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Towards Cavitation-Enhanced Permeability in Blood Vessel on a Chip

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Abstract. The development of targeted delivery systems releasing pharmaceutical agents directly at the desired site of action may improve their therapeutic efficiency while minimizing damage to healthy tissues, toxicity to the patient and drug waste. In this context, we have developed a bio-inspired microdevice mimicking the tumour microvasculature which represents a valuable tool for assessing the enhancement of blood vessel permeability due to cavitation. This novel system allows us to investigate the effects of ultrasound-driven microbubbles that temporarily open the endothelial intercellular junctions allowing drug to extravasate blood vessels into tumour tissues. The blood vessel on a chip consists of a tissue chamber and two independent vascular channels (width 200 μm, height 100 μm, length 2762 μm) cultured with endothelial cells placed side-by-side and separated by a series of 3 μm pores. Its geometry and dimensions mimic the three-dimensional morphology, size and flow characteristics of microvessels in vivo. The early stage of this project had a twofold objective: 1. To define the protocol for culturing of Human Umbilical Vein Endothelial Cells (HUVECs) within the vascular channel; 2. To develop a fluorescence based microscopy technique for measuring permeability. We have developed a reliable and reproducible protocol to culture endothelial cells within the artificial vessels in a realistic manner: HUVECs show the typical elongated shape in the direction of flow, exhibit tight junction formation and form a continuous layer with a central lumen that completely covers the channels wall. As expected, the permeability of cell-free device is higher than the one cultured with HUVECs in the vascular channels. The proposed blood vessel on a chip and the permeability measurement protocol have a significant potential to allow for the study of cavitation-enhanced permeability of the endothelium and improve efficiency in screening drug delivery systems.

INTRODUCTION

Most current conventional anticancer drugs do not accumulate selectively in the target organ, tissue or cell, because they typically spread through blood circulation in the body and only a small concentration of the therapeutic agents reaches the diseased site. Therefore, to achieve the required drug concentration in the region of interest, a large quantity of the drug must be administered as a significant amount is distributed in normal tissues causing undesirable side effects. As a result, conventional pharmaceuticals often damage healthy cells and cause toxicity to the patient [1]. Targeted drug delivery systems that selectively release the bioactive molecules to the desired site of action, reduce side effects of anticancer drugs and improve their therapeutic efficacy and patient compliance [1]. Currently, several targeted antitumour drug delivery systems are either on the market or in research phase, but there is still the need to improve their performances for instance in terms of drug loading, attachment of ligands to carriers, and cellular uptake in cancer cells [1]. Once the drug has reached the site of interest a problem often to be addressed is its inability to permeate the endothelium depending on the drug size which determines its efficiency in crossing the biological barrier. A non-invasive targeted drug delivery system based on the phenomenon of cavitation

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represents a valuable approach to improve the therapeutic efficiency and furthermore to treat central nervous system pathologies [2]–[4]. This method consists of microbubbles (MBs) injected into the bloodstream that oscillate and collapse in proximity of the endothelial layer under ultrasound irradiation, thus exerting a force on the endothelial cells. This mechanical action leads to a temporary increase of the inter-cellular spaces increasing the permeability of the barrier and allowing drug to extravasate blood vessel into malignant tissue. The aim of the early stage of the present project is to demonstrate the feasibility of an in vitro microdevice mimicking the realistic microvascular environment surrounding a tumour tissue, to be used for quantitatively assessing the effect of ultrasound-activated bubbles on endothelial cells permeability. The device [2] consists of a tissue compartment and two independent vascular channels placed side-by-side, mimicking the three-dimensional morphology, size and flow characteristics of micro-vessels in vivo. The tissue compartment and vascular channels are separated by a porous interface that allows for biochemical and cellular communication between the two compartments. The artificial vessels are lined by a culture of Human Umbilical Vein Endothelial Cells (HUVECs) in the vascular channels, where under flow of cell culture media cells develop a typical elongated shape in the direction of flow. Measurements of the endothelial layer permeability, using a confocal microscope in epi-fluorescence mode is then performed.

Small animal models are typically used to study the blood brain barrier permeability and to monitor the response to cancer therapy as they have the advantage to allow the study of therapeutics effects in natural environments. Nevertheless, these studies are expensive, time-consuming and difficult to perform. In this context, realistic in vitro models mimicking the cancer microenvironment (from nano to microscale) allow us to study the functioning mechanisms involved in drug permeation to tumours (for instance, the tumour vascular leakage [5]) as well as the screening of new pharmaceuticals. The novel system proposed here consists of a realistic tight endothelial barrier, which provides a platform for the study of the efficacy of cavitation which temporarily enhances the blood vessel permeability. Here we report on a feasibility study to validate both the protocol for culturing of HUVECs in the vascular channels and the fluorescence microscopy based methodology for measuring permeability in the chip.

CAVITATION

The basic principles of cavitation theory [6] are briefly described here to identify the physical phenomena leading to an increase of endothelial barrier permeability. MBs stably oscillate over time upon exposure to a low acoustic pressure, a process termed *stable cavitation*. These oscillations generate fluid flows surrounding the bubble, known as *acoustic microstreaming*. At higher acoustic pressures, this oscillation may become unstable where MBs oscillate more vigorously, leading to their violent collapse and destruction. This phenomenon is called *inertial cavitation* and can be accompanied by generation of shock waves in the medium close to the bubble. The ultrasound-induced collapse of MB can be asymmetrical, leading to the formation of high velocity jets [7]. The response of a MB to ultrasound waves depends on the acoustic parameters used, such as frequency, pulse repetition frequency, pressure amplitude and pulse duration [8]. Typically, the frequency to be used depends on the MB size and its resonance frequency [9]. For bubbles in the micrometer range, the natural resonance oscillation frequency can be estimated around 2 MHz [10], which is the typical ultrasound excitation frequency needed to achieve remarkable volume oscillation.

Stable and inertial cavitation are both already exploited to transiently open biological barriers, including the vascular endothelium [3], [11]–[13]. Nevertheless clinical application of this method requires further improvements, such as the assessment of ultrasound parameters to achieve the endothelium permeability enhancement avoiding irreversible damage to blood vessels and surrounding cells. A bio-inspired microdevice mimicking the microvasculature tumour environment, has the potential to facilitate the understanding and the consequent optimization of cavitation effects occurring inside blood vessels. It may be used also to better design MBs working as both cavitation nuclei and efficient drug carriers and therefore develop targeted therapeutics systems saving costs and time.

MATERIALS AND METHODS

Cell Line

Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Lonza (Walkersville, MD, USA). Cells were maintained at 37°C and 5% CO_2 in EGM-2 Endothelial Cell Growth Medium (EGM-2 Bulletkit) containing Endothelial Basal Medium-2 (EBMTM-2 Medium) and the following growth supplements: human

Epidermal Growth Factor (hEGF), 0.5 ml; Vascular Endothelial Growth Factor (VEGF), 0.5 ml; R3- Insulin-like Growth Factor-1 (R3-IGF-1), 0.5 ml; Ascorbic Acid, 0.5 ml; Hydrocortisone, 0.2 ml; human Fibroblast Growth Factor-Beta (hFGF-β), 2.0 ml; Heparin (0.5 ml); Fetal Bovine Serum (FBS), 10.0 ml; Gentamicin/Amphotericin-B (GA), 0.5 ml (Lonza, Walkersville, MD, USA). Prior experiments adherent cells are collected washing 1x with Dulbecco Phosphate Buffered Saline (PBS), detached using Trypsin-EDTA solution 1+ and blocked with Trypsin inhibitor from Glycine Max (Sigma-Aldrich, Missouri,USA).

Reagents

Fibronectin from human plasma and DAPI for acid nucleic staining were provided from Sigma-Aldrich, Missouri (USA). Texas Red-dextran (40 kDa) was from Life Technologies Corporation, Carlsbad (CA).

Seeding of HUVECs into the Blood Vessel on a Chip

The bio-inspired device produced by SynVivo (Alabama, USA) consists of a polydimethylsiloxane (PDMS) microfluidic chip placed on a microscope glass slide (Fig.1). A sketch of the resulting assembly is shown in Fig.2(a).

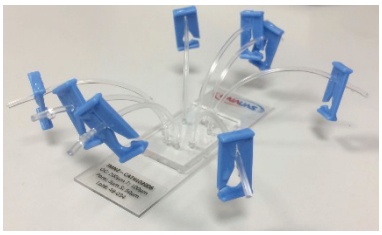
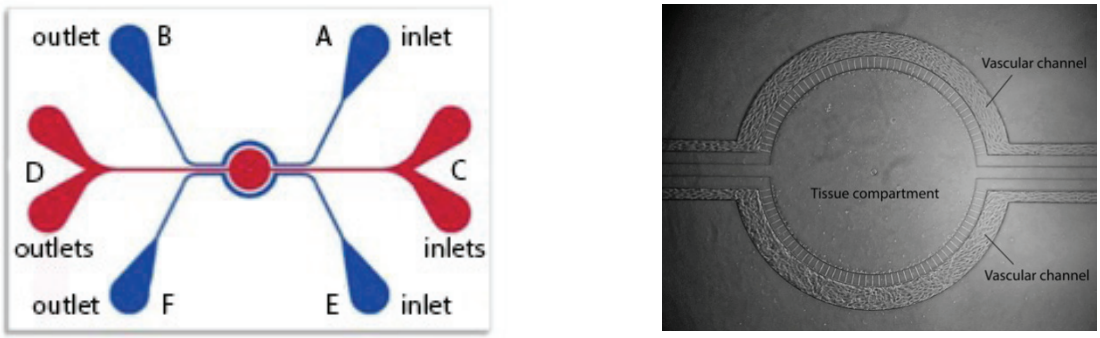


FIGURE 1. Photo of the SynVivo microdevice and Tygon tubing used to attach the system to pumps



(a) (b)

FIGURE 2. (a) Sketch of the SynVivo bio-inspired chip showing the tissue compartment (red) in the center of the device, surrounded by two independent vessel channels (blue). Inlets (A, C, E) and outlets (B, D, F) for flow access are also shown. (b) Bright-field image of the bio-inspired chip cultured with HUVECs.

This microdevice allows culturing cells (for instance, either brain or breast tumour cells) in a tissue compartment with dimensions of 1575µm x 100µm (diameter x height) and endothelial cells in two independent vascular channels (width x height x length: 200µm x 100µm x 2762µm) surrounding the tissue compartment (Fig.2(b)). An interface consisting of a series of pores along the length of the vascular channels (pore dimensions: 3µm x 3µm x 100µm, width x height x length, spaced every 50µm) separates the vessels from the tissue compartment. This porous membrane allows for biochemical and cellular communication between the two compartments [2].

Prior to endothelial cells seeding, the device is degassed, washed with PBS and then coated with fibronectin at 37°C and 5% CO_2 for two hours. Afterwards, HUVECs at an average concentration of $5 \cdot 10^6$ cells/ml in EGM media, are introduced in the vascular channels, using a programmable syringe pump (PhD ULTRA Syringe Pump, Harvard Apparatus, Massachussetts, USA). The inlets are clamped when the HUVECs reach a density of 80-90% in

the channels and the device is placed into a CO_2 incubator at 37°C for four hours, to let cells attach to the channels in the absence of flow. After incubation, using Tygon tubing (Saint Gobain PPL Corp., Pennsylvania, USA) with a total length 40-50 cm, the vascular channels are connected to two 1 ml syringes (Fisher Scientific, Pennsylvania, USA) mounted on a double syringe Harvard apparatus pump placed adjacent to the incubator and filled with EGM, being careful not to introduce air bubbles. A long part of the tube is inside the incubator thus warming up the media to 37°C before entering the vascular channels. At the end of 22 hours of flow, cells develop an elongated shape in the direction of low as shown in Fig.3.

To better visualize cells and provide direct evidence of uniformity, confluence and 3D tube formation a DAPI staining for cell nuclei was used (Fig. 4).

Measurements of Permeability

Being optically clear, the microdevice used here is suitable for direct measurements of the fluorescence dye diffusion from the vascular channel to the tissue compartment through the porous interface. Using a Harvard syringe pump placed next to the microscope, a fluorescent dye (Texas Red-dextran 40 kDa, 1mg/ml) is injected in the vascular channel at a flow rate of 0.2 µl/min. This flow rate, similar to that observed for blood flow in the microvessels in vivo, allows for the diffusion of fluorescent dextran from the vascular channel through the pores into the tumour compartment. As the fluorescent dextran accumulates in the tissue compartment in a time-dependent manner, permeability is estimated by measuring fluorescence signal over time using a confocal microscope (Olympus iX73 FluoView1200) operated in epi-fluorescence mode and a camera (Evolve 512 Delta EMCCD) with a multi-dimension acquisition tool. The time-lapse setting is 1 image per minute, for a total of 120 images captured at an exposure time of 30 ms. During the experiment, the microdevice is placed inside an incubator (OKOLAB, California, USA) specifically designed to fit on the microscope stage to keep cells at 37°C and 5% CO_2.

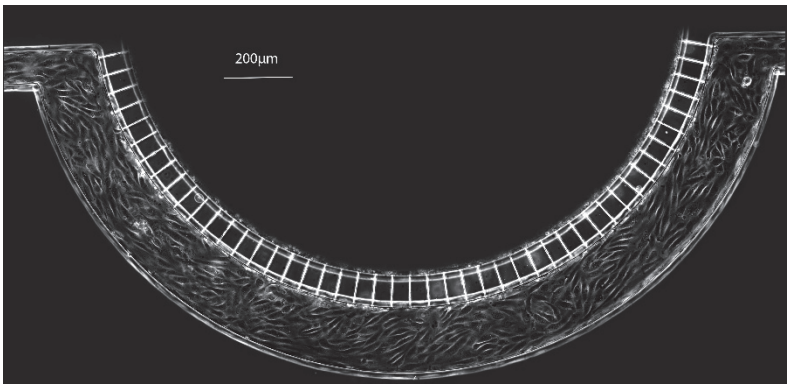


FIGURE 3. Reconstructed bright-field image of the bio-inspired chip: section of the vascular channel with HUVECs. The porous interface is also visible.

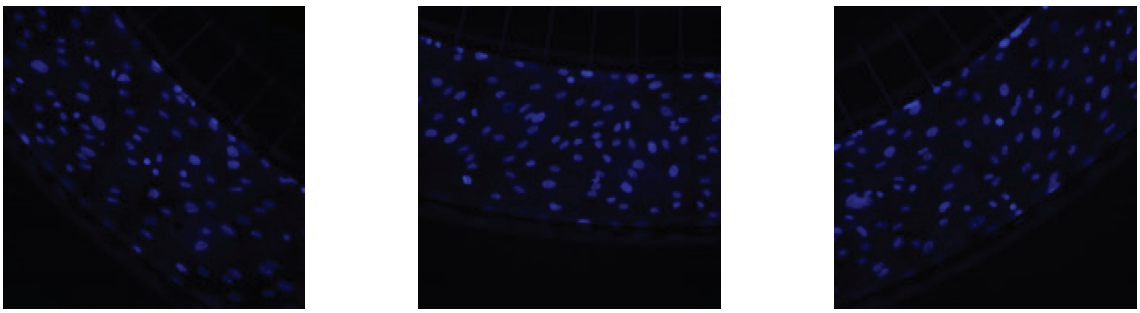


FIGURE 4. DAPI fluorescence images of HUVECs in the vascular channel of the microdevice indicate cell nuclei

Quantification of permeability is performed by post-processing the images with ImageJ software (Maryland, USA) to obtain the dye intensity in the tissue compartment as a function of time. Subsequently, the average intensity of the dye in the tissue compartment (I_t) is measured and normalized to its maximum intensity in the vascular channel (I_{v0}) by using MATLAB (The MathWorks, Massachussetts, USA). The slope of the line dI_t/dt is used to calculate the permeability (P) of dextran through the artificial endothelium using the following equation:

$$P = \frac{1}{I_{v0}} \frac{V}{S} \frac{dI_t}{dt} \tag{1}$$

where V/S is the ratio of vascular channel volume to its surface area [2].

RESULTS

The permeation of the fluorescent dextran from the vascular channel to the tissue compartment in a cell-free device was first evaluated to optimize the method for the quantification of permeability in the chip. Afterwards, the permeability of the cell-free device was compared to the average permeability of the ones with HUVECs cultured in the channel in order to assess the tightness of the endothelial barrier. As expected, a significant decrease in the permeability value in presence of the biological barrier is observed as it consists of realistic junctions between neighboring cells. In Fig.5, the difference in fluorescence dye accumulation in the tissue compartment for the two separate cases (with or without cells) may be observed as a function of time.

In line with the theory, the calculation of permeability is performed considering the intensity of vascular channel saturated to a constant value. For this reason, the first 20 images, corresponding to the time necessary to reach saturation in the vascular channel, are discarded before calculating the slope dI_t/dt. Permeability P is then calculated using Eq.1.

The results of a typical cell-free experiment (Fig.6) indicate that the tissue compartment intensity increases linearly with time. As expected, in the case of cell-cultured device, the fluorescent dextran needs more time to reach the tissue compartment because of the presence of the biological barrier (Fig.6), and the estimated value of the permeability in 5 separate experiments results one order of magnitude lower than the one measured in the cell-free chip.

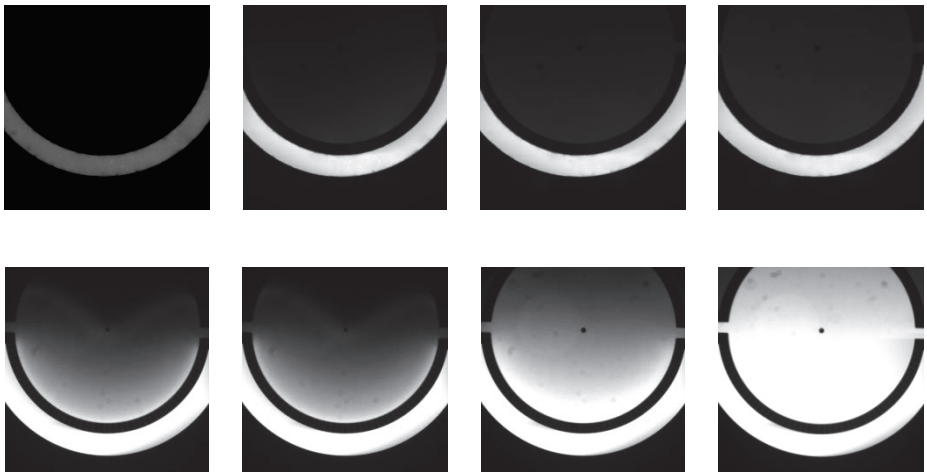


FIGURE 5. Real-time fluorescence images of the passage of dextran from the vascular channel to the tissue compartment for a free-cell device (bottom) and a cell-cultured device (top) after 15 min, 30 min, 60 min and 120 min from the start of the flow

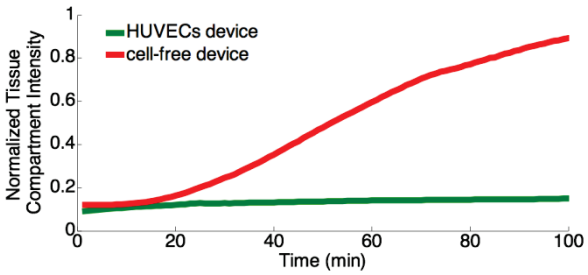


FIGURE 6. Comparison of normalized tissue intensity in a cell-free device and in a cell-cultured device

CONCLUSIONS AND FUTURE DEVELOPMENTS

We have developed a novel methodology to quantify the permeability of the endothelial barrier in a microfluidic chip mimicking the tumour microvasculature. Our preliminary results indicate that in presence of endothelial cells within the vascular channel, permeability decreases by one order of magnitude compared to a cell-free device. This is due to the presence of realistic tight-junctions between neighboring endothelial cells in the vascular channel. Since both the cell culture protocol and the permeability measurement methodology have been standardized and optimized, we next plan to develop a procedure to insonify MBs in the vessel channel under a microscope and observe and characterize how cavitation affects the permeability enhancement. We hypothesize that an increase in permeability in presence of ultrasound-driven MBs will be observed and that the efficiency of this method will depend not only on the acoustic parameters setting but also on sufficient accumulation of bubbles near the endothelium. The use of functionalized MBs that selectively bind to receptors expressed on the endothelial cell membrane [14], may contribute to an increase in the number of cavitation nuclei in proximity of the vessel wall. Furthermore, this methodology may be used to enhance the delivery of drug/gene carriers to tumours. Control over both ultrasound and MBs features is essential for proper optimization of cavitation used to temporarily open the tight-junctions of endothelial cells and avoid damages to the blood vessels and surrounding cells. We also plan to extend these studies into a model that will include the co-culturing of cancer cells (for instance, breast cancer cells lines) in the tissue compartment to fully exploit the potential of this novel microfluidic system to mimic the tumour environment and to characterize its response to cavitation when there are biochemical and cellular communications between the vessel and the malignant tissue which may affect their morphology and function.

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