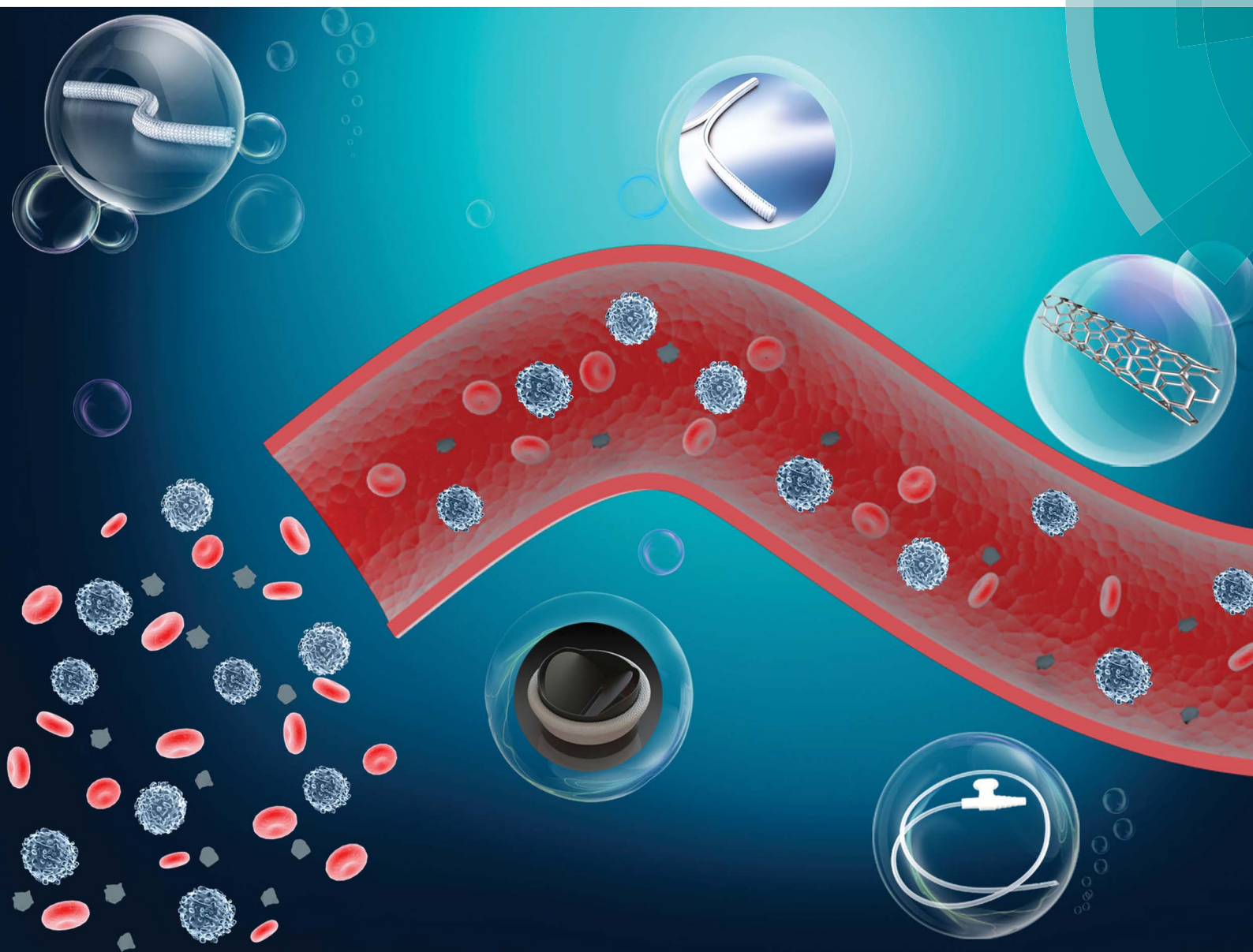


# Journal of Materials Chemistry B

Materials for biology and medicine

[www.rsc.org/MaterialsB](http://www.rsc.org/MaterialsB)



ISSN 2050-750X



## FEATURE ARTICLE

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Cite this: *J. Mater. Chem. B*, 2014, 2, 5718

Received 31st May 2014  
Accepted 16th July 2014  
DOI: 10.1039/c4tb00881b  
[www.rsc.org/MaterialsB](http://www.rsc.org/MaterialsB)

# Blood compatible materials: state of the art

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Devices that function in contact with blood are ubiquitous in clinical medicine and biotechnology. These devices include vascular grafts, coronary stents, heart valves, catheters, hemodialysers, heart-lung bypass systems and many others. Blood contact generally leads to thrombosis (among other adverse outcomes), and no material has yet been developed which remains thrombus-free indefinitely and in all situations: extracorporeally, in the venous circulation and in the arterial circulation. In this article knowledge on blood–material interactions and “thromboresistant” materials is reviewed. Current approaches to the development of thromboresistant materials are discussed including surface passivation; incorporation and/or release of anticoagulants, antiplatelet agents and thrombolytic agents; and mimicry of the vascular endothelium.

## 1. Introduction

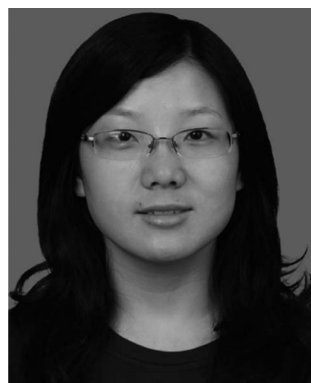
Blood compatible materials, *i.e.*, materials that can be used in contact with blood without causing harm, are required for a wide variety of medical devices. The range of applications is broad and covers implants in the cardiovascular system

(catheters, stents, valves, vessel grafts, circulatory assist devices) and extracorporeal blood treatments (apheresis, hemodialysis, oxygenation/heart-lung bypass). Despite several decades of effort, materials that are compatible with blood over time and in all situations have not been discovered or developed. Devices in use clinically at the present time require the use of medications such as anticoagulants; even then there is significant incidence of thrombosis and other complications. Harms caused by material–blood contact include platelet consumption, complement activation, and plasma protein depletion and denaturation. Material calcification leading to loss of mechanical properties is also a problem for chronically implanted devices, particularly tissue-derived prosthetic heart valves. Loss of material properties over time *via* biodegradation is similarly a

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potential longer term limitation. However clot and thrombus formation (and thrombo-embolization in the case of implants) remain the most serious and most intractable problems in the development of blood contacting devices. These phenomena cause, for example, the rapid occlusion of vascular grafts of diameter less than about 6 mm,<sup>1</sup> the blockage of blood sampling catheters,<sup>2,3</sup> and the failure of heart assist devices.<sup>4</sup>

A number of approaches to non-thrombogenic materials have been proposed. Examples are materials with incorporated anticoagulants (notably heparin)<sup>5</sup> and anti-platelet agents;<sup>6,7</sup> materials having porous surfaces to encourage limited thrombosis that can re-organize to an inert pseudo-endothelium;<sup>8</sup> and materials that attempt to mimic the vascular endothelium.<sup>9–11</sup> These and other approaches have so far met with only limited success; the search, therefore, continues.

The process of thrombus formation on artificial surfaces is complicated. When a biomaterial comes into contact with blood it rapidly becomes covered with a layer of proteins.<sup>12</sup> Platelets adhere to the protein layer, are activated and form aggregates; coagulation is initiated and fibrin is formed. The complex of platelet aggregates, fibrin and trapped red cells constitutes the thrombus.

Since blood–material interactions are strongly dependent on the chemical and physical properties of the material surface, surface modification has been investigated extensively. Three general strategies have been used and may be described as follows. (1) Surface passivation, *i.e.* prevention of blood–surface interactions, especially non-specific protein adsorption. Materials modified with various hydrophilic polymers<sup>13–30</sup> and zwitterionic polymers<sup>31–43</sup> have been shown to be strongly ‘protein resistant’. (2) Incorporation of bioactive molecules, *e.g.* anticoagulants,<sup>5</sup> platelet inhibitors,<sup>6,7</sup> and fibrinolytic agents.<sup>44</sup> (3) Mimicry of the vascular endothelium (the inner surface of intact blood vessels), the only surface that can truly be described as non-thrombogenic.<sup>10,11</sup>

In this article a brief introduction to surface-induced thrombosis is presented. The literature on anti-thrombogenic surfaces

is then reviewed. Topics include passive surfaces, anticoagulant and antiplatelet surfaces, thrombolytic surfaces, “topographic surfaces”, and endothelium mimicking surfaces. Other reviews of this area have appeared in recent years including those of Gorbet and Sefton,<sup>45</sup> Sefton *et al.*<sup>46</sup> and Li and Henry.<sup>47</sup>

## 2. Surface-induced thrombosis

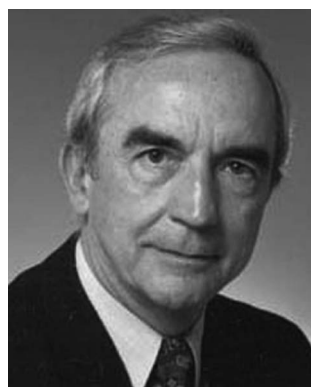
A schematic of blood–material interactions leading to thrombus formation is shown in Fig. 1.<sup>48</sup> Three adverse responses are triggered by the initially adsorbed protein layer: plasma coagulation *via* the intrinsic pathway, platelet adhesion and activation, and leukocyte interactions leading to inflammation and transient cell depletion.<sup>49</sup> These responses are not independent of one another. For example thrombin generated in coagulation is a potent activator of platelets,<sup>50,51</sup> and adherent platelets can provide phospholipid that acts as a catalyst in several of the coagulation reactions (see below). The thrombus consists mainly of fibrin, platelets and red cells. Initially surface-localized, it can embolize to downstream locations such as the heart (causing myocardial infarction) and the brain (causing stroke).

The initial protein layers have been studied extensively, including their composition and kinetics of formation. Knowledge remains sketchy, however, reflecting, perhaps, the daunting complexity of blood, containing as it does several hundred different proteins, lipoproteins, lipids, ions, and other species.<sup>52</sup> Regarding layer composition, which may be thought of as the ‘proteome’ of adsorbed proteins, it is fair to say that this is not known in detail for any surface, and this despite recent advances in proteomics technology including multi-dimensional gel electrophoresis and advanced mass spectrometry. A number of studies have been reported,<sup>53–56</sup> but in general only partial data on adsorbed protein ‘profiles’ have been obtained.

It is believed that the presence of certain proteins in the layer should be favourable, and of others unfavourable for anti-thrombogenicity and blood compatibility generally. Fibrinogen, fibronectin, vitronectin, and von Willebrand factor are seen as unfavourable since platelet adhesion occurs *via* receptor–ligand interactions involving specific amino acid sequences (*e.g.* RGD) in these proteins.<sup>57</sup> Albumin has been seen generally as favourable since it has been shown that platelets adhere minimally to albumin layers adsorbed on polymer surfaces.<sup>58,59</sup> More recently, however, it has been shown that platelets do adhere to layers of conformationally altered albumin<sup>60</sup> and that macrophages adhere to adsorbed albumin layers.<sup>61</sup>

The phenomenon known as the Vroman effect<sup>62–65</sup> indicates that the layer composition evolves with time, such that proteins of high relative abundance are adsorbed early (seconds) and are displaced later (minutes) by proteins of low relative abundance and high surface affinity. The classic Vroman effect example is of initially adsorbed fibrinogen being displaced by high molecular weight kininogen, a low concentration protein of the intrinsic coagulation pathway. Such dynamic effects are seen mainly for hydrophilic surfaces on which the proteins are less tightly bound than on hydrophobic ones.<sup>66</sup>

Fibrin formation (plasma coagulation) proceeds by two pathways, the intrinsic (all components within the blood) and



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site. The conversion of factor X and factor II (prothrombin) is accelerated by phospholipids (notably phosphatidyl ethanolamine on the surface of activated platelets, platelet factor 3), and calcium ions are required for these reactions.

Platelet involvement (Fig. 1) begins with adhesion to the adsorbed proteins, fibrinogen in particular.<sup>67</sup> The adherent platelets undergo shape change (discoid to spread with pseudopodial extensions), release granule contents (*e.g.* platelet factor 4, adenosine diphosphate, serotonin) and form aggregates which are stabilized by fibrin generated simultaneously. This process is analogous to platelet plug formation *in vivo* (hemostasis) following damage to the blood vessel wall.

The mechanism of platelet adhesion to biomaterials has been studied in considerable detail.<sup>45</sup> Interactions between receptors on the platelet surface and specific amino acid sequences in adsorbed proteins appear to be crucial. The glycoprotein receptors GPIIb/IIIa and GPIb/IX are of particular importance.<sup>68</sup> The integrin receptor GPIIb/IIIa ( $\alpha_{IIb}\beta_3$  integrin) is the most abundant platelet surface receptor; when platelets are activated, this receptor undergoes a conformational change and binds to RGD sequences in adsorbed fibrinogen, fibronectin, vitronectin, von Willebrand factor (vWF) and thrombospondin. GPIb/IX, also an abundant platelet receptor, interacts with adsorbed vWF on surfaces in flowing blood; shear stress in flow causes a conformational change in GPIb (CD42) that is required for binding to vWF. The primary activation of platelets that potentiates the membrane receptors is not unequivocally established but most likely is related to thrombin (a potent platelet activator) generated in the thrombotic process or to ADP released from red cells and platelets.

It is noted that platelets can interact with surfaces without adhering. Non-adhesive encounters can lead to platelet damage, premature elimination from the circulation and microparticle formation by budding from the platelet membrane. Hydrogels appear to be particularly prone to these effects.<sup>69,70</sup> The role of such non-adhesive interactions in material-induced thrombosis is not clear although platelet microparticles have been shown to be procoagulant.<sup>71</sup>

The challenge, then, for materials science is to design materials that prevent the activation of coagulation and platelets when blood comes into contact. A summary of attempts to do so constitutes the remainder of this article. We distinguish two general approaches to this problem: (1) the surface is designed such that blood interactions are prevented or minimized, *i.e.* the blood effectively does not 'see' the surface; this is referred to as surface 'passivation'; (2) it is accepted that adverse interactions will occur but may be inhibited by incorporation of appropriate bioactive species or mechanisms.

### 3. Surface modification for anti-thrombogenicity

#### 3.1 Surface passivation

The objective in passivation is to weaken (ideally eliminate) the interactions of proteins and cells with the surface. Most of the work in this area has focused on proteins, and since cells

interact with adsorbed proteins, not the bare surface, the emphasis on proteins is logical and appropriate.

Before discussing protein resistant surfaces in detail, it is useful to provide a "benchmark" for adsorbed quantities of proteins since much of the literature reports adsorption in these terms. The characteristic parameter is the quantity (usually reported as mass per unit area) in a monolayer of protein in the native state where coverage is complete. This will vary depending on the mass and dimensions of the protein and the exact configuration of the layer, *e.g.* close packed, molecules oriented end-on or side-on to the surface (asymmetric proteins). In general smaller proteins have smaller monolayer quantities and side-on orientations have smaller quantities than end-on. From available data on protein dimensions, monolayer quantities should lie in the range of  $\sim 0.2$  to  $\sim 1 \mu\text{g cm}^{-2}$ . Fibrinogen for example, with MW 340 000 Da and dimensions of  $450 \times 90 \times 90 \text{ \AA}$ , should be expected to have monolayer surface concentrations of  $0.70$  and  $0.14 \mu\text{g cm}^{-2}$  end-on and side-on, respectively. Reported data generally fall within the expected range for most surfaces and proteins. For protein resistance, values in the range of a few  $\text{ng cm}^{-2}$  are the goal, *i.e.* reductions greater than 99% from monolayer quantities. It has been suggested, for example, that fibrinogen adsorption from blood must be less than  $\sim 30 \text{ ng cm}^{-2}$  since greater quantities lead to significant (damaging) platelet adhesion.<sup>67</sup> As will be seen, values of this order have been achieved; however in no case can it be said that adsorption has been eliminated entirely. Also surfaces that appear to be resistant in simple media such as single proteins in buffer may be less so in physiologic fluids like blood. For example Gunkel and Huck<sup>72</sup> showed that polymer brush surfaces resistant to adsorption in simple media, adsorbed as many as 98 different proteins from plasma.

Two general approaches to protein resistant surfaces have been investigated. The first is the incorporation of various hydrophilic polymers. Polyethylene oxide (PEO), also referred to as polyethylene glycol (PEG), polyvinylpyrrolidone (PVP),<sup>73</sup> poly(hydroxyethyl methacrylate) (PHEMA),<sup>74</sup> poly(dimethyl-aminoethyl methacrylate) (PDMAEMA)<sup>75</sup> and polysaccharides such as dextran<sup>76</sup> are examples. The second is based on modification with zwitterionic polymers such as poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC),<sup>77</sup> poly(carboxybetaine acrylamide) (PCBAA),<sup>34,35</sup> poly(carboxybetaine methacrylate) (PCBMA),<sup>36</sup> and poly(sulfobetaine methacrylate) (PSBMA).<sup>36</sup> Protein resistant surfaces based on peptides and containing uniformly mixed positive and negative charges have also been reported.<sup>78</sup>

In an extensive review of this area by Chen *et al.*,<sup>79</sup> a list is provided of polymers that have been found to endow materials with some degree of protein resistance. From this list it is difficult to discern any fundamental principle that would explain or predict protein resistance. Ostuni *et al.* have proposed that resistant surfaces should meet four criteria: they should be hydrophilic, incorporate hydrogen bond acceptors, not include hydrogen bond donors, and be net electrically neutral.<sup>80</sup> Although many surfaces known to be resistant do satisfy these criteria, there are exceptions, *e.g.* P(DMAEMA)

surfaces with significant net positive charge;<sup>75</sup> and surfaces containing hydrogen bond donors such as hydroxyl groups.<sup>81</sup>

Important variables for the protein resistance of polymer-grafted surfaces are the graft molecular weight and density, density being the more important of the two. Resistance has been found, in general, to increase with graft MW up to a limiting value of a few thousand (in the case of PEO), and then to level off.<sup>82</sup>

Density is often described in terms of “brush” and “mushroom” configurations, brush referring to graft layers where the chain density is high and the chains must stretch towards their fully extended conformation, mushroom where the density is low enough that the chains can adopt the random coil conformation (Fig. 3). Protein resistance is greater for the brush case. However one must also take into account the protein size since small proteins may be able to penetrate the spaces between the grafts of a brush layer and reach the underlying substrate where larger ones would not.<sup>83</sup> Although from these considerations it might be assumed that resistance should increase with graft density, it has been shown for PEO that there may be an optimum density above which resistance decreases,<sup>84</sup> possibly due to dehydration of the polymer chains at higher density.

Many reports have emphasized the role of hydration and water structure in protein resistance, and as a general rule protein adsorption has been found to decrease with increasing surface hydrophilicity.<sup>85</sup> He *et al.* have suggested that zwitterionic surfaces are protein resistant due to hydration *via* ionic interactions, while PEO-based surfaces are resistant due to hydration *via* hydrogen bonding, the former giving superior protein resistance due to the specific associated water structure.<sup>86</sup> Water involvement in protein resistance has been

discussed in detail by Morra,<sup>87</sup> by Szleifer *et al.*<sup>88</sup> and others. Of the two commonly proposed explanations for the resistance of PEO/PEG, namely what may be called the water barrier hypothesis and the steric repulsion hypothesis, respectively, the water barrier concept appears the more plausible. Steric repulsion describes the situation where grafted polymer chains are flexible enough (*e.g.* PEO) to be significantly compressed as protein molecules approach the surface. The loss of entropy upon layer compression constitutes a free energy penalty and therefore a repulsive interaction. However chain flexibility will most likely be a significant factor only for longer polymer chains, whereas it has been shown that short PEO chains of a few EO units are able to confer protein resistance.<sup>89</sup> Equivalent to steric repulsion is the idea of “entropic shielding” proposed by Wörz *et al.*<sup>90</sup> These authors showed that crosslinked networks of various hydrophilic polymers when highly water swollen, are strongly protein resistant regardless of the chemical composition of the network (*e.g.* poly(ethyleneglycol methyl ether methacrylate); poly(dimethyl acrylamide)).

Of the hydrophilic polymers shown to be protein resistant PEO/PEG is the most extensively investigated and is regarded as something of a “gold standard”. It has been deployed on surfaces in a variety of ways including grafting, blending with matrix polymers, as self-assembled monolayers (SAMs) on gold, and as side chains on carbon backbone polymers, *e.g.* poly(oligoethyleneglycol methacrylate) (POEGMA). Work in our lab on PEO covers the gamut and involves materials prepared by end-tethering of PEO to gold;<sup>91,92</sup> chemical grafting of PEO on polyurethane;<sup>93,94</sup> grafting of poly(OEGMA) on silicon<sup>95</sup> and on polyurethane,<sup>96</sup> and blending of a PEO block copolymer with a polyurethane matrix.<sup>97,98</sup> The strongest protein resistance was shown by the PEO-PU blends; in these materials the PEO component migrates to the aqueous–material interface giving a dense PEO layer of unknown structure, not subject to description in terms of brushes, mushrooms *etc.* since the chains are not “pinned” as in grafts. Adsorption of fibrinogen from buffer as low as 50 ng cm<sup>-2</sup> (>95% reduction compared to the unmodified surface) was observed for this type of material.

As with other putatively protein resistant surfaces, PEO-modified surfaces perform less well in blood, plasma and other biofluids than in simple protein solutions. Thus Zhang *et al.*<sup>99</sup> showed that on a variety of resistant surfaces, including some based on PEO, total protein adsorption from plasma was several orders of magnitude greater than fibrinogen adsorption from buffer. Riedel *et al.*<sup>56</sup> found similar behaviour for a number of poly(ethylene glycol)-based surfaces, and identified between 9 and 24 proteins (depending on the method of PEO “display”) in the adsorbates from plasma. Apolipoprotein A-I, complement C3, fibrinogen, albumin and histidine-rich glycoprotein were present on all the surfaces. Similar findings were reported by Gunkel and Huck.<sup>72</sup> All of this work suggests that the goal of a surface that is completely protein resistant in blood contact has yet to be achieved.

The above discussion does not do justice to the very abundant literature on PEO surfaces though it is perhaps sufficient for the reader to appreciate the place of these surfaces in the

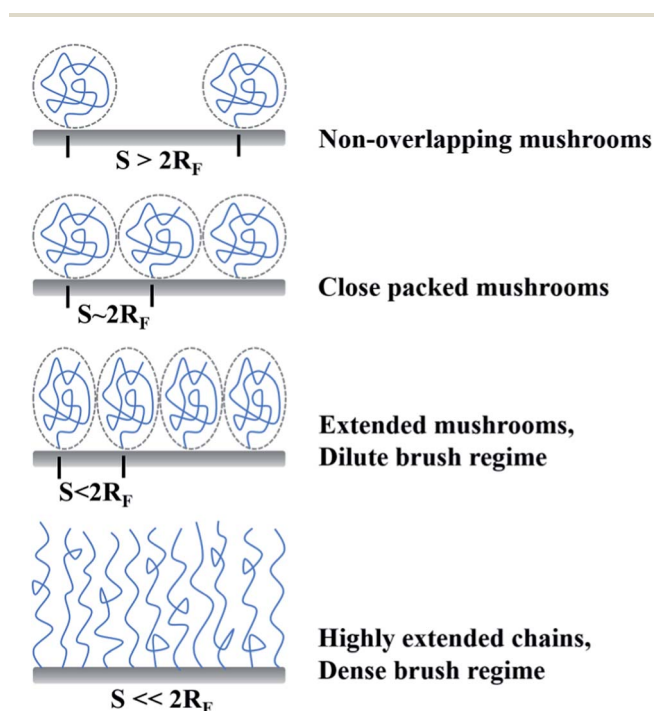
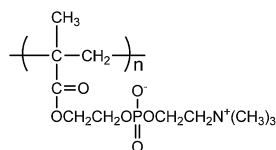


Fig. 3 Conformation and layer structure of tethered polymer chains.  $R_F$  is the Flory radius of the chains.

blood compatibility field. The reader is referred to other publications for more details.<sup>100–103</sup>

Hydrophilic polymers other than PEO (*e.g.* PVP, PVA, PHEMA) give surfaces that are protein resistant to similar extents<sup>79</sup> and it is difficult to rank the various polymers in this regard. However, it should be noted that PEO, with oxygen atoms repeating in the main chain, may be seen as unsuitable for longer term applications since it is known to decompose *via* metal ion-catalyzed oxidation.<sup>104</sup> The carbon chain polymers are not subject to these reactions. Also it has been shown that PEG in solution can activate the complement system<sup>105</sup> although complement activation associated with PEG surfaces in blood contact has not, to our knowledge, been reported.

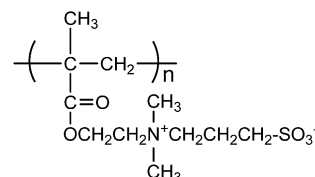
Surfaces modified with zwitterionic polymers have also been investigated for protein resistance. Dating from the early 1990s Ishihara *et al.*<sup>106</sup> have studied surfaces based on poly(2-methacryloyloxyethyl phosphorylcholine) (polyMPC), a carbon chain polymer with zwitterionic phosphorylcholine side chains:



This polymer has been shown to be strongly protein resistant when end-tethered to solid surfaces<sup>95</sup> and to be relatively blood compatible in various medical devices.<sup>107</sup> Initially it was proposed that blood compatibility was due to the preferential adsorption of phospholipids from blood to produce a “biomembrane-like” surface.<sup>33</sup> More recently water interactions have been invoked to explain the protein resistance of polyMPC. Kitano *et al.* have proposed that resistance is due to associated water that is minimally disturbed vis-à-vis its normal condition in the liquid state.<sup>108</sup> Along similar lines Ishihara *et al.* have suggested that the water associated with zwitterionic polymer surfaces is highly mobile and exchanges rapidly with bulk water, thereby weakening interactions with proteins.<sup>109</sup>

Extensive work on polymeric carboxy-, sulfo- and phosphobetaines has been carried out by Jiang *et al.*<sup>36,99,110</sup> Some of these materials have shown exceptionally low protein adsorption; for example a carboxybetaine polymer surface was found to adsorb  $\sim 1 \text{ ng cm}^{-2}$  (total protein) from undiluted human plasma.<sup>36</sup> In the same experiments a PEO-based self-assembled monolayer adsorbed  $\sim 600 \text{ ng cm}^{-2}$ . It should be recalled that protein monolayers contain  $\sim 1000 \text{ ng cm}^{-2}$ , so adsorption levels of  $\sim 1 \text{ ng cm}^{-2}$  representing  $\sim 0.1\%$  coverage may be considered very low, especially from plasma having a total protein concentration of  $\sim 90 \text{ mg mL}^{-1}$ .<sup>111</sup> The protein resistance of these zwitterionic materials has been attributed to water layers bound strongly *via* electrostatic interactions and to the exact balance between the positive and negative charges.<sup>112</sup> Jiang *et al.* have also studied surfaces containing mixed positive and negative charges (different molecules) and have reported strong protein resistance when the positive and negative charges are balanced and uniformly distributed at the molecular level.<sup>36</sup>

Work on zwitterionic surfaces has been reported by Smith *et al.*<sup>113</sup> and by Déjardin *et al.*<sup>114</sup> In the work of Smith *et al.* polyurethane catheters grafted with a polymeric sulfobetaine, *N*-(3-sulfopropyl)-*N*-methacryloxyethyl-*N,N*-dimethylammoniumbetaine):



were shown to be strongly protein- and cell-resistant. Of particular significance for blood compatibility, these catheters reduced thrombus formation *in vivo* (canine model) by 99% compared to unmodified catheters. Non-fouling properties were again attributed to the specific configuration of water associated with the zwitterionic motif. The surfaces of Déjardin *et al.* were prepared by grafting a phosphocholine phospholipid to siloxane films on glass or silicon. High densities of phospholipid were achieved, giving surfaces that were strongly hydrophilic and strongly resistant to several proteins including albumin, fibrinogen, avidin and lysozyme.

It is of interest to compare PEO-based materials and zwitterionic materials with respect to protein resistance. Using surfaces grafted with poly(OEGMA) and poly(MPC), work in our lab showed that protein resistance was similar when the graft lengths and densities were comparable.<sup>115</sup> With respect to comparing PEO and zwitterions it should again be mentioned that PEO is susceptible to oxidation and cannot be expected to remain stable over long periods.

It should be emphasized that knowledge of the performance of protein resistant materials *in vivo* is lacking, so it is not known whether protein resistance contributes significantly to blood compatibility. It is noted that poly(vinyl pyrrolidone) is used in the manufacture of polysulfone hemodialysis membranes presumably to aid in wetting;<sup>116</sup> it is possible that the PVP also reduces protein interactions with the membrane. Also PEO has been used in the manufacture of cardiopulmonary bypass circuits; it is reported that PEO-based coatings reduce platelet adhesion and activation and preserve platelet function (<http://www.medtronic.com>).

The above discussion has focused on minimization of the adsorbed quantity of proteins as a strategy for passivation. Several authors have concluded that the biologic and conformational status of the adsorbed proteins may also be important. Platelet adhesion to conformationally altered albumin, a protein generally believed to be unreactive to platelets, was mentioned above.<sup>58,59</sup> Lindon *et al.* suggested that platelet adhesion to adsorbed fibrinogen is correlated with the maintenance of a conformation recognizable by anti-fibrinogen antibodies.<sup>117</sup> Tsai *et al.* showed that platelet adhesion to polystyrene-based materials pre-exposed to plasma was correlated more to the exposure of the platelet-binding ligands in fibrinogen (RGD sequences) than to the quantity adsorbed.<sup>118</sup> More recently Latour *et al.* showed that platelet adhesion was strongly



correlated with the conformation of adsorbed fibrinogen determined by circular dichroism as well as with the quantity adsorbed.<sup>119</sup> These are potentially significant findings and may suggest that future passivation strategies should be based on designing the surface to be minimally ‘traumatizing’ of adsorbed proteins, possibly as well as to be minimally adsorbing. To our knowledge there have been no reports of such an approach. Surfaces that are resistant to adsorption may be seen as ‘minimally interacting’ generally and therefore may also be minimally ‘traumatizing’.

### 3.2 Bioactive surfaces

In this section we discuss approaches where bioactive components (usually biomacromolecules) are incorporated in the surface. These include anticoagulant and antithrombotic agents which are expected to inhibit thrombus formation, and profibrinolytics which are expected to promote destruction or lysis of the clot-thrombus before it is able to cause damage.

#### 3.2.1 Anticoagulants, antithrombotics

**3.2.1.1 Heparin.** By far the most extensively investigated anticoagulant in the blood compatibility field is heparin. Heparinized surfaces go back to the early 1960s starting with the work of Gott *et al.*<sup>120,121</sup> The Gott materials were referred to as ‘graphite benzalkonium heparin’ or ‘GBH’. Heparin was attached to graphite as substrate *via* electrostatic interactions with an intermediate layer of positively charged benzalkonium chloride. The five decades since GBH have seen a plethora of heparinized surfaces with a variety of designs based on type of substrate, method of surface attachment, and heparin variant. Some of these have been developed commercially and used clinically. The area of heparinized surfaces is extensive and merits a review of its own; only a few highlights are discussed in this article. It is first essential to discuss the fundamental mechanism by which heparin inhibits coagulation.

Heparin is a naturally occurring glycosaminoglycan (GAG) (Fig. 4). It is highly negatively charged and polydisperse with molecular weights in the range of 3 to 30 kDa. Typical unfractionated heparin available commercially has average MW 12–15 kDa.

Referring to Fig. 2 showing the blood coagulation pathways, heparin inhibits factors XIa, IXa, Xa and IIa (thrombin), the latter two being of particular importance. Essentially heparin acts as a catalyst of the reaction between antithrombin, an endogenous inhibitor of thrombin, factor Xa, and other

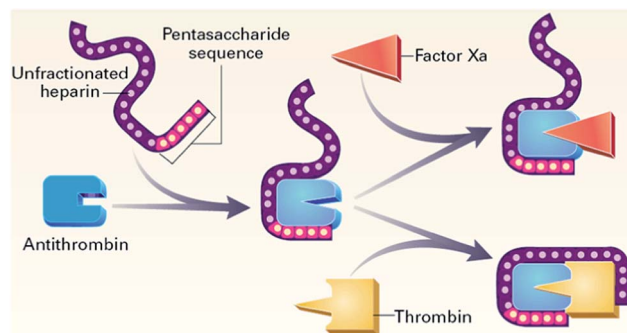


Fig. 5 Interactions of heparin, antithrombin, thrombin, and factor Xa. Adapted with permission from ref. 122, copyright 1997, Massachusetts Medical Society.

activated factors. When heparin binds to antithrombin a conformational change occurs that accelerates the thrombin–antithrombin and factor Xa–antithrombin interactions by a factor of approximately 1000.<sup>122</sup> (It should be noted that heparin co-factor II, present in blood, acts similarly to antithrombin.<sup>123</sup>) As coagulation proceeds, stable complexes of the two molecules (thrombin–antithrombin, or TAT, in the case of thrombin) are formed, and the activated factors are effectively eliminated. These interactions are depicted schematically in Fig. 5 which also indicates the specific pentasaccharide sequence that is the binding site for antithrombin. It is important to note that after the TAT and Xa-AT complexes are formed the heparin molecule is released and becomes free to repeat the process, *i.e.* it functions as a classical recycling catalyst. Similar interactions occur when heparin is attached to a blood contacting surface: the TAT complex is formed on the surface and released into the blood, leaving the heparin available to repeat the cycle. Surface heparinization is therefore an ideal strategy since in principle the bioactivity is preserved over time. This is in contrast to other approaches (discussed below) where the bioactivity is ‘consumed’ after a single thrombin-binding event.

As well as ‘standard’ or unfractionated heparin a number of heparin variants have been developed in recent years, for example low molecular weight heparin (LMWH) and isolated pentasaccharide sequence (Fig. 5), *e.g.* fondaparinux (Arixtra®). These are used as systemic anticoagulants and have not been developed to any extent for heparinization of surfaces. LMWH may be defined as a heparin preparation having an average molecular weight less than 8000 Da and for which the majority

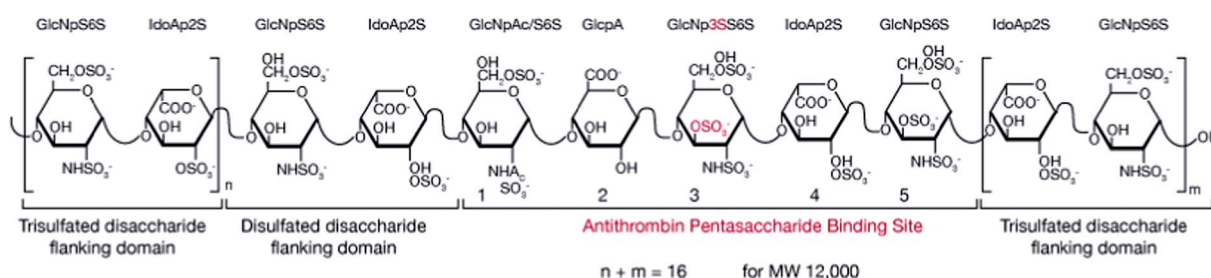


Fig. 4 Molecular structure of heparin. Adapted with permission from Heparin Science.



of the chains have a molecular weight less than 8000 Da. The main advantage of LMWH over standard heparin is improved control over efficacy (and therefore improved patient management). LMWH is more effective against factor Xa than against thrombin since formation of TAT requires longer chains than AT-Xa complex (Fig. 5). Fondaparinux is effective against factor Xa but less so against thrombin.<sup>124</sup>

Two general approaches to surface attachment of heparin have been followed: (1) attachment *via* electrostatic interactions, taking advantage of the high negative charge density of heparin; (2) covalent attachment. Electrostatically bound heparin is subject to loss by exchange with other ionic species in the blood, while covalently bound heparin may be inactivated by structural changes due to the attachment reactions. A limited selection from the very extensive body of research in this area is presented in the following. More detailed reviews are available.<sup>5,125,126</sup>

Modification of surfaces with amino groups for the subsequent covalent attachment of heparin by amide or sulfonamide bond formation is a widely used method. Amino groups have been introduced into poly(ethersulfone) (PES),<sup>126,127</sup> poly(DL-lactic acid) (PLA),<sup>128</sup> polyurethane (PU),<sup>129</sup> 316L stainless steel (SS)<sup>130</sup> and TiO<sub>2</sub> (ref. 131) surfaces, for subsequent immobilization of heparin. Also using carboxyl-amino reactions, Yu and coworkers modified poly(lactic acid) (PLA) films with heparinized microcapsules *via* layer-by-layer (LbL) self-assembly methods.<sup>132</sup> In a variation of this approach, heparin was functionalized with a photo-reactive moiety, 4-azidoaniline, which was then grafted to a 3-aminopropylphosphonic acid-modified titanium oxide substrate by UV irradiation to give specific heparin micropatterns. In contact with blood these surfaces were found to reduce platelet adhesion, and promote endothelial cell spreading and proliferation.<sup>133</sup>

More recently, dopamine, which has bioadhesive properties analogous to mussel proteins,<sup>134</sup> was used for surface attachment of heparin. This approach is attractive in that dopamine binds strongly to many types of surface without the need for pretreatment.<sup>135</sup> Dopamine-mediated heparin attachment was used to modify cobalt-chromium alloy disks,<sup>136</sup> and Park *et al.* attached heparin to stainless steel using a heparin- and dopamine-containing hydrogel;<sup>137</sup> the covalently bound hydrogel layer allowed enhanced retention of the heparin and its activity remained high as indicated by reduced platelet-surface interactions.

A strategy to improve the effectiveness of surface-attached heparin (and other biomolecules) is to interpose a molecular “spacer arm” between the heparin and the surface. The intent is to make the heparin more accessible for interactions with antithrombin and heparin cofactor II, and to avoid the possibility that the surface may “hide” the heparin binding sites. An early implementation of this concept was that of Hoffman *et al.*<sup>138</sup> in which heparin was bonded to PDMS using  $\epsilon$ -aminocaproic acid as a spacer. This approach allowed increased density of heparin although the anticoagulant activity was reduced. More recent work includes that of Kondo *et al.*,<sup>139</sup> where again it was shown that a higher density of heparin was achieved using hexamethylenediamine as spacer. Surfaces

modified with bis-amino-terminated poly(ethylene glycol) (BA-PEG) as spacer and grafted with heparin were shown to have improved antithrombin (AT) binding and anticoagulant properties compared to the same surfaces without the PEG spacer.<sup>140</sup> The PEG spacer provides a more hydrophilic environment and a reduction in non-specific protein adsorption as well as a greater level of conformational freedom for the heparin. Similarly, three-dimensional porous PLGA scaffolds modified with heparin *via* PEG as spacer suppressed non-specific protein adsorption, and growth factors could be tethered and presented to cells in a bioactive configuration.<sup>141</sup> Byun *et al.* compared the binding of thrombin and antithrombin to heparinized polymer surfaces where the heparin was attached either directly or through a PEO spacer. They showed that the direct surface bound thrombin but not antithrombin, whereas the PEO surface bound both, probably thereby accounting for the greater bioactivity of the PEO-heparin surface.<sup>142</sup>

Surfaces have also been prepared where heparin is combined with a second bioactive component. Yang and coworkers investigated titanium surfaces modified with heparin and fibronectin *via* either physisorption, electrostatic or covalent co-immobilization.<sup>143–145</sup> Their results suggested that co-immobilization of fibronectin and heparin improved the anticoagulant activity of the heparin. In other work heparin and phosphorylcholine groups were grafted onto polyurethane to improve hydrophilicity and blood compatibility.<sup>146</sup>

Significant disadvantages of unfractionated heparin (UFH) are its variable activity and its tendency to bind a large number of plasma proteins non-specifically, causing loss of activity.<sup>147</sup> Also heparin is unable to neutralize clot-bound (or surface bound) thrombin. With these limitations in mind, Chan *et al.* developed a covalent complex of heparin and antithrombin (ATH) which has increased active pentasaccharide content and high anticoagulant activity compared to UFH.<sup>148,149</sup> Other attributes of ATH include reduced non-specific binding of plasma proteins and the ability to inhibit surface-bound coagulation factors.<sup>150</sup> These advantages are important for surface modification; in addition, surface attachment is possible *via* chemical functions in the AT portion of the complex. It is important to note that in standard heparin preparations only one third of the molecules contain the active pentasaccharide sequence. In contrast, the ATH complex has at least one pentasaccharide per heparin molecule thereby giving higher thrombin inhibition rates. In addition it has dual activities against thrombin: *i.e.* direct activity through the antithrombin component and catalytic activity *via* the heparin.

We have developed ATH as a surface modifier using gold,<sup>151,152</sup> polyurethane<sup>153,154</sup> and PDMS<sup>155</sup> as substrates. The ATH was attached to these substrates using PEO as a spacer and for protein resistance. The methods used for attachment varied with the substrate. These include gold-thiol reactions<sup>151</sup> and isocyanate-hydroxyl reactions<sup>153</sup> to attach PEO to the substrate, and *N*-hydroxysuccinimide (NHS)-hydroxyl reactions to attach ATH to the distal end of the PEO.<sup>154</sup> These ATH-PEO surfaces showed improved antithrombotic activity (antithrombin binding, factor Xa inhibition, prolonged clotting time, reduced platelet adhesion). Interestingly it was found that the protein

resistance of the PEO was not diminished by the closely adjacent ATH. Also it was shown that along with providing resistance to non-specific protein adsorption, the PEO inhibited the specific adsorption of antithrombin, and that therefore an optimal balance between these two effects is required. A suitable balance was achieved using PEO of lower molecular weight (~600 Da).

Despite the limitations referred to above, heparin-modified materials have been developed commercially and have been used clinically in blood contacting devices. The commercial material with the longest history of clinical use is the CBAS® surface (CarmedaBioActive Surface) of Carmeda AB. This material evolved from the work of Larm, Larsson and Olsson<sup>156,157</sup> in which heparin was bound covalently to the substrate *via* the chain ends (“end point attached”, Fig. 6). The active pentasaccharide is therefore not involved in the binding and remains intact.

Heparin with an aldehyde group at the chain end is prepared by partial depolymerisation of standard heparin using nitrous acid; amino groups are incorporated into the substrate surface, for example by attachment of polyethylene imine. The aldehyde-amino reaction gives a Schiff's base which is reduced with sodium cyanoborohydride to give a N–C bond. A wide variety of substrates, including polyethylene, polycarbonate, polysulfone, polyurethane, PVC, silicone, stainless steel, PET, and glass have been used in this process.<sup>158,159</sup>

Other heparinized surfaces developed commercially and used clinically include Durafluo II (Baxter Corp) and Astute® (also referred to as Trillium, BioInteractions Ltd). The Durafluo II material is based on a heparin–surfactant complex that is soluble in organic solvents. The key attribute of this approach is the simple modification procedure, *i.e.* coating from solution, enabled by the organic-soluble form of the heparin. The

Astute® surface combines negative charge (sulfate and sulfonate groups), grafted PEO and heparin (covalently complexed to the PEO) ([http://www.biointeractions.com/pdfs/astute\\_technical.pdf](http://www.biointeractions.com/pdfs/astute_technical.pdf)).

*In vivo* experiments using a canine model with e-PTFE vascular grafts modified with CBAS showed that the CBAS grafts were significantly less thrombogenic, with improved patency, compared to controls.<sup>160</sup> The CBAS and Durafluo II materials were compared in a clinical trial involving patients undergoing coronary artery bypass surgery with reduced heparin dose.<sup>161</sup> Entire cardiopulmonary bypass circuits were treated with either CBAS or Durafluo. Both systems performed satisfactorily and no clinical differences were observed between them. The data indicated, however, that heparin leaked from the Durafluo materials into the blood. Antithrombogenic properties of the materials were indicated by a reduced patient requirement for heparin. A comprehensive review of clinical experience with heparinized materials, including CBAS, Durafluo II, and others was published by Wendel and Ziemer.<sup>162</sup> In general clinical experience with heparinized systems has been mixed.<sup>163–165</sup> It seems likely that reliable, reproducible methods of heparinization giving surfaces with clear benefits in terms of thrombogenicity have yet to be found.

**3.2.1.2 Direct thrombin inhibitors.** As indicated above heparin inhibits thrombin (and other activated clotting factors) indirectly by catalyzing the inhibition of thrombin by antithrombin. Other inhibitors act directly by binding to the active site of the enzyme. These include hirudin, hirudin analogues and peptides such as phe-pro-argchloromethylketone (PPACK).<sup>166–170</sup>

Some of these have been incorporated into biomaterials in attempts to provide an anticoagulant function. Hirudin, for example, is a small protein of molecular weight ~6.9 kDa found

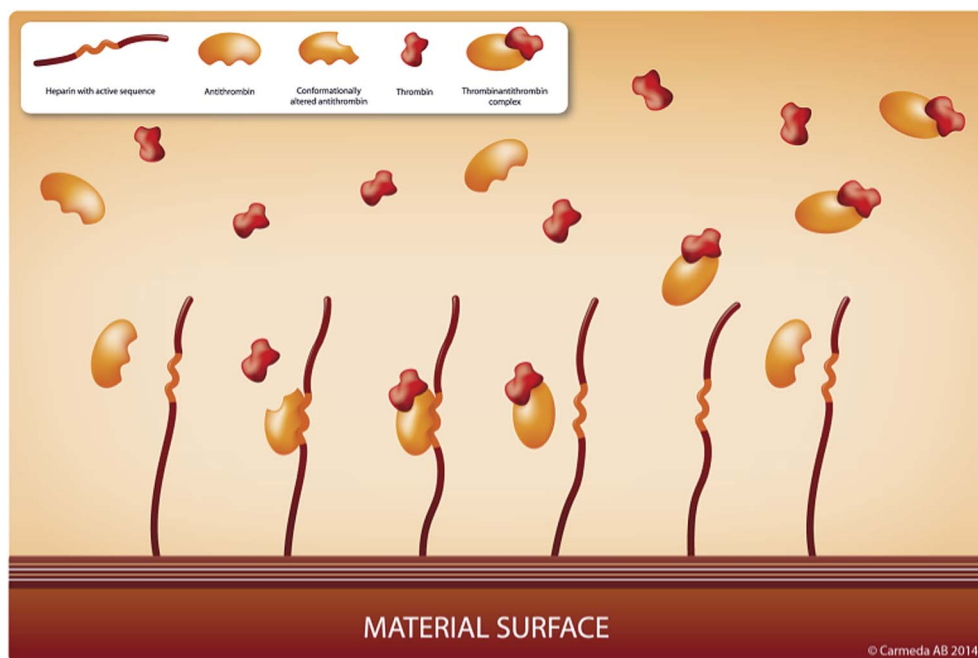


Fig. 6 CBAS end point attachment of heparin. Adapted with permission from Carmeda AB.

in the saliva of the medicinal leech *Hirudomedicinalis*. The N-terminal of hirudin binds to the apolar binding site of thrombin, and the C-terminal interacts with an anion-binding exosite.<sup>171</sup> The Pro46-Lys47-Pro48 sequence of hirudin occupies the basic specificity pocket near the active site of thrombin; hirudin is thus able to inhibit both free and fibrin-bound thrombin. Hirudin also interferes with the site for interaction of thrombin with platelets.<sup>172,173</sup>

As a surface modifier hirudin has been used with a variety of substrates including polyester,<sup>174,175</sup> polyurethane,<sup>176–178</sup> polyethylene,<sup>179</sup> nitinol metal (coronary stents)<sup>180</sup> and polytetrafluoroethylene.<sup>181</sup> Lahann *et al.*<sup>178</sup> reported that attachment *via* an epsilon-amino group of r-hirudin preserved full bioactivity of the hirudin; however, loss of activity occurred when the hirudin was selectively coupled *via* the N-terminal amino group. In later work they coated nitinol coronary stents with functionalized poly(paracyclophane) and attached hirudin to the coating. Prolonged clotting times and decreased platelet adhesion were observed for the hirudin-treated stents.<sup>180</sup> Phaneuf *et al.* attached hirudin to a polycarbonate-urethane<sup>177,182</sup> and to polyethylene terephthalate<sup>174</sup> and showed extensive thrombin binding on the modified surfaces. Berceli *et al.* coated polyester vascular grafts with hirudin and showed reduced local thrombin concentration using an *in vitro* assay.<sup>183</sup> Seifert *et al.* modified poly(lactideglycolide) with hirudin and observed decreased platelet adhesion and activation on these surfaces. Furthermore, the hirudin-modified surfaces showed prolonged clotting, similar to heparin-modified controls.<sup>184</sup> Surfaces modified with both polyethylene glycol and hirudin were developed by Alibeik *et al.* and were shown to have both anti-fouling and thrombin-neutralizing properties.<sup>185</sup>

As the work summarized above demonstrates, hirudin-modified surfaces are effective in scavenging and inhibiting thrombin, and in that sense they do have anticoagulant properties. However, a major limitation is that the thrombin–hirudin interaction is to all intents and purposes irreversible (dissociation constant  $\sim 2 \times 10^{-14}$  M (ref. 186)). As a consequence, each hirudin molecule is able to inhibit only one thrombin molecule and the anticoagulant effect is lost once all such interactions have occurred. This is in contrast to heparin where the active molecule is regenerated after each interaction with thrombin.

Hirudin derivatives such as bivalirudin have been developed which, unlike hirudin itself, bind thrombin reversibly. Bivalirudin, also known as hirulog, is a 20-amino acid peptide which contains the N-terminal residues and C-terminal residues of hirudin. The termini are connected by four glycine residues.<sup>187</sup> Bivalirudin is ‘bivalent’ in the sense that it inhibits both the active site and one of the two anionic binding sites of thrombin. It has been immobilized on 316L stainless steel using a bonding layer of polydopamine;<sup>188</sup> the resulting surfaces were shown to prolong clotting and inhibit the activation of platelets.

Several other direct thrombin inhibitors have been developed, *e.g.* argatroban,<sup>189</sup> that are used clinically as anticoagulants, for example where heparin is contra-indicated. Argatroban is ‘univalent’; it interacts only with the active site and not with the exosites of thrombin. These inhibitors have

not been used to any extent as surface modifiers for blood contacting materials.

**3.2.1.3 Thrombomodulin-protein C.** Thrombomodulin is a membrane protein of vascular endothelial cells. It acts as a catalyst-cofactor in the activation of circulating protein C by thrombin; activated protein C (APC) exerts an anticoagulant effect by inhibiting coagulation factors Va and VIIIa (see Fig. 2). In this sense thrombin is ‘modulated’ and its function is effectively reversed from pro- to anti-coagulant. Endothelial protein C receptor (EPCR) increases the rate of activation of protein C on the endothelium.

Efforts have been made to exploit these mechanisms in the development of blood compatible surfaces. For example Kador *et al.* attached both EPCR and thrombomodulin covalently to polyurethane and showed that APC generation was increased and *in vitro* clotting was delayed on these surfaces relative to surfaces with thrombomodulin alone.<sup>190</sup> Tseng *et al.*<sup>191</sup> prepared phospholipid monolayers containing varying quantities of thrombomodulin. In buffered solutions of thrombin and protein C these surfaces generated APC at rates which increased with thrombomodulin surface density. Thrombomodulin-protein C is a relatively unexplored approach in the search for antithrombogenic surfaces and may merit further investigation.

**3.2.1.4 Antiplatelet agents.** Clearly for a surface to be antithrombotic it should prevent platelet adhesion, activation and aggregation as well as coagulation. More effort has been focused on the anticoagulant side, and much of the platelet research has been on release of anti-platelet agents into the blood rather than immobilization on the surface. A few examples are discussed in the following.

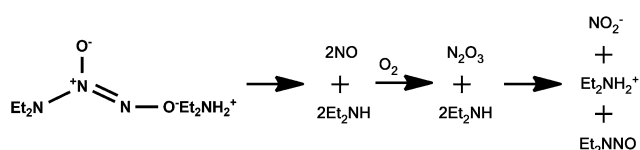
Dipyridamole (Persantin®) is an example of the immobilization approach. This molecule inhibits platelet activity by inhibition of cyclic phosphodiesterase which normally breaks down intracellular cyclic adenosine monophosphate (cAMP). The cAMP level is increased, thereby blocking the platelet aggregation response to adenosine diphosphate (ADP). Dipyridamole has been attached covalently to polyurethane either directly or through a spacer.<sup>6,7</sup> *In vitro* experiments showed reduced platelet adhesion on these materials. *In vitro* and *in vivo* experiments also indicated that the formation of endothelial cell layers may be supported on these surfaces.

Prostaglandins having antiplatelet activity have also been immobilized on biomaterials. For example, PGE1 inhibits platelet activity by increasing cAMP through augmented activity of adenylate cyclase. PTFE and dacron grafts with covalently immobilized PGE1 showed greatly reduced platelet adhesion in whole blood *in vitro* under both static and flow conditions compared to controls without PGE1.<sup>192</sup>

An innovative ‘immobilization’ approach involving the enzyme apyrase which degrades ADP, a well known platelet aggregation agent released from damaged red cells and platelets, was reported by Nilsson *et al.*<sup>193</sup> They demonstrated that a polystyrene surface with immobilized apyrase showed decreased platelet adhesion and activation compared to unmodified controls. Indications that coagulation was also inhibited, *e.g.* that formation of thrombin–antithrombin complex was reduced, were also noted.

Nitric oxide release. Nitric oxide has several beneficial functions in the cardiovascular system including vasodilation, anti-platelet activation-aggregation, anti-inflammation, anti-bacterial and pro-angiogenesis.<sup>194,195</sup> It is released in gaseous form from the endothelium of healthy blood vessels by the action of nitric oxide synthase (eNOS) on arginine. Release rates are of the order of  $10^{-10}$  mol cm<sup>-2</sup> min<sup>-1</sup>.

Nitric oxide is of interest in the blood compatible materials field as an antithrombotic agent due to its inhibitory effect on platelets, and materials have been developed that release NO at rates comparable to those occurring in the bloodstream. Two general approaches have been followed based, respectively, on N-diazoniumdiolates and S-nitrosothiols as NO release agents, the former being the more extensively explored of the two. N-diazoniumdiolates decompose under physiologic conditions to form NO and the corresponding amine.<sup>196</sup>



Handa *et al.*<sup>197</sup> investigated PVC modified by incorporation of diazoniumdiolated dibutylhexanediamine. They showed that the rate of NO release from modified catheters into a buffer solution was in the physiologic range and was sustained over periods of several days. The patency rate of arteriovenous shunts in rabbits was greater for the modified materials compared to controls.

S-nitrosothiols decompose to form NO and the corresponding disulfide:



As an example of this approach Seabra *et al.*<sup>198</sup> prepared a NO-releasing polyester containing S-nitrosothiol (S-NO) groups. In the form of a blend with poly(methyl methacrylate) this material was shown to release NO and to inhibit platelet adhesion from whole blood *in vitro*. Also Riccio *et al.*<sup>199</sup> prepared NO-releasing xerogels based on nitric oxide loading of a surface-localized thiolated silane precursor.

The recent review of Naghavi *et al.*<sup>200</sup> gives a comprehensive account of work on NO-releasing materials. This approach is appealing in the sense that it attempts to mimic an important antithrombotic function of the endothelium itself. However it seems that NO release alone will not be sufficient to confer effective, lasting anti-thrombogenicity on biomaterials and that other functions, for example as provided by active anti-coagulants, may be required. In this regard, Zhou and Meyerhoff<sup>201</sup> developed materials that incorporate both heparin and NO releasing capability; as well as releasing NO these materials were shown to have inhibitory activity against clotting factor Xa.

Other anti-platelet agents. Aspirin, vascular endothelial growth factor (VEGF) and GPIIb/IIIa receptor inhibitors have also been investigated as anti-platelet agents for release.<sup>202</sup> GPIIb/IIIa inhibitors are of particular interest since their mode of action is to block the receptor on activated platelets which

mediates adhesion of platelets to adsorbed fibrinogen (and to RGD motifs more generally in adsorbed proteins). A commercially available GPIIb/IIIa inhibitor is the anti-GPIIb/IIIa antibody abciximab (also known under the trade name ReoPro). Fontaine *et al.*<sup>203</sup> investigated stents that were coated with silicone and a layer of poly(paraxylylene) that was loaded “passively” with abciximab. In experiments on dogs the drug was released over a 16-day period and was found to reduce the thickness of the neointima formed on the stent. This effect was attributed to the antiplatelet effect of the abciximab, not in terms of anti-thrombosis but rather inhibition of smooth muscle cell proliferation. The reader is referred to the review of Kidane *et al.* for more details of research on the release of antiplatelet agents.<sup>202</sup>

An important application of the release of bioactive agents from blood contacting devices is in drug eluting stents (DES) and intra-vascular balloons. The main objective of drug eluting stents is to prevent re-stenosis or re-narrowing of the vessel lumen due to smooth muscle cell proliferation. Anti-proliferative drugs such as paclitaxel and sirolimus have been used for this purpose. Since the focus of the present article is prevention of thrombosis rather than re-stenosis, this area will not be discussed further.

**3.2.2 Surface modification for thrombolysis.** The discussion thus far has focused on prevention of thrombosis due to blood-foreign surface contact. In this section an alternative approach, namely destruction, or lysis, of the clot-thrombus once formed, is discussed. This approach exploits the body's fibrinolytic system which destroys hemostatic plugs when they are no longer needed. Hemostatic plugs are platelet-fibrin masses that form during the repair of damaged blood vessels; they are similar in composition to thrombi and are initiated by contact of the blood with sub-endothelial constituents, notably collagen. More generally, by lysing nascent clots, the fibrinolytic system plays an important role in the maintenance of blood flow in the vasculature.<sup>204,205</sup>

**3.2.2.1 Mechanism of fibrinolysis.** The mechanism of fibrinolysis is shown in simplified form in Fig. 7. Fig. 2 shows how fibrinolysis interacts with the coagulation pathways. The inactive proenzyme plasminogen (plasma concentration  $\sim 0.2$  mg mL<sup>-1</sup>, 2  $\mu$ M) is converted to the active enzyme plasmin by tissue-type plasminogen activator (t-PA). t-PA comes largely from damaged endothelial cells during hemostasis. Plasmin degrades insoluble fibrin clot giving soluble fibrin fragments referred to as fibrin degradation products (FDP). Plasmin is inhibited by endogenous  $\alpha$ -2-antiplasmin, and tissue plasminogen activator is inhibited by endogenous plasminogen activator inhibitor-1 (PAI-1) and by thrombin-activatable fibrinolysis inhibitor (TAFI).<sup>206</sup>

A more detailed schematic of fibrinolysis is shown in Fig. 8. Fibrinolysis may be viewed as a component of haemostasis which comprises both fibrinogenesis and fibrinolysis in an appropriate balance. The clot breaks down on the vascular wall. Plasminogen has two lysine binding sites (LBS) in kringle residues 1 and 4; these are used to bind to lysine residues in the clot. Upon binding to lysine, the tightly folded molecule “opens up” and becomes more susceptible to activation by t-PA released



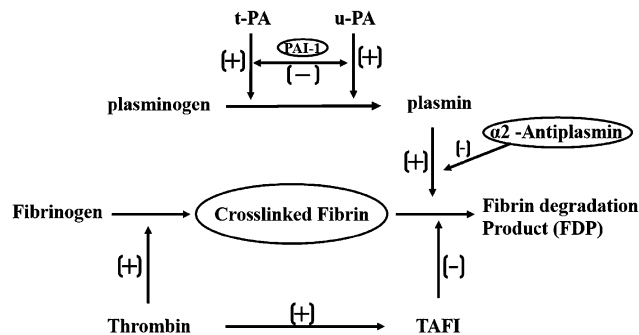


Fig. 7 Mechanism of fibrinolysis. Adapted with permission from ref. 205, copyright 2010, American College of Cardiology Foundation.

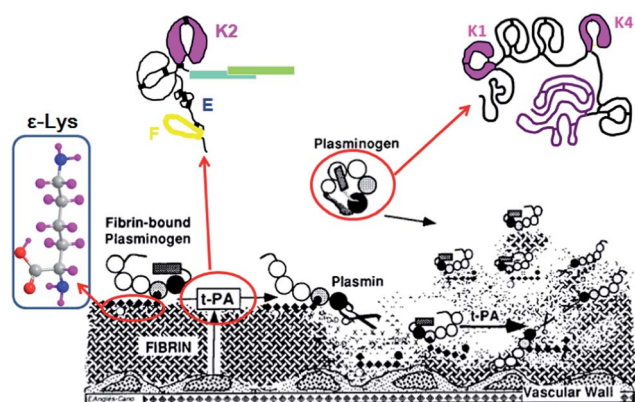


Fig. 8 Details of fibrinolysis. Adapted with permission from ref. 206, copyright 1994, Elsevier Ireland Ltd.

from damaged endothelial cells in the vessel wall. Plasmin is thus generated and the fibrin is broken down. It should be noted that the lysines that bind plasminogen in this way are C-terminal lysines in the degrading clot in which both the carboxyl and the  $\epsilon$ -amino groups are free. This carboxy-amino zwitterionic motif matches exactly to a similar one in the K1 and K4 kringles. We will refer again to this interaction in discussing the design of surfaces that promote fibrinolysis.

Fibrinolysis is exploited extensively in the treatment of cardiovascular disease, notably ischemic stroke and myocardial infarction (heart attack). Thrombolytic therapy consists of administration of plasminogen activators, most commonly t-PA, but also urokinase plasminogen activator (u-PA also referred to as urokinase). Several recombinant t-PAs have been developed and approved for clinical use including alteplase, reteplase, and tenecteplase.

For blood contacting biomaterials two approaches to fibrinolysis have been followed, namely mimicry of the physiologic fibrinolytic mechanism in surface design, and incorporation and release of t-PA into the blood. We have reviewed research in this area recently.<sup>44</sup>

**3.2.2.2 Capture of endogenous plasminogen and t-PA: lysine-derivatized surfaces.** Mimicking the physiologic mechanism of fibrinolysis (Fig. 8), a surface may be envisaged which can lyse

fibrin that begins to form on it. As indicated above, it is known that plasminogen and t-PA bind specifically to the surface of fibrin *via* carboxy-terminal lysine residues in which the carboxyl and  $\epsilon$ -amino groups are free (referred to as “ $\epsilon$ -lysines”).<sup>125,207–209</sup> Therefore, it is expected that an  $\epsilon$ -lysine-enriched surface could provide a substrate for the capture of plasminogen and t-PA from blood; the interaction of these two molecules should then lead to plasmin formation and thus to fibrinolysis.<sup>44,210</sup> This concept was first introduced by Brash and coworkers.<sup>211–213</sup> In initial work, lysine was directly immobilized on sulfonated surfaces. These were shown to adsorb plasminogen from plasma with some degree of selectivity and subsequently to lyse fibrin clot *in vitro* following exposure to t-PA.

Lysine density is the main factor determining the extent of plasminogen binding to these surfaces.<sup>214</sup> In one approach, surfaces were prepared using a coating reagent containing polyacrylamide and lysine. The lysine was conjugated to the polyacrylamide through the  $\alpha$ -amino group.<sup>215</sup> The  $\epsilon$ -lysine density achieved by this method ranged from 0.2 to 3.2 nmol cm<sup>-2</sup>, much higher than the highest lysine density of 0.0035 nmol cm<sup>-2</sup> achieved by direct binding. Plasminogen adsorption from plasma on these surfaces increased with increasing lysine density and reached a value of 1.2  $\mu$ g cm<sup>-2</sup> (in the monolayer range) for the surface with the highest lysine density; the other plasma proteins were all but excluded. This exceptional plasminogen specificity was attributed to the high  $\epsilon$ -lysine coverage and to the high affinity of the plasminogen  $\epsilon$ -lysine interaction. Polyethylene tubes coated with these lysine-containing polymers and exposed to t-PA were evaluated using an experiment in which non-anticoagulated whole blood was recirculated through a closed tubing loop (modified Chandler loop).<sup>216</sup> Thrombus generated on the  $\epsilon$ -lysine surface was lysed within minutes, while thrombogenesis continued on the control surfaces till the tubing was occluded and flow ceased.

The concept was further refined by designing the surface for both suppression of nonspecific protein adsorption and promotion of plasminogen adsorption. Polyethylene glycol (PEG) was used as a spacer-linker on either poly(dimethyl siloxane) (PDMS) or polyurethane to which lysine, with the  $\epsilon$ -amino group protected by t-BOC, was attached through the  $\alpha$ -amino group. The  $\epsilon$ -NH<sub>2</sub> was then deprotected to yield “ $\epsilon$ -lysine” surfaces.<sup>217–220</sup> These were shown to reduce nonspecific protein adsorption and platelet adhesion while binding plasminogen from plasma with a high degree of selectivity. When treated with t-PA and incubated in plasma, these lysine-derivatized surfaces were able to lyse plasma clots formed on them or adjacent to them. In addition, it was found that the rate of plasminogen adsorption and clot lysis increased with decreasing length of the PEG spacer.<sup>221</sup> To prepare a non-fouling, fibrinolytic surface of high lysine density, 2-hydroxyethyl methacrylate (HEMA) was graft polymerized (or copolymerized with other monomers) from a vinyl group-functionalized polyurethane surface. Lysine was then coupled to the hydroxyl groups in poly(HEMA) such that the  $\epsilon$ -NH<sub>2</sub> remained free.<sup>81</sup> This surface was demonstrated to be rich in lysine and to have increased plasminogen binding capacity and clot lysing ability. The preparation of lysine-rich non-fouling

surfaces was further simplified using an  $\epsilon$ -lysine-containing monomer, lysyl methacrylate (LysMA).<sup>222</sup> Lysine-containing surfaces were prepared by graft copolymerization of LysMA and HEMA from surface vinyl groups. Alternatively, copolymers of LysMA and HEMA were cast as films or blended with polyurethane. These methods avoid the multi-step surface modification procedures required in previous techniques. In particular they avoid the need for protection-deprotection of the  $\epsilon$ -NH<sub>2</sub> groups in lysine which exposes the materials to acid.<sup>223,224</sup> These surfaces were shown to be resistant to nonspecific protein adsorption and to have very high lysine density ( $\sim 9.85$  nmol cm<sup>-2</sup>). Furthermore, the plasminogen binding capacity could be closely regulated by varying the copolymer composition.

The lysine-plasminogen capture strategy has also been pursued by Samojlova and coworkers.<sup>225</sup> In their work polymer surfaces were coated with  $\epsilon$ -lysine-containing polyelectrolyte complexes at lysine densities of 2.2–5.5 nmol cm<sup>-2</sup>. These surfaces were demonstrated to take up plasminogen in significant amounts from plasma, and in experiments using a dog model, the mass of thrombus on the  $\epsilon$ -lysine containing materials was reduced by up to 90% compared to controls.<sup>226</sup>

Clearly plasminogen capture alone is insufficient to generate plasmin: activation of the captured plasminogen is required. Although  $\epsilon$ -lysine has affinity for t-PA as well as for plasminogen (albeit of a lesser magnitude), the concentration of t-PA in plasma is about 5 ng mL<sup>-1</sup> ( $\sim 7 \times 10^{-5}$   $\mu$ M)<sup>227</sup> compared to 2  $\mu$ M for plasminogen. Consequently endogenous t-PA capture on  $\epsilon$ -lysine surfaces is negligible compared to that of plasminogen. Indeed we showed<sup>228</sup> that t-PA adsorbed on a lysine-derivatized polyurethane was rapidly displaced by plasminogen in contact with plasma. Therefore a means of supplying plasminogen activator to these surfaces is required. Methods to do so are under investigation in our labs.

**3.2.2.3 Incorporation and release of plasminogen activators.** Controlled release of plasminogen activators pre-loaded into materials has also been explored. For example, Senatore *et al.* immobilized u-PA on the inner surface of fibrocollagen tubes using a glutaraldehyde entrapment process; the tubes were implanted as carotid-to-femoral artery grafts in dogs. Graft patency was higher in the urokinase grafts than in controls, and FDP production increased in the urokinase grafts but not in the controls.<sup>229</sup> Park *et al.* developed a t-PA-loaded porous poly-(L-glutamic acid)/PEG hydrogel which could dissolve fibrin clot by releasing t-PA. The hydrogel could presumably be applied as a coating on a substrate surface.<sup>230</sup> Our laboratory developed a polycation-modified polyurethane surface on which t-PA was loaded at pH 9.0. When released in contact with plasma, the t-PA retained its ability to generate plasmin and lyse clots.<sup>231</sup>

Recently, we reported a new t-PA releasing concept based on a unique protein-displacement triggering mechanism, taking advantage of the fact that plasminogen has higher affinity than t-PA for surface bound  $\epsilon$ -lysine.<sup>232,233</sup> This concept was implemented using a lysine-modified polyurethane material in the form of fibrous mats fabricated by electrospinning. t-PA was loaded *via* specific interaction with lysine residues and was displaced from the surface (released) by plasminogen when in

contact with plasma.<sup>234</sup> These t-PA-loaded materials have dual means of lysing clots, namely by t-PA release from the material to generate plasmin in the fluid phase, and by plasmin generated on the surface which can lyse surface localized fibrin.

During the last decade, biodegradable polymers have been used as carriers of drug molecules for subsequent release using external ultrasound as a trigger.<sup>235</sup> Torno *et al.* developed a carrier system consisting of a poly(lactic acid)-PEG diblock polymer.<sup>236</sup> Microspheres of the co-polymer loaded with t-PA were evaluated *in vitro* for thrombolytic activity. It was shown that, compared to normal t-PA, the t-PA loaded microspheres exhibited 2-fold greater clot lysis activity. Similarly, Uesugi *et al.* developed a PEG-gelatin nanocarrier for t-PA which prolonged the half life of the t-PA in blood and released it in response to ultrasound. In a rabbit thrombosis model ultrasound irradiation after administration of the nanocarrier resulted in complete re-opening of occluded arteries.<sup>237</sup> As far as we are aware, the ultrasound trigger method has yet to be exploited for release of t-PA from biomaterials used to construct blood contacting devices.

## 4. "Topographic" surfaces

The strategies discussed so far are based on surface chemical modification to achieve anti-thrombogenicity. However, it is increasingly recognized that not only surface chemical composition, but also topography and porosity influence biological interactions including protein adsorption,<sup>238–241</sup> platelet adhesion,<sup>242–245</sup> and cell behaviour generally.<sup>246–254</sup> Thus manipulation of surface topography offers an additional approach to improving blood compatibility, one that has not, to date, been much explored.

Surface roughness has been recognized for some time as a property that affects thrombogenicity.<sup>255,256</sup> Accordingly surface polishing was investigated as a method to reduce thrombogenicity.<sup>257,258</sup> Indeed it has been suggested that extreme smoothness is the main reason for the relatively good blood compatibility of prosthetic heart valves coated with highly polished pyrolytic carbon.<sup>259</sup> However it has been shown that platelet adhesion and thrombogenicity can in fact be reduced to levels lower than on the corresponding smooth surface by proper design of surface topography in the submicrometer to nanometer range.<sup>242–245,260–267</sup> This can be seen as a bioinspired strategy mimicking the multiscale micro/nano structures of the endothelial surface of natural blood vessels (Fig. 9). Such a strategy has been demonstrated to be useful for the development of anti-thrombogenic surfaces.<sup>245</sup> A variety of methods,<sup>268–271</sup> including mechanical and electrochemical, polishing/roughening, chemical etching and different patterning strategies (lithography, molding, embossing, imprinting, self-assembly), have been investigated for the fabrication and modification of surface topography.

In discussing the effects of surface topography, it should be kept in mind that topography is difficult to define in terms of simple parameters.<sup>273</sup> In the biomaterials field, it is customary to classify surface topography as macro (>10  $\mu$ m), micro (1–10  $\mu$ m) and nano (<1  $\mu$ m), according to the characteristic length

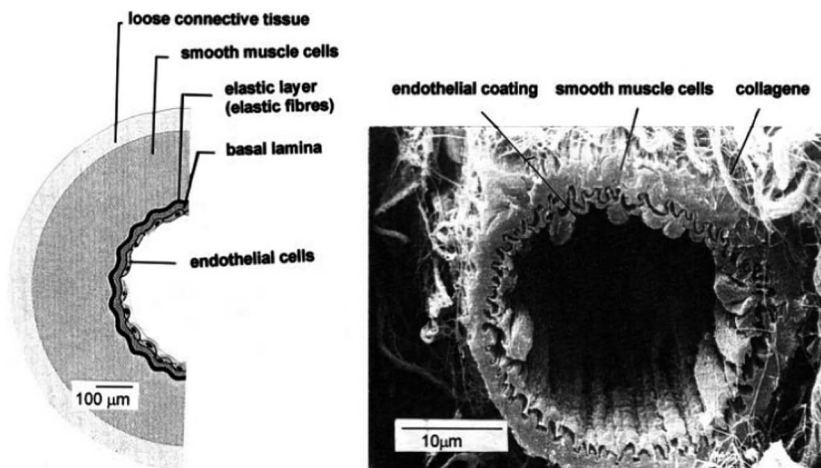


Fig. 9 Micro/nanomultiscale structures on the inner surface of a natural blood vessel: left, schematic; right, scanning electron micrograph. Clearly, the inner surface of the blood vessel is not smooth but rather rough at the scale of several microns, with micro-grooves aligned in the blood flow direction and submicron-scale protruberances on the ridges. Adapted with permission from ref. 272 copyright 1999, Springer Berlin Heidelberg.

scale,  $l$ , of the surface features. This length scale has been found to correlate strongly with implant–host interactions. A rule of thumb was proposed by Chen *et al.*<sup>245</sup> based on three regimes as follows:

(I)  $l > 2 \mu\text{m}$  (dimensions of platelets); increasing roughness results in more contact area for platelet adhesion and increasing thrombogenicity.

(II)  $2 \mu\text{m} > l > 50 \text{ nm}$  (dimensions of large proteins, *e.g.* fibrinogen); surface topographical features,<sup>242–244</sup> such as pillars and grooves, may reduce the effective area for platelet–surface contact, thus suppressing platelet adhesion and thrombus formation.

(III)  $l < 50 \text{ nm}$ ; the roughness features are smaller than the pseudopods of activated platelets, and the surface can be regarded as “smooth” with respect to platelet encounters. In this case, increasing roughness will have little influence on platelet adhesion and other factors will dominate.<sup>258</sup>

This analysis suggests that the scale of regime II should be optimal for suppression of platelet adhesion and activation.

Length scale is only one aspect of topography; shapes and spatial arrangements (patterns) of topographic features, taking into account the hydrodynamics of blood flow should also be considered.<sup>274,275</sup> Moreover, a synergistic combination of surface topography and surface chemistry may provide additional benefits. Understanding of how surface chemistry and topography relate to interfacial properties and biological interactions is clearly important but little understood.<sup>276</sup>

While this section focuses on the role of surface topography of relatively short length scale, it should be pointed out that in currently used synthetic vascular prostheses, such as those made from Dacron® (polyethylene terephthalate) and Goretex® (expanded polytetrafluoroethylene, e-PTFE), which perform well as large-calibre arterial replacements, pores or pore equivalents exist within the material, and in those cases it is recognized that surface porosity and texture influence blood interactions.<sup>8,277</sup>

Blood borne materials are deposited in the pores and remodel to give a stable “pseudo-endothelium” of limited thickness such that, in larger diameter vessels, the lumen remains open.

## 5. Surface endothelialization

As has frequently been pointed out, the inner surface of blood vessels, the vascular endothelium, is the only known surface that is truly blood compatible in all respects. Therefore considerable effort has been devoted to the “recreation” or simulation of the endothelium as an approach to blood compatible materials. The endothelium consists of a confluent layer of endothelial cells attached to a basement membrane and it is the properties of these cells that provide the essential functions of the endothelium, including the regulation of inflammation, thrombosis and fibrinolysis. With regard to thrombosis, protective effects include release of anticoagulant glycosaminoglycans (notably heparan sulfate),<sup>278</sup> release of nitric oxide, and the anticoagulant effect of the thrombomodulin-protein C system.<sup>279</sup> By releasing t-PA and u-PA, the endothelium also promotes fibrinolysis.

Surfaces have been developed based on emulation of one or another of these functions as has already been discussed. In this section attempts to design materials lined with functioning endothelial cells as the blood-contacting surface are briefly mentioned. This strategy can be referred to as “surface endothelialisation” and may be seen as more in the realm of tissue engineering than that of biomaterials *per se*, with the emphasis more on cell biology and less on materials chemistry. An extensive literature on endothelialization is available to which the reader is referred for more detailed information.<sup>9–11</sup> Broadly speaking these materials are of three types: (1) ECs are harvested and cultured on the substrate, referred to as EC “seed-ing” or “sodding”, the latter term referring to complete (confluent) coverage of the substrate by the cells; (2) the substrate is designed to encourage EC migration from adjacent

tissue and adhesion-proliferation on the substrate; this applies mainly to the case of vascular grafts; (3) the substrate is designed to capture endothelial progenitor cells (EPC) from the blood and to promote differentiation into functioning ECs (“blood borne endothelialization”).

### 5.1 EC seeding

Pre-culturing of autologous ECs on the blood contacting surface of implants, notably vascular grafts, has been investigated since the 1970s.<sup>280–284</sup> Initially there was great hope and enthusiasm for this approach, but in a review published in 2004, Pawlowski *et al.* wrote: “After 30 years of research in this area, this simple hypothesis has proven to be deceptively naive”,<sup>285</sup> suggesting that the initial promise of EC seeding has not been fulfilled. Two important considerations in cell seeding are the acquisition or harvesting of the cells, and the method of seeding on the substrate. The following brief discussion touches only on the seeding process itself.

Problem areas include the rate of surface coverage by the cells, and the stability of the cell layer in contact with flowing blood. Simple seeding including harvesting and attachment of cells may require times of the order of an hour. However growth to confluence and recovery of cell morphology, required for normal cell function, may take several days. These lengthy times are not compatible with clinical needs where a graft may be required at short notice. As a possible solution to this problem Pawlowski *et al.*<sup>285</sup> proposed that imposing a positive electrical charge on the graft surface should speed the seeding process, including maturation of the cells, since the cells are overall negatively charged. They showed that seeding on an e-PTFE graft was relatively rapid using this “electrostatic” process and that the cell layers persisted and led to reduced thrombogenicity in *in vivo* canine experiments.

In general the loss of seeded EC layers over time in contact with flowing blood has been problematic in these devices. Interestingly Poole-Warren *et al.*<sup>282</sup> showed, in sheep experiments with implanted e-PTFE and microporous polyurethane grafts, that while the loss of seeded ECs was in the range of 40–60% after one week, coverage increased to 80–90% after three weeks.

### 5.2 Endothelialization by migration from adjacent tissue

In vascular graft applications this may be described as “trans anastomotic endothelialization” (TAE). It should first be noted that, as has long been reported, TAE occurs readily in animal models,<sup>286</sup> but not, or at best to only a limited extent, in humans.<sup>287</sup> This “species difference” has remained a mystery and has frustrated researchers for the past several decades. Zilla *et al.*<sup>288</sup> have suggested that the explanation, at least in part, may lie not so much in fundamental biological differences between species but rather in differences between animal models used in research and clinical practice. In particular, grafts used in animal models are much shorter than typical clinical grafts, *e.g.* peripheral arterial grafts, and while TAE may occur over a small distance clinically, most of the graft remains devoid of endothelium. In the shorter grafts used in animal models, TAE

covers the entire graft. All of this notwithstanding, it is clear that there are indeed fundamental differences at the biological level between humans and other species with respect to TAE and that growth may be much faster in animals than in humans. Zilla *et al.*<sup>288</sup> have proposed that “transmural endothelialization” (TME) rather than TAE may be a more fruitful approach. TME occurs by transfer of material from contacting tissue across the graft wall and is governed mainly by the pore structure as opposed to the chemical composition and structure of the graft surface.

### 5.3 “*In situ*” endothelialization: selective binding of endothelial progenitor cells from blood

This approach is relatively recent and has the compelling advantage that it is the patient's own cells which provide the blood contacting surface, thereby eliminating the possibility of rejection by the body.<sup>11</sup> Endothelial progenitor cells (EPCs) are circulating cells that can differentiate into endothelial cells.<sup>289</sup> Vascular grafts seeded with EPCs have been shown to be relatively non-thrombogenic.<sup>290</sup> These cells have the ability to “home” on sites of vascular injury by the interactions of ligands on the EPC surface with molecules on, or secreted by, activated endothelial cells and platelets on the injured vessel wall. Such EPC homing or capture phenomena can be simulated on artificial graft surfaces, including incorporation of antibodies against cell surface receptors, EC-binding peptides (*e.g.* YIGSR, RGD) and aptamers. These approaches are in the very early stages of exploration, but they may hold significant promise as a solution for the blood compatibility conundrum.

## 6. Conclusion

The quest for blood compatible materials, in particular thromboresistant materials, has been ongoing and intense over the past four decades. That the focus has been maintained over such a period reflects the great importance of finding solutions. That no satisfactory solution has been *found* reflects the difficulty and complexity of the problem. In a sense an “artificial” material that is thromboresistant is a contradiction since the natural physiological “order” is that blood coagulates when it encounters a surface other than normal vascular endothelium.

Research continues because the stakes are high. Large numbers of life saving blood-contacting devices are used annually around the world, ranging from heart assists (thousands), to heart valves (hundreds of thousands), to coronary stents (millions), to hemodialysers (tens of millions), to catheters (hundreds of millions). And all of these devices are susceptible to thrombotic complications.

In a 1993 article entitled “The blood compatibility catastrophe”,<sup>291</sup> Ratner bemoaned the fact that “... we lack some guiding principle that will allow us to analyze and explain blood compatibility”, and pointed out that even a universally accepted definition of blood compatibility does not exist, and that no appropriate tests for assessing blood compatibility have been devised. A second similar article (“The catastrophe revisited”) from the same author appeared in 2007 (ref. 292) suggesting



that little progress had been made in the intervening years. As is clear from the present article, some of these issues are still with us. However we believe the article also shows that progress has been made.

A guiding principle that has emerged, perhaps not so new but by now inescapable, is that we must take our cues from the vascular system itself which, as described in Section 5, employs multiple ways to prevent thrombosis: incorporation and/or release of anticoagulants, antiplatelet agents and fibrinolytic agents. It is very unlikely that any one of these or any other single function alone will be sufficient for antithrombogenicity in an artificial material, suggesting that future research should focus on materials that are multifunctional. Materials that resist nonspecific interactions (anti-fouling) and have a specific bioactive function are currently under investigation. Building on this, one can envisage incorporating two or more bioactive functions similar to those of the endothelium, e.g. anticoagulant and antiplatelet functions.

Other than such new approaches to materials development *per se*, future needs include improved methods of materials evaluation for anti-thrombogenicity in blood contact, especially *in vivo* methods that can be readily correlated with the plethora of *in vitro* methods already in existence. In addition, and most importantly, knowledge of blood-material interactions needs to be deepened so that logically based approaches can be conceived.

It is the authors' hope that this article represents the state of the art of blood compatible materials research, and that, given the importance of such materials for the survival and the quality of life of millions of persons worldwide, research will continue until true solutions are found. We hope especially that the article will incentivize young investigators to enter the field and contribute new ideas for the solution of what has proved to be a frustrating, but hopefully not, ultimately, an intractable problem.

## Acknowledgements

Financial support by the Canadian Institutes of Health Research, the Natural Sciences and Engineering Research Council of Canada, the National Natural Science Foundation of China (21204005, 21334004 and 21304062), the Natural Science Foundation of Jiangsu Province (13KJA430006 and 13KJB430020), the China Postdoctoral Science Foundation (2013M541714) is gratefully acknowledged.

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