Directional Immobilization of Antibodies to Capture Circulating Cells

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Abstract

Vascular grafts and stents are currently used to bypass blocked arteries or reopen narrowed blood vessels, respectively, in patients with cardiovascular disease. While these prosthetic devices can be life-saving, complications such as restenosis and thrombosis can lead to complex revascularization procedures. An underlying cause of device occlusion is that these prosthetic materials lack the endothelial cell layer that maintains vascular homeostasis and provides antithrombotic properties to endogenous blood vessels. One approach to stimulate the formation of the endothelial cell layer is to promote the adhesion of endothelial progenitor cells (EPCs) to graft surfaces after implantation. Strategies to capture EPCs with surfaces functionalized with extracellular matrix (ECM) proteins, ECM-derived peptides or antibodies were shown to either lack specificity or to lead to incomplete endothelialization. To improve graft patency rates, the development of more effective surface modifications that maximize EPC capture efficiency and selectivity becomes imperative. The objective of this work was to develop a versatile platform to immobilize different antibodies on a substrate to study endothelial cell capture and behaviour. Different immunoglobulin G antibodies were successfully immobilized on commercially-available cell culture substrates. To achieve directional antibody immobilization, aminated polystyrene substrates were activated with an amine-to-sulfhydryl linking arm, followed by covalent conjugation of protein G, which binds to the constant domain of immunoglobulin G. The surface concentration achieved with this antibody immobilization strategy was significantly higher than immobilized antibody on adsorbed protein G onto aminated substrates. Antibody-modified surfaces did not exert adverse effects on the adhesion of human umbilical vein endothelial cells (HUVECs) in static cultures. The cell capture efficacy of antibody-functionalized surfaces was then assessed with a parallel-plate flow chamber. During capture studies, HUVECs were not arrested on surfaces functionalized with antibodies targeting endothelial cells markers such as anti-CD144 and anti-CD31. However, smaller-diameter mononuclear cells (CD14+) were captured on anti-CD14 surfaces, suggesting that inertial forces play an important role in the cell capture process. The versatile platform to immobilize antibody described in this work will allow further antibody screening to promote selective cell capture. This work

represents a first step towards engineering biomimetic vascular grafts with improved clinical outcomes.

Résumé

Les endoprothèses (stents) et les prothèses vasculaires sont actuellement utilisées pour, respectivement, rouvrir des vaisseaux sanguins et réaliser des pontages chez les patients atteints de maladies cardiovasculaires. Les prothèses vasculaires améliorent grandement la qualité de vie des patients. Toutefois, des complications comme les resténoses et les thromboses peuvent survenir et mener à des procédures de revascularisation complexes. Une cause sous-jacente de l'occlusion des dispositifs provient du faible taux d'endothélialisation de la surface qui maintient l'homéostasie vasculaire et fournit les propriétés anti-thrombogènes des vaisseaux sanguins endogènes. Une approche pour stimuler la formation de la couche endothéliale est de promouvoir l'adhésion des cellules progénitrices endothéliales (CPE) sur la surface des prothèses vasculaires implantées. Plusieurs stratégies de captation des CPEs avec des surfaces fonctionnalisées avec des protéines provenant de la matrice extracellulaire, des peptides dérivés de la matrice extracellulaire ou des anticorps se sont révélées incapables de produire une couche complète de cellules endothéliales dû au manque de spécificité. Pour améliorer la performance des prothèses, il est impératif de mettre au point des surfaces maximisant l'efficacité et la sélectivité de captation des CPEs. L'objectif du travail présenté était de développer une plateforme versatile pour immobiliser différents anticorps sur un substrat afin d'étudier la captation et le comportement des cellules endothéliales. Différents anticorps, de type immunoglobuline G (IgG), ont été immobilisés avec succès sur un substrat de culture cellulaire commercial. Une stratégie d'immobilisation directionnelle des anticorps a été employée en activant la surface aminée du substrat avec un bras d'ancrage reliant les amines aux groupements sulfhydryles, puis en conjuguant de façon covalente la protéine G, qui se lie au domaine constant des IgG. La concentration de surface obtenue avec cette stratégie d'immobilisation était significativement plus élevée que l'adsorption des anticorps sur les mêmes surfaces. Les surfaces modifiées par les anticorps n'ont pas eu d'effets indésirables sur l'adhésion des cellules endothéliales de veine ombilicale humaine (CEVOH) dans des cultures statiques. Au cours des études de captation réalisées dans une chambre d'écoulement à plaques parallèles, les cellules CEVOH n'ont pas été captées sur des surfaces fonctionnalisées avec des anticorps ciblant des marqueurs endothéliaux, tels

que l'anti-CD144 et l'anti-CD31. Cependant, des cellules mononuclées de plus petite taille (CD14+) ont été capturées sur des surfaces anti-CD14, suggérant que les forces d'inertie jouent un rôle important dans le processus de captation cellulaire. La plateforme versatile pour immobiliser les anticorps décrite dans ce travail permettra le criblage d'anticorps afin de favoriser la captation cellulaire sélective. Ce travail représente une avancée vers l'ingénierie de prothèses vasculaires biomimétiques avec de meilleurs résultats cliniques.

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Abbreviations

AFM	Atomic force microscopy
APTES	3-aminipropyltriethoxysilane
BMS	Bare metal stent
BSA	Bovine serum albumin
BVS	Bioresorbable scaffold
CAC	Circulating angiogenic cell
CD31	Platelet endothelial cell adhesion molecule
CD105	Endoglin
CD144	Vascular endothelial cadherin
Cys	Cysteine
DCC	Dicyclohexylcarbodiimide
DES	Drug-eluting stent
EC	Endothelial cell
ECFC	Endothelial colony forming cell
ECM	Extracellular matrix
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
Fab	Antigen-binding fragment
Fc	Fragment crystallizable region
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
IgG	Immunoglobulin G
IgY	Immunoglobulin Y
LDL	Low density lipoprotein
MAC	Myeloid angiogenic cell
MCAM	Melanoma cell adhesion molecule
NaOH	Sodium hydroxide
NHS	N-hydroxysuccinimide
NO	Nitric oxide
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCI	Percutaneous coronary intervention
PECAM	Platelet endothelial cell adhesion molecule
PGI_2	Prostacyclin

PLA	Poly-lactide acid
PLLA	Poly-L-lactide acid
PDLLA	Poly-D-L-lactide acid
PTFE	Polytetrafluoroethylene
QCM-D	Quartz crystal microbalance with dissipation monitoring
RO	Reverse osmosis
SDS	Sodium dodecyl sulfate
SMC	Smooth muscle cell
Sulfo-SMPB	Sulfosuccinimidyl 4-(N-maleimidophenyl) butyrate)
VE-Cadherin	Vascular endothelial cadherin
VEGFR-2	Vascular endothelial growth factor receptor-2
vWF	von Willebrand factor
WSS	Wall-shear stress
XPS	X-ray photoelectron spectroscopy

1 Introduction

Cardiovascular diseases are the primary cause of mortality in the world [1, 2]. Each year in Canada approximately 16 000 coronary artery bypass surgeries and 33 000 angioplasties or percutaneous coronary interventions (PCI) are performed by vascular surgeons [3]. PCI consists in reopening or reinforcing a narrowed blood vessel using a small mesh tube (a stent) following the accumulation of atherosclerotic plaque. The main complications after stent implantation are thrombosis (obstruction by a blood clot) and restenosis (re-narrowing of the blood vessel) due to inflammation and intimal hyperplasia – the thickening of the inner layer of the vessel (intima) (Figure 1) [4-7]. These complications lead to repeated vascularization procedures, further hospitalization, and patient anxiety [8, 9].

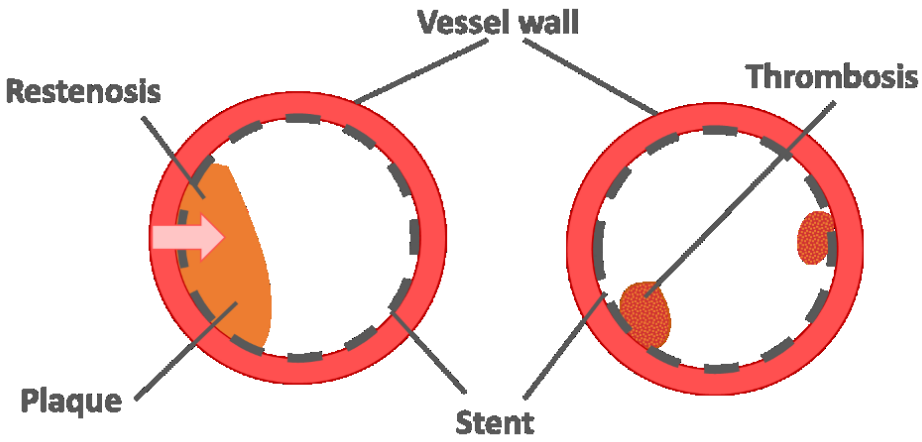

Figure 1. Schematic representation of restenosis and thrombosis after stent implantation into coronary arteries

In healthy blood vessels, endothelial cells (ECs) secrete molecules that inhibit thrombosis and intimal hyperplasia [10]. Rapid stent endothelialization has the potential to reduce the incidence of complications following PCI [8, 11-13]. Stent endothelialization may occur through the proliferation of ECs adjacent to stent struts, or from the recruitment of circulating endothelial progenitor cells (EPCs). Even without any surface modifications, different stent materials were found to impact the extent of endothelialization and the resulting stent performance [14]. Further, the material surfaces can be modified using

biomolecules that specifically capture EPCs from the circulation and promote EPC differentiation into mature ECs.

A variety of EPC capture stents have been developed to improve stent performance, including stents modified with surface-adsorbed cell adhesion peptides or capture antibodies. Vascular grafts with adsorbed RGD peptides demonstrated higher patency rates, likely through enhanced EC adhesion and inhibition of platelet attachment [15, 16]. However, the ubiquitous nature of cell adhesion to RGD limits the selectivity of this approach towards EPCs and ECs, potentially leading to the recruitment of immune cells to the graft. Some antibodies could potentially have a strong affinity towards EPCs and increase the selective capacity of capture stents. Anti-CD34 coated stents, the first commercialized EPC-capture stents (Genous™), were found to significantly increase the adhesion of cells expressing EC markers [17]. Although CD34 antibodies provide more specificity than RGD peptides, this marker is expressed not only by endothelial colony-forming cells but also by hematopoietic cells that can promote inflammation or clotting, potentially leading to graft failure [18].

This thesis describes the development of a method to immobilize a variety of antibodies onto aminated surfaces using surface-conjugated protein G. This method could be used to screen the effects of different putative EPC capture antibodies on EPC tethering, attachment, differentiation, and proliferation. This project provides a new tool to identify surface modifications promoting the endothelialization of vascular substitutes such as stents to improve their long-term performance.

2 Literature Review

To engineer an optimal biomaterial for vascular interventions, one potential approach is to mimic endogenous vascular regeneration mechanisms. This requires a good understanding of events leading to vascular dysfunction and atherosclerosis, and of the putative role of circulating cells in both inflammatory and repair mechanisms.

2.1 Role of the Endothelium in Vascular Homeostasis

All blood vessels, except capillaries, are composed of three layers surrounding a hollow core, called the lumen, through which the blood flows. The external layer, the tunica adventitia, is formed of connective tissue made of collagen fibers that protect and maintain the shape of the vessel. The middle layer, the tunica media, is the thickest layer made of smooth muscle cells (SMCs) attached to an annular and continuous sheet of elastin and collagen. This layer also contains neurofibers that allow the constriction and dilatation of the blood vessel. Finally, the inner layer, the tunica intima, is directly in contact with the blood and is composed of the endothelium and the basal lamina.

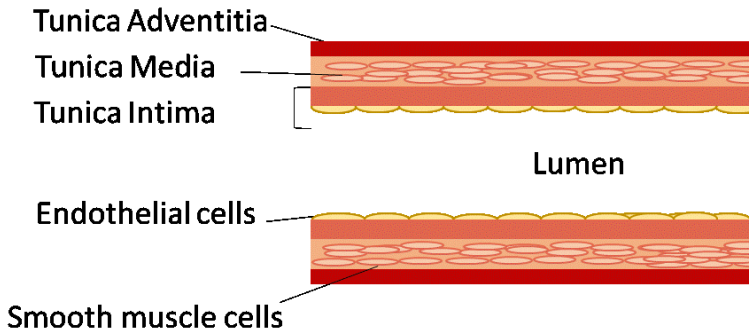

Figure 2. Schematic representation of cell layers in the artery wall

All blood vessels are lined by a monolayer of non-overlapping ECs. This endothelial layer forms an active tissue that creates a barrier between blood components and the underlying vessel and surrounding tissues. ECs secrete nitric oxide (NO), prostacyclin (PGI_2) and other factors that inhibit platelet activation and SMC proliferation, reducing the risk of thrombogenesis and intimal hyperplasia [19]. ECs also control vasodilation and

vasoconstriction through signals (e.g. NO) that impact the contraction of surrounding vascular cells (e.g. smooth muscle cells).

EC dysfunction or damage can disrupt the barrier and signaling functions of the intima, leading to atherosclerosis and cardiovascular disease. The atheroprotective function of ECs is maximized in the presence of undisturbed laminar flow on their apical surface, while their basal surface is attached via integrins and other receptors to extracellular matrix (ECM) proteins present in the basal lamina such as laminin and collagen [20, 21]. EC dysfunction is an early event in the progression of atherosclerosis, a disease that leads to the narrowing of blood vessels. Factors that contribute to EC dysfunction include cellular aging, hypertension, high levels of circulating low-density lipoproteins (LDL, also termed "bad cholesterol") compared to high-density lipoproteins, as well as disturbed flow patterns as seen in curved or branched segments. The loss of adequate EC barrier function can lead to the infiltration of monocytes and LDL deposition. Once in the intima, the monocytes differentiate into macrophages that ingest and oxidize lipoproteins. Lipid-filled macrophages become macrophage foam cells that release cytokines which will cause inflammation, attracting further monocytes to continue the cycle of plaque formation, promoting SMC proliferation. At this moment a visible fatty streak will appear. Plaque will continue to build up and SMCs will migrate to cover the plaque and create a barrier between the plaque and the blood. SMCs will form a fibrous cap and release calcium that will further calcify the fibrous cap and the plaque. The growing plaque eventually bulges into the lumen and reduces blood flow, increasing the risk of complete occlusion or thrombosis [19, 22-26]. The plaque may also detach and travel to occlude a downstream vessel, leading to a lack of oxygenation of downstream tissues (ischemia) such as the heart or brain – leading respectively to myocardial infarct (heart attack) or stroke.

2.2 Blood Cells and their Interactions with Endothelium

Designing a perfect blood-material interface is likely impossible, although significant strides have been made towards this ideal. As shown in Table 1, blood is a complex fluid containing a multitude of cells and proteins that interact with biomaterial surfaces and influence circulating cell fate [26, 27]. Proteins present in blood rapidly absorb to vascular

biomaterial surfaces to form a thin protein layer. This layer will undergo many modifications due to the dynamic interactions and competition of proteins on surfaces [28, 29]. In addition to protein adsorption, cells circulating in blood also interact with the surface and adsorbed proteins which can lead to surface alterations. Thus, biomaterial surfaces are in a perpetual flux where proteins, molecules, and cells continuously modify surface composition and properties. Surface modifications that facilitate biological events at the blood-biomaterial interface, such as surface endothelialization, should be viewed in the context of this complex and dynamic interplay.

2.3 Endothelial Progenitor Cells

Many terminologies have been used to describe and define EPCs, with a consensus statement recently proposed by authors from 14 different institutions [30]. In 1997, Asahara *et al.* were the firsts to use the terminology ‘‘putative EPCs’’. They suggested that a proportion of bone marrow-derived circulating progenitor cells can express endothelial markers *in vitro* and contribute to the endothelium healing process [31-33]. The Yoder group later demonstrated that the circulating angiogenic cells (CACs) isolated by Asahara are of myeloid lineage and are not endothelial progenitors [34-36]. The nomenclature proposed by Media *et al* for these cells is myeloid angiogenic cells (MACs). These cells express CD45, CD14 and CD31, but not CD146, CD133, and Tie2.

The second subtype of EPCs is described as endothelial colony forming cells (ECFCs). This population is important in the vascular healing process and of interest for this study due to their angiogenic capacity, their direct contribution to vascular repair and their ability to form vascular structures (vasculogenesis). They can be derived from umbilical cord blood or peripheral blood mononuclear cells (PBMCs) and are also present in vessel walls. Subsets of ECFCs display a high proliferative potential and express endothelial cell markers, such as CD105 (endoglin), CD144 (vascular endothelial cadherin; VE-cadherin), CD146 (melanoma cell adhesion molecule, MCAM), CD31 (platelet endothelial cell adhesion molecule, PECAM), endothelial nitric oxide synthase (eNOS) and von Willebrand factor (vWF). ECFCs also express the early hematopoietic cell marker CD34 but do not express CD14 or CD45 [18, 35, 37].

2.4 Vascular Substitutes in the Treatment of Cardiovascular Disease

The prevention of atherosclerosis starts by adopting a healthy lifestyle through healthy weight maintenance, physical activity, and avoiding smoking habits. Prevention of hypertension and control of diabetes will also help to decrease the risk of atherosclerosis development. When changes in lifestyle are insufficient to stop the progression of atherosclerosis, pharmaceutical intervention is possible through antihypertensive or cholesterol-lowering drugs. Medical procedures such as angioplasty and PCI may be needed when less invasive solutions have failed, or in emergency situations. This section describes some technologies approved for the treatment of atherosclerosis in humans.

2.4.1 Coronary Artery Angioplasty and Percutaneous Coronary Intervention

Coronary artery angioplasty is a procedure that is used to reopen a narrowed artery to avoid the complete occlusion of the vessel. During the intervention, a small balloon is inserted at the site of the occlusion with the help of a catheter. When in position, the balloon is inflated with high pressure to compress the plaque at the blood vessel wall and improve the blood flow. This intervention was first done by Andreas Grüntzig on September 16, 1977, in Zürich, Switzerland [38]. This intervention improved the survival rate of patients that were suffering from stenosis and it is still used nowadays. Nevertheless, the reported restenosis rate remained high, ranging between 20-35% [39]. The elastic recoil and rupture of the wall are potential causes of restenosis after angioplasty [40, 41].

2.4.2 Bare-Metal Stents

Bare-metal stents (BMS) were introduced in 1986 by Puel and Sigwart [41] to overcome abrupt vessel closure and restenosis observed after balloon angioplasty, lowering the rates of restenosis to around 31% compared to 42% with balloon angioplasty after six months [42]. A stent is a small mesh tube introduced at the occlusion site with the help of a catheter and deployed to reopen the vessel.

BMSs are made of 316 L stainless steel or nitinol and typically range between 2.5 mm to 4.0 mm in diameter after deployment. Other alloys, such as cobalt and platinum chromium, are also used. The reduction of struts thickness and size were shown to decrease the rates

of restenosis due to improved re-endothelialization and reduced trauma to the vessel wall [43].

Despite all technical improvements, restenosis is still a major problem with BMS even in combination with oral anticoagulation therapy.

2.4.3 Drug-Eluting Stents

To inhibit restenosis, drug-eluting stents (DES) were engineered in order to release pharmacological agents. Anti-proliferative drugs such as sirolimus and paclitaxel were first used to inhibit SMC proliferation and neointimal hyperplasia progression locally. These first-generation DESs significantly reduced 6-month restenosis rates to between 0%–9% with sirolimus compared to 25%–36% with BMSs [44, 45]. Since these drugs inhibit proliferation non-specifically, they can also negatively affect vessel healing and stent endothelialization [46]. The main concern related to DESs is the occurrence of late in-stent thrombosis which is believed to be provoked by the delayed stent re-endothelialization [47]. Everolimus and zotarolimus agents were also used in the development of second-generation DESs; however, late thrombosis remained problematic.

2.4.4 Bioresorbable Scaffolds

The last generation of stents appeared with the engineering of bioresorbable scaffolds. Since mechanical support may only be required temporarily after PCI, these stents were designed to provide support to the vessel wall for a limited time before slowly degrading. By liberating the treated vessel, the bioresorbable stent has the potential to overcome some of the limitations of BMSs and DESs by reducing chronic vascular inflammation caused by the foreign body response and allowing restoration of the endothelial cell lining. These stents are made of a variety of materials such as PLLA (poly-L-lactide acid), PDLLA (poly-DL-lactide acid) and magnesium [48-50]. The main source of long-term clinical data, which is critical to determine device safety, was derived from clinical studies of the PLLA everolimus-eluting stent produced by Abbott, called Absorb™. The risk of restenosis was lowered with the Absorb™ stent compared to metallic DES stents. However, the incidence of 2-year in-stent thrombosis events was higher for the Absorb™ stent [49]. One design challenge observed with the Absorb™ stent concerns the greater strut thickness required

to maintain mechanical integrity compared to metal stents. This limits the utilization of these stents for treating bifurcation lesions [51]. Moreover, the implantation procedure of this stent requires greater attention to reduce stent fracture and artery lesions during deployment. For all of these reasons, the bioresorbable technology requires development and optimization to improve vascular stent efficiency.

Table 1. Summary of coronary stent technologies

Stent	Incidence of Restenosis	Incidence of In-Stent Thrombosis	PRO	CON	Example
Bare-Metal Stents	25–36% after 6 months [44, 45]	24% early stent thrombosis in first generation BMS [52] 1–1,5% early stent thrombosis with dual antiplatelet therapy[53, 54]	Provide a stable scaffold with high radial strength Reduce the rate of vessel closure compared to balloon angioplasty	Chronic foreign body response Requirement for dual antiplatelet therapy Limited long-term efficacy due to in-stent restenosis	Bx-Velocity (316 L stainless steel)[55]
Drug-Eluting Stents	0–9% after 6 months [44, 45, 56]	0.35 % [57]Late in-stent thrombosis: 1.23%–1.7% for paclitaxel-eluting stents vs 0,42–1,0% for everolimus-eluting stents[56]	Reduce the risk of restenosis and early thrombosis 2nd generation of DES lower risk of in-stent thrombosis (Everolimus and zotarolimus-eluting stents)	Inhibit vessel healing and in-stent endothelialization Occurrence of late in-stent thrombosis which increases with time after implantation (up to 3% after four years [58])	Cypher ® (Sirolimus-eluting stent)[55, 59] Taxus™ (Paclitaxel-eluting stent) Xience™ (Everolimus-eluting stent) Endeavor® (Zotarolimus-eluting stent)
Bioresorbable Scaffolds	Similar to DES[40]	2–3% after two years[49]	Allow vessel healing and tissue reconstruction after stent implantation Complete resorption of the implanted scaffold	Low radial strength to support vessel wall Increased risk of device-related adverse events during implantation	Absorb™ (PLLA-everolimus)[49] PDLLA and Magnesium scaffolds developed and tested in porcine models[48, 50]
EPC Capture Stent	Similar to DES	3,1% after 2 years[60]	Fast surface endothelization	Lack of specificity to EPCs	Genous™ (Anti-CD34 coated stent-human trial) [61]

2.5 EPC Capture Stents and Surface Treatments to Promote Endothelialization

As described earlier, the endothelial lining of vascular arteries remains the ideal surface to interact with the blood and underlying vascular cells. To promote stent re-endothelialization, circulating EPCs could be captured by biomolecules attached to the stent surface such as antibodies, ECM-derived peptides or aptamers [62-64].

2.5.1 Surface Functionalization Strategies to Capture Circulating EPCs

2.5.1.1 Antibodies

The first human clinical attempt to capture EPCs with antibodies investigated the effects of CD34 antibody-coated Genous™ stents on in-stent late luminal loss at six months follow-up and demonstrated the safety and effectiveness of the stent for *de novo* coronary artery disease [61, 65]. Compared to DESs in a porcine model, the CD34 antibody-coated stents led to significantly higher levels of endothelialization after 28 and 90 days, but no significant differences in restenosis rates were observed [14, 60]. This was attributed to the lack of specificity of the anti-CD34 antibody since EPCs represent only a small proportion of the CD34+ cells found in blood. Some CD34+ cells can differentiate into monocytes and leukocytes which can induce pro-inflammatory events [64]. Endothelial cell captured using surfaces coated with anti-VEGFR 2 (vascular endothelial growth factor receptor 2) antibodies were also studied *in vitro* on glass coverslips [66]. Surfaces with immobilized anti-VEGFR-2 antibodies captured endothelial cells under low shear stress (shear rate at 50 s^{-1} in EBM-2 media with a viscosity of 0.78 cP at 37 ºC, ~0.4 dyn/cm^2) during a single flow pass. Moreover, two methods of antibody immobilization were compared, namely passive adsorption of the antibody versus oriented immobilization on adsorbed protein G. The oriented immobilization of antibodies via adsorbed protein G increased the surface density of antibodies as well as HUVEC capture compared to passive antibody adsorption. These studies suggest that antibody-functionalized surfaces can be used to target and capture EPCs from flow. However, antibody-based strategies are limited by the poor definition of cell surface markers that are specific to EPCs. In addition, adsorption is a reversible process in which the same amount of energy is used to adsorb and release the

molecule from the surface. Other proteins present in blood may compete for adsorption sites, leading to desorption of the surface-immobilized capture molecules, potentially decreasing cell capture efficiency and increasing the probability of activating the immune system. The advancing research on EPCs will improve, hopefully in the near future, our understanding and definition of EPCs to identify antibodies or biomolecules that target EPC surface markers with higher selectivity. With the help of surface engineering, these molecules can be attached to stent surfaces and improve vascular healing after PCI.

2.5.1.2 Extra-Cellular Matrix Derived Peptides

Peptides derived from ECM proteins were also studied to capture EPCs from flow and modify stent surfaces. ECM-derived peptides are of particular interest as they can influence cell phenotype and cell fate decisions. Many peptide sequences were isolated from fibronectin (REDV and RGD), laminin (YIGSR) and collagen due to their affinity to interact with cell receptors such as integrins [67]. The RGD sequence is widely studied in vascular biomaterials engineering due to its interactions with integrin receptors, promoting cell adhesion and proliferation. Cyclic RGD peptides covalently attached to glass surfaces were found to have a greater affinity to bovine aortic endothelial cell integrins than linear RGD motifs [63, 68]. Moreover, cyclic RGD-coated polymer stents significantly accelerated stent endothelialization, as well as, showing a reduction in neointimal area and stenosis area after 12 weeks compared to polymer stents without the cyclic RGD coating or BMSs when implanted into the right and left coronary arteries of adult domestic pigs [16]. However, the lack of specificity of the RGD peptide towards EPCs is of concern given that most adherent cell types express integrins that bind to this peptide. While other ECM-derived peptides may be more selective, no single peptide has so far been demonstrated to be specific towards EPCs compared to other circulating cells. An approach to improve peptide selectivity is to use a peptide or phage library to identify ligands that specifically target EPCs [69].

2.5.1.3 Aptamers

Aptamers are DNA or RNA sequences engineered to bind a specific target with high affinity. Hoffman *et al.* covalently attached aptamers with high affinity to EPCs on

star-polyethylene glycol-coated polydimethylsiloxane and polytetrafluoroethylene surfaces. *In vitro* studies with porcine blood demonstrated that circulating cells were captured by these surfaces [70]. When the surfaces were transferred to *in vitro* culture, the cells expanding on the surfaces adopted an endothelial phenotype based on morphology and CD31 expression. The aptamer technology is a promising approach to improve the selectivity of EPC capture biomaterials. However, DNA and RNA are likely to be degraded by RNases and DNases naturally produced in the body unless genetic modifications are developed to inhibit their cleavage. Further development and trials are now necessary to demonstrate the hemocompatibility of these molecules.

2.6 Engineering EPC Capture Surface via Antibody Immobilization

Surfaces functionalized with EPC-capture antibodies have shown great promise in reducing the incidence of restenosis by promoting the surface endothelialization of stents. In addition to challenges related to antibody selectivity, the immobilization strategy can significantly impact the binding affinity between the immobilized antibody and its antigen. As shown in Figure 3, antibodies are properly oriented and bioactive when they are immobilized by their fragment crystallizable regions (Fc region). In this position, both antigen binding sites, located in the antigen binding fragment (Fab), are available. Multiple techniques exist to immobilize capture antibodies, not only for EPC capture but also for biosensor development for the detection of proteins, biomolecules or cells.

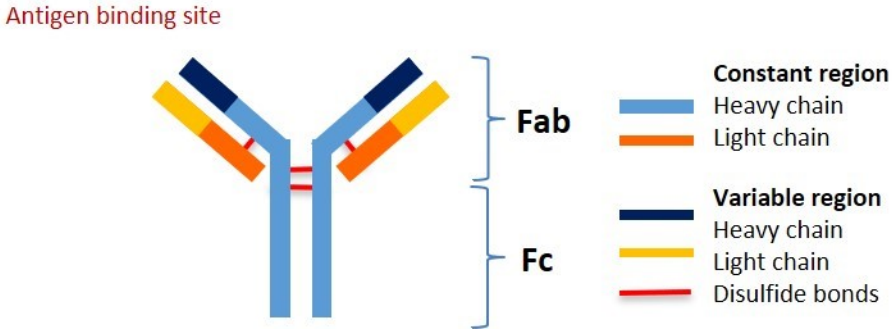

Figure 3. Schematic representation of an immunoglobulin G antibody

2.6.1 Antibody Immobilization by Adsorption

Passive physical adsorption is the most straightforward and frequently applied strategy for protein immobilization, although the reversible nature of protein adsorption phenomena limits the long-term stability of these coatings [13]. After implantation, proteins present in blood can compete for adsorption sites and lead to rearrangement or desorption of the pre-adsorbed biomolecules. Surface-adsorbed antibodies frequently undergo conformation changes, random immobilization orientation and denaturation that alter their antigen-binding capacities [71, 72]. Thus, more stable surface immobilization strategies are necessary to provide a controlled antibody attachment to maintain antibody affinity to the target ligand.

2.6.2 Direct Conjugation Antibody Immobilization

The covalent conjugation of antibodies on surfaces can create stable coatings, increase the antibody density and allow a strong attachment. Primary amines present on lysine side chains (ε-amine) and at the N-terminus of polypeptide chains (α-amine), as well as carboxyl groups present on aspartic acid and glutamic acid side chains and at the C-terminus of polypeptide chain can be targeted to conjugate antibodies to substrates. N-hydroxysuccinimide esters (NHS) can react with primary amines to form a stable amide bond. Proteins are often grafted to surfaces via a linking arm with an NHS group reacting with the protein and another (or the same) functional group reacting with a group present on the surface. Alternatively, NHS linkers can target surfaces with reactive amines, using another functional group on the biomolecule for surface coupling [73, 74]. Other reactive chemical groups, such as epoxide, aldehyde, and imidoester have also been used to activate surfaces, allowing protein immobilization by their amino groups [9, 75, 76]. For carboxyl groups, carbodiimides, such as EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) and DCC (dicyclohexylcarbodiimide) are zero-length linkers that activate carboxyl groups to react with primary amines and form stable amide bonds. They are often coupled with NHS linkers to graft biomolecules onto surfaces [77-79]. Carbohydrate chains are also used to immobilize antibodies. To conjugate anti-CD34 antibody on stainless steel substrates, Yuan *et al*. [80] oxidized antibody carbohydrates with periodate. Periodate can specifically oxidize the vicinal hydroxyl groups located in the Fc region to aldehydes which can further

react with amine groups present on the 3-aminipropyltriethoxysilane (APTES) functionalized substrate. This directional immobilization method increased antigen detection by 3-fold compared to a glutaraldehyde surface treatment which targets amino groups. While all of these methods lead to covalent antibody grafting, the relative ubiquity of carboxyl or amino groups on antibodies can lead to various antibody conformation on the surface and losses in antigen-binding capacity. For this reason, some groups have targeted the sulfhydryl moieties present in cysteine given the lower statistical content of these groups in proteins compared to carboxyl or amine residues. Targeting sulfhydryl can improve the oriented immobilization of proteins but depends on the availability of a free reduced cysteine in the desired location on the protein. To conjugate a protein via a sulfhydryl group, maleimide reagent can be used to form stable thioether linkages. In antibodies, all cysteines are participating in the intra- (twelve disulfide bonds) and inter-chain (four disulfide bonds, shown in Figure 3) structure of the antibody. Knowing their positions in the antibody structure favors the control of the linker location after reaction. Inter-chain cysteines present in disulfide bonds can be reduced to form reactive thiols and subsequently reacted with a maleimide-linker. With careful control over reducing conditions, the disulfide bonds in the antibody hinge region can be disrupted without affecting the disulfide bonds between the light and heavy (HL) chain, potentially allowing directional immobilization of HL fragments without disrupting the binding affinity of the Fab regions [81]. However, these techniques can be complex and will modify the antibody tertiary structure. Nevertheless, these methods showed an improvement in antigen binding capacity compared to non-directional antibody immobilization methods [72, 82, 83].

2.6.3 Bioaffinity-Based Antibody Immobilization

The ability of certain molecules to bind ligands specifically is called bioaffinity. An example of antibody immobilization via bioaffinity is the binding of biotin-labeled antibodies to avidin-modified surfaces [84]. However, the biotinylation is a complex multi-step reaction process which can alter antibody performance. In addition, the biotin tag can be introduced on any reactive group in the antibody, leading to non-directional antibody

immobilization [84]. Moreover, avidin molecules of bacterial origin raise concerns related to undesirable immune responses to the modified surfaces.

Another bioaffinity-based antibody immobilization technique involves Fc binding proteins such as protein A and protein G. These immunoglobulin-binding proteins contain five and three IgG binding sites, respectively, which specifically target the antibody Fc region, allowing directional immobilization. Protein G, in contrast to protein A, can bind to all human IgG subclasses [85]. Moreover, protein G has a higher affinity constant with respect to IgG than protein A (IgG K_a of 6.7 X 10^9 M^{-1} for protein G versus 1.4 X 10^8 M^{-1} for protein A) [86-88]. These two proteins were used in biosensor applications to control antibody orientation on surfaces using their IgG bioaffinity capacities [77, 82, 89-92]. Recombinant protein G lacking the albumin binding site is preferred for the surface functionalization to reduce the risk of interaction between the protein G and serum proteins. Several studies have shown improvement in antibody-antigen interaction when protein A and G were used to orient antibodies compared to passive adsorption or covalent conjugation [66, 81, 93-95]. Immobilization via protein A and G does not require antibody modification, keeping antibody binding functionality intact. Moreover, all incubation steps can be performed in aqueous solution avoiding damage to surfaces that may be sensitive to harsher solvents. To improve the stability of protein G on surface, a Cys residue can be added to the N or C-terminus of the protein G via recombinant DNA technology. Since the protein G sequence itself does not contain Cys residues [96], the recombinant Cys-protein G protein allows oriented conjugation of Cys-protein G onto surfaces via a disulfide bond or a thioether link [89-91]. The covalent conjugation of Cys-protein G via the free sulfhydryl group of a cysteine resulted in 2.2 fold higher IgG antibody binding efficiency compared to protein G conjugation via its lysine groups [97]. In another study, oriented IgG immobilization via covalently grafted Cys-protein G increased the IgG surface density by 4-fold over the surface density achieved with adsorbed protein G. To improve protein G-antibody complex stability, crosslinking reagents were also used to stabilize the complex and decrease the incidence of antibody dissociation from surfaces during biosensor utilization [95]. One drawback of protein A and G is the bacterial origin (*Staphylococcus or Streptococcus*) of the molecule, which may invoke an undesirable immune response

when used in the human body [88]. Strict hemocompatibility and immunogenicity tests are necessary to ensure the safe use of biomaterials modified with these molecules.

One way to overcome the undesirable immune response of the host body is to use only Fc-binding peptide sequences of protein A or G instead of the entire bacterial proteins. Phage and peptide library screens were used to identify peptide sequences that can bind to the Fc fragment [98, 99]. Jung *et al.* demonstrated IgG antibody immobilization via an Fc-binding peptide [100]. In addition to the ongoing research on Fc-binding peptides, aptamers were also developed to bind Fc fragment. Aptamers are oligonucleotide sequences, i.e. DNA or RNA chains, that fold to form a structure that can bind to specific proteins [101, 102]. This method can be used to develop ligands that can bind specifically to one subclass of IgG antibodies or, on the contrary, bind universally to all Fc fragments.

To summarize, the major challenges when engineering EPC-capture stents is the development of a stable coating that can specifically target EPCs and promote their adhesion. To do so, the coating should be firmly bound to the surface to avoid molecule release in the blood. Moreover, chosen molecules should reduce the risk of undesirable immune responses while having a strong affinity to EPCs. Finally, the binding affinity of the molecule should remain intact when entering into contact with proteins, platelets and other blood components.

3 Objective and Hypothesis

The long-term objective of the project is to develop biomimetic EPC capture stents that reproduce the sequential process of circulating cell recruitment to vessel walls, including tethering, adhesion, proliferation, and differentiation. The work performed in the scope of this thesis addresses the first of these steps. The hypothesis tested in this work was that directional immobilization of antibodies via protein G can allow selective capture of different circulating cell types. The general goal of this thesis was to develop a versatile method to immobilize antibodies on commercially-available cell culture surfaces to establish an EPC capture screening platform. Specifically, the aims of this project were to:

1. Create and optimize a grafting method to functionalize aminated polystyrene surfaces with antibodies.
2. Characterize antibody-functionalized surfaces by quantifying the amount of immobilized antibody and confirming the immobilization of different antibodies.
3. Determine whether this platform is suitable to selectively capture different circulating cell types.

Due to the low frequency of EPCs in blood, the selective capture of two cell types with similar properties to EPCs was tested. The first cell population is composed of monocytes which contains myelogenic angiogenic cells. Monocytes have properties such as cell diameters that are expected to be similar to circulating EPCs. The second cell population is composed of HUVECs, which express similar surface antigens as endothelial colony-forming cells derived from EPCs [30, 34].

4 Materials & Methods

4.1 Surface Modification and Antibody Immobilization

For cell capture experiments under flow, 2.5 cm × 3.0 cm surfaces were obtained by cutting aminated polystyrene Petri dishes (BD Purecoat™ Amine #354732, BD Biosciences, San Jose, USA) with a hot wire cutter (#K02B, Hot wire foam factory, Lompoc, USA). Surface edges were smoothed by removing imperfections and extra materials with a precision knife (X3201, X-Acto®, High point, USA) to obtain the desired dimensions to fit into the flow chamber. Surfaces were washed with reverse osmosis water (RO water) once and air-dried. The circumference of these cut samples was lined with Teflon™ tape (#3213-103, polytetrafluoroethylene (PTFE) thread sealant) to maintain solutions on surfaces during the reaction steps. All other surface modifications were performed directly in well plates. All incubation steps were performed in the dark with 90 RPM agitation on a rotary shaker (Ecotron, Infors HT) at room temperature. After each reaction step, solutions containing reactants were removed, and surfaces were rinsed twice with 0.2 µm-filtered phosphate buffered saline solution (PBS, #21600010, Thermo Fisher Scientific). First, the aminated surfaces were reacted for 2 hours with 150 µL/cm^2 of a 3 mg/mL suspension of sulfo-succinimidyl-4-(*p*-maleimidophenyl)-butyrate (sulfo-SMPB, #BC24, G-Biosciences) in PBS. Next, protein G was attached to the linking arm by adding 150 µL/cm^2 of a 5.5 µM recombinant Cys-protein G (protein G with an N-terminal Cys residue added to the recombinant protein sequence, #PRO-1328, Prospec-Tany Technogene Ltd) suspension in PBS for one hour. Finally, primary antibodies targeting cell surface antigens (mouse anti-human CD31 antibody #303101; mouse anti-human CD105 #323202; mouse anti-human CD144 #348502; and mouse anti-human CD14, anti-CD14, #367102; all from BioLegend, San Diego, US) were immobilized on the protein-G modified surfaces by adding 150 µL/cm^2 of antibody solution at 5 µg/mL in PBS for 1 hour. Surfaces were then rinsed twice with PBS, once with a 1% SDS-TRIS pH 11 solution (5% v/v of 20% sodium dodecyl sulfate #05030, from Sigma Aldrich and 2,4 % w/v TRIS base PBP151-500 from Fisher Scientific in reverse osmosis water, pH adjusted to 11 with 2N NaOH solution) to remove adsorbed molecules, twice with PBS, and finally rinsed with reverse osmosis (RO)

water. The surfaces were then air-dried and stored for at most one week before use. Adsorption controls followed the same surface modification scheme, except that surfaces were not activated with sulfo-SMPB prior to incubation with protein G. Figure 4 shows the surface functionalization steps to immobilize capture antibodies. Note that Alexa Fluor 488-conjugated goat anti-mouse secondary antibody (#A-11001, Thermo Fisher) was used to detect the primary capture antibodies by immunofluorescence.

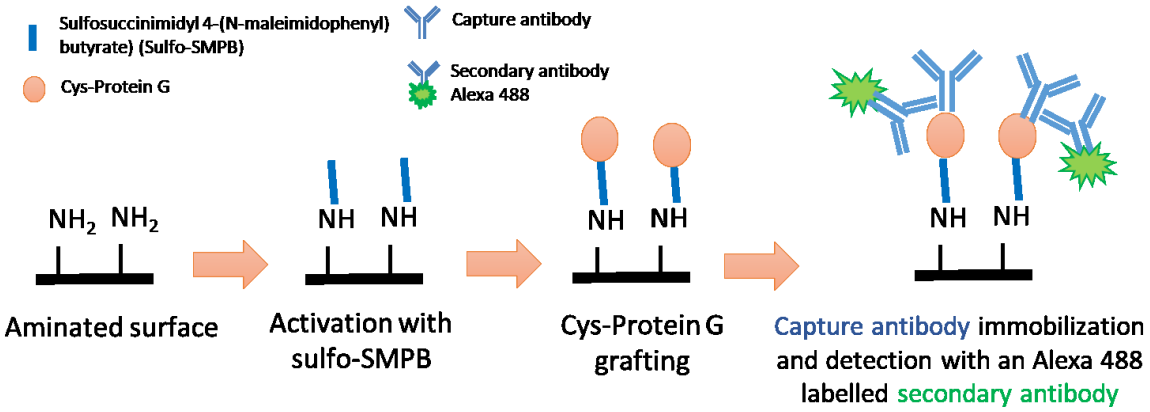

Figure 4. Schematic representation of the antibody immobilization process to functionalize polystyrene aminated surface for the capture of circulating cells.

4.2 Contact Angle Measurements

The contact angles between deionized RO water and functionalized surfaces were measured by the sessile drop method using an OCA 150 system (DataPhysics Instruments GmbH, Filderstadt, Germany). Water drops of 5 µL were deposited at a rate of 0.5 µL/s onto Purecoat ™ substrates, with or without sulfo-SMPB treatment. Images of the drops in contact with surfaces captured at the end of drop spreading were recorded. The average between the left and the right static contact angles was determined for each image using the SCA-20 software (DataPhysics Instruments).

4.3 Primary Amine Detection via the Orange II Assay

The surface concentration of primary amines was quantified using the Orange II assay [103, 104]. Under acidic conditions, the sulfonated Orange II dye is negatively charged and can electrostatically bind positively charged primary amines. After dye binding, surfaces are rinsed and immersed into an alkaline solution where the dye desorbs and is

released into solution. The absorbance of the solution can be measured and compared to a known reference to quantify amino groups.

Surfaces cut into 1 cm^2 surface-modified polystyrene pieces were transferred into 10 mL polystyrene tubes (#T406-2, Simport scientific, Beloeil, Ca). The samples were then submerged in Orange II sodium salt solution (14 mg/mL Orange II, 75370, Sigma Aldrich in RO water adjusted to pH 3 with 37% w/w HCl; 1.5 mL added per tube). After incubating for 30 minutes at 40°C, samples were rinsed with the acidic solution to remove all unbound dye and air-dried before being immersed in 1 mL of the alkaline solution (RO water adjusted pH to 12 with 5N NaOH solution) to desorb the dye. The pH of the desorbed dye solution was then readjusted to a pH of 3 by adding 1% v/v of 37% w/w HCl to each tube. All solutions were transferred to a cuvette where the solution absorbance was read at 484 nm by a Genova spectrophotometer (Jenway, Staffordshire, UK). Primary amines were quantified by comparing the absorbance obtained to a standard curve generated by adding Orange II dye in acidic solution at known concentrations ranging between 0.3 µg/mL and 140 µg/mL.

4.4 X-Ray Photoelectron Spectroscopy (XPS)

The chemical composition of the surface was investigated by XPS using a PHI 5600-ci spectrometer (Physical Electronics, Eden Prairie, MN). The main XPS chamber was maintained at a base pressure of $< 8\times10^{-9}$ Torr. A standard aluminum X-ray (Al Kα = 1486.6 eV) source was used at 300 W to record survey spectra with charge neutralization. The detection angle was set at 45º with respect to the normal of the surface and the analyzed area was 0.5 mm^2.

4.5 Enzyme-Linked Immunosorbent Assay for Protein G

A direct enzyme-linked immunosorbent assay (ELISA) was developed to detect and quantify protein G surface concentrations. Purecoat™ 96 well plates (BD Purecoat™ Amine # 356717, BD Biosciences) were functionalized as described above. To block further protein adsorption, 200 µL/well of 1% w/v BSA solution in PBS was introduced into each well and left to incubate for 90 minutes at 37ºC on a rotary shaker at 90 RPM. Wells were rinsed twice with washing buffer consisting of 0.05% v/v Tween-20 solution

in PBS (#P1379, Sigma-Aldrich, Germany). To detect protein G, a chicken immunoglobulin Y (IgY) anti-protein G was used. This antibody was selected due to the absence of affinity between protein G and the Fc fragment of IgY antibodies: only the antigen binding fragment of the IgY anti-protein G can interact with protein G, which should facilitate quantification of surface ligands. A volume of 100 µL of horseradish peroxidase-conjugated IgY anti-protein G secondary antibody (HRP anti-protein G, OAIA00498, Aviva systems biology) solution (0.02 µg/ml of HRP anti-protein G diluted in rinsing solution with 1% w/v BSA) was incubated in each well for 2 hours at room temperature. Wells were immediately rinsed once with 1% SDS-TRIS solution at pH 11 and twice with washing buffer adding 25 µl of PBS per well. To detect HRP, a volume of 100 µL of Slow TBM-ELISA substrate solution (#34024, Thermo Fisher) was added per well. After 25 minutes of incubation without agitation at room temperature, 100 µL/well of 1M sulfuric acid solution was added and absorbance measurements were taken at 450 nm on a Benchmark plate reader (Bio-Rad, Berkeley, USA).

4.6 Immobilized Antibody Detection and Quantification

Purecoat™ amine surfaces were modified as described above, except that only certain regions of test surfaces were treated with protein G by pipetting 0.5 µL of Cys-protein G solution at concentrations ranging between 0.055 µM and 55 µM. To assess the effect of adsorption on surface amounts of protein G, the spots were deposited on surfaces with (effect of covalent conjugation + adsorption) or without (effect of adsorption) sulfo-SMPB treatment. After 1-hour incubation, surfaces were rinsed with PBS and covered with primary antibody solution for 1 hour as described above. After two washes in PBS, surfaces were covered with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody solution at 20 µg/mL. After 1 hour of incubation at room temperature, surfaces were rinsed twice with 1% SDS-TRIS solution, twice with PBS and twice with RO water before air drying. Spots were then imaged using a laser scanning confocal microscope (Zeiss LSM 5 Exciter, Germany) at 10X with an argon laser (488 nm). A total of 10 images per spot were taken to obtain the mean fluorescence intensity of one spot. A total of 3 spots per replicate were studied to obtain the mean fluorescence intensity of each condition.

4.7 Human Umbilical Vein Endothelial Cells (HUVECs) Maintenance

HUVECs (#CRL-1730, ATCC) were thawed and cultured at 6000 cells/cm^2 in vented tissue culture treated T-25 and T-75 flasks (BD Biosciences) coated with gelatin in EGM-2 complete media (#CC-3162, Lonza, Basel, Switzerland) supplemented with fetal bovine serum (FBS, # SH3039603, Thermo Fisher Scientific) to reach a final concentration of 10% v/v FBS. For each passage, HUVEC cultures at 70–80% confluence in T-75 flask were detached from surfaces with 5 mL of TrypLE (#12604-021, Life Technologies, USA) before being resuspended in fresh EGM-2 complete medium and seeded at 6000 cells/cm^2 (~45 000 cell/mL). Cells were counted manually using a hemocytometer (Bright-Line hemocytometer, Hausser Scientific) after Trypan Blue staining (0.2% in PBS solution, Fisher scientific). The cells were maintained in flasks until passage 4 before being re-suspended in serum-free EGM-2 medium at 250 000 cells/mL for flow experiments (50 000 cells/mL final concentration in the entire flow loop including medium reservoirs) or 10 000 cells/cm^2 for static tests. The EGM-2 serum-free medium consisted in EBM-2 and EGM-2 Bullet kit supplements, except that the serum supplement from the EGM-2 kit was replaced by 0.4% w/v bovine serum albumin (BSA, #A9647, Sigma), 1.0 mg/mL recombinant human insulin, 0.55 mg/mL human transferrin and 0.5 µg/ml sodium selenite (100X ITS supplement diluted to 1X, Thermo Fisher Scientific). Antibiotics (50 units/ml penicillin and 50 µg/ml streptomycin from Thermo Fisher Scientific) were also added.

4.8 Peripheral Blood Mononuclear Cell (PBMC) Isolation

Human mononuclear cells were isolated from the whole blood of healthy volunteer donors. This research study was approved by the Institutional review board (IRB) of McGill University as IRB study number A06-M33-15A and was conducted according to ethical principles stated in the Declaration of Helsinki (2013). A volume of 120 mL of peripheral blood was collected from each donor in two 60 mL sterile Luer-lok™ tip syringes (BD Medical) coated with heparin (Sandoz). After 1:1 dilution in PBS, PBMCs were isolated according to the manufacturer's instructions using eight 50 mL Sep-mate™ tubes (#85450, Stemcell Technologies) pre-filled with 15 mL of Histopaque 1077 density gradient medium (#10771, Sigma Aldrich) and centrifuged at 1200× g for 10 minutes. Supernatants were

transferred into 50 mL conical tubes which were entirely filled with PBS before centrifuging at 300 × g for 8 minutes. Supernatants were discarded, and cell pellets were washed with PBS and pooled together in one 50 mL conical tube before centrifuging again at 300 × g for 8 minutes. The supernatant was discarded and isolated PBMCs were resuspended in CellGenix GMP DC medium (#20801, CellGenix) at a concentration of 2.5 × 10^6 cells/mL for the capture tests under flow (500 000 cells/mL final concentration in the flow loop).

4.9 HUVEC and PBMC Capture Under Flow

To study cell capture by antibody-modified surfaces under flow, functionalized surfaces were introduced into a custom parallel-plate flow chamber system with four independent chambers and flow paths, as previously described [105]. The flow chamber assembly was placed inside an incubator with humidified air maintained at 5% CO_2 and 37 ºC. Each chamber was pre-filled with 40 mL of warm medium and peristaltic pumps (Masterflex RK-7543-02 with Masterflex L/S two channels Easy Load II pump head using L/S 13 BPT tubing) were activated to fill all loops, to test the assembly for leakage and to allow temperature and pH adjustment of the medium before cell injection. After 30 minutes, the perfusion was stopped, and 10 mL of cell suspension, at concentrations previously described, were added to each loop through the injection port to reach a final volume of 50 mL in each loop. The perfusion was resumed for 1 hour and cells were circulated in the flow loop at a flow rate of 0.18 mL/s to obtain 1.5 dyn/cm^2 wall-shear stress. After 1 hour of circulation, cells were fixed using a 3.7% w/v paraformaldehyde solution for 10 minutes, rinsed once in PBS and stored in PBS for immunocytochemistry. Images of suspended ECFCs, CD14- cells, CD14+ cells and HUVECs in cell culture media before attachment were taken with a phase contrast microscope at 10X (Trinocular Inverted Microscope, VWR). Cell diameter means were obtained by measuring the diameter of 20 cells per image for each cell type.

4.10 HUVEC Adhesion Under Static Conditions

For adhesion studies, HUVECs were seeded into 6-well plates at 38 000 cells/mL (10 000 cells/cm^2) on functionalized surfaces in serum-free EGM-2 medium and then transferred to an incubator (humidified, 37ºC and 5% CO_2). The culture medium was pre-incubated in the same incubator prior to cell seeding to allow temperature and pH adjustment. After 3 hours, media were removed, and the surface was gently washed with PBS before fixing samples for 10 minutes in 3.7% w/v paraformaldehyde solution followed by immunohistochemistry.

4.11 Immunocytochemistry and Microscopy

Fixed cells were permeabilized for 15 min with 0.1% Triton X (VWR) in PBS. After rinsing in PBS, surfaces were blocked for 30 minutes using protein block solution (Dako), immediately followed by adding primary antibodies diluted in Antibody diluent (Dako). Primary antibodies used for PBMC capture studies were mouse anti-CD14 (diluted 1:200) and rabbit anti-CD144 (diluted 1:300) or rabbit anti-CD45 (diluted 1:300). Primary antibodies used in HUVEC capture studies were mouse anti-CD31 (diluted 1:200) and rabbit anti-CD144 (diluted 1:300) or rabbit anti-CD45 (diluted 1:300). After overnight incubation at 4ºC, surfaces were rinsed in PBS and secondary antibodies – goat anti-mouse AF555 (1:500) for anti-CD14 and anti-CD31 and goat anti-rabbit AF488 (1:500) for anti-CD144 and anti-CD45 – were added for 1 hour at room temperature. After washing, nuclei were stained with 1µg/ml DAPI (Sigma) diluted in RO water for 10 min. Slides were then rinsed with RO water and stored in PBS before being imaged on an inverted fluorescent microscope (Olympus IX81). Images were acquired at 10X and 20X in phase contrast and fluorescence using DAPI, FITC and TRITC filter sets. A total of 55 phase contrast images were acquired on each slide after cell capture experiment. Fluorescence imaging was performed for the same imaging areas to visualize cell nuclei (DAPI stained), CD14+, CD45+ and CD144+ cells for PBMCs, and CD31+, CD45+ and CD144+ for HUVECs. Captured cells were enumerated by manually counting all cell nuclei in the 55 images.

4.12 Statistics

Statistical analysis was performed with JMP Pro 13 software (SAS Institute, Cary, NC). Results were considered to be statistically significant at p-values<0.05. Unless otherwise mentioned, results represent the average ± standard error of the mean of 3 independent experiments. For water contact angle measurements, the reported values represent the average ± the standard error of the mean of 10 images per surface, replicated three times. For PBMC capture experiments, each experiment was performed with cells from a different donor. Analysis of variance (ANOVA), followed by the Fisher's least significant difference post hoc test, was performed for all experiments except for cell capture study where only the Fisher's least significant difference method with p-values<0.10 was executed.

5 Results

To develop a suitable antibody screening platform for cell capture, the proposed surface modification steps were first characterized, followed by testing the effect of different immobilized antibodies on EPC and monocyte cell capture under laminar flow.

5.1 Characterization of the Purecoat™ Substrate and sulfo-SMPB Activation of the Surface

Commercially-available aminated polystyrene plates were used as substrates to allow compatibility with standard cell culture platforms. Protein G was covalently conjugated onto these surfaces via the amine-to-thiol linking arm sulfo-SMPB. The NHS ester functional group presents on sulfo-SMPB was first reacted with the primary amines on the surface to create a stable amide bond. To confirm surface activation via sulfo-SMPB, the atomic composition of the surface was assessed by XPS. As expected, the nitrogen content decreased after sulfo-SMPB treatment at concentrations as low as 0.5 mg/mL incubated for 2 hours (Figure 5-A). The carbon content increased, and oxygen content decreased after the sulfo-SMPB reaction due to the elemental composition of the linking arm. Noël *et al* [103] demonstrated that the Orange II negatively charged sulfonated dye can be used to detect and quantify amine groups with positive charges. In this article, the dye method was compared to XPS analysis, and a correlation between the dye absorbance in solution after desorption from the surface and the concentration of amine groups detected by XPS was obtained confirming this convenient method for primary amine detection with near 1:1 stoichiometric ratio. The Orange II assay was applied to determine the presence of amino group on aminated Purecoat™ substrate compared to control polystyrene surfaces. As shown in Figure 5-B, the surface concentration of primary amines was significantly higher on the Purecoat™ amine substrates than on polystyrene surfaces from the same vendor. As expected, the primary amine surface density, obtained by the Orange II assay, decreased after applying sulfo-SMPB. No significant difference was observed between the 1 mg/mL and 3 mg/mL sulfo-SMPB solutions, suggesting that 1 mg/L of sulfo-SMPB solution is sufficient to reach saturation.

The static water contact angle with aminated and sulfo-SMPB activated surfaces provides information about changes in the surface wettability due to surface modification. As shown in Figure 5-C, the reaction of sulfo-SMPB with aminated surfaces for two hours at 1 mg/mL or 3 mg/mL increased the contact angle. The decreased surface hydrophilicity observed after sulfo-SMPB treatment is consistent with the decreased surface density of hydrophilic amino groups previously observed by XPS. Results from the XPS, Orange II assay and contact angle measurements are consistent with robust surface activation via sulfo-SMPB applied at 1 mg/mL to 3 mg/L for 2 hours.

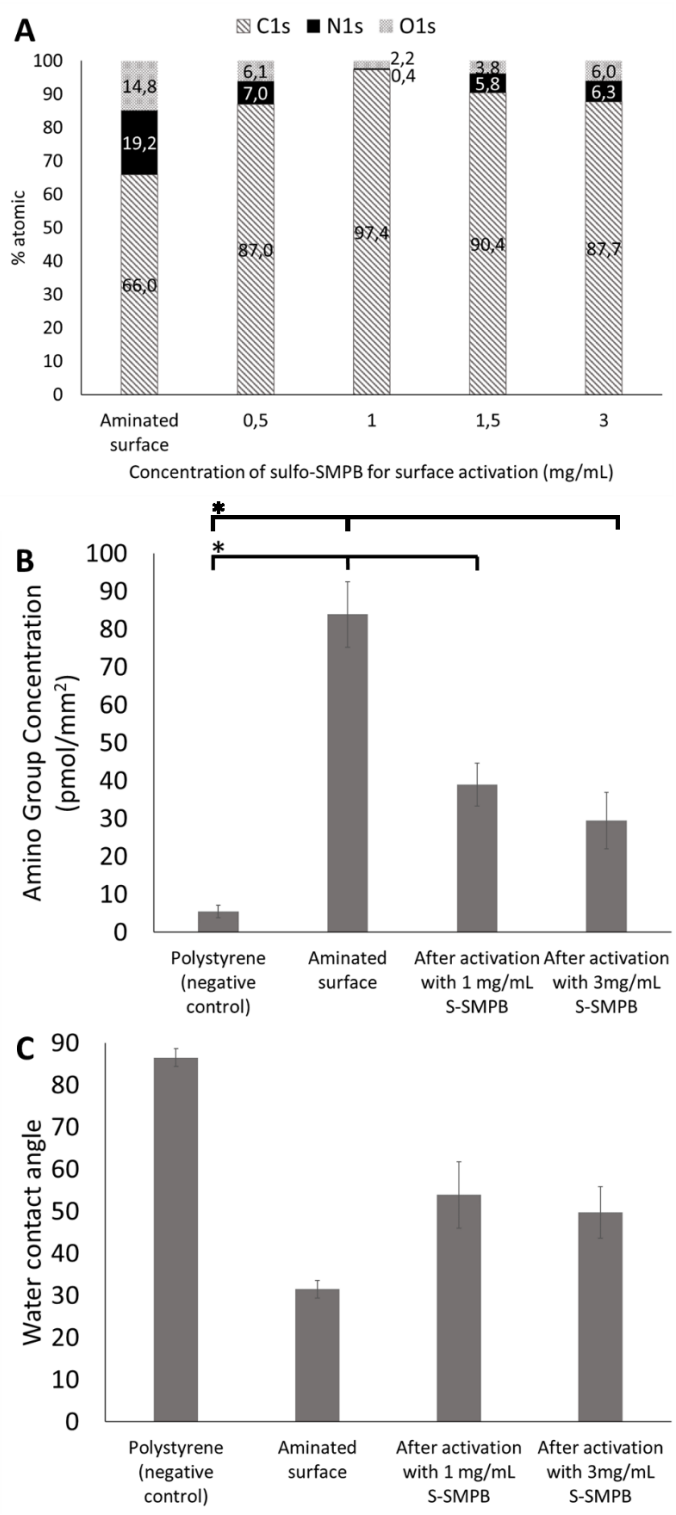

Figure 5. Characterization of the Purecoat™ surface (aminated surface) and sulfo-SMPB activated Purecoat™ surface.
(A) XPS chemical composition analysis, (B) Orange II assays for the amino group density detection, (C) water contact angle. *p<0.05 for polystyrene compared to other indicated groups.

5.2 Maximization of Protein G Grafting Efficiency

Next, Cys-protein G grafting was evaluated with a direct ELISA developed during this project to detect and confirm protein G presence on surfaces. An IgY HRP-conjugated anti-protein G antibody was used to target surface-immobilized protein G. After binding HRP-labeled anti-protein G to the immobilized protein G, a TMB solution (3,3',5,5'-tetramethylbenzidine) was used to detect the horseradish peroxidase activity.

Cys-protein G was grafted on surfaces using the maleimide reactive group of sulfo-SMPB. The added cysteine group on the N-terminal of the protein G provides the sulfhydryl group necessary to form the stable thioether bond with the maleimide group of the surface-grafted linking arm. Adsorbed protein G was also quantified and compared to the amount of grafted protein G. As shown in Figure 6, a positive correlation between protein G in solution and the absorbance signal was observed under covalent conjugation conditions. This correlation was not observed for adsorbed protein G in the absence of the linking arm. This strongly suggests that the covalent conjugation method with the sulfo-SMPB activation improved control over the amount of protein G present on surface compared to simple adsorption methods. Group means were compared by ANOVA using absorbance as a response variable and protein G concentration as the fixed factor, followed by the least significant difference test. This test indicated that absorbance was significantly different between the control, the aminated polystyrene surface without protein G, and the protein concentration at 0.55 μM, 5.5 μM and 55 μM in solution. These results show that sulfo-SMPB activation can be used to graft Cys-protein G in controlled amounts on aminated Purecoat™ surfaces.

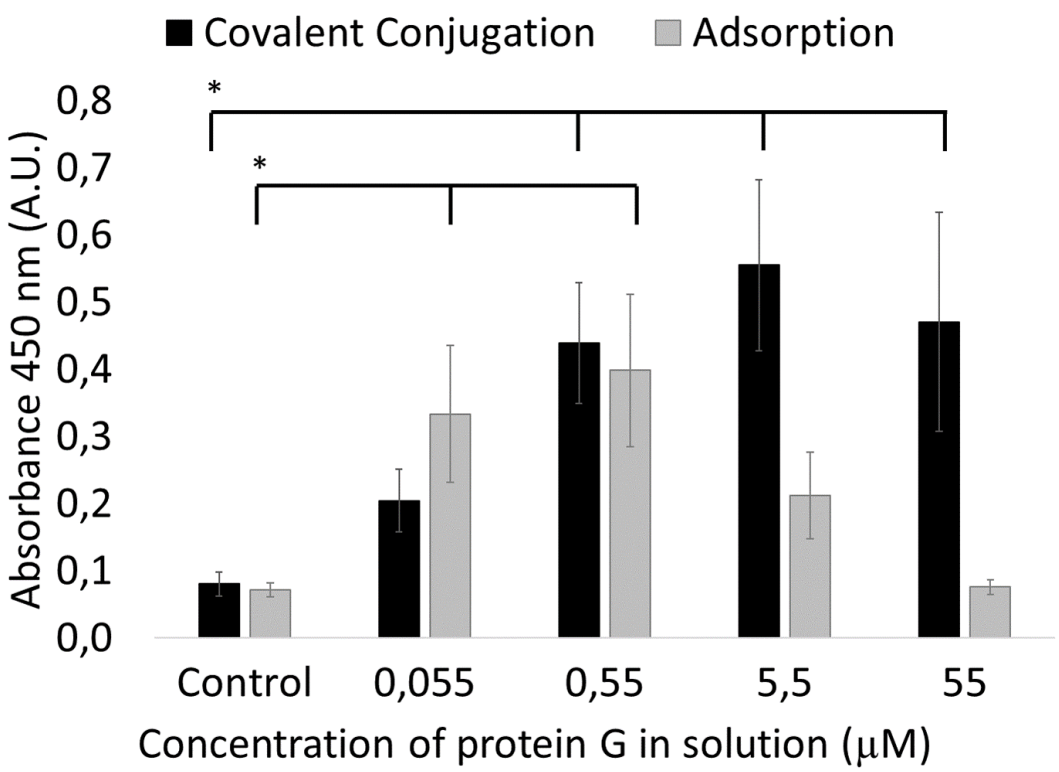

Figure 6. Detection of protein G by direct ELISA.
Covalent conjugation refers to surfaces functionalized with sulfo-SMPB followed by applying Cys-Protein G solutions. Adsorption refers to Cys-protein G solutions applied to aminated substrates without sulfo-SMPB activation. After applying test conditions, Cys-protein G was detected with an HRP-conjugated anti-protein G IgY antibody, followed by incubation in TMB substrate. N=3 independent experiments. *p<0.for controls (aminated substrate without protein G) compared to other indicated groups.

5.3 Antibodies Interact Specifically with Protein G Treated Surfaces

Having achieved covalent conjugation of protein G, the next step was to immobilize IgG antibodies onto the functionalized surfaces. As shown in Figure 7, anti-CD31 antibodies were successfully immobilized on surfaces functionalized with protein G, as detected by a fluorescent (Alexa Fluor 488 conjugated) secondary antibody. The fluorescence intensity increased with the protein G concentration applied to the polystyrene surfaces. This was not observed on surfaces with adsorbed antibodies (data not shown).

These experiments were repeated with four different antibodies targeting EC or monocyte surface markers. As expected, all IgG antibodies; anti-CD31, anti-CD105, anti-CD144, and anti-CD14, were immobilized on protein G and showed a significant difference with the immobilized anti-mouse secondary antibody control, as shown in Figure 8.

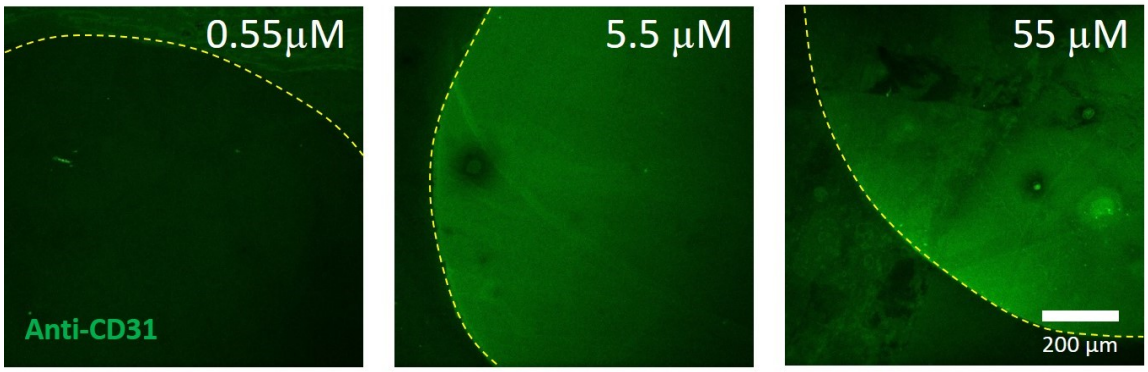

Figure 7. Immobilized anti-CD31 on conjugated protein G spots prepared at different concentrations.
Aminated substrates were completely covered in sulfo-SMPB solution and rinsed before applying Cys-protein G spots at different concentrations. The surfaces were then completely covered with anti-CD31 antibody solutions, followed by goat anti-mouse Alexa Fluor 488 antibody (green). Results demonstrate the specificity of anti-CD31 antibody immobilization on Cys-protein G spots. Fluorescence intensity increased with the protein G concentration used to create the spot on the sulfo-SMPB activated surface.

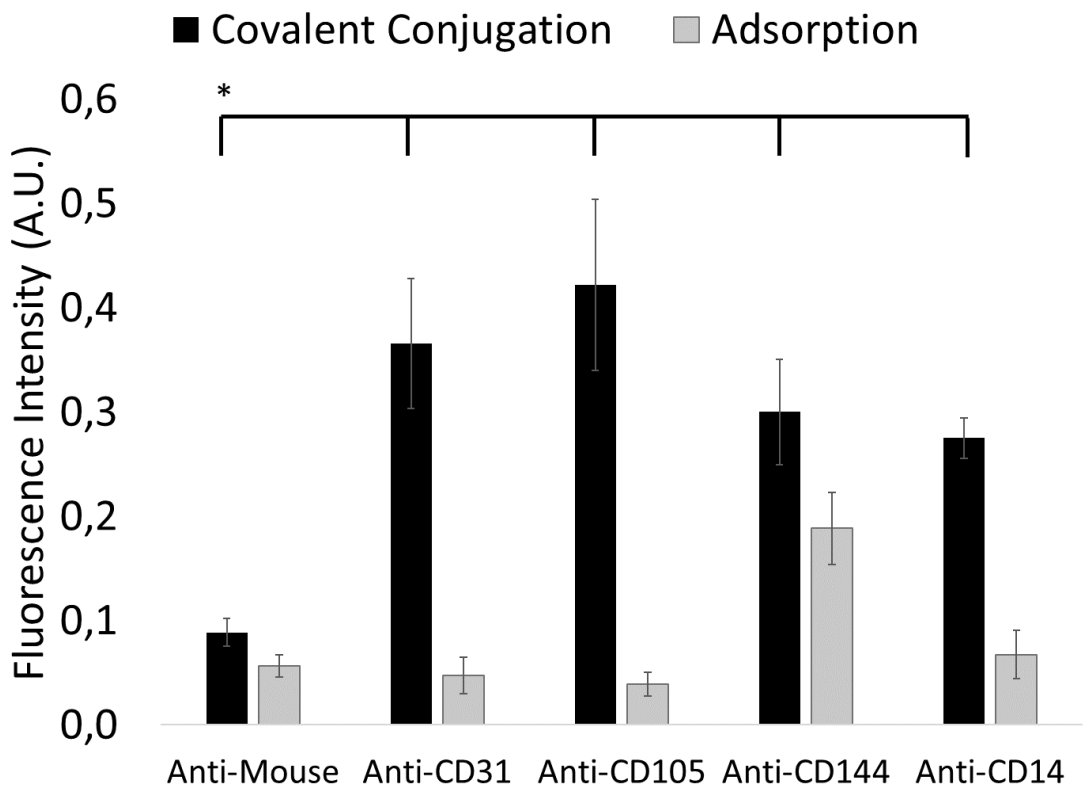

Figure 8. Surface immobilization of antibodies against various endothelial and monocyte cell surface markers.

Aminated substrates with (covalent conjugation) or without (adsorption) sulfo-SMPB activation were reacted with solutions containing 5.5 μM of different antibodies deposited as spots. After washing, the entire surface was immersed in Alexa Fluor 488-conjugated secondary antibodies. No significant differences were observed between antibodies. *p<0.05 for conjugation vs. adsorption for all indicated groups.

5.4 Antibody-Functionalized Surfaces can Capture Circulating Cells

To determine whether antibody-modified surfaces can mediate the capture of circulating cells such as EPCs, the capture of readily-available model cell types was assessed in a parallel-plate flow chamber. HUVECs were used as a first cell model due to their availability from a variety of commercial vendors and broad use in laboratories studying EC biology. HUVECs were injected in the loop of the flow chamber and were allowed to circulate for 1 hour at 1.5 dyn/cm^2 wall-shear stress. After 1 hour, cells were fixed and stained with DAPI before phase contrast and fluorescence microscopy. Surfaces with (1)

adsorbed anti-CD14, (2) adsorbed anti-CD144, (3) immobilized anti-CD14 on conjugated protein G and (4) immobilized anti-CD144 on conjugated protein G were tested in the flow chamber. HUVECs flowing over all functionalized surface were rarely seen to adhere at the end of the test. The minimal cell capture and adhesion on the surface indicate that anti-CD144 antibody did not mediate the capture of HUVECs by their CD144 surface markers in the test conditions selected. Tests with adsorbed anti-CD31 and immobilized anti-CD31 on conjugated protein G were also performed. CD31, also known as platelet endothelial cell adhesion molecule (PECAM), is expressed at higher levels than CD144 by HUVECs and therefore should enhance HUVEC capture. HUVECs were again rarely captured by the anti-CD31 functionalized surfaces. Thus, antibody-functionalized surfaces did not show any potential for the capture of HUVECs cells under laminar flow in the conditions applied (serum-free medium; 1.5 dyn/cm^2 wall shear stress; 60 minutes of flow).

One hypothesis that may explain the low HUVEC capture efficiency of the anti-CD144 or anti-CD31 functionalized surfaces is the large diameter of these cells compared to circulating cell types such as EPCs. As shown in Table 2, the cell diameter of mature endothelial cells such as HUVECs and passage 3 ECFCs is on the order of 24 µm – 26 µm which is significantly higher than the cell diameter of mononuclear cells observed in the CD14+ and CD14- cell population of PBMCs (7 µm – 11 µm). When considering forces acting on flowing cells, inertial effects may predominate over surface adhesion forces for larger cells such as HUVECs.

Table 2. Cell diameter of different cell types

	HUVECs	**ECFCs (Passage 3)**	**CD14+ fraction of PBMCs**	**CD14- fraction of PBMCs**
Cell diameter (µm)	26 ± 4	24 ± 4	11 ± 2	7 ± 3

To test this hypothesis, cell capture studies were conducted with smaller-diameter PBMCs isolated from the peripheral blood of healthy donors. Since the frequency of EPCs in PBMCs is very low, the capture antibodies tested included anti-CD144 (which targets

putative EPC surface proteins) but also anti-CD14 as a positive control. CD14 is a monocyte cell surface marker that is expressed by myelogenic angiogenic cells (so-called "early outgrowth EPCs") [30]. While it is undesirable to capture monocytes for most vascular applications, the relatively high frequency of these cells in the PBMC population compared to EPCs serves as a positive control to demonstrate circulating cell capture efficiency.

PBMCs were injected in the loop of the flow chamber and were allowed to circulate for 1 hour at 1.5 dyn/cm^2 wall shear stress. The test surfaces were (1) adsorbed anti-CD14, (2) immobilized anti-CD14 on conjugated protein G and (3) immobilized anti-CD144 on conjugated protein G. The anti-CD14 antibodies were immobilized on surfaces functionalized with 0.55 μM or 5.5 μM Cys-protein G solution concentrations to determine the effect of antibody surface density on cell capture efficiency. The trends observed in Figure 9, although not statistically significant, are consistent with selective monocyte capture on anti-CD14 functionalized surfaces compared to anti-CD144 when antibodies are immobilized on covalently grafted Cys-protein G. A trend ($p<0.1$) towards higher monocyte capture by anti-CD14 antibodies immobilized on covalently conjugated Cys-protein G (applied at 0.55 μM concentration) compared to adsorbed Cys-protein G (applied at 5.5 μM concentration) was also observed. As shown in Figure 10, the majority of cells captured on anti-CD14 functionalized surfaces expressed the hematopoietic CD45 and the monocyte CD14 cell surface markers, as expected. These markers were not highly expressed by cell captured on surfaces with immobilized anti-CD144 antibodies, indicating the selectivity of the immobilized antibodies. While further replication is required to achieve higher statistical power due to donor-to-donor variability, the proposed directional antibody surface immobilization strategy is a promising avenue to capture circulating PBMCs including EPCs.

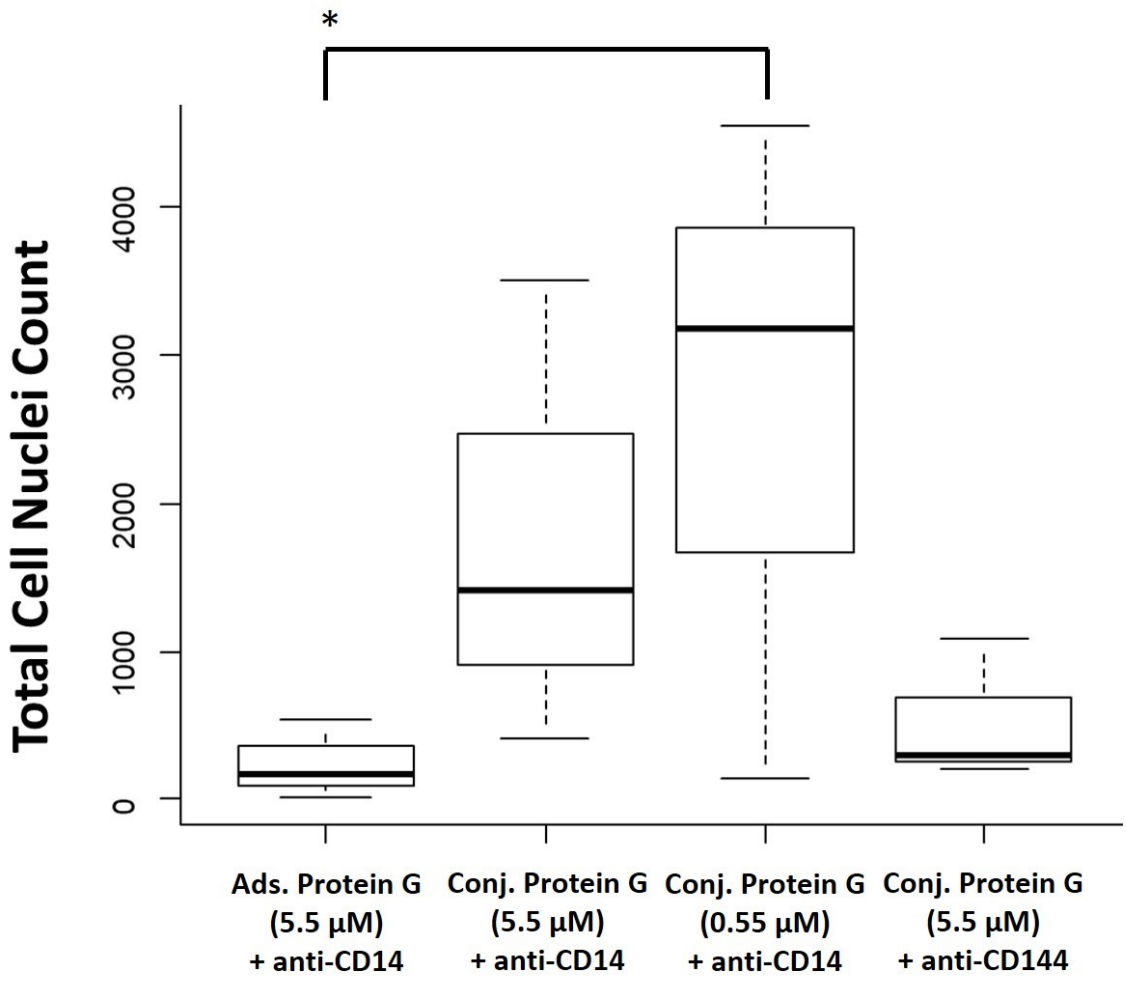

Figure 9. Evaluation of CD14+ monocyte cell capture by antibody-functionalized surfaces. The concentrations indicated represent the concentration of Cys-protein G applied to surfaces with (conj.) or without (ads.) sulfo-SMPB pre-treatment. Monocytes (CD14+ fraction of PBMCs isolated from healthy donors) were circulated at 1.5 dyn/cm^2 for 60 minutes in CellGenix GMP DC medium. Error bars represent the standard error. *p<0.10 for anti-CD14 immobilized on adsorbed Cys-protein G (5.5 µM) versus anti-CD14 immobilized on conjugated Cys-protein G (0.55 µM). N=3 independent experiments.

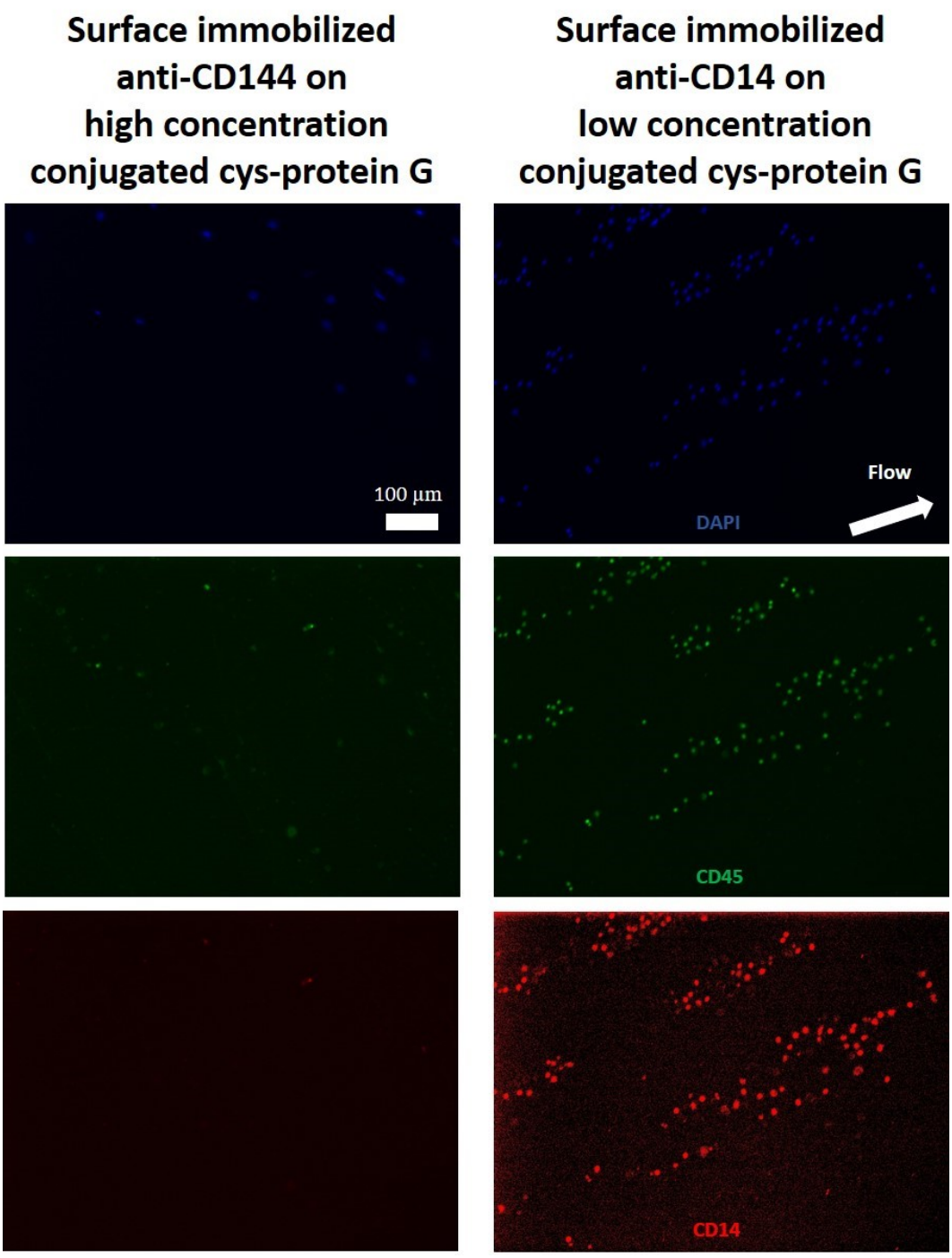

Figure 10. Representative images of mononuclear cells on surfaces with immobilized anti-CD144 and anti-CD14 antibody on conjugated protein G at low (0,55 µM) and high (5,5 µM) concentration.
Nuclei (blue) were stained with DAPI, CD45 hematopoietic marker was detected with a rabbit anti-CD45 primary antibody and an AF488 goat anti-rabbit secondary antibody, and CD14 monocyte marker was detected with a mouse anti-CD14 primary antibody and an AF555 goat anti-mouse secondary antibody.

5.5 HUVEC Adhesion on Antibody-Functionalized Polystyrene Surfaces

In addition to mediating circulating cell capture, the functionalized surfaces should ideally promote or at least not hinder EPC adhesion. To determine whether the functionalized surfaces exert any detrimental effects on this essential step in the endothelialization process, HUVEC were seeded onto test surfaces and left to adhere for 3 hours in serum-free medium in static conditions. The test substrates included unmodified aminated substrates, as well as surfaces with (conjugated) or without (adsorbed) sulfo-SMPB treated with Cys-protein G and then anti-CD14 or anti-CD144 antibodies. No significant differences were observed in the number or morphology of adherent HUVECs after 3 hours between any of the test conditions (Figure 11). The presence of protein G on the surface did not affect HUVEC adhesion compared to Purecoat™ surface, but HUVEC mobility appeared to be higher on surfaces with conjugated Cys-protein G compared to adsorbed Cys-protein G based on qualitative visual assessment. The lack of significant negative effects of the antibody-modified surfaces on HUVEC adhesion suggests that the proposed EPC capture strategy would not hinder their subsequent adhesion.

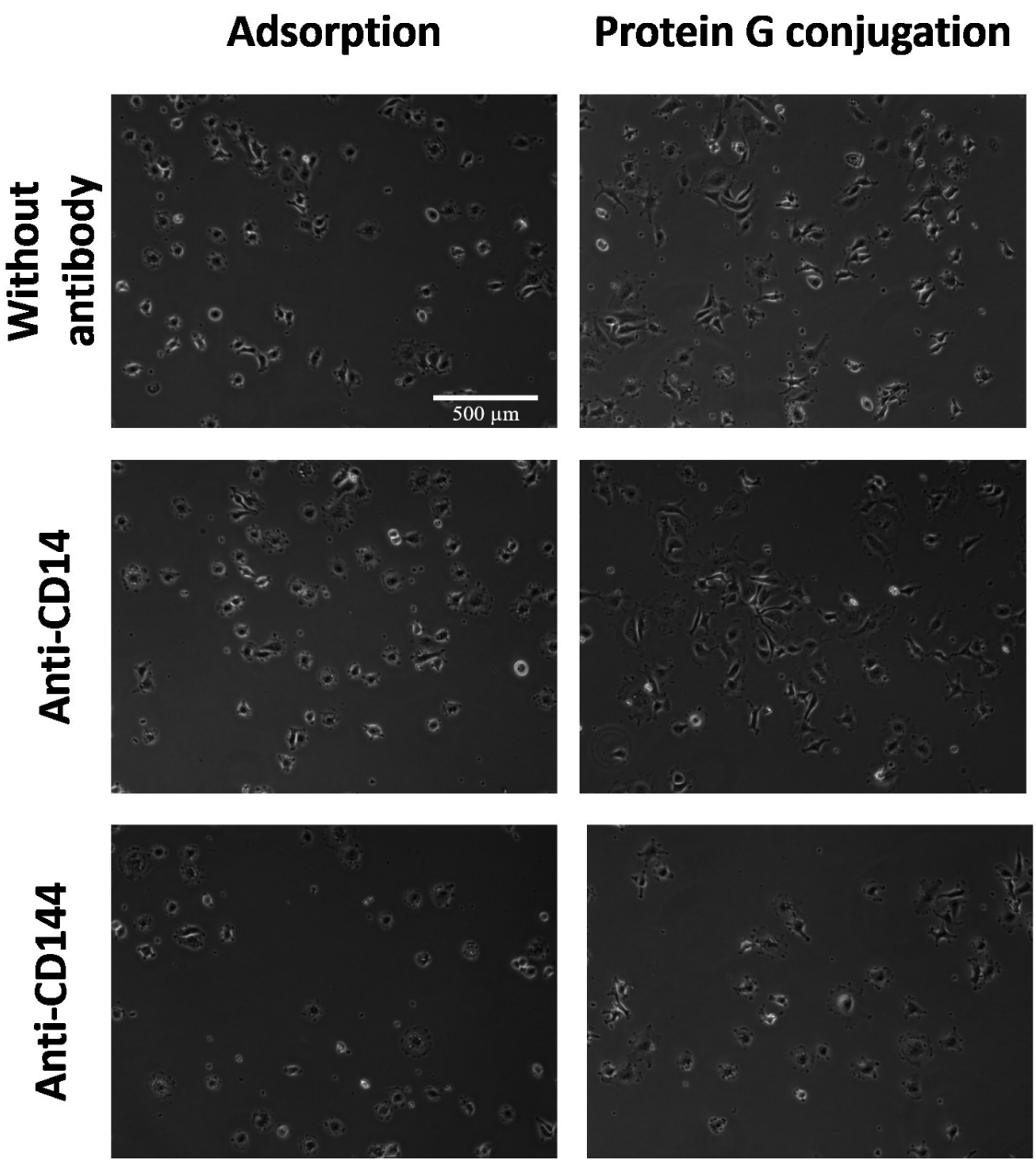

Figure 11. Representative images of HUVECs on antibody-functionalized surface.

Adhesion of HUVECs seeded at 10 000 cells/cm^2 in serum-free EGM2-medium on antibody-functionalized surface is not affected by the presence of either antibody and protein G on surfaces. No significant difference in cell number was observed on all surfaces compared to the Purecoat™ substrate (Adsorption without antibody). This test was performed for 3 hours in static culture. N=3 independent experiments.

6 Discussion

The surface modification of stents with biomolecules such as antibodies is a promising avenue to increase surface endothelialization and improve graft performance. Engineering a surface that promotes endothelial cell adhesion and proliferation while inhibiting immune cell interactions and inflammatory responses remains challenging. Currently, no clinically-approved stent has been found to entirely avoid long-term complications such as restenosis or late in-stent thrombosis. This study presents a versatile directional antibody surface immobilization method that could be applied to existing vascular substitutes such as stents. The method was developed on aminated polystyrene for *in vitro* screening, but it could potentially be applied to any surface with primary amines. The method consists of immobilizing antibodies via the Fc region to surface-conjugated protein G with an N-terminal Cys residue, previously reacted with aminated surfaces via a sulfo-SMPB bifunctional linking arm.

A direct ELISA was developed to detect and quantify protein G conjugated and immobilized on the surface. Using this assay, the surface amount of Cys-protein G was found to increase as a function of the concentration added in solution up to a saturation point, contrary to passive adsorption methods. Different IgG antibodies known to bind endothelial cell or monocyte surface markers were successfully immobilized on conjugated protein G, demonstrating the broad application of this method to a variety of antibodies and cell capturing strategies. To determine the capacity of the surfaces to promote endothelialization, the capture efficiency and adhesion of HUVECs on the surfaces were investigated. Surfaces functionalized with anti-CD144, an antibody targeting a surface marker expressed by HUVECs, did not enable HUVEC capture under flow at 1.5 dyn/cm^2 wall shear stress. The anti-CD144 surfaces did not hinder cell adhesion in static conditions, suggesting that the lack of attachment under flow was due to a lack of cell capture rather than a lack of affinity with the surface or anoikis. The lack of capacity to capture the cells was hypothesized to result from the large cell size of HUVECs, which would lead to significant cell inertia under flow compared to most circulating cells found in blood. To test this hypothesis, cell capture studies were conducted on PBMCs obtained from healthy

donors. The average diameter of cells in suspension in the CD14+ fraction from PBMCs was 11 ± 2 μm, similar to reported values for monocytes which range between 7.7 μm and 10 μm [106]. The average diameter size of CD14- cells isolated from PBMCs was 7 ± 3 μm. Although EPCs that generate ECFCs have never been isolated to 100% purity from peripheral blood, they were shown to reside in the CD14- population [11, 18]. The average diameter of these EPCs is therefore expected to be in the 7 μm diameter size range. By comparison, the diameters we measured for mature endothelial cells such as HUVECs and passage 3 ECFCs as diameters were of 26 ± 4 μm and 24 ± 4 μm, respectively. Considering cell diameter as an important factor for cell capture efficiency, the test with mononuclear cells (CD14+) is expected to be more reflective of the cell capture efficiency of circulating EPCs than the test with HUVECs. These tests show that CD14+ mononuclear cells were specifically captured on surfaces functionalized with immobilized anti-CD14 antibodies. The capture efficiency was not dependent on the protein G surface concentration over the range tested. The results of the mononuclear cells capture studies suggest that circulating EPCs and other small circulating cells of ~10 μm diameter or lower could potentially be captured by antibody-functionalized surfaces. This could help the re-endothelialization of vascular substitutes. This study also represents the first attempt to immobilize cell capture antibodies via covalently grafted protein G onto commercially available polystyrene vessels for cell culture. The platform developed in this work can be applied to a variety of *in vitro* cell capture studies.

Immobilized antibodies were used to specifically target and capture circulating cells expressing endothelial markers such as putative EPCs. The high-affinity interaction of an antibody against a cell antigen is important in this strategy. This interaction can promote the firm arrest of the cells from flow to drive subsequent adhesion to the prosthetic surfaces. Cell capture surfaces coated with anti-CD34, VEGFR-2 and VE-cadherin [14, 17, 66, 107-109] were previously shown to capture cells, but not with high efficacy. The antibody immobilization strategies used in these studies relied on passive adsorption either of the antibody or protein G. Many research groups have shown that directional immobilization of antibodies on surfaces improves the availability of antigen-binding sites as well as antibody-antigen interactions [66, 81, 93]. Moreover, antibody immobilization using

conjugated protein G increased the antibody binding efficiency of protein G and led to a higher antigen detection capability [97]. In this project, the focus has been on the covalent conjugation of protein G to reduce the incidence of protein denaturation, control the concentration of the protein grafted on the surface and to further immobilize antibodies in an oriented manner.

To perform the covalent conjugation of protein G, the surface was activated with sulfo-SMPB, an amine-to-sulfhydryl linking arm. This linking arm activates the surface, at slightly alkaline conditions (pH 7.2–8.5), by creating a stable amide bond between a primary amine on the surface and an active NHS-ester group. On the opposite side, during the second reaction step, the maleimide reactive group reacts, by a click reaction under near neutral pH conditions, to a reduced sulfhydryl to create a stable thioether linkage. The specificity of these reactions is only ensured at neutral pH. Under alkaline conditions (pH>8.5), maleimide groups will favour a reaction with primary amines or be hydrolyzed which will create maleamic acid [9, 110, 111]. Here, PBS buffer (pH 7.2-7.4) was used in this reaction to maintain the appropriate reaction condition and favour protein conjugation. The mild conditions (physiological pH, room temperature, aqueous conditions) of this surface modification step assures compatibility with a wide variety of cell culture substrates and biomaterials.

In addition to the formation of a stable bond between the biomolecule and the substrate surface, the sulfo-SMPB linking arm provides a spacer arm of approximately 11.6 Å, which allows molecule movement, reduces conformational changes that can be induced by steric hindrance and leaves bioactive binding sites available. Sulfo-SMPB is a versatile linking arm that can be used on different materials with aminated surface, for example, polytetrafluoroethylene (PTFE) [9], glass [111], poly (L-lactide (PLLA) and poly (ε-caprolactone) (PCL) [112]. However, to allow the formation of thioether bond, thiol groups should be reduced to allow reaction with maleimide. As thiols in solution tend to oxidize, biomolecules may tend to form disulfide bonds (S-S) in solution instead of reacting with the sulfo-SMPB. Higher concentrations of biomolecules with free thiols are expected to increase this effect, potentially explaining the trend towards lower protein G surface densities observed at high Cys-protein G concentrations added in solution (Figure 6). In

this context, the reaction environment and conditions should be extremely controlled, and fresh solutions of linking arm and protein should be prepared before each experiment to limit disulfide bond formation.

The stability of protein G surface conjugation was demonstrated by the resistance of the surface coatings to harsh washing steps (SDS in pH 11 TRIS buffer). SDS is a strong anionic surfactant which interacts with the protein structure and can neutralize cationic charges that are involved in protein adsorption. SDS is expected to disrupt weak protein-surface interactions and remove most weakly adsorbed or attached proteins. However, this method does not quantify binding forces involved in antibody immobilization or protein G grafting. Assessing these forces would be useful in comparing antibody surface affinity to other biomolecules which may compete with the immobilized antibodies once the surfaces are in contact with biological fluids such as blood. Quartz crystal microbalance with dissipation monitoring (QCM-D) could potentially provide information on the binding energy and conformation of proteins on surfaces [77, 113]. The binding energy could also be measured more directly with nanotweezers or by attaching test proteins (e.g. antibodies) to the tips of atomic force microscopy (AFM) cantilever arms [114, 115].

Surface conjugation via sulfo-SMPB led to better control over protein G surface density compared to protein G adsorption. When protein G was covalently grafted to surfaces, surface saturation was reached at approximately 0.55 μM protein G solution concentrations. Conversely, protein G adsorption was maximal at 0.55 μM, followed by decreased surface densities at higher concentrations. Following the Langmuir adsorption isotherm, the expectation was that the concentration of protein adsorbed on the surface reach a plateau when the first layer of protein occupied all available adsorption sites on the sorbent surface. As mentioned previously, a possible explanation for this trend is the ability of free cysteines on Cys-protein G to interact with each other in solution to form disulfide bonds which can produce dimers. The conformation or the larger diameter of these dimers may reduce surface affinity or packing density. Another explanation is related to the intermolecular interactions of protein G in solution. Higher concentration of protein G in solution will increase the solution potential energy, which may favour adsorbed protein G denaturation and spreading on the surface. Denatured protein G will modify the surface

energy and potentially reduce the quantity of protein G that can be adsorbed [116]. Therefore, the conjugation scheme with the sulfo-SMPB linking arm allowed better control over protein G surface density.

Next, IgG antibodies specific to different circulating cell types, including EPCs (CD144+CD105+CD31+ cells) and monocytes (which are CD14+), were immobilized to protein G functionalized surfaces. Protein G binds to all human and mouse IgG antibody subclasses via their Fc fragments while antigen-binding sites remain free to interact with cell surface proteins. This directional antibody surface immobilization strategy was selected to maximize the immunoaffinity of the antibodies compared to adsorption methods which can result in random orientation and reduced availability of antigen-binding sites. The four Fc fragment binding sites available on each protein G molecule can further amplify the number of antibodies and hence antigen-binding sites available to capture cells. The versatility of the antibody immobilization method by protein G is a good tool to screen different types of antibodies for cell capture. Once the protein G grafting is optimized, different IgG antibodies can be immobilized on the surface and tested for cell capture allowing fast screening of antibodies with high affinity to the targeted cells.

The major obstacle hampering the use of protein G in clinical applications is the possibility of provoking undesirable host immune responses [88]. In the case of vascular grafts, restenosis and thrombosis can be accelerated and induced through inflammation, which can lead to graft failure. Further development of antibody immobilization strategies via protein G for *in vivo* use will require further hemocompatibility and immunogenicity studies. Other ligands that can be used to replace protein G to immobilize antibodies are Fc-binding peptides, such as RRGW, HWRGWV and FYWHCLDE [88, 117-120].

While antibody binding to protein G should theoretically lead to directional immobilization, the orientation of antibodies should be confirmed in future studies. Directional antibody immobilization is expected to lead to different nanoscale topographies than random adsorption, which could be confirmed by techniques such as AFM. Well-oriented antibody functionalized surfaces tend to be rougher and present higher peaks in the general surface topography than surfaces with random antibody conformation [91, 121, 122].

The cell capture efficacy of the antibody functionalized surface was first investigated by flowing HUVECs over test substrates in parallel-plate flow chambers under uniform laminar flow. These experiments were conducted at a wall shear stress of 1.5 dyn/cm^2, which is much lower than physiological arterial flow values. These conditions were selected based on previous studies describing optimal flow conditions for cell capture *in vitro*. However, once appropriate capture antibodies have been identified in these conditions, the WSS level could be increased to study the effects of inertia and WWS on EPC capture efficiency.

HUVEC capture efficiencies were very low both with CD144 and CD31 capture antibodies. The low capture efficiency of HUVECs was correlated with the significantly larger diameter of these cells compared to PBMCs. Further cell capture tests were performed with PBMCs to investigate the impact of cell diameter on capture efficiency. As demonstrated, small CD14+ mononuclear cells were specifically captured on anti-CD14 functionalized surfaces. This illustrates the feasibility of arresting small circulating cells of ~10μm diameter on surfaces via antibodies targeted towards specific cell populations. These observations suggest that inertial forces may predominate over surface interaction forces for larger circulating cells. It is possible that optimizing the functionalization technique and the use of a high-affinity antibody against targeted cells would allow the capture of larger cells such as HUVECs or mature ECFCs.

The cell capture studies demonstrated that IgG antibodies immobilized on polystyrene surfaces via protein G retain their bioactivity. Preliminary observations indicate that inertial forces of flowing cells likely act as an important factor affecting successful cell capturing on the surface. With the current platform, cells with a diameter over 10 μm were not captured on surfaces functionalized with antibodies known to target surface antigens of the flowing cells. Conversely, mononuclear cells (CD14+) of smaller diameter were captured on anti-CD14 functionalized surfaces, demonstrating the feasibility of engineering cell capture strategies via antibody immobilization on surface-conjugated protein G. The development of this method to capture cell under flow represents a significant advancement towards improving EPC capture stent technologies.

7 Future Directions and Conclusions

7.1 Recommendations for Future Work

To continue this study, the capture of ECFCs under flow on surfaces functionalized with endothelial cell markers, e.g. with anti-CD31 and anti-CD144 antibodies, is recommended. As ECFCs are generally smaller than HUVECs, this test will demonstrate the feasibility of targeting circulating endothelial cells present in peripheral blood and reinforce the role played by inertial forces in cell arrest. ECFC capture tests will complete the picture of the EC capture ability of the anti-CD31- and anti-CD144-functionalized surface.

Another important factor to test is the subsequent expansion of the captured cells under flow, which is required for adequate and long-term surface endothelialization. An incomplete EC layer can lead to vascular graft failure. Studying the growth rates of ECFC or mature EC on different functionalized surfaces is crucial in selecting adequate surface modification strategies. The immobilized antibodies or other EPC capture strategies should ideally promote subsequent EPC attachment, proliferation and differentiation into a confluent and functional EC layer.

Coating stability under flow or in contact with blood is another important aspect to investigate. Covalent conjugation of protein G provides an anchor to the substrate conferring better stability of the surface coating compared to the adsorbed protein method, as shown with the SDS-TRIS washing test. However, this study does not demonstrate the stability of the coating under complex conditions such as the *in vivo* environment, where non-specific protein adsorption can occur. Proteins present in blood serum, such as albumin and fibrinogen can interact with the antibody coating and alter the desired cell capture effect. To study this effect, functionalized surfaces can be placed in contact with cell culture media supplemented with fibrinogen or blood in the flow chamber. Fluid flow on the surface can cause antibody detachment or undesired protein adsorption which can compete with the immobilized antibody. These events can reduce EC capture efficacy and must be studied prior to any *in vivo* cell capture tests.

The grafted protein G-functionalized surfaces can be used as a versatile platform to test a variety of IgG antibodies against EPCs. As the method is well characterized, many

antibodies can now be screened. Mixtures of antibodies could be applied to potentially improve selectivity towards EPCs. Alternatively, the platform can be modified and used for other applications, such as the development of microfluidic devices to capture specific proteins, enzymes or cells. Such devices are already developed as biosensors and diagnostic of diseases such as AIDS and cancer [123-125]. In the case of cancer, circulating tumour cells can be captured directly from blood flow and analyzed to optimize patient-specific treatments [126-128]. In this context, the platform developed in this study could be optimized to capture specific antigens from complex mixtures of cells and proteins such as blood.

The surface modification strategy developed in this work can be applied to other substrates with amino groups such as plasma-treated metal or PLA, two materials used to produce vascular stents. This characteristic improves the versatility of this functionalization technique to produce a cell capture surface. Moreover, modification of the linking arm can be achieved to extend the spacer arm between the two functional groups. These modifications can optimize antibody interactions with cell by reducing the steric hindrance effect cause by neighbouring antibodies or substrate proximity.

In the case of the further development of vascular biomaterials, the cell capture surface developed requires advanced tests to understand immunological reactions to the presence of protein G and antibodies on the surface. Pro-inflammatory signals need to be avoided at all cost on vascular biomaterials to extend the life of prostheses. In this context, hemocompatibility testing of the surface is recommended to assess the possibility of adverse effects that can be caused by leukocytes, platelets, or serum proteins.

Moreover, many options can be considered to improve EPC adhesion, proliferation, and differentiation on the surface. First, as introduced earlier, Fc-binding peptides can be used instead of protein G to immobilized antibody on surfaces while allowing oriented immobilization. This technique, like protein G, will help to keep both antibody antigen-binding site available. Another approach that can improve EPCs adhesion and differentiation is the combination of antibodies with ECM-derived peptides. This combination method will allow the capture of the targeted cell by the antibody while the peptide will favour cell spreading and differentiation.

Furthermore, inertial forces of cells under flow need to be assessed to determine the real capacity of the cell capture surface. To do so, tests with different bead sizes or weights can be performed to determine the critical size that limits the cell capture property of the functionalized surface. Moreover, the specificity and the limits of the cell capture surface can be tested with different cell types such as mature endothelial cells and other small mononuclear cells, such as monocyte and leukocytes. To confirm the cell capture specificity, a heterogeneous population of cell such as a mixture of one endothelial cell type (HUVECs or ECFCs) and one mononuclear cell type, such as monocytes or leukocytes, in a known concentration, could be used. The selective attachment of one cell type on the functionalized surface from a mixture of cells would represent a clear progress in the cell capture technology. Finally, all modified surfaces could be used in the parallel plate flow chamber to screen all chosen capture antibodies and molecule combinations to capture a specific cell type. The flow chamber is an ideal tool to test the cell capture property of a surface by allowing the circulation of cells in an environment where the flow, the pressure, the temperature, and the wall-shear stress are under control. This tool allows the assessment of the cell capture by the functionalized surface while minimizing the impact of external factors that can affect cell behaviour.

7.2 Conclusions

Cardiovascular diseases are the main cause of mortality in the world, leading to serious impact on patients and healthcare systems. Currently, vascular grafts and stents are used to bypass blocked arteries and reopen narrowed blood vessels, respectively. These vascular prostheses are improving patients' quality of life; however, they are still subject to complications such as restenosis and thrombosis that can lead to repeated revascularization procedures, other medical interventions, and in some cases mortality. One reason to explain graft failure is the lack of endothelial coverage on the prosthetic surface. The patency rate of prostheses could be increased if the inner surface of the prostheses is covered by the natural endothelial cell layer present in healthy arteries. This layer provides signals to the surrounding cells in order to maintain vessel homeostasis and anti-thrombogenic properties of the arterial wall. Several strategies were developed to promote EPC adhesion on vascular grafts after their implantation. Many groups have functionalized surfaces with ECM

proteins, ECM-derived peptides, and antibodies with the objective of capturing and enhancing EPC adhesion and proliferation on vascular prostheses. However, functionalized surfaces continue to demonstrate incomplete endothelialization and result in incidences of thrombosis and neointimal hyperplasia. The necessity to target endothelial cells with specific biomolecular interactions is becoming important for the development of vascular grafts with long-term patency. In this context, the development of a vascular graft surface that can specifically capture EPCs from flow is crucial. The use of antibodies to improve the surface re-endothelialization would allow the selective capture of cell types that favour regeneration such as EPCs by using unique antigen-antibody interactions. With that objective, this work has focused on the development of a versatile platform to immobilize antibodies on a substrate that can be used for *in vitro* analysis of EPCs capture. Specifically, this work shows that:

1. Aminated polystyrene substrates can be activated with the sulfo-SMPB linking arm to further covalently conjugate the IgG-antibody-binding protein G in a controlled manner.
2. Using this method, different IgG antibodies can be immobilized on conjugated protein G leading to higher surface densities of immobilized antibodies compared to antibody immobilization onto adsorbed protein G.
3. HUVEC adhesion and morphology were not affected by the presence of the protein G or the antibodies on the surface.
4. HUVECs were not captured under flow by anti-CD144 or anti-CD31antibody functionalized surfaces, but mononuclear cells (CD14+) were captured by anti-CD14 functionalized surfaces.

A versatile platform was designed in order to facilitate antibody screening to improve EPC capture from flow and study EPC adhesion, proliferation, and differentiation. The aminated polystyrene Purecoat™ substrate was functionalized to immobilize antibodies. Polystyrene is a well-known and widely used material in cell culture due to its excellent optical clarity, and ease to mold in various shapes. Moreover, polystyrene can be sterilized by irradiation and its surface can easily be modified to improve cell culture conditions. Protein G was covalently grafted onto these surfaces and an ELISA detection method was developed to

quantify protein G surface density. The covalent grafting method was compared to the protein G adsorption techniques and covalent grafting showed better control of protein G concentration on the surface. The four Fc binding site available on protein G were used to immobilize IgG antibodies in an oriented manner to keep antigen-binding sites available. Different types of IgG antibodies were successfully immobilized on surface-conjugated protein G and were in higher concentration on the surface compared to antibody immobilization on surfaces with adsorbed protein G. The antibody-modified surfaces did not hinder HUVEC adhesion or change their morphology. The cell capture potential of antibody-modified surfaces was next tested under laminar flow with uniform wall shear stress (1.5 dyn/cm^2). Surfaces functionalized with anti-CD144, or anti-CD31 antibodies did not significantly enhance HUVEC capture compared to controls, while CD14+ cells were successfully captured from PBMCs using anti-CD14 functionalized surface. These studies suggest that PBMC populations, potentially including EPCs, can be captured using the proposed directional antibody immobilization surface modification scheme.

The development of a versatile method to successfully study cell capture on antibody-functionalized surfaces under flow represents a significant advancement towards the development of biomimetic vascular grafts. This method will allow further antibody screening to determine the appropriate markers that can efficiently capture EPCs under flow. This work represents a step forward towards therapeutic interventions that can significantly improve the quality of life for patients with cardiovascular diseases. Moreover, this versatile platform can easily be modified and applied to other applications such as disease diagnostics and cell isolation. The developed method to covalently graft protein G and immobilize IgG antibody represents a stable and robust platform which can be optimized and used in many biomedical applications.

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