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Blodkompatibilitet hos biomaterial av rekombinant spindeltråd

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SUMMARY

Native spider silk is an attractive lightweight natural polymeric material with a unique combination of strength, extendibility and flexibility. Synthetic, recombinant spider silk is an attractive biomaterial with several possible fields of application.

When a foreign material is introduced to blood, this can for example cause unwanted coagulation and complement activation.

The aim of this study was to investigate if coatings of heparin or albumin could improve the blood compatibility of films of recombinant spider silk and to develop an initial method to do so. Hypothetically, objects like pacemakers, stents and dental implants could be covered with spider silk films of high blood compatibility to reduce potentially harmful foreign body reactions caused by the immune defense. Increased blood compatibility would likely lead to improvements for both the individual patients and the society thanks to reduced load on healthcare institutions.

Our objective was to assess the blood compatibility of both functionalized and non-functionalized recombinant spider silk by evaluating coagulation and activation of the complement system of the immune defense.

Coagulation was evaluated visually on glass slides and complement activation was analyzed by measuring the concentration of the complement system mediator C3a in blood samples that had been exposed to the different materials.

This study introduces a non-toxic and easy performed method of improving the blood compatibility of recombinant spider silk films by adding a heparin coating.

Heparin coated surfaces had statistically significantly higher blood compatibility than uncoated surfaces. The effects of albumin coating of films were less clear compared to the results of heparin coated films.

Complement activation could not be evaluated because of high activation in all samples prior contact with the materials that were included in the study.

SAMMANFATTNING

Naturlig spindeltråd är ett lätt material som är både flexibelt, elastiskt och starkt. Syntetisk rekombinant spindeltråd har flera möjliga användningsområden som biomaterial.

När ett främmande material kommer i kontakt med blod kan det leda till ogynnsam koagulation och komplementaktivering.

Syftet med den här studien var att undersöka om blodkompatibiliteten på filmer av rekombinant spindeltråd kunde förbättras med hjälp av beläggningar av heparin eller albumin som anlagts med enkla och giftfria metoder. Hypotetiskt skulle biomaterial och implantat kunna täckas av blodkompatibla filmer för att minska kroppens reaktion mot det som inte är kroppseget och på så sätt leda till ökat välbefinnande för den enskilda patienten och även för samhället i stort genom minskat vårdbehov.

Vårt mål var att undersöka blodkompatibiliteten hos både rekombinant spindeltråd med extra egenskaper och vanlig spindeltråd genom att övervaka aktiveringen av koagulationsprocessen och komplementsystemet.

Koagulationspåverkan studerades på objektsglas och komplementaktiveringen undersöktes genom att analysera koncentrationen av komplementfaktorn C3a.

Här presenteras en icke-toxisk och enkel metod för att öka blodkompatibiliteten hos spindeltrådsfilmer genom att belägga dessa med heparin. Albuminbeläggningens effekt på blodkompatibiliteten var mindre tydliga än effekten av heparinbeläggningarna, vars effekt gick att säkerställa statistiskt.

Aktiveringen av komplementsystemet kunde inte utvärderas på grund av för hög aktivitetsnivå i alla prover som analyserades.

INTRODUCTION

Background

Biomaterials

Definition and utilization of biomaterials

Biomaterials have been developed and used for a very long time, and their role in modern medicine has become more and more important (Binyamin et al., 2006; Ekdahl et al., 2011). Biomaterials are a wide group of diverse materials which can be defined and classified in many ways. The US National Institutes of Health has described biomaterials as "any substance (other than a drug) or combination of substances synthetic or natural in origin, which can be used for any period of time, as a whole or part of a system which treats, augments, or replaces tissue, organ, or function of the body". More recently, the definition also includes "any material used in a medical device intended to interact with biological systems" (Binyamin et al., 2006).

Spider silk as a potential biomaterial

When developing or selecting a new biomaterial one has to be aware of the mechanical and biological as well as the chemical properties of the material and the environment the material is intended to be used within. The biomaterials currently used can be grouped into four major groups; metals, polymers, ceramics and composites. The different materials fulfill different demands and should be selected based on their properties and intentions of use (Biehl et al., 2002; Binyamin et al., 2006).

Native spider silk is an attractive lightweight natural polymeric material with a unique combination of strength, extendibility and flexibility. Recombinant synthetic spider silk has been produced in a wide number of hosts, including bacteria, different mammals and plants (Rising et al., 2011; Widhe et al., 2012; Chung et al., 2012). Recombinant spider silk has successfully been processed into structures like foams, films and stable fibers for different biomedical applications (Kluge et al., 2008; Hedhammar et al., 2010; Rising et al., 2011, Spies et al., 2010; Widhe et al., 2012). The synthetic spider silk used herein is weaker than native spider silk but mimics the flexibility and elasticity of the native spider silk very well (Widhe et al., 2012).

Results from biocompatibility studies of recombinant and native spider silk is difficult to compare since available literature is based on results from several different spider silk proteins with different properties and different testing circumstances (Fredriksson et al., 2009; Moisenovich et al., 2011; Rising et al., 2011). However, it has been suggested that spider silk is more biocompatible than the commonly used silk worm silk (Rising et al., 2011). Spider silk has been used successfully in many different biomedical applications, for example as base material for cell culture plates and as support for neurological regeneration of peripheral nerves (Moisenovich et al., 2011; Rising et al., 2011; Widhe et al., 2012).

Blood compatibility of biomaterials

Physiological reactions to foreign objects and materials

Many of the first biomaterials used in vivo had to be abolished because they caused severe complications due to for example improper design or biological reactions caused by the implants (Binyamin et al., 2006). Anything introduced to blood or the body will be recognized as foreign and induce a physiological response. Today we know that material selection and design is crucial for the bio- and blood compatibility of objects, but still in modern time no completely biocompatible material is known or available (Gorbet & Sefton, 2004; Binyamin et al., 2006). One of the main problems with biocompatibility testing is that there is no definition of how much host response that is tolerable or harmful (Gorbet & Sefton, 2004). For this reason, much research has been performed to improve the compatibility properties of artificial surfaces (de Queiroz et al., 1997). Unfortunately, many in vitro results differ greatly from results in vivo (Li & Henry, 2011). This suggests that there are probably many complex and related reactions occurring in vivo that may not be present during in vitro tests. It is understandable that the in vitro models cannot fully represent the in vivo conditions, but these factors and relationships have to be considered when developing reliable and successful biomaterials (Seyfert et al., 2002; Gorbet & Sefton, 2004; Li & Henry, 2011).

Blood- and biocompatibility

Biocompatibility could be described as “how the body reacts to the surface of the material and the impact of the implant on the body” (Binyamin et al., 2006) or “the ability of a material to perform with an appropriate host response” (Gorbet & Sefton, 2004). Simply put, a material of greater biocompatibility will be better accepted by the host compared to a biomaterial with worse biocompatibility (Binyamin et al., 2006; Widhe et al., 2012). However, one could claim that the definition is too wide and that the exact meaning of biocompatibility is vague (Williams, 2008; Widhe et al., 2012). Widhe et al. (2012) discuss that the concept of biocompatibility actually consists of both biosafety and biofunctionality. Biofunctionality represents how well the implanted material is fulfilling its functional aims. Biosafety represents how the body reacts on the material and effects of the material on the host and includes the concept of blood compatibility.

When a biomaterial is introduced to viable tissue, serum proteins like albumin, fibrin and collagen will coat the surfaces of the biomaterial, creating a biofilm containing coagulation and inflammatory complement system components (de Queiroz et al., 1997; Nilsson et al., 2007; Li & Henry, 2011; Widhe et al., 2012; Jung et al., 2013).

Different materials attract different protein compositions that consequently affect the host response (Wilson et al., 2005; Widhe et al., 2012). It has been suggested that the host response to implants, which may affect the function and determine if the implant will be accepted or not, is triggered by this protein layer rather than by the actual biomaterial itself (Wilson et al., 2005; Anderson et al., 2008; Widhe et al., 2012).

The proteins may also denaturate or go through conformal changes when attached, influencing the response of the host (Weber et al., 2002). The composition of the protein layer may also change over time in a process called Vroman effect (Krishnan et al., 2004; Wilson et al., 2005; Jung et al., 2013).

However, the actual properties of the implant must still be considered together with other factors that will influence the host response, including potential tissue injury due to the implantation and inflammatory processes due to the continual presence and the properties of the biomaterial (Onuki et al., 2008). Furthermore, the place of implantation is also relevant since implants of the same material can cause different reactions in different tissues (Williams, 2008; Widhe et al., 2012**)**.

When a foreign material is introduced to blood, this can for example cause unwanted coagulation and complement activation (Gorbet & Sefton, 2004; Binyamin et al., 2006; Markiewski et al., 2007; Nilsson et al., 2007).

Introduction to the coagulation cascade

The conversion of fluid blood into a gelatinous mass or clot is called blood coagulation (Sjaastad et al., 2003; Rang et al., 2007). Normally, the blood in the vessels is in contact with vascular endothelium. Endothelium provides a non-thrombogenic, smooth and soft surface promoting non-turbulent flow of blood and plays an important role in preventing intravascular platelet activation and regulation of the coagulation processes (McGavin & Zachary, 2007; Rang et al., 2007; Li & Henry, 2011). However, different mediators can change the properties of the endothelium to promote coagulation and result in production of coagulation mediators (McGavin & Zachary, 2007; Rang et al., 2007).

The coagulation process relies on a complex series of sequential chain reactions between both non-enzymatical and enzymatical factors and is often called the coagulation cascade. The cascade is rigorously regulated by coordinated interactions between coagulation factors, fibrinolytic processes and released substances from blood vessels and platelets (Sjaastad et al., 2003; Jay et al., 2006; McGavin & Zachary, 2007; Rang et al., 2007).

Platelets can be activated by many factors (Gorbet & Sefton, 2004; Li & Henry, 2011). Sometimes adhesion to surface-bound proteins is enough (Li & Henry, 2011). Activated platelets express substances stimulating further direct or indirect actions by coagulation factors (Jay et al., 2006). Prevention of platelet adhesion and activation is critical for the production of non-thrombogenic objects (Li & Henry, 2011).

The coagulation factors are essentially circulating proenzymes that can be enzymatically activated to fully functional enzymes which in turn can activate other proenzymes, resulting in a cascade of enzymatic reactions. Because of this complex scheme of interconnected reactions, even a small initial stimuli can result in massive coagulation thanks to the possibility of amplification connected to every step in the coagulation cascade (Jay et al., 2006; Rang et al., 2007).

The coagulation cascade has traditionally been divided in an intrinsic and extrinsic pathway, converging with the activation of factor X (Jay et al., 2006; McGavin & Zachary, 2007). The activated factor X (abbreviated as factor Xa) is crucial in the final steps of the coagulation process, where the end product of the cascade is formed – fibrin (McGavin & Zachary, 2007; Rang et al., 2007). Factor Xa converts prothrombin to thrombin. Thrombin then both forms and stabilizes the produced fibrin (Sjaastad et al., 2003).

In the intrinsic pathway, all factors required are present in the blood. In the extrinsic pathway, factors that are not normally in the blood stream are introduced and activates coagulation (Sjaastad et al., 2003; Rang et al., 2007). The intrinsic pathway is also called the contact pathway since it relies on a series of reactions that occur during the first minutes of initial contact between blood and a surface (McGavin & Zachary, 2007; Rang et al., 2007; Li & Henry, 2011). Gorbet & Sefton (2004) questions the relevance of contact activation under physiological conditions, but it seems quite well established that the intrinsic pathway can be activated by artificial surfaces (Gorbet & Sefton, 2004; McGavin & Zachary, 2007; Rang et al., 2007).

More recent concepts of in vivo coagulation describes the process more like an connected web of reactions instead of the previous series of independent pathways (Gorbet & Sefton, 2004; McGavin & Zachary, 2007). Activation of the extrinsic pathway has been observed to stimulate the intrinsic pathway (Sjaastad et al., 2003), resulting in more efficient coagulation via a positive loop of generation of factor Xa (Jay et al., 2006) and manifesting the interaction and cooperation between the pathways (Rang et al., 2007).

Coagulation is intended to be a local process. Unwanted and uncontrolled activation of coagulation can lead to dangerous thrombi that may be fatal or cause serious tissue damages, and generalized or uncontrolled coagulation is indeed potentially dangerous (Sjaastad et al., 2003; Rang et al., 2007).

Introduction to the complement system

The immune system could be described as a system built of an innate and an acquired component that complement each other and collaborate to distinguish non-autologous (“non-self”) agents. The acquired component has high specificity for agents that the body has been exposed to previously. The innate component is unspecific and will be activated independently of previous exposure (Sjaastad et al., 2003; Tizard, 2009).

Both the innate and acquired immune system can start a complex cascade of biochemical and cellular reactions called the complement system (McGavin & Zachary, 2007; Tizard, 2009). The purpose of the complement system is to recognize, destroy and remove non-autologous agents upon discovery. The complement system is a complex and powerful system and its mechanisms are not fully understood (Nilsson et al., 2007). Activation of the complement system cascade results in generation of numerous biologically active molecules. The actions of the complement system are usually positive for the host but inappropriate activation of the complement system can cause serious host cell damage and tissue injury (McGavin & Zachary, 2007; Nilsson et al., 2007; Onuki et al., 2008; Tizard, 2009).

The complement system can be activated by three different ways, named the classical, lectin, and alternative pathways. The classical pathway can be triggered by antigen-antibody complexes as well as substances released from damaged cells. The (mannose binding) lectin pathway is activated by particles and structures (carbohydrates) found mainly on the surfaces of microorganisms (Sjaastad et al., 2003; Markiewski et al., 2007; McGavin & Zachary, 2007; Tizard, 2009).

The alternative pathway is triggered by foreign surfaces, substances and surface-bound proteins. Even though it has been proposed that the classical pathway and lectin pathway could be activated by biomaterials as well, and that the alternative pathway can amplify weak stimulus from the other complement pathways, the alternative pathway is believed to be of greatest importance regarding the biocompatibility of biomaterials (McGavin & Zachary, 2007; Nilsson et al., 2007).

Natural regulation and activation of the complement system in vivo is dependent on mediation of both plasma proteins and membrane bound proteins. The complement system contains more than 30 proteins that influence the action of its reactions, regulating inhibition and activation (Sjaastad et al., 2003; McGavin & Zachary, 2007; Nilsson et al., 2007; Tizard, 2009).

The mediators C3 and C5 are central for the activity and activation of the complement system (Tizard, 2009). Activation of factor C3 is required in all three pathways of complement activation and is needed to activate C5 to enable further actions, as shown in figure 1 (Nilsson et al., 2007; McGavin & Zachary, 2007; Tizard 2009). C3 and C5 are used here as examples because of their central role both in the complement system and biocompatibility although the basic principles could also be applied to the other factors of the complement system.

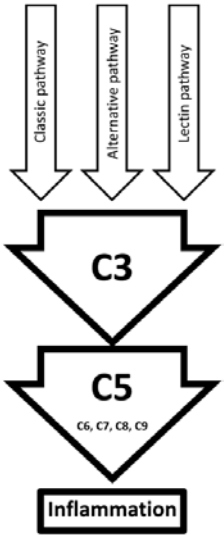

Figure 1. Schematic view on the central aspects of the complement system. The complement system can be activated by three pathways. All pathways rely on initial actions of complement factor C3 and C5 (based on Tizard, 2009).

Cleavage of complement factors by specialized convertases or other conformal changes can result in biologically active substances. C3a, C3b, iC3b and C5a are examples of mediators that can be derived from C3 and C5 (Nilsson et al., 2007; Tizard 2009). The active mediators can trigger many different actions of cells and other complement factors. C3a and C5a can stimulate leukocytes to release cytokines, and increase generation of leukotrienes and prostaglandins (Hong et al., 1999; Tizard, 2009). Surface bound C3b binds C5 that then divide into C5a and C5b, allowing further reactions and activation of other factors (Tizard, 2009). Because a single “upstream mediator” like C3b can activate several “downstream mediators” like C5, even small initial stimuli can have large impact (Sjaastad et al., 2003).

Complement activation caused by biomaterials is probably initiated by the biofilm-proteins on the biomaterial. Surfaces that bind more proteins results in higher levels of complement activation (Nilsson et al., 2007). Complement activation itself has also been reported to stimulate protein adhesion onto biomaterial surfaces (Nilsson et al., 2007). Complement activation results in adhesion of complement proteins onto the biomaterial surfaces (Hong et al., 1999; Nilsson et al., 2007) and a large portion (up to 20%) of the proteins bound to biomaterial surfaces has been assumed to be mediators of the complement system (Nilsson et al., 2007). For instance, Nilsson et al. (2007) reported that the protein layer absorbed to the surface of biomaterials upon exposure to blood also contains C3. These biofilm-attached C3 mimic the active mediator C3b (Andersson et al., 2002). Native C3b bind to the protein layer and trigger the alternative pathway. Activation of the complement system initiates a positive feedback loop of C3b generation causing amplified activation of the complement system (Nilsson et al., 2007). In addition to this, complement activation results in deposition of C3b and iC3b on the surface (Hong et al., 1999). C3b and iC3b has been suggested to be able to interact and bind covalently to plasma proteins like albumin (Nilsson et al., 2007).

In addition to biofilm-activation of the complement system, Nilsson et al. (2007) discuss the gas-fluid interactions of complement system activation. Oxygen, nitrogen and air have been reported to activate complement both in vivo and in vitro.

Interaction between complement and coagulation

Although the coagulation and complement system are specialized for different purposes, they have a lot in common on the biochemical and the enzymatical levels (Markiewski et al., 2007; Nilsson et al., 2007). Coagulation and complement activation are closely connected and can be seen as partners during inflammation, trying to be beneficial for the host (Gorbet & Sefton, 2004; Markiewski et al., 2007).

Different materials seem to cause different degrees of co-activation of the two systems (Arvidsson et al., 2007) and the relationship has been questioned (Biehl et al., 2002). However, direct interactions (“cross-talk”) between different parts of the respective systems, such as platelets and leukocytes, have been known for a long time (Nilsson et al., 2007).

A prothrombotic environment is also proinflammatory and complement factors can be generated by coagulation (McGavin & Zachary, 2007; Nilsson et al., 2007). It is well established that complement is activated during the clotting of blood (Markiewski et al., 2007). Activation of the coagulation system induces C3a activation (Nilsson et al., 2007). Furthermore, coagulation factor XIIa has been reported to induce activation of the classical pathway of the complement system (McGavin & Zachary, 2007). On the other hand, the complement system has several procoagulant properties (Markiewski et al., 2007). Activation of the complement system increases prothrombotic and reduces non-thrombotic mediators (Markiewski et al., 2007) and complement mediators have been reported to affect not only leukocytes but platelets as well (Nilsson et al., 2007; Tizard, 2009). The blood platelets can be activated by the complement system and for instance release substances if exposed to C3a and C5a (Gorbet & Sefton, 2004; Tizard, 2009).

Improving blood compatibility with albumin or heparin

Blood compatibility is a major issue in for example hemodialysis, cardiopulmonary bypass and extracorporeal circulation as well as with implanted cardiovascular grafts like stents and heart valves (Nilsson et al., 2007; Onuki et al., 2008; Williams, 2008; Li & Henry, 2011).

There have been attempts to develop biomaterials that degrade products formed by the host response against the biomaterial, like thrombi, but the most common and successful strategies today are focused on inhibition of the actual formation of the thrombi and like (Li & Henry, 2011). Except from selecting materials with suitable properties, the blood compatibility of biomaterials can be improved by addition of different coatings. Coatings can both inhibit activation of complement and coagulation and stimulate a cellular response favoring acceptance of the biomaterial, increasing the overall biocompatibility (Bos et al., 1999). Failure of coatings and loss of the integrity of the coating may occur over time if one is not aware of the properties of the current coating substance or its compatibility to the material that is being coated. Different materials have been coated with varying success and resistance of the finished coating (Arvidsson et al., 2007). In some cases it is desirable to intentionally make a coating that works like a temporary shield, until the surface has been populated by host cells. While coatings could be designed like this to be replaced with for example endothelial cells after a while, and others stimulate endothelial cell growth (Bos et al., 1999; Li & Henry, 2011), the focus here will be on coatings made of heparin and albumin, two molecules that have been used with varying success both alone and combined (Bos et al. 1999; Li & Henry, 2011; Jung et al., 2013).

Heparin

Heparin was discovered in the beginning of the 20th century and is now the most common anticoagulant for clinical use. It is also common practice to use heparin to stop blood from coagulating in blood samples (Sjaastad et al., 2003; Rang et al., 2007; Li & Henry, 2011). The heparin family consists of molecules of sulfated glycosaminoglycans with high heterogeneity (Rang et al., 2007; Honchel et al., 2011). The pharmacokinetics and pharmacodynamics of heparin are complex and depends on factors like the molecular weight and the concentration (Bose et al., 1999; Rang et al., 2007; Honchel et al., 2011).

Heparin may be capable of inhibiting coagulation by multiple mechanisms (Weber et al., 2002; McGavin & Zachary, 2007), but the major role of heparin is to accelerate the activity of the enzyme antithrombin III (ATIII) (McGavin & Zachary, 2007). ATIII is an important enzyme that regulates the coagulation processes together with heparin-like substances in the endothelium under physiological conditions, preventing adverse and generalized coagulation (Sjaastad et al., 2003; Rang et al., 2007). Coupled to heparin, this enzyme can degrade and inactivate the coagulation factor Xa. Heparin can also inactivate factor IIa (thrombin) when connected to both ATIII and factor Xa (McGavin & Zachary, 2007; Rang et al., 2007).

In addition to the anticoagulant effects, heparin has also been reported to have anti-inflammatory properties (van Bilsen et al., 2004; McGavin & Zachary, 2007; Nilsson et al., 2007; Lappegård et al., 2008).

A lot of the research in biocompatibility of biomaterials has been on heparin (Li & Henry, 2011). Heparin coatings reduce adhesion of platelets (Weber et al., 2002), and reduce activation of coagulation and inflammatory reactions like complement (Weber et al., 2002; McGavin & Zachary, 2007; Nilsson et al., 2007). For example, heparin coating of the commonly used biomaterial polyvinyl chloride (PVC), resulted in a substantially lower blood reactions compared to uncoated surfaces (Nilsson et al., 2007). It has also been reported that the composition of adsorbed proteins on a surface coated with heparin differs from a surface not coated, possibly reducing adhesion of pro-inflammatory proteins (Weber et al., 2002; van Bilsen et al., 2004; Lappegård et al., 2008).

Heparin is effective both in vivo and in vitro (Rang et al., 2007). Even though heparin coatings have been used successfully in vitro there are some questions regarding how well they work in vivo and how good the long-term effects really are (Gorbet & Sefton, 2004). Van Bilsen et al. (2004) suggest that heparin coatings may reduce the acute responses, but conclude that there was no significant difference between the long term success rate among patients that had been exposed to either uncoated or coated implants.

The potency of identical amounts of heparin may differ as a result of the molecular heterogeneity that follows from the production, since heparin is extracted from biological materials (Rant et al., 2007). The activity is carefully assayed (Rang et al., 2007), but since both the test protocols and calibration of the test methods varies worldwide and have changed over years, there have long been at least two different units describing the activity of heparin (Honchel et al., 2011). As of October 1st, 2009, efforts were made to harmonize the American USP unit with the international unit (IU), resulting in approximately 10% lower potency per USP unit compared to earlier (Honchel et al., 2011; U.S. Food and Drug Administration, 2011).

There are many methods of heparin immobilization and the process may influence the availability and hence the activity of heparin (Bos et al., 1999; Li & Henry, 2011). Furthermore, the potency of the heparin coating depends on the composition of the proteins that adhere to it and may be reduced if the coating is cluttered with different proteins like fibronectin (Bos et al., 1999).

Heparin can interact with many plasma proteins, possibly causing unwanted or surprising side effects (Ekdahl et al., 2011). Even though heparin coatings has been described to reduce platelet adhesion (Weber et al., 2002), Bos et al. (1999) reported that activated platelets were seen when evaluating surfaces coated with heparin.

Albumin

Albumin is the protein of highest concentration in the blood. It is produced in the liver and is important for the colloid osmotic pressure and also as a transport vessel for different substances (Grubb & Hansson, 2012). Albumin has been used as a coating for biomaterial surfaces of various objects and materials (Kinnari et al., 2004; Jung et al., 2013). The coatings have been claimed to improve surface properties, improve biocompatibility and prolong positive effects of implants (Kinnari et al., 2004). Albumin-treated surfaces have been called

platelet-resistant because the albumin can decrease adhesion and activation of platelets (Kottke-Marchant et al., 1989; de Queiroz et al., 1997; Bos et al., 1999; Baican et al., 2011). It is widely stated that albumin lowers the thrombogenicity of materials (de Queiroz et al., 1997; Ratner et al., 2004), but there are also reports suggesting that albumin coatings could reduce complement activation (Bos et al., 1999) or reduce bacterial adherence (Kinnari et al., 2004). The positive effect of albumin has been dedicated to albumin's lack of peptide sequences required for interaction with platelets and leukocytes or the enzymes in the coagulation cascade (Ratner et al., 2004).

Surface coating with albumin can make the surface smoother and more uniform (Kinnari et al., 2003; Baican et al., 2011). The coating is also said to inhibit cell adhesion (Kottke-Marchant et al., 1989; Kinnari et al., 2004) and adsorption of unwanted proteins like fibronectin (Kinnari et al., 2001; Kinnari et al., 2004). There are reports on positive impact of albumin coatings compared to uncovered implants even 8 months after implantation (Kinnari et al., 2004). However, when comparing the outcome after 8 months, no difference in success rate could be seen between the coated and uncoated implants (Kinnari et al., 2007) and the long-term effects of albumin coatings remain questionable (Ratner et al., 2004). Kinnari et al. (2003) suggested that it is likely that the albumin coating detach gradually with time and Ratner et al. (2004) enlightened that the albumin of a coating may be replaced with other proteins or if covalently bound, it may denature with time. Kinnari et al. (2003) observed that the effect of their albumin coating was diminishing with time, similarly to a report from Guidon et al. (1984) where signs of degradation of the albumin coatings could be observed 1-2 weeks after implantation.

Albumin coatings are affected by environmental parameters like temperature and humidity (Kinnari et al., 2003). Folding of albumin has been discussed regarding the efficacy and biocompatibility of albumin coatings (Jung et al., 2013). It has also been suggested that process of binding albumin to a surface could alter the properties of albumin (de Queiroz et al., 1997).

Aim of this study

The aim of this study was to investigate if coatings of heparin or albumin could improve the blood compatibility of films of recombinant spider silk and to develop an initial method for preparation of such coatings. Theoretically, objects like pacemakers, stents and dental implants could be covered with spider silk films of high blood compatibility to reduce potentially harmful foreign body reactions caused by the immune defense. Our objective was to assess the blood compatibility of both functionalized and non-functionalized recombinant spider silk by evaluating coagulation and activation of the complement system.

MATERIALS AND METHODS

Selection of surfaces

The recombinant spider silk protein 4RepCT was included both as coated test specimens and uncoated controls in all sessions since it is the base for all recombinant spider silk proteins tested herein. When combined with additional molecules that alter the properties of the

4RepCT, the spider silk is said to be functionalized. pLys-4RepCT is 4RepCT covalently bound to the peptide poly-L-Lysine (pLys). pLys has high density of positive charges and was selected to investigate if there was any difference between non-functionalized 4RepCT and pLys-4RepCT when coated with heparin, a molecule known to have pronounced negative charge. Similarly, pLys-coated glass slides were included in the heparin sessions and given a heparin coating as a substitute for a heparinized surface. ABD-4RepCT, a recombinant spider silk protein functionalized with the albumin binding domain ABD, was included to investigate if the ABD domain could improve the properties of an albumin coating. Uncoated films of ABD-4RepCT were included as controls to the albumin-coated films. They were also included to investigate if the ABD domain could bind and utilize the albumin available in the blood to improve the biocompatibility of the spider silk. Plain glass slides were used as positive controls in all sessions.

Preparation of recombinant spider silk films

pLys-4RepCT and 4RepCT were provided by Spiber Technologies AB. ABD-4RepCT was expressed within *E. coli* kept in shake flasks in a shake incubator for approximately 16 hours at a temperature of 14°C. The intracellulary produced spider silk proteins were retrieved after mechanical cell disruption using high pressure homogenization or enzymatically using lysozyme and DNAse. The proteins were purified using immobilized metal ion affinity chromatography (Chelating Sepharose Fast Flow, GE Healthcare, 17-0575-02) and dialyzed to 20 mM tris buffer before being stored in -20°C. The protein samples were later lipopolysaccharide (LPS) depleted as previously described by Hedhammar et al. (2010). Several reducing gel samples were taken during the process to evaluate the purity of the protein.

Preparations of glass tubes and glass slides

Ten sample tubes of glass (Schott Duran Jenaer Glas) were selected based on their wide base curvature, promoting good casting of films suitable for analysis of complement activation. The tubes were cut to a height of approximately 3-4 cm to allow faster drying and casting of the films. The glass tubes were subjected to extensive cleaning and washing followed by autoclaving for 15 minutes (120°C, 2.8 bar) prior first use. The glass tubes were reused after rigorous cleaning with HNO_3, and then prepared as previously described with washing and dH_2O rinsing, followed by autoclaving.

Plain glass slides (Menzel-Gläser, Thermo Scientific) were selected for the coagulation study. The glass slides were stored in a climate chamber (Binder KBF240 E5.2, 922-0150) over night to enhance the hydrophobic properties of the glass and thereby improving the interaction between the films and the glass slides.

Preparation of films

Determining film sizes and blood volume

Coagulation

Rigorous studies were performed to determine optimal volumes of blood and films sizes to use for the coagulation study. Aiming for a match between the height and the area of the

blood both on glass slides without films (controls) and slides with different films applied upon them, a liquid previously described to have properties mimicking blood (Yousif et al., 2009), was used. A blood volume of 120 µl was selected for studies of films made from 100 µl protein solution.

The pLys-coated control slides were hydrophilic and for that reason it was impossible to match the appearance of the blood on films and hydrophobic glass with the appearance on the pLys-coated controls. Instead, blood volumes on the two pLys-coated control slides were matched visually, resulting in addition of approximately 60-80 µl of blood to these slides.

Complement activation

A blood volume of 800 µl was selected to get a safe margin zone between the border of the film and the blood on a film casted from 1200 µl protein solution in the glass tubes used in the complement activation tests.

Casting of the films

Films were made from recombinant spider silk proteins stored in the freezer (-20°C) after purification. When fully thawed, the protein solutions were centrifuged and diluted when needed. Protein solutions were diluted with LPS-depleted 20 mM tris buffer (coagulation) or sterile filtered phosphate buffer (complement activation) to approximately match the concentration of the protein with lowest concentration (ABD-4RepCT). Prior to film casting, a sample for gel electrophoresis was taken and the absorbance (A_{280}) was measured to calculate the concentration of the protein solution. Acceptable concentrations for films used in this study were defined to 0.2-1.0 mg/ml.

Selection and storage of films on glass slides and in glass tubes

Coagulation

Films and control glasses were stored and let dry at least overnight in a climate chamber (30°C, 25% relative humidity) before use. Films of similar concentrations, appearances and shapes were selected for each test.

Complement activation

Tubes containing protein solution were stored in a climate chamber (30°C, 25% relative humidity) for at least four days. Film integrity was subject for visual evaluation before use.

Examination of the film integrity with Coomassie staining

Coagulation

4RepCT films were washed and then colored with Coomassie stain (Coomassie Brilliant Blue R250) to investigate if films were removed during washing with 20 mM phosphate buffer. Ready to use Coomassie was diluted an additional 5 times with deionized water and applied to films. The stain was left covering the films for 10 minutes and then removed by washing three times with deionized water before evaluating the results.

Complement activation

Glass tubes were stained with coomassie stain, as described above for films on glass slides, when the second complement activation test was finished and the blood had been removed from the tubes.

Treatment of silk films and glass surfaces to improve blood compatibility

Washing of films and surfaces

Coagulation

Films were washed carefully three times with 100 µl 20 mM phosphate buffer, added with a pipette, prior the start of the experiments. To avoid films running dry the last wash buffer was left on the films until final preparations were done and the test could proceed. pLys-coated glass slides were washed with filtered (0.2 µm) deionized water. Glass slides without any kind of coating (positive controls) were not washed at all in order to conserve their hydrophobic properties.

Complement activation

All tubes were washed three times prior addition of blood. All surfaces were washed with 20 mM phostphate buffer. The control tube without coatings was washed once with 20 mM phosphate buffer.

Preparation of the albumin coating solution

The albumin coating was made from 50 g/l stock solution of infusible human albumin (CSL Behring Gmbh; Apoteket AB, 494609). Prior use, required volume was diluted 2 times with sterile filtered (0.2 µm) 20 mM phosphate buffer in autoclaved Eppendorf tubes to a final concentration of 25 g/l.

Preparation of the heparin coating solution

The heparin coating was made from unfractionated heparin bought from Sigma Aldrich (Heparin sodium salt from porcine intestinal mucosa, Grade I-A, ≥180 USP units/mg, powder, BioReagent, suitable for cell culture, H3149-10KU). Every 4RepCT molecule was assumed to bind one heparin molecule each. Furthermore, the concentration of the solution was calculated so that 100 µl of the heparin solution should contain 10 times more heparin molecules compared to the number of molecules in a 4RepCT-film made of 100 µl protein solution with a concentration of 1 mg/ml. As a result, a sterile filtered (0.2 µm) heparin-phosphate-buffer solution with the concentration of 1.3 mg heparin/ml (≥ 229.5 USP/ml) and pH 7.4 was prepared. This solution was stored at 4°C and used in all experiments.

Application of coatings

Coagulation

Coating solutions were applied in volumes of 100 µl to films that had recently been washed once with 20 mM phosphate buffer. The coating solutions were incubated for 60 minutes and then carefully removed by pipetting.

Complement activation

All films where first washed once with 20 mM phosphate buffer. Coating solutions were then applied in volumes of at least 1200 µl each and incubated for 60 minutes before removal.

Poly-L-Lysine-coating of glass

Solutions of 15 µg/µl sterile filtered (0.2 µm) poly-L-lysine (pLys) (Poly-L-Lysine, Sciencell Research Laboratories, endotoxin concentration $\leq$ 20 EU/ml, 0413) were used as pLys-coating solution.

Coagulation

Non-hydrophobic glass slides were incubated in pLys-solution at approximately 37°C overnight. These slides were washed three times with sterile filtered (0.2 µm) deionized water during experiment sessions and then left until dry. Thereafter a heparin solution was applied in such a volume that half of the glass slides were covered. After 60 minutes the glass slides were washed three times with sterile filtered (0.2 µm) dH_2O and allowed to dry prior further actions.

Complement activation

pLys-coating of glass tubes were cast by filling two tubes closely to the brim with pLys solution. The filled tubes were then stored at 37°C until use in experiments the following day. At the start of the experiment sessions the tubes were emptied and washed three times. The last wash was left in the tube that was not to be coated with heparin in order to keep the pLys-coating moist until use.

Staining of heparin coating with toluidine blue

Films and glass slides were stained with toluidine blue to visually evaluate spontaneous heparin attachment to films of 4RepCT, pLys-4RepCT and glass slides coated with pLys. We evaluated if a staining protocol with neutral pH would result in sufficient staining compared to standardized acidic staining solutions. A PBS (phosphate buffered saline) solution with pH 7.4 was used in the neutral staining protocol and acetic acid with pH 2.4 was used in the standard protocol. Films were washed with 20 mM phosphate buffer before staining and addition of the heparin coating solution. 100 µl Toluidine blue solution was applied to the films and control slides coated with pLys were covered with stain solution for 10 minutes. Color intensity was evaluated both during the incubation by carefully rocking of the glass slides, and after removal of the stain solution and washing of the films and glass slides with dH_2O. Films without any heparin coating were used as controls.

Blood collection

Blood was collected at Uppsala University Hospital (Uppsala, Sweden), from healthy donors who had not been treated with any kind of pain-relievers or anti-inflammatory drugs (NSAID) within two weeks prior the donation.

Coagulation

Blood was collected into two Vacutainer tubes (either 4.5 ml or 6 ml tubes depending on availability) (Becton, Dickinson and Company) with citrate additive (0.129 M in 4.5 ml tubes and 0.105 M in 6 ml tubes) to inhibit coagulation. The blood was collected the same day as it was going to be analyzed. The blood was stored at room temperature and used within 4-6 hours from collection.

The blood tubes were gently rocked before blood was pipetted from them and applied to the glass slides and accessible films. Blood from one of the tubes was used for analyses of heparin coated films and the second tube was used for the albumin coated films. Analysis of albumin coated films was performed shortly after the analysis of the heparin coated films.

Complement activation

Blood was collected into two Vacutainer blood collecting tubes (Becton, Dickinson and Company, 6 ml) without any additive. Immediately after collecting a solution of heparin was added to the blood and carefully mixed with the blood to give a final heparin concentration of 7.13 µg/ml ($\geq$ 1.5 USP). The blood tubes were stored at 37°C in the lab before usage and was used within 2-3 hours from collection.

Execution of coagulation tests

Test setup

120 µl of whole blood with citrate additive was applied to different surfaces on glass slides. Signs of coagulation were observed during the session and clots were evaluated when the session was finished.

The different surfaces were divided in two groups depending on which type of coating (heparin or albumin) investigated, as shown in figure 2. The heparin series were examined first, followed by the albumin series.

Surfaces tested in the heparin series:

Seven surfaces were evaluated in the heparin part of the study:

- 4RepCT + Heparin (film)
- 4RepCT (film)
- pLys-4RepCT + Heparin (film)
- pLys-4RepCT (film)
- pLys-coated glass + Heparin (negative control)
- pLys-coated glass
- Hydrophobic glass (positive control)

Surfaces tested in the albumin series:

Five surfaces were evaluated in the albumin part of the study:

- ABD-4RepCT + Albumin
- ABD-4RepCT
- 4RepCT + Albumin
- 4RepCT
- Hydrophobic glass (positive control)

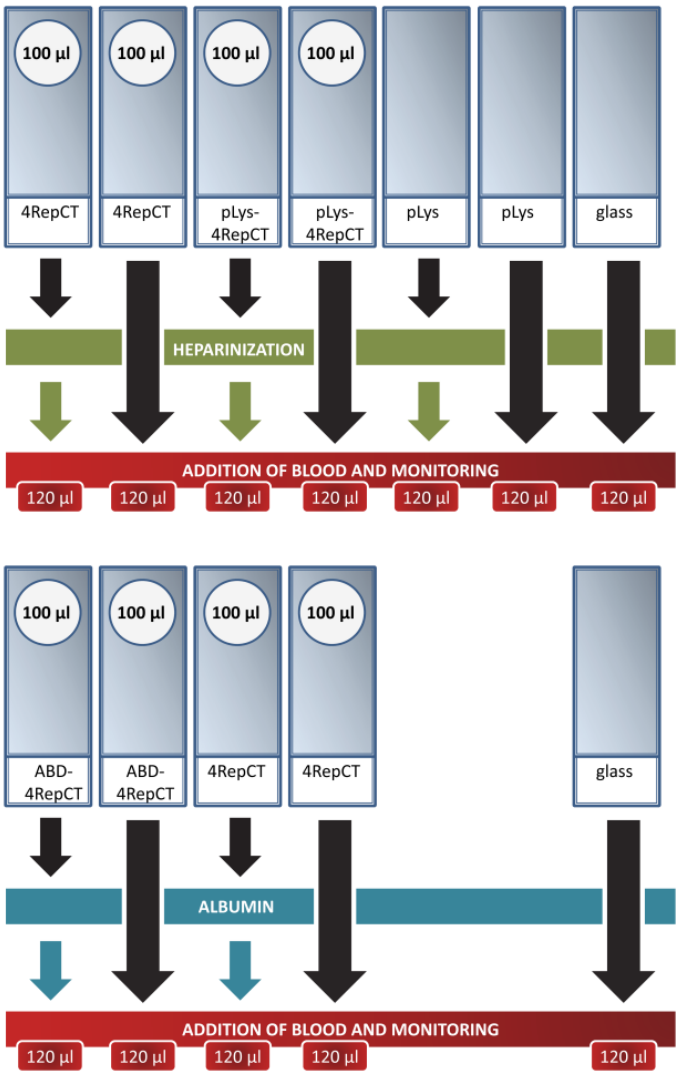

Figure 2. Setup of heparin (top) and albumin (bottom) series of coagulation tests.

Test environment and equipment

All test sessions were performed in a semi-sterile environment in a room separated from the ordinary lab to reduce risk of contamination. Solutions were either LPS-purified, filtered through 0.2 µm filter (Filtropur S 0.2, Sarstedt, 83.1826.001) or delivered sterile. Films were cast in a LAF bench and all pipette tips used were autoclaved or delivered pyrogen free.

During experiment sessions the LAF hood was inactive to reduce air flow and the glass slides were moved unrestrictedly during observation.

Wet paper towels and containers of hot (steamy) water placed in the proximity of the films and glass slides were used to raise the ambient humidity and thereby also to impede the drying of coatings during incubation, wash buffers during washing and blood drops during the actual experiment session. During the different experiment sessions the humidity varied between approximately 20-30 % (46% at most) and the ambient temperature was around 22-26 °C.

Monitoring coagulation and assessing final clots

The coagulation was reversibly inhibited by citrate, binding calcium. During coagulation tests, the coagulation was reactivated by addition of sterile filtered (0.2 µm) $CaCl_2$. 120 µl of blood was added to films recently covered with 10 µl of sterile filtered (0.2 µm) $CaCl_2$ solution (0.24 M), reaching a final concentration of 0.018 M of $CaCl_2$ in the added blood. The 0.24 M $CaCl_2$ solutions used were made from dilution of a recently prepared 1 M stock solution made of 1.1099 g $CaCl_2$ dissolved in 10 ml sterile filtered (0.2 µm) dH_2O.

When 120 µl blood had been applied to all films, changes in appearance of the blood were examined under a light source, repeatedly in a cycle-wise pattern. The analysis was performed until the clot formation and coagulation was believed to be clear enough for documentation and evaluation. During the first experiments it was concluded that a session length of 20 minutes was sufficient for both the heparin and albumin parts of the experiments.

Eight full sessions of the heparin series and six full sessions of the albumin series were performed (referred to as session A to F). Most sessions were run in single series where all slides were observed one by one. In session C and D the blood was applied to duplicates of the surfaces. The blood of one of the series in the current session was examined continuously one by one (C_1 and D_1). Both series (C_1, C_2, D_1 and D_2) were evaluated according to clot formation. Visual signs of coagulation were noted at each time point (data not shown).

At the decided end point the glass slides were carefully turn over and placed on paper towels to evaluate the blood clots. The size, thickness and shape of the blood clots formed were assessed visually and relatively compared to the other clots in the current experiment to make it possible to rank the formed clots between 0 (no clot) and 5 (massive clot). The clots were also documented on photographs.

Statistical analysis of the results from the coagulation tests

The results from the coagulation tests were compared to determine if there was any statistically significant difference between the tested surfaces. Statistical analyses were performed in Graphpad Prism 6 (GraphPad Software Inc., San Diego, California, USA). Surfaces with coating were compared with their uncoated control in a paired t-test, in which data from all successfully finished coagulation sessions was included. Coated surfaces were also compared to the positive control. Multiple factors were unique for each session and series (for example, temperature, humidity and the blood). For that reason, every session and series

were treated as individual and independent even though some sessions had more in common than others (for example, the heparin and base albumin solution). The confidence level was set to 95%.

Execution of the complement activation test

Test setup

Test setups and concept of the execution is summarized in figure 3. 800 µl of blood with heparin concentration of 7.13 µg/ml (≥ 1,5 USP) was applied to different surfaces of cut glass tubes. The following ten surfaces were included in each session:

- pLys-coated glass + Heparin (negative control)
- pLys-coated glass
- pLys-4RepCT + Heparin (film)
- pLys-4RepCT (film)
- 4RepCT + Heparin (film)
- 4RepCT (film)
- 4RepCT + Albumin (film)
- ABD-4RepCT + Albumin (film)
- ABD-4RepCT (film)
- Hydrophobic glass (positive control)

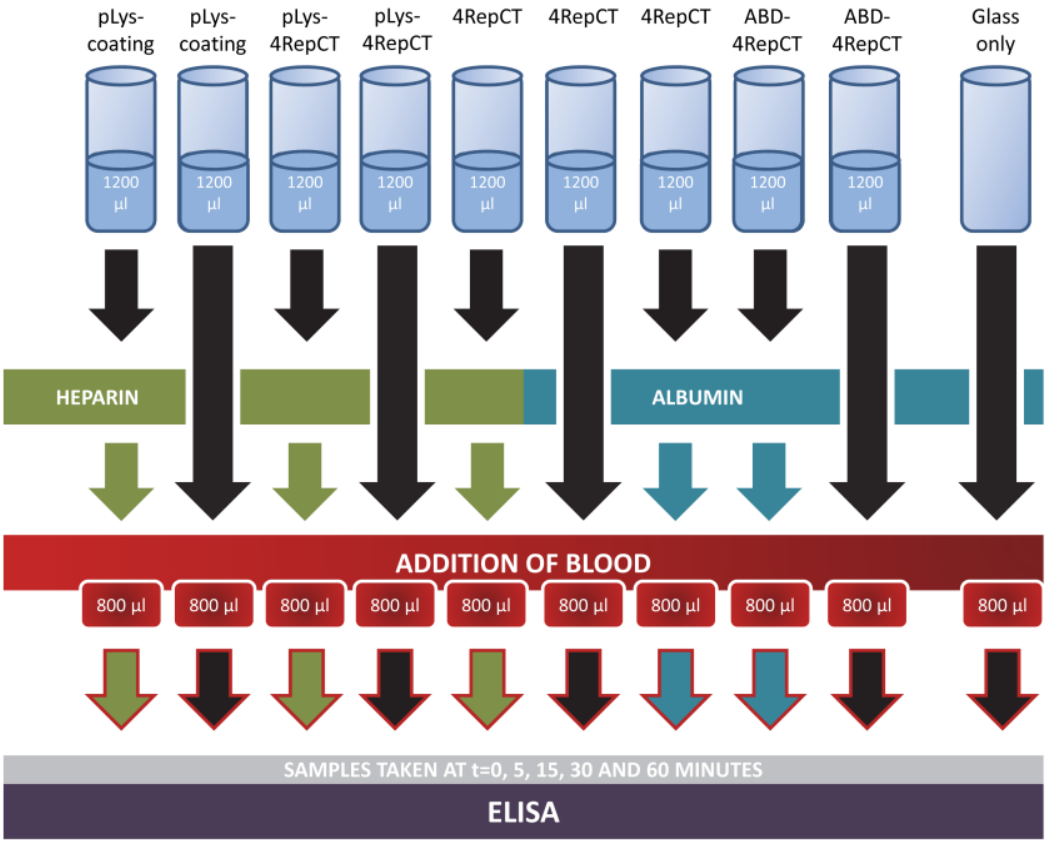

Figure 3. Setup and concept of execution of complement activation tests.

Collection of samples for complement activation analysis

Base level samples were taken directly from the blood collection tubes, and then 800 µl blood was added carefully to each glass tube with test surfaces. Thereafter the filled glass tubes were stored in 37°C. Samples (200 µl) were collected from each glass tube by careful pipetting after 5, 15, 30 and 60 minutes. The samples were transferred to 1.5 ml Eppendorf tubes on ice prepared with sterile filtered (0.2 µm) ethylenediaminetetraacetic acid EDTA solution (0.25 M, pH 7.4) to inhibit further activation of the complement system. For the first sample collection session, 2.7 µl EDTA was applied (resulting in concentration close to 1 g/l in the samples). During the second collection session, 5 µl EDTA was applied (resulting in a concentration close to 2 g/l in the samples). The samples were then centrifuged at 1000 x g for 15 minutes at 4°C. The supernatant (plasma) was transferred to new Eppendorf tubes on ice and stored in freezer (-70°C) awaiting ELISA analysis.

Analyzing and evaluating of the samples from the complement activation tests

C3a was selected as a biological indicator for complement activation. The complement activity was quantified using enzyme-linked immunosorbent assay (ELISA) technique to determine the concentration of C3a (C3a-desArg) in the samples (C3a ELISA, BD OptEIAHuman, 550499). The absorbance of the samples was measured at λ 450 nm (Tecan Sunrise-Basic, Tecan Austria GmbH, 16039400). For data collection, the software Magellan 6.5 (Tecan Austria GmbH) was used.

Two ELISA analysis were run to determine the complement activation of the samples. Samples from the positive controls (plain glass) were selected for initial analysis. The first ELISA was mainly performed to investigate how much the samples would have to be diluted to be analyzed. Both higher and lower dilutions than recommended (1:500) were used as specified in table 3. For the second ELISA analysis, all samples were diluted 5000 times with PBS (pH 7.4).

The concentration of C3a-desArg in the samples was calculated from standard curve equations, shown in figure 8. The standard curve was based on six samples with known concentration in ELISA 1. For ELISA 2, the standard curve was based on standard samples with the highest and lowest concentration to reduce the consumption of ELISA wells. The equations were generated by Microsoft Excel 2010 (Microsoft Corporation, Redmond, Washington, USA).

In the first ELISA analysis were samples from 5, 15, 30 and 60 minutes of the first complement activation tests session analyzed together with samples collected from the blood collecting tube prior to session start (base level samples). In the second ELISA analysis, samples from both complement activation test sessions were analyzed, but only base level samples and samples from blood that had been exposed to plain glass for 30 minutes were included in the analysis.

RESULTS

Within this study, the blood compatibility of films made of different functionalized recombinant spider silk proteins were investigated.

Expression and purification of recombinant spider silk

A recombinant spider silk protein functionalized with the albumin binding domain (ABD-4RepCT) was successfully expressed in *E. coli.* Two different procedures for cell disruption (in order to retrieve the intracellulary produced protein) were tried; one mechanical (high pressure homogenization - HPH) and one enzymatical (lysozyme and DNAse). The mechanical method resulted in a higher (mean value of 23%) yield of the target protein after subsequent purification (data not shown). Pooling of purified HPH-fractions with high concentrations only, resulted in a substantial gain in mean concentration of the finished protein solution (from 0.23 mg/ml to mean value of 1.31 mg/ml) without need for concentration processes. Gel analysis of samples from different batches showed pure proteins (data not shown).

Preparation of films

The purified spider silk proteins were used to prepare films on two different substrates, suitable for analysis of coagulation (glass slides) and complement activation (glass tubes), respectively. Further analysis of film properties were done on films on glass slides, due to the smaller sample volume needed and more facile analyses.

All protein solutions were sampled for gel electrophoresis prior casting of the films (see figure 4). The respective proteins showed excellent similarity between different castings, verifying that the tested surfaces were identical in all test sessions.

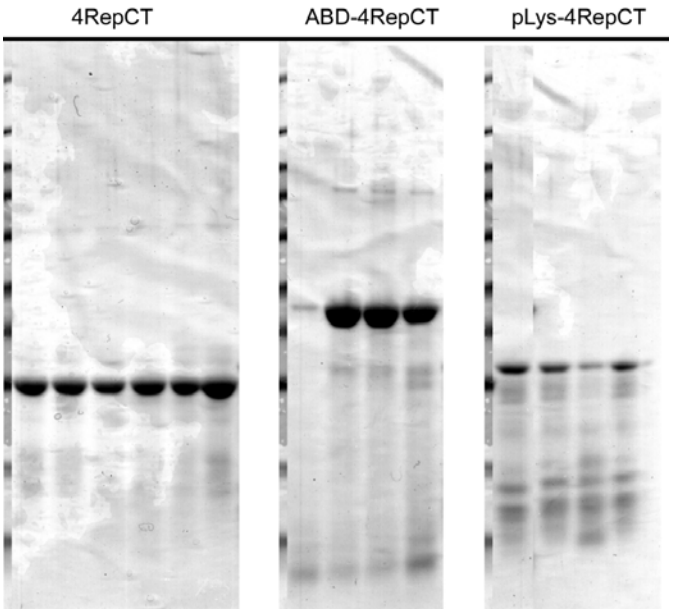

Figure 4. Gels of the recombinant spider silk proteins that the tested films were cast from. A size marker is included to the left of every group of proteins. All instances of respective proteins appeared to be largely identical.

Optimal size for films and blood volumes applied to hydrophobic glass slides

Based on rigorous studies on films of different sizes and several glass slides with different surface properties, an optimal size of 100 µl films made on hydrophobic glass slides was determined. These films had a volume-bearing capacity of 200-400 µl, before liquid floated out from the film surface. 110-130 µl liquids applied to films had a similar appearance (height and area) to equal volumes of liquid applied to plain hydrophobic glass (used as control). Films made on hydrophilic glass slides were fragile and unreliable, and therefore not used within this study.

Examination of the film integrity

In order to confirm that the films were maintained throughout the analyses, their integrity was investigated after a washing procedure that partly replicates the conditions that the films would be subjected to during analysis. All liquids were carefully applied to the films and only kept within the borders of the films. Subsequent staining with Coomassie blue (colors proteins blue) showed that the films did resist washing with phosphate buffer (data not shown).

A gently dipping and moving of the pipette tip in the applied wash buffer volume resulted in formation of fiber-like structures between the liquid and pipette tip during wash of some films on glass slides, mostly those made of ABD-4RepCT.

Staining of heparin with toluidine blue solution

Films and glass slides were stained to evaluate spontaneous heparin attachment to films of 4RepCT, pLys-4RepCT and glass slides coated with pLys. Generally, Toluidine staining protocols for staining of the heparin in mast cell granules require use of solutions with low pH. Since acids could be used to remove spider silk films from surfaces a protocol with neutral pH would be preferable because of reduced risk of film damage during staining. For this reason, we evaluated if a staining protocol with neutral pH would result in sufficient staining. During staining, films previously coated with heparin were more colored and grainy than films which had not been coated with heparin as described in figure 5 (data not shown). The difference in color intensity between non-heparinized controls and heparinized films was less apparent when the neutral staining solution was used compared to when the acidic solution was used (data not shown). However, as expected, low pH staining interfered more with the integrity of the films than neutral staining. No color was seen on the pLys-coated glass slides with and without heparin coating following the low pH protocol. Intensely colored dots were seen on one of the two pLys coated slides coated with heparin. Small dots could be seen on glass slides without heparin coating.

Figure 5. Schematic representation of the outcome when staining a film without coating and a heparin-coated film with Toluidine blue.

Coagulation of blood exposed to surfaces of interest

Blood was applied to different surfaces on glass slides. Signs of coagulation of blood that had been exposed to the surfaces of interest were observed visually to evaluate the blood compatibility of the surfaces. If any clot formation was seen it was evaluated and the clots were given a clot score on a five-grade scale as shown in table 1 and table 2. The score was based on thickness and size of the clot. Higher values represent higher activation and expected lower blood compatibility. The score system was relative and based on the other films in the current experiment session. Seven heparin sessions and six albumin sessions were performed and are referred to as session A to F.

Results from the heparin sessions

The results from the heparin sessions are summarized in table 1 and figure 6. Generally, films coated with heparin caused less clot formation than films without coating (see figure 6). This was seen in session A, B, F and to certain extent in session E. In session E, 4RepCT was given the same clot score as 4RepCT with heparin but pLys-4RepCT with heparin got a lower score than pLys-4RepCT without coating.

4RepCT with heparin had significantly better blood compatibility compared to uncoated 4RepCT (P: 0.0363) and the plain glass control (P: 0.0081). The difference between heparin-covered pLys-4RepCT and heparin-covered 4RepCT was not considered to be substantial even though heparin coated pLys-4RepCT got slightly lower total accumulated clot score than 4RepCT coated with heparin (table 1). Furthermore, no statistically significant difference could be observed comparing the heparin-coated pLys-4RepCT film with uncoated pLys-4RepCT (P: 0.0828). However, heparin-coated pLys4RepCT was still statistically significantly better than the hydrophobic glass (P: 0.0082).

pLys coated glass slides with heparin coating were selected as negative controls and was given the least clot score 0 in all sessions except from A, where a it was given a score of 1. Heparin coating improved the blood compatibility of the pLys coated glass slides (P: 0.0062 compared to uncoated pLys slide; P: 0.0105 compared to positive control), but it should be noted that the blood volumes on the control slides were lower than the volume used on films and hydrophobic glass slides in all sessions.

Session C and D consisted of duplicates from the same donor and the other sessions consisted of just single replicates. In session C and D almost no clot formation was seen when evaluating coagulation after 20-30 minutes. Very small micro clots could possibly be seen on a few glass slides and films in session C. In session D, larger but still very thin and fragile, clots were seen.

Table 1. Evaluation of coagulation extent ("clot scores") of tested surfaces from heparin session A-F, indicating size and thickness of the clots (higher values indicate thicker or larger clots)

Surface	Experiment session								Total
	A	B	C_1	C_2	D_1	D_2	E	F	
4RepCT + Heparin	2	0.5	0	0	0.5	0	2	2	7
4RepCT	4	2	0	0	1	0.5	2	3	12.5
pLys-4RepCT + Heparin	2	0.5	0	0.5	0	0	1	2	6
pLys-4RepCT	4	1.5	0	0	0	0	3	3	11.5
pLys coated glass + Heparin	1	0	0	0	0	0	0	0	1
pLys coated glass	3	3	0.5	0.5	0.5	0.5	2	1.5	11.5
Hydrophobic glass	5	3	0.5	0.5	0.5	1	4	5	19.5

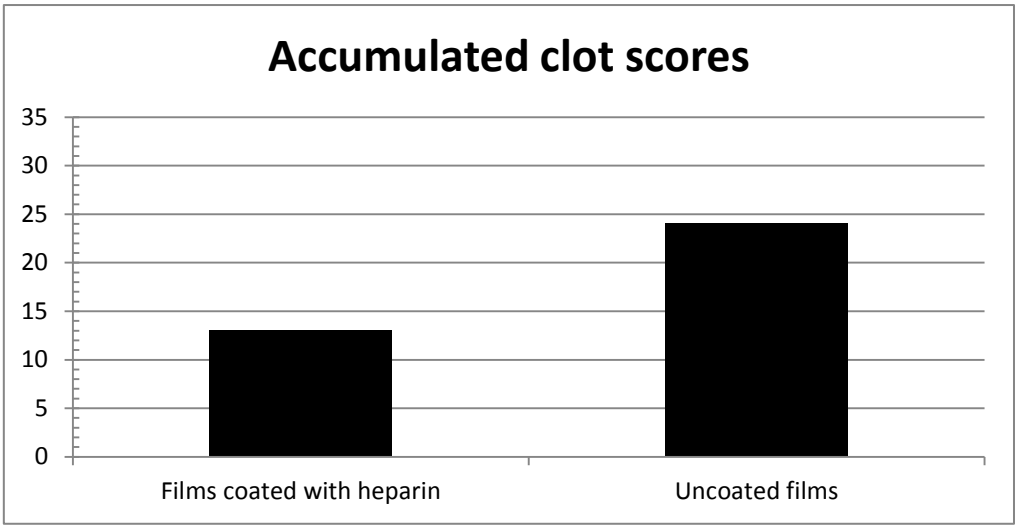

Figure 6. Accumulated clot scores from all heparin sessions. Films coated with heparin got lower clot scores than films without coating. Higher clot scores represent more coagulation.

Results from the albumin sessions

The results from the albumin sessions are summarized in table 2 and figure 7. Compared to uncoated ABD-4RepCT, coating with albumin on ABD-4RepCT films resulted in lower scores in 3 of 6 sessions (A, B and E), but slightly higher clot scores in 2 sessions (D_1 and D_2). In session F, coating ABD-4RepCT with albumin resulted in the same score as the positive control (hydrophobic glass) without any coating. Albumin coating of ABD-4RepCT did not result in a statistical significant difference compared to the uncoated ABD-4RepCT films (P: 0.8560) or the positive controls (P: 0.1152).

Coating of 4RepCT with albumin resulted in lower clot scores in all except one session (E). Consequently, there were also statistically significant differences between 4RepCT with and without albumin coating (P: 0.0066) as well as between the albumin coated 4RepCT and the positive controls (P: 0.0093). Generally, 4RepCT films got lower clot scores than ABD-4RepCT films. This was seen in all sessions except from session E.

Table 2. Evaluation of coagulation extent ("clot scores") of tested surfaces from albumin session A-F, indicating size and thickness of the clots (higher values indicate thicker or larger clots)

Surface	Experiment session							Total
	A	B	C	D1	D2	E	F	
ABD-4RepCT + Albumin	3	3	-	2.5	2.5	0.5	4	15.5
ABD-4RepCT	4	4	-	2	2	1.5	2.5	16
4RepCT + Albumin	2	1	-	2	1	2	1.5	9.5
4RepCT	3	2.5	-	3	2.5	2	2.5	15.5
Hydrophobic glass	2	4.5	-	4	4	3.5	4	22

ABD-4RepCT + Albumin was ruined during session A. The clot score recorded were taken from a reserve film of ABD-4RepCT (+Albumin) that was run when the other films of session A had been evaluated.

Session C was cancelled because of low reactivity of the tested blood.

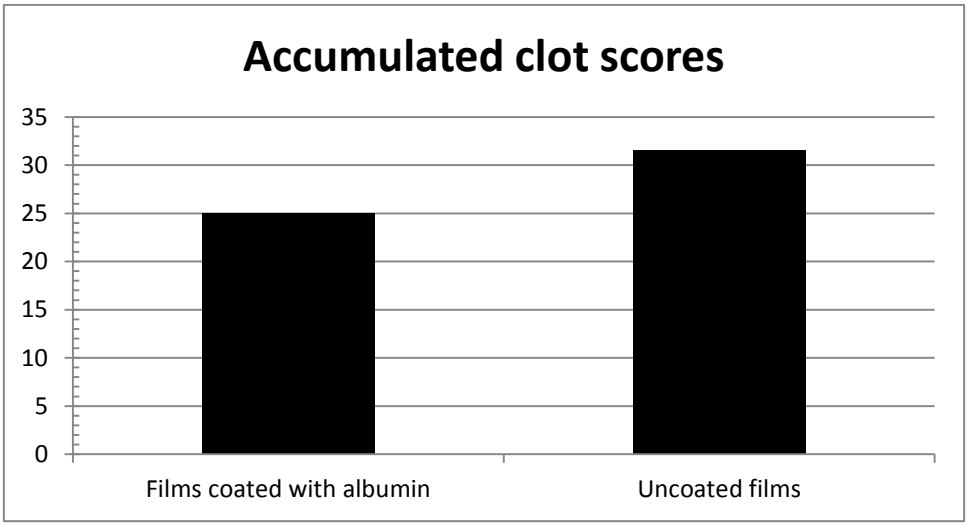

Figure 7. Accumulated clot scores from all albumin sessions. The difference between uncoated and coated films was small. Higher clot scores represent more coagulation.

Complement activation of blood exposed to surfaces of interest

Activation of the complement system was measured and quantified using ELISA analysis of samples from surface-exposed blood from two separate sessions (referred to as ELISA 1 and ELISA 2). The concentration of the complement mediator C3a in the form of C3a-desArg was selected as a biological indicator for complement activation. Samples from the surface-exposed blood and samples taken directly from the blood collection tube shortly before session start were compared to standard curves based on samples with known concentration of C3a-desArg included in the ELISA kit.

Results from ELISA 1

A standard curve was made from six samples with known concentrations of C3a-desArg ranging from 2.5 ng/ml to 0.08 ng/ml (shown in figure 8).

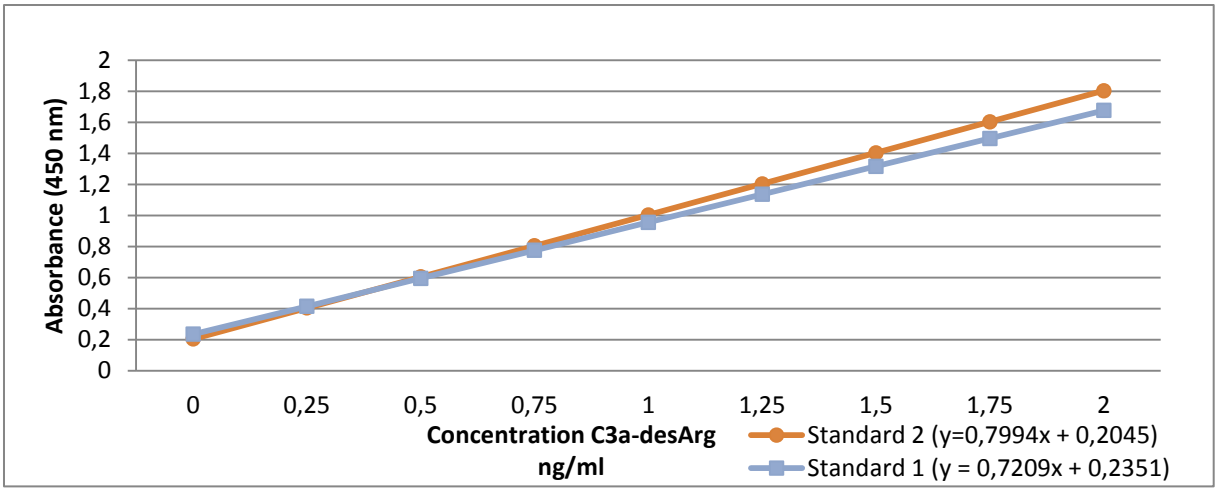

Figure 8. Graph of the standard curve equations from ELISA 1 (standard 1) and ELISA 2 (standard 2).Based on the graphs of the two standard curve equations, the standard curve made for the ELISA 2 which was based on two samples only, matches the six-point standard curve well.

Samples from plain glass from the first sample collection were selected to determine which sample dilution to use for the rest of the samples, as we expected highest levels of activation in these. As shown in table 3, the measured values from all samples except one were higher than the measured values from standard series samples. This only sample within the standard curve interval (30^1) was taken 30 minutes after the start of the session and was diluted 1000 times.

Table 3. Measured absorbance and calculated concentrations of C3a-desArg of samples analyzed in ELISA 1

Sample	Dilution factor	Absorbance (λ 450nm)	Concentration C3a (ng/ml)
0^1	500	3.589	2326
0^1	250	4.474	1470
0^1	100	5.939	791.2
5^1	500	3.835	2497
15^1	500	4.064	2656
30^1	500	4.005	2615
30^1	700	2.536	2234
30^1	1000	1.395*	1609
60^1	500	4.176	2733

Samples taken n minutes after introduction of blood to a plain glass surface
0^1- Reference (base level) samples taken direct from the blood collecting tubes
n^1- Samples from experiment session 1
* - within interval of standard curve (0.254-2.011)

Results from ELISA 2

A second ELISA was run in a new attempt to determine optimal sample dilution. A standard curve was made from the maximum (2.5 ng/ml) and minimum (0.08 ng/ml) standard samples and PBS to save wells in the ELISA plate. The standard curves from the first and second ELISA were comparable (figure 12). Samples from plain glass tubes from both the first and the second experiment were analyzed. The measured values of samples are shown in table 4 and were within the standard interval when diluted 5000 times.

Table 4. Measured, normalized absorbance and calculated concentrations of C3a-desArg in samples analyzed in ELISA 2.

Sample	Dilution factor	Absorbance (λ 450nm)	Normalized absorbance	Concentration (ng/ml)
0^1	5000	0.596	0.360	973
30^1	5000	0.527	0.291	541
0^2	5000	0.514	0.278	460
30^2	5000	0.478	0.242	235

Samples taken n minutes after introduction of blood to a plain glass surface
Normalized absorbance – Absorbance of PBS subtracted from samples
0^1 and 0^2 - Reference (base level) samples taken direct from the blood collecting tubes
n^1- Samples from experiment session 1
n^2- Samples from experiment 2

No significant differences were seen comparing base level samples taken straight from the blood tubes (0^1 and 0^2) with samples taken after 30 minutes (30^1 and 30^2) from plain glass. All measured values from samples showed high levels of complement activation, even the samples taken prior contact with the surfaces of interest (0^1 and 0^2). The other samples collected during the both complement activation experiments were expected to show similar activation and for that reason were not analyzed.

DISCUSSION

Coagulation test

The coagulation study was made in an attempt to investigate if the short-term blood compatibility of recombinant spider silk could be improved by adding coatings of heparin or albumin.

Heparin sessions

Although with exceptions and varying clot scores between sessions, one could summarize that blood contact with films coated with heparin was inducing lower degree of clot formation than films missing heparin coating (visualized in figure 6).

The difference between pLys-4RepCT films with and without heparin coating was not statistically significant but the difference between 4RepCT with and without heparin coating was. Despite this, it is not possible to certainly conclude which heparin-coated film that had the best blood compatibility due to the limitations of the used evaluation method and small actual differences during tests. It should also be pointed out that the standard deviation was quite substantial (figure 9).

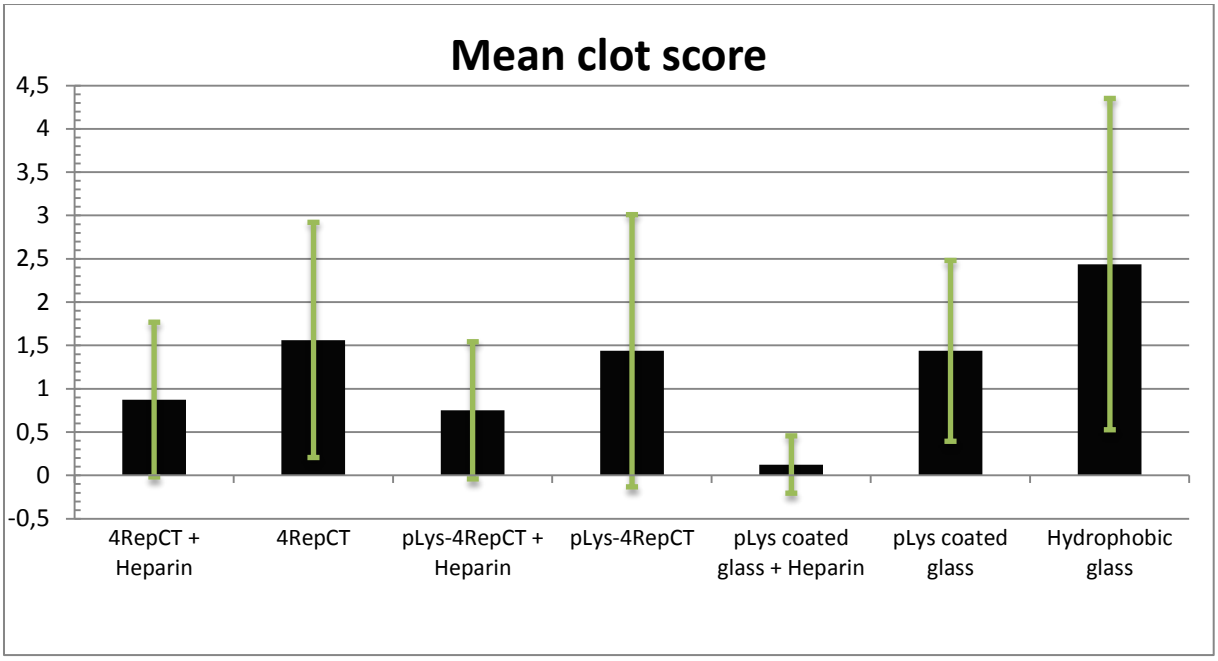

Figure 9. Mean values and standard deviation of clot scores for each surface in the heparin sessions.

Differences between different donors were expected due to the properties of blood as previously mentioned. However, differences were also present in the sessions with duplicates with blood from the same donor (C and D). This could for example be explained by the subjective nature of the visual assessment of coagulation, inevitable differences regarding how and when the blood and reagents were applied and different degrees of movement of the glass slides when observing coagulation upon the light source.

Even though heparin is probably one of the most extensively used and well-documented anticoagulant and blood compatibility enhancing substance used, there are still concerns. One concern regarding heparin is that its molecular length-dependent biological activity makes it difficult to determine which concentration to use in for example coating solutions. Furthermore, it is not known how spontaneous attachment of unfractionated heparin to recombinant spider silk films or pLys-coated glass affects its activity or availability. In addition to this, both endurance and resistance of the heparin bound to the investigated surfaces are unknown. Since biomaterials need to be reliable and safe, this has to be studied in greater detail if intentions are to use films coated this way in medical applications. Staining of washed heparin-coated films suggested that there was heparin left on the films but it is not known if parts of any heparin are removed during the process. When staining heparin on

pLys-coated glass slides, almost nothing was colored. The absence of colored heparin probably shows that the pLys-coated glass, heparin and the acidic staining solution were incompatible since the results from the coagulation tests suggested that the heparin coating was maintained and effective during sessions.

Albumin sessions

Albumin is the most abundant protein in blood. If one could utilize autologous or non-autologous albumin to cover and "hide" biomaterials from detection by the complement system, one could expect that such a material would have superior blood compatibility.

Albumin coatings could improve multiple properties, including the blood compatibility, of biomaterials according to several authors (Kottke-Marchant et al., 1989; de Queiroz et al., 1997; Bos et al., 1999; Kinnari et al., 2001; Kinnari et al., 2003; Kinnari et al., 2004; Ratner et al., 2004; Baican et al., 2011).

However, this was not clearly seen in our tests. The effect of coating films with albumin resulted in both higher clot scores (prominent coagulation) and lower scores compared to uncoated films. The results varied quite much (see table 2 and figure 10) and compared to the results from the heparin studies, the overall results from the albumin sessions were less promising and less predictable (compare figure 6 with figure 7). We believe that these inconsequent and varied results were caused mainly by other factors than the evaluation method and discrepancies in assessment between sessions.

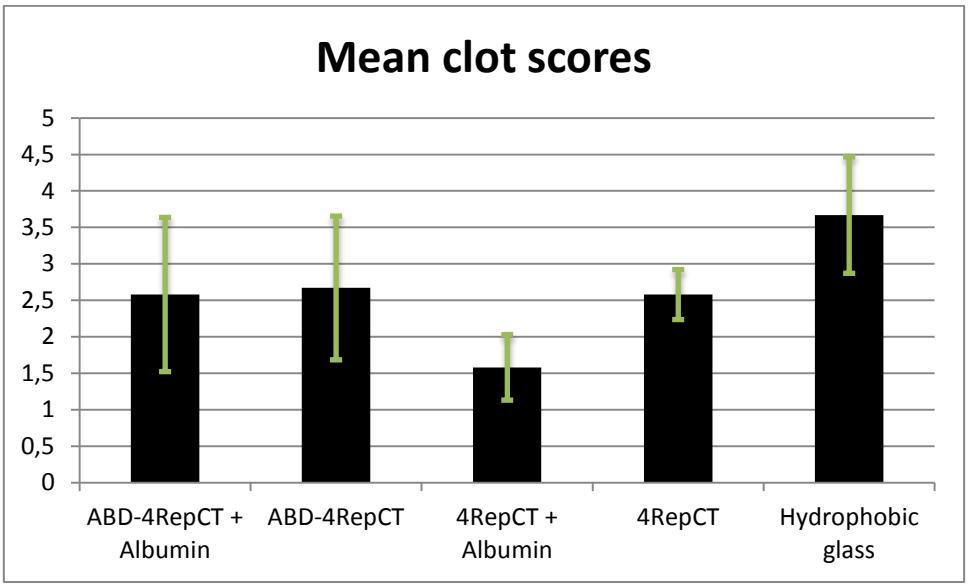

Figure 10. Mean values and standard deviation of clot scores for each surface in the albumin sessions.

Albumin is a protein that interacts with many substances and particles, including both physiological molecules and drugs (Grubb & Hansson, 2012). Biomaterial-bound albumin may interact with and activate the complement system, according to reports from Hong et al.

(1999) and Nilsson et al. (2007). Even though not investigated, it is possible that such interactions could be one of the reasons behind our varied results.

ABD-4RepCT, functionalized to bind albumin, generally got higher clot scores, indicating worse blood compatibility compared to 4RepCT. 4RepCT with albumin had slightly lower clot scores compared to 4RepCT without additional albumin in 4 of 6 sessions. The difference between 4RepCT and albumin coated 4RepCT was statistically significant but no statistically significant difference was calculated between ABD-4RepCT with and without the albumin coating. This suggests that albumin coating of 4RepCT is better than albumin coating of ABD-4RepCT. However, the observed differences were mainly small and indistinct and one should not make too broad conclusions from these results. Theoretically, this could for example be due to unspecific interactions to ABD-4RepCT-bound albumin and blood, or interactions caused by faulty folded albumin bound to ABD-4RepCT and the coagulation or complement system.

It is possible that ABD-4RepCT would be able to bind albumin from blood, thereby possibly improving blood compatibility and reducing risk of non-self reactions to foreign (pre coating) albumin. However, this cannot be concluded from our results since ABD-4RepCT films without a pre-coating of albumin had worse (higher) clot scores in 4 out of 6 sessions compared to ABD-4RepCT films that were pre-coated with non-autologous albumin.

The binding of albumin to ABD-4RepCT is likely more dependent on time than concentration according to previous results (data not shown). Our albumin solution had a concentration of 25 g/L which is lower than the physiological serum albumin concentration of blood (34-46 g/L) (Grubb & Hansson, 2012). It should be noted that the reference values for serum albumin concentration vary depending on the analyzing lab, age and gender (Grubb & Hansson, 2012) but the concentration of our solution was calculated to be available in great excess (data not shown).

Complement activation

The C3a ELISA was run as an attempt to objectively quantify the impact and blood compatibility of recombinant spider silk, more specifically investigating the activation of the complement system and its signal substances, represented by C3a. Unfortunately, the C3a-activation was much higher than expected in all investigated samples, including the reference samples taken directly from the blood collecting tubes.

It was necessary to dilute all the samples ten times more (1:5000) than the standard suggestion dilution (1:500 times) to perform reliable measurements. Although the blood reactivity was expected to be individual and the ELISA protocol used was originally designed for evaluation of C3a concentrations in human blood rather than blood compatibility testing, this suggests a high activation prior contact with the recombinant spider.

We expected the complement activation to rise with time, following patterns similar to those reported by others (Weber et al., 2002; Johansson et al., 2005). Surprisingly, when the samples were analyzed we observed higher activation in the reference samples taken directly

from the blood collection tubes than in the samples collected after 30 minutes on films. The differences between samples were considered non-significant and believed to be a result of amplification of small differences and noise rather than any actual differences relevant for the blood compatibility. Together with the high levels of activity of the base level samples it was impossible to come to any reliable conclusions regarding the complement activation.

Body-biomaterial interactions and biomaterial-body interactions

Short- and long term biocompatibility

It is not only interesting how the material influences the physiological environment. It is also of interest to know how the physiological conditions and physical forces alter the material. In this study the focus was mainly on short-term reactions upon short and/or initial exposure of the material to blood rather than long-term reactions. These brief contacts may be similar to materials and objects used during surgery, but if the material is to be used in or on implants like stents, one need to study the blood compatibility of the material both during long as well as short periods of time.

Film integrity during washing

Resistance of the coatings of recombinant spider silk has not in detail been investigated in this study. However, films on glass slides were stained to visually prove that they could successfully resist careful washing with phosphate buffer.

During washing some of the films appeared to be forming fiber-like structures between the pipette tip and wash buffer solution. This implies that proteins from the films were released in the wash buffer. However, it has not been fully investigated if this was just excess proteins that were not actually bound to the surface of the glass slides or if it was essential parts of the films. It is also unknown how big proportions of the films which were removed during each washing step. Films were washed at least three times prior use. If washes are found to cause actual loss of integrity of the films, one has to investigate the impact on blood compatibility of the (potentially damaged) film coating the object, the object itself and the effect of the released proteins in vivo. In addition to this, it would be of interest to evaluate if different concentrations of films result in different film- and blood compatibility properties.

Coomassie staining and binding of proteins to films

During washing of the glass tubes in the second ELISA session there were signs of flakes of films loosening from the glass. To investigate the integrity of the films after the experiment they were stained following the same protocol used to investigate the integrity of films on glass slides. Staining showed no obvious flaws in the films resembling previous concerns of integrity but several scratch marks in the films were evident. Most of these were believed to have been introduced during the removal of blood and wash prior staining. Some of the scratches could have been introduced as a result of accidental touching of the films with the pipette tips during sample collection. The impact of such events on complement activation is not known.

Proteins in glass tubes coated with pLys were colored less intense compared to glass tubes coated with films of recombinant spider silk. There are several possible explanations for this which would need to be investigated further. Even though pLys-coating indeed consists of peptides and one could suspect that the less intense staining is due to that the pLys-coating is thinner (and/or contains less molecules available for staining) than the spider silk films, other explanations are still possible. For example, one could propose that proteins from the blood adhered to the films and pLys-coating. According to this, the higher stain intensity on spider silk films could be explained as that more proteins were absorbed onto the spider silk films compared to pLys-coated glass.

Staining of the films and coatings prior introduction of blood could maybe clarify if the staining is due to bound plasma proteins or the coatings/films themselves. However, Coomassie staining is a non-specific and qualitative method only showing presence of alkaline amino acids lacking objective and quantitative interpretation. The actual quantity of bound protein and occurrence of different binding properties of different variants of spider silks, if any, has for this reason not been investigated. Since the composition of absorbed proteins is of high importance for complement activation and blood compatibility (Wilson et al., 2005; Widhe et al., 2012; Nilsson et al., 2007) it would be of interest to study, quantify and compare the interaction and protein binding properties of different coatings and films of spider silk in detail with other methods in future studies.

Sources of error and suggested improvements

Coagulation

Imprecise evaluation method

The coagulation study could be improved by adding more objective and quantitative evaluation tools with higher precision. The coagulation was evaluated visually and formed clots were difficult do document and save in a representative manner. Naturally, bigger and thicker clots were easier to distinguish and give clot scores than thin and fragmented clots, possibly exaggerating higher scores and missing small reactions. In addition to this, previous results probably caused the interpretation of the latter sessions to be biased, resulting in lower clot scores. Even though there actually were statistically significant differences between some of the samples, one should note that the actual visual experience of the differences not always was as clear as the apparent outcome of the statistical analysis. To summarize, a setup enabling objective and quantitative analysis of the coagulation would be preferred.

Controls not matching samples during heparin sessions

During the heparin sessions, it was impossible to match the appearance (height and area) of blood added to the two pLys-coated glass slides with the appearance of the blood on films or hydrophobic glass slides (the positive control). Even though a lower volume of blood was added to these slides it spread over a much wider area. The volume and concentration of $CaCl_2$ was not adjusted, causing a higher and varying end concentration in the blood. This reduces the relevance of the slides as controls, and should be taken into account when comparing the results with other slides. For future research, this could maybe be overcome by using glass slides with pre formed wells, removing the need for hydrophobic enhancement and increasing conformity of the surfaces investigated according to size and shape.

Older blood was used for albumin sessions

Albumin sessions were always performed when the corresponding heparin session had been finished, sometimes more than 4 hours after blood collection. The temperature of the blood was not controlled or measured. A shorter time period between blood collection and experiment start would be better. Even though we observed normal coagulation, we cannot guarantee that one would get identical results if tests had been performed earlier. For example it is possible that components of the blood (including albumin and coagulation factors) change or degrade with time and interfere with final results (see below).

Complement activation

Since blood components can be very reactive to circumstances needed during the tests, like volume-area-ratio during pipetting (Jung et al., 2013), it was anticipated that the highly reactive complement system would inevitably be activated by pipetting, manual handling and storage. However, we did not expect such to cause as high activation as observed, and we rather believe that the activation observed already at time point zero was of another origin. Since the standard curves of both ELISA 1 and ELISA 2 were comparable we assume that the results from ELISA analyzes are reliable.

The concentration of EDTA in standard EDTA blood collecting tubes (Becton, Dickinson and Company) is said to be 1.8 mg/ml. We used a blood concentration of 1-2 mg/ml EDTA during the complement activation sessions. Since no evident differences were seen between samples from the first session where a concentration of 1 mg/ml was used and samples from the second session, where a concentration close to 2 mg/ml was used, we think that the high C3 levels were caused by another factor.

A possibility is that something was wrong with our heparin-solution. Use of standard tubes with pre-added heparin (with activity around 15-20 IU/ml) was expected to totally inhibit complement activation. For that reason, we prepared a solution with a concentration with an calculated activity of 1.5 USP/ml. Heparin solutions with concentrations in a range from 0.5 to 3 IU have been used in similar investigations of complement activation (Hong et al., 1999; Weber et al., 2002; Nilsson et al., 2007; Sieb et al., 2012). Unfortunately, our solution was not as effective as desired.

Because of absent visual signs of coagulation during the sessions, we assume that the heparin solution was sufficiently distributed in the blood collection tube and that the concentration of the heparin solution was high enough to inhibit coagulation. Although coagulation and complement activation has been described to have connected reactions and common way of actions, it has also previously been reported that inhibition of complement activation requires higher concentration than coagulation inhibition (Gong et al.,1996). We now believe that the concentration or activity of the heparin was too low to inhibit the pre-activation of the complement system sufficiently to make it possible to measure any differences between samples in our study. The complement activation during the 2 hour period from blood collection to test start was too high.

During the first ELISA experiment the heparin solution was added within approximately 10-20 seconds after blood collection and in the second ELISA experiment it was added approximately after 30-60 seconds. In other studies investigating complement activation of foreign materials, the main activation has been seen after a couple of minutes or even longer periods rather than instantly (Hong et al., 1999; Weber et al., 2002; Johansson et al., 2005). We assume that blood collection tubes are designed to reduce unwanted blood activation even though the complement is known to be highly reactive. In addition to this, samples from both the first and second experiments were similar. For these reasons it seems less likely that the complement activation was caused during the time before heparin was added to the blood. Rather, we believe that the blood was activated during the transport or storage prior to the actual experiments. To quantify this unwanted activation one could collect blood in a tube with EDTA additive in addition to the heparin tubes to get a true value of the base level of activation.

Gaining more knowledge about the blood

Collection of blood and individual differences

First of all, blood is a complex fluid with a wide range of dynamic properties that likely are influenced both by intrinsic as well as extrinsic factors acting on the individual. There are differences between sexes in blood composition (Sjaastad et al., 2003; Grubb & Hansson, 2012). Examples of intrinsic factors are biological variations between individuals regarding natural number of inflammatory and other blood cells, thrombocytes, enzymatic activity, mineral balance and hydration status. Medical drugs, chemicals from the environment and infections are examples of extrinsic factors which could possibly affect the properties of blood. Other extrinsic factors depend on the way the blood is collected and treated after collection. For example, Grubb & Hansson (2012) claims that the concentration of albumin in the blood could increase by 10% if the blood is collected from a standing individual due to hydrodynamic transitions.

Unwanted activation of platelets and inhibition of blood activation

Platelets can be activated by many factors (Gorbet & Sefton, 2004; Li & Henry, 2011) including events that may occur during the collection of blood. Efforts must be made to reduce collection-induced activation (Seyfert et al., 2002). Tourniquets used during blood collection change the blood flow as does the introduction of foreign objects like venous catheters into the blood vessels, interfering with the normal laminar blood flow and interaction with the non-thrombogenic endothelial surface of the blood vessels (Gorbet & Sefton, 2004; McGavin & Zachary, 2007)

The material of, and additives added to, the collecting tubes can be selected to reduce further activation and improve the conservation of the blood after collection. However, the blood should be used in tests within a minimal delay since some properties of blood change with time (Seyfert et al., 2002) due to biological processes and degradation. For example, the reactivity of platelets diminishes quickly, having a reduced agonist-response within just 60 minutes after collection (Jung et al., 2013).

In the current study, the blood was used within approximately 2-6 hours from collection. This interval was considered to be acceptable based on the aim and execution of the current study. However, for future studies it would be preferable to emphasize methods where experiments are performed within a shorter and more well-defined time interval to get more reliable results.

Avoiding misinterpretations by exploring critical blood parameters by blood analyzes

Although not expected to be essential for this study, one could suggest that it is better the more you know about the blood used and the conditions during collection to get maximal yield out of the results from blood compatibility studies. In addition to documentation of the personal details of the donor, a full analysis of the donor's blood could be performed. This way one could discover any correlation regarding how the blood reacts and for example sex, age, blood group or any other intrinsic or extrinsic variables of interest.

Enhancing physiological resemblance in vitro

Although the testing initially has to be done in vitro, it must be emphasized that the blood compatibility testing should be carried out under optimal conditions, resembling a physiological environment as much as possible (Seyfert et al., 2002). Any compromises from the natural conditions have to be carefully considered when interpreting the results before making decisions of how and if to proceed to in vivo testing.

Temperature of the blood

In the current study we had to compromise several physiological circumstances due to practical reasons. For example, the temperature of the blood was not monitored and allowed to adapt to ambient temperature during the coagulation tests. The ambient temperature varied during different sessions but was always substantially lower than 37°C. It is likely that some reactions are temperature-dependent and that the low temperature affected the outcome of the study even though it is hard to evaluate.

We expected blood coagulation to occur within minutes of exposure to the surfaces during the coagulation sessions. Surprisingly, we did not observe any blood clots until after 20-30 minutes. During session C almost no coagulation was seen, not even on our supposed positive controls (plain hydrophobic glass). Lower activity of enzymes due to below-physiological temperature could maybe partly explain that the coagulation processes were slower but does not fully explain the abundance of coagulation during some sessions. It is likely that more thorough blood analyses would be of high value to better understand events like this.

Mimicking blood flow

Blood contains three main types of cells. As long as blood is flowing through the smooth and nonthrombogenic blood vessels, the cells are relatively evenly distributed in the plasma (Sjaastad et al., 2003; Rang et al., 2007). Changes in the blood flow affects the rate of coagulation cascade (Gorbet & Sefton, 2004) and a dynamic model have been said to be important for biomaterial testing since biomaterials contact blood mainly under flow conditions in vivo (Jung et al., 2013). If the blood is kept in a test tube and blood is prevented from coagulating, the distribution will change. If a blood sample is left alone, the plasma will be on top, resting on a layer of blood platelets and leukocytes, with the erythrocytes on the bottom (Sjaastad et al., 2003).

Motion of glass slides during visual evaluation of coagulation

Since blood was applied to films in form of drops in the coagulation tests, and then monitored, the blood flow was not physiological. During the cycle-like monitoring of coagulation, the glass slides were moved over a light source. There was no way of measuring how every single slide was moved but the glass slides were likely moved in different ways. Moving the slides may, inevitably, have caused motions in the blood volumes. This ripple may have resulted in introduction of more blood to the surface or mixing of the blood components. Mixing of activated blood and non-activated blood could possibly impact the pattern, the rate and level of coagulation reactions caused by the foreign material. However, it is not known how great impact – if any – this movement has on the activation of coagulation and actual outcome of the results. Differences were observed even with experiments on blood from the same donor (session C and D). Even though these differences could be caused by previously described imprecise visual evaluation we do not know if the slight differences observed could also have been a result of some slides being moved more than others. A more standardized setup where all samples are treated identically and where physiological blood flow is mimicked would be preferred.

Sedimentation during the ELISA tests

During the ELISA tests we observed signs of sedimentation within the blood volumes added into the glass tubes. The impact this had on the results was not investigated. However, it is likely that separation and sedimentation occurring within the blood volumes alters the distribution of important cells as platelets and erythrocytes as well as the distribution of proteins and other substances. Distribution changes like this will most likely affect the behavior and activation of the blood, including the complement system and coagulation cascade. Distribution changes would without doubt make the in vitro results less representative for in vivo blood compatibility and make result interpretation more demanding.

It is possible that the results would have been more representative if a setup of spider silk films and pLys coatings in rotating tubes, similar to previously described setups for blood compatibility testing (Seyfert et al., 2002; Weber et al., 2002; Nilson et al., 2007; Lappegård et al., 2008), had been used. If the tubes had been placed rotating in a water bath with a temperature of 37°C, both natural blood flow as well as physiological temperature would have been mimicked. On the other hand, one can expect that excess or careless movement could activate blood as well. In the current study, manipulation of the blood was minimized and the equipment was selected to evoke as little reactions as possible. Efforts were made to treat all samples equal so that blood activation would be caused by the materials of interest rather than handling. This way, it would be possible to distinguish the reactions caused by materials from background activity inevitably induced during the tests.

In addition, it would be advantageous with a more closed system than those used in our setup. A closed system reduces the risk of contamination from particles in the air and accidental contact with foreign materials other than those which are to be studied, including air-induced complement activation discussed by Nilsson et al. (2007).

CONCLUSIONS

This study introduces a non-toxic and easy performed method of improving the blood compatibility of recombinant spider silk. Adding a heparin coating onto films of spider silk reduces activation of coagulation and formation of blood clots.

Heparin coated surfaces had statistically significantly higher blood compatibility than uncoated surfaces. The effects of addition of albumin to the films were not clear enough to draw any final conclusion from these studies.

The blood compatibility results from this study are based on subjective evaluation by vision. It would be preferable to use objective methods for quantitative evaluation of coagulation and complement activation caused by recombinant spider silk.

The impact of spider silk on the complement system could not be evaluated due to high base level activity. More studies have to be performed to evaluate and quantify the impact of recombinant spider silk on the complement and coagulation system.

There is still a lot of exciting studies and promising opportunities of research left to be done to better understand the blood compatibility of recombinant spider silk. Future research will hopefully be able to show how to turn recombinant spider silk into a reliable biomaterial.

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