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Modulation of lymphatic distribution of subcutaneously injected poloxamer 407-coated nanospheres: the effect of the ethylene oxide chain configuration

S.M. Moghimi*

Molecular Targeting and Polymer Toxicology Group, School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton BN2 4GJ, UK

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Abstract **Lymphatic distribution of interstitially injected poloxamer 407-coated nanospheres (45 nm in diameter) is controlled by surface configuration of the ethylene oxide (EO) segments of the adsorbed copolymer. At low poloxamer surface coverage, EO tails spread laterally on a nanosphere surface and assume a 'flat or mushroom-like' configuration. Such entities drain rapidly from the subcutaneous site of injection into the initial lymphatic, when compared to uncoated nanospheres, and subsequently are captured by scavengers of the regional lymph nodes. In vitro experiments have also confirmed that such entities are prone to phagocytosis. When the equilibrium poloxamer concentration is at 75 μg/ml or greater the EO chains become more closely packed and project outward from the nanosphere surface. These surface-engineered nanospheres drain faster than those with EO chains in mushroom configurations into the initial lymphatic, escape clearance by lymph node macrophages, reach the systemic circulation, and remain in the blood for prolonged periods. These experiments provide a rational approach for the design and engineering of nano-vehicles for optimal lymphatic targeting and are discussed.**

Key words: Adsorption thickness; Drug carrier; Lymphatic; Macrophage; Poloxamer; Targeting

1. Introduction

The thin-walled and fenestrated lymphatic microvessel is easily penetrated by colloidal particles after injection into the extracellular space [1]. Once inside the vessel, particles that are transported with lymph are usually cleared by macrophages located in the lymph nodes [1]. These means of particulate transportation and clearance mechanisms can be taken into clinical advantage. For example, interstitial delivery of colloidal diagnostic agents has been of benefit in determining regional spread of cancer and assessing lymphatic function either by lymphoscintigraphy or indirect lymphography [2–5].

The behaviour of particles following interstitial administration is controlled by a number of physicochemical and biological factors [2]. Physicochemical considerations include particle size and their surface characteristics [1,2,6–8]. Generally, the size of the particles must be larger than 20 nm to prevent their leakage into the blood capillaries. Although larger particles (>100 nm) may carry a considerable amount of therapeutic or diagnostic agents they move very slowly from the site of injection into initial lymphatics; the drainage often takes a number of days [2]. This slow transit may induce local inflammation and renders particles susceptible to phagocytosis or macropinocytosis at the injection site. Even the movement of small particles (20–100 nm range) from the interstitial sites into regional lymph nodes is generally slow and uptake values hardly exceed 10% of the administered dose even 24 h post injection [1,2]. This is usually due to particle aggregation and/or interaction with the ground substance of interstitium [1]. Previous work initiated by the author [7] demonstrated that by using the concept of steric stabilisation one could successfully control the rate of particle drainage from the subcutaneous injection site and manipulate their lymphatic distribution. For example, steric stabilisation of model polystyrene nanoparticles can be achieved by surface coating with poloxamers, which are ABA type block copolymers [9]. The 'A' segment is comprised from ethylene oxide (EO) units, whereas the central 'B' region is formed from propylene oxide (PO) units. The longer the EO chain of the poloxamers (while maintaining the length of the hydrophobic PO segment), the less tendency there is for particle aggregation as well as interaction with the interstitial elements. This results in rapid particle drainage into the initial lymphatics [7]. In addition, the length of the EO chain was shown to control the extent of particle–macrophage interaction. For example, the strong steric barrier of poloxamer 407, which arises from 98 EO units per chain, suppresses particle opsonisation in lymph and/or interaction with macrophage receptors [7]. Such engineered entities drain rapidly into the initial lymphatics, escape clearance by lymph node macrophages, reach the systemic circulation, and remain in the blood for prolonged periods of time [7]. To enhance simultaneously both rapid particle drainage from interstitium and capture by regional lymph node macrophages, particles require coating with poloxamers containing 4–15 EO units per chain (e.g. poloxamers 401 and 402) [7].

In view of these findings, it is imperative to further examine the relationship between physicochemical properties of poloxamer-coated nanoparticles and their lymphatic distribution and establish requirements for optimal targeting. Therefore, this manuscript examines the effect of surface density and the resultant configuration of the EO chains of the poloxamers on

*Corresponding author. Fax: (44)-1273-679333.
E-mail address: s.m.moghimi@brighton.ac.uk (S.M. Moghimi).

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the extent of drainage and lymphatic distribution of model polystyrene nanoparticles following interstitial injection.

2. Materials and methods

2.1. Particles and surface modification procedures

Polystyrene nanospheres, 45.4 ± 0.7 nm (polydispersity index 0.08 ± 0.01), manufactured by Polysciences (USA) were obtained from Park Scientific (Northampton, UK) and surface-labelled with Na[^{125}I] (Amersham Pharmacia Biotech, UK) by a radiation-induced bonding of idoine as described in detail previously [7,10]. The labelled particles were cleaned by dialysis prior to surface modification with copolymers. Poloxamers 401, 402 and 407 (Table 1) were the kind gifts of BASF (Mt. Olive, NJ, USA) and were dissolved in 10 mM McIlvaine buffer, pH 7.2. In accordance with previous experiments [7] nanospheres were coated by preincubation in a 0.1% (w/v) solution of various poloxamers at room temperature overnight. The equilibrium concentration of poloxamers used in nanosphere coating (0.1% w/v) is well above the final plateau region of their respective adsorption isotherm [7]. In some experiments, nanospheres were incubated with different concentrations of poloxamer 407 in order to achieve different percentages of surface coverage at points below the top plateau region of the adsorption isotherm (the top plateau of the adsorption isotherm is reached at an equilibrium poloxamer concentration of 180–210 μg/ml at 25°C with the designated batch of nanospheres). The adsorbed layer thickness of poloxamers on polystyrene nanospheres was measured by photon correlation spectroscopy, using a Zetasizer 3000 (Malvern Instruments, UK), in 10 mM McIlvaine buffer, pH 7.2, at 24°C as described in detail elsewhere [11]. Briefly the measurements were determined at a wavelength of 633 nm, scattering angle of 90°, dispersant viscosity of 0.89 cP and refractive index of 1.35.

2.2. Lymphatic distribution studies

Groups of three male Wistar rats, body weight 150 ± 10 g, were injected subcutaneously into the dorsal surface of the left footpad with either uncoated or poloxamer-coated polystyrene nanospheres (in a total volume of 100 μl containing 0.1 mg polystyrene nanoparticles). Rats were sacrificed either at 2 or 6 h post injection and the nanosphere-associated radioactivity was measured in the footpad (whole foot), regional lymph nodes (whole popliteal or primary, and iliac or secondary nodes), blood and organs of the reticuloendothelial system, RES, (liver and spleen) using a gamma counter. Due to macrophage heterogeneity the whole liver and spleen were counted for radioactivity. No radioactivity was detected in the right footpad (dermal region) and its associated regional lymph nodes. To determine the amount of radioactivity in the blood, a total blood volume per rat of 7.0% of body weight was assumed [12]. The correction factor for the blood content of the injected footpad was determined by subtracting the amount of radioactivity associated with the non-injected footpad. The correction factor for the blood content in the liver and the spleen was determined as previously [12].

2.3. Nanosphere uptake by peritoneal macrophages

Resident peritoneal macrophages were obtained by washing the peritoneal cavity of rats with Hanks' balanced salt solution (HBSS), followed by centrifugation at 150×*g*, 5 min [13]. Contaminating red cells were lysed by Tris-ammonium chloride buffer (pH 7.2) [13]. Cells were washed twice in HBSS and resuspended in the same buffer.

Table 1
Structure of the A_1-B-A_2 type block copolymers (A_1=A_2)

Poloxamer type	A_1 block (EO units)	B block (PO units)	A_2 block (EO units)
Poloxamer 401	5	67	5
Poloxamer 402	11	67	11
Poloxamer 407	98	67	98

Macrophages were plated in 48-well tissue culture dishes at a density of 5×10^5 cells per well in HBSS containing 10% v/v foetal calf serum. After 24 h incubation at 37°C, ^{125}I-labelled polystyrene nanospheres of different surface characteristics (10^{10} particles) were added and the cells were left to incubate for an additional 3 h. Prior to cell incubation, free poloxamer molecules were removed from the nanoparticle suspensions by centrifugation at 200 000×*g* for 30 min and subsequent washings. All incubations with polystyrene nanoparticles contained 10% v/v serum, thus to account for possible opsonisation events [11]. At the end of the incubation, cells were washed three times with HBSS, then solubilised with KOH. The total radioactivity associated with cell lysate was determined using a gamma counter. For each nanoparticle type triplicate incubations were performed. Uptake of uncoated nanoparticles was considered as 100%. The results of polymer-treated nanospheres are expressed as % uptake of uncoated particles.

3. Results and discussion

The data in Table 2 demonstrate the adlayer thickness of poloxamers on polystyrene nanospheres as well as the lymphatic localisation of polymer-coated nanospheres following interstitial injection into rat footpad. It has generally been accepted that the surface density of the adsorbed block copolymers can affect polymer configuration, e.g. 'mushroom' versus 'brush' configurations [11,14,15]. In addition, the thickness of the coating layer can reflect surface configuration of the adsorbed or grafted polymers on nanoparticles [11,14–17]. Since the equilibrium concentration of the poloxamers used in Table 2 for the surface modification of nanospheres is in the plateau region of their respective adsorption isotherm [7], the EO chains are thought to project from the surface of polystyrene nanospheres and assume 'mushroom-brush' intermediate and/or 'brush-like' configuration(s) [11,14,15]. Although, the fastest drainage from the injection site into the initial lymphatic occurs with the poloxamer 407-coated systems, nanosphere capture by lymph node is minimal even at 6 h post injection. Conversely, the rate of drainage is considerably slower with poloxamer 401- and 402-coated systems, but particle capture by regional nodes is maximised. In contrast, the bulk of uncoated nanospheres remain at the injection site. The differential biodistribution of particles is due to the extent of

Table 2
Biodistribution of uncoated and poloxamer-coated polystyrene nanospheres following subcutaneous injection

Coating	Coating thickness	Sacrifice time (h)	Distribution (% of injected dose)				
			Footpad	1° node	2° node	RES	Blood
None	–	2	79.3 ± 3.2	1.7 ± 0.6	0.8 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
		6	71.6 ± 2.8	3.8 ± 0.2	1.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
401	1.40 nm	2	64.3 ± 1.7	13.9 ± 2.8	4.6 ± 1.1	2.9 ± 0.3	1.2 ± 0.6
		6	51.7 ± 3.6	16.3 ± 1.7	8.8 ± 1.3	5.3 ± 0.3	0.4 ± 0.1
402	2.59 nm	2	60.8 ± 3.7	17.6 ± 3.6	8.2 ± 1.9	1.7 ± 1.0	0.9 ± 0.2
		6	48.7 ± 1.9	19.6 ± 1.3	14.3 ± 2.6	2.1 ± 0.6	0.8 ± 0.3
407	8.84 nm	2	34.5 ± 1.6	0.7 ± 0.4	0.2 ± 0.1	2.6 ± 0.1	49.6 ± 2.1
		6	13.7 ± 0.8	0.5 ± 0.1	0.2 ± 0.1	4.4 ± 0.7	60.3 ± 2.5

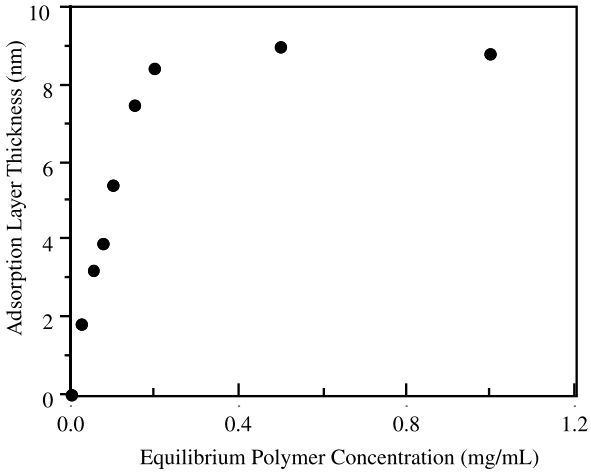

Fig. 1. Adlayer thickness of poloxamer 407 on polystyrene nanoparticles as a function of the equilibrium poloxamer concentration.

surface opsonisation or direct interaction with macrophage receptors, and these are controlled by the efficacy of the imposed steric barrier of the poloxamer EO chains, as discussed previously [7]. Unlike the uncoated system, where the major site of nanosphere accumulation in the regional lymphatic is the primary lymph node (popliteal node), surface coating with both poloxamers 401 and 402 further enhances particle retention in the secondary (illiac) node (Table 2). Therefore, it appears that for rapid and effective lymph node targeting the projected thickness of the coating polymer should not exceed 3.0 nm (as in the case of poloxamer 402-coated nanospheres).

On the basis of the above suggestions it should be possible to simultaneously enhance the drainage of poloxamer 407-coated particles from the subcutaneous injection site and their retention in regional lymph nodes. This could be achieved if the adlayer thickness of poloxamer 407 on the surface of polystyrene nanospheres is adjusted to 1.5–2.5 nm (a thickness layer similar to those of poloxamers 401 and 402, see Table 2). Therefore, the adlayer thickness of poloxamer 407 on polystyrene nanospheres as a function of the equilibrium poloxamer concentration was examined. The data in Fig. 1 demonstrate that the adlayer thickness of poloxamer 407 on polystyrene nanospheres increases with increasing the equilibrium polymer concentration and is in accordance with the previous suggestions of Baker and Berg [14]. The thickness rises rapidly at low concentrations and levels off at bulk polymer concentrations corresponding to the plateau adsorption coverage (the adsorption isotherm is not shown). These observations indicate changes in configuration of the EO chains with increasing polymer concentration. At low surface coverage, EO tails are less densely packed and gyrate although, eventually finding bare patches on the particle surface causing them to spread laterally assuming a 'flat or mushroom-like' configuration [16]. This mode of adsorption explains why the adlayer thickness values are very low (less than 3.5 nm) at equilibrium poloxamer concentrations below 0.05 mg/ml. Interestingly, the measured adlayer thickness of poloxamer 407 on nanospheres at polymer concentrations below 0.05 mg/ml is very similar to those of poloxamers 401 and 402, where the equilibrium polymer concentration was in the plateau region of the adsorption isotherm (Table 2). Next, the lymphatic distribution of nanospheres was tested following interstitial injection. Remarkably, the extent of drainage and lymph node localisation of particles pre-coated with poloxamer 407 at concentrations below 0.05 mg/ml are similar to those of poloxamer 401- or 402-coated nanospheres both at 2 and 6 h post injection (Table 3). These observations suggest that the EO chains of poloxamer 407 in 'flat or mushroom' configuration are not only effective in suppressing nanosphere aggregation at the interstitial sites, but also the assumed configuration fails to efficiently camouflage particles against macrophage recognition. It should be emphasised that similar observations are obtained with poloxamer 401 and 402 but with EO chains in a brush-like configuration (Table 2). Therefore, it seems that partial surface coverage (in the region of 11–15% of total surface area) with poloxamer 407 is sufficient to minimise nanoparticle aggregation at interstitial sites and simultaneously enhance clearance by lymph node scavengers. This statement is further supported by in vitro experiments with peritoneal macrophages, as these cells were able to recognise poloxamer-coated particles with EO chains in a predominantly mushroom configuration (Fig. 2). However, these particles are recognised significantly to a lesser extent than their uncoated counterparts, and this further supports the presence of the relatively hydrophilic EO chains in close proximity to the surface. These observations are also in agreement with a recent report from this laboratory where microspheres coated with methoxypoly(ethylene glycol)-$_{5000}$ in a mushroom regime were found to be prone to phagocytosis in vitro [11].

As the equilibrium concentration of poloxamer 407 increases, the thickness of the adsorbed layer also increases (Fig. 1) [14,15]. This is believed to be due to repulsion between EO and PO chains [16]. The repulsion causes EO chains to desorb from the surface and assume 'mushroom-brush' intermediate and/or 'brush-like' configuration(s) [16]. Such surface

Table 3
The effect of equilibrium concentration of poloxamer 407 on biodistribution of subcutaneously injected polystyrene nanospheres

[Polymer] (mg/ml)	Coating thickness	Sacrifice time (h)	Distribution (% of injected dose)				
			Footpad	1° node	2° node	RES	Blood
None	–	2	82.1 ± 3.2	1.4 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
		6	76.3 ± 2.8	2.8 ± 0.7	1.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
0.025	1.85 nm	2	57.4 ± 3.9	15.6 ± 1.7	10.2 ± 2.1	4.3 ± 1.1	1.1 ± 0.4
		6	45.3 ± 1.9	17.3 ± 1.1	13.4 ± 0.9	5.1 ± 0.2	0.9 ± 0.1
0.050	3.20 nm	2	50.3 ± 2.7	11.1 ± 3.3	8.8 ± 1.9	5.2 ± 0.2	0.9 ± 01
		6	39.6 ± 1.9	12.6 ± 1.2	13.3 ± 0.7	7.3 ± 0.6	0.8 ± 0.3
0.075	3.92 nm	2	42.9 ± 3.2	3.2 ± 1.5	2.8 ± 1.9	7.7 ± 1.2	32.1 ± 2.3
		6	19.6 ± 1.7	4.1 ± 0.6	3.1 ± 0.2	10.2 ± 2.1	45.6 ± 2.1
0.100	5.40 nm	2	36.3 ± 1.8	0.7 ± 0.6	0.3 ± 0.2	2.1 ± 0.2	50.2 ± 2.4
		6	11.8 ± 0.9	0.8 ± 0.4	0.3 ± 0.1	3.1 ± 0.4	66.1 ± 1.3

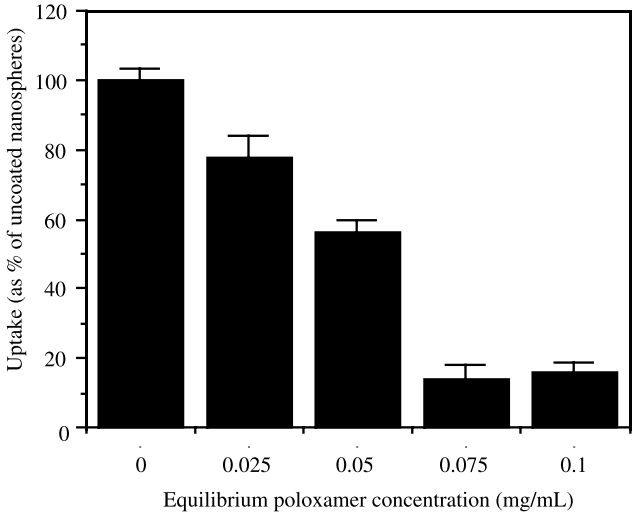

Fig. 2. Interaction of nanoparticles with rat peritoneal macrophages. Uptake of uncoated nanoparticles is considered as 100%. The results of poloxamer-coated nanoparticles are expressed as % uptake of uncoated nanoparticles.

configurations are highly effective in preventing particle aggregation at the injection site as well as particle capture by lymph node scavenger cells. The effect is observed at an equilibrium poloxamer 407 concentration of 0.075 mg/ml, corresponding to an adlayer thickness of 3.92 nm (Table 3). The relationship between the adlayer thickness (hence, the EO configuration) and phagocytosis is further illustrated by in vitro experiments where nanoparticles with surface EO chains in brush configuration show resistance to macrophage recognition (Fig. 2).

In summary, this work has established an interesting correlation between the surface characteristics of colloidal particles and their lymphatic distribution. By controlling the surface configuration of the EO segments of poloxamers, and hence the adlayer thickness, or vice versa, the lymphatic distribution of sterically stabilised particles can be modulated (e.g., lymphoscintigraphic tracing versus lymph node mapping). The described relationship between surface properties of nanoparticles and their biodistribution provides a simple but a rational means for the design and engineering of lymphotropic entities, rather than choosing coating materials on a random basis [18]. Therefore, this report for the first time shows that a stealth polymeric coating can be used to design carrier systems that can be targeted efficiently and rapidly to the desired lymph node elements (for drug delivery or medical imaging) following interstitial injection. These suggestions may also be applicable to liposome engineering for lymphatic targeting. Incorporation of 5–7 mol% monomethoxypoly-(ethyleneglycol)$_{2000}$-phosphatidylethanolamine (mPEG$_{2000}$-PE) into liposomal lipid bilayer is known to dramatically enhance the drainage of interstitially injected vesicles into the initial lymphatics [6,19]. However, such vesicles are recognised poorly by scavengers of the regional lymph nodes and are passed into the blood stream [19]. Based on measurements of ultrasound velocity and absorption in liposome suspensions it appears that at a concentration of 5–7 mol% mPEG$_{2000}$-PE, the surface grafted mPEG$_{2000}$ assumes an intermediate mushroom-brush conformation, whereas at lower concentrations (up to 4 mol%) the surface PEG molecules display a non-overlapped mushroom regime [20]. Therefore, to optimise the lymphatic targeting of sterically stabilised liposomes a combination of an appropriate quantity of mPEG-lipid (up to 4 mol%) and targeting ligand (e.g. mannose or antibodies grafted to phospholipids or to the distal end of PEG molecules) is proposed.

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