A Biomicrofluidic Screening Platform for Dysfunctional Endothelium-Targeted Nanoparticles and Therapeutics

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The internal surfaces of all blood and lymphatic vessels are lined with an endothelium, which tightly controls and regulates the permeability of biological molecules. A dysfunctional endothelium (Dys-En) is a hallmark of many diseases, including atherosclerosis. Dys-En in atherosclerosis leads to loss of adherens junctions between cells, thus enhancing permeability and upregulation of adhesion receptors such as vascular cell adhesion molecule 1 (VCAM-1). Both this enhanced permeability of the endothelium and associated upregulated endothelial cell surface receptors can be exploited in nanomedicine targeting to atherosclerotic plaques. However, the relationship between targeting ligand and nanoparticle (NP) size is not well understood within this context. Herein, a biomicrofluidic model of Dys-En is developed and this platform is used to screen VCAM-1 targeted NPs. Screening of NPs with varying properties under flow shows that size plays a dominant role in NP targeting, with NPs in the range of 30–60 nm showing increased targeting to Dys-En. Moreover, treatment of Dys-En-on-a-chip with Annexin A1, as a novel proresolving mediator of inflammation, results in restoration of adherens junctions and normalization of the barrier integrity. The results demonstrate utility of using “Dys-En-on-a-chip” as a screening platform for Dys-En-targeted nanomedicines and biologics.

1. Introduction

Cardiovascular diseases (CVDs) are the leading causes of death worldwide, leading to 18 million deaths annually.1 CVD is estimated to remain the main cause of mortality, resulting in 24 million global deaths per year by 2030.2 The dominant cause of CVD is atherosclerosis, which is an inflammation-driven disease of the arteries.3 This number-one killer disease results in the formation of atherosclerotic plaques on arterial walls and can eventually cause thrombosis, myocardial infarction, and ischemic stroke.4,5 Despite the growing need for new and improved atherosclerotic therapies, the FDA approvals for new atherosclerotic therapeutics decreased during the past decade.6 One of the reasons behind this problem is the lack of predictive and effective disease models to test novel therapeutics prior to clinical studies.6–7 In vivo models are lengthy, costly, labor intensive, ethically problematic, and poor predictors of human responses.8 In contrast, conventional in vitro models cannot fully replicate the pathophysiological condition of this disease.8 A suitable vascular disease model should be able to mimic the physiological condition of a vessel (e.g., vessel dimensions, flow rate, shear stress, etc.) and the relevant pathological condition of the disease. To overcome the shortcomings of the conventional in vitro and in vivo models, biomicrofluidic vascular models have recently gained significant attention.8–11

To develop a biomimetic and biomicrofluidic model for atherosclerotic research, we focused on dysfunctional endothelium (Dys-En) as one of the main characteristics of atherosclerosis. In a healthy vessel, nutrients and oxygen are supplied to the intima by their diffusion from the lumen. Furthermore, a microvascular network called “vasa vasorum,” supplies oxygen and nutrients to the outer compartment of the vessel walls10 (Figure 1a). Due to the plaque formation in an atherosclerotic

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Dys-En cannot regulate the barrier integrity, which
It therefore, normalization and restoration of a compromised and
leaky endothelium by novel anti-inflammatory and resolving
mediators (e.g., Annexin A1) can be a potent therapeutic strategy
for atherosclerosis[12,13] (Figure 1d).

In this study, we first fabricated a three-layered microchip,
consisting of two polydimethylsiloxane (PDMS) layers separated
by a semipermeable membrane layer. Next, endothelial cells
were cultured on the membrane using microfluidic cell culture
techniques to develop a biomimetic model of endothelium-on-a-
chip (En-on-a-chip). The established En-on-a-chip was infected
using a proinflammatory cytokine to develop a dysfunctional-
endothelium-on-a-chip (Dys-En-on-a-chip), which could success-
fully mimic the enhanced permeability, overexpression of
VCAM-1, and the shear condition of a Dys-En. Then, we used
the developed microfluidic models to 1) screen and understand
VCAM-1-targeted NP permeability and binding to Dys-
En as a function of NP biophysicalchemical properties for more
effective drug delivery to the plaque and 2) investigate the restor-
ative effect of Annexin A1 on Dys-En. A few studies have
investigated binding of VCAM-1-targeted NPs to Dys-En under
microfluidic shear conditions.[34–37] However, a comprehensive
study to investigate the effect of NP properties (i.e., size, VCAM-1
peptide density, zeta potential, and polydispersity index [PDI]) on
NP permeability and binding across healthy and Dys-En-on-a-
chip under flow conditions is yet to be reported. VCAM-1 is
not only overexpressed in atherosclerosis, and vascular inflam-
mation manifests in a wide range of other disease conditions,[4]
where our screening chip can therefore have a much broader util-
ity, and it is why we chose to investigate VCAM-1 targeting in this
instance. In addition, we chose to investigate the proresolving
effects of Annexin-A1 on Dys-En because this protein has
recently attracted significant attention in the field of anti-
inflammatory drug discovery.[31,38,39] Annexin A1 can mimic
how inflammation naturally subsides in the body; thus, it has
fewer side effects than current anti-inflammatory drugs.[33] It
has also been reported that Annexin A1 can show positive effects
to restore the damaged blood–brain barrier (BBB).[40–42]
However, the restorative and proresolving effects of Annexin
A1 on Dys-En as therapeutic strategies for CVD are yet to be
investigated.

2. Results and Discussions

2.1. Development of En-on-a-Chip and Dys-En-on-a-Chip

In the first instance, we developed a microfluidic platform incor-
porating a monolayer of endothelial cells exposed to flow and
shear stress, to be able to mimic the physiological environment
of a vessel. For this purpose, we used a microfluidic chip consist-
ing of three layers. The upper and lower layers were made of
PDMS and fabricated by standard photolithography (Figure S1, Supporting Information) and soft lithography

![Figure 1. a) Structure of a vasa vasorum on a healthy vessel wall. b) Structure of a neovessel sprout from a vasa vasorum into an atherosclerotic plaque. c) Targeted nanoparticles (NPs) are being intensively investigated for diagnosis and treatment of atherosclerosis.[12,13] d) Targeted drug delivery systems to atherosclerotic plaques based on unique targeting opportunities provided by Dys-En such as leaky vessels and upregulated receptors (Figure 1c) and 2) restoration and normalization of Dys-En by an anti-inflammatory and resolving mediator (i.e., Annexin A1).](image-url)
Each PDMS layer contained a channel with the dimensions of $6 \times 0.4$ mm as the main channel, which was connected to two side channels with the dimensions of $3 \times 0.4$ mm. Two circles (2 mm diameter) were considered as the access points to the channels to prevent leakage at the inlet and outlet points. A thin layer of semipermeable polyethylene terephthalate (PET) membrane was bonded between these PDMS layers. This membrane not only separated the upper and lower channels, but also provided suitable support for culturing of endothelial cells. The schematic and photographic representation of the fabricated chip can be seen in Figure 2a,b–d, respectively. After fabrication of the chip, the porous membrane was treated with fibronectin to promote the cell attachment. Human umbilical vein endothelial cells (HUVECs) were then cultured on the membrane by microfluidic cell culture techniques. The schematic representation of the developed biomicrofluidic system and the full coverage of the membrane by the cells can be seen in Figure 2e,f, respectively. Various drugs (e.g., Annexin A1) and NPs (e.g., VCAM-1-targeted NPs) can be perfused through the upper channel and their interaction with the endothelium under physiological shear conditions can be studied not only by collecting the samples from the lower channel, but also using microscopy techniques. The components of the microfluidic cell culture setup developed in this study are shown in Figure S2a, Supporting Information. The system allows long-term culture of HUVECs under physiological flow conditions, inspection of the cells after seeding and during their growth, and accurate control of the flow rate and thus shear stress. This setup was used to run the experiments for eight channels (i.e., four chips), simultaneously. A photographic representation of the setup can be seen in Figure S2b, Supporting Information.

Based on in vivo measurements and fluid mechanical models, vessels prone to atherosclerosis and dysfunction show decreased shear stress ($<4–5$ dyne cm$^{-2}$).\cite{43,44} Decreased shear stress can disrupt vascular wall functions (e.g., reduction in endothelium repair, decreased production of nitric oxide synthase [NOS], etc.)

**Figure 2.** Development of the En-on-a-chip. a) Schematic representation of the three-layered chip fabricated in this study and the dimensions of the chip and channels. In this figure, a and b show the inlet and outlet of the upper channel and c and d show the inlet and outlet of the lower channel. Photographic representation of the chip from b) top view, c) perspective view, and d) side view. e) Schematic representation of the chip after culturing the cells on the membrane. f) Confocal image showing the top view of the upper channel proves the full coverage of the membrane by HUVEC cells (flow rate: 10 $\mu$L min$^{-1}$, scale bar: 100 $\mu$m). Cells were stained for nucleus (blue), VE-cadherin (green), and F-actin (red).
In contrast, obstructions in advanced atherosclerosis can significantly increase shear stress. In this study, we aimed to focus on early-stage atherosclerosis. Therefore, we selected to use lower shear stress (<4–5 dyne cm\(^{-2}\)). The optimized shear stress for microfluidic cell culture not only should be in line with reported in vivo conditions, but also should result in elongation of the cells along the direction of the flow and support a healthy cell culture without disruption of the cellular integrity. To find an optimized shear stress, we performed the microfluidic cell culture using the shear stress values of 0, 0.1, 1, 2, and 4 dyne cm\(^{-2}\). Based on Equation (1), the required flow rates for the shear stresses of 0, 0.1, 1, 2, and 4 dyne cm\(^{-2}\) were calculated to be 0, 1, 10, 20, and 40 \(\mu\)L min\(^{-1}\), respectively. As can be seen in Figure 3, the low shear stress (0 and 0.1 dyne cm\(^{-2}\)) did not result in the complete elongation of the cells along the direction of the flow. In contrast, high shear stress (2 and 4 dyne cm\(^{-2}\)) disrupted cellular connections and did not support healthy cell culture and growth. Moderate shear stress (1 dyne cm\(^{-2}\)) showed elongation of the cells and complete barrier integrity. Therefore, we selected the shear stress of 1 dyne cm\(^{-2}\) (flow rate of 10 \(\mu\)L min\(^{-1}\)) as an optimized condition for HUVEC culture and development of the En-on-a-chip and Dys-En-on-a-chip in this study.

After culturing En-on-a-chip, tumor necrotic factor-\(\alpha\) (TNF-\(\alpha\)) was used to induce Dys-En. TNF-\(\alpha\) is a proinflammatory cytokine, which is highly involved in the pathogenesis of cardiovascular disorders. TNF-\(\alpha\) plasma levels in patients suffering from cardiovascular disorders were reported to be \(\approx 20\) ng mL\(^{-1}\). In this study, media containing 20 ng mL\(^{-1}\) of TNF-\(\alpha\) was perfused through the developed En-on-a-chip, and then three indicators of Dys-En, including the loss of vascular endothelial cadherin (VE-cadherin), formation of actin stress fibers, and overexpression of VCAM-1 receptors, were investigated by immunostaining and fluorescent microscopy (Figure 4).

VE-cadherin is a cell–cell adhesion protein, which plays a key role in endothelial barrier integrity and vascular permeability. The loss of VE-cadherin in endothelial cells results in endothelial barrier disruption and thus enhanced permeability. In this study, the established En-on-a-chip showed the assembly of VE-cadherin molecules at the cellular periphery, where they form tight borders between endothelial cells. In contrast, Dys-En-on-a-chip showed patchy expression of VE-cadherin and loss of VE-cadherin connectivity at the cellular borders, which is an indicator of intercellular gap formation and enhanced permeability (Figure 4a). Furthermore, actin networks participate in endothelial barrier integrity by binding to junctional proteins and stabilizing them. Therefore, healthy En shows the arrangement of actin fibers at the cellular borders to establish endothelial junctions. In contrast, Dys-En shows the formation of actin fibers in central parts of the cells, known as actin stress fibers. These fibers cause transcellular tensions and cell contractions and finally disrupt the barrier integrity. In this study, the developed En-on-a-chip showed the arrangement of actin fibers at the cellular periphery to form actin cortical rims. In contrast, the developed Dys-En-on-a-chip could successfully show the formation of actin stress fibers, which were prolonged in the central domain of the endothelial cells (Figure 4b). Moreover, VCAM-1 immunostaining was performed to investigate the expression of this molecule on the developed En-on-a-chip and Dys-En-on-a-chip. VCAM-1 is an immunoglobulin-like adhesion molecule, which is normally absent on healthy endothelial cells but rapidly shows overexpression on inflamed and dysfunctional endothelial cells. The developed Dys-En-on-a-chip, in this study, successfully showed a significant increase in VCAM-1 expression compared with En-on-a-chip (Figure 4c). To quantify the fluorescent signals, imageJ software was used. The quantification of VE-cadherin, F-actin, and VCAM-1 signals, respectively, showed 2.2-fold decrease, 1.9-fold increase, and 7.8-fold increase in Dys-En-on-a-chip compared with En-on-a-chip. Therefore, the quantification results confirmed the loss of VE-cadherin, formation of elevated production of reactive oxygen species [ROS], and higher permeability of lipoproteins and leukocytes) and therefore results in Dys-En and early-stage atherosclerosis. In contrast, obstructive plaques in advanced atherosclerosis can significantly increase shear stress. In this study, we aimed to focus on early-stage atherosclerosis. Therefore, we selected to use lower shear stress (<4–5 dyne cm\(^{-2}\)). The optimized shear stress for microfluidic cell culture not only should be in line with reported in vivo conditions, but also should result in elongation of the cells along the direction of the flow and support a healthy cell culture without disruption of the cellular integrity. To find an optimized shear stress, we performed the microfluidic cell culture using the shear stress values of 0, 0.1, 1, 2, and 4 dyne cm\(^{-2}\). Based on Equation (1), the required flow rates for the shear stresses of 0, 0.1, 1, 2, and 4 dyne cm\(^{-2}\) were calculated to be 0, 1, 10, 20, and 40 \(\mu\)L min\(^{-1}\), respectively. As can be seen in Figure 3, the low shear stress (0 and 0.1 dyne cm\(^{-2}\)) did not result in the complete elongation of the cells along the direction of the flow. In contrast, high shear stress (2 and 4 dyne cm\(^{-2}\)) disrupted cellular connections and did not support healthy cell culture and growth. Moderate shear stress (1 dyne cm\(^{-2}\)) showed elongation of the cells and complete barrier integrity. Therefore, we selected the shear stress of 1 dyne cm\(^{-2}\) (flow rate of 10 \(\mu\)L min\(^{-1}\)) as an optimized condition for HUVEC culture and development of the En-on-a-chip and Dys-En-on-a-chip in this study.

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actin stress fibers, and overexpression of VCAM-1 in the developed Dys-En-on-a-chip in this study (Figure 4d–f).

2.2. Permeability and Binding of VCAM-1-Targeted NPs Across En-on-a-Chip and Dys-En-on-a-Chip

To gain understanding of which biophysicochemical properties of NPs (i.e., size, charge, PDI, and targeting peptide density) have the most influence on NP binding and translocation across the Dys-En under biomimetic microfluidic conditions, VCAM-1-targeted polystyrene NPs with different biophysicochemical properties were synthesized. We selected polystyrene NPs as model NPs for our experiments. These NPs are widely used as model and reference NPs in vitro and in vivo due to their low cytotoxicity, ease of synthesis, ease of size and surface modification over a broad range, commercial availability, low polydispersity, and high colloidal stability.[51–57] In this study, amino-functionalized and rhodamine-labeled polystyrene NPs with the size of 30, 60, 120, and 250 nm were abbreviated as NP1, NP2, NP3, and NP4, respectively. The NPs were first PEGylated using maleimide polyethylene glycol N-hydroxysuccinimidy l ester (Mal-PEG-NHS) and methoxy polyethylene glycol N-hydroxysuccinimidy l ester (m-PEG-NHS) (Figure 5a). Subsequently, the PEGylated NPs were conjugated with a VCAM-1-targeting peptide (VHPKQHR-GGGC) (Figure 5b). After NP preparation, they were characterized for their size, zeta potential, PDI, and peptide density. Based on the dynamic light scattering (DLS) technique, NP sizes for NP1, NP2, NP3, and NP4 were, respectively, 49.3 ± 1.1, 73.4 ± 1.2, 122.9 ± 2.5, and 281.1 ± 3.5 nm (Figure 6a). Furthermore, zeta potentials for NP1, NP2, NP3, and NP4 were respectively 8.1 ± 0.9, 7.5 ± 0.4, −8.3 ± 0.8, and −10.4 ± 1.3 mV (Figure 6b). In addition, this technique showed PDIs of 0.11 ± 0.014, 0.02 ± 0.015, 0.10 ± 0.001, and 0.04 ± 0.005 for NP1, NP2, NP3, and NP4, respectively (Figure 6c). Based on UV spectroscopy, the number of peptides per NP for NP1, NP2, NP3, and NP4 were, respectively, 67.87 ± 11.64, 437.84 ± 30.72, 2996.51 ± 150.89, and 75366.97 ± 638.03. Therefore, peptide density per surface unit of NPs can be calculated, which showed the density of 0.003 ± 0.0006, 0.009 ± 0.0006, 0.017 ± 0.0009, and 0.078 ±

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**Figure 4.** Confocal images of a) VE-cadherin, b) F-actin, and c) VCAM-1 for En-on-a-chip and Dys-En-on-a-chip. To establish Dys-En, HUVECs were cultured on the chip (10 μL min⁻¹, 48 h) and then stimulated with TNF-α (20 ng mL⁻¹, 10 μL min⁻¹, 12 h). The images are representative of three independent experiments (scale bar: 50 μm). Quantification of d) VE-cadherin, e) F-actin, and f) VCAM-1 signals confirmed the loss of VE-cadherin, formation of actin stress fibers, and overexpression of VCAM-1, which are known as hallmarks of Dys-En. Data show mean ± SD (n = 5–7) and *** represents statistically significant difference (p-value < 0.001).
for NP1, NP2, NP3, and NP4, respectively (Figure 6d). After NP characterization, the permeability and binding of the NPs across En-on-a-chip and Dys-En-on-a-chip were studied (Figure 6e–h).

The permeability of NP1, NP2, NP3, and NP4 across En-on-a-chip was 2.01 ± 0.46%, 1.69 ± 0.88%, 1.21 ± 0.04%, and 0.21 ± 0.10%, respectively. For Dys-En-on-a-chip, the permeability of all NPs was higher than their permeability across En-on-a-chip due to intercellular gaps, disrupted junctions, and compromised integrity of Dys-En. The permeability of NP1, NP2, NP3, and NP4 across Dys-En-on-a-chip was, respectively, 7.87 ± 1.08%, 6.05 ± 0.77%, 3.88 ± 1.04%, and 0.51 ± 0.10% (Figure 6e). The binding of the NPs to En-on-a-chip and Dys-En-on-a-chip was investigated by fluorescent microscopy. The representative images can be seen in Figure 6f. To quantify the results, mean fluorescent intensity (MFI) of rhodamine (i.e., MFI of the NPs) and the exact number of nuclei (i.e., the exact number of cells) were obtained and then MFI/cell was calculated. The binding of NP1, NP2, NP3, and NP4 to En-on-a-chip was, respectively, 2.5 ± 0.41, 2.2 ± 0.38, 1.6 ± 0.27, and 1.8 ± 0.9. However, their binding to Dys-En-on-a-chip was found to be 20.02 ± 0.8, 18.7 ± 0.9, 8.3 ± 0.6, and 11.7 ± 1.2 (Figure 6g).

Consequently, based on the above-mentioned data: 1) permeability across Dys-En was in the following order: NP1 > NP2 > NP3 > NP4. So, the smaller the NPs, the higher their permeability. The larger NPs showed lower permeability, even though they had higher peptide density. These results suggest that size governs NP permeability regardless of peptide density. 2) NP binding to Dys-En was in the following order: NP1 and NP2 (no significant difference) > NP4 > NP3. So, the smaller NPs showed the highest binding even though they had the lowest peptide density. NP4, which had the highest peptide density among all groups, was in the next place, followed by NP3. Therefore, the results suggest that NP binding is first governed by size and then peptide density. Finally, 3) in this study, no trends were found between permeability and binding data with PDI and zeta potential. The summary of the results can be seen in Figure 6h, which shows the effect of the NP properties on their permeability and binding across a Dys-En-on-a-chip.

Incorporating a porous membrane into a microfluidic device has been previously used to study the permeability of fluorescent tracers across endothelial layers. Although the results of these studies can be informative about the integrity and disruption of various endothelial layers, they cannot be used in the field of nanomedicine to design optimal NPs to traverse these
barriers. The reason is that the hydrodynamic diameter of these tracers is much smaller than the diameter of typical NPs used in nanomedicine. Only a few studies have been reported on the permeability of NPs across healthy and/or Dys-En under microfluidic flow. For instance, Wang et al. studied the permeability of two types of NPs with different stiffnesses but similar sizes (≈70 nm) across TNF-α-stimulated HUVECs. The results showed similar permeability for these NPs, which indicates the dominant effect of size on permeability. Also, Kim et al. studied the permeability of lipid NPs with a size of ≈70 nm across nonstimulated and TNF-α-stimulated HUVECs. The results showed a significant increase in NP permeability across TNF-α-stimulated cells compared with nonstimulated cells, which is in line with our findings. However, none of these studies investigated the effect of NP properties such as size and peptide density on their permeability. In addition, Sasaki et al. developed a mathematical model to investigate the relationship between the size of NPs and their permeability across a microporous membrane under microfluidic flow. Their model confirms the higher permeability of smaller NPs, but the main limitation of their study is that the membrane was without any cells. Therefore, the effect of NP–cell interactions on permeability was not considered.

Figure 6. Characterization and screening of the NPs across En-on-a-chip and Dys-En-on-a-chip in this study. Characterization of the NPs for their a) size, b) zeta potential, c) PDI, and d) peptide density. e) Permeability of the NPs across En-on-a-chip and Dys-En-on-a-chip. f) Representative images showing the binding of the NPs to En-on-a-chip and Dys-En-on-a-chip. Red and blue colors show NPs and cell nuclei, respectively (scale bar: 20 μm). g) Quantification of the binding results. h) Heat map summarizing the effect of NP properties on their permeability and binding across Dys-En-on-a-chip. The results suggest that NP size is the most dominant factor, and smaller NPs show higher permeability and binding. In this figure, data show mean ± SD of at least three independent experiments. *, **, and *** represent statistically significant differences with p-values of <0.05, <0.01, and <0.001, respectively. ns represents nonsignificant differences.
Various studies have demonstrated that models used to study NP binding to cell adhesion molecules (CAMs) should reflect the flow microenvironment and hemodynamics. For instance, Bhowmick et al. showed that flow and physiological adaptation to flow can control binding and endocytosis of NPs targeted to intercellular adhesion molecule 1 (ICAM-1) in vitro and in vivo. In a similar study, Calderon et al. showed that ICAM-targeted NPs tend to bind to capillary beds, where shear stress is minimal,
rather than binding to vascular areas subjected to significant shear stress, where NPs need to overcome high hydrodynamic forces.\cite{65} Furthermore, Han et al. investigated the importance of flow on targeting of NPs to platelet endothelial cell adhesion molecule 1 (PECAM-1). Their studies showed that chronic and acute flow microenvironments can affect the NP targeting mechanisms.\cite{66,67} These studies emphasize the importance of flow microenvironment for binding of CAM-directed NPs to endothelial cells. Moreover, the VCAM-1 binding to its ligand has been reported to show catch-bond characteristics.\cite{34,58,70–72} The lifetime of a catch-bond initially increases by force because the molecules deform and lock more tightly and then decreases like in a normal bond.\cite{69} Therefore, any mechanical force like shear stress can affect VCAM-1 binding. Despite the important role of shear in this field, only a few studies have used shear-generating systems when studying binding of CAM-targeted NPs.\cite{34,58,70–72} For instance, Kolhar et al. investigated the effect of NP shape on ICAM-1 binding. They reported higher binding of polystyrene nanorods compared with polystyrene nanospheres with the same size and peptide density.\cite{71} Muro et al. studied the effect of dose and peptide density on binding of ICAM-1-targeted NPs and reported the dose-dependent binding of NPs to TNF-α-activated En.\cite{72} Kusunose et al. synthesized a VCAM-1-targeting liposome and studied the effect of peptide density on VCAM-1 binding. The results showed that the NP binding significantly increases by increasing the peptide density.\cite{14} Furthermore, Campos et al. investigated the effect of NP shape on VCAM-1 binding under microfluidic flow. The results revealed that the binding of rod-like NPs was significantly higher than the binding of spherical NPs.\cite{70} However, all studies were based on single-sized NPs. To the best of our knowledge, the effect of NP size on VCAM-1 binding under flow microenvironment has not been reported.

2.3. Restorative Effect of Annexin A1 on Dys-En-on-a-Chip

To study the effect of Annexin A1 on Dys-En, we first measured the translocation of Lucifer Yellow, as an endothelial permeability marker, across the developed En-on-a-chip, Dys-En-on-a-chip, and Annexin A1-treated Dys-En-on-a-chip (Figure 7a). Lucifer Yellow is a hydrophilic and fluorescent tracer molecule, with wide Stokes shift and distinct excitation/emission wavelength, making it an ideal marker for robust permeability studies in cell models.\cite{73} The average intensity of the translocated Lucifer Yellow across Dys-En-on-a-chip was 3.5-fold higher than En-on-a-chip, which indicates the enhanced permeability and compromised integrity of the developed Dys-En compared with the healthy En. However, Annexin A1 treatment could significantly lower the Lucifer Yellow translocation across the treated Dys-En. The average intensity of translocated Lucifer Yellow across Annexin A1-treated Dys-En-on-a-chip was 1.8-fold lower than the nontreated Dys-En-on-a-chip, which confirmed the endothelial tightening and the rescuing effect of Annexin A1 on permeability of Dys-En (Figure 7b).

Furthermore, immunostaining of VE-cadherin was performed to investigate the distribution of this adherens junction component for Dys-En-on-a-chip before and after Annexin A1 treatment (Figure 7c–d). The results of confocal microscopy displayed a patchy distribution of VE-cadherin and loss of cell–cell connectivity in nontreated Dys-En-on-a-chip. However, the treated Dys-En-on-a-chip showed more distribution of VE-cadherin molecules at the cellular borders and higher connectivity of the junctions (Figure 7c). The quantification of fluorescent signals by ImageJ confirmed a ≈ 1.7-fold increase in VE-cadherin signals in treated Dys-En-on-a-chip, compared with nontreated Dys-En-on-a-chip (Figure 7d). Therefore, Annexin A1 treatment of the developed Dys-En-on-a-chip could inhibit the loss of VE-cadherin and/or enhance the expression of this critical component of adherens junctions, further confirming the potential use of Annexin A1 as a valuable drug candidate for CVDs.

In addition, we measured transendothelial electrical resistance (TEER) for healthy En, Dys-En, and Annexin A1-treated Dys-En, using an automated multowell CellZscope device. TEER measurement is a nondestructive and label-free method to study barrier integrity. Intact and tightly connected endothelial cells are resistant to the passage of electrical current but disrupted endothelial layers allow the passage of the electrical current more easily. Therefore, there is a direct relationship between the measured TEER and the integrity of the endothelial layer.\cite{74} Figure 7e shows the real-time TEER, and Figure 7f shows the measured TEER at the end of the experiment (after 24 h), termed “endpoint TEER.” As shown in these figures, Annexin A1 showed a positive effect on the integrity of endothelial layers. The endpoint TEER for Dys-En was threefold less than healthy En, which confirms the disrupted barrier integrity of the developed Dys-En model. However, Annexin A1 treatment could increase the TEER values, and the endpoint TEER for treated Dys-En was 1.5-fold higher than nontreated Dys-En, which indicates the restorative effect of this protein on endothelial integrity.

3. Conclusion

In this study, a microchip consisting of two parallel channels separated by a semipermeable membrane, which is a simple but robust design for on-chip permeability studies, was utilized to establish a Dys-En-on-a-chip model. The developed biomicrofluidic model not only can mimic the relevant pathological shear stress but also showed loss of adherens junctions at cellular borders, change of cytoskeleton organization to form actin stress fibers, and upregulation of VCAM-1 on endothelial cells, which are all indicators of a successful Dys-En biomimetic in vitro model. The developed platform was used to screen several VCAM-1-binding NPs with different properties. Based on the NP translocation assessments and microscopy studies, smaller NPs showed higher permeability and binding, and NP size dominated the NP targeting to Dys-En. Moreover, the restorative effect of Annexin A1 on Dys-En was investigated. Treatment of Dys-En-on-a-chip with Annexin A1 resulted in restoration of adherens junctions and enhancement of the barrier integrity, which confirm the potential application for this drug to be used clinically for the treatment of CVD such as atherosclerosis.

4. Experimental Section

Materials: SU-82.075 and PDMS (Sylgard 184) were purchased from MicroChem (USA) and Dow Corning (USA), respectively. (3-aminopropyl)
streptomycin, C, which is fl for 1/C2 1. Brie fl ÷ fl 1 cm for four chips, which were bonded to microscopy 2 cm was fabricated using polymethyl/C3 is the width and μ/C2 is the viscosity of the W 10 is the height of the microchannel. Q fl 1¼ for the channel with this aspect ratio is reported. For the f fi in culture media) fl H buffer saline (PBS), fetal bovine serum (FBS), penicillin–streptomycin, for fibronectin from human plasma, trypsin, ethylene diamine tetracetic acid (EDTA), Triton X-100, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), cytoeine, Lucifer Yellow, and ethanol were provided by Sigma-Aldrich (Denmark). HUVECs (PCS-100-013), vascular cell basal medium (PCS-100-030), and VE growth factor (VEGF) kit (PCS-100-041) were obtained from American Type Cell Culture (ATCC, USA). Transwell inserts, containing a PET porous membrane with the pore size of 1 μm, were purchased from Corning (UK). TNF-α and recombinant human Annexin A1 were obtained from R and D systems (USA). Alexa fluor488 VE-cadherin antibody was provided by Santa Cruz Biotechnology (USA). Alexa fluor647 Phalloidin, mouse monoclonal IgG1 anti-VCAM-1 antibody, and Alexa fluor555-conjugated goat anti-mouse IgG1 antibody were obtained from Invitrogen (Denmark). Polyether ether ketone (PEEK) tubing, Tygon tubing, PEEK microtight adaptor, parafomaldehyde solution, and Hoechst 33342 were obtained from Thermo Fisher Scientific (Denmark). Polyurethane NPs, which were functionalized by amine groups and labeled by rhodamine B, were provided in four different sizes (i.e., 30, 60, 120, and 250 nm) by Micromod (Germany). VCAM-1-targeting peptide (VHPKQHR) by cleavage using 1% BSA solution for 30 min at the same flow rate. After preparation and sterilization of the setup, it was placed in a cell-culture incubator (17 °C, 5% CO2) for the rest of the experiment duration. Then, 100 μg mL−1 fibronectin solution was perfused through the upper channels of the chips with the flow rate of 100 μL min−1 for 2 min, to coat the membranes. Then, the system was statically incubated with the perfused fibronectin for 1 h. Following this, HUVECs (1 × 107 cells mL−1) were perfused through the upper channels of the chips. The cell distribution was inspected using the microscope to ensure complete and even seeding of the cells on the membrane. The cells were incubated for 3 h under static condition to settle and adhere to the membrane. Subsequently, the continuous flow of the culture media was started at the flow rate of 10 μL min−1 (unless otherwise indicated). The condition of the cells was regularly inspected using the microscope of the system. After 48 h, the cells were found to reach confluency and establish a healthy En-on-a-chip. Development of Dys-En-on-a-Chip: HUVECs were grown to confluency as mentioned earlier. Subsequently, TNF-α (20 ng mL−1 in culture media) was perfused through the upper channel for 12 h. Shear Stress Calculation: To calculate shear stress from the flow data in a microchannel with a rectangular cross section, Equation (1) was used.

\[
\tau = \frac{6\eta Q}{W H} \times \left( 1 + \frac{H}{W} \right) \times f_s \times \frac{H}{W}
\]

In this equation, \( \tau \) and Q are shear stress and volumetric flow rate, respectively. \( \eta \) is the viscosity of the fluid and assumed to be equal to the viscosity of water at 37 °C, which is \( \approx 10^-3 \) Pa.s. W is the width and \( \eta \) is the height of the microchannel. \( f_s (\frac{H}{W}) \) is a correction factor, which depends on the channel dimensions and the aspect ratio.\(^{76} \) For the designed microchannel in this study, the height-to-width ratio \( \frac{H}{W} \) is 0.375. The calculated \( f_s \) for the channel with this aspect ratio is reported to be 0.7.\(^{76} \)

Immunostaining and Quantification: For all steps of immunostaining experiments, the reagent solutions were perfused through the upper channel at the flow rate of 100 μL min−1 for 2 min to deliver the reagents to the cells. Then, the cells were incubated with the reagents under a stopped-flow condition at room temperature. The solutions were introduced to the cells in this order: PBS to rinse away the culture medium, 4% parafomaldehyde solution for 20 min to fix the cells, PBS to rinse, 0.1% Triton X-100 for 30 min to permeabilize the cells, PBS to rinse, and 1% BSA solution for 1 h to block nonspecific bindings. Subsequently, cells were labeled with an immunostaining antibody. For VE-cadherin staining, cells were incubated with Alexa fluor488 VE-cadherin antibody (diluted 1:1000 in PBS) for 40 min. In the case of F-actin staining, cells were incubated with Alexa fluor647 Phalloidin (diluted 1:40) in PBS) for 1 h. For VCAM-1 staining, cells were first incubated with mouse monoclonal IgG1 anti-VCAM-1 antibody

Development of En-on-a-Chip: First, all connectors, fittings, tubing, filters, reservoirs, and caps were sterilized by autoclaving. Then, they were connected to the chips and the flow system inside a laminar flow hood under sterilized conditions. To ensure the sterilization of the chips and all components, the entire system was sterilized by perfusing 70% ethanol for 30 min at the flow rate of 20 μL min−1. Subsequently, PBS was perfused for 1 h at the same flow rate. After preparation and sterilization of the setup, it was placed in a cell-culture incubator (17 °C, 5% CO2) for the rest of the experiment duration. Then, 100 μg mL−1 fibronectin solution was perfused through the upper channels of the chips with the flow rate of 100 μL min−1 for 2 min, to coat the membranes. Then, the system was statically incubated with the perfused fibronectin for 1 h. Following this, HUVECs (1 × 107 cells mL−1) were perfused through the upper channels of the chips. The cell distribution was inspected using the microscope to ensure complete and even seeding of the cells on the membrane. The cells were incubated for 3 h under static condition to settle and adhere to the membrane. Subsequently, the continuous flow of the culture media was started at the flow rate of 10 μL min−1 (unless otherwise indicated). The condition of the cells was regularly inspected using the microscope of the system. After 48 h, the cells were found to reach confluency and establish a healthy En-on-a-chip. Development of Dys-En-on-a-Chip: HUVECs were grown to confluency as mentioned earlier. Subsequently, TNF-α (20 ng mL−1 in culture media) was perfused through the upper channel for 12 h. Shear Stress Calculation: To calculate shear stress from the flow data in a microchannel with a rectangular cross section, Equation (1) was used.

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(diluted 1:50 in PBS containing 1% BSA) for 1 h as the primary antibody. Following that, cells were rinsed with PBS and incubated with Alexa fluor555-conjugated goat antimouse IgG1 antibody (diluted 1:1000 in PBS) for 1 h as the secondary antibody. For all experiments, nuclei staining was performed using Hoechst (diluted 1:2000 in PBS) for 30 min. Finally, confocal images were captured using a laser scanning confocal microscope (LSM780, Zeiss, Germany).

To quantify the signals, three independent experiments were performed. 5–7 images of each sample were obtained, and then all images were quantified by image software (ImageJ 1.47, USA). To quantify the VE-cadherin signals in an image, the threshold value was selected by the software and applied to the images. Then, the fluorescent intensity above the threshold value was calculated by the software, normalized to the number of nuclei in the image, and expressed as average VE-cadherin signal/cell. Using a single threshold value for all samples within an experiment, the average VE-cadherin signal/cell was calculated for En-on-a-chip and Dys-En-a-chip. Finally, data obtained from Dys-En-a-chip were normalized to the data obtained from En-on-a-chip. To quantify F-actin and VCAM-1 signals, a similar quantification method was performed.

Preparation of VCAM-1-Targeted Polystyrene NPs: For this purpose, polystyrene NPs, which were functionalized by amine groups and labeled by rhodamine B, were purchased from Micromod (Germany). NP solutions were first washed twice in phosphate buffer (20 mM, pH 8). For this purpose, NPs were centrifuged at 10 000 rpm, 4 °C (30 min for NP1, NP2, NP3, and 15 min for NP4), using Amicon ultra-0.5 centrifugal filter units (10 kDa cutoff, Millipore, UK) and then dissolved in 200 μL phosphate buffer. Then, NPs were PEGylated by a mixture of MAL-PEG-NHS and m-PEG-NHS. In this study, we used MAL-PEG-NHS and m-PEG-NHS with a molar ratio of 1:5 to avoid remaining of MAL groups on the surface of the NPs, which can react with thiol groups on cells and cell culture proteins. The PEG linkers were dissolved in DMSO and then mixed with NP solutions at room temperature overnight. The molar ratio of amine groups to PEG in these reactions was 1:3. Next, the VCAM-1-targeting peptide was conjugated to the surface of the NPs. For this purpose, the PEGylated NPs were washed twice in PBS buffer containing 1 mM EDTA and then reacted with an excess amount of the peptide overnight at room temperature. Then, unreacted maleimide groups were quenched using 20 mM cysteine solution. Finally, the NPs were washed twice in PBS using Amicon centrifugal filter units as mentioned earlier.

Characterization of VCAM-1-Targeted Polystyrene NPs: Size, zeta potential, and PDI of the NPs were measured by DLS technique (DLS, Nano-ZS, Malvern, UK). To quantify the peptide density on the surface of the NPs (number of peptides per nm² of NP surface or #peptide/nm²), the UV absorbance of the NPs was measured at 205 nm before and after peptide coupling using a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, UK) and the difference of the absorbance (ΔA205) was obtained. Moreover, the extinction coefficient (ε205) of the peptide at 205 nm was calculated based on a model reported by Anthis et al. and their online computation program (available at: http://spin.niddk.nih.gov/clone). Based on this model, the extinction coefficient of VHPKQHR-GGCC at 205 nm was 40 640 M⁻¹ cm⁻¹. Therefore, the concentration of the peptide was calculated based on ε205 and ΔA205 results, using the Beer–Lambert law. Finally, we calculated #peptide/nm² by knowing the concentration of the peptide, NP size, and NP density (1.03 g cm⁻³).

Permeability of VCAM-1-Targeted NPs across En-on-a-Chip and Dys-En-on-a-Chip: NP solutions (100 μg mL⁻¹ in culture medium) were perfused through the upper channel at the flow rate of 5 μL min⁻¹. Moreover, the culture medium without any NPs was perfused through the lower channel at the flow rate of 1 μL min⁻¹. After 6 h, the solution containing translocated NPs was collected from the collection reservoir of the lower channel. This solution was analyzed using a fluorescence plate reader (Spark, Tecan Life Science, Switzerland) at the excitation/emission wavelength of 552/580 nm to measure the fluorescent intensity of the translocated NPs. Furthermore, standard curves were prepared to indicate the relationship between the intensity and NP concentration. Based on the intensity results and the standard curves, the concentration of the translocated NPs was calculated. Finally, the concentration of the translocated NPs was divided by the initial concentration of the NPs to obtain percent permeability.

Binding of VCAM-1-Targeted NPs to En-on-a-Chip and Dys-En-on-a-Chip: For this purpose, the NP solution at the concentration of 100 μg mL⁻¹ was perfused through the upper channel at the flow rate of 5 μL min⁻¹ for 2 h. Subsequently, the cells were washed extensively to remove any unbound NP, fixed by 4% paraformaldehyde solution for 20 min, stained for nuclei by Hoechst (diluted 1:2000 in PBS) for 30 min, and then imaged using a laser scanning confocal microscope (LSM780, Zeiss, Germany). Zen Intellisence software (Zeiss, Germany) was used to quantify the images and obtain the MFI of NPs and the exact number of cells for each image. Then, MFI/cell was calculated.

Modulation of Dys-En-on-a-Chip by Annexin A1: First, Dys-En-on-a-chip was developed as mentioned earlier. Then, 20 μg mL⁻¹ recombinant human Annexin A1 was added to the TNF-α-containing medium and perfused through the upper channel at the flow rate of 10 μL min⁻¹ for 12 h.

Lucifer Yellow Permeability Assay: Lucifer Yellow solution (5 μg mL⁻¹ in culture medium) was perfused through the upper channel. The culture medium without any fluorescent tracer was perfused through the lower channel. The flow rate for both channels was set at 5 μL min⁻¹. After 30 min, the samples were collected from the collection reservoir of the lower channel. The fluorescence intensity was measured using a fluorescence plate reader (Spark, Tecan Life Science, Switzerland) with excitation/emission wavelength of 428/536 nm. For comparison, data were normalized to the fluorescence intensity of the translocated Lucifer Yellow across a bare membrane (a chip without any cells).

TEER Measurements: TEER was measured using CellZeScope (NanoAnalytics, Germany). First, cells were cultured on Transwell inserts, containing a PET porous membrane with pore size of 1 μm. For this purpose, the membranes were treated with 100 μg mL⁻¹ fibronectin for 1 h at 37 °C. Then, HUVECs at the cell density of 0.2 × 10⁶ cell inserts seeded on the membranes and incubated for 24 h to form confluent endothelial layers (En group). Then, the media in the inserts was exchanged with the culture media containing 20 ng mL⁻¹ TNF-α, and the inserts were incubated for 12 h (Dys-En group). Subsequently, 20 μg mL⁻¹ Annexin A1 was added to the inserts (Annexin A1 treated Dys-En group). Finally, 3–5 inserts of each group were placed inside the CellZeScope device and the TEER was measured every 30 min, for the duration of 24 h. The data obtained from the Dys-En group and Annexin A1-treated Dys-En group were normalized to the data obtained from the En group.

Statistical Analysis: OriginPro 10 (OriginLab, USA) was used to perform statistical analyses. All data were derived from at least three independent experiments and reported as mean ± SD. The comparisons between groups were made by two-sample t-test. The p-values of <0.05, <0.01, and <0.001 were shown as *, **, and ***, respectively. The symbol “ns” showed nonsignificant differences.

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.