

PDMS SURFACE FUNCTIONALIZATION FOR SUSTAINED CELL ADHESION IN MICROFLUIDIC ORGAN-ON-CHIP MODELS



A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF
MASTER OF SCIENCE
IN
MATERIALS SCIENCE AND NANOTECHNOLOGY

By
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September 2025

**PDMS SURFACE FUNCTIONALIZATION FOR SUSTAINED CELL
ADHESION IN MICROFLUIDIC ORGAN-ON-CHIP MODELS**

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

PDMS SURFACE FUNCTIONALIZATION FOR SUSTAINED CELL ADHESION IN MICROFLUIDIC ORGAN-ON-CHIP MODELS

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M.S. in Materials Science and Nanotechnology

Advisor: Gürkan Yeşilöz

September 2025

The inherent hydrophobicity of Polydimethylsiloxane (PDMS) poses significant challenges for its use in cell culture applications, particularly in organ-on-chip technology. This study presents a novel, versatile, and biocompatible surface treatment method for polydimethylsiloxane (PDMS), designed to significantly improve its cytocompatibility and shear stability in organ-on-chip applications. Four tailored surface functionalization protocols were systematically explored using 3-(Trimethoxysilyl)propyl methacrylate (TMSPMA), optimized to enhance wettability, surface roughness, and chemical functionality, leading to robust and sustained cell adhesion. The efficacy of these treatments was confirmed via comprehensive surface characterizations of contact angle measurements, scanning electron microscopy (SEM), atomic force microscopy (AFM), and X-ray photoelectron spectroscopy (XPS). The changed hydrophilicity supported robust cell adhesion, proliferation and viability that were determined using three distinct human cell types: HUVECs, MCF-7, and Human-derived induced pluripotent stem cells (iPSCs). Importantly, our findings show that the treated PDMS supports long-term adhesion and proliferation under both static and dynamic microfluidic conditions, with minimal cell detachment even under flow rates

corresponding to physiological shear stress (~ 1.5 dyne/cm²). Numerical simulations further validate the experimental flow conditions, strengthening the model's physiological relevance. This study underscores the effectiveness of TMSPMA treatment in improving PDMS compatibility for cell culture, offering an accessible, tunable, and scalable solution for researchers aiming to create more reliable and biologically relevant microfluidic systems.



Keywords: Microfluidics, TMSPMA, PDMS Functionalization, Cell Adhesion, Organ-on-chip, Surface Treatment, Surface Characterization.

ÖZET

MİKROAKIŞKAN ÇİP-ÜSTÜ-ORGAN MODELLERİNDE HÜCRE TUTUNMASI İÇİN PDMS YÜZEY FONKSİYONELLİĞİ

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Malzeme Bilimi ve Nanoteknoloji, Yüksek Lisans

Tez Danışmanı: Gürkan Yeşilöz

Eylül 2025

Polidimetilsiloksan'ın (PDMS) doğasında bulunan hidrofobiklik, özellikle çip-üstü-organ teknolojisinde olmak üzere hücre kültürü uygulamaları için önemli zorluklar teşkil etmektedir. Bu çalışma, çip-üstü-organ uygulamalarında PDMS'in uyumluluğunu ve akış altındaki stabilitesini önemli ölçüde artırmak amacıyla tasarlanmış, yenilikçi, çok yönlü ve biyouyumlu bir yüzey işlem yöntemi sunmaktadır. Bu amaçla, 3-(Trimetoksisilyl)propil metakrilat (TMSPMA) kullanılarak dört özel yüzey fonksiyonelleştirme protokolü sistematik olarak araştırılmıştır. Bu protokoller, ıslanabilirlik, yüzey pürüzlülüğü ve kimyasal fonksiyonellik gibi özellikleri artıracak şekilde optimize edilmiştir ve güçlü, uzun süreli hücre tutunmasına olanak sağlamıştır. Uygulanan yüzey işlemlerinin etkinliği, temas açısı ölçümleri, taramalı elektron mikroskobu (SEM), atomik kuvvet mikroskobu (AFM) ve X-ışını fotoelektron spektroskopisi (XPS) gibi kapsamlı yüzey karakterizasyon teknikleriyle doğrulanmıştır. Artan hidrofiliklik, insan kaynaklı üç farklı hücre tipi (HUVEC, MCF-7 ve indüklenmiş pluripotent kök hücre iPSC'ler) kullanılarak belirlenen güçlü hücre tutunması, çoğalması ve canlılığını desteklemiştir. Önemli olarak, elde ettiğimiz veriler, işlem görmüş PDMS yüzeyinin hem statik hem de dinamik mikroakışkan koşullar altında uzun süreli hücre tutunması ve çoğalmasını desteklediğini, fizyolojik kayma gerilmesine (~ 1.5 dyne/cm²)

karşılık gelen akış hızlarında dahi minimum hücre kopmasıyla çalıştığını göstermektedir. Sayısal simülasyonlar, deneysel akış koşullarını doğrulayarak modelin fizyolojik geçerliliğini daha da güçlendirmiştir. Bu çalışma, TMSPMA ile yapılan yüzey işleminin PDMS'in hücre kültürüne uygunluğunu artırmadaki etkinliğini vurgulamakta ve daha güvenilir, biyolojik olarak anlamlı mikroakışkan sistemler oluşturmayı hedefleyen araştırmacılar için erişilebilir, ayarlanabilir ve ölçeklenebilir bir çözüm sunmaktadır.



Anahtar sözcükler: Mikroakışkanlar, TMSPMA, PDMS Yüzey Fonksiyonelleştirilmesi, Hücre Tutunması, Çip-üstü-Organ, Yüzey İşlemi, Yüzey Karakterizasyonu.

Acknowledgement

I want to start by thanking Assoc. Prof. Dr. Gürkan Yeşilöz. I am grateful for his support, guidance and mentorship during a new chapter in my life. He not only played a significant role in my academic growth but also in my personal growth. All the things I have learned and experienced here, have created the foundation for my future as a scientist, and for that my thanks go to my supervisor.

I want to thank all the members of Yeşilöz Research Group; Faruk, Ali, Servin, Aslı, Faiqa, Eylül and Dr. Munir; it was a pleasure to work with all of you, get to know you, and find laughter in our good and bad times. I have learned many things from each one of you, thank you for being there for me in the good and the bad. I would like to open a parenthesis here to also specially thank my 2 super-PhDs; Faruk and Servin. They have helped me build this project from scratch and it was a pleasure working alongside very bright and sincere scientists.

I gratefully acknowledge the generous financial support provided by the Scientific and Technological Research Council of Türkiye (TÜBİTAK) BİDEB International Outstanding Researchers Program (Project No. 118C263) and the TÜBİTAK 1001 Program (Project No. 123E157), granted to Dr. Yeşilöz. Also, I am thankful to National Nanotechnology Research Institute Center (UNAM), and Bilkent University.

To my family, whom I always felt that they were there for me. I am so unbelievably lucky to have so supportive and insightful parents and I am forever so grateful to you. My beautiful mother, İrem Erdoğan, who have taught me so much on how to survive basically! I don't remember how many times I have called her for the smallest things. I always look up to the person that you are and hope to become a strong person like you. My loving father, Batur Erdoğan. As I grow older, I feel so thankful for your insights and much needed criticism. I could not have grown into this person without your guidance and unwavering support that I have felt every step in my life. My baby bro, İzo. Even though you are now taller and larger than me, I still see you as a baby and probably continue to do so. It was a blessing to grow up and have fun with you and now to watch you grow. I love you all so so much, couldn't have done this without you.

To my best friend, Derin Akman. I am so lucky to have such a kind soul in my life to know all of me and care for me so deeply. We have literally been inseparable from the moment we met and we have grown together through all these 8 years. To my best friend, İrem Kasapoğlu. My emotional, physical and mental rock. You have always been there for me whenever I come to you and I always admire how strong and ambitious you are. Let's move onto, Çağlar Temiz. You are too funny Çağlar ya! (at least you were during our bachelors). I am so happy to have someone I can banter with nonstop but also have sincere support. My Master buddies; Beyza Nur Küçük and Ahmet Göktuğ Özkurt. I did not imagine meeting great people such as you guys and I can say you made my time here in Bilkent certainly more enjoyable and bearable. Thank you all for your impacts in my life.

To my boyfriend, Mehmet Eşmeli. We have crossed paths in a surprising time and way, but I am so lucky to have someone in my life that is so calm, understanding and fun. Thank you for all your support during my stressful moments and for always putting a smile on my face.

As I am writing these sentences, I realized how lucky I am to have formed these incredible connections in my life that have helped me put together this thesis one way or another. I can say this thesis is not just about my academic efforts but rather a product of all the support and love I have received from incredible people.

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CHAPTER 1

1.INTRODUCTION

1.1 2D and 3D Cell Cultures

Biological research utilizes cell culturing, in order to study the cell response and behavior in laboratory environment in vitro. It is first found in 1900s by Ross Harrison and has been used widely among many different biological research purposes. [1] The main ideology behind cell culture is the cultivation of cells that have been obtained and originated from a known source, providing them with the necessary factors such as temperature, oxygen, nutrients and metabolites mimicking their natural physiological environment. This controlled environment allows cells to proliferate and behave as they would do in vivo. [2]

Starting from the invention of cell culturing, cell have been cultured on planar surfaces which would create a 2-dimensional environment. On this planar surface, cells would attach and grow where nutrient and oxygen supplies would be equally distributed in the population. However, this is not the case in the native tissues. Tissues are made up of huge number of cells that are positioned in a very dynamic environment, interacting with surrounding tissues, cells and environmental cues. This intracellular and extracellular interactions play a critical role in the behavior of cells such as viability, differentiation, stress response, gene and protein expression. [3] Although, 2D culture environment has been widely utilized, this technique fails to recapitulate the same sophisticated and interactive environment cells inside the organism face. Therefore, due to this limitation, cell behavior findings from 2D culture are deviating significantly from the real-life case. [4] Starting from the need to better mimic the physiological

environment, 3D culture systems have been brought up. In the 1970s, the initial attempts to culture cells in soft agar medium started the 3D culture systems. [5] Cells cultured in a 3-dimensional sense have the ability to interact with adjacent cells in all 3 directions and not only in the x-dimension that 2D culture allows. Moreover, 3D culture gives the freedom to co-culture cells from different lineages to facilitate the intercellular interactions which is not very applicable in the case of 2D culture. [6]

The design of 3D culture models can be classified as scaffold-based where cells are interacting with a substrate and adhere to or scaffold-free models that does not provide a substrate for cells to attach, forcing to be aggregated. In the latter model, spheroid and organoid formation is achieved mimicking cell cluster and more complex self-assembled tissue-like models, respectively. [7], [8] Aside from obtaining cellular interactions, the novel platforms have emerged to increase physiological relevance in 3D culture. These platforms help to tackle another limitation arising from 2D culturing in petri dishes or culture plates, that is the absence of dynamic environment. In our tissues, cells are subjected to shear stress induced from fluid flow, different interstitial pressures in different parts of tissues and varying levels of available nutrient, oxygen and metabolite supply. These parameters are also found to critically effect the cell behavior in a similar manner as cell interaction does. [9] Newly found ways to culture mammalian cells have paved the way for acquiring more realistic outcomes to what happens in vivo bridging the gap between animal studies and in vitro studies. [10]

Table 1. Comparison between 2D and 3D cell culture.

<i>Culture Method</i>	<i>2D Culture</i>	<i>3D Culture</i>	<i>References</i>
<i>Time Required for Culture</i>	Faster - Within Minutes to Hours	Slower - Few hours to Few days	[11], [12]
<i>Cost of Culturing</i>	Cheap	Expensive	[13], [14], [15]
<i>Access to essential components</i>	Oxygen, nutrients, signaling factors and metabolites are always homogenously available. (Contrasting <i>in vivo</i>)	Oxygen, nutrients, signaling factors and metabolites are available in different degrees. (Similar to <i>in vivo</i>)	[2], [16]
<i>Cell Properties</i>	Morphological and proliferative changes; cell polarity and phenotypic variations are lost.	Morphology and proliferation are preserved; cell polarity and different phenotypes are achieved.	[17], [18], [19]
<i>Cell Interactions</i>	Cell-cell and cell-ECM interactions are restricted; loss of microenvironment and niche presence.	Cell-cell and cell-extracellular matrix interactions are allowed; formation of proper microenvironment and niches like <i>in vivo</i> .	[18], [20], [21], [22]
<i>Molecular Mechanisms</i>	Different gene and protein expressions.	Gene and protein expression levels are corresponding to <i>in vivo</i> .	[23], [24], [25]
<i>Ease of utilization</i>	Simple culturing technique with high performance, reproducible, long-term culturing is available, the analysis is easily done.	Difficulty in culturing and interpretation of results, performance and reproducibility is low.	[26]
<i>In vivo Imitation</i>	Does not represent the natural form of tissues and organs.	3D form of tissues and organs like <i>in vivo</i> .	[27]

1.2 Microfluidic Technology for Organ-on-Chips

Microfluidics is the technology where small amount of liquids are analyzed, processed and manipulated through micro to nanometer sized channels and geometries. The idea of microfluidics first emerged in semiconductor field and later spread to microelectromechanical systems (MEMS). The microscale platforms were referred as miniaturized total analysis systems (mTAS) or lab-on-a-chip (LoC) systems. [28] At microscale, liquid tends to behave different than those in macroscale due to the dominating forces differing. For instance, the gravitational forces become less significant in the smaller scale and due to higher surface to sample-volume ratio, viscous forces become predominant rather than inertial forces. Moreover, surface tension and capillary forces become prominent as well. From these forces laminar flow profile is obtained and variety of tasks are achieved through the utilization of these fluid properties. [29] As 20th century was coming close to an end, microfluidics was used as a biological device for the first time. [30] Since this novel incorporation, this multi-disciplinary approach has progressed and it is continuing to progress, opening new paths for researchers to manipulate miniscule volumes of fluids inside nanoscale platforms. To name a few applications utilized in those platforms are, pumping liquid through those channels, achieving multiphase streams, filtering and removing certain analytes, forming dispersed droplet are some of it. This control freedom allows microfluidic technologies to address many problems and applications. [31] These devices were highly utilized in biology as it provided many advantages such as reducing the total consumed reagent and sample volumes. therefore, reducing the cost. Moreover, microfluidic platforms provide researchers with more control over the cell microenvironment in terms of its spatio-temporal dynamics. It also decreases the analysis time as volume it decreased. Moreover, analysis can be done in batches similar to multi-well plate setting with high-throughput results gleaned. [32], [33]

As discussed in the previous chapter, 3D culture systems provided researchers to cultivate cells in a physiologically relevant environment that allowed to obtain outcomes that draw near to real situations. [27] However, 3D culture also has limitations. Such as difficulty to maintain organoids in fixed positions for long periods of time or difficulty to

sampling contents for further analyses. [34] Furthermore, these systems lack the tissue interfaces such as how there is an interface between vascular endothelial structures and their tissue, supporting cells; carrying an important aspect in their functionality. Finally, even though scaffolds used in 3D cultures create a dense environment mimicking the extracellular matrix surrounding the tissues and organs, cultured cells still lack the mechanical cues present in our bodies. [35] Fluid shear stress, pressure, compression is some of the examples of these cues. These drawbacks highly suggest the need for a novel platform to be incorporated in 3D culture studies. This is where microfluidic technology meets the biology field to incorporate innovative and modular platforms to level up the research possibilities. [36]

The term “organ-on-chip” refers to devising small units of organs that can successfully function as the whole organ would and recapitulate the organ or tissue functions. Moreover, microfluidic-based organ-on-chips indicate the use of microfluidic devices to achieve this goal. These devices allow the cells to be kept in microchannels or microchambers, allowing cells to be grown in specific compartments or geometries and kept under continuous perfusion. Flexibility in designing the environment of cell culture creates the suitable tool to model the physiological features of tissues and organs. [37]

The design of the microfluidic device can be as simple as one single perfused microfluidic channel to culture one kind of cell type to show the function of one tissue type. In order to portray more complex environments; according to the need of your research, one may create multiple microchannels with varying geometries that connect or interact through porous materials to culture different kinds of cells. [38] These complex designs showcase the interfaces between different tissues like in tissue-vascular interface.

Aside from the microfluidic platform design, physical forces can also be incorporated to the microchip such as fluid flow to create a dynamic environment and introduce physiologically relevant shear stress levels on cells, cyclic strain or mechanical compression of the chambers, all to create the necessary environment. [39] This permits the ability to acquire organ-specific responses, in reactions to drugs, toxins or other environmental cues and stresses. In more sophisticated cases, multiple chips mimicking different organs can be linked fluidically to transfer the factors produced as a response of one organ to another for it to show its immediate effect on the subsequent organ. [40]

1.3 Fabrication and Components of Microfluidic Chips

The fabrication of a microfluidic chip starts on choosing the appropriate substrate material for your device. [41] As there are many diverse applications carried out with the microfluidic chip, the substrate choice carries utmost importance. The materials preferred directly effects the fabrication technique to be followed thus the ease of fabrication is changed according to it. [42] Other properties to keep in mind while choosing the material is whether your application requires a robust and durable device or if transparency is needed for visualization. [43] If biological compounds are going to be used in the chip, the material should be biocompatible and if certain chemicals are going to be applied on the surface of your channels, chemical compatibility should be considered. [44] Moreover, your material should withstand the temperature and pressure setting you will utilize your platform in. [45], [46]

Various materials are used to manufacture microfluidic chips, most common ones currently are glass, silicon, metals and polymers. [42], [47] Each of these comes with specific advantages and disadvantages in terms of what they will be used for. [48] Metal is a cheap option that is easily accessible and easy to graft your desired geometries onto. [47] Moreover, they are very durable and can withstand high temperature and chemicals. [49] Metal microfluidic chips are found to be useful to generate size-tunable nanoparticle synthesis applications. [50] Silicon is preferred commonly for its many advantages such as availability, ease of fabrication, chemical and thermal compatibility. [51] It gives the freedom to be used for many designs and modify its surface. Nevertheless, optical properties of silicon limit its use for visualization purposes in visible and UV regions. [52], [53] It is also a fragile material with high elastic modulus being prone to breakages. This property may cause issues if for instance syringe pumps were to be installed. [51] Lastly, silicon is not a cost-effective material but its advantages usually outweighs the limitation and it is still used for many biological applications and point-of-care devices. [54] Glass also provides many advantages for microfluidic research. It is chemically inert, rigid, stable under different thermal conditions and easy to functionalize the surface. [51], [55] Compared to silicon, it has great optical transparency allowing for visualization

analyses. [47], [53], [54] Moreover, it is cheaper and as mentioned above active components such as pumps and valves can be successfully incorporated to glass microdevices. [42], [51] However, as all materials do, using glass for fabrication comes with its own limitations. Microfabrication of glass is difficult and the process is expensive and laborious, restricting its application. [51], [52] Paper-based microfluidics is another cheap alternative for portable rapid tests, point-of-care devices that are commercialized for medical screening. [56], [57] It is simple and accessible, [58] with highly porous and adsorptive nature. [52], [43] Moreover, the fluid flow is achieved through capillary forces without the need of active components. [59] The introduction of biomolecules is also relatively easy as paper is naturally biocompatible, similar to ECM and easy to sterilize and modify. [52], [60] However, its mechanical strength is decreased when wetted and transparency is acquired only in specific thicknesses. [52]

The emergence of using polymers for microfluidic platforms started relatively newly, and it shifted the interest from silicon and glass for widespread use. Polymers offer an inexpensive option that is easy and cheap to manufacture. [47] They come in transparent or semi-transparent options allowing the use of optical experiments when necessary and their thermostability under high temperature is also an advantage. [50] These properties of polymers make them an ideal candidate to be used in a broader scope of applications from nanomaterial synthesis to fluid manipulations. Examples of polymers used in microfluidic studies are as follows, polydimethylsiloxane (PDMS), polymethylmethacrylate (PMMA), fluoropolymers, cyclo-olefin polymers and copolymers (COPs/COCs), and Thiol-ene polymers (TEs). [45]

Among the polymer class, polydimethylsiloxane (PDMS) is a widely used elastomer for microchip manufacturing. PDMS is a cheap material that has many good qualities for biology-related research such as easy to handle and mold, highly elastic, has optical transparency, gas-permeability, biocompatibility, low autofluorescence and so on. [41], [42] These properties allow to carry out long term cell culturing, cell screening, bacteria and disease detection, biomimicking of organ and tissue environments. [57] However, the nature of PDMS may create limitations in its usage. PDMS is a porous elastomer that creates advantages as well as disadvantages such as molecule adsorption, swelling, evaporation and hydrophobicity. [53], [61]

Microfluidic chips can be fabricated with a variety of approaches. As explained above, many material choices also available for microfluidic platform fabrication each having properties specific to them. [62] Therefore, the choice of fabrication method should be chosen according to your material in order to get the highest efficiency and resolution of nanometer geometries. Another point to keep in mind while choosing the fabrication method is the cost. [63] Microfluidic devices are usually used one time and discarded after use because of the difficulty to clean them effectively. Thus, if the fabrication method is expensive, it would create a financial toll on researchers to fabricate many chips. [51] The fabrication methods used nowadays can be investigated under 2 sections: methods that remove the material to fabricate and methods that deposit the material to fabricate. [46] Material removing techniques rely on creating a structure via removing local parts of material from the bulk. [64] Some of the examples can be laser ablation and mechanical etching techniques. For laser ablation, the material should be transparent at the chosen laser's wavelength in order for laser photons to be absorbed by the material and increase its internal temperature and finally evaporate the material. [65] In the etching techniques, material is removed either with plasma or particle beams which is called dry etching or with liquid solvents that called wet etching. Silicon, glass and metal materials can be etched to fabricate the platforms. The downside of this group of techniques is the loss of material while removing, which can be a problem when the substrate used is expensive. [66] The second group material depositing techniques simply applies an amount of material in designated locations. This can be achieved only if the material is applicable to be deposited in a place and solidified in said place. For instance, polymer materials used for deposition can be in the form of semi-completed polymerization of precursor or is dissolved in a solution, that is later allowed to polymerize after deposition or removing the solvent after deposition to leave the solid material in place, respectively.

The most commonly preferred material deposition technique used to fabricate microfluidic chips are lithography techniques. The process "lithography" was first invented in the 1800s by Senefelder. [67] It involves the use of hydrophilic and hydrophobic surface areas in specific patterns, which absorbs and rejects the ink applied, respectively. [68] Today, photolithography technique is used frequently which uses the same logic of lithography but does the job with UV light. For this method, a photoresist

should be applied on the substrate, commonly used is SU-8. When this photoresist is subjected to radiation with UV through a photomask, the subjected areas either peel off or become cured and stable, etching the desired channels and structures into the substrate. [69] Photolithography has several advantages, including high resolution and the ability to create complex structures. Once the mask is generated, the mass production of chips can be achieved. However, it can be expensive and time-consuming. [70] Soft lithography is another frequently used technique for manufacturing. In order to fabricate your chip with soft lithography, the choice of substrate carries importance, as this technique can only be used with flexible polymer substrates. [71] The steps of the process- namely, generating the hard-master mold, depositing liquid substrate onto the mold, curing the substrate with heat and peeling off the substrate. This way, the polymer becomes a replica of the design on the master mold. Soft lithography enables high-resolution replicas to be made with low cost and rapid production. [72] There are new advancements to fabricate microfluidic chips with molding approach such as hot embossing replica or inject molding, utilizing the same logic as soft lithography. [73] Last technique in the deposition group is 3D printing technology. This is done with a specialized printer that is controlled by a computer depositing a specific resin or filaments onto the surface following the design of the microchannels. New 3D printing technologies are invented nowadays to advance the printing capabilities such as inkjet printing, multijet printing and so on. The most important limitation of this technique is the resolution problems especially in the z-dimension. [46], [74]

1.4 Properties of PDMS and Its Limitations

In the past, the utilization of glass and silicon was prominent for the fabrication of microfluidic chips. [1], [2] However, their fabrication process was time-consuming, expensive and required professional elements that was not very common for all labs to have. [3], [4] Therefore, the need for a substrate that could be shaped easily was carrying utmost importance. This search for a material was concluded in the twentieth century, when poly(dimethyl)siloxane was discovered. [79] Nowadays, PDMS is the most

commonly used elastomer polymer for the microfluidic chip fabrication. The widespread use of PDMS also comes from the fabrication method that is coupled with, soft lithography. [80] As explained in the previous section, soft lithography is a very easy method that has few simple steps that is compatible with rapid prototyping. [72] However, soft lithography solely is not the reason for the popularity of PDMS among biology-related researches. Its intrinsic properties with tunability freedom make the perfect material for diverse applications.

PDMS is labeled as the simplest polymer in the silicone family that is classified via the $-R_2Si-O$ repeating unit. Moreover, polymers that contains the specific $-Si-O-$ unit is called the siloxane. [81], [82] In PDMS, this flexible siloxane unit give thermal and chemical stability, moreover, it allows chains to be arranged easily to place methyl groups at their surfaces. [83] The most common commercial PDMS product is available as “Sylgard 184[®]” and its composition is as follows: dimethylsiloxane oligomers with vinyl end groups, silica filler, tetra(trimethylsiloxy) silane and ethylbenzene and a platinum catalyst. In order to polymerize the PDMS base a curing agent containing a cross-linking agent is utilized. [84], [85], [86]

The elasticity and stiffness of PDMS can be tailored according to the need of research via changing the base to curing agent ratio. [87], [88] The surface stiffness can change the behavior of cells cultured such as T-cell activation or proliferation, therefore it carries importance for the sake of mimicking cell response ideally. [89], [90] The stiffness can be changed with curing temperature, [91] UV exposure as well. [92] Moreover, PDMS is gas-permeable that allows effective cell culture studies to be carried out in PDMS-based microfluidic devices. [93], [94] Transparent and low auto-fluorescent nature also makes the polymer compatible with microscopy and spectroscopy techniques, very important for the validation of cell studies.[95], [96], [97] If required, the transparency can also open to manipulation via changing polymer composition or surface modifications. [98], [99], [100]

The last important property of PDMS is intrinsic hydrophobicity, which can be advantageous or disadvantageous for some research. If one were to model the cardiac extracellular matrix, which is rich of hydrophobic proteins, PDMS can be used directly. [101], [102] However, if cell adhesion is required, hydrophobicity would prevent the fluid flow inside microchannels via capillary force. [103], [104] That limitation can be

mitigated through surface modifications. [105] There are other concerns for using PDMS in biological research, such as the presence of uncross-linked oligomers becoming cytotoxic when leaked into the medium [106] and the hydrophobicity resulting in absorption of small hydrophobic molecules. [107], [108] These limitations can interfere with the cell viability or signaling changing the outcomes. New approaches are being made in terms of surface treatment and PDMS compositions to overcome these side effects and achieving the effective microphysiological systems.

However, “hydrophobic PDMS” is not a single, invariant property. All the parameters used during its fabrication process such as the base: curing-agent ratio, curing temperature and time, and any post-cure thermal aging, change network crosslink density, the amount and mobility of low-molecular-weight oligomers, and the effective roughness/compliance of microstructures. All these factors together set both the *intrinsic* surface energy and the *apparent* wettability of the resulting PDMS. On smooth slabs, varying the Sylgard-184 mixing ratio mainly tunes mechanics; the native water contact angle remains near $\sim 100\text{--}110^\circ$ with only modest shifts before any surface treatment.[109] Later, once micro/nanotopography is introduced; softer, low-crosslink PDMS shown to not support micropillars and they collapse under capillary forces. This can be explained as changing the interface from a Cassie state toward Wenzel state, which amplifies the material’s intrinsic character and increases contact-angle hysteresis with.[110], [111] These effects explain why nominally identical chemistries can yield different priming and bubble behaviours after replica molding from the same master.

Curing temperature and time further modulate wettability dynamics by controlling how the complete network forms and how many mobile oligomers remain. After oxygen-plasma or UV/ozone activation, a more completely cured PDMS retains hydrophilicity longer with slower recovery, because mobile species that otherwise migrate to and “heal” the surface are reduced or immobilized; classic measurements showed that simple thermal aging markedly delays the rebound of the contact angle after plasma[112], consistent with other studies of corona/plasma-oxidized PDMS and the role of crosslink density in recovery.[113], [114] Practically, a sufficiently hot/long cure followed by a short post-cure improves the time window for downstream chemistry and yields more reproducible surface energy from device to device.

Formulation and cure also influence bulk–interface transport. Under-cured or off-ratio networks retain more uncrosslinked chains and exhibit greater swelling and sorption. As a result, hydrophobic small molecules partition from the medium into PDMS and can be released later, altering delivered doses; this partitioning and its consequences for microfluidic biology are well documented.[108], [115] Thermal post-treatments that drive off or immobilize oligomers reduce solvent swelling and mitigate sorption/leaching, improving concentration control in long-term culture and dosing experiments.[116], [117]

These changes in the structural, chemical and mechanical properties of PDMS directly effects the success or failure of the surface treatments. For instance, oxygen plasma produces surface silanols (Si–OH) that must be clean and accessible for silanization. Networks that are under-cured or rich in mobile species foul those sites during or shortly after activation, narrowing the window for effective coupling and yielding patchy silane layers. By contrast, well-cured, thermally aged PDMS supports more uniform grafting.[118]

1.5 Hydrophobicity and the PDMS–Cell Interface

The intrinsic chemical structure of any material or molecule defines its polarity. If different atoms present in the molecule is able to cancel out their different electronegativity with symmetry, then the molecule appears to be a non-polar molecule with no net charge. On the contrary, if these electronegative charges cannot be balanced out in the molecule, then molecule will have a net charge, appear apolar and carries a permanent dipole moment. Water is a polar molecule; electronegativity differences between hydrogen and oxygen atoms generate partial charges and a permanent dipole. This structure enables strong intermolecular interactions through hydrogen bonding and van der Waals forces.[119] In turn, molecular polarity underlies hydrophobic and hydrophilic behavior; non-polar materials tend to be hydrophobic repelling water, whereas polar materials are hydrophilic attracting water and other polar molecules. This phenomenon is also captured from a thermodynamics perspective on the interfacial

energy of a surface interface. When a water droplet contacts on a hydrophobic surface, it will stay in its round shape minimizing the surface interacting with the water. Because in hydrophobic surfaces, replacing the solid–air interface with a solid–water interface increases the Gibbs free energy ($\Delta G > 0$) and this is energetically unfavorable. On the other hand hydrophilic surfaces this replacement lowers the interfacial energy ($\Delta G < 0$) so water can spread and reduce the contact angle.[119]

At hydrophobic interfaces, non-polar surfaces lack hydrogen-bond donors/acceptors or polar sites that would interact with water and therefore have little affinity for water. Water within a hydrophobic boundary cannot maintain its preferred hydrogen-bond network, and occupies a higher-free-energy state. The system lowers its free energy by expelling that interfacial water from the surface with hydrophobic interactions. This attraction is not a new chemical bond; it is an entropically driven, solvent effect that exceeds van der Waals interaction under aqueous condition.[120], [121], [122] Oppositely, hydrophilic interfaces present polar or ionic groups with hydrogen-bond donors/acceptors. They are able to orient the water molecules and promote strong hydrogen-bonding interactions, resulting in a structure hydration layer. This interfacial structuring lowers the solid–water interfacial free energy and water is able to wet the entire surface.[120], [121] Taken together, hydrophilic surfaces accommodate water by building hydration shells, whereas hydrophobic surfaces exclude water creating an effective attraction that favors their approach. These behaviors map cleanly onto the engineering language of contact angles.[119]

Building directly on this framework, when native PDMS meets serum-containing medium, the proteins in the medium with non-polar sides, for instance albumin, present those sides to the PDMS surface and readily bond with the surface. This replaces the unfavorable interfacial water adjacent to the hydrophobic wall and is thermodynamically favored; adsorption is therefore rapid and strong through hydrophobic interactions. Talking from the albumin perspective, as it is the most prominent protein in the medium, it covers the PDMS surface denaturing during the process and creates largely non-adhesive film that presents hydrophobic patches and masks bioactive motifs. Later, other extracellular-matrix (ECM) proteins that would facilitate cell adhesion with specific ligand sites can not interact with the surface and therefore cell attachment could not be achieved. [123] On hydrophobic PDMS, this conditioning layer would be dominated by

denatured or misoriented proteins, so an important ECM protein to facilitate cell adhesion, integrins fail to engage productively. Cells remain rounded, form few and fragile focal adhesions, and any apparent attachment is easily perturbed.[120], [121], [124]

The surface chemistry of the materials modulates which adhesive proteins deposit, how they are oriented and whether additional modified chemical or peptide are displayed, directly affecting the engagement of cell adhesion molecules such as integrins. Increasing the hydrophilicity of a surface promotes the necessary protein adsorption. Once such proteins are adsorbed to the surface, cells can begin to attach and spread on the substrate, a result of integrin ligation and initiation of signaling cascades that instruct the cells to attach and proliferate. [122]

In the context of microfluidics and biointerfaces, this explains why hydrophilic treatments enable reliable priming, controlled wetting, and the formation of stable, ligand-presenting interfacial layers, whereas untreated hydrophobic PDMS tends toward water exclusion, uncontrolled protein films, and unstable, poorly reproducible interfaces.[123], [124]

1.6 Importance of Surface Treatment and Existing Treatment Options

As mentioned in the previous section, the inherent hydrophobicity of PDMS creates poor cell adhesion, prevention of fluid flow and absorption of protein and small molecules causing swelling. [30]-[35] To address this, various techniques have been developed to modify the PDMS surface. Each technique offers unique advantages tailored to specific applications, with inevitable limitations. Thus, the search for novel surface modification techniques is still a hot topic, as each developed technique aims to enhance cell adhesion therefore the further organ-on-chip applications and diminish the shortcomings to create a generalized approach for all applications. The most important factors to keep in mind while validating the success of surface treatment are, the stability

of treatment ie. inhibiting hydrophobic recovery, the increase in the wettability of the surface and the biocompatibility of treatment for organ-on-chip applications. [125], [126]

In this section, the current surface modification techniques will be explained and analyzed on how widespread can they be used or on what terms they are successful. Various techniques have been proposed for PDMS treatment, those can be categorized into 2 groups on how the process is carried out. The first group of PDMS surface modification method is gas-phase processing where the surface is activated by deposited radical groups. The most widely used method from this group is plasma treatment. It involves exposing PDMS to ionized gases such as oxygen, argon, or nitrogen, generating reactive species that bond with the surface and introduce functional groups like hydroxyls. [127] These groups enhance hydrophilicity, reducing contact angle values and improving cell adhesion and protein binding. [128] Plasma treatment is simple, efficient, and time-effective. However, a major drawback is its rapid hydrophobic recovery, where contact angles revert to near-original values within hours to days, limiting its applicability to short-term microfluidic studies. [129], [130] Ultraviolet (UV) treatment is another gas-processing method for modifying PDMS surfaces, often combined with oxygen or ozone to generate reactive species. [131] This increases surface wettability, but hydrophobic recovery remains an issue after some time. Moreover, the process is slower, requiring 30 minutes to several hours, yet often results in incomplete treatment. [132] Additionally, UV exposure induces mechanical stress, leading to shrinkage and cracking, particularly in thicker PDMS layers. The modification depth is also limited, restricting its application to thin membranes or surface layers rather than thicker PDMS structures. [105] Chemical Vapor Deposition (CVD) is the final gas-processing modification method, where gaseous reactants chemically react with the PDMS surface in a reaction chamber, forming a thin film. [133] This technique enables the production of conformal, pinhole-free coatings on complex geometries, offering high stability and potential for advanced functionalization. [134], [135] However, CVD requires specialized equipment and precise control of temperature, pressure, and precursor flow rates to achieve uniform deposition. Additionally, the coating can be heterogeneous, with thickness variations across the surface, posing challenges for consistent modification in microfluidic applications. [136]

The second category of surface modification technique is modifying the PDMS surface through physisorption of wet chemicals. This category includes techniques such

as Layer-by-Layer (LBL) assembly, Sol-gel and Silanization. LBL involves alternating deposition of oppositely charged polyelectrolytes, proteins, or nanoparticles, forming polyelectrolyte multilayers (PEMs). This method allows precise control over coating thickness and composition, enabling customization with biofunctional molecules like collagen and fibronectin to enhance cell adhesion. [137] However, LBL coatings face stability issues since electrostatic interactions are weaker than covalent bonds, making them prone to degradation or delamination. Factors such as ionic strength, solvent compatibility, and pH influence PEM integrity. Additionally, organic solvents used in LBL assembly can cause PDMS swelling, disrupting coating uniformity and stability. [138] The sol-gel technique involves hydrolyzing and condensing metal oxides like tetraethyl orthosilicate (TEOS) or methyltriethoxysilane (MTES) on surface, enhancing chemical resistance, wettability, and biocompatibility properties of PDMS. [139] They are applied as a sol mixture, later it solidifies and bonds to the surface through Si-O-Si covalent bonds. [140] Despite its benefits, precise control of reaction parameters, such as temperature and precursor concentration, is crucial for successful modification, adding complexity to the process. [141] Also, sol-gel treatment creates a protective layer, the process itself can disrupt PDMS. The precursor solution may cause swelling, and improper optimization can lead to uneven coatings and cracks, reducing effectiveness and limiting its applicability. [142] Silanization is the last technique in the wet chemical modification category. It involves the reaction of alkoxysilanes with hydroxyl (-OH) groups, forming strong Si-O-Si covalent bonds. Since -OH groups are essential for bonding, an oxidation step is required, typically achieved via plasma, UV treatment, or in-situ oxidation. [118] The most commonly used silane for this technique is the 3-aminopropyltriethoxysilane (APTES) introducing amine (-NH₂) groups. [143] APTES-treated PDMS improves wettability, reducing water contact angles and enhancing endothelial cell adhesion and proliferation. [144] The introduced amino groups also promote protein adsorption, stabilizing cell-substrate interactions. [145] However, APTES-based treatment faces limitations, including hydrophobic recovery due to PDMS oligomer migration and incomplete hydrolysis and condensation of ethoxy groups can lead to non-uniform coatings and residual unreacted molecules, affecting surface consistency and long-term stability. [146]

The last preferred method for PDMS surface modification is via using Extracellular Matrix (ECM) proteins. Commonly used to coat PDMS and create natural component for cell adhesion are collagen, fibronectin and gelatin. [128] ECM proteins self-assembled on PDMS surface through covalent bonding and allowed cell adhesion altering the surface roughness. [127], [147] Collagen, for instance, is a major structural protein in our body, and a specific type of it, Collagen I, found to increase the hydrophilicity of PDMS the most while having the highest adsorption rate among other ECM proteins. [148], [149] However, long-term cell culture on collagen-coated PDMS is often suboptimal. Achieving a uniform coating is difficult: collagen adsorbs unevenly on hydrophobic PDMS, leaving patchy regions where the protein cannot interact effectively with the surface. Because collagen is a large, fibrillar protein, perfusing it through microchannels can also clog channels and trap air bubbles. Moreover, the collagen that does adsorb frequently undergoes conformational changes and partial denaturation, which reduces the availability of cell-binding motifs and accelerates further loss of bioactivity. Together, these issues compromise coating stability and, ultimately, cell attachment over time.[123], [146] Also, Collagen type IV does not achieve effective wettability of the surface therefore it is not preferred. [150] Gelatin is another ECM protein used for similar purposes. It is a thermoresponsive collagen derivative. Gelatin coated PDMS showed strong cell adhesion and endured shear stress. [151] Compared to collagen and fibronectin, gelatin can maintain the cell activity for longer periods.[152] However, using gelatin itself is not enough to provide a good adhesive surface such as collagen, limiting its usage to some extent. [153] The last ECM protein commonly used is Fibronectin. It has specific domains that interact with Endothelial cell membrane receptors to provide cell adhesion. [154] Moreover, inside microfluidic network, fibronectin is found to be better at supporting sheep carotid arterial ECs, primary porcine aortic ECs, HUVECs and valve ECs. [153], [155], [156] However, studies shown that only fibronectin is not enough for long-term culturing as cells detach. [104]

1.7 The Active Chemical: 3-(trimethoxysilyl)propyl methacrylate

Organosilanes are silicone-based monomers, that have at least one direct bond between a silicone atom and a carbon atom. They are the silicone analogs of alcohol class. A typical organosilane molecule consists of an organic functional group and an inorganic functional group, both are attached to the silicon atom. The inorganic functional groups interact with inorganic surfaces especially when the substrate contains silicon or heavy metals in its structure, such as metal or glass. [157] This interaction occurs very rapidly and dynamically in 2 reactions. The first reaction is the hydrolysis of alkoxy groups either with a water addition or from the residual water on the surface. The second reaction is the silanol coordination with hydroxyl groups on the surface to form oxane bonds with condensation reaction. Through this bonding, a stable polymeric siloxane network is achieved on the inorganic substrate. The resulting chemical interface is more stable, and therefore more resistant to moisture, temperature or mechanical stress. [158]

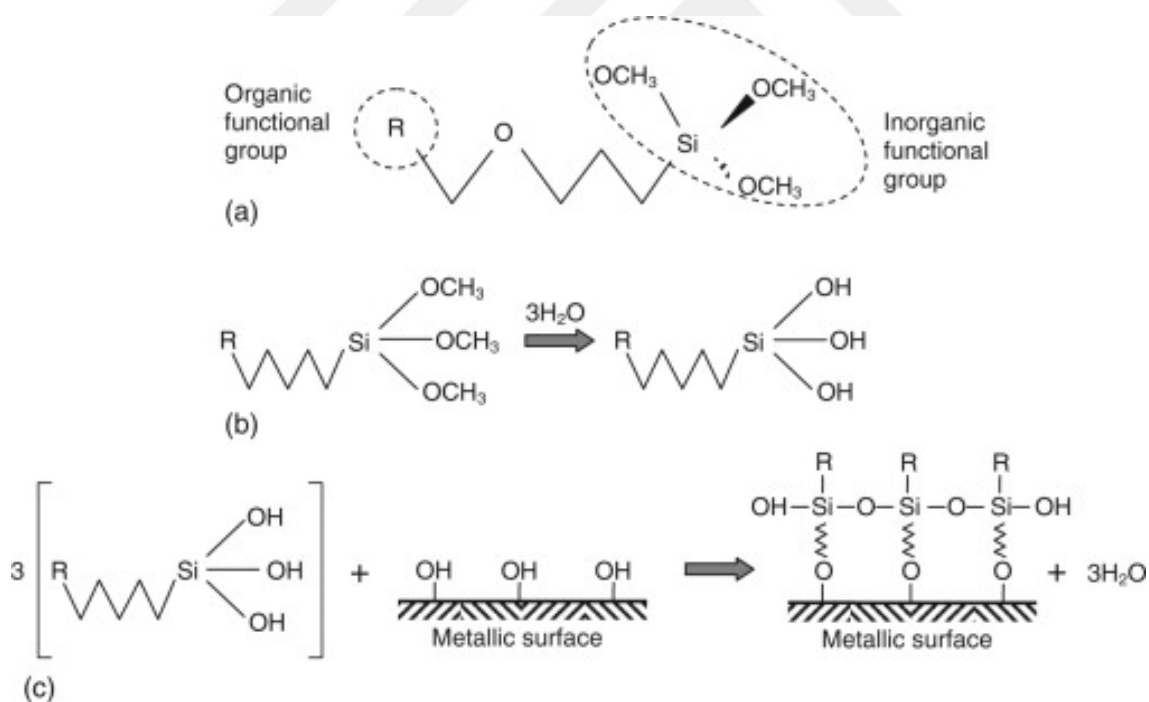


Figure 1. (a) Schematic of an organosilane monomer. (b) Hydrolysis of silane monomer. (c) Covalent bonding to inorganic substrate. Reproduced from [159].

3-(trimethoxysilyl)propyl methacrylate (TMSPMA) is an organosilane monomer. As mentioned above, the chemical composition of TMSPMA is made up of 3 functional

groups: first is the methacrylate group ($-\text{CH}_2=\text{C}(\text{CH}_3)\text{COO}-$), that corresponds to the organic functional group, allowing the covalent attachment to organic matrices such as polymers. The inorganic functional group is comprised of silane group ($-\text{Si}(\text{OCH}_3)_3$) facilitating the siloxane (Si-O-Si) bonds with inorganic hydroxyl-containing surfaces through condensation reactions. Lastly a propyl linker chain ($-\text{CH}_2\text{CH}_2\text{CH}_3-$) acting as a spacer between the 2 functional groups to provide stability and flexibility to the molecule.

TMSPMA undergoes hydrolysis reaction in aqueous or acidic environments explained in Fig 3b., generating reactive silanol ($-\text{SiOH}$) groups. Those silanol groups readily bond to hydroxyl-rich inorganic surfaces such as glass, silica, and metal. The free organic functional side offers a dual functionality to the TMSPMA, which makes it particularly useful in bioengineering and material science applications.

In the literature, TMSPMA is found to be used in very diverse research projects. For instance, it is used as a silane coupling agent to improve the adhesion between PDMS and LiNbO_3 wafers. [159] Coupling agents are compounds that function as adhesion promoters between organic and inorganic interfaces. Organosilanes are commonly used for the purpose of chemically and physically wedding these different materials to a strong bond. [160] Another study used TMSPMA to treat TiO_2 nanoparticles to increase its hydrophilicity and enhance its dispersion. TMSPMA with 2 functional carbon groups ($\text{C}=\text{C}$ and O-C=O) provided a stronger bond to the nano- TiO_2 . [161] Similarly, Zirconium Oxide (ZrO_2) nanoparticles have been silanized with TMSPMA to modify its surface adhesive properties. [162] Moreover, TMSPMA is commonly preferred in modern clinical dentistry as well. The tri-alkoxy groups present in this chemical offers strong adhesion between the inorganic mineral surfaces. [163] TMSPMA is tried out to coat different materials, utilizing its organosilane composition to form stable bonds with the surface. The applicability of TMSPMA in biological or microfluidic platform has yet to be discovered. The multiple alkoxy groups and with its dual reactive sides with silane and methacrylate groups, it can offer a good candidate for PDMS surface modification.

Therefore, in this project, our goal is to perform a comprehensive assessment of PDMS surface modification using 3-(Trimethoxysilyl)propyl methacrylate (TMSPMA). This new proposed surface functionalization technique will enable a robust, tunable, and biocompatible surface treatment compatible with organ-on-chip applications. We will cover all assessment areas from protocol optimization and proposing multiple protocols

to characterize the surface and cell behavior ensuring the necessary environment created to ensure long-term cell adhesion and viability. Once validated, this new effective treatment protocol will be applied in a functional biological model to demonstrate its translational potential. Among the many physiological interfaces in the human body, the vasculature plays a central role in homeostasis, molecular transport, and disease progression. Thus, in the next chapter, we extend our work to show the importance and working mechanism of the vasculature system, to create a complex, dynamic barrier system, that not only creates physiologically relevant and application-driven setting but also will show the performance of the optimized TMSPMA treatment.



CHAPTER 2

2.1 Barriers in Human Body

All living organisms must have a shield as a frontier between the internal and the external world. These specialized physiological barriers guard against pathogens, toxins, and help maintain internal homeostasis. Moreover, it allows to maintain their identity and compartmentalize contents that reduces the entropy. [164] Without these organism-intrinsic barriers, subcellular, cellular compartments would not exist and therefore the electrophysical or chemical gradients would fail to be established. Examples can vary from the level of organelles, cell membranes or internal barriers such as blood barrier and barriers in contact with the outside such as intestine, skin and so on. As expected, each barrier has unique structural features and protective functions. They serve as organizing structures, they generate a compartmentalization where ingredients of insides and outsides are not allowed to mix freely and their interaction is tightly controlled. The compounds inside or outside the barriers can be necessary for survival or development, but they can also be harmful viral components. Thus, these biological barriers function as guards to allow and prevent the passage of molecules depending on their origin and need. [165]

To name few examples of our internal barriers; skin barrier is the largest organ in the human body. It covers the entire external surface and serves many functional roles for a health organism. It is constituted of multilayer and the outermost layer, the stratum corneum, is a formidable physical and chemical barrier. It prevents dehydration and blocks entry of most foreign substances. Typically, only a small percentage of a topically applied drug penetrates into the inner layers, into epidermis. This protects against environmental pathogens and toxins, but limits transdermal drug delivery.[165], [166]

Another very important barrier is the intestinal barrier, composed of epithelial layer, mesenchymal layer and mucus, forming a crucial barrier between the external lumen and internal body. A healthy intestinal barrier permits nutrient and fluid absorption while

blocking harmful microbes and toxins. The first line of defense against infections and microbes is antimicrobial peptide containing mucus. [167] Aside from serving as a defensive barrier, the tight junction complexes linking the intestinal epithelial cells, play a crucial role in preventing uncontrolled passage of luminal antigens and bacteria. If this barrier becomes “leaky,” it is implicated in diseases from inflammatory bowel disease to Parkinson’s disease. [168] For orally administered drugs, the intestinal epithelium is the major obstacle to absorption into circulation. Only certain small molecules can permeate paracellularly or via transporters, and many macromolecules (like peptide drugs) are normally blocked. [169] Another very important and very specialized barrier is the Blood-brain barrier (BBB). Formed by brain microvascular endothelial cells joined by tight junctions, along with astrocytes and pericytes, the BBB strictly regulates entry of substances into the central nervous system. This barrier is one of the most selective barriers in the body, as it shields the brain from blood-borne toxins and pathogens. Any irregular number of molecules or pathogens reaching to the brains results in neurological diseases. [170] BBB also severely limits drug penetration, over 98% of small-molecule drugs and essentially all large therapeutics cannot cross the intact BBB. Only very small (<400–600 Da), lipophilic molecules diffuse transcellularly across the BBB. While this exclusion protects neural tissue, it complicates pharmaceuticals targeting neural diseases. [171]

Lastly, one of the largest structures in our body with intricate and complex structure creates our vasculature. Vascular structure of human body is comprised of huge number of vessels that play an integral role in blood transport throughout the circulatory system. It is an extensive network of arteries, capillaries and veins that maintains cellular functions, absorption of nutrients and waste removal for cell homeostasis. Without a healthy transport across blood vessel that reaches to each organ individually, organs and tissues would not be able to get the nutrients they require to sustain their activities or remove their waste that otherwise would intoxicate themselves. [172]

These barriers profoundly impact drug delivery strategies. Therapeutics must be designed or delivered in ways that circumvent or exploit each barrier’s properties. For example, the BBB’s selective transport mechanisms have inspired “Trojan horse” drugs that hitchhike on nutrient transporters or transferrin receptors to enter the brain. Oral drugs are formulated with enteric coatings or absorption enhancers to improve crossing

of the gut epithelium. Transdermal drugs employ either potent lipophilic molecules or mechanical aids (like microneedles) to breach the skin. Understanding the structure and function of each barrier enables the development of such targeted delivery systems. Unfortunately, the barriers in our body are vital for the protection and homeostasis for the body yet they create obstacles to therapeutic intervention, requiring innovative solutions to achieve effective drug diffusion to target sites. [166], [169], [171]

Therefore, in the scope of this thesis, we wanted to model the blood vasculature inside microfluidic chip to study the transport mechanism and essentially obtain an in vitro model that can be used to study pharmacokinetics. After acquiring a simple barrier model, the incorporation of other types of cells to model the disease environment will be helping making the model more realistic and legitimate for drug testing. In the next sections, only mechanisms of blood vasculature will be introduced.

2.2 Molecular Transport Across Vasculature

Once a drug or molecule is in the bloodstream, it must traverse the vascular wall to reach tissues. Transport across the endothelium cell lining of blood vessels occurs via two primary routes: paracellular and transcellular pathway, transport between the endothelial cells and transport through the cells, respectively. Both are playing a critical role in maintaining physiological homeostasis and might contribute to pathological conditions if problems arise. [173]

In the transcellular pathway, there is many different approaches for transporting substrates across the vasculature that is based on the nature molecule. Small lipophilic molecules such as gases, steroid hormones, simply diffuse across the cell membranes down their concentration gradients in a process called passive diffusion. Slightly larger or hydrophilic molecules may cross via transcellular pores or carriers, some examples of it, aquaporin channels for water, or GLUT1 transporters for glucose on the BBB endothelium. Molecules can also be transported actively via pumps that works to the concentration against gradients. [174] On the other hand, larger macromolecules such as peptides, proteins and nanoparticles generally require vesicular transport where the

endothelial cell engulfs the cargo in a vesicle and shuttles it across in a process called transcytosis. There are 2 different forms of transcytosis: receptor-mediated transcytosis and adsorptive transcytosis. Transcytosis can be used for both hydrophobic and hydrophilic molecules most abundantly used for LDL and albumin transportation. [175], [176]

In healthy capillaries, inter-endothelial tight junctions severely limit paracellular diffusion to only very small hydrophilic solutes or water can slip through, and even then at a slow rate. In tissues with fenestrated or discontinuous capillaries, paracellular gaps or fenestrae allow easier passage of fluids and macromolecules. The paracellular openings can be adherent junctions, tight junctions or gap junctions. Paracellular permeability can be dynamically regulated by endothelial junction proteins; claudins, occludin, VE-cadherin and so on. Various stimuli can cause these junctions to open and increase the permeability of endothelial lining. [174], [176], [177]

As mentioned above, endothelial permeability is not static and it responds to pathological and physiological conditions. For instance, tumor-associated blood vessels are found to be notoriously leaky. This is due to the rapid and disordered angiogenesis driven by the overexpressed growth factors. The resulting vessel structures have defective endothelial junctions, fenestrations, and lack supporting pericytes. Solid tumors are characterized by this leaky vasculature and impaired lymphatic clearance, leading to accumulation of large drug carriers in the tumor. Another condition that highly known to modify the permeability is inflammation. Pro-inflammatory cytokines like TNF- α and interleukins, as well as inflammatory mediators (histamine, bradykinin), act on endothelial cells to loosen junctions and initiate vesicular pathways. TNF- α , for instance, triggers signaling that disrupts the VE-cadherin and tight junction complexes, leading to gaps between cells. The result is plasma proteins and immune cells extravasate to the tissue, which is helpful for fighting infection but also causes swelling. [178], [179]

Aside from pathology or factor-driven permeability changes, environmental cues can also later the permeability of vasculature. The mechanical force of blood flow and thus, shear stress can modulate endothelial barrier function. Laminar shear stress found in straight, healthy arteries have an anti-permeability, vessel-stabilizing effect. It induces endothelial expression of genes such as KLF2 and Nrf2 that strengthen cell junctions and promote alignment of cells with flow. High laminar shear encourages production of

robust glycocalyx and tight adheren junctions, thereby reducing permeability. By contrast, low or oscillatory shear stress that occurs at arterial branches or areas of turbulent flow is known to impair the endothelial glycocalyx and disrupt the cytoskeleton and junctions, increasing permeability. This contributes to atherosclerosis – regions of low shear develop leakier endothelium that allows LDL cholesterol and immune cells to infiltrate the arterial wall, spurring plaque formation. [180], [181] Hypoxia is another environmental factor to change the behavior of the vasculature. Endothelial cells respond to hypoxia by stabilizing HIF-1 α , which in turn upregulates VEGF and other factors that affect permeability. Hypoxia may also weaken junctions directly or via oxidative stress pathways. Interestingly, some studies suggest HIF-2 α can enhance barrier integrity in certain contexts, indicating a complex role of hypoxia signaling. In tumors, chronic hypoxia creates a pro-permeability, pro-angiogenic environment that exacerbates the already leaky vasculature. [182]

Understanding these transport mechanisms and their modulators is crucial for developing therapies that either reinforce the vascular barrier or transiently open it to enhance drug delivery to tissues.

2.3 Vasculature-on-Chip Studies

As explained above, the blood vessel interface portrays a crucial structure in our body that is regulated in a dynamic environment with many physical and chemical cues. This complex nature of the vasculature requires the need for new study models to be developed. Thus, in the recent years, vasculature-on-a-chip technologies have emerged as powerful platforms for studying vascular biology and disease in vitro. These models are designed to study endothelial barrier function, drug and molecular transport, vascular remodeling, and disease progression, particularly in conditions such as atherosclerosis, and cancer formation. Microfluidic platforms became a good opportunity to design and modify the platforms with channels or chambers lined with endothelial, epithelial cells under controlled flow conditions, often incorporating multiple cell types and 3D matrix components to mimic the microenvironment of real vessels. By precisely tuning flows

(shear stress), chemical gradients, and cell co-cultures, researchers can recreate key features of human vasculature and observe cellular behaviors that were previously difficult to capture in static dishes or animal models. [183], [184]

These models can be developed to deeply understand the underlying mechanisms that is regulating the permeability of vasculature. In a study by Polacheck et al. a human engineered microvessel (hEMV) model is introduced where endothelial cells (ECs) were seeded to form a lumen inside a collagen hydrogel, closely mimicking native microvasculature. This system incorporated fluid flow to generate physiological shear stress and allowed co-culture with pericytes, which play a key role in regulating vascular barrier function. By using fluorescent tracers, the researchers quantitatively measured vascular permeability and demonstrated that Notch1 signaling stabilizes endothelial junctions in response to shear stress, reducing leakage. These findings about the underlying mechanisms of microvessels has the utmost importance to uncover the disease progressions as well as targeted drug developments. [185]

Another major application of vasculature-on-chip systems is to model human disease conditions in vitro for research and drug testing. For instance, atherosclerosis is a complex disease that is directly related to dysfunctionalities in the vasculature. Recently, researchers developed a 3D arterial wall-on-a-chip that includes a perfusable endothelial tube surrounded by a chamber with SMCs and embedded macrophages to simulate an intima layer. These cells are arranged to recreate the architecture of an arterial vessel with early atherosclerotic lesions. Under flow, LDL cholesterol could be perfused through the endothelial channel, and investigators observed monocyte adhesion, transmigration, and formation of foam cells in the vessel wall analog. This plaque-on-chip model successfully mirrored key events of atherogenesis. [186]

Inflammation is another very prominent stress condition that changes the state of the vasculature. So, in order to model the inflammation and study the immune cell migration, researchers have developed an “inflammation-on-a-chip” model of the capillary bed using a 3D microfluidic platform (OrganoPlate®). Chemoattractants and human neutrophils were introduced to simulate an inflammatory signal. The neutrophils were observed adhering to activated endothelium and migrating across the endothelial barrier into a collagen matrix, much like they would exit blood vessels into tissues during inflammation. The chip allowed quantification of transmigration efficiency and real-time

imaging of the sequence of events and providing insight into how different conditions influence the route of extravasation. [187]

Vasculature-on-chip studies are revolutionizing our understanding of vascular physiology and pathology. The impact of these microfluidic vascular models is profound, they offer a level of control (over flows, cell makeup, and mechanical forces) and visualization that is nearly impossible in vivo, while maintaining human biology that animal models lack. These in vitro blood vessel systems are now hot topic research in drug screening, disease modeling, and personalized medicine, allowing researchers to investigate vascular responses to drugs, tumor metastasis mechanisms, and endothelial dysfunction in cardiovascular diseases. Future advancements in 3D bioprinting, integration of immune cells, and patient-derived cell sources will further enhance the complexity and translational relevance of vascular-on-chip platforms, bridging the gap between preclinical studies and clinical applications. [184]

2.4 Aim of Study

In this study an innovative PDMS surface treatment with 3-(Trimethoxysilyl)propyl methacrylate (TMSPMA) is presented. The crucial need for surface treatment of PDMS lies in the hydrophobic nature of PDMS, inhibiting the microfluidic organ-on-chip applications. To overcome this limitation of PDMS, we designed this surface treatment to increase the hydrophilicity and cell attachment. Our approach allows fast, easy, low-cost and a stable treatment that outperforms many other available treatment options.

We have investigated four protocols with TMSPMA, namely protocol 1, 2, 3 and 4 with varying solvents, TMSPMA concentrations, treatment durations, and treatment conditions, each of which can be applied interchangeably. In order to support the effect of treatments, we have characterized the surface of PDMS with scanning electron microscopy (SEM), atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), and contact angle measurements. These characterizations resulted in significant alterations in topography, surface chemistry, and wettability. Each different protocol

yielded results that are deviating from each other with small differences, which supports the interchangeable usage possibility. As our goal is to create a surface treatment that would be used for organ-on-chip applications, our proposed treatment should not interfere with or disrupt the cell proliferation and behavior mechanisms. Biocompatibility and cell adhesion assays were conducted in two approaches using three cell lines with completely different natures: human umbilical vein endothelial cells (HUVECs), MCF-7 human breast cancer cells, and human-derived induced pluripotent stem cells (iPSCs). Initially, these cells were cultured on PDMS-coated 96-well plates to assess whether the biocompatibility of the modified surfaces is achieved. Enhanced cell attachment and proliferation were observed on the treated PDMS surfaces compared to untreated controls. Furthermore, live cell staining and cell proliferation was assessed to find cell viability continue to be high several days post-culture and proliferation rate was within the expected and normal rate. After concluding the healthy nature of cells cultured on the treated surfaces, we moved our assessments to microfluidic platforms. Cells were cultured in TMSPMA-treated PDMS-based microfluidic channels, where improved cell adhesion and proliferation were observed, with no detectable cytotoxicity. We have done staining for these cells to show their healthy morphology. Notably to mimic a dynamic environment inside microfluidic platforms, HUVECs were cultured under regulated, continuous flow. These cells found to be maintaining their strong attachment, underscoring the effectiveness of the TMSPMA treatment in promoting robust cell adhesion under physiologically relevant conditions.

This study demonstrates the potential of TMSPMA treatment as a promising strategy for modifying the physicochemical properties of PDMS surfaces, thereby promoting cell adhesion and proliferation. Additionally, the versatile and flexible protocols developed provide customizable solutions to accommodate diverse experimental needs, with broad potential for applications in organ-on-chip technologies.

We want to explore the translational potential of TMSPMA in an organ-on-chip model. Specifically, we have chosen to mimic the vascular barrier with HUVEC endothelial cells in a layered chip studying the molecule and drug transport mechanisms. This application will shed light on the limitation of in vitro drug testing platforms since vascular barrier poses a big problem on the drug efficacy as it does not allow the passage of every molecule.

CHAPTER 3

3.MATERIALS AND METHODS

3.1 Materials

For cell culture studies, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12), DMEM with High Glucose, L-Glutamine, Penicillin-Streptomycin, Fetal bovine Serum (FBS) and Trypsin-EDTA (0.25%) were supplied from Serena Europe GmbH (Brandenburg, Germany). For iPSC culture, mTeSR Plus Basal medium and Accutase cell detachment solution were supplied from StemCell Technologies (Canada). For staining cells, Calcein-AM reagent and F-actin dye were acquired from Invitrogen, Thermo Fisher Scientific Inc. (USA). DAPI dye was acquired from Sigma Aldrich (USA). Cell proliferation rate was assessed with the Cell Counting Kit (WST-8) from Abcam (UK). Pluripotent Stem Cell 4-Marker Immunocytochemistry kit was used from Molecular Probes, Invitrogen, Thermo Fisher Scientific Inc (USA).

3.2 TMSPMA Treatment Preparation

In this thesis, we have designed 4 different protocols of using TMSPMA to modify the PDMS surface to enhance cell attachment and proliferation. In **Table 2.**, the components of the solution applied to the surface and the duration of incubation is listed. These protocols have been inspired from available methods in the literature. [188], [189] As explained in the previous chapter, TMSPMA is a well-known organosilane that have been used as an adhesive and coating agent in the literature for varying purposes.

However, its utilization on polymer or specifically PDMS-based microfluidic chips have not been investigated. Therefore, our research carries the novelty to use TMSPMA in a whole new application that can open new application possibilities.

The first protocol contains TMSPMA solution along with ethanol and acetic acid as solvents. The preparation steps are rather simple, we add 1 mL of TMSPMA (Sigma-Aldrich, USA) to 200 mL of pure ethanol. Separately, we prepared the acetic acid solution as 1:10 and added 6 mL from that solution to the TMSPMA + Ethanol mixture. This should be done just before use to have a fresh and not completely hydrolyzed TMSPMA solution. After our PDMS blocks, PDMS-covered wells and microfluidic chip platforms have been oxidized with plasma (Plasma Etch, USA), prepared solution is added to the surface. The existing protocol for this one suggested the 3-minute incubation in the room temperature. However, we have optimized it to 10 minutes after deciding it would be too short for especially microfluidic chips as it needs to flow and cover whole the surface first. By simply combining ethanol and acetic acid, synergistic effects are achieved due to their complementary roles in the reaction process. Ethanol increases the solubility of TMSPMA thereby facilitating uniform coverage on the PDMS surface. Acetic acid, on the other hand, acts as a catalyst by promoting the hydrolysis of TMSPMA, leading to the rapid formation of silanol groups and subsequent condensation into a stable siloxane network [190], [191]. This combination not only enhances the overall efficiency of the surface modification but also allows for more precise control over reaction time and surface uniformity, making it a highly effective method for achieving rapid and consistent PDMS modification.

The second protocol utilized a TMSPMA solution comprising deionized water and acetic acid as solvent. The preparatory steps start with adjusting the pH of deionized water from 7.0 to 3.5 by adding small amounts of acetic acid and measuring simultaneously with our pH meter. After achieving the desired pH, we have added 4 mL of TMSPMA to 1 L of deionized water. The complete TMSPMA solution is added onto the PDMS and this time 1 hour of incubation time in room temperature is employed. In this protocol, water is not as effective as ethanol in dissolving TMSPMA and promoting its reactions, thus we extended the treatment time to compensate for the lower solubility and reactivity of TMSPMA in water compared to ethanol and achieve adequate surface modification. By removing ethanol, the protocol minimizes exposure to such solvents,

which can be advantageous for applications where the use of milder, less aggressive substances is preferred making it more biocompatible.

Lastly, protocol 3 and 4 are the same protocol that are comprised of two different (1% and 5%, respectively,) TMSPMA concentrations. The reasoning behind our choice of utilizing the same protocol twice is to show the how higher concentration of TMSPMA changes the surface properties with higher silane groups affects the surface modification, while still showing biocompatibility and non-toxicity to cells. Moreover, all the remaining protocols aside from protocol 4, had similar concentrations of 1% or 0.5% TMSPMA and it was crucial to add a different concentration to make a comprehensive study. We have also tried out 10% TMSPMA, however, in that case we have come across cell death to conclude that much high concentration of TMSPMA is not suitable for cells. Their TMSPMA solutions are prepared in one step, adding measured amount of TMSPMA to pure ethanol solutions to acquire specific concentrations. This time solution is incubated for 2 hours in 80°C. The increased incubation temperature and period substantially promote the annealing of TMSPMA coating on PDMS. In all cases, the channels were immersed in their respective solutions and subsequently washed multiple times with either ethanol (for protocols 1, 3, and 4) or deionized water (for protocol 2) to remove any residual chemicals.

Our developed protocols, incorporating various solvents, temperatures, treatment durations, and TMSPMA concentrations, were designed to identify optimal conditions for modifying the PDMS surface. Additionally, these protocols provide flexibility and versatility to accommodate different application preferences, including the avoidance of high temperatures, extended incubation periods, and harsh chemicals, thereby meeting a wide range of research needs and requirements.

Table 2. The recipes and protocols of the prepared TMSPMA solutions.

	Recipe	Protocol
Protocol 1	0.5% TMSPMA in Ethanol & Acetic Acid	10 mins reaction at RT
Protocol 2	1% TMSPMA in dH ₂ O & Acetic Acid	60 mins reaction at RT
Protocol 3	1% TMSPMA in Ethanol	120 mins reaction at 80°C
Protocol 4	5% TMSPMA in Ethanol	120 mins reaction at 80°C

3.3 PDMS Surface Characterization

To characterize the morphology, chemical composition, topography and wettability of the PDMS surfaces before treatment and after TMSPMA treatment, 4 characterization analyses were employed: Scanning Electron Microscope (SEM), X-Ray Photoelectron Spectroscopy (XPS), Atomic Force Microscopy (AFM) and Contact angle. During these experiments, we have prepared PDMS blocks with 3mm heights and cut them to 1-2mm square shapes. They were polymerized on smooth Si Wafers, so their surface did not have any impurities or shapes. After we have carried out the TMSPMA treatments as explained in the previous section, we have washed the blocks with ethanol and left it to air dry for 24 hours to ensure complete dryness. Same washing steps were done for the bare PDMS samples as well.

3.3.1 Scanning Electron Microscopy (SEM)

Scanning Electron Microscope (SEM) was used to characterize the surface morphology of TMSPMA-treated (across all four protocols) and untreated PDMS surfaces as our control group. In SEM high energy electrons are sent to the surface and interact with the electron and photons on the surface. Some of those are released from the surface in various angles and the detector of SEM collects these electron signals and uses them to produce the image. Relying on atomic number and contrast, secondary electrons, material morphology, and backscattered electrons can be used to estimate an atomic composition. In order to carry out SEM analysis, the sample should be conductive. In our case, PDMS is a non-conductive sample, therefore we need to coat the surfaces before starting the analysis. For this thesis, Scanning Electron Microscope (SEM, FEI Quanta 200F) equipped with a field emission gun (FEG-SEM) was utilized. The SEM was operated in environmental mode (ESEM) at an accelerating voltage of 30 kV. To coat the PDMS surface, a 10 nm layer of gold/palladium was sputter-coated onto the surface before imaging. The coated samples were then mounted on stubs using carbon-conductive adhesive tape for analysis.

3.3.2 Atomic Force Microscopy (AFM)

In order to provide a more detailed description of the surface features of the PDMS samples treated with TMSPMA, Atomic Force Microscopy (AFM) analysis was performed using a PSIA AFM system (Oxford Instruments, U.K). PDMS samples were prepared as 1 cm × 1 cm slabs, cured at 70 °C for 2 hours, and gently cleaned with isopropanol before imaging. Measurements were carried out under ambient conditions (~22 °C, 45% relative humidity). A Nanosensors NCLV-A cantilever with a force constant of 48 N m⁻¹ was used in AC mode for air topography scans. The tip was engaged until the Z voltage reached ~70 V, and scans were performed at a rate of 0.6 Hz over 5 μm × 5 μm areas with 512 × 512 pixel resolution. At least three distinct regions were imaged for each sample. The resulting topography images were analyzed using Gwyddion software, without applying any data manipulation such as filtering or smoothing. Surface roughness parameter, root mean square roughness (Rq) was extracted to quantitatively compare untreated and TMSPMA-treated surfaces.

3.3.3 X-ray Photoelectron Spectroscopy (XPS)

We wanted to analyze the chemical compositions of unmodified PDMS and treated PDMS surfaces. In principle, X-ray photoelectron spectroscopy (XPS) identifies the chemical elements present on the sample surface and provides the binding energy values of those elements detected. The surface is subjected to high-energy X-ray beams that excite electrons and some are ejected. Through this data, we can identify the specific chemical bonds present on our samples. In our analysis, we used X-ray photoelectron spectroscopy (XPS, K-Alpha XPS, Thermo Fisher Scientific, USA). The PDMS samples were secured with copper tapes and XPS examination was performed with monochromated aluminum Kα 945 radiation. The untreated PDMS surface served as the control group, denoted as bare PDMS. Among our four TMSPMA protocols, we detected Carbon 1s, Oxygen 1s, and Silicon 2p scans to support the chemical treatment success.

All parameter settings and data collection were done through the software (Thermo Advantage).

3.3.4 Contact Angle

To analyze the wettability of a surface, we performed contact angle measurements for untreated PDMS and TMSPMA-treated PDMS surfaces (across all four protocols). Through this analysis, we were able to identify the different characteristics of unmodified surface and treated surfaces. Since hydrophilicity is required for cell adhesion and fluid flow, it is necessary for our microfluidic platform to obtain this property. To carry out this characterization method, we employed Contact Angle Measurement System (Dataphysics, OCA 30, Filderstadt, Germany). In this procedure, our PDMS blocks were placed on a planar surface and raised the stage to bring it closer to the needle that was positioned in the upper region. From that needle, a 2 μL droplet of ultrapure water was dispensed onto the surfaces at room temperature, and the resulting contact angles were recorded using the custom in-house software. At least three images were taken for accurate assessments.

3.4 PDMS Well-Plate and Microfluidic Platform Fabrication

We have carried out our PDMS studies in 2 approaches. For the first one we used 96-wells, to confirm the biocompatibility of TMSPMA treatment. The reason behind our choice to start our research with a traditional culture plate is our aim to test many treatment concentrations and protocols at the same time in a high-throughput manner.

For this approach, we have covered the bottom of 96-well plates with PDMS. We prepared 10:1 ratio PDMS with curing agent, mixed thoroughly, and degassed the solution to remove bubbles that emerged in the mixing part. The liquid uncured polymer

solution was then poured to each well with 1000 μL micropipette. We cut the tip of the pipette tip to decrease the viscous forces, to make it easier for us to inject the PDMS solution easily. Each well had approximately 500 μL of PDMS solution to cover the whole surface of the wells. After discarding, we checked each well for bubbles and popped them via a syringe needle. The prepared wells were kept in the room temperature for few hours to let PDMS settle down to the bottom from the walls of the well. Then we moved the plates to hot plates to thermally cure the PDMS at 75°C for 2 hours. This concluded the preparation step for the PDMS covered 96-well plates.

For the second approach in our study, we have used microfluidic chips, since our main goal behind the generation of this surface treatment protocol is to be used in microfluidic studies. To fabricate the microfluidic chips, we have utilized standard soft lithography method similar to our previous studies [192], [193]. The microchannel design was created using CAD software and transferred onto a photomask. After that a silicon wafer is covered with SU-8 substrate and it is spin coated to create a smooth surface. Later UV shine from the top, through the photomask to pattern the design of our microchannel design onto the silicon wafer. **Figure 2.** summarizes the fabrication of the developed microfluidic device.

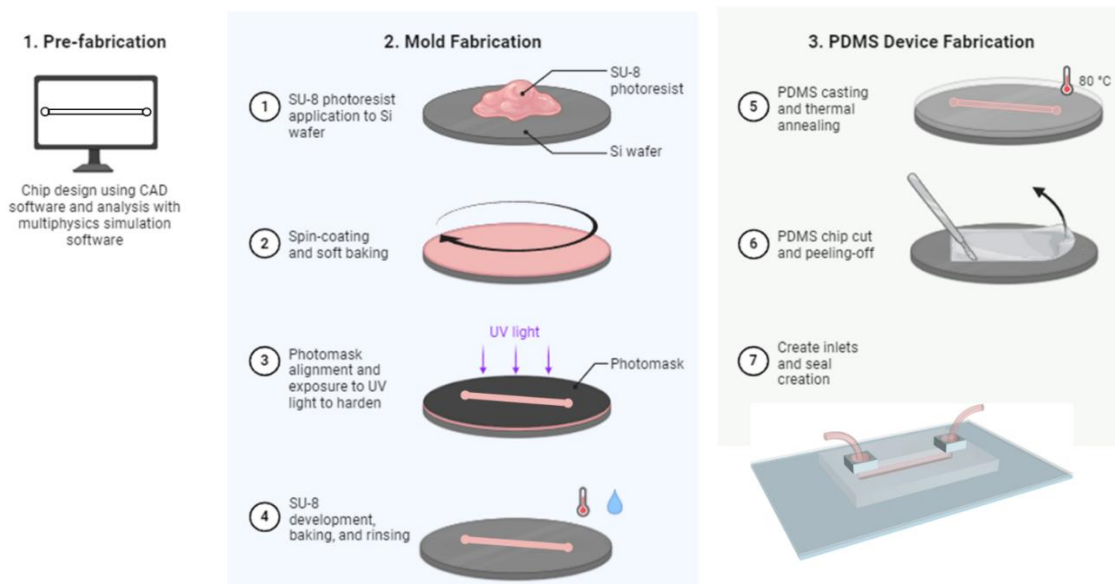


Figure 2. Fabrication steps for the developed microfluidic platform.

Our design is a single, straight channel that is 1.19 mm long, 1.24 mm wide and 150 μm high. Same as previous, Polydimethylsiloxane (PDMS) was prepared at a 10:1 ratio with a curing agent, thoroughly mixed, degassed, and then poured onto the master mold, which is the patterned silicon wafer. After PDMS is distributed among the wafer homogenously, it was thermally cured at 75°C for 2 hours. Once cured, the PDMS was carefully cut and peeled off from the mold. As discussed in the introduction, the fabrication steps and parameters play central role in the properties of the obtained PDMS. Our goal behind adopting the standardized 10:1 ratio and curing in high temperature is to show our protocol in the most commonly used kind of PDMS to maximize the reproducibility and improve cell adhesion outcomes.

Channels were carefully cleaned with ethanol to remove any dust or PDMS particles occurred while peeling. The PDMS block was turned over to have the channels facing upward. Inlet and outlet ports were created using 1.5 mm biopsy puncher. For the bottom of the channel, a glass slide coated with a thin layer of PDMS was fabricated using a spin coater at 5000 RPM for 15 seconds. Both the channel and PDMS-coated glass slide were carefully cleaned with ethanol then dried with air purge followed by treatment with oxygen plasma for 2 minutes to facilitate the bonding process. After plasma treatment, channels were bonded to the glass slide to create rectangular channels that is PDMS in all 4 surfaces. The bonded platform is then kept on hot plate for a few minutes for a complete sealing. In order to have extra reservoirs for media, two PDMS cubes (4 mm thick) were prepared, and they were punctured in the center using an 8 mm biopsy puncher. The reservoirs were then cleaned with ethanol, dried, and treated with oxygen plasma for bonding. Once prepared, the PDMS cubes were bonded to the inlet and outlet of the channel, forming the final microfluidic platform. The microchannel 3D schematic, fabricated layers and the completed chip is shown in **Figure 3**.

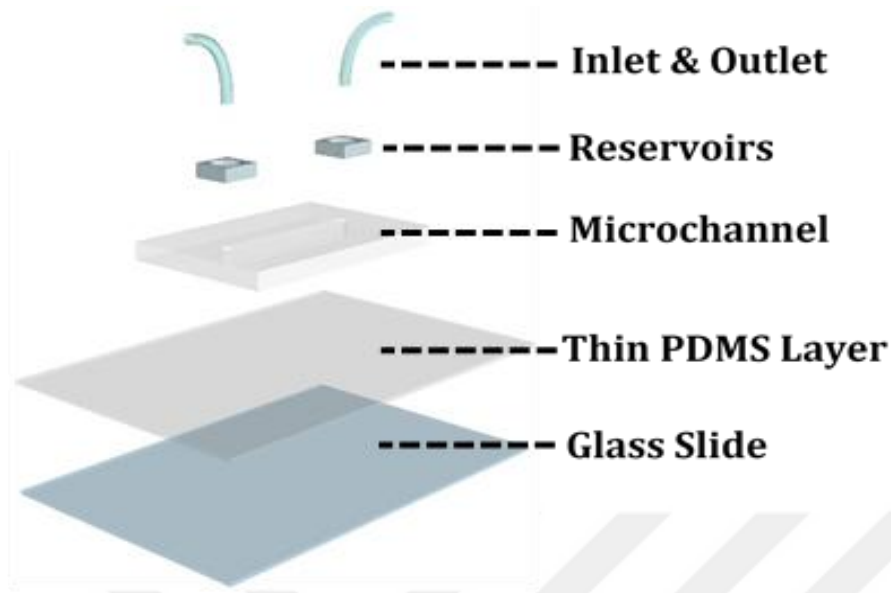


Figure 3. 3D schematic of the microfluidic platform.

3.5 Multilayered Barrier-on-chip Microfluidic Platform Fabrication

After comprehensively assessing TMSPMA treatment, its translational potential will be demonstrated on a vascular barrier-on-chip platform. The device will be composed of three layers: an upper and a lower PDMS layer, each containing straight channels of 1.19 mm length, 1.24 mm width, and 150 μm height, as described in the previous section. Between these layers, a porous PDMS membrane will be integrated, featuring pores of 30 μm diameter spaced 50 μm apart. Inlet and outlet ports will be introduced into the channel layers using a 1.5 mm biopsy punch, while the middle PDMS layer will be punched with an 8 mm tool to create an interface for monitoring molecular transport. The porous membrane itself will be fabricated via soft lithography: a CAD-designed pillar pattern transferred to a photomask and then molded into PDMS.

All layers will be thoroughly cleaned with ethanol, dried with an air purge, and subjected to a 2-minute oxygen plasma treatment to facilitate bonding. The assembled

device will be sealed on a hot plate to ensure complete adhesion. **Figure 4.** illustrates the barrier-on-chip platform, presenting both the physical layers and their schematic design.

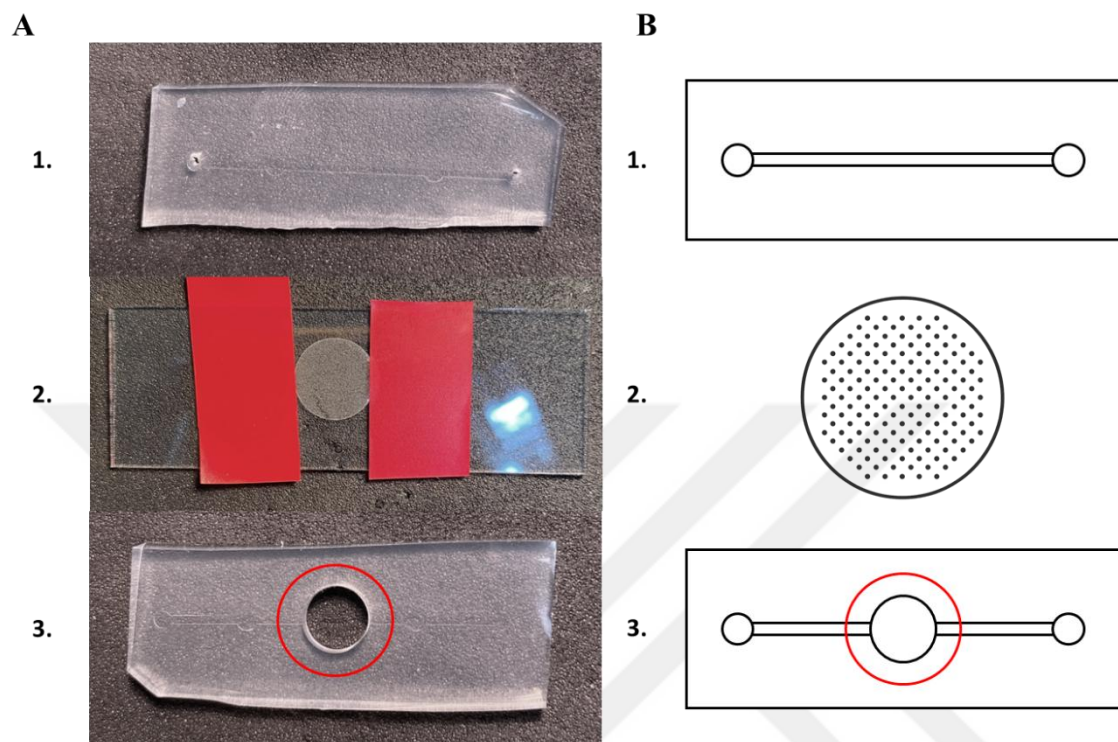


Figure 4. Barrier-on-chip platform, A: The fabricated layers are shown. B: Layers were demonstrated. 1. The upper layer with a straight channel, 2. PDMS porous membrane layer, 3. The second layer with the same straight channel, the red circle showing the punched region.

3.6 Cell Culture

In this project, we have used 3 cell lines to test the efficiency of our surface treatment. Human umbilical vein endothelial cells (HUVECs) were maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM-F12), supplemented with 1 mM L-Glutamine, 10% Fetal bovine serum (FBS), and 1% penicillin-streptomycin. MCF-7 cells were cultured in DMEM high glucose, 1 mM L-Glutamine medium supplemented with 10% FBS, and 1% penicillin-streptomycin. The plating of these 2 cells lines are carried out as follows: Cell stocks for MCF-7 and HUVECs were taken out of -80°C, and their vials were thawed in the water bath. After the vials are

melted, the cell suspensions were added to a falcon that contains 7 mL of prewarmed media. The falcon is centrifuged at 175 Rcf for 5 minutes, the supernatant that contains the freezing medium is discarded and the pellet is resuspended in 1 mL of the corresponding media of cell line. The cell suspension is then added to the 10-cm cell culture treated petri dish that has 10 mL of culture medium. The last cell line we used in our study is the Human-derived induced pluripotent stem cells (iPSCs). They were cultured in mTeSR Plus basal supplemented with 1% penicillin-streptomycin on Matrigel hESC-qualified matrix-coated well plates. Accutase cell detachment solution was utilized for dissociation of iPSCs into single cells before cell seeding. 10 μ M Y-27632 was added to the medium for a period of 24 hours after single cell culturing of iPSCs. All media are changed every 2 days, for iPSC medium without Y-component is used in the following days. All cell lines were kept in humanified cell culture incubators in 5% CO₂ and 37°C until they reach full confluency and are ready to be seeded onto PDMS.

3.6.1 Cell Culture in 96-well Plate

Cells are passaged and seeded to PDMS-covered 96-well plates after they have been cultured on petri dishes for 1 week. For HUVEC and MCF-7 cells, we prepared 5 conditions with 4 replicates each: 4 protocols of TMSPMA treatments and 1 untreated PDMS. Cells are seeded in the concentration of 5×10^3 cells/well and topped off with 200 μ L media. For iPSCs cells, we had the control case of Matrigel + PDMS and protocol 1 and 2 as the linker between PDMS and Matrigel. iPSCs are seeded to well to have 5×10^3 cells per well too.

From these experiments, we gathered information on the biocompatibility of treatment, the cell adhesion, viability and proliferation rate to conclude TMSPMA is not toxic to cells and can be used to treat the surface of PDMS.

3.6.2 Cell Proliferation Assay

To assess the proliferation rate of the cells, the Cell Counting Kit was utilized according to the manufacturer's instructions. After cells are seeded to 96-well plates, the proliferative ability of cells was quantified up to 4 days after culturing. For each cell line, one well was used from the replicates each day. We discarded the culture media from wells and added the WST-8 solution diluted with appropriate media, then cells were incubated at 37°C humanified cell culture incubator for approximately four hours. After incubation the clear solution turns to orange according to the cell population density. Then the solution is drawn and transferred into an empty well-plate where its optical density (OD) values of absorbance is measured at 460 nm using a microplate reader. All measurements were taken 3 times for good measures.

3.6.3 Cell Viability Assay

Again, the cells seeded to 96-well plates were stained for cell viability. This analysis was performed via live cell staining with Calcein-AM reagent. For this analysis, cells were incubated with 2 μ M Calcein-AM reagent for 15 minutes at 37°C, then they were washed with Phosphate- Buffered Saline (PBS). We visualized the stained cells under fluorescence microscope with the filter set for Alexa Fluor 488. The viable cells absorbed the stain to appear green.

3.6.4 Immunofluorescent Staining of iPSCs

In order to show that iPSCs were still portraying their pluripotent nature after culturing them on 96-well plates, Immunofluorescent staining of iPSCs was performed in collaboration with Assist. Prof. Dr. Burcu Özçimen at Hacettepe University, Stem Cell

Research and Application Center (PEDI-STEM) and our PhD student Servin Bagheralmoosavi. They were stained with Pluripotent Stem Cell 4-Marker Immunocytochemistry' kit. This kit contains antibodies specific to three pluripotency-related proteins, Oct3/4, SOX2 and TRA-1-60 specifically. First cell culture media is removed from the wells, and they were washed with PBS and fixed with 4% PFA (paraformaldehyde) for 20 minutes at room temperature, followed by a 5-minute PBS wash. To permeabilize the cells, they were incubated with 0.1% Triton X-100 in PBS for 45 minutes. After this step, the cells were incubated for 1 hour in blocking buffer (0.1% Triton-X and 5% FBS in 1X PBS) to prevent non-specific binding before antibody application. The primary antibody solutions (anti-OCT4, anti-SOX2, anti-TRA-1-60) were prepared in blocking buffer (1:200). After overnight incubation at +4°C, the cells were washed three times with wash solution (0.1% Triton-X in 1X PBS). This is followed with the wash step with PBS for 5 minutes three times. The secondary antibody incubation solutions were prepared freshly in blocking buffer, and incubated with Alexa Fluor™ 555 donkey anti-rabbit; for anti-OCT4 (1:250), Alexa Fluor™ 488 donkey anti-rat; for anti-SOX2 (1:250), and Alexa Fluor™ 594 goat anti-mouse IgM; for anti-TRA-1-60 (1:250) for 1 hour at dark at room temperature. After secondary antibody incubation, the cells were washed five times with wash solution and then incubated with DAPI (10 µg) nuclear stain for 5 minutes. A final PBS wash was done before fluorescence microscope analysis.

3.6.5 Static Culture in Microfluidic Chip

The first chip experiments were carried out in the fabricated chips as explained above in static conditions. This means that the active flow of media is not applied to the microchannels thus cells were not subjected to shear stress. The media flow is only facilitated by capillary forces (i.e. zero flow) from the reservoirs at inlet and outlet down to channels. After microchannel surfaces were treated with 4 protocols of TMSPMA-treatment, PBS flowed through the channels to wash and equilibrate the surfaces. Since the treatments were carried out outside of the sterile safety cabinet environment, it is crucial to sterilize the chip before introducing the cells. This is achieved by subjecting the

platform to high-intensity UV for 1 minute. Then cells were trypsinized and counted with hemocytometer. According to the total cell number, a final concentration of 1×10^7 cells/mL was prepared. Then the cell suspension is slowly infused into the channels, avoiding injecting air bubbles into the channels. In order to not sweep the cells before they attach to the surface, channels were then incubated at the incubator without supplementing media to the reservoirs. After waiting approximately four hours, cells can be visualized under light microscope to make sure they have settled down. If so, equal amounts of media were supplemented to the reservoirs. From that point, cells are checked under the microscope to monitor their condition and growth. The media of the reservoirs are replenished daily, and cells are cultured for a few days until full confluency is achieved.

3.6.6 F-actin/DAPI Staining

When cells cover the whole surface of microchannel, we stained them with F-actin/DAPI to visualize their healthy morphology. We start by removing the cell culture medium, then wells were washed with PBS and fixed with 4% paraformaldehyde (PFA) for 20 minutes. Then, to remove the remaining PFA a PBS wash is done and permeabilization solution is applied with 0.2% Triton X-100 for 5 minutes. After washing cells with PBS, they were incubated with 2.5% F-actin dye (Invitrogen, Thermo Fisher Scientific Inc. USA) in 1X PBS for 30 minutes at dark. After removal of F-actin dye, cells were washed with PBS and then incubated with 10 μ g DAPI for 10 minutes at dark. Solution was then removed, and cells were washed with PBS and analyzed under fluorescence microscope with appropriate wavelengths. All the steps were performed at room temperature.

3.6.7 Dynamic Culture in Microfluidic Chip

The second part of microfluidic chip experiments entails dynamic culture environment modelling. For this part, only HUVEC cell line was utilized, as these cells are part of the vasculature system in our body and subjected to shear stress from continuous blood flow. HUVECs were seeded to microchannels and cultured as we did for the static conditions, until they formed a confluent monolayer. Once the monolayer was established, dynamic culture experiments were initiated.

Before starting, two 1.5 mm tubings, for the inlet and outlet, and a small vial to collect the media from the outlet were sterilized with ethanol was and high-intensity UV exposure, after sterilization tubings were moved to safety cabinet. The necessary amount of media was calculated with the flow rates we plan to apply, and a 5 mL syringe to hold that media was selected and opened inside the safety cabinet. Lastly, syringe pump (New Era Pump Systems Inc., USA) was employed to introduce cell culture media from the syringe into the microchannel through the inlet. The diameter of the selected syringe was measured to enter the value to syringe pump, so the pump will automatically calculate how much force to apply to the syringe in order have the wanted flow rate. **Figure 5.** shows the laboratory setup used for the dynamic culture experiment. After all the preparations are made, the syringe pump was started at the lowest flow rate, in our case it is 50 $\mu\text{L/h}$. We waited until there was a droplet forming at the tip of the inlet syringe pump, after that we inserted the tube to the inlet. By doing this, we prevent the introduction of an air bubble into the channel. One side of the outlet tube is placed to the outlet opening of the channel and the other one is connected to the waste vial. This whole setup is prepared inside the safety cabinet to ensure sterility. After all the connections are made, we move the whole setup to the microscope. In the microscope's software we have prepared tiles, to take pictures of the whole channel throughout the process. Then we began our flow experiment, applying increasing flow rates from 50 $\mu\text{L/h}$ up to 2500 $\mu\text{L/hr}$. The shear stress applied on HUVECs is calculated with respect to channel dimensions, media viscosity and flow rate. We wanted to show physiologically relevant shear stress levels as cell covering the blood vessels would face.

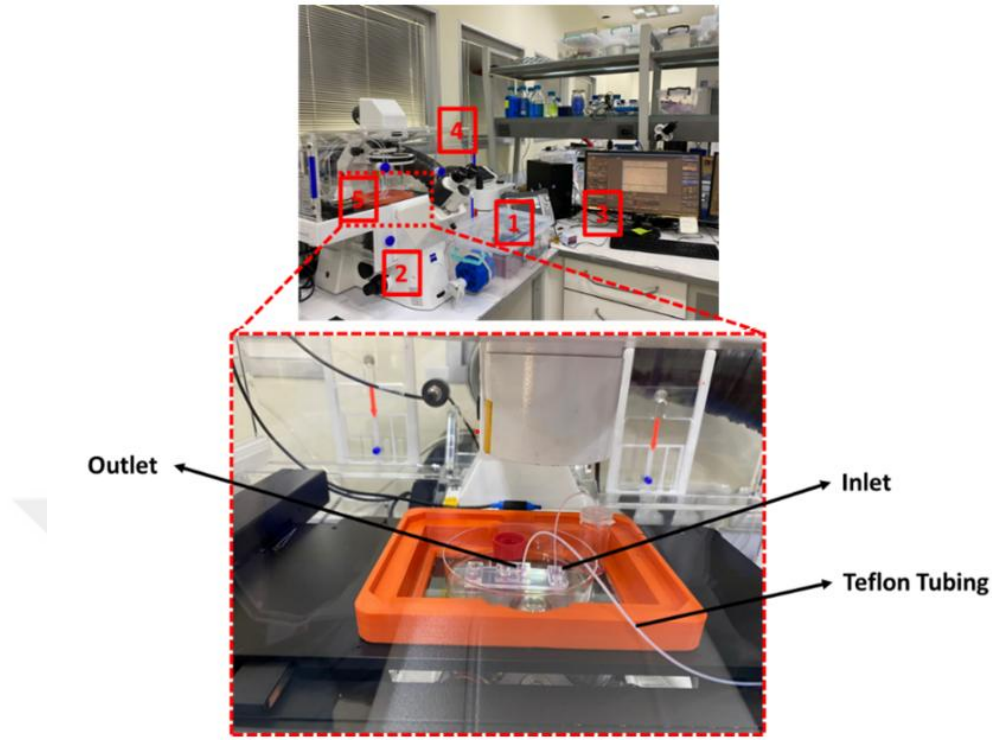


Figure 5. Experimental setup used for the dynamic culture experiments. 1. Syringe pump inside a custom-made incubation system. 2. Inverted light microscope. 3. Monitor connected to the CCD camera for visualization. 4. Incubation system for microscope.

3.6.8 Numerical Modelling

We also wanted to numerically model the dynamic environment we are creating inside the microchannels with the help of our PhD Faruk Aksoy. The Navier-Stokes equations, continuity and conservation of momentum equations (Equations **1.1** and **1.2**) are solved numerically to acquire the pressure, velocity, and shear stress profiles. It is assumed that the fluid (cell media - DMEM with 10% FBS at 37°C) has a dynamic viscosity of 0.930 mPa.s and a density of 1.009 g/cm³ [194]. This fluid is considered Newtonian and incompressible under a steady-state pressure-driven flow [195], [196], [197],

$$\nabla \cdot \mathbf{u} = 0 \quad (1.1)$$

$$\rho \left(\frac{\partial \mathbf{u}}{\partial t} + (\mathbf{u} \cdot \nabla) \mathbf{u} \right) = -\nabla p + \mu \nabla^2 \mathbf{u} \quad (1.2)$$

where ρ is the fluid density (kg/m³), $\mathbf{u}$ is the velocity vector (m/s), t is time (s), p is the pressure (Pa), and μ is the fluid viscosity (Pa.s). The boundary conditions for the walls of the microchannel are defined as no-slip. Initially, the velocity field and pressure in the channel are defined as 0 in the x and y directions at $t=0$. For the inlet boundary condition, a fully developed flow is prescribed in terms of a volumetric flow rate (m³/s) [198], [199]. The outlet is described as a suppressed backflow with 0 pascals. The equation governing the flow at the outlet is described as follows:

$$[-p\mathbf{I} + \mathbf{k}]\mathbf{n} = -\widehat{p}_o\mathbf{n}, \text{ and } \widehat{p}_o \leq p_o \quad (1.3)$$

Where $\mathbf{I}$ is the identity matrix, which ensures isotropic stress contribution from pressure, $\mathbf{k}$ represents the viscous stress tensor, accounting for internal fluid friction (shear stress), $\mathbf{n}$ is the normal vector at the boundary, perpendicular to the outlet surface, and p_o is the pressure at the outlet. The condition $\widehat{p}_o \leq p_o$ introduces a suppressed backflow constraint, meaning that the outlet is designed to prevent any reverse flow into the domain and maintain a pressure equal to or lower than the specified value (0 Pascals in this case) [198], [199]. The numerical analysis was carried out using the finite element software COMSOL Multiphysics (Burlington, MA, US)

CHAPTER 4

4. RESULTS

4.1 Surface Characterization Results

4.1.1 Scanning Electron Microscopy (SEM) Results

We started characterizing the treated PDMS surfaces with Scanning Electron Microscopy. In **Figure 6**, SEM images of all our samples have been presented. We have used bare untreated PDMS as our control group and the other samples are the 4 protocols we have utilized. The bare PDMS surface appears as smooth without any apparent structure or clumps found, which is consistent with the findings from literature. [200],[201] However, when we move onto the treated PDMS surfaces, we can see a repeating pattern on the surface is now introduced. In protocol 1 and 2, which included 1% and 0.5% TMSPMA concentration dissolved in acetic acid and deionized water, respectively. The low concentration of TMSPMA created mesh-like appearance on the PDMS surface for these 2 cases. They look very similar while protocol 1 is showing more protrusions that could be an indication of the slightly higher TMSPMA concentration. The others, protocol 3 and 4, is creating again very similar structures on the PDMS surface. This time the structures are more prominent with wavy patterns repeated. Protocol 4 showed the most pronounced wave patterns that look like a certain depth is introduced due to a thicker layer of TMSPMA. This is expected as Protocol 4 contains the highest concentration of TMSPMA with 5%. Aside from TMSPMA concentration effect, the solvents and temperature are also demonstrating differences in the surface morphology. For instance, between protocol 1 and protocol 3, TMSPMA concentration

does not change. Moreover, protocol 1 is applied on room temperature while protocol 3 is left on 80°C. These changes suggest that increased temperature may have facilitated extensive surface modification and cross-linking, resulting in a more textured surface. The choice of solvent did not directly affect the surface modification degree; it was just useful for decreasing reaction time.

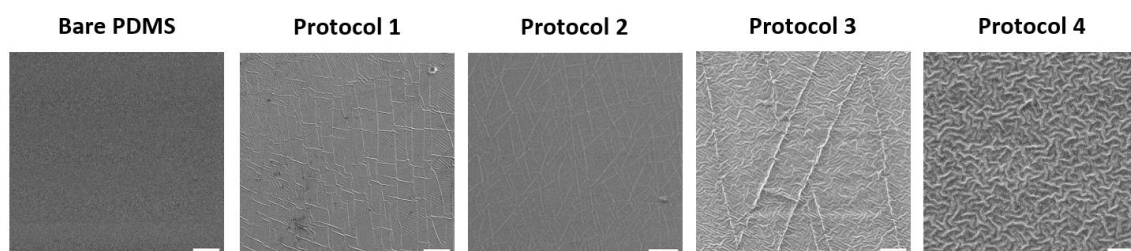


Figure 6. Scanning electron microscopy results of TMSPMA treated and untreated PDMS surfaces. Scale bars are 5 μm .

4.1.2 Contact Angle Results

Wettability is another important feature of PDMS we hope to achieve alterations in. We did our analyses on 5 samples this time as well. In **Figure 7A**, captured images of droplets on different surfaces are presented. Just by looking at these images, we can conclude the increase in the wettability property of PDMS after treatment. Droplet is staying on bare PDMS as if it is not touching the surface, portraying almost a perfect spherical shape. When the surface is hydrophobic, the contact angle between the water droplet interface and surface interface is expected to be above 90°. This is the case in bare PDMS sample, when the degrees are meticulously calculated, 112° is found. In the other samples, a reduction in the contact angle to below 90° for all TMSPMA-treated PDMS samples is achieved. This confirms the surfaces' transition to hydrophilic behavior compared to untreated PDMS. All the degrees of 5 sample wettability results are plotted in **Figure 7B**. The contact angle of untreated PDMS, which was initially 112°, significantly decreased to 78° following Protocol 1, 70° with Protocol 2, 74° with Protocol 3, and 77° with Protocol 4. These findings clearly support TMSPMA treatment as it is sufficient to enhance the hydrophilic properties of the PDMS surface. The

reduction in contact angles with almost a 40° decrease, is within desired range. Sometimes in other treatments this decrease is very little, resulting in a partial hydrophilic surface or rapid hydrophobic recovery. [125] Our findings are consistent with previous studies, a similar reduction in contact angle and improvement in surface hydrophilicity were observed following silane-based treatments on PDMS [202], [203].

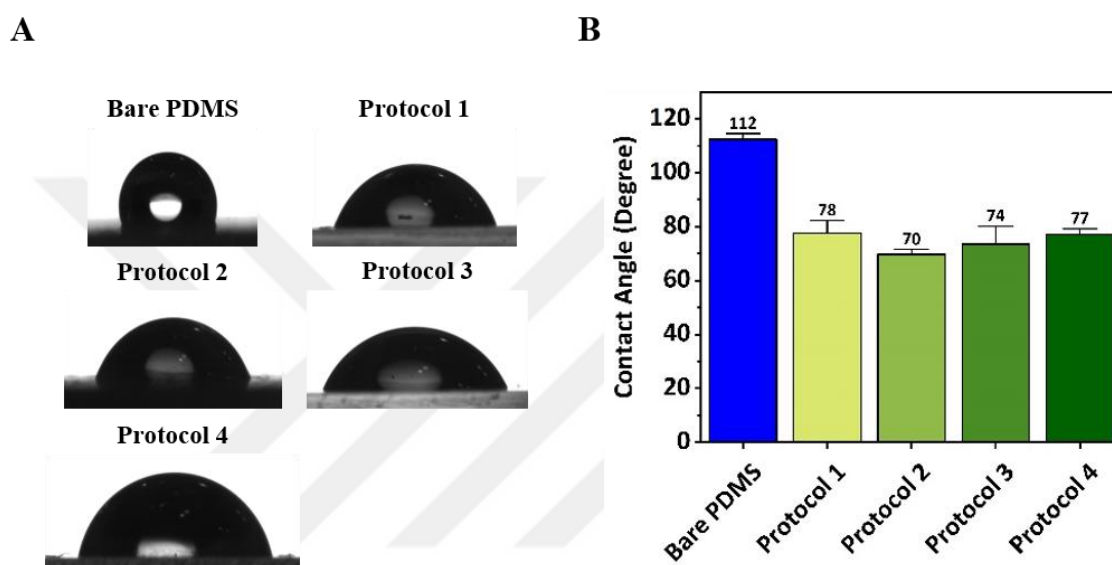


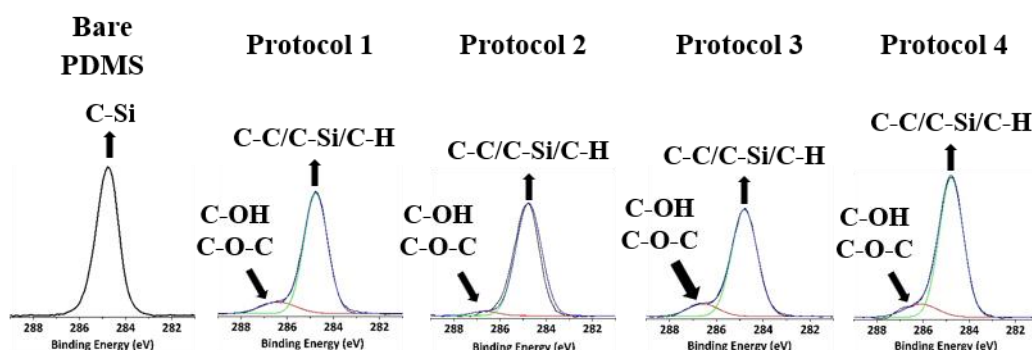
Figure 7. Contact angle results of TMSPMA treated and untreated PDMS surfaces. A: Droplet images, B: Bar graphs of contact angle degrees.

4.1.3 X-Ray Photoelectron Spectroscopy (XPS) Results

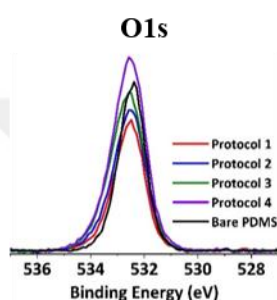
In order to precisely characterize the surface of PDMS, we need to understand the elemental composition of the treated surfaces. Through an XPS analysis, elemental composition and chemical states of the atoms were identified. The XPS analysis is scanned for the Carbon 1s, Oxygen 1s, and Silicone 2p elements for both the bare PDMS (control group) and four protocols, displayed in **Figure 8**. Chemical composition and bonds are known to give peaks in specific binding energy (B.E.) values and through this knowledge, we were able to pinpoint the chemical bonds TMSPMA treatment introduced to the PDMS surface. In **Figure 8A**, we are presenting the C1s spectra separately for each

sample because there are many different carbons found in the surface. Both treated and untreated PDMS surfaces exhibited a common peak between 284.0-285.0 eV B.E., which corresponds to C-Si bond. In PDMS, carbon and silicon atoms are present thus, this bond is coming from them. This observation aligns with previous studies reported in the literature [204]. Moreover, the TMSPMA-treated PDMS samples are deviating from the bare PDMS samples in some cases. TMSPMA introduced additional carbon atoms with peaks at around 284.78 eV and 286.28 eV corresponding to C-C/C-H and C-O bonds, respectively. We can see the rise in relative C1s elemental composition ratios, as TMSPMA have C elements new different carbon bonds regarding the concentration of TMSPMA are introduced. The higher the concentration of TMSPMA solutions, the greater the number of C-based bonds are obtained. The highest C1s peaks and compositional ratios were got from protocol 4, as expected. The second highest carbon bond is achieved from Protocol 1. The effect of TMSPMA concentration played a role in this ratio, via that we get a higher amount of Carbon than protocol 2. However, Protocol 1 also displayed a higher carbon ratio than protocol 3, which has the same TMSPMA amount as it. This increase can be the result of the addition of acetic acid, bringing additional carbons from its chemical composition.

A



B



C

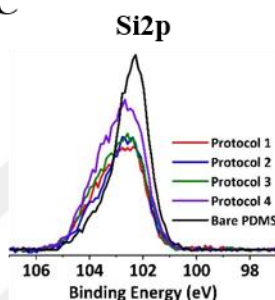


Figure 8. X-ray photoelectron spectroscopy results of C1s, O1s, and Si2p scans of the TMSPPMA-treated and untreated (bare) PDMS surfaces.

Moving onto the O1s spectra, after TMSPPMA treatment, a distinct peak is observed at approximately 532.0 – 533.0 eV, which corresponds to overlapping contributions from C–O, C=O, and Si–O bonds. Similarly, the Si2p spectra of all samples showed a peak centered around 102–103 eV contributing to Si–O bonds as well. In Si2p spectra of bare PDMS, we have seen the largest peak for Si–O. This could be due to all Silicon atoms resting free in the polymer matrix. While the nature of TMSPPMA explained, the possible interaction mechanism was also introduced. The interaction of TMSPPMA with the PDMS surface is facilitated by oxygen plasma. After plasma treatment the PDMS surface acquires OH- radical groups that are bonded to Silicon atoms. When TMSPPMA is introduced to the surface, this OH- is replaced by Si–O coming from the chemical. This may lead to a decrease in the free Si–O bonds of PDMS. After treatment, the intensity of the Si–O peak decreased across all protocols indicating a change in the surface chemistry following TMSPPMA treatment. Specifically, protocol 4 (5% TMSPPMA concentration, the highest) showed the greatest intensity among the treated samples, which is correlated with the high number of TMSPPMA being introduced, thus high number of new Si–O bonds. Whereas protocols 1, 2, and 3 (0.5%, 1%, and 1% TMSPPMA concentration, respectively) showed relatively similar peak intensities. The

overall trend indicates that TMSPMA treatments reduce the surface Si–O bonding detected by XPS, due to the formation of a surface layer involving the silicon from the TMSPMA. From these analyses, we were able to investigate the new chemical groups introduced to the surface and how native PDMS were changed with treatment.

4.1.4 Atomic Force Microscopy (AFM) Results

Lastly, the surface topography and roughness were examined with AFM to show the difference between bare and treated surfaces. In **Figure 9**, produced topographic images are displayed both with upper and 3D view. For the bare PDMS, AFM images showed noisy surface morphology without any pattern visible, like our SEM results. AFM provided us with a deeper insight into the surface modifications, showing the height of surface structure or the roughness values we could not understand from SEM visualization. After protocol 1 treatment, the surface of PDMS becomes smoother. In bare PDMS, the protrusions reach up to 11.4 nm whereas in protocol 1 it is decreased to 4.18 nm, supporting a more uniform surface. The same case is observed in the case of protocol 2 as well, with its topography peak reaching 3.25 nm at max. These results are in line with the root mean square (RMS) roughness values that indicate the surface roughness values as well as average surface height variations. Values are shown in **Table 3**.

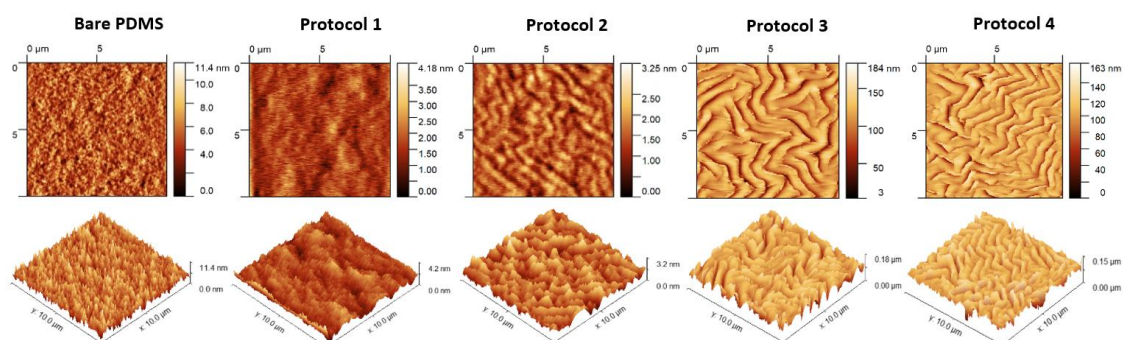


Figure 9. Atomic Force Microscopy results of the TMSPMA-treated and untreated PDMS surfaces.

When we check the topography images of protocol 3 and 4, we observe a wavy and labyrinth-like structure, which could indicate surface strain or a phase separation during treatment, seen in both SEM and AFM. As for the roughness of the surface, we are seeing very elevated numbers for the highest region on the surface, with 184 nm and 163 nm. The roughness of the surface is supported by both the topographical section and RMS values. The difference of roughness between protocol 1 and protocol 3 suggests the involvement of parameters other than TMSPMA concentration. It may be due to acetic acid addition, which would achieve more efficient hydrolysis of TMSPMA that resulted in a homogenous and smooth surface coating.

Table 3. Root mean square (RMS) roughness measurements from AFM.

Protocol	Bare PDMS	Protocol 1	Protocol 2	Protocol 3	Protocol 4
RMS (nm)	1.406 ± 0.1406 nm	0.3471 ± 0.066 nm	0.3911 ± 0.062 nm	21.33 ± 2.822 nm	16.03 ± 2.75 nm

4.2 Cell Culture Analysis

4.2.1 Biocompatibility Studies in 96-well Plate

Our cell studies started with PDMS-covered TMSPMA-treated 96-well plates. Starting with our control group, all 3 cell lines, HUVEC, MCF-7 and iPSC, were seeded on bare PDMS. **(Figure 10.)** No cell adhesion or viability was observed from these experiments. Cells stayed in their circular clump shapes and did not portray their healthy morphology after 24 hours. This finding is consistent with previous reports in the literature. [205], [206] This phenomenon is due to the hydrophobic nature of the PDMS. PDMS's hydrophobicity causes it to absorb small soluble biomolecules such as proteins present in the cell culture medium or secreted by the cells, which can lead to protein denaturation and depletion. Therefore, the lack of proteins and biomolecules can disrupt the specific signaling mechanisms required for proper cell adhesion and subsequent cellular functions. [183]

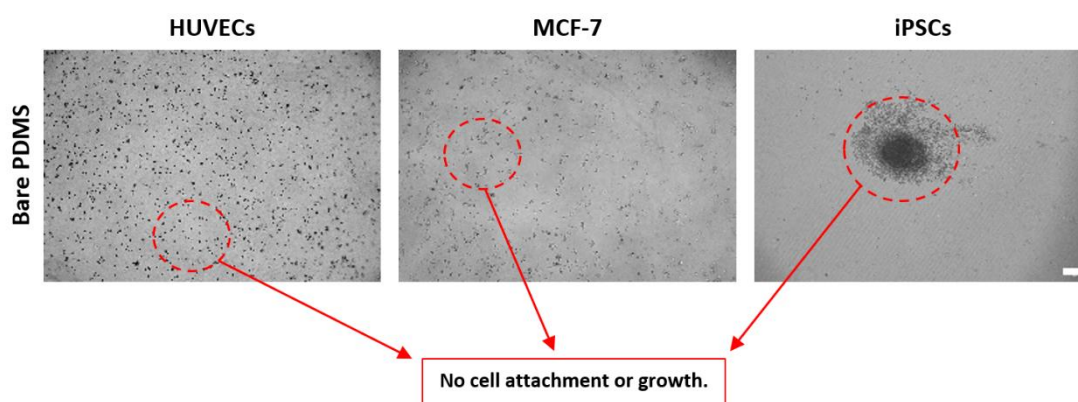


Figure 10. Brightfield images of cells on the bare PDMS surface 24 hours after cell cultivation.

Then, 4 TMSPMA protocols were utilized to treat the PDMS surfaces and subsequently HUVEC and MCF-7 cells were seeded directly on top of the TMSPMA treatment. Both HUVECs and MCF-7 cells showed proper adhesion, distribution and spreading on the PDMS surfaces treated according to protocols 1, 2, and 3 presented in **Figure 11**. Additionally, approximately 48 hours after cell growth, we have analyzed the cell viability by live cell staining. The results revealed high cell viability for both HUVECs and MCF-7 on the surfaces treated with protocols 1, 2, and 3 as depicted in **Figures 11**, respectively. This underscores the biocompatibility of our protocols, indicating their suitability and effectiveness for use in biological applications. Surface roughness is a well-known factor influencing cell adhesion, as documented in the literature [184], [185], [186]. However, in our study, while two of our protocols (protocols 1 and 2) resulted in smoother surfaces and one (protocol 3) resulted in a rougher surface compared to bare PDMS, cells adhered to all these surfaces, nonetheless. This suggests that surface roughness is not the sole reason behind successful cell adhesion, instead, surface chemistry, hydrophilicity, and protein adsorption may also play significant roles. Even with variations in surface roughness, if the surface chemistry and hydrophilicity are favorable, cells can still attach effectively. Thus, the successful cell attachment observed with our protocols indicates that these treatments effectively modified the PDMS surface to support cell adhesion. On the other hand, even though surface characterization results

of protocol 4 with 5% TMSPMA yielded similar results particularly to protocol 3, cells did not adhere to the surface (data not shown). This points only one parameter that is completely different from other protocols to be the reason behind this failure, the TMSPMA concentration. It is known that our protocols did not use pure TMSPMA as it is essentially a chemical that would be hazardous to cell lines. Therefore, the lack of adhesion on these surfaces can be explained by elevated cytotoxicity associated with higher concentrations of TMSPMA. This observation highlights the importance of TMSPMA concentration to ensure biocompatibility and proper cell adhesion with minimal cell toxicity. Overall, while surface roughness, surface chemistry, and hydrophilicity are important factors influencing cell adhesion, none of these factors alone can guarantee successful cell attachment. Instead, it is the synergistic interaction between these factors that collectively determines the effectiveness of cell attachment. Therefore, an optimal combination of these elements must be achieved to promote successful cell adhesion, highlighting the need for a balanced approach. In the following parts, protocol 4 will not be mentioned as it did not pass the biocompatibility test of our treatment.

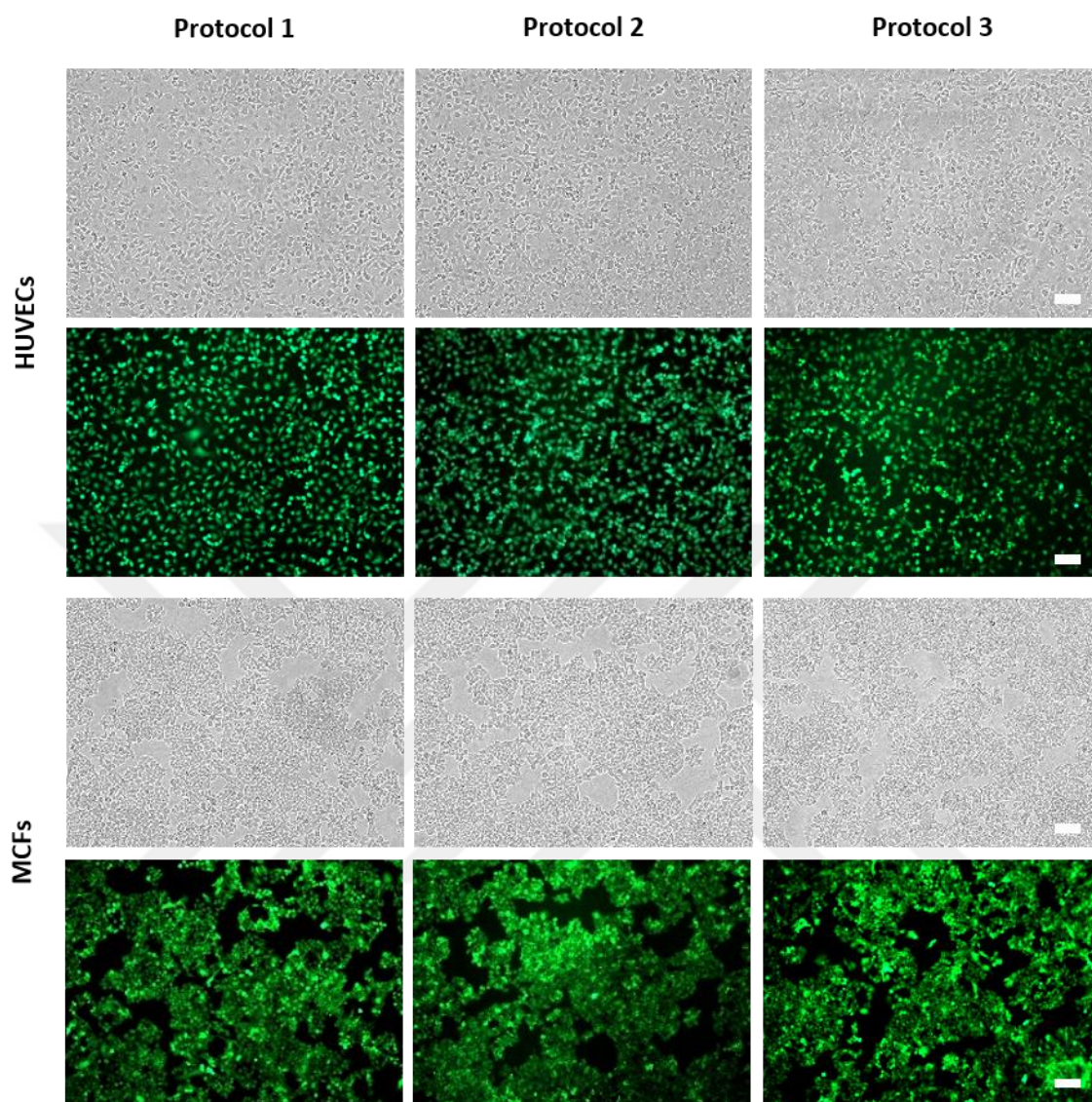


Figure 11. Brightfield and fluorescence images showing cell adhesion and viability of HUVECs and MCF-7 on the surfaces of the TMSPMA-coated PDMS based on protocols 1, 2, and 3.

To culture iPSCs in vitro, specific requirements needed to be met to maintain cell viability and proliferation in their undifferentiated state. Cell behavior is shaped according to their environment thus in stem cell research a specific matrix protein rich organic mixture that is called Matrigel. [207] These requirements led us to wonder if TMSPMA can enhance the binding of Matrigel to PDMS, working as a linker. As explained before, TMSPMA has dual-reactivity nature, one side of its chain can reach

with organic substances where the other can interact with inorganic ones. Utilizing its distinct property, we wanted to enhance Matrigel binding, because it is prominent that linking an ECM protein-based treatment to PDMS surface is a challenge and sometimes patchy attachments or detaching occurs.[104], [146], [153] We first checked if similar to ECM proteins, Matrigel is also able to successfully bind to PDMS surface and continue to support iPSCs adhesion and viability. We coated the surface of PDMS with Matrigel, seeded iPSCs and followed their state for 5 days. Although the cells were initially able to attach to the surface on day 1, they were unable to proliferate and maintain their healthy morphology starting from day 3 and at day 5 they were not viable anymore as shown in **Figure 12**. This proved our concerns of challenges in culturing stem cells in PDMS platforms. Therefore, we carried out our “linker” experiments with protocol 1 and 2. PDMS surface modifications by TMSPMA using protocol 1 and 2 could properly support the Matrigel coating and cell attachment for longer culture periods as shown. Protocol 3 did not allow the binding of Matrigel successfully; thus, cells were not viable in this case (data not shown). We hypothesized that the reason for failure of protocol 3 is most likely due to the heterogenous and uneven coating of TMSPMA on the surface (represented by SEM and AFM results) which prevented effective binding of Matrigel to the surface and hindered proper cell binding. Upon Matrigel coating on PDMS surfaces treated with protocol 1 and 2, iPSCs were successfully able to generate huge colonies approximately 5 days after single cell seeding. These results demonstrate that this environment not only supported the initial iPSCs attachment but also maintained their viability, growth, and proliferation.

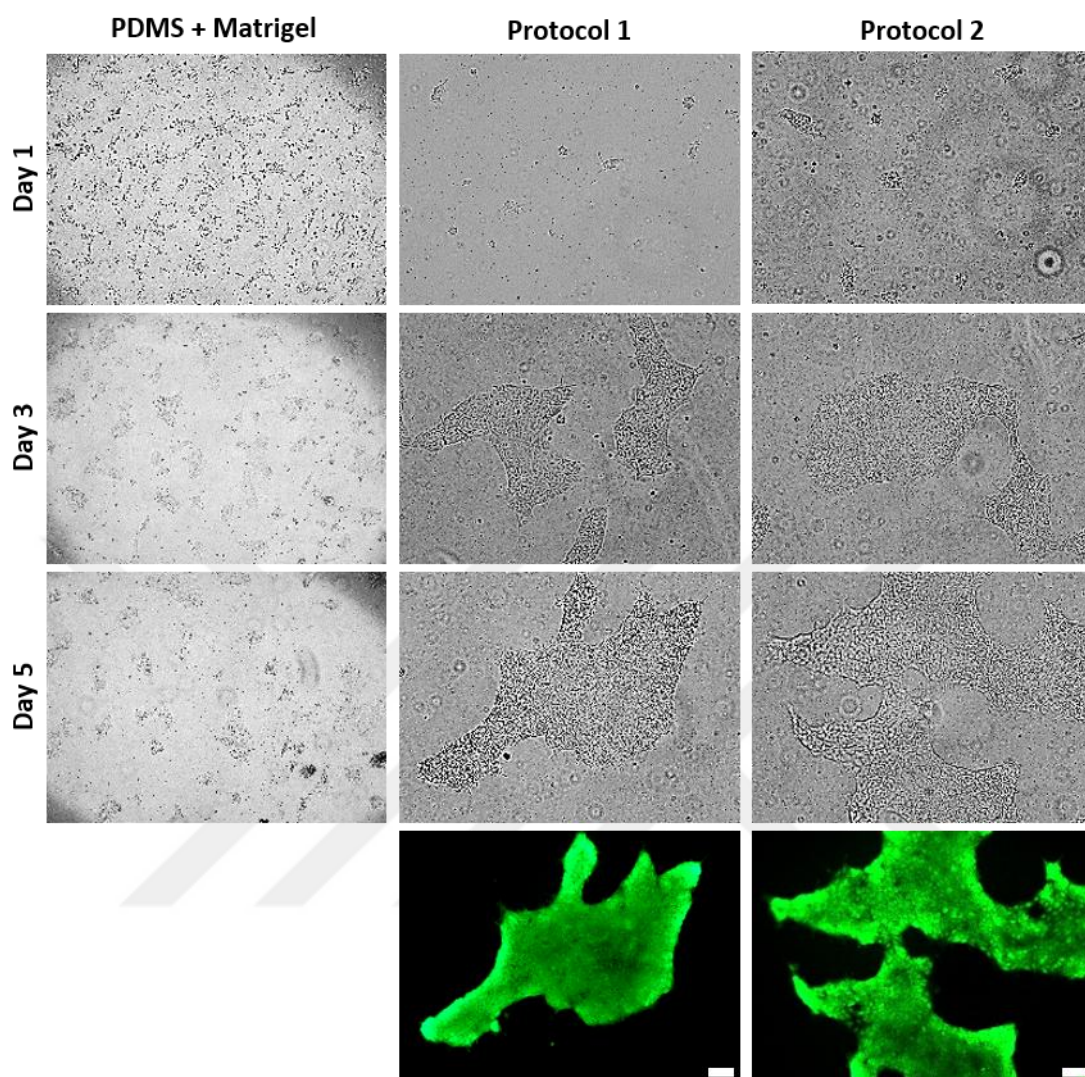


Figure 12. Brightfield images cells on the Matrigel-coated bare PDMS surface, adhesion, viability, and colony formation of iPSCs from single cell on the TMSPMA-treated Matrigel-coated PDMS surfaces based on Protocol 1 and Protocol 2.

4.2.2 Cell Proliferation Results

Up until now, we have evaluated the biocompatibility of TMSPMA-treated PDMS surfaces. We had very promising results; therefore, we wanted to quantify the proliferation rate of cell cultured on these surfaces. We were able to observe the expanding and multiplying of cells under microscope but quantifying it will allow us to compare it with the previously reported cases. We have performed cell proliferation

analysis using WST-8 assay for 4 days after cultivation to show cells were active after long period of culturing times. All the cell lines' absorbance results are given in **Figure 13**.

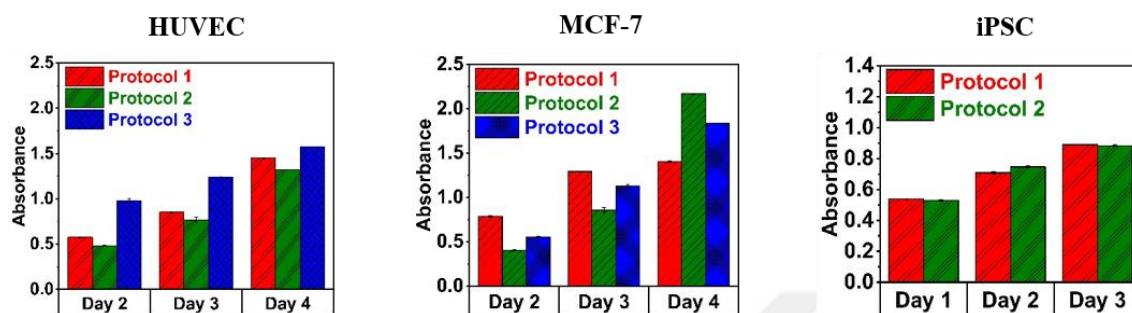


Figure 13. Cell proliferation rate 3-4 days after cell cultivation on TMSPMA coated PDMS surfaces.

HUVECs have reached max 1.5 absorbance value in day 4 when they started at 0.5 in day 2, tripled their cell number in 2 days. (Figure 12A.) Among the 3 protocols, there is not much change in their increment, for protocol 2, for instance the starting cell number was low, therefore at the end the least cell number was still in this protocol. Moreover, in the case of MCF-7, the increase of cell number is higher than HUVECs. (Figure 12B.) This time 4 times increase is observed in all protocols; high rate of proliferation can be explained due to its cancerous nature. The only abnormal result is observed in protocol 2 at day 4, when the starting number of cells were the least but increased over 4 times in 2 days. This can be because of cancer nature or the used wells were not very similar in cell count initially. Lastly, iPSCs showed the slowest proliferation rate among all cell lines, but it was consistent within all the protocols. So, it is safe to say, protocol differences did not affect the rate of growth. From these results we concluded that the chemical modification of the PDMS surfaces not only enables initial cell attachment but also supports the cellular functions which are essential for cell proliferation and growth in long-term cultivation.

4.2.3 Immunofluorescent Staining Results

Nonetheless, maintaining stem cells in an undifferentiated state is crucial for preserving their ability to self-renew and differentiate when needed. To ensure that the TMSPMA-treated PDMS surfaces did not induce any unwanted differentiation, we assessed the pluripotency state of iPSCs by evaluating the normal expression of stem cell markers octamer-binding transcription factor 4 (OCT4), (sex determining region Y)-box 2 (SOX2), and tumor-rejection antigen (TRA-1-60) upon culturing on the TMSPMA-treated PDMS surfaces based on protocols 1 and 2 as illustrated in **Figures 14A and B**, respectively. Our analysis revealed normal expression levels of these markers, indicating that the cells maintained their undifferentiated state. This suggests that the TMSPMA treatment created a surface environment that supports stem cell maintenance without affecting their fundamental characteristics.

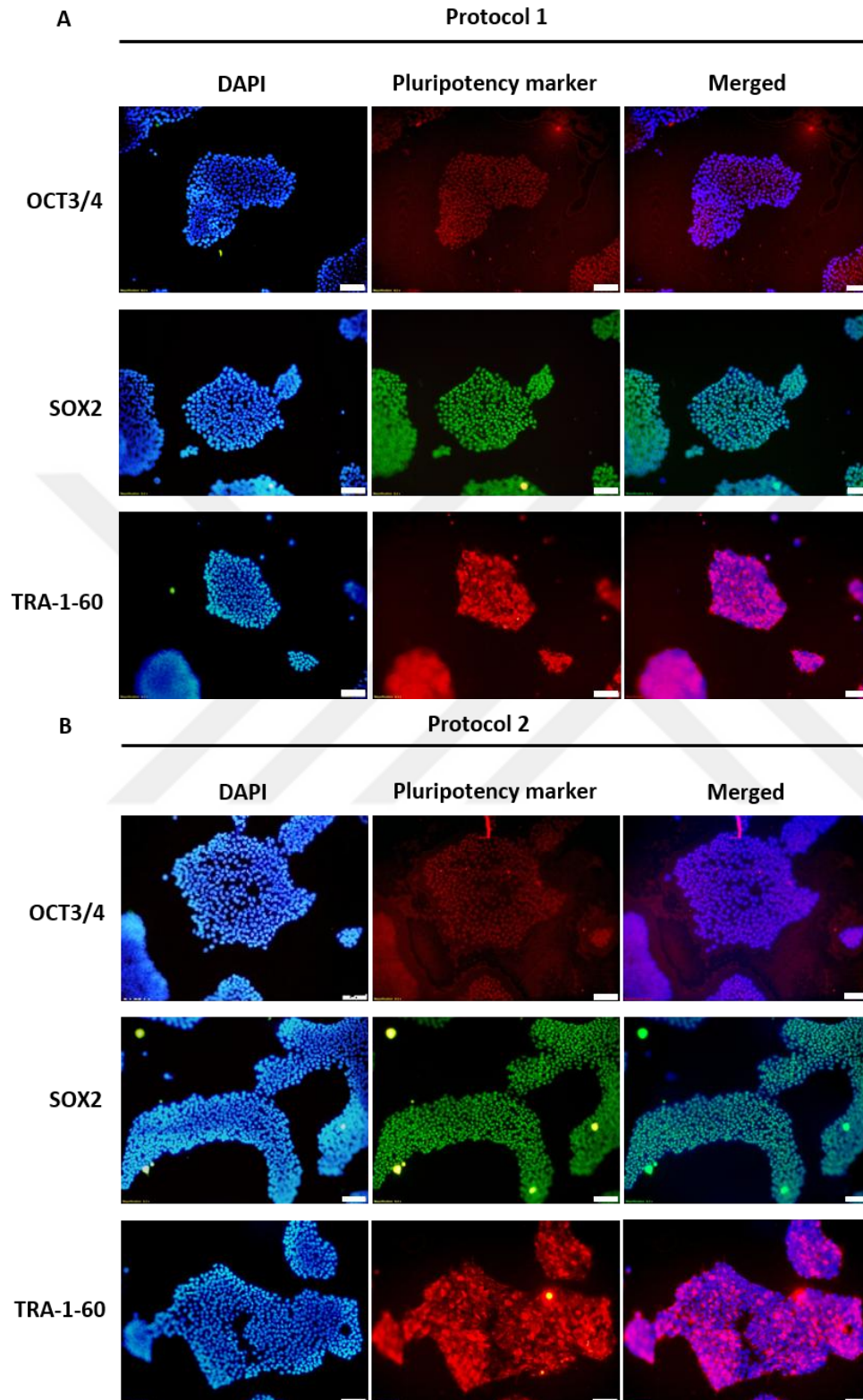


Figure 14. Detection of pluripotency-related protein expressions in iPSCs cultured on TMSPMA-treated PDMS surfaces according to A: Protocol 1 and B: Protocol 2. Scale bars are 100 μm .

4.2.4 Static Culture in Microchannels

After confirming that TMSPMA treatment is indeed biocompatible, not only does not harm the cells, but also promotes cell adhesion and proliferation, we moved our analysis to microfluidic platforms. As our primary goal to explore the use of TMSPMA for organ-on-chip applications, the stability and behavior of cells in a more complex environment that is the microfluidic channels carried utmost importance. For the assessment of on-chip culturing, we used a relatively simple channel geometry with a single layered straight channel, as the purpose of our microfluidic platform is to show the viability and proliferation of our cultured cells. The employed microfluidic platform design is shown in **Figure 3**.

Our investigations were done on the same experimental groups as we did on 96-well plates. We used HUVEC and MCF-7 cells to test the efficiency of TMSPMA treatments of Protocols 1-3, the negative control for this group was bare (untreated) PDMS. For the iPSC, we used TMSPMA treatment of Protocol 1 and 2 and applied Matrigel on top of these treatments to show the linker efficiency of TMSPMA. The negative and Matrigel control for this group is bare PDMS without any treatment and Matrigel coating applied on the PDMS, respectively.

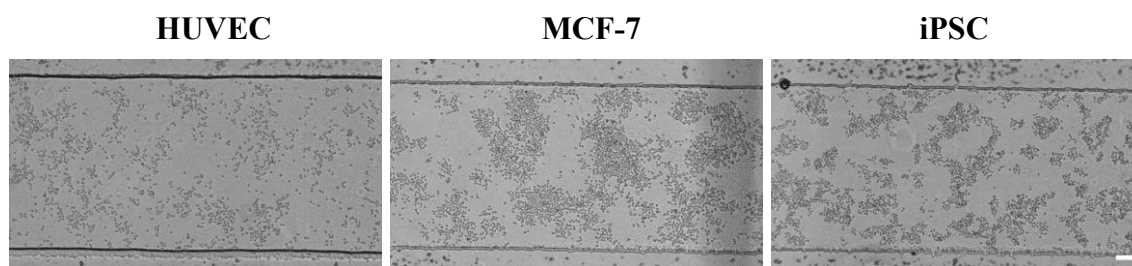


Figure 15. Brightfield images of cells on the surface of bare PDMS 24 hours of cell cultivation on microfluidic channels. Scale bars are 200 μm .

The morphology of cells changes evidently after they form a healthy attachment to the channel surface. In our negative control groups, where we seed all our cell types on top of bare PDMS, cells appeared rounded and in clumps as supported with the brightfield images in **Figure 15**. The images are taken 24 hours after seeding and from their morphology we can deduce that they were unable to attach to the bare PDMS thus,

not viable anymore. [208] Moreover, the unattached cells were mobile and were flowing due to the media flow across the channels. However, after approximately 4 hours, cells can settle down, attach to the channel surface and display their morphology. After this incubation period of 4 hours, we add the media to the reservoirs. We add equal amounts of them to create minimal flow inside the channel. From there on the media was changed daily and we have followed the proliferation and coverage of cells through microscope imaging.

In **Figure 16.** the brightfield images of HUVEC are presented for all 3 tested protocols. In each protocol, the channel image of culture time of 24 hours and 96 hours were shown to understand the increase in the confluency of cells. After they created a good monolayer on the channel surface, we have fixed the cells to stain them with F-actin/DAPI. The staining results show the healthy morphology HUVECs have and the empty areas in the channel. In the brightfield images, the channel looked almost fully confluent but by increasing the contrast through staining we were able to interpret the growth in the channels as well. Finally, the brightfield images as well as the F-actin/DAPI staining images showed no notable differences to support the interchangeable use of our proposed protocols for the same purposes.

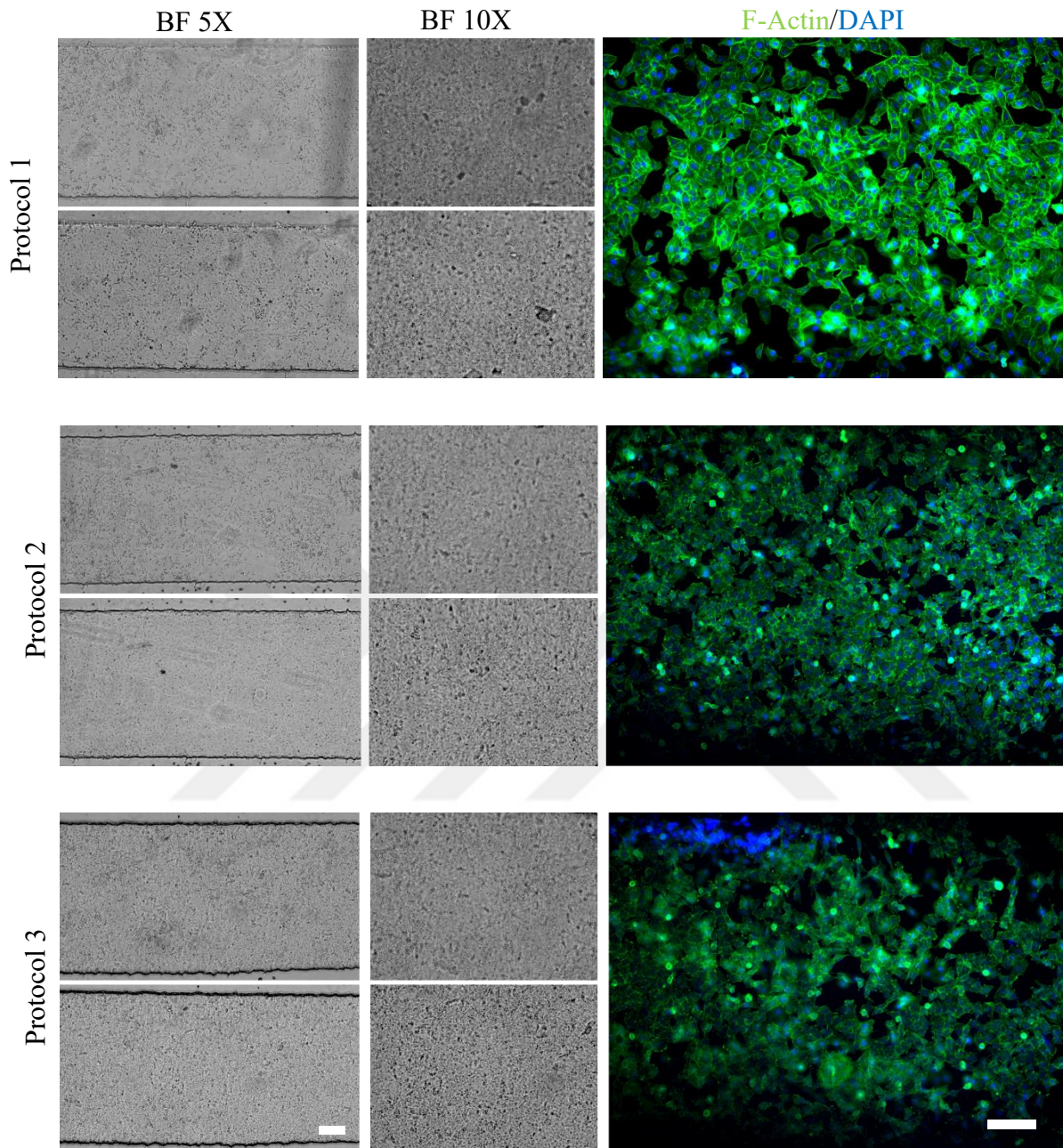


Figure 16. The cell attachment and proliferation on the PDMS-based TMSPMA-coated microfluidic channel shown in brightfield and fluorescent images of HUVEC (Green: cell cytoskeleton, Blue: cell nuclei). PR: Protocol. Scale bars are 200 μm .

For MCF-7, similar investigations are carried out with daily media refreshments and brightfield images until 96 hours have passed for on-chip culture (**Figure 17**). Cells were proliferating and covering the surface as well without any problems. However, MCF-7s have portrayed a slower growth profile compared to HUVECs when we compare the daily microscope images. At the end of our investigations, after 96 hours, we could

see some empty areas larger than what we observed in HUVECs case. Our observations were verified when we stained the cells after 96 hours. The fluorescent images showed more evident blank spots in the channel. This can be due to the different origins of the cell lines where HUVEC cells originate from the inner linings of umbilical cords where they are constantly subjected to blood flow, therefore shear stress.

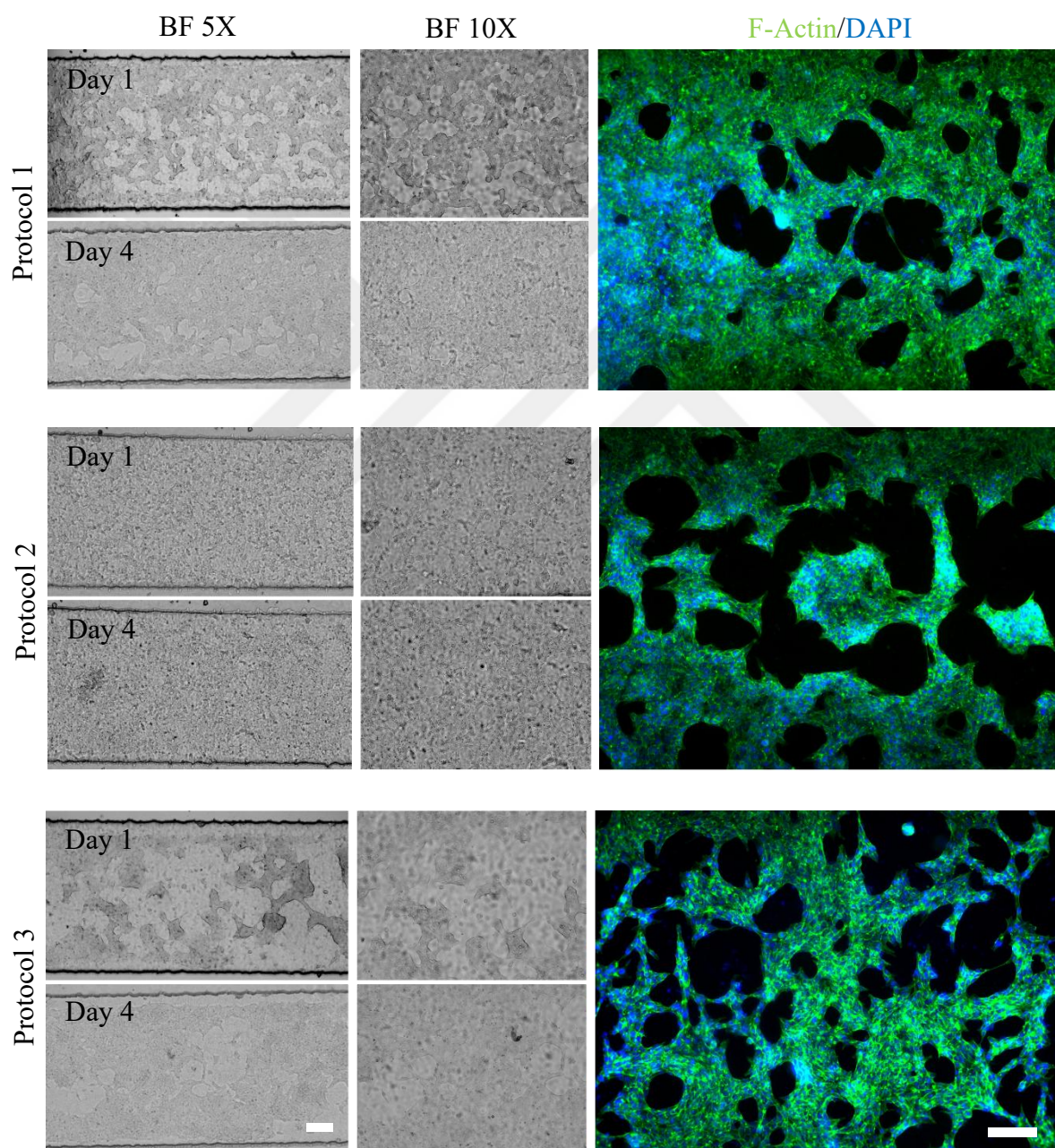


Figure 17. The cell attachment and proliferation on the PDMS-based TMSPMA-coated microfluidic channel shown in brightfield and fluorescent images of MCF-7 (Green: cell cytoskeleton, Blue: cell nuclei). PR: Protocol. Scale bars are 200 μm .

On the other hand, MCF-7 breast cancer cells grow in clusters or clumps that do not face flow in the body, so the microfluidic channel does not provide them with the physiologically relevant environment. Moreover, the geometry of the microfluidic channel also creates a similar environment for HUVECs to proliferate easier than MCF7.

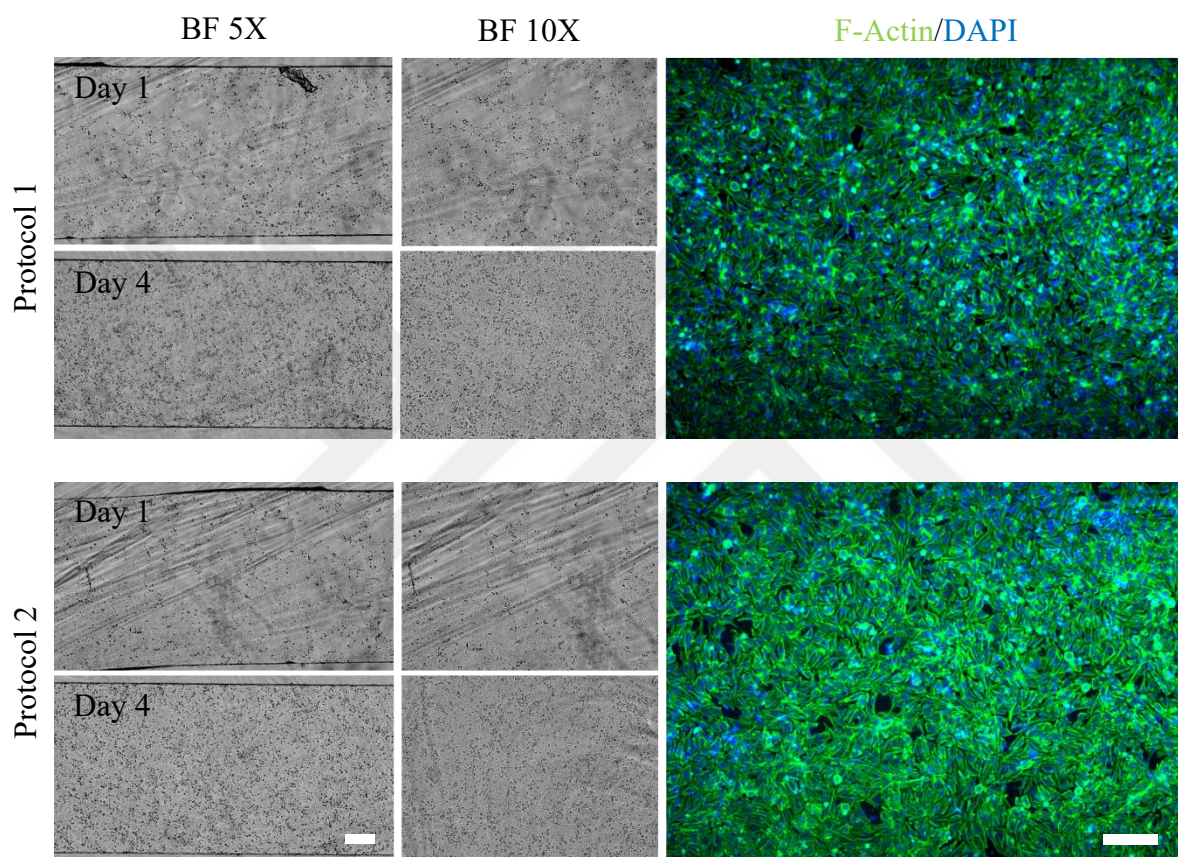


Figure 18. The cell attachment and proliferation on the PDMS-based TMSPMA-coated microfluidic channel shown in brightfield and fluorescent images of iPSC (Green: cell cytoskeleton, Blue: cell nuclei). PR: Protocol. Scale bars are 200 μm .

Lastly, we have investigated the behavior of iPSC cultured inside microfluidic chips. The findings of our negative control with bare PDMS have been discussed for all cell lines. Additionally, we have a Matrigel control group, where we wanted to see how stable Matrigel binds to bare PDMS to understand the need for a linker molecule. As we suspected, Matrigel on top of PDMS is not enough to support the growth of iPSCs. Similar outcome to the 96-well case, cells attached to the Matrigel but after time has passed, cells have become non-viable. (data not shown) This points to the need for a linker layer to

enhance the stability of Matrigel. After our controls, the PDMS channels were treated with TMSPMA and subsequently coated with a layer of Matrigel with Protocols 1 and 2. We did not apply Protocol 3 as we did not get any positive result from the 96-well studies.

Protocol 1 and 2 yielded successful results, showing stem cells were able to attach to the channel surface and proliferated healthily to form a monolayer in 4 days. In **Figure 18**, the brightfield images are shown as well as the cytoskeleton and nuclei staining images. The coverage of stem cells was the highest among the other cell types, the surface was almost fully covered. From the staining images, the coverage and the morphologies of the stem cells appear to be same, showing no significant difference among the different protocols.

Up until this point in our investigation, we have only discussed the cell coverage from the brightfield and fluorescent images. To further strengthen the success of our protocols, we wanted to show a quantitative analysis of cell growth inside the microfluidic channels. (**Figure 19.**) As the proliferation assay done within the 96-well culture requires the solution to be collected after incubation, we could not pursue the same assay inside microfluidic chips. Therefore, we chose an indirect analysis to understand the cell coverage.

This was assessed by comparing images taken 24 hours and 96 hours after initiating the static culture. For HUVECs, protocol 1 began with cells occupying 23.30% of the microchannel surface and nearly tripled to cover 63.55% of the microchannel area after 96 hours. Protocol 2 exhibits a comparable growth pattern, with an increase from 21.98% to 63.11%. Finally, in protocol 3, the initial coverage after 24 hours was 36.95% and it increased to 66.75% after 96 hours, indicating a twofold growth rate with a comparatively slower rate of proliferation. Upon examining the cell coverage of MCF-7 cells, it was seen that it duplicated in microchannels treated with protocols 1 and 2 within a span of 96 hours. However, the microchannel treated with protocol 3 exhibited a slightly greater rate of cell proliferation compared to the other protocols. Moreover, iPSCs cultivated in the microchannels treated with protocol 1 exhibited a 2.3-fold increase in their cell number over a span of 3 days, whereas that of protocol 2 resulted in a doubling of cell number during the same timeframe. The proliferation rate of all cell lines shown previously in **Figure 13**. revealed similar profiles as compared to the coverage analysis results on microfluidic channels. This indicates that the proliferation rate remains rather

stable even in a microfluidic channel setting, and the cells were able to multiply as they would in a 2D culture environment. Moreover, coverage analysis validated our claim that the protocols can be used interchangeably, as it showed no significant difference between the growth profiles.

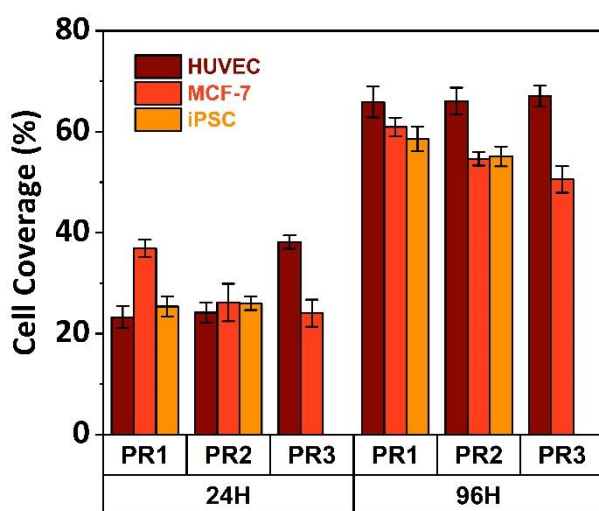


Figure 19. Cell coverage analysis for the cells cultured on PDMS-based TMSPMA-coated microfluidic channel. PR: Protocol.

4.2.5 Dynamic Culture in Microchannels

Additionally, to show the robust cell attachment achieved due to our treatment and to replicate the natural environment of our body where fluid flow plays a crucial role in some of the physiological phenomena, such as nutrient and oxygen delivery, waste removal, and shear stress-mediated endothelial function, we introduced flow conditions. As we previously done our biocompatibility and static condition analysis with multiple cell lines, we wondered if we can continue the dynamic condition analysis with the same cell lines. However, as shear stress induces novel expressions on the affected cells, it would be hard to maintain the pluripotency of our iPSCs and they would most certainly differentiate into primary germ layers; endoderm, mesoderm and ectoderm. As for the MCF-7 cells, physiologically, breast cancer cells are not subjected to flow, therefore they would not endure the shear stress effects successfully. Thus, we removed iPSC and MCF-

7 cell lines from this assay, as these cell types are not naturally exposed to fluid flow in the body and lack the physiological adaptations to withstand shear stress. Including them in this flow-based setup would likely result in poor viability or non-representative responses, making their use in this context irrelevant. In contrast, HUVECs can be included in this assay because they are endothelial cells that naturally reside in blood vessels and are constantly exposed to shear stress from fluid flow. Their physiological role involves sensing and responding to flow, making them highly relevant and well-suited for modeling flow-based environments.

We have started our experiments as we would do in the static conditions, with cell seeding and daily media change. Until the cells have reached approximately full confluency, we did not introduce any flow to the microfluidic channels and cultured them statically. After the desired coverage is obtained, we initiated the experiment with a low flow rate of 50 $\mu\text{L/h}$ for a duration of 1h and monitored the dynamics under microscope. Then the flow rates were increased gradually to 250, 500, 1000, 1200, 1400, 1600, 1800, 2000 and 2500 $\mu\text{L/h}$ with each flow rate being applied for 1 hour ensuring that the flow rate is stabilized and shear stress effects on cells are prominent.

Upon the exposure at every flow rate, the brightfield image of whole channel is taken for direct comparison of cell coverage among different flow rates. For simplicity, only the images taken after 1 hour of 50 $\mu\text{L/h}$ and 2500 $\mu\text{L/h}$ is shown for all 3 TMSPPMA protocols is shown in **Figure 20**. The experiments were initiated with estimations of the wall shear stress in vivo demonstrated that cells tolerate a range of shear stress at different locations. Typically, cells experience shear stress ranging from less than 1 to 10 dynes/cm² in large veins. [209], [210], [211] In our experiments, the shear rate reached up to 1.5 dyne/cm², which is within an acceptable range for simulating conditions in large blood vessels. Despite this high-moderate shear rate, the cells successfully resisted the applied shear rate and maintained their monolayer for over an hour. This result can be seen in all the protocols which demonstrates that all our protocols effectively establish a surface that supports initial cell adhesion and ensures their stability, even under the challenging conditions caused by mechanical forces.

In order to further support this claim of robust attachment, we performed coverage analysis similar to one we did for the static condition. For this, we analyzed the images captured for each flow rate which was after they were applied for one hour and calculated

how much of the channel surface was covered with cells. After calculating all the coverage percentages, we created a line graph to show the rate of cell detachment shown in **Figure 21**.

In the case of Protocol 1 TMSPMA treatment, the cell coverage was 72.18% at the beginning as a dense monolayer. After total 10 hours of various flow rates applied, the cell coverage regressed to 67.03%. For Protocol 2, initial coverage was 79.40% and decreased to 75.99% after 10 hours. So up to this point, 5% reduction has been observed in Protocol 1 and 3% reduction in Protocol 2. The numbers are not signaling to a significant cell loss and the final channel images are still showing highly dense coverages. Lastly, in Protocol 3 we got the most robust cell attachment and least amount of detachment rate with 2%. This can conclude that all the treatments performed efficiently in terms of offering robust cell attachment and capability to withstand shear stress and pressure.

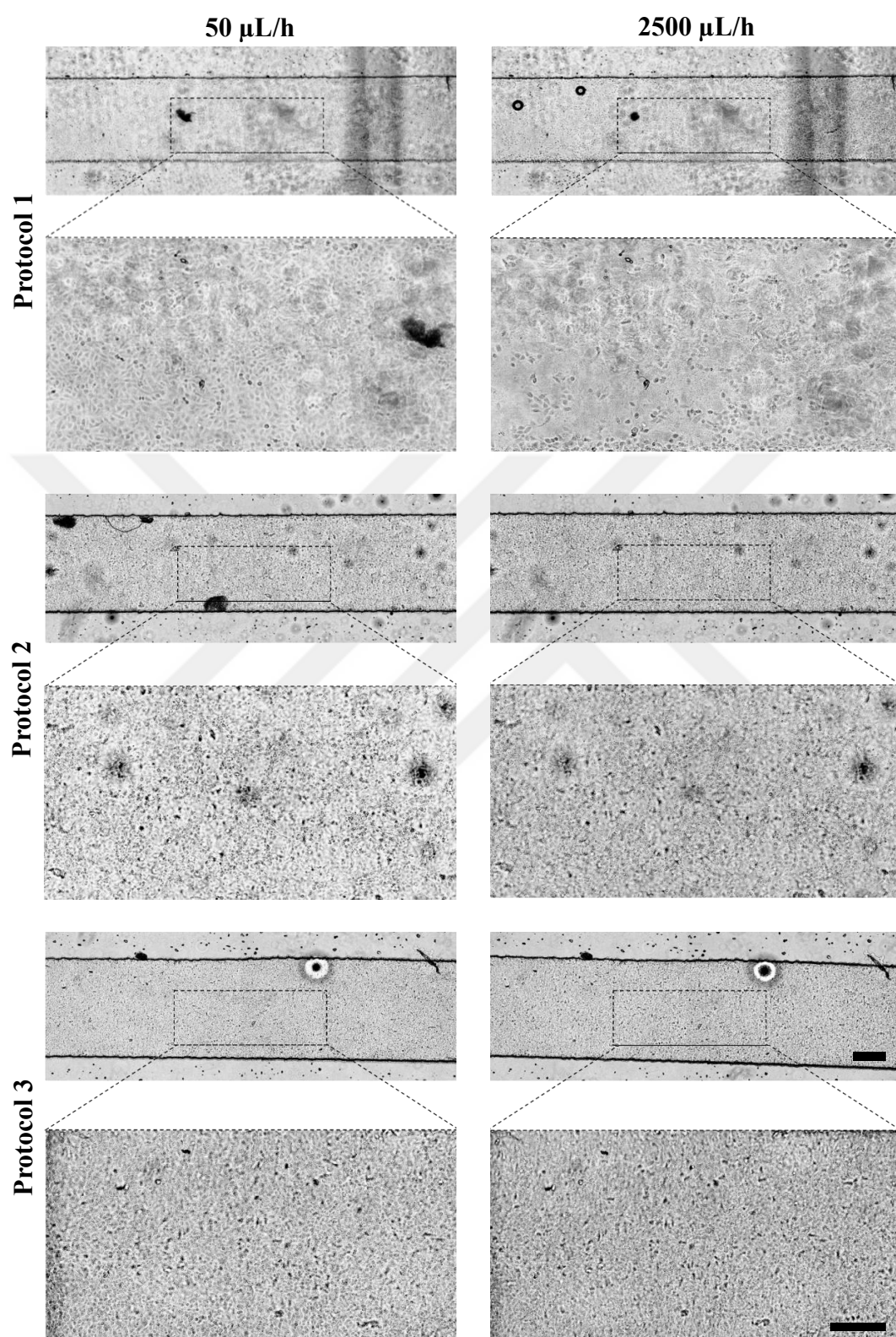


Figure 20. Cell attachment under increasing flow rates in the PDMS-based TMSPMA-coated microfluidic channel for Protocol 1, Protocol 2, and Protocol 3. Scale bars are 500 μm

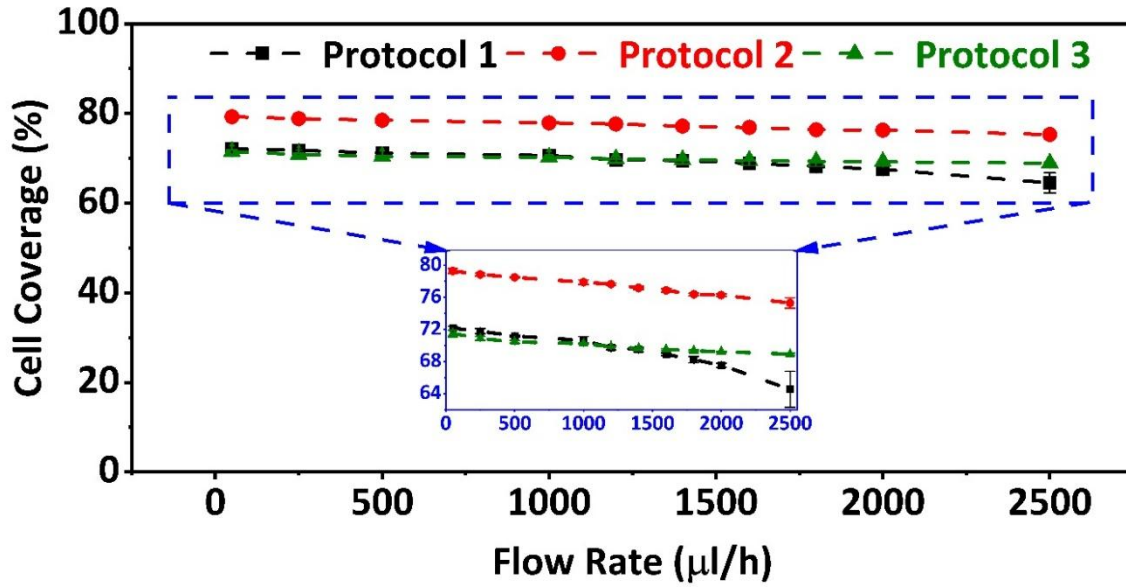


Figure 21. The cell coverage percentage along the treated-PDMS surface (Protocols 1, 2, and 3) with respect to flow rate.

4.2.6 Numerical Modelling of Shear Profile

Attached cells experience shear forces generated by the fluid flow under dynamic flow conditions. Studying these shear stresses is important because they are a driving factor in how cells behave, and in particular when modelling the in vivo environment, where cells are subjected to these forces in blood vessels.[212] Visualization or measurement of shear stress into micro channels is not an easy job experimentally. To cope with this treatment, a 3D rectangular microchannel, such as that of the experiments, is simulated and a COMSOL Multiphysics analysis is performed in order to systematically investigate the physical mechanisms inside, so that the velocity, pressure, and shear stress profiles both at the wall and in the entire microchannel could be obtained. For this purpose, a detailed three-dimensional (3D) numerical model in **Figure 22A and 22B** with dimensions and boundary conditions has been constructed. As shown in **Figure 22C**, the flow rate is changed between 0 and 2500 $\mu\text{L/h}$, and the corresponding velocity and pressure values are calculated for each setting. The behavior in was linearly proportional to flow rate for both velocity and pressure as theoretically predicted.[213], [214] In addition, the velocity and pressure profiles are shown in **Figure 22D** at a flow

rate of 50 $\mu\text{L/h}$. The velocity profile we calculated exhibited a classical parabolic flow meaning the maximum velocity is at the center of the channel, and the velocity approaches zero around the boundaries, which is consistent with previous profile reports.[215]

The shear stress distribution of the microchannel is described in **Figure 22E** showing the shear stress is the highest along the wall regions and the lowest at the center regions. To examine the influence of various shear stresses on cell adhesion, the flow rate was adjusted between 0 and 2500 $\mu\text{L/h}$, and the shear stress exerted on cells was regulated. The shear stress (τ) was calculated as $\tau = \frac{6\mu Q}{wh^2}$, where μ is the fluid dynamic viscosity, h and w are the height and width of the microchannel, respectively.[216], [217] The shear stress results measured using the simulation at the varied fluid flow rates also met the theoretical values. (**Figure 22E**) To further visualize the shear stress distribution profile in all the applied flow rates inside the microchannel, a cross section in the x-z plane taken into consideration for plotting. In **Figure 22F**, the shear stress is shown at the maximum point reaching 1.6 dyne/cm^2 in the highest flow rate and almost negligible at the center parts. This comprehensive analysis allows for a more detailed understanding of the shear stress dynamics within the microchannel and provides a foundation for understanding what cells are enduring under varying shear stress conditions.

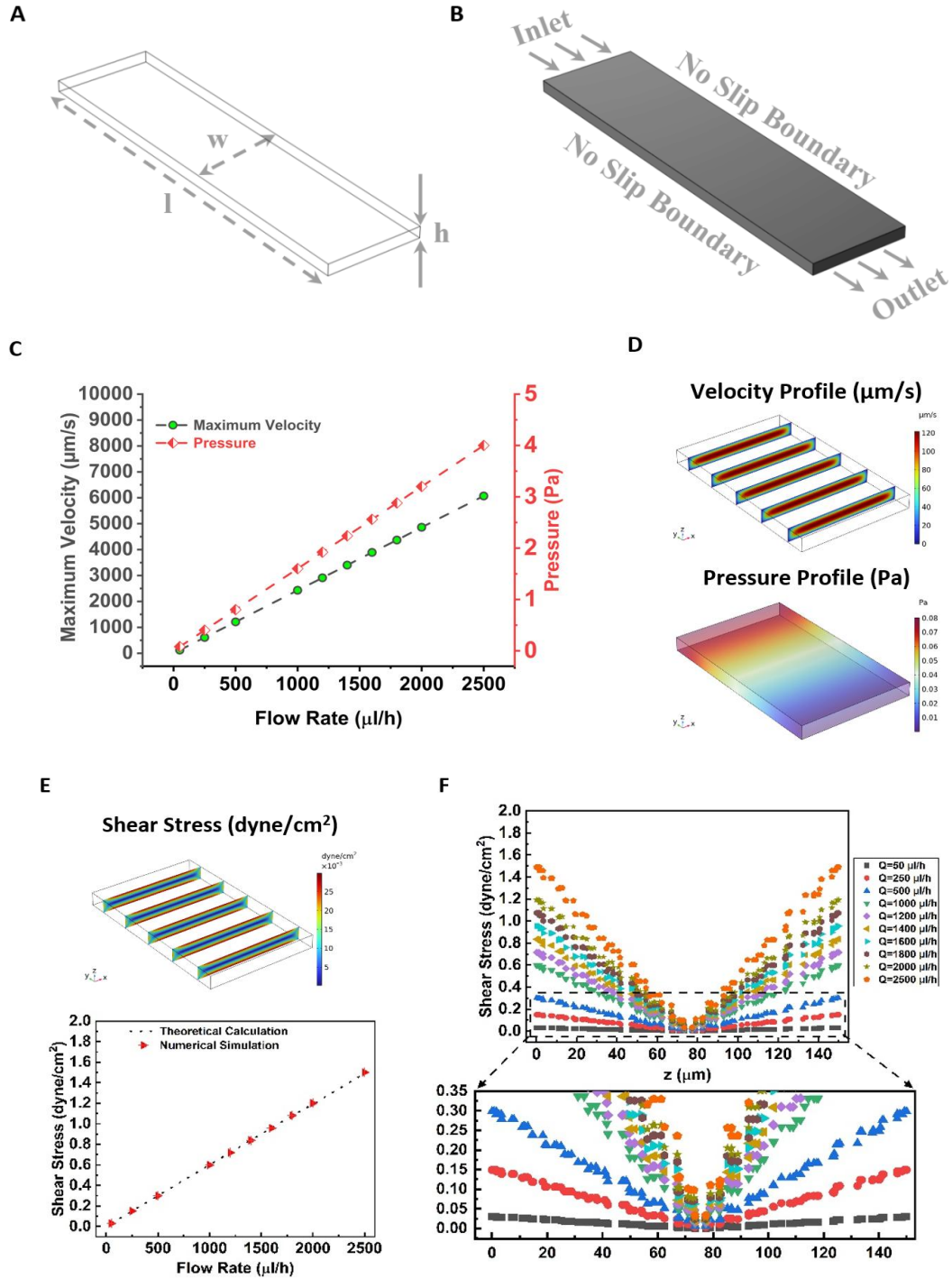


Figure 22. Numerical Simulation results for the 3D microfluidic platform. A: The dimensions of the microfluidic chip ($w=1.24$ mm, $l=19$ mm, and $h=150$ μm) and B: its boundary conditions. C: The velocity and pressure values with respect to varying flow rate. D: Velocity and pressure profiles within the microchannel at 50 $\mu\text{l/h}$. E: Shear stress profile at 50 $\mu\text{l/h}$ and comparison between theoretical calculation and numerical simulation of shear stress with varying flow rate. F: The shear stress profile from the side view (along the x - z plane) shows the distribution of the shear stress with a cross-section showing different values for the corresponding flow rate.

CHAPTER 5

5.CONCLUSION AND FUTURE PROSPECTS

This study introduces a new method for modifying PDMS surfaces with 3-(Trimethoxysilyl)propyl methacrylate (TMSPMA), which is the first application of this treatment to the organ-on-chip field. For improving PDMS surface conditions, such as hydrophilicity, roughness, and chemical groups with higher-performance cell adhesiveness, was the first required objective. Four specific treatment protocols were systematically examined with variations including type of solvent, TMSPMA concentration and treatment environment, leading to a wide applicability to a variety of biomaterials utilized in microfluidic devices. The effect of these treatments was confirmed by extensive surface characterization methods. X-ray photoelectron spectroscopy (XPS) was employed to study surface chemistry alterations, and wetting behaviour was characterized by contact angle measurements. Moreover, surface topography and roughness were characterized by scanning electron microscopy (SEM) and atomic force microscopy (AFM). After characterization, biocompatibility was evaluated through cell adhesion and proliferation assays using three different cell lines: human umbilical vein endothelial cells (HUVECs), human breast cancer line (MCF-7), and human-induced pluripotent stem cells (iPSCs). Preliminary cell culture studies using treated and coated 96 well plates demonstrate good cell attachment and proliferation. Significantly, the pluripotency of iPSCs was retained as evidenced by continued expression of markers specific for stem cells. These encouraging results were further confirmed in microfluidic channel configurations replicating the organ-on-chip in vivo context. HUVECs seeded in the microfluidic channels were specifically subjected to medium flow to test cell adhesion strength. All four protocols increased the surface wettability and nano-roughness capabilities, but only three provided a suitable surface for robust cell attachment under these vortex conditions. Findings of both the surface analysis

and biocompatibility studies indicate that the formation of effective cell adhesion depends on the integrated effects of surface chemistry, hydrophilicity and roughness.

In conclusion, this study demonstrates that TMSPMA surface treatment represents a highly effective and adaptable approach for PDMS modification. Its versatility makes it particularly suitable for organ-on-chip applications requiring strong cell adhesion under dynamic culture conditions. Given the importance of physiological barriers in drug delivery and disease modelling, future research should explore leveraging this optimized PDMS treatment to develop sophisticated vasculature barrier models. These models could simulate blood vessel structures and incorporate dynamic flow conditions, enabling the creation of advanced multilayered in vitro drug screening platforms featuring endothelial cells in upper compartments and cancer spheroids in lower compartments. This approach holds significant promise for enhancing drug screening efficacy, toxicity assessments, and disease modelling accuracy.

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Appendix A

Appendix

A.1 Declaration of Copyright Clearance Agreements

Reference Number	License Supplier	License Number
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