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Interactions Between Multiple Cell Types in Parallel Microfluidic Channels: Monitoring Platelet Adhesion to an Endothelium in the Presence of an Anti-Adhesion Drug

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Abstract

A simple method for immobilizing endothelial cells in the channels of a microfluidic device fabricated with soft lithography is presented that requires no surface oxidation of the substrate material used in conjunction with the microfluidic device and is operable even with a reversible seal. Specifically, optimal conditions for culturing bovine pulmonary artery endothelial cells (bPAECs) to the surface of a Petri dish were investigated. The parameters investigated included fibronectin concentration, temperature, seeding density, and immobilization time. To enhance the utility of the device, all optimization studies, and studies involving platelet adhesion to the immobilized endothelium, were performed in parallel channels, thereby enabling improved throughput over a single channel device. The optimal conditions for cell immobilization included coating the petri dish with 100 μg/mL fibronectin, a seeding cell density of 1.00×10^5 cells mL^{-1} and an immobilization time of 90 min at 37 °C. The device was then employed to monitor the physical interaction (adhesion) of platelets to the immobilized endothelium in the presence of a known platelet activator (ADP) and a drug inhibitor of platelet activation. The number of platelets adhering to the endothelial cells in the channels increased from 17.0 ± 2.3 in the absence of ADP to 63.2 ± 2.4 in the presence of 5.00 μM ADP. Moreover, the data presented here also shows that inhibition of endothelium nitric oxide (NO) production, a recognized inhibitor of platelet adhesion to the endothelium, increased the number of platelets adhering to the surface to 35.4 ± 1.0. In the presence of NO inhibition and 5.00 μM ADP, the affect on platelet adhesion was further increased to 127 ± 5.2. Finally, this device was employed to investigate the effect of a drug known to inhibit platelet adhesion (clopidogrel) and, in the presence of the drug, the platelet adhesion due to activation by 5.00 μM ADP decreased to 24.0 ± 3.8. This work is the first representation of multiple cell types physically interacting in the channels of a microfluidic device and further demonstrates the potential of these devices in the drug discovery process and drug efficacy studies.

Introduction

It is known that while traversing the circulation, red blood cells (RBCs) release nanomolar to micromolar amounts of adenosine triphosphate (ATP) due to deformation and other stimuli such as hypoxia, pharmacological agents or, more recently, metal-activated C-peptide.[1-7] The importance of this *in vivo* release of ATP from the RBC is that ATP is a known stimulus of nitric oxide synthase (NOS), which catalyzes the production of nitric oxide (NO) in various cell types.

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NO is not only responsible for blood vessel dilation[8-11] but is also an inhibitor of platelet activation and subsequent aggregation.[12-15] Although platelets flow through the circulation and do not generally adhere to the endothelium, upon vascular injury, subendothelial collagen is exposed and stimulates platelet activation. Upon activation, the platelet shape changes allowing for adhesion to the vascular walls and even subsequent recruitment of additional platelets. This process is mediated by the activation of protein kinase G from NO;[16] however, left uncontrolled, adhered platelets are a determinant in thrombus formation and can lead to vessel blockage.

There have been previous reports of microfluidic systems that enable certain features of the circulation to be investigated. Models of the blood brain barrier have been reported and successfully implemented for studies involving transport across an endothelium layer.[17] Other reports of a cell culture analogue, a device containing different tissue types connected by a series of fluid channels, have also been reported.[18, 19]

Many groups, including our own, have demonstrated the ability to culture or immobilize cells in the channels of various microfluidic devices. Previously, we demonstrated the ability to image NO production by endothelial cells immobilized in the channels of a mircofluidic device, as well as determine the concentrations of ATP released from RBCs flowing through microfluidic channels.[20, 21]

In an extension of previous reports involving immobilized endothelial cells in microfluidic channels, we report on the ability of platelets to adhere directly to endothelial cells in a microfluidic device. In this study, we investigated the parameters for optimal bPAEC adhesion to a fibronectin coated substrate. However, rather than immobilizing the cells directly onto the microfluidic channel, the microfluidic device was reversibly sealed to a Petri dish. In this construct, the microfluidic device channels are simply used to direct reagent and fluid flow while the endothelial cells are immobilized to the surface of a Petri dish. Upon immobilization, these cells then have platelets directed over them through the channels of the microfluidic device under various conditions. Importantly, the channels are in parallel creating a high-throughput device capable of optimizing cell immobilization conditions. Therefore, the work presented here demonstrates the ability of multiple cell types (an endothelium and platelets) to interact directly in the channels of a microfluidic device. Such a device should prove useful for investigating those pharmaceutical substances whose mechanism of action is to prevent platelet activation *in vivo*.

Experimental

Preparation of the microfluidic device

PDMS channel structures were produced following previously published methods.[22] Briefly, masters for the production of PDMS microchannels were made by coating a 4-in. silicon wafer (Silicon, Inc., Boise, ID) with SU-8 10 negative photoresist (MicroChem Corp., Newton, MA) using a spin coater (Brewer Science, Rolla, MO) operating with a spin program of 2000 rpm for 20 s. The photoresist was prebaked at 95 °C for 5 min prior to UV exposure with a near-UV flood source (Autoflood 1000, Optical Associates, San Jose, CA) through a negative film (2400 dpi, PageWorks, Cambridge, MA), which contained the desired channel structures. All channel structures were drawn in Freehand (PC version 10.0, Macromedia, Inc. San Francisco, CA). Following this exposure, the wafer was postbaked at 95 °C for 5 min and developed in Nano SU-8 developer (Microchem Corp.). The thickness of the photoresist was measured with a profilometer (Alpha Step-200, Tencor Instruments, Mountain View, CA), which corresponded to the channel depth of the PDMS structures.

A 20:1 mixture of Sylgard 184 elastomer and curing agent (Ellsworth Adhesives, Germantown, WI) was used to increase the adhesiveness of the PDMS to aid in the reversible bonding procedure. This degassed mixture was poured onto the silicon wafer and cured at 75 °C for approximately 10 – 12 minutes. Another mixture, 5:1, was prepared and poured onto the 20:1 mixture and baked for an additional 10 min. After this time, the thermally sealed PDMS layers were removed from the master and inlet holes were punctured (using a 20 gauge luer stub adapter) through the chip as well as 1/8″ exit holes. A chip containing channels of 100 μm depth × 200 μm width × 2 cm length was used for all studies reported here. The channel depth corresponds to the height of the master, which was measured with the aforementioned profilometer.

Cell culture

Unless otherwise stated, all chemicals and reagents for cell isolation and culture were purchased from Lonza (Walkersville, MD), fluorescent dyes were from Invitrogen (Carlsbad, CA), and all other materials were purchased from Fisher Scientific (Pittsburgh, PA).

Bovine pulmonary artery endothelial cells (bPAECs) were thawed and expanded in Endothelial Cell Basal Medium (EBM) supplemented with 0.1% gentamicin sulfate/amphotericin (GA-1000), 0.1% human epidermal growth factor (rhEGF), 0.1% hydrocortisone, 0.4% bovine brain extract (BBE), and 5% fetal bovine serum (FBS). All reported experiments used bPAECs between passages 2 and 10.

Isolation and purification of platelets

Rabbits were anesthetized with ketamine (8 mL/kg, im) and xylazine (1 mg/kg, im) followed by pentobarbital sodium (15 mg/kg, iv). A cannula was placed in the trachea, and the animals were ventilated with room air. A catheter was then placed into a carotid artery for administration of heparin and for phlebotomy. After heparin (500 units, iv), animals were exsanguinated and the whole blood collected in two 50 mL tubes.

Generally, 70 mL of blood were collected from the animal. Blood was centrifuged at 500 × *g* at 37 °C for 10 min. The platelet-rich plasma (PRP) was decanted for the subsequent isolation of platelets. Platelets were isolated from the PRP by adding 1 mL of acid citrate dextrose (ACD) to 9 mL of the PRP and centrifuging at 1500 × *g* at 37 °C for 10 min. The harvested platelets were then washed twice by centrifugation in a mixture containing Hanks' balanced salt solution (HBSS, H1387 from Hanks' Balanced Salt, Sigma) and ACD (6:1 v/v). The washed platelets were then resuspended with HBSS and counted using a hemacytometer; based on the hemacytometer count, the platelets were adjusted to a count of 3.0×10^8 platelets mL^{-1} in HBSS. Platelets were generally harvested and prepared on the day of use. Our group has found that, if kept in the PRP, the platelets can be purified within 1-2 days after the surgical procedure and still produce reliable data. However, the platelets are generally consumed through experimental procedures on the day of harvesting from the rabbit. All studies involving animal use were approved by the Animal Investigation Committee at Michigan State University.

Reagent preparation

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and used as received without further purification unless otherwise noted. *N*-γ-nitro-L-arginine methyl ester hydrochloride (L-NAME) was prepared as a 10 mM stock solution in HBSS. Adenosine diphosphate (ADP) was prepared as a 100 μM stock solution in HBSS. Clopidogrel was prepared as a 1.0 mM stock solution by dissolving a 35 mg tablet in 100 mL DDW, while the working solution reported here was prepared by dilution of the stock into HBSS. Solutions were prepared approximately 2 h before experimentation and stored at room temperature.

Immobilization procedure

The microfluidic device was placed with the array of channels down into a 100 × 20 mm Petri dish (Fig. 1). The Petri dish is of the same nature to tissue flasks used to culture bPAECs and other cell types. Prior to bPAEC loading, the microchannels were coated with a cell adhesion protein, bovine plasma fibronectin (FN, Invitrogen). FN was prepared in 1 mL of distilled, deionized water to a concentration of 1000 μg mL^{-1}. For optimization studies, five solutions of FN were prepared from stock solution having concentrations of 100, 50, 25, 10, and 7.5 μg mL^{-1}. To coat the microchannels, 10 μL of FN were added to the top of the inlet of the microchannel device and vacuum was applied at the exit hole to draw solution into each microchannel. An additional 10 μL of FN solution was added to the exit reservoir to keep the channel from drying due to evaporation. The device was incubated at 37 °C for 45 min, allowing for sufficient adsorption of the protein onto the surface. After removing excess FN solution from the inlets and reservoirs by pipet, the channel was dried with a stream of clean dry air (AirGas, East Lansing, MI), covered, and exposed to UV light for approximately 10 min. The device is subjected to a 10 minute UV exposure in order to ensure any potential bacterial contamination is removed from the system.

Cells were washed with 10 mL HEPES, treated with 5 mL trypsin, and neutralized with 10 mL trypsin neutralizing solution (TNS). The solution of trypsin/TNS containing the cells was collected from the flask and centrifuged at 2200 × *g* for 5 min at 25 °C. The supernatent was removed and the pellet suspended into 200 μL of equilibrated EBM. The concentrated cell solution was simultaneously loaded into the microchannels in the same manner as FN and the device was incubated at 37 °C and 5% CO_2 for 60 min. The channels were rinsed with equilibrated EBM 1 h after initial seeding to remove any non-adherent cells. Additional cells were loaded into the channel and the above was repeated until the channels were >75% confluent. On average, the time elapsed was approximately 2 h from intital seeding of the channel with cells.

For the optimization studies, the cells were tagged with Hoechst 33342 nuclear dye (5 μg mL^{-1} in HBSS) after adhesion was complete. The dye was incubated for 20 min at 37 °C, after which time the solution was rinsed away with equilibrated HBSS. Regarding the platelet adhesion studies, platelets were tagged with Cell Tracker Green CMFDA (1 μM in HBSS) approximately 2 h before the studies were performed. Once the channels were confluent, platelets were hydrodynamically pumped over endothelium for 10 min. The device was incubated at 37 °C and 5% CO_2 for 20 min. before the channels were rinsed with an equilibrated HBSS solution. Images were acquired using an Olympus IX71 Microscope (Olympus America, Melville, NY) with an electrothermally cooled CCD (Orca, Hamamatsu) and Microsuite software (Olympus America). The microscope is fitted with a FITC filter cube (Chroma Technology Corp.) containing the excitation (460-500 nm) and emission (505-560 nm) filters.

Results and Discussion

Immobilization optimization

Composed of 5% carbohydrates, FN is a high molecular weight glycoprotein known to aid in cell adhesion. Other common adhesion proteins include collagen, vitronectin, and laminin. It has been previously shown that a confluent layer of bPAECs can be immobilized into a microchip channel and successfully monitor events that occur *in vivo*, including ATP-stimulated production of endothelial cell-derived NO by amperometry[23] and fluorescence microscopy.[20] Although neither paper investigated the role of FN, recent work by Wheeler showed successfully cultured porcine aortic and aortic valve endothelial cells (pAEC and pAVEC, respectively) on FN and type I collagen while monitoring cell spreading and adhesion

to the proteins.[24] The results here are for immobilization directly to a Petri dish while using the microfluidic device simply as a tool to direct fluids over the cells.

The data in Fig. 2 illustrates an increase in the number of adhered bPAECs to a coated surface as a function of increasing concentrations of FN. In order to maximize bPAEC adhesion to the PDMS surface, different concentrations of FN were examined in the microchannel array. An increase of adherent cells, as a result of increased FN concentration, was observed in the array device (Fig. 2a). A FN concentration of 100 μg mL^{-1} generally produced a microchannel that was >75% confluent and the remaining studies utilized this optimal concentration. It should be noted that concentrations of FN above 100 μg mL^{-1} did not result in an increase in adherent cells. Moreover, the data in Fig. 2 were obtained by using the array of channels. Specifically, different concentrations of FN were added to each channel and the resultant cell counts were determined. Therefore, the optimization of the FN concentration occurred in a parallel format, thereby reducing the amount of time required to determine the optimal concentration.

It is established that bPAEC culturing is most successful in a controlled temperature environment of 37 °C, thus an investigation of the dependency of FN seeding temperature on cell adhesion was also performed and the results are shown in Fig. 2b. Although multiple concentrations (7.5 – 100 μg mL^{-1}) were examined, there was little statistical difference ($p > 0.025$) in the FN incubation temperature.

Using the array device, the optimal seeding time for the endothelial cells was also determined (Fig. 2c). A solution of concentrated bPAECs was pipetted onto each inlet, pulled through, and incubated over a range of times: 15, 30, 45, 60, 75, 90, and 120 min. After the prescribed time, equilibrated EBM solution was pushed through the channel to remove any excess or non-adherent cells. Cell adhesion increased with increasing incubation periods until a maximum was reached. The data suggests that optimal cell adhesion occurs approximately at 90 min and cells incubated for 120 min showed no statisical difference from those incubated for 90 min.

Finally, optimal endothelial cell density was also examined in the array device to determine if a highly concentrated solution of cells can overpopulate and prevent proper cell attachment. The data illustrates that a concentrated solution of cells (1.5×10^6 cells mL^{-1}) provides an adequet number of cells without leading to cellular overcrowding (Fig. 2d).

Endothelial cell lines are known for their ‘cobblestone’ appearance when confluent on a surface. In a situation where there is an excess of cells, they will adhere to the surface, but stay rounded in apperance and will not spread into confluent, viable monolayers. This round shape is similar to their appearance after trypsinization. Investigating bPAEC seeding time and density was important in understanding the factors involved in creating confluent layers in the device. Notably, the data in Fig. 2c and 2d indicate optimal cell seeding time is 90 min, which is in agreement with previous reports,[23] and cell density was determined to be approximately 1×10^5 cells mL^{-1}, although more concentrated cell solutions were not examined. Figure 2e is a fluorescence microscopy image of stained endothelial nuclei in a portion of a microfluidic channel employing the determined optimal conditions in 2a-d.

Platelet adhesion

To date, quantitative research involving platelet activation has been confined to cuvettes[25, 26] and electrodes.[27] These techniques focus on the platelet only and, while important in understanding platelet activity in the form of aggregation and the molecules secreted by these activated platelets, do not enable the monitoring of actual adhesion to an endothelium under the conditions of flow. Recently, a microfluidic device was used to control the flux of platelet agonists into flowing blood containing platelets, although no endothelium was employed to enable cell to cell interactions.[28]

To demonstrate the ability of platelets to adhere to an immobilized endothelium upon activation, platelets were incubated in solutions containing various concentrations of ADP and hydrodynamically pumped through an endothelium-lined microchannel for 10 min. After introduction to the channel, the platelets were allowed to incubate for 20 min at 37 °C and 5% CO_2. Following this incubation period, the channel was washed with a solution of equilibrated HBSS and the resultant number of adhered platelets was determined by utilizing fluorescence microscopy to count tagged cells. A representation of platelets adhering to the immobilized endothelial cells in the presence and absence of ADP is shown in Fig. 3. In figure 3, the images on the left are bright field images of the immobilized endothelium while the images on the right are the corresponding fluorescent images of the platelets adhering to the endothelium. In addition, the images were acquired for platelets alone and platelets in the presence of ADP. Finally, to demonstrate the importance of endothelium-derived NO on platelet adhesion, images were also obtained after the endothelial cells in the microfluidic channels had been incubated with L-NAME, a recognized inhibitor of endothelial nitric oxide synthanse and subsequent NO production. An examination of the fluorescent platelets in each image clearly shows the increased platelet adhesion due to ADP activation. Moreover, the data in figure 3 also suggest that a decrease in NO production by the endothelium increases the number of platelets adhering to the endothelial cells.

The data in Fig. 4 is a summary of the results represented in figure 3 and additional concentrations of ADP that were investigated. The number of platelets adhering to the immobilized endothelium in the absence of ADP was 17.2 ± 2.3; however, the number of adhered platelets approximately doubled to 35.4 ± 5.2 at an ADP concentration of 1.0 μM. The number of adhered platelets continues to increase as a function of increasing amounts of ADP added to the platelets.

To demonstrate the utility of the microfluidic device for future studies involving drug discovery or drug efficacy, platelets were activated with increasing concentrations of ADP in the presence and absence of clopidogrel, a pharmaceutical often prescribed to prevent platelet aggregation. The mechanism of action of clopidogrel is thought to be via its ability to block a variant of the P_{2y} receptor, which is a receptor for ADP, on the platelet surface. As shown in figure 5, platelet adhesion studies that were performed over the course of 3 days (using platelets from 3 different rabbits) resulted in an adhesion number of 15.3 ± 1.4. However, when this study was repeated using an aliquot of the platelets that had been incubated in 40 μM clopidogrel for 30 min., the number of platelets adhering to the endothelium decreased to 9.70 ± 1.4. This decrease in platelet adhesion was even more pronounced in the presence of higher concentrations of platelet-activating ADP. For example, in the presence of 1.0 μM ADP, the number of platelets adhering to the endothelium was 31.3 ± 2.0. In the presence of clopidogrel and 1.0 μM ADP, the number of platelets adhering to the endothelium is decreased to a value (16.7 ± 1.3) that statistically overlaps with the number of platelets adhering to the endothelium in the absence of any ADP. Similar effects from the clopidogrel are also seen at higher concentrations of ADP. Importantly, these results show that clopidogrel has the ability to prevent adhesion of platelets to an endothelial surface. Therefore, the parallel device has the capacity to not only monitor the adhesion of platelets to an endothelium, but also the effects of an platelet inhibitor.

Conclusion

Here, we report the creation of an *in vitro* mimic of an *in vivo* process, platelet adhesion to an endothelium, and successfully used this device to monitor the adhesion activity of platelets. The construction of the device is rather simplistic. Specifically, the use of a petri dish as the substrate to which the endothelial cells are immobilized requires no pre-treatment of the substrate (such as plasma oxidation). Moreover, the PDMS-chip was sealed to the substrate in

a reversible manner, which also simplified construction of the microfluidic system in comparison to previous systems employed in our labs.

The device incorporates an important constituent of the bloodstream (platelets) and enables the direct monitoring of their adhesion when activated. Such a device will enable future studies involving pharmaceutical candidates for reducing platelet activation to be investigated in a high throughput manner using conditions that more closely approximate conditions in the bloodstream *in vivo*.

In response to stimuli that result in platelet activation, platelets have the ability to release ATP[15] and produce their own NO (via stimulation of NOS)[29, 30] in response to ATP stimulation.[25,26] It is known that activated platelets are involved in vessel blockage and that NO regulates this process as an inhibitor of platelet activation; therefore the development of a device capable of monitoring such platelet activity is important for studies involving pharmaceutical agents or cellular communication that inhibit adhesion. This importance of such a device is exemplified when considering work by Freedman[15] resulting in the conclusion that NO production from the endothelium prevents platelet adhesion to the endothelium. In turn, NO production by platelets may prevent platelets aggregating to each other, a process known as platelet recruitment. The measurements reported here provide strong evidence that the conclusion involving the role of endothelium-derive NO is correct.

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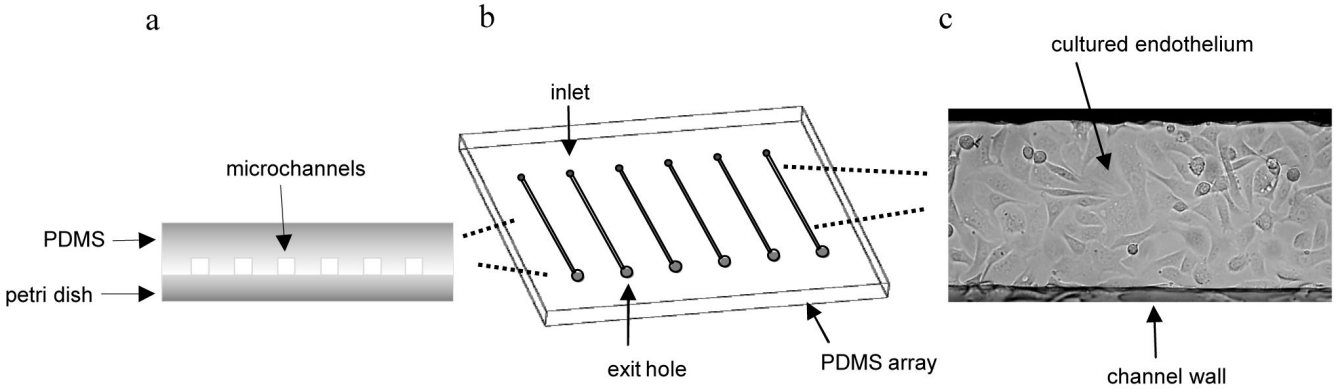

Figure 1.

(a) Cross section of the microfluidic array; each channel has dimensions of 100 μm width and depth. (b) PDMS array with inlet and exit holes for introducing flow to the system. (c) A solution of bPAECs is pushed through the channel to create an immobilized endothelium on the Petri dish.

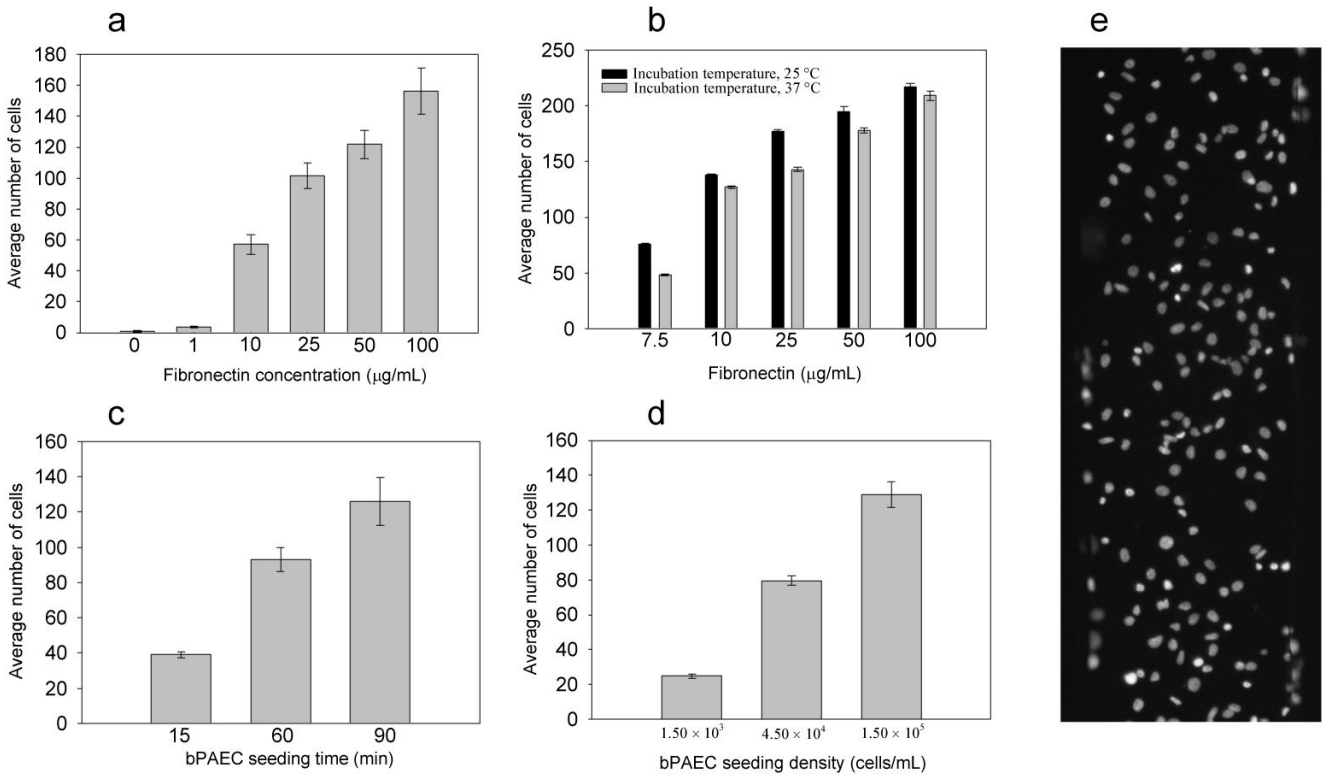

Figure 2.

Determining the optimal parameters for bPAEC adhesion. Each successive experiment incorporates the optimal variable from previous experiment. Error bars represent standard error of the mean (SEM) from n = 4 separate chip experiments. (a) Varying concentrations of FN coated into a 6 channel chip. (b) Effect of variable concentrations of FN incubated at different temperatures. (c) Determination of the seeding time of bPAECs. (d) Effect of varying the seeding density of bPAECs. (e) Confluent channel of bPAECs resulting from 100 μg mL^{-1} FN application with bPAECs incubated at 37 °C, seeded for 90 min. as illustrated by cell nuclei staining.

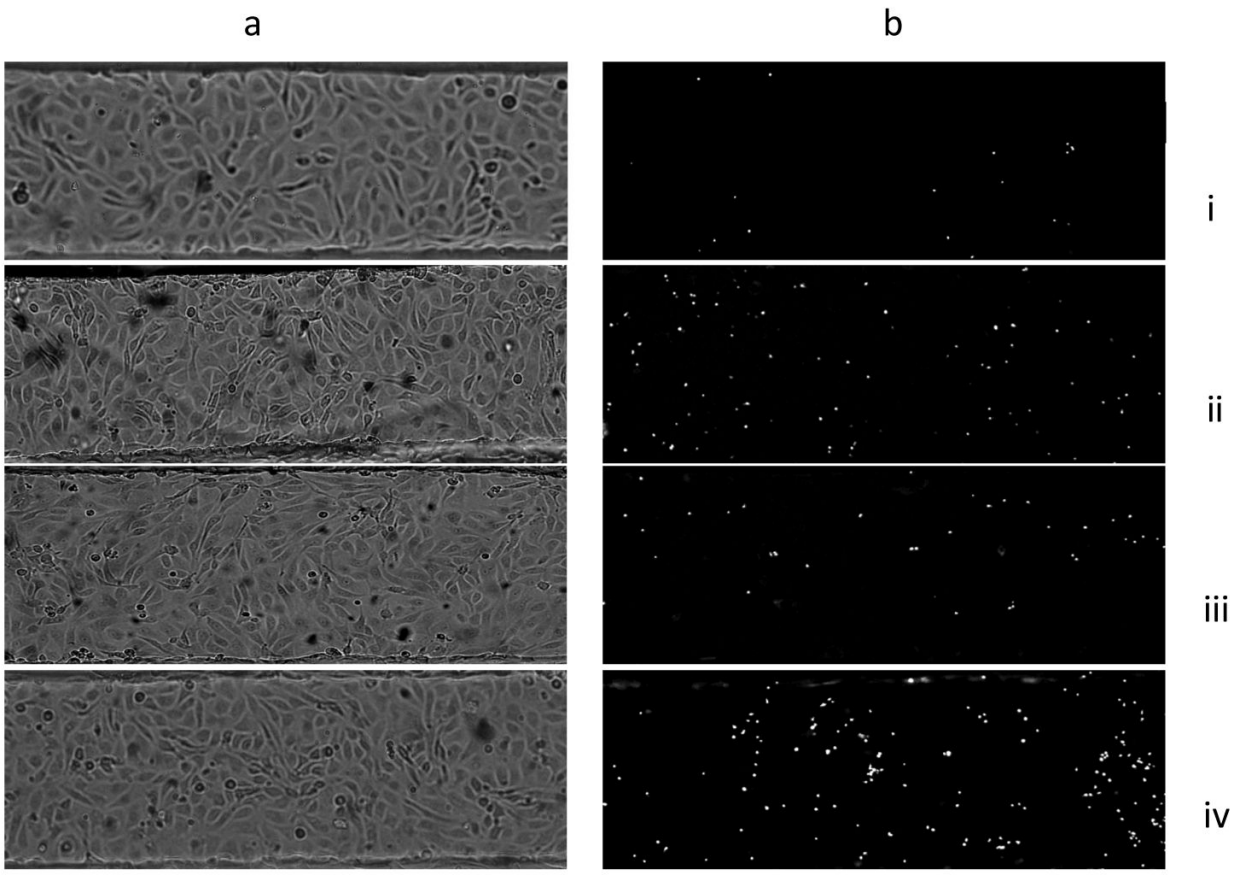

Figure 3.

The effect of ADP activation and L-NAME on platelets pumped over a confluent channel of bPAECs. A stream of equilibrated medium was pumped over the endothelium to ensure any non-adherent platelets were removed before averaging the platelet count. The images in the left column (a) represent bright field images, while those on the right (b) represent the corresponding fluorescence images. (i) Untreated platelets, (ii) platelets incubated in 5 μM ADP, (iii) untreated platelet adhering to a 10 mM L-NAME treated endothelium, and (iv) platelets activated with 5 μM ADP adhered to a 10 mM L-NAME treated endothelium.

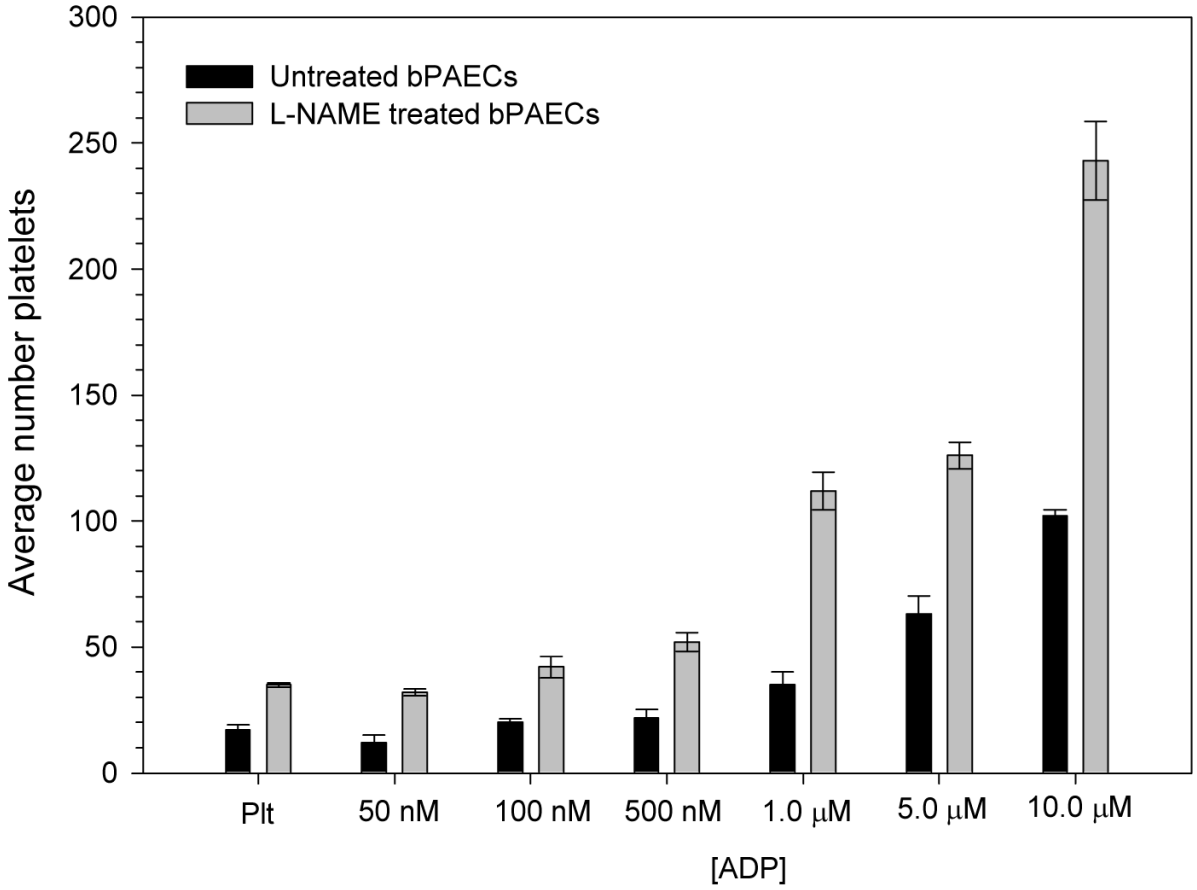

Figure 4.
Varying concentrations of ADP from 0 nM to 10 μM affects the number of platelets adhered to an untreated and L-NAME treated bPAEC monolayer. Non-activated platelets were also examined on similar untreated and treated surfaces. Error bars represent SEM for platelets obtained from n = 5 different rabbits.

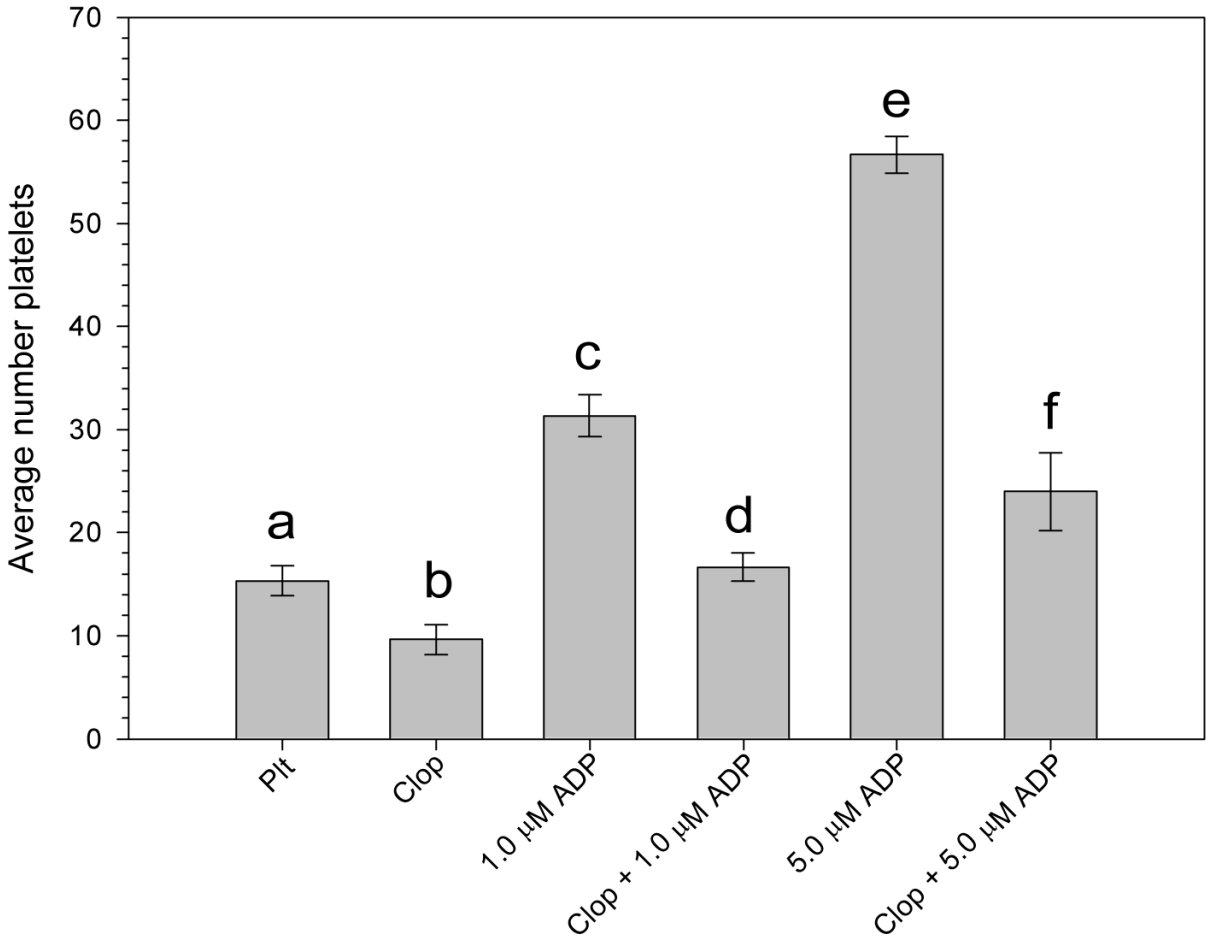

Figure 5.
The effect of an anti-platelet drug, clopidogrel (Clop), on platelet adhesion to a bPAEC monolayer. The number of adhered platelets is shown in the absence (a) and presence (b) of clopidogrel. In addition, the number of platelets adhering to the endothelium in the presence of 1.0 μM ADP is shown in the absence (c) and presence (d) of clopidogrel. Similar studies are shown for 5.0 μM ADP in the absence (e) and presence (f) of clopidogrel. The error bars are the SEM for platelets obtained from n = 5 rabbits.