The ability to target nanoparticles to the dysfunctional endothelium may present a new and innovative therapy for cardiovascular disease. In this work, an anti-inflammatory gene therapy nanoparticle targeted to VCAM-1, a protein overexpressed by inflamed endothelial cells located within atherosclerotic plaque, is developed. Targeting is accomplished via a peptide-grafted coating for the nanoparticles. Nanoparticle binding to VCAM-1 is probed via surface plasmon resonance with imaging to determine kinetics and binding dynamics. Targeted nanoparticles are internalized by transfected inflamed primary endothelial cells more than nontargeted controls (80% vs 30% positive cells) under both static and dynamic flow conditions. The nanoparticles also bind specifically to VCAM-1+ endothelial cells within atherosclerotic plaque from mouse models in both the aortic sinus and whole aorta. Taken together, this nanoparticle formulation may be an effective and specific strategy for anti-inflammatory therapy within the context of atherosclerosis.

1. Introduction

Cardiovascular disease, whose clinical manifestations largely result from atherosclerosis, has become the leading cause of mortality and morbidity worldwide. It is estimated that roughly 70% of cardiovascular clinical events cannot be treated with the currently available therapies, including statins,[1] necessitating the development of new and innovative strategies. As research continues to implicate the immune system as a main driver of atherosclerosis,[2] there is also a need to target specific sites of inflammation within the blood vessels for novel anti-inflammatory therapies. Nonviral gene delivery using synthetic polymer nanoparticles (NPs) has the potential to induce the safe and endogenous expression of anti-inflammatory proteins, such as interleukin-10 (IL-10), that attenuate inflammation.[3,4] The authors have previously developed poly(β-amino ester) (PBAE) NPs for the delivery of interleukin-10 (IL-10) plasmid DNA (pDNA) in primary human and mouse endothelial cells (mECs)[5] as a proof-of-concept anti-inflammatory strategy for atherosclerosis. While it could be shown that PBAE NPs can successfully transfuse cells in vitro and tissues in vivo through local administration,[6] targeting these NPs to specific disease sites via intravenous (IV) administration would boost their potential for clinical translation while minimizing off-target binding.

There have not yet been any reports exploring the use, and especially not targeting, of PBAEs in the context of atherosclerosis,[7,8] however, other NP materials have shown some success for this application,[9,10] which highlights the potential of PBAEs. On the other hand, early attempts to target PBAE NPs to noncardiovascular tissues employed large (20+ amino acid) peptides featuring block sections of negatively charged amino acids that anchored the peptide to cationic NPs and RGD-targeting amino acid sequences that served to localize NPs to mouse models in vivo. RGD-coated PBAE NPs accumulated in and successfully transfected liver endothelial cells and Kupffer cells in wild-type mice as well as tumor tissue in a mouse cancer model.[11] RGD is known to bind many different integrins and cell types[12] and thus, particle localization using this approach is relatively nonspecific. Another targeting strategy incorporated mannose as a ligand into the PBAE backbone and has shown success in transfusing antigen-presenting cells in vivo. RGD-coated PBAE NPs accumulated in and successfully transfected liver endothelial cells and Kupffer cells[13] in wild-type mice as well as tumor tissue in a mouse cancer model.[14] RGD is known to bind many different integrins and cell types[15] and thus, particle localization using this approach is relatively nonspecific. Another targeting strategy incorporated mannose as a ligand into the PBAE backbone and has shown success in transfusing antigen-presenting cells and cancer cells[16] in vitro. In a more recent and specific strategy, T cells were targeted in vivo using PBAE NPs containing plasmids encoding chimeric antigen receptors (CAR) that sensitized the T cells to cancerous cells in situ.[17] They included a poly(glutamic
acid) (PGA)-based coating functionalized with antibodies specific for lymphocytes to accomplish this.

Although these studies have shown successful application of PBAEs for targeted gene delivery, the kinetics and binding of targeted PBAE NPs in physiological environments were not thoroughly investigated. This information could help validate the effect of common formulation components, such as poly(ethylene glycol) (PEG) chains or ligand concentration, in addition to the specificity of the ligands. NP binding and uptake has been studied using model materials, such as polystyrene, with cell-based assays. However, extracting the binding properties from these assays is challenging since cells and tissues express many different receptors that could interact with NP ligands. Furthermore, gene delivery NPs, including PBAEs, are fabricated from soft and rapidly biodegradable materials, requiring real-time and sensitive techniques to measure binding events. The investigation of potential gene delivery strategies for atherosclerosis should not only involve the incorporation of targeting components but also characterization of their ligand binding properties to a target receptor. In atherosclerosis, the endothelium lining of blood vessels becomes inflamed, dysfunctional, and is typified by the overexpression of inflammatory cell recruitment receptors, such as VCAM-1. Thus, an NP formulation targeting VCAM-1 and containing an anti-inflammatory gene, such as IL-10, could localize to plaque endothelial cells specifically and reduce the inflammatory component of atherosclerosis.

In this study, a PGA-based coating containing PEG and a peptide specific for VCAM-1 was synthesized and applied to PBAE:plasmid DNA NPs containing plasmid DNA encoding IL-10. The aim of the study was to develop a VCAM-1-targeted PBAE NP and evaluate its binding to VCAM-1 at different levels and in various physiological conditions. Binding of the NPs to the VCAM-1 protein alone was initially investigated using surface plasmon resonance with imaging (SPRi) to determine specific binding and kinetic behavior. SPRi is a real-time, label-free biosensing technique traditionally used to measure the binding properties of biomolecules, such as nucleic acids, proteins, and peptides. Thus, it is uniquely suited for studying the binding kinetics of targeted NPs made of biodegradable and sensitive materials. Surprisingly, few studies have taken advantage of SPRi to investigate NP targeting to specific proteins and to the best of the authors' knowledge, this study is the first to do so with gene delivery NPs. NP uptake and transfection were then investigated in inflamed endothelial cells overexpressing VCAM-1 in vitro. The binding of NPs was then investigated on atherosclerotic plaque-containing tissues from mice ex vivo as well as in vivo via live 2D and 3D imaging. This study thus presents the development and characterization of a nonviral gene delivery NP strategy capable of specifically targeting inflamed endothelial cells via VCAM-1.

2. Results

2.1. Synthesis and Characterization of PGA-PEG-VHPK and PGA-PEG-Cys Coatings

Two different PGA-based coatings (targeted and nontargeted) were synthesized from three components: a PGA backbone, PEG linker, and VCAM-1 targeting peptide (or l-cysteine in the case of nontargeted coating, Figure 1). The PEG linker (NH2-PEG-Mal) was conjugated to PGA via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysuccinimide (EDC/NHS) chemistry at a molar ratio of 20% PEG-maleimide to PGA carboxylic acid groups. Comparing the ratio of maleimide peak at 6.8 ppm to PGA peak at 4.25 ppm, the 1H-NMR analysis revealed that roughly 12 mol% of carboxylic acid groups were functionalized for the PGA-PEG-Mal for the VHPK coating and around 8 mol% for PGA-PEG-Mal for the Cys coating (Figure 2A). The completion of the coating was accomplished by adding an amount of VHPK peptide or l-cysteine equimolar to that of the maleimide (as determined by 1H-NMR). The terminal thiol group from either the VHPK peptide or l-cysteine reacted with the maleimide double bond to form a stable thioether due to the high specificity and 100% reaction efficiency of maleimides; this was shown by the disappearance of the maleimide signal at 6.8 ppm (Figure 2A). The completion of the coating was accomplished by adding an amount of VHPK peptide or l-cysteine equimolar to that of the maleimide (as determined by 1H-NMR). The terminal thiol group from either the VHPK peptide or l-cysteine reacted with the maleimide double bond to form a stable thioether due to the high specificity and 100% reaction efficiency of maleimides; this was shown by the disappearance of the maleimide signal at 6.8 ppm (Figure 2A). Peptide peaks are indicated by stars on the spectra for PGA-PEG-VHPK (Figure 2A). The completion of the coating was accomplished by adding an amount of VHPK peptide or l-cysteine equimolar to that of the maleimide (as determined by 1H-NMR). The terminal thiol group from either the VHPK peptide or l-cysteine reacted with the maleimide double bond to form a stable thioether due to the high specificity and 100% reaction efficiency of maleimides; this was shown by the disappearance of the maleimide signal at 6.8 ppm (Figure 2A). Peptide peaks are indicated by stars on the spectra for PGA-PEG-VHPK (Figure 2A). In the PGA-PEG-Cys spectra, the disappearance of the maleimide signal, presence of the PEG and PGA signals, and absence of the starred peptide peaks of PGA-PEG-VHPK indicated synthesis of the desired product.
2.2. Physicochemical Properties of NP Formulations

PBAE and pDNA solutions were mixed to form NPs, which were then coated by adding PGA-PEG-VHPK (NP-VHPK) or PGA-PEG-Cys (NP-Cys) (Figure 1). The application of the PGA-PEG-VHPK and Cys coatings reduced the NP surface charge from +30 to −10 mV without having a significant effect on the size, polydispersity, or concentrations of the formulations (Figure 2B,C and Figure S4, Supporting Information). Size measurements with dynamic light scattering (DLS) were consistent with those of NTA, although DLS showed an increase of 40 nm with both coatings as compared to uncoated NPs (Figure S4, Supporting Information).

2.3. SPRi Biosensing for the Assessment of Binding Kinetics of NP and VCAM-1

To determine the ability of nanoparticle formulations to target VCAM-1, binding kinetics between the VCAM-1, functionalized on the SPRi chip, and the NP-Cys (control) or NP-VHPK formulations were investigated. Changes in the refractive index (measured in refractive index unit (RIU)) on the VCAM-1 coated chip were directly related to NP binding, where increased signal indicated increased binding. As can be seen in Figure 3A, NP-VHPK reached an RIU of $7 \times 10^4$ at maximum concentration, whereas the max RIU achieved by NP-Cys was an order of magnitude lower at $5 \times 10^3$. To assess the binding affinity of the NP formulations for VCAM-1, the changes in refractive index were plotted as a function of the NP concentration. For the NP-VHPK particles, a typical Hill behavior was observed; the apparent dissociation constant ($K_d$) and Hill coefficient could be deduced by fitting the Hill model in Equation (1) with the plasmonic RIU signal being a function of the log concentration:[24] (Figure 3B).

\[
(NP - VHPK) + VCAM \rightarrow (NP - VHPK - VCAM)
\]  

The fitting parameters to determine $K_d$ for NP-VHPK are reported in Table S1 (Supporting Information) and the data used to fit kinetic models for NP-VHPK and NP-Cys can be found in Tables S2 and S3 (Supporting Information), respectively. From
fitting this model, the $K_d$ for NP-VHPK was found to be $60.3 \pm 3.0 \, \mu g \, mL^{-1}$, which is equivalent to a theoretical VHPK peptide concentration of $662.8 \times 10^{-9} \, m$. In addition, the Hill coefficient of NP-VHPK was determined to be $n = 1.9 \pm 0.2$. As in this case, a Hill coefficient greater than 1 indicates that NP-VHPK binds to VCAM-1 in a manner indicating positive cooperativity and that binding of one NP-VHPK facilitates the binding of subsequent others to VCAM-1.\textsuperscript{25} Unsurprisingly, the RIU signal did not increase with increasing concentrations of NP-Cys, denoting a weak and/or nonspecific binding of these NPs to VCAM-1.

2.4. Cellular Uptake and Transfection with Targeted Gene Delivery NPs

The proposed anti-inflammatory gene therapy NPs were engineered to target VCAM-1, which is overexpressed in inflamed endothelial cells that overlie the atherosclerotic plaque.\textsuperscript{20} To simulate this condition in vitro, primary mECs were challenged with TNF$\alpha$ to overexpress VCAM-1, as verified by western blot (Figure 4A). NP formulations were incubated under both static and dynamic flow conditions at a shear stress of 15 dyn cm$^{-2}$. Under static conditions without TNF$\alpha$, there was negligible uptake of the NP-VHPK formulation (5% positive cells, Figure 4B). However, with the addition of TNF$\alpha$, NP-VHPK uptake significantly increased (80% positive cells). For the NP-Cys formulation, there was more uptake without TNF$\alpha$ (50% cells positive) and this did not significantly increase upon the addition of TNF$\alpha$ (30% positive). The overall fluorescent signal of NPs taken up by cells can also be seen in Figure S3 (Supporting Information), where NP-VHPK labeled a greater proportion of cells, along with a higher intensity of signal, than NP-Cys. Thus, in the inflammatory state (with TNF$\alpha$), there was significantly increased uptake of NP-VHPK as compared to NP-Cys. As expected, there was little-to-no uptake of the pDNA only without any polymer (Figure 4B). Under dynamic flow, NP-VHPK were also significantly taken up by TNF$\alpha$-activated mECs (Figure S5, Supporting Information).

Figure 3. Binding kinetic analysis of NP formulations assessed via SPRI biosensing. A) SPR responses of the VCAM biosensor as a function of NP-Cys and NP-VHPK concentrations. B) Binding affinity curve in PBS-T as a function of the log NP-VHPK concentration. Solid line corresponds to Hill curve nonlinear fitting using Gauss–Newton algorithm ($n = 13$ spots per experiment, $R^2 = 0.9795$).

Figure 4. Cellular uptake and transfection of mECs from targeted and nontargeted gene delivery NPs. A) The expression of VCAM-1 in mECs in the presence and absence of TNF$\alpha$ via western blotting; $^*p < 0.05$ as determined by Student’s $t$-test, $N = cells$ from three different mice. B) The uptake of NPs containing Cy3-labeled IL-10 plasmid as measured via flow cytometry; $^*p < 0.01$, $^**p < 0.001$ as determined by two-way ANOVA with Sidak’s multiple comparisons test, $N = cells$ from at least three different mice. C) Fluorescent microscope images showing transfection of mECs with coated PBAE NPs containing GFP plasmid DNA with and without the presence of TNF$\alpha$. Scale bars = $100 \mu m$. 

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Figure 5. Binding of NP-VHPK and control NP-Cys within the plaque isolated from atherosclerotic mouse models. A) NP-VHPK and B) NP-Cys both containing Cy3-labeled IL-10 (red) were incubated on tissue sections counterstained with VCAM-1 (green) and DAPI (blue). C) Quantification of background-corrected fluorescence ratio of NPs to VCAM-1 and D) quantification of colocalization-thresholded Manders coefficient tM1. Confocal images were taken at 63× from six mice per group with data analyzed by ImageJ. *p < 0.05 and n.s. not significant via Student’s t-test.

To assess whether NP uptake results translated into actual gene expression, mECs were challenged with TNFα (20 ng mL⁻¹ overnight) before incubation with NPs containing a pMAX-EGFP plasmid. NP-VHPK transfected some cells without TNFα activation, but transfection remarkably increased after stimulation with TNFα (Figure 4C). Interestingly, although NP-Cys was taken up by many of the cells with and without TNFα, this did not translate into much transfection as shown by low GFP expression (Figure 4C).

2.5. Targeted NPs Bind Endothelial Cells within the Plaque Ex Vivo

To investigate whether NP formulations also specifically bound to endothelial cells in atherosclerotic plaque, NP formulations were incubated on plaque-containing murine tissue slices from LDLR⁻/⁻ mice that had been on a high fat diet for 9–12 weeks. NPs containing Cy3-labeled IL-10 plasmid were incubated on the aortic sinus, washed off, and sections were costained for VCAM-1 to identify activated endothelial cells within the plaque (Figure 5). Within the plaque-dense regions of the aortic sinus, NP-VHPK were seen as a bright and continuous pattern along the edge of the plaque where the expression of VCAM-1 was also observed (Figure 5A). NP-Cys staining was not as prevalent on the endothelium (Figure 5B). The images were analyzed to compare the background-subtracted fluorescence of the NPs to that of the region of interest defined by VCAM-1 fluorescence, the endothelium (Figure 5C). NP-VHPK fluorescence was significantly brighter on the endothelium than the NP-Cys fluorescence. The colocalization of NP and VCAM-1 fluorescence was also examined using the Coloc2 plugin from ImageJ. Although on average, NP-VHPK colocalized with VCAM-1 fluorescence slightly more than NP-Cys in the endothelium region of interest, this increase was not significant (Figure 5D).

Similarly, to evaluate the specificity of NP-VHPK binding plaque versus nonplaque regions on the same tissue section, NPs were incubated on whole aortas removed from atherosclerotic mice. En face confocal microscope images of the interior curvature of the aortic arch (Figure 6A), which is the area prone to oscillatory shear stress, inflammation, and plaque formation,[20] showed much higher expression of VCAM-1 than images from the descending region (Figure 6B) of the same aorta. The increased VCAM-1 expression observed in the inner aortic arch ECs was accompanied by an increase in binding of NP-VHPK formulated with Cy5-labelled IL-10 plasmid (Figure 6A). Because the entire aortic segment was incubated freely in nanoparticle and VCAM-1 solutions, verification that staining was only observed in the endothelial layer of the tissue was necessary. A rotated 3D confocal image of the aortic segment incubated with NP-VHPK and VCAM-1 antibodies indeed confirmed that the staining was present on only the top (endothelial) layer of the tissue (Figure 6C).

2.6. Targeted NPs Localize to Thoracic Region in LDLR⁻/⁻ Mice In Vivo

To assess the targeting and biodistribution of both NP-VHPK and NP-Cys, LDLR⁻/⁻ mice on HFD for at least nine weeks were
injected intravenously with NPs containing 25 µg Cy5-labeled IL-10 plasmid DNA. Figure 7A illustrates the whole mouse distribution of NP formulations 2 h after injection. Cy5 signal can be observed in the thoracic region near the heart for the NP-VHPK formulation. Both NP formulations have a similar biodistribution profile and are mainly found in the liver with minor accumulation in the kidneys, spleen, and lungs (Figure 7B). Signals from the NP formulations are brightest 2 h following injection and decrease significantly by 24 h.

To further investigate the fluorescent signal in the thoracic cavity arising from the Cy5-plasmid delivered by the two NP formulations, fluorescence imaging tomography (FLIT) was used to take images at various planes and reconstruct a 3D image using the IVIS imaging system and Living Image software. The mouse skeleton and heart were added in by the software to approximate organ location. White arrows in Figure 8 point to fluorescent signal measured in the thoracic cavity near the heart in the NP-VHPK formulation 2 h following injection, in the approximate location of the aorta. This fluorescent signal was not observed for the NP-Cys formulation. Otherwise, signal was observed in the abdominal cavity, near the aforementioned biodistribution organs (liver, kidneys, and spleen) for both formulations.

3. Discussion

Although a number of studies have explored NP treatments within the context of atherosclerosis,[8] no reported study could be found utilizing a DNA gene therapy approach for this pathology. Targeted binding of gene delivery NPs to inflamed endothelial cells was accomplished here using PBAE:pDNA NPs with a three-part PGA-PEG-VHPK coating. PGA served as the base layer, and its highly anionic charge anchored it to the cationic PBAE:pDNA NPs. To confer protection against nonspecific uptake and increase particle stability and stealth, a 2 kDa PEG chain was added to the PGA base. PEGylation of NPs has been shown to increase circulation time and reduce nonspecific uptake; however, at high grafting ratios, PEG can prevent particle uptake and cargo delivery.[26] A PEG grafting ratio of 12% of the PGA was chosen for the final NP formulation. Initial experiments using a 5% PEG-grafted PGA coating indicated that these required more coating to be stable as evidenced by DLS analysis where the combination of a small size and negative charge was assumed to indicate stability (Figure S6, Supporting Information). 5% PEG-grafted coatings were also taken up less by cells than the 12% coated NPs (Figure S7, Supporting Information). Finally, the targeting peptide sequence (VHPK) was selected as it has previously been shown to bind VCAM-1 with high affinity.[9,27,28]

Besides binding effects, the overall size and charge of NPs have also been shown to influence their uptake.[29] Upon formation, the uncoated NPs possessed a highly cationic surface charge (+30 mV), which can lead to toxicity in vivo.[30] After coating, the charge decreased to −10 mV. Similar strategies have developed targeted anionic NPs by grafting full protein antibodies to PGA[16] or long-chain polyanion peptides containing anionic polypeptides preceding the targeting sequence;[11,31] however, antibodies and long-chain peptides can be expensive to produce and optimize, which may limit clinical translation.

To better understand the NP-VHPK kinetics and strength of binding to VCAM-1, an in vitro model was developed using SPRi biosensing. This label-free and real-time approach could
Figure 7. Evaluation of NP targeting and biodistribution in vivo with NP-VHPK and NP-Cys formulated with Cy5-labeled IL-10 compared to vehicle injection (sodium acetate buffer). A) LDLR−/− mice on HFD (12 weeks) were imaged 2 h after injection for NP localization within the whole mouse. B) Organs were removed and imaged for Cy5 signal using IVIS imaging system and quantified using Living Image software at both 2 and 24 h for C) NP-VHPK and D) NP-Cys. N = 3 mice per condition.

Figure 8. 3D fluorescence tomography (FLIT) was performed on LDLR−/− mice 2 h following injection of NP formulations using IVIS imaging system and Living Image software. A) White arrows point out Cy5 signal from labeled IL-10 plasmid within NP formulations near vasculature surrounding heart. N = 3 mice/group.

measure the interactions between a species in solution and one immobilized on a surface with high sensitivity. Though typically utilized to probe the interactions between proteins, it offers advantages in nanomedicine, specifically for sensitive particles that may degrade rapidly, as is often required for DNA gene therapy nanoparticles.[32] Surprisingly, few studies have taken advantage of its applicability to study nanoparticle targeting specifically.[22,23]

As observed in the SPRi analysis, NP-Cys bound to VCAM-1 faster than NP-VHPK ($k_{on} = 2.5 \pm 0.5 \text{ min}$ compared to $4.5 \pm 0.5 \text{ min}$ for NP-VHPK), but the signal from this initial binding did not last long, as it did for NP-VHPK (Figure 3A,B; Tables S2 and S3, Supporting Information). VCAM-1 was grafted to the biosensor via EDC/NHS chemistry, which is not a site-specific reaction and could link the protein to the chip using any of the many amine-containing amino acids in the protein. These random arrangements of the protein may explain differences in the initial NP interactions and may give NP-Cys more opportunities for fast, yet nonspecific binding. Although slower to initially bind VCAM-1, data from the Hill model fitting indicated that NP-VHPK interacted with VCAM-1 receptor binding sites in a more complex and stable manner.
For binding, a $K_\text{d}$ of $\approx 660 \times 10^{-9}$ m for the NP-VHPK formulation was determined. There are few studies available for comparison, but Chakraborty et al. used FRET to deduce the affinity of Very Late Antigen-4 (VLA-4), the protein with homologous sequence to the VH PK peptide, and VCAM-1 to be $41.8 \pm 2.4 \times 10^{-9}$ m. It is probable that the peptide does not have as high a $K_\text{d}$ as the homologous protein (VLA-4), and similarly, the peptide grafted on a nanoparticle may have even less affinity. Thus, characterization of the binding affinity and kinetics of targeted NPs was of high importance to understand how NP formulations interact with their targets. Studying binding properties could also be used for comparing different targeting strategies and formulations or to select NPs that bind in a desirable range for targeting, below which NP s may not be effective and above which, tightly bound NPs may induce untoward physiological side effects or compete with desirable cell binding.

NP-VHPK were preferentially internalized and led to increased transfection in endothelial cells stimulated to express VCAM-1, compared with unstimulated cells. This was observed under dynamic flow as well (Figure S5, Supporting Information), which bridges a crucial gap in literature for gene therapy (and other NP s) formulations that are destined for IV injection, though more in-depth studies are required. There was also appreciable uptake of the NP-Cys formulation without TNF\(\alpha\) even though it was shown to not bind to VCAM-1 specifically via SPR\(\text{i}\) analysis or endothelial cells. There are many nonspecific mechanisms that could explain this result. For example, NPs may have hydrophobic and/or charged domains that resemble other commonly endocytosed molecules, such as lipoproteins. Endothelial cells are also known to internalize lipids, such as acetylated LDL, and in inflammatory conditions, can act as phagocytes and increased scavenger receptor activity. Furthermore, although NP-VHPK and NP-Cys are matched in both charge and size, the PEG-Polymer system used to make PGA-PEG-Cys had slightly less grafting at 8% versus the 12% for PGA-PEG-VH PK. This may lead to a slight difference in the proteins adsorbed on the particle surface, as transfections are conducted in full serum media, and may influence NP uptake as it also means NP-VHPK had slightly more PEG than NP-Cys on the surface. Further studies are required to elucidate the relationship between the protein corona and NP uptake and transfection, particularly with the added complexity that inflammation brings. However, NP-VHPK was shown to have an increased uptake in the presence of TNF\(\alpha\) in addition to specific VCAM-1 binding at the protein and tissue level in vivo. Interestingly, uncoated NPs, which had a highly positive charge, were also significantly internalized by mECs upon activation with TNF\(\alpha\) but did not bind to endothelial cells at the plaque (Figure S8, Supporting Information).

Within the endothelial cells at the plaque, only NP-VHPK bound specifically in the VCAM-1+ area ex vivo, further confirming the in vitro and SPR\(\text{i}\) results. However, the exact overlap of pixels could not be shown via colocalization analysis (Figure 5D). It is likely that the tissue images were not high enough quality for this precise analysis, which is more often performed in vitro with cellular monolayers. Furthermore, it is not currently known exactly where the VH PK peptide binds to VCAM-1. Thus, a potential overlap of NP-VHPK and any VCAM-1 antibody for immunofluorescent analysis would greatly influence colocalization.

To further probe the specificity of NP-VHPK, some ex vivo slides were also incubated with NP-VHPK and co-stained with CD68 to label phagocytic cells, and there was no overlap in signal (data not shown). The lack of NP-VHPK binding to other plaque-associated cells, such as macrophages and foam cells (CD68+) and smooth muscle cells (intimal layer), strengthens the specificity of this approach. Indeed, in the in vivo evaluation, NP-VHPK, but not NP-Cys, localized in the thoracic region in the vicinity of the aorta as seen via 3D fluorescence imaging.

Taken together, these results imply that NP-VHPK may be a candidate for future targeted anti-inflammatory gene delivery strategies, and the experimental work performed to arrive at this conclusion may be further exploited in developing targeted nanomedicine strategies involving nonviral DNA delivery. Adaptation of the polymer and coating may diversify the applications to include delivery of drugs, RNA, peptides, and imaging moieties.

4. Conclusion

In this study, a PEGylated peptide-targeted coating was developed and employed to bind gene delivery NPs (NP-VHPK) to VCAM-1, an important target in atherosclerosis. Cellular uptake and gene delivery studies in primary endothelial cells were complemented with SPR\(\text{i}\) biosensing, which allowed for the real-time investigation of interactions between the particles and targeted VCAM-1 receptor. NP-VHPK similarly bound to inflamed endothelial cells (VCAM-1 high) in atherosclerotic plaque regions from the aorta and aortic sinus of mice. Additionally, injection of NP-VHPK in vivo showed its localization in the thoracic region near the heart and blood vessels in a mouse model of atherosclerosis. These results showed that the coating permitted efficient targeting observed at the protein, cellular, and tissue levels without disturbing the main function of the NPs, gene transfection. The strategies identified herein could be exploited for screening other types of targeted particles (e.g., by varying the targeting ligand, cargo, and physicochemical properties) or targeting other cell receptors (multiplexing the protein receptors or whole cells or extracted cell membranes on the SPR\(\text{i}\) chip). Finally, NP-VHPK containing IL-10 plasmid DNA could be further investigated as a targeted nonviral vehicle for specific gene delivery strategies to attenuate atherosclerotic inflammation.

5. Experimental Section

Materials: All materials were purchased and used without modification unless otherwise noted. Kits were used as per manufacturer instructions. PAAE monomers were 1,4-butanediol diacrylate (B4) (Sigma, CA, 411744), 5-amin o-1-pentanol (S5) (Sigma, CA, 123048), and 1-(3-Aminopropyl)-4-methylpipерazине (E7) (Alfa Aesar, USA, L04876). EDC and NHS for coating synthesis were from Sigma Canada. PGA was purchased from Sigma Canada ($M_\text{w}$ 50–100 kDa, P4886). NH$_2$-PEG$_{2000}$-maleimide (NH$_2$-PEG-MaI) was purchased from JenKem USA ($M_\text{w}$ 2 kDa, AS005-1). The VH PK peptide (VHKQHRGGSGC) was custom synthesized by Sigma Canada (Saint Laurent, QC, Canada) with >95% purity. L-cysteine was purchased from Sigma Canada ($M_\text{w}$ 52-89-1). The IL-10 plasmid (pIL-10) used was produced as previously described. Labeling kits to fluorescently tag IL-10 plasmid were purchased from Mirus Bio (Label IT (USA, MIR3700 for Cy5, MIR3600 for Cy3). For SPR\(\text{i}\) studies, recombinant mouse chimera VCAM-1 protein (with human IgG) was....
obtained from R&D Systems (USA, 643-VM-050). Self-assembled monolayer (SAM) solutions were SH-PEG-OH (Triethylene glycol mono-11-mercaptoundecylyl ether, Sigma Canada #673110) and SH-PEG-COOH (O-(2-carboxyethyl)-O’-(2-mercaptoethyl)heptaethylene glycol, Sigma Canada #672688).

Synthesis and Characterization of Targeted and Control Coatings: The coating reaction was adapted from a previous study[39] to use a different PEG-maleimide reagent. Briefly, 50 mg PEA, equivalent of 387 µmol —COOH groups, was dissolved in 2 mL HEPES buffer (10 × 10⁻³ M, pH 6.5) and added to a small round bottom flask. 5 mg NHS and 33 mg EDC were each dissolved in 1 mL HEPES buffer and added to the flask. The flask was then placed under nitrogen. 140 mg NH₂–PEG2000–maleimide, equivalent of 68 µmol amine group (17.5% of PEG carboxylic acid groups), was dissolved in 4 mL HEPES buffer and injected into the flask, which was left to stir overnight at room temperature. The solution was diazided against 10 × 10⁻³ M NaCl (>2 buffer changes) and then milliQ H₂O (>2 buffer changes) for 48 h using a SpectraPor 4 (New Jersey, USA) RC membrane (12–14 kDa MWCO). The solution was frozen at −80 °C overnight and then lyophilized. 1H-NMR was performed to quantify the grafting in D₂O supplemented with a few drops of sodium deuteroxide (NaOD 30 wt% in D₂O, Sigma 164488) to help polymer dissolution. To complete the coating, PEA-PEG-Mal was dissolved in HEPES buffer (pH 7.4) at a concentration of 20 mg mL⁻¹ and added to a solution of the VHPK peptide in the HEPES buffer at a 1:1 molar ratio of peptide to maleimide (as determined by 1H-NMR). The solution was stirred overnight at room temperature and then dialyzed, freeze dried, and characterized via NMR as mentioned above.

The same process was used to synthesize the cysteine-terminated coating except in the last step, cysteine was used instead of the VHPK peptide.

Polymer Synthesis and NP Formation: The PBAE polymer B4S5E7 was synthesized as previously reported[40] using the E7 end-group instead. NPs were formed by first diluting PLL-10 and B4S5E7 in sodium acetate buffer (25 × 10⁻³ M, pH 5.2) with a PLL:PEL ratio of 30:1. 60 µL of these solutions were mixed, vortexed for 10 s, and incubated for 10 min at room temperature for particle formation. An equal volume of coating (w/w ratio coating:PELA of 0.5:1.0) PEG-PeVHVK or PEG-PeVHPK/Cys diluted in sodium acetate buffer was then added, briefly vortexed, and then incubated five more minutes at room temperature to complete NP formation (Figure 1). Sodium acetate buffer alone was used to maintain consistent volumes between formulations in the case of uncoated NPs.

Characterization of NP Physicochemical Properties: DLS and zeta potential measurements were performed using a Brookhaven ZetaPALS light scattering analyzer (New York, USA). NP solutions were diluted in running mobile phase for polydispersity measurements. 10 µL PBS for zeta potential measurements. For NTAs, NPs were formed by mixing 10 µL of each component as mentioned above and diluted 1:500 in PBS. Measurement videos were recorded and analyzed using a Nanosight NS300 (Malvern, UK). All NP measurements were performed in duplicate on at least three different days. For transmission electron microscopy (TEM) imaging, 25 µL NP-VHPK solution was deposited on a glow discharge coated grid and left to air dry before imaging. Images were acquired on a FEI Tecnai G2 Spirit 120 kV TEM.

SPRI Biosensing Analysis of NP Binding VCAM-1:

Surface Immobilization of VCAM-1 onto SPRI Chips: Glass slides were coated with a thin gold layer using electron-beam physical deposition. The surfaces were cleaned and immersed in a solution containing 9 × 10⁻³ M SH-PEG-OH and 1 × 10⁻³ M SH-PEG-COOH in absolute ethanol overnight to form a SAM on the slides. Slides were then cleaned and mounted on an SF11 equivalent prism (Horiba, Japan, n = 1.765) using a refractive index matching liquid to create an SPRI chip, and immediately placed in the SPRI instrument for functionalization. The substrate was thoroughly rinsed with PBS-T (0.002% Tween-20) until a stable baseline was obtained. Covalent surface functionalization with VCAM-1 was performed by adapting a protocol from Gobi et al.[41] Briefly, 200 µL of a solution containing 2 mg mL⁻¹ each of EDC and NHS as well as 5 µg mL⁻¹ of VCAM-1 in deionized (DI) water was flown onto the SAM slide inside the SPRi apparatus (Figure S1, Supporting Information). The peristaltic pump speed was adjusted to achieve a 1 h incubation time. The pump speed was then increased to 15 µL min⁻¹ for all subsequent injections. Unreacted —COOH groups on the SPRI chip surface were quenched by injecting 200 µL of 1 M ethanolamine in water (pH 8.5). Grazing was confirmed by RIU signal changes, which did not happen without EDC/NHS (Figure S2, Supporting Information). The VCAM-1-coated SPRI chips were used for all experiments unless otherwise noted.

SPRI Instrumentation and Measurement: The SPRI biosensor (SPRI-Lab+, Horiba Scientific-Genoscripts, France) was used in an incubator (Memmert Peltier, Rose Scientific, Canada) set at 25 °C during all experiments. To analyze the kinetics of NP binding, the slope of the plasmon curves was computed by the manufacturer’s software to facilitate the selection of the working angle, which corresponds to the point of the plasmon curve where the slope is maximum. After baseline stabilization by running mobile phase PBS-T, NP solutions were prepared as described in Section 2.3, diluted into PBS-T, and injected into the SPRI mobile phase via an injection loop with 200 µL volume to determine the binding kinetics. Once a stable signal was observed (i.e., a plateau in the RIU signal was reached), NPs could be disassociated from the VCAM-1 protein, regenerating the binding sites for subsequent NP injections at different concentrations. To accomplish this, the SPRI chip surface was subjected to sequential 30 s injections of two regeneration solutions (R1: 2 m MgCl₂ + 0.02% SDS and then R2: 100 × 10⁻³ M NaOH) (Figure S1, Supporting Information). Up to 20 spots were defined for measurement on the SPR image of the chip surface in the software. Statistical analyses for affinity and kinetics were performed for at least three independent experiments. Binding is reported in RIU. All the data treatment, analyses, and statistics were performed using a custom R script based on the tidyR package, which can be found in the Supporting Information.

Cell Culture: Primary mECs were isolated from the lungs of C57Bl/6 or GFP+ mice 6–10 weeks old as previously described.[42] Cells were maintained in a 1:1 mixture of EGM-2 (Lonza, Canada, CC-3162) and DMEM/F12 (Wisent, QC, Canada) containing 10% FBS and 1% penicillin/streptomycin (P/S). mECs were cultured in flasks or plates coated with 0.1% gelatin (EMD Millipore E006-08-B) and used before passage 5.

VCAM-1 Activation and NP Administration: To activate VCAM-1 expression on mECs, cell culture media was changed to DMEM/F12 (10% FBS, 1% P/S) containing 20 ng mL⁻¹ mouse TNFα (Peprotech, USA, 315-01A) overnight. VCAM-1 expression was assayed with western blotting of cell lysates as mentioned previously[21] on a 10% SDS-PAGE gel using a VCAM-1 primary antibody (1:500, Santa Cruz, CA, Sc-8304). For NP uptake, mECs were seeded into six-well plates at 26 000 cells cm⁻² and challenged with 100 ng mL⁻¹ TNFα overnight as mentioned above. Nanoparticles were then formed containing a total of 5 µg well of Cy3-labeled PLL-10 and added directly into wells containing cells and medium with 10% serum. 2 h later, cells were washed twice with PBS, trypsinized, washed again in PBS, and then resuspended in PBS with 2% FBS for flow cytometry measurements. An example gating strategy to identify Cy3+-cells can be seen in Figure S3 (Supporting Information).

Flow Cytometry: Flow cytometry was performed on a BD LSR Fortessa (BD Biosciences, USA) using a 561 nm laser (LSB9/15 filter) for Cy3 fluorescence and 405 nm laser (450/50 filter) for DAPI, which was added 5–10 min before running the sample at a final concentration of 1.67 µg mL⁻¹ to discard dead cells. A total cell count of 10 000 live cells was considered for analysis. Data were analyzed by gating singlets, live cells, and finally Cy3+ cells using Flowjo software (BD Biosciences).

Animal Care: All animal experiments were approved by McGill University-Lady Davis Institute’s animal ethics committee. Mice were maintained on a 12 h light/dark cycle with unlimited access to food. LDLR⁻⁺ mice (C57BL/6 background) were placed on a high-fat diet (0.5% cholesterol, 13% cocoa butter) for 9–12 weeks to develop atherosclerotic lesion. Mice were weighed on 12 h light/dark cycle with unlimited access to food. LDLR⁻⁺ mice (C57BL/6 background) were placed on a high-fat diet (0.5% cholesterol, 13% cocoa butter) for 9–12 weeks to develop atherosclerotic lesion. Mice were weighed on a weekly basis and sacrificed via CO₂ asphyxiation to preserve blood vessel integrity within tissues. No distinction was made between animal sex as both male and female mice of these models develop sufficient plaques for the purpose of this study.
Ex Vivo Binding of NPs to Mouse Atherosclerotic Tissue Sections:

Aortic Sinus: For preparing aortic sinus slices, the heart was removed along with the brachiocephalic artery (BCA) and connecting aorta in one piece. Tissues were fixed in 4% PFA overnight at room temperature and then switched to PBS + 30% sucrose and left an additional 24 h at 4 °C. The heart was isolated, and the bottom half cut away to create a flat surface. The top half of the heart containing the aortic sinus was embedded in optimal cutting temperature (OCT) compound (TissueTek, USA). 7 µm sections were sliced using a cryostat (Leica, Germany, CM3050S), placed onto Superfrost Plus slides (Fisher Scientific, USA), and stored at −80 °C until use. Tissue sections were thawed at room temperature for at least 30 min, blocked (PBS, 5% goat serum, 0.3% Triton X-100) for 1 h and then incubated with VCAM-1 (clone D8U5V, Cell Signaling Technologies, USA, #39036) diluted 1/400 in PBS, 1% BSA, 0.3% Triton X-100 overnight at 4 °C. Slides were then washed 3× (5 min each) with PBS and incubated 1 h protected from light with goat anti-rabbit AlexaFluor 488 secondary antibody (ThermoFisher Life Technologies, USA, A11008) diluted 1/500 in PBS (1% BSA, 0.3% Triton X-100). Slides were again washed 3× (5 min each) and incubated with targeted or nontargeted NPs (as prepared above) containing 10 µg Cy3-labeled IL-10 plasmid for 2 h protected from light. After washing 3× (5 min each) in PBS, slides were incubated in a final solution of DAPI (1/50 000 in PBS) for 10 min protected from light. A final wash was performed as above and slides were mounted with Dako mounting medium (Agilent Dako, USA, S3023). Images were acquired with a Zeiss LSM 780 confocal microscope using Zen Black 2012 software and analyzed using ImageJ (NIH, USA).

En Face Aortas: En face preparations of mouse aortas were achieved as previously described.[4] Briefly, a midline incision was made and then the ribs were cut laterally to the sternum to reveal the thoracic cavity. A gravity perfusion setup was constructed consisting of 60 mL syringes of NP-VHPK, NP-Cys, or vehicle control (Na-acetate buffer, 25 × 10^{-3} M, pH = 5.0). NP solutions contained 25 µg Cy5-IL10 plasmid. The solution was then changed to 4% PFA and allowed to flow through the vasculature until the liquid coming out of the femoral artery was clear. The solution was then changed to 4% PFA and allowed to flow through the vasculature for 5 min. The aorta was then cleaned of all perivascular fat and carefully removed, all the while taking care not to overstrectch or rip the aorta, which can affect endothelial cell morphology. The aorta was then removed and placed in a petri dish with PBS under a dissecting microscope to make the en face cuts. The opened vessels were then transferred to each well of a 12-well plate and permeabilized in PBS + 0.1% Triton X-100 for 10 min with rocking at room temperature (RT), washed briefly with PBS, and blocked in TTBS (10% goat serum, 2.5% Tween-20) for 30 min with rocking at RT. Aortas were then incubated with VCAM-1 antibody (as mentioned above) in TTBS (10% goat serum, 2.5% Tween-20) overnight at 4 °C with rocking. After washing 3× (10 min each) in TTBS at RT with rocking, secondary goat anti-rabbit AF488 (as mentioned above) was incubated in TTBS (10% goat serum, 2.5% Tween-20) for 1 h at RT with rocking protected from light. DAPI was included in this solution at a final concentration of 1 µg mL^{-1}. Wells were washed as above and targeted or nontargeted NPs containing 10 µg Cy3-labeled pl-l-10 were added and incubated 1 h with rocking at RT protected from light. Aortas were finally washed with TTBS as mentioned above and rinsed a final time in PBS before being placed endothelium-side-down on a cover slip containing mounting media, followed by the slide and some paper towels. Roughly 3 kg of weight was then applied onto the slide for at least 5 min to flatten out the tissue as much as possible before sealing the slide with nail polish. Images were taken on a Zeiss LSM 780 confocal microscope using Zen Black 2012 software and analyzed using ImageJ.

In Vivo Injections and Imaging: LDLR^{-/-} mice on HFD at least nine weeks were injected intravenously via the retro-orbital sinus with 175 µL solution of NP-VHPK, NP-Cys, or vehicle control (Na-acetate buffer, 25 × 10^{-3} M, pH = 5.0). NP solutions contained 25 µg Cy5-IL10 plasmid. The mice were imaged either the morning on drawing or the morning of imaging using a combination of an electric razor and depilatory cream. Two or 24 h after injection, mice were imaged live in a supine position using the IVIS imaging system and data were analyzed using Living Image software (Version 4.5.2.18424). The following imaging sequence was used to differentiate Cy5 signal from tissue autofluorescence (Ex./Em.: 605/660, 605/680, 605/700, 640/680, 640/700, 640/720, 640/740). Mice were then sacrificed via CO2 asphyxiation and organs (liver, lungs, kidney, and spleen) were collected and similarly imaged for biodistribution data.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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