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# Surface engineered nanospheres with enhanced drainage into lymphatics and uptake by macrophages of the regional lymph nodes

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**Abstract**

The concept of steric stabilization as used in colloid science is applied to carefully manipulate the drainage and lymphatic distribution of subcutaneously administered model polystyrene nanospheres. A wide range of synthetic polyoxyethylene (POE)/polyoxypropylene (POP) block co-polymers of poloxamine and poloxamer series have been used to produce sterically stabilized nanospheres. We have found a correlation between the length of the stabilizing POE chains of the block co-polymers and nanosphere drainage and passageway across tissue lymph interface in dermal lymphatic capillaries in the rat footpads; the longer the POE chains, the faster the particle drainage. Nanospheres conditioned with block co-polymers of POE chains of 5–15 ethylene oxide units are effectively opsonized in lymphatics; a process which dramatically enhances sequestration (up to 40% of the administered dose) by macrophages of the regional lymph nodes. If the dimensions of the stabilizing POE chains of the poloxamines and poloxamers exceed the range of the Van der Waals force of attraction, opsonization fails to occur and rapidly drained engineered vehicles escape clearance by macrophages of the regional nodes, reach the systemic circulation and remain in the blood for prolonged periods. These observations suggest that a lymphatic delivery composition based on polymer-coated particles will be advantageous for many applications in clinical and experimental medicine.

*Key words:* Lymphatic system; Lymph node; Nanosphere; Macrophage; Poloxamine; Poloxamer; Drug carrier

## 1. Introduction

An interstitially administered carrier system for the delivery of diagnostic and therapeutic agents to the regional lymph nodes should have two characteristics. Firstly, it should spread well from the injection site and, secondly, it should provide good uptake in regional nodes. In addition to the integrity of the lymphatic system, it is known that both the particle size and its surface characteristics can influence the rate of colloid drainage from the subcutaneous injection site into the dermal lymphatic capillaries and phagocytosis by lymph node macrophages [1–3]. Particles up to 100 nm in diameter are preferentially transported into the lymph capillaries and phagocytosed in the lymph nodes, whereas larger particles will, for a long time, be trapped in the interstitial space [2]. However, because of poor drainage, even uptake of small particles into regional lymph nodes is generally low and values from 1–10% of the administered dose 2–24 h after subcutaneous administration have been reported [1–3]. Here we report that by using the concept of steric stabilization [4], it is possible to control the rate of drainage from the subcutaneous injection site and manipulate the lymphatic distribution of a model particle following subcutaneous administration.

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## 2. Materials and methods

Polystyrene nanospheres, 60 nm in diameter, were purchased from Polysciences (UK) and surface labelled with Na[$^{125}$I] (Amersham International, UK) as described in detail elsewhere [4,5]. All poloxamines and poloxamers were a gift from BASF, USA. The weight average polyethylene glycol/polyethylene oxide equivalent molecular mass of poloxamines was determined by the method of raised temperature gel-permeation chromatography (RAPRA Technology, UK) as described in detail elsewhere [6] and was 2,079, 3,193 and 22,560 Da for poloxamine 901, 904 and 908, respectively. The ethylene oxide content (mol%) of poloxamines was measured by NMR spectroscopy (Brucker 360 MHz) in deuterated chloroform by a comparison of the integration from the methyl peak (generated from the POP segment of the molecule) to the total ethylene oxide/propylene oxide signal [6]. The corresponding values were 20.8%, 50.7% and 88.0% for poloxamine 901, 904 and 908, respectively. Microspheres were coated by preincubation in a 0.1% (w/v) solution of various poloxamines at room temperature overnight. Adsorption of the polymers onto the surface of nanospheres was confirmed by measuring the surface potential of nanospheres in 10 mM McIlvaine buffer, pH 7.0, [4] by means of Laser Doppler Anemometry as described in detail previously [4]. The corresponding zeta potentials were $-15.6 \pm 0.3$ mV, $-5.0 \pm 0.2$ mV and $-1.9 \pm 0.1$ mV for poloxamine 901-, 904- and 908-coated nanospheres, respectively. Excess polymer was removed by dialysing the nanosphere suspension (Spectrum Medical Industries, USA; $M_r$ cut-off 100,000) against a large volume of de-ionized water. Removal of excess polymer was monitored by the iodine assay [7]. The quantity of the polymer adsorbed onto the surface of nanospheres was determined from an adsorption isotherm [4] and was $74 \times 10^{-8}$, $50 \times 10^{-8}$ and $9 \times 10^{-8}$ mol/m$^2$ for poloxamine 901, 904 and 908, respectively. These values represent a surface density of approximately 2.2, 3.3 and 18.5 nm$^2$ of polystyrene/molecule of poloxamine 901, 904 and 908, respectively.

Groups of three male Wistar rats, body weight $150 \pm 10$ g, were injected subcutaneously into the dorsal surface of the left footpad with either uncoated or polymer-coated nanospheres (100 $\mu$l equivalent to


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0.1 mg of latex nanospheres). 5 min before sacrifice 50 μl of patent blue dye was injected into the footpad to facilitate easy localization of the regional lymph nodes. Finally, rats were sacrificed at various time points and the nanosphere-associated radioactivity was measured in the footpad, regional lymph nodes (which is defined as popliteal, inguinal, iliac and renal nodes), blood and organs of the reticuloendothelial system. No radioactivity was detected in the right footpad and its associated regional nodes.

Two hours after subcutaneous administration of either uncoated or poloxamine coated (904 and 908) nanospheres the intradermal tissue, popliteal and iliac nodes were cut into small blocks and immersed in fixative (2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) for 4 h. After initial aldehyde fixation the samples were further processed in the conventional manner as described in detail elsewhere [4]. Micrographs were taken with an electron microscope (JEOL 1200EX) operated at 80 kV. Polystyrene spheres do not scatter electrons effectively and are thus visualized in the electron microscope as translucent spheres. In order to determine whether artifacts were produced by tracer particles, animals were injected with 100 μl of water for injection or a solution of poloxamine 904 and the tissue was fixed and processed for ultrastructural studies. Non-injected animals were also examined.

## 3. Results and discussion

In accordance with previous studies [8] we have used small-sized polystyrene nanospheres (60 nm in diameter) as model particles since they are preferentially transported into the lymph capillaries and phagocytosed in the lymph nodes (Fig. 1a and b). Specimens observed at 2 h after subcutaneous injection of control uncoated nanospheres revealed extracellular aggregation of the particles at the footpad (Fig. 2a) and as a result the drainage of nanospheres into initial lymphatics was slow. Even at 24 h post-administration approximately 70% of the dose remained at the interstitial site (Fig. 1a). Our electron microscopic observations were in accord with the earlier studies of Leak [9] and demonstrated that the intercellular cleft between adjacent sinusoidal endothelial cells (patent junction) provided the major route for the passage of spheres (micrographs not shown). Macrophages of the regional nodes frequently phagocytosed the drained aggregates of nanospheres (Fig. 2b); approximately 10% and 2% of the dose were sequestered by popliteal and iliac nodes, respectively, at 24 h post-administration (Fig. 1a and b). Serum opsonization of control nanospheres reduced the tendency for aggregation at the injection site (micrograph not shown) and resulted in more rapid dermal clearance (Table 1).

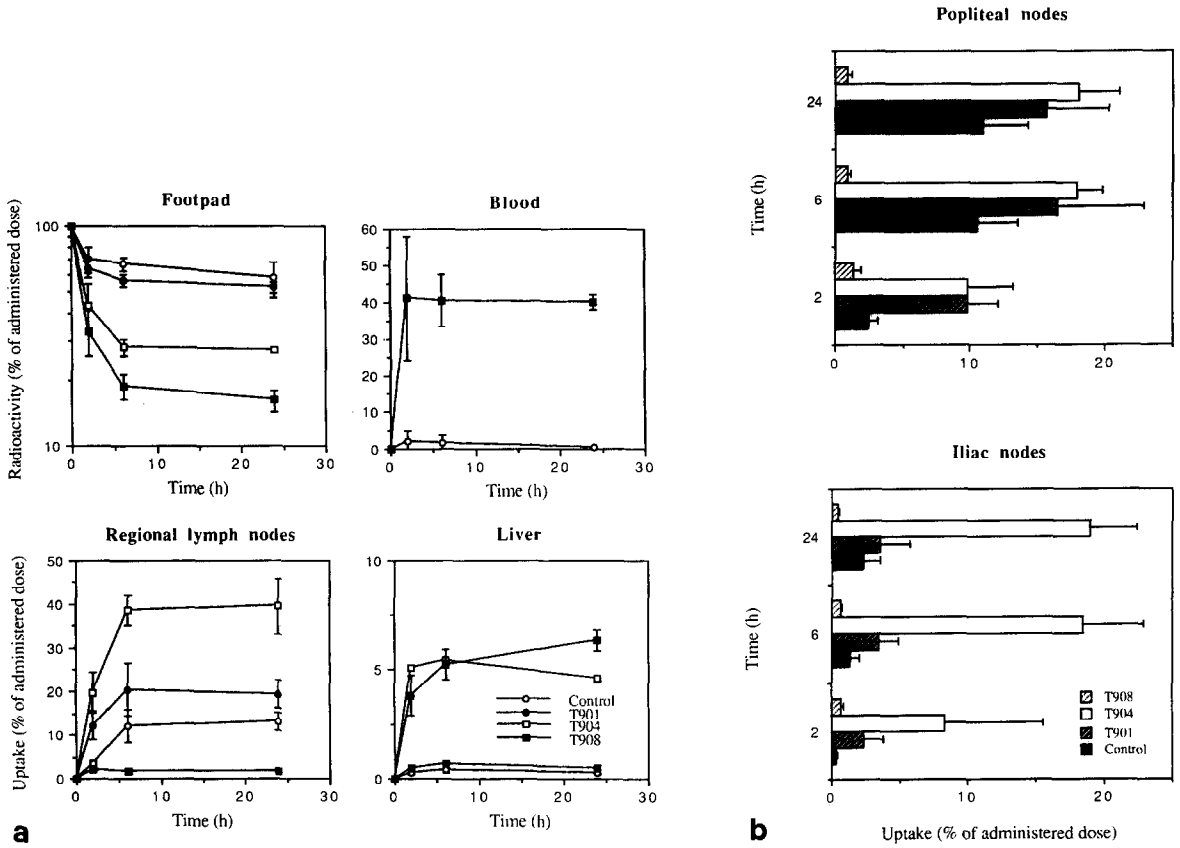


Fig. 1. Kinetics of lymph node and organ distribution of uncoated and poloxamine-modified polystyrene spheres following subcutaneous administration into rat footpads. The results are presented as a percentage of the administered dose ± S.D. In some cases the error bars are hidden within the symbols. Blood values are only shown for uncoated (control) and poloxamine 908-coated (T908) nanospheres since corresponding blood values for poloxamine 901- (T901) and poloxamine 904- (T904) coated particles overlaps with that of control nanospheres. In control experiments, 2 h following subcutaneous administration of free radioactive iodine together with either unlabelled nanospheres or poloxamines or with both nanospheres and poloxamines resulted in accumulation of less than 0.2% of the initial dose of radioactivity in the regional lymph nodes. The free radiolabel was rapidly cleared from the injection site and became associated with thyroid and bladder and, subsequently, excreted in urine.

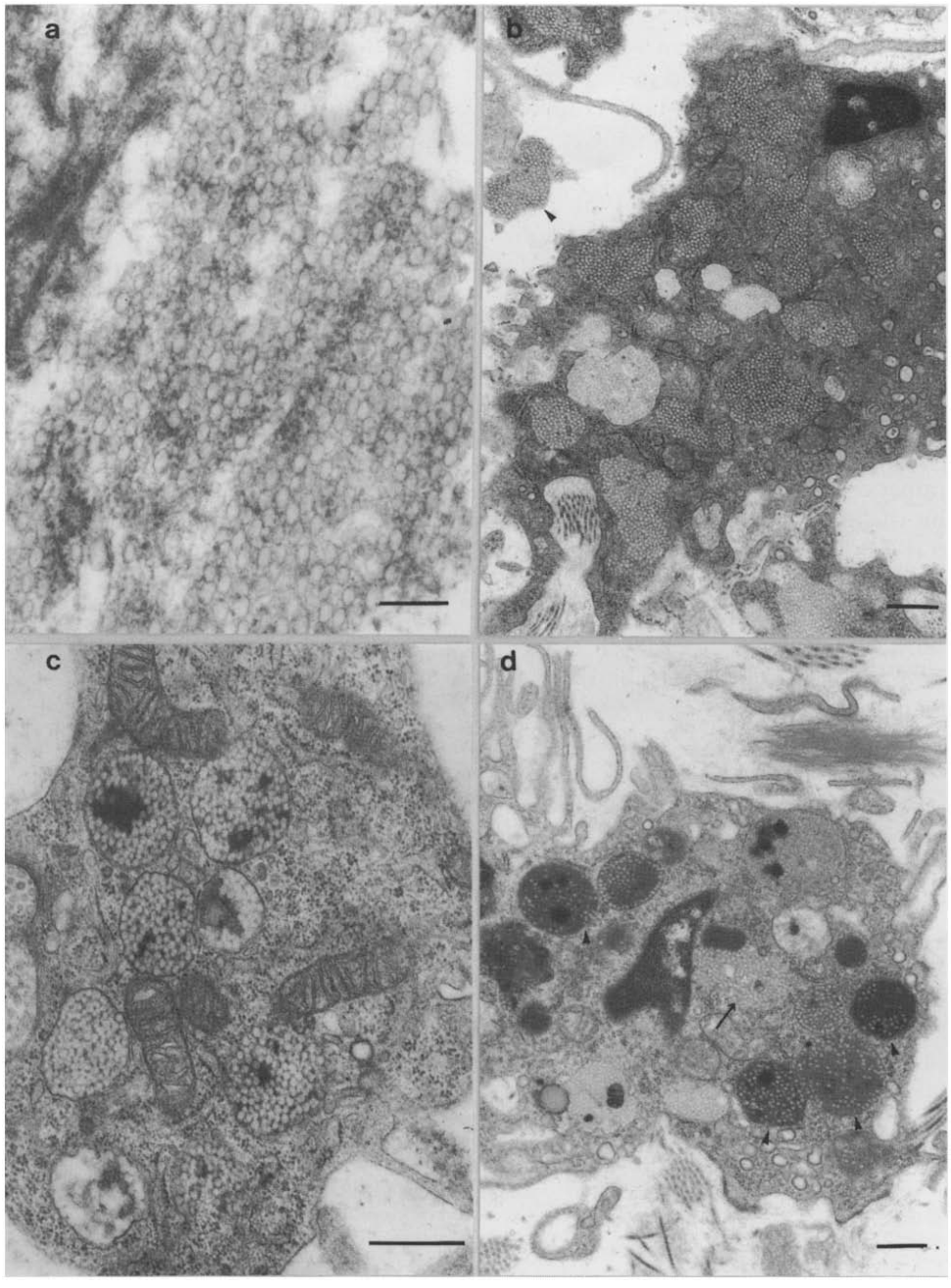


Fig. 2. Electron micrographs of intradermal tissue and regional lymph node phagocytic cells. (a) Large aggregates of uncoated nanospheres which were often found in the intradermal tissue; this process can greatly be minimized by coating the nanospheres with poloxamines and poloxamers; the longer the POE chains of the block co-polymers the lesser the tendency for aggregation. Bar = 200 nm. (b) A phagocytic cell of the subcapsular sinus from popliteal node containing large amounts of uncoated nanospheres; the cell appears to have sequestered spheres in aggregates since no well-defined lysosomal structures are apparent. Note extracellular aggregates of nanospheres (arrow head) similar to those observed intradermally. Bar = 500 nm (c) A phagocytic cell from subcapsular sinus of an iliac node containing large amounts of phagocytosed poloxamine 904-coated nanospheres in well-defined lysosomal structures. Bar = 500 nm. (d) A phagocytic cell at intradermal tissue containing phagocytosed nanospheres which were opsonized prior to administration; spheres appear to be taken up both in aggregates (arrow) and in non-aggregate form (arrow heads). Bar = 500 nm.

Both the drainage of intradermally injected spheres and uptake by the lymph node macrophages can be manipulated by conditioning their surface with synthetic POE/POP block co-polymers (poloxamines and poloxamers), Fig. 1a and b. These polymers can adsorb strongly onto the surface of polystyrene spheres via their hydrophobic POP segments while their hydrophilic POE segments extend from the nanosphere surface and provide stability to the particle suspension by suppressing aggregation (steric stabilization) [4]. We suggest that the

rapid drainage of the tested poloxamine-coated nanospheres from the intradermal tissue, when compared to that of uncoated particles, may be related to this 'steric-stabilization' phenomenon (Fig. 1a and b) [4]. Thus, the hydrophilic POE segments of the poloxamines may minimize the interaction of microspheres with the amorphous ground substances or gel-like matrix of the interstitium and facilitate nanosphere movement through the water-rich/colloid-poor and colloid-rich/water-poor phases, which co-exist within the interstitium [10], into the lymphatics. For effective stabilization of polystyrene nanospheres with polyethyleneglycol type materials, it is essential that the dimensions of the stabilizing chains of the polymers exceed the range of the Van der Waals attraction force [4]. These conditions are best met for poloxamine 908, rather than that of poloxamine 901 and 904, and as a result the drainage from the injection site of poloxamine 908 is most rapid when compared to that of other poloxamines (Fig. 1a and b) at all time points (poloxamines 901, 904 and 908 contain 4, 15 and 119 ethylene oxide units, respectively, per POE chain, whereas they all share a central POP region consisting of 68 propylene oxide units) [4,5]. These observations can also be reproduced with the related poloxamer series 401, 402 and 407 (Fig. 3).

Localization of the poloxamine-modified nanospheres in regional lymph nodes, particularly in popliteal and iliac nodes, is dramatically enhanced for poloxamine 904 and to a lesser extent for that of poloxamine 901 when compared to that of uncoated control spheres at 2 h post-administration (Fig. 1a and b). This distribution was even maintained after prolonged hours. Histological observations demonstrated that the phagocytic cells of the subcapsular sinus of cortex and the classical macrophages of the medula in both the popliteal and iliac nodes were mainly responsible for the clearance of these poloxamine-coated nanospheres (Fig. 2c). In contrast to these poloxamines with low POE chain lengths, coating of nanspheres with a poloxamine of a longer POE chain (poloxamine 908) dramatically reduced lymph node localization and showed increased blood concentration when compared to that of uncoated control spheres at all time points (Fig. 1a and b). This observed difference in lymph node sequestration among the poloxamine-coated particles may be attributed to their different surface characteristics. The steric barrier imposed by hydrophilic POE chains of the poloxamines has been shown to suppress both opsonization processes and interaction of particles with phagocytic cells; a process which is directly proportional to its chain length (the shorter the POE chain, the lesser the steric-barrier activity) [4,5]. Therefore, it is not surprising that lymph node macrophages can efficiently filter the drained poloxamine 901- and 904-coated particles rather than that of poloxamine 908-coated particles during the course of the experiment. This concept is further supported by the observation that the interstitially administered poloxamine 908-coated nanospheres can reach the systemic circulation, remain within the blood, and avoid extensive clearance by Kupffer cells and spleen macrophages [4,5,8] even at 24 h post-administration (Fig. 1a). In contrast, the short POE chains of the poloxamine 901 and 904 may, thus, allow the opsonization processes to occur, presumably in the initial lymphatics, and hence facilitate recognition of nanospheres by the node macrophages. This suggestion is based on the opsonization experiments (Table 1). Opsonization of nanospheres in serum prior to administration not only enhanced particle retention in both popliteal and iliac nodes but also stimulated phagocytosis by macrophages at the injection site (Fig. 2d) when compared to unopsonized control nanospheres. Again, the lymph node distribution of nanospheres is not poloxamine specific and can be reproduced with the related poloxamer family of the appropriate POE chains (Fig. 3).

In summary, we have demonstrated that the concept of steric stabilization is beneficial in enhancing lymphatic absorption of particulates from the subcutaneous injec-

Table 1
Lymphatic distribution of uncoated and poloxamine-coated nanospheres opsonized in serum

| Sites | Uptake (%) of administered dose) | | | | | |
|---|---|---|---|---|---|---|
| | Uncoated | | 904-coated | | 908-coated | |
| | −serum | +serum | −serum | +serum | −serum | +serum |
| Footpad | 74.3 ± 17.2 | 52.2 ± 11.3 | 45.6 ± 7.0 | 32.3 ± 5.8 | 33.4 ± 7.8 | 37.4 ± 3.6 |
| *Regional nodes* | | | | | | |
| Popliteal | 3.9 ± 0.4 | 16.4 ± 1.6 | 12.1 ± 1.3 | 10.8 ± 2.4 | 1.3 ± 0.6 | 1.3 ± 0.4 |
| Iliac | 0.5 ± 0.1 | 9.6 ± 5.4 | 13.9 ± 2.8 | 18.4 ± 2.3 | 0.7 ± 0.2 | 0.4 ± 0.1 |

Polystyrene nanospheres (60 nm in diameter) were incubated in 50% (v/v) of autologous serum for 5 min at room temperature before subcutaneous administration into rat footpads. Animals were killed 2 h after injection. For experimental details see the legend to Fig. 1. Prior opsonization of nanospheres in serum had no effect on circulation time and clearance by organs of the reticuloendothelial system (liver and spleen) and the results were comparable to those of unopsonized nanospheres as illustrated in Fig. 1. Total radioactivity recovered at the end of the experiment was more than 85% of the administered dose in all cases.

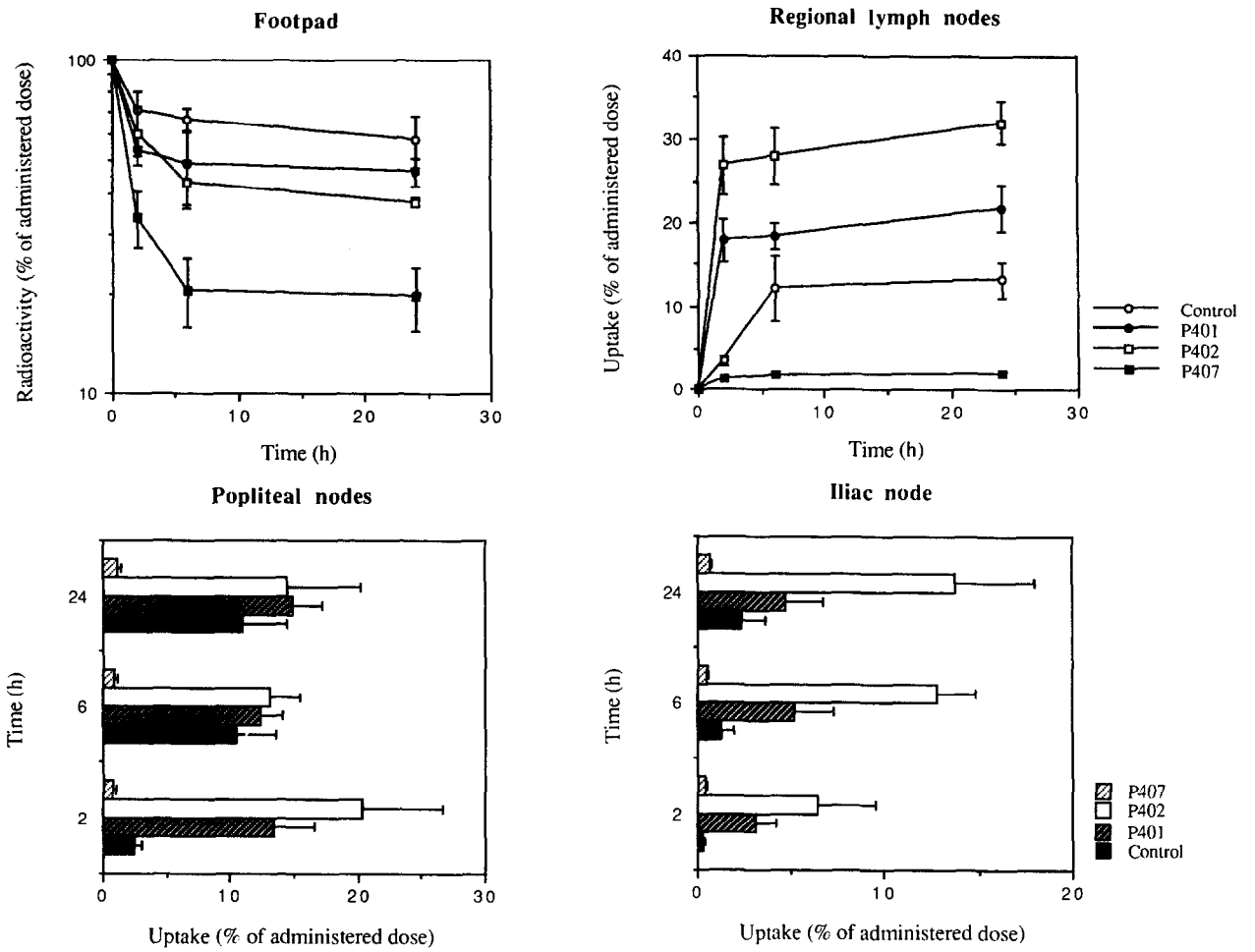


Fig. 3. Kinetics of lymph node distribution of uncoated and poloxamer-modified nanospheres following subcutaneous administration into rat footpads. Poloxamers 401, 402 and 407 contain 5, 11 and 98 ethylene oxide units, respectively, per POE chain, whereas they all share a central POP region consisting of 67 propylene oxide units. The corresponding zeta potential values were $-8.3 \pm 0.8$ mV, $-16.3 \pm 1.1$ mV and $-1.9 \pm 0.1$ mV for poloxamer 401-, 402- and 407-coated nanospheres, respectively. These polymers were selectively used since their central POP region contain a similar number of propylene oxide units to those of the tested poloxamines. Such a high content of propylene oxide units are necessary to anchor the polymers firmly onto the surface of nanospheres. For this reason polymers with low content of POP were not used since they can be displaced easily from the surface of nanospheres in biological environments [5,15]. Block co-polymers of poloxamine and poloxamer types containing 20–80 ethylene oxide units but sharing a central POP region consisting of 65–70 propylene oxide units are not commercially available. For experimental details see legend to Fig. 1.

tion site, but the steric barrier activity should not be too strong as this will limit the ability of the regional lymph nodes to sequester such engineered particles. We envisage that a lymphatic delivery composition based on polymer-coated particles will be advantageous for many applications to include vaccine design, studying the kinetics of lymph node macrophage re-population following selective elimination of lymph node phagocytic cells, enhanced imaging and visualisation modalities (lymphoscintigraphy and X-ray), radiation therapy for ablation of metastatic diseases, and site-specific delivery of drugs and therapeutic agents for prevention of tumour metastases and for treatment of various lymph node diseases particularly of those agents which accumulate or hide in macrophages (such as human immunodeficiency virus, filariasis, and brucellosis) [2,3,11–13]. Biodegradable carriers based on poloxamine 908 or poloxamer 407 may provide an alternative route for the delivery of therapeutic agents to the systemic circulation and may be used as a sustained release system [14].

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