Surface Modification of Poly(lactide-co-glycolide) Nanospheres by Biodegradable Poly(lactide)-Poly(ethylene glycol) Copolymers

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The modification of surface properties of biodegradable poly(lactide-co-glycolide) (PLGA) and model polystyrene nanospheres by poly(lactide)-poly(ethlene glycol) (PLA:PEG) copolymers has been assessed using a range of *in vitro* characterization methods followed by *in vivo* studies of the nanospheres biodistribution after intravenous injection into rats. Coating polymers with PLA:PEG ratio of 2:5 and 3:4 (PEG chains of 5000 and 2000 Da, respectively) were studied. The results reveal the formation of a PLA:PEG coating layer on the particle surface resulting in an increase in the surface hydrophilicity and decrease in the surface charge of the nanospheres. The effects of addition of electrolyte and changes in pH on stability of the nanosphere dispersions confirm that uncoated particles are electrostatically stabilized, while in the presence of the copolymers, steric repulsions are responsible for the stability. The PLA:PEG coating also prevented albumin adsorption onto the colloid surface. The evidence that this effect was observed for the PLA:PEG 3:4 coated nanospheres may indicate that a poly(ethylene glycol) chain of 2000 Da can provide an effective repulsive barrier to albumin adsorption. The *in vivo* results reveal that coating of PLGA nanospheres with PLA:PEG copolymers can alter the biodistribution in comparison to uncoated PLGA nanospheres. Coating of the model polystyrene nanospheres with PLA:PEG copolymers resulted in an initial high circulation level, but after 3 hours the organ deposition data showed values similar to uncoated polystyrene spheres. The difference in the biological behaviour of coated PLGA and polystyrene nanospheres may suggest a different stability of the adsorbed layers on these two systems. A similar biodistribution pattern of PLA:PEG 3:4 to PEG 2:5 coated particles may indicate that poly(ethylene glycol) chains in the range of 2000 to 5000 can produce a comparable effect on *in vivo* behaviour.

KEY WORDS: poly(lactide)-poly(ethylene glycol) copolymers; poly(lactide-co-glycolide); biodegradable; nanospheres; steric stabilization; surface characterization; biodistribution; blood clearance.

INTRODUCTION

The rapid removal of intravenously administered colloidal drug carriers by the cells of the mononuclear phagocyte system such as the Kupffer cells of the liver and the spleen macrophages has been identified as the major barrier to target delivery of drugs to other organ or tissue sites within the body. Extensive investigations have been conducted by various research centres into the optimisation of carrier size and surface characteristics for successfully bypassing this obstacle (1,2,3). It has been shown by our own laboratory for model polystyrene nanospheres of 60 nm in diameter that coating with amphipathic triblock or star shaped copolymers, such as copolymers of polyoxyethylene-polyoxypropylene and polyoxyethylene-polyoxypropylene ethylene diamine, commercially available as Poloxamer and Poloxamine surfactants, can significantly alter the biodistribution of the nanospheres (4). Hence, coating with Poloxamer 407 and Poloxamine 904 resulted in a reduction in the level of liver/spleen accumulation and in a redirection of a significant portion of the administered particles to the bone marrow endothelial cells, while Poloxamine 908 coated particles exhibited a prolonged circulation time in the vascular compartment (5,6,7). Such discrimination in delivery has been suggested to be due to the nanosphere surface characteristics influencing the interaction with and thereby determining the degree and balance of opsonins and dysopsonins being adsorbed at the nanosphere surface (8).

The copolymers are bound to the polystyrene surface by hydrophobic interactions of polyoxypropylene chains with the hydrophilic polyoxyethylene chains protruding into the surrounding medium creating a sterically stabilizing layer (9). The formation of the copolymer layer results in a decrease in negative surface potential and an increase in surface hydrophilicity (10). Such coated nanospheres have *in vivo* biodistribution properties which depend on the particle size and coating properties. The exploitation of this knowledge to prepare biodegradable and bioresorbable carriers with surfaces that target specifically to predetermined sites in the body is currently under investigation in this laboratory.

The concept of modifying the surface by coating colloidal particles with amphipathic copolymers has also been applied to lipid stabilized emulsions (11) and liposomes (12,13,14). The latter systems have been prepared by either coating, adding polyoxyethylene lipid derivatives during the production, or coupling polyoxyethylene to preformed vesicles containing surface active groups. Prolonged circulation and reduced uptake by the cells of the mononuclear phagocytic system were shown for polyoxyethylene modified liposomes, and these effects were virtually independent of the lipid components of liposomes (15).

The aim of the present work was to assess the use of poly(lactic acid) (PLA) and monomethoxypoly(ethylene glycol) (PEG) copolymers (PLA:PEG) to achieve the surface modifications of polymeric nanospheres and hence changes in biodistributions comparable to those obtained for Poloxamines and Poloxamers coated polystyrene latex. These materials were described by Churchill and Hutchinson (16,17) and copolymers with various PLA:PEG ratios, molecular weights and structure have been synthesized. The copolymers have been discussed for their possible application as carriers to achieve drug targeting (16,17) and have been used as surface active agents in the preparation of poly(lactide/

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glycolide) nanospheres (18). Recently, Langer and coworkers reported nanospheres prepared from the blends of poly(lactic-co-glycolic acid) and diblock copolymers of poly(lactic-co-glycolic acid)-polyoxyethylene and showed a prolonged half-life of these nanospheres in the blood (19). The water soluble PLA:PEG copolymers (PLA:PEG 2:5 and 3:4) were selected for this study. These are AB-block copolymers with a relative hydrophobic PLA block and a relative hydrophilic PEG block. This structure makes the PLA:PEG copolymers good candidates for achievement of surface adsorption and effective steric stabilization. The advantage of the PLA:PEG copolymers over Poloxamers and Poloxamines is in replacing the polyoxypropylene moiety of the latter polymers with the biodegradable PLA chain.

The PLA:PEG polymers were used for coating of two nanosphere systems, model polystyrene particles which have been used extensively in previous modelling studies, and biodegradable poly(lactide-co-glycolide) (PLGA) nanospheres. The PLGA nanospheres were prepared either in the presence of the PLA:PEG copolymers or Poloxamine 908, or "surfactant free"—without the stabilizing copolymers. The latter system was, as well as the polystyrene nanospheres, subsequently incubated with an aqueous solution of the stabilizing copolymers, and the effect on the surface characteristics of the particles evaluated by *in vitro* methods. The nanosphere systems were characterised in terms of surface potential, surface layer thickness, flocculation properties and interaction with model protein and the biological fate of the nanospheres was determined after intravenous injection into rats.

MATERIALS AND METHODS

Poly(D,L-lactide-co-glycolide) (PLGA, 75:25) was purchased from Boehringer Ingelheim as Resomer® RG 755. According to the manufacturer's specification, the molecular weight of the copolymer as determined by gel permeation chromatography in chloroform using polystyrene standard is 63 kDa. Poly(lactic acid)-poly(ethylene glycol) (PLA:PEG) copolymers 2:5 and 3:4 were synthesized and supplied by Zeneca Pharmaceuticals plc, Macclesfield, Cheshire, U.K. The copolymers were synthesized by ring opening polymerisation of D,L-lactide in the presence of the corresponding methoxypolyethylene glycol and stannous octoate as a catalyst (16,17). Methoxypolyethylene glycol with 5000 Da molecular weight was used in the synthesis of PLA:PEG 2:5 and 2000 Da for PLA:PEG 3:4. The weight average molecular weight of the copolymers is 9500 Da for PLA:PEG 2:5 and 5400 Da for PLA:PEG 3:4 as determined by gel permeation chromatography using polyoxyethylene as standard. The copolymers were used as obtained. Poloxamine 908, ABA star shaped copolymer of polyoxyethylene-polyoxypropylene ethylene diamine, was chosen for the comparison as the molecular weight of one of its polyoxyethylene chains is comparable to that of PEG chain of PLA:PEG 2:5 copolymer. The polymer was purchased from BASF Corporation, USA, and used as obtained. As specified by the supplier, the average molecular weight of the copolymer is 25,000 Da and molecular weight of each of the four polyoxyethylene chains is 5000 Da. All other chemicals used were of HPLC purity grade. Deionized water used was chromatographically purified (Elgastat, Elga Ltd., U.K.). Polystyrene nanospheres of particle size 156 nm ± 4.2% (9.9% w/v dispersion) were obtained from Interfacial Dynamics Corporation, Portland, USA. The dispersion, prepared by an emulsion polymerization of styrene, can be considered free of any residual emulsifier.

Preparation of PLGA Nanospheres, Sterically Stabilized During the Production

To assess whether the PLA:PEG copolymers can be used in the production of PLGA nanospheres these were prepared by the precipitation-solvent evaporation method, as described previously (20), employing either PLA:PEG 2:5, 3:4 or Poloxamine 908 aqueous solutions (1% w/v) as the continuous phase. The PLGA was dissolved in acetone (10 ml, 20.0 mg/ml) and a mixture of deionized water and ethanol (1:1) was added dropwise (25G syringe needle) into the copolymer solution stirred by magnetic stirrer (Ika-Labortechink, Germany), until turbidity indicative of copolymer precipitation was visually observed. The suspension of these preformed nanospheres was then added to an aqueous surfactant solution (15 ml, 1% w/v) placed in a glass beaker (50 ml) and agitated by a magnetic stirrer at ambient temperature until complete evaporation of the organic solvent had taken place.

Incubation of Nanospheres with Copolymers

In order to characterise the coating layer of the PLA:PEG copolymers and Poloxamine 908, a dispersion of PLGA nanospheres (0.3% w/v) was prepared by the same method as described above, but without stabilizer in the aqueous phase, and subsequently coated. Polystyrene nanospheres were diluted to a 0.3% w/v dispersion with deionized water. Both types of nanospheres were coated by mixing the dispersion of the nanospheres with an equal volume of an aqueous solution of the stabilizer (2% w/v) and incubation overnight at room temperature. The PLGA and polystyrene nanospheres subsequently coated in this way with the stabilizing copolymers were used throughout the study, except where otherwise stated.

Morphological Characterization of Nanospheres by Transmission Electron Microscopy

The nanosphere dispersion was stained with phosphotungstic acid and the dried sample examined with an electron microscope (JEOL 1200 EX12, Japan).

Determination of Particle Size and Adsorbed Layer Thickness

The nanospheres were sized by photon correlation spectroscopy (PCS) using a Model K7025 multibit correlator (Malvern Instruments Ltd, Malvern, U.K.) in combination with a Commodore PET 3008 computer and Malvern spectrometer employing a helium laser (Siemens, Germany) as previously described (21). The samples were diluted with filtered deionized water and twenty measurements of each sample performed. The mean value and standard deviation

were calculated. The copolymer layer thickness was determined by comparing the radii of coated and uncoated nanospheres. The polydispersity was derived from Koppel's method of cumulants (22) and has been used to express polydispersity numerically. Using this approach, monodisperse latices should have a polydispersity index of 0.03 but correlative data from electron microscopy suggest that values less than 0.1 reflect particles sizes that are narrowly distributed.

Determination of the Surface Zeta Potential

Measurements were carried out using a Malvern Zetasizer IV (Malvern Instruments, UK), as described previously (23). The nanospheres were suspended in 10 ml of a low ionic strength buffer, 0.001M HEPES (Sigma, U.K.) adjusted to pH 7.4 by 0.1 M HCl. Four readings were taken for each sample and the mean values and standard deviations calculated.

Hydrophobic Interaction Chromatography (HIC) of Copolymers and Coated Nanospheres

The ability of the PLA:PEG copolymers to modify a particle surface in comparison to Poloxamine 908, was assessed applying HIC on the polystyrene nanospheres coated with the copolymers. HIC was carried out using alkylagarose gels (Chromatography affinity media, Kit MAA-8, prepacked in 2.5 ml columns, Sigma, USA). For these studies a range of gels from ethyl to dodecyl were used to characterize the copolymers, and butyl and pentyl gels were used for the coated particles. Samples (150 μl of 1% w/v solution of the copolymers or 0.3% w/v particles dispersion) were added to the columns and washed with a buffer which promotes the hydrophobic interaction (0.01M Tris-HCl in 1.5 M NaCl, equilibration buffer). To elute the copolymers from the gels deionized water was used to provide a decreasing salt gradient, whilst for the nanosphere samples, 0.1% of Triton X-100 in deionized water was used (elution buffer). The copolymer eluent was passed through a refractive index detector (Gilson model 131, France), and a UV detector at 380 nm (Linear readout ultraviolet spectrophotometer, Cecil Instruments) was used for the particle eluent. The signals were recorded via a chart recorder (Autograph S, Shandon Southern), and the areas under the equilibration and elution buffer chromatographic curves (AUC) obtained by the cut and weight method. The experiment was done in duplicate and the results shown are the mean value.

Stability of Nanosphere Dispersions

The stability of the nanosphere dispersions was monitored by measuring their turbidity as a function of the electrolyte concentration. Sodium sulfate solution (2.0 ml, 0.05, 0.1, 0.2, 0.4, 0.45, 0.50, 0.55, 0.6, 0.8 mol/l) was added to the nanosphere dispersions (0.250 ml, 0.15% w/v) in test tubes placed on a horizontal shaker (Ika-Vibrax, Ika-Labortechnik, Germany) and the turbidity of the dispersions was measured after 15 minutes (Uvikon 860 spectrophotometer, Kontron Instruments, U.K.). The results were also confirmed by measuring changes in the particle size by photon correlation spectroscopy. From a plot of dispersion turbidity versus electrolyte concentration the critical coagulation/flocculation concentration was determined.

The effect of pH on the stability of the nanosphere dispersions was determined by measuring the turbidity of the dispersions (0.250 ml, 0.15% w/v) in buffers of different pH (2 ml, 0.005 M McIlvaine buffer adjusted to various pH values with 0.1 M HCl or NaOH).

Adsorption of Albumin onto Nanospheres

Albumin solution (10 mg/ml) was prepared by diluting Albutein (20%, Alpha Therapeutics Ltd., U.K.) with 0.005 M McIlvanes buffer, pH 7.4, and was added dropwise (1 ml) to the nanosphere dispersions (0.5 ml, 0.15% w/v) in test tubes placed on a horizontal shaker (Ika-Vibrax, Ika-Labortechnik, Germany). After 2 and 24 hours incubation, the samples were analyzed by photon correlation spectroscopy, as described above. The radii of the control (the nanosphere dispersions in the buffer) and the incubated nanospheres were compared.

Preparation of 111In-labelled PLGA Nanospheres

PLGA nanospheres were radiolabelled by entrapment of the gamma-emitting isotope Indium-111 within the nanosphere during the preparation, as described previously (24). 50 μl of an Indium-111-oxine complex (37 MBq/ml at the activity reference date, Amersham International, U.K.) was added to the acetone solution of PLGA. The nanospheres were then prepared by the precipitation-solvent evaporation method without the surfactant in the aqueous phase and separated from the free label by gel permeation chromatography (radioactivity was assayed using an LKB 182 Compugamma CS, LKB Wallac, Finland) in aliquots taken prior to and after the nanospheres separation to assess the efficiency of the labelling process. The assessment of the labelling efficiency showed that 70.2% of the label was associated with the nanospheres.

Radiolabelling of Polystyrene Nanospheres

For *in vivo* studies polystyrene nanospheres were surface labelled with NaI-125 using a procedure based on that of Huh et al. (25). 2.5 μl of NaI-125 solution (3.7 GBq/ml at the activity reference date, Amersham International, U.K.) was mixed with 1 ml of the particle dispersion (0.6% w/v) and exposed to a Cesium-137 source (activity 5.54 × 104 ± 0.67% Rads per hour on 19/1/90) for 48 hours. After irradiation, excess free iodine was removed by dialysis (Spectropor® dialysis tubing, molecular weight cut off 100 kDa) against double distilled water, for three days with daily changes of the dialysis medium. The evaluation of the radioiodination efficiency revealed that 50% of the label was bound to the nanospheres.

Incubation of Radiolabelled Nanospheres with Copolymers

For *in vivo* studies the radiolabelled polystyrene and PLGA nanospheres were incubated with coating polymers as described above for non-labelled spheres.

Biodistribution Studies

For each system, three Wistar rats (150 ± 10g) were injected via the tail vein with a dispersion containing 1 mg of the nanospheres. 20 μl samples of blood were taken from the tail vein at various time intervals (15, 30 minutes, 1, 2 and 3 hours). The rats were sacrificed after 3 hours using 0.3 ml of pentobarbitone solution (60 mg/ml) and the liver, spleen, lungs, kidney and one femur (left hind leg) were removed. The thyroid was taken from the rats injected with NaI-125 labelled nanospheres. The organ and blood associated activity was counted using a gamma counter (LKB 182 compugamma CS, LKB Wallac, Finland). To determine the amount of nanosphere associated activity in the blood, a total blood volume per rat of 7.5% of body weight was assumed (26). The results are expressed as a percentage of the injected dose and are a mean of the three rats ± standard deviation (S.D.). The data for lung, kidney and femur are not presented, since the radioactivity associated with these organs was found to be negligible.

RESULTS AND DISCUSSION

Hydrophobic Interaction Chromatography (HIC) of PLA:PEG Copolymers

HIC was employed to assess the relative hydrophilicity/hydrophobicity of the PLA:PEG copolymers, in comparison to Poloxamine 908. Applied on the most hydrophilic gel, ethyl-agarose, the copolymers were washed through with the equilibration buffer in the void volume of the column, indicating an absence of hydrophobic interactions with the gel. When more hydrophobic gels were used the chromatograms, as illustrated in Fig. 1, were recorded. The areas under the equilibration and elution curves of the chromatograms were examined, and the results presented in Table I. They show that the sum of equilibration and elution AUC for all gels decreases in the following order: Poloxamine 908 > PLA:PEG 2:5 > PLA:PEG 3:4. The observed decrease indicates that some portion of the latter copolymers was bound to the gel by strong hydrophobic interactions and could not be removed under the conditions employed. For instance, using the dodecyl column, the area under the curve for PLA:PEG 3:4 copolymer indicates that a small portion of the copolymer was washed through the column by the equilibration buffer, and it would therefore have been expected that a large portion of the sample would be washed out by the elution buffer. However, the small area under the curve for the elution buffer indicates that the copolymer was only partly eluted and that a large fraction remained in the column. It should be noted that an exact comparison of chromatograms from different chromatographic gels is not possible due to slight variations in flow rate through the columns which caused the differences in recorded chromatograms. A comparison of the AUCs sum obtained on one gel type was therefore taken as indicative of differences in copolymer hydrophilicity. Hence, the copolymers could be placed in order of decreasing hydrophilicity: Poloxamine 908 > PLA:PEG 2:5 > PLA:PEG 3:4, which is in accordance with the molecular characteristics.

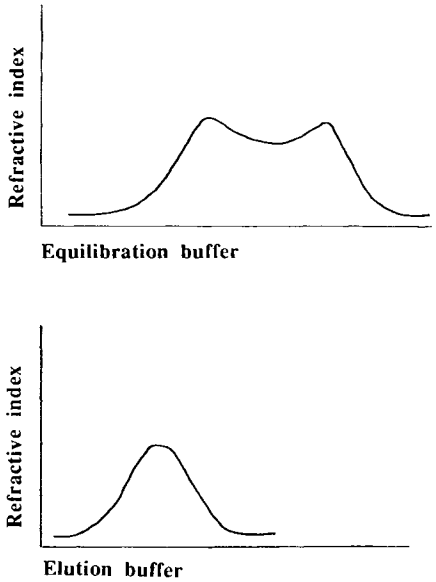

Figure 1. Hydrophobic interaction chromatogram on octyl-agarose column for PLA:PEG 2:5 copolymer.

Hydrophobic Interaction Chromatography of Coated Polystyrene Nanospheres

HIC was also applied to assess the ability of the PLA:PEG copolymers to alter the surface hydrophobicity/hydrophilicity of the coated polystyrene nanospheres (Table I). For the uncoated polystyrene nanospheres, the whole population was retained in the column when the equilibration buffer was used for washing, and the portion was washed through using elution buffer containing detergent. As could be expected from the above results the coating of the polystyrene nanospheres with the PLA:PEG's and Poloxamine 908 increased the hydrophilicity of the particle surface as compared to uncoated nanospheres. The PLA:PEGs coated polystyrene nanospheres were significantly less hydrophilic than the Poloxamine 908 coated colloid. The polystyrene nanospheres coated with the PLA:PEG 2:5 could be considered to be more hydrophilic than the corresponding 3:4 coated nanospheres. It should be mentioned that the shape of the chromatograms recorded (not shown, similar to Fig. 1) clearly demonstrates a heterogenicity in all the systems regarding nanosphere surface hydrophobicity.

Preparation of Nanospheres in the Presence of the Stabilizing Copolymers

Fig. 2 is a transmission electron micrograph of the PLGA particles prepared in the presence of PLA:PEG 2:5, and is also illustrative for the particles prepared with PLA:PEG 3:4 copolymer. The photograph demonstrates that the particles formed are discrete and spherical, in a nanometre size range. The particle size analysis by means of photon correlation spectroscopy of each batch prepared, showed that the average particle size between batches varies from 120 to 140 nm, but within each batch the nanospheres have a size and a polydispersity index (between 0.08 and 0.13) indicative of a relatively narrow particle size distribution. The TEM micrograph also shows the presence of the copolymer micelles in the sample. Studies investigating the behav-

Table I. Areas Under Curve (AUC) for the Equilibration and Elution Buffer of Hydrophobic Interaction Chromatograms for the PLA:PEG Copolymers and Poloxamine 908 and Polystyrene Nanospheres Coated with These Copolymers

	AUC for equilibration: AUC for elution buffer (summa of AUC)			
	Butyl-	Pentyl-	Hexyl-	Dodecyl-agarose
Copolymer				
PLA:PEG 2:5	549:161 (701)	850:315 (1165)	550:180 (730)	605:220 (825)
PLA:PEG 3:4	410:80 (490)	400:135 (535)	390:125 (515)	275:90 (365)
Poloxamine 908	720:265 (985)	815:360 (1175)	770:260 (1030)	760:490 (1250)
Nanospheres coated with:[a]				
PLA:PEG 2:5	995:735 (1700)	610:1600 (2210)	—	—
PLA:PEG 3:4	730:325 (1055)	385:332 (717)	—	—
Poloxamine 908	1890:1485 (3375)	1210:1855 (3065)	—	—

[a] Uncoated polystyrene nanospheres could not be washed through the column with the equilibration buffer.

iour of PLA:PEG copolymers in aqueous medium, including the formation of micelles, are currently being undertaken and will be reported in a separate paper.

Coating Layer Thickness of the Copolymers on Nanospheres

In order to assess the adsorbed layer thickness of the stabilizing copolymers the nanospheres were prepared in the absence of surfactant during the production, and subsequently incubated with the stabilizing copolymers. As preliminary studies showed a batch to batch variation of about 20 nm for PLGA nanospheres, all comparative experiments used nanospheres from the same batch.

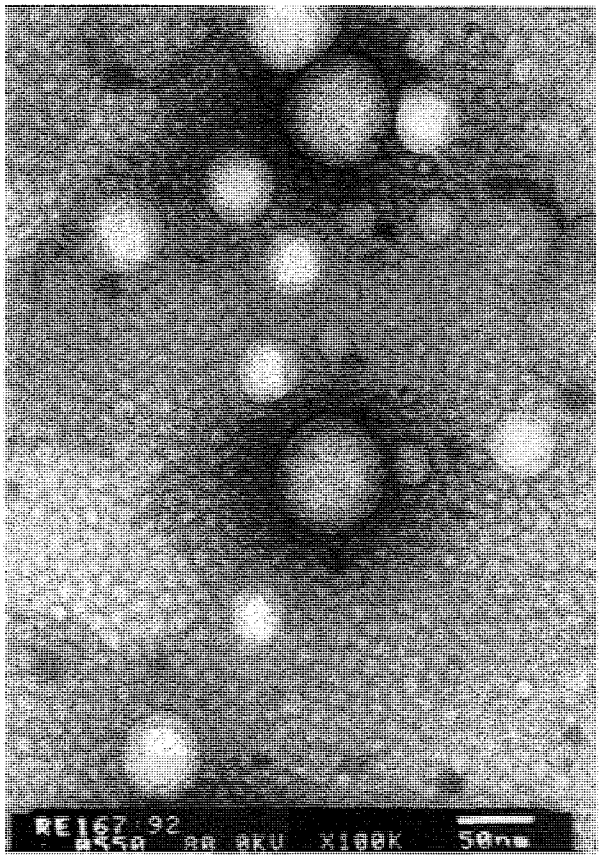

Figure 2. Transmission electron micrograph of PLGA nanospheres prepared in a presence of PLA:PEG 2:5 copolymer.

Table II shows the particle size of polystyrene and PLGA nanospheres before and after the incubation with PLA:PEG 2:5 and 3:4 copolymers, as well as with Poloxamine 908. An increase of the nanosphere size was observed, indicating a formation of a coating layer. Incubation of both the polystyrene and PLGA nanospheres with PLA:PEG copolymers resulted in significant increases in average nanosphere radii which may be directly related to the length of the PEG chain in the PLA:PEG molecule. These findings suggest that when adsorbed on both the polystyrene and PLGA nanospheres, PLA:PEG copolymers orientate with the PLA part towards the surface of the nanosphere and with the hydrophilic PEG part protruding into the surrounding media. The adsorption layers were thicker on the PLGA than on the polystyrene nanospheres, i.e. 10.5/3.2 and 7.2/1.9 respectively for the 2:5 and 3:4 polymers, whilst Poloxamine 908 layer thickness was virtually the same on both nanospheres.

The Nanosphere Surface Charge

The effect of the incubation of the nanospheres with PLA:PEG copolymers and Poloxamine 908 on the surface

Table II. The Effect of the Incubation of the Polystyrene and PLGA Nanospheres with Aqueous Solution of PLA:PEG 2:5 and 3:4 Copolymers and Poloxamine 908 on the Particle Size and Zeta Potential

System[a]	Particle size ± S.D./nm (Coating layer thickness.nm)	Zeta potential ± S.D./mV
Polystyrene		
Uncoated	164 ± 3.2	−44.7 ± 1.7
PLA:PEG 2:5	178 ± 3.5 (7.2)	−21.3 ± 1.6
PLA:PEG 3:4	168 ± 3.2 (1.9)	−32.2 ± 1.5
Poloxamine 908	188 ± 2.6 (9.2)	−15.8 ± 2.2
PLGA		
Uncoated	140 ± 2.7	−40.3 ± 2.3
PLA:PEG 2:5	161 ± 3.7 (10.5)	−18.5 ± 1.4
PLA:PEG 3:4	147 ± 3.6 (3.3)	−26.9 ± 1.3
Poloxamine 908	160 ± 3.8 (9.8)	−14.9 ± 1.8

[a] Uncoated and nanospheres coated with the copolymers.

charge of the nanospheres is also shown in Table II. In all cases, a decrease in the zeta potential for the coated systems is seen in comparison to the uncoated nanospheres. A decrease of 52.3, 27.9 and 64.6% was observed after coating polystyrene latex with PLA:PEG 2:5 and 3:4, and Poloxamine 908, respectively. For PLGA nanospheres coating with the same copolymers gave a decrease in zeta potential of 45.1, 33.3, and 63.0%, respectively. It should be noted that the net surface charge of the coated systems studied differ, and have values in the range of −32.2 to −14.9 mV.

A comparison of the results for the coating layer thickness and the zeta potential (Table II) reveals that an increase in the coating layer thicknesses of the copolymers on the polystyrene and PLGA nanospheres is accompanied by a decrease in the surface charge. This is assigned to the formation of an adsorbed layer of the copolymer on the surface of the nanospheres, which shifts the plane of shear to the outer boundary of the layer, resulting in a reduction of the zeta potential.

The Effect of the Copolymers on Stability of the Nanosphere Dispersions

To examine the ability of the PLA:PEGs to provide an effective steric barrier flocculation studies were performed where the effect of the addition of electrolyte or changes in the pH was compared to those of the uncoated nanospheres. Fig. 3 shows changes in the turbidity of uncoated and coated PLGA nanosphere dispersion after addition of an increasing amount of the electrolyte. A similar graph was obtained for the polystyrene nanospheres (graph not shown). The results reveal that for both uncoated polystyrene and PLGA nanospheres the addition of a small amount of sodium sulfate (0.05 mol/l) produced changes in turbidity indicative of agglomeration. The results were confirmed measuring the particle size by photon correlation spectroscopy (the particles size increased to few μm while polydispersity index values were 1–2). When the nanospheres were coated with the stabilizing copolymers, flocculation was observed to occur at significantly higher concentrations of the electrolyte. The PLA:PEG 3:4, 2:5 and Poloxamine 908 coated polystyrene nanospheres flocculated at sodium sulfate concentrations of 0.45, 0.50 and 0.55 mol/l, respectively. PLGA nanospheres coated with PLA:PEG 3:4 and 2:5 flocculated at 0.45 mol/l sodium sulfate, while Poloxamine 908 coated spheres appeared to be slightly more stable than the former systems, as indicated by a higher salt concentration, 0.55 mol/l, required for flocculation.

The stability of uncoated and coated nanospheres towards changes in the pH is illustrated in Fig. 4. An increase in turbidity of the dispersions of the uncoated particles at low pH values indicates that these systems flocculate in acidic media. The pH at which maximal changes in the turbidity was observed varies with the type of the nanosphere, pH 2 for uncoated polystyrene nanospheres and around pH 4 for uncoated PLGA nanospheres. A previous study (20) suggested that stability of a dispersion of PLGA nanospheres prepared without stabilizer is achieved by electrostatic repulsion due to the presence of carboxyl groups in the polymer endgroups on the surface of the nanospheres. The polystyrene latex dispersion used in this study is specified by the manufacturer to be electrostatically stabilized by the presence of sulfate groups on the surface. The results in Fig. 4 correspond to this difference in nature of the surface ionizable groups. If we assume ionization constants of 10^{-2} for sulfate group, and 10^{-4} M for carboxyl group, the results then show that the coagulation occurred at the pH of almost complete deionization of these groups. As expected, the coagulation of the nanospheres was not observed at neutral and alkaline pH values, where the surface groups are ionized. Hence, it can be concluded that the coagulation of uncoated polystyrene and PLGA particles at low pH is due to deionization of the surface ionizable groups and a consequent diminished electrostatic repulsion between the particles which stabilized the dispersion. In contrast, when the nanospheres were coated with the stabilizing copolymers, no changes in the stability of the dispersions could be observed even at very low pH values.

The differences in the stability of uncoated and coated particles upon the addition of the electrolyte and changes in pH of the medium, demonstrate that in the presence of the polymeric stabilizers, steric forces, in combination with

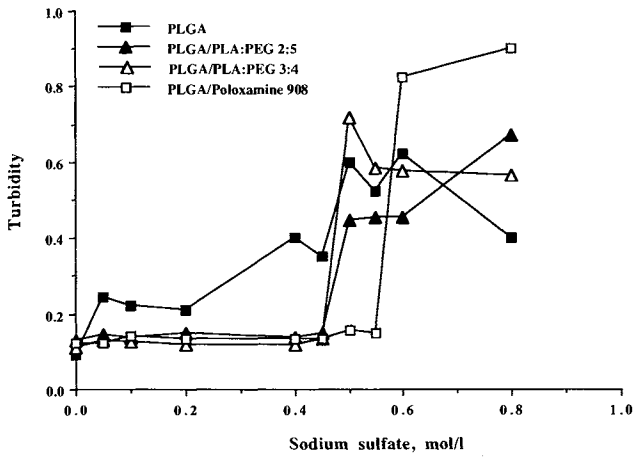

Figure 3. Critical flocculation concentration for dispersions of uncoated and PLGA nanospheres coated with the stabilizing copolymers.

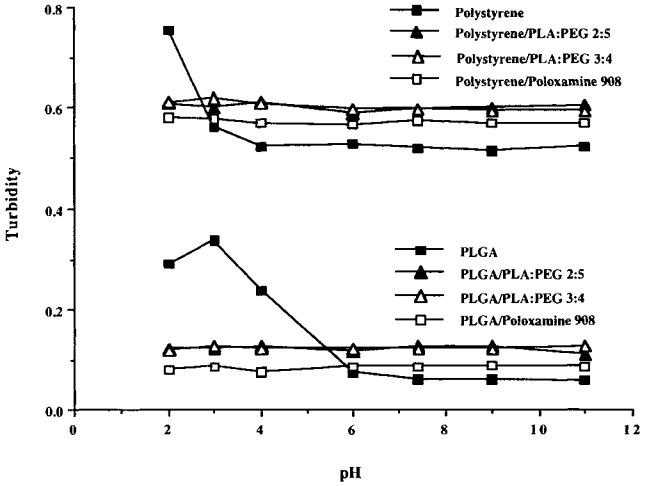

Figure 4. Turbidity of the dispersions of polystyrene and PLGA nanospheres at different pH.

electrostatic repulsion, acted to achieve stability of the nanosphere dispersions, providing an electrosteric stabilization. In view of the distinct advantages of steric over electrostatic stabilization, where there is the relative insensitivity to the presence of electrolytes, changes in pH, reversibility of flocculation, the stability tests suggest an advantageous stability of the PLA:PEG coated as compared to uncoated nanospheres. Moreover, the results of the stability tests show that the stability of polystyrene and PLGA nanospheres coated with PLA:PEG copolymers is only slightly lower than that of the model polystyrene-Poloxamine 908 system.

Adsorption of Albumin

The objective of the albumin adsorption study was to assess whether coating of the nanospheres with the stabilizing polymers can provide a repulsive barrier against protein adsorption. The results shown in Table III clearly reveal that the incubation of uncoated particles resulted in a significant increase in the nanosphere size, indicating a formation of an adsorbed layer of protein around 10 nm in thickness. After incubation with protein solution no significant changes in the particle size could be observed for any of the coated systems. Hence, the results indicate that coating the particles with any of the stabilizing copolymers can produce a surface which significantly decrease albumin adsorption. Interestingly, the results also indicate that PLA:PEG 3:4 copolymer, which has a PEG chain of molecular weight 2000 Da and produces a coating layer of around 3 nm, can achieve a similar effect on protein adsorption as Poloxamine 908 and PLA:PEG 2:5, which have a PEG chain of molecular weight 5000 Da and produce coating layers around 9 and 7 nm, respectively. This is in agreement with the findings of other authors (27,28) who suggest that there appears to be an optimal molecular weight of PEG which is necessary for reduced protein adsorption, and that further increase in the molecular weight does not result in a significant reduction in protein adsorption. The phenomenon has been attributed to a folding of the PEG chain into the hydrated coil which forms a repulsive hydrated layer. This folding can occur only above a certain molecular weight of the PEG chain, with molecular weights of 3500 and 1500 Da being suggested as the critical values (27,28). It should be noted that the PCS can only provide a rough estimate of whether adsorption has taken place or not since a layer of less than 1–2 nm would not be detectable and that although the PCS did not detect a layer of protein on the sterically stabilized nanospheres other methods would most likely have shown a much decreased amount adsorbed as shown for Poloxamine coated polystyrene particles (29).

Table III. The Effect of the Incubation of Uncoated and Coated Polystyrene Nanospheres with Albumin on the Particle Size

System	Particle size ± S.D./nm[a]		
	Control	Incubated with albumin	Layer/nm
Polystyrene uncoated	171 ± 3.3[b]	191 ± 2.5	10.1
	171 ± 3.2[c]	193 ± 3.6	11.1
Polystyrene/PLA:PEG 2:5	184 ± 3.6	182 ± 3.5	nsd[d]
	186 ± 1.9	185 ± 2.7	nsd
Polystyrene/PLA:PEG 3:4	177 ± 4.1	176 ± 3.8	nsd
	178 ± 3.4	177 ± 3.3	nsd
Polystyrene/Poloxamine 908	189 ± 3.6	188 ± 4.4	nsd
	191 ± 4.1	190 ± 3.7	nsd

[a] Polydispersity indices for all the systems were not higher than 0.068.
[b] The particle size after 2 hours incubation.
[c] The particle size after 24 hours incubation.
[d] Not significantly different.

In Vivo Biodistribution Studies

The blood clearance curves for uncoated and coated polystyrene nanospheres are shown in Fig. 5. The blood data reveals that coating either with PLA:PEG 2:5 or PLA:PEG 3:4 reduces the rapid clearance from the circulation observed for uncoated nanospheres. The PLA:PEG 2:5 coated particles show initial high blood circulating levels comparable to Poloxamine 908 coated nanospheres, while the values for the PLA:PEG 3:4 are slightly lower. In contrast to Poloxamine 908 coated particles, the circulating levels for both PLA:PEG coated systems were dramatically reduced between one and two hours post administration. The radioactivity associated with the liver, spleen and blood three hours after administration of the nanospheres is shown in Table IV. The results reveal that nanospheres coated with PLA:PEG copolymers are removed from the circulation by the liver to the same extent as uncoated nanosphere, but for the coated nanospheres a higher splenic clearance was observed. This difference in splenic deposition might be a result of the higher initial circulating levels of PLA:PEG coated systems, and has been shown for Poloxamine 908 coated nanospheres (8).

The coating of PLGA nanospheres with the PLA:PEG copolymers resulted in extended circulating levels, as shown in Fig. 6, which, in contrast to polystyrene were maintained over the duration of the experiment. The resulting blood clearance profiles are similar to that for particles coated with Poloxamine 908. Table IV reveals that for both the PLA:PEG

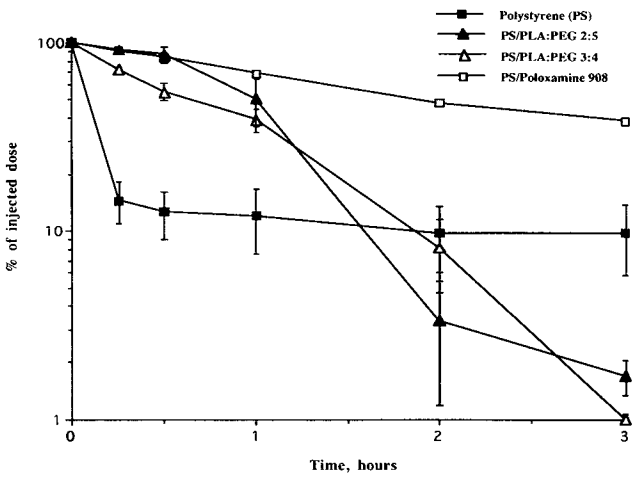

Figure 5. Blood clearance profiles for uncoated and PLA:PEG 2:5, PLA:PEG 3:4 and Poloxamine 908 coated polystyrene nanospheres in rats post intravenous injection.

Table IV. The Effect of Coating of Polystyrene and PLGA Nanospheres with PLA:PEG 2:5 and 3:4 Copolymers and Poloxamine 908 on Organ Deposition in Rat at Three Hours Post Intravenous Injection

System	Radioactivity associated with the organ/%		
	Liver	Spleen	Blood
Polystyrene uncoated	50.4 ± 3.1	5.6 ± 2.6	9.8 ± 4.0
Polystyrene/PLA:PEG 2:5	50.4 ± 0.5	11.8 ± 4.0	1.7 ± 0.4
Polystyrene/PLA:PEG 3:4	46.4 ± 3.3	17.3 ± 1.8	1.0 ± 0.1
Polystyrene/Poloxamine 908	14.0 ± 0.5	23.5 ± 1.2	38.6 ± 1.5
PLGA uncoated	63.8 ± 0.1	2.2 ± 0.3	4.6 ± 0.5
PLGA/PLA:PEG 2:5	22.7 ± 1.0	10.7 ± 1.9	28.5 ± 9.3
PLGA/PLA:PEG 3:4	20.9 ± 5.6	8.4 ± 1.1	17.5 ± 1.8
PLGA/Poloxamine 908	33.4 ± 0.4	12.0 ± 0.5	20.3 ± 0.8

copolymers and Poloxamine 908 coated systems a similar pattern of deposition is seen in the liver and spleen.

The reason for the difference in blood clearance and organ distribution of PLA:PEG coated polystyrene and PLGA nanospheres is unknown. An explanation could be in a difference in stability of the PLA:PEG coatings on polystyrene and PLGA nanospheres. In vitro results for the adsorption of albumin onto the surface of uncoated and coated polystyrene nanospheres indicate that the stability of the coating layer and its ability to provide an effective barrier is sufficient to prevent the adsorption of this protein up to 24 hours (Table III). However, *in vivo* results suggest that the coating layer is only effective in preventing opsonisation of the nanospheres and their subsequent sequestration by the liver for the initial 2 hours post administration. This might be explained, as it has been suggested by Senior et al. (2) for the poly(ethylene glycol) coated liposomes, by a gradual plasma protein adsorption onto the surface of the coated nanospheres, so that the whole surface is not instantaneously coated with plasma proteins as appeared to occur with uncoated latex. It is possible that the PLA part of the PLA:

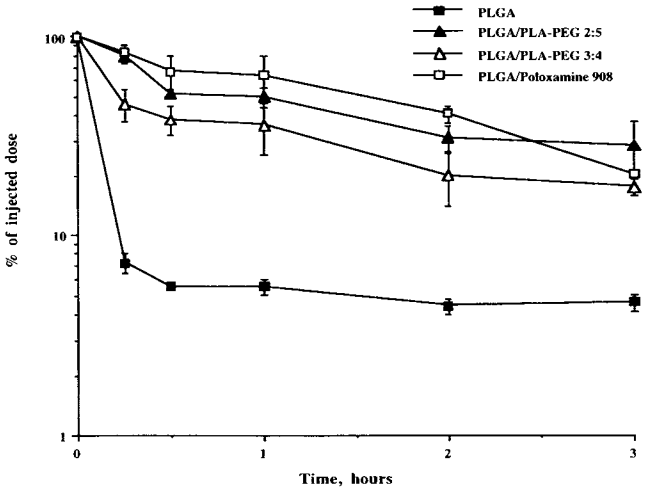

Figure 6. Blood clearance profiles for uncoated and PLA:PEG 2:5, PLA:PEG 3:4 and Poloxamine 908 coated PLGA nanospheres in rats post intravenous injection.

PEG copolymers would be a better anchor group when the copolymers are adsorbed onto the surface of a similar structure, i.e. PLGA nanospheres and would therefore provide the coating layer stable to desorption and displacement which prevents any gradual adsorption of plasma proteins.

In conclusion, the results have shown the PLA:PEG copolymers can be exploited as coatings for biodegradable PLGA nanospheres to obtain sterically stabilised particles with reduced protein adsorption and dramatically increased blood circulation time and decreased hepatic uptake in the rat model as compared to naked PLGA nanospheres.

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REFERENCES

1. L. Illum and S. S. Davis. The organ uptake of intravenously administered colloidal particles can be altered using nonionic surfactant (poloxamer 338), *FEBS Lett.* 167:70–82 (1984).
2. J. Senior, C. Delgado, D. Fisher, C. Tilcock and G. Gregoriadis. Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles. *Biochim. Biophys. Acta,* 1062:77–82 (1991).
3. R. H. Muller, K. H. Walis, S. D. Troster, and J. Kreuter. In vitro characterization of poly(methyl-methacrylate) nanospheres and correlation to their in vivo fate. *J. Control. Rel.* 20:237–246 (1992).
4. L. Illum, S. S. Davis, R. H. Muller, E. Mak and P. West. The organ distribution and circulation time of intravenously injected colloidal carriers sterically stabilised with block-copolymer-poloxamine 908. *Life Sci.* 40:367–374 (1987).
5. S. M. Moghimi, L. Illum and S. S. Davis. Organ specific targeting of synthetic and natural drug carriers. In: B. R. Jetter (ed.) *Homing Mechanisms and Cellular Targeting,* Advances in Molecular and Cell Biology, Vol. 9, pp 263–282
6. I. S. Muir, S. M. Moghimi, L. Illum, S. S. Davis and M. C. Davies. The effect of block copolymeres on the uptake of model microspheres by Kupffer cells—in vitro and in vivo studies. *Biochem. Soc. Trans.* 19:329S (1991).
7. C. J. Porter, S. M. Moghimi, L. Illum. and S. S. Davis. The polyoxyethylene/polyoxypropylene block co-polymer Poloxamer-407 selectively redirects intravenously injected microspheres to sinusoidal endothelial cells of rabbit bone marrow. *FEBS* 305:62–66 (1992).
8. S. M. Moghimi, I. S. Muir, L. Illum, S. S. Davis, and V. Kolb-Bachofen. Coating particles with a block co-polymer (poloxamine 908) supress opsonization but permits the activity of dysopsonins in the serum. Biochim. Biophys. Acta. 1179:157–165 (1993)
9. J. B. Kayes and D. A. Rawlins. Adsorption characteristics of certain polyoxethylene-polyoxypropylene block co-polymers on polystyrene latex. *Coll. Polym. Sci.* 257:622–629 (1979).
10. L. Illum, L. O. Jacobsen, R. H. Muller, E. Mak and S. S. Davis. Surface characteristics and the interaction of colloidal particles with mouse peritoneal macrophages. *Biomaterials* 8:113–117 (1987).
11. L. Illum, P. West, C. Washington, and S. S. Davis. The effect of stabilizing agents on the organ distribution of lipid emulsions. *Int. J. Pharm.* 54:41–49 (1989).
12. J. H. Senior. Fate and behaviour of liposomes in vivo: A review of controlling factors. *Crit. Rev. Ther. Drug Carrier. Syst.* 3: 123–193 (1987).
13. S. M. Moghimi, C. J. H. Porter, L. Illum, and S. S. Davis. The effect of poloxamer-407 on liposome stability and targeting to bone marrow: comparison with polystyrene microspheres. *Int. J. Pharm.* 68:121–126 (1991).
14. A. L. Klibanov, K. Maruyama, V. P. Torcelini, and L. Huang.

Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding target. *FEBS Lett.* **268**:235–237 (1990).
15. M. C. Woodle and D. D. Lasic. Sterically stabilized liposomes. *Biochem. Biophys. Acta* **1113**:171–191 (1992).
16. J. R. Churchill and F. G. Hutchinson. Biodegradable amphipathic copolymers. European Patent Application, 0 092 918, 1983.
17. J. R. Churchill and F. G. Hutchinson. Biodegradable amphipathic copolymers. European Patent Application 0 166 596, 1986.
18. G. Spenlehauer, M. Veillard and T. Nerrechia. Pharmaceutical microspheres containing biocompatible and biodegradable surfactants and polymers. PCT Int. Appl. WO 91 15, 193, 1991.
19. R. Gref, Y. Minamitake, M. T. Perachchia, V. Trubetskoy, A. Milshteyn, J. Sinkule, V. Torchilin and R. Langer. Biodegradable PEG-coated stealth nanospheres. *Proceed. Intern. Symp. Control. Rel. Bioact. Mater.* **20**:131–132 (1993).
20. S. Stolnik, M. C. Davies, L. Illum, S. S. Davis, M. Bousta and M. Vert. *J. Control. Rel.* **30**:57–67 (1994).
21. S. J. Douglas, L. Illum, S. S. Davis and J. Kreuter. Particle size and size distribution of poly(butyl-2-cyanoacrylate) nanoparticles. *J. Colloid and Interface Sci.* **101**:149–158 (1984).
22. H. Z. Cummins and E. R. Pike (eds.) *Photon Correlation and Light Beating Spectroscopy*, NATO-ASI Series, Plenum Press, New York, 1974.
23. L. Illum, S. S. Davis, R. H. Muller, E. Mak, and P. West. The organ distribution and circulation time of intravenously injected colloidal carriers sterically stabilised with block-copolymer-poloxamine 908. *Life Sci.* **40**:1553–1560 (1987).
24. P. D. Scholes, M. C. Davies, L. Illum and S. S. Davis. Radiolabelling of biodegradable microspheres for site specific drug delivery. *Proceed. Inter. Symp. Control. Rel. Bioact. Mat.* **19**:154–155 (1992).
25. Y. Huh, W. Donaldson and F. J. Johnson. A radiation induced bonding of iodine at the surface of uniform polystyrene particles. *Radiation Research* **60**:42–53 (1974).
26. H. M. Patel, S. N. Tuzel and B. E. Ryman. Inhibitory effect of cholesterol on the uptake of liposomes by liver and spleen. *Biochim. Biophys. Acta.* **761**:142–151 (1983).
27. C. G. Golander, J. N. Herron, K. Lim, P. Claesson, P. Stenius and J. D. Andrade. Properties of immobilized PEG films and interacton with proteins: I Experiments and modeling. In J. M. Harris (ed.) *Poly(ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications*, Plenum Press, 1992, pp. 221–245.
28. W. R. Gombotz, W. Guanghui, T. A. Horbert and A. S. Hoffman, Protein adsorption to and elution from polyether surfaces. In: J. M. Harris (ed.), *Poly(ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications*, Plenum Press, New York, 1992, pp. 249–261.
29. M. E. Norman, P. Williams and L. Illum. Influence of block copolymers on the adsorption of plasma proteins to microspheres. *Biomaterials* **14**:193–202 (1993).