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Lymph node localisation of biodegradable nanospheres surface modified with poloxamer and poloxamine block co-polymers

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Abstract Studies were performed to develop a sub-100 nm biodegradable colloidal system for the efficient delivery of drugs and diagnostic agents to the lymphatic system. Nanospheres of poly(lactide-co-glycolide) were prepared by interfacial polymer deposition. The nanospheres were coated with block co-polymers in order to modify their surface characteristics. Radiolabelling of the nanospheres for in vivo tracing was achieved by the incorporation of the lipophilic complex ^{111}In-oxine during nanosphere preparation. In vitro stability of the radiolabelled nanospheres was determined in rat serum at 37°C. The lymphatic distribution of the nanospheres was determined after subcutaneous administration to the rat. Lymphatic uptake of all coated systems was enhanced compared to the uncoated nanospheres, and a maximal uptake of 17% of the administered dose in the regional lymph nodes was achieved. These observations suggest that the nanospheres are suitable for diagnostic and therapeutic applications in clinical and experimental medicine.

Key words: Lymphatic system; Lymph node; Nanosphere; Poly(lactide-co-glycolide); Poloxamer; Poloxamine

1. Introduction

The uptake of colloidal drug delivery systems from an interstitial site into the lymphatic system is governed by the physicochemical characteristics of the colloid and the pathophysiological structure of the interstitial space and lymphatic system [1]. A colloid, administered interstitially, for lymphatic targeting purposes should drain well from the site of injection and be well retained in the regional lymph nodes. The concept of surface modification, wherein model polystyrene nanospheres have been coated with block co-polymers that provide a sterically stabilised surface, has been used previously to control the rate of drainage from a subcutaneous injection site and to manipulate the lymphatic distribution [2].

Targeting of nanospheres to specific body sites for the delivery of therapeutic agents to man requires that the delivery system is both biocompatible and biodegradable [3]. Liposomes have been investigated extensively by other workers for lymphatic delivery but with disappointing results: 6.3% of the administered dose represented total lymph node uptake [4]. More recently, liposomes incorporating a PEG 1900 Da layer have been investigated following s.c. administration to mice [5]. Maximum reported levels in the lymph nodes were approximately 250 cpm/mg tissue normalised to 10^6 cpm of radiolabel injected per mouse after 48 h. Due to such poor lymphatic delivery of liposomes, poly(lactide-co-glycolide) (PLGA) was chosen in the present work as a suitable biodegradable polymer for the investigation of nanosphere preparation and biodistribution. Nanospheres were radiolabelled by the incorporation of ^{111}Indium-oxine and their surface modified by coating with adsorbed block co-polymers. The block co-polymers were poloxamers and poloxamines from series with a constant length of polypropylene oxide chains and increasing chain length of polyethylene oxide. The serum stability of the radiolabel was also determined.

2. Materials and methods

Poly(DL-lactide-co-glycolide 75:25) (Resomer RG755) of average molecular weight 50000 was obtained from Boehringer Ingelheim (Germany). The poloxamines 901, 904 and 908 and the poloxamers 401, 402, 403, and 407 were a gift from BASF (USA). Their composition and molecular weight characteristics are given in Table 1. ^{111}Indium-oxine was purchased from Amersham International (UK) as a solution in HEPES buffer at a pH of 6.5–7.5, with a specific activity of 37 MBq/ml. All other chemicals were of analytical grade and used as obtained.

Nanospheres were prepared by modification of a method described previously [6]. PLGA was dissolved in 10 ml acetone to a concentration of 0.1% w/v, and 3 MBq of the ^{111}In-oxine added. This organic phase was then added dropwise to 20 ml of de-ionised water during magnetic stirring. After evaporation of the organic phase, the resulting nanospheres were concentrated by ultrafiltration (Amicon Inc., USA) to 0.2% w/v. Particles were then coated by incubation overnight in a 0.2% w/v solution of the various block co-polymers at room temperature. Excess polymer and radiolabel were subsequently removed by passage of the nanospheres through a 10 cm column of Sepharose CL-4B (Pharmacia, Sweden).

The particle size of the resultant nanospheres was determined by photon correlation spectroscopy (PCS) using a Malvern 4700 instrument (Malvern, UK). The adsorbed layer thickness of the coating layer was determined from the difference in radii of coated and uncoated systems. The zeta potential of the nanospheres was determined by laser doppler anemometry using a Malvern Zetasizer IV (Malvern, UK) in 10 mM McIlvaine buffer at pH 7.

To determine the stability of the radiolabel, nanospheres, cleaned of excess radiolabel and polymer, were incubated at 37°C in 50% v/v rat serum over a time course of 6 h. Samples (0.5 ml) were collected in duplicate at selected times of 5 min, 1, 3, and 6 h and were passed down columns of sepharose CL-4B (Pharmacia, Sweden) using PBS at pH 7.4, (Sigma, UK), as eluent to separate the dissociated radiolabel from the nanospheres. Twenty-five consecutive fractions of 0.5 ml were collected and counted for associated radioactivity in a gamma counter (LKB 1282 Compugamma CS, LKB, Finland). The stability was expressed as the percentage of activity remaining associated with the nanospheres. The total activity applied to the column was taken to be the denominator for the calculations.

Nanospheres prepared without radiolabel were incubated overnight at room temperature with free radiolabel and then cleaned of excess radiolabel. These nanospheres were then incubated with serum in the same manner as above. In this way any association of the radiolabel to the nanosphere surface could be determined and the stability of surface bound radiolabel monitored.

The biodistribution of the free radiolabel and the various labelled PLGA nanosphere systems was determined in groups of three male Wistar rats, body weight 150 ± 10 g. Biodistribution of free radiolabel was determined either alone or when co-administered with naked nanospheres (0.1 mg), poloxamine 908 (0.1%), or a combination of

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nanospheres and poloxamine 908. All systems were injected subcutaneously (100 μl) into the dorsal surface of the left hind footpad, under halothane anaesthesia. After 6 h (2, 6 and 24 h for uncoated PLGA nanospheres), a blood sample was taken from the tail vein and the rats killed by cervical dislocation under halothane anaesthesia. Associated radioactivity was measured in the footpad (injection site), the regional lymph nodes (popliteal, inguinal, iliac and renal nodes) as defined by Hebel and Stromberg [7], and the organs of the reticuloendothelial system. Total blood volume was calculated assuming that blood in the rat represented 7.5% v/w of body weight [8,9].

3. Results and discussion

Particle size, as determined by PCS, showed that the nanospheres of PLGA were in the sub-100 nm range with a low polydispersity index (Table 2). The adsorbed layer thickness of the poloxamers and poloxamines on the surface of the nanospheres was related to the number of polyethylene oxide (PEO) units in their structures. An increase in thickness was found for an increased number of PEO units in the co-polymer chain. Poloxamines 901, 904, and 908 contain 4, 15 and 119 PEO units, respectively, per PEO chain, whereas they all share a common anchoring polypropylene oxide (PPO) chain of 68 PPO units. The related series of poloxamers each share a common anchoring chain of 69 PPO units with 5, 11, 20 and 98 PEO units per PEO chain for poloxamers 401, 402, 403 and 407, respectively (Table 1). Since the adsorbed layers measured were small in comparison to the standard deviation of the size measurements, confirmation of nanosphere coating was obtained by measuring the surface potential of the nanospheres (Table 2). A general reduction in zeta potential was observed when the nanospheres were coated with the block co-polymers. This is attributed to the masking of the inherent negative charge of the nanospheres (carboxyl groups in the polymer end groups [10]). The PEO chains bring about a shift in the shear plane to a distance further from the nanosphere surface [11].

In order to determine the in vivo distribution of the nanospheres, an associated radiolabel should be well retained under physiological conditions. After an initial burst release of surface associated radiolabel from the nanospheres, the in vitro release should be minimal or stop [12]. The PLGA nanospheres with surface adsorbed label exhibited a rapid initial burst release resulting in only 60% of the radioactivity remaining associated with the nanospheres after 5 min (Fig. 1). Thereafter, there was a continued loss of radiolabel from the nanosphere surface with time, such that after 6 h only around 35% of the label remained with the nanospheres. This dissociation of label from the nanosphere surface was unaffected by the presence of a coating polymer.

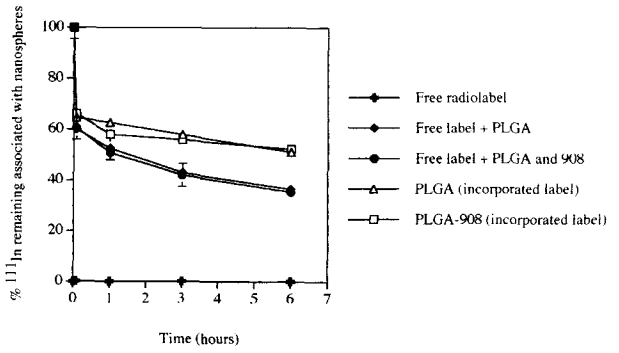

Fig. 1. Stability of ^{111}Indium radiolabelled nanospheres.

Nanospheres, labelled by the incorporation of the label into the nanosphere structure during preparation, also exhibited a rapid burst release of the radiolabel after 5 min (Fig. 1), probably as a result of dissociation of surface bound radiolabel. However, further loss of the radiolabel was less pronounced with only a further 14% of the label being lost from the nanospheres over the following 6 h. Thus, around 50% of the activity remained associated with the nanospheres at this time whether the nanospheres were uncoated or coated with poloxamine 908. This figure was similar after 6 hours incubation with serum. A slow release of ^{111}In-oxine from PLGA nanospheres, after an initial burst, has been observed previously [12] and has been attributed to the slow leaching of the radiolabel from the porous nanosphere structure. The porous nature of the nanospheres has also been suggested as a factor that allows the access of biological fluids into the nanosphere core [13].

Nanospheres were radiolabelled by the incorporation of the lipophilic, gamma emitting complex ^{111}In-oxine during nanosphere preparation. The method was developed by Scholes [14] following unsuccessful attempts to label the nanospheres by surface labelling with ^{125}Iodine or incorporation of ^{125}Iodine cholesteryl aniline or neutron activation following incorporation of samarium acetylacetonate. The conclusion from this work was that of these only ^{111}In-oxine could be employed for radiolabelling of PLGA nanospheres prepared by the polymer interfacial deposition technique.

Other workers [15] have radiolabelled poly(lactic acid) using ^{14}C, in which the radiolabelled PLA was synthesised from D,L-lactic acid [carbonyl ^{14}C]. Nanospheres were then prepared by a precipitation method. The radiolabel was reported to be released and excreted as carbon dioxide from the lungs only after degradation of the nanospheres. However, the ideal radiolabel for ease of detection would be a gamma emitter.

Table 1
Composition and molecular weight characteristics of poloxamers 401, 402, 403 and 407 and poloxamines 901, 904 and 908

Polymer	Molecular weight	% Ethylene oxide	Number of PPO units per chain[a]	Number of PEO units per chain[a]
Poloxamers				
401	4400	10	69	5
402	5000	20	69	11
403	5750	30	69	20
407	12600	70	69	98
Poloxamines				
901	4700	10	17	4
904	6700	40	17	15
908	25000	80	17	119

[a]Poloxamines contain four chains of both PEO and PPO; poloxamers have two PEO chains but only one PPO.

Table 2
Characterisation of PLGA nanospheres ($n=6$)

Polymer	Mean size (nm)	Mean polydispersity	Adsorbed layer thickness (nm)	Mean zeta potential (mV)
Uncoated	85.1 ± 3.2	0.154 ± 0.034	–	−35.9 ± 2.0
Poloxamers				
401	88.5 ± 1.6	0.126 ± 0.038	1.7	−12.7 ± 0.9
402	87.0 ± 2.2	0.127 ± 0.030	1.0	−14.9 ± 1.6
403	87.7 ± 2.7	0.146 ± 0.042	1.3	−15.3 ± 1.3
407	84.1 ± 2.4	0.122 ± 0.052	4.5	−7.4 ± 1.4
Poloxamines				
901	flocculation			
904	88.6 ± 2.1	0.128 ± 0.015	1.8	−15.4 ± 2.2
908	95.2 ± 3.2	0.125 ± 0.035	5.1	−5.7 ± 1.4

PLGA has been radiolabelled using ^{111}Indium which was chelated with diethylenetriamine pentaacetic acid stearyl amide (DTPA-SA) [16]. Serum stability studies showed less than a 2% loss of label during 4 h. However, the nanospheres were prepared by an emulsification method and replication of the results using the polymer interfacial deposition technique herein have proved unsuccessful.

Free ^{111}In-oxine, administered in aqueous solution, was rapidly drained from the injection site, with only 14% of the administered dose remaining after 6 h (Table 3). The label was seen to pass through the lymphatics to the bloodstream with insignificant uptake by the lymph nodes. Since only 50% of the administered dose was recovered, it can be assumed that the remainder had been excreted in both the urine and faeces [17].

Free label co-administered with naked PLGA nanospheres exhibited a reduced rate of clearance from the injection site with 41% of the administered dose remaining in the footpad after 6 hours (Table 3). It is thus evident that some of the free label had become associated with the nanospheres; the results reflecting uptake of nanospheres as well as distribution of free label. The lymph node uptake of the label was higher than for the free label alone, with 3% of the dose sequestered by the nodes. Levels of activity in the blood and liver were comparable to free label alone. The recovery levels for this system were much higher than for the free label, indicating that the label associated with the nanospheres was not so rapidly excreted.

Free label co-administered with an aqueous solution of poloxamine 908 exhibited a distribution profile comparable to free label alone, indicating that there was no association between the free poloxamine and the free label. When free label was co-administered with both PLGA nanospheres and poloxamine 908, the levels of administered dose observed at the injection site were around 30%, which is greater than for free label alone, but less than for free label administered with naked PLGA nanospheres. This may be due to a reduced association of the label with the nanospheres as a result of the steric barrier created by the adsorption of poloxamine 908 to the nanosphere surface, or may be a reflection of the increased drainage of poloxamine coated nanospheres if the label is associated with the nanosphere surface.

Naked PLGA nanospheres, with incorporated label, were seen to be retarded at the injection site for long periods of time. Even 24 hours after administration, 65% of the administered dose was retained in the footpad (Table 4). Since colloid passage through the interstitium to the initial lymphatics is facilitated by aqueous channels [18], it is not surprising that the nanosphere movement was inhibited, considering the hydrophobicity of the PLGA nanospheres. Previous work has investigated the mechanism of uptake of colloids from the interstitium into the initial lymphatics [2,19,20]. Two pathways of colloid transport were proposed, one being via extracellular dispersed particle flow directly into the initial lymphatic vessels, and the other by intracellular carriage after phagocytosis by macrophages within the interstitium. Nanospheres which do drain into the lymphatic system are effectively sequestered by the regional lymph nodes, with the majority of lymphatic uptake in the primary, popliteal node. The phagocytic cells of the subcapsular sinus of the cortex and the classical macrophages of the medulla in both the popliteal and iliac nodes are mainly responsible for the phagocytosis of nanospheres [2]. Any nanospheres which are then not sequestered by the lymph node macrophages and reach the general circulation can be removed by the Kupffer cells of the liver.

The coating of the naked nanospheres with block co-polymers of the poloxamer or poloxamine series resulted in an enhanced drainage from the injection site compared to uncoated nanospheres (Table 5). It is believed that the surface of the nanospheres was more acceptable for passage through the aqueous channels of the interstitium. Therefore, a greater number of nanospheres were presented to the macrophages of the lymph nodes. If the nanosphere surface was recognisable to the macrophages as foreign then the nanosphere would be retained. However, nanospheres with a sufficient steric barrier to prevent opsonisation with plasma proteins and minimise

Table 3
The biodistribution of free ^{111}In-oxine 6 h after s.c. administration into the rat hind footpad

System	Injection site	Total lymph nodes	Liver	Blood	Recovery
Free ^{111}In-oxine	14.1 ± 3.1	1.0 ± 0.2	4.2 ± 0.5	11.5 ± 2.0	51.3 ± 8.5
Co-administered with:					
PLGA nanospheres	41.2 ± 2.9	3.3 ± 1.1	4.2 ± 0.6	18.5 ± 5.6	92.3 ± 9.2
Poloxamine 908	15.2 ± 2.7	0.9 ± 0.1	5.4 ± 0.4	16.3 ± 3.8	62.9 ± 7.3
Nanospheres+P908	29.0 ± 4.4	6.9 ± 0.5	8.6 ± 1.7	11.6 ± 2.8	76.5 ± 10.4

Data are expressed as % of initial dose ($n=3$).

Table 4
The biodistribution of naked PLGA nanospheres, 2, 6 or 24 h after s.c. administration

Time (h)	Injection site	Total lymph nodes	Liver	Blood	Recovery
2	73.9 ± 7.1	2.4 ± 0.3	0.3 ± 0.1	3.7 ± 0.5	82.8 ± 7.0
6	67.9 ± 5.9	3.0 ± 0.4	1.0 ± 0.1	3.7 ± 0.5	82.7 ± 5.6
24	65.9 ± 5.0	4.9 ± 1.0	2.5 ± 0.4	2.8 ± 0.4	87.2 ± 6.0

Data are expressed as % of initial dose ($n = 3$).

cell-nanosphere interaction [21] should escape recognition by the lymph node macrophages and enter the general circulation. All coated systems exhibited an enhanced lymph node uptake compared to naked uncoated nanospheres. Increased hydrophilicity of the nanospheres, as reflected in the increased PEO content of the coating polymer, did not result in large differences in injection site drainage but did result in an increase in lymphatic uptake; the maximum uptake was around 11% of the administered dose for poloxamer 407 and 17% for poloxamine 908 by the lymph nodes (Fig. 2). The popliteal node was responsible for the greatest uptake by the lymph nodes in each case.

The coating of polystyrene nanospheres (60 nm) with the same block co-polymers has been reported previously [2]. In these experiments there was a greater drainage from the injection site with increasing hydrophilicity. High lymph node accumulation was demonstrated for nanospheres coated with block co-polymers possessing intermediate PEO chain lengths such as poloxamine 904. A greater degree of block co-polymer hydrophilicity was required for the poloxamers and poloxamines adsorbed to PLGA nanospheres. This may be a result of the PLGA nanospheres being larger in size, but is more likely to be due to smaller adsorbed layer thicknesses of the block co-polymers on the nanospheres, resulting in greater hydrophobicity. For example, poloxamine 908 produced a layer of 9.6 nm on polystyrene nanospheres compared to only 5.1 nm on PLGA nanospheres. The masking of negative surface charge of the nanospheres and the hydrophobic PLGA core is, therefore, less effective (zeta potential of poloxamine 908 on polystyrene was −1.8 mV compared to −5.7 mV for PLGA).

It is noted that the initial burst release of ^{111}In-oxine from the nanospheres will result in a biodistribution profile which is not totally representative of the nanospheres alone. Since free label remains at the injection site to some degree (14%), the injection site values for the PLGA nanosphere systems may be slightly overestimated. Conversely, free radiolabel is not taken up by the lymph node macrophages and thus the loss of radiolabel will result in an underestimation of the lymph node uptake for the nanosphere systems. Moreover, the results are considered to be well indicative of a situation where an incorporated 'drug' is being delivered to the regional nodes for therapeutic effect. Free radiolabel ('drug') does not accumulate in the lymph node macrophages. In contrast, radiolabel ('drug') detected in the lymph nodes after dissection must have been delivered there by the nanospheres. It is also possible, but considered unlikely, that the nanospheres, empty of label ('drug') could have then passed through to the circulation.

The results herein are presented in terms of percentage uptake of the administered dose. Other workers in this field often express the results in terms of percentage uptake per gram of tissue. The average weight of a popliteal lymph node is around 12 mg. For poloxamine 908 coated PLGA nanospheres, the uptake by the popliteal lymph node was 10% of the administered dose. This equates to 1000%/g. For the iliac node, 4.6% of the administered dose was taken up. Taking the weight of this node to be 13 mg, then the uptake equates to 354%/g. Other workers, investigating the lymphatic uptake of a lymphotrophic polyglucosylated macrocomplex following intravenous administration, report 35–45%/g in the central lymph nodes of rats [22]. Accumulation of ultrasmall superparamagnetic iron oxide (USIOP) particles in the lymph nodes has also been reported, with 3.6%/g in the mediastinal nodes 24 h after intravenous administration to the rat [23]. Following subcutaneous administration of liposomes, the highest reported lymph node uptake was 4.5% of the injected dose for the primary node (popliteal) and 1.8% for the secondary node (iliac) [4]. Using the weight of nodes used above, this equates to 375%/g and 138%/g for the primary and secondary nodes respectively. The results presented herein for PLGA nanospheres are, therefore, far superior to previous results obtained with other biodegradable nanospheres. Only results obtained with model polystyrene nanospheres have produced higher lymph node uptake [2].

In conclusion, the results show that it is possible to deliver therapeutic and diagnostic agents to the regional lymph nodes using biodegradable nanospheres following interstitial administration. We are presently studying alternative methods to label PLGA nanospheres as well as alter animal models displaying pathological states that involve the regional lymphatics.

Table 5
The distribution of surface modified PLGA nanospheres, 6 h after subcutaneous administration to the rat

Polymer	Injection site	Total lymph nodes	Liver	Blood	Recovery
Poloxamer					
401	54.7 ± 9.9	6.6 ± 2.0	2.1 ± 0.2	8.8 ± 1.6	84.3 ± 15.4
402	64.4 ± 5.1	7.2 ± 0.8	2.5 ± 0.3	9.8 ± 2.5	96.6 ± 1.4
403	66.7 ± 2.0	9.4 ± 5.7	2.0 ± 0.3	10.1 ± 4.0	98.4 ± 6.3
407	45.9 ± 4.1	11.3 ± 4.6	8.8 ± 1.9	10.3 ± 0.9	90.0 ± 3.1
Poloxamine					
901	Flocculation				
904	69.7 ± 7.2	12.4 ± 2.3	1.9 ± 0.7	4.5 ± 0.8	96.3 ± 7.5
908	49.9 ± 5.7	16.8 ± 3.3	5.4 ± 0.6	10.8 ± 2.6	95.2 ± 2.9

Data are expressed as % of initial dose ($n = 3$).

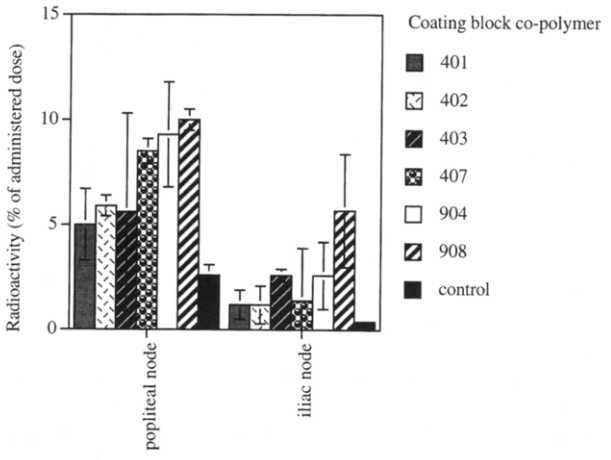

Fig. 2. Regional lymph node uptake of PLGA nanospheres.

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