becomes a viable alternative to other dosage forms only if the physical presence of the system produces minimal unwanted effects. Thus it must have no toxicity or, at least, a level of toxicity which is preferable to drug delivery systems presently in use. The toxicity of PLA has been investigated previously (Sjoholm & Edman 1979) and, amongst others, Schwope et al (1976) reported that PLA implants were free of any harmful tissue reaction. The extrapolation of this conclusion to PLA microspheres, particularly at a size below 10 μm, is difficult. It is necessary, therefore, to use histological techniques to view any possible damage to lung tissue following administration of the microspheres.

Colloidal gold is probably the most popular cytochemical marker system in use and a previous study (Armstrong et al 1993) has reported the possibility of its incorporation into PLA microspheres. However, successful incorporation is difficult and unpredictable and, therefore, an alternative may be found in the use of fluorescent dyes. Fluorescein and related compounds are widely used as markers or tracers in medicine. Cohen et al (1991) have investigated the use of PLA for long-term delivery of water-soluble proteins and prepared polymers incorporating fluorescein isothiocyanate-labelled bovine serum albumin. Fluorescein has also been incorporated into PLA microspheres to study the clearance of the dye from the eye, to study adverse reactions (Khoobehi et al 1992) and to determine phagocytosis in retinal glial cells (Mano & Puro 1990). Hence it may be possible to adopt a similar approach and incorporate fluorescein and other histological dyes into PLA microspheres to monitor the distribution and histopathological effects of the microspheres, following intra-pulmonary delivery.

Materials and Methods

Materials

Polyvinyl alcohol of molecular weight 14000, fluorescein, malachite green, eosin Y, methylene blue and silica gel were

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supplied by the Aldrich Chemical Co., Gillingham, Dorset, UK. Dichloromethane, acetone, sodium dihydrogen orthophosphate dihydrate, disodium hydrogen orthophosphate, sodium chloride were supplied by BDH, Poole, UK. Hypnorm was obtained from Janssen Pharmaceuticals Ltd, Oxford, UK. PLA molecular weight 27000, was prepared in this laboratory. All reagents used were of Analar grade or equivalent.

Dye compatibility studies

Because of the techniques involved in the microsphere preparation, and the nature of the histological studies, the dye used must fulfil the following criteria: it must be compatible with the polymeric material; it must be sufficiently soluble in the organic solvent used in the microsphere production; it must be sufficiently soluble in water to allow efficient washing of the microspheres to remove extra-microspherical dye; and it must exhibit minimal toxicity. The following dyes were selected for examination to determine their suitability for incorporation into PLA microspheres: eosin Y, malachite green, methylene blue and fluorescein.

All dyes were compatible with PLA and sufficiently soluble in water to allow suitable washing procedures. Eosin Y was insoluble in dichloromethane and was, therefore, rejected. Malachite green was reported as toxic to lung tissue and was not investigated further. The remaining dyes, methylene blue and fluorescein, were reported to exhibit minimal toxicity when used as biological markers. Fluorescein, however, is so powerful a fluorescing agent that it can be detected in a dilution of 1 part in 4×10^7, thus allowing smaller quantities to be used to achieve the desired effect. For these reasons fluorescein was selected for use in all further experiments.

Preparation of microspheres

Microspheres were prepared by variations of the following general method: 250 mL 0·24% w/v aqueous polyvinyl alcohol and 7 mL dichloromethane containing PLA and fluorescein (total weight 1·0 g) were emulsified for 2 h at room temperature (21°C), using a Silverson Laboratory Mixer Emulsifier operating at 300 rev min^{-1}. The mixture was allowed to stand overnight and the microspheres collected by centrifugation, washed with distilled water and dried under vacuum over silica gel.

Visualization of microspheres

Microspheres were coated by a sputter-coating technique, employing gold as the coating material, and photographed using a Jeol JSM-840 scanning electron microscope. Microspheres were also examined by light microscopy using a Vickers M41 photoplan light microscope.

Particle size determination

Microspheres were sized using a Coulter Electronics Ltd, Coulter Counter, model TAII.

Determination of fluorescein content of microspheres

Samples (100 mg) of fluorescein-PLA microspheres were dissolved in 5 mL acetone and the UV absorbance of the resulting solutions at 496 nm, using acetone as reference, was used to determine the quantity of fluorescein in the samples.

Stability of fluorescein-PLA microspheres in physiological buffer

PLA microspheres (100 mg) containing 15% w/w theoretical loading of fluorescein were placed in a dialysis bag and immersed in 100 mL phosphate/saline buffer (pH 7·4), which was maintained at 37°C using a Gallenkamp Orbital Incubator, operating at 100 rev min^{-1}. Aliquots (4 mL) of the buffer solution were removed every 24 h and examined for the presence of fluorescein using UV spectroscopy at 496 nm and phosphate/saline buffer as reference. The 4-mL aliquots were replaced by 4 mL buffer solution to maintain sink conditions.

Stability of fluorescein-PLA microspheres in liquid nitrogen

PLA microspheres (100 mg) containing 15% w/w theoretical loading of fluorescein were examined by scanning electron microscopy (SEM). The microspheres were then immersed in liquid nitrogen at −70°C for 7 days and re-examined using SEM to determine whether any defects occurred in the structures as a result of this process.

In-vivo studies

Experiments were performed on fifteen Californian rabbits (2–3·5 kg). Rabbits were predosed with Hypnorm (0·3 mL kg^{-1} intramuscularly) and allocated into one of three experimental groups. The first group inhaled PLA microspheres (20 mg) containing 15% w/w theoretical loading of fluorescein, suspended in 4 mL 0·15 M saline. A second group of animals inhaled unlabelled PLA microspheres (20 mg in 4 mL 0·15 M saline). The third group was dosed with 4 mL 0·15 M saline only, as a control. Microspheres were delivered using a jet nebulizer (Medicaid Ltd) with 100% oxygen at a rate of 7 L min^{-1}. The approximate duration of the delivery period was 20 min. Twenty-four hours after administration the animals were killed, the lungs removed and fixed by immersing in liquid nitrogen before their storage at −70°C. Serial sections of 25-μm thickness were taken from each lobe for examination using a Vickers M41 photoplan light microscope.

Results and Discussion

Incorporation of fluorescein into PLA microspheres

The results indicate clearly that fluorescein-PLA microspheres of suitable size and quality were produced by this technique (Fig. 1). Table 1 details variations in the general production method. The use of a small volume of organic solvent produced microspheres of good quality with an appropriate size range (2–5 μm), whereas the size range increased when larger volumes were used. A small volume of organic solvent ensures the precipitation of all the solutes into the aqueous phase within a short period of time, thus allowing the production of microspheres of a similar size, hence a narrow size range. Small volumes of organic solvent also decrease the probability of any solvent remaining in the final product. Microsphere size was also affected by the amount of fluorescein present. When fluorescein was absent the size range of the microspheres is 7–10 μm, but under the

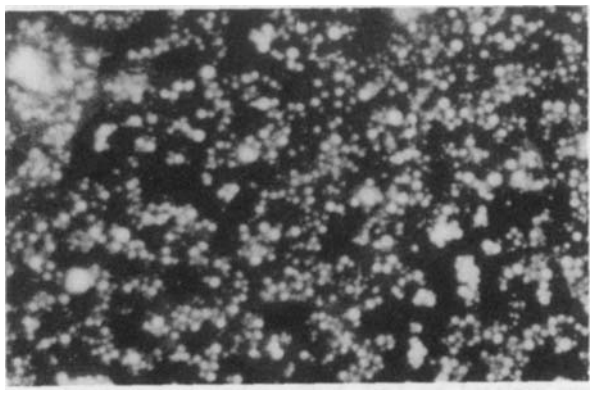

FIG. 1. Photograph of PLA-fluorescein microspheres as viewed using a Vickers M41 photoplan light microscope.

same conditions with a theoretical loading of 15% (w/w) fluorescein, the resultant size range is 2–5 μm. However, as the theoretical content of fluorescein was decreased, the size range tends towards 7–10 μm such that at the 7% theoretical loading there is no difference in size range between PLA microspheres containing the dye and those which did not (Table 1). The precipitation of the fluorescein into the aqueous phase as the solvent evaporates means that some of the dye becomes present as particulate matter, and it is probably the abrasiveness of these particles which result in the production of smaller microspheres.

It may be noted (Table 2) that there is a considerable difference between the theoretical and actual content of fluorescein in the microspheres. Because fluorescein is soluble in the organic phase, like the polymer, it is introduced into the aqueous phase by precipitation, and it is this co-precipitation which gives rise to a matrix formation in which the dye is uniformly dispersed. The results would indicate, however, that much of the fluorescein precipitated

Table 1. Effect of variations in microsphere production method on microsphere size range.

Volume of dichloromethane (mL)	Theoretical fluorescein content (%, w/w)	Microsphere size range (μm)
10	15·05	2–5
15	15·03	3–8
20	15·02	3–11
40	15·03	no spheres
10	13·02	2–5
15	13·03	3–9
20	13·02	3–11
40	13·01	no spheres
10	11·04	3–8
15	11·05	4–12
20	11·04	4–15
40	11·02	no spheres
10	9·07	6–9
15	9·06	5–12
20	9·07	5–16
40	9·05	no spheres
10	7·05	8–12
15	7·05	7–15
20	7·05	8–20
40	7·04	no spheres
10	1·12	8-12
15	1·14	7–16
20	1·16	7–22
40	1·13	no spheres

Table 2. Theoretical and actual fluorescein content of prepared microspheres.

Theoretical fluorescein content (%, w/w)	Actual fluorescein content (%, w w*)	Visible by light microscopy
15·07	0·36 ± 0·03	YES
13·04	0·32 ± 0·04	YES
11·06	0·26 ± 0·05	YES
9·04	0·17 ± 0·03	YES
7·05	0·11 ± 0·03	YES
1·12	0·00 ± 0·00	NO

*Mean ± s.e.m., n = 5.

is not incorporated into the PLA. There appears to exist a triphasic equilibrium involving the fluorescein, related to the solubility of the dye in the organic solvent and the polymer. This necessitates the presence of an excess of undissolved fluorescein in the system to facilitate incorporation of the dye into the polymer matrix, as attempts to produce microspheres with a low theoretical loading of 1% led to little or no incorporation of the dye. Decreasing the theoretical content resulted in a decrease in actual content, even though there was still an excess of fluorescein present in the system. The amount of undissolved fluorescein would appear to be critical as the quantity incorporated was always greater when larger volumes of organic solvent were used.

Stability of microspheres

No leaching of fluorescein from the microspheres was detectable when stored for 7 days in either physiological buffer (pH 7·4) or liquid nitrogen at −70 C. There was no evidence of any change in the shape or surface characteristics of the microspheres during these studies. The fluorescein-PLA microspheres should, therefore, remain intact during the period of the in-vivo study. This is a requirement if the labelled microspheres are to be used to examine distribution and tissue damage within the lungs.

In-vivo distribution studies

Fluorescence microscopy revealed that fluorescein-labelled microspheres were distributed throughout all four lung lobes in each of the rabbits (Fig. 2). A comparison of the number of microspheres contained in each of the four lung lobes is given in Table 3. Whilst there was no significant

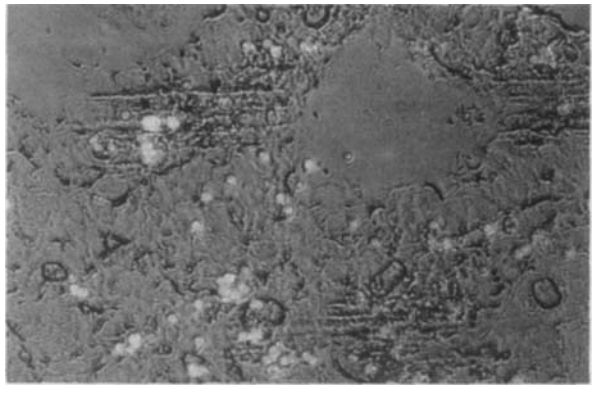

FIG. 2. Photograph of PLA-fluorescein microspheres located in rabbit lung tissue as viewed using fluorescence microscopy.

Table 3. Mean numbers of PLA microspheres in each of four lung lobes.

Lung lobe	Right upper	Left upper	Right lower	Left lower
Microspheres per mm^2 of lung tissue	357 ± 20	364 ± 21	307 ± 19	307 ± 41

Mean ± s.e.m., n = 6.

difference between the average number of microspheres per lung lobe, this should not be construed as evidence of an homogenous distribution of individual microspheres. Observations revealed that the larger microspheres (4–5 μm) were being deposited in the two upper lobes and that the smaller microspheres (2–3 μm) tended to penetrate into the two lower lobes. The microspheres were observed to cluster in discrete groups in the lung tissue and so were not evenly distributed. These observations are in accordance with published data relating to microsphere distribution in the lungs (Swift et al 1979; Kellaway & Farr 1990).

Histological studies

Histological examination of serial sections from the lung tissue, adjacent to that in which microspheres had been identified, produced evidence of inflammation. Pooled data from each lung lobe in each experimental group demonstrated inflammatory responses to both fluorescein-labelled and unlabelled PLA microspheres. There was a significant difference ($P < 0.05$) with respect to both neutrophil infiltration and the incidence of haemorrhage in each of the test groups, compared with the corresponding changes in rabbits treated with saline alone (Table 4). Although the number of eosinophils was greater in both of the test groups, the increases were not significantly different ($P > 0.05$) from those in saline-treated animals (Table 4).

However, analysis of non-pooled data (i.e. average data from individual lung lobes) revealed a significant difference ($P < 0.05$) between eosinophil counts in the left upper and left lower lobes of rabbits treated with fluorescein-labelled microspheres, compared with those receiving saline nebulization only (Table 5). Infiltration by neutrophils was significant in three of the four lung lobes when treated with labelled microspheres. Moreover, the unlabelled microspheres also evoked neutrophil infiltration in three lobes.

Significant haemorrhage was also observed in three lung lobes, the left upper and right lower lobes following administration of unlabelled microspheres, and in the right lower lobe following administration of labelled microspheres. There was little evidence of haemorrhage in the lungs of rabbits treated with saline only.

These results demonstrated that the microspheres were

Table 4. Comparison of histological changes evoked by fluorescein-labelled and unlabelled PLA microspheres with those evoked by saline alone, in the whole rabbit lung.

Treatment groups	Neutrophil infiltration (scored on a scale 0–5)	Haemorrhage (scored on a scale 0–5)	Eosinophil infiltration (scored on a scale 0–5)
Labelled microsphere-treated animals	2·3 ± 0·1*	1·8 ± 0·2*	1·6 ± 0·1
Unlabelled microsphere-treated animals	2·4 ± 0·1*	2·0 ± 0·1*	1·3 ± 0·1
Saline-treated animals	1·7 ± 0·1	1·2 ± 0·1	1·1 ± 0·1

Mean ± s.e.m., n = 5 for all treatment groups. *$P < 0.05$ compared with the saline control group.

Table 5. Comparison of unpooled data from the histological analysis of each of the four lung lobes in the three experimental protocols.

Lung lobes	Right upper	Left upper	Right lower	Left lower
Neutrophil infiltration (scored as 0–5)				
Labelled microspheres	2·5 ± 0·2*	2·3 ± 0·2*	2·6 ± 0·3*	2·0 ± 0·2
Unlabelled microspheres	2·6 ± 0·2*	2·4 ± 0·1*	2·1 ± 0·1	2·4 ± 0·2*
Saline	1·8 ± 0·2	1·8 ± 0·2	1·7 ± 0·2	1·5 ± 0·2
Eosinophil infiltration (scored as 0–5)				
Labelled microspheres	1·6 ± 0·3	1·8 ± 0·2*	1·6 ± 0·2	1·6 ± 0·1*
Unlabelled microspheres	1·4 ± 0·1	1·3 ± 0·1	1·1 ± 0·1	1·3 ± 0·1*
Saline	1·2 ± -0·2	0·9 ± 0·1	1·3 ± 0·2	0·9 ± 0·1
Haemorrhage (scored as 0–5)				
Labelled microspheres	1·8 ± 0·3	1·7 ± 0·3	2·3 ± 0·3*	1·6 ± 0·3
Unlabelled microspheres	2·0 ± 0·2*	1·8 ± 0·2	1·9 ± 0·2	2·3 ± 0·2*
Saline	1·2 ± 0·2	1·3 ± 0·2	1·3 ± 0·3	1·2 ± 0·2

Mean ± s.e.m., n = 5 for all treatment groups. *$P < 0.05$ compared with saline control group.

not biologically inert and that they evoked a significant inflammatory response. They produced a significant influx of both neutrophils and eosinophils into the lung tissue adjacent to the sites of impacted microspheres. The time course of the infiltration (within 24 h) is commensurate with that of an acute inflammatory response. This response was not induced by the fluorescein label as there was no difference between the incidence of acute inflammation in rabbits treated with labelled microspheres and those treated with unlabelled microspheres.

Conclusion

The incorporation of low percentages of fluorescein into PLA microspheres, which can then be viewed using light microscopy, has proved beneficial in the tracking of microspheres administered to the respiratory tract. The ability to locate such microspheres proved invaluable in the assessment of tissue damage arising from the presence of the microspheres. The histopathological data give cause to doubt the supposed non-toxic nature of PLA microspheres when administered to the respiratory tract, a conclusion which supports two earlier reports of inflammatory responses to PLA microspheres (Ratcliffe et al 1984; Smith & Hunneyball 1985).

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