Phagocytosis of poly(L-lysine)-graft-poly(ethylene glycol) coated microspheres by antigen presenting cells: Impact of grafting ratio and poly(ethylene glycol) chain length on cellular recognition

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Microparticulate carrier systems have significant potential for antigen delivery. The authors studied how microspheres coated with the polycationic copolymer poly(L-lysine)-graft-poly(ethylene glycol) (PLL-*g*-PEG) can be protected against unspecific phagocytosis by antigen presenting cells, a prerequisite for selective targeting of phagocytic receptors. For this aim the authors explored the influence of PLL-*g*-PEG architecture on recognition of coated microspheres by antigen presenting cells with regard to both grafting ratio and molecular weight of the grafted PEG chains. Carboxylated polystyrene microspheres (5 μm) were coated with a small library of PLL-*g*-PEG polymers with PLL backbones of 20 kDa, grafting ratios from 2 to 20, and PEG side chains of 1–5 kDa. The coated microspheres were characterized by their ζ-potential and resistance to IgG adsorption. Phagocytosis of these microspheres by human monocyte derived dendritic cells (DCs) and macrophages (MΦ) was quantified by phase contrast microscopy and by analysis of the cells' side scattering in a flow cytometer. Generally, increasing grafting ratios impaired the protein resistance of coated microspheres, leading to higher phagocytosis rates. For DC, long PEG chains of 5 kDa decreased the phagocytosis of coated microspheres even in the case of considerable IgG adsorption. In addition, preferential adsorption of dysopsonins is discussed as another factor for decreased phagocytosis rates. For comparison, the authors studied the cellular adhesion of DC and MΦ to PLL-*g*-PEG coated microscopy slides. Remarkably, DC and MΦ were found to adhere to relatively protein-resistant PLL-*g*-PEG adlayers, whereas phagocytosis of microspheres coated with the same copolymers was inefficient. Overall, PLL(20)-[3.5]-PEG(2) was identified as the optimal copolymer to ensure resistance to both phagocytosis and cell adhesion. Finally, the authors studied coatings made from binary mixtures of PLL-*g*-PEG type copolymers that led to microspheres with combined properties. This enables future studies on cell targeting with ligand modified copolymers. © *2006 American Vacuum Society.* [DOI: 10.1116/1.2409645]

I. INTRODUCTION

Microparticulate carrier systems such as liposomes, microspheres, or microcapsules are of considerable interest in drug delivery, vaccination, and as diagnostic agents.[1–3] Notably, microparticulate antigen delivery systems have been shown to elicit cytotoxic T lymphocyte immunity as well as antibody and helper T cell responses[4–7] through phagocytosis by antigen presenting cells (APCs) and subsequent presentation of the antigen on MHC I (i.e., cross presentation) or MHC II.[8] [The abbreviations used are MPS, mononuclear phagocytic system; APCs, antigen presenting cells; DCs, dendritic cells; MΦ, macrophages; PEG, poly(ethylene glycol); PLL, poly-L-lysine; PLL(x)-g[y]-PEG(z), copolymer of PEG (MW=z kDa) grafted to PLL (MW=x kDa) at a grafting ratio y; grafting ratio, g, number of lysine monomers divided by the number of PEG side chains; IgG, immunoglobulin G; PS, polystyrene; HEPES buffer, 10 mM 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid, *p*H 7.4; PBS buffer, 10 mM phosphate buffered saline, *p*H 7.4; RPMI medium, RPMI 1640 (Roswell Park Memorial Institute) with L-glutamine; and SSC, side scattering (measured by flow cytometry).]

It is generally recognized that surface modifications of microparticulate antigen delivery systems with suitable peptides, proteins, oligosaccharides, or nucleic acids are of potential interest to control their first encounter with APC and could thus affect the type of immune response that will be elicited.[9–12] In order to specifically target APC, ligand mediated interactions with suitable receptors would be necessary. *In vivo*, however, injected particulate drug carriers are instantly recognized as foreign material and efficiently removed via ligand-unspecific phagocytosis by the MPS,

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which comprises phagocytic cells such as DCs and MΦ, among others.[13] The extent and rate of phagocytic clearance are dependent on size and surface properties. For instance, phagocytosis is enhanced by charged or hydrophobic surfaces.[14–16] The adsorption of opsonic proteins onto the surface of hydrophobic particles promotes phagocytosis.[17–19] Therefore, receptor specific targeting requires a microparticulate carrier system that is able to escape the MPS due to a surface modification that renders them resistant to opsonization. Only under this condition can the decoration of the particles' surface with specific ligands lead to a specific targeting of APC via surface receptors.

There are various approaches to prevent unwanted serum protein adsorption and cellular recognition.[20–22] Most strategies rely on the immobilization of PEG coatings, either through covalent or noncovalent PEGylation of the surface. Due to its low toxicity and immunogenicity, PEG is highly suitable for biomedical applications.[23,24] PEG is an uncharged, hydrophilic polymer that is soluble in water as well as in many organic solvents. In an aqueous environment, its extensive hydration, good conformational flexibility, and high chain mobility cause a steric exclusion effect.[23] Besides steric stabilization, hydration due to PEG-coupled, structured water is believed to contribute to the protein resistance of PEGylated surfaces.[25,26] As demonstrated theoretically[26–29] and experimentally[30–35] protein resistance of PEG-based coatings depend on both chain length and density, the product of which determines the thickness of the PEG adlayer. Protein resistance becomes increasingly efficient the more the PEG chains overlap,[33] and longer chains have been found to have the ability to fill in the gaps between less densely grafted PEG chains.[34] Theoretical studies have revealed two modes of protein adsorption:[29] (i) primary adsorption at the PEG-substrate interface and (ii) secondary adsorption at the top of the PEG brush. Small proteins may penetrate the PEG brush and adsorb onto the underlying surface. Increasing PEG chain density is expected to reduce the degree of primary adsorption. Resistance to larger proteins, which may undergo secondary adsorption, requires a sufficiently thick PEG layer to screen long range, e.g., electrostatic protein-substrate interactions.

A convenient method for the noncovalent immobilization of PEG on negatively charged surfaces is the adsorption of poly(L-lysine)-graft-poly(ethylene glycol) (PLL-*g*-PEG) copolymers, consisting of a polycationic poly(L-lysine) backbone whose side chain amino groups are partly grafted with PEG chains. Such polymers were previously found to spontaneously adsorb from aqueous solution onto negatively charged metal oxide surfaces.[36,37] Due to its positive charge at physiological *p*H, the PLL backbone adsorbs strongly onto negatively charged substrates through electrostatic interaction, leading to a monolayer with PEG chains stretched out perpendicularly to the surface.[36] Simulations using a self-consistent-field approach revealed the PEG end-segment distribution to be farther displaced from the PLL backbone for decreasing grafting ratios (defined as the ratio of the number of L-lysine monomers to the number of PEG side chains) as a consequence of increased stretch-out of the PEG chains due to increased PEG-PEG steric repulsion.[38] In addition, the PEG side chains can be functionalized with bioligands while retaining resistance to unspecific protein adsorption.[39–42] Appropriate choice of polymer architecture and assembly conditions renders such coated surfaces protein resistant even in the presence of full human serum.[35,36] Optimum protein resistance of PLL-*g*-PEG adlayers on metal oxide surfaces was observed for architectures with a 20 kDa PLL backbone, 2 kDa PEG side chains, and a grafting ratio of approximately 3.5.[35,37]

PEGylation of nano- and microparticles does not only reduce plasma protein adsorption but also inhibits the nonspecific internalization by phagocytes.[22,43–45] Recent publications report the modification of microparticles with PLL-*g*-PEG and ligand modified PLL-*g*-PEG polymers that showed excellent protein resistance.[39,46] In addition, it was shown for DC and MΦ cell cultures that phagocytosis of such particles could be abolished with PLL-*g*-PEG coatings.[47] However, these studies were performed only with the aforementioned "optimum" architecture, while no evaluation of the influence of polymer structure was reported so far.

In this work, we explored the influence of PLL-*g*-PEG architecture on phagocyte recognition of microspheres with regard to both grafting ratio and molecular weight of the grafted PEG chains. PLL-*g*-PEG coated polystyrene microspheres were first characterized by their ζ-potential and degree of IgG adsorption. In a second step, phagocytosis of PLL-*g*-PEG coated microspheres was examined *in vitro* in DC and MΦ by a microscopy-based counting protocol and by analysis of the cells' light scattering characteristics. In the second part of the study, we investigated the effect of PLL-*g*-PEG coatings on the spreading of DC and MΦ on flat surfaces and correlated the effect of surface chemistry on phagocytosis with cell adhesion.

II. MATERIALS AND METHODS

A. Materials

As microparticulates we used carboxylated PS microspheres (5 μm diameter) from Micromod Partikeltechnologie GmbH, Rostock, Germany. PLL-*g*-PEG copolymers were synthesized as outlined by Pasche *et al.*[35] The notation used for PLL(x)-*g*[y]-PEG(z) copolymers indicates the average molecular weights of PLL (x) and PEG (z) and the grafting ratio $g(y)$, which specifies the total number of lysine monomers divided by the number of PEG side chains as determined by ^{1}H NMR. The PLL-*g*-PEG copolymers consisted of a PLL backbone of 20 kDa with grafted PEG chains of 1, 2, or 5 kDa and a grafting ratio of 6.5 (for 1 kDa), 2.2, 3.5, 5.7, 10.1 (for 2 kDa), and 18.7 (for 5 kDa). All chemicals used in this study were of analytical grade (from Fluka, Buchs, Switzerland) unless otherwise specified. Ultrapure water (NANOpure Diamond™, Skan AG, Allschwil, Switzerland) was used for buffer preparation.

B. Methods

1. DC and MΦ cell culture

DC and MΦ were obtained from human peripheral blood monocytes according to Sallusto *et al.*[48] Briefly, peripheral blood monocytes were isolated from buffy coats (Bloodbank Zurich, Zurich, Switzerland) by density gradient centrifugation of Ficoll-Paque™ PLUS (Amersham Biosciences, Uppsala, Sweden). Isolated peripheral blood monocytes were washed in PBS (Sigma, Buchs, Switzerland) and resuspended in RPMI 1640+L-glutamine (Invitrogen, Basel, Switzerland) supplemented with 10% heat-inactivated (pooled) human serum (Bloodbank Zurich, Zurich, Switzerland), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Basel, Switzerland), and then allowed to adhere for 2 h in culture flasks (25 cm^2). Nonadherent cells were removed, whereas adherent cells were further cultured in RPMI medium supplemented with 5% heat-inactivated (pooled) human serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in the presence of 1000 IU/ml interleukin-4 (Sigma, Buchs, Switzerland) and 50 ng/ml human granulocyte-macrophage colony stimulating factor (R+D Systems Europe Ltd., Abingdon, UK) to obtain DC. MΦ were obtained without additional supplements.[14] These media will further be referred to as supplemented RPMI media. Cultures of DC and MΦ were kept at 37 °C in 5% CO_2-humidified atmosphere. For phagocytosis studies, cells were mechanically removed from the flasks after 24 h and reseeded into 24-well plates (300 000–350 000 cells per well).

Surface antigen expression of DC and MΦ obtained by this protocol were previously validated by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ).[47] Cells were incubated for 45 min at 4 °C with the following antibodies: CD11b (Mac-1, Clone ICRF44, Pharmingen, San Diego, CA), CD14 (Clone UCM-1, Sigma), CD83 (Clone HB15e, Pharmingen), CD86 (Clone IT2.2, Pharmingen), mouse isotype control antibody IgG2a (UPC-10, Sigma), and subsequently (after washing) with the secondary antibody (antimouse IgG R-phycoerythrin conjugate, Sigma). For further identification, the cells were also challenged with lipopolysaccharide (1 μg/ml, Escherichia coli 055:B5, Sigma) 48 h before the antibody labeling, which causes maturation of DC only. More than 90% of the cells were identified as DC, and in MΦ cultures, more than 90% of the cells were $DC14^+$ and $CD83^-$, even in the presence of lipopolysaccharide.[47]

2. Adhesion studies

Adhesion of DC and MΦ on modified surfaces was tested on glass coverslips coated with the respective PLL-*g*-PEG polymers. To rule out variances originating from donor-to-donor variability, experiments on each cell batch were always performed in parallel with all six polymers simultaneously. Before coating, 2-propanol cleaned glass coverslips (12 mm diameter) were oxygen-plasma treated in a plasma cleaner/sterilizer PDC-32G instrument (Harrick Scientific Corporation, Ossining, NY) for 2 min. Coating with PLL-*g*-PEG was performed in 24-well plates under aseptic conditions with 0.1 mg/ml of the respective polymers dissolved in 10 mM HEPES buffer, *p*H 7.4, for 30 min on a shaker. All solutions were sterile filtered through a 0.2 μm membrane filter before use. Control coverslips were incubated in HEPES buffer only. After coating, the coverslips were rinsed twice for 5 min with PBS (Sigma, Buchs, Switzerland) and once with supplemented RPMI medium. Either DC or MΦ incubated for 1 week in cell culture flasks was randomly added to each well (200 000–300 000 cells in 600 μl of cell medium) containing either coated or control coverslips. After 4 h cells were washed twice (DC) or three times (MΦ) with supplemented RPMI medium, and cellular adhesion was checked by microscopy (Axiovert 35, Zeiss, Germany). Five to six randomly acquired images were taken at tenfold magnification using a charge-coupled device (CCD) camera (CF 8/1 DXC, Kappa, Gleichen, Germany).

3. Coating of microspheres

PS microspheres (5 mg/ml) were dispersed under aseptic conditions in sterile filtered HEPES buffer (10 mM, *p*H 7.4). The relatively low ionic strength of the buffer was chosen to avoid loop formation of the PLL backbone upon adsorption of the polymer to the microsphere surface.[49] For microsphere coating the dispersions were mixed at equal volumes with the respective PLL-*g*-PEG or mixed PLL-*g*-PEG solutions (1 mg/ml in sterile filtered HEPES buffer) and incubated under gentle mixing for 15 min at room temperature. Surface-coated microspheres were centrifuged (5 min, 2655 $\times g$, Eppendorf, Centrifuge 5417R, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) and redispersed in half of the previous volume in sterile filtered HEPES buffer.

4. ζ-potential of PLL-g-PEG coated microspheres

PLL-*g*-PEG coated and uncoated PS microspheres (65 μg/ml) were dispersed in filtered HEPES buffer (10 mM, *p*H 7.4), HEPES buffer supplemented with 150 mM NaCl, or RPMI medium. Measurements were performed on a Zetasizer 3000 HS (Malvern Instruments, Worcestershire, UK; five measurements per sample).

5. Protein resistance of PLL-g-PEG coated microspheres

To determine the protein resistance of surface-coated PS microspheres, we used a fluorescence labeled immunoglobulin, TxRed-goat antirabbit IgG(H+L) (Molecular Probes Inc., Eugene, OR). The protein solution was diluted in filtered HEPES buffer (10 mM, *p*H 7.4) to yield a final concentration of 150 μg/ml, and added to an equal volume of microsphere dispersion (1 mg/ml in filtered HEPES buffer). After 45 min of incubation in the dark, samples were centrifuged (10 min, 2655*g*, Eppendorf, Centrifuge 5417R, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany), washed, and redispersed in filtered HEPES buffer at 250 μg/ml. Fluorescence of the microspheres as a consequence of antibody binding was determined in a flow cyto-

meter (FACSCalibur from Becton Dickinson, Franklin Lakes, NJ). For data analysis, median fluorescence values were calculated using the Cytomation SUMMIT software (v3.1, Cytomation Inc., Fort Collins, CO). Relevant quenching of IgG-TxRed fluorescence on the particles was excluded by an incubation experiment with concentrations up to 160 μg/ml, resulting in an approximately linear relationship of fluorescence intensity and IgG-TxRed concentration.

6. Phagocytosis studies

Studies were performed with both DC and MΦ. To rule out variances originating from donor-to-donor variability, experiments on each cell batch were always performed in parallel with all six polymers simultaneously. MΦ cultured in 24-well plates were washed once with RPMI medium and then covered with 600 μl of supplemented RPMI medium. Because DC adhered less strongly to the cell culture plates, they were not washed but directly exposed to the microspheres. To exclude compromised viability, we stained the DC with annexin-V-APC (BD Biosciences, Basel, Switzerland) and propidium iodide (BD Biosciences, Basel, Switzerland) according to the manufacturer's protocol. Analysis by flow cytometry revealed that more than 80% of the cells turned out to be viable upon double staining, and less than 15% were positive for propidium iodide.

For the assessment of phagocytosis, microspheres dispersed in filtered HEPES buffer were added to either DC or MΦ cultures and incubated at 37 °C in 5% CO_2-humidified atmosphere for 4 h. The medium was carefully exchanged to remove nonadherent cells. Cells were immediately inspected for phagocytosis by light microscopy (phase contrast; Axiovert 35, Zeiss, Germany). Seven to eleven randomly acquired images were taken from three samples per experiment at 20-fold magnification using a CCD camera (CF 8/1 DXC, Kappa, Gleichen, Germany). The number of phagocytosed microspheres per cell was counted manually. To account for the variance of phagocytotic activity of cells from different donors, numbers of microspheres per cell were normalized to PS positive controls.

Following microscopic analysis, the cells were mechanically recovered in a test tube and additionally evaluated by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ).[50] For data analysis, mean SSC values were calculated with Cytomation SUMMIT software (v3.1, Cytomation Inc., Fort Collins, CO).[51]

7. Significance testing

For evaluation of statistical significance, samples were evaluated by analysis of variance followed by a *post hoc* assessment using the Tukey HSD method. Differences were considered significant when equal or less than $p=0.05$ using SPSS software (SPSS Inc., Chicago, IL).

TABLE I. Microsphere characterization. ζ-potential of and IgG adsorption on PLL-*g*-PEG coated microsheres in 10 mM HEPES buffer. ζ-potential in mV (±SD); IgG adsorption in % of uncoated control microspheres as determined by flow cytometry (median of fluorescence±SD).

Polymer	ζ (mV)	IgG (% of PS)
PLL(20)-[2.1]-PEG(2)	1±1	2.6±0.5
PLL(20)-[3.5]-PEG(2)	6±2	3.8±0.0
PLL(20)-[5.7]-PEG(2)	7±2	8.5±1.3
PLL(20)-[10.1]-PEG(2)	5±2	18.2±6.5
PLL(20)-[6.5]-PEG(1)	14±6	16.9±2.5
PLL(20)-[18.7]-PEG(5)	4±2	6.8±0.4
PS microspheres	−44±2	100±0.0

III. RESULTS

A. ζ-potential of coated microspheres

We measured the ζ-potential to monitor the coatings of PLL-*g*-PEG copolymers on carboxylated polystyrene microsphere surfaces. Uncoated microspheres showed a strongly negative ζ-potential (−45±5 mV in 10 mM HEPES buffer). All coatings gave rise to a slightly positive ζ-potential of the microspheres when measured in 10 mM HEPES buffer (pH=7.4), thus providing evidence for effective coating of the microspheres (Table I). While the ζ-potentials of most of the polymers were around 5 mV, PLL(20)-[2.2]-PEG(2) and PLL(20)-[6.5]-PEG(1) yielded slightly different values of 1±2 mV and 14±6 mV, respectively. Only the latter value, however, was statistically different from the others. When the ζ-potential of coated microspheres was determined at the higher ionic strength used in the cell assays, the data were no longer statistically significant: The ζ-potentials of coated microspheres were all 5±2 mV when measured in 10 mM HEPES supplemented with 150 mM sodium chloride (pH =7.4) or in cell culture medium (RPMI medium supplemented with 5% human serum).

B. Resistance of coated microspheres to IgG adsorption

The protein resistance of the PLL-*g*-PEG coated microspheres was assessed by incubation with a goat-antirabbit IgG(H+L) serving as a model protein (Table I and Fig. 1). For polymers with PEG chains of 2 kDa, we found the adsorption of IgG to increase linearly with the grafting ratio. Very low amounts of IgG adsorbed on coatings of polymers with the lowest grafting ratios (<4% of the amount detected on uncoated polystyrene microspheres), while a significant amount of protein adsorbed at the highest grafting ratio [18.2(±6.5%)]. Interestingly, the intermediate grafting ratio (g=5.7) that had been qualified as protein resistant in the literature[35] showed considerable protein adsorption.

At constant ethylene-glycol-monomer surface densities no significant difference in IgG adsorption was found between a PEG chain length of 1 kDa [16.9(±2.5%)] and 2 kDa [18.2(±6.5%)]. Interestingly, much lower adsorption of IgG was found with 5 kDa PEG chains [6.8(±0.4%)].

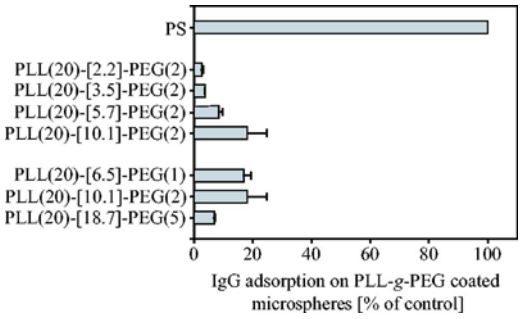

FIG. 1. Protein resistance of PLL-*g*-PEG coated PS microspheres. Fluorescence of PLL-*g*-PEG coated microspheres after incubation with TxRed-goat antirabbit IgG(H+L) in 10 mM HEPES buffer and subsequent washing. Data represent median fluorescence values from flow cytometry normalized to uncoated control microspheres.

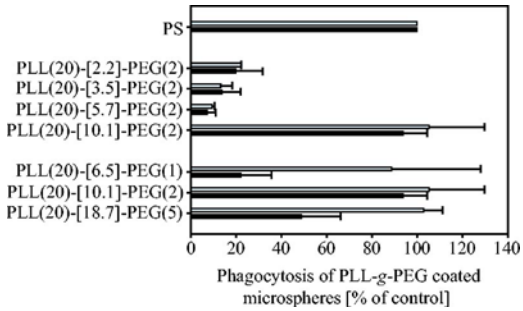

FIG. 2. Phagocytosis of PLL-*g*-PEG coated PS microspheres. Phagocytosis of PLL-*g*-PEG coated microspheres by DC (black) and MΦ (gray). Numbers of internalized microspheres were counted after 4 h of incubation and normalized to uncoated control microspheres.

C. Phagocytosis of PLL-*g*-PEG coated PS microspheres by DC and MΦ

To investigate whether PLL-*g*-PEG coatings would render microspheres resistant to phagocytosis, we coated PS microspheres (5 μm) and incubated them in DC or MΦ cultures, respectively. The extent of phagocytosis was first analyzed by microscopy (Table II and Fig. 2).[52] As a reference we used uncoated microspheres which were efficiently internalized within the time frame of the experiment (4 h) by about 70% of the cell population. The effect of the grafting ratio of PLL-*g*-PEG type copolymers was examined using four copolymers of the general architecture PLL(20)-*g*-PEG(2) with grafting ratios of 2.2, 3.5, 5.7, and 10.1, respectively. The first three have been reported to resist protein adsorption but not the latter.[35] As expected, the numbers of PLL(20)-[10.1]-PEG(2) coated microspheres that were subject to phagocytosis were close or equal to those for uncoated reference microspheres, while polymers classified by Pasche *et al.*[35] as protein resistant reduced the phagocytic uptake to about 10%–20%. Interestingly, the lowest numbers of phagocytosed microspheres (about 10%) were observed for $g=5.7$ and increased for smaller grafting ratios, though without statistical significance. No difference in phagocytosis was found between DC and MΦ.

In addition to the effect of the grafting ratio, the influence of the PEG chain length was investigated as well. For this purpose, we chose polymers with PEG chains of 1, 2, and 5 kDa, which result in roughly the same ethylene-glycol-monomer surface density when assembled on a surface (as determined by Pasche *et al.* on Nb_2O_5 surfaces[35]), i.e., PLL(20)-[6.5]-PEG(1), PLL(20)-[10.1]-PEG(2), and PLL(20)-[18.7]-PEG(5). Equal to the $g=10.1$ polymer, relatively high amounts of serum proteins were observed to adsorb on flat surfaces coated with the other two polymers.[35] Consistently, microspheres coated with any of the three polymers were internalized by MΦ about as efficiently as reference microspheres. However, DC and MΦ behaved differently: While microspheres coated with the 2 kDa PEG chain polymer were phagocytosed to a similar extent as uncoated reference microspheres, both longer (5 kDa) and shorter (1 kDa) PEG chains significantly ($p<0.01$) impaired the microspheres' internalization to about 50% and 20%, respectively.

TABLE II. Adhesion and phagocytosis. Cell adhesion (number of adherent cells) on PLL-*g*-PEG coated glass slides in % of uncoated glass by microscopy (mean counts±SD); phagocytosis of PLL-*g*-PEG coated microspheres determined by flow cytometry (mean of SSC±SD) and by microscopy (mean counts±SD) in % of uncoated microspheres. Evaluation after 4 h of incubation.

	Adhesion (% of glass)		Phagocytosis (% of PS)		
Polymer	DC microscopy	MΦ microscopy	DC flow cytometry	DC microscopy	MΦ microscopy
PLL(20)-(2.1)-PEG(2)	72±19	121±5	10±28	20±12	22±0
PLL(20)-[3.5]-PEG(2)	1±1	1±1	4±27	14±8	13±6
PLL(20)-(5.7)-PEG(2)	47±41	73±25	−3±25	7±4	10±1
PLL(20)-(10.1)-PEG(2)	86±17	88±2	95±13	94±11	105±25
PLL(20)-[6.5]-PEG(1)	66±20	83±14	19±10	22±14	89±40
PLL(20)-(18.7)-PEG(5)	84±7	79±3	46±34	49±18	103±9
PS microspheres	···	···	100±0	100±0	100±0
Glass (uncoated)	100±0	100±0	···	···	···

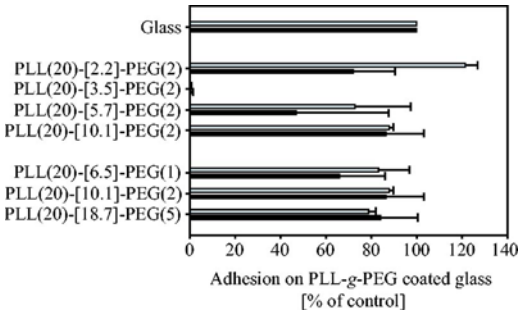

FIG. 3. Adhesion of PLL-*g*-PEG coated glass slides. Adhesion of DC (black) and MΦ (gray) on PLL-*g*-PEG coated glass slides. Numbers of adherent cells were counted after 4 h of incubation and normalized to uncoated control slides. Both cell types did not adhere to glass slides coated with PLL(20)-[3.5]-PEG(2).

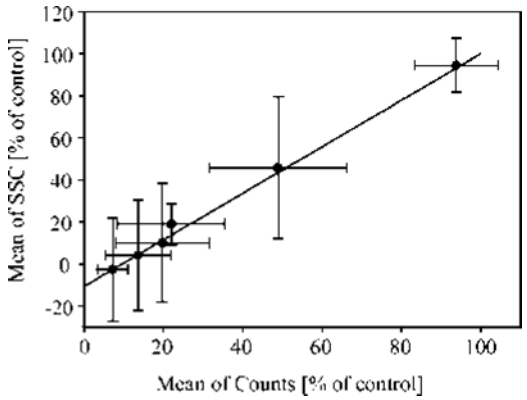

FIG. 4. Correlation of flow cytometry and microscopy. Correlation of DC phagocytosis data obtained from flow cytometry (mean of SSC±SD) vs data obtained from phase contrast microscopy (mean counts±SD). PS microspheres coated with PLL-*g*-PEG copolymers of different architectures were incubated with DC for 4 h. Samples were successively analyzed by both techniques.

D. Quantification of phagocytosis by side scatter analysis

For the quantification of phagocytic efficiency we focused not only on phase contrast microscopy but also sought for a less time consuming technique. Therefore we successfully established a flow cytometric protocol that was based on the shift of the side scattering signal as a result of phagocytosis.[51,53,54] Because MΦ adhere very strongly to cell culture dishes and are difficult to remove without damaging them, only DC were used in these assays. Our results (Table II and Fig. 3) demonstrate an excellent correlation between the mean of the side scatter and the values obtained by microscopic analysis. Thus side scatter analysis by flow cytometry can be used as a convenient method to quantify phagocytosis, at least within the experimental framework of our study and for DC.

E. Influence of polymer mixtures on phagocytosis of coated microspheres

In addition to varying the architecture of the PLL-*g*-PEG, it is also possible to assemble the polymers from mixtures of two polymers, which may result in microspheres with surface characteristics that lie either between those of the individual polymers or are dominated by one of them. As an example we investigated the phagocytosis of microspheres coated with varying ratios of the two polymers PLL(20)-[5.7]-PEG(2) and PLL(20)-[10.1]-PEG(2), the former being the most phagocytosis resistant one among the polymers used in this study, while microspheres coated with the latter were internalized about as efficiently as uncoated reference microspheres. Up to a fraction of 40% of the nonresistant polymer we observed a direct dependence of phagocytic efficiency and polymer proportion. Above that proportion phagocytosis was close to 100% of control (Fig. 4).

F. Adhesion of DC and MΦ on PLL-*g*-PEG coated glass slides

To investigate the effect of polymer coatings on the adhesion of APC on flat surfaces, glass coverslips coated with the respective polymers were used as substrates and subsequently incubated with DC or MΦ for 4 h prior to evaluation by microscopy (Table II and Fig. 5). As a reference uncoated coverslips were used, to which both cell types showed a high affinity as indicated by good spreading and strong adhesion.

As expected for the group of 2 kDa PEG chains, the numbers of adherent cells on the polymer with the relatively high grafting ratio of 10.1 were close or equal to the number on the reference glass slides. Interestingly, although both $g=3.5$ and 5.7 yielded significantly ($p<0.01$) reduced numbers of adherent cells, only the polymer with a grafting ratio of 3.5 was found to completely abolish cellular adhesion for both cell types. Both the higher and the lower grafting ratios of 2.2 and 5.7 were found to be less resistant to cellular adhesion, with a high variance between different samples and positions in the case of $g=5.7$.

Coatings of the non-protein-resistant group of polymers of different PEG chain lengths were not expected to impede

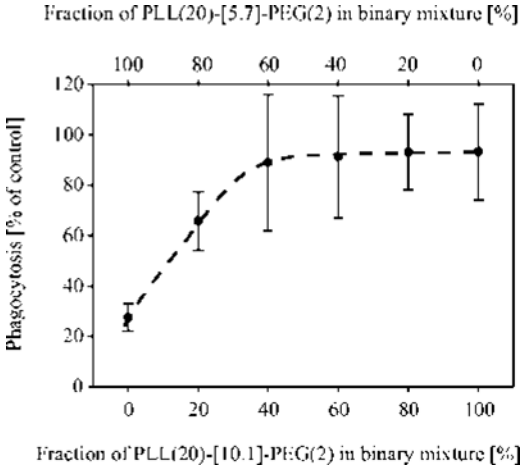

FIG. 5. Phagocytosis of PS microspheres coated with binary PLL-*g*-PEG mixtures. PS microspheres coated with binary mixtures of PLL(20)-[5.7]-PEG(2) and PLL(20)-[10.1]-PEG(2) were incubated with DC. Phagocytosis was quantified by side scatter analysis (mean of SSC±SD) after 4 h of incubation and normalized to uncoated control microspheres.

cellular adhesion to a great extent. Consistently, all polymers showed about the same numbers of cellular adhesion, which were close to control values. Only the polymer with the short PEG chains (1 kDa) and the relatively low grafting ratio of $g=6.5$ featured significantly ($p<0.05$) less DC adhesion.

IV. DISCUSSION

Recognition and subsequent phagocytosis by APC represent important steps in the first encounter of microparticulate antigen delivery systems with the innate immune system. Unspecific recognition of microparticulates by APC is supported by prior adsorption of opsonizing serum proteins on their surface.[17,18] With the aim of establishing design rules for PLL-*g*-PEG surface coatings in terms of their ability to shield microspheres from recognition by APC, we studied the impact of PLL-*g*-PEG copolymer architecture on protein resistance and phagocytosis of coated PS microspheres. In a first set of experiments, we examined four polymers of the general architecture PLL(20)-*g*-PEG(2) having grafting ratios *g* of 2.2, 3.5, 5.7, and 10.1. The first three polymers have previously been reported to provide protein resistant flat surfaces, while the latter was not.[35] However, earlier work suggested that PLL-*g*-PEG copolymers with a grafting ratio of 5–6 were less protein resistant than those with the grafting ratio of 3.5.[35,37] In addition to the effect of the grafting ratio, we were also interested in the potential effect of the PEG chain length. To this end, we chose polymers with PEG chains of 1, 2, and 5 kDa. As protein resistance on flat surfaces has previously been related to the amount of ethyleneglycol-monomer density per surface area rather than to PEG chain length or grafting ratio alone,[35] we chose three polymers having about the same ethylene-glycol-monomer density per surface area, i.e., PLL(20)-[6.5]-PEG(1), PLL(20)-[10.1]-PEG(2), and PLL(20)-[18.7]-PEG(5). As mentioned above for the $g=10.1$ copolymer, the protein resistance of flat surfaces coated with these polymers was relatively low.[35]

To monitor the effects of PLL-*g*-PEG coatings on phagocytosis, we used carboxylated PS microspheres as core material. Microspheres of 5 μm in diameter were chosen, the size of which is within the optimum size range for phagocytosis by APC.[7,55] Due to their negative surface charge, carboxylated PS microspheres adsorb PLL-*g*-PEG copolymers by electrostatic interaction resulting in a PEG corona.[36,47]

For the quantification of phagocytic efficiency, we first focused on phase contrast microscopy. Counting the numbers of phagocytosed microspheres per cell from several statistically acquired images has the advantage of yielding absolute numbers without the need of further sample processing. However, manual acquisition of statistically significant cell counts by a counting protocol turned out to be extremely time consuming. Flow cytometry has been routinely used for fast and reliable quantification of fluorescently labeled microspheres.[56] A less common flow cytometric approach to monitor particle internalization takes advantage of the high sensitivity of the side scattering signal to changes in cell morphology as a consequence of phagocytosis,[51,53,54] thus bypassing the need for fluorescent dyes. The direct use of fluorescently labeled microspheres was discarded because of an interference of the PLL-*g*-PEG coatings with the label, potentially due to inhomogeneities in the negative surface charge distribution on such spheres. Because MΦ adhere very strongly to cell culture dishes, we exclusively used DC to examine the feasibility to quantify particle internalization by evaluation of the side scattering signal. Our results demonstrate an excellent correlation between the mean of the side scatter and the values obtained by microscopic analysis. It is a general drawback of flow cytometry that arbitrary units are obtained instead of the absolute numbers of phagocytosed microspheres per cell. However, this is not necessarily a disadvantage as numbers can be normalized to unmodified control microspheres. In addition to being less time consuming, the developed protocol also yields statistically more reliable data than microscopy, based on the several thousands of cells per sample that can be analyzed in the former case.

A. Characterization of coated microspheres

The coating process could be conveniently monitored by measuring the microspheres' ζ-potential. Upon PLL-*g*-PEG adsorption the strongly negative surface charge of uncoated carboxylated polystyrene microspheres is counterbalanced by the positive charges of the PLL backbone. Furthermore, remaining interfacial charges are shielded by ions within the PEG corona. Thus, coating of the particles is expected to yield a ζ-potential close to zero.[46] Experimentally, slightly positive values (5 ± 2 mV) were obtained for all coated microspheres when measured in solutions of physiological ionic strength. At lower ionic strength (10 mM HEPES), a statistically significant difference was only observed for PLL(20)-[6.5]-PEG(1). The slightly higher ζ-potential obtained for this polymer (14 ± 6 mV) probably results from its shorter 1 kDa PEG chains, which may be assumed to be less efficient in shielding the remaining positive interfacial charge than the 2 or 5 kDa PEG chains of the other coatings. However, the measured increase in surface charge is small compared to formulations in other studies that observed an enhanced phagocytosis for positively charged particle formulations.[14–16] Hence, a major influence of the surface charge on the results of our cell adhesion or phagocytosis experiments is unlikely. Overall, our observations are consistent with the successful formation of a polymeric adlayer on PS microspheres, whose surface is efficiently shielded by a PEG corona.

In principle, polymeric coatings can be characterized by the size increase of the coated microspheres. PLL-*g*-PEG, however, has been shown to form a monolayer when adsorbed on a negatively charged surface[36] with a thickness of less than 10 nm.[57] Therefore, the size increase during the coating of the microspheres was too small to be detected by light scattering. Moreover, the negligible size increase discards size as a factor for phagocytic efficiencies in our study.

In its physiological context, it is well established that phagocytosis of microspheres requires prior opsonization by immunoglobulins and small proteins of the complement

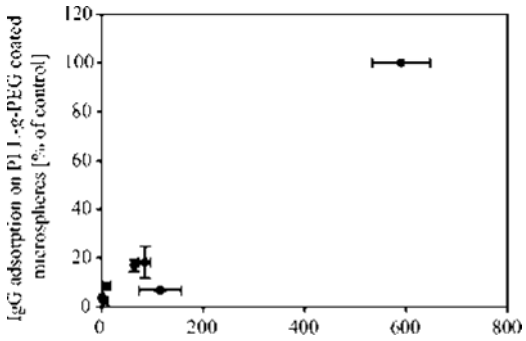

FIG. 6. Correlation of IgG adsorption on coated PS microspheres with serum adsorption on coated flat Nb_2O_5. Median fluorescence values (±SD) from flow cytometry after incubation of uncoated and PLL-*g*-PEG coated PS microspheres with TxRed-goat antirabbit IgG(H+L) were normalized to uncoated control microspheres and correlated with published data on protein mass adsorbed from whole serum on flat Nb_2O_5 surfaces coated with PLL-*g*-PEG copolymers of the same architectures [from Pasche *et al.* (Ref. 35)].

system.[8,18] The amount and nature of adsorbed proteins are believed to affect the extent and rate of phagocytosis.[13,19,43,58] To mimic opsonization *in vitro* we chose immunoglobulin G, goat-antirabbit IgG(H+L), as model protein. Despite the variable charge and size of proteins present in full serum, IgG is an established model to check for resistance to serum protein adsorption.[39,45] In particular, class G immunoglobulins are the principal "heat-stable" opsonic proteins.[17–19] In addition, opsonization with complement was found to be insufficient for phagocytosis unless the studied particles also contained IgG.[59]

Hypothetically, a direct relationship between the previously published adsorption of whole serum on flat, polymer-coated surfaces[35] and our data of IgG adsorption on microspheres coated with the same type of polymers could be expected. However, the number of available data pairs does not allow a statistically significant confirmation (Fig. 6). Deviations from such a direct relationship may be due to the different serum proteins used in both essays, i.e., IgG or whole serum. For example, in the case of surfaces with dense PEG chains (low $[g]$ value), the very small gaps between the PEG chains would be expected to be accessible for the primary adsorption of very small proteins only. As well, a steric effect of long PEG chains (5 kDa) could be assumed. Indeed, this seems to be the case for the 5 kDa PEG copolymer used in this study. As its grafting ratio (18.7) is higher than that of any of the 2 kDa PEG copolymers (10.1), and IgG adsorption was found to increase with increasing grafting ratio, even higher amounts of IgG would be expected to adsorb on microspheres coated with the 5 kDa PEG copolymer. However, we found a reduced IgG adsorption, which thus can only be attributed to the longer PEG chains. This would be in accordance with a recent publication claiming that longer PEG chains ($\geqslant$5 kDa) have the ability to fill in effectively the space between less densely spaced PEG chains.[34] In contrast to IgG with its rather large molecular size, smaller serum proteins may still be able to reach the surface, thus leading to an enhanced protein adsorption as documented in a previous study in serum.[28,35] It is interesting to note that we found a linear increase in IgG adsorption with increasing grafting ratio, whereas previous studies[35,37] led to ambiguous results for $g=5.7$.

B. Cellular recognition of coated microspheres: Phagocytosis

The major aim of our study was to investigate the capacity of PLL-*g*-PEG coatings to resist phagocytosis of the PS microspheres. We hypothesized that this should correlate with the resistance of the polymeric adlayers to unspecific protein adsorption. The latter has previously been shown to depend primarily on the ethylene-glycol-monomer surface density, which is a function of both grafting ratio and PEG chain length of the polymer.[35] To verify this hypothesis, both DC and MΦ were incubated with surface-coated microspheres. Numbers of internalized microspheres per cell were determined by phase contrast microscopy and normalized to uncoated control microspheres.

Concerning the effect of the grafting ratio, while keeping the general architecture of PLL(20)-*g*-PEG(2) polymers constant, we found relatively low phagocytosis rates (about 10%–20% of control levels) with grafting ratios of 2.2, 3.5, and 5.7, whereas the phagocytosis efficiency of PLL(20)-[10.1]-PEG(2) coated microspheres was close or equal to the uncoated control. This is in general agreement with the previous work on flat surfaces of Pasche *et al.*, who qualified the first three as protein resistant (i.e., $\leqslant 10$ ng/cm^2 adsorbed serum), but not the latter.[35] Within the $[g]$ range of 2.2–5.7, however, IgG protein adsorption was found to increase with the grafting ratio g, which in turn should have led to increasing average numbers of phagocytosed microspheres even for these small values. Experimentally, with respect to phagocytosis, the data showed an opposite trend to the one seen in the IgG adsorption experiments. (Although statistically insignificant, the same tendency was found in most experiments.) A possible explanation for this apparent discrepancy would be the assumption of a preferential adsorption of dysopsonins between the grafted PEG chains for polymers with $g=5.7$ when exposed to the cell culture medium. Although difficult to identify, dysopsonins have been shown to impair the phagocytosis of various particulates such as latex microspheres or liposomes.[17,18,60–62] In a recent study, a PEG corona was demonstrated to not only reduce protein adsorption but also shift the composition of the adsorbed proteins towards dysopsonins.[43] In particular, albumin is abundant in body fluids and has been shown to have dysopsonizing properties.[19,43] Further studies should therefore concentrate on the identification of the adsorbed proteins. Another important issue to consider is the polymer surface coverage. Pasche *et al.*[35] showed for Nb_2O_5-coated surfaces that the surface coverage of the polymer with the very low grafting ratio, $g=2.2$, was significantly reduced, while the polymers with $g\geqslant 3.5$ were approximately identical. This was ascribed to the smaller positive charge of the PLL backbone and especially to the onset of strong steric repulsion between the

densely packed PEG chains. Concomitantly, a slight increase in serum adsorption relative to the polymer with the next higher grafting ratio, $g=3.5$, was observed. Thus, internalization of microspheres coated with this polymer would be expected to increase relative to those with a slightly higher grafting ratio.

In a second set of experiments we varied the PEG chain length from 1 to 5 kDa, while the grafting ratio was adjusted such that the ethylene-glycol-monomer surface density was kept approximately constant. The rationale of this procedure was to examine a potential effect of the PEG chain length with polymers of comparable ethylene-glycol-monomer density. By choosing a set of non-protein-resistant polymers we expected to find more drastic effects, if any, than for polymers that resist the adsorption of proteins. Interestingly, in this set of experiments we encountered differences between the two cell types. In the case of MΦ, coatings of the copolymers PLL(20)-[6.5]-PEG(1), PLL(20)-[10.1]-PEG(2), and PLL(20)-[18.7]-PEG(5) yielded no significant deviation from control microspheres. This was not surprising, given the fact that relatively high amounts of protein adsorbed on all three coatings. For DC, however, the results were unexpected in the sense that both the 1 and 5 kDa PEG grafted polymers significantly reduced microsphere internalization. In fact, the 1 kDa PEG copolymer reduced phagocytosis to nearly the same extent as the protein-resistant ones. These findings clearly indicate that although the ethylene-glycol-monomer density per surface area seems to be the decisive factor for protein resistance,[35] it is not a sufficient criterion to predict phagocytic efficiency of APC. As discussed above, this effect cannot be ascribed to differences in the microspheres' surface charge because these were all found to be quite low for the coated particles. In addition, residual positive charges would have led to the opposite result since cationic surfaces are believed to enhance phagocytosis.[14–16] In the case of the 5 kDa PEG copolymer, its long PEG chains may constitute a steric barrier around the microspheres, reducing the degree of interaction with the opsonins.[63] This was corroborated by our IgG adsorption study in which unexpectedly low levels of IgG were seen to adsorb onto the microspheres coated with this long PEG chain copolymer. On the other hand, microspheres coated with the 1 kDa PEG copolymer adsorbed high amounts of protein. Thus, the explanation for the highly reduced phagocytosis of these microspheres must probably be rather found in the type and composition of the adsorbed proteins. Indeed, the grafting ratio of this polymer ($g=6.5$) is close to the grafting ratio of PLL(20)-[5.7]-PEG(2), for which we speculated that it would favor preferential adsorption of dysopsonins. The fact that these observations only apply to DC but not to MΦ is likely to reflect the generally superior phagocytic efficiency of MΦ.[14,64]

In summary, two main conclusions can be drawn from our phagocytosis study: First, microsphere resistance to recognition by phagocytes is primarily correlated with resistance of its surface to protein adsorption. While high PEG chain densities are crucial for low cellular recognition, lower PEG chain densities may, to some extent, be balanced if the PEG chains are sufficiently long (5 kDa). Secondly, our data suggest that for polymer-coated surfaces with low but not negligible protein adsorption, the nature of the adsorbed proteins could be crucial. Although further studies with a variety of proteins would be needed to corroborate this assumption, in special cases adsorption of dysopsonins could be even more effective in reducing phacocytic activity than minimal protein adsorption.

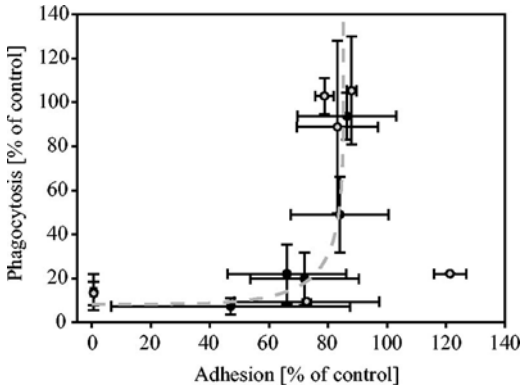

FIG. 7. Correlation of phagocytosis of PLL-*g*-PEG coated PS microspheres with APC adhesion on PLL-*g*-PEG coated glass slides. Correlation of data obtained from phagocytosis (mean counts±SD) vs data obtained from adhesion experiments (mean counts±SD). DC (black) and MΦ (gray) are plotted in the same graph. The outlier data point refers to PLL-(20)-[2.1]-PEG(2). The dashed curve indicates trend of data.

C. Phagocytosis of microspheres versus cellular adhesion

As both phagocytosis of microspheres (that do not expose specific ligands) and cellular adhesion rely primarily on protein binding to the respective surfaces prior to recognition by APCs, a correlation between both processes could be expected depending on the physicochemical properties of the respective surfaces, i.e., the architecture of the PLL-*g*-PEG polymer in our case. Preliminary results from Faraasen *et al.*[47] supported this hypothesis. With the more extensive data set of this work, we found, however, that the correlation between phagocytic activity and cell adhesion is nonlinear. As seen in Fig. 7, both DC and MΦ were able to adhere to some extent to more or less protein-resistant polymer adlayers, whereas phagocytosis of microspheres coated with the same copolymers was yet inefficient. Two factors may account for this finding: First, cells that are seeded onto a surface undergo sedimentation and secrete proteins to remodel the surface for improved adhesion. The resulting high interfacial concentration of proteins will increase the probability of protein adhesion to structural defects in the coating, resulting in an increase of cell adhesion to the remodeled surfaces. Moreover, cellular adhesion on PEG coated surfaces has been attributed to reversible adsorption of proteins, which was reported to support weak, reversible cellular adhesion.[30] Second, cells require a minimum surface density of "adhesive spots" for efficient spreading,[65] while phagocytosis relies on a minimum number of activated receptors to

recognize the microspheres as a foreign material.[8] Obviously, these numbers need not be the same, and it is possible that higher ligand densities are needed for phagocytosis than for spreading. In addition, receptors that mediate adhesion are not necessarily active in phagocytosis, and dysopsonins may interfere with microsphere recognition.

Thus, adhesion studies yield supplementary information rather than merely confirming phagocytosis data. In the present study, only PLL(20)-[3.5]-PEG(2) showed no cellular adhesion, which is another hint that this is the only polymer, within the tested series, with an architecture that ensures optimum protein resistance. This finding corresponds with results from other authors on comparable flat or microparticulate systems.[35,37,47]

D. Binary polymer mixtures

In the previous section we showed that the bioresponsiveness of surface coatings of microspheres could be tailored by using PLL-*g*-PEG with different architectures. In addition, we were able to demonstrate that the degree of resistance to phagocytosis can be quantitatively tailored by assembling mixed PLL-*g*-PEG coatings based on two different polymers rather than just one type. In the present study, binary mixtures consisting of a protein-resistant copolymer (PLL(20)-[5.7]-PEG(2)) and a nonresistant copolymer (PLL(20)-[10.1]-PEG(2)) were tested. We observed microspheres to become increasingly resistant to phagocytosis with increasing proportion of the protein-resistant PLL-*g*-PEG copolymer in the mixed adlayer. The correlation is, however, not linear (Fig. 4), with the phagocytotic activity increasing most steeply at the lowest fraction of the (non-protein-resistant) PLL(20)-[10.1]-PEG(2), and leveling off once the proportion of this polymer in the coating has reached 60%. Since protein adsorption is expected to correlate approximately linearly with the fraction of the non-protein-resistant polymer, this implies that (much) less than a monolayer of opsonins at the microsphere surface is required for efficient recognition and uptake by APC.

V. CONCLUSION

In this work, we studied the impact of PLL-*g*-PEG copolymer architecture on the resistance of coated microspheres to protein adsorption and phagocytosis by APC. As expected, protein adsorption was found to increase with increasing grafting ratio. Lower adsorption was found for longer (5 kDa) PEG chains, which suggests a steric effect of the long PEG chains on the adsorption of the relatively big model protein, IgG.

Two conclusions could be drawn from phagocytosis and cellular adhesion studies: Firstly, microsphere resistance to recognition by phagocytes was found to be mostly dependent on the protein resistance of the surface coatings. Although a too low PEG density could, to some extent, be balanced by longer PEG chains, high PEG chain densities were crucial for low cellular recognition. From the copolymer architectures used in this study, PLL(20)-[3.5]-PEG(2) was identified as optimal to ensure resistance to phagocytosis. Second, our data suggested that for polymers with low but not negligible protein adsorption, the nature of these proteins would be of essential importance. Although rather speculative at the moment, in special cases adsorption of dysopsonins seemed to be even more effective to impair phagocytosis than minimal protein adsorption. In addition, PLL-*g*-PEG surface coverage may become important for very high PEG chain densities as in the case of PLL(20)-[2.2]-PEG(2), where the PEG interchain repulsion is believed to reduce the surface coverage of the copolymer, thereby decreasing the protection from cellular recognition.

In this work, we successfully established quantification of DC phagocytosis by side scatter analysis using a flow cytometer. Application of this technique to microspheres coated with binary PLL-*g*-PEG mixtures revealed the feasibility to modify microsphere surfaces with a coating made from a mixture of different PLL-*g*-PEG type copolymers. This is of particular interest for the future use of these microspheres in cell targeting. For instance, it would be possible to decorate the microspheres' surface with a mixture of cell targeting ligands, which would be individually linked to PEG side chains of PLL-*g*-PEG copolymers. Different surface compositions could then be obtained by variation of the proportions of differently functionalized and unfunctionalized copolymers in the coating mixture. Thus our microsphere system would allow for a convenient adjustment of ligand density and composition. At the same time, a stealth background could be ensured by the choice of the appropriate copolymer architecture.

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