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PEGylation of microspheres generates a heterogeneous population of particles with differential surface characteristics and biological performance

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Abstract Surface PEGylation of polystyrene microspheres with methoxy-poly(ethylene glycol)-5000 (mPEG-5000) generated a heterogeneous population of entities that differed in surface characteristics and in vitro biological performance (phagocytosis and complement activation). Surface heterogeneity was determined by hydrophobic interaction chromatography, measurements of particle electrophoretic mobility in a defined field and adlayer thickness of the projected mPEG chains. The particle population separation by hydrophobic interaction chromatography demonstrated a remarkable linear relationship between the particle zeta potential and phagocytosis by J774 A1 macrophage-like cells. Microsphere populations bearing a predominant surface of mPEG molecules as high-density mushroom–brush intermediate and/or brush configuration were most resistant to phagocytosis and activated the human complement system poorly. Conversely, those populations with predominant surface mPEGs in a mushroom regime were potent activators of the complement system and were prone to phagocytosis. Therefore, surface heterogeneity explains why a fraction of intravenously injected 'long-circulating' nanoparticles is cleared rapidly by macrophages of the reticuloendothelial system. Hydrophobic interaction chromatography can readily assess the extent of surface heterogeneity of PEGylated particulate drug delivery systems and pre-select particles with optimal retention times in the blood. These observations may also be relevant with respect to successful surface camouflaging of cells, drug depots and implantable devices.

Key words: Complement activation; Surface camouflaging; Long-circulating particle; Macrophage; Microsphere; Poly(ethylene glycol)

1. Introduction

Recently, we described a simple two-step approach for surface enrichment of polystyrene particles with methoxy-poly-(ethylene glycol)-5000 (mPEG-5000) in order to generate entities which are resistant to macrophage recognition and hence exhibit prolonged circulation times in the blood [1]. Here, the surface of polystyrene particles was first enriched with bovine serum albumin (BSA) at the top plateau region of the adsorption isotherm, where BSA molecules form a close-packed monolayer. The nucleophiles of the adsorbed BSA monolayer were then conjugated with molar excess quantities of cyanuric chloride-activated mPEG-5000. However, within the first hour of intravenous injection into rats, a significant fraction of these PEGylated particles, corresponding to 25% of the initial dose, was captured by the liver macrophages [1]. This phenomenon is also similar to biodistribution of other studied surface-camouflaged particles such as PEG-grafted liposomes and nanoparticles coated with PEG-R-type copolymers where R is poly(lactic acid) or poly(lactide-co-glycolide) (reviewed in [2]). Biophysical characterisation of PEGylated particles demonstrated that the preparation was composed of two different populations of entities of similar size distribution but with different surface hydrophilicity [1]. The minor population, corresponding to approximately 20% of the total, was prone to phagocytosis and hence did not exhibit prolonged circulation times in the blood. Differences in hydrophilicity indicate variant surface coverage of modified particles by mPEG molecules. Therefore, variable surface heterogeneity explains why a significant fraction of intravenously injected mPEG-grafted particles is rapidly sequestered by macrophages in contact with blood.

From a cancer therapeutic point of view, macrophage sequestration of PEGylated particles may be problematic. For example, following uptake and subsequent intracellular release of the encapsulated anti-cancer drug macrophage apoptosis is induced, a process which leads to depletion of a significant number of the host's defence cells [3,4]. In the case of the liver, restoration of Kupffer cells may take up to 2 weeks; this period of Kupffer cell deficiency may be associated with conditions such as bacteraemia, which is potentially fatal in some patients [4]. Therefore, it is necessary to refine surface engineering strategies that yield a single population of particles with a homogeneous macrophage-repelling surface.

This article examines physicochemical parameters which determine surface heterogeneity of well-defined model BSA-coated polystyrene particles following PEGylation with activated mPEG-5000. Therefore, we have studied how the mode of BSA adsorption (a close-packed monolayer versus a disorganised surface structure) on to polystyrene particles can affect surface PEGylation in terms of the number of attached mPEG molecules per adsorbed BSA molecule, mPEG configuration (brush versus mushroom or flat regimes), and particle population heterogeneity. These approaches have led us to successfully establish a correlation between surface parameters and the biological performance (complement activation and

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phagocytosis) of the engineered particles. We also provide essential guidelines for the design of nanospheres with optimal macrophage-resistant properties.

2. Materials and methods

2.1. Surface engineering of polystyrene particles (protein adsorption and PEGylation)

Polystyrene particles (2.5% w/v), 1.0 μm in diameter, manufactured by Polysciences (Warrington, PA, USA), were purchased from Park Scientific (Northampton, UK). In some experiments polystyrene particles were surface labelled with Na[^{125}I] (Amersham Pharmacia Biotech, Amersham, UK) as described previously [1]. The surface of the polystyrene particles was modified with BSA. Initially, an adsorption isotherm of BSA on polystyrene lattices was constructed as described in detail elsewhere [1,5]. Briefly, protein solutions were prepared in phosphate buffer, pH 7.4 and ionic strength of 0.15. Protein-coated particles were prepared by adding 2.5 mg of polystyrene particles, corresponding to 4.25×10^9 particles, with a total surface area of 0.014 m^2, to protein solutions ranging from 0 to 650 μg/ml BSA in a total volume of 1.0 ml in triplicate Eppendorf tubes. All tubes were shaken gently (100 rpm) for 24 h at 24°C in a water bath. The suspensions were then centrifuged for 30 min at $16\,000\times g$. For each protein concentration a control tube was prepared without any polystyrene particles to account for any protein loss due to adsorption to the Eppendorf tubes. Protein concentrations were determined by Bio-Rad assay for the resulting supernatants using a standard curve run concurrently with the adsorption experiment. In a series of further experiments, polystyrene particles incubated in 250 and 600 μg BSA/ml were centrifuged for 30 min at $16\,000\times g$; after four washing steps and subsequent centrifugation no more protein was detected in the supernatant as assessed by the Bio-Rad protein assay solution. No polystyrene particles were lost in supernatants as monitored by light scattering during the centrifugation steps. The cleaned particles were finally resuspended in either phosphate buffer or 0.1 M sodium tetraborate at pH 9.2, and gently shaken for 24 h at 24°C to determine whether any protein could desorb. These experiments demonstrated strong association of the adsorbed proteins with polystyrene particles as no protein was detected in the supernatant. Finally, protein-coated particles were suspended in 0.1 M sodium tetraborate at pH 9.2. Cyanuric chloride-activated mPEG-5000 was added to the suspensions in excess (in 50-fold molar excess to the adsorbed albumin) and incubated for 1 h at 4°C; the total volume was kept to 1.0 ml. The chemical basis of this reaction is discussed elsewhere [1]. Unattached PEG molecules were removed by centrifugation at $16\,000\times g$ for 30 min. Finally, particles were suspended in 10 mM phosphate buffer, pH 7.2, and washed three times to ensure the complete removal of unconjugated PEGs. Removal of free PEG and the quantity of adsorbed conjugated mPEG–BSA were determined by the colorimetric assay of Nag et al. [6].

2.2. Particle size and electrophoretic mobility

The hydrodynamic radius and the electrophoretic mobility of particles were determined by photon correlation spectroscopy (PCS) and laser Doppler electrophoresis, respectively, in 10 mM McIlvaine buffer, pH 7.2, at 24°C using a Zetasizer 3000 system (Malvern Instruments, UK) as described previously [1]. PCS measurements (based on CONTIN analysis) were determined at a wavelength of 633 nm, scattering angle of 90°, dispersant viscosity of 0.89 cP and refractive index of 1.35. For electrophoretic mobility measurements the conditions were as follows: dielectric constant 78.3, current 1.4 mA, fluid refractive index of 1.35, cell field of 29.3 V/cm, viscosity of 0.89 cP and conductivity of 0.7 mS/cm.

2.3. Hydrophobic interaction chromatography

The interaction of PEGylated particles in the hydrophobic column was investigated by adsorption to and elution from pentyl-agarose [1]. For this, pre-packed columns containing 2.5 ml pentyl-agarose (Sigma-Aldrich Company, Poole, UK) were used. In all experiments particles (0.5 mg) were suspended in 10 mM phosphate buffer (pH 7.2) with 1.0 M sodium chloride to promote interaction. The column was equilibrated with the same buffer. Elution was made by decreasing the concentration of sodium chloride and concurrently increasing the concentration of Triton X-100 (from 0.0 to 0.1% v/v). No aggregation of the sample was observed before application onto the column. The elution pattern was read using a spectrophotometer ($\lambda=450$ nm) or by radioactivity (when particles were surface labelled with ^{125}I). Pooled fractions were concentrated by centrifugation for 60 min at $16\,000\times g$ and washed twice with 10 mM phosphate buffer prior to further analysis. In some experiments pooled fractions were concentrated by centrifugation and after cleaning incubated in 0.1% w/v solution of poloxamine 908 (BASF, Mt. Olive, NJ, USA) for 1 h at room temperature. Following incubation, the suspension was centrifuged and washed twice to remove free poloxamine 908 molecules.

2.4. Residual total complement haemolytic assay

Freshly obtained human serum was diluted with an equal volume of isotonic veronal-buffered saline (containing 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$ and 0.1% w/v gelatin). Diluted human serum samples (50 μl) were incubated with an equal volume of particle suspensions (0.2 mg polystyrene) at 37°C for 1 h. After the incubation period, the mixtures were diluted with 150 μl of isotonic veronal-buffered saline and kept on ice. Residual total complement haemolytic activity of particle-treated serum was measured according to established methods [7]. Briefly, sheep erythrocytes were sensitised with rabbit anti-sheep erythrocyte antibody and suspended to 1.5×10^8 cells/ml in isotonic veronal-buffered saline. In triplicate, 50 μl antibody-sensitised cells were incubated with 50 μl of particle-treated serum for 30 min at 37°C. Samples were then diluted by the addition of 2 ml of dextrose–gelatin veronal-buffered saline containing 40 mM EDTA. Unlysed erythrocytes were pelleted by centrifugation and the amount of haemoglobin released into the supernatant was quantified spectrophotometrically at 414 nm. Blanks and 100% lysis controls were also performed. The residual complement haemolytic activity was also assessed in heat-treated serum (56°C, 30 min) as well as zymosan-treated serum. The residual complement haemolytic activity of the treated serum is expressed as a percentage of the total haemolytic level as determined in the 100% lysis control. The above experiments were repeated three times and each time serum was obtained from a different healthy individual.

2.5. Phagocytic assay

Phagocytosis was monitored using ^{125}I-labelled polystyrene particles. The J774 A1 murine macrophage-like cell line was plated in 48-well tissue culture dishes at a density of 5×10^5 cells per well in RPMI medium containing 10% v/v foetal bovine serum. In order to remove non-adherent cells, cells were washed 1 h after plating with fresh RPMI medium. Cell viability, as determined by dye exclusion, was greater than 95% after 24 h of incubation at 37°C. After 24 h incubation at 37°C, polystyrene particles of different surface characteristics (5×10^8 particles per well) were added and the cells were left to incubate for an additional 6 h. All incubations with polystyrene particles contained 10% v/v foetal bovine serum, thus to account for possible opsonisation events. At the end of the incubation, cells were washed three times with RPMI medium, then solubilised with KOH, and total radioactivity associated with cell lysate was determined using a gamma counter. For each polystyrene particle type triplicate incubations were performed.

3. Results

3.1. Surface engineering and particle characterisation

In agreement with previous studies [1,5] the results in Fig. 1 demonstrate that at a pH of 7.4 and ionic strength of 0.15, BSA has a high affinity for the polystyrene particle surface at low concentrations. The isotherm is trimodal. After an initial sharp increase, the isotherm breaks at approximately 0.6 mg/m^2, then begins to rise again and levels out around 1.1 mg/m^2 and finally reaches a plateau limit of approximately 2.0 mg/m^2. All features of this isotherm have been confirmed by repeated experiments. The size of uncoated polystyrene particles as measured by photon correlation spectroscopy was 1173.2 ± 12.4 nm. The average increase in the hydrodynamic radius for a BSA-coated polystyrene particle is 4.0–4.5 nm and is irrespective of samples taken at various points along

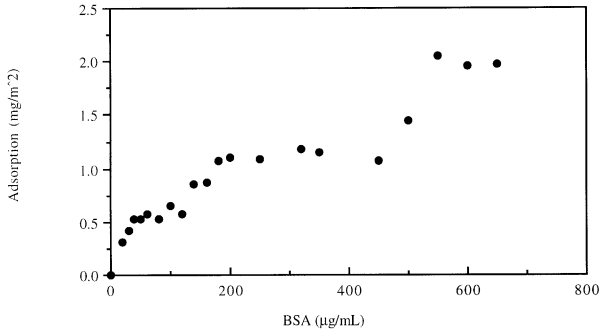

Fig. 1. Adsorption isotherm for BSA at pH 7.4, $I = 0.15$, $T = 24°C$.

the adsorption isotherm (100, 250 and 600 μg/ml) (Table 1). Therefore, the position on the isotherm has little effect on the observed Stokes radius. By considering the approximate ellipsoid dimensions of BSA ($3.8 \times 3.8 \times 14.0$ nm) [1,5] these observations correlate closely with the side-on mode of BSA adsorption at all points along the isotherm. These observations are in accordance with previous experiments using submicrometre polystyrene particles [1,5].

Protein-coated polystyrene particles, taken at BSA equilibrium concentrations of 250 and 600 μg/ml, were subjected to PEGylation with cyanuric chloride-activated mPEG-5000. The incubation containing 250 mg BSA/ml generates polystyrene particles with 3.05×10^4 surface-bound BSA molecules (occupying 48.5% of the total surface area) (PS_{250}), whereas incubation at an equilibrium concentration of 600 μg BSA/ml generates particles bearing 5.57×10^4 surface-adsorbed BSA molecules (occupying 89.9% of the total surface area) (PS_{600}). Following the PEGylation step we have calculated an average of 7.3 ± 0.2 and 4.1 ± 0.3 μmol PEG/μmol protein for polystyrene particles coated with 3.05×10^4 and 5.57×10^4 BSA molecules, respectively.

We have also assessed surface hydrophilicity and population heterogeneity of BSA-coated as well as the two PEGylated particulate preparations by hydrophobic interaction chromatography. Both types of BSA-coated particles adsorbed strongly to the column at high salt concentrations; approximately 3 and 12% of PS_{250} and PS_{600} particles were eluted under 1.0 M salt concentration (Fig. 2). The bulk of BSA-coated particles were eluted at a low salt concentration (0.25 M NaCl and 0.05% w/v Triton X-100), thus demonstrating their relative hydrophobic nature. Passing a suspension of PEGylated PS_{250} particles in 1.0 M sodium chloride through a hydrophobic column retained the majority of particles; only 20% of the total particles were eluted (F1a fraction) (Fig. 3a). When the sodium chloride concentration was reduced to 0.5 M with a concomitant increase in Triton X-100 concentration, multiple peaks were observed spectrophotometrically on elution from the column. The peaks collected under F2a and F3a fractions correspond to 33% and 43% of the total amount of particles placed on the column. The electrophoretic mobility of the three collected fractions was considerably lower than the BSA-coated particles under identical conditions (Table 2). This is due to the steric protection by the surface mPEG molecules. Among the three fractions, the electrophoretic mobility of F1a particles was considerably less negative than those of F2a and F3a. These results clearly indicate surface heterogeneity, with three distinct populations, among the PEGylated PS_{250} particles under these experimental conditions. Interestingly, according to particle size analysis PEGylation had no significant effect on increasing the hydrodynamic layer thickness of F2a and F3a particles when compared to PS_{250} particles (Table 2). Since the electrophoretic mobility of these two populations is considerably different from PS_{250} particles (Table 2), conjugated mPEG chains themselves could be adsorbed to the particle surface, therefore exhibiting a predominantly non-overlapped or weakly overlapped mushroom configuration [8]. On the other hand, the considerable increase in the hydrodynamic radius of F1a particles (~4 nm) suggests that the attached mPEG-5000 molecules are projected from the surface in mushroom–brush and/or brush configurations [8,9]. Further PEGylation of the three fractions had no significant effect on hydrodynamic layer thickness and the electrophoretic mobility of the particles (data not shown) thus suggesting complete surface PEGylation during the first step.

In contrast to PEGylated PS_{250} particles, the passage of PEGylated PS_{600} particles in 1.0 M sodium chloride through the hydrophobic column failed to retain the majority of the particles (F1b fraction) (Fig. 3b). The remaining particles (20% of the total) were eluted (F2b) following reduction in sodium chloride concentration. Again, the electrophoretic mobility measurements (Table 2) confirmed that particles collected in the F1b fraction have the least negative value and hence are more hydrophilic when compared to those eluted under the F2b fraction. Again, the significant increase in hy-

Table 1
The size of uncoated polystyrene particles and those coated with varying amounts of BSA as determined by photon correlation spectroscopy

Equilibrium protein concentration (μg)	Particle diameter (nm)	Hydrodynamic layer thickness of BSA (nm)
0.0	1173.2 ± 12.4	0.0
100.0	1181.3 ± 9.4	4.1
250.0	1182.9 ± 7.5	4.8
600.0	1182.1 ± 9.3	4.5

Polydispersity index was less than 0.07 for all measurements.

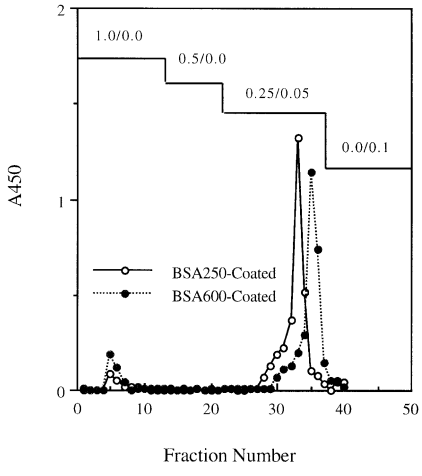

Fig. 2. Hydrophobic interaction chromatography of BSA-coated polystyrene microspheres on a column of pentyl-agarose. The final equilibrium concentration of BSA was either 250 μg/ml (BSA 250-coated) or 600 μg/ml (BSA 600-coated). The elution gradient is also shown (solid lines), the two numbers above each gradient step represent the molarity of sodium chloride (left) and %v/v of Triton X-100. The above profile was reproducible and tested with three different preparations of BSA-coated particles.

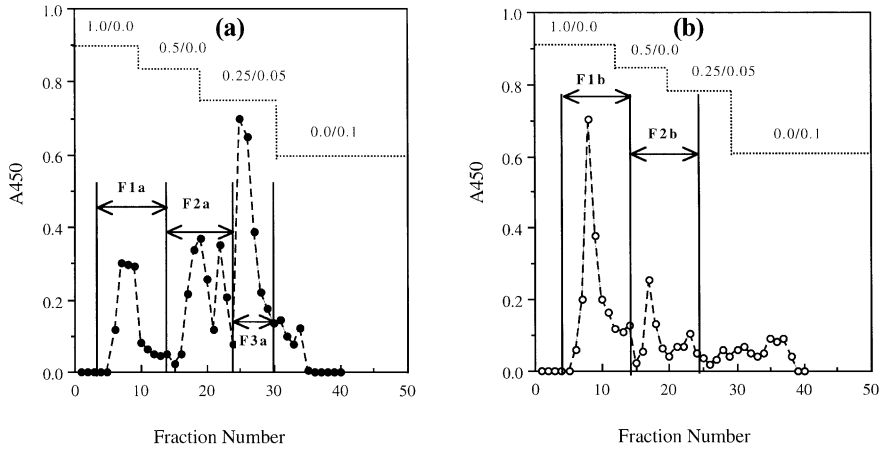

Fig. 3. Hydrophobic interaction chromatography of mPEG-5000-grafted BSA-coated polystyrene microspheres on a column of pentyl-agarose. In panel a microspheres were coated with BSA at a final equilibrium concentration of 250 μg/ml prior to PEGylation and in panel b microspheres were coated with BSA at a final equilibrium concentration of 600 μg/ml prior to PEGylation. The elution gradient is also shown (dotted line), the two numbers above each gradient step represent the molarity of sodium chloride (left panel) and %v/v of Triton X-100 (right panel). The above profile was reproducible and tested with three different preparations of PEGylated BSA-coated particles. Designated fractions were pooled, concentrated and washed for further biophysical analysis.

drodynamic radii (approximately 4–6 nm) of both F1b and F2b particles in comparison to PS_{600} particles (Table 2) closely correlates with surface-projected mPEG molecules in a predominantly mushroom–brush/brush configuration [9].

3.2. Particle phagocytosis

The kinetics of association of uncoated polystyrene particles with J774 A1 murine macrophage-like cells was characterised by a rapid initial increase at 37°C followed by a gradual decline reaching saturation level at 6 h (data not shown). Incubation of cells with polystyrene particles at 4°C, which only allows for surface adsorption, demonstrated that 85% of the total particles are internalised by the macrophage-like cells after 6 h incubation at 37°C. The phagocytic uptake of protein-coated and PEGylated particles was also compared to that of uncoated polystyrene particles (Fig. 4). The uptake of BSA-coated particles was similar to those of uncoated entities regardless of the amount of surface-adsorbed BSA. Among the three fractions of PEGylated PS_{250} particles the F1a fraction was most resistant to phagocytosis, whereas the uptake of both F2a and F3a particles was reduced by only 20% when compared to control incubations containing uncoated particles (Fig. 4a). Further treatment of F2a and F3a PEGylated particles with poloxamine 908 significantly suppressed phagocytosis (approximately by 80% as compared to incubations with uncoated particles). In contrast to these, the macrophage uptake of poloxamine-treated F1a particles was reduced by a further 10% in comparison to non-treated F1a particles.

Similar to F1a particles, the two fractions of PEGylated PS_{600} particles (F1b and F2b) were also poorly recognised by macrophages and among them F1b particles were most resistant to phagocytosis (approximately 30% of uncoated control) (Fig. 4b). The results in Fig. 4c demonstrate a remarkable linear relationship between the zeta potential of particles in buffer and the extent of phagocytosis by the macrophage-like cells. Thus, the extent of phagocytosis can be predicted by measuring the zeta potential of the particles. Cells exposed to all types of particles appeared to be morphologically intact, and when assayed for viability using trypan blue, greater than 95% of cells excluded the dye.

3.3. Complement activation

Complement haemolytic assays were used to detect activation of the human complement system by surface-engineered particles. Human serum was incubated with particles for 1 h at 37°C and the residual complement haemolytic activity was quantified. A reduction in the residual complement haemolytic activity of serum signifies activation of the complement system by particles. The detection of this reduction is sensitive to the initial levels of complement present in serum. The results in Fig. 5 show that the residual complement haemolytic activities of human serum exposed to uncoated and BSA-coated particles are similar; following exposure 40% of residual complement activity remained. This indicated the complement-activating nature of both uncoated and BSA-coated particles. However, PEGylated particle fractions were poor activators of the complement system with the majority of

Table 2
Physical characteristics of BSA-coated and mPEG-grafted microspheres following hydrophobic interaction chromatography

Microsphere type	Hydrodynamic diameter (nm)	Electrophoretic mobility (10^{-8} m^2/Vs)	Zeta potential (mV)
PS_{250}	1183.2 ± 7.5	−6.01 ± 0.15	−76.7 ± 1.8
PS_{250} (F1a)	1190.3 ± 3.4	−2.76 ± 0.06	−35.3 ± 0.3
PS_{250} (F2a)	1184.6 ± 6.2	−4.16 ± 0.03	−47.8 ± 1.3
PS_{250} (F3a)	1184.3 ± 8.4	−4.68 ± 0.28	−59.7 ± 3.6
PS_{600}	1181.1 ± 4.6	−6.39 ± 0.11	−81.5 ± 1.4
PS_{600} (F1b)	1192.1 ± 5.7	−2.34 ± 0.10	−29.8 ± 1.3
PS_{600} (F2b)	1189.7 ± 3.4	−2.97 ± 0.06	−37.8 ± 0.4

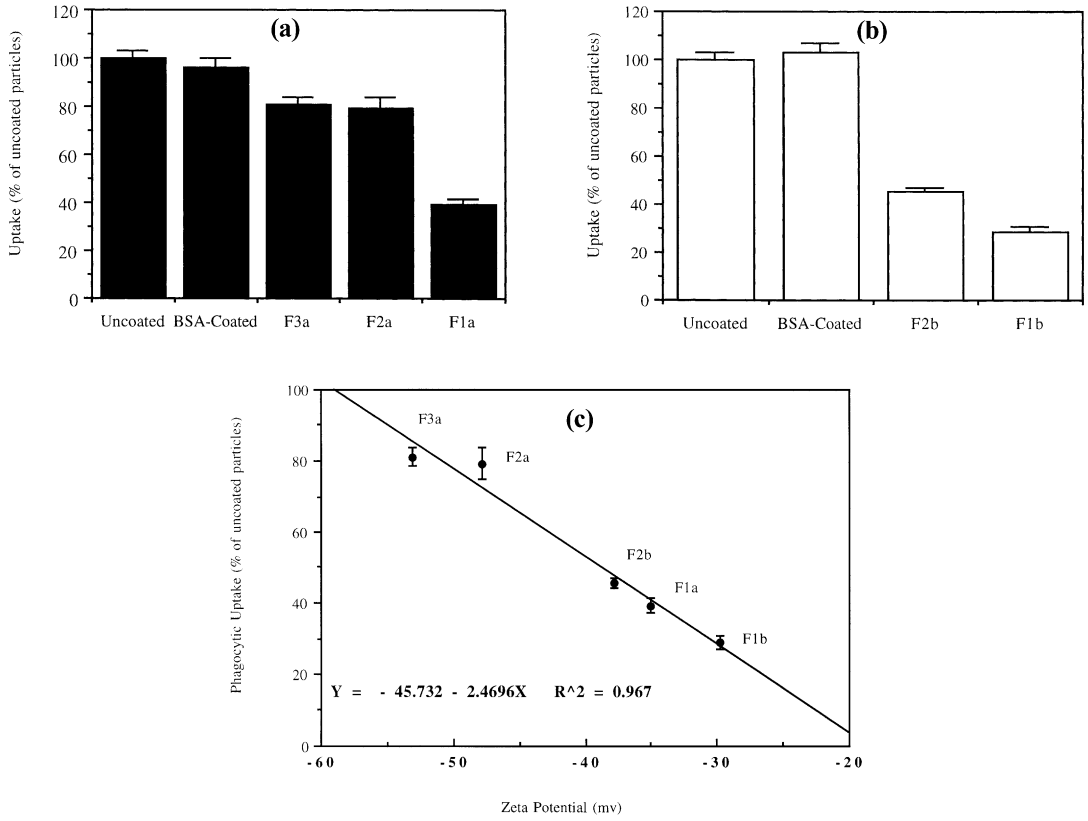

Fig. 4. Microsphere uptake by J774 A1 cells. Uptake of uncoated particles is considered 100%. The results of test microspheres are expressed as % uptake of uncoated particles. In panel a microspheres were coated with BSA at a final equilibrium concentration of 250 μg/ml and in panel b microspheres were coated with BSA at a final equilibrium concentration of 600 μg/ml. Microspheres F1a, F2a, F3a, F1b and F2b are PEGylated and represent pooled fractions from the hydrophobic interaction chromatography experiment (see Fig. 3). In panel c the relationship between microsphere zeta potential and phagocytic uptake is presented.

the residual complement activity remaining following exposure of the most hydrophilic particles (F1a and F1b fractions), or particles with predominant mushroom–brush/brush mPEG configuration. It is of note that even the most hydrophilic particles (F1b fraction) could still activate the complement system. Crossed immunoelectrophoresis of complement component C3 also confirmed C3 cleavage with all tested systems (data not shown).

4. Discussion

The PEGylation reaction demonstrated covalent attachment of more activated mPEG-5000 molecules, on an average basis, per surface-bound BSA molecule for PS_{250} rather than PS_{600} particles. These observations may be due to differences in the mode of BSA adsorption which vary at different plateau levels of the adsorption isotherm. Previous studies have

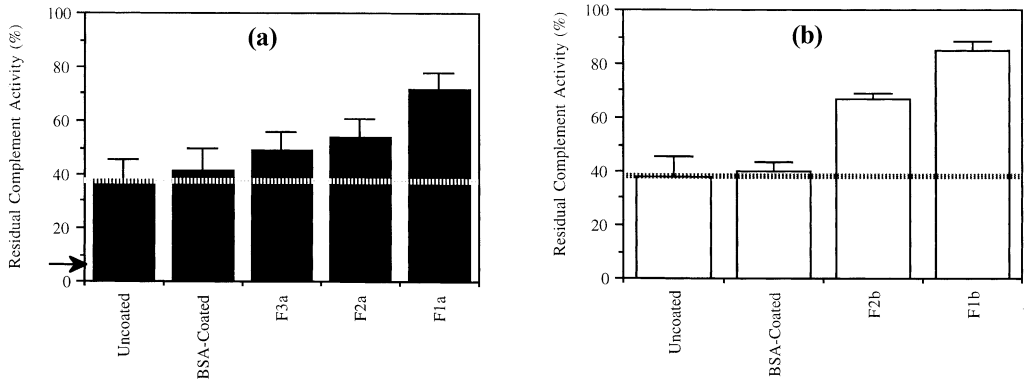

Fig. 5. Effect of mPEG-5000 on human complement activation by surface-engineered microspheres. In panel a microspheres were coated with BSA at a final equilibrium concentration of 250 μg/ml and in panel b microspheres were coated with BSA at a final equilibrium concentration of 600 μg/ml. Microspheres F1a, F2a, F3a, F1b and F2b are PEGylated and represent pooled fractions from the hydrophobic interaction chromatography experiment (see Fig. 3).

established that the surface-adsorbed BSA is in a form close to its native conformation, in both the lower and final plateau regions of the adsorption isotherm [5]. At low equilibrium concentrations (e.g. 250 μg/ml), BSA adsorption to the particles proceeds by a random uncorrelated irreversible mode, which may or may not involve conformational change [5]. However, at the final plateau a transition to a co-operative mode is believed to occur in which a close-packed protein monolayer is formed [5]. Therefore, close proximity of BSA molecules in a close-packed monolayer, as in PS_{600} particles, may generate geometric factors and surface dynamics (e.g. steric factors), which are less favourable for coupling to more activated mPEG-5000 molecules when compared to PS_{250} particles.

PEGylation of both types of BSA-coated polystyrene particles generated a heterogeneous population of entities that differed in surface characteristics and in vitro biological performance. It seems that the mode of BSA adsorption to the polystyrene surface as well as the extent of proximity of adsorbed protein molecules to each other can control the extent of surface heterogeneity of particles following PEGylation. Even higher starting concentrations of activated mPEGs (up to 200-fold molar excess to the adsorbed albumin) failed to reduce or totally abandon the heterogeneity phenomenon [1]. Hydrophobic interaction chromatography data suggested that the majority of particles bear a relatively homogeneous hydrophilic surface (F1b fraction) when a close-packed BSA monolayer is PEGylated. The close proximity of adsorbed BSA molecules appears to generate an effective 'molecular PEG cloud' to resist phagocytosis. The thickness of the 'molecular PEG cloud' is approximately 4 nm. This implies that the grafted mPEG molecules are predominantly in a brush-like configuration [9]. Regardless of surface topology both particle recognition by macrophages and complement activation are not totally inhibited. This indicates that a fraction of the particles (which cannot be separated by hydrophobic interaction chromatography) have surface defects, which allows complement activation and phagocytosis to occur, albeit at much lower levels when compared to uncoated particles of identical size distribution. Conversely, surface homogeneity of PEGylated particles decreases substantially when BSA is adsorbed below the upper plateau region (e.g. when 50% of the total surface area is covered by BSA). Here, three different populations of PEGylated particles (F1a, F2a and F3a) were classified by hydrophobic interaction chromatography. Among them, the F1a particles exhibited biological performance (resistance to phagocytosis and poor complement activating ability) similar to that of the F1b and F2b fractions of PEGylated PS_{600} particles. Therefore, we can speculate that these three particle populations differ in surface PEG density as well as conformation. For instance, the F1a-type particles may express random regions of high PEG density in mushroom–brush and/or brush-like conformation (arising from closely associated PEGylated albumin molecules) separated by poor or non-PEG-enriched (naked) areas. In contrast, frequency of expression of high PEG density regions on F2a and F3a particles must be considerably lower than that of the F1a fraction. These particles are likely to have large surface areas, which are either naked and/or shielded by isolated rather than closely associated mPEG–BSA conjugates with PEG chains adsorbed onto the particle surface (a mushroom configuration). These speculations are supported by the higher electrophoretic mobility values of F2a and F3a particles compared to F1a particles as well as differences in the hydrodynamic mPEG layer thickness. Since surface PEGylation of all three populations is complete, the mode and the proximity of the adsorbed proteins seem to control the configuration of surface-attached mPEG molecules. Experiments with poloxamine 908 further support the above hypothesis, as poloxamine is able to mask naked (BSA-coated) surface patches on the F2a and F3a particles resulting in poor particle recognition by macrophages [10]. Indeed, poloxamine can adsorb efficiently onto the surface of protein-coated polystyrene particles [10,11]. According to suggestions of McPherson et al. [8] surface-adsorbed mPEG molecules, as in the case of F2a and F3a particles, are expected to block the adsorption sites for proteins but the results show potent complement activation by such surfaces. Therefore, for effective suppression of complement activation mPEG molecules must be in a predominant brush-like configuration as this can sterically suppress deposition of large C3 convertases (for instance dimensions of the convertase C3bBb are about 14×8 nm) [12]. We strongly emphasize that the generation of all designated populations of PEGylated PS_{250} and PS_{600} particles was reproducible; this may be indicative of some spontaneously controlled self-assembly surface phenomenon. This heterogeneity is not specific to BSA-coated polystyrene particles. Previously, we demonstrated that PEGylation of IgG-coated polystyrene particles also produces a heterogeneous population of entities with respect to surface characteristics [1].

The electrophoretic mobility measurements (or zeta potential) are routinely used for characterisation of sterically stabilised particles [13–16]. Interestingly, the results of the current study demonstrated a linear relationship between the zeta potential and the extent of particle uptake by macrophages. The failure of previous studies in establishing a direct correlation between surface properties of long-circulating particles and phagocytosis appears to be due to population heterogeneity and the proportion of each population within the mixture [13–16].

The results of this work may also be relevant to poorly understood biological behaviour of PEGylated liposomes. For example, Doxil®, a PEGylated liposomal formulation containing doxorubicin, has a biphasic circulation half-life of 84 min and 46 h in humans, respectively [17]. In addition to this, Doxil® is a potent activator of the human complement system with activation taking place within minutes [18]. Doxil® contains 5–7 mol% mPEG–phospholipid with surface-projected mPEG chains in a mushroom–brush configuration, which explain the prolonged circulation times of the vesicles. However, the macrophage clearance of a small fraction of Doxil® within 1–2 h post injection and its complement activating nature may arise from vesicle heterogeneity in a scenario similar to population heterogeneity of PEGylated BSA-coated particles. Our preliminary hydrophobic interaction chromatography studies with PEGylated liposomes, with identical lipid composition to Doxil®, have indicated considerable surface heterogeneity with some populations poorly protected by mPEG chains (unpublished data). In summary, our data strongly suggest that surface PEGylation of particles spontaneously generates a heterogeneous population of entities with different biological performance. Hydrophobic interaction chromatography can readily assess the extent of surface heterogeneity of PEGylated particulate drug

delivery systems and identify particles with optimal biological behaviour (e.g. resistant to phagocytosis, minimising complement activation and possibility of complement-mediated pseudoallergic responses). For optimal macrophage-resistant property, a homogeneous mPEG-grafted surface in a predominant mushroom–brush/brush configuration is essential. Our observations may also be relevant with respect to successful surface camouflaging of depot and implantable devices.

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