Preparation of Biodegradable, Surface Engineered PLGA Nanospheres with Enhanced Lymphatic Drainage and Lymph Node Uptake

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Purpose. Nanospheres can be utilised for the targeting of drugs and diagnostic agents to the regional lymph nodes. The surface modification of model polystyrene, (PS), and poly(lactide-co-glycolide),(PLGA), nanospheres by poly(lactide)-poly(ethylene glycol), (PLA:PEG), copolymers has been assessed by *in vitro* characterisation and *in vivo* biodistribution studies following subcutaneous administration of the nanospheres to the rat.

Methods. Three PLA:PEG copolymers were investigated, with PEG chain lengths of 750, 2000 and 5000 Da. The PLA:PEG copolymers were either coated onto the surface of PS and PLGA nanospheres or used as a co-precipitate in the formation of PLGA-PLA:PEG nanospheres. Coating of the nanospheres was confirmed by an increase in their particle size and a corresponding decrease in the surface potential. The kinetics of injection site drainage and lymph node retention was determined over a 24 hour time course for naked, coated and co-precipitated nanosphere systems.

Results. Dependent on the surface characteristics, the distribution of the nanospheres can be significantly modified and the lymph node localisation dramatically enhanced by coating their surfaces with PLA:PEG copolymers or by producing co-precipitate nanospheres of PLGA and PLA:PEG.

Conclusions. A fully biodegradable nanosphere system has been developed with excellent lymph node targeting characteristics.

KEY WORDS: lymph node targeting; subcutaneous administration; poly(lactide-co-glycolide); poly(lactide)-poly(ethylene glycol) copolymers; polystyrene; nanospheres.

INTRODUCTION

The uptake of subcutaneously administered nanospheres by the lymphatic system is governed by the rate of drainage of the nanospheres from the injection site and the recognition of the nanospheres and subsequent uptake by the lymph node macrophages (1). Extensive investigations have been conducted by various research groups into the optimisation of the carrier size and surface characteristics, whether liposomes or nanospheres, so as to identify the parameters that can lead to successful drainage from the interstitium and enhanced node uptake (2–6). The maximum lymph node uptake reported to date for a liposomal system is about 6% of the administered dose (7).

In contrast it has been demonstrated within our own laboratory that the lymph node localisation of model polystyrene nanospheres of 60 nm diameter can be improved dramatically by coating with block co-polymers of the poloxamer and poloxamine series (2). A relationship was demonstrated between the effectiveness of the steric barrier created by these polymers and the lymph node uptake, with an optimum lymph node uptake of 40% of the administered dose being achieved with nanospheres coated with a poloxamine of intermediate hydrophilicity, poloxamine 904. The exploitation of these results for the preparation of biodegradable and bioresorbable carriers, with surfaces that specifically target to the regional lymph nodes, is currently under investigation in this laboratory. Recently it has been found, with poly(lactide-co-glycolide) (PLGA) nanospheres coated with poloxamines and poloxamers, that a maximal lymph node uptake of 17% of the administered dose, can be achieved with nanospheres coated with poloxamine 908. As far as we are aware, this figure represents the highest lymph node uptake of a biodegradable colloidal system obtained to date (8).

We have also found that extended circulation times can be achieved for PLGA nanospheres, administered intravenously, by coating the nanospheres with block co-polymers, such as poloxamine 908, as well as novel poly(lactide)-poly(ethylene glycol) (PLA:PEG) copolymers with PEG chains of 2000 Da (9). The PLA:PEG coatings were found to adsorb to the surface of the nanospheres more efficiently, resulting in a prolongation of circulation times as compared to poloxamine 908 coated nanospheres.

The aim of the present work was to assess the use of PLA:PEG copolymers to determine whether it is possible to surface engineer nanospheres in order to achieve changes in lymph node distribution and a greatly enhanced lymph node uptake, as previously achieved using poloxamer and poloxamine coated polystyrene latex (2). The copolymers were used both as coatings for model polystyrene nanospheres and biodegradable PLGA nanospheres as well as co-precipitates in the production of PLGA-PLA:PEG nanospheres. The nanospheres were characterised in terms of size, (surface layer thickness for the coated systems) and surface potential. The biological fate of the nanospheres was determined after subcutaneous administration into rats. The biodistribution was determined over a 24 hour time course.

MATERIALS AND METHODS

Materials

Polystyrene nanospheres of nominal size 60 nm were purchased from Polysciences Ltd (UK) at a concentration of 2.5% w/v. Poly(DL-lactide-co-glycolide 75:25), as Resomer RG755, of average MW 50,000 was obtained from Boehringer Ingelheim (Germany). PLA:PEG copolymers 1.5:0.75, 1.5:2 and 2:5 were synthesised and supplied by Zeneca Pharmaceuticals (U.K). The copolymers were synthesised by ring opening polymerisation of D,L-lactide in the presence of the corresponding methoxypolyethylene glycol and stannous octoate as a catalyst (9). The characteristics of the polymers are outlined in Table I. All polymers were used as received. ^{125}Iodine, in the form of sodium iodide, and ^{111}Indium-oxine, as a solution in HEPES buffer at a pH of 6.5–7.5, were purchased from Amersham International (UK). All other chemicals used were of analytical grade and used as received.

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Table I. Composition and Molecular Weight Characteristics of PLA:PEG co-polymers 1.5:0.75, 1.5:2 and 2:5

PLA:PEG	Average molecular weight (Da)[a]	Molecular weight of PEG chain (Da)
1.5:0.75	2219	750
1.5:2	3518	2000
2:5	6307	5000

[a] As determined by gel permeation chromatography using polyoxyethylene as standard.

Preparation of PLGA and PLGA-PLA:PEG Nanospheres

PLGA nanospheres were prepared and radiolabelled as described previously (10). Briefly, 3 MBq of ^{111}In-oxine was added to a solution of 0.1% w/v PLGA in acetone (10 ml). The mixture was then added dropwise to 20 ml of magnetically stirred water. After overnight evaporation of the organic phase at room temperature the nanospheres were concentrated to 0.2% w/v by ultrafiltration through a filtration membrane of 300,000 molecular weight cut off (Amicon Inc, USA). Nanospheres were then coated by incubation of a 0.2% w/v suspension of the nanospheres in a 0.2% w/v solution of the polymers at room temperature. Both excess polymer and free radiolabel were then removed by gel permeation chromatography using a 10 cm column of Sepharose CL-4B (Pharmacia, Sweden).

The PLGA-PLA:PEG nanospheres were prepared and labelled in a similar manner by co-precipitation of the PLGA and PLA:PEG 1.5:0.75 (total amount 10 mg) from the organic phase into the water. The percentage of PLGA in the mixture ranged from 75% to 55%.

The stability of the radiolabel in serum and the biodistribution of free radiolabel have been determined previously (8). The results demonstrated that there was an initial burst release of radiolabel from the nanospheres, attributed to surface bound label. However, radiolabel was shown not to accumulate in the lymph nodes. Thus, any radioactivity detected in the lymph nodes must have been associated with the nanosphere carriers.

Radiolabelling of Polystyrene Nanospheres

Polystyrene nanospheres (1 ml) were surface labelled with NaI^{125} (20 MBq) by irradiation in a ^{137}Cesium source (activity $5.54 \times 10^4 \pm 0.67\%$ Rads per hour on 19/1/90) for 48 hours according to a method described previously (11). Unbound iodine and reaction products were removed by extensive dialysis against a large volume of de-ionised water using tubing of MWCO 100,000 (Spectrum medical industries, U.S.A.). Nanospheres were then coated with the PLA:PEG co-polymers in the same way as for PLGA and excess polymer removed by a further period of dialysis.

Determination of Nanosphere Size and Zeta Potential

The particle size of the different nanosphere systems was determined by Photon Correlation Spectroscopy (PCS) using a Malvern 4700 instrument (Malvern, U.K), as previously described (9). The adsorbed layer thickness of the polymer on the nanosphere surface was determined by comparing the radii of coated and uncoated nanospheres. The coating of the nanospheres was confirmed by the measurement of surface charge, in 10mM McIlvaine buffer at pH7, by Laser Doppler Anemometry (LDA) using a Malvern Zetasizer IV instrument (Malvern, UK). The mean value and standard deviation were calculated from 6 replicate measurements of each sample.

Biodistribution of Nanospheres

Groups of three male Wistar rats, body weight 150–180 g, each received a subcutaneous injection (100 μl equivalent to 0.1 mg) of the various nanosphere systems into the dorsal surface of the left hind footpad, under halothane anaesthesia. At various times after administration, a 20 μl sample of blood was removed via the tail vein. Animals were then sacrificed by cervical dislocation and the regional lymph nodes (popliteal, inguinal, iliac and renal), liver, spleen and footpad removed for measurement of associated radioactivity in a gamma counter (LKB 1282 Compugamma CS, LKB, Turku, Finland). To determine the amount of nanosphere associated activity in the blood, a total blood volume of 7.5% of body weight was assumed (12).

RESULTS AND DISCUSSION

Nanosphere Characterisation

The uncoated nanospheres of PLGA or polystyrene were found to be in the sub-100 nm size range required for lymphatic targeting purposes (13,14) (Table II). The nanospheres were highly negatively charged at their surfaces as reflected in their zeta potentials (Table II). This high negative charge has been attributed to the presence of sulphate groups remaining from the production process for polystyrene (15) and carboxyl groups on the surface of PLGA (9).

Adsorption of polymers to the nanosphere surfaces resulted in an increased nanosphere size; this being attributed to the

Table II. Characterisation of Nanospheres by Photon Correlation Spectroscopy and Laser Doppler Anemometry

Polymer	Mean size/nm (Coating layer thickness.nm)	Mean polydispersity index[a]	Mean zeta potential/mV
Uncoated PS	74.7 ± 2.8	0.029 ± 0.076	−40.6 ± 2.2
Uncoated PLGA	85.1 ± 3.2	0.154 ± 0.034	−35.9 ± 2.0
Polystyrene			
1.5:0.75	83.7 ± 4.4(4.5)	0.043 ± 0.089	−22.6 ± 2.5
1.5:2	84.6 ± 3.6(5.0)	0.034 ± 0.042	−10.9 ± 3.1
2:5	92.1 ± 2.5(8.7)	0.032 ± 0.028	−3.0 ± 3.2
PLGA			
1.5:0.75	84.8 ± 3.4(0)	0.137 ± 0.037	−18.8 ± 1.6
1.5:2	90.3 ± 2.8(2.6)	0.142 ± 0.044	−10.9 ± 1.6
2:5	99.3 ± 4.0(7.1)	0.114 ± 0.029	−4.3 ± 1.2
PLGA-PLA:PEG	(1.5:0.75)	Co-precipitate	
75% PLGA	85.2 ± 0.4	0.133 ± 0.013	−19.4 ± 1.8
65% PLGA	85.9 ± 1.0	0.150 ± 0.033	−14.7 ± 0.1
55% PLGA	92.1 ± 1.5	0.241 ± 0.053	−10.3 ± 0.2

[a] A measure of the narrowness of the particle size distribution, with an index of less than 0.2 indicating a monodisperse particle size.

formation of a coating layer. The adsorbed layer thickness formed was related to the molecular weight of the polyethylene glycol (PEG) chain in the PLA:PEG molecule. It has been shown that the PLA:PEG copolymers are orientated at the surface of the nanosphere such that the PLA portion adsorbs to the surface of the nanosphere and the PEG portion protrudes into the surrounding media (9).

A decrease in the effective surface charge (a result of a shift of the shear plane to a greater distance from the nanosphere surface) was observed for the coated systems, compared to uncoated nanospheres. The value of the zeta potential could be related to the length of PEG chain at the nanosphere surface; with longer PEG chains resulting in lower zeta potentials. The zeta potential results are, therefore, a confirmation of the presence of a coating layer on the nanosphere surface. The zeta potential of the coated systems were comparable whether the nanosphere core was composed of polystyrene or PLGA.

Nanospheres produced by the co-precipitation of PLGA and PLA:PEG 1.5:0.75 exhibited a decreasing zeta potential as the amount of PLA:PEG in the mixture was increased; with the zeta potential for 75% PLGA co-precipitate nanospheres having an identical zeta potential to that of PLGA nanospheres coated with 1.5:0.75 PLA:PEG. Interestingly, when the amount of PLA:PEG in the nanospheres was increased, as in the 55% PLGA co-precipitate nanospheres, the zeta potential decreased to −10 mV, which is similar to the value found for PLA:PEG 1.5:2 coated PLGA nanospheres (Table II). The size of the nanospheres was independent of the ratio of PLGA to PLA:PEG up to content of 35% PLA:PEG. The decrease in zeta potential with increased PLA:PEG content may be related to an increased density of PEG on the surface of the nanosphere.

The polydispersity index indicated that the polystyrene nanospheres were monodisperse in nature with values less than 0.1 for both naked and coated systems (Table II). The polydispersity indexes for the PLGA nanospheres were higher but could still be considered to indicate practically monodisperse systems whereas the polydispersity of the PLGA-PLA:PEG nanospheres were seen to increase with increasing content of PLA:PEG.

In Vivo Distribution of Nanospheres Coated with PLA:PEG Co-polymers

The PLA:PEG co-polymers may have advantages over the poloxamers and poloxamines used previously as particle coating agents for intravenous studies (16) or lymphatic studies (2,8) since they could be more acceptable to the regulatory authorities due to their favourable biodegradation characteristics (9). In accordance with our previous work (2), model polystyrene nanospheres were investigated as control nanospheres in terms of their drainage from the injection site and lymph node accumulation (Figures 1 and 2). In agreement with these previous studies, it was noted that control, uncoated, nanospheres were retained at the injection site for extended periods of time (Figure 1). The drainage of the nanospheres was improved by the presence of PEG on the nanosphere surface (Figure 1). The rate of drainage from the injection site was related to the length of the PEG chain.

The mechanism of transport of colloids through the aqueous channels of the interstitium to the initial lymphatics may either be extracellular, by dispersed particle flow directly into

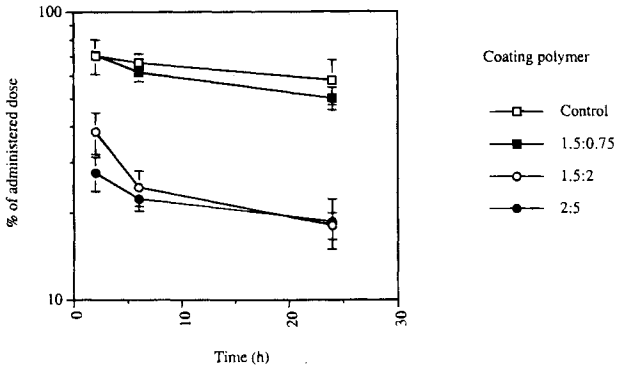

Fig. 1. Kinetics of drainage of polystyrene nanospheres from a subcutaneous injection site.

the initial lymphatic vessels, or by intracellular carriage after phagocytosis by macrophages resident in the interstitium (17). Sterically stabilised nanospheres have properties that lead to a reduction in the interaction with the amorphous ground substance of the interstitium. Passage of the coated nanospheres through the aqueous channels of the interstitium into the initial lymphatic channels is thereby facilitated.

The enhanced movement of the nanospheres into the lymphatic channels results in an increased number of nanospheres presented to the macrophages resident in the regional lymph nodes. However, a balance in the properties of the surface between hydrophobic and hydrophilic character is required. Only those nanospheres with steric barriers sufficient to provide a hydrophilic surface to increase injection site drainage, but still hydrophobic enough to provide an opportunity for recognition by the lymph node macrophages, will exhibit high lymph node uptake. In the case of the PLA:PEG co-polymers studied in this work, these requirements are best met by PLA:PEG 1.5:0.75 where the PEG length is 750 Da. This length of PEG chain does not constitute a sufficiently large steric barrier to prevent macrophage interaction. Polystyrene nanospheres coated with this polymer were observed to accumulate in the lymph nodes, to a total of around 15% and 20% of the administered dose after 2 hours and 6 hours, respectively (Figure 2). The distribution was maintained over the 24 hour time course studied. This value compares well with previous studies and is

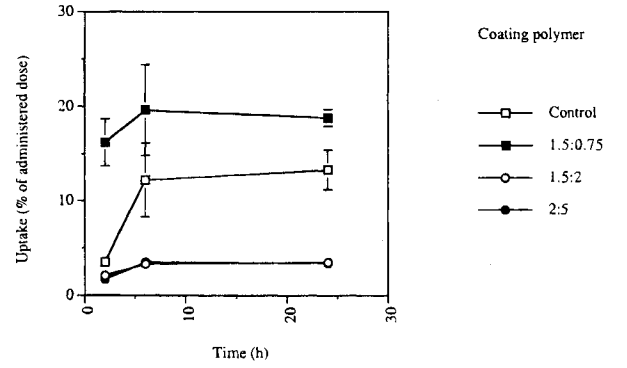

Fig. 2. Kinetics of uptake of polystyrene nanospheres into the regional lymph nodes following subcutaneous administration.

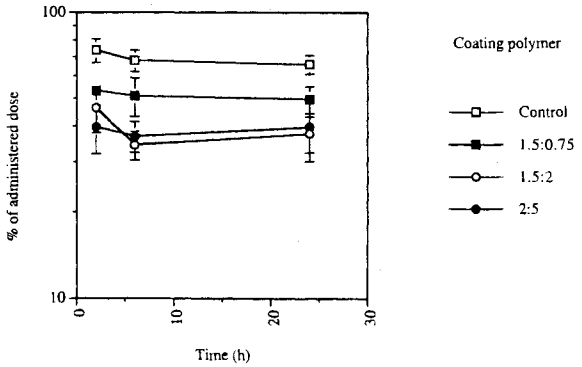

Fig. 3. Kinetics of drainage of PLGA nanospheres from a subcutaneous injection site.

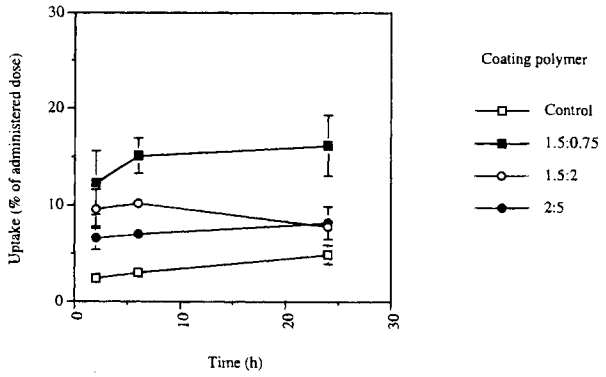

Fig. 4. Kinetics of uptake of PLGA nanospheres into the regional lymph nodes following subcutaneous administration.

much higher than that found for liposomes incorporating a PEG moiety of 1900 Da size (18).

The steric barriers created by PLA:PEG 1.5:2 and 2:5 on the surface of polystyrene nanospheres were apparently "too hydrophilic" and were sufficient to prevent nanosphere capture by the lymph node macrophages. This resulted in a reduced lymph node localisation compared to uncoated nanospheres. After 2 hours, these hydrophilic nanosphere systems were seen to accumulate in the general circulation and then to be removed from the circulation by the liver at subsequent time points (data not shown). This may be attributed to a gradual desorption of the polymers from the polystyrene surface with time, through the competitive adsorption of plasma proteins, as previously suggested in studies on the direct intravenous administration of similar nanospheres (9).

The coating of PLGA nanospheres with the PLA:PEG copolymers also resulted in an enhanced drainage of the nanospheres from the injection site (Figure 3) compared to uncoated nanospheres, although the increase in rate of drainage was less pronounced than that observed for the coated polystyrene systems. All coated systems exhibited an enhanced lymph node localisation compared to the control uncoated nanospheres. Lymph node uptake after 24 hours was comparable (15%) for both polystyrene and PLGA nanospheres carrying adsorbed PLA:PEG 1.5:0.75, but for the copolymers with larger PEG chains, the lymph node localisation was higher for PLGA coated systems than for the polystyrene systems. The reason for this difference is unknown, but may be a result of the smaller adsorbed layers of the co-polymers on PLGA than on polystyrene nanospheres (Table II), resulting in a smaller steric barrier and more effective nanosphere capture by lymph node macrophages. Alternatively a different conformation of the PLA:PEG on the surface of PLGA as compared to polystyrene nanospheres could be an explanation of the different result. A similar difference in the lymphatic uptake of PLGA and polystyrene nanospheres coated with poloxamers and poloxamines has previously been found (8).

The *In Vivo* Distribution of Nanospheres Prepared by Co-precipitation of PLGA with PLA:PEG 1.5:0.75

The biodistribution profiles of nanospheres produced by co-precipitation of PLGA with PLA:PEG 1.5:0.75 are shown in Table III. These nanospheres would be expected to have an increased density of PEG on the surface when the percentage of copolymer used in their preparation was increased, thus resulting in a more effective steric barrier. This was confirmed by measurement of the zeta potentials (Table II). The inherent negative charge of the naked nanospheres had been masked by the shift in the shear plane brought about by the copolymer.

Enhanced drainage of the nanospheres from the injection site was demonstrated *in vivo* for increasing percentages of PLA:PEG in the nanospheres (Table III). The incorporation of the copolymer into the nanospheres resulted in increased lymph node uptake at all percentages of the copolymer as compared to the naked nanospheres (Table III). The uptake was maximal for nanospheres with a 75% and 65% PLGA content (16% and 17% lymph node uptake respectively). With a greater incorporation of polymer the lymph node localisation was seen to decrease, probably as a result of an improved steric barrier,

Table III. Biodistribution of Nanospheres Prepared by Co-Precipitation of PLGA with PLA:PEG 1.5:075, 6 Hours After Subcutaneous Administration to the Rat

Polymer	Injection site	Total lymph nodes	Liver	Blood	Recovery
Naked PLGA	67.9 ± 5.9	3.0 ± 0.4	1.0 ± 0.1	3.7 ± 0.5	82.7 ± 5.6
Co-precipitated					
75% PLGA	55.7 ± 6.6	16.1 ± 4.9	5.2 ± 1.2	7.0 ± 1.0	93.5 ± 8.4
65% PLGA	42.3 ± 1.3	17.2 ± 3.9	10.8 ± 1.9	8.7 ± 3.1	88.6 ± 6.3
55% PLGA	43.4 ± 1.0	10.8 ± 0.5	12.4 ± 0.5	12.0 ± 0.9	98.4 ± 2.7

Note: Data expressed as % of initial dose (n = 3).

and hence a surface that was less easily recognisable by the lymph node macrophages.

Previous studies have investigated the effect of PEG surface density on the circulation time of intravenously administered particles. The density of covalently attached PEG onto the surface of polystyrene nanospheres was demonstrated to enhance the circulation time (19). This may be attributed to the improved efficiency of the steric barrier to prevent particle opsonisation and, therefore, in this case to prevent uptake by the Kupffer cells of the liver.

In conclusion, the results have shown that the PLA:PEG copolymers can be exploited as coatings for biodegradable PLGA nanospheres in order to obtain sterically stabilised particles with highly improved drainage from an interstitial injection site and enhanced lymph node localisation in the rat model, as compared to naked PLGA nanospheres.

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