Brief Overview on Blood Clotting

Medical implants used today can incur thousands of dollars in cost to the patient and often require invasive methods of maintenance and eventual replacement (see Fig. 1) to correct unintended physiological responses by the body (bio-response). While many of these complications stem from the implant design, broad limitations exist in designing proper material interfaces that can coexist the dynamic environment of the body and its complex biochemical response to foreign surfaces. Therefore, the design of proper biomaterials requires a fundamental understanding of the bio-response mechanism from the body and its ultimate effects at the interface of the material surface.

Figure 1. Prosthetic carbon-based mechanical heart valve, (left) before implantation, (right) after implantation rejected by the body.
 Courtesy of T. Horbert (University of Washington)
To understand the body’s response to implanted materials, it is insightful to first study how the body responds to normal internal and external wounds (lacerations), as well as imperfections in everyday functional tissues via blood clotting. The formation of a blood clot is the result of a concerted interplay between various blood components, such as the platelets, or thrombocytes. The platelets are cells in the blood that are involved in the cellular mechanisms of the primary blood clotting process, the hemostasis.

Initial response to a laceration begins when platelets from the blood plasma aggregate at the wound site, to create a clot that impedes blood loss. Blood platelet aggregation is assisted at the wound site by a protein known as the von Willebrand factor (vWF). The von Willebrand factor found in both tissue cells as well as the blood stream supports the clotting factor VIII. When people show a deficiency in the von Willebrand factor, factor VIII can weaken and cease to perform its function in blood clotting leading to excessive bleeding upon injury. With von Willebrand factor, platelet cells are biochemically stimulated to bridge exposed tissue cells that further initiates clotting factors to jumpstart the coagulation phase of the clotting response. Generally, the intrinsic response is contained within the blood plasma itself and responsible for a larger part of clot formation while the extrinsic response is by the surrounding tissue cells. This is meant to supplement the intrinsic pathway to accelerate clot formation. These two pathways ultimately converge to arrive at the common response. This common pathway, as highlighted in Fig. 2a, activates crucial proteins involved in forming an adhesive matrix to bind and solidify the existing platelets, forming what is known as a “hard clot.” As with all biochemical processes, the formation, usage and ultimate degradation of the numerous clotting factors is self-regulated via feedback mechanisms recognized by the factors themselves at each individual reaction stage.

**Figure 2a (left)** Blood clotting pathway for tissue injuries with emphasis on fibrinogen activation and regulation, circled. **2b (right)** Specific mechanism of the activation of fibrinogen by thrombin, and SFM image of a fibrin clot on highly oriented graphite.
The common pathway is responsible for the formation of an adhesive protein based gel that interacts with and further coordinates the final stages of clotting. Here, a common product from both intrinsic and extrinsic pathways, clotting factor X\textsubscript{a}, transforms an existing factor ‘prothrombin’ to its active form ‘thrombin’. This active form then proceeds to activate another factor, fibrinogen, by breaking specific intramolecular connections in a process known as cleavage. Active fibrinogen, referred to as ‘fibrin monomer’, is responsible for polymerizing with itself at the previously cleaved sites to rapidly form an adhesive gel to support the surrounding platelet aggregation in what is known as a ‘soft clot’. Thus, the common pathway and resulting fibrin polymer is the product of both the intrinsic and extrinsic pathways and the main driving force in the clotting cascade’s coagulation phase. The mechanism of fibrin polymer formation is shown in Figure 2b, which also provides a visualization of the fibrin matrix on a model implant surface (graphitic carbon) by scanning force microscopy (SFM).

**Fibrinogen Structure and Functioning Mechanism**

Fibrinogen in its inactive form is 340 kD (~47.5 nm) in size and exists as a covalently bound two part molecule (known as a “dimer”) associated through three disulfide bridges, as shown in Fig. 3. It is comprised of three intertwined strands of amino acids shown in Fig. 3b as the A, B, and C strands. These strands associate with each other to form several functional domains, including the terminal sticky $\alpha$, $\beta$, and $\gamma$ domains (shown as tangled lines in Fig. 3b) of the protein as well as the rigid spacer linking the two portions of the dimer together. From the center, strands A and B contain short sequences of amino acids which together form the thrombin cleavage site, shown as stemmed circles in Fig. 3b. After the short sequences (known as ‘fibrinopeptides’) are cleaved off, the newly vacant sites (pathway shown in Fig. 2b) are now free to interact specifically with the sticky $\alpha$ and $\beta$ domains from adjacent fibrin monomers for polymerization and the formation of a ‘soft clot’. Further, polysaccharides contained within the terminal sticky ends of fibrin (shown as black hexagons in Fig. 3b) help provide an even stronger fibrin polymer through a process called cross-linking (off-axis bonding) to ultimately form a ‘hard clot’ via a factor known as XIIIa. These domains of fibrin are spaced ~16 nm from the center domain via a structured triple-helix spacer domain, where the three strands are intertwined to give fibrinogen and the resulting clot a rigid structure.

The last domain, the $\gamma$-sticky end also plays a crucial role (as seen in Fig. 4a) in interacting with platelet cell surface receptors to ultimately incorporate the existing platelets into the fibrin clot. Typically, both inactive and active forms of fibrinogen can mediate adhesion via $\gamma$-domain interaction with platelet surface-bound factors known as GPIIb/IIIa. When these surface receptors are bound, platelets switch from inactive to active form and begin to secrete cofactors (a factor designed to work with another factor) and signaling proteins (including fibrinogen and vWF) which act as positive feedback agents to further promote clot formation. As covered in the next section, this interaction plays a crucial role in implant rejection due to the lack of need for an active form of fibrinogen to initiate a clotting cascade.
Bio-Response toward Implant Devices and Foreign Bodies

Many of the same factors play a role in the identification and isolation of foreign material surfaces in the body, which leads to ‘rejections’. One major difference, however, is the lack of vWF or other existing extrinsic pathways to supplement or jumpstart the
bio-response cascade as in normal blood clotting. The initial response to foreign surfaces is predominately intrinsic and stems from the material surface’s abilities to adsorb and aggregate various factors and proteins, which then, jumpstart the clotting mechanism and ultimately the formation of a fibrous capsule that walls off the implant from the rest of the body. One example of this is by fibrinogen, which can recruit platelet cells in its inactive form (from Fig. 4b) via its \( \gamma \) domain and GPIIIa/IIb platelet-surface protein receptor interactions. Thus, if the implant surface displays affinity towards aggregation of fibrinogen, it will also display affinity towards platelet cells in the blood plasma. As shown in Fig. 4a, this protein aggregation phase is one of the primary factors in initiating the implant bio-response cascade.

Another differentiating factor between foreign body response and blood clotting is the involvement of certain immune system elements in the cascade. Shown in Fig. 5, the lack of normal extrinsic pathway signals can persuade the body to identify a material as foreign and attack it with immune cells, such as ‘neutrophils,’ ‘macrophages’ and others. This attack typically ends with the formation of an encapsulating cell caused by fusion of macrophages, and known as a ‘foreign body giant cell’. The foreign body giant cell engulfs the entire surface and recruits connective tissue cells known as fibroblasts to the implant site. The fibroblasts then form a dense fibrous capsule to wall off the implant from the rest of the body in a stage termed ‘fibrosis’. This stage of bio-response is also termed ‘thrombosis’ and can occur within 3 weeks of the initial response. It is useful here to note that the non-specific adsorption of various bodily factors towards any material surface will likely initiate a bio-response cascade by the body and ultimately complications in the lifetime of the implant device. This has been one of the main challenges fueling the development of novel engineered biomaterial systems.

![Figure 5. Stages of implant rejection over time, beginning with protein aggregation and ending with capsule formation](image)

**Implant Material Design**

It is widely accepted that the prevention of non-specific protein adsorption can be highly correlated with improved implant lifetime and viability. To understand strategies in camouflaging material surfaces chemically, it is useful to understand the general properties of physiological proteins. The body, comprised of \(~70\%\) water, is a highly aqueous environment in which proteins have evolved in to function. In the course of evolution, the majority of proteins found in humans are folded to exhibit hydrophilic exteriors and hydrophobic cores, as illustrated in Fig. 6, much like lipid micelles found in
soaps. Without this phase segregated property, proteins would tend to precipitate out of solution and lose the ability to travel in the blood stream or any aqueous environment as needed for bodily functions.

![Figure 6](image)

**Figure 6.** Schematic representing a protein backbone (black outline) with corresponding charged hydrophilic side-chains facing outward (dark blue region) and water-fearing aliphatic groups facing inward (light blue region).

Many foreign surfaces act as condensers of proteins due to their insolubility with aqueous environments, causing proteins to denature at the interface and aggregate. Thus, for specific properties of aggregation prevention, solvability is a key factor in surface engineering for improved biomaterials. In particular, an effective strategy for implant surface chemistry design has been to maximize the hydrophilicity of implant surfaces to tightly bind layers of water in place of potential proteins. This also orients the tightly bound water molecules towards the biological environment so blood proteins see no significant difference between the blood and the implant surface, effectively camouflaging the implant device.

For this reason but also because of their mechanical strength, common biomaterials used for today’s implants include hydrophilic metal-oxides such as titanium, cobalt, chromium, and some stainless steels. Where softer plastics and gels are used, hydrophilic polymers such as poly-ethylene glycol (where oxygen in the carbon backbone enhances polarity and water affinity) and even protein coatings such as heparin are used to prevent aggregation.

Lastly, a major factor in implant design is concerned about the biomaterial surface topography and total surface area of the exposed material. As seen in Fig. 7, roughness and porosity play key roles in bioactivity. In general, smooth surfaces present less surface area of the material chemistry to the environment and also maintain a lower surface energy. Metallic grain boundaries and porosity often introduce increased densities of unsatisfied bonding which increase the overall surface energy and interaction with the environment. Rough surfaces also tend to obstruct flow patterns in the bloodstream, increasing the likelihood of biological agents in contacting the foreign surface due to the generation of flow turbulences. These properties, particularly the surface energy and resulting roughness, play significant roles in the performance when considering a bio-interface.
A material that is often used for prosthetic heart valves is pyrolytic carbon because of its enhanced properties of toughness and apparent bio-inertness to high amounts of blood flow. Due to its large and atomically flat surface, graphite remains inert both because of its relatively low surface energy and also little disruption to the bloodstream flow. From previous clinical study[2], an as-deposited layer of pyrolite brand carbon is significantly rough and is observed to elicit a decrease in thromboresistance (the tendency to resist blood clotting). The polished version, on the other hand, is commonly used in implants today with low levels of inflammation and bio-response. These observations confirm the previously discussed principles of implant material design, where topography and surface energy play key roles in bio-inertness. While the failure rate is moderately low for these carbon prostheses, many cases of patient rejection still occur. This is because proteins and cofactors still have some affinity towards a graphitic surface, as illustrated in Figure 8.
Characterization of Adsorption Processes

Surface adsorptions processes are manifold depending on variables such as solute concentrations, pressures, temperatures, and deposition environments. Furthermore, the bonding mechanism is influenced by the level of molecular and surface interactions, surface diffusion, and local and integral substrate properties and morphologies.

A straightforward molecular model for adsorption is the Langmuir model, developed by Irving Langmuir in 1916. It describes the dependence of the surface coverage of an adsorbed inert gas on the pressure (or partial pressure) of the gas above the surface at a fixed temperature (isothermal state). While this so-called *Langmuir Isotherm* provides one of the simplest models, it offers a good starting point towards a molecular understanding of adsorption processes. Although developed for non-interacting simple gases, it is extensively employed to analyze macromolecular adsorption processes in biology.

With the Langmuir model we assume the following:

1. All surface sites have the same activity for adsorption.
2. There is no interaction between adsorbed molecules.
3. All of the adsorption occurs by the same mechanism (e.g., physisorption or chemisorption), and each adsorbent complex has the same structure.
4. The extent of adsorption is no more than one monolayer.

To illustrate the model, we shall assume a surface with a fixed number of active adsorption sites for molecule $A$ that is exposed to a gas containing $A$. If we define $\theta$ as the fraction of surface sites covered by adsorbed molecules then $(1- \theta)$ is the fraction of surfaces sites that are still active; i.e., not bound to $A$. Depending on the gas, we will use either the gas pressure $P$ for a monomolecular gas, or the partial pressure $p_A$ for a multicomponent gas mixture. We can expect that with increasing gas pressure, the rate with which the surface is covered will increase linearly, according to:

$$ r_a = k_a p_A (1- \theta). $$

(1)

Thereby, we assumed dealing with a gas mixture and implied that the rate of adsorption can be expressed in the same manner as any kinetic process with a kinetic order of one. In other words, the adsorption rate is linearly expressed with the applied partial pressure via the adsorption rate constant $k_a$. Analogous, we express the desorption rate constant as

$$ r_a = k_d \theta, $$

(2)

where $r_a$ is the desorption rate and $k_d$ is the rate constant for desorption. $k_a$ and $k_d$ are determined from kinetic experiments.
At equilibrium, we can equate Eqs (1) and (2), which yields
\[ \theta^* \frac{k_d}{k_d} A_p = 1 - \theta^* \]
(3)

Thereby, \( \theta^* \) defines the equilibrium fractional surface coverage at a particular partial pressure \( p_A \). Substituting the rate constant ratio \( k_d/k_d \) with the binding equilibrium constant \( K_d \) (also referred to as equilibrium association constant), the fractional surface coverage can be expressed as
\[ \theta^* = \frac{K_d p_A}{1 + K_d p_A} \]
(4)

which is the analytical expression for the *Langmuir Isotherm*. For a typical equilibrium experiment, adsorption data are gathered over a range of partial pressures, and final coverages are plotted with respect to concentration as illustrated in Fig. 9. Equation (4) yields for the equilibrium association constant
\[ K_d = \frac{\theta^*}{(1 - \theta^*) p_A} \]
(5)

**Figure 9. Langmuir Isotherm** yielding an equilibrium binding constant \( K_d \) of \( 2 \times 10^{-5} \text{ Pa}^{-1} \).

Accordingly, it is common to analyze adsorptions from solutions with the Langmuir model, expressing \( \theta^* \) as:
\[ \theta^* = \frac{K_d [X][S]}{[S] + K_d [X][S]} = \frac{K_d [X]}{1 + K_d [X]} \]
(6)

Thereby, we considered the reaction
\[ X + S \xrightarrow{k_d} XS \]
(7)

\([X]\) and \([S]\) representing the solute (e.g., protein) concentration and the substrate immobilized active site concentration, respectively. \([XS]\) is the compound concentration at the surface. The equilibrium constant for association \( K_a \) and dissociation \( K_d \) are related via \( K_a = [XS]/[X][S] = 1/K_d \). Note, the equilibrium constants results from both (i) the protein-surface interaction, and (ii) the protein-surface interaction with the buffer solution (solvent). Thus, instead of the partial pressure, it is the free adsorbate concentration \([X]\), which we assume to be constant that is the variable parameter in the *Langmuir Isotherm*. The *Langmuir Isotherm* provides the equilibrium constant \( K_d \), from which the standard free energy of adsorption \( \Delta G = -RT \ln(K_d) \) can be determined.
Under transient (non-equilibrium) conditions, where the coverage is changing over
time, we can express the change in the fractional surface coverage in terms of the reaction
and desorption coefficient as
\[
\frac{d\theta}{dt} = r_A - r_d = k_a p_A (1 - \theta) - k_d \theta .
\] (8)

After integration, the transient fractional surface coverage is given as
\[
\theta(t) = \theta^* [1 - e^{-t/\tau}] ,
\] (9)

where \(\theta^*\) is the equilibrium fractional surface coverage provided in Eq. (4), and
\[
\tau = \frac{1}{k_a p_A + k_d}
\] (10)
is the process relaxation time. \(k_a p_A + k_d\) reflects the observed rate constant. Equation (10)
is used to fit the data collected in time-varied experiments. A time-dependant illustration
of a Langmuir adsorption process is illustrated in Figure 10. Here, the surface is initially
unoccupied and undergoes a rapid population until sites are screened (sterically) from
the free particles above, resulting in a slow saturation towards the equilibrium surface
coverage asymptote.

Although the Langmuir equation and its derivatives provide a method to quantify the
interaction strengths of inert molecular adsorption processes, it holds certain limitations
that restrict its applicability. Proteins, in particular, have been shown to possess unique
aggregation mechanisms at solid interfaces exhibiting adsorption curves that largely
deviate from Langmuir. Also surface imperfections, with preferential sites for adsorption
as on stepped graphite surfaces (see Fig. 8) modify the adsorption kinetics. Thus, the
Langmuir Isotherm method is to be understood as a primer to building a foundation for
further exploration of peculiar adsorption mechanisms, or as an initial assessment of
general affinity without regard for specific interaction mechanisms.
Artificial Nose or Biosensor

The Langmuir adsorption isotherm provides a useful foundation for understanding a variety of applications. One such application is a novel scanning force microscopy (SFM) tool known as the "artificial nose" that has also found applications as a biosensor. With this tool, molecular concentrations on the picomolar scale can be "rapidly" sensed. Briefly, the working principle is as follows: An array of free-standing cantilevers that are coated or "functionalized" for sensitivity to adsorption of molecules (see Figure 10a) is exposed to either a gas or a buffer solution, respectively. While cantilever material coatings, typically polymers, serve as adsorption (more precisely absorption) membranes for gaseous solutes, chemical functional materials act as adsorption sites (receptors) for liquid buffer dissolved solutes, Fig. 10a. Due to the single side coating and adsorption process of the cantilever probes, the cantilevers will be asymmetrically strained, which leads them to bend, Fig. 10b. This degree of bending is captured by the laser beam deflection scheme of the SFM, as illustrated in Figure 10.

![Figure 10. Working principle of a functionalized SPM biosensor; (a) before and (b) after adsorption of bio-molecules.](image)

With Stoney's formula applied to a cantilever beam, the tensile surface stress $\sigma$ acting on the lever can be related to the cantilever properties and normal deflection $\Delta z$, as:

$$
\sigma = \frac{4}{3} \left( \frac{L}{W} \right) \frac{\Delta z}{D} k_N \left( 1 - \nu \right) = \frac{4}{3} \left( \frac{L}{W} \right) \frac{\Delta F_N}{D(1-\nu)} = \theta \sigma^* 
$$

(11)

where $L$, $W$ and $D$ are the lever size dimensions (length, width and thickness), $k_N$ and $\nu$ are the cantilever normal spring constant and Poisson’s ratio, respectively, and $\Delta F_N = k_N \Delta z$ is the normal force acting on the lever. Thereby, changes in $\sigma$ can be assumed to be directly proportional to changes in the fractional surface coverage $\theta$ with $\theta = \sigma/\sigma^*$, where $\sigma^*$ is the equilibrium stress imposed by the adsorbed film for infinite exposure time. Surface stresses imposed by a monolayer adsorption of macromolecules such as proteins are on the order of tens of dyne/cm ($10^{-3}$ N/m). This translates according
to Eq. (11) to ~10 nN normal cantilever deflection forces for a Poisson’s ratio of 0.23 (silicon lever), and cantilever dimensions \((L, W, D)\) of 100 \(10\) and 0.1 \(\mu m\), respectively.

References
6. For an adsorbed film of thickness \(t_F\), the surface tensile stress \(\sigma [N/m]\) is related to the stress in the adsorbed film along the cantilever beam \(\sigma_F [Pa = N/m^2]\) via \(\sigma = \sigma_F / t_F\).