Published in final edited form as:

ACS Nano. 2016 December 27; 10(12): 11290–11303. doi:10.1021/acsnano.6b06451.

Tailoring Nanostructure Morphology for Enhanced Targeting of Dendritic Cells in Atherosclerosis

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Abstract

Atherosclerosis, a leading cause of heart disease, results from chronic vascular inflammation that is driven by diverse immune cell populations. Nanomaterials may function as powerful platforms for diagnostic imaging and controlled delivery of therapeutics to inflammatory cells in atherosclerosis, but efficacy is limited by nonspecific uptake by cells of the mononuclear phagocytes system (MPS). MPS cells located in the liver, spleen, blood, lymph nodes, and kidney remove from circulation the vast majority of intravenously administered nanomaterials regardless of surface functionalization or conjugation of targeting ligands. Here, we report that nanostructure morphology alone can be engineered for selective uptake by dendritic cells (DCs), which are

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Author Contributions

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Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.6b06451.

Supplemental figures (PDF)

critical mediators of atherosclerotic inflammation. Employing near-infrared fluorescence imaging and flow cytometry as a multimodal approach, we compared organ and cellular level biodistributions of micelles, vesicles (*i.e.*, polymersomes), and filomicelles, all assembled from poly(ethylene glycol)-*b*-poly(propylene sulfide) (PEG-*b*-PPS) block copolymers with identical surface chemistries. While micelles and filomicelles were respectively found to associate with liver macrophages and blood-resident phagocytes, polymersomes were exceptionally efficient at targeting splenic DCs (up to 85% of plasmacytoid DCs) and demonstrated significantly lower uptake by other cells of the MPS. In a mouse model of atherosclerosis, polymersomes demonstrated superior specificity for DCs ($p < 0.005$) in atherosclerotic lesions. Furthermore, significant differences in polymersome cellular biodistributions were observed in atherosclerotic compared to naïve mice, including impaired targeting of phagocytes in lymph nodes. These results present avenues for immunotherapies in cardiovascular disease and demonstrate that nanostructure morphology can be tailored to enhance targeting specificity.

Graphical Abstract

Keywords

polymersome; biodistribution; near-infrared fluorescence imaging; flow cytometry; dendritic cells; targeted delivery; atherosclerosis

An unsolved challenge for the controlled delivery of therapeutics is nonspecific cellular uptake by the mononuclear phagocytes system (MPS), which consists of phagocytic cells in the liver, spleen, lymph nodes (LNs), kidneys, and blood.¹ These monocytes, macrophages, and dendritic cells (DCs) readily clear nanomaterials from circulation regardless of their engineered surface chemistries or targeting ligands, which can result in decreased efficacy and adverse effects.² Certain populations of MPS cells serve as professional antigen presenting cells (APCs), which process and present nano- and microscale pathogens for the generation of controlled inflammatory and immune responses. The function and inflammatory potential of each APC subset are distinct, and differences in preferred mechanisms of uptake and surface receptor concentration as well as organ location contribute to the efficiency of pathogen and nanomaterial endocytosis and the resulting generation of directed immune responses.^{3–6} All of these factors can be influenced by nanostructure morphology, *i.e.*, the geometry (size, shape and aspect ratio), which can determine cell membrane interactions, transport through biological fluids and tissues, circulation time, and intracellular delivery.^{7–9} We therefore hypothesized that by mimicking the distinct nanostructures (NS) of viruses while maintaining the same surface chemistry, different MPS cell populations could be targeted by nanomaterials to different degrees without the use of a targeting ligand. Enhanced targeting of specific MPS subsets by

nanomaterials may decrease off-target effects of drug therapy, improve targeted immunotherapy, and offer treatments for inflammation-driven pathologies like cardiovascular disease (CVD).^{10–13}

Atherosclerosis is an inflammatory condition within the walls of arterial vessels and a principal cause of CVD. Accumulation of inflammatory cells and their products induces maturation of atheromas, or plaques, ultimately resulting in plaque rupture, leading to ischemic stroke or myocardial infarction.¹⁴ Since monocytes and macrophages are primary mediators of lipid accumulation within arterial vessel walls, they have become the focus of targeted delivery for imaging and treatment of atherosclerosis.¹² However, atherosclerotic lesions contain a complex mixture of diverse immune cell populations, including T cells, neutrophils, eosinophils, and DCs.^{15–17} Activated by a uniquely diverse range of pattern recognition receptors,¹⁸ DCs progress from preDC precursors to mature DCs, which are marked by heightened expression of cytokines, chemokines, and cell surface coreceptors that activate diverse T cell subsets and drive atherosclerotic inflammation. DCs are found within atheromas at all stages of lesion development, and although their accumulation correlates with the level of plaque instability, they have been found to be both atherogenic as well as atheroprotective, likely a result of their heterogeneity.^{19–23} Much attention has been generated for the targeting of DCs in cancer immunotherapies,^{11,24,25} but few therapeutic strategies have focused on targeting these cells in CVD.

Self-assembled NS formed from poly(ethylene glycol)-*b*-poly(propylene sulfide) (PEG-*b*-PPS) have been shown to be versatile vehicles for intracellular delivery to and modulation of DCs.^{9,26,27} Depending on the individual block lengths, these copolymers can be engineered to assemble into a variety of different nanostructures in aqueous solutions, including spherical micelles, vesicles (*i.e.*, polymersomes), and filamentous wormlike micelles (*i.e.*, filomicelles).^{28–30} The highly hydrophobic PPS block drives the self-assembly and is responsible for aggregate stability and the stable retention of lipophilic payloads, which has proven advantageous for both *in vitro* and *in vivo* imaging using hydrophobic fluorescent dyes.^{26,27,31} Furthermore, the low critical micelle concentration of $\sim 10^{-7}$ M and lyotropic character of the PPS core contribute to the previously observed stability of PEG-*b*-PPS copolymer NS under physiological conditions.^{8,9,29,32–35} The hydrophilic PEG fraction (f) of the total block copolymer molecular weight dictates aggregate morphology, as demonstrated by $f > 45\%$ forming micelles, $f < 25\%$ forming inverted microstructures, and $45\% > f > 25\%$ forming vesicles.³⁶ PEG-*b*-PPS is nonimmunogenic and noninflammatory, possessing an immunostimulatory potential based solely upon their selected molecular payloads.²⁷ Contributing to this inertness is the dense outer PEG corona that provides a neutrally charged, highly hydrated surface to resist protein adsorption, modulate the protein corona, and minimize nonspecific cellular interactions with PEG-*b*-PPS NS.^{5,37–39} Although PEG provides an excellent platform for chemical modification and conjugation, maintaining a consistent surface chemistry, regardless of the encapsulated payload, can enhance reproducibility during therapeutic applications.^{5,40,41} On the contrary, PEG-functionalized nanomaterials without surface conjugated targeting ligands can stealthily evade cellular interactions, but still cannot avoid eventual uptake by the constitutively phagocytic cells of the MPS, in part due to dynamic changes in their protein corona.^{1,42}

DCs are present at considerably lower concentrations than monocytes and macrophages within organs of the MPS and atheromas, yet can contribute extensively to the local and systemic maintenance of inflammation and the progression of atherosclerosis.^{1,16,21,22} Here, we investigated nanostructure-enhanced targeting (NSET) of DCs compared to other MPS cells and applied our findings toward the improved targeting of DCs in atherosclerosis in the absence of surface-conjugated targeting moieties. We synthesized ~20 nm micelles (MC), ~100 nm polymersomes (PS), and ~50 nm × micron length filomicelles (FM) using PEG-*b*-PPS block copolymers to maintain a consistent surface chemistry between all NS. The organ and cellular biodistributions of each morphology were respectively assessed qualitatively by near-infrared fluorescence (NIRF) imaging and quantitatively by flow cytometry following intravenous (i.v.) injection into naïve and atherosclerotic C57BL/6 mice. Our results demonstrate that assessing the influence of nanostructure morphology on cellular biodistributions under different disease conditions may provide an additional method of enhancing cellular targeting as an alternative or supplement to surface conjugated targeting ligands.

RESULTS AND DISCUSSION

Fabrication and Characterization of PEG-*b*-PPS Nanostructure Morphologies

To investigate the influence of nanostructure morphology on *in vivo* uptake by diverse cell subsets, we synthesized three different NS, which were all self-assembled from PEG-*b*-PPS block copolymers and possessed the same surface chemistry: PEG₄₅-*b*-PPS₂₀ MC, PEG₁₇-*b*-PPS₃₀ PS, and PEG₄₄-*b*-PPS₄₅ FM. Figure 1 shows the representative size and morphology of the NS as determined by cryogenic transmission electron microscopy (CryoTEM). The hydrodynamic size and size distributions of MC and PS were further characterized by dynamic light scattering (DLS) (Table 1).

To qualitatively explore the influence of nanomaterial morphology on biodistributions at the organ level, assembled NS were loaded with NIRF imaging agent indocyanine green (ICG). The incorporation of ICG into nanomaterials has been demonstrated to improve its optical properties and stability.⁴³ We characterized the stability and loading of ICG within PEG-*b*-PPS MC *via* CryoTEM, UV–vis absorbance, and fluorescence spectroscopy. ICG encapsulation efficiencies of 80%–90% were achieved for all three NS using thin-film hydration, which is a robust method of loading and assembling PEG-*b*-PPS NS.^{9,26,27} The emission spectrum shifted in wavelength and increased in intensity for the same concentration of ICG when comparing ICG-loaded NS to free ICG (Figure 2A,B). The absorbance steadily increased as the loaded ICG concentration increased within the NS (Figure S1A). We calculated the optimal loading for the strongest IR signal to be 2.3 μ M, or a 33:1 molar ratio of copolymer:dye (Figure 2C). Self-quenching of the IR emission was observed at higher concentrations and likely due to j-aggregate-induced static quenching.⁴³ CryoTEM images revealed no changes in size nor morphology after incorporation of ICG into any of the NS (Figures 2D and S1B,C).

A Distinct Organ Biodistribution Was Observed for Each Nanostructure Morphology

Nanomaterials have been widely used to deliver therapeutic agents *in vivo*, and their tissue distribution can either limit or enhance such applications. ICG-loaded MC, PS, and FM were therefore assembled at a 33:1 molar ratio of copolymer:dye, and their biodistributions were compared to control injections of free-form ICG and PBS in naïve C57BL/6 mice. Liver, spleen, kidneys, lung, and heart were harvested and investigated with an IVIS optical imaging system. Following i.v. injection, ICG usually has a half-life of <10 min primarily due to removal from circulation in the liver.⁴⁴ As expected, we observed immediate accumulation of ICG in the liver after 1 h and no observable fluorescence at the 24 h time point (Figure 2E). In contrast, mice injected with ICG-loaded NS revealed strong fluorescent signals from multiple organs at 1, 24, and 48 h post-injection, indicating an extension of the circulation time of ICG due to encapsulation within NS (Figure 2E). At all time-points, stronger fluorescence from liver, spleen, and kidneys of mice treated with PS and MC was detected compared to mice treated with FM, which revealed minimal accumulation in organs at the 24 h time point (Figure 2E). Furthermore, significantly more PS were distributed in spleen than MC, FM, and free ICG ($p < 0.01$) 1 and 24 h post-injection (Figure S2A, B). Surprisingly, only PS were detectable in the spleen 48 h after injection ($p < 0.001$) (Figures 2E and S2). As the largest lymphatic organ and home to diverse immune cell populations, the spleen has been regarded as a promising target for vaccination and immunotherapy, and our results suggest PS to be an excellent nanocarrier for these applications.

The minimal detection of FM in all organs after the 1 h time-point suggested decreased uptake of FM by the MPS compared to MC and PS. This observation is consistent with previous studies comparing filamentous to spherical morphologies.^{8,45,46} Since our NS are all formed from PEG-*b*-PPS copolymers of identical surface chemistry, our results further support that morphology alone can dramatically affect the biodistribution of nanomaterials *in vivo*. In summary, PS preferentially targeted spleen for over 48 h, while spherical MC had higher accumulation in liver and kidneys up to 24 h post-injection, and FM had negligible presence within MPS cells after 1 h.

A Distinct Biodistribution Was Observed within Inflammatory Cell Populations for Each Nanostructure Morphology

To understand and predict the effects of delivered therapeutics, it is essential to investigate not only the general organs that are targeted but also the specific cell populations.^{24,25} Inflammatory responses and many drug-induced side effects are generated by cells within both the MPS and broader immune system. Therefore, we further investigated the influence of nanostructure morphology on the cellular distribution within organs of the MPS and immune system following i.v. administration. In order to both track and quantify the biodistributions of each NS without modifying their PEG surface chemistry or self-assembly, we covalently conjugated the lipophilic fluorophore DyLight 650 maleimide onto the thiol-functionalized ends of the hydrophobic PPS blocks of the PEG-*b*-PPS copolymers after their assembly in aqueous solution. The DyLight 650-labeled NS were verified to be stable and unchanged in size and morphology by CryoTEM (Figure S1B).

Multicolor flow cytometry was performed to evaluate the uptake of our NS within cells of the innate and adaptive immune system. Mice were administered DyLight 650-labeled PS, MC, and FM by tail vein injection. After different time points (1, 24, and 48 h), spleen, LN, liver, kidneys, and blood were collected and prepared into single cell suspensions for analysis (gating strategies shown in Figure S3). We separately measured the percentages of NS positive (NS⁺) cells within each major inflammatory cell population, which allowed us to compare the uptake of NS between high and low concentration cells in each organ. For all organs evaluated, NS were predominantly associated with phagocytic cells in the MPS (Figures 3 and S4). In accordance with our organ-level ICG analysis, significantly higher PS association was observed for immune cells in the spleen, where all NS associated strongly with macrophages and DCs and minimally with natural killer (NK) cells, granulocytes, and T cells (Figure 3A,E,I). Among these, between 30%–40% of isolated macrophages and DCs were PS positive (PS⁺) at the 1 h time point, which were significantly higher than for MC ($p < 0.0001$) and FM ($p < 0.005$) (Figure 3A). Although no significant differences were observed between PS uptake and MC uptake for macrophages, significantly higher percentages of DCs were PS⁺ 24 h ($p < 0.01$) and 48 h ($p < 0.005$) post-injections (Figure 3E,I). For MC and FM, the cellular distribution showed no significant difference after 24 h (Figure 3E,I).

Intravenously injected nanomaterials require more time to reach LNs, since they must first exit circulation and drain from peripheral tissue. As expected, uptake of NS by immune cells in LNs was delayed compared to the spleen and liver, and minimal association with cells was detected until 24 to 48 h post-injection (Figure 3B,F,J). Significantly more PS⁺ macrophages and PS⁺ DCs were found compared to micelle positive (MC⁺) macrophages ($p < 0.005$), MC⁺ DCs ($p < 0.005$), filomicelle positive (FM⁺) macrophages ($p < 0.01$), and FM⁺ DCs ($p < 0.005$) 24 and 48 h after administration (Figure 3F,J).

As the main blood-filtering organ, the liver is enriched in macrophages, DCs, NK cells, and T cells, but contains minimal granulocytes.⁴⁷ All NS were detected at high levels in hepatic macrophages (Figure 3C,G,K), which represent up to 80–90% of the total body macrophage pool.⁴⁸ At 1 and 24 h post-injection, PS⁺ and MC⁺ macrophages and DCs showed no significant difference in uptake (Figure 3C,G). However, after 48 h administration, MC was found to target over 90% of macrophages and 65% of DCs in the liver, which is significantly more than PS (targeting 40% of macrophages, $p < 0.0001$ and 35% of DCs, $p < 0.005$) and FM (targeting 25% of macrophages, $p < 0.0001$ and 40% of DCs, $p < 0.005$) (Figure 3K).

FM exhibited low association with immune cells in the MPS, but were taken up by significantly higher percentages of monocytes, granulocytes (predominantly neutrophils), and macrophages in blood compared to PS and MC (Figure 3D,H,L). FM associated with approximately 90% of blood neutrophils 1 h after i.v. administration and up to 80% of blood monocytes after 24 h (Figure 3D,H). This rapid uptake by blood cells may have contributed to the significantly lower serum concentration of FM at the 1 h time point compared to MC and PS ($p < 0.005$) (Figure S5). The relatively steady FM blood concentration over the course of 48 h is possibly due to minimal association with MPS cells in the liver, spleen, and LN, allowing longer blood circulation and increased opportunity for uptake by blood phagocytes.

In summary, PS exhibited superior targeting of DCs in lymphoid organs (spleen and LN), while MC showed preferential uptake by macrophages in the liver. FM were rapidly taken up by blood-resident monocytes and neutrophils and exhibited longer circulation times and decreased uptake by MPS cells in the liver, spleen, and LN. These results highlight the promising potential of NSET for detection and modulation of distinct inflammatory cell populations and in particular for the targeting of DCs by the PS morphology.

Polymersomes Target Relevant DC Subsets in Lymphoid Organs of Naïve Mice

Although of critical importance for understanding and predicting inflammatory responses, very little research has been performed to explore the cellular biodistribution of nanomaterials within DC subsets. We therefore used flow cytometry to probe the influence of morphology on the *in vivo* uptake of our NS by several key DC populations. Multiple subsets of DCs, which possess different cell surface markers, cytokine expression, and immunological functions, have been identified.⁴⁹ DCs can be broadly divided into classical DCs (cDCs) and plasmacytoid DCs (pDCs).⁵⁰ Murine cDCs are further divided into myeloid DCs (CD11b⁺ DCs) and lymphoid DCs (CD8⁺ DCs), which are respectively analogous to human CD1c⁺/cDC2 that have diverse Th1, Th2, or Th17 polarized antigen/adjuvant-dependent responses and CD141⁺/cDC1 cells that efficiently cross-present antigen for cytotoxic T cell activation.^{1,49,51} pDCs both activate athero-protective regulatory T cells (Tregs) in an IDO-dependent manner as well as release proatherogenic type I interferons (IFN).^{52,53} Thus, it is critical to understand which specific subsets of DCs are targeted by nanomaterials when developing strategies for therapy and diagnosis.

Since PS associated with higher percentages of DCs overall compared to MC and FM, we further investigated whether this held true across the major DC subsets. PS demonstrated significantly higher association with all DC subsets than MC and FM (Figure 4), which is consistent with our previous data showing more PS⁺ DCs than MC⁺ and FM⁺ DCs in the spleen and draining LNs (Figure 3). Furthermore, unlike MC and FM, PS associated with mature DCs to a significantly higher degree than preDCs at all time points in the spleen and 24 h post-injection in the LN. Although this may suggest that uptake of PS induced DC maturation, PEG-*b*-PPS NS are non-immunogenic and noninflammatory unless loaded with antigen or adjuvant.^{26,27} An alternative explanation is that the preferred mechanism for uptake of PS were receptor-mediated endocytosis or phagocytosis, while MC and FM entered cells primarily *via* macropinocytosis, since macropinocytosis is dramatically reduced following DC maturation.⁵⁴ Receptor-mediated endocytosis is known to be dependent on nanomaterial shape and size and has been modeled as a function of cell surface receptor surface density and membrane diffusion.⁴

The percentage of PS⁺ pDCs was significantly higher than that of MC⁺ and FM⁺ pDCs at all time points tested in the spleen and 24 h post-injection in the LN (Figure 4). At only 1 h after injection, over 60% of isolated pDCs were PS⁺ (Figure 4A). Most strikingly, PS showed very high capacity to target pDCs (up to 85% in spleen and 75% in LNs) compared to CD11b⁺ DCs (~40% in spleen and 45% in LNs) and CD8⁺ DCs (~20% in spleen and LNs) (Figure 4B,E). Overall, distinct nanostructure morphologies revealed significantly different distributions in DC populations, with PS exhibiting a significantly higher capacity

to target all DC subsets in lymphoid organs (spleen and LNs) with a particular affinity for pDCs. In addition to their well-documented role in responding to viral infection with a massive production of type I interferon (IFN), pDCs are attracting more attention as targets in immunotherapies due to their roles in tolerance and atherosclerosis.^{49,55} PS may therefore serve as an excellent vehicle for nanomaterial-based immunotherapies.

Polymersomes Target DCs in Atherosclerotic Lesions and Lymphoid Organs of *Ldlr*^{-/-} Mice

Studies have demonstrated that numerous immune cells including lymphocytes, macrophages, DCs, and neutrophils play critical roles in the development and maturation of atherosclerotic lesions. Nanomaterials incorporated with small molecules, chelated ions, or metals have been established as promising components of strategies for diagnosis and treatment of atherosclerosis.⁵⁶ The accumulation and internalization of nanomaterials by specific immune cell populations could therefore substantially increase diagnostic and therapeutic efficacy and reduce risk factors.⁵⁷ Since atheroma-resident DCs increase in number during lesion development and both these and splenic DCs can induce tolerance against autoantigens in the arterial micro-environment to suppress T cell activation and proliferation, DCs are promising therapeutic targets for immunomodulation of atherosclerosis.^{12,53,57} Given the marked differences in uptake of NS by immune cells in naïve mice and the promising DC targeting capacity of PS, we further explored the cellular distribution of these three separate NS among pathological lesions and lymphatic organs of atherosclerotic mice. Atherosclerotic lesions were induced by keeping *Ldlr*^{-/-} mice on a high-fat diet for 16 weeks. After 24 h, i.v. injections of MC, PS, and FM, aorta, spleen, LNs, and liver from *Ldlr*^{-/-} mice were harvested and analyzed by flow cytometry as previously described. A significantly higher percentage of PS⁺ DCs ($p < 0.01$ and $p < 0.005$) was detected compared to MC⁺ and FM⁺ DCs in the aorta of atherosclerotic mice (Figure 5A). However, MC and FM were shown to be taken up in much higher percentages by macrophages ($p < 0.0001$), B cells ($p < 0.005$; $p < 0.005$), and NK cells ($p < 0.005$; $p < 0.005$) than PS in atherosclerotic aorta (Figure 5A). We additionally analyzed NS⁺ DCs and other immune cells in the spleen, LNs, and liver of *Ldlr*^{-/-} mice. It was found that NS were predominantly associated with APCs (macrophages and DCs) in the spleen and liver, while they were minimally internalized by immune cell populations in LNs (Figure 5B–D). Within APC subsets, PS were taken up by over 30% of splenic DCs and up to 72% of DCs in the liver, which were significantly more than MC ($p = 0.027$ and $p < 0.005$) and FM ($p < 0.005$ and $p < 0.0001$) (Figure 5B,D). However, macrophages in spleen displayed much higher association with MC ($p = 0.022$) and FM ($p < 0.005$) compared to PS (Figure 5B). Thus, these findings further suggested that the PS morphology could be used to enhance targeting of splenic and lesion-resident DCs in atherosclerosis.

Polymersomes Target Significantly Lower Percentages of Macrophages in Spleen while Maintaining High Levels of Uptake by Dendritic Cells in Athero-sclerotic vs Naïve Mice

The importance of DCs in innate and adaptive immunity is widely acknowledged in numerous inflammatory diseases, including atherosclerosis. Given the attractive capacity of PS to target DCs in the spleen and LNs in naïve mice, we further studied the cellular distribution of PS in atherosclerotic *Ldlr*^{-/-} mice on a C57BL/6 background.⁵⁷ To compare

with naïve C57BL/6 mice, *Ldlr*^{-/-} mice fed a high-fat diet for 16 weeks were injected intravenously with the same concentration of DyLight 650-labeled PS. Since our previous data indicated peak uptake of PS by DCs at the 24 h post-i.v. injection time point (Figure 4), spleen, LN, liver, kidneys, and aorta were harvested after 24 h and analyzed by flow cytometry as before (Figure 6). The percentages of PS⁺ macrophages were significantly lower in spleen ($p < 0.005$) of *Ldlr*^{-/-} mice, while uptake of PS by DCs remained unchanged (Figure 6A). As a result, uptake of PS by splenic DCs was significantly higher than for macrophages ($p = 0.006$) in atherosclerotic mice, and a similar effect was observed in the liver ($p = 0.016$) (Figure 6A,C). In LNs, however, PS displayed a significant decrease in the association with both macrophages ($p < 0.0001$) and DCs ($p < 0.005$) in atherosclerotic compared to nonatherosclerotic mice (Figure 6B). Our data showed no significant difference of association among splenic DC subsets (Figure 7A), but a consistent and significant decreased association was observed among all tested DC subsets in LNs (Figure 7C).

Monocytosis is a common observance during atherosclerosis wherein the bone marrow and spleen overproduce monocytes that enter the blood circulation and contribute to hypercholesterolemia.⁵⁸ We hypothesized that increased levels of circulating monocytes in blood might contribute to the decreased presence of PS⁺ macrophages in the spleen and liver of atherosclerotic mice. To confirm this hypothesis, we investigated the association of PS with circulating monocytes in blood of *Ldlr*^{-/-} and naïve mice. In *Ldlr*^{-/-} mice, a 1.9-fold significant increase ($p = 0.025$) in blood monocyte concentration was observed, and 30% of circulating blood monocytes were PS⁺ ($p < 0.005$), while <2% were PS⁺ in naïve mice (Figure 7B). In addition, PS were found to associate more with Ly6C⁺ monocytes than Ly6C⁻ monocytes ($p = 0.039$) (Figure 7D). Ly6C⁺ monocytes are the primary source of inflammatory lipid-laden foam cells that are found in atherosclerotic lesions.⁵⁹ These data verified that blood monocytes were more efficiently removing PS from circulation in atherosclerotic mice, which combined with monocytosis may explain the decreased available PS for uptake by macrophages in the liver of atherosclerotic mice. Overall, our results demonstrate significant differences in nanomaterial uptake under conditions of atherosclerosis notably in the blood and LNs. This suggests that clinical strategies involving nanomaterials, particularly for vaccination and immunotherapy that require targeting of LN-resident cell populations, may have different efficacy under conditions of atherosclerosis, which has been shown to impair lymphatic fluid transport.⁶⁰

Polymersomes Target Resident Dendritic Cells in Lesions of Atherosclerotic Mice

The pool of circulating DCs has been shown to decrease as DC numbers increase in vulnerable inflamed vascular tissue of atherosclerotic mice and humans.⁶¹ These DCs contribute to atherosclerotic inflammation by activating T cells that weaken the plaque boundary and by releasing chemokines and cytokines to respectively attract and activate additional inflammatory cells. While the targeting of monocytes and macrophages with nanomaterials has been extensively investigated for the treatment of heart disease, atheroma-resident DCs present an untapped and under investigated target for both detecting vulnerable plaques and treating the local inflammation.¹² We therefore investigated the targeting ability of PS to DCs in the aorta, which is recognized as a primary location for vascular lesions in *Ldlr*^{-/-} mice. Over 25% of DCs were observed to be PS⁺ in the aorta of atherosclerotic

mice, which was significantly higher than macrophages, monocytes, and all other isolated immune cell populations ($p < 0.005$) (Figure 8A). All DC subsets investigated in the lesions were targeted equally (between 15 and 30% PS⁺), with the exception of CD8⁺ DCs, which are typically nonmigratory and restricted to lymphoid organs (Figure S6).^{49,51}

To further verify the targeting of DCs by PS in aorta, we next performed immunofluorescence studies of aorta from naïve C57BL/6 mice and *Ldlr*^{-/-} mice 24 h after injection with DyLight 650-labeled PS. Cross-sectional histology stained with DAPI and Alexa-488 conjugated CD11c antibody was observed under spinning disk confocal microscopy. CD11c is a key marker of DCs, and a significant increase in the number of CD11c⁺ cells was detected in the intimal region of *Ldlr*^{-/-} aortic lesions, while minimal CD11c⁺ cells were observed in the aorta of control mice (Figure 8B,C). Furthermore, PS were found to accumulate extensively in the lamina adventitia of only the *Ldlr*^{-/-} mice. In Figure 8B, Z-stacks showed that fluorescence from both the PS (red) and CD11c⁺ DCs (green) was seen in the intima, which further demonstrated the targeting effects of PS to DCs in the aortic lesion of *Ldlr*^{-/-} mice. These results indicated that in the absence of surface conjugated targeting ligands, the PS morphology was sufficient to enhance targeting of aortic DCs in atherosclerotic mice. The localization of PS within the aorta to primarily the adventitia (Figure 8C) suggests that the PS may enter arterial lesions *via* the vasa vasorum, which is consistent with the proposed mechanism used by other nanomaterials to enter and image atherosclerotic plaques.⁶²

CONCLUSION

Conjugation of a targeting ligand to the surface of a nanomaterial typically results in only an incremental improvement in specificity, as this strategy cannot influence nonspecific uptake by the MPS. We demonstrate here that changing the nanostructure morphology, while maintaining the same surface chemistry, can significantly impact both nonspecific and specific uptake by inflammatory cell subsets as a function of both time and disease state. PS were the only NS found to consistently associate with a higher percentage of DCs in comparison to other MPS cells. Switching from a MC to a PS morphology resulted in a decrease in uptake by liver macrophages by over 50% while improving the targeting of splenic DCs by up to 35%. Liver resident macrophages represent the largest MPS population, and decreasing their uptake can significantly improve therapeutic targeting of lower concentration cells such as DCs. We therefore selected PS for further investigation as a vehicle to target both splenic and atheroma-resident DCs in an *Ldlr*^{-/-} mouse model of atherosclerosis.

Applying NSET under the diseased condition of atherosclerosis resulted in several additional advantages that may improve diagnosis and immunotherapeutic treatment of heart disease. The PS morphology enhanced the specificity of targeting DCs in both spleen and aortic plaques compared to MC and FM, with the added benefit of targeting Ly6C⁺ monocytes in the blood that accelerate the progression of CVD. Furthermore, we observed significantly decreased uptake of PS by LN-resident DCs, which may have important implications for the application of immunotherapeutic strategies under conditions of atherosclerosis or high-fat diets. These differences in endocytosis likely reflect complex

atherosclerosis-related changes in the cell activation state, surface receptor expression, preferred mechanisms of endocytosis, and lipid content in biological fluids. Our data advocate that nanostructure morphology and systemic abnormalities due to disease state deserve equal attention as surface chemistry when designing nanomaterials for therapeutic delivery. These results open avenues for therapeutic intervention of atherosclerotic inflammation by modulating DCs and suggest that NSET warrants further investigation for improved nanomaterial targeting.

MATERIALS AND METHODS

Materials

All chemicals were purchased from Sigma-Aldrich, unless otherwise stated. Antibodies, Zombie Aqua fixable cell viability kit, cell staining buffer, and cell fixation buffer were purchased from BioLegend.

Synthesis of PEG-*b*-PPS Block Copolymers

Distinct NS were fabricated based on the controlled self-assembly of PEG-*b*-PPS block copolymers. A variety of different architectures can be obtained by controlling the molecular weight (MW) ratio of the hydrophilic PEG to hydrophobic PPS blocks. The assembled size of each NS is influenced by the total MW of the block copolymer, with higher MWs producing thicker PPS membranes and PEG coronas. The diameters of vesicular NS can be further controlled *via* extrusion through nanopore membranes and solvent conditions that influence the aggregation number.^{63,64} Block copolymers PEG_m-*b*-PPS_n were synthesized as previously described.³⁰ Briefly, PEG thioacetate initiator was deprotected by sodium methoxide to reveal the initiating thiolate. Propylene sulfide was added as necessary to polymerize the desired block lengths, and the polymerization was end-capped by 2,2'-dithiodipyridine or protonated with CH₃COOH to create the PPS thiol-end groups for subsequent fluorophore conjugation. The obtained block copolymers (PEG₁₇-*b*-PPS₃₀, PEG₄₅-*b*-PPS₂₀, and PEG₄₅-*b*-PPS₄₄) were purified by double precipitation in cold diethyl ether or methanol and then characterized by ¹H NMR (CDCl₃) and gel permeation chromatography (ThermoFisher Scientific) using Waters Styragel columns with refractive index and UV-vis detectors in a tetrahydrofuran mobile phase.

Assembly of PEG-*b*-PPS Nanostructures and Loading with ICG

Three different NS, PEG₄₅-*b*-PPS₂₀ MC, PEG₁₇-*b*-PPS₃₀ PS, and PEG₄₅-*b*-PPS₄₄ FM, were assembled and loaded with the lipophilic NIRF imaging agent ICG using the thin-film hydration method as previously described.⁶⁵ Briefly, 8.6 mM of each block copolymer was dissolved in 150 μ L dichloromethane within 1.8 mL clear glass vials (ThermoFisher Scientific). After desiccation to remove the solvent, the resulting thin films were hydrated in 1 mL of phosphate-buffered saline (PBS) or 1 mL of ICG solution (0.258 mM in PBS solution) under shaking at 1500 rpm overnight. The single-layer PS were obtained by extrusion multiple times through 0.2 μ m and then 0.1 μ m nucleopore track-etched membranes (Whatman). The ICG-loaded NS were purified from free ICG by Zeba Spin Desalting Columns (7K MWCO, ThermoFisher Scientific) equilibrated with PBS solution,

and dialyzed against PBS using Slide-A-Lyzer Dialysis Cassettes (7K MWCO, ThermoFisher Scientific).

Covalent Fluorescent Labeling of PEG-*b*-PPS Nanostructures

Maleimide-functionalized fluorescent dye was conjugated to NS *via* free thiol-end-functionalized moieties on the PPS core. Block copolymers protonated with CH₃COOH were first assembled into nanostructures using thin-film hydration in PBS as previously described. Solutions of MC, PS, and FM at 30 mg/mL were covalently labeled with 0.07 mM of DyLight 650-maleimide (Fisher) under shaking at room temperature for 12 h. Excess unreacted DyLight 650-maleimide was removed by Zeba Spin Desalting Columns (7K MWCO) equilibrated with PBS solution and dialysis against PBS using Slide-A-Lyzer Dialysis Cassettes (7K MWCO, ThermoFisher Scientific). The degree of fluorescence labeling was determined by diluting samples in PBS solution and measuring the fluorescence in a spectrophotometer (SpectraMax M3, Molecular Devices). The degree of DyLight 650 labeling for various nanomaterials was: 0.100 μ g/mg for MC, 0.067 μ g/mg for PS, and 0.073 μ g/mg for FM.

Characterization of PEG-*b*-PPS Nanostructures

The size distribution and zeta potential of the nanostructures were analyzed by Zetasizer Nano (Malvern Instruments) with a 4 mW He-Ne 633 nm laser at 1 mg/mL in PBS. The polydispersity index (PDI) was calculated using a two parameter fit to the DLS correlation data. The morphology of each nanostructure were determined by CryoTEM. The ICG concentration of different nanoparticles was measured by UV-vis spectroscopy (SpectraMax M3, Molecular Devices) after sample dilution in PBS solution. To characterize the stability and fluorescent properties of ICG-loaded NS, different molar ratios of polymer: ICG (1:10, 1:25, 1:33, 1:50, 1:75 and 1:100) were prepared with 10 mg/mL of PEG-*b*-PPS. Solutions of ICG-loaded NS were diluted 1:50 in PBS solution prior to the generation of the absorbance spectrum. Wavelengths from 250 to 850 nm were scanned with 10 nm increments. The fluorescent spectrum was measured by the excitation of 780 nm and emission from 700 to 850 nm with 5 nm increments. In all cases, ICG-loaded NS were matched with free ICG (in PBS solution) at the same concentrations of ICG. Before animal studies, all samples were verified to be endotoxin-free (<0.01 EU/mg) by the TLR4 activation HEK Blue LPS detection assay (Invivogen).

Animals

C57BL/6 female mice, 6–8 weeks old, were purchased from Jackson Laboratories. The low-density lipoprotein (LDL) receptor-deficient mice (*Ldlr*^{-/-} mice) with C57BL/6 background were obtained from The Jackson Laboratory at 4 weeks old and fed a high-fat diet (HFD, Harlan Teklad TD.88137, 42% kcal from fat) starting at 6 weeks old for 16 weeks until sacrificed. The control diet for naïve mice was TD.08485 with 13% kcal from fat. All mice were housed and maintained in the Center for Comparative Medicine at Northwestern University. All animal experimental procedures were performed according to protocols approved by the Northwestern University Institutional Animal Care and Use Committee (IACUC).

Assessment of Organ-Level Biodistributions of PEG-*b*-PPS Nanostructures in Naïve Mice

The ICG-loaded MC, PS, and FM were prepared with the optimal molar ratio of PEG-*b*-PPS:ICG (33:1) and suspended in PBS. Free ICG (50 $\mu\text{g/mL}$ in PBS solution) served as a control. C57BL/6 mice ($n = 6$) were injected intravenously with 150 μL of free ICG, MC, PS, and FM (7.5 μg of loaded ICG in each sample). At different time points (1, 24, and 48 h post-injection), whole-body NIRF imaging was performed using an IVIS Lumina (Center for Advanced Molecular Imaging, Northwestern University) with $\lambda_{\text{exc}} = 745 \text{ nm}$, $\lambda_{\text{em}} = 810 \text{ nm}$, exposure time = 2 s, and $f/\text{stop} = 2$. For organ NIR fluorescence imaging, animals were euthanized by carbon dioxide, and liver, spleen, kidney, heart and lung were harvested in Petri dishes and imaged by the IVIS Lumina with the same parameters as previous.

Immune Cell Biodistributions of PEG-*b*-PPS Nanostructures in Naïve Mice

C57BL/6 female mice ($n = 8\text{--}12$) were injected intravenously with 150 μL of MC, PS, and FM with block copolymer concentration of 15 mg/mL. At different time points (1, 24, and 48 h post-injection), mice were anesthetized by intraperitoneal injection of a mixture of ketamine/xylazine followed by exsanguination. Blood was collected by retro-orbital puncture with BD Microtainer tubes and dipotassium EDTA (BD Biosciences). Serum was separated by centrifugation at 3000 rpm at 4 °C for 25 min. Fluorescence intensity of each nanostructure was normalized by diluting NS solutions in PBS and measuring the fluorescence using a spectrophotometer. The fluorescence of blood serum was measured with an excitation of 655 nm and the emission of 675 nm and correlated with the normalized standard curve for each DyLight 650-labeled NS. To prepare white blood cell suspensions, blood cells were washed twice with 10 mL PBS and treated 3 $\times$ with ammonium-chloride-potassium (ACK) lysis buffer (Invivogen) to eliminate red blood cells. Liver, spleen, LN (popliteal, inguinal, axillary, and brachial), and kidneys were harvested, gently dissociated, and incubated in a 12-well plate with each well of 2 mL collagenase D (2 mg/mL) for 45 min at 37 °C and 5% CO_2 . Single-cell suspensions were prepared by mechanical dissociation and passing through a 70 μm cell strainer. Antimouse CD16/CD32 was used to block FcRs, and Zombie Aqua fixable viability dye was used to determine live/dead cells. For flow cytometric analysis, cells were stained using cocktails of fluorophore-conjugated antimouse antibodies: panel 1: CD45-FITC, CD3-APC/Cy7, CD4-PE, CD8 α -PE/Cy7, NK1.1-PerCP-Cy5.5, CD19-Pacific Blue; panel 2: I-A/I-E-FITC, CD11c-Pacific Blue, CD8 α -PE-Cy7, CD11b-PerCP-Cy5.5, CD45RB/B220-PE, Gr-1-APC-Cy7; and panel 3: F4/80-FITC, CD11c-Pacific Blue, CD11b-PerCP-Cy5.5, Ly6C-APC/Cy7, Ly6G-PE/Cy7. After washes, cells were fixed by IC cell fixation buffer (Biosciences). Flow cytometry was performed with FACSDiva on a LSRII flow cytometer (BD Biosciences), and data were analyzed with FlowJo software.

Assessment of PEG-*b*-PPS Nanostructure Biodistributions in Atherosclerotic Mice

Ldlr^{-/-} mice fed a HFD for 16 weeks were used as a model of atherosclerosis. The *Ldlr*^{-/-} mice ($n = 8\text{--}12$) were administered 150 μL of PS, MC, and FM at a concentration of 15 mg/mL *via* tail vein injection. Blood was drawn by retro-orbital bleeding, and organs (spleen, LNs, liver and kidneys) were collected at 24 h post-injection. Aortas were carefully harvested after perfusion with PBS solution under a microscope. To prepare a single cell

suspension, the aortic tissue was cut into small pieces and digested in Aorta Dissociation Enzyme Solution (ADES) (125 U/mL collagenase type X1, 60 U/mL hyaluronidase type 1-s, 60 U/mL DNase I, and 450 U/mL collagenase type I, in 2.5 mL of RPMI-1640 medium without FBS, modified from ref 66) for 1 h at 37 °C and 5% CO₂. The other organs were dissociated and digested with collagenase D (2 mg/mL) as described above. Single-cell suspensions were obtained by mechanical dissociation and passing through a 70 μ m cell strainer. FcRs were blocked with antimouse CD16/CD32 and cells stained with Zombie Aqua fixable viability dye prior to antibody staining. Cells were then stained with multiple cocktails of fluorophore-conjugated antimouse antibodies as described above. Samples were analyzed by flow cytometer and FlowJo software. The gating strategy to identify the immune cell subsets and percentages of each cell type that were NS⁺ is depicted in Figure S3. The NS without a fluorescent label were set as negative control gates in the analysis.

Immunofluorescence

Ldlr^{-/-} mice and C57BL/6 naïve mice (as controls) were injected intravenously with 150 μ L of PS (15 mg/mL) as previously described. After 24 h, the heart and aorta were perfused with 4% paraformaldehyde (PFA)/5% sucrose in PBS solution for 10 min. The aorta was harvested and fixed in 4% PFA/5% sucrose PBS solution 12 h at 4 °C. The isolated aortic arch-derived arteries were immersed in 15% sucrose solution for 12 h and then 30% sucrose solution for 24 h. The resulting specimens were embedded in Tissue-Tek OCT and frozen at -80 °C. Tissue blocks were sectioned at 5 μ m thickness and stained with 4',6-diamidino-2-phenylindole (DAPI) and Alex-488-anti-CD11c antibody. Images were taken on a spinning disk confocal microscope (Leica). Z-stacks were performed at the inner aortic tissue at 63 $\times$ magnification using MetaMorph software.

Statistical Analysis

A minimum of two independent experiments were studied, with 3–6 mice per treatment group in each experiment ($N = 6$ –12). Two-tail Student *t* tests were performed to determine statistical significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank C. Reardon and G. Getz for advice, training, and protocols regarding the extraction and analysis of mouse aortas. We also thank J. Remis for CryoTEM assistance and the following facilities at Northwestern University: Robert H. Lurie Comprehensive Cancer Center Flow Cytometry Core; Center for Advanced Molecular Imaging; Biological Imaging Facility; Mouse Histology and Phenotyping Laboratory. This work was supported by the National Institutes of Health Director's New Innovator Award (grant no. 1DP2HL132390-01), the American Heart Association (grant no. 14SDG20160041), Chemistry of Life Processes Institute Postdoctoral Fellows Program, the Chemistry of Life Processes Institute, the Louis A. Simpson and Kimberly K. Querrey Center for Regenerative Nanomedicine Catalyst Award, National Institutes of Health Predoctoral Biotechnology Training Grant (grant no. T32GM008449).

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Figure 1.

Nanostructure morphologies assembled from PEG-*bI*-PPS copolymers. (A) PEG₄₅-*bI*-PPS₂₀ micelles (MC) (scale bar = 100 nm), (B) PEG₁₇-*bI*-PPS₃₀ polymersomes (PS) (scale bar = 100 nm), and (C) PEG₄₄-*bI*-PPS₄₅ filomicelles (FM) (scale bar = 200 nm) were imaged by CryoTEM. The chemical structure of each copolymer is inserted into the lower left corner of the images. The hydrophilic PEG fraction (f_{PEG}) of the total block copolymer molecular weight and assembled nanostructure morphology are shown above each CryoTEM image.

Figure 2.

Assessment of organ-level biodistributions of PEG-*b*I-PPS nanostructures. An IVIS optical imaging system was used to investigate the biodistributions of NIRF imaging agent ICG-loaded nanostructures in C57BL/6 mice. (A) Absorbance and (B) fluorescence spectrum of free ICG and ICG-loaded PEG-*b*I-PPS micelles at an excitation wavelength of 780 nm. ICG emission increased and red-shifted upon loading. (C) The fluorescence intensity of ICG-loaded micelles at an excitation of 780 nm and emission of 810 nm at different ICG loading concentrations. (D) DLS measurements verified that mean diameter of micelles was not influenced by the concentration of loaded ICG. CryoTEM images can be viewed in Figure S1. (E) Fluorescence images were obtained for representative organs (liver, spleen, kidneys, heart, and lung) at 1, 24, and 48 h after i.v. injection of micelles (MC) polymersomes (PS) and filomicelles (FM); $N = 3-6$.

Figure 3.

Assessment of cellular-level biodistributions of PEG-*b*/PPS nanostructures. Flow cytometric analysis of the association of MC, PS, and FM with immune cells isolated from spleen (A, E, I), lymph nodes (LNs) (B, F, J), liver (C, G, K), and blood (D, H, L) of C57BL/6 mice after time points of 1 h (A–D), 24 h (E–H), and 48 h (I–L) following tail vein injection. Histograms show the average percentages $\pm$ standard deviation (SD) of nanostructure positive (NS⁺) cells for each indicated cell type in different tissue. Macrophages: CD11b⁺F4/80⁺; dendritic cells (DCs): CD11c⁺; B cells: CD45⁺CD19⁺; natural killer (NK) cells: CD45⁺CD49b⁺; granulocytes: Gr-1⁺CD11b⁺; CD4 T cells: CD45⁺CD3⁺CD4⁺; CD8 T cells: CD45⁺CD3⁺CD8⁺; monocytes: Ly6G[−]CD11c[−]CD11b⁺; and neutrophils: CD11b⁺Ly6G⁺. Refer to gating strategies in Figure S3. Data were obtained from two independent experiments and contain $N = 6$ –8 for each group. Statistical significance: * $p < 0.01$, ** $p < 0.005$, *** $p < 0.0001$.

Figure 4.

Polymersomes target relevant DC subsets in lymphoid organs of naïve mice.

Biodistributions of MC, PS, and FM in DC subsets from spleen (A–C) and LNs (D–F) after 1 h (A, D), 24 h (B, E), and 48 h (C, F) following i.v. injection were analyzed by flow cytometry. Histograms show the average percentages $\pm$ SD of NS⁺ cells for each indicated cell type in different lymphoid organs. PreDC: I-A/I-E[−]CD11c⁺; mature DCs: I-A/I-E⁺CD11c⁺; plasmacytoid DCs: I-A/I-E⁺CD11c⁺Gr-1⁺B220⁺; CD8⁺ DCs: I-A/I-E⁺CD11c⁺CD8⁺; CD11b⁺ DCs: I-A/I-E⁺CD11c⁺CD11b⁺. Data were obtained from two independent experiments and contain *N* = 6–8 for each group. Statistical significance: **p* < 0.01, ***p* < 0.005, ****p* < 0.0001.

Figure 5.

Polymersomes target DCs in aorta and lymphoid organs of atherosclerotic mice. MC, PS, and FM were compared for association with immune cell populations from aorta (A), spleen (B), LNs (C), and liver (D) of *Ldlr*^{-/-} mice 24 h following i.v. injection. Histograms show the average percentages $\pm$ SD of NS⁺ cells for each indicated cell type in different tissue as analyzed by flow cytometry. Macrophages: CD11b⁺F4/80⁺; DCs: CD11c⁺; B cells: CD45⁺CD19⁺; NK cells: CD45⁺CD49b⁺; granulocytes: Gr-1⁺CD11b⁺; CD4 T cells: CD45⁺CD3⁺CD4⁺; CD8 T cells: CD45⁺CD3⁺CD8⁺. Data were obtained from two independent experiments and contain $N = 8$ –12 for each group. Statistical significance: * $p < 0.01$, ** $p < 0.005$, *** $p < 0.0001$.

Figure 6.

Biodistributions of polymersomes within immune cell populations differed in atherosclerotic *vs* naïve mice. Flow cytometric analysis of Dylight 650-labeled PS uptake by various immune cell populations of spleen (A), LNs (B), liver (C), and kidneys (D) 24 h after i.v. injection in naïve C57BL/6 mice and atherosclerotic (*Ldlr*^{-/-}) mice on a C57BL/6 background. Macrophages: CD11b⁺F4/80⁺; DC: CD11c⁺; B cells: CD45⁺CD19⁺; NK cells: CD45⁺CD49b⁺; granulocytes: Gr-1⁺CD11b⁺; CD4 T cells: CD45⁺CD3⁺CD4⁺; CD8 T cells: CD45⁺CD3⁺CD8⁺. Data were obtained from two independent experiments and contain *N* = 6–8 for each group. Statistical significance: **p* 0.01, ***p* 0.005, ****p* 0.0001.

Figure 7.

Polymersome biodistribution in dendritic cell subsets and circulating monocytes of atherosclerotic vs naïve mice. Spleen (A), blood (B, D) and LNs (C) 24 h after i.v. injection of Dylight 650-labeled PS were analyzed by flow cytometry. Histograms show the average percentages $\pm$ SD of NS⁺ cells for each indicated cell type in different lymphoid organs. PreDC: I-A/I-E⁻CD11c⁺; mature DCs: I-A/I-E⁺CD11c⁺; plasmacytoid DCs: I-A/I-E⁺CD11c⁺Gr-1⁺B220⁺; CD8⁺ DCs: I-A/I-E⁺CD11c⁺CD8⁺; CD11b⁺ DCs: I-A/I-E⁺CD11c⁺CD11b⁺. (B) Flow cytometric analysis showed significantly higher uptake of polymersomes by circulating monocytes in *Ldlr*^{-/-} mice than C57BL/6 mice 24 h after i.v. injection. (D) Distribution of polymersomes in circulating Ly6C⁻ and Ly6C⁺ monocytes in *Ldlr*^{-/-} mice 24 h after i.v. injection. Ly6C⁻ monocytes: Ly6G⁻CD11b⁺CD11c⁻Ly6C⁻; Ly6C⁺ monocytes: Ly6G⁻CD11b⁺CD11c⁻Ly6C⁺. Data were obtained from two independent experiments and contain *N* = 6–8 for each group. Statistical significance: **p* 0.01, ***p* 0.005, ****p* 0.0001.

Figure 8.

Polymersomes target aortic dendritic cells in atherosclerotic mice. (A) Distribution of polymersomes within immune cell populations in the aortas of atherosclerotic (*Ldlr*^{-/-}) mice at 24 h post-injection, as analyzed by flow cytometry. Macrophages: CD11b⁺F4/80⁺; DC: CD11c⁺; B cells: CD45⁺CD19⁺; NK cells: CD45⁺CD49b⁺; granulocytes: Gr-1⁺CD11b⁺; CD4 T cells: CD45⁺CD3⁺CD4⁺; CD8 T cells: CD45⁺CD3⁺CD8⁺; monocytes: Ly6G⁻CD11c⁻CD11b⁺; and neutrophils: CD11b⁺Ly6G⁺. *N* = 3 for each group; two independent experiments. Statistical significance: ***p* < 0.005. (B) Spinning disk confocal fluorescence images (Z-stacks) of aortic tissue from an *Ldlr*^{-/-} mouse after i.v. injection of Dylight 650-labeled PS 24 h prior to excision. The nuclei were stained with DAPI (blue); DCs were stained with Alexa 488 dye conjugated to anti-CD11c antibody (green); Dylight 650-labeled PS (red). Scale bar = 10 μ m. (C) Confocal immunofluorescence images of aortas from C57BL/6 mice (left) and *Ldlr*^{-/-} mice (right) following i.v. injection of PS-Dylight650 (red) 24 h prior to excision. Serial cross sections (5 μ m thickness) were stained with DAPI (blue), and Alexa 488 dye conjugated antibodies against CD11c (green) for detection of DC. Scale bar = 50 μ m.

Table 1Physicochemical Characteristics of PEG-*b*-PPS Nanostructure Morphologies in PBS Solution (pH = 7.4)

name of samples	average diameter (nm)	polydispersity index (PDI)	zeta potential (mV)
micelles (MC)	22.5	0.132	-1.35 ± 0.38
polymersomes (PS)	113.7	0.179	-0.20 ± 1.68
filomicelles (FM)	N/A	N/A	-1.40 ± 0.31