Overcoming Nanoparticle-Mediated Complement Activation by Surface PEG-Pairing

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ABSTRACT. Many PEGylated nanoparticles activate the complement system, which is an integral component of the innate immunity. This is of concern, since uncontrolled complement activation is potentially detrimental, and contributes to disease pathogenesis. Here, it is demonstrated that in contrast to carboxyPEG₂₀₀₀-stabilized poly(lactic-co-glycolic acid) nanoparticles, surface camouflaging with appropriate combinations and proportions of carboxyPEG₂₀₀₀ and methoxyPEG₅₅₀ can largely suppress nanoparticle-mediated complement activation through the lectin pathway. This is attributed to the ability of the short, rigid methoxyPEG₅₅₀ chains to laterally compress carboxyPEG₂₀₀₀ molecules to become more stretched, and assume an extended, random coil configuration. As supported by coarse-grained molecular dynamics simulations, these conformational attributes minimize statistical protein binding/intercalation, thereby affecting sequential dynamic processes in complement convertase assembly. Furthermore, PEG-pairing has no additional effect on nanoparticle longevity in the blood, and macrophage uptake. In conclusion, PEG-pairing significantly overcomes nanoparticle-mediated complement activation without a need for surface functionalization with complement inhibitors.

KEYWORDS: nanoparticles; surface engineering; PEG conformation; protein absorption; complement lectin pathway

The complement system is an enzymatic defense cascade.¹ Complement activation can prime particulate intruders with opsonic C3b and iC3b components, which aid their recognition by the blood leukocytes and tissue macrophages.¹⁻⁴ Surface camouflaging of nanoparticles with hydrophilic polymers, such as poly(ethylene glycol) (PEG) and related copolymers reduces complement activation, but polymer surface density and conformation apparently regulate the mode of complement activation.^{2,6-10} For instance, changes in surface polymer architecture from ‘mushroom’ to intermediary “mushroom-brush” and ‘brush-like’ configurations not only reduce the extent of complement activation, but further shift complement activation from the classical and alternative pathways to the lectin pathway.⁶ Changes in polymer conformation may affect the extent, the type and the mode of protein binding, where some bound proteins through conformational changes trigger complement activation or expose functionalities that allow protein-C3 adducts formation (opsonization).^{3,9,10} Thus, such dynamic processes might explaining the differential complement activation phenomenon.¹⁰ However, the total bound proteins does not determine the efficacy of complement activation and C3 opsonization.¹⁰

Improved surface engineering initiatives are therefore needed to suppress non-specific blood protein deposition, which in turn may diminish or overcome complement activation. When a protein molecule approaches the surface of a polymer-coated nanoparticle, the number of available conformations of surface projected polymer chains is reduced due to compression or interpenetration of polymer chains.¹¹ This develops an osmotic repulsive force due to loss of conformational freedom of the polymer chains. If the polymer surface density is high, it is probable that compression is preferred to interpenetration, while if the density is low interpenetration is likely to dominate. Therefore, surface conditions that generate polymer compression may reduce complement activation processes due to lesser protein

intercalation.^{7,12,13} Here, we have tested this hypothesis through a ‘polymer-pairing’ surface engineering process (combinations of short and long PEG chains), since such combinations can modulate projected polymer chain conformation and distancing.¹⁴

Spherical PEGylated poly(lactic-co-glycolic acid) (PLGA) nanoparticles (SPNs) were considered as a reference system. These nanoparticles have been extensively tested *in vitro* and *in vivo* for diverse biomedical and targeting applications.^{15–18} SPNs were synthesized via an established nano-precipitation method,¹⁵ resulting in a hydrophobic core of PLGA externally stabilized by a monolayer of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-carboxy-PEG₂₀₀₀ conjugate (carboxyPEG₂₀₀₀-DSPE). The carboxylic acid functionality was chosen as earlier studies suggested that nanoparticle-mediated complement activation in human sera may be minimized depending on the carboxylic acid surface density and patterning, which could promote factor H binding (a complement control protein, which processes cofactor activity for Factor I mediated C3b cleavage).^{19–21} In line with these suggestions, our preliminary experiments with PEGylated liposomes has further confirmed the superiority of carboxy-terminated PEGs over their methoxy-terminated counterparts in suppressing complement activation (**Figure S1**). Furthermore, carboxylic functionality may allow for PEG conformational sensing/shielding through electrophoretic mobility measurements. Two different SPN preparations were synthesized: SPN₂₀₀₀, which are surface decorated nanoparticles with carboxy-PEG₂₀₀₀-DSPE (PEG Mw = 2,000 Da); SPN_{2000/550}, which are surface decorated nanoparticles with a combination of carboxyPEG₂₀₀₀-DSPE and methoxyPEG₅₅₀-DSPE (PEG Mw = 550 Da). The methoxy functionality was chosen for PEG₅₅₀-DSPE as to minimize electrostatic interactions with smaller proteins that might penetrate through the carboxyPEG₂₀₀₀ cloud. In the SPN₂₀₀₀ preparation, the

carboxyPEG₂₀₀₀-DSPE concentration varied between 5 and 40% of the total nanoparticle weight. In the case of SPN_{2000/550}, a library of nanoparticles was generated by pairing a fixed amount of carboxyPEG₂₀₀₀-DSPE (20% of the total particle weight) with varying quantities of methoxyPEG₅₅₀-DSPE, ranging from 5 to 20% of the total nanoparticle weight. A schematic representation for the two SPN preparations is provided in **Figure 1a,b**. In the sequel, PEG₂₀₀₀, herein, is considered with a carboxy termination, whereas PEG₅₅₀ is referred to as with a methoxy termination.

The SPN size distribution was examined by both Dynamic Light Scattering (DLS) and Nanoparticle Tracking Analysis (NTA). Typical DLS size distribution profiles are provided in **Figure 1c** and in **Figure S2**, whereas particle sphericity is confirmed by electron microscopy (**Figure 1d**). No significant change in SPN mean hydrodynamic size was detected by DLS with increasing PEG₂₀₀₀ and PEG₅₅₀ levels. For SPN₂₀₀₀, the hydrodynamic diameter ranged between 64.0 ± 9.0 nm and 70.0 ± 10.0 nm, whereas for SPN_{2000/550}, the hydrodynamic diameter varied between 56.0 ± 1.0 nm and 60.0 ± 2.0 nm.

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Nanoparticle size distribution was further studied by NTA. The data in **Figure 2** presents the particle size distribution vs nanoparticles concentration (top rows) and intensity of scattered light obtained in terms of particle concentrations versus size and overall size distribution (bottom rows). For SPN₂₀₀₀, NTA revealed a decrease in SPN mean size on increasing PEG₂₀₀₀ content (**Figure 2a–c**). Specifically, the NTA particle mean size reduced from 81.0 ± 5.0 nm, for the 5%PEG₂₀₀₀, to 68.0 ± 3.0 nm, for the 20%PEG₂₀₀₀, and 52.0 ± 3.0 nm, for the 40%PEG₂₀₀₀ (table

in **Figure 2g**), respectively. The decreasing size trend is in line with a decrease in particle mode and further reflected in gradual loss of nanoparticle subpopulations larger than 100 nm in size, an overall increase in particle number, and a more negative ζ potential (table in **Figure 2g**). The proportion of nanoparticle subpopulation >100 nm were ~20% for the 5%PEG₂₀₀₀, became ~10% for the 20%PEG₂₀₀₀, and, eventually, ~5% for 40%PEG₂₀₀₀ (**Figure 2a-c, upper row**). Scatterplots of relative intensity versus particle size further show an increase in the proportion of nanoparticles <80 nm in size, with higher scattering above the median scattering intensity (> 0.175 arbitrary unit; Q1 and Q2 quadrants) (**Figure 2a-c, lower row**). Considering the significant increase in nanoparticle numbers on increasing carboxyPEG content, the observed changes in ζ potential values may indicate changes in surface projected PEG conformation with better exposure of carboxylic acid functionality (i.e., a more stretched “brush-like” configuration). In contrast to NTA, DLS measurements (table inset in **Figure 2g**) registered comparable mean sizes for all three SPN preparations but with considerably higher values than NTA. These discrepancies may be due to the polydisperse nature of SPN suspensions and presence of a fraction of nanoparticles >80 nm in size, since the measure of total scattered light from an ensemble of SPNs in DLS is heavily weighted towards larger particles. NTA size estimates are generally more accurate, since, unlike DLS, NTA measures the Brownian motion by tracking the movement of individual particles.²² Nevertheless, the decrease in polydispersity index (PDI) on increasing carboxyPEG levels is in line with NTA observations.

On PEG-pairing, the presence of 5% PEG₅₅₀ dramatically reduced mean sizes from 68.0 ± 3.0 nm, for SPN₂₀₀₀ with 20% PEG₂₀₀₀ and 0% PEG₅₅₀, to 46 ± 1 nm, for SPN_{2000/550} with 5% PEG₅₅₀, as determined via NTA (table inset in **Figure 2g**). For the other two combinations, corresponding to 10% and 20% PEG₅₅₀, the mean NTA particle size was very similar with values

of 40 ± 1 nm and 44 ± 1 nm, respectively. This was again reflected in nanoparticle mode reduction, an increase in nanoparticle number and a more negative ζ potential (table in **Figure 2g**). As such, in the SPN_{2000/550}, PEG₂₀₀₀ chains are expected to be laterally supported, and constrained by the short and more ‘rigid’ PEG₅₅₀ chains. This would lead to a more compact and tightly packed configuration, where the PEG₂₀₀₀ chains would extend away from the particle surface in a staple but more stretched ‘brush-like’ conformation with exposed carboxy termini. Differently, in the SPN₂₀₀₀, the long and more flexible PEG₂₀₀₀ chains are expected to partially collapse on the nanoparticle surface, leading to a ‘mushroom-like’ and/or “mushroom-brush” conformation with partially exposed carboxy moieties. This would explain the differences in hydrodynamic size and surface ζ potential documented above in the side-by-side comparison between SPN₂₀₀₀ and SPN_{2000/550}. A further increase in PEG content, however, did not dramatically affect nanoparticle properties. The significant size reduction in PEG-paired SPN_{2000/550} compared with SPN₂₀₀₀ may further imply higher nanoparticle stability.

The NTA results also show that nanoparticle mean size, mode and total concentration remain comparable for all three different SPN_{2000/550} configurations. However, increasing the PEG₅₅₀ content increases the proportion of nanoparticles smaller than 80 nm in size with higher scattering above median scattering intensity (> 1 arbitrary unit; Q1 and Q2 quadrants). Another exception is a substantial decrease in ζ potential when PEG₅₅₀ content is increased to 20%. The reason for this is unclear, but may be a reflection of some PEG₂₀₀₀-conjugate displacement from the surface by increasing numbers of PEG₅₅₀ molecules that compete better for the available space. The observed trends in NTA are also reflected in DLS studies confirming smaller mean sizes and narrower polydispersity on PEG pairing compared with the corresponding native SPN₂₀₀₀ carrying 20% PEG₂₀₀₀. Finally, serum stability of SPNs was assessed in diluted (50%

v/v) and undiluted FBS at 37 °C. There was no observable sign of nanoparticle aggregation within 2h of incubation in serum compared with non-PEGylated SPNs, which rapidly aggregated (**Figure S3**).

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Nanoparticle-mediated complement activation in human sera was assessed by monitoring the generation of anaphylatoxin C5a and the soluble form of C5b-9 (sC5b-9), which are the two established markers of the terminal pathway of the complement system.⁶⁻⁸ The results in **Figure 3** show nanoparticle-mediated rises of C5a and sCb-9 on the basis of equivalent surface area (estimated through NTA). For each nanoparticle type, increasing surface area resulted in higher generation of both markers (**Figure 3a-b**). At the highest surface area tested (1.04 m²/mL serum) complement activation was more profound with 40%PEG₂₀₀₀ SPNs compared with the corresponding nanoparticles of lower PEG content. This may be a reflection of the smaller size of 40%PEG₂₀₀₀ SPNs as compared to 20% and 5%PEG₂₀₀₀ SPNs, since nanoparticle curvature plays a significant role in modulating the conformation of bound immunoglobulins and/or complement sensing molecules. For instance, nanoparticle size was shown to affect IgM binding and conformation,²³ where adsorbed IgM on smaller nanoparticles (e.g., 100 nm) is strained and assumes a staple conformation that triggers activation of the first complement protein (C1), and hence activation of the classical pathway. On the other hand, adsorbed IgM on larger nanoparticles assumes a planar conformation, which does not activate C1.²³ The results in **Figure 3c,d**, further show that complement activation by all SPNs is Ca²⁺-dependent, and does not involve any direct activation through the alternative pathway, which is Mg²⁺-dependent.⁶

In contrast to mono-PEGylated nanoparticles, PEG-pairing dramatically reduced complement activation (**Figure 3e-f**), where incorporation of a minimum of 10% PEG₅₅₀ was sufficient to dramatically decrease C5a and sC5b-9 generation; however, with inclusion of 20% PEG₅₅₀

complement inhibition became more profound. The SPN_{2000/550} with 20% PEG₂₀₀₀ and 20% PEG₅₅₀ hold a total of 5.22×10^{16} PEG-phospholipid molecules/mg nanoparticle, which is slightly more than 20%PEG₂₀₀₀ SPN (4.33×10^{16} PEG-phospholipid molecules/mg nanoparticle), but considerably lower than 40%PEG₂₀₀₀ SPN (8.66×10^{16} PEG-phospholipid molecules/mg nanoparticle), yet this intermediate PEG-lipid level substantially overcome complement activation. By considering the observation that these nanoparticles exhibit similar size distribution profiles, the observed reduction in complement activation is presumably the result of altered PEG₂₀₀₀ spacing and stretching on PEG-pairing. Thus, PEG stretching might not only have generated end-terminal carboxy clusters of appropriate architecture that overcomes complement activation,¹⁹ but also could have limited protein intercalation into PEG₂₀₀₀ cloud, thereby sterically minimizing complement convertase assembly.

PEG-pairing had no direct effect on alternative pathway activation, since complement activation was halted in the presence of EGTA/Mg²⁺ (**Figure 3e,f**). To delineate the role of calcium-sensitive pathways, complement experiments were repeated with both 20%PEG₂₀₀₀ nanoparticles and SPN_{2000/550} (20% carboxyPEG₂₀₀₀ and 20% methoxyPEG₅₅₀) in the presence of 2.25 μ M (final concentration) C1 esterase inhibitor (an inhibitor of the classical pathway components C1r and C1s, the contact system, and fibrinolytic proteases).¹⁰ With all nanoparticle types, no inhibition was observed and equivalent quantities of C4d (an established marker for activation of both classical and lectin pathways)^{6,7} were liberated (serum C4d background level of 3.8 ± 1.1 μ g/mL, and 12.8 ± 1.6 μ g/mL and 13.2 ± 0.9 μ g/mL C4d in the absence and presence of C1 esterase inhibitor for 20%PEG₂₀₀₀ nanoparticles, and 4.9 ± 0.6 μ g/mL and 4.7 ± 0.9 μ g/mL C4d in the absence and presence of C1 esterase inhibitor for SPN_{2000/550}, respectively). These observations not only exclude the role of classical pathway in complement activation, but also confirm the lesser ability of PEG-paired nanoparticles in activating complement. Therefore, the observed complement activation is most likely through the lectin pathway, and this is further supported by lack of C4d liberation on nanoparticle addition to serum pretreated with Futhan (150 μ g/mL), which is a broad-spectrum serine

protease inhibitor capable of inhibiting lectin pathway mannose-binding lectin (MBL) (or collectins/ficolins)/MBL-associated serine proteases (MBL-MASPs) ($2.8 \pm 0.6 \mu\text{g/mL}$, $3.1 \pm 0.5 \mu\text{g/mL}$, and $3.3 \pm 0.8 \mu\text{g/mL}$ for background, 20%PEG₂₀₀₀ and 20%PEG₂₀₀₀/20%PEG₅₅₀–paired nanoparticles, respectively). Therefore, PEG-pairing interferes with the action of MBL/ficolins/collectins-MASPs, presumably by minimizing their intercalation into the PEG cloud.

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To further gain insight into the above mentioned observations, coarse-grained (CG) Molecular Dynamics (MD) simulations within the Martini framework^{24,25} were performed to predict the organization of PEG chains and the interaction with blood molecules. Following the experimental cases above, five different configurations were tested (**Figure 4a**): 20%PEG₂₀₀₀ and 40%PEG₂₀₀₀, representing conventional camouflaging approaches with long PEG chains solely; 10%PEG₅₅₀-20%PEG₂₀₀₀ and 20%PEG₅₅₀-20%PEG₂₀₀₀, representing PEG-pairing approaches with short and long PEG chains together; 20%PEG₅₅₀, representing a control configuration with low density, short PEG chains.

The results of the simulations are presented in **Figure 4b** in terms of lipid concentration, water molecules and PEG₂₀₀₀ content along the z-coordinate. Moving away from the surface, it is observed a sharp decrease in lipid concentration from $1,100 \text{ kg/m}^3$ on the inner most layer to zero at about 3.5 nm, an increase in the density of water molecules from nearly zero in the core to $\sim 1,000 \text{ kg/m}^3$ in the bulk fluid, and a biphasic variation in PEG₂₀₀₀ density reaching a maximum of 4–5 nm. The sharp reduction in the lipid content is ascribed to the fact that lipid chains are localized only in the inner most layer (red layer in **Figure 4a**), whereas the outer

region is mostly occupied by polymer chains and water molecules. Significant variations for the water and PEG concentrations are observed for all configurations. Specifically, as the concentration of PEG₂₀₀₀ and PEG₅₅₀ increases, the density of water molecules adjacent to the lipid layer ($z > 2$) reduces, while the center of mass for the PEG₂₀₀₀ distribution progressively moves towards larger z values (from 4 to 5 nm). Importantly, for the 20%PEG₅₅₀-20%PEG₂₀₀₀ paired configuration, the maximum PEG₂₀₀₀ density is at $z = 5$ nm. Simulations also allowed to quantify the end-to-end distance for the PEG₂₀₀₀ chains (**Figure 4c**). For the 20%PEG₅₅₀-20%PEG₂₀₀₀ configurations, PEG₂₀₀₀ chains appeared more elongated with an end-to-end distance of 5.3 ± 0.1 nm as compared to the conventional system 20%PEG₂₀₀₀ returning a value of 4.9 ± 0.1 nm. Collectively, these results further support the notion that the inclusion of PEG₅₅₀ guides the PEG₂₀₀₀ chains to predominantly display a ‘stretched brush-like’ architecture.

MBL/ficolins/collectins-MASPs are large complexes and their intercalation into the PEG cloud is expected to be more challenging compared with smaller proteins, such as albumin. Thus, in order to estimate the effect of PEG conformational displays on protein intercalation, the separation distance between albumin molecules and the lipid-layer was monitored over time for the five different cases (**Figure 4d**). MD simulations predicted three distinct behaviors: protein adsorption at the PEG coating-water interface; partial protein intercalation within the PEG chains; deep protein intercalation into the PEG cloud. At first instance, protein persistence at particle surfaces is modulated by chain mobility and softness of the PEG outer-layer.^{26,27} At the PEG-water interface (between 5 and 10 nm), the paired combination of 20%PEG₅₅₀–20%PEG₂₀₀₀ induced an overall larger fluctuation in albumin separation distance compared with conventional 20%PEG₂₀₀₀ and 40%PEG₂₀₀₀ platforms (**Figure S4**). This would suggest an overall higher outer-layer deformability for the PEG-paired configuration that could more efficiently repel any

approaching protein, and possibly limit protein intercalation. At the opposite extreme, albumin would intercalate deep into the PEG cloud in the case of a low density, short PEG₅₅₀ configuration (20%PEG₅₅₀ cyan line in **Figure 4d**). A similar behavior was also observed with the 20%PEG₂₀₀₀ configuration, displaying a low density long PEG₂₀₀₀ chains (black line in **Figure 4d**). As short PEG₅₅₀ chains were inserted into a lipid layer with 20%PEG₂₀₀₀, albumin intercalation was delayed and occurred partially (10%PEG₅₅₀-20%PEG₂₀₀₀ blue line and 20 PEG₅₅₀-20%PEG₂₀₀₀ red line in **Figure 4d**; **Movie S1** and **S2**, respectively). A maximum in steric hindrance was observed in the case of lipid platforms exhibiting 40%PEG₂₀₀₀ (green black line in **Figure 4d**) for which the separation distance of the albumin from the lipid-layer was only slightly larger than in the case of the PEG-paired 20%PEG₅₅₀-20%PEG₂₀₀₀ (red line in **Figure 4d**). It should be emphasized that although the dense 40%PEG₂₀₀₀ surfaces appear to block more efficiently albumin intercalation compared with the PEG-paired 20%PEG₅₅₀-20%PEG₂₀₀₀ platforms, simulations do not account for the contribution of surface curvature on PEG conformation, or the overall particle stability in suspension. On a curved surface, the projected long PEG₂₀₀₀ chains would tend to open up more than short PEG₅₅₀ chains, thus facilitating protein intercalation. Collectively, these simulations would indicate that surface PEG-pairing efficiently limits binding and/or intercalation of medium- and small-sized proteins compared to PEG₂₀₀₀ grafted surfaces. Indeed, this is in line with the complement activation studies documented in **Figure 3**, and would support the notion that MBL/ficolins/collectins-MASPs intercalation into the PEG-paired cloud is impaired.

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It has long been known that the steric hindrance of the surface projected PEG chains can minimize nanoparticle-phagocyte contact, despite opsonization.^{3,4} Accordingly, we sought to investigate whether PEG-pairing can suppress nanoparticle uptake by J-774.A1 macrophages (which express complement receptor 3). The data in **Figure 5a** support the suppressive effect of increasing PEG₂₀₀₀ density on SPN₂₀₀₀ internalization by macrophages (reduced red fluorescent intensity with increasing PEG₂₀₀₀ surface density from 5% to 40%). Thus, by considering that complement activation (and presumably C3b/iC3b opsonization) proceed better with 40%PEG₂₀₀₀ SPNs compared with nanoparticles of lower PEG density (**Figure 3**), these observations confirm that PEG conformation (and steric hindrance) plays a modulatory role in nanoparticle-cell interaction, and therefore interfere with ligation of surface-bound opsonins with macrophage receptors. Flow cytometry was also employed to quantitatively determine differences in cell uptake between 20%PEG₂₀₀₀ SPNs and PEG-paired nanoparticles. No significance difference was detected among 20%PEG₂₀₀₀ SPNs and 20%PEG₂₀₀₀ SPNs carrying either 5% or 20%PEG₅₅₀ (**Figure 5b,c**). Again, this supports the notion that the PEG steric barrier is the dominant factor in modulating the extent of nanoparticle-cell interaction,^{3,4} and its efficacy remains unaffected on surface PEG-pairing. In line with these observations, comparable blood clearance kinetics was also observed between single and PEG-paired Cu⁶⁴-DOTA-labelled SPNs upon intravenous injection (**Figure 5d**). The blood clearance kinetics is similar to the previously described PEGylated nanosystems.^{29,30} In this respect, it is important to highlight that the proposed PEG-pairing is different from other hierarchical PEG surface structures, such as the double-PEG layer,²⁷ in that the main objective here is minimizing complement activation.

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In conclusion, this report showed that surface PEG-pairing of nanoparticles could minimize complement activation without further affecting macrophage clearance and circulation profile. Considering these properties and the observation that PEG-paired stabilized SPNs generate considerably less C5a in comparison to conventional PEGylated species, PEG-pairing may offer a better strategy for design and engineering of safer nanomedicines. Indeed, intratumoral complement activation by extravasated PEGylated nanomedicines could potentially promote tumor growth, since the build-up of a sufficient C5a gradient helps with recruitment of immunosuppressive cells.³¹ Thus, PEG-pairing may be considered as an alternative cost-effective approach for overcoming complement activation compared with nanoparticles grafted with complement inhibitors. Finally, the residual level of complement activation products by PEG-paired SPNs may be ascribed to the heterogeneity of the current system, and presumably arising from the presence of a small subpopulation of larger nanoparticles (>80 nm) with poorer PEG shielding. Improved nanoprecipitation procedures (e.g., through microfluidic processes)³² may eventually yield a more homogenous population of complement-evading PEG-paired nanoparticles.

Supporting Information

The following files are available free of charge: PDF file (Methods and Materials and Supporting Figures S1–S4) and Movies S1 and S2.

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M.P. performed MD simulations; S.E., J.K., and S.A. synthesized and characterized SPNs; L-P.W. performed complement and NTA studies; all authors contributed to experimental planning, and data analysis. S.M.M. and P.D. conceived the idea, supervised the work, and wrote the manuscript with contributions from all co-authors.

Notes

The authors declare no competing interests.

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Figure legends:

Figure 1. Geometrical configuration and physicochemical characterization of SNP₂₀₀₀ and SNP_{2000/550}. Schematic representation of conventional SNP₂₀₀₀ consisting of a poly(lactic acid-co-glycolic acid) (PLGA) core, a lipid monolayer shell and a hydrophilic coating of carboxyPEG₂₀₀₀-phospholipid chains on the surface (PEG₂₀₀₀-DSPE). **(b)** Schematic representation of PEG-paired SNP_{2000/550} consisting of a PLGA core, a lipid monolayer shell and a hydrophilic coating of carboxyPEG₂₀₀₀-phospholipid and methoxyPEG₅₅₀-phospholipid chains on the surface (PEG₂₀₀₀-DSPE and PEG₅₅₀-DSPE). **(c)** Hydrodynamic diameter (nm) of SNPs with different carboxyPEG₂₀₀₀ contents, measured by dynamic light scattering (DLS). **(d)** A representative scanning electron micrograph of SNP₂₀₀₀. The scale bar in inset = 100 nm.

Figure 2. Size distribution profiles of different SPN₂₀₀₀ and SPN_{2000/550} determined by Nanoparticle Tracking Analysis (NTA). **(a–c)** Size distribution profile (upper row) and 2D plots of relative light scattering intensity of particles versus the estimate of the particle size for different SPNs₂₀₀₀ (lower row). **(d–f)** Size distribution profile (upper row) and 2D plots of relative light scattering intensity of particles versus the estimate of the particle size of different SPN_{2000/550} (lower row). **(g)** List of key geometrical features for SPNs determined via NTA and DLS analyses.

Figure 3. Mediated complement activation in human serum for SPNs. Panels **(a)** and **(b)** show surface area-dependent SPN₂₀₀₀-mediated rises of serum sC5b-9 and C5a above the background. Zymosan (1 mg/mL) was used as a positive control for monitoring complement activation. Panels **(c)** and **(d)** distinguishes complement activation between calcium-sensitive pathways and the alternative pathway turnover. Panels **(e)** & **(f)** show serum sC5b-9 and C5a

levels on SPN_{2000/550} addition. * $p < 0.05$; ** $p < 0.01$. Similar results were obtained in three other tested sera.

Figure 4. Molecular dynamics simulations for PEG-paired SPNs. (a) Computational domain (left) for the 20%PEG₅₅₀ configuration, including PEG₂₀₀₀ chains (green), PEG₅₅₀ chains (cyan), lipids (red). List of different computed configurations (right). (b) The density of lipids (lipid layer line), water molecules (water line), and PEG₂₀₀₀ (PEG₂₀₀₀ line) is plotted along the z-coordinate, normal to the surface. (20%PEG₂₀₀₀ – black; 40%PEG₂₀₀₀ – green; 10%PEG₅₅₀-20%PEG₂₀₀₀ – blue; 20%PEG₅₅₀-20%PEG₂₀₀₀ – red; 20%PEG₅₅₀ – cyan). (c) End-to-end distance for PEG₂₀₀₀ chains. (d) Distance between an albumin molecule (blue) and the center of mass of the lipid layer headgroups over a period of 4 μ s. The table inset shows mean distance values computed over the last 2 μ s. The black circle inset shows an albumin molecule interacting with a 20%PEG₂₀₀₀ platform. The red circle inset depicts an albumin molecule interacting with a 20%PEG₂₀₀₀-20%PEG₅₅₀. These images were back-mapped from a coarse-grained to an atomistic representation (gromos54a7 forcefield).²⁸

Figure 5. Cell uptake and in vivo blood longevity of SPNs. (a) Confocal microscopy images of J-774.A1 macrophages incubated for 2h with 5%, 20%, 40%PEG₂₀₀₀ SPNs. (SPN₂₀₀₀ labeled with red fluorescent dye (PE-RhB), and cell nuclei are labeled with 4',6-diamidino-2-phenylindole (DAPI)). (b) Flow cytometry analysis showing the extent of SPN_{2000/550} association with J-774.A1 cells at 1h post incubation. (c) Flow cytometry analysis of SPN_{2000/550} association with J-774.A1 phagocytes at 1 and 2 h post incubation, as a function of the PEG₅₅₀ content (0%, 5% and 20%). In all cases, nanoparticles carried 20%PEG₂₀₀₀. (d) Blood concentration of Cu⁶⁴-

labeled SPN₂₀₀₀ and SPN_{2000/550}, with different PEG₅₅₀ content (0%, 5% and 20%) in healthy mice following tail-vein injection (n = 3).