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The blood compatibility challenge Part 2: protein adsorption phenomena governing blood reactivity

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Abstract

The adsorption of proteins is the initiating event in the processes occurring when blood contacts a “foreign” surface in a medical device, leading inevitably to thrombus formation. Knowledge of protein adsorption in this context has accumulated over many years but remains fragmentary and incomplete. Moreover, the significance and relevance of the information for blood compatibility are not entirely agreed upon in the biomaterials research community. In this review, protein adsorption from blood is discussed under the headings “agreed upon” and “not agreed upon or not known” with respect to: protein layer composition, effects on coagulation and complement activation, effects on platelet adhesion and activation, protein conformational change and denaturation, prevention of nonspecific protein adsorption, and controlling/tailoring the protein layer composition.

Keywords

Surface; protein adsorption; blood coagulation; thrombosis; complement activation; blood compatibility; antifouling

1. Introduction

The adsorption of proteins is the first significant event following blood contact with “foreign” surfaces. Protein adsorption in blood contact is thus the precursor to subsequent adverse (usually catastrophic) effects that include plasma coagulation, platelet adhesion, platelet activation, immune responses and inflammation. Coagulation and platelet adhesion

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This paper is part 2 of a series of 4 reviews discussing the problem of biomaterial associated thrombogenicity. The objective was to highlight features of broad agreement and provide commentary on those aspects of the problem that were subject to dispute. We hope that future investigators will update these reviews as new scholarship resolves the uncertainties of today.

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and activation constitute, together, the principal events in surface-induced thrombosis, which is the most serious limitation on the use of blood-contacting devices.

This problem remains unsolved despite extensive research over the past many years [1-3]. Understanding and thereby acquiring the ability to control protein adsorption as the initiating event would seem to be a *sine qua non* for the solution of this seemingly intractable problem. Knowledge of protein-surface interactions in general, and in the context of blood in particular, is substantial, but despite extensive investigation, it remains fragmentary and lacking in cohesion.

The key aspects considered in this article are named in the following and sketched in Figure 1:

- The composition of the adsorbed layer, i.e., which of the several hundred protein species in blood [4] are adsorbed, what are the relative quantities, and how does the layer composition evolve over time.
- The effect of the layer composition on coagulation and complement activation.
- The effect of the layer composition on platelet adhesion and activation.
- The role of protein conformational change and denaturation.
- Elimination of nonspecific protein adsorption and determination of whether a protein-free surface in blood is likely to be generally passive and in particular non-thrombogenic.
- Controlling/tailoring the layer composition to achieve blood compatibility.

This overview on protein adsorption is part 2 of a short series of four review articles (including an introductory article written largely from a clinical point of view), which discuss both generally accepted and more controversial viewpoints on the interactions between blood and biomedical devices.

2. What is the composition of the protein layer adsorbed from plasma or blood?

Agreed upon:

1. Protein adsorption occurs on all surfaces unless preventive measures are taken.
2. Adsorption occurs immediately upon blood contact and the surface is covered in a few seconds. The layer composition evolves with time. Although variation in the adsorbed layer in the short term is well understood and often results in a decrease in initially adsorbed fibrinogen, the variation in the adsorbed layer at longer times, especially clinically relevant times such as days and weeks, is poorly understood.
3. The detailed layer composition on any given surface is unknown and unpredictable. The layer is complex and contains many of the proteins in plasma. Adsorbed quantities of a given protein vary widely among surfaces.

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4. The adsorbed layer composition generally does not reflect the protein composition of the plasma.

5. Proteins present on most surfaces investigated include albumin, fibrinogen, immunoglobulins, vitronectin and apolipoproteins.

Discussion:

1. The “universality” of protein adsorption (all proteins, all surfaces) is well documented [5-7]. In the context of surfaces in contact with blood, extensive work by Brash et al. using immunoblotting methods on a range of surfaces including hemodialysis membranes [8], hydrophobic and hydrophilic polymers [9], putatively protein resistant surfaces [10], heparinized surfaces [11], and liposomes [12] may be cited in this regard. Many other labs have contributed to the establishment of the protein adsorption “axiom” [13].

2. It is expected that proteins will move to and arrive at the surface ahead of cells due to size and concentration differences. Indeed it has been shown that protein layers are formed typically in times of the order of seconds [14, 15]. There is evidence that the layer composition varies with time [16]. Perhaps the most extensive information on time dependence comes from studies of the Vroman effect [17-19]. Initial work showed that fibrinogen is adsorbed at short time but at longer time it is displaced by other plasma constituents. Vroman et al. suggested that proteins of the intrinsic coagulation pathway, particularly high molecular weight kininogen (HMWK), are mainly responsible for fibrinogen displacement [20, 21], a suggestion supported by work of Brash et al. [22]. More generally Vroman proposed that in plasma, adsorption is sequential and that over time more abundant proteins of lower surface activity are replaced by less abundant ones of higher surface activity [23]. However, it is clear that such a sequence does not occur on all surfaces [15].

   A reasonable summary statement on kinetics of adsorption from plasma is that behavior is dependent on surface properties such as hydrophilicity-hydrophobicity. Transients may occur at short time (< 1 min). Thereafter it appears that the composition of the layer remains fairly constant.

3. The protein layers adsorbed from blood are complex, and it is not possible to predict *a priori* the composition on any given surface. Brash et al., using immunoblotting methods for protein identification, have shown that on many surfaces all proteins probed for are found to be present [8-12]. Extensive data have come recently from studies of the “protein corona” that forms on the surfaces of nanoparticles introduced into the bloodstream [24-26]. The protein corona has been studied mainly using proteomics methods. The information content of these methods is very high and, as an example, has allowed the identification of around 800 individual protein species on a series of silver- and gold-based nanoparticles, with an average of about 70 proteins on any one particle (surface) type [27]. Also Lundqvist et al. [26] showed that the composition of the plasma corona on a series of modified polystyrene-based

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nanoparticles was significantly dependent on the size and charge of the particles. The number of proteins identified on these materials ranged between 30 and 50. One of us (TAH) has serious concerns here and thinks that these data at least partly reflect proteins retained in carryover of bulk phase proteins, not adsorbed proteins. The protein corona work on nanoparticles needs very close examination as it may be seriously compromised by incomplete rinsing to remove bulk phase proteins entrained in interstices of packed or aggregated particles. It is difficult to remove bulk proteins because convective flow of the rinse buffer is heavily inhibited around packed particles. Such packing must occur if particles are filtered or spun down so that rinse buffer can be separated from the particles.

Concerning blood interactions with nanoparticles more generally, it should be noted that the protein corona, containing immunoglobulins and complement proteins, decreases the lifetime of the nanoparticles in the circulation, thus negating the specific function for which they were designed, typically targeted drug delivery. It is premature elimination, rather than coagulation and thrombosis, which bedevils the use of nanoparticles in blood. There has therefore been considerable research aimed at suppressing or limiting corona formation. The use of passivating or protein-resisting agents such as PEO/PEG and zwitterionic polymers, has been shown to be effective for this purpose. Protein resistance is discussed in detail in section #6 of this article. As noted above, the protein composition of the corona may depend to some extent on particle size as discussed by Lundqvist et al. [26]. It is difficult to discern, however, any general trend or what exactly the nature of the dependence is.

4. A simple, physically based view of adsorption from blood might suggest that proteins would be adsorbed based on their relative quantities in the plasma, so that the layer composition would match the plasma composition. This is generally not the case. Albumin, for example, which constitutes about 50% by weight of the plasma protein complement, is in general under-represented in the layers adsorbed from plasma [9]. Also it has been shown that fibrinogen, constituting about 5% by weight of the protein complement, is over-represented at short times [28]. It is clear that some proteins are more surface active than others.

5. Albumin, fibrinogen, immunoglobulins, vitronectin and apolipoproteins are usually present in significant quantities regardless of adsorbing surface properties [29]. These proteins (or their fragments) run on reduced SDS-PAGE in the molecular weight range 50-80 kDa and from that point of view appear to dominate the adsorbed layers. Data on the nanoparticle corona support this observation. For example Göppert and Müller [30] showed that albumin, fibrinogen and apolipoproteins AI and AII were prominent in the layers adsorbed to solid lipid nanoparticles. Also it has been shown [31, 32] that on PEG-based and other protein resistant surfaces, the proteins that do adsorb include those in this size range, in particular the clusterin proteins, also known as apolipoprotein J.

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Not agreed upon or not known:

1. The extent of protein adsorption in blood contact: “adsorption stops after approximately a monolayer” versus “the layer continues to build indefinitely”.
2. The dependence of layer composition on adsorbing surface properties, e.g., charge, hydrophobicity, micro/nano topography [33-35].
3. The dependence of layer composition on protein properties, e.g., size, charge, conformational stability.

Discussion:

1. Knowledge of the total quantity of adsorbed protein, as well as the layer composition, is important. From the literature on adsorption in simple systems of one or a few proteins it can be concluded that adsorption is generally limited to monolayers with surface concentrations in the 1 μg/cm^2 range [5-7]. The solution concentrations used in these studies are generally of the order of a few mg/mL whereas the total protein concentration in plasma is about 90 mg/mL [36]. Nonetheless it has been found that adsorption from blood and plasma is also often in the monolayer range. For example Gore et al. [37] showed that adsorption from whole blood to poly(vinyl chloride) was less than 1 μg/cm^2, and to a heparinized surface (on which antithrombin accounted for ~40% of the adsorbed layer) about 2.6 μg/cm^2. Although data at longer times (days, weeks, months) are lacking, it appears from available data that the protein layers are relatively “thin”, suggesting that the influence of the surface falls off markedly after it becomes fully covered.
2. Although it is clear that layer composition varies from surface to surface, the nature of the dependence on properties such as surface charge and hydrophobicity is not well understood. Hydrophobicity/hydrophilicity is a case in point. Compelling evidence for single protein systems that adsorption increases with increasing hydrophobicity was provided by studies with hydrophobicity gradient surfaces [38]. This trend has not been observed consistently, however. For example, Zhao et al. [39] showed that although adsorption of fibrinogen on a series of fluorinated polymer surfaces (water contact angles in the range of 80-110°) increased with increasing surface hydrophobicity, the adsorption to all of the hydrophobic surfaces studied was much lower than on a relatively hydrophilic PMMA surface (contact angle ~70°). Others have observed continuously increasing adsorption of single proteins as hydrophobicity increases, but with superhydrophobic surfaces (water contact angle > 100°) showing very low adsorption [40]. One of us (RAL) points out that superhydrophobic surfaces function by the presence of trapped air. While proteins may tend to adsorb to the air-plasma interface, it is likely that this process is not stable and that the protein easily washes off under flow, with the surface apparently showing very low protein adsorption. It may even be possible that the air-plasma interface actually denatures proteins that come in contact with it, possibly then even inducing emboli formation downstream of the surface.

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Thus superhydrophobic surfaces should be treated as a separate category not grouped with hydrophobic surfaces that do not trap air pockets, but have intimate atomistic-level contact between blood and the material surface itself.

In a very nice review of the interaction of superhydrophobic materials with blood, Jokinen et al. [41] make several important observations, including the possible role of trapped air in activating platelets and how stable the trapped air pockets may be in the longer term in the face of a complex mixture of proteins and lipids at the bubble interface that may lead to wetting and adsorption, or even simple dissolution of the bubble into the surrounding liquid. Another very nice comprehensive review of biological interactions with superhydrophobic materials was provided by Falde et al. [42], who also comment on instability: "Moreover, proteins and other biological surfactants can themselves promote air removal from the superhydrophobic surfaces in two ways. Proteins are surface-active molecules (surfactants) that decrease the interaction energy at the material-water interface through reduction of the surface tension of water. Further, proteins will adsorb to the low energy material surface over time with denaturing-and-binding events, thereby increasing the sur-face energy of the material to favor the interaction with water… [so that] water infiltration is promoted with further protein binding."

Protein adsorption to flat hydrophobic surfaces is often extremely rapid, particularly in the initial phase when incoming protein molecules interact with a largely empty surface, and the rates of adsorption can be at or near the so called diffusion limit, i.e. they adsorb at the same rate as they arrive as all hits result in sticks. In this regime, the effects of both diffusion and convection govern the observed adsorption rate, so that observed adsorption rate increases with the flow rate. These phenomena are very well described and analyzed in the publications of Robertson et al. [43].

However, protein adsorption to hydrophobic surfaces with rough surfaces displays different behavior, namely increasing the flow rate and thus the shear rate at the surface causes the "adsorbed" proteins to be removed. Thus, Koc et al. [44] concluded that "Although adsorption of BSA from solution was not reduced by using superhydrophobic surfaces under static conditions, the ease by which it could be removed was, particularly on nanostructured surfaces with a fluorocarbon coating, where complete removal was observed within measurement error." But it is important to stress that these surfaces trap air bubbles in the surface asperities, preventing direct contact of the protein solution with the solid surface, and that the application of shear in this case removes the attached air bubbles. Koc et al proposed that bubble detachment would strip away any adsorbed protein on the tips of the asperities (which are uncovered by the air bubble) by surface tension forces. However, these authors failed to recognize another strong possibility, namely that protein adsorbed to the interface of the protein solution and the air bubble will be carried off with the bubble. It is well known that proteins attach to the air water interface. More generally, it should be pointed out that many other investigations of protein

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adsorption and blood interaction with superhydrophobic surfaces did not take into account that both proteins and blood interact strongly with air bubbles, although it was explicitly discussed in a review article by Jokinen et al. (see above).

3. Differences in protein properties such as size and charge might be expected to influence which of the proteins in plasma are adsorbed on a given surface. As already indicated the plasma is effectively "fractionated" in the adsorption process and the layer composition is not the same as the plasma composition. However, the influence of protein properties, *per se,* has not been systematically investigated and is not well understood.

3. How does the protein layer composition affect coagulation and complement?

Agreed upon:

1. Negatively charged surfaces attract activated blood proteins which initiate the intrinsic pathway of coagulation. Recent data indicate positively charged surfaces may trigger coagulation via Factor VII Activating Protease (FSAP).

2. Complement proteins are deposited from blood plasma onto already adsorbed blood plasma proteins. Complement and coagulation are interconnected.

3. Coagulation and complement activation are minimized at charge-neutral, strongly water-binding surfaces possessing high substrate surface mobility.

Discussion:

1. The intrinsic pathway of coagulation can be activated in a calcium-independent manner via interactions between four plasma proteins: factor XII (FXII), prekallikrein (PKK), high molecular weight kininogen (HMWK) and factor XI (FXI) (Figure 2). It is triggered close to, or at negative surfaces in general [45] via the autoactivation of FXII to FXIIa [46, 47]. Coagulation progresses thereafter from the surface and outwards into the fluid phase[48]. Excess fibrinogen has the capability, however, to attenuate the autoactivation of the intrinsic pathway [49]. Silicon, a hydrophilic negatively charged surface, was shown to adsorb a 3-5 nm thick layer of proteins from plasma within 1 min [50]. After only a few seconds of plasma contact fibrinogen and HMWK became antibody-detectable on this surface. The antibody detectability of fibrinogen (e.g., on silicon) vanishes, however, very rapidly due to the displacement of fibrinogen by HMWK, FXII and other plasma proteins (Vroman effect) [20]. Upon adsorption to negative surfaces both HMWK and FXII become cleaved and activated, and expose thereafter positively charged histidine rich domains. In less than 1 min of plasma contact, anti-HMWK totally dominates the antibody detection signal on silicon.

Contact activation in plasma is also triggered and propagated on hydrophobic and positively charged surfaces, though much slower than on negative ones [48].

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Hydrophobic surfaces bind proteins more indiscriminately and more tightly than hydrophilic surfaces, and tend to retain the antibody detectability of fibrinogen, albumin, IgG and several other plasma proteins but not for the four contact activation proteins. The Vroman effect is thus less evident on these surfaces. The physiological role in diseases of contact activation of coagulation is not well understood as the pathway is not required for the initiation of hemostasis *in* vivo [51]. Today it is, however, accepted that polyphosphate aggregates (negative aggregates) released from platelet alpha-granules in vivo have the capacity to activate Factor XII [52], and may play an important role in blood clotting during inflammatory and immune processes [52]. Interestingly, positively charged polyethylene imine (PEI) and poly L-lysine surfaces activated whole blood coagulation, tentatively via adsorbed Factor VII Activating Protease (FSAP) [53]. However, currently the details of this activation mechanism remain obscure although the study indicates strongly that both negative and positive surface charge activate blood coagulation, although via different mechanisms. Coagulation is further discussed in part 3 of this series.

2. The increasing thickness of the total protein layer with time is likely explained by immune complement binding on top of other adsorbed and partially denatured proteins and many bound protein degradation fragments.

The spontaneous tick-over cleavage of complement C3 in plasma generates reactive C3b* and soluble C3a [54]. This is likely the origin of the early surface deposition of C3b (Figure 2) onto plasma-protein covered surfaces that subsequently form C3-convertases and activate complement via the alternative pathway (AP). The dominant C3 binding sites are partially denatured surface bound plasma proteins and other nucleophilic sites [55, 56]. But, alternative pathway convertases can also be formed on surface deposited and partially denatured pure C3. Excessive activation is suppressed by factors H and I. Classical pathway activation (CP) is triggered via adsorbed and accessible IgG, IgM, C1q, or lectins. The Chenoweth complement activation model indicates that nucleophilic surfaces activate complement via the alternative pathway only [57]. This model is, however, challenged by the fact that surfaces with particular chemistries (e.g., immobilized mercapto-glycerol, mercapto-ethanol) and adsorbed, accessible IgG and IgM activate complement via the classical pathway [50, 58-60]. Failure to detect bound CP proteins (e.g., C1q and IgG) immunochemically at biomaterial surfaces may explain the previously held belief that biomaterials activate complement via the AP only. However, surface CP activation is very rapid and is always immediately followed by activation of the effector pathway, the alternative pathway [60].

Well-known biomaterials with varying protein adsorption patterns all demonstrate increasing C3b deposition with time *in vitro*. For instance, hydrated aluminum (point of zero charge, pzc ~8–9) showed a rapid and strong anti-IgG binding after incubation in blood plasma and activation via the classical pathway [50], spontaneously oxidized titanium (pzc ~5–6) activated also rapidly but with a lower anti-IgG surface binding [50], and silicon (pzc ~2–3) apparently

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activated in a delayed manner (5-10 minutes lag phase) and with no anti-IgG deposition via the spontaneous and alternative pathway [50], These and other “model” experiments suggest that reactive complement proteins bind mainly to already adsorbed blood proteins [55, 56]. This interpretation is entirely reasonable since complement is an integral part of the humoral clearance system, the functions of which include the removal of immune complexes, denatured proteins and cellular debris. Protein adsorption and denaturation on foreign surfaces thus facilitates complement-based opsonization of biomaterials, similar to that of foreign or abnormal cells.

Recent literature also suggests strongly that coagulation- and the innate immunity systems are interconnected. For example, activated lectin complement pathway serine proteases (MASP-1/2) activate coagulation *in vitro* via cleavage of the coagulation factors prothrombin to active thrombin, fibrinogen to fibrin and fibrinogen degradation products (FDPs), factor XIII to active factor XIIIa, and thrombin-activatable fibrinolysis inhibitor (TAFI) to its active form [61-63]. This activation is triggered by activated platelets and by the generation of fibrin during thrombotic reactions in vitro and in vivo [63].

The clinical significance of immune complement activation on biomaterials is, however, still poorly understood [64]. Complement is further discussed in part 3 of this series.

3. As opposed to simply being a passive molecule that represents the major component of physiological fluids, water plays an extremely important role that mediates the adsorption of proteins and other biomolecules to material surfaces. The interaction of water with biomaterial surfaces has been extensively addressed by Latour [65] and Ratner [66]. To summarize, when a biomaterial is placed in contact with a biological fluid, water is the first molecule that adsorbs to the functional groups of the biomaterial surface. The thermodynamic state of the adsorbed water determines whether biomolecules in solution are able to displace the adsorbed layer of water and adsorb to the surface. Because of the lack of hydrogen-bondable functional groups on nonpolar hydrophobic surfaces, the initial surface-adsorbed layer of water exists in a highly structured, high free-energy state compared to water molecules in the bulk solution. This condition results in the rapid displacement of this layer of adsorbed water by nonpolar functional groups of biomolecules (e.g., proteins), leading to a large decrease in the free-energy state of the system and strong biomolecule adsorption as the adsorbed water is displaced from the surface back into the bulk solution. Surfaces with charged functional groups resulting in a net surface charge (i.e., other than net-neutral zwitterionic functional groups) tend to more strongly interact with oppositely charged functional groups of biomolecules than water. This again results in the thermodynamically favorable adsorption of oppositely charged biomolecules to the surface. In contrast to this, neutrally charged highly hydrophilic surfaces, such as polyethylene glycol and many zwitterionic surfaces, bind to water in a manner causing the free-energy state of the adsorbed water to be sufficiently low that the displacement of the water by biomolecules in

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solution is thermodynamically unfavorable, leading to surfaces that are highly resistant to protein adsorption.

Coagulation and complement activation can often be minimized at charge-neutral, strongly water binding surfaces with high substrate surface mobility [67-71]. Such moieties are widely used to inhibit protein adsorption and immune recognition in circulating nano/microcarriers, and to avoid surface-triggered blood coagulation on biomaterials in general [69, 72, 73]. It should be noted however that in the case of PEG/PEO and PEG/PEO-like surfaces there is conflicting evidence regarding complement activation. Thus some authors have reported that PEG/PEO based materials are complement activating [74, 75] and others that they are not [76-78]. More details on material surfaces that are designed to minimize protein adsorption from blood plasma can be found in part 4 of this article.

Not agreed upon or not known:

1. Factor XII activation during surface induced coagulation is not fully understood.
2. The combined roles of coagulation and immune complement in blood and tissue biocompatibility is not understood.
3. Activation of the tissue factor pathway of coagulation at biomaterials surfaces is not well understood.

Discussion:

1. The FXII activation mechanisms during surface-induced coagulation remain obscure. *In vitro* experiments with purified FXII in buffer solution and negative hydrophilic or hydrophobic surfaces revealed, surprisingly, that autoactivation of FXII was enhanced on *both* types of surfaces [79]. However, when plasma contact was initiated, both the activation rate and yield were significantly attenuated on hydrophobic surfaces but increased on hydrophilic surfaces. Also, contact activation of FXII in the presence of proteins unrelated to the plasma coagulation cascade was accelerated on negative hydrophilic surfaces but suppressed on hydrophobic surfaces [80]. The impact of contact activation proteins and other adsorbed plasma proteins was obviously significant although the rate limiting mechanisms are not well understood and are not yet associated with specific proteins. It is, however, tempting to speculate that strongly adsorbed plasma proteins on hydrophobic surfaces will hinder access of FXII; in contrast on hydrophilic, negative surfaces protein binding is weaker and attractive interactions with the histidine-rich domains of FXII become possible, thus facilitating access to the surface and thus autoactivation of FXII.

   Recently it was suggested that FXII activation *in vivo* is caused by negatively charged polyphosphates (polyP) released from platelet dense granules [81]. These results could, however, not be confirmed in later experiments [82], although it is now well accepted that polyP aggregates released from platelet alpha granules (negative surfaces) have the ability to activate FXII [52]. It is not

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understood, however, under what circumstances and in what species this phenomenon occurs. The surface protein layer seems not important in this case.

2. The combined roles of coagulation and complement activation in blood compatibility is not well investigated. Reactive C3b* deposition from plasma is apparently not dependent on the adsorbed protein layer composition, although pure surface immobilized fibrinogen and fibrin films were shown to inhibit complement deposition [83]. It is also known that adsorbed pure C3 and monocytes in combination activate the tissue pathway of coagulation during cardiopulmonary bypass, and provide a direct link between coagulation and complement at foreign surfaces [84]. The interconnection between the systems is further complicated by the fact that currently we do not know if, and if so when, molecules that activate the lectin pathway trigger MASP 1-3 to bind to artificial surfaces. If so, then coagulation is activated via the above-mentioned mechanisms. In summary, very little is known about interactions between the protein cascade systems and how these in turn are related to inflammatory and immune cells, wound healing and biomaterials integration.

3. Tissue factor (TF) and tissue-pathway-triggered coagulation have not been reported at biomaterial surfaces in flowing blood *in vitro.* Interestingly, the tissue pathway of coagulation was instead connected to complement activation [85, 86]. Complement C3 and C5 may be necessary for monocyte- and/or granulocyte-mediated release of TF with a subsequent risk of anti-phospholipid antibody-mediated thrombophilia [85] and of C5a generation during thrombus formation at endothelial surfaces [85]. Hence, the anaphylatoxin C5a appears to be an important molecule for the terminal complement pathway (lysis of bacteria and fungi), during inflammatory/immune conditions, and possibly for the activation of the tissue-factor pathway of coagulation. The implication for biomaterials is that coagulation, and thereby wound healing, are probably modulated in part via the complement and the tissue factor pathways acting in concert. The significance of these effects as well as their complicated interrelations remain largely unexplored and it will be of fundamental interest in future *in vivo* blood compatibility studies to investigate surface-triggered coagulation and immune complement activation in concert, rather than independent phenomena.

4. How does the adsorbed protein layer composition affect blood cell (particularly platelet) adhesion and activation?

Agreed upon and/or known:

1. The biological basis for platelet adhesion and activation on biomaterials is the presence of adhesion receptors on platelets that mediate the adhesion of platelets to proteins adsorbed from blood to the biomaterial surface (Figure 2).

2. Since unactivated platelets do not bind to soluble fibrinogen but do bind to fibrinogen adsorbed to biomaterials, the ability of adsorbed fibrinogen to bind to

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platelets is thought to be due to conformational changes induced in adsorbed fibrinogen that result in the exposure of platelet binding regions of fibrinogen.

Discussion:

1. The receptor GP IIb/IIIa is an integrin receptor that binds to fibrinogen and mediates platelet aggregation of activated platelets by using fibrinogen to bridge between the platelets, allowing platelet plugs to form and arrest bleeding in injured arteries [87, 88]. The other major adhesion receptor on platelets is the receptor for von Willebrand's factor (vWf), GPIb-V-IX, a non-integrin receptor responsible for platelet adhesion under shear [89]. Because of the fibrinogen and vWf adhesion receptors on platelets, the nonspecific competitive adsorption of these proteins to biomaterials from plasma is the reason that platelets adhere to biomaterials. Thus, for example, platelet adhesion to biomaterials preadsorbed with afibrinogenemic plasma is very low but is restored if exogenous purified fibrinogen is added to the deficient plasma used for preadsorption [90]. Other adhesion proteins such as fibronectin or vitronectin that can promote adhesion when preadsorbed in pure form do not appear to play a role when adsorbed from plasma as there is no decrease in adhesion to surfaces preadsorbed with plasmas selectively deficient in these proteins [91]. Although vWf adsorption has no effect on adhesion in the absence of shear [91, 92], preadsorption of biomaterials with vWf-deficient plasmas greatly reduces platelet adhesion under shear flow and adhesion is restored by addition of purified vWf to the deficient plasma used for preadsorption [92-95]. Adhesion to biomaterials also appears to activate platelets because adherent platelets are more procoagulant than nonadherent platelets, as indicated by the shortening of clotting time of plasma exposed to adherent platelets and by direct measurement of thrombin generation rates by adherent platelets [96, 97].

2. The ability of adsorbed fibrinogen to mediate platelet adhesion varies with surface type and adsorption conditions due to differences in availability of platelet binding domains. Thus, fibrinogen adsorption is necessary for adhesion, but adhesion is not proportional to the quantity of adsorbed fibrinogen. Instead it correlates well with reactivity of the adhesion domains as measured with monoclonal antibodies that bind to the adhesion motifs [98, 99]. More recent evidence for the role of changes in conformation of adsorbed fibrinogen that are needed for platelet adhesion was provided by Sivaraman and Latour [100]. These investigators directly measured losses in helical content of adsorbed fibrinogen using circular dichroism, and showed a direct correlation between platelet adhesion and the degree of adsorption-induced fibrinogen unfolding on a series of surfaces varying widely in surface chemistry. Many other studies report conformational changes in adsorbed proteins, e.g., a recent study showing the effect of various protein-repellent zwitterionic coatings on conformational alterations in several model proteins interacting with nanoparticles [101]. Conformational changes are further discussed in much greater detail elsewhere in

this series (see "What is The Role of Adsorbed Protein Conformation and Denaturation?") and in a recent overview of work on fibrinogen adsorption [102].

The strict requirement for adsorbed, recognizable fibrinogen in mediating platelet adhesion to biomaterials represents a design criterion for blood compatible surfaces, i.e. surfaces that eliminate fibrinogen adsorption should be more blood compatible because of the prominent role of adsorbed fibrinogen in mediating platelet adhesion. Similarly, any surface with low vWf adsorption should prove highly resistant to platelet adhesion under shear flow conditions.

Not agreed upon or not known:

1. The role of specific adsorbed proteins such as fibrinogen or vWf, including the relative amounts of these proteins, in promoting blood clotting on biomaterials *in vivo* is largely unknown, particularly in longer-term blood contact.
2. Adsorbed albumin was long considered to passivate biomaterials in regard to platelets, but this idea requires some refinement in the light of studies showing that conformationally altered albumin supports platelet adhesion.
3. The role of other proteins present in the adsorbed protein layer on many biomaterials, e.g., HMWK, Factor XII (FXII), and lipoproteins, in platelet-surface interactions, is not well understood.

Discussion:

1. A few *ex vivo* studies have shown that preadsorption of biomaterials with adhesion proteins (fibrinogen, vWf, and fibronectin) enhanced the deposition of platelets in the acute phase [103]. However, most studies showing a strong role for adsorbed proteins in supporting platelet adhesion were carried out *in vitro.* Thus, the role of these adhesion proteins in longer term *in vivo* events, such as steady state platelet turnover or clotting on stents, is still unknown. Studies of the protein layer on materials exposed to blood for long periods of time have shown that many of the adsorbed proteins are present in proteolytically degraded forms [104, 105] but the role, if any, of degraded versions of adhesion proteins to support platelet interactions is not known. For example, while it might be expected that degradation of the initially adsorbed adhesion proteins would reduce their platelet reactivity, there is no direct evidence that this is the case.

   Other large gaps in our understanding of the role of adsorbed proteins in hemocompatibility *in vivo* exist, e.g., knowledge of the threshold quantity of adsorbed fibrinogen below which platelet adhesion and activation do not occur. The ability of plasma deposited tetraglyme coatings to resist fibrinogen adsorption can readily be varied by varying the power used for plasma deposition. Similarly, the quantity of adsorbed fibrinogen “allowed” in other nonfouling coating systems can be varied. Thus, testing a series of materials varying in fibrinogen adsorption appears to be a way to better establish the role of fibrinogen adsorbed quantity in causing variations in platelet reactivity in *in vivo* testing.

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2. Many *in vitro* studies [90, 91] and at least one *ex vivo* shunt study [103] have shown that adsorbed albumin inhibits platelet adhesion. Furthermore, it is standard procedure in cell adhesion studies to use albumin "blocked" surfaces as controls to which the same surface preadsorbed with a protein being tested for its platelet adhesiveness (e.g. fibrinogen) is compared. However, as discussed in detail elsewhere in this article (see "What is The Role of Adsorbed Protein Conformation and Denaturation?") it has been found that conformationally altered albumin adsorbed from solution can promote platelet adhesion and activation [106] and that this interaction involves the GPIIb/IIIa receptor [107].

   However, the role of adsorbed albumin in supporting platelet adhesion and activation from whole blood under clinical conditions would seem to be minor. This statement is based on two considerations. First, platelet adhesion on surfaces exposed to plasma deficient in fibrinogen is very much reduced but can be restored to “normal” by addition of exogenous fibrinogen [90, 108]. The surfaces used in these studies are highly likely to have adsorbed albumin, due to the high concentration of this protein in both the plasma used for the preadsorption step and in the blocking solution used after plasma preadsorption to suppress nonspecific adhesion to exposed substrate sites. Such studies indicate a major role of adsorbed fibrinogen, and suggest that any role of albumin must be minor. Second, albumin adsorbed from plasma or albumin-blocking solution would be in a densely packed layer that would inhibit unfolding. This idea is well supported by studies showing that conformationally altered albumin caused platelet adhesion and that albumin adsorbed at higher concentrations was less platelet reactive [106]. In summary, under clinically relevant conditions, there is very strong evidence of the dominance of adsorbed fibrinogen in mediating platelet adhesion, and it seems likely that any contribution from adsorbed albumin due to conformational alterations will be very small because conditions do not favour albumin unfolding, at least in the short term. Longer term adsorption studies have shown that a layer of albumin that is adsorbed with minimal denaturation and does not support platelet adhesion response initially can undergo aging-induced unfolding over a six-month period and then does support platelet adhesion [109]. Thus it is possible that adsorbed albumin (and other proteins) may play a role in platelet adhesion after longer periods of blood contact.

3. There is some evidence for the role of other proteins in platelet interactions with surfaces. It has been reported that adsorbed HMWK inhibits platelet adhesion [110]. In this regard, HMWK seems similar to most of the proteins in plasma in the sense that it is a passivating protein that inhibits rather than promotes platelet adhesion. However HMWK may play a bigger passivating role than its low plasma concentration might suggest because of its ability to displace adsorbed fibrinogen (Vroman effect). Siedlecki et al. [79, 80, 111] have investigated interactions involving proteins of the contact pathway of coagulation. They showed that FXII becomes activated upon adsorption leading to initiation of the contact pathway, and that contact activation and platelet adhesion act

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synergistically in the process of thrombus formation on surfaces [79, 80, 111]. Also lipoproteins have been shown to adsorb to many surfaces, including protein repellent surfaces [112, 113], and thus may play a role in blood-biomaterial interactions. However there is little evidence that adsorbed lipoproteins promote platelet adhesion and activation (see section: "Can nonspecific protein adsorption be eliminated; would a protein resistant surface in blood be generally passive and non-thrombogenic?").

5. What is the role of adsorbed protein conformation and denaturation?

Agreed upon:

1. Protein adsorption and adsorption-induced changes in protein conformation on biomaterial surfaces play a key role in the contact activation of the intrinsic blood coagulation cascade.

2. Although nonactivated platelets do not interact strongly with fibrinogen in solution, it is apparent that they are able to adhere to and activate against surface-adsorbed fibrinogen. This behavior suggests that platelet adhesion-activation on adsorbed fibrinogen is mediated by mechanism(s) related to adsorption-induced changes in fibrinogen conformation [2, 98, 114-118].

3. Although it was previously believed that a layer of adsorbed albumin provides resistance to platelet adhesion and activation under all conditions because of the lack of platelet binding sites in the albumin structure, recent research has shown that under certain conditions platelets can adhere to and become activated on adsorbed layers of albumin [106].

Discussion:

1. Proteins often undergo conformational changes when they adsorb onto biomaterial surfaces, with the degree of conformational changes influenced by the chemistry and topology of the surface as well as the inherent structural stability of the protein. Studies investigating the influence of surface chemistry on adsorbed protein structure have shown that nonpolar hydrophobic surfaces tend to induce the greatest degree of protein unfolding while neutrally charged hydrophilic surfaces tend to induce the least amount of unfolding [5, 65, 119, 120]. Studies of the influence of surface topology on the conformational behaviour of proteins have been conducted by treating similar surfaces to vary surface roughness as well as using nanoparticles of different diameters to vary surface curvature [35, 121]. These studies have clearly documented that surface topology influences both the amount and conformation of adsorbed proteins, with the adsorption response also being dependent on the type of protein adsorbed.

   With respect to blood compatibility, contact activation on biomaterial surfaces is understood to be mediated by adsorption-induced conformational changes of adsorbed proteins leading to the activation of factor XII, prekallikrein, and high-molecular-weight kininogen, which initiate blood coagulation via the intrinsic

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pathway. This issue is addressed in this series under the topic "How does the protein layer composition affect coagulation and complement."

2. Early studies suggested that platelet response to adsorbed fibrinogen is related to its conformation in the adsorbed state [2, 98, 114-118]. More recently, conformation effects were studied directly using circular dichroism spectropolarimetry (CD) to measure adsorption-induced changes in fibrinogen structure [100, 122]. It was shown that platelet adhesion was strongly and directly correlated with the degree of adsorption-induced unfolding of fibrinogen. Platelet adhesion and activation were found to occur only to a nominal extent when the adsorbed fibrinogen retained its near native structure, but as the extent of unfolding increased, adhesion and activation increased by up to 5-fold [100]. Additionally, these studies showed that platelet adhesion to the adsorbed fibrinogen was mediated by the platelet GPIIb/IIIa receptor under all conditions.

3. Recent studies using CD to measure the degree of adsorption-induced changes in the structure of human serum albumin (HSA) showed that platelet adhesion to surface-adsorbed HSA was negligible as long as the near-native structure of the HSA was retained [106]. However, when adsorption conditions resulted in HSA being unfolded to an extent corresponding to greater than about 33% loss in α-helicity, platelet adhesion and activation were observed in direct proportion to the degree of HSA unfolding [106], similar to the fibrinogen case. Further studies showed that platelet adhesion to adsorbed and unfolded HSA is mediated by the same receptor that mediates adhesion to adsorbed fibrinogen—i.e, the GPIIb/IIIa receptor [107]. Platelet adhesion to adsorbed HSA is of particular interest because it has no known GPIIb/IIIa binding sites in its native-state structure. These results thus imply that GPIIb/IIIa binding sites are somehow created when adsorbed HSA is significantly unfolded.

Not agreed upon or not known:

1. Is more than one mechanism involved in platelet adhesion and activation when platelets interact with adsorbed proteins? For example, is there one mechanism that mediates binding via GPIIb/IIIa in non-activated platelets to fibrinogen adsorbed in its near-native-state, and an entirely different one that mediates such binding to fibrinogen adsorbed in an unfolded state?

2. It has been shown that an irreversibly adsorbed layer of HSA in which the native state conformation (as determined by CD) was retained was highly resistant to platelet adhesion [109]. However, upon aging in physiological saline over a 6-month period, the HSA underwent a substantial degree of unfolding, with subsequent loss of platelet "resistance." If this turns out to be a general mechanism for all irreversibly adsorbed proteins, then it may be expected that any surface that adsorbs a layer of protein irreversibly will eventually become thrombogenic on continued exposure to blood, even if it were initially resistant to platelet adhesion.

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3. To prevent aging-induced conformational changes, and consequent platelet adhesion and activation and thrombus formation, is it required that proteins be adsorbed reversibly, or at least that they be continuously exchanging with other proteins (Vroman effect) without denaturation [109, 115]?

Discussion:

1. As indicated, studies investigating the relationship between the degree of adsorption-induced protein unfolding and platelet adhesion showed that platelets adhered to adsorbed fibrinogen even without any detectable change in its structure, with adhesion increasing 5-fold as unfolding increased [100]. In contrast, negligible platelet adhesion was observed on adsorbed HSA which retained its near-native-state structure. However similar to the fibrinogen case, adhesion increased as HSA unfolding increased beyond a critical extent (corresponding to ~33% loss in helicity) [106].This behavior suggests that two separate mechanisms may be involved in platelet adhesion. In one mechanism, adhesion is mediated by platelet-binding motifs that are present in the native-state structure of the protein. This mechanism would explain why platelet adhesion occurs on adsorbed fibrinogen even in its near-native-state structure (e.g., via the C-terminus of the γ-chains), whereas adhesion does not occur on adsorbed HSA in its near-native-state since native HSA does not possess any platelet-binding motif [106]. In a second, completely different mechanism, the adsorption-induced creation of platelet-binding motifs may occur; this would explain the increase in platelet adhesion to both fibrinogen and HSA as adsorption-induced unfolding increases [100, 106]. It has been proposed that this mechanism involves adsorption-induced separation of “salt-bridged” amino acids causing them to transition from RGE-like to RGD-like motifs [106].

2. Proteins tend to adsorb to most materials irreversibly. While Vroman effects are known to result in the exchange of adsorbed proteins, it is likely that these processes stop or become negligible within a short period of time as the surface equilibrates with the surrounding protein-containing environment (e.g., blood). It can be expected that the adsorbed proteins will then undergo aging-induced unfolding over time. Therefore, if in general platelets adhere to adsorbed proteins that are unfolded, it is reasonable to speculate that surfaces that adsorb proteins irreversibly such that their native-state structure is retained and platelet adhesion is negligible initially, will begin to exhibit platelet adhesion and activation as the adsorbed proteins unfold over time. It seems possible that late thrombus formation on drug-eluting stents [123] may be due to such aging-induced protein unfolding.

3. If protein unfolding is one of the primary mechanisms that mediates platelet adhesion to adsorbed proteins, and if irreversibly adsorbed proteins can be expected to undergo aging-induced unfolding over time, then it may be desirable that biomaterial surfaces should adsorb proteins reversibly and in a manner that does not substantially alter the proteins’ native-state structures. This may be achieved in two ways. Firstly, by designing the surface to have high resistance to

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protein adsorption, indicating that the protein adsorption process is inherently reversible. Secondly, by designing the surface such that exchange of adsorbed proteins with proteins in the blood is continuous, with the additional condition that no significant conformational changes occur in the proteins that interact with the surface.

In conclusion with respect to protein conformation/denaturation, it is widely recognized that platelet adhesion to adsorbed proteins is substantially influenced by adsorption-induced changes in protein structure. However, the clinical consequences of this behavior are not yet understood. Most importantly, there is still much to be learned regarding how the understanding that we presently have can be exploited in the design of blood-contacting surfaces to provide improved blood compatibility for vascular and cardiovascular applications.

6. Can nonspecific protein adsorption be eliminated; would a protein-free surface in blood be generally passive and non-thrombogenic?

Agreed upon:

1. The adsorption of proteins to biomaterial surfaces can be reduced by a variety of chemical modifications of the surface.
2. Surfaces have been developed that are highly resistant to nonspecific protein adsorption, e.g., reducing fibrinogen adsorption to below 5 ng/cm^2.

Discussion:

1. Many strategies have been used to prepare materials that are resistant to nonspecific protein adsorption [2, 124-130]. These include incorporation of polyethylene glycol (PEG), other hydrophilic polymers (e.g., poly(hydroxyethyl methacrylate) (pHEMA), and zwitterionic molecules. Early work on PEO-containing materials by Merrill and coworkers showed, using *in vitro* platelet adhesion measurements [131] and an *ex vivo* baboon shunt model [132], that PEO reduced blood-surface interactions. Suppression of protein adsorption to metal oxide surfaces (TiO_2 and $Si_{0.4}Ti_{0.6}O_2$) was achieved with poly(L-lysine)-PEG copolymers (PLL-PEG) that spontaneously adsorbed to these materials to form stable coatings [71]. It was also found that maximum suppression was achieved when the radius of gyration of the PEG chains was greater than the distance between attachment points of the PEG chains, i.e., at high packing density. The clotting times of whole blood and plasma were 2-to-3 times longer on the PLL-PEG surfaces than on uncoated titanium [73]. In a different approach, PEO copolymers were blended with a polyurethane [133], giving materials that adsorbed as little as 26 ng/cm^2fibrinogen from whole blood, with correspondingly low platelet densities of ~10^4 per cm^2, compared to 600 ng/cm^2 fibrinogen and 10^6 platelets/cm^2 on the unmodified polyurethane, with good correlation between fibrinogen adsorption and platelet adhesion. Nonfouling materials based on PEO, PEO-like polymers, and zwitterionic species, were reviewed by Szott and Horbett [134]. A recent, brief review of nonfouling

materials was also included in a review of fibrinogen adsorption studies [102]. Typically for such materials it has been found that although protein adsorption is reduced, it is never completely eliminated.

2. Materials with very high resistance to protein adsorption, greater than that of PEO materials, have recently been reported. For example, poly(carboxybetaine acrylamide) (polyCBAA) grafted surfaces were shown to reduce fibrinogen adsorption from plasma to well under 1 ng/cm^2, i.e. substantially below the 5 ng/cm^2 thought to be required to support platelet adhesion [135]. Furthermore these materials do not cause a foreign body reaction when implanted in soft tissue [136]. It is worth emphasizing that all previously tested materials, including other zwitterionic materials that adsorb higher amounts of fibrinogen [137], do elicit a foreign body reaction. Thus, it may be a worthwhile objective to evaluate the *in vivo* performance of this and other materials that exhibit extremely low fibrinogen adsorption. Testing in both the short term and long term should be carried out since as of the present time, the relationship between short- and long-term blood compatibility testing in animals is unknown. More recently, a novel method has been described to coat hydrogel surfaces with zwitterionic polymers; this also results in greatly reduced fibrosis around the surfaces after 14 days in the peritoneal cavity of mice, although fibrinogen adsorption was not reported [138].

   A recent study reported that a novel type of non-fouling coating formed by chemical vapor deposition of hydroxyl poly-p-xylylene and subsequent ring opening polymerization with an epoxide resulted in hyperbranched polyglycerol coated surfaces that strongly resisted protein adsorption and cell adhesion [139]. Finally, a study of the interactions of PEG and zwitterionic polymers with model proteins in solution designed to better understand the fundamental nature of protein interactions with these types of polymer was conducted using several spectral methods to determine whether the proteins were affected by the polymers [140]. These methods showed the existence of weak hydrophobic interactions between PEG and proteins, while there were no detectable interactions between the zwitterionic material (poly(sulfobetaine methacrylate) and proteins.

Not agreed upon or not known:

1. Is resistance to nonspecific protein adsorption, resulting in resistance to platelet adhesion and activation and the formation of thrombus and thromboemboli, necessary and sufficient to provide a high degree of biomaterial blood compatibility *in vivo?*

2. How stable are any of the protein resistant surfaces over time with regard to prevention of nonspecific protein adsorption?

3. Are highly hydrophilic surfaces that tend to be highly resistant to nonspecific protein adsorption also highly complement-activating?

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4. Is the elimination of fibrinogen adsorption sufficient to provide blood compatibility or must surfaces also be resistant to other proteins, including proteins that may be involved in promoting blood clotting?
5. How "protein repellent" must a surface be to be considered "blood compatible"?
6. Are protein-repellent surfaces that are resistant to platelet adhesion also resistant to blood coagulation via the intrinsic pathway of the clotting cascade?

Discussion:

1. The ability of protein-resistant surfaces to prevent clot promoting events such as platelet adhesion and activation of the intrinsic clotting cascade has so far been evaluated almost exclusively using *in vitro* methods. It remains to be determined whether these properties will be maintained under clinically relevant conditions. Currently, blood clotting and thrombosis associated with devices used clinically, such as stents, is minimized by systemic anticoagulant therapy. However, increases in mortality due to clotting in patients with drug eluting stents (DES) compared to those with bare metal stents (BMS) [141] clearly show a continuing need for improved materials [142]. The urgent need to extend the testing of protein repellent surfaces to more clinically relevant *in vivo* testing in animals is also highlighted by the stent experience.
2. Most protein resistant materials have not been evaluated with regard to the long-term maintenance of their properties. Some of them, in fact, appear to be chemically unstable. Thus PEG/PEO has been shown to undergo metal ion-catalyzed oxidation over time causing it to lose its protein resistance [2]. On the other hand, at least one type of zwitterionic protein-resistant surface seems to have good long term functional stability since it eliminated the foreign body reaction around implants for 30 days [136]. Nonetheless, it is generally not known if protein resistant (nonfouling) surfaces are only useful in short-term applications (e.g., temporary catheters, hemodialysis systems, blood oxygenators for heart-lung bypass surgery), or if they will retain protein resistance over time sufficient for long-term applications (e.g., vascular prostheses, stents, ventricular assist devices). Whether there are specific surface chemistries and designs that are inherently more stable and resistant to change over time is also unknown.
3. With regard to complement activation by protein resistant surfaces, PEG and PEG-modified surfaces have been shown to activate complement [2, 143, 144]. Similarly, coatings of plasma-polymerized tetraglyme, a PEG like material, were shown to be strong activators of complement [74]. Whether other highly protein-resistant surfaces, in particular zwitterionic materials, are also subject to complement activation is currently unknown.
4. While much work on protein resistant surfaces has focused on reducing fibrinogen adsorption because of its role in mediating platelet adhesion, there is evidence that at least some surface treatments that are highly resistant to the adsorption of fibrinogen and other proteins nonetheless adsorb LDL quite strongly from buffer [112], plasma [113], and whole blood [145, 146].

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Furthermore, in one of these studies albumin and fibrinogen were found in the adsorbate from plasma, but not in that from buffer, suggesting that the adsorption of albumin and fibrinogen from plasma was mediated by other proteins such the apolipoproteins contained in LDL [145]. While early studies indicated that platelets have receptors for lipoproteins and thus bind soluble LDL [147-150], and while one study showed that lipoproteins adsorbed to biomaterials promote platelet adhesion [151], there is insufficient evidence to indicate a definitive and significant role for adsorbed lipoproteins in platelet-surface interactions.

5. The degree of protein resistance required to achieve blood compatibility is not well established. However, if adsorption is not completely eliminated, compatibility will surely be compromised because it appears that platelets need relatively little adsorbed fibrinogen to support adhesion. Thus, Tsai et al. [90] observed that replenishment of afibrinogenemic plasma with varying amounts of exogenous fibrinogen restored full scale platelet adhesion to polystyrene surfaces preadsorbed with the plasmas. However, much less than the normal concentration of fibrinogen was needed, so that as little as 5 ng/cm^2 of adsorbed fibrinogen was sufficient to restore full adhesion levels [90]. The extreme sensitivity of platelets to adsorbed fibrinogen was confirmed in subsequent studies in which exogenous fibrinogen was added to serum used to preadsorb several types of surfaces [91]. These observations led to the idea that biomaterials with ultralow fibrinogen adsorption would be needed to prevent platelet interactions [91].

   It must be emphasized that protein resistance should be evaluated in contact with blood or plasma, not simple protein solutions. Thus, for example, plasma-deposited tetraglyme coatings [125, 152] and several zwitterionic materials [153] were shown to adsorb less than 5 ng/cm^2 from simple media but adsorption was well above this level from full strength plasma. Few biomaterials have been evaluated under appropriate conditions, although certain zwitterionic types have “passed” the full strength plasma test, with fibrinogen adsorption from plasma shown to be well under 1 ng/cm^2 [135].

6. It has been found that some, but not all, materials that resist fibrinogen adsorption and platelet adhesion are also known to be less active in promoting clotting via the intrinsic system. For example, the clotting of recalcified plasma was much slower on plasma-deposited tetraglyme-coated surfaces than on controls [125]. Extended clotting times were also reported for several types of zwitterionic and ethylene-glycol self-assembled-monolayer (SAM) surfaces [153], and for zwitterionic sulfobetaine-grafted poly (vinylidene fluoride) membranes [154]. On the other hand, in a series of zwitterionic glycosyl-modified polyethersulfone membranes with good resistance to protein adsorption, the clotting times were not greatly increased compared to controls [155].

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7. Can the protein layer composition be controlled and "tailored" to achieve blood compatibility?

Given that the adsorbed protein layer determines the outcome of blood-material contact, the question arises: "can we design a surface to control the properties of the layer, particularly its composition, to achieve blood compatibility?"

Agreed upon:

1. Certain proteins are undesirable in the adsorbed layer, others are desirable.
2. Surfaces should be designed to capture specific desirable proteins and repel all others. Desirable proteins include albumin, antithrombin, plasminogen, t-PA, thrombomodulin and protein C.

Discussion:

1. It is clear that fibrinogen is undesirable, as are other adsorbed proteins that mediate platelet adhesion and activation, e.g., fibronectin and von Willebrand factor. Proteins that may initiate and/or propagate coagulation, primarily the proteins of the intrinsic pathway, are likewise undesirable. Proteins that are desirable include inhibitors of coagulation, e.g. antithrombin, thrombomodulin and protein C (in its activated form) [156]. Proteins such as plasminogen and t-PA that may promote fibrinolysis are also desirable, as is albumin based on its reputed resistance to platelet adhesion as discussed elsewhere in this article.
2. Following from point #1, an overall design approach may be formulated, namely that the layer should contain proteins which promote desirable biological effects and should resist all others. Attempts to implement this idea involve surfaces designed both to resist nonspecific protein adsorption, and to adsorb specifically proteins that promote anticoagulation, fibrinolysis and other desirable responses. For example a number of designs have been investigated where resistance to nonspecific adsorption is based on modification with poly(ethylene oxide)-poly(ethylene glycol) (PEO/PEG) [10, 157-159] or zwitterionic species [160]. Some of these surfaces have been designed, in addition, to capture specific proteins: e.g., plasminogen and t-PA by modification with lysine to promote fibrinolysis[161-164]; thrombin by attachment of hirudin to prevent fibrinogenesis [165]; antithrombin by attachment of an antithrombin-heparin complex [159] or heparin [166] to inhibit coagulation; and factor XIIa by modification with corn trypsin inhibitor to prevent contact-system activation [167]. Protein C, which in activated form interacts with thrombin and thrombomodulin on the endothelial surface to inhibit factors Va and VIIIa should also be mentioned in this connection [168, 169].

Not agreed upon or not known:

1. Albumin attached to a surface renders it 100% passive to cell adhesion, in particular to platelet adhesion.

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2. Surfaces with “tailored” protein layers will have limited stability over time. Priming with polydopamine or other “bioadhesive” may/may not be useful in this regard.

Discussion:

1. The literature on adsorbed albumin as a passivating agent in blood contact is extensive [170,171] and in particular there is widespread belief that albumin layers are unreactive to platelets since they do not possess amino-acid sequences required for binding platelet receptors. Sivaraman and Latour [106] showed that platelets do adhere to albumin layers in which the albumin is unfolded to expose amino acid sequences that bind to integrin platelet receptors. Also it is not clear that albumin can give complete protection over long time, possibly due to loss by exchange with other blood components.

2. As discussed above protein-surface interactions in blood contact are known to be dynamic. Therefore it seems unlikely, even if a surface can be tailored to be initially selective for specific proteins, that the layer will retain its composition over long periods as generally required in blood contacting devices. However, if the capture mechanism is such that the protein can be continuously renewed (e.g., heparin capture of antithrombin), then protein layer “tailoring” may still be a valid approach.

   Also relevant in this regard is the use of “bioglues” to bind the protein more tightly to the surface. Perhaps the best known example of a bioglue is polydopamine, related to the adhesive protein of mussels. A considerable body of work has shown that dopamine is easily bonded to surfaces by simple solution coating methods [172-175]. Dopamine undergoes oxidative polymerization, resulting in a layer of polydopamine (PDA) which acts as a “bioglue” for the attachment of biomolecules containing thiol or amino groups [172, 176]. Proteins attached to surfaces via PDA, given that the bonding is covalent, may have relatively long half lives in blood. The “downside” of PDA layers is that they are strongly adsorbing of proteins generally, so that the PDA must be fully covered by the bioactive species to avoid nonspecific adsorption [177].

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Statement of significance

This paper is part 2 of a series of 4 reviews discussing the problem of biomaterial associated thrombogenicity. The objective was to highlight features of broad agreement and provide commentary on those aspects of the problem that were subject to dispute. We hope that future investigators will update these reviews as new scholarship resolves the uncertainties of today.

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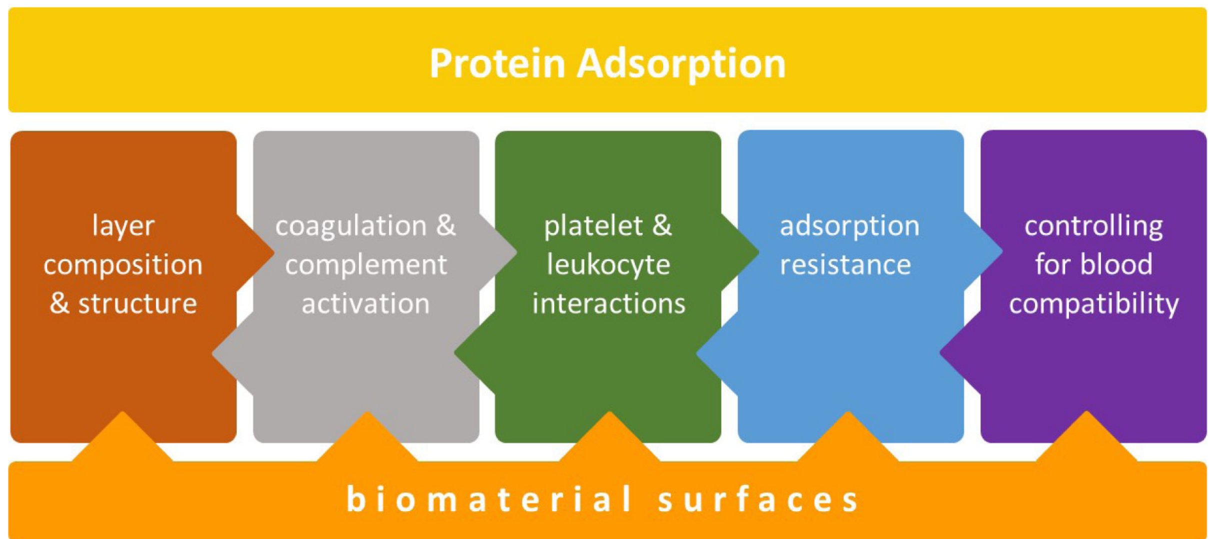

Figure 1.
Key aspects of protein adsorption on biomaterial surfaces

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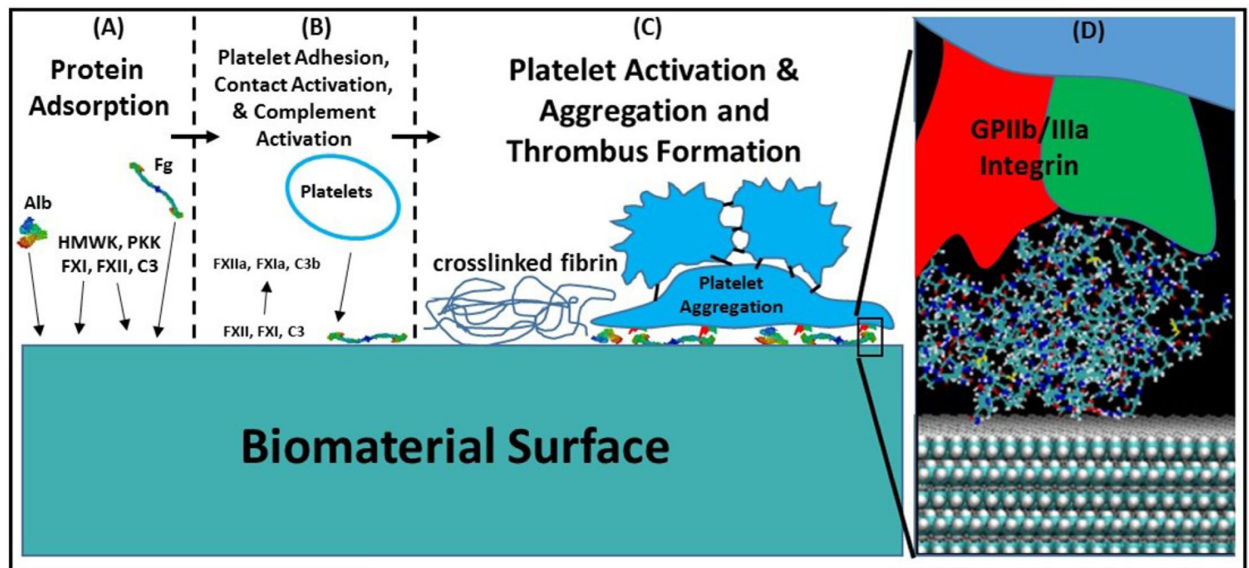

Figure 2.

Overview of protein adsorption and subsequent events induced on biomaterial surfaces. (A) Adsorption of proteins to biomaterial surface as initial event (Alb: albumin, Fg: Fibrinogen, HMWK: high-molecular weight kininogen, PKK: prekallikrein, FXI & FXII: factor XI and factor XII intrinsic blood coagulation cascade proteins, C3: complement protein). (B) Non-activated platelets adhere to layer of adsorbed proteins, adsorbed FXI and FXII initiate contact activation processes, and C3 is activated to C3b. (C) Activated platelets, contact activation proteins, and complement activation synergistically interact to induce thrombus formation. (D) Platelet interaction with an adsorbed protein via a GPIIb/IIIa integrin receptor; protein is illustrated as adsorbed on a polyethylene surface.