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Quantitation of nanoparticle accumulation in flow using optimized microfluidic chambers

J. Kusunose, M. K. J. Gagnon, J. W. Seo, and K. W. Ferrara

Department of Biomedical Engineering, University of California, Davis, CA, USA

Abstract

Background—The vascular cell adhesion molecule-1 (VCAM-1) targeting peptide sequence, VHPKQHR, is a promising moiety for targeting atherosclerosis through incorporation into nanoparticles such as dendrimers and liposomes.

Purpose—We aim to develop VCAM-1-targeted nanoparticles that effectively accumulate on the endothelium under shear conditions and to develop robust microfluidic chambers able to house sufficient cells for flow cytometric measurements.

Methods—Carboxyfluorescein-labeled monomeric VHP-peptide, tetrameric VHP-dendrimers (bisbidentate or radial architecture, with or without N-terminal acetylation) and VHP-peptide liposomes were prepared. Human umbilical vein endothelial cells were treated with nano-particles under 0 or 2.9 dyne/cm² shear, and particle binding was quantified. Flow chambers cured at various temperatures, with or without glass backings were fabricated, characterized for deformation and applied in experiments.

Results—Although liposomes accumulated with highest efficiency, dendrimers also demonstrated specific binding. N-terminal acetylation significantly reduced dendrimer binding, and despite shorter movement range, bisbidentate dendrimers outperformed radial dendrimers, suggesting multiple epitope presence within its estimated arm-span of 57 Å. Under shear, while liposome binding increased 300%, dendrimer binding to cells decreased 65%. Through higher temperature curing and glass backing insertion, polydimethylsiloxane flow chambers maintaining rectangular cross-section with aspect-ratio as low as 1:111 were achieved.

Conclusion—Optimized dendrimers and liposomal nanocarriers specifically accumulated onto cells within microfluidic chambers.

Keywords

Atherosclerosis; dendrimers; in vitro model; liposomes; peptide; targeted drug delivery

Introduction

Cardiovascular diseases are responsible for one in every three deaths in the United States and, in particular, atherosclerosis plays a major role in disease etiology [1]. The disease is characterized by the chronic formation and remodeling of fatty plaques on the arterial walls leading to stenosis. The disease progression is gradual, remaining asymptomatic (or “silent”) over decades, until the fibrin cap separating the fatty lesion inside the vulnerable plaque and the arterial lumen ruptures. Upon rupture, highly thrombogenic material, such as tissue factor [2], is released into the lumen, leading to thrombi formation, which can then lead to myocardial infarction or stroke. Molecular imaging of and selective delivery of therapeutics is necessary for the diagnosis, treatment and monitoring of atherosclerotic sites; however, such vehicles are currently lacking. Vascular cell adhesion molecule-1 (VCAM-1) is an inflammatory marker that plays an integral role in the monocyte recruitment observed in early atherogenesis [3] and is prevalent on the microvasculature [4] and smooth muscle cells [5] in atherosclerotic plaques, making it an attractive target for molecular imaging or targeted drug delivery. A VCAM-1 targeting cationic heptapeptide, VHPKQHR [6] (VHP), has been identified through *in vivo* phage display and has been successfully incorporated into multivalent nanoparticles for VCAM-1 imaging using magnetic resonance imaging [7] and positron emission tomography [8]. We incorporate this peptide into our particles in order to compare the resulting avidity and accumulation. The avidity of such peptide-mediated delivery vehicles is affected not only by multivalency [9] but also by the structure [10] and peptide terminus. Peptide modifications, such as C-terminal amidation, sulfation, phosphorylation and glycosylation, and N-terminal acetylation occur naturally and have profound effects on biological activity [11].

Flow environment plays an integral role in atherosclerosis. High shear stress (>15 dyne/cm²) protects the vessels from the formation of atheroma, while disturbed flow or low shear stress (<4 dyne/cm²) promotes atherogenesis by altering protein levels, including vasodilators/constrictors, antioxidant enzymes and inflammatory mediators [12]. The target receptor in this study, VCAM-1, has been shown to be maximally up-regulated after cells have been exposed to a shear stress of approximately 2 dyne/cm² [13]. Certain ligand-receptor pairs involved in leukocyte recruitment during atherogenesis have been identified as catch bonds [14,15], allowing for leukocytes to bind under flow in a shear stress range of 1.5 ~ 4.0 dyne/cm² [16].

In developing nano-carriers targeting atherosclerosis, where carriers will be delivered to the vascular walls through the blood stream, *in vitro* characterization of the particles in the presence of physiological shear flow is crucial. Particle migration toward and adherence to chamber walls is traditionally assessed by flowing particles through parallel plate flow chambers (PPFCs) or microfluidic chambers and counting the number of particles bound per cell or unit area. However, quantification can be laborious and challenging when working with nanoparticles of size below the optical threshold. We aimed to build a robust, quantitative, easy to use device that conserve resources and allows for the quantification of particle capture to cells under flow. Flow cytometry is a fast and quantitative method suited for assessing the association of fluorescent nanoparticles per cell, but requires a sizable population of cells to be collected. For maximal cell exposure to physiologically relevant,

uniform shear stress while minimizing the volume of treatment solution, a low aspect-ratio flow chamber with maximal surface area and minimal chamber height is desirable. When working with polydimethylsiloxane (PDMS), it is recommended that microfluidic chamber aspect ratios be kept above 1:10 in order to prevent collapse [17]. Previous studies show that, although chambers with aspect ratios as low as 1:80 can be fabricated and function without collapsing, the chamber ceiling experiences significant sagging. Deformation in the chamber cross-section is problematic as flow profile uniformity can no longer be assumed, and the shear stress magnitude cannot be characterized using the Navier–Stokes equation for rectangular chambers ($\tau_w = \frac{6\mu Q}{h^2 w}$). PPFCs on the other hand do not suffer from deformation, but their chamber height is limited by gasket thickness (127 μm for the PPFC commercially available from Glycotech (Leland, NC)), which is far greater than that routinely achieved through soft lithography (<50 μm). In this article, we develop PDMS flow chambers with glass inserts as supports with differing curing temperatures to create micro-fluidic chambers with wide, parallel surfaces with chamber height on the order of tens of microns. Using these chambers, we studied the effects of shear stress on nanoparticle binding to cells.

Recent simulations suggest that the phenomenon of shear-enhanced binding is not necessarily limited to those with catch bonds but that similar effects can be achieved in multivalent nanoparticles depending on the valency, shear stress and bond compliance [18]. Previous experiments involving peptide-targeted liposomes have demonstrated that when VCAM-1 expressing cell monolayers were treated under shear the binding of VCAM-1-targeted liposomes increased, while those targeting aminopeptidase N, even with equivalent valency, experienced a decrease in binding [17]. We hypothesized that this effect is due to the combination of high valency and the effective catch bond-like effect of the VCAM-1 targeting VHP peptide. In this study, the accumulation of VHP-peptide targeted constructs is compared with those decorated with control peptides: ARALPSQRSR (ARAL), a cationic peptide of equal charge, and QAHPHVD (QAH), a neutral, scrambled peptide.

In this study, we compared the delivery efficacy of monomeric VHP-peptide, VHP-tetrameric dendrimers and VHP-coated nanoparticles to a monolayer of VCAM-1-expressing human umbilical vein endothelial cells (HUVEC) and studied the effects of shear stress on each construct. Furthermore, ligand/receptor interactions are governed by steric and electrochemical pharmacophore features, such as hydrogen bond donors and acceptors, and hydrophobic regions. Alternative dendrimer scaffold structures and terminal chemistries were explored to optimize binding. We compared the accumulation of N-terminally acetylated peptides with those terminated by their native amine (a hydrogen bond donor). In order to quantify the fluorescence delivered per cell under flow, a non-deforming low-aspect ratio PDMS microfluidic chamber with a large treatment area to enable cell collection for analysis by flow cytometry was developed and characterized. The chamber was designed to be robust, such that it can be applied over a monolayer of confluent cells in a conventional 35 mm Petri dish, and to consume significantly less treatment volume than the commercially available PPFC while producing comparable shear stress.

Materials and methods

Dendrimer, monomer and lipo-PEG-peptide synthesis

Peptides were synthesized on Rink amide MBHA resin (100–200 mesh, 0.56 mmol/g) (NovaBiochem, Beeston, UK) using solid phase peptide synthesis with standard Fmoc chemistry. Fmoc-amino acids and peptide coupling reagents were purchased from NovaBiochem, with the exception of Fmoc-NH-(PEG)27-COOH, which was purchased from EMD Chemicals (Darmstadt, Germany). Solvents and other reagents of analytical purity were purchased from Sigma-Aldrich (Milwaukee, WI) and VWR (Brisbane, CA).

For the VCAM-1 targeting VHPKQHR (VHP) sequence, four dendrimers (VD1, VD2, VD3 and VD4), a monomer (VM) and a lipo-PEG-peptide (LPP) (LPP-VHP) were synthesized (Figure 1). In addition, two sequences were used as controls; QAHPHVD (QAH), a neutrally charged peptide sharing many of the same amino acids as VHP, and ARALPSQSR (ARAL) [19], a non-specific peptide with the same +3 charge as VHP. A dendrimeric version of ARAL (AD) and LPP versions of QAH and ARAL (LPP-QAH and LPP-ARAL, respectively) were synthesized. VD1, VD2, VD3, VD4, AD and VM were labeled with a single 5,6-carboxyfluorescein (FAM) molecule each.

VHP dendrimers were synthesized by building the peptide on a tetrameric base built on an ivDde protected lysine and composed of lysines and short polyethylene glycol chains (miniPEG). The base structure of VD1 and VD3 was based upon the selective high affinity ligand (SHAL) antibody mimics [10] with the arms arranged in bisbidentate form (Figure 1A). VD2 and VD4 have radially extending arms, and their dendrimer base structure was modeled upon the VHP poly-lysine dendrimer published by Nahrendorf et al. [8] but with the arm length extended through the addition of a miniPEG to match the arm span of VD1 and VD3 (Figure 1B). The amine ends of the VHP peptide of VD1 and VD2 were reacted with acetic anhydride to form an acetyl cap. In synthesizing VD3 and VD4, a Boc-protected valine was used instead, which resulted in a free amino group on the N-terminus post-cleavage. The sequence for each VHP monomer and dendrimer on resin is as follows: VM = Boc-VHPKQHRGGSK(ivDde)GC, VD1 = [(Ac-VHPKQHR)-K(RHQKPHV-Ac)]miniPEG-K([(Ac-VHPKQHR)-K(RHQKPHV-Ac)]miniPEG)-K(ivDde), VD2 = [(Ac-VHPKQHR-miniPEG)-K(miniPEG-RHQKPHV-Ac)]-K[(Ac-VHPKQHR-miniPEG)-K(miniPEG-RHQKPHV-Ac)]-K(ivDde), VD3 = [(Boc-VHPKQHR)-K(RHQKPHV-Boc)]miniPEG-K([(Boc-VHPKQHR)-K(RHQKPHV-Boc)]miniPEG)-K(ivDde) and VD4 = [(Boc-VHPKQHR-miniPEG)-K(miniPEG-RHQKPHV-Boc)]-K[(Boc-VHPKQHR-miniPEG)-K(miniPEG-RHQKPHV-Boc)]-K(ivDde). FAM labeling was accomplished by removing the ivDde with 2% hydrazine in dimethylformamide to reveal the amine, followed by conjugation with FAM. FAM-labeled ARAL dendrimer was synthesized using a modified method from previous literature. The FAM-labeled dendrimer base carrying four aminoxy groups and levulinic acid-amido-dPEG4-ARAL were synthesized individually on solid phase and were ligated together in liquid phase to form the FAM-labeled ARAL dendrimer. Each monomer carried a single FAM molecule and a single targeting peptide, while each dendrimer molecule carried one FAM molecule and four targeting peptides. Products were cleaved off resin, purified using HPLC (Varian, Palo Alto, CA) and the

molecular mass was confirmed using matrix-assisted laser desorption ionization time of flight spectrometry 4700 (Applied Biosystems, Carlsbad, CA).

Unlabeled monomeric peptides were coupled to form LPP conjugates using methods previously described by Zhang et al. [20]. In brief, after synthesis of the desired peptide sequence, Fmoc-NH-(PEG)27-COOH (three units), an Fmoc-Lys(Fmoc) and stearic acid were coupled using Fmoc chemistry onto the peptide through a free amine. For ARAL, the N-terminus amine was used, and for VHP and QAH, the amine exposed at the lysine following ivDde removal was used. This resulted in a PEGylated lipid-like structure, which could then be incorporated into liposomes (Figure 1C). The sequence for each LPP is as follows; LPP-VHP =Boc-VHPKQHRGGSK-(PEG3600-[Stearic acid-K(Stearic acid)])GC, LPP-QAH =Boc-QAH PHVDGGSK-(PEG3600-[Stearic acid-K(Stearic acid)])GC and LPP-ARAL =stearic acid-K(stearic acid)PEG3600-ARALPSQSR.

Dendrimer arm span estimation

The range of motion between adjacent dendrimer arms for VD3 and VD4 were estimated from the number of amino acids, PEG monomers and additional bonds (such as in the lysine side chain) present. Each amino acid was treated to be 3.6 Å [21], each PEG to be 3.5 Å [22] and each bond outside of these were approximated to be 1.17 Å (3.5 Å/3).

Targeted liposome fabrication

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000), mini extruders, membranes, and filters were purchased from Avanti Polar Lipids (Alabaster, AL), FAM was obtained from Sigma-Aldrich (St Louis, MO) and Sephadex G-75 was obtained from GE Healthcare (Little Front, UK). Liposomes were prepared using methods reported previously [20]. In brief, 3 mg total of DPPC, DSPE-PEG2000 and LPP dissolved in chloroform were combined in a glass vial at 94:4:2 molar ratio, dried to a thin film under a stream of nitrogen and lyophilized overnight. Lipids were then re-suspended in 300 µl of 10 mM FAM in DPBS+/+ solution (pH 7.4, 300 mOs) through repeated heating, vortexing and light sonication. Lipid solutions were extruded through polycarbonate membrane filters with 100 nm pores 21 times, and the formed liposomes were purified *via* size exclusion chromatography through a column of Sephadex G-75. Liposome size was verified using a Nicomp 380ZLS particle sizer (Nicomp PSS, Santa Barbara, CA). Each 100 nm liposome is decorated with approximately 1000 targeting peptides on the surface and is loaded with approximately 3000 FAM molecules.

Cells and cell culture

HUVEC were purchased from Clonetics (San Diego, CA) and were cultured in endothelial growth media supplemented with 0.1% (v/v) human endothelial growth factor, 0.1% (v/v) hydrocortisone, 0.1% (v/v) GA-1000, 0.4% (v/v) bovine brain extract and 10% (v/v) fetal bovine serum, all from Lonza (Portsmouth, NH) inside a 95% air/5% CO₂ atmosphere incubator maintained at 37 °C. Well-plates and Petri dishes for experiments were pre-treated with 50 µg/ml collagen (Type 1, rat tail, BD Biosciences, Bedford, MA) in 0.02 M acetic

acid for 1 h, aspirated and rinsed with sterile DPBS—/— prior to plating with HUVEC of passage four to six. Experiments were conducted when confluence was reached.

PDMS chamber fabrication

PDMS chambers were designed and fabricated as previously reported [17] but with several modifications. In brief, chambers were designed in AutoCADLT2000 (Autodesk, San Rafael, CA), converted to photomasks by Output City (Brandon, OR), and developed into PDMS molds (SU-8 2050 (Microchem, Newton, MA) on silicon wafer), following the manufacturer's instructions for 50 μm feature heights. Sylgard 184 (Dow Corning, Midland, MI) pre-polymer and hardener were mixed at a 10:1 weight ratio and cured at 70 or 85 $^{\circ}\text{C}$, with or without a glass slide imbedded into the PDMS over the chamber. Two glass piece sizes were investigated; a wide piece covering the chamber and over parts of the vacuum network (Figure 2A, left), and a fitted piece covering only the main chamber (Figure 2A, right). Chambers with glass insertions were cured in two steps; first for 60 min with only the bottom half of the mold filled with polymer, and then for another 60 min with the glass backing placed over the chamber and the remaining space filled with pre-cured PDMS. Chambers without glass insertions underwent a single 120-min cure. Once hardened, an inlet, outlet and two vacuum holes were punched through the PDMS using a sharpened 16-gauge blunt needle. House vacuum was applied to the vacuum holes, evacuating the air out the spider web, reversibly binding the chamber to the Petri dish. During experiments, the treatment solution was placed directly over the inlet, and the outlet was connected to a PHD2000 microinjector (Harvard Apparatus, Holliston, MA), which drew treatment solution through the chamber at controlled flow rates.

PDMS chamber characterization

To characterize any chamber deformation during treatment, the height along the cross-section at one-quarter, half and three-fourth way through the PDMS flow chamber was measured under a microscope (Olympus IX71, Tokyo, Japan), while solution was flown through at 10 $\mu\text{l}/\text{min}$. To measure the height, the underside of the PDMS chamber (both inside and outside of the flow chamber) was tagged with a commercial permanent marker at one-quarter, half, and three-fourth way through the flow chamber. The markings inside the chamber were used for determining the ceiling depth, while the markings outside the chamber (where the PDMS came in direct contact with the Petri dish surface) were linearly extrapolated to determine the floor depth across the cross section. The differential between the ceiling and floor depth was reported as the local chamber height. Depth measurements were made every 250 μm . A minimum of six cross-sections were measured for each sample group.

Flow cytometry and analysis

Flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ) was used to quantify the FAM associated with the cells in each cell population. Cells were analyzed using the FL1 channel ($530 \pm 30 \text{ nm}$) to match FAM (495/518). All experimental sample groups were replicated in triplicate or more. Median fluorescence values were recorded and normalized to the no treatment control group, and the mean and standard errors were reported. Analysis of variance tests were applied; and after significant differences were noted, a Bonferroni-

corrected t test (two-tailed, unequal variance) was used to compare each group. A *p* value of less than 0.05/(no. of groups × (no. of groups – 1)/2) was considered significant.

Particle binding studies

Static studies (for VD1, VD2, VD3 and VD4 comparison)—HUVEC were grown to confluence in 24-well plates and treated with 10 ng/ml of tumor necrosis factor- α (TNF α) in media for 4 h. Cells were incubated in 0.2% BSA DPBS+/+ for 30 min, treated with 10 μ M FAM-labeled dendrimer in 0.2% BSA DPBS+/+ for 30 min, and then incubated in 0.2% BSA DPBS+/+. Treatments/incubations were conducted at 37 °C. Cells were rinsed twice with DPBS–/–, dissociated using an enzyme-free cell dissociation buffer (Gibco, Invitrogen, Carlsbad, CA) and collected for FACS analysis.

Static studies (for comparing different nanoparticle carrier types against dynamic studies)—HUVEC were grown to confluence in 48-well plates and treated with 1 ng/ml of TNF α in media for 4 h. In comparing nanoparticles of different composition and size, we anticipate that some constructs will be internalized into the cells more rapidly than others, affecting the apparent particle binding on the cell surface. To prevent internalization during treatment, cell treatments with FAM-labeled monomer, dendrimer or liposomes were conducted at 4 °C for 5 min. Treatment solutions were normalized by cargo concentration (10 μ M FAM), such that the relative cargo delivery efficacy for each nano-carrier could be directly compared. For the monomer and dendrimer, which carry a single FAM molecule each, 10 μ M monomer and dendrimer treatment solutions were prepared in 1% BSA DPBS +/+. For liposomes, which carry on the order of 3000 FAM molecules each, the FAM-matched treatment solution is approximately 3 nM. FAM concentration was verified by measuring fluorescence after lysing the liposomes with Triton X, measuring the fluorescence and comparing it against a standard. Following treatment, cells were rinsed with cold DPBS–/– twice, and incubated in 0.2% BSA DPBS+/+ at 37 °C for 1 h. Cells were then rinsed twice with DPBS–/–, dissociated using TrypLEeTM (Invitrogen), collected and analyzed *via* FACS.

Dynamic studies—HUVEC were grown to confluence in 35 mm Petri dishes and were pre-treated with 1 ng/ml TNF α in media for 4 h. Cells were then chilled to and maintained at 4 °C until and throughout treatment. PDMS flow chambers were affixed over the cell-covered dishes *via* house vacuum, and the cells then underwent a 10 μ l/min flow treatment at a shear stress of 2.9 dyne cm^{–2}, which is in the atherogenic shear stress range, where leukocyte binding would take place. For each flow treatment experiment, cells were treated with rinsing solution (0.2% BSA DPBS+/+) for 1 min, nanoparticle solution (as described above in static studies) for 5 min and rinsing solution for 2 min. Chambers were removed, and the cells outside the treatment region were carefully scrubbed off. Cells were rinsed with cold DPBS–/– twice and incubated in 0.2% BSA DPBS+/+ at 37 °C for 1 h. Cells were dissociated and collected using TrypLEeTM and analyzed through FACS.

Results

Flow chamber construction

The mean and standard deviation of the chamber height across the flow chamber cross-section is illustrated in Figure 2. The mean and standard deviation of all height measurements from each sample group, along with the standard deviation normalized to the mean is tabulated in Table 1. Both the higher curing temperature and the insertion of glass backing material into the PDMS resulted in a significant reduction in flow chamber deformation (increased overall chamber height and decreased variance), with the curing temperature having the greater effect of the two. The insertion of a wide glass backing, combined with the higher curing temperature (PCWG85) led to the rapid degradation in the chamber's ability to form a proper seal over cell surfaces. When the glass was fit to cover only the main chamber such as in PCFG85, effective seals were formed. For maintaining both a low-aspect ratio rectangular cross-section, reliable seals and minimizing flow volume, we found the chamber embedded with a fitted glass piece and cured at 85 °C (PCFG85) to be optimal.

By employing both techniques, sagging was nearly eliminated, from 14.1% standard deviation for PCNG70 to 3.8 and 3.4% for PCFG85 and PCWG85, respectively. Although PCWG85 exhibited the least amount of deformation (and was therefore most favorable), its ability to effectively adhere to the Petri dish surface through vacuum diminished rapidly after fabrication and often became unusable after a week. On the other hand, PCFG85 remained effective at reversibly adhering to surfaces even after several months. A final aspect ratio of 1:111 ($35.9\text{ }\mu\text{m} \times 4000\text{ }\mu\text{m}$) was achieved for PCFG85.

Effect of dendrimer arm configuration and end capping

Several versions of the VHP dendrimer were synthesized and characterized for binding optimization to HUVEC under static conditions (Figure 3). Accumulation was greatest with VD3, the bisbidentate dendrimer without the acetyl cap, and was greatly reduced for VD2, the capped radial dendrimer (90% reduction in accumulation between VD3 versus VD2). Addition of the acetyl cap to the bisbidentate dendrimer (VD1) resulted in a 74% reduction in accumulation when compared to the uncapped version (VD3), while changing the arm structure from bisbidentate to radial (VD3 versus VD4) resulted in a 26% reduction in accumulation. All differences were found to be significant ($p < 0.00167$).

Peptide specificity

The peptide specificity of the VHP peptide was tested by comparing the delivery of liposomes (Figure 4A) and dendrimers (Figure 4B) targeted with the VHP-peptide against those containing control peptides (ARAL and QAH) under static and dynamic conditions. For the VHP-dendrimer, VD3 was used for comparison with the other particles. A 10- and 14-fold difference in accumulation was observed between VHP- and ARAL-targeted liposomes under static and dynamic conditions, respectively, with minimal accumulation of QAH-liposomes observed under either condition (Figure 4A). A 14-fold difference in accumulation was observed between VHP- and ARAL-dendrimer under static conditions, but only a threefold difference was observed under dynamic conditions (Figure 4B).

Targeted-particle accumulation under static versus dynamic conditions

The delivery efficacy of FAM to HUVEC through monomeric, tetrameric and liposomal constructs targeted with the same VHP-peptide was directly compared under static and dynamic conditions (Figure 5). Under both conditions, liposomes were able to most effectively deliver FAM, followed by dendrimers (VD3) and then monomers. However, while dendrimer accumulation decreased by 65% when treated under shear stress, VHP liposome accumulation increased by 300%. All differences, with the exception of static monomer versus dynamic monomer, were significant ($p < 0.0033$).

Discussion

Quantitative evaluation of the accumulation of nanometer-sized constructs in flow is complicated by the paucity of devices within which cells can be exposed and assayed. In this study, we optimized the engineering of a flow chamber and applied these chambers to evaluate constructs with diameters from a few nm to 100 nm and with a range of architectures and peptide terminus. The dendrimeric structures studied here are of great interest for the treatment of cancer and cardiovascular disease due to their small size, potential to penetrate tissue and minimal toxicity. Alternatively, 100-nm liposomal structures can carry a far greater payload and offer enhanced valency. We applied the optimized chamber and demonstrated the ability to provide a direct comparison between these approaches.

The VHP peptide is an amino acid sequence identified through *in vivo* phage display and is a homolog of the very late antigen-4 (VLA-4; $\alpha_4\beta_1$) [6], a leukocyte integrin that binds to VCAM-1, facilitating monocyte extravasation. We sought to synthesize and optimize a dendrimeric nanoparticle targeting VCAM-1 using this peptide sequence. The impact of peptide arrangement on particle avidity was explored by synthesizing and comparing the binding of tetrameric dendrimers with arms presented in bisbidentate or radial arrangement and with amino ends exposed or capped. The VCAM-1 protein is built of seven immunoglobulin-like domains, of which domain 1 and domain 4 are homologous, and both are active binding sites to VLA-4. Blocking studies using antibodies specific to domain 1 (but not to domain 4) have been shown to prevent VLA-4 presenting Ramos cells from binding to VCAM-1, suggesting a close link between the two active domains [23]. The bisbidentate dendrimer consists of two pairs of peptides bridged by a PEG chain and is based upon the SHAL antibody mimics [10] designed for targeting two separate but closely positioned epitopes on a single ligand receptor, much like antibodies. Alternatively, the radial dendrimer consists of four peptide-PEG chain arms bound by a lysine tetramer base and maximizes the individual arm's freedom from each other. Using the methods described earlier, we estimated the maximal distance between the two terminal valines in the bidentate peptides to be 57 Å, and the maximal distance between the valines of adjacent arms in the radial dendrimers to be 79 Å. We found that the bisbidentate dendrimers, despite the shorter range of movement, outperformed the radial dendrimers, suggesting the presence of multiple epitopes within a close range.

Acetylation is the most common covalent modification observed in eukaryotic proteins [24]. Approximately 85% of eukaryotic proteins are acetylated at the N-terminus, and many are

reversibly acetylated at the epsilon-lysine residue, such as in the case of histones, high mobility group proteins, transcription factors, nuclear receptors and alpha-tubulin [24]. The biological effect of acetylation can be profound: N-terminal acetylation is responsible for blocking the transport of newly translated protein into the endoplasmic reticulum [25], and the acetylation of epsilon-lysine residues on histones affect histone–DNA binding, interactions between nucleosomes and between histones and regulatory proteins [26]. In terms of the effect on binding avidity, acetylation has been found to both improve [27] and inhibit [28] binding to target proteins. In our studies, the N-terminal acetylation was found to significantly reduce VHP dendrimer binding (76% for bisbidentate and 87% for radial). Although the acetylation reduces the dendrimer's overall charge from +12 to +8, it is unlikely that the charge was the primary driving force behind the VHP-dendrimer's avidity, as the equally cationic ARAL dendrimer (+12) exhibited minimal binding. In highly multivalent situations such as the 2 mol% LPP liposomes, the effect of charge is more apparent, as the charged ARAL liposomes, though not nearly as effective as the VHP-liposomes, bound significantly better than the uncharged QAH-liposome. Rather, it is likely that the terminal valine or the amine itself plays a key role in the binding of the VHP peptide to VCAM-1. Analyzed as a pharmacophore, the amine is a hydrogen bond donor, and therefore its acetylation would result in a loss of a functional group.

Monomeric, dendrimeric and liposomal forms of the VHP-peptide were synthesized and labeled with FAM, and their ability to deliver cargo (FAM) was compared under static and dynamic conditions. Treatment solutions were normalized by FAM concentration in each. As anticipated from various studies regarding multivalency [8,9], increasing the number of targeting peptides from one to four led to a significant (30-fold) increase in particle delivery under static conditions. Liposomes with 2 mol% peptide loaded with 10 mM FAM were able to deliver four times as much FAM to HUVEC than dendrimers with a single FAM molecule under static conditions. When cells were treated with particles flown over at 2.9 dyne/cm², monomer binding was not significantly affected, dendrimer binding decreased by 65% and liposome accumulation increased by 300%. In our previous work with peptide-targeted liposomes, we showed that, given similar size and peptide concentration, the accumulation of liposomal nanoparticles in the presence of shear stress is very much dependent on the peptide-ligand receptor pair [17]. In this study, comparing the VHP-dendrimer and VHP-liposome's response to shear stress, it is clear that the nanoparticle's response to shear flow is dependent not only on the targeting moiety but also on the avidity and particle size.

We designed a robust PDMS microfluidic chamber optimized for quantifying nanoparticle accumulation to cells under physiological shear stress while minimizing the reagent volume. We synthesized and compared the adhesion of various atherosclerosis-marker targeting carriers to cells, from monovalent to highly multivalent nanoparticles, under static and dynamic conditions using the new chamber. By curing PDMS at a higher temperature and introducing a glass backing, we were able to maintain a rectangular cross-section for flow chambers with final aspect ratios as low as 1:111 (35.9 μm×4000 μm, for PCFG85), creating a chamber with uniform shear stress profile while minimizing treatment volume. This robust microfluidic chamber with low-aspect ratio and rectangular cross section was applied for

cell analysis *via* FACS, combining the advantages of PPFC with those of PDMS microfluidic chambers. Cells over a significant surface area were treated with relatively uniform shear stress characterized by the Navier–Stokes equation. A chamber height on the micron scale ($>50\ \mu\text{m}$) was achieved, significantly reducing the treatment volume required for producing physiological shear stress. The new flow chamber, with dimension of $4\ \text{mm} \times 15\ \text{mm} \times 36\ \mu\text{m}$ (width $\times$ length $\times$ height), can treat a surface area of $0.6\ \text{cm}^2$ at $2.9\ \text{dyne/cm}^2$ with reagent consumption of just $10\ \mu\text{l/min}$. A similar PPFC with $127\ \mu\text{m}$ gasket thickness would require $12.4\times$, or $124\ \mu\text{l/min}$ of reagent to produce an equivalent shear stress. The area treated is comparable to that of a single well from 48-well plate, which has a surface area of $0.75\ \text{cm}^2$. The chamber can be vacuum-affixed over a fully grown monolayer of cells in a standard 35 mm Petri dish, is inexpensive to fabricate, and can be used repeatedly. Though chamber dimensions were optimized for FACS, we anticipate that the flow treatment area can be further expanded without deformation to accommodate studies that require even more cells to be collected, such as those involving cellular protein extractions for downstream protein expression testing post flow treatment.

Conclusion

In developing nano-carriers targeted to vascular diseases such as atherosclerosis, the *in vitro* characterization of the particles in the presence of physiological shear flow is essential. We designed a robust, non-deforming, low aspect ratio microfluidic chamber optimized for quantifying nanoparticle accumulation to cells under physiological shear stress while consuming less than $1/10$ of the reagent typically consumed. We synthesized and compared the adhesion of various atherosclerosis targeting carriers to cells, from monovalent to highly multivalent nanoparticles, under static and dynamic conditions. As expected, among monomers, dendrimers and liposomes, delivery efficiency generally increased with valency, with liposomes being the most efficient carrier. Treating the cells under a shear stress of $2.9\ \text{dyne/cm}^2$ led to an increase in liposome accumulation by 300% and a decrease in dendrimer accumulation of 65%, demonstrating that a nanoparticle's response to shear flow is dependent not only on the targeting moiety but also on the avidity and particle size.

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Figure 1.

Structures of the VHP nanoparticles synthesized for this study. (A) Bisbidentate VHP dendrimer (R' : NHCOCH₃ (VD1), R' : NH₂ (VD3)). (B) Radial VHP dendrimer (R' : R' : NHCOCH₃ (VD2), R' : NH₂ (VD4)). (C) Lipo-PEG-peptide structure, where the peptide is linked to a pair of stearic acids through a PEG-lysine chain.

Figure 2.

(A) Schematic of PDMS chambers with wide (left) or fitted (right) glass embedded into the PDMS over the channel. (B) Chamber heights throughout various PDMS chambers. Measurements are of chambers cured at low (70 °C) or high (85 °C), with or without a glass piece of backing embedded into the PDMS. <□> low temp, no backing (PCNG70), <△> low temp, wide glass backing (PCWG70), <■> high temp, no backing (PCNG85), <◆> high temp, fitted glass backing (PCFG85), <▲> high temp and wide glass backing (PCWG85). Deformation decreased with higher curing temperature and the addition of a glass support. At least six measurements were made per data point.

Figure 3.

Binding comparison of FAM-labeled VHP peptide dendrimers to HUVEC under static conditions, at 37 °C for 30 min. In general, uncapped and bisbidentate dendrimers bound better than acetyl capped and radial dendrimers. A sample size of $N=6$ was employed for each group. $p<0.00167$ for all groups.

Figure 4.

Comparing the VHP peptide with control peptides on (A) liposomes and (B) dendrimer accumulation to VCAM-1 expressing HUVEC under static and dynamic conditions. For the VHP-peptide dendrimer, VD3, the bisbidentate dendrimer with free N-terminal amine was used. The accumulation of particles decorated with the VHP peptide were compared with those coated with ARAL, a peptide with equal charge (+3 charge), and QAH, a scrambled peptide with neutral charge. Both ARAL liposomes and dendrimers bound significantly less than VHP counterparts under static and dynamic conditions. Mean and standard error are displayed. All differences not specifically marked were significant ($p < 0.0033$ for A, $p < 0.0083$ for B).

Figure 5.

Flow cytometry results of HUVEC treated with FAM-labeled VCAM-1 targeted nanoparticles under static and dynamic (2.9 dyne/cm²) conditions. VD3 was used as the VHP dendrimer. All treatment solutions were normalized by fluorescence prior to treatment. A minimum of 12 samples were used for each group. Mean and standard error are displayed. While the treatment of cells under flow reduced dendrimer accumulation, it increased liposome accumulation. Difference in monomer accumulation under static versus dynamic conditions was not significant. For all others, $p < 0.0033$.

Table 1

Average and standard deviation of all chamber height measurements made for each group.

Chamber name	Glass insert	Curing temperature (°C)	$\mu \pm \sigma$ (μm)	σ/μ
PCNG70	N/A	70	27.53 ± 3.87	0.141
PCWG70	Wide	70	30.06 ± 2.81	0.093
PCNG85	N/A	85	32.94 ± 1.78	0.054
PCFG85	Fitted	85	35.93 ± 1.35	0.038
PCWG85	Wide	85	38.76 ± 1.34	0.034