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Ligand-Specific Targeting of Microspheres to Phagocytes by Surface Modification with Poly(L-Lysine)-Grafted Poly(Ethylene Glycol) Conjugate

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Purpose. The purpose of this study was to demonstrate specific receptor-mediated targeting of phagocytes by functional surface coatings of microparticles, shielding from nonspecific phagocytosis and allowing ligand-specific interactions via molecular recognition.
Methods. Coatings of the comb polymer poly(L-lysine)-*g*-poly(ethylene glycol) (PLL-*g*-PEG) were investigated for potential to inhibit 1) nonspecific spreading of human blood-derived macrophages (MOs) and dendritic cells (DCs) on glass and 2) nonspecific phagocytosis of PLL-*g*-PEG-coated, carboxylated polystyrene (PS) or biodegradable poly(D,L-lactide-*co*-glycolide) (PLGA) microspheres. Coating was performed by adsorption of positively charged PLL-*g*-PEG on negatively charged microparticles or plasma-cleaned glass through electrostatic interaction. The feasibility of ligand-specific interactions was tested with a model ligand, RGD, conjugated to PEG chains of PLL-*g*-PEG to form PLL-*g*-PEG-RGD and compared with inactive ligand conjugate, PLL-*g*-PEG-RDG.
Results. Coatings with PLL-*g*-PEG largely impaired the adherence and spreading of MOs and DCs on glass. The repellent character of PLL-*g*-PEG coatings drastically reduced phagocytosis of coated PS and PLGA microparticles to 10% in presence of serum. With both MOs and DCs, we observed ligand-specific interactions with PLL-*g*-PEG-RGD coatings on glass and PS and PLGA microspheres. Ligand specificity was abolished when using inactive ligand conjugate PLL-*g*-PEG-RDG, whereas repellency of coating was maintained.
Conclusions. Coatings of PLL-*g*-PEG-ligand conjugates provide a novel technology for ligand specific targeting of microspheres to MOs and DCs while reducing nonspecific phagocytosis.

KEY WORDS: poly(D,L,-lactide-co-glycolide) (PLGA) microspheres; surface modification; poly(L-lysine)-grafted-poly(ethylene glycol) (PLL-*g*-PEG); phagocytosis; RGD-peptide.

INTRODUCTION

For almost two decades injectable, biodegradable microspheres have been developed into an established platform for the sustained delivery of therapeutics for localized or systemic action. After injection of such microspheres, the embodied drugs can be protected from proteolytic cleavage and follow a controlled and prolonged release regimen that is governed by the bioerosion of the polymer carrier. Particularly, microspheres prepared from poly(D,L-lactide-*co*-glycolic acid) (PLGA) have a proven track record for drug delivery. They are highly biocompatible and biodegradable, and their rates of bioerosion and release of encapsulated drugs can be tailored by modulating their composition (1,2). This makes the microencapsulation of drugs in PLGA microspheres an approved and safe technology for the long term delivery of therapeutics.

Phagocytic cells, such as macrophages (MOs), may recognize injected particulate drug carriers as foreign materials and remove them quickly and efficiently via nonspecific phagocytosis by the mononuclear phagocytic system (MPS). The MPS represents a main hurdle that needs to be overcome to achieve selective targeting of particulates to selected organs, tissues, or cells. The phagocytosis of microspheres is stimulated by their surface properties and is believed to be enhanced by charged or hydrophobic surfaces (3). Furthermore, the extent of phagocytosis is enhanced by the adsorption of opsonic plasma proteins to their surface (4). To protect particulates from phagocytosis, poly(ethylene glycol) (PEG) coatings have been investigated for a wide array of biomedical applications. Immobilization of PEG on surfaces has long been known to decrease protein adsorption (5,6). Approaches to immobilize PEG on the surface of microspheres involve covalent grafting (7–9), or adsorption of PEG-containing nonionic surfactants, such as poloxamers, through hydrophobic interaction (10,11). Such approaches not only reduce plasma protein adsorption, but also inhibit the nonspecific uptake by phagocytes (5,8,10,12).

Nevertheless, upon uptake of water, adsorbed nonionic surfactants may quickly desorb from the surface, especially when microspheres were prepared from fast biodegrading, low molecular weight PLGA polymers (13). However, the chemical derivatization of PLGA is restricted to the end groups of the polymer chain and therefore likely to affect the physicochemical and bioerosion characteristics of the polymer. This renders the development of drug-loaded microspheres from modified PLGA more complex because it requires case-to-case adjustments of composition and process parameters for microencapsulation. To avoid this complexity, surface coatings of PLGA microspheres would be highly desirable to modify their biodistribution and reduce their physiologic clearance by the MPS.

Recently, a new class of grafted comb copolymers based on poly(L-lysine)-*g*-poly(ethylene glycol) (PLL-*g*-PEG) was found to spontaneously adsorb from aqueous solution onto several metal oxide surfaces and onto heterogeneous biologic surfaces (6,14). The architecture of the comb copolymer has been demonstrated to play an important role for its behavior. When adjusted accordingly, the resulting adsorbed layers are highly effective in reducing the adsorption of plasma proteins. Adsorptive immobilization of the protective PEG-moiety is suggested to occur through interaction of the positively charged primary amino groups of PLL bound to the negatively charged surface (6,14).

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PLGA microspheres display a hydrophobic and at the same time highly negatively charged surface (15,16). They have been demonstrated to be subject to extensive plasma protein adsorption (12) and are efficiently phagocytosed by the MPS, and by antigen presenting cells like MOs and dendritic cells (DCs) (15).

At first, this work aim to investigated the potential of a protein-repellant, PLL-*g*-PEG-based surface coating to prevent the nonspecific uptake of PLGA microspheres by phagocytic cells. For this purpose, we studied the uptake of model polystyrene (PS) and PLGA microspheres by human-derived peripheral blood MOs and DCs, upon coating the microspheres with PLL-*g*-PEG. As a second step, we investigated the effect of a model peptide ligand, RGD (17), grafted to PLL-*g*-PEG, on the receptor-mediated phagocytosis of suitably coated microspheres by MOs and DCs. The RGD motif serves as a model ligand for the specific interaction of the coatings with integrin receptors, which are expressed on the surface of phagocytic cells. Thus, our study represents a proof-of-concept to demonstrate that specific, receptor-mediated recognition between suitably coated microspheres and phagocytes is feasible. Provided that selective phagocyte specific ligands can be identified, the microspheres would have potential, e.g., as a platform for the specific targeting of particulate vaccine delivery systems to professional antigen-presenting cells, such as DCs.

MATERIALS AND METHODS

Materials

Carboxylated PS that were 4.5 μm in diameter and carried a negative charge were obtained from Polysciences Europe, Basel, Switzerland. PLGA-type polymer (Resomer RG502H, ratio lactide:glycolide 50:50, molecular weight 14,000, uncapped end groups) was obtained from Boehringer Ingelheim (Germany). The PLL-*g*-PEG polymer used in this study consisted of a PLL-backbone of 20 kDa with grafted PEG chains of 2 kDa and a grafting ratio of lysine units to PEG side chains of 3.3 (6). Further modification of this polymer was by conjugation with the RGD adhesion motif to yield PLL-*g*-PEG-RGD, and RDG as negative control for PLL-*g*-PEG-RDG, both with an approximate peptide coverage of 0.1 pmole/cm^2 (17). All PLL-*g*-PEG polymers were synthesized as outlined by Huang *et al.* (18). All chemicals used in this study were of analytical grade (from Fluka, Switzerland) unless otherwise specified.

Methods

PLGA Microspheres

PLGA microspheres were prepared by spray drying as previously described (19). Briefly, PLGA-type polymer was dissolved in dichloromethane (w/w 5%) and was spray-dried in a laboratory spray-dryer (Model 190, Büchi, Switzerland) at an inlet temperature of 45°C. The spray-flow was set at 500 NL/h and the product feed was 3 ml/min. The microspheres were washed with water, collected on a 0.45-μm cellulose acetate membrane filter and dried under vacuum for 24 h. The microspheres were stored at 4°C. Fluorescence-labeled PLGA microspheres were prepared by adding 6-coumarine (5 mg/g polymer, Acros Organics, Belgium) to the polymer solution. Size distribution of the microspheres was measured by laser light scattering (Mastersizer X, Malvern Instruments Ltd., Worcestershire, UK; equipped with a 45-mm lens). Calculation of particles size and distribution was based on Mie's theory. The particle size distribution was presented in the volume-weighted mode and revealed particles in the range of 1–10 μm as previously described in detail (15,19).

MO and DC Cell Culture

MOs and DCs were obtained from human peripheral blood monocytes according to Sallusto *et al.* (20). Briefly, peripheral blood monocytes were isolated from buffy coats (Bloodbank Zurich, Switzerland) by density gradient centrifugation of Ficoll-Paque (Pharmacia Biotech, Buchs, Switzerland). Peripheral blood monocytes were resuspended in RPMI 1640 supplemented with 10% heat-inactivated (pooled) human serum (Bloodbank Zürich, Switzerland) and then allowed to adhere for 2 h in culture flasks (25 cm^2). Nonadherent cells were removed and adherent cells were further cultured in RPMI 1640 supplemented with 5% heat-inactivated (pooled) human serum in the presence of 1000 IU/mL interleukin-4 (Sigma, Switzerland) and 50 ng/mL granulocyte macrophage-colony stimulating factor (R+D Systems) to obtain DCs. MOs were obtained without additional supplements (21). Cultures of MOs and DCs were kept at 37°C in 5% CO_2-humidified atmosphere. For particle uptake studies, cells were mechanically removed from the flasks after 24 h and reseeded onto coverslips in 24-well plates (300,000–400,000 cells per well) for further studies.

Surface antigen expression of MOs and DCs was analyzed by flow cytometry (22). 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 DCs only. The cells were incubated (45 min, 4°C) with each of the following primary anti-human antibodies: CD11b (Mac-1, ICRF44; 44), CD83 (Clone HB15e), CD86 (B70/B7-2, Clone IT2.2; all three from Pharmingen, Lucerne, Switzerland), and CD14 (Clone UCHM-1, Sigma). Control cells were processed similarly with mouse isotype control IgG2a (UPC-10, Sigma). Cells were washed three times and subsequently incubated with the secondary antibody (anti-mouse IgG, R-Phycoerythrin conjugated, Sigma) for 45 min at 4°C, washed three times, transferred to FACS tubes, and analyzed on an FACScan type from Becton Dickinson (Switzerland). According to these criteria, more than 90% of the cells were identified as DCs, and in MOs cultures more than 90% of the cells were $CD14^+$ and $CD83^-$, even in the presence of lipopolysaccharide.

Adhesion Studies

The adhesion of MO and DC on modified surfaces was first tested on glass surfaces coated with PLL-*g*-PEG and PLL-*g*-PEG-RGD or PLL-*g*-PEG-RDG, respectively. Before coating, the glass coverslips (12 mm in diameter) were oxygen-plasma cleaned in a Plasma Cleaner/Sterilizer PDC-32G instrument (Harrick Scientific Corporation, Ossining, NY) for 7 min. Coating was performed in 24-well plates under aseptic conditions with 1 mg/mL of the respective polymers dissolved in 10 mM HEPES buffer, pH 7.4, for 15 min. All

solutions were 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 phosphate-buffered saline (PBS) and once with RPMI 1640 medium. MOs or DCs incubated for 1 week in cell culture flasks were added to each well (250,000 cells in 600 μL of cell medium) containing either coated or control coverslips and cell adhesion was checked after different time points by microscopy (Axiovert 35, Zeiss, Germany). Images were taken using a CCD camera (CF 8/1 DXC, Kappa, Gleichen, Germany).

Coating of Microspheres

Microspheres were dispersed in filtered HEPES buffer at a concentration of 5 mg/mL (polystyrene particles) or 1 mg/mL (PLGA microspheres). The dispersions were mixed at equal volumes with PLL-*g*-PEG, PLL-*g*-PEG-RGD or PLL-*g*-PEG-RDG (1 mg/mL in filtered HEPES buffer) and incubated under gentle mixing for 15 min at room temperature. The coated microspheres were centrifuged (5 min, 7,400 × g, Eppendorf, Centrifuge 5417R, Eppendorf-Netheler-Hinz GmbH, Germany) and redispersed in half of the previous volume in filtered HEPES buffer by slightly vortexing.

Phagocytosis Studies

MOs cultured on coverslips in 24-well plates were washed once with RPMI 1640 medium and then covered with 1 mL of RPMI 1640 medium with or without serum. Microspheres dispersed in HEPES buffer were added and the cells were incubated at either 37°C or at 4°C for 4 h. The cells were washed twice with RPMI 1640 medium and subsequently fixed with 3% paraformaldehyde in PBS for 30 min at room temperature. The samples were stored in PBS at 4°C. Because DCs only slightly adhered to the cell culture plates or to coverslips, these cells were not washed and the microspheres were added directly to the cells. Also the washing step after incubation was avoided and approximately 300 μL of medium was added to each well to allow a better verification of phagocytosed microspheres. These samples were analyzed immediately without prior fixation.

Sample Analysis by Fluorescence Microscopy

The amount of phagocytosed microspheres per cell was assessed by fluorescence microscopy (Axiovert 35, Zeiss, Germany; filter set: excitation 450–490/emission 520, beamsplitter 510). A minimum of three samples was checked per experiment and the number of microspheres taken up per cell was counted in 50 cells per sample. Images were taken using a CCD camera (CF 8/1 DXC, Kappa, Gleichen, Germany). To assure cell viability some of the samples were stained with ethidium bromide homodimer-1 (EthD1, 2 μL/mL, Molecular Probes, Lucerne, Switzerland) in PBS for 30 min at room temperature (250 μL/well). Viability staining was performed before fixing the cells. Cellular uptake of EthD1 is restricted to nonviable, necrotic cells with leaky cell membranes. Staining results from nuclear intercalation with DNA. Necrotic cells were detected by fluorescence microscopy (Axiovert 35, Zeiss, Germany; excitation 546/emission 590, beam splitter 580).

Sample Analysis by Confocal Laser Scanning Microscopy (CLSM)

For CLSM, the cells were stained with rhodamine-labeled phalloidine (tetramethylrhodamine isothiocyanate-phalloidine, Sigma) to visualize the actin cytoskeleton of the cells. All incubation steps were performed at room temperature. After fixation, the cells were incubated in 0.1 M glycin in PBS for 5 min. The samples were washed once with PBS, incubated for 40 min with 0.2% Triton X-100 to permeabilize the cell membrane, and then washed once again with PBS. Rhodamine-phalloidine, diluted 1:10 in 3% BSA (bovine serum albumin) in PBS, was added and samples were incubated for 90 min in the dark. The samples were washed three times for 5 min with PBS and subsequently mounted on coverslips for microscopic evaluation using a Zeiss LSM 510 (Zurich, Switzerland; lasers He-Ne 543 nm, Ar 488/514 nm).

Significance Testing

The significance of the results was tested using a t test (assuming unequal variances).

RESULTS AND DISCUSSION

Inhibition of Cellular Interactions by PLL-*g*-PEG-Modified Flat Surfaces

In earlier investigations, the comb polymer system PLL-*g*-PEG has been demonstrated to be highly efficient in reducing the adsorption of serum proteins on flat metal oxide surfaces (6,18). PLL-*g*-PEG consists of a PLL backbone, which is highly cationic at a physiologic pH, and spontaneously adsorbs from aqueous solutions onto negatively charged surfaces by electrostatic interactions (6). At high grafting density, the PEG chains form a densely packed brush, providing the protein-resistant function of the graft. This performance was found to depend on the molecular weight of the PEG chains, the grafting ratio of the PLL-*g*-PEG copolymer, and the surface coverage of the polymer (6,14,18). Based on previous results (6), we used a PLL backbone of 20 kDa with grafted PEG chains of 2 kDa and a molar grafting ratio of Lys units to PEG side chains of 3.3.

To test the repellent effect of PLL-*g*-PEG on professional phagocytic cells, MO and DC, we coated glass surfaces with PLL-*g*-PEG and tested the adhesion of the cells in comparison with untreated glass. All samples were checked by microscopy after 1, 4, 24 and 48 h. After only 4 h, we observed a clear difference between untreated glass and PLL-*g*-PEG-coated glass. Adhesion appeared to be complete after 24 h. Both cell types displayed high affinity to untreated glass, as indicated by significant cell adhesion and spreading (Fig. 1a, e). In contrast, PLL-*g*-PEG treatment completely abolished cellular adhesion and spreading, with the remaining cells typically adopting a spherical cross-section at a reduced diameter (Fig. 1 b, f). Thus, in addition to their protein-repellent character, PLL-*g*-PEG coatings limited the biologic interactions and recognition of the surfaces by professional phagocytes.

Ligand-Specific Interactions by PLL-*g*-PEG-RGD-Modified Flat Surfaces

The next aim was to convert the repellent surface to an interface with specific bioreactivity, while maintaining its re-

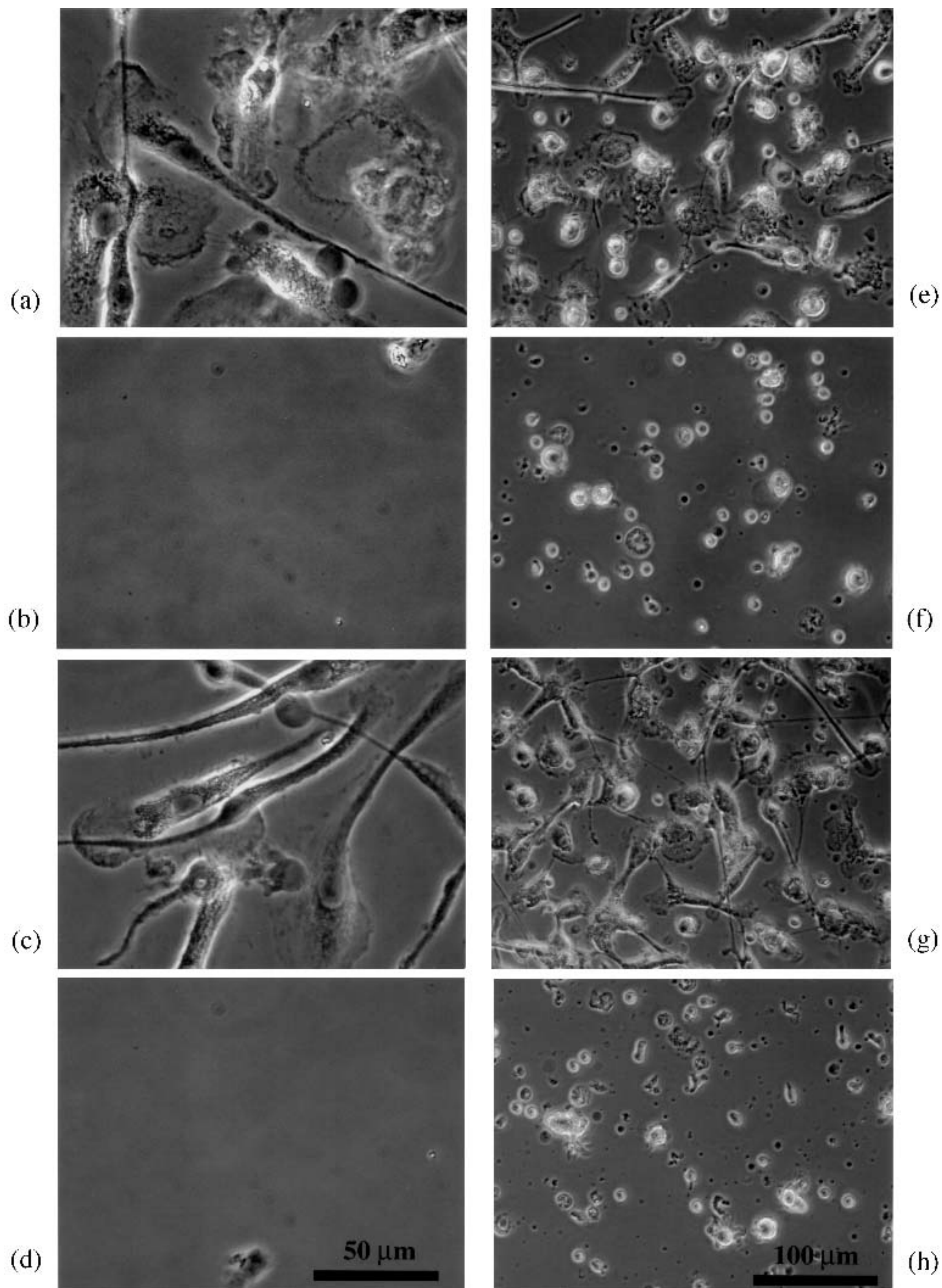

Fig. 1. Adhesion and spreading of macrophages (a–d) and dendritic cells (e–h) on untreated glass surfaces (a, e), and on glass surfaces coated either with PLL-*g*-PEG (b, f), PLL-*g*-PEG-RGD (c, g), or PLL-*g*-PEG-RDG (d, h). Photographs were taken after 24 h of incubation.

pellent characteristic toward nonspecific interactions. PLL-*g*-PEG was modified at the terminal position of the PEG chains to provide additional reactive sites for the specific interaction with cellular surfaces or cellular receptors. As a model ligand, we used an RGD-containing peptide that has been suggested to mediate receptor specific interactions with MOs and DCs because of its affinity to integrin receptors expressed on the surface of these cells (23–25). The RGD-containing peptide was covalently bound to the PEG chains (PLL-*g*-PEG-RGD; 17) with an approximate coverage of 0.1 pmol/cm^2. We found ligand-specific adhesion and spreading of the cells after coating the glass surface with PLL-*g*-PEG-RGD (Fig. 1c, g). In contrast, coating with PLL-*g*-PEG conjugated to the inactive control RDG peptide (PLL-*g*-PEG-RDG) resulted in the same repellent character as PLL-*g*-PEG (Fig. 1d, h), supporting the ligand-specific character of the interaction with PLL-*g*-PEG-RGD. The data demonstrate that the PLL-*g*-PEG system can be used as a platform to generate surfaces displaying

a selective interaction with specific cellular binding sites on a highly protein-resistant background.

Inhibition of Nonspecific Phagocytosis by Surface Modification with PLL-*g*-PEG

Intensive investigations have been undertaken to inhibit nonspecific uptake of polymeric micro- and nanospheres by phagocytic cells of the MPS. Polystyrene particles were previously used as model systems and were either coated with nonionic surfactants by hydrophobic interaction, such as polymers of the poloxamer and poloxamine series (10,11,26) or were made of polystyrene covalently linked to PEG chains (7), resulting in bulk modified microspheres exposing a repellent surface. All approaches resulted in the inhibition of phagocytosis down to 20–35% in the presence of serum. Moreover, experiments *in vivo* demonstrated that coatings with hydrophilic surfactants such as PEG, poloxamers, poloxamines or poly(vinylalcohol) indeed prolong significantly the circulation time and alter the body distribution of synthetic carriers (27–31).

To investigate the phagocytosis-inhibiting effect of PLL-*g*-PEG coatings, we used carboxylated polystyrene particles with a particles size of 4.5 μm, which were previously reported to be efficiently phagocytosed by MOs and DCs (21). Because they exhibit a negative surface charge (21), they were expected to be efficiently coated by the positively charged PLL-*g*-PEG polymer because of electrostatic interactions. We analyzed by microscopic examination the uptake of noncoated and PLL-*g*-PEG-coated polystyrene particles in MOs and DCs. A typical photomicrograph is given in Fig. 2. No particle uptake was detected when the incubation was performed at 4°C. Different amounts of particles were added to the cells (2×10^6, 10^7, 2×10^7, 4×10^7 particles/well) and saturation was obtained with 10^7 particles/well which corresponds to an average of approximately 25 particles per cell (data not shown). Thus, throughout we performed further experiments by adding 10^7 particles/well. A typical distribution of internalized particles per cell is shown in Fig. 3a. Because of its broad statistical distribution, the data was presented as box plots (Fig. 3b and c).

Coatings of the microspheres with PLL-*g*-PEG efficiently reduced phagocytosis by MOs and DCs ($p \leq 0.05$) to about 10% of the average number of phagocytosed particles observed with noncoated particles (Fig. 3). The repellent character of the PLL-*g*-PEG coating was maintained up to 4 d at either 4 or 37°C and was independent of the presence of serum proteins (data not shown). Thus, inhibition of phagocytosis by PLL-*g*-PEG coatings due to electrostatic interactions was comparable or even superior to coatings consisting of nonionic surfactants (10,11,26). Our data thus suggests that the repellent character of PLL-*g*-PEG surface coatings may be maintained for a relevant time span that might be sufficient to significantly alter the body distribution of the microspheres and enhance their circulation time *in vivo*. Such experiments are considered for future studies.

Induction of Specific Phagocytosis by Surface Modification with PLL-*g*-PEG-RGD

Targeted delivery of micro- and nanospheres to a selected tissue or a specific receptor has raised increasing interest for the administration of potent drugs (32–40). In addition, addressing a specific receptor may induce distinct signaling pathways in the target cells, such as in immunomodulatory antigen-presenting cells (23,41–44). To avoid other pathways, nonspecific phagocytosis needs to be limited at the same time in order to enable specific interactions with the target tissue or the selected receptor. In accordance with the experiments with flat glass surfaces, we aimed to convert the PLL-*g*-PEG-coated repellent particle surface to an interface with specific bioreactivity, while maintaining its repellence toward nonspecific phagocytosis. Indeed, coating of the polystyrene particles with PLL-*g*-PEG-RGD fully abolished the repellent nature of the particles and led to ligand-specific phagocytosis. In contrast, no bioreactivity was observed when the inactive RDG peptide was conjugated to the polymer ($p \leq 0.05$; Fig. 3). Coatings of inactive RDG modified PLL-*g*-PEG were equally repellent as PLL-*g*-PEG coatings.

Phagocytosis of Surface-Modified PLGA Microspheres by MOs and DCs

Whereas polystyrene particles were only used as model particles, microspheres prepared from biodegradable PLGA are pharmaceutically more meaningful. They have been extensively studied for the encapsulation and delivery of therapeutics, and were successfully marketed for human use (2,45). PLGA microspheres prepared by spray-drying were previously reported to be in the size range of 1–10 μm and to display a strongly negative surface charge (16). In another study, we confirmed the feasibility to achieve stable PLL-*g*-PEG coatings on the surface of PLGA microspheres by simply incubating the microparticles in an aqueous solution of PLL-*g*-PEG (46). Moreover, PLL-*g*-PEG-coated PLGA microspheres showed a drastic decrease in protein adsorption by two orders of magnitude in comparison to noncoated microspheres due to the high surface density of PEG chains corresponding to 15–25 ethylene-glycol monomers per nm^2 (46).

In accordance with the studies with polystyrene particles, we analyzed the uptake of noncoated and PLL-*g*-PEG-coated fluorescent-labeled PLGA microspheres in MOs and DCs by microscopic examination. Again, no uptake was seen when the incubation was performed at 4°C. Different amounts of particles added to the cells (20, 100, 200, and 400 of μg microspheres per well) indicated saturation at about 100 μg microspheres per well. Thus, all further experiments were performed at 100 μg microspheres per well. In correspondence with the data on polystyrene particles, coating of the PLGA microspheres with PLL-*g*-PEG significantly reduced phagocytosis by MO and DC (Fig. 4) to about 10% of the values with noncoated particles.

Phagocytosis of noncoated and PLL-*g*-PEG-coated PLGA microspheres was further investigated by CLSM to verify the intracellular localization of the microspheres in contrast to those attached to the outer membrane. The shape of the phagocytes was visualized by staining their actin cytoskeleton with rhodamine-phalloidine (red), while the microspheres were labeled with 6-coumarin (green; Fig. 5). The result is a clear difference between the phagocytosis of noncoated vs. PLL-*g*-PEG-coated PLGA microspheres. Quantification of uptake by counting the number of microspheres in the cells resulted in numbers, which were in reasonable agreement with the data obtained by fluorescence microscopy (data not shown).

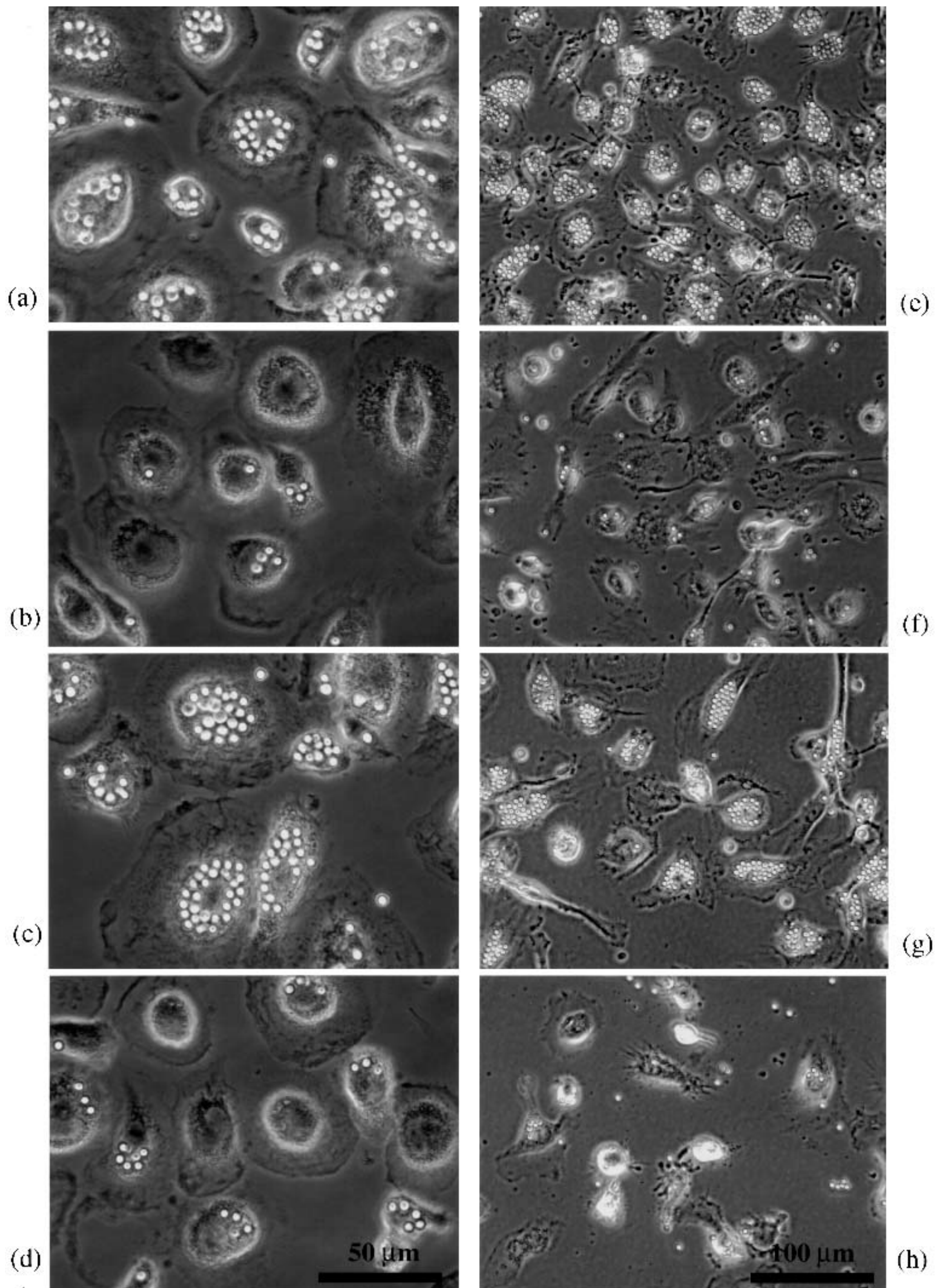

Fig. 2. Phagocytosis of polystyrene particles by macrophages (a–d) and dendritic cells (e–h) upon the addition of noncoated particles (a, e), and particles coated with PLL-*g*-PEG (b, f), PLL-*g*-PEG-RGD (c, g), and PLL-*g*-PEG-RDG (d, h). Photographs were taken after 4 h of incubation at 37°C.

A preliminary estimation of the cellular toxicity of the coatings was also performed. In control experiments, less than 5% of the MOs or DCs were found to be necrotic, irrespective of whether noncoated or PLL-*g*-PEG-coated microspheres were used. This corresponds to previous investigations on toxicity (14), immunogenicity, immunomodulatory potential, pyrogenicity and biodegradation (47–49) of PLL-*g*-PEG and suggests good biocompatibility of the coatings.

Previous attempts to inhibit phagocytosis of PLGA and poly(L-lactide) (PLA) micro- and nanospheres by surface modification focused on coatings with poloxamer (11), high molecular weight PEG (50), or on the use of di-block and tri-block co-polymers of PLA/PLGA and PEG to prepare bulk modified micro- or nanospheres (8,9,51). For instance, the circulation time of PLA-PEG nanospheres was extended up to 6 h and they were found in increasing amounts in the heart, in blood vessels and in the lung (52). Inhibition of phagocytosis in the presence of serum or plasma was down to

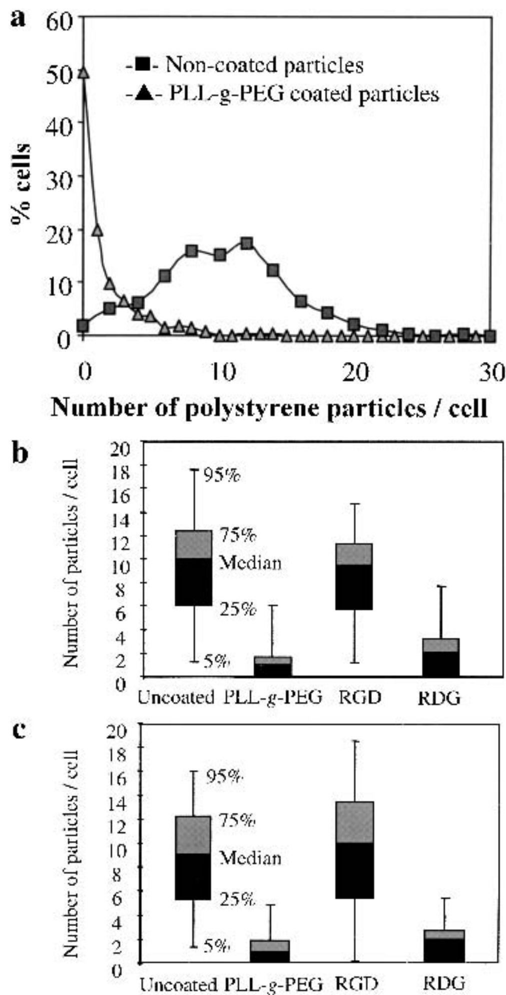

Fig. 3. Phagocytosis by macrophages (MOs) and dendritic cells (DCs). Typical distribution (a) showing the uptake of noncoated and PLL-*g*-PEG-coated polystyrene particles in MOs. Box plots for the phagocytosis by MOs (b) and DC (c) of noncoated particles and particles coated with either PLL-*g*-PEG, PLL-*g*-PEG-RGD, or PLL-*g*-PEG-RDG. Significance testing revealed differences between noncoated and PLL-g-PEG-coated particles ($p \leq 0.05$) and between PLL-*g*-PEG-RGD- and PLL-*g*-PEG-RDG-coated particles ($p \leq 0.05$) for both MOs and DCs.

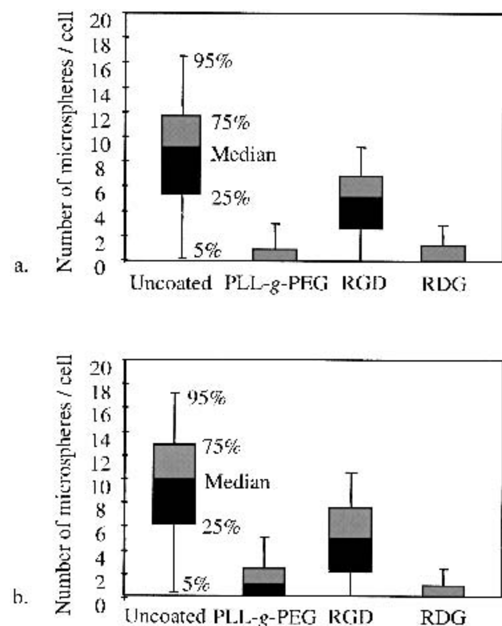

Fig. 4. Phagocytosis of noncoated PLGA microspheres and microspheres coated with either PLL-*g*-PEG, PLL-*g*-PEG-RGD, or PLL-*g*-PEG-RDG by macrophages (a) and dendritic cells (b) presented as box plots. Significance testing revealed differences between noncoated and PLL-*g*-PEG-coated particles ($p \leq 0.05$) and between PLL-*g*-PEG-RGD- and PLL-*g*-PEG-RDG-coated particles ($p \leq 0.05$) for both macrophages and dendritic cells.

20–55%, revealing a comparable or even superior effect of the PLL-*g*-PEG-coated microspheres used in this study. Moreover, at both 4 and 37°C the repellent character of the PLL-*g*-PEG coatings on PLGA microspheres was maintained for up to 4 days (data not shown). In contrast, when in contact with water, the coatings of nonionic surfactants are subject to enhanced desorption followed by water uptake and subsequent swelling, especially with microspheres prepared from fast degrading low molecular weight PLGA polymers (13). From a fabrication point of view, the spontaneous adsorption of PLL-*g*-PEG polymers to the carriers appears to be a superior technology to the covalent PEGylation of the polymer bulk. The combination of two separate platforms, i.e., the established PLGA microsphere technology and the PLL-*g*-PEG based surface modification, may allow separate development and optimization of carrier and coating. Conversely, the use of PEG-modified bulk polymers is expected to affect the physicochemical properties and the bioerosion of the polymer matrix, and requires the composition and process parameters for microencapsulation to be optimized on a case-to-case basis.

In addition to the inhibition of nonspecific phagocytosis and in correspondence with the data from polystyrene particles, we found that coatings of the PLGA microspheres with PLL-*g*-PEG-RGD resulted in ligand-specific phagocytosis by MO and DC (Fig. 4). As compared with noncoated microspheres the extent of phagocytosis of PLL-*g*-PEG-RGD-coated microspheres was decreased, which may be explained by some degree of microsphere agglomeration observed in the preparation. To our knowledge, this is the first demonstration of a technology that enables the targeting of biodegradable PLGA microspheres to specific cell surface receptors while simultaneously preventing nonspecific phagocytosis.

CONCLUSIONS

In this study, we demonstrated a simple and reproducible approach for the surface modification of biodegradable PLGA microspheres for targeting purposes. Coating with positively charged PLL-*g*-PEG by electrostatic interaction with negatively charged microspheres drastically reduced the nonspecific uptake by professional phagocytes such as MO and DC. Conjugation of a specific ligand to PLL-*g*-PEG enables receptor-specific targeting while maintaining the repellent character of PLL-*g*-PEG coatings. This should preclude rapid physiologic clearance of the microparticles by the MPS. The biospecificity of the interaction was proven by using the inactive nonspecific RDG-conjugated polymer as a control. Overall, our findings provide a potential technology to diminish nonspecific phagocytosis of micro- and nanoparticles and

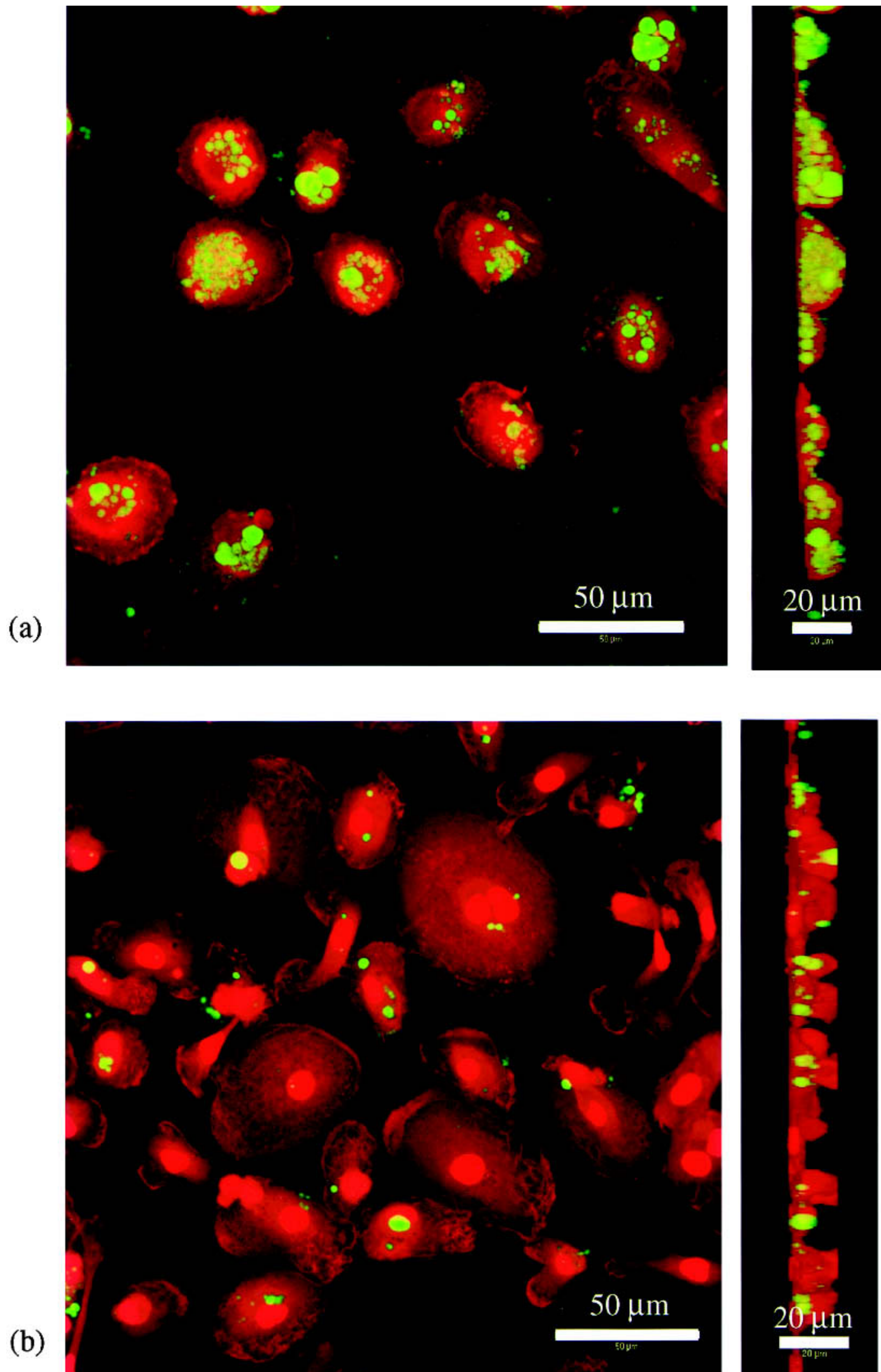

Fig. 5. Phagocytosis of noncoated (a) and PLL-*g*-PEG-coated (b) PLGA microspheres in macrophages as analyzed by confocal laser scanning microscopy. The outline of the individual cells was visualized by staining the actin cytoskeleton with rhodamine-phalloidin (red). Microspheres were labeled with 6-coumarin (green). Optical sections (xy, left panel) and yz-projections (right panel) allow to distinguish between extracellular and internalized microspheres.

at the same time target them to specific receptors. As a proof-of-concept this study was restricted to a model ligand, RGD. For the future we expect larger benefit of this novel technology to result from the use of more selective ligands, e.g., for the specific targeting of DC rather than MO (53). Moreover, in case suitable ligands can be provided, the technology may be also of use to target particulate drug carriers to other tissues and cells. This needs yet to be demonstrated by experiment.

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