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Engineering PEGylated polyester nanoparticles to reduce complement-mediated infusion reaction

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Abstract

Translation of intravenously administered nanomaterials to the clinic is limited due to adverse infusion reactions. While these reactions are infrequent, with up to 10% prone to experiencing infusion reactions, the reactions can be severe and life-threatening. One of the innate immune pathways, the complement activation pathway, plays a significant role in mediating this response. Nanoparticle surface properties are a relevant design feature, as they control the blood proteins the nanoparticles interact with and allow the nanoparticles to evade the immune reaction. PEGylation of nano-surfaces is critical in improving the blood circulation of nanoparticles and reducing opsonization. Our goal was to understand whether modifying the surface architecture by varying the PEG density and architecture can impact the complement response in vitro. We utilized block-copolymers of poly(lactic acid)-b-poly(ethylene glycol) prepared with poly(ethylene glycol) macroinitiators of molecular weights 3400Da and 5000Da. Tracking the complement biomarker C5a, we monitored the impact of changing PEGylation of the nanoparticles. We also investigated how the changing PEG length on the nanoparticle surface impacts further strengthening the stealth properties. Lastly, we determined which cytokines change upon blood incubation with nanoparticles in vitro to understand the extent to which inflammation may occur and the crosstalk between the complement and immune responses. Increasing PEGylation reduced the generation of complement-mediated anaphylatoxin C5a in vitro, with 5000Da PEG more effectively reducing levels of C5a generated compared to 3400Da PEG. The insights gathered regarding the impact of PEG density and PEG chain length would be critical in developing stealth nanoparticles that do not lead to infusion reactions upon intravenous administration.

Keywords

PEGylation, poly(lactic acid) nanoparticles, stealth-properties, complement-mediated hypersensitivity response, infusion reaction

Introduction

Infusion reactions are complex immune responses that are set in motion minutes after intravenous infusions.¹ While only 10% of humans appear to exhibit infusion responses,² the symptoms can range from mild hypersensitivity issues to severe cardiovascular complications and fatality in a subset of the population.³ Intravenously administered nanomedicines are engulfed in proteins as soon as they contact bodily fluids, forming a protein corona.^{4 5} The immune system can recognize either the nanoparticle surface or the protein corona formed, which initiates the complement pathways resulting in infusion reactions.⁴

The complement system is a vital component of the innate host defense⁶ and is associated with the onset of hypersensitivity reactions and subsequent clearance of

nanoparticles from the bloodstream.¹ The complement activation occurs through either the classical, alternative, or lectin pathway.^{7, 8} The initiating molecule is different for each of the pathways. The classical pathway is initiated through C1 binding to antigen-antibody complex⁹, while the spontaneous hydrolysis of C3 triggers the alternative pathway.¹⁰ The lectin pathway is initiated through plasma protein mannose-binding lectins attaching to carbohydrate structures present in the surface of pathogens.¹¹ Each pathway converges with the formation of initial C3 convertase, eventually leading to the generation of anaphylatoxins C3a and C5a.¹⁰ The anaphylatoxins are responsible for many of the symptoms associated with the onset of infusion reactions, including the degranulation of mast cells and production of histamines,^{9, 12} vasodilation, and increased permeability of blood vessels.¹³ Hence, a change in the anaphylatoxins can be a suitable biomarker for tracking the immune response to nanoparticles upon intravenous infusion. While identifying stealth properties, tracking cytokines, especially pro-inflammatory chemokines, can be helpful to evaluate the immunotoxicity.¹⁴ However, therapeutics delivered intravenously results in immediate protein corona formation and receptor recognition for subsequent clearance, and data regarding how the biomolecules governing inflammation change when nanoparticles encounter blood matrices is inadequate. It should also be noted that the impact of complement activation in vivo is not entirely well understood. Some of the earlier and recent works have studied the impact of complement on circulation times by comparing the pharmacokinetics between control and complement deficient^{15, 16} or complement knock-out mice¹⁷ and found that the complement cascade alone was not a predictor for the circulation times. However, when it comes to the uptake of nanoparticles by different leukocytes, there are differences observed between control and complement knock-out mice, and the differences are C3 dependent.¹⁸ Complement activation impacts safety for nanomedicine, especially in large animal models that are more prone to the response by resulting in cardiopulmonary distress and other physiological manifestations of complement.¹⁹ Liposomal formulations such as AmBisome and Doxil can cause drastic changes in cardiopulmonary vitals in minutes after infusion in pigs.²⁰ More insight is required to understand further the complexities of complement activation in vivo in nanomedicine. Even studying complement in vitro can lead to challenges, particularly in translating the outcomes to humans. As highlighted by a study that identifies the differences in complement activation pathways in mouse and human serum, the observations from the mouse system may not be entirely translatable to the human system.²¹ Hence, sensitive in vitro models that are more relevant to the human system are required to accurately study the impact of surface properties of nanomaterials on complement activation.

PEGylation refers to covalently grafting, entrapping, or adsorbing Poly(ethylene glycol) (PEG) chains to a molecule or material.²² One of the earliest theories by Jeon et al. regarding why PEGylation of surfaces leads to protein resistance is because PEG has the lowest refractive index among water-soluble synthetic polymers, resulting in low van der Waals force.²³ Based on molecular weight, grafting density, the hydrophilic nature, and flexibility of the PEG chains, PEGylation leads to a more extended conformation in the presence of water molecules.^{22, 23} Owing to the hydrophilic nature and higher chain flexibility based on molecular weight, the PEG coating can lead to the formation of an impermeable cloud-like conformation even from a small number of PEG chains and prevent opsonization.²⁴ PEGylation of liposomal nanoformulations has increased the half-life during circulation, decreased plasma clearance,²⁵ and reduced clearance due to reticuloendothelial systems.²⁶ While PEGylation has been extensively studied for making stealth nanomaterials, the details of the density and molecular weight of the chains that are critical for this, particularly in response to infusion reactions, have not been studied thoroughly. Surface coverage of poly (lactic-co-glycolic acid) (PLGA) nanoparticles with PEG can impart stealth properties by forming a weak complex with blood albumen leading to the chameleon effect and repulsion to biomolecules in the blood responsible for rapid clearance of the nanoparticles.²⁷ Surface modification with polyphosphoester,²⁸ and

polysaccharides combined with PEG²⁹ can lead to surface enrichment with clusterin. This dysopsonin protein helps avoid clearance by macrophages.³⁰ Increased molecular weight and grafting density for PEG have been found to enhance clusterin adsorption while also reducing cellular internalization.³¹ This is because the grafted PEG chains on the surface impact complement activation and the activation pathways modulating access between nano surfaces and blood proteins.³² Thus, particles with brush-like PEG (highly dense) or dense dextran brushes are more resistant to binding complement proteins than their counterparts with mushroom-like/ or lightly enveloped surfaces.^{32, 33} Moreover, how PEG remains attached to the surface also plays a pivotal role in determining the extent to which opsonization is blocked, as surface adsorption is reversible, and surface conjugation may lead to insufficient surface coverage.²² Owing to its hydrophilicity and chain flexibility, even PEGylation with PEG of molecular weight as low as 2000 Da lowers protein adsorption on poly(lactic acid) nanoparticles by 57%.³⁴

Because degradable polyesters have been used extensively in developing drug delivery systems, we were interested in understanding the details of how PEGylation might reduce or eliminate infusion reactions. To investigate the impact of PEGylation on polyesters, we developed polylactic acid (PLA) nanoparticles and a series of PLA-based nanoparticles with different densities and architectures of PEG arms. We quantified C5a, a biomarker of the complement activation, in vitro, as we incubated the surface-modified nanoparticles with the blood matrices. Our goal was to understand how the extent of surface PEGylation modulates the innate immune response and further comprehend what role PEG chain length plays in imparting the stealth properties. Moreover, we investigated the impact of PEGylation on cytokine levels upon in vitro incubation to better understand the overall immune response between blood matrices and PLA-*b*-PEG nanoparticles.

Results

Characterization of nanoparticles with changing amount of surface PEGylation

First, we tracked the change in complement protein C5a for PLA nanoparticles. The nanoparticles prepared had an average size of 324.3 ± 4.7 nm and a zeta-potential of -25.4 ± 0.66 mV. Our goal was to design a set of nanoparticles with different amounts of PEGylation on the surface to compare with the complement response observed for the PLA nanoparticles. The polymers used, poly(l-lactic acid)-*b*-(polyethylene glycol) and poly(d-lactic acid), were prepared through ring-opening polymerization. The molecular weight of the synthesized polymers PDLA, PLLA-*b*-PEG5000, and PDLA-*b*-PEG3400 were 24,000-31,293 Da. (Figure S1) Based on the molecular weight of the PLLA-*b*-PEG5000 polymer, with a macroinitiator of 5000Da PEG, polymer consists of 18.8wt% PEG, and PDLA-*b*-PEG3400 with a 3400 Da PEG macroinitiator consists of 10.9wt% PEG. We blended block copolymer of poly(l-lactic acid)-*b*-(polyethylene glycol) and poly(d-lactic acid) at different ratios to generate these nanoparticles. (Figure 1) The hydrodynamic diameter and zeta-potential of the nanoparticles were determined through DLS, while SEM was also used to determine the average diameter. (Figure S2) Compared to the hydrodynamic diameters observed, the average diameters of the nanoparticles with different PEG densities on the surface are within the range of 311-359 nm and similar core size, and the higher values observed for hydrodynamic diameter could be due to the PEGylation and its behavior in aqueous phase PBS.

Varying amount of PEG on the surface of the nanoparticles

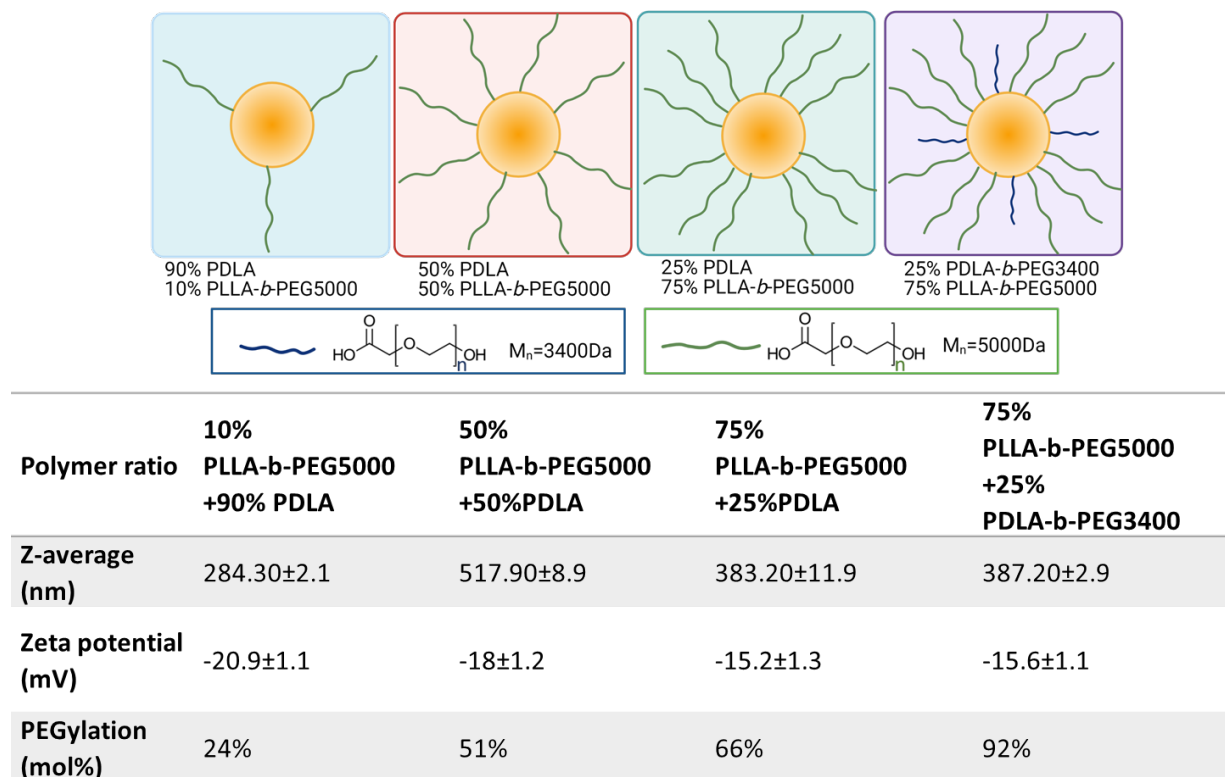


Figure 1: Summary of characterization data for nanoparticles with varying amounts of surface PEG

To further understand how PEGylation can help generate stealth properties, we also prepared nanoparticles combining PEG of different molecular weights on the surface. To achieve this variation, we combined block copolymers of poly(L-lactic acid)-*b*-(polyethylene glycol) with PEG molecular weights of 5000Da and 3400Da. The hydrodynamic diameter was determined via DLS, and the zeta-potential was determined via the Malvern zeta sizer using a zeta potential load cell. (Figure 2). For nanoparticles with the highest degree of PEGylation, SEM was also used to determine the average diameter. (Figure S3) SEM images show a uniform core size for the nanoparticles ranging from 310-341 nm, while the observed hydrodynamic diameters are larger, most likely due to the PEG corona. This phenomenon has been seen previously with PEGylated polyester nanoparticles³⁵.

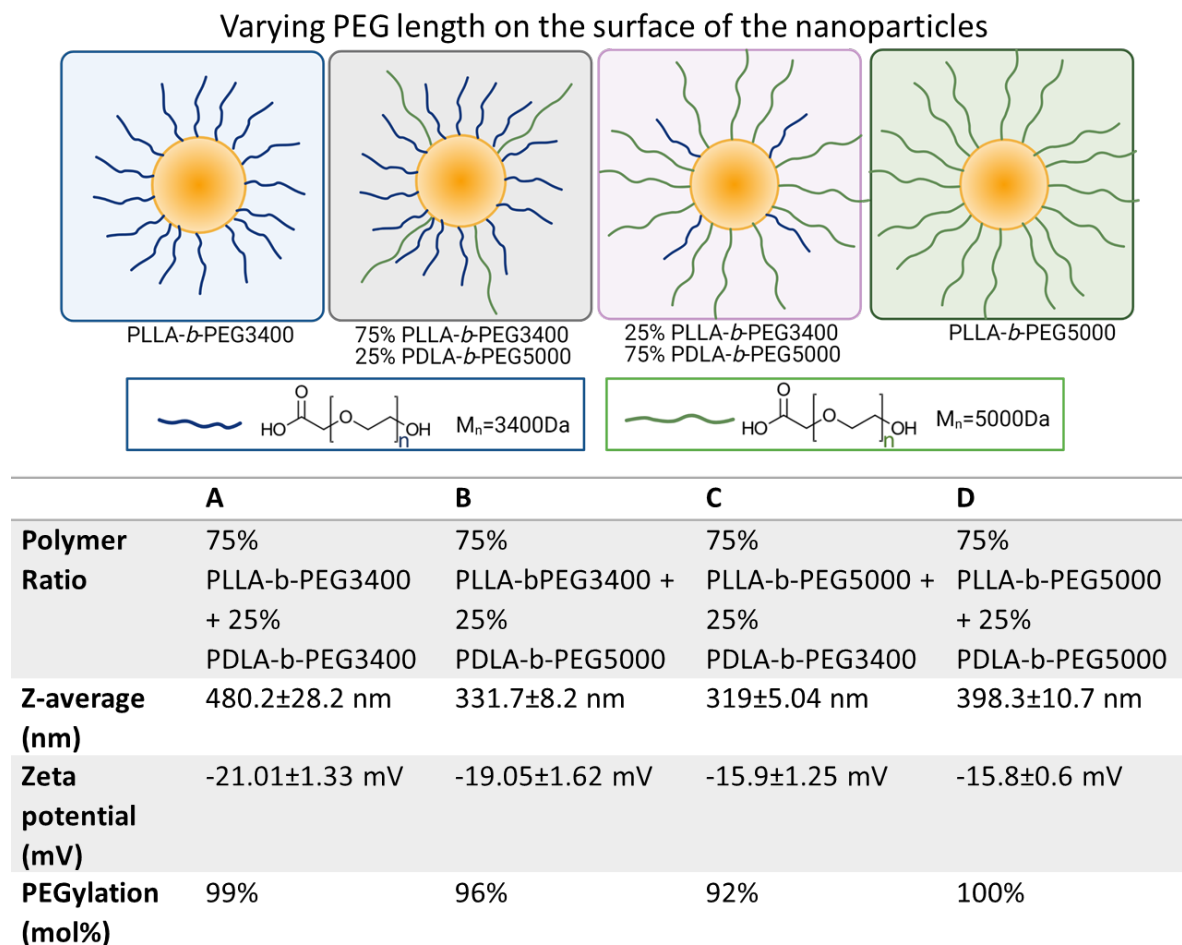


Figure 2: Summary of DLS data for nanoparticles with a combination of PEG lengths in the corona

The nanoparticles prepared were characterized using dynamic light scattering (Malvern ZetaSizer (Nano ZS90), Malvern Panalytical Ltd). The nanoparticles' average size and the Zeta-potential in 10mM KCl solution are summarized below in Figure 1 and Figure 2. As the extent of PEGylation increases, the zeta-potential increases, as the more negatively charged surface of the PLA nanoparticles getting cloaked by the PEG chains. The extent of PEGylation calculated using ^1H NMR is also summarized in these tables. The extent of PEGylation is measured as the percentage of moles of PLA with a PEG chain attached to it. We determined the values based on the ^1H NMR peak integrated areas observed for PLA segments and PEG segments in deuterated Chloroform (Figure S4 and S5). While the nanoparticles were prepared with poloxamer-188 for the in vitro assays, we determined the PEGylation levels for nanoparticles both with and without poloxamer-188 using ^1H -NMR in deuterated chloroform (Figure S6.) Poloxamer 188 contains PEG blocks which could confound the results. The differences in the PEGylation levels observed with and without the presence of poloxamer-188 are shown in table 1.

Table 1: Differences in PEGylation levels observed for nanoparticles prepared with and without poloxamer-188 as stabilizer

	Very low-density PEG brush	Low-density PEG brush	Medium-density PEG brush	High-density PEG brush
Polymer ratios used (wt%)	10%PLLA-b-PEG(5000)+90%PDLA	50%PLLA-b-PEG(5000)+50%PDLA	75%PLLA-b-PEG(5000)+25%PDLA	75%PLLA-b-PEG(5000)+25%PDLA-b-PEG(3400)
Nanoparticles prepared with poloxamer-188	24%	51%	66%	92%
Nanoparticles prepared without poloxamer-188	5%	31%	52%	73%

Change in complement protein C5a in vitro for different PEG corona structures.

We measured the change in complement protein C5a in vitro in human whole heparinized blood and complement protected human serum by incubating the blood matrices with the nanoparticles suspended in PBS at a concentration of 0.25mg/ml. As a positive control, Zymosan was also used at a concentration of 0.25mg/ml. The preparation method is based on our previously established protocol for sensitively quantifying complement proteins in vitro.³⁶ The assay was carried out following the kit manufacturer's provided guideline, and the calibration curve was fitted using 4 parameter logistic equation. The complement-mediated response is highly donor sensitive. Heparinized whole blood is more sensitive as the complement pathways are not impacted by the anticoagulant itself,³⁷ while in serum, as the coagulation cascade is no longer intact, the complement pathway is not suppressed, due to the absence of cells leading to biological turnover.³⁸⁻⁴⁰ As a result, in serum, complement protein levels are high due to initial activation, and the level change is faster.⁴¹ Hence the complement-mediated response was measured in both heparinized whole blood and complement protected human serum.

Change in complement protein C5a due to PLA nanoparticles in vitro

We investigated the impact of non-PEGylated poly(lactic acid) nanoparticles. When incubated with heparinized whole blood, poly (lactic acid) nanoparticles without any surface modifications led to the highest amount of complement protein C5a generated within the samples. (Figure 3) The level observed was comparable to the amount of C5a generated for the positive control zymosan. Statistical analysis was carried out using the Chi-square analysis, and the change in C5a detected was significantly different for the positive control Zymosan and the PLA nanoparticles.

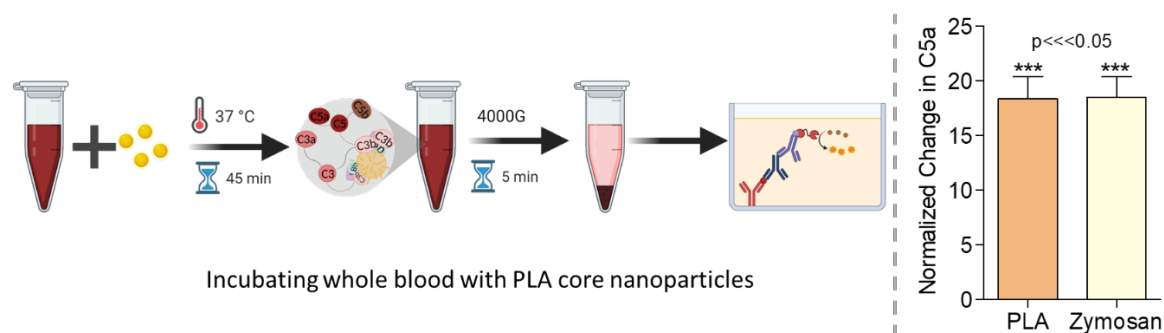


Figure 3: Change in complement protein C5a upon incubation of heparinized whole blood with PLA nanoparticles. Using Chi-square analysis, p-value obtained was less $p < 0.05$ when compared against an expected value of 1 for the level in sample incubated with PBS.

Impact of PEG density on surface of nanoparticles on generating complement protein C5a in vitro

As we cloaked the surface with the PEG corona, the increasing extent of PEGylation on the surface leads to a decrease in C5a detected within the samples in heparinized human whole blood. Even the lowest amount of PEGylation tested led to 10 times lower C5a generation within the sample. As the extent of PEGylation increases, the C5a generation further decreases with the samples. The result summarized is the response observed in whole heparinized blood. (Figure 4). Based on the Chi-square analysis, for the PEGylated nanoparticles, a p-value lower than 0.05 was obtained, indicating the significant difference for the nanoparticles with 51% PEGylation. However, the p-value was greater than 0.05 for 66 and 92% PEGylation, indicating lower differences observed than the sample incubated with PBS. Hence, the increasing extent of PEGylation can control the C5a generation in vitro and prevent complement activation. In complement-protected human serum, the nanoparticles with high-density PEG had a lower change in C5a when compared to the nanoparticles with 66% PEGylation (Figure S7). Based on the Chi-square analysis, the observed change in C5a was not significantly different from the serum incubated with PBS, as observed in the whole blood.

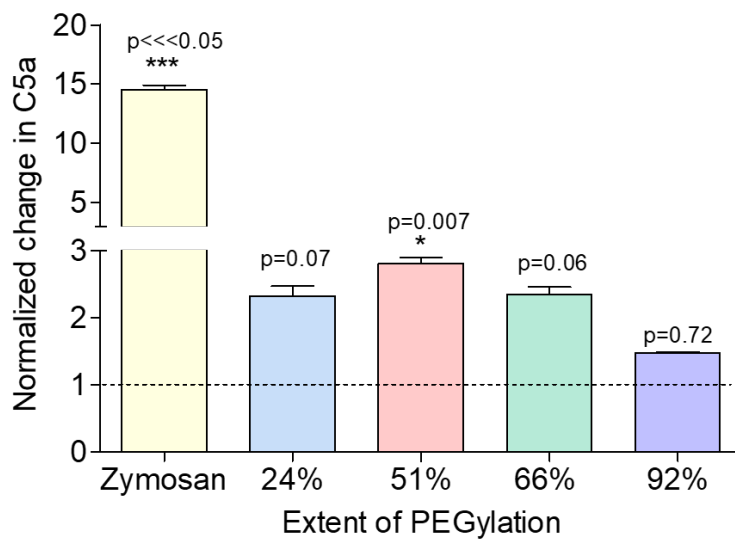
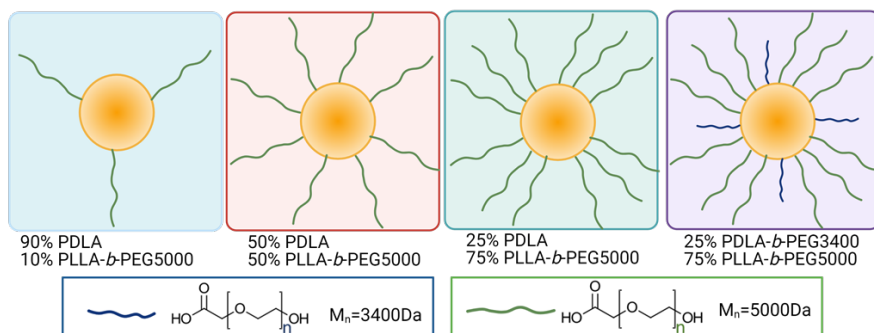


Figure 4: Impact of the extent of PEGylation on the normalized change in complement protein C5a in vitro upon incubation of heparinized human blood with nanoparticles at a concentration of 0.25mg/ml. As a positive control, Zymosan was used. The normalized change was calculated in comparison to the sample incubated with PBS. Using Chi-square analysis, p-value obtained was less $p < 0.05$ when compared against an expected value of 1 for the level in sample incubated with PBS for the 51% PEGylation while the change was highly significant for Zymosan but not for the other groups.

Change in complement response in vitro for PEG polymers of different lengths

While increasing the extent of PEGylation reduced changes in C5a, we also investigated the impact of the PEG chain length. We compared four variations, ranging from highly PEGylated nanoparticles with a PEG chain length of 3400 Da to nanoparticles with a dual PEG brush combining 3400 and 5000Da PEG chains and nanoparticles with a PEG chain length of 5000 Da. We investigated this change in whole blood and complement protected human serum at a 0.25mg/ml nanoparticle concentration. The C5a levels detected were lowest for nanoparticles with 5000Da PEG in human whole heparinized blood (Figure 5) and complement-protected human serum (Figure S8) compared to 3400 Da PEG. In the case of the dual-PEG corona, the molecular weight of the dominant PEG in presence seemed to be the controlling factor. For nanoparticles with a higher presence of 3400Da PEG chain, the C5a detected was higher than the amount detected for nanoparticles with a higher fraction of 5000Da PEG chain in both blood and serum. The C5a level detected remained close to the levels detected in the control sample incubated with PBS only. There was no significant difference in the means observed; however, the means were significantly different from the positive control Zymosan in all the cases. This was confirmed through the Chi-square analysis, where calculated p-values were greater than 0.05.

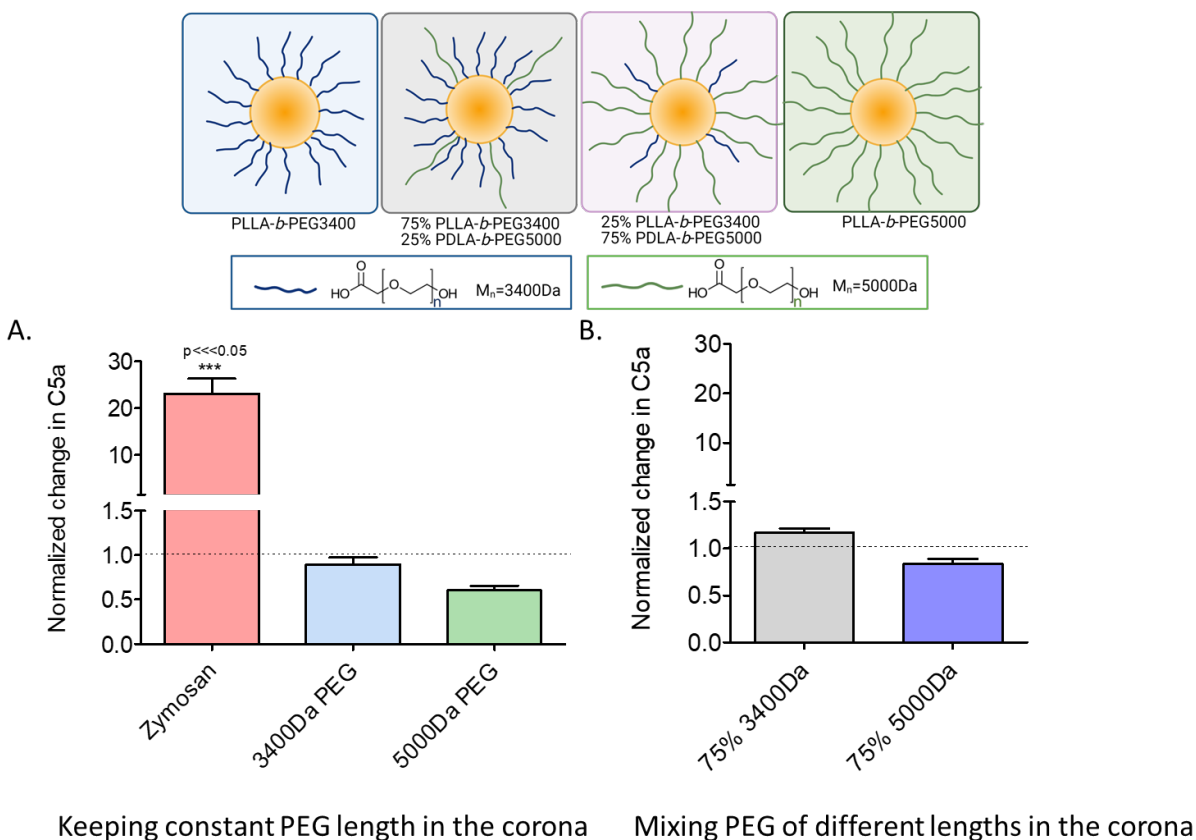


Figure 5: Impact of PEG chain length on normalized change of complement protein C5a in vitro upon incubation of heparinized human blood with highly PEGylated nanoparticles at a 0.25mg/ml concentration. As a positive control, Zymosan was used. The normalized change was calculated in comparison to the sample incubated with PBS. A. The normalized change in C5a was observed for PEG lengths of 3400Da and 5000Da. B. The normalized change in C5a was observed for mixing different PEG lengths. Using the Chi-square analysis, the p-value>0.05 was obtained, indicating the C5a levels observed are similar to the levels observed in samples incubated with PBS.

Overall, a comparison between C5a levels observed in human heparinized whole blood (Figure 5) and complement protected human serum (Figure S8) shows that 3400 Da PEG leads to higher C5a level compared to 5000Da PEG. Also, when present together, the C5a level in both cases is lower for nanoparticles with higher ratio of 5000Da PEG. Hence in both the blood matrices, the nanoparticles lead to a same pattern of change in the complement protein C5a.

Change in cytokine response for PEGylated nanoparticles

To better understand the crosstalk between complement and the other inflammatory responses in vitro, we quantified the changes in cytokine levels in heparinized human whole blood through dot blot using a cytokine array kit. We incubated the nanoparticles with heparinized human whole blood at a concentration of 0.25mg/ml to detect the presence of the cytokines. We compared the impact of PEG corona conformation and identified whether the cytokines were upregulated or downregulated for PEGylated and non-PEGylated nanoparticles. (Figure 6). We also tracked the changes for Zymosan, a known complement activator, and lipopolysaccharide (LPS), a potent activator of macrophages and monocytes.⁴²

The change observed in C5/C5a biomarker was the greatest, with the value being significantly higher for the non-PEGylated PLA nanoparticles and Zymosan in donor blood 1. PEGylation of PLA-*b*-PEG nanoparticles lowered the levels of C5/C5a detected. The levels observed were significantly higher based on the Chi-square analysis. Whereas for LPS, C5/C5a levels were much higher in comparison to Zymosan in donor blood 2. Anaphylatoxin C5a is a potent mediator of inflammation.⁴³ As we compared the cytokine profile for two different donors, we notice differences in some of the pro-inflammatory biomarkers that were detected. The cytokines detected in donor blood 1 (Figure 6A) primarily include IL-8 and IL-16. In the case of donor blood 2 (Figure 6B), IL-18 and IL-16 are detected, where IL-18 is only observed in blood incubated with LPS and PLA. Interleukin-16 (IL-16) is a chemoattractant factor,⁴⁴ released in response to mitogens, antigens, or vasoactive amines such as histamine or serotonin.⁴⁴ IL-16 is responsible for the upregulation of pro-inflammatory cytokines (IL-1 β , IL-6, and TNF- α) or cytokines that can cause pro-inflammatory activity.⁴⁵ The levels observed remained low for IL-16 in all cases for donor blood 1, however in donor blood 2, levels were significantly elevated for PLA and LPS. Receptor antagonist for IL-1 (IL-1ra) dampening inflammation,⁴⁶ can be downregulated due to complement activation.⁴⁷ The anti-inflammatory cytokine IL-1ra remains close to levels observed for MEM in donor blood 1. In donor blood 2, IL-1ra was significantly higher for LPS and PLA and slightly higher for the PLA-PEG nanoparticles compared to MEM. Chemokine protein CXCL-12 is known to trigger anti-inflammatory molecular cascades.⁴⁸ It is critical in the recruitment of leucocytes and fundamental in inflammation.⁴⁹ While CXCL-12 levels remain similar for PLA, PLA-PEG, and MEM, the detected level is lower for Zymosan in donor blood 1, whereas in donor blood 2, levels are significantly higher for PLA and LPS. The regulators for the innate and acquired immune system, Macrophage migration inhibitory factor (MIF), remain lower for the PEGylated nanoparticles, and the level of downregulation is significant. MIF is a critical upstream regulator of the innate and acquired immune response,⁵⁰ induces pro-inflammatory cytokine expression.⁵⁰ PAI-1 levels also remain close to the level observed in blood incubated with MEM, slightly decreasing for both Zymosan as well as LPS. In

both donor blood 1 and 2, the cytokines detected remained lower, and for most parameters, close to the observations for MEM for the PLA-PEG nanoparticles suggesting PEGylation helps in suppressing the cytokine upregulation upon exposure of nanomaterials to blood in vitro.

For intravenous infusion, it is critical to understand how the cytokines may change upon exposure to nanoparticles in vivo. As a preliminary assessment, we sought to understand how the biomarkers change in vitro in whole heparinized blood and whether complement-mediated infusion reactions and inflammation can be prevented. The method of detecting the cytokine is pseudo-quantitative, as the absolute values are not obtained, rather the intensities are determined. It should also be noted that using whole human blood introduces variability in response, as the response could be donor-specific. However, for detecting an overview of cytokine profiles, whole blood assays are found to be as effective as quantifications were done using peripheral blood mononuclear cells.⁵¹ It should also be noted that the collected blood storage time may impact the sensitivity of the assay as well. Hence, the detection of upregulation or downregulation of cytokines used is mainly a risk assessment tool. Overall, the nanoparticles did not upregulate the pro-inflammatory cytokines in the whole heparinized blood. The greatest change was observed for C5/C5a, and the trend observed is similar to what we have seen in ELISA as well, where PLA and Zymosan had resulted in the highest level of changes.

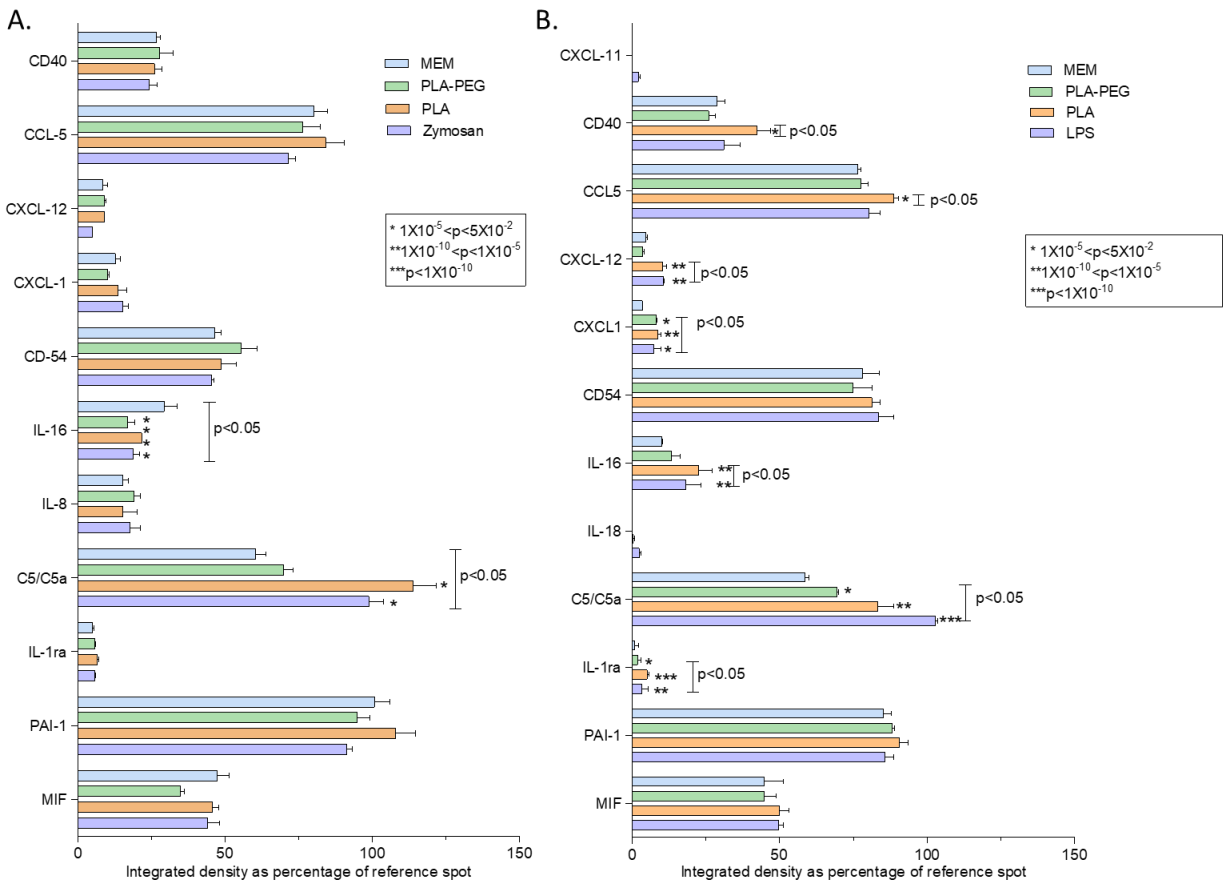


Figure 6: Impact of extent of PEGylation of nanoparticles on the cytokine levels in heparinized human whole blood. A. Changes in cytokine levels with Zymosan. B. Changes in cytokine levels with lipopolysaccharide.

Change in IL-6 for nanoparticles incubated with rat endothelial cells

We further quantified the changes in IL-6 levels when rat endothelial cells were incubated with PLA and PLA-PEG nanoparticles. As a positive control, Zymosan was used and resulted in the highest level of IL-6 observed. The normalized change was calculated compared to the observations for rat endothelial cells incubated with MEM. The change was statistically significant as well based on the chi-square analysis resulting in $p < 0.05$. Both PLA and PLA-PEG nanoparticles did not lead to significant changes in IL-6, and the values remained even lower than the observation for the MEM. (Figure 7)

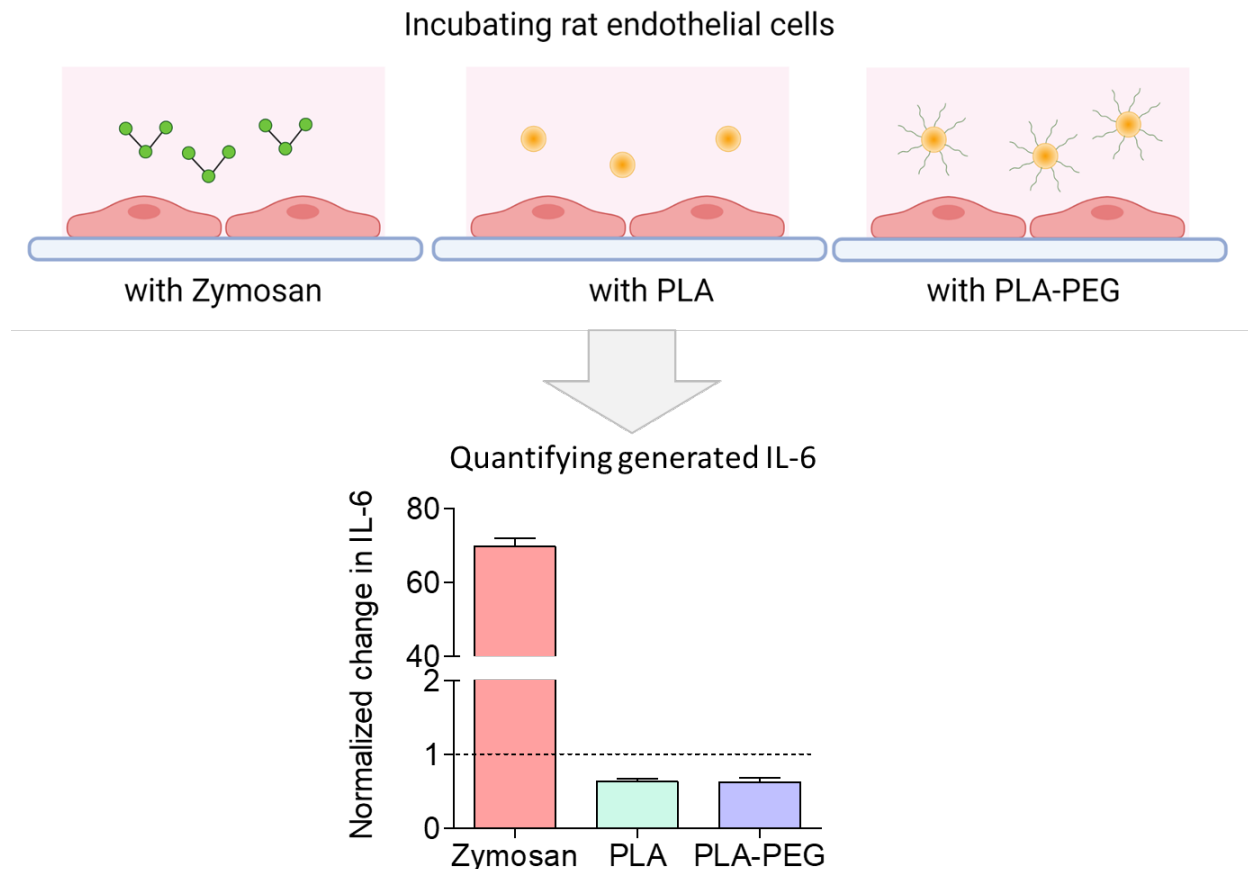


Figure 7: Change in IL-6 due to encounter of nanoparticles with rat endothelial cells. The change in IL-6 was significantly higher for the positive control Zymosan, but remained low for the PLA and PLA-PEG nanoparticles.

Discussion

PEGylation has been essential in preventing aggregation of nanoparticles, opsonization, and phagocytosis.⁵² Due to the highly hydrophilic nature and chain flexibility, PEG chains of molecular weight as low as 2000Da can lower protein adsorption on poly(lactic acid) nanoparticles by 57%.³⁴ Our goal was to understand how the extent of PEGylation impacts changes in complement proteins and cytokines in vitro. We hypothesized that increasing the extent of PEGylation will lead to cloud-like conformation covering the maximum surface and imparting stealth properties to develop nanoparticles that avoid complement-mediated infusion reactions. We also investigated the role PEG chain length plays in imparting the stealth property. The biomarker tracked in vitro was the complement protein C5a, the anaphylatoxin

produced due to complement activation. We also quantified the pro-inflammatory cytokines and chemokines generated in vitro upon contact with blood matrices to further understand the crosstalk between the complement system and the immune system and what that may imply upon in vivo administration.

First, we generated nanoparticles with varied amounts of PEG on the surface. We focused on the impact for PEG chain lengths of 3400Da and 5000Da as they could be used for functionalization with peptides or moieties of interest. Based on previous data for similar polyester nanoparticles, a molecular weight of 4600 Da leads to better molecular interaction with targets for peptide moieties conjugated to the PEG chain ends.³⁵ Moreover, beyond the molecular weight of 5000Da, higher PEG molecular weights increase the chance of shielding functional moieties of interest.³⁴

It is critical how PEG is attached to the nanoparticle. Adsorption is a reversible process, and it can often lead to desorption and result in exposed nano surface, while covalent attachment can often lead to insufficient surface coverage.²² We prepared block copolymer of poly(lactic acid)-*b*-poly(ethylene glycol) with the heterobifunctional PEG as the macroinitiator through ring-opening polymerization.^{53, 54} By mixing ratios of PLA and PLA-*b*-PEG, we generated nanoparticles with different extents of PEGylation. The extent was a measure of the density of the PEG corona. The highest density conformation was for the nanoparticle formulation with a combination of PLLA-*b*-PEG and PDLA-*b*-PEG.

Upon quantifying the biomarker C5a in vitro, we found the C5a levels were lowest for nanoparticles with the greatest extent of PEGylation. The density of surface graft is a crucial component of surface protein adsorption and subsequent complement activation. As the surface density changes from mushroom-like lateral chains to brush-like conformations, nanoparticles lead to lower complement activation products.³² The brush conformation can be confirmed even for the lowest density, which leads to a 4.8wt% PEG on the nanoparticles. In the case of PLGA-PEG nanoparticles with 3wt% PEG on the surface, a brush configuration was observed, with the brush getting denser for 5wt% PEG.⁵² While PEGylation can reduce the biomolecule adsorption it cannot prevent it entirely. Compared to bare PLA nanoparticles with the most significant change in C5a levels, all the PEGylated nanoparticles resulted in lower complement activation, .i.e., generation of biomarker C5a. Hence, we identified that increased PEGylation leads to the lowest complement protein adsorption and subsequent complement activation in vitro. This agrees with our previous observation of PLGA-PLL-PEG nanoparticles with 10-15% PEGylation¹⁹ showing higher levels of C5a as well in vitro.³⁶

After determining that combining PLLA-*b*-PEG and PDLA-*b*-PEG resulted in the lowest complement activation, quantified through the change in biomarker C5a, we investigated the role PEG chain length plays. While it may seem that the longer the chain, the higher the flexibility would lead to better repulsion, the range above 5000Da has shown no significant decrease in plasma protein adsorption.³⁴ Whereas, as PEG chain length increased from 2000Da to 5000Da, 50% reduction was seen in adsorbed protein amounts.³⁴ Based on the pattern, we investigated whether increasing PEG chain length from 3400Da to 5000Da would reduce the change in complement protein C5a. The C5a detected in samples incubated with 5000Da PEG was lowest in both blood and serum. At the same time, we also explored the approach of combining two different PEG lengths. The concept has been applied for microbubbles, the shorter PEG layer leading to steric repulsion prohibiting the approach and adhesion of opsonin, lipoproteins, and other liposomes, and the more extended PEG layer providing target specificity.⁵⁵ Recently, PEG pairing has also been explored to support longer chains with the shorter chains and project the longer chains further to prevent protein surface adsorption.⁵⁶ We found that in combining 5000Da PEG and 3400Da PEG, the impact of chain

length of the PEG present in a higher ratio was dominant. When the ratio of 5000Da PEG to 3400Da PEG increased, the complement protein C5a detected decreased in both blood and serum.

We tracked how cytokine levels change in vitro in heparinized human whole blood to validate the stealth property of the nanomaterial surfaces. The physiological properties of nanoparticles and the cytokine profiles observed are mostly related.¹⁴ Understanding how the changing PEGylation impacts cytokine levels can help further engineering the nanoparticles to evade complement-mediated infusion reactions. Complement proteins like C5a can stimulate Plasminogen activator inhibitor-1 (PAI-1), leading to vascular injury.⁵⁷ The release of cytokines has been detected for several nanoformulations as well. Iron-based nanomedicine and carbon nanotubes depending on the physicochemical properties of the final formulation can lead to an elevation in IFN- γ and TNF- α along with IL-1 β .⁵⁸⁻⁶⁰ We did not detect any changes in the mentioned pro-inflammatory cytokines that are found to be elevated for the iron and carbon nanotubes. For the detected cytokines, the levels observed are not significantly different in heparinized human whole blood compared to the observations for the negative control MEM, showing statistically significant differences mainly for the complement protein C5/C5a only. Based on our observations, immediately after nanoparticles encounter blood, the cytokine levels do not change significantly for the pro-inflammatory cytokines in whole heparinized blood.

We also quantified changes in IL-6 when endothelial cells are exposed to the nanoparticles, mainly as anaphylatoxins like C5a can induce the generation of the pro-inflammatory cytokines IL-6.⁶¹ On further assessment with rat endothelial cells, we determined that the cytokine IL-6, a potent biomarker of inflammation, was upregulated for Zymosan; however, it remained downregulated for the PLA and PLA-PEG nanoparticles. While quantifying IL-6, we had used Zymosan as a positive control. Zymosan can bind with endothelial cells (such as human umbilical vein endothelial cells) and lead to a complement-mediated IL-6 secretion.⁶²

While nanoparticle PEGylation primarily increases blood circulation time and prevents opsonization, it can also be utilized to inhibit complement-mediated infusion reactions. Since surface architecture is crucial for surface coverage and subsequent cloud-like conformation that can reduce protein adsorption, we tracked this change with changing amount of PEG on the surface. Once we figured that higher PEGylation leads to lesser complement activation, we investigated the role of the PEG molecular weight in further extending that stealth property. Looking into the cytokine profiles, we get a picture of the pro and anti-inflammatory chemokines that are elevated or downregulated upon nanoparticles encountering blood. Further incorporating the nanoparticle core role and similarly exploring coatings other than PEG, along with this study, can be vital to developing nanoparticles that do not lead to complement-mediated infusion reactions upon intravenous administration.

Conclusion

We have developed a study to track the impact of PEGylation on complement activation in vitro. To achieve that, we quantified the changes in anaphylatoxin C5a, produced upon complement activation, as a biomarker. We utilized block copolymers of PLA-b-PEG to generate the nanoparticles. The highest possible PEGylation with this block copolymer led to the least amount of C5a detected in the blood in vitro. We investigated the impact of PEG chain length as well. The increasing PEG length from 3400Da to 5000Da leads to the least amount of C5a within the sample in vitro. How PEG pairing impacted the change in C5a was intriguing as we can leverage this to design stealth nanoparticles with better targeting capability. Lastly, we generated the cytokine profiles in vitro for these nanoparticles to mark the chemokines critical in the inflammation process. As nanoparticles' physiochemical properties are essential regulators of the interaction between the nanomaterials and blood proteins, we can further extend this

study to include the nanoparticle core's role. Combining the knowledge gathered can lead to the detection of the properties that strengthen the stealth behavior to prevent nanotherapeutics' complement-mediated infusion reactions once they encounter blood proteins.

Materials and Methods

Materials

Poly(lactic acid)-*b*-poly(ethylene glycol) block copolymer was synthesized using L-lactide from Polysciences Inc. (Warrington, PA), heterobifunctional poly(ethylene glycol) with 5000 Da and 3400Da molecular weight from Laysan Biosciences (Arab, AL), and D-lactide from Purac Biomaterials (Corbion, Amsterdam, Netherlands). 1,3-Bis-(2,4,6-trimethylphenyl)imidazole-2-ylidene (iMES) obtained from Sigma Aldrich was used as a catalyst for the ring-opening polymerization. All solvents used were of ACS grade and obtained from Fisher Scientific. In vitro assays used for quantifying C5a, cytokine profile and IL-6.

We monitored Complement protein C5a using a C5a human ELISA duo kit (DY2037) obtained from R&D Systems (Minneapolis, MN). We also measured cytokine levels in vitro using Proteome Profiler Human Cytokine Array Kit (R and D Systems, catalog number ARY005B). For assessing changes in IL-6 in rat endothelial cells, we used the Rat IL-6 Quantikine ELISA Kit (R&D systems, R6000B).

Handling of blood products

Heparinized human whole blood and complement-protected human serum were obtained from Innovative Research Inc. (Novi, MI) for the in vitro complement studies. In whole blood, blood was collected in heparinized collection tubes from a single donor and shipped using the same collection vial to avoid exposure to multiple surfaces and lead to unwanted complement activation. Blood was handled with care, stored, and shipped at 4°C, and only mixed gently, and stored on ice while running the assay. The complement-protected human serum was shipped frozen and stored at -80°C until used for the assays. The complement-protected serum was thawed immediately before being used at 37°C, and stored on ice until the assay was run. To avoid freeze-thaw associated complement activation, serum was only thawed once and used up for the in vitro assays.

Synthesis of nanoparticles

Block copolymers of poly(lactic acid)-*b*-poly(ethylene glycol) with either 3400 or 5000Da PEG macroinitiators were synthesized through a ring-opening polymerization reaction.^{53, 54} Poly(D-lactic acid) (PDLA) was also synthesized using a ring-opening polymerization but with methanol as the initiator. The molecular weight was determined using gel permeation chromatography in Tetrahydrofuran (THF).

The nanoparticles were prepared using different ratios of poly(L-lactic acid)-*b*-poly(ethylene glycol) (PLLA-*b*-PEG5000, PLLA-*b*-PEG3400), Poly(D-lactic acid)-*b*-poly(ethylene glycol) (PDLA-*b*-PEG5000, PDLA-*b*-PEG3400) and PDLA through nanoprecipitation.⁵⁴ The nanoparticle groups used in this study, along with the variation in the polymers used, are summarized in table 2 and table 3. The polymer dissolved in the organic phase, i.e., Tetrahydrofuran (THF), at a concentration of 20mg/ml, was added dropwise to double the phosphate-buffered saline's volumetric amount leading to nanoparticles. After stir-hardening for 3 hours, excess THF was removed by exposing the solution to air, and poloxamer-188 was added as the stabilizer. The nanoparticles were then collected through centrifugation at 4000XG for 10 minutes at 4°C and resuspended in phosphate-buffered saline.

Non-PEGylated PLA nanoparticles were prepared by dissolving the polymer in chloroform (20 mg/ml) and adding to a PVA solution (5 wt%) dropwise. The mixture was briefly

sonicated and then added to a larger volume of PVA solution (1 wt%) to further stir harden the nanoparticles overnight. The nanoparticles were collected via centrifugation at 10,000rpm for 10 minutes.

Table 2: Altering amount of surface PEG by varying ratio of block copolymer PLLA-*b*-PEG5000 and PDLA (%w/w)

	Polymers used
Very low-density PEG brush	10% PLLA- <i>b</i> -PEG5000 +90%PDLA
Low-density PEG brush	50% PLLA- <i>b</i> -PEG5000 +50%PDLA
Medium-density PEG brush	75% PLLA- <i>b</i> -PEG5000 +25%PDLA
High-density PEG brush	75% PLLA- <i>b</i> -PEG5000 +25% PDLA- <i>b</i> -PEG3400

Table 3: Combining surface PEG of different molecular by varying ratio of block copolymer PLA-*b*-PEG5000 and PLA-*b*-PEG3400 (%w/w)

	PEG length	Polymers used
A	3400Da PEG	75% PLLA- <i>b</i> -PEG3400+ 25% PDLA- <i>b</i> -PEG3400
B	75% 3400Da PEG	75% PLLA- <i>b</i> -PEG3400+ 25% PDLA- <i>b</i> -PEG5000
C	75% 5000Da PEG	75% PLLA- <i>b</i> -PEG5000+ 25% PDLA- <i>b</i> -PEG3400
D	5000Da PEG	75% PLLA- <i>b</i> -PEG5000+ 25% PDLA- <i>b</i> -PEG5000

Characterization of the nanoparticles

The particles were characterized through dynamic light scattering for determining the hydrodynamic diameter where nanoparticles resuspended in PBS was used, and the zeta-potential for nanoparticles was determined in dilute potassium chloride solution (10mM). The extent of PEGylation was determined through ¹H-NMR as well. For ¹H-NMR, the nanoparticles were dissolved in deuterated chloroform, and peaks were obtained for further analysis. To address the presence of poloxamer-188 added as a stabilizer, ¹H-NMR data was also obtained for nanoparticles prepared without any poloxamer. SEM images were also captured using the FEI Nova NanoSEM 450 after gold coating of the samples, and imageJ was used to determine the diameter of the nanoparticles from the images.

Generating complement response in vitro and quantification through complement assay

The change in complement protein C5a was quantified following previously established protocol.³⁶ For generating the complement response, human heparinized whole blood and complement protected human serum were used. The blood matrix was incubated with nanoparticles suspended in Dulbecco's PBS (without calcium and magnesium). As a positive control, Zymosan was used. The dosage used for the nanoparticles and Zymosan was 0.25mg/ml in the blood matrices. The samples were incubated at 37°C for 45 minutes and then centrifuged at 4000g for 5 minutes to separate the nanoparticles. The plasma/serum was aliquoted in clean tubes and stored on ice until assay was carried out using the C5a ELISA assay duo kit (R&D Systems). The optical density for the samples and standards were measured at a wavelength of 450 nm using SpectraMax M2 Microplate Reader (Molecular Devices LLC) with background correction done using reading obtained at 540 nm. The normalized change was measured by comparing the level of C5a observed in the sample incubated with PBS.

$$\text{Normalized Change} = \frac{\text{Biomarker in sample incubated with Zymosan or nanoparticles}}{\text{Biomarker in sample incubated with PBS}}$$

Generating immune response in vitro and quantification through cytokine array

Nanoparticles resuspended in Minimum essential medium (MEM) were added to human heparinized whole blood to reach a final concentration of 0.25mg/ml. As positive controls, Zymosan and Lipopolysaccharide were used to reach a final concentration of 0.25mg/ml. The ratio of blood to the nanoparticle in MEM was 5:1. The blood was gently pipetted with the nanoparticle resuspension and incubated for 45 minutes at 37 °C in a rotating shaker. Immediately after incubation, samples were placed on ice and then centrifuged at 4000G for 5 minutes. The supernatant plasma was collected in clean microcentrifuge tubes. For the cytokine array panel, 200µl of plasma was used to prepare samples following the protocol, and subsequent steps given in the protocol were followed for the human cytokine array panel (R&D Systems). The final image was taken using a Bio-Rad imager. For further analysis, ImageJ was used. Integrated density gives the sum of total pixels within a region. Following this method, each dot blot within the membrane was quantified for each sample. The levels of the detected cytokines were expressed as a percentage of the pixel density determined for the reference spot.⁶³

Detecting the levels of IL-6 generated from rat endothelial cells exposed to PLA and PLA-PEG nanoparticles

Rat endothelial cells were isolated from epididymal fat pads of Sprague Dawley rats and cultured in 5% bovine aortic endothelial cell conditioned medium until passage 10. For further passages, Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS, 10 mM HEPES, 1mM sodium pyruvate, 1mM L-glutamine, and 1% penicillin/streptomycin was used. Rat endothelial cells were seeded at a density of 150,000 cells/well in tissue culture treated 24-well plates and incubated overnight at 37C and 5% CO₂. Nanoparticles resuspended in MEM or Zymosan resuspended in MEM was added to reach a concentration of 0.25mg/ml and incubated further for 24 hours. The supernatant was removed and centrifuged to separate the nanoparticle pellet, and the collected supernatant was used to quantify the IL-6 levels using the Rat IL-6 Quantikine ELISA Kit (R&D systems, R6000B). The normalized change was determined as follows.

$$\text{Normalized Change} = \frac{\text{Biomarker in sample incubated with Zymosan or nanoparticles}}{\text{Biomarker in sample incubated with MEM only}}$$

Statistical Analysis

We used Chi-square analysis to calculate the p-value within groups. The C5a level for heparinized whole blood or complement protected human serum incubated with PBS or MEM was used as the expected value.

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Supporting Information

A supporting information document can be found online that includes the characterization of polymers used and nanoparticles prepared, NMR spectra for the nanoparticles, and complement protein C5a levels in complement protected human serum.

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