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Effect of the Poly(ethylene glycol) (PEG) Density on the Access and Uptake of Particles by Antigen-Presenting Cells (APCs) after Subcutaneous Administration

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Abstract

Lymphatic trafficking of particles to the secondary lymphoid organs, such as lymph nodes, and the cell types that particles access are critical factors that control the quality and quantity of immune responses. In this study, we evaluated the effect of PEGylation on the lymphatic trafficking and accumulation of particles in draining lymph nodes (dLNs) as well as the cell types that internalized particles. As a model system, 200 nm polystyrene (PS) particles were modified with different densities of poly(ethylene glycol) (PEG) and administered subcutaneously to mice. PEGylation enhanced the efficiency of particle drainage away from the injection site as well as the access of particles to dendritic cells (DCs). The accumulation of particles in dLNs was dependent on the PEG density. PEGylation also enhanced uptake by DCs while reducing internalization by B cells at the single cell level. Our results indicate that PEGylation facilitated the trafficking of particles to dLNs either through enhanced trafficking in lymphatic vessels or by enhanced internalization by migratory DCs. This study provides insight into utilizing PEGylated particles for the development of synthetic vaccines.

Keywords

synthetic vaccines; lymphatic trafficking; dendritic cells; nanomaterials; PEGylation

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ASSOCIATED CONTENT

Supporting Information

Additional figures and table as discussed in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Notes

The authors declare no competing financial interest.

INTRODUCTION

Synthetic vaccines enable more precise control of immune responses, which can potentially improve the efficiency and safety of current vaccines. The quality and quantity of immune responses induced by a given vaccine are affected by the level of antigens available to be presented to naive T cells by antigen-presenting cells (APCs), and the type of APCs that internalize antigens.^{1,2} The interaction of naive T cells with APCs mainly occurs in lymphoid organs, including lymph nodes (LNs) and the spleen.

Several strategies have been investigated to increase the level of synthetic vaccines in lymphoid organs. Those strategies can be broadly categorized as direct access through intralymphnodal injection^{3–5} or distal access by improving trafficking and accumulation of vaccines in lymphoid organs upon systemic administration (i.e., subcutaneous injection).^{6–19} The latter strategy is more practical and simple for mass vaccination as it does not require specialized techniques of injections. Several design parameters that affect the trafficking and accumulation of synthetic vaccines (e.g., liposomes or nanoparticles) in lymphoid organs have been studied, such as particle size,^{6–8} surface chemistry (charge,^{9,10,20} hydrophobicity^{11–15}), and cell-targeting moieties (e.g., antibody¹⁰).

In addition to the level of vaccines able to access lymphoid organs, the ability to access relevant APCs can dictate subsequent immune responses.¹⁷ B cells, macrophages, and DCs are the major types of APCs. DCs are considered as the most critical APCs given their unique ability to prime both CD4⁺ and CD8⁺ T cells and engage the innate immune system.^{1,21} DCs can be categorized as migratory and lymph-node-resident based on their anatomical locations.^{22,23} Migratory DCs reside in peripheral tissues, such as mucosal or epidermal surfaces, capturing pathogens and migrating to the secondary lymphoid organs such as dLNs. Lymph-node-resident DCs sample pathogens that are transported to the LNs through lymphatic vessels, blood, or other cells. Each DC subset displays characteristic surface molecules and different functions.^{24,25} All the murine DC subsets express CD11c.²⁶ They can be further distinguished by the surface molecules CD8 and DEC205.²⁷ CD8⁺ DCs, known as CD8 α DCs, mostly reside in LNs and are resident DCs while CD8[–] DCs, including Langerhans cells (LCs) (DEC205^{high}) and dermal DCs (DEC205^{intermediate}), mostly reside in peripheral tissues and are migratory DCs. DCs which do not express both CD8 and DEC205 are called double negative DCs (DN DCs). DN DCs mostly reside in dLNs and are categorized as resident DCs.²⁸ CD8 α DCs are superior in priming CD8⁺ T cells compared to other DCs, while CD8[–] DCs, including LCs and dermal and DN DCs, mostly facilitate the generation of CD4⁺ T cells.^{21,29} Thus, the accessibility of vaccines to different DC subsets can influence the quality and quantity of immune responses.

PEGylation has been utilized to modify the surface chemistry of synthetic vaccines.^{6,30–33} Poly(ethylene glycol) (PEG) can alter the hydrophobicity of particles and affect plasma protein adsorption and particle–cell interactions.³⁴ The effects of both PEG polymer length and surface density in terms of lymphatic trafficking and biodistribution have been studied by using liposomes,^{14,15,35} polymer nanoparticles,^{11,12} or colloidal magnetite.¹³ PEGylation has been shown to reduce the retention of particles at the injection sites while enhancing the level of particles transported to and retained in dLNs.^{11–14,35} PEGylated particles have recently been utilized for both systematic and mucosal vaccinations.^{7,36,37}

In this study, we examined the effects of the PEGylation on the access and cellular uptake of particles by APCs as well as specific subsets of DCs. Fluorescent polystyrene (PS) nanoparticles (200 nm) were modified with different PEG densities and administered subcutaneously *in vivo*. Our results confirmed previous studies that PEGylation could enhance the particle drainage to the LNs. The major APCs that internalized PEGylated

particles were B cells. Increasing the PEG density enhanced the access of particles to DCs and the cellular uptake of individual DCs. Thus, PEGylation of synthetic vaccines not only can enhance access of particles to lymphoid organs but also can passively target DCs. This study provides insight into how PEGylation can affect access to APCs that may subsequently influence the quality and quantity of both humoral and cell-mediated immune responses.

EXPERIMENTAL METHODS

Materials

200 nm fluorescent carboxylated polystyrene (PS) beads were purchased from Polysciences Inc. (Warrington, PA). Amine-terminated PEG (5000 Da) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma (St. Louis, MO). *N*-Hydroxysulfosuccinimide (sulfo-NHS) was purchased from Pierce (Rockford, IL). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Fixable Viability Dye eFluor 780, anti-mouse CD11c eFluor 450 (clone N418), anti-mouse CD8a APC (clone 53-6.7), anti-mouse CD3e APC (clone 145-2c11), anti-mouse CD205 PE (clone 205yekta), anti-mouse F4/80 antigen PerCP-Cy5.5 (clone BM8), and anti-human/mouse CD45R (B220) PE-Cy7 (clone RA3-6B2) were all purchased from eBioscience Inc. (San Diego, CA).

Conjugation and Characterization of PS Particles with Varying Densities of PEG

Varying amounts of PEG were covalently coupled to 200 nm carboxylated PS beads (5×10^6 , 5×10^5 , 5×10^4 , 0 PEG/particle). Briefly, 1 mg of carboxylated beads was first activated in 0.4 mL of 2-(*N*-morpholino)-ethanesulfonic acid solution containing 50 mM sulfo-NHS and 50 mM EDC for 1 h at room temperature. 0–9 mg of PEG was added to the mixture and incubated in 0.4 mL of phosphate buffered saline (PBS) for an additional 2 h at room temperature. Particles were then centrifuged, and the reaction solution was removed. The particles were then quenched by incubating with 0.5 mL of PBS containing 100 mM of glycine for 30 min. PEGylated particles were washed and stored in PBS at 4 °C. The actual PEG density of each type of particle was quantified by nuclear magnetic resonance (NMR) spectroscopy (300 MHz ^1H Bruker spectrometers). Zeta potential and diameter of particles were characterized by dynamic light scattering on a Malvern ZetaSizer Nano S90 (Worcestershire, U.K.).

Mice

6–8 week old female C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). Mice were given 2×10^{10} particles with different PEG densities either in 20 μL of PBS via the hind footpad or in 50 μL of PBS via the lateral torso subcutaneously. For the control groups, either PBS or bare PS particles were administered. At the indicated time points, mice were sacrificed. The dLNs (for footpad administration, popliteal lymph nodes; lateral torso, brachial and axillary lymph nodes) and tissues of injection sites were collected. All procedures used in this study complied with federal guidelines and institutional policies, and were approved by the University of Washington Institutional Care and Animal Use Committee.

Evaluation of Draining and Retention of Particles

The tissues of injection sites and dLNs were minced and then dissolved in 1 or 0.7 mL of a mixture of chloroform and DMSO (1:1), respectively. The tissues were incubated on a rotary shaker for 2–2.5 h. Tissues were centrifuged, and the fluorescence of the supernatant was determined by a SpectraMax M5 Multi-Detection Microplate Reader (Ex: 441 nm, Em: 514

nm). The amount of particles was calculated using a standard curve established by determining the fluorescence of known amounts of FITC-PS particles.

Evaluation of the Uptake of PEGylated Particles by Cells in dLNs

The dLNs were digested in 2 mg/mL collagenase D (Roche) and 30 μ g/mL DNase I (Roche) in RPMI medium (containing 1% penicillin–streptomycin, 2 mM L-glutamine, 50 μ M 2-mercaptoethanol, and 10% fetal bovine serum (FBS)) at 37 °C for 30 min. Tissues were collected by centrifugation, resuspended in Hanks balanced salt solution (HBSS) containing 5% fetal bovine FBS and 5 mM EDTA, and incubated at 37 °C for 5 min. A single cell suspension was prepared by grinding the tissues with the plunger of a 3 mL syringe through a 70 μ m cell strainer. The cells were washed by FACS buffer (DPBS containing 1% FBS) twice. The cells were stained with the viability dye at 4 °C for 30 min, washed, and then stained with the antibody cocktail composed of antimouse CD11c eFluor 450, anti-mouse CD8a APC, anti-mouse CD205 PE, anti-mouse F4/80 antigen PerCP-Cy5.5, and antihuman/mouse CD45R (B220) PE-Cy7 at 4 °C for 20 min. Cells were then washed and analyzed by flow cytometry (BD LSR II). Flowjo was used for data analysis. Particles associated with the cell surface were quenched using trypan blue before flow cytometric analysis. No significant differences were observed between quenched and nonquenched samples, indicating that particles were internalized.

Evaluation of Nanoparticle Distribution in Lymph Nodes

10¹¹ PEGylated particles were injected through the hind footpad subcutaneously. After 18 h the dLNs were collected, flash-frozen, and cryosectioned into 10 μ m sections. Sections were first incubated with anti-mouse CD16/CD32 antibody for 30 min at room temperature to block the Fc receptor. Then the sections were incubated with rat antibodies against mouse CD45R/B220 (Biolegend, CA) at a concentration of 10 μ g/mL in blocking buffer for 1 h at room temperature. Sections were washed and then incubated with 10 μ g/mL goat AlexaFluor 647 anti-rat IgG for 1 h at room temperature. Then sections were washed and fixed with 4% paraformaldehyde in DPBS for 20 min at room temperature. The dLN sections were then washed and mounted onto glass coverslips with hard-set mounting medium (Vector Laboratories Inc., CA). The sections were imaged with a confocal laser scanning microscope using a 10 $\times$ objective (LSM 510 Meta, Zeiss).

Uptake of Particles by Cells Isolated from Lymph Nodes

Single cell suspensions of popliteal lymph node cells were obtained as previously described. Cells were plated in a 96 well plate with a concentration of 5×10^5 cells/well. Particles at 5×10^3 particles/cell were added to cells and further incubated at 37 °C for 2 h. Cells were washed, stained for surface markers, and analyzed as previously described. Particles associated with the cell surface were quenched using trypan blue. No significant differences were observed between quenched and nonquenched samples, indicating that the majority of particles were internalized.

Statistical Analysis

Student's *t* test was used to compare two groups. *P* value < 0.05 was considered to be significant. All the experiments were repeated two to three times independently.

RESULTS

Characterization of PEGylated Particles

Fluorescent PS beads, 200 nm in diameter, were conjugated with varying amounts of PEG. The PEG density was quantified by NMR (Figure S1 in the Supporting Information).

Different feed ratios used in the conjugation ($(0.5 \text{ to } 50) \times 10^5$ PEG/PS particle) yielded varying surface PEG densities: 3800, 5700, and 13300 PEG/PS particle. Accordingly, the calculated surface area of a PEG molecule on the particle surface decreased from 33.3, 22.0 to 9.33 nm², indicating that the PEG conformation on the particle surface transitioned from a “mushroom” to a “brush” structure.³⁸

The diameters of particles, as measured by DLS, increased from ~220 to ~240 nm as the PEG density increased (Figure 1A). PEGylated particles had zeta potentials of -11.47, -48.97, -54.33 mV with the PEG/PS ratio of 13300, 5700, 3800 perparticles, respectively, compared to -69.77 mV of the bare PS particles (Figure 1B). The zeta potential of particles increased with the increasing density of PEG per particles as previously reported.^{12,13} These results indicated that particles with varying PEG densities were obtained.

Effects of PEGylation on the Drainage of Particles from Injection Sites

The quantity of particles remaining at injection sites were evaluated at 4 h and 1, 3, and 7 days postadministration for two different injection sites: hind footpad (Figure 2A) and lateral torso (Figure 2C). The amount of bare PS particles at the footpad did not decrease significantly over a period of 7 days. Nearly 90% of injected particles remained in the hind footpad. In comparison, only 50%–80% of injected PEGylated particles remained at the hind footpad (Figure 2A). Though the difference of drainage between non-PEGylated and PEGylated was not statistically different, similar trends were observed in three independently repeated experiments. Thus, PEGylation enhanced the drainage of particles away from the injection site. Similar trends were observed when particles were administered subcutaneously on the lateral torso of mice. Approximately 60–80% of bare PS particles remained at the injection site while 50–75% of PEGylated particles remained (Figure 2C). Again, for all the particle types, no significant differences were observed at different time points postadministration.

Effects of PEGylation on the Accumulation of Particles in dLNs

As shown in Figure 2B,D, less than 2.5% of injected particles accumulated in dLNs regardless of the time postadministration, PEG density, and injection sites. For both injection sites, the amount of particles accumulated 4 h after injection was too low to be detected. For the injection at the hind footpad (Figure 2B) at day 1, about 0.2–0.5% of injected particles were detected in dLNs for all particle types. At days 3 and 7, increasing amounts of particles were detected in dLNs. Compared to the injection at the hind footpad, fewer particles accumulated in the dLNs after subcutaneous administration at the lateral torso (Figure 2D). With increasing duration, more particles were present in dLNs. At both sites of injection, there was no clear trend observed for the effect of PEGylation on the accumulation of particles in dLNs. Interestingly, PEGylation slightly increased accumulation of particles in dLNs for both injection sites at a density of 5700 PEG/PS particle, but did not exhibit any statistically significant difference from bare PS particles.

Effects of PEGylation on the Access of Particles to Different Cell Types in dLNs

In order to examine the effect of PEGylation on the access of particles to different cell types, the particle-containing cells from the hind footpad dLNs were phenotyped in terms of DCs, B cells, and macrophages using flow cytometry. Particle-positive cells were first identified as non-DCs (CD11c⁻) and DCs (CD11c⁺)²⁶ (Figure 3A). The non-DC population was further divided into B cell (B220⁺F4/80⁻)³⁹ and macrophage (B220⁻F4/80⁺)⁴⁰ (Figure 3B). The majority of particle-containing cells were B cells, followed by DCs and other cells (Figure 3C). We further identified “other” cells by using anti-CD3 and anti-CD169, which identify T cells and subcapsular sinus macrophages, respectively. The population of “other” cells mainly consisted of CD3⁺ T cells. Less than 1% of cells were CD169⁺ macrophages

(data not shown). Interestingly, PEGylation enhanced the access of particles to DCs and reduced their access to B cells. DCs were only 4% of particle-containing cells for bare PS particles but increased approximately 5-fold to 21% for particles with 13300 PEG/PS particle. In contrast, the percentage of B cells decreased from 87% to 62% of total particle-containing cells with increasing PEG density. PEGylation had little effect on the access of particles to F4/80⁺ macrophages (Figure 3C). We also examined the distribution of cell types of particle-positive cells after 4 h (Figure S2B in the Supporting Information). At this shorter time point, particles are expected to reach lymph nodes mainly through lymphatic drainage but not by migratory cells at the site of injection.⁴¹ However, a similar trend was observed compared to the distribution of cell types after 18 h.

Effects of PEGylation on the Access of Particles to Different DC Subsets

We next examined how PEGylation affected the access of particles to different subsets of DCs. Based on the expression of two surface molecules, CD8 and DEC205, DCs can be categorized into four subsets: Langerhans cells (LCs) (CD8⁻DEC205^{high}), dermal DC (CD8⁻DEC205^{intermediate}), double-negative (DN) DC (CD8⁻DEC205^{low}), and CD8 α DC (CD8⁺DEC205^{intermediate})²⁷ (Figure 4A). The first two cell types are migratory DCs while the latter two are resident DCs.^{8,26,27} As shown in Figure 4B, PEGylation slightly increased the access of particles to either dermal or Langerhans cells. As the PEG density increased, the total percentage of particle-containing CD8 α ⁺ DCs was reduced from 30% to 15% while that of DN DCs increased from ~32 to ~52%.

Effect of PEGylation on the Uptake of Particles at the Single Cell Level

We next quantified the level of particle uptake for each cell type using flow cytometry (Figure 5). Cell types were gated on particle-positive cells as above, and the geometric mean fluorescence intensity of particle-positive cells was determined. PEGylation reduced the level of particles internalized by B cells while it significantly increased that by DCs and macrophages (Figure 5A,B). For DCs, PEGylation enhanced the internalization by all DC subsets (Figure 5C). Many *in vitro* studies have shown that PEGylation of particles reduced the cellular uptake by phagocytic cells, such as macrophages.^{42–44} The discrepancy may arise from the differential access of non-PEGylated and PEGylated particles to different cell types *in vivo*, which results in different local concentrations of particles around each individual cell type.

Effect of PEGylation on the Overall Level of Particles Associated with Each Cell Type

We determined the overall level of particles associated with each cell type by considering both the fraction of each cell type that particles access and the uptake of particles by individual cell type at the single cell level. As shown in Figure 6A, PEGylation enhanced the overall level of particles associated with DCs but reduced that with B cells. DCs can be distinguished by their ability of priming either CD4⁺ or CD8⁺ T cells. Dermal and DN DCs and LCs mainly prime CD4⁺ T cells while CD8 α DCs mainly prime CD8⁺ T cells. We refer to the former group of DCs as CD4 DCs. PEGylation enhanced the overall level of particles associated with both CD4 DCs and CD8 α DCs (Figure 6B).

DISCUSSION

Lymphatic trafficking and retention of synthetic vaccines, in particular particulate-based vaccines, in lymphoid organs have been considered as one of the major challenges in improving their efficacy in inducing robust and long-lasting immunity.^{7,17} Enabling the access of synthetic vaccines to relevant APCs could result in more controlled immune responses for increased safety.^{16,17,35,45–47} The effects of various physicochemical factors of vaccine delivery vehicles, such as size, shape, and surface chemistry, have been examined

for their role in lymphatic trafficking.^{6–15} Reddy et al. developed ultrasmall particles (25 nm) that showed much higher draining efficiency than 100 nm particles upon interstitial tail vein administration, and effectively targeted lymph-node-resident DCs.⁷ Although the use of ultrasmall particles may enable more efficient access to cells in the dLNs, nanosized particles have been previously shown to be inefficient in terms of cross-presenting antigen.^{48,49} Alternatively, direct intralymphnodal injection can potentially bypass the inefficiency of lymphatic trafficking and enhance local concentration of vaccines to APCs.⁵⁰ Intralymphnodal injection of a peptide-based vaccine has been shown to substantially enhance immunogenicity compared to intradermal, subcutaneous, or intravenous injections.⁵¹ However, this approach may be limited in practice due to the increased difficulty in administering such injections.⁵⁰ Thus, strategies to enable more efficient transport of synthetic vaccines to lymphoid organs require further exploration. Enhancing the access of vaccines to DCs can lead to more robust and controlled immune responses.^{19,45–47} It has become clear that DCs are composed of several subsets with different anatomical locations and functions. Thus, in evaluating the lymphatic transport, it is critical to assess the access of synthetic vaccines to different DC subsets.

In this study, we examined the effect of PEGylation on the transport of synthetic vaccines to dLNs. As a model synthetic vaccine, PS particles of 200 nm in diameter were used. We assessed the type of APCs that particles accessed and the level of uptake by different types of APCs. Furthermore, we identified specific DC subsets that internalized PEGylated particles. The effect of PEGylation on lymphatic trafficking and accumulation in dLNs is in agreement with previous studies.^{11,12,14,35} Increasing PEGylation enhanced the transport of particles away from the injection site while only slightly increasing the accumulation in dLNs (Figure 2).

The transport of particles with defined size to dLNs and their accumulation in dLNs are affected by various factors such as the physiology of injection sites and the interaction of particles with extracellular matrix (ECM) and cells at both the injection sites and dLNs. The inflammation due to tissue injury and engagement of the innate immune system can also significantly affect both the transport and the accumulation of particles. In our study, inflammation was negligible. PEGylation of PS particles did not significantly alter the particle diameters (particle size increased from 204 to 244 nm, Figure 1A) and density (Table S1 in the Supporting Information), thus ruling out the effect of particle size and density on lymphatic trafficking and accumulation. Increased PEG density on the particle surface led to increased zeta potential (Figure 1B) as previously reported^{12,13} and hydrophilicity.⁵² Both could lead to different levels of opsonization, or adsorption of proteins, of PEGylated particles.^{34,53} Thus, varying degrees of PEGylation can selectively target particles to particular cell types, such as migratory DCs, via their adsorbed proteins. Previous studies have shown that more hydrophilic particles drain away from the injection site more effectively.^{13,54} It was hypothesized that the increased hydrophilicity and neutralization of surface charge reduced the interaction of particles with the ECM, which is composed of a network of collagen fibers and negatively charged glycosaminoglycans (GAGs).⁵⁵ As a result, PEGylated particles may be more dispersed in the gel-like ECM and provide higher probability for the drainage through lymphatic vessels and phagocytosis by migratory DCs. Both charge and hydrophilicity of particles can influence their interaction with lymphoid resident cells, trafficking inside the dLNs, and the level of particles carried by migratory DCs, all of which might contribute to the retention of particles in dLNs. Collectively, PEGylation plays multiple roles in modulating the lymphatic transport and accumulation of particles in dLNs though the detailed mechanisms are still not so clear yet. Others have suggested that there exists an optimal density of PEG per particle to balance between the effects of enhanced transport through physical barriers and reduced internalization by cells with increasing PEG density.^{12,13} In our case, particles with a PEG

density of 5700 molecules/particle showed slightly greater, but not statistically significant, accumulation in dLNs after 3 and 7 days than other particles (Figure 2). We examined the percentage of particle-positive cells at 4 and 18 h. PEGylation enhanced the percentage of particle-positive cells in dLNs at 4 h, in particular at the highest PEG density ($p < 0.05$), but not at 18 h (Figure S2A in the Supporting Information).

For the PEGylated PS particles used in this study, the majority of particles (~40–90%) still remained at the injection site and the accumulation in LNs slightly increased after day 7 (Figure 2). Other studies have shown similar observations.^{11–13} Particles administered through the hind footpad drained away from the injection site more effectively compared to the lateral torso administration. The difference may be due to the properties of interstitial space, lymphatic vessels, and presence of migratory DCs between injection sites.^{55,56} Inefficient drainage from the injection site over time indicates that the lymphatic trafficking of particles may be limited by other factors, such as the formation of larger aggregates and the hindered diffusion through ECM upon the drainage of solvent away from the injection site. Larger aggregates, as large particles, may be trapped within the ECM upon injection.^{16,17,57} They may also prevent the infiltration of migratory cells into the packed particles.

Regardless of the administration routes and PEG density, the particles retained in dLNs consisted of a small fraction of total particles that drained away from the injection site (Figure 2). It is possible that particles drained to other LNs and spleen or reached systemic circulation.^{12,13} Others' and our results on the lymphatic trafficking and the retention of particles in dLNs leave two questions: First, how can we improve the retention of particles in lymphoid organs in addition to improving the drainage of particles from administration sites? Second, how will the distribution of particles among different lymphoid organs affect the quality and quantity of immune responses?

We also examined how PEGylation affected the level of particles associated with APCs. PEGylation affected both the access of particles to different APCs and the level of internalization of particles by each cell type at the single cell level. We examined these two aspects independently (Figure 4 and 5). B cells were the major cell type that particles gained access to, and PEGylation diminished this preference. Interestingly, the access of particles to DCs was enhanced with PEGylation. For bare PS particles, only 4% of particle-positive cells were DCs. With PEGylation, the percentage increased to approximately 22%. PEGylation did not change the access of particles to macrophages significantly. Our results differ from previous observations where DCs and macrophages were the main cell types that internalized nanoparticles.⁶ This discrepancy may be due to the difference in particle size, route of administration and the method of quantification. Particles used in the previous study were less than 25 nm in diameter while the particles in our study had diameters of 200 nm. Different sizes of particles access different regions of dLNs and thus different cell types.^{8,17}

We further examined the subsets of DCs that particles gained access to. DCs were differentiated into four phenotypes, including Langerhans cells, dermal DCs, DN DCs, and CD8 α DCs, based on the expression of two cell surface markers, CD8 and DEC205.²⁷ The former two DCs are migratory DCs while the latter two types mainly reside in dLNs. PEGylation did not change the ability of particles to access migratory DCs over resident DCs but altered the distribution of particles among resident DCs (Figure 4B). These results indicate that PEGylation may change the transport of particles within dLNs.

DC subsets exhibit distinct ability of processing antigens and priming T cell responses and differential profiles of pattern recognition receptors.^{21,24,25,58} PEGylation can potentially modulate immune responses by varying the access of synthetic vaccines to DC subsets. For

instance, CD8 α DCs are more equipped for cross-presenting antigens and priming CD8 $^{+}$ T cells than other DC subsets.^{21,29} More robust and sustained CD8 $^{+}$ T cell responses require the help from CD4 $^{+}$ T cells, which can be primed by other DC subsets.^{25,29} Different DC subsets have varying expression of pattern recognition receptors, such as toll-like receptors.^{21,58} These receptors serve as a first-line defense against pathogens and provide a crucial link between innate and adaptive immune responses.⁵⁹ Responses to synthetic vaccines that incorporate both antigens and immune-stimulators can potentially be altered by accessing relevant cell types through PEGylation.

We also quantified the uptake by B cells, macrophages, and DC subsets at a single cell level. The cellular uptake by B cells was reduced by PEGylation, which is in agreement with results obtained *ex vivo* or in cell lines that have shown that PEGylation reduces the uptake of particles by phagocytic cells.^{44,60,61} However, PEGylation significantly enhanced cellular uptake of all DC subsets and macrophages (Figure 5). The results were rather surprising and not consistent with studies conducted *ex vivo* or with cell lines. We also examined the uptake of particles using cells isolated from mouse dLNs *ex vivo* (Figure S3 in the Supporting Information). PEGylation reduced the uptake of B cells, DCs, and macrophages, which is consistent with previous studies.^{43,59,60} In *ex vivo* uptake conditions, all cells types were exposed to the same concentration of particles, while under *in vivo* conditions, PEGylation affected the level of particles that cells were exposed to. This may be due to the compartmentalization of particles in the lymph nodes (Figure S4 in the Supporting Information), which could contribute to the differential uptake of particles by different cell types at the single cell level. We would like to note that both tissue sectioning and confocal microscopy analysis at a low magnification prevented us from definitively identifying each compartment of lymph nodes and comparing the compartmentalization between different types of particles. More extensive studies are required to address this issue.

The magnitude and maintenance of immune responses are affected by the level of antigens available for priming T cells.⁶² Considering both the access of particles to different APCs and the level of internalization of particles by each cell type at the single cell level, PEGylation enhanced the level of antigens presented by DCs (Figure 6A). Given the potency of DCs in priming T cells, it is expected that PEGylated particles can generate a higher magnitude of T cell responses. Both CD4 DCs and CD8 α DCs acquired more particles as the PEG density increased, and the effect of PEG density on the overall uptake by CD4 DCs was more significant compared to CD8 DCs. The balance between CD4 $^{+}$ and CD8 $^{+}$ T cell responses can thus change with the PEGylation. Since CD4 $^{+}$ T cells provide help for the generation and maintenance of CD8 $^{+}$ T cells, it is possible that both CD4 $^{+}$ and CD8 $^{+}$ T cell responses can be enhanced and prolonged. PEGylation reduced the level of particles internalized by B cells. It is expected that the humoral responses may be compromised. However, CD4 $^{+}$ T cell responses provide help for the generation of humoral responses.⁶³ It is likely that the humoral responses will be enhanced and prolonged.

Our results suggest that PEGylation of particulate vaccines can potentially offer a simple approach to modulate the strength, duration, and quality of immune responses though further studies are required to enable its translation for clinical applications.

CONCLUSIONS

In this study, the effects of PEGylation on the lymphatic trafficking of 200 nm PS particles and their access to different APCs and DC subsets were evaluated. PEGylation enhanced the transport of particles away from the injection sites at two different administration routes, and increased the access of particles to DCs. PEGylation enhanced the uptake by DC subsets while reducing that of B cells at the single cell level. PEGylation had a more profound effect

on the overall level of particles associated with CD4 DCs than CD8 α DCs. Our results together with previous studies indicate that PEGylation has multifaceted effects on synthetic particulate vaccines, and its effects on the quality and quantity of immune responses require careful evaluations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of PEGylated particles. (A) Diameter and (B) zeta potential of PEGylated polystyrene particles with different PEG densities. PEG: poly(ethylene glycol). PS: fluorescent polystyrene particle. * denotes p value < 0.05 .

Figure 2.

Effect of PEGylation on particle drainage and accumulation in dLNs (4h, 4 h; 1d, 1 day; 3d, 3 days; 7d, 7 days). (A) Percentage of particles that remained at the hind footpad injection site. (B) Percentage of particles that drained to popliteal LNs. (C) Percentage of particles that remained at the lateral torso injection site. (D) Percentage of particles that drained to inguinal, brachial, and axillary LNs. PEG: poly(ethylene glycol). PS: fluorescent polystyrene particle. Data shown is representative of 3 independent experiments. * denotes p value < 0.05 . The amount of particles draining to dLNs after the injection at the lateral torso was too low to be detected.

Figure 3.

Evaluation of the access of PEGylated particles to different cell types. (A) Particle-positive cells were first distinguished as non-DCs ($CD11c^-$) and DCs ($CD11c^+$) based on the expression of CD11c. (B) The non-DC population was further divided into B cells ($B220^+F4/80^-$) and macrophages ($B220^-F4/80^+$) based on the expression of B220 and F4/80, respectively. (C) Distribution of cell types among total particle-positive cells in dLNs for different PEG densities. The dLNs were collected 18 h after subcutaneous administration at the hind footpad. The underlined numbers indicate number of PEG/number of PS. Data shown is representative of 3 independent experiments. The mice injected with PBS are used as negative controls.

Figure 4.

Identification of DC phenotypes in particle-positive DCs and the percentage of four different DCs phenotypes in particle-positive DCs at the varying PEG density. (A) Based on the expression of two cell surface markers, CD8 and DEC205, particle-positive DCs were categorized into four subsets, Langerhans cells ($CD8^-DEC205^{high}$), dermal DC ($CD8^-DEC205^{intermediate}$), double-negative (DN) DC ($CD8^-DEC205^{low}$), and $CD8\alpha$ DC ($CD8^+DEC205^{intermediate}$). (B) The percentage of the four different DCs phenotypes in particle-positive DCs with varied densities of PEGylated particles were evaluated with flow cytometry. The dLNs were collected 18 h after the subcutaneous administration at hind footpad. PEG: poly(ethylene glycol). PS: fluorescent polystyrene particle. Data shown is representative of 3 independent experiments.

Figure 5. Quantification of the uptake of particles by different cell types in dLNs. The dLNs were collected 18 h after the subcutaneous administration at the hind footpad. (A, B) Flow cytometry histogram and geometric mean fluorescence intensity (gMFI) for different cell types that internalized particles. (C) Cellular uptake, quantified as geometric mean fluorescence intensity (gMFI), in different DC phenotypes with different density of PEG. PEG: poly(ethylene glycol). PS: fluorescent polystyrene particle. Data shown is representative of 3 independent experiments.

Figure 6.

Effect of PEGylation on the overall level of particles associated with each cell type. The dLNs were collected 18 h after the subcutaneous administration at the hind footpad. (A) The overall level of particles associated B cells and DCs, quantified by considering both the average cellular uptake of individual cells and the particle-positive cell fraction of each cell type in total cells. For example, [uptake of particles by B cells] = [% of particle-positive B cells in total cells] $\times$ [no. of particles per B cell (gMFI)]. The uptake by macrophages was negligible. (B) The overall level of particles associated with CD8 α DCs and CD4 DCs with varying density of PEG. CD4 DCs includes Langerhans cells, dermal cells, and DN DCs. The uptake of particles by DC subsets was the same as the previous calculation. PEG: poly(ethylene glycol). PS: fluorescent polystyrene particle. Data shown is representative of 3 independent experiments.