Published in final edited form as:

Acta Biomater. 2018 October 01; 79: 283–293. doi:10.1016/j.actbio.2018.09.001.

PEGylation of Model Drug Carriers Enhances Phagocytosis by Primary Human Neutrophils

William J. Kelley,

University of Michigan, Department of Chemical Engineering

Catherine A. Fromen,

University of Michigan, Department of Chemical Engineering

Genesis Lopez-Cazares, and

University of Michigan, Department of Chemical Engineering

Omolola Eniola-Adefeso

University of Michigan, Department of Chemical Engineering

Abstract

Targeted drug carriers are attractive for the delivery of therapeutics directly to the site of a disease, reducing systemic side effects and enhancing the efficacy of therapeutic molecules. However, the use of particulate carriers for drug delivery comes with its own set of challenges and barriers. Among these, a great deal of research effort has focused on protecting carriers from clearance by phagocytes via altering carrier surface chemistry, mostly with the use of polyethylene glycol (PEG) chain coatings. However, few papers have explored the effects of PEGylation on uptake by freshly obtained primary human phagocytes in physiological conditions. In this work, we investigate the effect of PEGylation on particle uptake by primary human neutrophils *in vitro* and compare these effects to several cell lines and other model phagocytic cells systems. We find that human neutrophils in whole blood preferentially phagocytose PEGylated particles (e.g., ~40% particle positive neutrophils for PEGylated versus ~20% for carboxylated polystyrene microspheres) and that this effect is linked to factors present in human plasma. Model phagocytes internalized PEGylated particles less efficiently or equivalently to carboxylated particles in culture medium but preferentially phagocytosed PEGylated particles in the human plasma (e.g., ~86% versus ~63% PEGylated versus carboxylated particle positive cells, respectively). These findings have significant implications for the efficacy of PEGylation in designing long-circulating drug carriers, as well as the need for thorough characterization of drug carrier platforms in a wide array of *in vitro* and *in vivo* assays.

lolaa@umich.edu.

Supporting Information

Supporting information is available and includes PEG density calculations, phagocytosis of PEGylated silica and PEG-based hydrogel particles, flow cytometry gating, PEGylated particle uptake by human monocytes and mouse blood cells, and experiments probing the potential role of PEG antibodies and immunoglobulins as well as various uptake mechanisms in the enhanced uptake effect reported here.

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Keywords

Nanoparticles; Microparticles; PEGylation; opsonization; neutrophils; phagocytosis; particle drug carriers

1. Introduction

Particulate drug delivery systems remain a relevant platform to improve the delivery of a variety of therapeutics for various diseases. The advantages of particulate drug carriers are numerous,[1] yet only a handful of such formulations have gained FDA approval.[2] Much of the perceived barriers to success include the inability to achieve targeted, localized delivery due to rapid clearance by professional phagocytes. Specifically, serum proteins rapidly adsorb onto the surface of particulate drug carriers as they enter the bloodstream in a process referred to as opsonization. These proteins can include any serum proteins but are most commonly complement factors and immunoglobulins.[3] This opsonin adsorption process quickly marks the particles as foreign, where receptors on phagocytes (monocytes, macrophages, and neutrophils) bind to the opsonins to initiate the process of phagocytosis or cellular eating.[4] Once internalized by cells, the particles are removed from the bloodstream, preventing them from achieving the goal of targeted drug delivery.[3] As a result, numerous studies have examined the effects of particle size,[5,6] shape,[7–9] and surface chemistry[10–12] on the phagocytic uptake of particulate carriers in an attempt to limit clearance via phagocytosis.

A typical approach to mitigating opsonization, and thus phagocytosis, involves the modification of particle surfaces with non-fouling, biocompatible polymers, which reduce opsin protein adsorption. Hydrophilic, high molecular weight polyethylene glycol (PEG) has been widely studied in this regard. Indeed, coupling PEG to the surface of a variety of particle drug carriers at high density has been shown to reduce macrophage uptake.[10,13] Due to this effect, many studies have demonstrated the long-term circulation of PEGylated particles in mice.[14,15] However, to date, no PEGylated polymeric formulations have been approved for clinical use, though there is a handful currently in clinical trials.[16] While some PEGylated particle formulations have gained FDA approval by demonstrating increased circulation half-life,[17] these formulations were liposomes rather than polymeric particles. The lack of a widespread clinical translation of PEGylated polymeric carriers could be due to the bulk of the evidence regarding the success of these having primarily been gathered *via* immortalized cell lines with non-physiological media conditions and rodent *in vivo* models.[15–18] Only a few preclinical studies have explored particle uptake of PEGylated particles in freshly-obtained blood with primary human blood cells,[18,19] which is crucial to understanding the impact of PEGylation on particle phagocytosis *in vivo* in humans. For one, the exact cellular composition and serum proteins present can dramatically change the effectiveness of PEG in preventing phagocytosis, which the human blood environment is not recapitulated with mouse models and immortalized cell line assays. Of the limited studies using primary human blood cells, PEG modification has delivered mixed results. One study suggests that the reduction in uptake due to PEGylation for primary blood cells is heavily dependent on PEG grafting density, with the requirement

that the Flory radius (R_F) is at least twice as large as the distance between PEG grafts[18]. Other studies have reported no reduction in uptake from PEGylation compared to other surface coatings such as pluronic and chitosan in primary human monocytes in culture medium (RPMI) with fetal bovine serum (FBS).[19] More importantly, the bulk of the prior studies investigating primary cell phagocytosis focus solely on phagocytosis by macrophages and ignore phagocytic contributions from neutrophils. While macrophages are critical professional phagocytes, neutrophils account for approximately 50-70% of cells in the human bloodstream[20]; and therefore are the first cells to be encountered by intravenously administered particulate drug carriers. Indeed, our recent work has demonstrated that these cells are significant contributors to the particle clearance process *in vivo*[21], which follows logically given their known role in pathogen removal. Furthermore, recent studies suggest that neutrophils may be activated and undergo degranulation in the presence of PEGylated surfaces.[22–24] Thus, it is crucial to investigate the impact of PEGylation on drug carrier phagocytosis by primary human neutrophils, as this remains a fundamental question in the literature.

In this study, we evaluate the impact of PEGylation on phagocytosis by primary human neutrophils across a range of PEG grafting densities and particle sizes, 2 μm , 500 nm, and 200 nm, and compare obtained results to the uptake by model phagocytes. The 2 μm particles are included as micron-scale particles have been shown to exhibit better margination and vascular wall adhesion *in vitro* and *in vivo* [25,26], and a number of published drug carrier platforms feature particles sized between 100 and 500 nm (see[27–30]). We report a striking difference between the effect of PEGylation on phagocytosis by primary human neutrophils versus cultured phagocytic cells. In particular, we observe that the addition of PEG increases particle uptake specifically by primary neutrophils in whole blood and human plasma contrary to the observation with THP1 monocytes, mouse blood cells, and mouse bone marrow-derived macrophages. Furthermore, we note that the effect of PEGylation depends heavily on the cellular species of origin, as mouse neutrophils behave different from human neutrophils, and that factors in human plasma are at least partially responsible for this difference. These results have implications for researchers interested in designing PEGylated nanoparticles for drug delivery, highlighting the need for thorough characterization of particle coatings on uptake by phagocytic cells.

2. Materials and Methods

2.1 Study Approvals:

Human blood was obtained from healthy donors via venipuncture per a protocol approved by the University of Michigan Internal Review Board. Informed, written consent was obtained from donors prior to blood collection.

Mouse blood was obtained via cardiac puncture per a protocol approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC) and in accordance with the National Institute of Health guidelines for the care and use of laboratory animals. Mice were obtained from Jackson Laboratories, and were either male BALB/c or male C57BL/6.

2.2 Particle Functionalization:

Fluorescent carboxylated polystyrene (2 μm , 500 nm, and 200 nm, Polysciences, Inc.), PLGA (~2 μm , fabricated in-house), and silica (500 nm, Microspheres-Nanospheres, Inc.) were conjugated with aminated, methoxy-terminated polyethylene glycol (PEG, 5k molecular weight, Fisher) using *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC, Sigma) chemistry. Particles (1% w/v) were suspended in 50mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer and incubated with PEG at varying concentrations for 15 minutes on a rotator. After 15 minutes, EDC (20mg) was added, and the particles were rotated for 20 hours. Glycine (4mg, Sigma) was added to quench the reaction. After conjugation, particles were washed 3x with PBS $-/-$ (pH 7.4), and the particle concentration was determined using a hemocytometer on a fluorescent microscope.

2.3 PEG Quantification:

Rhodamine-labeled, amine-terminated PEG (Nanocs) was conjugated to particles as described above. A rhodamine standard curve was used to determine the amount of PEG present on particles by taking fluorescent readings on BioTek Synergy H1 microplate reader. Then, the PEG density was calculated using the method described by Perry *et al.*[31] (Supplementary Table 1). For PEG chains with variable end groups, it was assumed that swapping out small functional groups on the end of the PEG chain will not alter the conjugation chemistry, and thus the same chemistry was used to conjugate PEGs of variable end groups.

2.4 Bone Marrow-Derived Macrophage Isolation:

BALB/c and C57BL/6 mice were euthanized via CO_2 and their hind legs were removed with surgical scissors. The bones were flushed with FACS buffer (PBS $-/-$ with 2% FBS); fluid containing bone marrow progenitor cells was collected as described previously.[32] Cells were centrifuged at 500 $\times$ G for 5 minutes and suspended in RBC lysis buffer (eBiosciences) for 1 minute. Cells were diluted in FACS buffer again, centrifuged at 500 $\times$ G for 5 minutes, and suspended in DMEM medium + L929 conditioned media to promote differentiation into macrophages, and plated on T-75 cell culture flasks for 6 days prior to use.

2.5 Particle Uptake Studies:

Various particle types (described above) were incubated with heparinized blood, primary neutrophils in plasma, primary neutrophils in RPMI, THP1 monocytes, bone marrow-derived macrophages, mouse blood, and HL-60 derived neutrophils at varying concentrations (1E7/mL for 2 μm particles, scaled concentrations to match total particle mass for smaller particles) and times at 37°C and 5% CO_2 . For whole blood studies, samples were removed from the incubator and stained (CD45, CD11b, CD14, BioLegend) for 30 minutes on ice. The 1-step Lyse/Fix solution (eBiosciences) was then applied to lyse the RBCs and fix the WBCs. Fixed samples were washed with FACS buffer (PBS $-/-$ + 2% FBS, pH 7.4) before flow cytometry analysis. For primary neutrophil/monocyte incubation studies, the incubation steps were performed as described above, after which cells were stained for 30 minutes on ice and then fixed using 2% paraformaldehyde (Sigma) solution. For cultured cell studies, cells were seeded in 96-well plates at a concentration of 10⁶ cells/

well in medium and incubated with particles at 37°C and 5% CO₂. THP1 cells were removed from the wells and transferred to FACs tubes, centrifuged and resuspended in 2% paraformaldehyde solution for fixation. Bone marrow-derived macrophages were trypsinized for 5 minutes and then removed from the wells, centrifuged and resuspended in 2% paraformaldehyde solution. Cells were washed 3x with FACS buffer before analysis via flow cytometry. Fluorescent microscopy images of particle uptake (Supplemental Figure 1) were obtained with isolated neutrophils in plasma, where cells were exposed to particles at the same concentrations as all other uptake studies and imaged live.

2.6 Flow Cytometry:

All flow cytometry was performed on an Attune Acoustic Focusing Cytometer and results analyzed using FlowJo software (Tree Star). Human whole blood samples were stained with CD45 and CD11b (BioLegend) on ice before addition of Lyse/Fix solution. Cells identified as CD45+/CD11b+ were considered to be granulocyte neutrophils and monocytes, and the two populations were sorted by FSC/SSC (Supplemental Figure 2). Mouse whole blood samples were stained with CD45, CD11b, and Ly6G (BioLegend) on ice before addition of Lyse/Fix solution. Cells identified as CD45+/CD11b+/Ly6G+ were considered to be neutrophils, while CD45+/CD11b+/Ly6G- cells were considered to be monocytes. Single-cell populations (Bone Marrow-Derived Macrophages, THP1 monocytes, and HL-60 derived neutrophils) were not stained before analysis via cytometry. Gating was applied to remove doublets. For all samples, particle-positive cells were identified by increased FITC MFI from untreated samples.

2.7 Differentiation of HL-60 Derived Neutrophils:

HL-60 cells were differentiated into neutrophils by culturing the cells in RPMI with 10% FBS and 1.3% DMSO, as previously reported.[33] Cellular differentiation was monitored by shifting CD11b expression over time, as measured by flow cytometry. Neutrophil-like cells were used after the population exhibited a uniform CD11b expression peak, generally 4-5 days after culture in DMSO.

2.8 SDS-PAGE Gels:

Particles were incubated in human plasma (1.83E7/mL) or buffer for 1 hour at 37°C, as done previously.[34] Then, particles were washed 3x in PBS -/- and resuspended in 50uL SDS stain (Pierce SDS-PAGE Sample Prep Kit). Particles were placed in a Bio-Rad T100 Thermal Cycler and boiled to strip proteins from the particle surface. Particles were then centrifuged and the supernatant solution was run on a Novex WedgeWell 4-20% Tris-Glycine Gel alongside a protein ladder. Gels were then stained using EasyBlue Safe Stain, and destained by shaking overnight in deionized water. Images were taken using a Bio-Rad EZ Gel Imager. Specific bands were cut out, digested, and analyzed via LCMS at the University of Michigan proteomics core.

2.9 Size and Zeta Potential Measurements:

2 µm carboxylated and PEGylated particles were incubated in plasma or deionized water at a concentration of 1E8/mL for 1 hour at room temperature; plasma-incubated particles were

centrifuge and resuspended in deionized water and particle size was measured using a Malvern ZEN3600 zetasizer. Zeta potential was measured across a range of concentrations (5E7-2E8/mL) and an average taken to determine zeta potential for both 2 μ m carboxylated and PEGylated particles.

2.10 Statistical Methods:

Data from uptake studies is representative of at least two independent experiments, with two or three technical replicates each. Human whole blood trials (where aggregated) represent n = 3 independent donors, performed in duplicate. For all studies, all data points were included in the analysis. Data are plotted with standard error. Significance was determined by ANOVA with Tukey's post-test performed in GraphPad Prism. Asterisks indicate p values of: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. n.s. indicates "not significant".

3. Results

3.1 Uptake of PEGylated Particles by Primary Human Cells

To determine how PEGylation affects the uptake of particulate drug carriers by primary human blood cells, we incubated model polystyrene drug carriers with whole blood samples taken from healthy donors in heparinized whole blood. In these studies, we compared the uptake of 2 μ m carboxylated polystyrene particles with PEGylated particles of the same size. Particles were determined to have PEG grafted in the "dense brush" conformation (Supplementary Table 1).[18] Additionally, these studies we carried out across 16 donors, utilizing blood from both male and female donors (10 male, 6 female). Individual donor experiments for neutrophils are shown in Figure 1A. Except for Donor 1, there was a significant increase in the uptake of PEGylated particle by neutrophils over the carboxylated particles for all blood samples, which is demonstrated visually in Supplemental Figure 1. Additionally, Fig 1B illustrates the impact of PEG on particle uptake by neutrophils over time, showing that PEGylated and carboxylated particles were initially taken up at a similar rate, but PEGylated particle uptake was eventually higher at longer timescales. No significant differences were observed between male and female donors.

We observe that a brush PEG formation on the particle surface increases uptake by human neutrophils across a range of particle properties. Figure 1C demonstrates that this effect held true across a variety of particle sizes, and in fact, the impact of a brush PEG formation on particle uptake was more extensive for smaller particles. An average of ~37% of neutrophils internalized 2 μ m PEGylated particles versus ~17% for carboxylated particles (aggregated results for all donors in Figure 1A). Meanwhile, ~98% of human neutrophils internalized PEGylated 500 nm particles versus ~38% for carboxylated 500 nm particles, and ~90% of human neutrophils internalized PEGylated 200 nm particles versus ~40% for carboxylated. The increased uptake of PEGylated particles was maintained across a range of particle concentrations from 1E6/mL to 1E8/mL (Figure 1D), as well as across multiple other particle materials, including PLGA and PEG-based hydrogel particles commonly explored for various drug delivery applications (Supplemental Figure 3). Notably, this effect was not observed in primary human monocytes, which demonstrated no difference in uptake between PEGylated and carboxylated polystyrene particles (Supplemental Figure 4).

Because PEG surface density is known to impact particle *in vivo* circulation time,[31,35] we sought to explore whether neutrophils' preferential phagocytosis of PEGylated particles remained consistent across an array of PEG grafting densities. Thus, we reacted 2 μ m, 500 nm, and 200 nm polystyrene particles with a range of PEG concentrations, resulting in a range of PEG densities (characterized by R_F/D ; the ratio of the Flory radius of the PEG polymer to the distance between PEG chains – a higher R_F/D value indicates a denser PEG grafting). These particles were then incubated in whole blood at concentrations matching those in above studies.

For 2 μ m particles, all PEG densities from “dense brush” to “loose brush” were associated with a significant increase in particle uptake (Figure 2A). For 500 and 200 nm particles, all PEG densities except one were associated with a significant increase in particle uptake (Figure 2B-C). For the R_F/D of 2.15 for 500 nm particles, we observe a non-significant increase in PEGylated particle uptake relative to the carboxylated particles. For the R_F/D of 0.58 for the 200 nm particles, we see the same level of particle uptake between carboxylated and PEGylated particles. These results suggest that, across a range of particle sizes and PEG grafting densities, human neutrophils largely preferentially uptake PEGylated particles compared to carboxylated counterparts. Interestingly, for the 200 nm particles at the lowest PEG density, it appears that the uptake behavior approaches that of carboxylated particles, which may imply that there is a threshold amount of PEG, above which neutrophils will preferentially phagocytose pegylated particles. The exception to this rule seems to be with 500 nm particles at the highest PEG density ($R_F/D = 2.15$). However, it is difficult to extrapolate based on this data point whether the nonsignificant increase in uptake for that particle type implies a change in behavior at extremely high PEG densities, although this was the highest PEGylation density we were able to achieve with our coupling chemistry for the 500 nm particles. Notably, 2 μ m particles with the $R_F/D > 2.15$ still exhibit increased uptake by primary neutrophils.

Because surface chemistry can dictate protein adsorption profiles and particle interactions with cellular membranes, it is possible that the PEG end group could play a role in modulating neutrophil phagocytosis. Thus, we examined the neutrophil uptake of PEGylated particles with methoxy, hydroxyl, and carboxyl end groups (Figure 2D). In agreement with the above experiments, neutrophils preferentially took up PEGylated particles, regardless of which end group was present. Additionally, we sought to explore the impact of PEG chain length on this phenomenon, as other groups have shown that PEG chain length can impact the performance of PEGylated particles *in vivo*. [36] As shown in Figure 2E, altering the PEG chain length between 2000 kDa and 10000 kDa, at R_F/D values approximately equal to those used for the 5000 kDa, had no impact on the increased uptake observed for the 2 μ m particles with human neutrophils, i.e., all chain lengths resulted in an increased uptake over carboxylated surfaces. Similar results were obtained for the 500 nm and 200 nm particles with the 2000 kDa and 10000 kDa PEG relative to the 5000 kDa PEG (Supplemental Figure 5).

3.2 Uptake of PEGylated Particles by Other Phagocytic Cells

The results in Figures 1 and 2 were initially quite surprising, as we had hypothesized that PEGylation would reduce particle uptake. Therefore, we next examined the uptake of PEGylated particles in a variety of other cell types frequently used for characterizing particle uptake, with the hypothesis that these studies should mostly match those presented in prior publications (that is, PEGylation would reduce particle uptake). For these studies, we used the same particle sizes and concentrations as used above for neutrophils. We incubated carboxylated and PEGylated particles with mouse whole blood and with THP1 monocytes and mouse bone marrow-derived macrophages (BMM; from both BALB/c and C57BL/6 mice) in RPMI with 10% FBS. For all the experimental conditions, PEGylation either reduced uptake by model phagocytes or showed no significant difference from carboxylated particles, except for C57BL/6 mouse neutrophils taking up PEGylated 200 nm particles more than their carboxylated counterparts (Supplemental Figure 6). Overall, these experiments matched prior reporting that PEGylation reduces particle uptake.

Given that one reason neutrophils are less widely-studied for phagocytosis is that there are few model neutrophil cell lines with which to work, we wanted to explore the uptake of PEGylated particles by one of the models that exist: HL-60 derived neutrophils. The HL-60 is a human promyelocytic leukemia cell line that is capable of differentiation to a neutrophil-like phenotype in the presence of DMSO or retinoic acid.[37] We hypothesized that HL-60 derived neutrophils in the presence of human plasma should behave similarly to primary human neutrophils with respect to PEGylated particle uptake.

The HL-60 derived neutrophils were incubated with particles as above for primary neutrophils in either culture medium (RPMI + 10% FBS) or human plasma (Figure 3). Interestingly, HL-60 derived neutrophils seemed to preferentially take up carboxylated particles in the culture medium, in line with similar phagocytic studies of other secondary cells. However, in the presence of human plasma, HL-60 derived neutrophils behaved similarly to human neutrophils, with a significant increase in particle-positive cells for 2 μ m and 200nm PEGylated particles versus their carboxylated counterparts. For 500 nm particles, both particle types resulted in approximately 100% particle uptake, suggesting that the combination of particle size and concentration dominated any effect PEG might have had on the uptake. These results, combined with the human whole blood uptake studies in Figures 1 and 2 strongly suggest that factors in human plasma drive an increased uptake of PEGylated particles by human neutrophils.

3.3 Impact of Human Plasma on PEGylated Particle Uptake

To further explore a potential human plasma effect as observed with the HL-60 cells, we incubated carboxylated and PEGylated particles with human plasma before incubation with BMMs from both BALB/c and C57BL/6 mice. The results of these studies are shown in Figure 4A and Figure 4B, respectively. For both mouse strains, the incubation of particles with human plasma prior to addition to cells increased PEGylated particle uptake. Specifically, in RPMI with 10% FBS, PEGylated particles were phagocytosed by approximately 20-40% fewer macrophages, while PEGylated particles were phagocytosed at the same level as carboxylated particles with the addition of human plasma. Additionally, we

cultured THP1 monocytes in human plasma and evaluated the uptake of particles soaked in human plasma (Figure 4C). Similar to the BMMs, we saw an increase in uptake of PEGylated particles by THP1 monocytes, eliminating any significant differences between carboxylated and PEGylated particles. These cell-line results suggest that factors in human plasma help to drive PEG particle uptake, resulting in a mismatch between many cultured cell uptake assays and what can potentially occur in human *in vivo*.

Given the appearance of a significant role played by human plasma in the increased uptake of PEGylated particles, we investigated potential differences in plasma proteins acquired by carboxylated and PEGylated particles. First, we measured particle zeta potential of the 2 μ m carboxylated and PEGylated particles, which yielded a value of ~ -41.4 and ~ -17.4 mV, respectively (Supplemental Figure 7), confirming the positive impact of PEGylation on the polystyrene particle surface charge. Accordingly, an SDS-PAGE analysis demonstrated that PEG was successful in reducing the total protein on particles surface, repelling many of the low molecular weight plasma proteins (Figure 5). However, PEGylated particles demonstrated a clear band in the ~ 50 kDa and the ~ 250 kDa range that was not present for carboxylated particles. The presence of these bands indicates that there may be specific proteins in plasma that preferentially bind to PEG particles and confer the preferential uptake of these particles. The higher molecular weight band corresponds to immunoglobulin (Ig) proteins; However, neutrophil uptake assays performed in Ig-depleted plasma still demonstrated increased uptake of PEGylated particles (Supplemental Figure 8), suggesting that there may be other specific plasma proteins responsible for the PEGylation induced uptake effect. Additionally, inhibitor studies (using chlorpromazine to inhibit clathrin-mediated phagocytosis, sodium azide and 2-deoxyglucose to inhibit ATP-driven phagocytosis, and lovastatin to inhibit FC receptor-mediated phagocytosis) performed to probe the driving mechanism of PEGylated particle uptake by human neutrophils were inconclusive (Supplemental Figure 8), but did point toward an ATP-driven process at play. Further, to explore whether PEG antibodies could be responsible for the increased PEGylated particle uptake, we spiked whole blood samples with free PEG before introducing particles (Supplemental Figure 8). However, we did not observe any noticeable antibody-blocking effect, suggesting that PEG antibodies not be a primary driver of the observed increase in particle uptake. Overall, we did not find a notable change in the size of the particles, bare or PEGylated, with plasma incubation (Supplemental Figure 9), suggesting that a change in particle size with protein adsorption is not responsible for the positive uptake of PEGylated particles relative to the bare polystyrene particles.

3.4 Probing the Role of Complement and Serum Albumin in PEGylated Particle Uptake

Another possibility suggested by the SDS-PAGE analysis is that complement proteins and/or serum albumin might be responsible for the difference in uptake between carboxylated and PEGylated particles. Complement proteins were present in the bands that appeared in the corona for PEGylated particles but not carboxylated particles, and their role in particle phagocytosis has been well-documented.[38–40] Conversely, human albumin was heavily present on the carboxylated particles, relative to PEGylated particles, and could be driving decreased particle uptake. Thus, we examined the uptake of carboxylated and PEGylated 2 μ m polystyrene particles by isolated human neutrophils in RPMI with 10% FBS, RPMI with

complete human complement sera (9.67 CH50 units/mL), RPMI with human complement C3 (119 $\mu\text{g/mL}$), and RPMI with human serum albumin (HSA, 35 g/L). We also examined particles that were incubated for 1 hour in serum albumin before introduction to the neutrophils. Complement C3 was chosen for its known role in phagocytosis of foreign bodies by neutrophils and its presence in the SDS-PAGE analysis.[41]

As shown in Figure 6a, when carboxylated particles were exposed to human complement only, they were phagocytosed much more readily by neutrophils than in plasma (~93% for complement-exposed particles, versus ~35% for particles in plasma). Additionally, carboxylated particles exhibited an increase in uptake when exposed to C3 alone (~49%). Notably, carboxylated particles exposed to HSA only exhibited the same level of uptake as carboxylated particles in whole plasma and decreased uptake (~26%) when presoaked in HSA for 1 hr before adding to cells.

Similar to their carboxylated counterparts, when PEGylated particles were only exposed to HSA, i.e., no other protein in their corona, they exhibit drastically decreased uptake compared to the level seen in plasma (Figure 6b; ~3% in RPMI + HSA versus ~75% in plasma). Conversely, when PEGylated particles were exposed to complete human complement, they exhibit a slightly increased uptake (~88%) relative to whole plasma. However, PEGylated particles exhibit decreased uptake in C3 alone (~4%). Taken together, these results, along with the SDS-PAGE gel result, suggest that there is indeed an important role played by human complement in particle uptake by human neutrophils, and, more precisely, the increased affinity for complement by PEGylated particles is responsible for their increased uptake by human neutrophils relative to non-PEGylated particles, which may be mitigated by albumin.

4. Discussion

PEGylation is often employed as a strategy to reduce phagocytosis and clearance of particle drug carriers by circulating blood cells (i.e., neutrophils and monocytes), extending the blood circulation time of such particles. This strategy has been explored by numerous research groups in a variety of *in vitro* and *in vivo* assays,[12,13,15,18,31,36] demonstrating the potential of PEGylation to extend the lifetime of particles in the bloodstream. However, most of these studies are performed in artificial systems in the absence of human plasma, in model phagocytes such as cultured monocytes and macrophages, which are tissue-resident, rather than circulating, or in rodent models *in vivo*. Additionally, the phagocytic contributions of neutrophils are frequently overlooked due to the lack of model neutrophils systems and their difficulty in handling; yet, neutrophils typically make up 60% or more of leukocytes in circulating human blood [42], making them the first WBCs to be encountered by IV-delivered drug carriers.

In this work, we thoroughly characterize the uptake by human neutrophils of model particle drug carriers with and without PEG coatings. Surprisingly, we find that PEG dramatically increases the uptake of particles by primary human neutrophils, regardless of particle size, particle material, PEG density, or end group (Figures 1-2). It is unlikely that this result is an effect of surface charge variations given the reported zeta potential values for the 2 μm

carboxylated and PEGylated particles of ~ -41.4 and ~ -17.4 mV, respectively (Supplemental Figure 7). Notably, we do not observe a similar phenomenon in primary human monocytes (Supplemental Figure 4) that are also present in circulating blood. It has been previously established that a high absolute value of the zeta potential (either positive or negative) is a driver of phagocytosis, which would suggest that the carboxylated particles should be taken up more efficiently.[43,44] Additionally, size measurements before and after particle incubation in plasma revealed no substantial difference between carboxylated and PEGylated particles (Supplemental Figure 10). Instead, we find that human plasma plays a role in the observed phenomenon, resulting in higher uptake of PEGylated particles when introduced to model phagocytes, including HL-60 derived neutrophils, and BMMs from different mouse strain backgrounds (Figures 3-4).

The results presented in this work were initially a surprise. Upon closer inspection of the literature, we found that, although PEG has been widely studied as a protective coating against protein adsorption and immune cell uptake, very few studies have explored the uptake of PEGylated particles in human neutrophils. We found one previous report demonstrating that very high-density PEG ($R_g/D > 2.8$) grafting results in reduced particle uptake in human neutrophils[18], but these cells were purchased as already-prepared buffy coats and incubated in culture medium with human serum added rather than in whole blood or the presence of complete human plasma. Additionally, the concept of anti-PEG antibodies has recently introduced another complication into the field of particle drug delivery. The application of PEG in a wide variety of personal care products (e.g., shampoos and cosmetics) can result in the development of anti-PEG antibodies which recognize and bind to PEG chains. These antibodies have been detected in humans by ELISA[45], and further research suggests that anti-PEG antibodies are not specific to the PEG head group, but to the PEG chain itself.[46] While our study spiking PEG into the media to saturate any anti-PEG antibodies does not support the contribution of anti-PEG antibodies to the effect reported here, taken together with the increased uptake of PEGylated particles by human neutrophils, these concerns surrounding PEGylated particles have dramatic implications for their effectiveness in humans *in vivo*.

To ensure the surprising result obtained in our assays with primary neutrophils are not an artifact of our protocol, we characterized the uptake of our PEGylated particles in a variety of phagocytic cells typically used in assays to assess uptake. In these conventional *in vitro* tools, we find that PEGylation does, in fact, reduce uptake. Even using the most-accurate model cell for human neutrophils, HL-60 cells, we do not observe uptake resembling the primary cell type unless these are cultured using human plasma. Thus, one conclusion from the presented results is that the conventional *in vitro* cell tools are incapable of fully capturing the behavior of primary human neutrophils. Often, groups choose a convenient phagocytic cell-line to model the uptake of their engineered particle systems *in vitro*, with the underlying assumption that the behavior of those cell lines will approximate the behavior of human phagocytes *in vivo*. Given time and financial constraints, this is understandable, but the results here highlight a need for comprehensive characterization of particle uptake properties in a variety of cell types and, more importantly, in physiological media such as complete plasma. Indeed, it has been reported that differences in plasma protein corona formation between species have an impact on the efficacy of targeted drug carriers,[47] as do

alternative complement pathway activation and neutrophil phagocytosis of bacteria and apoptotic T-cells[54–56], but its role in the phagocytosis of drug carriers remains relatively unexplored. Gelsolin and α -2 macroglobulin have also been explored in their capacity to impact neutrophil function and phagocytosis[57,58], but also have gone unexplored in the context of drug carrier phagocytosis. A thorough study of the impact of these proteins on human neutrophil phagocytosis of various particulate drug carriers could yield enlightening information as to how to best design a drug carrier to avoid phagocytic clearance.

One limitation of this study is the lack of demonstration of the preferential uptake of PEGylated particles by neutrophils *in vivo*. While *in vivo* studies are indeed important, in this work we notice a distinct difference between the behavior of mouse cells and human cells. As shown in Supplemental Figure 6, in general, there is a decrease in the uptake of PEGylated particles by mouse blood cells, which is in line with historically published works, yet we find the opposite occurs specifically for human neutrophils. Furthermore, we provide evidence that the increased phagocytosis of PEGylated particles is due to factors in human plasma. Therefore, an *in vivo* animal study, which would typically be conducted in mice, would likely not give any more information on that front. One potential way to further probe the positive impact of PEGylation on human neutrophil phagocytosis would be to explore a spatial coupling strategy, i.e., use of Janus particles in which PEG is coated only on one hemisphere, and employ live visualization to determine whether neutrophils preferentially orient the particles on the PEGylated side during the phagocytic process. Achieving this type of spatial surface coupling would require specialized particle fabrication techniques, which is outside the scope of our experimental capabilities but could be explored in future collaborative work to gain a more thorough understanding of neutrophils' apparent preference for PEGylated particles.

Importantly, these results may have implications for the effectiveness of PEGylation as a strategy for creating long-circulating particulate drug carriers in humans. Recent work has shown that circulating neutrophils phagocytose drug carriers within the bloodstream and play a significant role in the clearance of particle drug carriers.[21] While PEG has been widely used because it imparts long circulation times in mouse studies, it has not proven to be the “magic bullet” it was once expected to be in the particulate drug delivery field, and this effect likely plays some role in that result. Other non-fouling coatings, like zwitterionic coatings, may prove to be more effective in this regard. Indeed, zwitterionic coatings have shown some promise in significantly reducing protein adsorption on nanoparticle surfaces, but most uptake studies using such particles have neglected to characterize their uptake in primary human blood cells in the presence of complete plasma.[59] More thorough characterization of their uptake properties is required to determine whether zwitterionic coatings can have the impact PEG was once expected to have, which we plan to explore in future studies. Overall, it is imperative to investigate the factors driving neutrophil phagocytosis of drug carriers more fully and to develop coatings or other strategies to combat the removal of drug carriers by circulating neutrophils.

5. Conclusions

Despite decades of research into the impacts of non-fouling PEG coatings on particle uptake by model phagocytes, there is a dearth of research into the impacts of these coatings on primary human phagocytes. In particular, neutrophils are understudied due to their inconvenience and a lack of immortalized cell lines to study them and their sensitivity *ex vivo*. In this work, we thoroughly characterize human neutrophil uptake of model particle drug carriers with and without PEG coatings, and the results are quite surprising. We find that PEG significantly increases the uptake of particles by primary human neutrophils, regardless of particle size, PEG density, or end group. We found that human plasma, particularly human complement proteins, plays a role in this phenomenon, resulting in higher uptake of PEGylated particles when introduced to model phagocytes. Finally, we see that HL-60 derived neutrophils behave similarly to primary human neutrophils in the presence of human plasma. These results suggest a need for the characterization of phagocytosis of drug carrier platforms in primary cells and human plasma to become standard practice. In the future, researchers should take care to characterize particle uptake in a wide variety of cell types and media conditions to develop a more robust understanding of the impact of particle properties on leukocyte behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the National Science Foundation Graduate Research Fellowship Program (W.J.K.), the University of Michigan's Postdoctoral Fellowship (C.A.F), and NIH R01 HL115138 (O.E.A.).

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Statement of Significance:

The work in this manuscript is highly significant to the field of drug delivery, as it explores in-depth the effects of polyethylene glycol (PEG) coatings, which are frequently used to prevent phagocytic clearance of particulate drug carriers, on the phagocytosis of such carriers by neutrophils, the most abundant leukocyte in blood circulation.

Surprisingly, we find that PEGylation enhances uptake by primary human neutrophils, specifically in the presence of human plasma. This result suggests that PEGylation may not confer the benefits in humans once thought, and may help to explain why PEG has not become the “magic bullet” it was once thought to be in the field of particulate drug delivery.

Figure 1—.

Uptake of carboxylated and PEGylated 2 μm polystyrene particles by primary human neutrophils, as determined by flow cytometry. (a) Uptake by neutrophils from individual human donors. (b) Neutrophil uptake of 2 μm carboxylated and PEGylated polystyrene particles. (c) Aggregated neutrophil particle uptake for 2 μm, 500 nm, and 200 nm carboxylated and PEGylated polystyrene particles. (d) Neutrophil uptake of 2 μm carboxylated and PEGylated polystyrene particles at varying concentrations.

Figure 2—

Characterization of the Impact of PEG Density and Chain Length on PEGylated Particle Uptake in Whole Blood. Uptake of (a) 2 μ m, (b) 500 nm, and (c) 200 nm carboxylated and PEGylated polystyrene particles of varying PEG densities. (d) Uptake of 2 μ m PEGylated polystyrene particles with varying PEG chain endgroups. (e) Uptake of 2 μ m PEGylated polystyrene particles with varying PEG chain lengths.

HL-60 Particle Uptake - Effect of Human Plasma

Figure 3—.

Uptake of 2 μm, 500 nm, and 200 nm carboxylated and PEGylated polystyrene particles by differentiated HL-60 derived neutrophils in the presence of human plasma.

Figure 4—.

Uptake of carboxylated and PEGylated polystyrene particles by various cell types in presence of human plasma. Uptake by (a) bone marrow-derived macrophages from BALB/c mice of particles soaked in human plasma; (b) bone marrow-derived macrophages from C57BL/6 mice of particles soaked in human plasma; and (c) THP1 monocytes cultured in human plasma; particles soaked in human plasma.

Figure 5—.
SDS PAGE analysis of proteins adsorbed to carboxylated vs PEGylated 2 μ m polystyrene particles and proteins of interest found in bands exclusive to each particle.

Figure 6—.

Uptake of (a) carboxylated and (b) PEGylated polystyrene particles by primary human neutrophils in plasma, RPMI + 10% FBS, RPMI + human complement sera, RPMI + human complement C3, RPMI + human serum albumin, and RPMI + human serum album with particles soaked in human serum albumin.