Biomaterials Science

PAPER

View Article Online
View Journal | View Issue

Cite this: *Biomater. Sci.*, 2019, **7**, 1411

Disparate effects of PEG or albumin based surface modification on the uptake of nano- and micro-particles†

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Received 30th November 2018,
Accepted 9th January 2019

DOI: 10.1039/c8bm01545g

rsc.li/biomaterials-science

Surface modification of particulate systems is a commonly employed strategy to alter their interaction with proteins and cells. Past studies on nano-particles have shown that surface functionalization with polyethylene glycol (PEG) or proteins such as albumin increases circulation times by reducing their phagocytic uptake. However, studies on surface functionalized micro-particles have reported contradictory results. Here, we investigate the effects of surface functionalization using polystyrene particles with 4 different diameters ranging from 30 nm to 2.6 μm and coating them with either albumin or PEG. Our results show that with increasing particle size, surface functionalization has less to no effect on altering phagocytic uptake. The data also suggest that these differences are observed with a dense arrangement of molecules on the surface (dense brush conformation for PEG conjugation), appear to be independent of the serum proteins adsorbing on particle surfaces, and are independent of the endocytic uptake pathway. These results provide insight into the differences in the ability of surface modified nano- and micro-particles to avoid phagocytic uptake.

1. Introduction

Nano- and micro-particle systems are being developed for a wide range of biological applications that include, but are not limited to, *in vivo* imaging,[1,2] drug delivery[3,4] and vaccination strategies.[5–8] Such diverse uses are achieved by modulating both physical (shape and size) and chemical (bulk material and surface properties) characteristics of the particulate system.[9,10] Importantly, while the bulk composition of particles may determine their function, the surface characteristics are a major determinant of biological compatibility.[11,12]

Surface properties of particulates dictate their interaction with plasma proteins, *in vivo* circulation times, drug release kinetics, and phagocytosis by immune cells.[13–17] Of the many surface characteristics, the most commonly studied are charge and hydrophobicity–hydrophilicity. For example, it has been shown that particles with highly hydrophobic[18] or positively charged surfaces[19,20] are taken up in larger quantities by phagocytic cells, while coating nano-particle surfaces with hydrophilic molecules such as PEG is known to reduce their phagocytic uptake.[21–24] Similarly, studies have shown the dysopsonization effects of albumin coatings.[25,26] However, a majority focus on nano-particles, and the few that study surface functionalization of micro-particles show contradictory results. Some of these reports show lowered phagocytosis following PEGylation of micro-particles.[22,24,27] In contrast, others observe no effect of micro-particle PEGylation on the uptake levels.[28,29] The aforementioned studies relied on qualitative microscopy and error-prone flow cytometry-based side-scatter measurements for their analysis of phagocytosis. To understand these conflicting results and re-assess the effect of decorating micro-particle surfaces with albumin or PEG, a systematic and quantitative study needs to be performed.

Utilizing fluorescently tagged particles and albumin/PEG molecules, here, we studied the effect of surface modification on phagocytosis. While surface modification affects the uptake of nano-particles as expected,[15,30] it does not affect the uptake of micro-particles. The altered uptake is not a result of changes in the surface charge or density of molecules on the surface. We also assessed the effect of serum proteins and inhibitors of various endocytic pathways on the uptake of surface modified nano- and micro-particles, and observed no major differences. Further similar effects were observed in *ex vivo* and *in vivo* uptake studies with surface-modified micro-particles being taken up at levels similar to non-modified particles.

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†Electronic supplementary information (ESI) available. See DOI: 10.1039/c8bm01545g

2. Materials and methods

2.1 Materials

The following materials were used in this study and were obtained from a variety of manufacturers as indicated in parentheses: *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) (40 mM, SRL Chemicals, India), *N*-hydroxysuccinimide (NHS) (50 mM, SRL Chemicals, India), MES (Merck, USA), fluorescein isothiocyanate (FITC) labelled bovine serum albumin (BSA) (Merck), polyethylene-glycol (PEG) (2 kDa, Creative PEGWorks, USA or NANOCS), SDS (SRL Chemicals, India), Amico Ultra-15 Centrifugal filters (100 kDa, Merck Millipore), 2.6 μm polystyrene (PS) particles (Bangs Laboratories Inc., USA), 500 nm PS particles (Bangs Laboratories Inc.), 100 nm PS particles (Thermo Fisher Scientific, USA), 30 nm PS particles (Merck), Count Bright absolute counting beads (Thermo Fisher Scientific), PLGA (55–65 kDa, from PolySciTech, USA), RAW 264.4 murine macrophage cell line (Merck, USA), DMEM (CellClone, India), fetal bovine serum (US origin, Thermo Fisher Scientific), antibiotic–antimycotic solution (Thermo Fisher Scientific), Zombie NIR and Zombie Aqua (BioLegend, USA), propidium iodide (PI) (Merck), CD115 (clone AF598, Biolegend, USA), F4/80 (clone BM8, Biolegend, USA), Ly6G (clone 1A8, Biolegend, USA), and CD14 (BD Biosciences, USA).

2.2. Covalent conjugation

Carboxyl groups on particle surfaces were activated using 40 mM EDC and 50 mM NHS prepared in MES buffer, pH 5.5. After activation, the particles were washed and incubated in PBS containing unlabeled or FITC labelled BSA or PEG for 3–4 hours. Finally, the particles were washed three times with PBS to remove unbound BSA/PEG. Washing of 30 and 100 nm particles was performed using centrifugal filters while 500 nm and 2.6 μm particles were washed by centrifugation. For adsorption, the particles were incubated with unlabeled or FITC labelled BSA in the absence of any other chemical for 3–4 hours in PBS. The following numbers of particles were labeled in each batch: 2.6 μm – 3×10^7, 500 nm – 1.4×10^9, 100 nm – 2×10^{12}, 30 nm – 6×10^{12}, and 15.74 μm (rhodamine-PLGA) – 9.43×10^5.

To confirm the surface modification of 2.6 μm particles, their fluorescence was measured on a flow cytometer (BD-FACS Canto II or BD-FACS Celesta) and the data were analyzed using FlowJo (Tree Star, Ashland, OR, USA). Fluorescence imaging of 500 nm particles was performed to confirm the presence of surface molecules post-conjugation and adsorption of BSA/PEG. To determine the amount of BSA/PEG on particle surfaces, the fluorescence of conjugated or adsorbed particles was measured using a spectrophotometer (Tecan Microplate Reader, Infinite 200 PRO), along with a standard curve of the respective molecule in PBS with appropriate controls. To differentiate particles modified through covalent conjugation and adsorption, 2.6 μm surface modified particles were incubated in PBS containing 1% SDS for one hour at room temperature.

Following labeling and washing, the conc. of 2.6 μm particles in the final suspension was determined on the flow cytometer using counting beads. For 30, 100 and 500 nm fluorescent PS particles, equal volumes were taken for each kind of surface modification and labelled as described earlier, and it was assumed that an equal number of particles were present following surface modification.

2.3. *In vitro* cellular uptake

A RAW 264.4 murine macrophage cell line was cultured in DMEM media containing 10% fetal bovine serum and 1% antibiotic–antimycotic solution. For uptake studies, 1×10^5 cells were seeded in 12 well cell culture plates and incubated at 37 °C (in an incubator with 5% CO_2) for two hours to allow them to adhere. After the cells had adhered, 2.6 μm PS particles were added to the cells at a ratio of 1 : 2 (cells to particles) in duplicate (other ratios such as 1 : 4 and 1 : 8 were also tested with similar results, but data are not shown) and volumetrically equivalent amounts of particles were added for all other sizes. The cells and particles were incubated for 60, 120 and 1440 min. At the end of incubation, cells were scraped from the wells and the suspension spun down at 350*g* for 4 min. In order to remove particles adhered to the cell membrane, cells were washed twice with ice-cold PBS. Finally, cells were re-suspended in PBS containing PI at a concentration of 2 μg ml^{-1}. For 100 nm particles, staining with Zombie NIR was performed to distinguish live and dead cells. The cells were analyzed on the flow cytometer (BD FACS-Celesta or BD FACS-Canto, Becton-Dickinson, USA) to determine the number of live cells that had taken up particles in each case (a minimum of 10 000 events of live cells among the single cell population was recorded).

2.4. Incubation in biological fluid mimics

Particles were incubated in either PBS, or in PBS containing 10% FBS (ser-PBS), or PBS containing 10% human plasma (pla-PBS) at room temperature or 37 °C on a roto-spin. At specific times (0, 10, 30, 60, 120, 480 and 1440 min), particles were pelleted through centrifugation, washed twice, and re-suspended in PBS and their fluorescence was measured on the flow cytometer. To study the interaction of albumin/PEG coated (both through physical adsorption and covalent conjugation) 3 μm PS particles with proteins in solution, the modified particles were incubated in PBS containing 10% serum and 100 μg ml^{-1} of BSA-FITC. Particles were then washed twice and re-suspended in PBS and the amount of FITC labeled albumin on the surface of the particles was measured on the flow cytometer.

2.5. Effect of serum and endocytic inhibitors on cellular uptake

RAW 264.4 cells were cultured as mentioned previously. To study the effect of serum on the uptake of particles, the media were removed from the wells, and cells were washed once with PBS and re-suspended in media without serum (incomplete media). Particles were added to the wells and co-incubated

with cells for 120 min and their uptake analyzed by flow cytometry as described previously.

To study the effect of inhibitors on the uptake of the particles, after adherence, cells were treated with different pharmacological endocytic inhibitors for one hour at following concentrations: chlorpromazine hydrochloride (20 μM) which blocks clathrin mediated uptake, nystatin (10 μM) which blocks caveolae mediated uptake, methyl-β-cyclodextrin (10 mM) which is believed to affect both clathrin and caveolae mediated uptake, cytochalasin D (1 μM) which inhibits actin polymerization and affects macropinocytosis, and nocodazole (10 μM) which inhibits microtubule assembly (all chemicals from Merck). After this pre-incubation of cells with inhibitors, particles were added and incubated for 120 min, following which the samples were prepared for flow cytometry as mentioned previously.

2.6. *In vivo* experiments

The experiments were performed in agreement with the Control and Supervision Rules, 1998 of the Ministry of Environment and Forest Act (Government of India), and approved by the Institutional Animal Ethics Committee of the Indian Institute of Science. BALB/c mice (7 to 10-week-old) bred at the Indian Institute of Science central animal facility were used for the experiments. Surface modified (or control) fluorescent 2.6 μm particles were injected intraperitoneally at a concentration of 3×10^6 per mouse. After 1 hour, mice were euthanized by cervical dislocation. Peritoneal exudates were collected, passed through a 100 μm filter, incubated in RBC lysis buffer, and finally suspended in flow cytometry buffer (PBS containing 1% BSA and 4 mM EDTA). Spleen, mesenteric lymph nodes and mediastinal lymph nodes were also collected. Single-cell suspensions of the three were prepared through mechanical breakdown, followed by RBC lysis and suspension in flow cytometry buffer before further analysis.

Single cell suspensions from various organs were stained with the following antibodies for 20 min at 4 °C: CD115, F4/80 and Ly6G (clone 1A8, Biolegend, USA). The cells were finally stained with PI to distinguish between live and dead cells before being run on the flow cytometer.

2.7. Cellular uptake by human monocytes

Studies involving human blood were approved by the Institutional Human Ethics Committee at the Indian Institute of Science (approval number 5-15032017). After obtaining informed consent, blood was collected at the Indian Institute of Science Health Centre from volunteer donors in EDTA coated tubes. Isolation of PBMCs was performed using Histopaque density gradient centrifugation. Briefly, 7.5 ml of Histopaque was taken in a 15 ml tube and 5 ml blood was overlaid on it. The tube was then centrifuged at 500× rcf for 20 min (with the brake turned off) at room temperature. The upper yellow layer of plasma was aspirated and stored at −80 °C until further use. PBMCs were collected, counted, seeded in a cell-culture well plate, and allowed to adhere for one hour. Surface modified and non-modified particles (30 nm and 2.6 μm) were added to the cells and co-incubated for 120 min. Cells were scraped, stained with Zombie Aqua (for live/dead distinction) for 20 min and fixed with 2% PFA for 30 min at RT. Finally, staining was done with a fluorescently tagged antibody against human CD14, which is a marker for monocytes in PBMCs, for 30 min at 4 °C and cells were analyzed on the flow cytometer with at least 10 000 events of live monocytes recorded.

2.8. Release kinetics of rhodamine-loaded PLGA particles

Rhodamine encapsulated PLGA micro-particles were prepared using a double-emulsion evaporation technique, and lyophilized. Their size was determined using a Master sizer 3000 (Malvern). The volume averaged diameter was calculated to be 15.74 μm, D_{10} to be 7.38 μm and D_{90} to be 17.64 μm. Rhodamine release in both PBS and ser-PBS was monitored over a period of 10 days. The amount of rhodamine released was quantitatively determined using a spectrophotometer by reading the absorbance at 540 nm (λ_{max} of rhodamine B).

2.9. Statistics

All data were analyzed, and graphs generated using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Data from biological duplicates of a single independent experiment are reported through a single mean value, and at least (unless stated otherwise) 3 independent experiments were performed. For data involving comparisons between 2 groups, Student's *T* test was used. For data involving comparisons between multiple groups, repeated measures ANOVA followed by the Bonferroni *post-hoc* test was used for statistical comparisons.

3. Results

3.1. Surface modification

Fluorescently tagged albumin or polyethylene glycol (PEG) was either covalently grafted or physically adsorbed onto carboxylated polystyrene (PS) particles. As measured using flow cytometry, for 2.6 μm PS particles, a higher amount of albumin was observed on particle surfaces following covalent conjugation as compared to physical adsorption (ESI Fig. 1A†). To confirm that the molecules are covalently conjugated, surface labelled 2.6 μm particles were treated with sodium dodecyl sulfate (SDS), which is known to disrupt physical interactions without affecting the covalent bonds. Following SDS-treatment, the fluorescence intensity dramatically reduced for particles on which albumin was physically adsorbed but did not change significantly for covalently conjugated particles (ESI Fig. 1A†). Surface modification of 500 nm particles was confirmed by imaging the particles after surface modification. The presence of BSA-FITC/PEG-FITC could be observed as green fluorescence on the particle surface (ESI Fig. 1B†).

3.2. *In vitro* uptake

To determine the effect of surface modification on the particle–immune cell interaction, phagocytic uptake studies were performed using 30, 100, 500 nm and 2.6 μm fluorescent PS particles. Following uptake, nano-particles (30 and 100 nm)

showed a unimodal distribution of fluorescence associated with cells, while sub-micro- and micro-particles (500 nm and 2.6 μm) showed a multimodal distribution of fluorescence associated with cells (Fig. 1). Hence uptake was quantified as the median fluorescence intensity for nano-particles, and as the percentage uptake for micro-particles (a direct comparison of percentage uptake is also reported in ESI Table 1†). Furthermore, confocal microscopy was used to confirm that the fluorescence associated with cells was a result of the intracellular uptake of particles (500 nm and 2.6 μm) (ESI Fig. 2†), which was also confirmed for 30 nm sized particles by performing flow-based fluorescence measurements after quenching with trypan blue (data not shown). Similar to prior reports in the literature on nano-particles,[21,22] our studies show that the surface modification of 30 and 100 nm PS particles through covalent conjugation of albumin or PEG

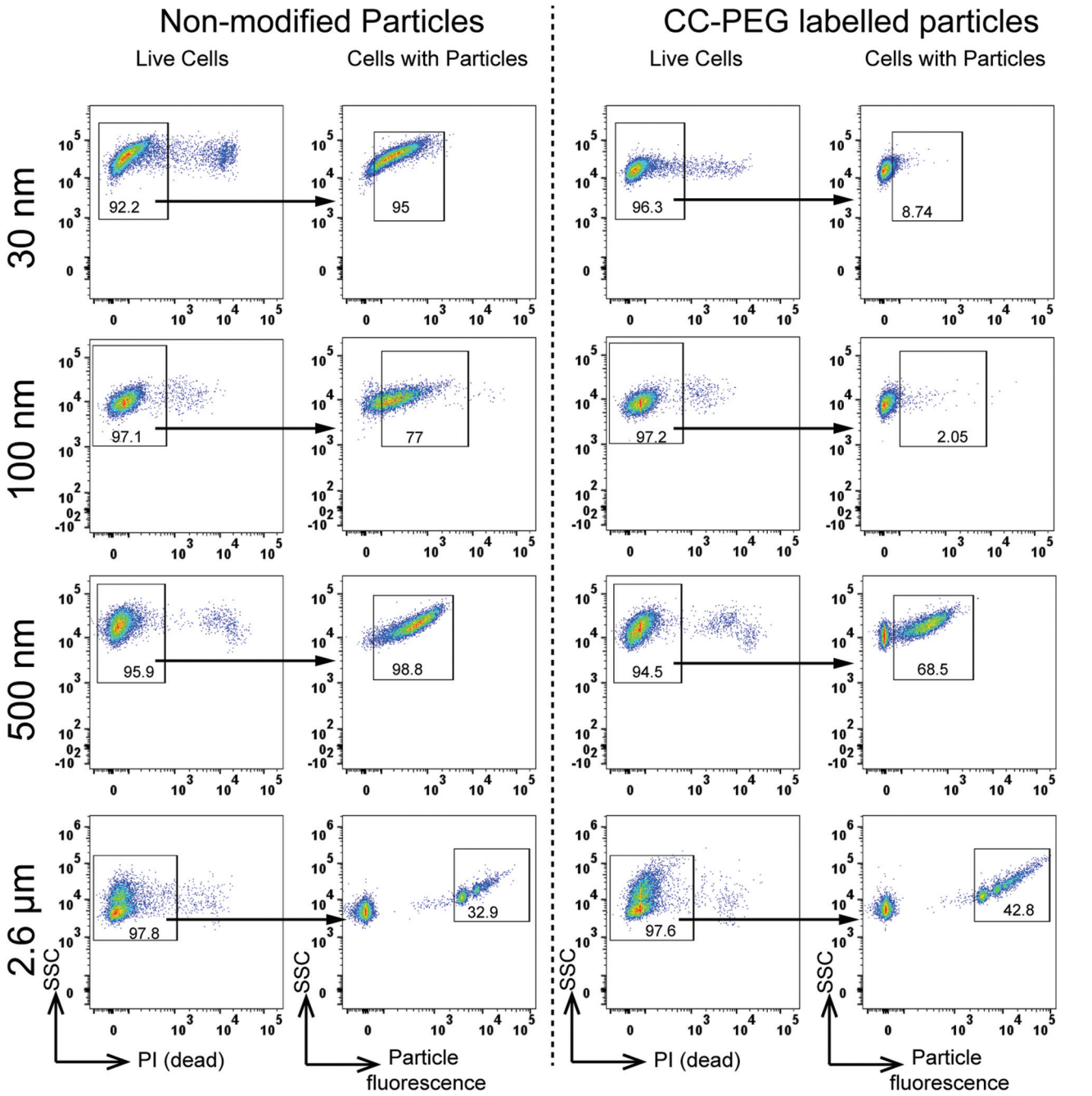

Fig. 1 Analysis of uptake using flow cytometry. Representative flow cytometry dot plots describing the gating scheme used for *in vitro* uptake experiments. Following gating on live cells (dot plots on the left), the percentage of cells containing particles was determined (gates and associated numbers in the dot plots on the right) for particles with or without PEG based surface modification. A similar gating strategy was followed for particles modified with albumin. PI represents propidium iodide, which was used to differentiate live and dead cells. SSC indicates side scatter.

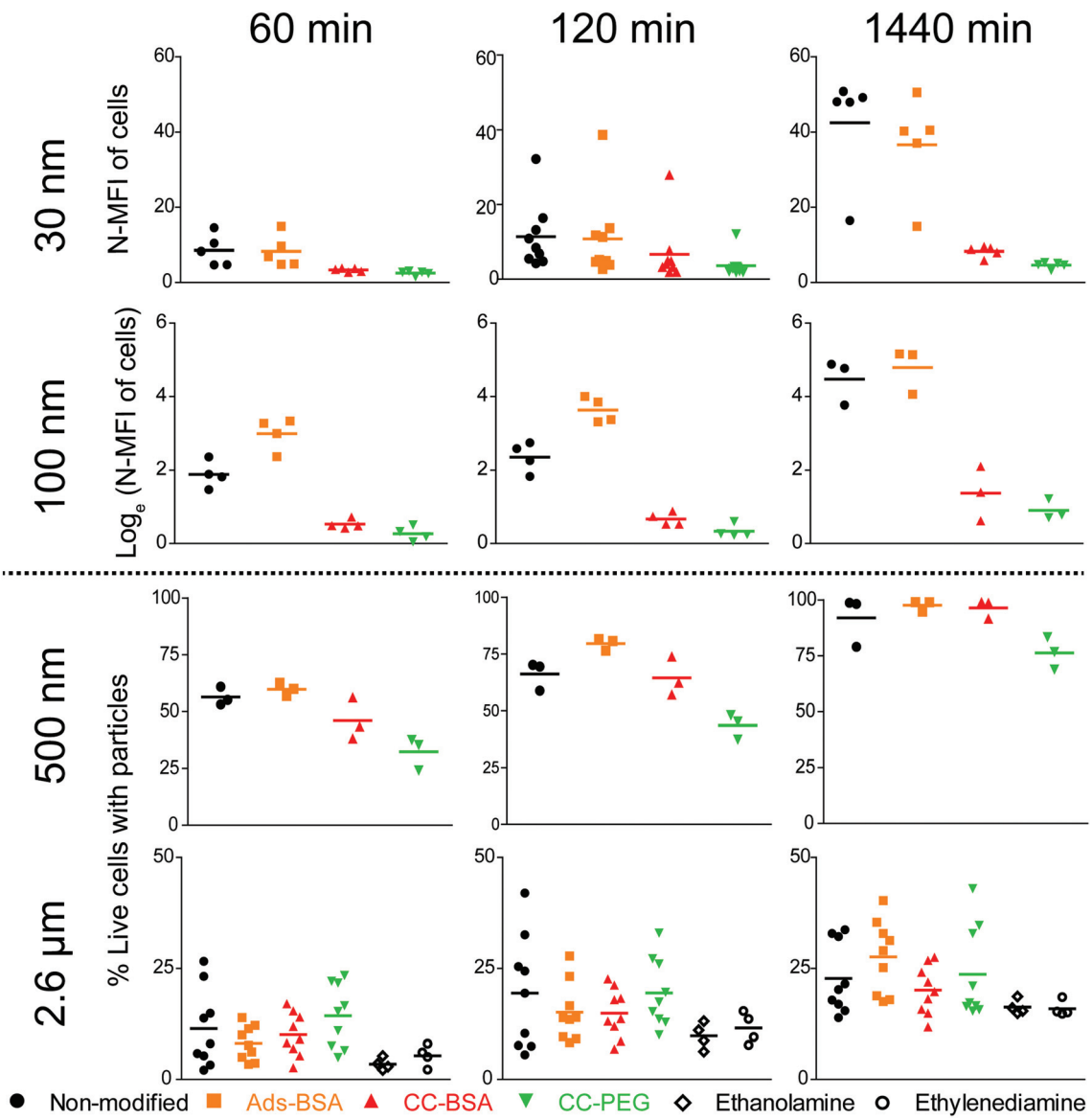

Fig. 2 *In vitro* uptake by the RAW 264.4 murine macrophage cell line. Uptake is quantified as either a fold increase in the median fluorescence intensity (MFI) of cells with particles over cells without any particle addition (30 nm and 100 nm), or percentage of cells that have taken up particles (500 nm and 2.6 μm). Particles were added to the cells at a ratio of 1 : 2 (cells to particles) for 2.6 μm particles, and volumetrically equivalent amounts of particles were added for all other sizes. Data sets are representative of $n \geq 3$ experiments. *Significant differences were observed in 30, 100 nm, and 500 nm particle uptake, and the corresponding p values are shown in ESI Table 1.†

results in dramatically reduced uptake (Fig. 2). However, the same was not true for larger particles. The surface modification of 500 nm particles resulted in no change in uptake following albumin conjugation and a slight decrease (~20%) following PEG conjugation. This effect was further diminished in 2.6 μm particles, where conjugation with neither albumin nor PEG resulted in changes in phagocytic uptake when compared to the non-modified particles (Fig. 2 and ESI Table 2†). Additionally, surface modifications did not inhibit the uptake of multiple particles by cells (ESI Fig. 3†). Furthermore, the fluorescence intensity of the surface tagged molecules (albumin and PEG) dropped over time, suggesting potential processing of the surface tagged molecules (ESI Fig. 4†). The absence of an effect of surface modification on the phagocytic uptake of micro-particles could be due to the change in the surface charge following modification, or the low density of surface labeling, or due to serum proteins that adsorb onto modified surfaces, or due to altered mechanisms of uptake.

3.3. Surface charge and labeling density

The neutralization of the surface charge on particles following surface conjugation with albumin or PEG could be one possible reason for the absence of reduced uptake in larger microparticles. To ascertain if the surface charge was a causative

Table 1 PEG grafting density on the particle surface

Particle diameter	Flory radius (R_F)	No. of PEG molecules per particle	Distance (D) between PEG grafts
2.6 μm	3.5 nm	$1.09 \pm 0.19 \times 10^7$	1.59 ± 0.15 nm $D < R_F$
500 nm	3.5 nm	$1.42 \pm 0.10 \times 10^6$	0.84 ± 0.03 nm $D < R_F$

factor, 2.6 μm particles were modified with a neutrally charged (ethanolamine) or a positively charged (ethyl-diamine) small molecule. Modifications with these molecules did not result in a significant change in uptake, when compared to non-modified particles. The higher than expected uptake of surface modified micro-particles could also be a result of the low concentration of PEG molecules present on the surface (a mushroom-like arrangement of PEG on the surface).[31,32] To determine if this was the case in our system, the number of PEG molecules on the surfaces of 500 nm and 2.6 μm particles was quantified following covalent conjugation (Table 1). Based on the number of PEG molecules per particle,[31] the distance between individual PEG molecules on the surface was calculated, and was found to be less than the 'Flory' radius of a 2 kDa PEG molecule, suggesting that they are arranged in a dense brush like conformation[31] in our system. Similarly, the number of albumin molecules present on the surface of a single 2.6 μm particle following covalent conjugation was calculated to be $5.24 \pm 1.07 \times 10^6$.

3.4. Stability in biological fluid mimics and adsorption of proteins

The effect of serum proteins on the molecules present on PS particle surfaces was investigated next. For *in vitro* non-cellular studies, we chose non-fluorescent 3 μm PS particles. Post surface-modification, these particles were incubated in PBS, or PBS containing 10% fetal bovine serum (ser-PBS), or PBS containing 10% human plasma (pla-PBS). In all three fluids, the amounts of covalently conjugated albumin or PEG on the particle surfaces did not change appreciably (ESI Fig. 5A†). As might be expected, on surfaces that were modified through physical adsorption, the amount of albumin dropped by ~70%, following incubation in PBS. In contrast, similar decreases were not observed following incubation in ser-PBS or pla-PBS. While an ~50% decrease was observed in ser-PBS, no change was observed in pla-PBS. Given that physically adsorbed albumin was not being displaced as might be expected, it was postulated that serum/plasma proteins (such as albumin itself) might adsorb onto modified particle surfaces and prevent the first layer of albumin from being displaced. To determine if this might occur, particles whose surfaces were modified with untagged albumin/PEG were incubated in ser-PBS containing 100 μg ml^{-1} albumin-FITC. As hypothesized, albumin-FITC was observed on surface-modified particles, and the amount present was comparable to that of non-modified particles (ESI Fig. 5B†).

The observation that albumin (and possibly other serum/plasma proteins) adsorbed equally well on particles with different surface modifications led us to question how serum proteins might affect the uptake of particles. Serum proteins have been known to modulate the interaction between particles and macrophages, and the surface properties of particles play a significant role in determining the type of protein adsorbing onto the particle surface.[15,33] Uptake studies were performed in the absence of serum and compared to the uptake observed in the presence of serum. For non-modified PS particles of sizes 100 nm and above, the absence of serum in the culture did not affect the levels of uptake (Fig. 3 and ESI Fig. 6†). Similar results were observed for PEG-modified PS particles of all sizes. However, 30 nm PS particles showed increased uptake by cells in the absence of serum when compared to cultures in the presence of serum for all surface modifications (Fig. 3). These studies suggested that the uptake of surface-modified PS is not significantly affected by serum proteins, and if it is affected, as in the case of 30 nm particles, it results in lowered uptake.

3.5. Effect of endocytic inhibitors on cellular uptake

Another possible reason for the lack of reduced uptake of PEG/BSA modified micro-particles could be the pathway used by cells to take up these particles. To study if the surface modification alters the pathway of endocytosis, we performed uptake experiments in the presence of small molecule inhibitors that block specific endocytic pathways. In the presence of inhibitors, regardless of surface-modification, the uptake of PS particles of all sizes was reduced when clathrin-dependent endocytosis was blocked using chlorpromazine (Fig. 4). Additionally, inhibiting actin polymerization by treatment with cytochalasin D resulted in a strong inhibition of 2.6 μm particle uptake (Fig. 4).

3.6. *In vivo* uptake and uptake by human monocytes

Next, we sought to determine if the effects observed in the culture with RAW macrophages would be replicated in mouse models *in vivo*, and in primary monocytes isolated from humans. Surface modified or non-modified 2.6 μm PS particles were injected intraperitoneally into BALB/c mice, and one hour after injection, the cellular uptake of these particles was analyzed. The cellular uptake of non-modified and surface modified particles in these studies (Fig. 5A and ESI Fig. 7†) were consistent with the *in vitro* data, suggesting that the surface modification of micro-particles does not affect their phagocytic uptake. Additionally, it was observed that these particles were primarily taken up by macrophages, and there was no difference in the uptake of surface modified or non-modified particles by both macrophages and neutrophils (ESI Table 3†). Also, similar to *in vitro* experiments, cells were able to take up multiple particles and there was no major difference based on surface modification (ESI Fig. 8†). Furthermore, cells associated with particles were not observed in the spleen, mesenteric lymph node and mediastinal lymph node at this early time point.

To determine if the aforementioned results hold true for primary human monocytes, PBMCs were isolated from blood and uptake experiments were performed *ex vivo*. In these

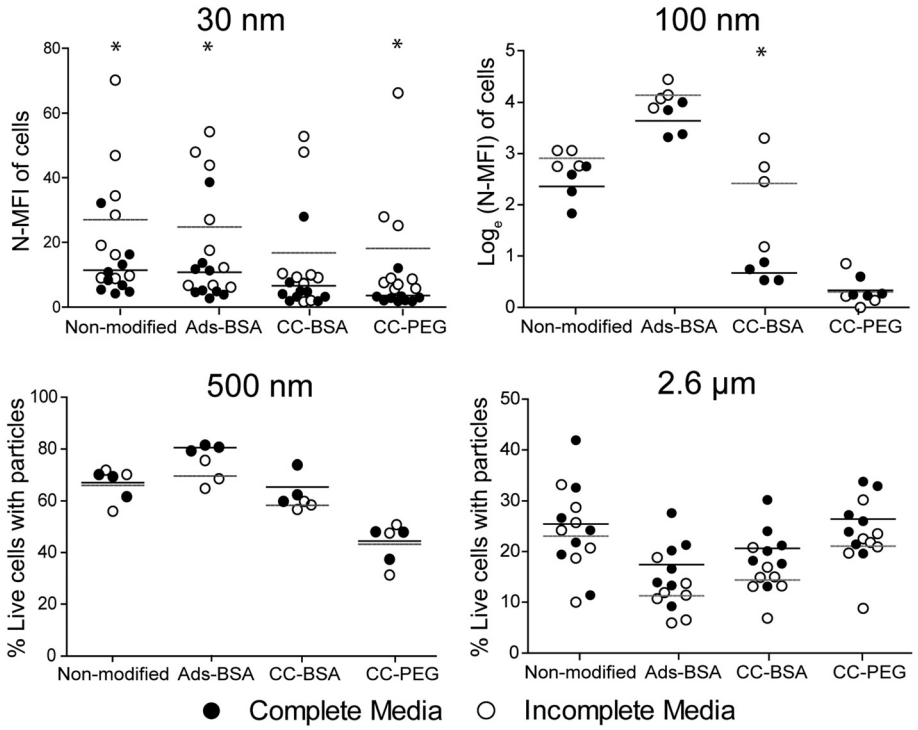

Fig. 3 Effect of serum on *in vitro* phagocytic uptake. To study the effect of serum on the uptake of particles, particles were co-incubated for 120 min with cells cultured in either complete or incomplete media (media without serum) and their uptake was analyzed by flow cytometry. Data sets are representative of $n \geq 3$. * Represents $p < 0.05$ when comparing uptake in complete and incomplete media, calculated using Student's *t*-test.

studies too, it was observed that CD14 expressing cells phagocytosed surface modified and non-modified 2.6 μm PS particles equally well (Fig. 5B and ESI Fig. 9†). Finally, to determine if the above results were applicable to other particle functions as well, the release kinetics of the model drug rhodamine from surface modified or non-modified rhodamine-loaded PLGA particles was measured. Here too, no differences were observed in release kinetics among non-modified, albumin-adsorbed and albumin-conjugated particles over a 10-day period (ESI Fig. 10†).

4. Discussion

Micro-particles with sizes ranging from 1 to 20 μm have found a variety of uses in research laboratories and in the clinic. Their ability to deliver larger drug doses, mimic cellular interactions and passively target phagocytic cells makes them useful for applications involving the modulation of immune cell function. Such functions may at times mandate avoiding immune cell interactions and at other times require enhanced phagocytosis by the same cells. These dichotomous functions may be achieved by the same micro-particle system through surface decoration strategies that dictate much of the interaction with immune cells. Of note in this context is the modification of surfaces with PEG and albumin, which have been shown to reduce phagocytosis in studies involving nano-particles, but have shown various results with micro-particles. To understand these conflicting reports, the effect of surface modification on phagocytic uptake was studied using polystyrene particles of various sizes as a model system.

As expected,[21–23] PEG or albumin-based surface modification resulted in lowered uptake of nano-particles. Importantly, this reduction was only relative (in comparison with non-modified particles or particles with surface modified with adsorbed albumin), as a large number of cells still took up the PEG or albumin conjugated particles. Additionally, the surface modification of sub-micrometer particles also resulted in lower uptake, but not to the same extent of the nano-particles. Furthermore, the surface modification of micro-particles had no effect on uptake. The observed lack of an effect was not a result of the surface charge (Fig. 2) or density of molecules on the particle surface (Table 1).

Proteins that adsorb onto particle surfaces have been shown to influence cellular interactions and uptake by immune cells.[30,34,35] To study the effect of serum proteins in the context of our experiments, the stability of surface conjugated/adsorbed molecules on micro-particles was evaluated in various biological fluids. Physically adsorbed proteins on particle surfaces are thought to be displaced over time following incubation in serum or plasma, while the same is not expected to occur following covalent conjugation.[36] Our experiments confirmed that covalently conjugated albumin/PEG is not displaced from the surface of micro-particles, but we also observed that physically adsorbed albumin was not entirely displaced. The likely reason for this observation is the adsorp-

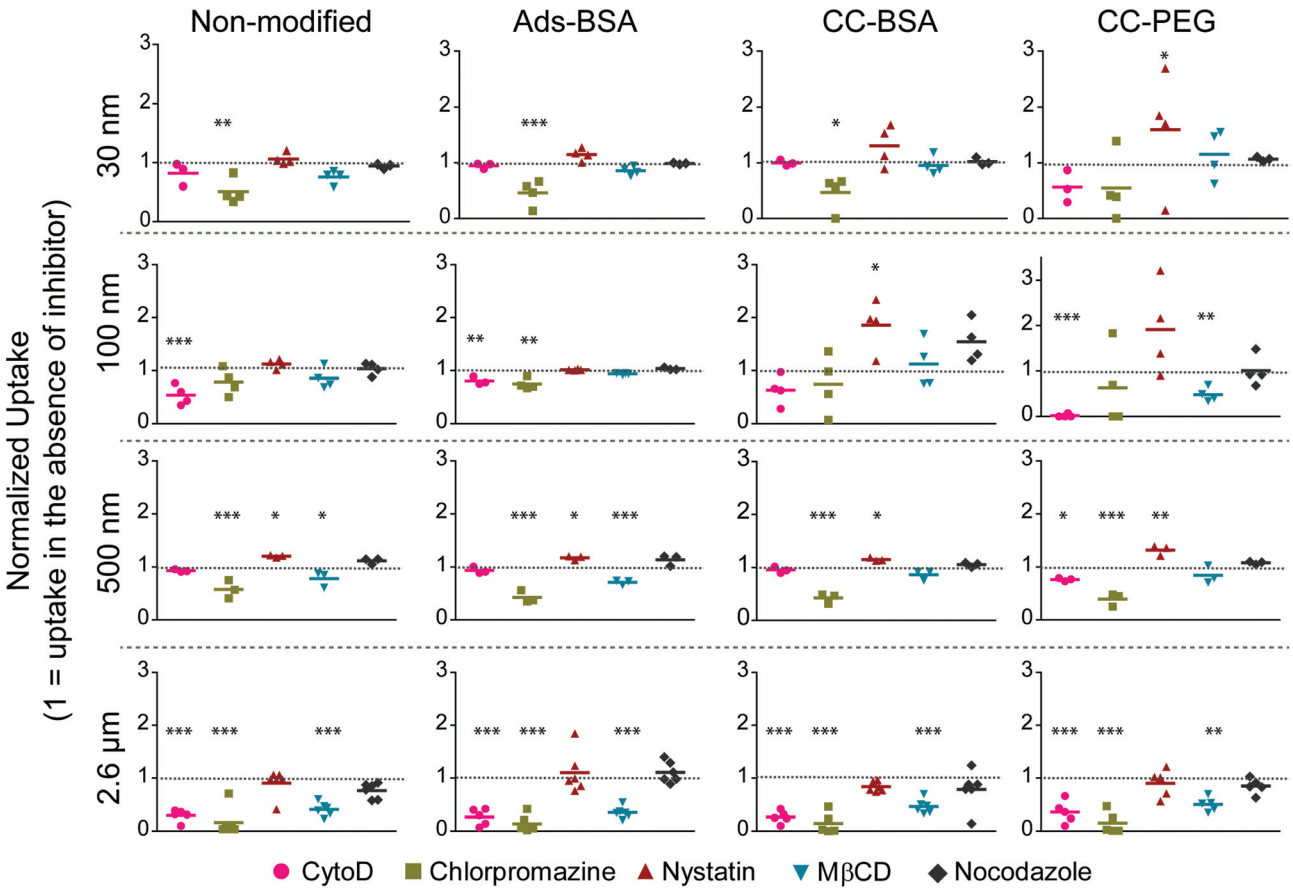

Fig. 4 Effect of endocytic pathway inhibitors on *in vitro* phagocytic uptake. To study the effect of inhibitors on particle uptake, cells were treated with different pharmacological endocytic inhibitors for one hour: cytochalasin D, nystatin, methyl-β-cyclodextrin, chlorpromazine hydrochloride and nocodazole. After pre-incubation of cells with inhibitors, particles were added and incubated for 120 min. Flow cytometry was performed to determine the MFI of cells in the particle fluorescence channel for 30 and 100 nm particles, and the percentage of cells with particles for 500 nm and 2.6 μm particles. For graphical representation, normalization was performed by dividing the uptake value obtained for a given inhibitor by the uptake value in the absence of the inhibitor. Data are representative of $n \geq 3$ experiments. *** indicates $p < 0.001$, ** indicates $p < 0.01$ and * indicates $p < 0.05$ measured using one-way ANOVA followed by a Bonferroni post-test comparing specific inhibitor treatment to the untreated case.

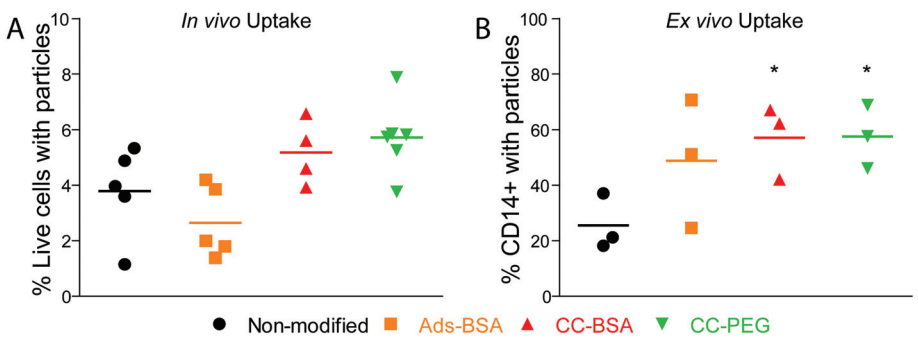

Fig. 5 *In vivo* and *ex vivo* phagocytic uptake. A – Surface modified or non-modified 2.6 μm fluorescent particles were injected intraperitoneally in BALB/c mice. One hour after injection the peritoneal exudate was collected, and cells that had phagocytosed particles were determined using flow cytometry. Data are represented as the total number of live cells isolated from the peritoneal cavity with particles. Breakdown of uptake by individual immune cell sub-types is provided in ESI Table 2.† Data are based on $n \geq 4$ mice per type of particle. B – PBMCs were isolated from blood and uptake experiments were performed by incubating surface modified or non-modified 2.6 μm fluorescent particles with cells for two hours. Percentage of CD14 expressing cells (monocytes) with particles was determined using flow cytometry. Data are based on $n = 3$ independent experiments (each performed in duplicate). *$p < 0.05$ measured using one-way ANOVA followed by a Bonferroni post-test comparing specific surface modified particles to non-modified particles.

tion of proteins from serum or plasma as a second layer on top of the first layer of adsorbed albumin, which was confirmed by the observation that albumin adsorbed onto micro-particles with or without surface modification (ESI Fig. 5B†). This result contrasts the observation of lowered serum proteins with nano-particle surfaces that are covalently modified with albumin/PEG.[37]

Having observed no difference in the amount of protein adsorbing on micro-particles with modified surfaces, the effect these adsorbed proteins might have on phagocytic uptake was evaluated by performing uptake studies in the absence of serum proteins. In these experiments it was observed that the uptake of both non-modified and PEG-modified micro-particles remained unchanged in the absence of serum (Fig. 3). This result was expected for non-modified particles as the absence of a change or an increase in uptake under serum-free conditions has been previously reported for other particle types.[38–40] This result was surprising as surface modification with PEG is thought to result in altered protein corona, which is the primary driver for altered cellular interactions including lower phagocytic uptake.[41–43] Our data suggest that for PS particles, lowered uptake of nano-particles because of PEG modification may occur due to the protein corona formed on the surface, but for micro-particles uptake is independent of the protein corona.

Phagocytes utilize a variety of endocytic pathways to internalize the extracellular material.[44] Our experiments to elucidate the pathway utilized for the uptake of particles showed that internalization was clathrin dependent for particles of all sizes and surface modifications tested (Fig. 4). Additionally, as shown in previous studies,[45] the uptake of larger particles (2.6 μm) was also actin-dependent. Importantly, a surface-modification dependent trend in the pathway used for uptake was not observed in particles of any size.

The results observed *in vitro*, using a macrophage cell line, hold true in *in vivo* and *ex vivo* primary cell culture systems too. Intraperitoneal injection of non-modified or surface-modified micro-particles in mice showed similar uptake levels of all types of particles. Results from our studies on *ex vivo* uptake by primary monocytes from human blood were in accordance with both *in vitro* and *in vivo* studies involving micro-particles (Fig. 5). Further, no differences were observed between non-modified and surface modified particles in another system involving larger micro-particles. Using rhodamine encapsulated PLGA micro-particles, we show that covalent conjugation or physical adsorption with albumin does not alter *in vitro* release kinetics when compared to non-modified particles, in both PBS and serum containing PBS solutions (ESI Fig. 10†). Put together, these data suggest that the surface modification of micro-particles with PEG or albumin does not result in altered biological interactions when compared to the non-modified particles.

The question regarding why there is less of a difference in the uptake of micro-particles following surface modification still remains. One of the reasons could be that PEG or albumin modification of micro-particles does not significantly affect protein adsorption (ESI Fig. 5†), which in turn might result in the lack of a difference in uptake by phagocytic cells. However, the absence of a difference in uptake is also seen under serum free conditions, suggesting that protein adsorption alone does not explain the observations. Another possibility is that a minimum number of interactions between particle surfaces and receptors on the cell membrane (or other membrane components) is required for uptake. PEGylation lowers particle–cell membrane interactions, and for nano-particles that have a lower surface area per particle such lowering is sufficient to prevent uptake. However, for micro-particles which have a relatively higher surface area per particle, PEG modifications may not result in the lowering of interactions below the minimum threshold. This hypothesis remains to be experimentally proven. An alternative possibility is that given the size of micro-particles being similar to membrane ruffles in macrophages, the particle–cell interaction is dominated by the curvature of the particle[9] with very little to no effect of surface ligands.

Notably, the effect of surface chemistry being more pronounced for nano-particles as compared to micro-particles has been observed by a few others. A study by Pacheco *et al.* showed that while the internalization efficiency of small polystyrene particles (0.5 μm to 2 μm) is affected by changes in the Fc ligand density, particles greater than 2 μm showed little correlation between internalization and Fc density.[46] Similarly Parak and colleagues show that the surface charge plays a crucial role in nano-particle uptake,[47] but the effects are diminished for micro-particles.[48] Nevertheless, it should be noted that the results described here are primarily based on studies using polystyrene particles. It remains to be seen if these results will be replicated in other polymeric particles and in non-polymeric systems. Furthermore, we have not looked at the effect of the PEG molecular weight in this study. PEG chains of different lengths have been shown to affect particle properties, and studying these properties would be of relevance for future studies.

In conclusion, we show that the surface modification of micro-particles with PEG or albumin does not alter its functional properties in terms of protein adsorption, phagocytic uptake and drug release kinetics. These data highlight the differences between micro-particles and nano-particles in their protein and cellular interactions, and the need for further experimentation to improve the performance of micro-particle systems.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

We thank Aritra Chatterjee, Jayashree V., Manjyot Kaur Chug and Varsha Gadiyar for assistance with cell culture and flow cytometry. We also acknowledge the support of personnel at

the central animal facility, and the imaging and flow cytometry core facility, Indian Institute of Science. This work was supported by the Department of Science and Technology, Govt. of India fellowship to SJ (Ramanujan Fellowship – SB/S2/RJN-135/2015), and partly by a Science and Engineering Research Board, DST, Govt. of India grant (ECR/2016/000629) to SJ. P.S. is supported by a University Grants Commission (Govt. of India) fellowship. SJ is in receipt of the R.I. Mazumdar Young Investigator Fellowship at the Indian Institute of Science.

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