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International Journal of Pharmaceutics 298 (2005) 315–322
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Particle size and surface charge affect particle uptake by human dendritic cells in an in vitro model

Camilla Foged [a,b,*], Birger Brodin [a], Sven Frokjaer [a], Anne Sundblad [b]

[a] *Department of Pharmaceutics, The Danish University of Pharmaceutical Sciences, Universitetsparken 2, DK-2100 Copenhagen, Denmark*
[b] *Division of Hematology, Centre for Molecular Medicine, Karolinska University Hospital and Institute, S-17176 Stockholm, Sweden*

Received 3 December 2004; received in revised form 16 March 2005; accepted 21 March 2005
Available online 14 June 2005

Abstract

Current vaccine development includes optimization of antigen delivery to antigen presenting cells, such as dendritic cells (DC). Particulate systems have attracted increasing attention in the development of vaccine delivery systems. In the present study, we investigated DC uptake of model fluorescent polystyrene particles with a broad size range and variable surface properties. Localization of particles was investigated using confocal laser scanning microscopy and uptake was quantified by flow cytometry. Immature DC were generated from mononuclear cells isolated from human blood. The polystyrene particles interacted with the DC throughout the tested diameter range of 0.04–15 μm in a time- and concentration-dependent manner. The optimal particle diameter for fast and efficient acquisition by a substantial percentage of the DC was 0.5 μm and below. The surface of 1 and 0.1 μm polystyrene particles was covalently modified with different polyaminoacids/proteins, yielding particles with varying surface charge. Uptake of 1 μm particles was greatly enhanced when particles displayed a positive surface charge. In general, the present findings establish that particle diameters of 0.5 μm and below were optimal for DC uptake; however uptake of larger particles could be greatly enhanced by rendering the particle surface positive. Whether increased particle uptake is correlated with increased immune responses, remains to be established.

Keywords: Dendritic cells; Antigen delivery; Particle uptake; CLSM; Flow cytometry

Microspheres possess pharmaceutical and immunological advantages as vaccine delivery systems for subunit vaccines based on peptides, protein or DNA (Raychaudhuri and Rock, 1998). Particulate systems are naturally targeted to antigen presenting cells (APC) since their dimensions are comparable to those of microorganisms, and particles can through phagocytosis deliver antigens to APC 1000–10,000-fold more efficiently than soluble antigen (Kovacsovics-Bankowski et al., 1993; Vidard et al., 1996). Presentation of processed, particulate antigens by major histocompatibility complex (MHC) class I and class II can

* Corresponding author. Tel.: +45 35 30 64 02; fax: +45 35 30 60 30.
E-mail address: cfo@dfuni.dk (C. Foged).

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doi:10.1016/j.ijpharm.2005.03.035

activate $CD4^+$ and $CD8^+$ T-cell responses. Particulate antigens might therefore mediate the induction of both humoral and cellular immunity.

Dendritic cells (DC) represent the main APC lineage able to induce primary immune responses and immunological memory (Banchereau and Steinman, 1998). Antigen acquisition by DC is mediated by several uptake mechanisms such as receptor-mediated endocytosis, macropinocytosis and phagocytosis, depending of the nature of the antigen (Reis e Sousa et al., 1993; Sallusto et al., 1995; Steinman and Swanson, 1995). Interactions between particulate formulations and cells in general depend on particle characteristics such as size and surface properties, including surface charge and hydrophobicity. In the present study we addressed the importance of particle size, charge and concentration for efficient interactions between particles and human DC.

DC were generated from human peripheral blood monocytes and characterized by flow cytometry, according to previous reports (Foged et al., 2004; Romani et al., 1996; Sallusto and Lanzavecchia, 1994). The study was approved by the local ethics committee (KF01-020/00). The 0.1, 0.5, 1.0 and 4.5 μm Fluoresbrite™ carboxylated yellow-green microspheres were purchased from Polysciences Europe GmbH, Eppelheim, Germany. 0.04, 10 and 15 μm yellow-green fluorescent (505/515) carboxylate-modified FluoSpheres® were obtained from Molecular Probes, Europe BV, Leiden, The Netherlands. Protamine sulphate (PS) (grade III), poly-L-lysine (PLL) M_W 5000–10,000, wheat germ agglutinin (WGA) from *Triticum vulgaris* and poly-D-L-alanine (PA) M_W 1000–5000 were purchased from Sigma Aldrich A/S, Vallensbaek Strand, Denmark. Tetanus toxoid (TT) was from Statens Serum Institut, Copenhagen, Denmark (Batch XXVII, 669 lf/mg, 2.23 mg/ml). The 0.1 and 1.0 μm carboxylated polystyrene particles were coated with PLL, PS, PA, WGA and TT by covalent coupling of the molecules (400 μg) to the free carboxyl groups on the particle surface using the Carbodiimide Coupling Kit for Large Microspheres according to manufacturers instructions (Polysciences Europe GmbH, Eppelheim, Germany). For the 0.1 μm particles, a special Carbodiimide Kit with Hollow Fiber Filtering System was applied to concentrate the particle suspension (Polysciences Europe GmbH, Eppelheim, Germany). Amounts of bound peptide/protein was measured indirectly by determination of non-bound material in the supernatants by the bicinchoninic acid protein assay kit according to manufacturers instructions (Sigma–Aldrich, Denmark A/S). The average size of unmodified particles was measured in 10 mM NaCl by dynamic light scattering with a Zetasizer 4 from Malvern Instruments, Sweden. The zeta potential (surface charge) was determined in Milli-Q water using a Zeta Master (Malvern Instruments Nordic AB, Uppsala, Sweden). The relative concentration of surface-modified particles was adjusted by comparison of fluorescence intensity with non-conjugated particles of known concentration using a Luminescence Spectrometer (LS50B, Perkin-Elmer, excitation wavelength 458 nm, emission wavelength 540 nm, and emission filter 515 nm).

For particulate uptake studies, DC were harvested, counted and viability was determined by trypan blue staining. The cells were seeded in 24-well culture plates at a density of 2×10^5 cells/well, and particles were added in a small volume with a micropipette after vigorous vortexing for 1 min. The tissue culture plates were swirled gently to distribute particles evenly in the wells and incubated at 37 °C. Cells were then harvested by addition of ice-cold PBS and by pipetting. Staining for surface MHC class II (human leukocyte antigen (HLA) DR) was done with phycoerythrin conjugated mouse anti-HLA-DR (or control IgG2a) as reported previously (Foged et al., 2004). Cell samples were fixed in 1% paraformaldehyde prior to acquisition and analysis by a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using the CellQuest software (Becton Dickinson). Dead cells were gated out based on their light scattering properties. For confocal laser scanning microscopy (CLSM) DC incubated with particles were fixed for 15 min in 1% paraformaldehyde on ice. Staining was performed with a monoclonal mouse anti human CD1a antibody (Immunotech, Beckman Coulter, Marseille, France) followed by a rhodamine-conjugated donkey anti-mouse IgG (AffiniPure, Jackson Immunoresearch Laboratories Inc., West Grace, PA, USA) in PBS + 4% BSA. The cell preparations were washed with PBS and mounted using the SlowFade™ Antifade Kit (Molecular Probes Europe BV, The Netherlands). Fluorescence microscopy was performed with a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Jena, GmBH, Germany) equipped with an argon laser (458

and 488 nm) and a HeNe laser (543 nm) using the LSM 510 software and a 63× oil objective.

1. Results and discussion

Polystyrene spheres were chosen as model particles for the continued analyses of specific physicochemical variables of importance in particle-DC interactions, since such microspheres are available in many different and well-defined sizes. Initially, interaction between 1.0 μm fluorescein isothiocyanate (FITC) labelled, carboxylated polystyrene spheres and DC was analyzed by flow cytometry after culture of immature DC with different concentrations of particles (5, 20 and 80 times excess of particles numbers to DC numbers) for up to 24 h. Fluorescent particles bound to or taken up by DC appeared as discrete populations by flow cytometry analysis (Fig. 1A) allowing for measurements of the efficiency of microsphere-association with DC by determination of the frequency of $FITC^+/HLA\text{-}DR^+$ double positive cells. A concentration- and incubation time-dependent DC-particle interaction was observed (Fig. 1B). Accordingly, the mean fluorescence intensity (MFI) per cell increased in a similar manner (results not shown). However, only a limited fraction of the DC population interacted with the polystyrene spheres of 1.0 μm size, reaching approximately 10% of double positive DC (Fig. 1B) after incubation with the highest particle concentration after 24 h. Increasing the particle concentration above 80 times excess of particles numbers to DC numbers did not further increase the percentage of double positive cells (results not shown). Confocal analysis confirmed the sparse interaction; limited numbers of particles were associated with the cell membrane after 2 h, or were present in the cytoplasm close to the cell membrane (Fig. 1C). After 24 h incubation, the localization of particles had not changed.

Since only a limited fraction of immature DC was observed to interact with microspheres of 1.0 μm size under the present culture conditions, DC-particle interaction was examined by incubating DC with a range of particle with diameters of 0.1, 0.5, 1.0 and 4.5 μm. There is no straight-forward method for evaluating uptake when comparison is to be made between microspheres of different sizes (Tabata and Ikada, 1988). The extent of uptake may be evaluated using either a fixed number of particles or by using a fixed volume of particles. In this work both identical numbers and identical volumes of different-sized polystyrene microspheres were added to the DC-cultures. However, due to the large size differences and the resulting large differences in fluorescence intensity between the different-sized spheres, it was not possible to compare uptake when using fixed numbers of spheres per DC. Therefore, a fixed volume of the variably sized spheres was added per DC (particle volumes corresponding to the 1:80 addition for the 1.0 μm spheres).

The results showed consistently that limiting the particle size increased the binding to DC as determined by the frequency of double positive cells (Fig. 1A) which was paralleled by an increase in MFI per cell (data not shown) when using identical total volumes of particles. Very few DC associated with 4.5 μm particles while approximately 30% of the DC interacted with 0.5 μm particles and 60% interacted with 0.1 μm particles after 24 h incubation (Fig. 2A). The cells were further examined by CLSM. DC were able to take up all tested particle sizes, and the particles were present in the cell cytoplasm or, in some cases, associated with the cell membrane (Fig. 2B). Very few DC were able to internalize spheres in the size range of 10–15 μm (results not shown). In addition, 0.04 μm particles were tested and uptake of these particles was comparable to the uptake of 0.1 μm spheres (data not shown).

The surface of the 0.1 and 1.0 μm polystyrene spheres was modified by attaching different polyaminoacids/proteins covalently by carbodiimide-catalyzed binding between amine groups and surface carboxyl groups. The polypeptides poly-L-lysine (PLL) and poly-D-L-alanine (PA) were chosen to introduce positive charge and a more neutral molecule respectively at the surface without introducing large sterical differences due to the molecular 3D-structure. In addition the highly positively charged small nucleoprotein protamine sulphate (PS) composed of more than 90% arginines was chosen, the lectin wheat germ agglutinin (WGA) and finally tetanus toxoid (TT) were selected, the latter intended for use in future antigen presentation studies. The characteristics of the polystyrene spheres are shown in Table 1. The highest change in surface charge was observed when coupling PS and PLL that introduced positive charge. Coupling of PA with a net neutral charge did not change the surface charge, probably because the same number of

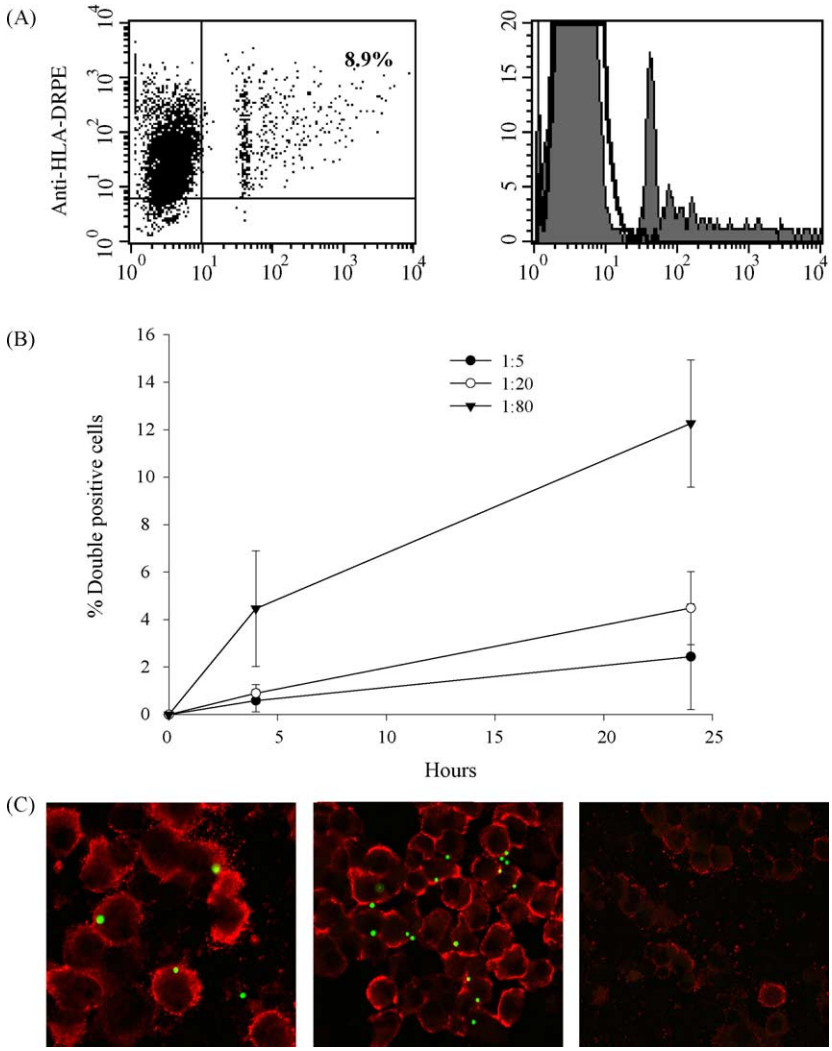

Fig. 1. Flow cytometry and CLSM analysis of interaction between DC and 1.0 μm particles. (A) Dot plot and histogram for DC interaction with 80-fold excess of particles for 24 h. The percentage of HLA-DR$^+$/particle$^+$ double positive cells is indicated (left). (B) Percentage of HLA-DR$^+$/particle$^+$ double positive cells as a function of incubation time and particle concentration. Closed circles: 1:5. Open circles: 1:20. Triangles: 1:80. Data represent triplicate experiments where blood from three different donors was used to generate DC. (C) Confocal analysis: two colour immunofluorescence of immature DC incubated for two (left) or 24 (middle) h with polystyrene spheres (green), fixed and stained for CD1a (red). Right: Background fluorescence, isotype-matched irrelevant antibody.

negative charges is preserved after coupling due to the carboxylic end group of PA. Coupling efficiencies for the 1.0 μm spheres was in the range of 4–11 μg/mg polymer, while the efficiencies for 0.1 μm spheres was in the range of 5–56 μg/mg polymer. The size of the modified particles was not measured. However, the particle size might be influenced by modification of the surface, and it cannot be excluded in these studies that surface modification can cause some degree of particle aggregation.

The interaction between DC and polystyrene spheres with surface modifications was determined for 1.0 and 0.1 μm sized particles for fixed particle:DC ratio (80:1 for the 1.0 μm spheres and a corresponding

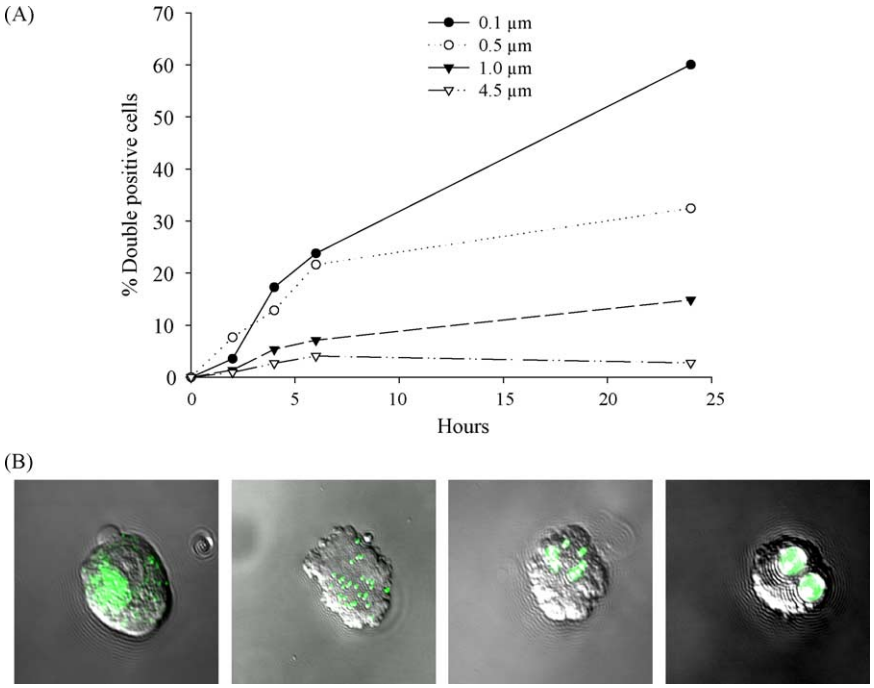

Fig. 2. (A) Flow cytometry analysis of DC incubated with 0.1, 0.5, 1.0 and 4.5 μm FITC-labelled, carboxylated polystyrene spheres for 2, 4, 6 or 24 h. For the 1.0 μm particles, an 80-fold excess of particles to numbers of DC was used. Particle-volumes corresponding to the 1:80 addition for the 1.0 μm spheres were added with respect to the other particle sizes. The percentage of HLA-DR$^+$/particles$^+$ double positive cells as a function of incubation time is shown. (B) One colour immunofluorescence of DC incubated for 24 h with polystyrene spheres (green). The confocal pictures of the particles are overlaid with transmission pictures to indicate the cell size. From left to right: 0.1, 0.5, 1.0 and 4.5 μm spheres.

Table 1
Characteristics of polystyrene spheres

Diameter[a] (μm)	Coupling moiety	Zeta potential[c] (mV)
0.099 ± 0.006	–	−60.6 ± 1.0
0.1 ± 0.003[b]	PLL	20.1 ± 0.7
0.1 ± 0.003[b]	PA	−44.4 ± 1.4
0.1 ± 0.003[b]	TT	−26.9 ± 2.6
0.486 ± 0.003	–	−54.1 ± 1.4
0.947 ± 0.012	–	−60.1 ± 0.4
0.949 ± 0.017[b]	PLL	12.4 ± 0.3
0.949 ± 0.017[b]	PS	41.4 ± 1.2
0.949 ± 0.017[b]	PA	−57.4 ± 1.1
0.949 ± 0.017[b]	WGA	−53.0 ± 0.8
0.949 ± 0.017[b]	TT	−42.9 ± 1.2
4.465 ± 0.142[b]	–	−66.9 ± 1.9

[a] Values represent mean ± S.D., $n = 3$.
[b] Values as given by manufacturer (mean ± S.D.).
[c] Values represent mean ± S.D., $n = 5$.

volume ratio for the 0.1 μm spheres, as also used in Fig. 2) after 4 or 24 h of incubation. The negatively charged 1.0 μm particles interacted with a low percentage of the HLA-DR positive DC (10% after 24 h, Fig. 3A) as previously observed for the uncoated polystyrene spheres (Fig. 1). Coating the spheres with TT almost doubled the percentage of FITC/HLA-DR positive cells. The interaction of DC with positively charged PS- and PLL-coated spheres resulted in 10-fold increase in percentage of FITC/HLA-DR positive cells as compared to uncoated, carboxylated spheres after 4 h. Only a minor rise in percentage of double positive cells was noted after 24 h indicating that for the present conditions saturating levels were reached after a few hours. The increase in DC-percentage interacting with particles was accompanied by an increase in MFI per cell, with the exception of the PS-coated spheres, for which a decrease in mean fluorescence per cell was observed after 24 h suggesting that this type of coating

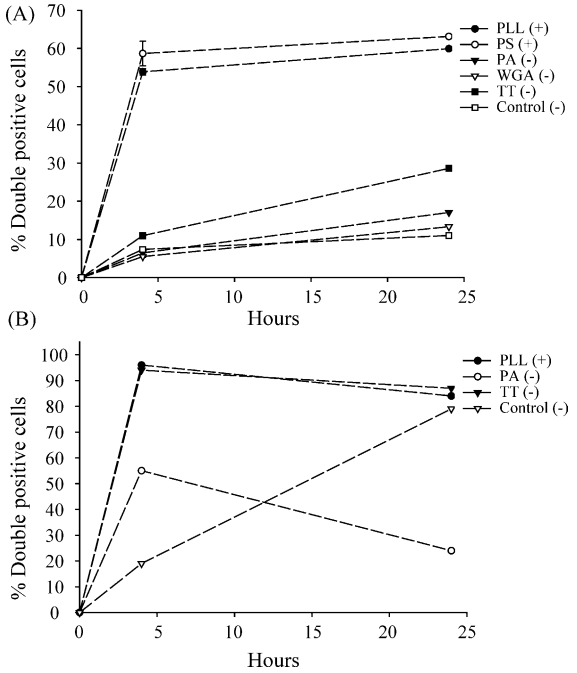

Fig. 3. (A) DC were incubated for 4 or 24 h with an 80 times excess of particles to DC of coated or uncoated 1.0 μm FITC-labelled polystyrene spheres, stained for surface HLA-DR and analyzed by flow cytometry. Incubation for 4 h was done in triplicate cultures, while 24-h incubations were carried out as single incubations. (B) DC were incubated with the same volume of 0.1 μm PLL-, PA- and TT-coated and uncoated 0.1 μm FITC-labelled polystyrene spheres as for the 1.0 μm spheres.

leads to expelling or degradation of particles and/or fluorescence (results not shown). For the 0.1 μm sized PLL-, PA- and TT-coated spheres a different pattern was observed: after 4 h incubation, PLL- and TT-coated spheres were taken up by almost the entire DC population as determined by frequency of double positive DC (Fig. 3B). MFI per cell was higher for the positively charged PLL-coated spheres than for the negatively charged TT-coated spheres (results not shown). PA-coated and uncoated spheres were taken up to a smaller extent (Fig. 3B) with comparable lower MFI per cell that PLL- and TT-coated spheres (results not shown). After 24 h incubation, no further increases in DC binding were observed for the PLL-, PA- and TT-coated spheres, while the uptake of uncoated spheres increased (Fig. 3B). The spatial localization of the 0.1 μm particles was examined by CLSM (results not shown). The PLL- and TT-coated spheres had a tendency to cluster at the cell surface after 4 h while longer incubation led to translocation of the particles to the cell interior. Anionic uncoated and, to a smaller extend, PA-coated particles where more evenly distributed inside the cells.

Taken together, the results showed that DC bound to and internalized particles in the tested size range of 0.04–15 μm in diameter with different efficiencies. In general, the smaller the particle size, the higher percentage of the DC interacted with the polystyrene spheres. The kinetics of the association rate of particles to DC was influenced by surface charge and the numeric relation of particles:DC. Increasing the ratio of particles per DC under non-saturating conditions enhanced the binding as expected. Alteration of surface charge had most effects upon DC association for the larger particle sizes (>0.5 μm in diameter). The latter is in accordance with previous studies of 1 and 4.5 μm sized particles (Thiele et al., 2001).

However, the present results suggest that the influence of particle surface charge is different for particles below the size of 0.5 μm upon interaction with DC. Thus, 0.1 μm sized particles (except for the uncoated particles) were rapidly associated to DC and bound to a large percentage of the DC within 4 h of incubation, while only positively charged particles of 1 μm size reached similar (but lower) degrees of DC association when adding identical volumes of particles (Fig. 3). Such a function could imply an advantage in vaccine design if small sized particles, also with negative surface charge as exemplified with the tested TT-coated particles, could efficiently interact with DC. Administration of cationic particles may result in non-specific interaction with any cell type in vivo, due to electrostatic interaction with the negatively charged cell surfaces. Small-sized, negatively charged particles might therefore be superior to cationic particles due to reduction of non-specific interaction with other cell types. Specific uptake by DC could further be increased by addition of an active targeting moiety to such anionic particulate antigen formulations.

Surprisingly, DC internalized polystyrene particles as large as 15 μm. However, beads in the size range of 1–15 μm were only phagocytosed by a minority of the DC as reported in the literature (Reece et al., 2001; Thiele et al., 2001). The 0.04 and 0.1 μm beads are probably taken up by macropinocytosis (fluid phase

endocytosis) and the larger spheres by phagocytosis, but this has to be proven experimentally. This limited extent of particle uptake in DCs compared to macrophages (Thiele et al., 2003) has been suggested to reflect the different roles that these two cell types play for immune induction: whereas macrophages scavenge foreign material with high efficiency leading to clearance, DCs are suggested only to take up antigens to an extent that is necessary to initiate an immune response (Kiama et al., 2001).

Surface hydrophobicity is an equally important particulate physicochemical factor and may as well play a crucial role for the interaction between particles and DC. Polystyrene is a hydrophobic polymer compared to commonly used biodegradable alternatives such as poly(lactide) and poly(lactic-co-glycolides). Future studies should address the dependency of this factor, and particles of different hydrophobicities could be characterized for example by measuring the angle of contact of water or oil towards the particles.

In addition to antigen uptake, other important mechanisms determine how efficient DC prime T-cells. Future characterization of particulate vaccine formulations in DC culture systems should therefore also include functional assessments of antigen presentation, which is stimulation of T-cells, including measurements of proliferation and effector functions.

In conclusion, the results indicate an optimal particle size range, which in addition to incubation time, charge and concentration dependency, were important factors for efficient binding and uptake of particles by human DC. Particle diameters of 0.5 μm and below were optimal for DC uptake; however uptake of larger particles could be greatly enhanced by rendering the particle surface positive. The applied methodology could be useful for further analyses of the interaction in vitro between DC and particulate antigen formulations. Whether increased particle uptake is correlated with increased immune responses, remains to be established.

Acknowledgements

This work was supported by the Danish Technical Research Council, Centre for Drug Design and Transport (DUPS) and Torsten and Ragnar Söderberg Foundation, Sweden. The Schering-Plough Research Institute, U.S.A. is acknowledged for supply of hrIL-4. Margareta Andersson is thanked for excellent technical assistance and Masih Ostad is acknowledged for help with DC culture techniques. The Lundbeck Foundation is acknowledged for co-funding the Zetasizer 4 from Malvern Instruments.

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