An Integrated Biometric Platform for Evaluation of Targeted Drug Delivery

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ABSTRACT

Endothelial cells (ECs) form the inner lining of blood vessels and are exposed to various factors like hemodynamic conditions, biochemical signals and interaction with other cell types. Blood vessel functions are regulated by interactions among these factors. The occurrence of pathological conditions lead to localized upregulation of cell adhesion molecules on the endothelial lining of blood vessels. This process is promoted by cytokines such as tumor necrosis factor-alpha (TNF-α), which leads to expression of intercellular adhesion molecule-1 (ICAM-1) on the EC surface among others. We developed a novel biomimetic blood vessel model by culturing confluent, flow aligned, ECs in a microfluidic platform, and performed real-time *in situ* characterization of localized pro-inflammatory endothelial activation. The model mimics the physiological phenomenon of cytokine activation of endothelium from tissue side and studies the heterogeneity in localized surface ICAM-1 expression and F-actin arrangement. Fluorescent antibody coated particles were used as imaging probes for identifying cell surface ICAM-1 expression. The binding properties of particles were evaluated under flow for different particle sizes and antibody coating densities. This allowed the investigation of spatial resolution and accessibility of ICAM-1 molecules on ECs, along with their sensitivity in receptor-ligand recognition and binding. This work has developed an *in vitro* blood vessel model that integrate various heterogeneous factors to effectively mimic a complex and physiologically relevant endothelial microenvironment.

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Keywords: Vascular pathology, Microfluidics, Receptor-ligand recognition, Endothelial microenvironment, Real-time study

1 INTRODUCTION

The local vascular dynamics of ECs under disease conditions these changes and their induction remain largely enigmatic, in part due to the difficulties of modeling. *In vivo* studies most adequately reflect the pathophysiological context, but the system complexity is beyond our current means to accurately dissect specific aspects. Parameters of the heterogeneous physical, chemical, and biological pathways influencing the blood vessel add to the complexity [1]. On the other hand, the biological relevance of conventional cell culture models is limited. Therefore, both understanding of pathophysiology and designing adequate interventions demand alternative bio-mimetic model platforms.

This study aims to develop a bio-mimetic blood vessel to study dynamics of endothelial activation induced by local action of pro-inflammatory cytokines. To achieve this goal, we combined endothelial microfluidics/flow adaptation approach with local cytokine application via a semi-permeable "sub-endothelial compartment" and employed antibody-coated nanoparticles as imaging probes. This approach allowed us to define flow-mediated local heterogeneity of endothelial activation by cytokines that appears to reflect the pathophysiology of the process. The platform facilitated real-time microscopic analysis of cellular characteristics and particle binding as well as differentiation between localized differences in cell responses to treatment. Furthermore, the results of this study support design of drug delivery systems based on affinity nanocarriers targeted to the pathological endothelium.

2 MATERIALS AND METHODS

2.1 Fabrication and EC culture on bio-mimetic blood vessel model

Blood vessel mimicking channels are photolithographically fabricated and BAOEC culture is attained as explained prior [2]. The upper and lower channels are casted out of Sylgard 184 polydimethylsiloxane (PDMS) (Dow Corning Corp.). The upper channel is 20 mm long, 350 µm wide and 100 µm tall, and the lower channel is 5 mm long, 1000 µm wide and 100 µm tall. A polycarbonate (PC), track-etched thin clear membrane (Whatman, GE Healthcare) with 1 µm diameter pores and an average calculated pore density of $1.5*10^7$ pores/cm^2 is embedded between two PDMS channels. The bottom PDMS channel layer is bonded on to a thin glass slide by exposing the sides in contact to oxygen plasma. Upper PDMS channels is bonded to the membrane by using a thin PDMS mortar film (10:1 ratio of base and curing agent with toluene in equal proportion). The upper channel bonded to the membrane is then bonded to lower channel after making sure the channels are aligned. After each step the components are placed in an oven at 60oC for the appropriate time to enhance bonding. Inlet and outlet ports were punched to provide access to upper and lower channels.

The device was sterilized in UV light overnight and the upper channel of the device was coated with 50 µg/ml fibronectin solution (Sigma-Aldrich) overnight at 37oC. BAOECs cells were seeded in the upper channel at a density of 2*107 cells/m and let to attach on the semi-permeable membrane. Cell seeded devices were placed in an incubator under standard culture conditions (37oC and 5% CO2) overnight to allow cell attachment and spreading on the membrane. On reaching confluence, the BAOECs were subjected to a FSS of 12 dyne/cm^2. The flow was brought about using a high precision and extremely low pulsation peristaltic pump (ISMATEC, IPC-N series) and the entire setup was placed in standard culture conditions. Recirculation of media was not permitted while thrombin and tracer molecule was flowed through the channels. To calculate the volumetric flow rate that correspond to the required maximum FSS experienced by the ECs, the following equation was used [3]. $\tau_{cell} = \frac{6\mu Q}{wh^2}$

2.2 TNF- α treatment

The dynamics of endothelial activation and ICAM-1 expression by local action of TNF-α were studied. BAOECs were subjected to 24 hrs or more of flow at 12 dyne/cm^2 and were finally activated with TNF-α to study surface ICAM-1 expression. TNF-α was mixed in media at a concentration of 10 Units (U)/ml and the treatment was performed for 2 hrs on a confluent EC layer (>80%). BAOECs were locally activated by introducing TNF-α in the lower channel of the device which has direct access to a section of the upper channel. TNF-α diffused from the lower to the upper channel through the pores of the membrane and locally activated the endothelial layer from the basal side.

2.3 Particle binding study

The particles were introduced to the flow in the upper channel of the device and after their designated flow time unbound particles were removed by flushing with a buffer solution. The wash buffer contains a plasma membrane stain (CellMask™, Life technologies) to fluorescently tag the BAOECs. The particle bound BAOECs were then fixed in paraformaldehyde (3.7%). Particle binding was analyzed by phase contrast and fluorescence microscopy (FV1000-IX81, Olympus) and image analysis was performed using ImageJ software.

To demonstrate the applicability of our platform towards real time *in situ* studies we characterized dynamic binding of anti-ICAM-1 coated NPs to BAOECs being subjected to TNF-α treatment. The enclosed nature of the biomimetic blood vessel platform allowed maintenance of sterile conditions. We performed a continuous particle binding study over time where the flow set-up was assembled on a microscope table. BAOECs were subjected to 12 dyne/cm^2 of FSS for 6 hrs before localized TNF-α treatment from the lower channel while a steady flow is continued in the upper channel. Real time binding of anti-ICAM-1 coated 210 nm particles (232.5 ± 25 anti-ICAM-1/particle) was analyzed to characterize the dynamic nature of ICAM-1 expression by BAOECs. The cells were cultured in HEPES buffered media and such cultures do not require a controlled gaseous atmosphere [4]. The pump controlled the flow rate of the media and an in-line solution heater (Warner Instruments, SH-27B) maintained the media temperature at 37^{o}C.

3 RESULTS AND DISCUSSION

3.1 On-chip cell culture

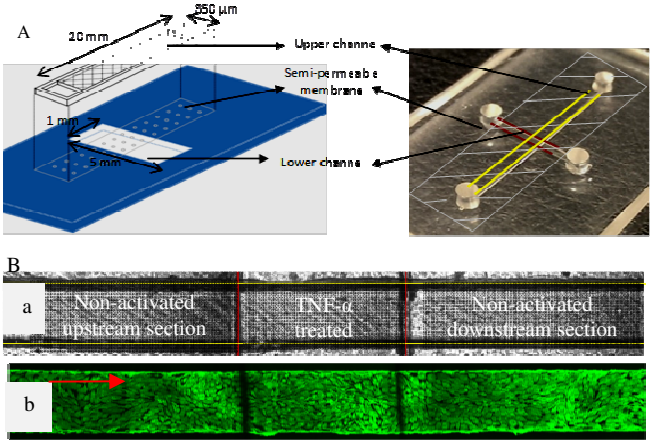


Figure 1: A, Schematic and photograph of the bi-layer device showing the upper (yellow lines) and lower (red lines) channel separated by the porous membrane; B (a) Bright field image of the upper (yellow lines) and lower (red lines) channel defining the upstream, TNF-α activated and downstream sections, (b) Fluorescence labeled actin cytoskeleton (FITC phalloidin) images of confluent BAOEC layer aligned to flow (12 dyne/cm^2 FSS for 24 hrs). The cells grow on the semi-permeable membrane in the upper channel of the device. Arrow shows flow direction

Pro-inflammatory and proliferative signaling pathways of vascular ECs become down-regulated when the EC layer is exposed to unidirectional sustained FSS [5]. Because the quiescent phenotype of ECs is critical to the success of a biomimetic blood vessel model and subjecting the ECs to physiologically relevant FSS is important [6]. A confluent and flow-aligned BAOEC monolayer was maintained for 5 days and the F-actin arrangement pattern was comparable to that of BAOECs subjected to 12 dynes/cm^2 FSS for 24 hrs.

3.2 BAOEC stress fiber alignment under FSS

To further examine the EC layer subjected to flow in our device, their actin assembly characteristics were studied. ECs exposed to FSS change shape and microfilament network to align with the direction of flow [7]. We examined this by staining F-actin stress fibers of BAOECs growing in the upper channel. The exposure of cells to 12 dynes/cm^2 FSS resulted in alteration of cell shape from the

typical cobblestone pattern to fusiform as observed in ECs *in vivo* [8-9]. The cells and their actin stress fibers were uniformly aligned in the direction of flow (Fig. 2 B). The control (no flow) case had the cortical actin and F-actin fibers arranged in a radial pattern (Fig. 2 A). To better depict the relationship between cell orientation and flow direction, the angle (α) between the orientation of stress fibers and the width (short-axis) of the microchannel was plotted. It is observed that for the control case the stress fibers align at an average angle of 51.4°, while the cells exposed to flow show an average angle of 90.1° (Fig. 2 C).

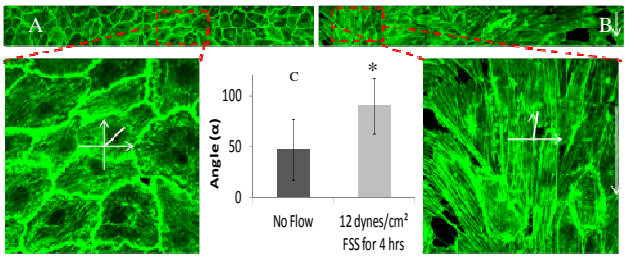


Figure 2: Organization of F-actin stress fibers (green) in BAOECs assessed by confocal microscopy (A) Static case; Cell stress fibers tend to align parallel to the width of the channel. (B) After 4 hrs of flow at 12 dynes/cm2 FSS; Cell stress fibers tend to align parallel to the flow direction (C) Quantitative measurement of stress fiber alignment angle (α) to the width of the channel under static and flow shear stress case (*p<0.001, by Student's t test)

3.3 Characterization of flow-mediated localized BAOEC activation using fluorescent probes

ICAM-1 expression was evaluated by studying specific binding of anti-ICAM-1 coated particles to ECs in the upper channel. Saturated anti-ICAM-1 coating on 210 nm particles produced a high antibody coating density of 232.5 ± 25 anti-ICAM-1/particle. This was brought down to a low antibody coating density of 112.9 ± 19 anti-ICAM-1/particle using control IgG antibody. 210 nm nanoparticles coated with anti-ICAM-1 or control IgG were perfused at 6 dyne/cm^2 FSS.

Having both direct cytokine-activated and non-activated ECs in the same channel allowed investigation of the heterogeneous nature of ICAM-1 expression and the hemodynamic control of the marginal zone between inflammation foci and relatively normal vasculature. As the ECs were fluorescently tagged, the boundaries of individual cells were marked and the number of particles binding per cell was counted using ImageJ software. The binding of fluorescent particles to ICAM-1 in the upstream, TNF-α treated and downstream areas of the channel were studied using antibody coated 210 nm and 1 µm particles. Flow rates of 6, 12 and 18 dyne/cm^2 were employed after the BAOECs were treated with TNF-α for 2 hrs under a steady and sustained FSS of 12 dyne/cm^2.

BAOECs in the TNF-α treated section of the upper channel have around 4-5 times higher particle binding density compared to the upstream section for 210 nm particle with a coating density of 232.5 ± 25 anti-ICAM-1/particle (Fig. 3 B) ($p<0.01$). A similar trend was observed for the lower antibody coating density case (Fig. 3 A) and this was consistent for all the flow cases for both particle antibody coating densities. This clearly showed a significant increase in surface ICAM-1 expression in BAOECs at the TNF-α treated section. The p value is <0.01 by one way ANOVA test for particle binding data compared between TNF-α treated, upstream and downstream regions for both 210 nm particle antibody coating density cases. The downstream section of the channel also showed significantly higher (around 2 times) particle binding density compared to the upstream section ($p<0.01$).

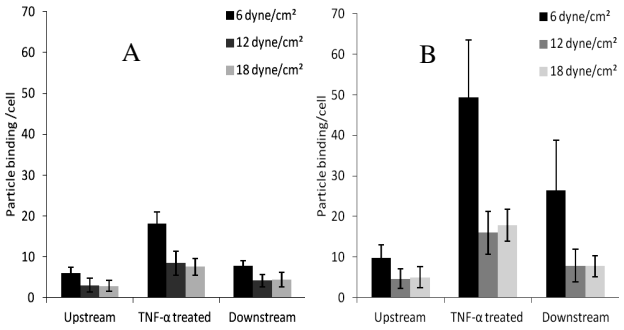


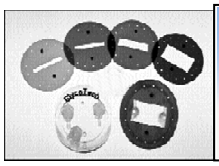
Figure 3: Targeted binding of anti-ICAM-1 coated particles of different antibody coating densities on BAOECs in the upstream, TNF-α treated and downstream sections of the channel at different shear rates. Quantification of particle binding density per cell at 6, 12 and 18 dyne/cm^2 for 210 nm particles of; (A), Anti-ICAM-1 coating density of 112.9 anti-ICAM-1/particle and; (B), 232.5 anti-ICAM-1/particle. $p<0.01$ by one way ANOVA test for particle binding data compared between upstream, TNF-α treated and downstream regions for both 112.9 and 232.5 anti-ICAM-1/particle cases. Data are shown as mean ± S.D. (n=100 cells from three independent experiments for each case).

3.4 Advantages and improvements over existing methods, devices or materials

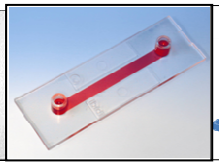
Gold standards platforms currently used to model blood vessels *in vitro* are on a petridish and flow chambers. Competitors in today's market for microfluidic technology similar to ours are “Cellix LTD”, "Ibidi GmbH" and “CFD Research Corporation”. These companies offer microfluidic platforms on which cells can be cultured to study different phenomena/processes. The microfluidic chips they offer have some features that match our technology. But our product has several other key features not offered by our competitors like, the ability to closely mimic *in vivo* healthy and diseased blood vessel conditions in the same channel, ability to grow multiple cell lines, tunable geometry etc.

The introduction of a bi-layer system allows us to locally trigger receptor expression on cells that mimic vascular disease, grow two types of cells in the same system, and test targeted drug particle delivery and uptake. Our product allows control and target cells to be grown on the same

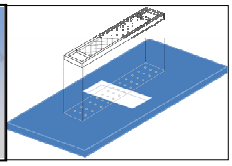
device, integrated *in situ* imaging and testing, patient specific vascular geometry and flow condition, and fast parametric evaluation with minimal sample volume. The introduction of the bi-layer design supported by the semi-permeable membrane allows for an advantage in studies related to exchange, uptake and delivery, above and beyond what the competitors offer making the platform well suited for studying targeted drug delivery and other phenomena.



Flow channel with preset dimensions in flow chambers
Courtesy: Glycotech



Current single layer platforms
Courtesy: Ibidi



Our platform with the bi-layer advantage and tunable geometry

Flow chambers	Single layer channel	Our bi-layer channel
~ 1000 $ Reusable Tedious to set-up Not a lot of options Higher running cost: Need more sample volumes	~30 $/device Not reusable Single layer cell culture Customer specific channels not available Can only do one cell line	~10 $/device Not reusable Bi- layer cell culture Customer specific channels available Capable of doing multiple cell lines (top & bottom channel)

Table 1: Comparison of existing *in vitro* blood vessel models to our bi-layer platform

4 CONCLUSION

Our invention is a bio-mimetic microfluidic platform that can prototype a blood vessel outside human body. This platform integrates an EC layer in a microfluidic channel, which can be subjected to specific flow and chemokine stimulations. It consists of a top and bottom channel separated by a semi-permeable, porous, cell culture friendly membrane. ECs that form the inner lining of blood vessels are coated on the semi-permeable membrane in the top channel. Our platform has the capability to access specific sections of the top channel from the bottom channel, which allows spatially controlled simulations of these ECs from the basal side. This *in vitro* blood vessel model has the capacity to be applied as a generic platform to study various topics on, targeted drug delivery (ligand-receptor specificity, drug carrier features, hemorheology factors), cardiovascular conditions (atherosclerosis, drug eluting stents), immunology (inflammation, leukocyte adhesion and migration), as well as perform explicit studies on patient specific blood vessels (design channel geometries specific to patient blood vessel; integrate ECs from patient) to understand the best treatment strategy for a disease condition or study various factors that culminated in the onset of a disease condition, etc. This model understands the current/upcoming need of a blood vessel model that can cater to the demands for patient specific therapeutics, as well as provide a more realistic platform to enhance current conventional research studies.

REFERENCES

1. Staton, C.A., et al., *Current methods for assaying angiogenesis in vitro and in vivo.* International Journal of Experimental Pathology, 2004. **85**(5): p. 233-248.
2. Antony Thomas, H.D.O.-Y., Linda Lowe-Krentz, Vladimir R. Muzykantov, Yaling Liu,, *Biomimetic channel modeling local vascular dynamics of pro-inflammatory endothelial changes.* Lab on a chip: Under Review, 2015.
3. Khan, O. and M. Sefton, *Endothelial cell behaviour within a microfluidic mimic of the flow channels of a modular tissue engineered construct.* Biomedical Microdevices, 2011. **13**(1): p. 69-87.
4. Shipman, C., *Evaluation of 4-(2-Hydroxyethyl)-1-piperazineëthanesulfonic Acid (HEPES) as a Tissue Culture Buffer.* Experimental Biology and Medicine, 1969. **130**(1): p. 305-310.
5. Chien, S., *Effects of Disturbed Flow on Endothelial Cells.* Annals of Biomedical Engineering, 2008. **36**(4): p. 554-562.
6. Wasserman, S.M. and J.N. Topper, *Adaptation of the endothelium to fluid flow: in vitro analyses of gene expression and in vivo implications.* Vascular Medicine, 2004. **9**(1): p. 35-45.
7. Malek, A.M. and S. Izumo, *Mechanism of endothelial cell shape change and cytoskeletal remodeling in response to fluid shear stress.* Journal of Cell Science, 1996. **109**(4): p. 713-726.
8. Wong, A., T. Pollard, and I. Herman, *Actin filament stress fibers in vascular endothelial cells in vivo.* Science, 1983. **219**(4586): p. 867-869.
9. Kim, D.W., A.I. Gotlieb, and B.L. Langille, *In vivo modulation of endothelial F-actin microfilaments by experimental alterations in shear stress.* Arteriosclerosis, Thrombosis, and Vascular Biology, 1989. **9**(4): p. 439-45.
10. Goldblum SE, D.X., Campbell-Washington J., *TNF-alpha induces endothelial cell F-actin depolymerization, new actin synthesis, and barrier dysfunction.* Am J Physiol., 1993. **264**(4 Pt 1): p. 894-905.
11. Peng, J., et al., *Protein kinase C-alpha signals P115RhoGEF phosphorylation and RhoA activation in TNF-alpha-induced mouse brain microvascular endothelial cell barrier dysfunction.* Journal of Neuroinflammation, 2011. **8**(1): p. 28.
12. McKenzie, J.A.G. and A.J. Ridley, *Roles of Rho/ROCK and MLCK in TNF-α-induced changes in endothelial morphology and permeability.* Journal of Cellular Physiology, 2007. **213**(1): p. 221-228.