Development of Systems for Targeting the Regional Lymph Nodes for Diagnostic Imaging: *In Vivo* Behaviour of Colloidal PEG-Coated Magnetite Nanospheres in the Rat Following Interstitial Administration

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Purpose. Nanoparticles can be utilised for targeting drugs to the regional lymph nodes or as diagnostic agents. The surface modification of magnetite nanospheres with poly(ethylene glycol) (PEG) has been assessed by *in vitro* characterisation and *in vivo* studies following subcutaneous administration to the rat.

Methods. Magnetite nanospheres were prepared with a grafted PEG layer using various PEG lengths from 350 to 1000 Da. Thermogravimetric analysis was utilised to measure the adsorbed amount of PEG. Colloid stability was confirmed by measurement of the particle size and electrophoretic mobility. The kinetics of injection site drainage and lymph node retention were determined 2 hours after subcutaneous administration, for nanospheres coated with PEG lengths of 350, 550, 750, and 1000 Da. For the 750 PEG coated nanospheres, the kinetics of distribution was determined over a 48-hour time course.

Results. The distribution of the nanospheres was modified and the lymph node localisation enhanced by altering the surface coverage of PEG on the magnetic surface.

Conclusions. PEG-coated magnetite nanospheres with different surface characteristics can be utilised to target a diagnostic agent to regional lymph nodes.

KEY WORDS: lymph node targeting; subcutaneous administration; magnetite; poly(ethylene glycol); nanospheres; diagnostic imaging.

INTRODUCTION

The uptake of subcutaneously administered nanoparticles by the lymphatic system is governed by the rate of drainage of the nanoparticles from the injection site and their recognition and subsequent uptake by the macrophages resident in lymph nodes (1). Interstitially administered colloids with a size less than 100 nm, will drain through the interstitium to the initial lymphatic vessels, as determined by the diameter of the aqueous channels within the interstitium (2). Once within the lymphatic vessels, the passage of colloids is unhindered under normal conditions, until the material reaches the lymph nodes (1). Colloids may then be captured within the lymph nodes either by phagocytosis by resident macrophages or by a mechanical filtration process (1). Colloidal particles will pass through the lymph nodes if they are sufficiently small to avoid the filtration processes and if they have a surface coating that permits them to avoid capture by macrophages (3). Such particles will eventually reach the systemic circulation and behave as though they were administered by the intravenous route. Thus a colloid, administered interstitially for lymphatic targeting purposes, should drain effectively from the site of injection and be well retained in the regional lymph nodes. The concept of surface modification, wherein model polystyrene nanospheres are coated with block co-polymers that provide a sterically stabilised hydrophilic surface, has been used previously to control both the rate of drainage of the colloid from a subcutaneous injection site and to manipulate lymphatic distribution (4). This previous work resulted in a reported maximum uptake of 40% of the amount administered by normal regional lymph nodes for polystyrene nanospheres, of 60 nm diameter, coated with the block co-polymer poloxamine 904 (4). Later, this concept has been applied successfully to biodegradable nanospheres of poly(lactide-co-glycolide) that were surface modified with poloxamer and poloxamine block co-polymers (5) and to co-polymers of poly(lactide)-poly(ethylene glycol), (PLA:PEG) (6).

The concept of surface modification of subcutaneously administered colloids can also be applied to the diagnostic field for the development of magnetite as a contrast agent in diagnostic magnetic resonance imaging (MRI). The lymph nodes can be the site of spread of metastatic disease and a pathway for the growth of most malignancies. It is, therefore, of importance to be able to detect the spread of cancer to lymph nodes and to differentiate between enlarged hyperplasic lymph nodes and metastatic nodes. Among the imaging techniques available, neither MRI nor Computed Tomography (CT) alone can normally accurately detect metastases in lymph nodes (7) and imaging could be improved with lymph node targeted contrast agents.

Following the successful targeting of lymph nodes using PEG coated colloidal systems, the aim of the present work was to assess the utility of PEG coated magnetites for lymph node imaging. PEG moieties of various chain lengths have been used to alter the surface characteristics of magnetite, and candidate systems have been evaluated as to their ability to target the regional lymph nodes, following subcutaneous administration to rats.

To date, the synthesis of polymer coated magnetite, including those containing derivatives of PEG, has been achieved by forming magnetite particles in an aqueous phase in the presence of a polymer species such as dextran (8) or α, ω-dicarboxymethyl PEG (9–11). This method is simple to perform and produces superparamagnetic particles with good colloidal stability that are ideal for use as MRI agents. However, for the case of PEG-coated magnetite, it is difficult to manipulate the amount of polymer adsorbed to the particles. A novel method of producing PEG-coated magnetite particles, involving the grafting of a PEG monolayer to magnetite by treatment with trimethoxysilane-PEG in an aqueous environment, has been developed (12) and has been utilized in this work.

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MATERIALS AND METHODS

Materials

End-substituted trimethoxysilane-PEG (SiPEG) with molecular weights of 350, 550, 750, and 1000 Da were obtained from Shearwater Polymers (USA). ^{59}Iron, in the form of iron chloride in a solution of 1M hydrochloric acid, with a specific activity of 37 MBq/ml, was purchased from Amersham International (UK). All other chemicals used were of analytical grade and were used as received.

Preparation of Colloidal Magnetite

Magnetite was prepared according to the method of Massart (13) and has been described in detail in Butterworth *et al.* (12). Typically, radiolabelled iron (III) chloride ($^{59}FeCl_3$) was added to a fresh mixture containing 1 ml of 1M iron (III) chloride ($FeCl_3 \cdot 6H_2O$) and 0.25 ml of 2M iron (II) chloride ($FeCl_2 \cdot 4H_2O$) dissolved in 2M hydrochloric acid. The resulting mixture was then added to 25 ml of 1M ammonium hydroxide (NH_4OH) with rapid stirring. The precipitated magnetite was immobilised with a permanent magnet and the aqueous supernatant decanted and discarded. The remaining slurry was then centrifuged for 2 min at 3000 rpm and redispersed in 10 ml of 1M tetra-methyl ammonium hydroxide $N(Me)_4OH$. The resulting colloid was dialysed against a large volume of de-ionised water to reduce the pH to around 11–12.

Magnetite-citric acid complexes were prepared for use as an uncoated control system, since they are colloidally stable at pH 4. Typically, citric acid (1M) was slowly added to colloidal magnetite until the pH was reduced to around 2. The reduction in pH resulted in flocculation of the particles due to the shift of the iso-electric point of the particles. However, the particles could be resuspended to form a stable colloid around pH 4 by titration with $N(Me)_4OH$.

Preparation of PEG-Coated Magnetite

Aqueous dispersions of radiolabelled magnetite (Fe_3O_4) in $N(Me)_4OH$ at pH 12 were coated by treatment with trimethoxysilane-PEG (12). The active coating reagents, formed in aqueous conditions, were presumed to be the corresponding hydroxyl silanes, which react via a condensation reaction with iron hydroxyl groups at the particle surface to give a siloxane linkage. For each coating reaction, the trimethoxysilane-PEG was added directly to stirred magnetite with the pH maintained above 11 by titration with NMe_4OH until dissolution was complete. The pH was then adjusted to 10.5, by addition of NMe_4OH or perchloric acid ($HClO_4$), as required, and the reaction mixture was stirred at 80°C for 4 hours.

For cleaning purposes, the particles were precipitated by the addition of di-sodium sulphate (Na_2SO_4). Using a powerful magnet to immobilise the flocculated particles, the excess soluble reagents were cleaned from the particles by carefully washing with water and decanting the supernatant ten times. The particles were resuspended as the electrolyte was removed from the particles by successive washing. After the final wash, the particles were dispersed simply by removal of the magnet followed by gentle swirling. The stability of the chemisorbed trimethoxysilane-PEG layer on the magnetite particles was previously confirmed (12).

Characterisation of Nanospheres

The amount of PEG adsorbed to the particle surface was determined by thermogravimetric analysis (TGA) using a Perkin-Elmer TGA7 instrument scanning from room temperature to 800°C at 20°C/min (12). The weight of the polymer component of the nanospheres was assumed to represent the difference between the percentage weight of the sample and the magnetite control, with a correction factor applied, as necessary, to account for the water content of the nanosphere. Using the specific surface area and density for magnetite, the polymer coverage (Γ) expressed in $\mu mol/m^2$, was calculated. Full details of this method are given in Butterworth *et al.* (12).

The particle size of the magnetite nanospheres was determined by Photon Correlation Spectroscopy (PCS) using a Malvern 4700 instrument (Malvern, UK), as previously described (14). The particle size was measured as a function of pH to assess the stability of the colloid. The magnetite nanospheres were diluted with 10^{-3} M potassium chloride and the pH adjusted using NMe_4OH or $HClO_4$, as appropriate. The electrophoretic mobility was measured as a function of pH using a Malvern Zetasizer 4 (Malvern Instruments).

Biodistribution of Nanospheres Following Subcutaneous Administration

Groups of three male Wistar rats, body weight 150–180g, each received a subcutaneous injection (100–150 μl equivalent to 0.1 mg) of the various magnetite systems into the dorsal surface of the left hind footpad, under halothane anaesthesia. A blood sample was collected from the tail vein prior to sacrifice of the animals by cervical dislocation at various time points after dose administration. The regional lymph nodes (popliteal, inguinal, iliac, and renal), liver, spleen, kidney, and footpad were removed for measurement of associated radioactivity in a gamma counter (LKB 1282 Compugamma CS, LKB, Turku, Finland). Residual radioactivity associated with the carcass was counted in a Well Counter (John Caunt Scientific, Eynsham, UK). For determination of the total dose in the blood, a total blood volume of 7.5% of body weight was assumed (15,16). Results were calculated as the percentage of the administered dose and are the mean of the three animals (± standard deviation (SD)).

RESULTS AND DISCUSSION

PEG Coverage

The chemisorption of the trimethoxysilane-PEG polymers of different PEG chain length onto magnetite is shown in Figure 1 where polymer coverage (in $\mu mol/m^2$) is plotted against equilibrium concentration. The isotherms are Langmuirian in nature (12). The polymer adsorption reached a plateau value at high values of c_{eq}, probably due to the formation of a monolayer on the nanosphere surface. The molar coverage (Fig. 1) decreased with an increase in polymer molecular weight. This is believed to be due to an entropy effect, which tends to oppose the polymer coating reaction (12). The adsorption of trimethoxysilane-PEGs with PEG moieties of 350 and 550 Da were similar, reaching a plateau of about 1.3 $\mu mol/m^2$, while plateau values of around 0.8 and 0.6 $\mu mol/m^2$ were obtained for PEG lengths of 750 and 1000 Da, respectively.

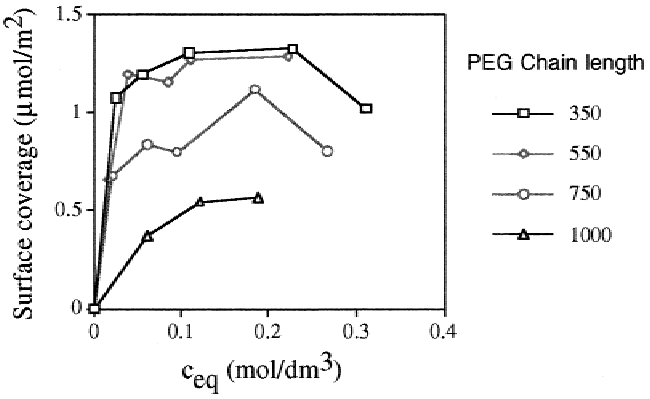

Fig. 1. Adsorption isotherms for the chemisorption of trimethoxysilane-PEG polymers onto magnetite; effect of PEG molecular weight.

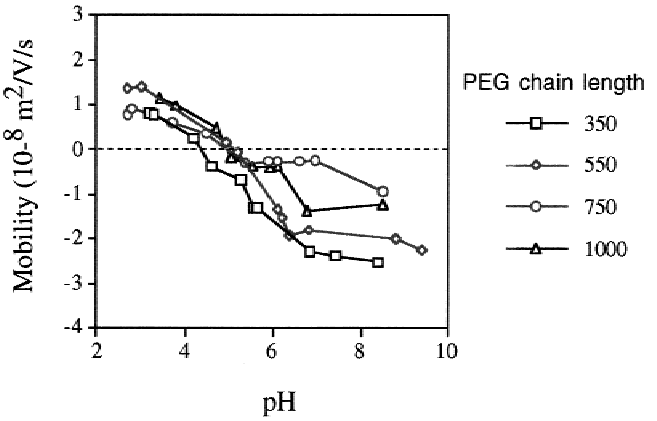

Fig. 3. The effect on pH on the electrophoretic mobility of magnetite particles coated with adsorbed layers of trimethoxysilane-PEG.

The area per attached PEG chain on the magnetite and the wt % of silicon can be calculated from the maximum adsorbed amounts for SiPEG 350, SiPEG 550, SiPEG 750, and SiPEG 1000 (12). The values for area per PEG chain were 1.26, 1.30, 2.08, and 2.91 nm^2, respectively, and for silicon content were 0.47, 0.46, 0.29, and 0.21 wt %, respectively. These latter values are relatively low, so that the quantity of silanol in a total dose of particles used in diagnostic imaging should not pose problems in terms of toxicity. The toxicity of silanols has been well reviewed by Iler *et al.* (17).

Particle Size and Surface Charge

The effectiveness of the steric stability of a colloidal suspension may be determined by the induction of particle aggregation/flocculation by the addition of increasing amounts of electrolyte, which will reduce the solvency of the stabilising chains to a point where the system becomes unstable. The effectiveness of the steric barrier will depend on the length of the hydrophilic chain adsorbed to the nanosphere surface, the molecular structure, the chain packing density and the surface coverage of the polymer (18). In this work the effectiveness of the PEG layer to stabilise magnetite was determined by measurement of the particle size and particle size distribution and the electrophoretic mobility. Data for the PEG-coated magnetite nanospheres are presented in Figures 2 and 3 and are plotted as a function of pH. The size of the nanospheres was in the range of 40–50 nm diameter and was generally consistent over the pH range investigated, indicating that extensive flocculation did not occur (Fig. 2). The apparent increase in particle size in the pH range 4–6 can be associated with the point of zero charge on the particles (see below). The electrophoresis results, presented in Figure 3, demonstrate that the PEG coated magnetite had a point of zero charge between pH values of 4.5 to 5.5. The magnitude of the mobility of the nanospheres over the pH range investigated was generally low. This is due to the shielding of the surface charge of the particles by the attached PEG layer and is dependent on both the thickness and density of the PEG layer. In general, the thickness of the PEG layer will increase with increasing molecular weight (PEG chain length). The PEG density is a function of molar coverage and increases with the affinity of adsorption. This was shown to increase as the molecular weight of the PEG decreased (Fig. 1). The net effect is that the shielding of the magnetite is greater at the extremes of PEG molecular weight due either to a thick PEG layer (PEG 1000) or a dense PEG layer (PEG 350).

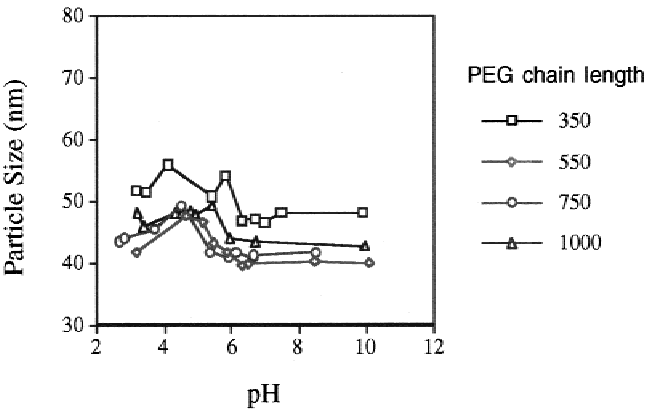

Fig. 2. The effect of pH on the particle size of magnetite particles coated with adsorbed layers of trimethoxysilane-PEG.

Interstitial Administration

Time Course Studies

Initial *in vivo* distribution experiments were performed in order to follow the time course of clearance of particles from the injection site and their appearance in the regional lymph nodes. Data for the clearance of magnetite particles coated with PEG 750 over a period of 48 hours are shown in Figure 4. For this system more than 50% of the labelled material had left the injection site by 2 hours. For most systems, no significant difference in remaining activity was found for later time points. Lymph node uptake appeared to reach a steady value at this time (data not shown). Thus, a time period of 2 hours was used in subsequent experiments.

The Effect of PEG Concentration and Chain Length

PEG coated magnetite particles prepared at different concentrations of PEG for different PEG chain lengths were prepared and administered interstitially to the rat. The data are summarised in Table I. In the majority of cases the mass balance figures are acceptable. For the uncoated particles, a large proportion of the dose (about 80%) remained at the injection site 2 hours after administration and lymph node

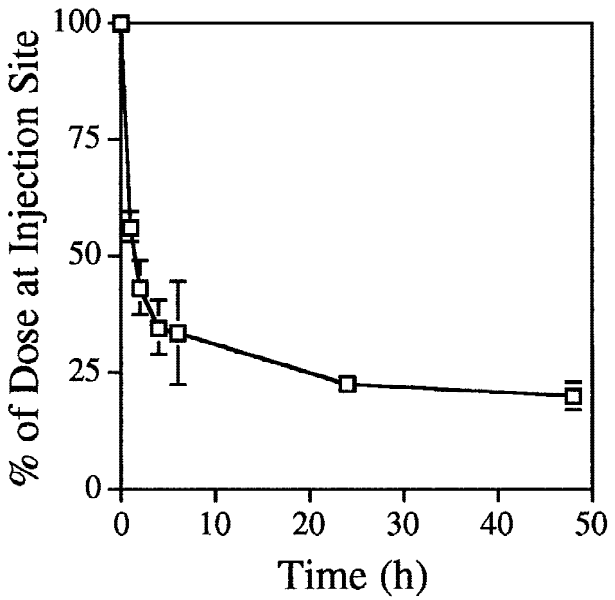

Fig. 4. Kinetics of drainage of magnetite particles coated with trimethoxysilane-PEG 750 from a subcutaneous injection site in the rat (PEG concentration 0.18 mol/dm^3).

uptake was low at 1.8% of the administered dose. Coating the particles with PEG generally gave rise to a pronounced reduction in the quantity remaining at the injection site, an increase in the quantity of the material found in the lymph nodes, and increased quantities in the blood circulation and the liver. Thus, the attachment of PEG to the magnetite particles provided an opportunity for such particles to be cleared more effectively into the lymphatics and a proportion of the particles could then be sequestered in the lymph nodes (to a maximum value 6.5%). A significant proportion of the dose was able to pass through the nodes into the blood for all systems tested. The amount found in the liver should reflect particles free in the blood pool (25% of circulating blood is found in this organ in mammals) as well as particles taken up by liver cells, Kupffer cells, and hepatocytes.

Generally, the attachment of the low molecular weight PEGs 350 and 550 to magnetite particles led to an increase in the injection site clearance and a slight increase in the lymph node uptake as compared to the control. With the highest molecular weight PEG the quantity remaining at the injection site at 2 hours was less than 40% but the particles appeared to have passed readily into the bloodstream and to remain in the circulation. It is well known that the attachment of PEG of a molecular weight of 2000 D or greater to a colloidal nanosphere will result in the particle being poorly recognised by macrophages and, if injected intravenously, to have a long circulation time (19). The attached PEG layer provides a steric barrier to the conditioning (opsonisation) of the particles by blood components. Apparently a molecular weight of 1000 Da is sufficient to provide a similar effect for particles administered interstitially.

Hawley *et al.* (6) have proposed that, in order to achieve good clearance from the injection site and for satisfactory uptake in the regional lymph nodes, the colloidal particles need to have a small size (less than 100 nm) and a surface that is neither too hydrophobic nor too hydrophilic. The hydrophobic particles will remain largely at the injection site (probably due to aggregation), while the hydrophilic particles will leave the injection site and pass unrecognized through the lymph nodes. A surface of intermediate properties should allow the particle to move into the lymphatic system (i.e., it should be sufficiently hydrophilic to prevent aggregation but sufficiently hydrophobic to be conditioned by blood compo-

Table I. The Biodistribution of Magnetite Nanospheres Prepared with Various Concentrations of SiPEG, 2 Hours After Subcutaneous Administration to the Rat[a]

PEG conc. (mol/dm^3)	Injection site	Total lymph nodes	Liver	Blood	Carcass	Mass balance
Uncoated	79.5 ± 3.4	1.8 ± 0.3	1.5 ± 0.3	6.6 ± 7.7	2.3 ± 1.0	91.7
PEG 350						
0.014	47.7 ± 0.6	0.4 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	48.2
0.025	77.3 ± 6.9	2.8 ± 1.3	2.3 ± 0.9	2.9 ± 0.9	1.3 ± 0.5	86.6
0.033	58.1 ± 8.2	4.7 ± 0.1	14.7 ± 1.4	0.0 ± 0.0	2.6 ± 0.2	80.1
0.068	56.4 ± 6.9	4.6 ± 0.6	10.6 ± 1.8	0.0 ± 0.0	1.6 ± 0.5	73.2
PEG 550						
0.06	49.7 ± 15.7	1.5 ± 0.1	23.3 ± 4.0	14.7 ± 3.5	7.6 ± 0.7	96.8
0.10	45.9 ± 8.8	1.3 ± 0.3	15.6 ± 0.7	29.1 ± 6.3	3.2 ± 0.6	95.1
0.16	44.5 ± 3.4	0.4 ± 0.1	6.0 ± 1.0	22.4 ± 3.6	12.3 ± 2.3	85.6
PEG 750						
0.010	87.0 ± 13.0	3.1 ± 1.2	0.3 ± 0.3	0.9 ± 0.2	0.0 ± 0.0	91.3
0.017	43.0 ± 10.1	3.7 ± 0.9	23.8 ± 4.4	16.3 ± 1.6	11.0 ± 0.9	97.8
0.031	78.4 ± 8.3	4.4 ± 0.6	1.1 ± 0.5	0.1 ± 1.0	0.6 ± 0.8	84.6
0.040	60.4 ± 6.4	5.9 ± 1.2	24.7 ± 3.0	2.9 ± 0.9	4.6 ± 0.7	98.5
0.058	72.7 ± 3.1	6.3 ± 1.1	11.1 ± 1.3	0.8 ± 0.3	4.4 ± 3.3	95.3
0.066	39.2 ± 8.0	0.9 ± 0.3	14.4 ± 2.1	26.9 ± 4.3	18.7 ± 0.2	100.1
0.100	63.5 ± 9.4	6.5 ± 1.3	14.9 ± 1.5	0.0 ± 0.0	4.1 ± 1.6	89.0
0.180	43.0 ± 5.6	0.8 ± 0.2	10.1 ± 1.6	44.2 ± 7.4	19.8 ± 2.9	117.9
PEG 1000						
0.058	37.8 ± 1.0	0.9 ± 0.0	6.9 ± 0.1	38.6 ± 7.5	15.2 ± 2.7	99.4
0.156	34.0 ± 1.9	0.7 ± 0.2	3.7 ± 1.0	36.7 ± 7.9	18.1 ± 4.0	93.2

[a] Mean percentage of administered dose (n = 3).

nents). The suggested optimal chain length of 750 Da found in the present work with magnetite compares well with results from previous studies using different colloidal particles.

For polystyrene particles coated with block copolymers, the material poloxamine 904 was found to be optimal (4). This block copolymer contains 4 hydrophilic PEG groups each as a chain length of 730 Da. For self-assembling PLA-PEG particles, a PLA-PEG ratio of 1.5:0.75 was found to provide good lymph node targeting (6). This polymer had a molecular weight of about 2000. The molecular weight of the PEG chain was 750 Da.

The results obtained for the magnetite particles coated with PEG 750 are generally encouraging, and lymph node uptake values representing greater than 6% of the dose have been obtained (Fig. 5). However, some of the systems prepared using PEG 750 demonstrated behaviour closer to that found for PEG 1000 (i.e., good injection site clearance but low lymph node uptake). It is noted that the values for the standard deviations for each group of 3 rats are acceptable, as are the mass balance values. Thus, the variability found is attributed to slight variations in the actual manufacturing process of producing the coated particles. It should be noted that a separate batch of particles was produced for each experiment involving 3 rats. It is suggested that the packing of the PEG chains at the surface could be slightly different from batch to batch even though we know from the data presented in Figure 1 that the systems were prepared at the plateau in the adsorption isotherm.

At a PEG molecular weight of 750, the coated magnetite system may present a surface that is close to some critical condition. Interestingly, in our previous studies with polystyrene particles coated with block copolymers, a well-defined relationship between lymph node uptake and PEG chain length (as expressed in terms of surface hydrophobicity) was found (4). The relevant figure from that paper is reproduced here in Figure 6 and shows that small changes in surface hydrophobicity (i.e. PEG chain packing) could lead to dramatic differences in lymph node uptake. It is suggested that the coated magnetite systems produced using PEG 750 in the present work are close to a similar discontinuity in their properties.

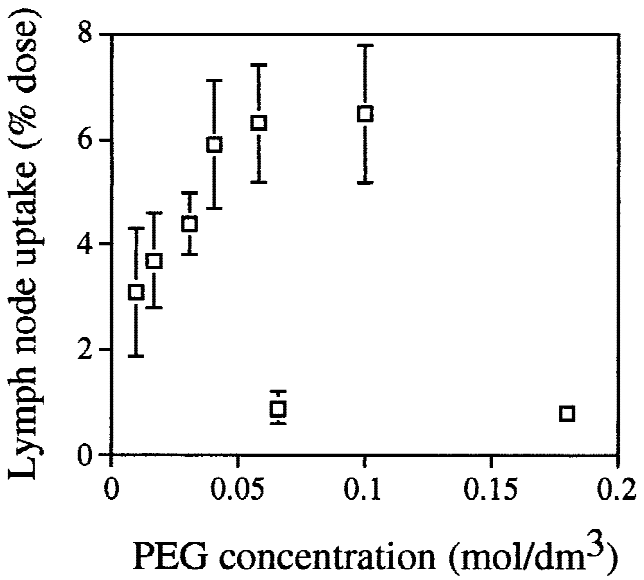

Fig. 5. Uptake of magnetite particles coated with trimethoxysilane-PEG 750 in regional lymph nodes following subcutaneous administration in the rat.

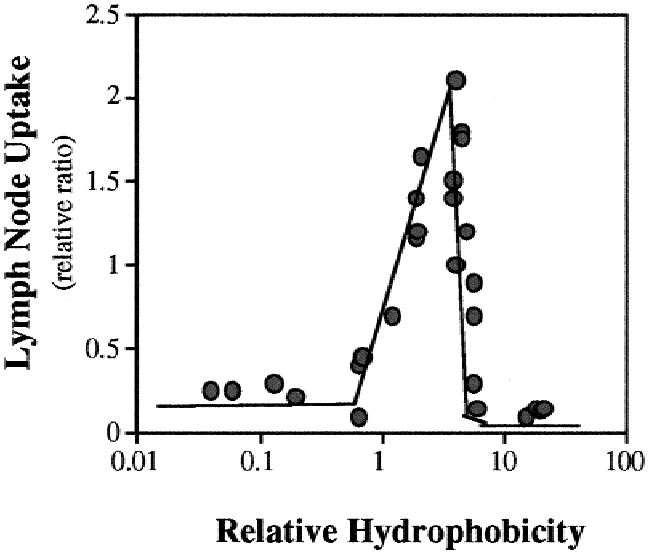

Fig. 6. Relationship between lymph node uptake of coated polystyrene nanoparticles and relative surface hydrophobicity (derived from reference 4).

The values for lymph node uptake obtained in the present work are much less than found previously for model particles in the form of polystyrene microspheres and for PLGA microspheres and PLA-PEG self-assembling systems. However, the results do represent a substantial improvement over the values found for uncoated magnetite particles. The values are similar to those obtained using PEGylated liposomes (20).

Further *in vitro* studies are planned in order to provide a more detailed understanding of the process of coating of the particles using trimethylsiloxane-PEG.

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