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Review Article

Surface Functionalization and Plasma-Based Approaches in Microfluidic Models of Leukocyte Adhesion in Atherosclerosis

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Abstract

Atherosclerosis is a chronic inflammatory disease characterized by endothelial dysfunction, with leukocyte adhesion primarily driven by interactions between P-selectin and PSGL-1, which play a crucial role. Capturing these rolling interactions under physiological shear stress is essential for understanding disease progression. However, traditional *in vivo* models and static assays often fail to replicate the dynamic blood flow conditions. Microfluidic platforms have become valuable tools, enabling real-time studies of leukocyte behaviour under precisely controlled flow environments. A major challenge remains achieving stable and functional protein immobilization on microchannel surfaces. This review thoroughly examines both covalent and non-covalent surface functionalization techniques designed to incorporate reactive chemistries that boost ligand retention, reduce detachment, and maintain biological activity. When combined with protein micropatterning, these methods allow spatial control over adhesion molecules, better mimicking the complex heterogeneity of endothelial surfaces. Despite their promise, current approaches still face issues with reproducibility, long-term stability, and application in complex biological systems, such as live-cell rolling assays or synthetic leukocyte models using nanoparticles. Nonetheless, plasma-assisted microfluidic platforms present an exciting, largely unexplored opportunity for high-fidelity modelling of cardiovascular inflammation and leukocyte–endothelial interactions.

Keywords: Leukocyte Adhesion, Microfluidics, P-Selectin, PSGL-1, Endothelial Dysfunction, Plasma Surface Modification, Atherosclerosis.

Introduction

Atherosclerosis is a key example of cardiovascular disease, where fats, cholesterol, and other materials build up as plaque on the inner walls of arteries [1]. This plaque buildup triggers chronic low-grade inflammation and endothelial dysfunction, causing diseases like myocardial infarction (heart attack) and stroke [2]. Risk factors such as aging, high cholesterol, smoking, and hypertension damage the endothelium, activating the immune response. Immune cells such as mast cells, macrophages, and endothelial cells release signals that attract leukocytes to inflamed areas [3]. Nitric oxide (NO) produced by endothelial cells relaxes the muscles of blood vessels, thereby widening them and increasing blood flow. This lowers blood pressure and improves oxygen and nutrient delivery. As an endothelium-derived relaxing factor (EDRF), NO plays a crucial role in maintaining vascular health, thereby preventing hypertension and atherosclerosis. Shear stress influences the endothelial response to vasodilation by stimulating NO release through endothelial nitric oxide synthase (eNOS) [4]. In atherosclerosis, an imbalance occurs between the production and consumption of NO, with LDL and other factors accumulating. This causes abnormal endothelial activation, particularly in bifurcations, which reduces shear stress and increases turbulence. This inflammation leads to fatty streaks—early signs of atherosclerosis with lipid buildup in cells, progressing to increased vessel permeability, platelet and leukocyte activation, adhesion, and further inflammation, which promotes plaque formation and arterial damage (Figure 1) [5].

Leukocyte Migration in Inflammation

Leukocytes reach injury sites via postcapillary venules through extravasation, starting with rolling under chemical signals such as TNF-α, IL-1, or LPS, which are triggered by adhesive events and shear forces from the damaged endothelium. Rolling speed depends on blood flow and bond dynamics. Molecular interactions, especially bond breaking and reforming, are key to rolling [6].

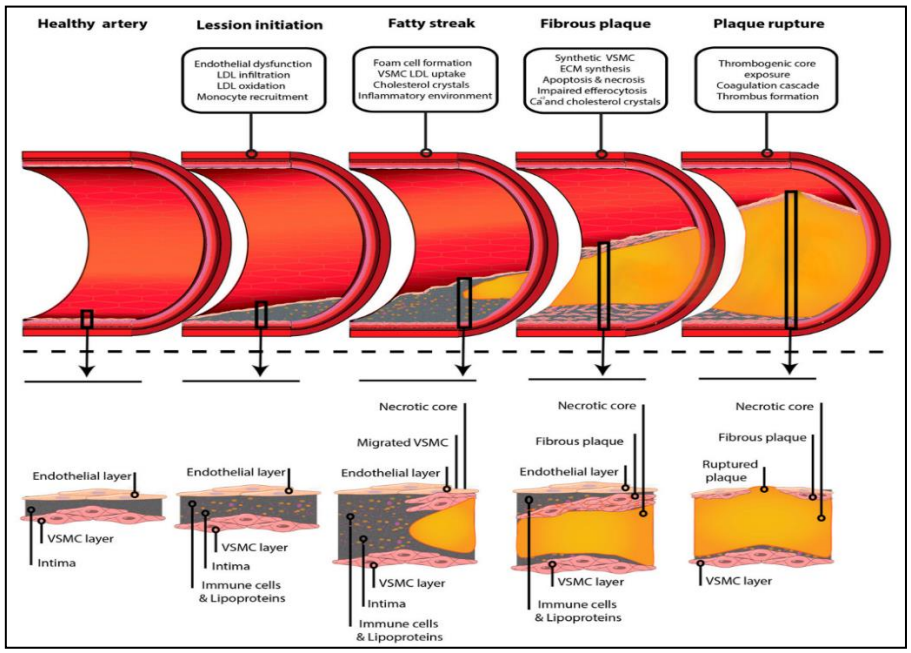

Figure 1. Development of atherosclerosis - a healthy artery on the left gradually accumulates fatty deposits, leading to narrowing on the right [5]

Selectins, glycoproteins on cell membranes, enable leukocyte rolling through adhesion, allowing neutrophils or cancer cells to bind to ligands such as glycans on blood vessel walls. These calcium-dependent bonds are C-type lectins, which are classified into three main types: E-, P-, and L-selectin, found on various cells. They recognize carbohydrates, such as sialyl Lewis X, on leukocytes and their receptors. P-selectin, on the endothelium, initiates leukocyte rolling, extending 40 nm with a lectin, EGF domain, and nine CR repeats [7].

While several reviews, such as those by Andersen *et al.* (2015) and Singh *et al.* (2024), have summarized leukocyte adhesion and microfluidic approaches, they mainly focus on general designs or molecular interactions without emphasizing surface functionalization strategies. This review, however, specifically concentrates on plasma-based surface functionalization techniques used in microfluidic models and their effects on leukocyte adhesion under flow conditions. It also presents recent findings on protein immobilization stability, micropatterning techniques, and offers a systematic comparison between covalent and non-covalent binding methods – the topics that have not been thoroughly covered before. This approach provides a current, targeted perspective that connects surface chemistry, microfluidic design, and leukocyte biomechanics, offering valuable insights for both basic research and translational applications.

Modeling Leukocyte Rolling In Vivo

Investigating leukocyte rolling *in vivo* is complex due to the dynamic nature of the vascular environment, which is affected by some factors such as blood pressure, endothelial integrity, and systemic inflammation. Intravital microscopy enables the observation of leukocyte behaviour across organs, revealing the geometry of blood vessels, flow patterns, and the dynamics of leukocytes.

However, each tissue presents unique challenges: the mesentery can exhibit artifactual rolling from surgical exposure; skin chambers may alter vascular function; the liver's sinusoids can obscure interactions; the lung's capillaries are hard to access and may involve different adhesion mechanisms; and in the brain, leukocytes may roll on platelets instead of endothelium due to the absence of P-selectin [8, 9]. To address these challenges, researchers utilize *in vitro* models to enable precise control of shear stress and cellular interactions (shown in Table 1), thereby eliminating surgical and tissue artifacts and facilitating a clearer understanding of leukocyte-endothelial dynamics. Table 1 summarizes these models, showing their design, uses, advantages, and limitations. Static models are easy to use and allow high-throughput testing but lack *in vivo*-like flow. These traditional chemotaxis assays have limitations that restrict their clinical use. They offer poor control over chemokine gradients, rely solely on isolated neutrophils, and require large blood volumes and extensive processing, which can alter cell phenotypes and compromise the reliability of migration measurements.

Transition to Microfluidic Models in Leukocyte Adhesion Studies

Microfluidic chips offer a powerful alternative to traditional leukocyte adhesion assays, integrating laboratory processes into compact platforms that reduce reagent use, accelerate reactions, and facilitate high-throughput analysis. Made from materials such as PDMS, glass, or PMMA, they feature microscale channels (10–100 μm) with fluid flow controlled by inlets, outlets, and external pumps or valves.

Table 1. *In vitro* models (static and flow-based) used for leukocyte studies

Model	Static/Flow	Design	Application	Advantages	Disadvantages	Ref.
Boyden Chamber	Static	Two chambers with a porous membrane	Chemotaxis	Simple, high throughput, widely used, maintains a controlled environment, and is cost-effective.	Manual handling, poor stability, limited imaging, and variable cell density.	[10, 11]
Zigmond Chamber	Static	Glass slide + coverslip with narrow gap	Gradient Chemotaxis	Easy gradient setup, simple design, and better control	Transient gradients, hard to control accurately.	[12]
Dunn Chamber	Static	Glass slide with two concentric wells	Chemotaxis	Stable gradients, compatible with live cell tracking	Low throughput, limited temporal resolution, and automation	[13]
Micropipette	Static	Manual gradient creation using a micropipette	Chemotaxis	Simple and low cost	Low precision; poor reproducibility.	[14]
Parallel Plate Flow Chamber	Flow	Two flat plates with a gasket and a syringe pump	Rolling and adhesion	Real-time imaging, controlled shear, easy setup.	Low throughput, high reagent use, leakage, slow setup, and flow issues.	[15]
Multi-well plate microfluidic system	Flow	Microfluidic chip in standard well-plate format, uses air-pressure pumps for flow control.	Rolling, adhesion, and chemotaxis	High-throughput shear flow, automated multiplexed assays, compatible with readers and imaging.	Complex operation, air bubble issues, high cost, and calibration required.	[16]
Capillary Tubes	Flow	Glass or plastic tubes, like blood vessels, often have an endothelial coating.	Leukocyte rolling and adhesion	Cost-effective, simple setup, ideal for basic assays.	Limited to low shear rates, lacks complexity.	[17]
Microfluidic Flow Chambers	Flow	Microchannels simulate blood vessel conditions.	Leukocyte rolling, adhesion, and transmigration	Precise shear control, physiological relevance, and high throughput.	Complex fabrication, expensive equipment, and limited mimicry.	[18]

Formats such as continuous-flow, droplet-based, and paper-based systems enable real-time observation of leukocyte behaviour under physiological shear stress (1–10 dyn/cm^2), using minimal sample volumes. These devices enable precise control over ligand density, flow rates, and channel geometry, allowing for the replication of vascular microenvironments associated with inflammation and disease. Surface functionalization is key: while non-covalent methods are standard, covalent attachment provides greater protein stability under flow. This review uniquely compares covalent and non-covalent strategies; highlights advance in plasma-assisted micropatterning and addresses translational challenges in leukocyte rolling assays. Together, these approaches enhance ligand presentation, mimic endothelial heterogeneity, and improve the physiological relevance of *in vitro* models. [19, 20].

Surface Functionalization Methods in Microfluidic Systems

Surface functionalization is crucial in microfluidic devices for simulating cellular interactions; however, common substrates such as PDMS, glass, and thermoplastics lack the necessary biochemical properties for stable attachment of biomolecules. Surface treatment enhances hydrophilicity, reduces nonspecific binding, and allows for immobilization of adhesion proteins. While non-covalent methods, such as biotin–streptavidin and antibody–antigen interactions, are widely used, covalent immobilization is preferred in high-shear or prolonged-flow conditions, offering superior mechanical stability and long-term performance. Covalent attachment also enables precise control over ligand density, orientation, and spacing, all of which are crucial for maintaining the native protein conformation and ensuring functional binding (Figure 2). To optimize these surface modifications, advanced characterization techniques such as atomic force microscopy (AFM) are employed to analyse the nanoscale surface morphology and roughness.

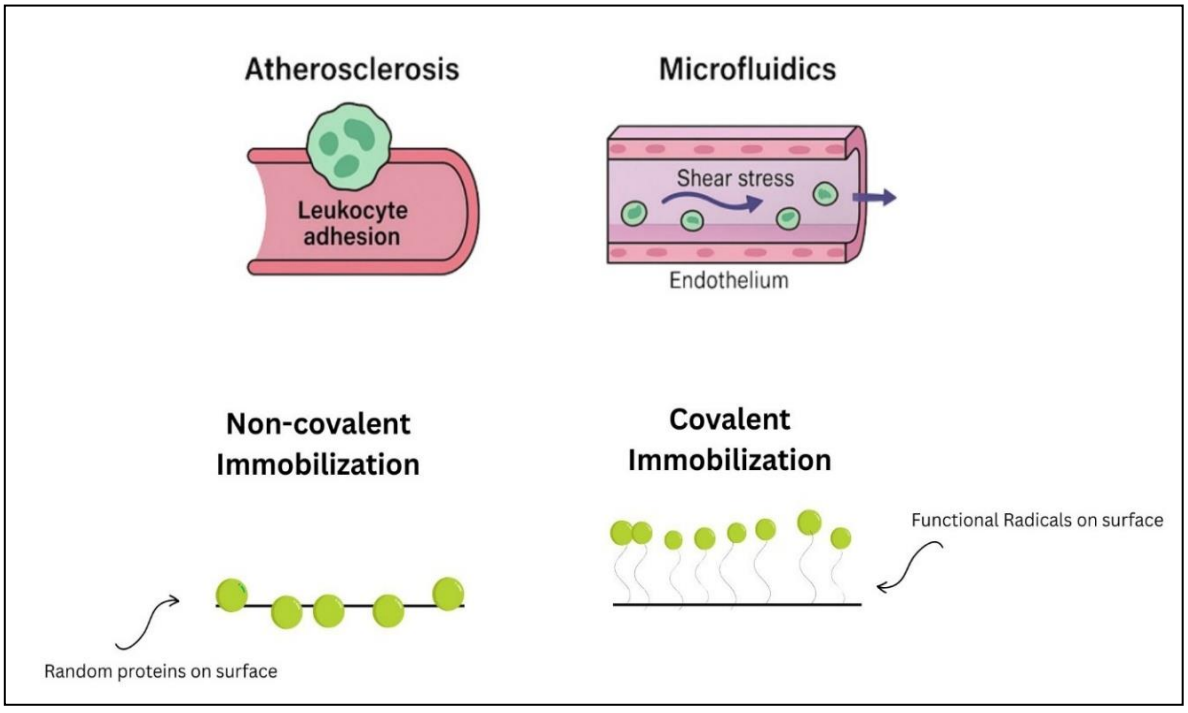

Figure 2. Schematic of non-covalent and covalent immobilization of biomolecules on a microfluidic surface (schematic created using Canva Pro subscription)

Recent studies combining AFM with flow injection analysis have demonstrated the ability to quantitatively assess nanoparticle distribution and surface roughness under continuous microfluidic conditions, providing valuable insights into surface topography that influence protein immobilization and function [21]. However, overly rigid tethering can block active sites, which is why flexible linkers are often used to elevate proteins from the surface. Plasma-treated surfaces support this strategy, though linker length must be optimized, as longer chains may hinder activity. In some cases, direct attachment yields better functionality. Maintaining the active conformation of proteins such as P-selectin and PSGL-1 under flow is key for replicating *in vivo* adhesion, making covalent functionalization, particularly with plasma activation, the most robust approach [20]. The shear resistance values could not be found that are specifically reported for leukocyte studies. However, Table 2 summarizes common surface functionalization methods used in microfluidic systems for biomolecule immobilization. These techniques vary in bond type, stability, and suitability under shear flow conditions typical of rolling assays.

Table 2. Several functionalization techniques used in microfluidic studies of biomolecules

Method	Bond Type	Definition	Advantages	Disadvantages	Suitability for Shear Flow / Rolling Assay	Ref.
Physical adsorption	Physical	Protein adsorbs onto surface via van der Waals/hydrophobic forces	Simple, no complex chemistry; quick	Unstable under flow; desorption likely; batch-to-batch variation	Low shear (~0.5–1 dyn/cm^2); not ideal for long assays vs. physiological 1–1.5 Pa (10–16 dyn/cm^2)	[22, 23]
APTES silanization	Covalent (via silane)	Amine groups added to glass/PDMS via (3-Aminopropyl) triethoxysilane, followed by protein linking	Moderate stability; enables ECM coating	Hydrolysis over time; moisture-sensitive; limited stability without crosslinking	~0.153–93 dyn/cm^2	[24-26]
APTES + Glutaraldehyde	Covalent	APTES base, followed by glutaraldehyde crosslinker for strong protein attachment	Stronger covalent linkage; better stability under flow	Toxic reagents; requires careful handling	20-150 dyn/cm^2	[27]
O_2 Plasma Activation	Reactive surface (silanol OH groups)	Treats glass/PDMS to introduce –OH groups for bonding or adsorption	Rapid hydrophilicity; good for initial bonding; used for PDMS-glass bonding	Hydrophobic recovery within hours; needs coating quickly post-treatment	0-23 dyn/cm^2	[24, 28]

APPJ and PAC	Covalent; surface radicals + reactive groups (amines, carboxyls, hydroxyls)	APPJ activates glass; PAC deposits reactive coating for protein immobilization	Safer than vacuum plasma, reagent-free, stable coating, high protein retention	Not ideal for PDMS unless optimized; SDS wash may be needed to confirm covalent binding	0.007-12.7 dyn/cm^2	[29]
Streptavidin–Biotin	Non-covalent (strong affinity)	Streptavidin immobilized on the surface binds biotinylated proteins	High specificity; strong interaction (Kd ~10^{-15} M)	Irreversible; can be sensitive to surface presentation and density	3.5 to 11 piconewtons (pN)	[30]
Thiol–maleimide and NHS–amine	Covalent (via heterobifunctional GMBS linker)	Amine–carboxyl crosslinking via EDC/NHS chemistry	Stable covalent link; widely used in protein immobilization	Requires pH control; side reactions possible	0.75-10 dyn/cm^2	[31]

Benefits and Limitations of Micropatterning Using Plasma Treatment in Microfluidics

Micropatterning enables the creation of microscale biomolecule or cell patterns on surfaces; however, few studies have specifically applied protein micropatterning to leukocyte research. For instance, Eddie A. Shimp *et al.* developed a rapid microfluidic method to stripe mP-selectin, cutting coating time to just five minutes—thirty times faster than traditional static methods. They found that mP-selectin binding increased with shear stress, revealing flow-dependent kinetics, unlike fibronectin or fibrinogen, which adsorbed independently of shear. This indicates that P-selectin interactions are sensitive to flow, similar to other lectins involved in shear-dependent adhesion [32]. Accordingly, another study used microfluidic patterning to precisely control the spatial deposition of multiple adhesion proteins, including P-selectin and von Willebrand factor (vWF), on a single substrate.

By perfusing whole blood at a physiological shear rate of 200 s^{-1}, they demonstrated the selective rolling and adhesion of leukocytes on P-selectin lines and platelets on von Willebrand factor (vWF) lines, which were distinguishable by cell size despite identical fluorescent labelling. This technique enables the simultaneous investigation of different cell types under flow, thereby enhancing experimental efficiency and facilitating the exploration of intercellular crosstalk within defined spatial arrangements [33]. A recent study developed a microfluidic device featuring Au nanopatterns spaced by SiO_2, fabricated via colloidal lithography, which enables precise nanoscale protein immobilization. Using thiol chemistry and PLL-g-PEG/PLL-g-PEG-NTA functionalization, they created dual protein patterns within microchannels for selective, reversible attachment of streptavidin-biotin and HIS-tagged proteins. Fluorescence microscopy confirmed high pattern fidelity with minimal nonspecific binding. The platform co-localized adhesion molecules—ICAM1, P-selectin, E-selectin—in distinct patterns, mimicking the endothelial surface for leukocyte rolling and adhesion. Tests with activated THP1 cells under flow showed specific rolling and arrest on P-selectin/ICAM1 patterns, validating it as a biomimetic tool for studying leukocyte recruitment at nanoscale [34]. Plasma

treatment further enhances protein micropatterning on microfluidic surfaces by modifying material properties—particularly of PDMS—rendering them more hydrophilic and thus better suited for protein immobilization and cell adhesion. This treatment introduces polar groups that reduce water contact angles, improving wettability and promoting protein adsorption. Additionally, plasma treatment strengthens the bonding between device layers, such as PCTE membranes and PDMS, ensuring structural integrity. While plasma can be harnessed to create complex protein patterns through selective ablation, these effects are often temporary and may require further surface modifications to maintain long-term stability [35]. Despite these advances, micropatterning on plasma-treated microfluidic surfaces still faces challenges: achieving high spatial resolution, maintaining protein stability and activity, preventing nonspecific adsorption, and scaling up production. Recent progress addresses these challenges by employing improved materials, optimized immobilization protocols, advanced patterning techniques such as microcontact printing and laser-assisted methods, and the development of suitable bioinks for complex three- dimensional structures. Plasma treatments with gases such as oxygen, nitrogen, or argon introduce functional groups that enable covalent protein bonding, thereby mitigating PDMS's inherent hydrophobicity and its tendency for nonspecific adsorption and hydrophobic recovery. For example, oxygen plasma has been shown to enhance wettability in PDMS–PCTE–PDMS chips, while atmospheric plasma treatments improve cell growth and fluidic functions, such as capillary pumping [36]. Maintaining protein stability after immobilization remains critical. Approaches such as genetic engineering, protein nanocages, and covalent organic frameworks (COFs) facilitate the immobilization of proteins while preserving their function. However, some activity loss is possible, requiring careful optimization of protocols and enzyme engineering. Microfluidic platforms incorporating these refined micropatterning techniques also enable single-cell analysis, revealing cell heterogeneity, and support multiplexed, ultrasensitive immunoassays essential for clinical diagnostics [37, 38]. Moreover, these micropatterning strategies are increasingly being explored in regenerative medicine, where immune-modulating biomaterials and engineered microenvironments can guide tissue regeneration and integration. As highlighted by Haddadi *et al.*, novel biomaterial-based methods capable of mimicking antigen-specific tolerance are gaining attention, particularly in the contexts of transplantation and tissue repair. These microengineered systems, especially when combined with plasma-functionalized surfaces, offer a promising approach to controlling immune responses, enhancing cell–material interactions, and developing next-generation regenerative therapeutics [39].

Conclusion

Leukocyte adhesion plays a crucial role in cardiovascular inflammation, which is regulated by complex molecular interactions and biomechanical forces. Microfluidic platforms that utilize plasma-based covalent immobilization offer superior control and more accurately mimic *in vivo* endothelial environments compared to simpler noncovalent methods, which are less stable under flow conditions. Techniques such as plasma-activated coatings (PAC) and atmospheric pressure plasma jets (APPJ) facilitate durable biomolecule attachment, thereby enhancing model accuracy. However, challenges remain in optimizing surface chemistries to maintain protein stability over time and in replicating the diverse nature of

endothelial cells. Understanding how plasma-treated surfaces affect leukocyte rolling dynamics is crucial for maximizing the potential of these technologies. Future research should combine micropatterning with plasma-activated microfluidics to simulate complex vascular microenvironments more effectively, thereby broadening applications in personalized medicine and therapeutic development. These interdisciplinary platforms, which combine biology, materials science, and engineering, have the potential to revolutionize cardiovascular research by providing precise disease models and targeted treatments. Continued collaboration across these fields is vital to bring such innovations from the lab to clinical practice.

Conflict of Interest

No conflicts of interest were reported in this study.

Consent for Publications

All authors read and approved the final manuscript for publication.

Authors' Contributions

S. Inam compiled, wrote, and formatted the manuscript, while M. Waqar and S. Ikram reviewed it.

Ethics Approval and Consent to Participate

This article was a review and did not include any original research involving human participants or animals. Therefore, it did not require ethical approval.

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