Diese Dissertation haben begutachtet:

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DISSERTATION

Characterization of potentially immunogenic properties of a novel PEGylated FVIII preparation

ausgeführt zum Zwecke der Erlangung des akademischen Grades eines Doktors der Naturwissenschaften unter der Leitung von

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Kurzfassung

Hämophilie A ist eine erblich bedingte Blutgerinnungsstörung, bei der es durch ein Fehlen von funktionellem Gerinnungsfaktor VIII (FVIII) zu einem Defekt in der Koagulationskaskade kommt. Die derzeitige Therapie besteht in der Substitution des fehlenden Gerinnungsfaktors mit plasmatischen oder rekombinanten FVIII Konzentraten. BAX 855 ist ein chemisch modifiziertes FVIII Präparat, das durch eine verlängerte Halbwertszeit eine reduzierte Behandlungsfrequenz ermöglicht. Eine der schwersten Komplikationen im Patienten ist die Entstehung von neutralisierenden Antikörpern gegen FVIII, welche die Therapie unwirksam machen. Da der zugrundeliegende Mechanismus noch nicht entschlüsselt worden ist, muss BAX 855, wie alle neuen FVIII Präparate, im Zuge der präklinischen und klinischen Entwicklung ausführlich auf die Immunogenität getestet werden.

In der vorliegenden Thesis wurden verschieden Aspekte zur möglichen Induktion einer Immunantwort von BAX 855 im Patienten beleuchtet. Der erste Teil dieser Arbeit beschäftigt sich mit der potentiellen Immunogenität von Proteinaggregaten. Dabei wurden die Quantität und Qualität dieser Proteinaggregate von BAX 855 und ADVATE™ mittels einer neu etablierten durchflusszytometrischen Methode verglichen. ADVATE™ ist das Basismolekül für BAX 855 mit einer langjährigen, therapeutischen Sicherheit. In unserer Studie war kein Unterschied zwischen beiden Präparaten festzustellen. Dadurch ist zu erwarten, dass ein möglicher Einfluss von Proteinaggregaten auf die Immunogenität für BAX 855 und ADVATE™ vergleichbar groß ist. Der zweite Teil dieser Arbeit beschäftigt sich mit der potentiellen Immunogenität von Polyethylenglykol (PEG), einem Bestandteil von BAX 855. Unsere Resultate zeigen, dass ein Teil der gesunden Bevölkerung IgG und IgM Antikörper entwickeln, die aber keine offensichtliche Symptomatik verursachen. Außerdem deuten unsere Daten darauf hin, dass PEG an ein immunogenes Protein gekoppelt sein muss, um eine Immunantwort auszulösen. Daher würde man in jenen Patienten, die mit BAX 855 behandelt werden, die Entstehung von PEG Antikörpern erwarten, die wiederum eine Immunantwort gegen FVIII entwickeln. Unsere Erfahrungen mit PEG Antikörpern in gesunden Spendern sowie weiterführende Studien, die gezeigt haben, dass diese Antikörper nicht mit endogenen Antigenen kreuzreagieren, weisen aber auf ein vernachlässigbares Risiko hin. Zusammenfassend zeigen die gefundenen Daten, dass für BAX 855 ein vergleichbares Sicherheitsprofil wie für ADVATE™ im Patienten zu erwarten ist.

1. Abstract

Hemophilia A is a congenital bleeding disorder characterized by the lack or insufficient amount of functional factor VIII (FVIII) leading to a defect in the coagulation cascade. Patients are currently treated with a replacement therapy using plasma-derived or recombinant FVIII. BAX 855 is a novel PEGylated FVIII preparation with a prolonged half-life of FVIII in the circulation of patients. The formation of neutralizing antibodies against FVIII, so called FVIII inhibitors, remains the major challenge in current hemophilia A care. Why some patients develop FVIII inhibitors while others do not is far from being understood. Therefore it is important that BAX 855, like all novel FVIII preparations, need to be tested for its immunogenic potential during non-clinical and clinical development.

In the present thesis we established suitable technologies to address different aspects of the potential of BAX 855 to induce unwanted immune responses and to analyze different components of the immunogenicity of BAX 855. In the first part we focused on sub-visible particles, which were recently linked to enhance the immunogenicity of protein therapeutics. We established a flow-cytometry-based approach for the analysis of sub-visible particles and compared the content and quality of sub-visible particles within BAX 855 and ADVATE™, the base molecule of BAX 855 with a proven safety record. In our study we could not identify any differences between both molecules and concluded that the potential impact of sub-visible particles on the immunogenicity is expected to be similar for both, BAX 855 and ADVATE™. In the second part we investigated the potential antibody responses against polyethylene glycol (PEG), which is a major compound of BAX 855, and the possible mechanisms on their induction. Our study indicates a development of IgM and IgG antibodies against PEG in healthy individuals without any pathology. The induction of anti-PEG antibodies seems to require the attachment of PEG to immunogenic peptides or proteins. Therefore, we expect that patients who recognize the protein part of BAX 855 as immunogenic protein are at risk to develop antibodies against PEG. Our experience with anti-PEG antibodies in healthy individuals and the negative outcome of a tissue cross-reactivity study however indicate that these anti-PEG antibodies are unlikely to be of any safety concern. Based on the findings in this thesis, BAX 855 can be expected to have a safety profile in patients similar to that of ADVATE™.

2. Introduction

2.1 Hemostasis

Hemostasis is the physiological process that keeps the blood fluid and prevents blood loss upon injury by combined activity of vasculature, platelet, and plasma factors. The immediate response after trauma is local vasoconstriction of the damaged blood vessel to minimize the blood loss. Vasoconstrictors are released from surrounding nerve endings (neurogenic reflex vasoconstriction) and damage of smooth muscle cells lead to their contraction (myogenic vasoconstriction). In addition, injured endothelial cells secrete factors like endothelin which further enhance this process [1].

2.1.1 Primary hemostasis

Healthy endothelial cells promote blood fluidity by secreting several factors like nitric oxide, prostacyclin and ADP phosphatase that prevent binding and activation of platelets. The disruption of the vascular endothelium leads to the absence of these mediators and subsequently to the adhesion of platelets to the now exposed extra cellular matrix (ECM). Initially, platelets bind to von Willebrand's factor (VWF) that is released into the subendothelium from damaged endothelial cells [2,3]. Binding of VWF via the platelet specific membrane glycoprotein Ib (GPIb) stimulate the activation of platelets which leads to the assembly of glycoproteins IIb and IIIa on the platelet surface membrane. Fibrinogen binds to the glycoprotein IIb/IIIa complexes of adjacent platelets, connecting them into aggregates. Furthermore, platelets undergo degranulation upon activation and release among others adenosindiphosphate (ADP), thromboxin A2, serotonin, fibrinogen, coagulation factors, VWF and calcium. Some of these factors cause further vasoconstriction, platelet activation and aggregation as well as attraction of more platelets to the area of injury, promoting the formation of a platelet plug called primary hemostatic plug. Calcium is essential for the activation of several plasma factors within the coagulation cascade, marking the final step in transforming liquid blood into a blood clot [4].

2.1.2 Secondary hemostasis

The primary hemostatic plug formed by platelets is relatively unstable and needs to be supported by a fibrin network. Platelets provide surfaces for the assembly and activation of

blood coagulation factors resulting in the generation of thrombin at the end of the pathway. Thrombin converts soluble fibrinogen to fibrin. Fibrin strands bind aggregated platelets thereby forming a stable clot called secondary hemostatic plug. The coagulation system is a proteolytic cascade with more than a dozen different coagulation factors involved. The clotting proteins consist of several enzymes that are present in the blood in their inactive form (zymogen). Depending on their activation trigger there are two different pathways in the coagulation cascade: the intrinsic and extrinsic pathway, which converge on a single common pathway that forms the stable fibrin clot (Figure 1).

2.1.2.1 The intrinsic coagulation pathway

The intrinsic coagulation pathway, also called contact activation pathway, can be initiated by contact of blood with a negatively charged surface like glass or collagen on the exposed surface of an injured blood vessel. In the first step factor XII (XII), prekallikrein and high-molecular-weight kininogen (HMWK) assemble on negatively charged surfaces. In presence of calcium and phospholipids prekallikrein convert into kallikrein. This leads to the activation of FXII by proteolytic cleavage. FXIIa in turn is able to hydrolyze prekallikrein into kallikrein resulting in a positive feedback loop within the initial phase of the intrinsic pathway. Kallikrein is also involved in the release of the potent vasodilator bradykinin [5]. Next, FXIIa activates FXI which then can activate FIX. Under physiologic conditions the intrinsic pathway is activated by thrombin cleavage of FIX [6]. In the presence of calcium and phospholipids the activated factor IX together with the activated co-factor VIII (FVIIIa) and factor X forms the tenase complex, which results in the activation of FX and initiates the common pathway [6].

2.1.2.2 The extrinsic coagulation pathway

The extrinsic pathway, also called tissue factor pathway, is initiated upon exposure of extravascular tissue factor (factor III, TF) to the blood coagulation factor VII (FVII). TF is a glycoprotein present in almost every human cell including smooth muscle cells, pericytes and fibroblasts. An exposure of TF to blood can happen after tissue damage but also after induced expression of TF by inflammatory stimuli [7]. TF activates FVII and the complex of TF/FVIIa initiates the coagulation cascade by activating FX and FIX [8]. The extrinsic coagulation pathway is very rapid, resulting almost instantaneously in the activation of FX [9], which leads to an initial burst of thrombin, while the intrinsic pathway results in an amplification of the

coagulation cascade. In the in vivo situation, coagulation happens mainly via the extrinsic pathway [10], which is also reflected by the fact, that people with deficiencies in FXII, HMWK or prekallikrein have no bleeding abnormalities [5,11,12].

2.1.2.3 The common pathway

The activation of FX by the intrinsic or extrinsic pathway marks the start of the final common pathway where FXa forms the so called prothrombin complex together with activated co-factor V (FVa) and prothrombin (factor II) in the presence of calcium and phospholipids. FXa is then able to convert prothrombin into thrombin. Thrombin has a lot of functions within the coagulation cascade, but mainly cleaves fibrinogen into fibrin that finally forms a stable clot [13]. Moreover, thrombin can activate factor XIII, which is required to crosslink the fibrin polymers within the clot, as well as factors V, VIII and XI or cells like platelets that are involved in the coagulation cascade. This underlines the importance of thrombin in the propagation of the coagulation [14].

2.1.3 Regulators of coagulation

A complex system of regulatory mechanisms is in place to ensure that the clotting process is locally contained and to prevent an uncontrolled amplification of the coagulation. One way of regulation is the inactivation of coagulation factors. Antithrombin III (ATIII) is the most prominent factor that acts inhibitory on thrombin, FXa, FXIa and FIXa by forming complexes with the active site of the protease enzymes making them inaccessible for their usual substrates. Heparin is a natural anti-coagulant that enhances the inhibitory activity of ATIII by a factor of 1000 [15].Other plasma protease inhibitors are for example 2-macroglobulin, 1-antitrypsin or heparin cofactor II. Two vitamin K dependent proteins are responsible for the inactivation of coagulation co-factors FVIII and FV: Protein C acts in combination with free protein S to form a complex and subsequent proteolyze FVIII and FV after activation of protein C by thrombin bound to thrombomodulin on endothelial cell surfaces [16]. Another regulator of coagulation is the Tissue Factor Pathway Inhibitor (TFPI). It is a protease inhibitor that binds both to FXa and to tissue factor-FVIIa complex. If a large amount of tissue factor is present, as in a vascular injury, this inhibition of TFPI is overcome. Another important regulatory function is executed by the fibrinolytic pathway. The stable formed secondary hemostatic clot at the end of the coagulation cascade has to be removed after the

injured vessel has been repaired. Fibrinolysis dissolves the clot by using the proteolytic enzyme plasmin to catalyze the degradation of fibrin. Fibrinolysis is a process that is initiated immediately after the endothelial cells have been damaged and releases tissue plasminogen activator and urokinase. To balance the system and maintain the clot temporarily until the vessel has been repaired, fibrinolysis is controlled by plasminogen activator inhibitors (PAIs; e.g. PAI-1) and plasmin inhibitors (e.g., α2-antiplasmin) [17] that are released from vascular endothelial cells and activated platelets.

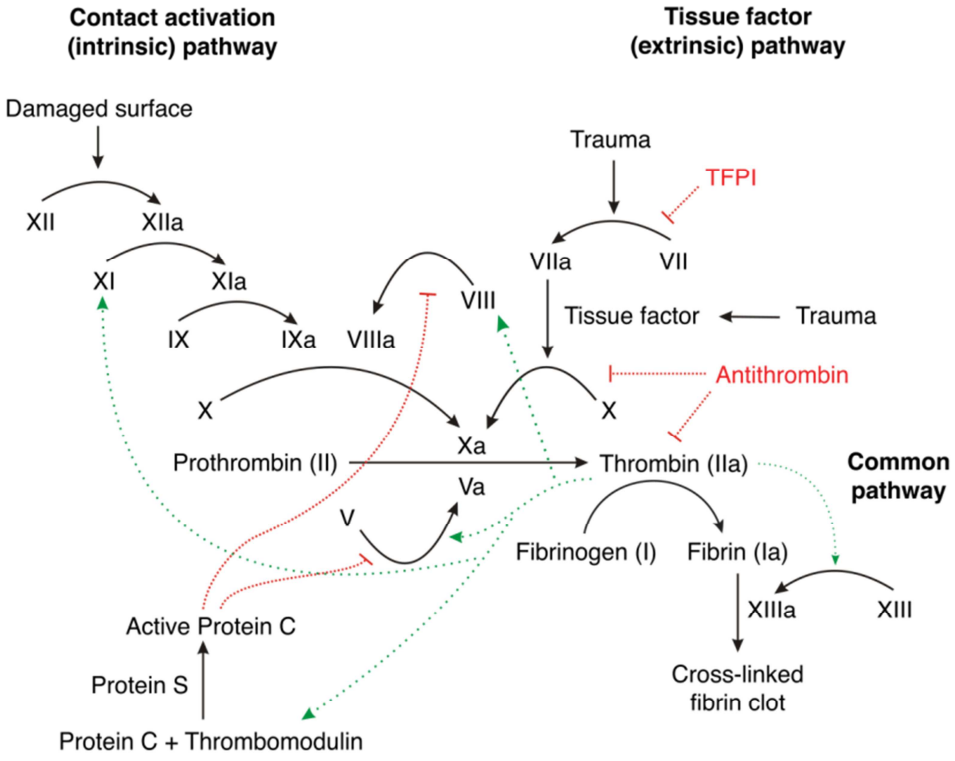


Figure 1: Overview of the coagulation cascade

Source: http://en.wikipedia.org/wiki/File:Coagulation_full.svg; downloaded 2015-03-29

2.2 Hemophilia A

Hemophilia A is the most frequent bleeding disorder caused by genetic mutations of the coagulation factor VIII (FVIII) with a prevalence of about 1 of 5000-10000 men [18,19]. The mutations in the FVIII coding gene are very heterogeneous and cover the entire possible spectrum: complete or partial depletions, insertions, sequence duplications, frame shifts, splice junction alterations, nonsense and missense mutations [20]. The most frequent

mutations are inversions of intron 22 [21] and of intron 1 [22]. In hemophilia A patients these mutations result in the lack or in insufficient amount of functional FVIII leading to a defect in the coagulation cascade. The symptoms of this disease range from easy bruising and abnormal clotting after trauma to spontaneous hemorrhage [23]. Even though it is known that the phenotype of bleedings in patients is rather heterogeneous [24], patients are classified into three categories [25] depending on the residual FVIII activity, which reflect the severity of clinical symptoms (Table 1).

Classification	Factor VIII activity in plasma	Spontaneous bleedings
severe	< 0.01 IU/mL (<1% of normal)	Frequent spontaneous joint and muscle bleedings
moderate	0.01 -0.05 IU/mL (1-5% of normal)	Spontaneous bleedings are rare; bleedings into joints and muscles after minor injuries
mild	0.05 - 0.40 IU/mL (>5-40% of normal)	No spontaneous bleedings

Table 1: Classification in hemophilia A; adopted from White et al 2001 [25]

2.2.1 Factor VIII (FVIII)

The gene for FVIII (F8) has a size of about 186 kb with 26 exons [26] and encodes for a precursor protein of 2351 amino acids [27]. The cleavage of the signal peptide leads to the mature, functional protein with a final size of 2332 amino acids. Gitschier et al described in 1984 that the gene for FVIII is located on the X-chromosome [28]. This discovery and the fact that hemophilia A is recessively hereditary transmitted, explains why primarily male individuals are affected by this disease. FVIII is a large glycoprotein made up of different domains in the following order: A1-A2-B-A3-C1-C2 [29] (Figure 2A). The tertiary structure of FVIII has so far only been resolved for activated FVIII by Ngo et al [30] and Shen et al [31] (Figure 2B).

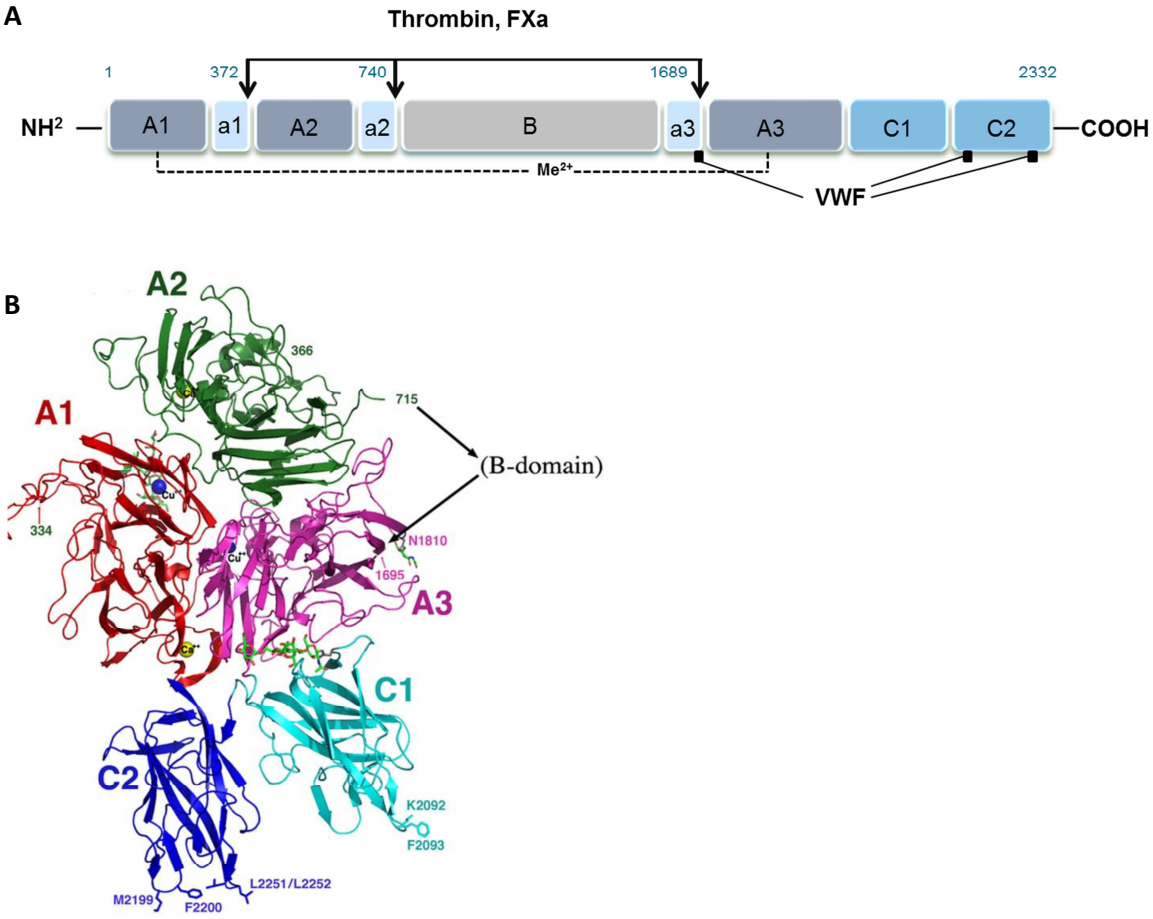


Figure 2: Structural Domains of FVIII (A) and tertiary structure of activated FVIII adopted from Shen et al 2008 [31] (B)

FVIII is mainly synthesized and released into the circulation by sinusoidal endothelial cells (LSECs) in the liver [32], but FVIII mRNA has been shown to be also present in other tissues [33]. Before the protein is released into the circulation, proteolytic cleavage leads to the formation of a FVIII hetereodimer consisting of a heavy chain including the A1-A2-B domain and a light chain with the A3-C1-C2 domain [27]. The heterodimer is non-covalently held together through the A1 and A3 domain via a divalent Ca^{2+} ion. After FVIII is released into the circulation it immediately forms a non-covalent complex with von Willebrand factor (VWF) via the light chain. VWF plays an important role within the coagulation process. On the one hand it is essential as an adhesion protein for circulating platelets to the site of vascular injury. On the other hand it protects FVIII from degradation and guides FVIII to the subendothelium as well as platelets [34,35]. Thrombin activates FVIII by cleaving FVIII at the acidic regions (a1, a2, and a3) neighboring the A domains that contain clusters of aspartic

and glutamic residues. Upon activation VWF is released and FVIII forms the tenase complex with FIX, FX and phospholipids. After activation of FX the complex dissembles and FVIII gets deactivated by spontaneous dissociation of its subunits or via activated protein C. Clearance of FVIII happens via binding to catabolic receptors on scavenger cells (Figure 3).

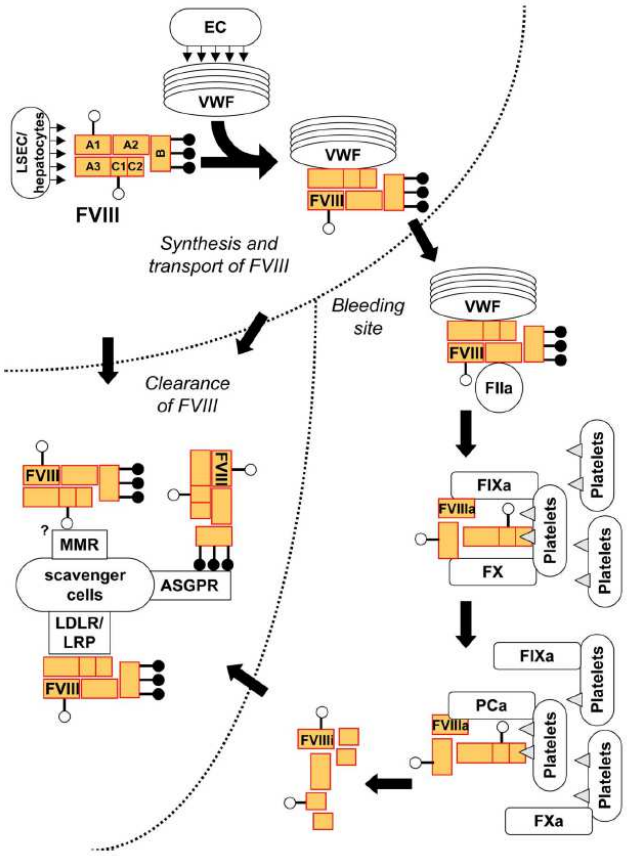


Figure 3: Life cycle of FVIII under physiologic conditions adapted from Lacroix-Desmazes et al 2008 [36]

2.2.2 Treatment of hemophilia A patients

A replacement of the non-functional FVIII is the obvious therapy in hemophilia A but it was not until the early 1960's that this approach was successful in patients. Pool et al discovered in 1964 that the cryoprecipitated fraction from plasma contained considerable amounts of FVIII [37]. This was a breakthrough in hemophilia A care. Before, the only possible treatment for bleeding episodes was infusions with whole blood or fresh plasma from healthy donors. However, it was not possible to stop major bleedings due the low plasma levels of FVIII. Therefore, most of the patients already died in childhood or in early adulthood as a result of

hemorrhages after surgery or trauma or bleedings in vital organs [38]. Using cryoprecipitate it was possible to infuse enough FVIII protein in relatively small volumes to control severe bleedings and allow treatment at home. A major side effect of this treatment was the high risk of infections with blood borne pathogens. In the early 1980's almost all treated patients with severe hemophilia A were infected with the hepatitis C virus (HCV) and 60-70 % of hemophiliacs became infected with the human immunodeficiency virus (HIV) [39]. To meet the need for a safe treatment, novel technologies were implemented: Viral inactivation techniques were developed for the production of FVIII concentrates and the purification process was further enhanced. All this innovation finally led to the treatment of the first hemophilia A patients with recombinant FVIII products in the late 1980's [40].

2.2.3 Treatment regimens in hemophilia A patients

Initially, the intention of a replacement therapy was the control of bleeding episodes in patients on demand. However, it became soon clear that this treatment regimen cannot prevent the degeneration of the patient's joints (hemophilic arthropathy). It is believed that iron deposits originating from accumulated blood in the joints trigger an inflammatory reaction, release of oxidative products, and vascular proliferation [41]. This leads to recurring bleeding episodes into the same joint and to an exacerbation of this condition, resulting in chronic arthritis with progressive damage of cartilage and bone. Physicians in Sweden recognized that patients with a moderate or mild form of hemophilia A do not bleed recurrently into joints and almost none of these patients develop hemophilic athropathy [42]. A new prophylactic treatment regimen was designed with the goal to maintain a certain level of functional FVIII in the circulation of patients. The new treatment regimen has been proven to be very effective in reducing the frequency of total and joint bleeds and preventing damage to the joint on a long-term perspective [43-47]. Although the benefit of prophylactic treatment is not in question, there is still debate about the intensity of the treatment and when to start treating the patients, especially when taking the heterogeneity of bleeding phenotypes into account. Moreover, two major disadvantages are at the moment hindering the spread of prophylactic treatment within the hemophilia community. First, prophylaxis is a very expensive treatment: Manco-Johnson et al calculated 300.000$ annual costs for a boy of 50 kg weight [48]. Second, patients on prophylaxis usually have to be treated three times a week with intravenous infusions to keep the FVIII at a sufficient level

due to the short half-life of FVIII in circulation of about 12 hours [24]. Especially for young patients a venous access is necessary, bearing the concomitant risks of infection and thrombosis.

2.3 FVIII inhibitors in hemophilia A patients

One of the major remaining complications in hemophilia A treatment today is the formation of neutralizing antibodies against FVIII, so called FVIII inhibitors [49,50], rendering replacement therapy ineffective. The prevalence of FVIII inhibitors is ~ 20%-32% in patients with severe hemophilia A and about 3%-13% in patients with moderate or mild hemophilia A [51,52]. Inhibitors usually develop in patients with severe hemophilia A within the first 50 exposures to FVIII products which happen at an average age of 1-2 years [53]. FVIII inhibitors are typically of the IgG isotype and target functional active sites of FVIII (Figure 4), hindering thereby the interaction of FVIII with other coagulation factors like FIIa, FIXa, FX and VWF as well as phospholipids [54,55].

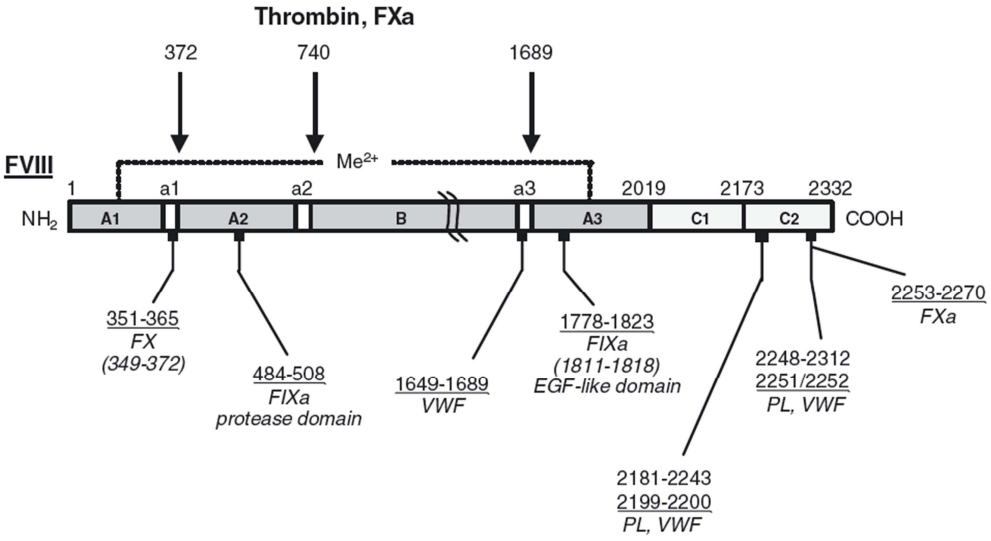


Figure 4: FVIII Structure and epitopes of FVIII inhibitors from Reipert et al 2007 [56]

The current standard of care aims to eradicate the inhibitory antibodies in these patients via immune tolerance induction (ITI) therapy. Since the first successful therapy in 1977 [57] several different protocols have been established [58] but three are mainly used: The original Bonn protocol [57], the Van Creveld protocol [59] and the Malmö protocol [60,61]. These protocols differ

in their treatment regimen as well as FVIII doses but in general FVIII is given to the patients in short intervals over a longer period of time to induce immune tolerance towards FVIII. This therapy is overall effective in about only 70% of the cases [62], leaving a large number of hemophilia patients with no effective replacement therapy.

The treatment of patients with inhibitory antibodies in case of severe hemorrhages is difficult since the treatment is dependent on the inhibitor titer. While low-titer inhibitors can be overwhelmed with a large dose of FVIII, patients with high-titer inhibitors need bypassing agents like activated prothrombin complex concentrates (e.g. FEIBA) or recombinant FVIIa (e.g. NovoSeven). Although both FEIBA and NovoSeven have an overall efficacy rate of > 80% [63,64], they are not as effective as FVIII replacement therapy and do not work equally in each patient. Therefore, the risk of increased morbidity caused by bleeding episodes, that are difficult to arrest, as well as the substantially increasing treatment costs for these patients lead to a reduced quality of life [65,66].

2.3.1 Development of FVIII-specific antibodies is regulated via T-cell-dependent and -independent pathways

A prerequisite for the humoral immune response against a protein antigen like FVIII is the recognition of antigenic epitopes via a membrane bound immunoglobulin molecule on mature, naïve B lymphocytes, the B-cell receptor (BCR). This initial event can lead to the activation of B-cells and their terminal differentiation into antibody-producing plasma cells. The current understanding of this process (Figure 5) involves additional activation signals provided either from cognate $CD4^+$ T helper cells or from T-cell-independent pathways as well as activation of the innate immune system [67]. T-cell-independent pathways occur in splenic marginal zone B-cells as well as in B-cells that differentiate outside of B-cell follicles of lymphoid organs [67,68]. These B-cells may circumvent their need for co-stimulation by T-cells if the protein antigen provides repetitive patterns that are able to cross-link the BCR [69] or by stimulation via pattern recognition receptors (PRRs) that sense different, non-specific danger signals. Emerging antibody-producing plasma cells from these pathways are usually short-living and antibodies are of low affinity [67,68]. In contrast, plasma cells deriving from B-cells that differentiated within germinal centers inside of the B-cell follicle with the help of $CD4^+$ -T-cells are most of the time long-lived plasma cells. Resulting antibodies are of higher affinity and switched classes from IgM mainly to IgG and IgA [70]. $CD4^+$ T-cells are immune

response mediators that need activation before they can carry out their role and support B-cells. As for B-cells, the primary activation of T-cells needs two independent signals. The first signal involves the recognition and binding to antigenic peptide-HLA II (Human Leukocyte Antigen Class II) molecules on professional antigen presenting cells (APCs) like dendritic cells via the T-cell receptor (TCR). This requires that the APC encounter and uptake the antigen and travel to the next secondary lymphoid organ. Extracellular antigen uptake occurs in APCs via three main mechanisms: non-selective macropinocytosis, receptor-mediated endocytosis and phagocytosis [71]. All three mechanisms lead the antigen into compartments of the endosomal pathway, where the antigen is partially proteolized, loaded on HLA II molecules and subsequently recognized on the surface of the APC by the TCR and CD4 receptor on T-cells. The uptake of the antigen leads to the maturation of APCs, resulting in reduced antigen recognition by down regulating surface receptors, while other surface molecules that are important for the interaction with T-cells are up regulated in context of additional danger signals like for example toll-like receptor (TLR) ligands [72]. These activating co-stimulatory molecules are the second signal required for T-cell activation. One of the best studied co-stimulatory interactions is the binding of B7-1 and B7-2 molecules on APCs with CD28 on T-cells [73]. Additional, cytokines are released from the APCs which have substantial influence on the type of immune response and trigger differentiation of $CD4^+$ T-cells into the different effector subsets.

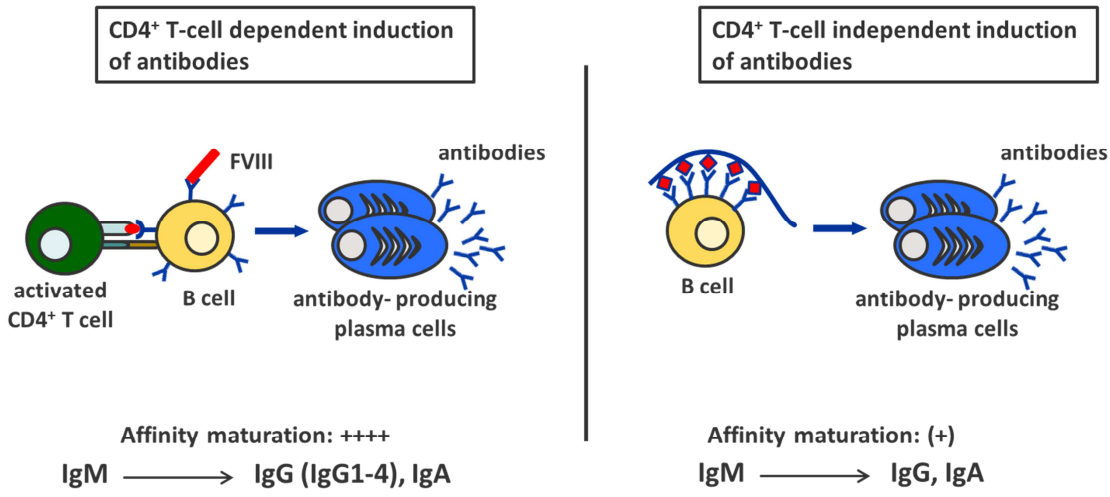


Figure 5: Regulation of FVIII-specific antibody responses: adapted from Reipert et al 2011 [74]

Although it has been shown in hemophilia A that development of high affinity, neutralizing antibodies against FVIII depends on $CD4^+$ T-cell help [75,76], T-cell-independent pathways may be the initial step in an immune response against FVIII or enhance the T-cell dependent pathway. Non-neutralizing antibody responses from T-cell- independent pathways could allow a more efficient uptake of FVIII by APCs either via the formation of immune complexes or via the activation of the alternative complement cascade resulting in the presentation of T-cell epitopes [77]. However, it was recently shown, that binding FVIII-antibodies with restricted Ig isotypes and IgG subclasses and of low affinity are found in healthy human individuals [78,79]. The biological relevance of these low-affinity antibodies in healthy individuals is not clear at the moment. One possible mechanism has been recently proposed and suggests that this natural occurring autoimmunity is part of a physiological mechanism called the immunological homunculus. In this model, autoimmune T-cell and B-cell repertoires as well as innate receptors sense the current state of the immune system and ensure immune homeostasis [80].

2.3.2 Genetic risk factors for FVIII inhibitor development

The risk for patients to develop FVIII inhibitors are potentially influenced by several different gene polymorphisms that alter or modulate the immune response or cells of the immune system [81]. First of all, the type of F8 mutation and respectively the F8 haplotype has a strong impact. The repertoire of $CD4^+$ T-cell specificities is formed during their development within the thymus. T-cells that recognize self-antigens in the process of negative selection are deleted [82]. Therefore, patients with large deletions and subsequently truncated FVIII proteins will have more FVIII reactive T-cells in circulation than patients were the FVIII protein is more similar to the replacement product. Accordingly, different F8 haplotypes found in different ethnicities may also increase the risk of developing a FVIII inhibitor. For example, a certain F8 haplotype that was only found in African Americans was associated with an increased risk in these patients [83]. However, recent data from Gunasekera and colleagues suggests that although African Americans with an intron-22 inversion of FVIII have a 3 times higher risk to develop an inhibitor than Caucasians with the same mutation, this higher inhibitor risk did not correlate with the F8 haplotype [84]. Therefore it is not clear at the moment if FVIII products that do not match the specific F8 haplotype of a patient's ethnicity contribute to FVIII inhibitor development.

In addition to F8 haplotypes, polymorphisms of genes that are involved in immune regulation or antigen presentation were associated with the formation of FVIII inhibitors. The HLA class II gene region has a very high degree of polymorphisms. As already mentioned, HLA II molecules play an important role during an immune response by selecting peptides that are presented to CD4+ T-cells [82]. Surprisingly only three haplotypes have been associated with 90% of all human autoimmune diseases [85] and only one was associated with higher risk of FVIII inhibitor formation [86-88]. Other genes that are important in the formation of FVIII inhibitors are involved in the activation state of the innate immune system or on the expression level of co-stimulatory molecules of leukocyte subsets [81]. So far the influence of immunoregulatory genes on FVIII antibody development could be demonstrated for heme oxygenese-1 [89,90], IL 10, TNF-α [88,91,92] and CTLA-4 [93]. Recently Astermark and colleagues showed the complexity of genetic polymorphisms that may influence antibody responses against FVIII [94].

2.3.3 Non-genetic risk factors for inhibitor development

Despite the genetic and ethnic background other factors are maybe also involved in the development of FVIII antibodies (Figure 6). These factors were recently summarized in general for protein therapeutics [95] and include the length of the treatment, aggregation of the protein therapeutic, route of administration, dosage, impurities in the formulation and specific biological activity. Especially the dosage could be a critical parameter in hemophilia, because in case of strong bleeding episodes or during surgery the amount of given FVIII can increase dramatically. High doses of FVIII in combination with tissue damage could eventually break peripheral tolerance without T-cell help leading to the production of antibodies against FVIII as already discussed in chapter 2.3.1. In addition, sub-visible particles, protein misfolding and protein aggregation were recently linked to the immunogenicity of protein therapeutics [96-98] and have to be considered as a potential risk factor in FVIII inhibitor development.

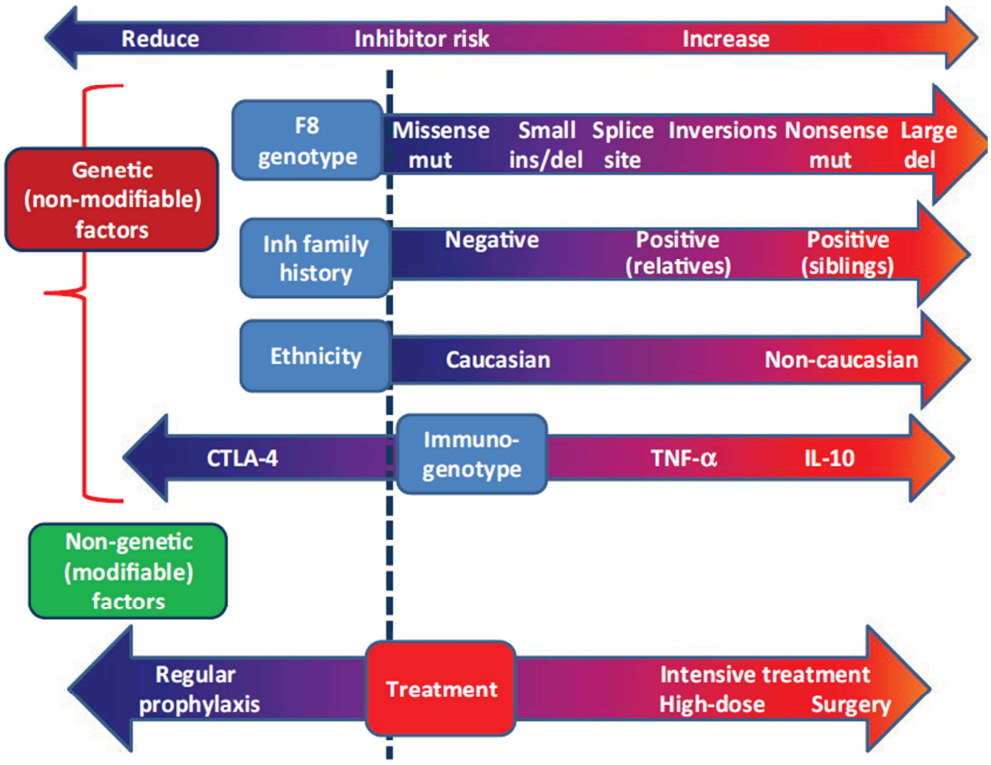


Figure 6: Protective or predisposing effects of genetic and non-genetic factors that may potentially influence the risk of inhibitor development. Adapted from Coppola et al 2010 [99]

2.4 Development of longer acting FVIII products

Various approaches have been made in the last couple of years to improve the treatment of hemophilia A [100], especially with regards to their pharmacokinetic profile. Such longer acting FVIII products would allow patients on prophylactic treatment to maintain appropriate clotting factor activity levels with fewer infusions.

2.4.1 BAX 855, a PEGylated rFVIII product with prolonged activity

One of the currently investigated bioengineer strategies to extend the half-life of rFVIII is the conjugation of Polyethylene glycol (PEG) to the clotting factor. This so called PEGylation has become one of the most commonly used methods in overcoming major shortcomings in protein based therapeutics like immunogenicity, proteolytic degradation, low solubility and short circulating half-life. PEG is a chemically inert, synthetic polymer with a repetitive chemical structure [HO–(CH_2–CH_2–O)n–H] that is soluble not only in aqueous but also in organic solutions. Furthermore, it has a highly flexible backbone chain and the ability to bind

water molecules [101-103]. These factors have been suggested as the reason that PEG exhibits the ability to precipitate proteins [104], exclude proteins and cells from surfaces [105], reduce immunogenicity and antigenicity [106] and prevent degradation by mammalian cells and enzymes [107]. BAX 855 is the stable PEGylated form of Baxalta's full length, native rFVIII product ADVATE™ for treatment of hemophilia A. The PEGylation reagent used for conjugation to rFVIII has a branched glycerol based structure containing two PEG chains of 10 kDa each and an active N-hydroxy succinimide (NHS) ester group (Figure 7).

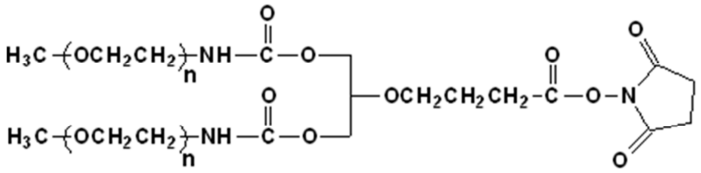


Figure 7: PEG reagent used for conjugation to rFVIII: (1,3-bis(methoxy poly(ethylene glycol) MW 10,000 carbamoyl)-2-propanoxy)-4-succinimidyl butanoate

The PEGylation process used to produce BAX 855 is based on the reaction of the PEG NHS ester with accessible primary amino groups on the FVIII protein to form a stable amide bond (Figure 8). The conjugation reaction mainly target ε-amino groups of the lysine residues and the reaction was optimized resulting in an average molar ratio between PEG and rFVIII of approximately 2 (mol/mol) [108]. BAX 855 has an increased half-life of about 1.5 fold compared to ADVATE™ [109]. PEGylation occurs predominantly at the B-domain which is cleaved off upon activation by thrombin [108]. Moreover it was shown that the chemical modification with PEG led to a decrease in binding to low-density lipoprotein receptor related protein (LRP) [110,111]. This could explain the extended half-life of BAX 855 because FVIII is mainly cleared in the liver via LRP [112-114].

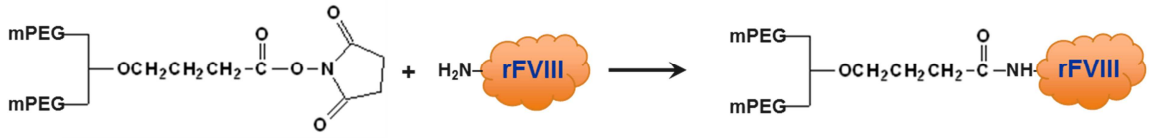


Figure 8: Reaction sequence for the PEGylation of rFVIII

2.5 Objectives of the PhD thesis

The development of neutralizing antibodies against FVIII is the major complication in treatment of patients with hemophilia A. Although latest research has considerably contributed to our understanding of the essential determinants of the immunogenicity of FVIII, we are still not able to predict the likelihood of a patient developing an unwanted immune response.

BAX 855 is a PEGylated form of Baxalta's recombinant full-length FVIII product ADVATE™ that has been developed with the aim to extend the half-life of FVIII in the circulation of patients. Like all novel FVIII preparations, BAX 855 needs to be tested for its immunogenic potential during non-clinical and clinical development. For this purpose, suitable technologies need to be established to address different aspects of the potential of BAX 855 to induce unwanted immune responses and to analyze the different components of the immunogenicity of BAX 855. The objectives of this thesis were the following:

1. To establish a new flow cytometry based method for the assessment of sub-visible particles in protein therapeutics. Sub-visible particles contained in protein formulations are linked to an increase in immunogenicity of protein therapeutics.
2. To assess potential antibody responses against PEG, a major component of BAX 855, and the mechanisms of their induction.
3. To apply the newly established technologies for the assessment of sub-visible particles in protein formulations and for the assessment of antibodies against PEG in the overall immunogenicity assessment of BAX 855 during non-clinical development.

3. Results

3.1 A Flow-Cytometry-Based Approach to Facilitate Quantification, Size Estimation and Characterization of Sub-visible Particles in Protein Solutions

3.1.1 Abstract

Purpose: Sub-visible particles were shown to facilitate unwanted immunogenicity of protein therapeutics. To understand the root cause of this phenomenon, a comprehensive analysis of these particles is required. We aimed at establishing a flow-cytometry based technology to analyze the amount, size distribution and nature of sub-visible particles in protein solutions.

Methods: We adjusted the settings of a BD FACS Canto II by tuning the forward scatter and the side scatter detectors and by using size calibration beads to facilitate the analysis of particles with sizes below 1 µM. We applied a combination of Bis-ANS (4,4'- dianilino-1,1'-binaphthyl-5,5'-disulfonic acid dipotassium salt) and DCVJ (9-(2,2-dicyanovinyl)julolidine) to identify specific characteristics of sub-visible particles.

Results: The FACS technology allows the analysis of particles between 0.75 and 10 µm in size, requiring relatively small sample volumes. Protein containing particles can be distinguished from non-protein particles and cross-β-sheet structures contained in protein particles can be identified.

Conclusions: The FACS technology provides robust and reproducible results with respect to number, size distribution and specific characteristics of sub-visible particles between 0.75 and 10 µm in size. Our data for number and size distribution of particles is in good agreement with results obtained with the state-of-the-art technology micro-flow imaging.

3.1.2 Abbreviations

Aβ 1–40	Amyloid beta 1–40 peptide
Bis-ANS	4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt
DCVJ	9-(2,2-dicyanovinyl)julolidine
DMSO	Dimethyl sulfoxide
D-PBS	Dulbecco's Phosphate-Buffered Saline
preparation Non-Prot	Non-protein particles
preparation Prot	Protein particles without crossß-sheet structures
preparation Prot-Crossß	Protein particles containing crossß-sheet structures
rFVIII	Recombinant human factor VIII
TEM	Transmission Electron Microscopy
ThT	Thioflavin T
WFI	Water For Injection

3.1.3 Introduction

Since the introduction of recombinant technologies, an increasing number of protein therapeutics have become available, providing new treatment options for a wide range of diseases [1]. Experience with protein therapeutics has shown that many of them bear the risk of inducing unwanted immune responses in patients which can be associated with severe clinical consequences such as a reduction or loss of efficacy, hypersensitivity reactions or neutralization of the natural counterpart. Often, these events are infrequent and only become recognized at a late stage during clinical development or after marketing authorization [2]. The most common event is the development of anti-drug antibodies which can diminish drug efficacy, alter the pharmacokinetic profile of a drug, cause hypersensitivity reactions or cross-react with an endogenous protein. The immunogenic potential of each protein therapeutic is determined by a multiplicity of different factors, which may be patient-, product- or treatment related [3–7]. Although research has added considerably to our understanding of the essential determinants of the immunogenicity of protein therapeutics, we are still not able to predict the likelihood of a patient developing unwanted immune responses to a particular protein therapeutic.

Recently, protein misfolding and aggregation were suggested to facilitate the immunogenic potential of protein therapeutics and negatively impact clinical performance [8–10]. Misfolding associated with aggregation of endogenous proteins has previously been recognized as a serious problem associated with a number of severe diseases [11]. These diseases are characterized by the occurrence of protein aggregates with a highly ordered structure known as cross-spine or cross-β sheet [12]. The histology of the resulting insoluble protein aggregate deposits has been called amyloid [11]. Maas et al. demonstrated that a number of protein therapeutics contain amyloid-like protein aggregates and proposed that these aggregates determine the risk of protein therapeutics inducing unwanted immunogenicity and toxicity [13]. This proposal has been supported by findings indicating that amyloid-like protein aggregates can activate the innate immune system, which could be an essential trigger of unwanted immunogenicity [14, 15]. However, there is a wide variety of soluble and insoluble protein aggregates, ranging from 1 to 100 nm in size for soluble aggregates and 0.1 to 100 µm for insoluble aggregates (particles) [16]. Sub-visible particles are defined as particles between 0.1 and 50 µm in size [17]. A more thorough understanding

of the potential contribution of the different types of aggregates to the risk of protein therapeutics inducing unwanted immunogenicity is required.

Misfolding and aggregation are intrinsic propensities of proteins when exposed to a number of stress factors which may occur during the manufacturing process of protein therapeutics. Exposure to air-water interface [18], pH changes, light and temperature fluctuations, lyophilization [19], sonication [20], contact with packaging material [21] and sample agitation [22] have been described as potential risk factors for protein misfolding. Moreover, non-protein particles present in protein therapeutics may provide nucleation sites and facilitate protein aggregation [23–27]. In addition, protein interaction with silicone oil, which is widely used to prevent protein fouling on surfaces and as lubricant, may promote protein aggregation [28, 29]. Therefore, manufacturers of protein therapeutics are expected to develop protein formulations that adequately minimize the impact of the various stress factors on the native protein structure [8, 30]. For this purpose, suitable analytical tools are required to closely monitor and characterize protein aggregates and non-protein particles. Currently available analytical technologies such as nanoparticle tracking analyses, flow microscopy, electrophoretic light scattering, light obscuration, electrospray differential mobility analyses and resonant mass measurement have provided important information. However, these technologies cannot simultaneously characterize the composition, structure, chemistry, amount and size of protein aggregates in a single sample. Thus, there is a need for additional analytical tools which facilitate both the detection of a wide size range of protein aggregates and an assessment of their composition and structural properties.

More than two decades ago, flow-cytometry was already used to analyze amyloid plaques [31], but it was only recently that Mach et al. [32] and Ludwig et al. [33] introduced flow-cytometry as a suitable analytical tool to characterize sub-visible particles. They provided evidence that flow-cytometry might be a promising technology for efficient monitoring of sub-visible particles in therapeutic protein formulations. Here, we describe a flow-cytometry-based approach which facilitates the simultaneous analysis of amount, size distribution and nature of sub-visible particles in protein solutions. We paid particular attention to the analysis of potential cross-β-sheet structures because they were suggested to determine the risk of protein therapeutics inducing unwanted immunogenicity and toxicity [13]. Furthermore, we present data demonstrating the applicability of flow-

cytometry to assess the interaction of proteins with silicone oil droplets without the need for labeling the interaction partners.

3.1.3 Materials and methods

Sample Preparation for Analysis of Sub-visible Particles

Non-Protein Particles: Silicone Oil Droplets (Preparation Non-Prot)

Silicone oil droplets were prepared as described by Mach et al. [32]. In brief, 10 ml Dulbecco's PBS (D-PBS; Invitrogen Corporation) were filled into a 30 ml BD Syringe Plastipak (BD Bioscience) which contains special silicone lubrication to facilitate smooth plunger moves. Silicone oil particles were produced by vertically shaking the filled syringe manually for 2 min.

Protein Particles Without Cross-ß-Sheet Structures: A Complex Consisting of Biotinylated Bovine Serum Albumin and Avidin (Preparation Prot)

Biotinylated bovine serum albumin (Biotin-BSA) and avidin from egg white (both Sigma-Aldrich) were mixed together at concentrations of 50 µg/ml Biotin-BSA and 12 µg/ml avidin. The mixture was incubated at room temperature for 60 min. The resulting Biotin-BSA-avidin complex was diluted 1:10 with D-PBS and stored at ≤−60°C prior to analysis. The absence of cross-β-sheet structures was confirmed using a thioflavin T assay as described below in the section "Thioflavin T (ThT) assay" (Fig. 1a). If not otherwise indicated, the final working concentration of preparation Prot was 5.4 µg/ml.

Protein Particles Containing Cross-ß-Sheet Structures: Aggregated Amyloid Beta 1–40 Peptide (Preparation Prot-Crossß)

One milligram amyloid beta 1–40 peptide (Aβ 1–40; Bachem AG) was dissolved in 115 µl water for injection (WFI; Pharma Hameln) to obtain a final concentration of 2 mM. This solution was incubated at 2–8°C for 168 h. The resulting material was diluted with WFI to a final concentration of 15 µg/ml, aliquoted and stored at ≤−60°C prior to analysis. The presence of cross-β-sheet structures was confirmed using a ThT assay and transmission electron microscopy as described below in the sections "Thioflavin T (ThT) assay" and

"Transmission electron microscopy" (Fig. 1a, b). If not otherwise indicated, the final working concentration of preparation Prot-Crossß was 13.1 µg/ml.

Thioflavin T (ThT) Assay

ThT binding assays were done as described [34]. A stock solution of ThT (Sigma-Aldrich) was prepared at a concentration of 1 mM in D-PBS and stored at 4°C, protected from light. For ThT fluorescence measurements, protein samples were diluted with D-PBS to a final protein concentration of 10 µg/ml and mixed with the ThT stock solution to a final concentration of 20 µM. Three replicates were analyzed for each sample. The ThT fluorescence measurements were done on a Synergy™4 Hybrid Microplate Reader (BioTeK) using clear bottom, black, special optic plates (Corning Life Sciences). The signals were recorded from the bottom of a microtiter plate, with the excitation set at 440±9 nm and the emission set at 485±9 nm.

Transmission Electron Microscopy (TEM)

TEM micrographs were collected using a JEM-120B microscope (JEOL) operating with an accelerating voltage of 80 kV. Typical nominal magnifications ranged from 40,000 to 80,000 X. Samples were deposited on formvar-coated 400-mesh copper grids and negatively stained with 1% aqueous uranyl acetate (Sigma-Aldrich).

Human Recombinant Factor VIII

The human recombinant factor VIII (rFVIII) was production intermediate obtained from Baxter BioScience. It was dialyzed against D-PBS containing calcium- and magnesium chloride (Invitrogen Corporation) and stored at ≤−60°C prior to analysis.

Comparison of MFI and FACS Technologies

Solutions of rFVIII (20 µg/ml) containing increasing amounts of spiked silicone oil droplets were used.

Interaction Studies of Silicone Oil Droplets and Protein

A solution of rFVIII (73.4 µg/ml) in D-PBS was filtered through a 0.22 µm pore filter (Merck Millipore) and subsequently mixed with D-PBS enriched with silicone oil droplets. The

mixture was prepared 10 min prior to analysis. The rFVIII concentration in the final solution was 20 µg/ml. Control samples were prepared by diluting the filtered rFVIII solution and by diluting the D-PBS enriched with silicone oil droplets with equal volumes of D-PBS.

Detection, Size Estimation, Characterization and Quantification of Sub-visible Particles by Flow-Cytometry

All samples were analyzed using a 3-laser (405, 488 and 630 nm) 8-color BD FACS Canto II flow cytometer (BD Bioscience) which was calibrated on a weekly basis using CS&T beads (BD Bioscience). A low and constant background signal, essential for reproducibility and stable performance of the analysis, was achieved by appropriate cleaning and equipment preparation using BD FACS Clean and Rinse solutions (BD Bioscience). The flow rate of the cytometer was set to the lowest level (~10 µl/min) while acquiring and recording each sample for 300 s.

Settings of the BD FACS Canto II Flow Cytometer

Thresholds on FSC and SSC were set to 250; PMT Voltages: 405 V for FSC and 210 V for SSC; PMT 530/30 (blue laser 488 nm): 360 V; PMT 450/50 (violet laser 405 nm): 380 V; Window Extension: 7

Detection and Size Estimation of Sub-visible Particles

To verify the resolution capability of 0.75 µm particles, voltages for forward scatter (FSC) and side scatter (SSC) as well as the threshold levels for these channels were adjusted using Fluoresbrite®YGCarboxylate Size Range beads (Polyscience Inc.). The size of sub-visible particle was estimated by setting different size ranges using beads with defined diameters of 0.75, 1, 2, 4.5, 6 and 10 µm. In most experiments, size ranges were set between the peak maxima of each bead population in a FSC histogram. When the flow-cytometry-based technology was compared with micro-flow imaging, the size ranges for the flow-cytometry approach were set from each peak maxima up to the right end of the scale. Size ranges were adjusted for each experiment.

Characterizing the Nature of Sub-visible Particles

To distinguish protein from non-protein sub-visible particles, samples were stained with the fluorescent dye 4,4'-dianilino-1, 1'-binaphthyl-5,5'-disulfonic acid dipotassium salt (Bis-ANS; Sigma-Aldrich) which binds to hydrophobic patches contained in proteins [35]. Bis-ANS was excited with the violet laser (405 nm). The emitted fluorescence which has a peak maximum at 515 nm, was detected with a 450/50 bandpass filter in the violet detector array. Protein sub-visible particles were further characterized using Bis-ANS in combination with the fluorescence dye 9-(2,2-Dicyanovinyl)julolidine (DCVJ; Sigma-Aldrich) which binds to cross-β-sheet structures present in aggregated proteins [35–37]. DCVJ was excited with the violet laser (405 nm) and the blue laser (488 nm). The emitted fluorescence which has a peak maximum at 480–505 nm (35), was detected with a 530/30 band-pass filter in the blue octagon detector array.

Bis-ANS was dissolved in WFI to a final concentration of 1 mM. DCVJ was dissolved in 73% DMSO/WFI to a final concentration of 4mM. Both dye stock solutions were covered with aluminum foil and stored in the dark at +4–8°C. They were stable for at least 3 months.

Quantification of Sub-visible Particles

CountBright™ Absolute Counting Beads (Invitrogen Corporation) were used to quantify particles. The bead solution was prepared according to the manufacturer's instructions and added together with Bis-ANS (final concentration 20 µM) and DCVJ (final concentration 25 µM) to each sample prior to analysis.

Data Analysis

Data for each sample was stored as an FCS 3.0 file and subsequently analyzed by FlowJo 10.0.6. (Tree Star Inc.) using the following gating strategy [see Supplement Figure 1 for further details]: CountBright™ Absolute Counting Beads were gated using the 660/20 band-pass filter in the red detector array. Gating on protein sub-visible particles was done in the Bis-ANS channel (violet detector array, 450/50 band-pass filter) by acquiring a non-protein sub-visible particle control (preparation Non-Prot) as a negative control reference. Protein sub-visible particles bearing a cross-β-sheet structure were acquired in the DCVJ channel (blue detector array, 530/30 band-pass filter) using preparation Prot as a negative reference control and preparation Prot-Crossß as a positive control for cross-β-sheet structures.

Each sample was analyzed in 3 replicates. The average concentrations of sub-visible particles for total sub-visible particles, non-protein sub-visible particles, protein containing sub-visible particles and cross-ß-sheet containing protein sub-visible particles were calculated taking the dilution factors of the dyes and the CountBright™ Absolute Counting Beads into account. If not otherwise indicated, the average concentration of sub-visible particles refers to particles between 0.75 and 10 µm in size.

Single Particle Detection Versus Swarm Detection

To verify that the flow-cytometry based technology detects single particles rather than swarms of multiple particles, we serially diluted preparation Prot-Crossß and analyzed total particles, protein particles and cross-β-sheet-containing protein particles for each dilution step. We compared the total particle concentrations obtained for each dilution step taking the dilution factor into account.

Validation of the Flow-Cytometry-Based Technology

We evaluated the reliability and accuracy of the flow-cytometry-based technology by assessing multiple properties as suggested for this purpose by current regulatory guidelines for bioanalytical method validation [38, 39]. Assessed properties included potential carry-over effects of the dye or the protein, specificity, precision, accuracy, dilution linearity and relative sensitivity. Except for carry-over effects and relative sensitivity, the samples for validation of the technology were prepared as described above in the section “Detection, size estimation, characterization and quantification of sub-visible particles by flow-cytometry”.

Assessing Potential Carry-Over Effects of Protein Particles and Fluorescence Dyes

To assess potential carry-over effects of protein particles and dyes, we analyzed preparation Prot-crossß with and without washing the tubing system after analysis of each of three replicates and compared the average concentrations of sub-visible particles for total sub-visible particles, non-protein sub-visible particles, protein containing sub-visible particles and cross-ß-sheet containing protein sub-visible particles. To assess carryover effects of the dyes, we analyzed an unstained Prot-crossß sample directly after the three replicates of the stained Protcrossß sample. The unstained Prot-crossß sample was not stained with Bis-ANS

or DCVJ, therefore, any fluorescence staining would reflect a carry-over effect of the dyes which are still present in the tubing system.

Assessing Specificity

The specificity of the technology was verified using positive and negative controls for the dyes (preparations Non-Prot, Prot, Prot-Crossß).

Assessing Precision

The precision of the technology was defined as inter- and intra-assay variation. We analyzed preparation Prot-Crossß in 6 independent experiments for inter-assay variation and in 6 experiments run in parallel for intra-assay variation. The coefficients of variation (CV %) for inter- and intra-assay were calculated individually for total particles, for protein particles and for cross-β-sheet-containing protein particles.

Assessing Accuracy

The accuracy of the method (in CV %) was calculated as variability in the number of total sub-visible particles detected for the different size ranges. For this purpose, the same sample was analyzed 6 times on the same day.

Assessing Dilution Linearity

The linearity of the technology was assessed as described in the section “Single particle detection versus swarm detection”.

Assessing the Relative Sensitivity

The relative sensitivity of the method was calculated as the minimum concentration of particles which could be analyzed with a maximum CV of 25%. For this purpose, beads of the Fluoresbrite® YG Carboxylate Size Range Kit I & II were serially diluted with D-PBS. These beads were not stained with Bis-ANS or DCVJ but emitted light in the respective fluorescence PMTs. Each dilution was analyzed ten times and CVs% were calculated for each dilution step for the PMTs detecting total sub-visible particles, non-protein sub-visible particles, protein containing sub-visible particles and cross-ß-sheet containing protein sub-visible particles.

Micro-Flow Imaging

Micro-flow imaging (MFI) was done using a DPA4200 Flow Microscope (Brightwell Technologies). The pump speed was set to 0.17 ml/min during sample measurement. The flow cell was flushed with water before each analysis. Optimized illumination of the background was done using product filtered through 0.22 µm pores. Baseline runs with WFI to verify and quantify system cleanliness were done before the first and after the last sample was measured. 800 µl of a sample was needed for each sample run with an analyzed volume of approximately 450 µl. During each sample run, images were stored for evaluation of particles and for use of the electronic filter settings afterwards. After analyses, particle concentrations were categorized according to the particle sizes ≥1, ≥2, ≥5, ≥10, ≥25, ≥50 and ≥70 µm.

To compare state-of-the-art MIF technology and our flow-cytometry approach, we analyzed samples of rFVIII with or without silicone oil droplets using both technologies. rFVIII was freshly reconstituted before measurement. The silicone oil droplets were generated as previously described, diluted to two concentrations (non-Protα; non-Protβ) and mixed with rFVIII prior to analysis. We compared the concentration of total sub-visible particles of different size ranges. For this purpose, the size ranges of the flow-cytometry-based technology were adapted to those covered by MFI, namely ≥ 1, ≥ 2, ≥ 5 and ≥ 10 µm. rFVIII without non-protein silicone particles was included as a control. Samples to be analyzed by flow-cytometry were prepared as described in section “Detection, size estimation, characterization and quantification of sub-visible particles by flow-cytometry”.

Statistical Analysis

The CV% expresses the assay variation as a percentage of the mean and was calculated as follows: (standard deviation/ mean)∗100.

The standard deviation was calculated using

$$\sqrt{\frac{\sum(x-\bar{x})^2}{(n-1)}}$$

where x is the sample mean (number1, number2,…) and n is the sample size.

3.1.4 Results

Detection, Size Estimation and Quantification of Sub-visible Particles Using Flow-Cytometry

The BD FACS Canto II flow cytometer is usually set up to analyze cells between 5 and 20 µm in size. We adjusted the settings of the BD FACS Canto II to facilitate the analysis of sub-visible particles with sizes below 1 µm. We achieved the best signal to noise ratio by setting the thresholds of FSC and SSC to 250 and by setting the voltages of these detectors to 405 V for FSC and 210 V for SSC. When we compared Height and Area for the recording of FSC and SSC signals, we did not see any difference in outcome which is why we used Area for the rest of the studies. A representative example for the appearance of sub-visible particles using these settings is shown in Fig. 2a which reflects the analysis of a preparation of aggregated Aß1-40 peptide (preparation Prot-Crossß). When we used the same settings for the analysis of size calibration beads, we could establish a reproducible working size range of 0.75 to 10 µm which is reflected in Fig. 2b. The average concentrations of sub-visible particles refer to particles between 0.75 and 10 µm in size. Next, we established more specific size ranges using the peak maxima for each size as determined by the size calibration beads in the FSC channel, e.g. 0.75–1, 1–2, 2–4.5, 4.5–6 and 6–10 µm (Fig. 2c). When we applied these specific size ranges to the analysis of preparation Prot-Crossß, we obtained the following results for the size distribution of sub-visible particles in this preparation: 789,374 particles/ml with sizes between 0.75 and 1 µm; 246,767 particles/ml with sizes between 1 and 2 µm; 163,747 particles/ml with sizes between 2 and 4.5 µm; 20,970 particles/ml with sizes between 4.5 and 6 µm and 10,284 particles/ml with sizes between 6 and 10 µm.

Characterization of Sub-visible Particles

After establishing specific size ranges, we were interested to differentiate particles containing protein from non-protein particles and to identify cross-β-sheet structures in protein containing particles. We tested different combinations of fluorescent dyes which bind to specific structures in proteins to find the optimum combination with respect to minimal spectral overlap, absence of dye-induced artifacts and reproducibility of staining behavior. We selected an optimized combination of Bis-ANS and DCVJ. Bis-ANS binds to hydrophobic patches on protein surfaces resulting in about 100-fold increase in its

fluorescence intensity [35]. The results presented in Fig. 3a–c reflect representative examples using Bis-ANS as a protein stain. Presented are the analyses of silicone oil particles (preparation Non-Prot, Fig. 3a), of a complex containing biotinylated BSA and avidin (preparation Prot, Fig. 3b) and of aggregated Aß1-40 peptide (preparation Prot-Crossß, Fig. 3c). While the protein-containing samples (preparations Prot and Prot-Crossß) showed binding to Bis-ANS associated with an increase in the fluorescence signal (Fig. 3b and c), staining of silicone oil droplets (preparation Non-Prot) with Bis-ANS did not cause an increase in the fluorescent signal (Fig. 3a). In addition to silicone oil particles, we tested other non-protein particles such as glass beads, polystyrene beads and latex beads, which generated negative results similar to those obtained with silicone oil particles.

DCVJ binds to cross-β-sheet structures in aggregated proteins [35–37]. Data presented in Fig. 3e–g reflect representative examples using DCVJ staining. Whereas preparation Prot-Crossß (Fig. 3g) contained cross-ß sheets, preparations Non-Prot (Fig. 3e) and Prot (Fig. 3f) did not contain cross-ß sheets. The Prot-Crossß preparation bound DCVJ which was associated with an increase in the fluorescence signal (Fig. 3g). The preparations Non-Prot and Prot did not bind DCVJ (Fig. 3e and f).

After establishing the suitability of Bis-ANS staining for the differentiation of protein particles from non-protein particles and the suitability of DCVJ staining for the staining of cross-ß sheets in protein particles, we tested the combination of both dyes. The results of these studies are shown in Fig. 3d and h, presenting overlays of preparations Non-Prot, Prot and Prot- Crossß. The results illustrate that the combination of Bis-ANS and DCVJ is suitable to facilitate the differentiation of protein particles from non-protein particles and the identification of protein particles that contain cross-ß sheets.

Single Particle Detection Versus Swarm Detection

Using flow-cytometry, the analysis of individual particles with a size below 1 µm can be challenging because it involves the measurement of dim signals which are close to the detection limit of the instrument. Recently, the phenomenon of swarm detection was reported which describes the detection of several small particles at the same time resulting in an underestimation of the absolute number of particles [40, 41]. To verify the capacity of the FACS technology to detect single particles, we serially diluted a solution of aggregated Amyloid beta 1–40 peptide (preparation Prot-Crossß) and quantified the numbers of total

particles, protein containing particles without cross-β- sheet structures and protein containing particles with cross-β- sheet structures for each dilution step. Results presented in Fig. 4 confirm the linearity of the FACS technology and indicate a linear relationship between the protein concentration of the analyte and the detected particle count for each of the particle types analyzed. Moreover, the total concentrations of particles were independent of the dilution factor. When considering the total concentration of particles (0.75–10 µm) calculated for each of the six dilution steps, we calculated a mean of 2,235,748 particles /ml with a CV of 10.71% for total particles; a mean of 2,142,220/ml with a CV of 14.95% for protein particles without cross- β-sheets and a mean of 2,048,453 with a CV of 18.21% for cross-β-sheet containing protein particles. These results indicate that the number of counted particles is independent of the dilution factor which supports the detection of single particles rather than swarms of small particles.

Validation of the Flow-Cytometry-Based Technology

To verify reproducibility and accuracy of the FACS technology we validated it by assessing parameters suggested by current regulatory guidelines for bioanalytical method validation [38, 39].

Carry-over effects for both the dye and the protein particles were observed when two samples were analyzed without a washing step of the tubing system in between. Therefore, we included a washing step after each sample acquisition using BD FACS Rinse for 60 s at the highest flow rate.

The specificity of the technology was validated by assessing a range of control preparations: preparation Non-Prot containing silicone oil particles, preparation Prot containing protein particles without cross-ß sheets and preparation Prot- Crossß containing protein particles with cross-ß sheets. Our validation data indicate that Bis-ANS binds to protein particles (preparations Prot and Prot-Crossß) but not to non-protein particles (preparation Non-Prot). Moreover, Bis- ANS does not bind to glass beads, polystyrene beads or latex beads. DCVJ only binds to preparation Prot-Crossß containing protein particles with cross-β sheets, but not to preparations Non-Prot or Prot (Fig. 3 and Supplement Figure 1).

The precision of the technology was validated as inter- and intra-assay variation. We analyzed preparation Prot-Crossß containing protein particles with cross-ß sheets in 6 independent experiments for inter-assay variation and in 6 parallel experimental runs for

intra-assay variation. The coefficient for inter- and the intra-assay variation (CV %) was calculated for total particles, for protein-containing particles and for cross-β-sheet-containing protein particles. The CVs for interassay variation were 10.32% for total particles, 10.36% for Bis-ANS positive protein particles and 16.32% for DCVJ positive cross-ß-sheet-containing particles (Supplement Table 1A). The CVs for intra-assay variation were 3.92% for total particles, 3.79% for Bis-ANS positive protein particles and 2.05% for DCVJ positive cross-ß-sheet-containing particles (Supplement Table 1B).

The accuracy of the method (in CV %) was calculated as the variability in the number of total particles detected in 1ml for the different pre-defined size ranges. For this purpose, the same sample was analyzed 6 times on the same day. The CVs in the respective size ranges were 13.87% for the size range 0.75–1 µm; 15.18% for the size range 1–2 µm; 19.67% for the size range 2–4.5 µm; 20.13%for the size range 4.5–6 µm and 20.56% for the size range 6–10 µm (Supplement Table 1C). Based on the validation results, we conclude that all CVs% for intra- and inter-assay variation as well as for accuracy met the pre-defined acceptance criteria which were set to a maximum of 25%, which is in compliance with current regulatory guidelines [38, 39].

The linearity of the technology was assessed by serially diluting preparation Prot-Crossß and analyzing total particles, protein particles and cross-β-sheet-containing protein particles for each dilution step. Our results as presented in Fig. 4 indicate a direct relation between the concentration of the analyte and the particle count for each type of particle tested.

The relative sensitivity of the technology was defined as the minimum number of particles in 1ml which can be reproducibly detected by the flow cytometer when a maximum CV of 25% is accepted. We serially diluted beads from the Fluoresbrite® YG Carboxylate Size RangeKit I & II, measured each dilution step 10 times and calculated the CV% for each dilution step. The lowest bead concentration which could be analyzed with a maximum CV of 25% was 1500 beads per ml.

In summary, the validation results confirm that the flow-cytometry based technology for the analysis of sub-visible particles with a size range 0.75–10 µm meets all requirements for bioanalytical methods as suggested by current regulatory guidelines [38, 39].

Comparative Analysis of Sub-visible Particles Using Flow-Cytometry and State-of-the-Art Micro-Flow Imaging

Next, we were interested to know how results obtained with the flow-cytometry-based technology compare to results obtained with the state-of-the-art technology micro-flow imaging. We used a preparation of rFVIII mixed with two different non-protein silicone particle preparations (preparation Non- Protα and Non-Protβ, Fig. 5a and b) and analyzed the concentration of total sub-visible particles in different size ranges. For this purpose, the size ranges of the flow-cytometry-based technology were adapted to the size ranges covered by the micro-flow imaging technology, namely ≥1, ≥2, ≥5 and ≥10 µm. The results obtained with the micro-flow imaging technology (Fig. 5a) were in good agreement with those obtained with the flow-cytometry-based technology (Fig. 5b). Both technologies showed a concentration-dependent increase in sub-visible particle count after adding non-protein silicone oil particles to the preparation of rFVIII. While the size distribution of sub-visible particles was similar for both technologies, the flow-cytometry-based technology appeared to be more sensitive, counting higher absolute numbers of sub-visible particles than themicro-flow imaging technology for all size ranges (Fig. 5a and b).

Interaction Studies of Proteins with Silicone Oil Droplets

Silicone oil is one of the most abundant sources of non-protein sub-visible particles present in protein therapeutics [42]. Therefore, we were interested to know if the flow-cytometry-based technology could be used to study interactions of proteins with silicone oil droplets. We used rFVIII as a protein source and D-PBS buffer enriched with silicone oil sub-visible particles. The rFVIII solution, which was filtered through 0.22 µm pores, contained only very few sub-visible particles (Fig. 6a). In contrast, the D-PBS sample enriched with silicone oil particles contained a substantial number of sub-visible particles (Fig. 6b). We mixed the rFVIII solution with silicone oil particles, incubated the mixture for 10 min and subsequently analyzed it for sub-visible particles (0.75–10 µm). The results of this analysis as presented in Fig. 6c and d indicate that the incubation of the rFVIII solution with silicone oil particles generated an increased number of protein-containing particles, as demonstrated by binding to Bis-ANS. At the same time, the number of non-protein particles which did not bind to Bis-ANS was substantially reduced, indicating that silicone oil particles were covered with rFVIII and appeared as protein-containing particles. Figure 6d shows an overlay of Fig. 6b and c for

better comparison. Our results demonstrate that the FACS technology can detect sub-visible particles with a non-protein core (e.g. silicone oil) and a protein surface without the need for prior labeling of either the protein or the silicon oil droplets.

3.1.5 Discussion

Recently, sub-visible particles present in protein therapeutics have received increasing attention because of their potential contribution to the unwanted immunogenicity of protein therapeutics [16]. Although a couple of mechanisms have been proposed, the root cause for the induction of unwanted immune responses by sub-visible particles is not completely understood. The design of scientifically sound studies has been complicated by the limited availability of technologies suitable to quantify and characterize the wide variety of sub-visible particles which can be present in protein solutions. Here, we present a flow-cytometry-based technology which uses a combination of forward and side scattering together with fluorescence staining of specific particle features to determine the number, size distribution and specific characteristics of sub-visible particles between 0.75 and 10 µm in size. Flow-cytometry has only recently been introduced as a potential technology platform for the analysis of sub-visible particles present in solutions of therapeutic proteins [32, 33]. Mach et al. used SYPRO Orange dye, which binds to hydrophobic surfaces of partially destabilized proteins, to preferentially stain protein-containing particles and measure their numbers in therapeutic preparations of monoclonal antibodies [32]. The authors used a Beckman-Coulter FC-500 flow cytometer equipped with a 488-nm argon laser and combined the detection of SYPRO Orange fluorescence with forward and side scattering. Thus, they were able to detect protein particles with a size of ≥1 µm. Ludwig et al. applied flow cytometry for the detection and characterization of sub-visible particles in silicone-oil-contaminated protein formulations [33]. They used a BD FACS Calibur equipped with two lasers (488 and 635 nm) and combined the detection of two fluorescence dyes (AlexaFluor 647 and BODIPY 493/503) with forward and side scattering. Using proteins pre-labeled with Alexa Fluor 647 and silicone oil pre-stained with BODIPY 493/503, the authors demonstrated that flow cytometry has the ability to discriminate between homogenous protein aggregates and heterogeneous particles made up of silicone oil and protein. They estimated that they were able to analyze sub-visible particles with a size ≥1.8 µm. Nishi et al. described the use of flow-cytometry for the label-free detection of sub-visible aggregates in liquid IgG1

antibody formulations prepared under different stress conditions [43]. The authors used a BD FACS Canto II flow cytometer equipped with two lasers (488 and 633 nm), quantified the particle counts by acquiring all samples for a fixed time and compared their counts with results obtained with MFI and with light obscuration. The authors concluded that all three applied methods provided similar results for total particle counts.

We further explored the suitability of flow cytometry for the analysis of sub-visible particles by using this technology for the simultaneous detection, quantification, size estimation and characterization of sub-visible particles. For this purpose, we optimized the settings of a 3-laser 8-color FACS Canto II flow cytometer by tuning the forward scatter and the side scatter detectors and by using size calibration beads to facilitate the analysis of sub-visible particles with sizes below 1 µm. This way, we could reproducibly detect and characterize particles as small as 0.75 µm. This lower limit of detection for the particle size might be further reduced to 0.1 µm by using specialized flow cytometers with improved optics and an optimized detector design [44]. Importantly, the particle size is always an estimate because the refractive index of the particle, which influences the size determination by flow cytometry [45], is different for each particle and therefore unknown. Moreover, the morphology of the particle influences size determination. In contrast to amorphous particles, the detected size for a particle with a needle-like structure depends on the angle of the particle when it passes the interrogation point of the flow cytometer. Taking both aspects into consideration, it is important to emphasis that the presented flow-cytometry-based technology facilitates comparative analysis of samples containing the same protein. It should not be used to compare samples containing completely different proteins.

We paid particular attention to the capacity of the FACS technology to detect single particles rather than swarms of small particles. Analyzing serial dilutions of particle solutions, we generated data sets which support this capacity. However, we are aware that protein aggregates are in a thermodynamic equilibrium [46]. They can change their morphology or return to their monomeric structure upon dilution. The linear decrease of particle count and size distribution, in correlation with the samples dilutions, demonstrated that the protein aggregates remained intact and did not separate into their building blocks when diluted under our experimental set ups. It is accepted, that protein aggregates, especially those containing cross-β-sheet structures, are at local energetic minimum, which facilitates the use of analytical tools for in vitro analysis of these aggregates [46]. To further minimize any

potential destabilizing effect of the analytical procedure on the stability of protein aggregates, the analysis should preferentially be done in the final protein formulation. The presented FACS technology is well suited to analyze samples in the final protein formulation. Flow cytometry data related to the size distribution of particles in protein solutions were in good agreement with data generated with the state-of-the art technology micro-flow imaging. However, the flow-cytometry-based technology seemed to be more sensitive which was reflected by counting higher absolute numbers of particles for each size range. We believe that the use of fluorescent dyes may circumvent detection problems that are related to translucent proteins [47, 48] which could be the reason for the higher sensitivity of the flow-cytometry-based technology.

A major advantage of flow-cytometry-based technologies for the analysis of sub-visible particles is the possibility to combine forward and side scattering with fluorescence detection. The BD FACS Canto II we used for our analysis is equipped with 3 lasers (405, 488, and 630 nm) and offers the maximum capacity of analyzing 8 different fluorescence colors on one particle. This setting would allow a comprehensive assessment of the major characteristics of each sub-visible particle provided that suitable fluorescence dyes which bind to specific structures of protein and non-protein particles become available.

When we initiated this study our major objective was to combine a fluorescence dye which binds to proteins and another dye which binds to cross-ß-sheet structures contained in proteins. We tested several different combinations of dyes and eventually chose Bis-ANS as a protein stain and DCVJ as a stain for cross-β-sheet structures in proteins. Given our optimized instrument settings for the BD FACS Canto II, the combination of these two dyes showed the best performance with respect to signal to noise ratio, reproducibility of staining and minimal cross-talk of the dyes into other filters. Nevertheless, we could not completely prevent some limited cross-talk between the two dyes. DCVJ fluorescence, the stain for cross-β-sheet structures shows some cross-talk into the channel for protein detection by Bis-ANS fluorescence. However, this cross-talk should not generate any analytical problem because all cross-β-sheet positive particles are protein particles.

Although we were able to differentiate all proteins included in our study from non-protein particles, we are aware that the binding of fluorescent dyes is influenced by the nature of the protein and the nature of the protein aggregate. Therefore, we would like to re-emphasize that the flow-cytometry-based technology facilitates comparative analysis of

samples containing the same protein. It should not be used to compare samples containing completely different proteins.

Using the flow-cytometry-based technology presented in this manuscript, we could clearly distinguish between non-protein particles such as silicone oil droplets and protein particles and could also study the interaction of proteins with silicone droplets. For method development, we used artificially high concentrations of silicone oil droplets. However, we do not consider these high concentrations to affect interactions between proteins and silicone oil droplets. Silicone oil presents one of the major sources for non-protein particles in formulations of protein therapeutics, especially if the proteins are formulated in prefilled glass syringes. These syringes are usually lubricated with silicone oil, which is sprayed onto the interior surfaces of the syringe during the syringe manufacturing process, to allow for smooth plunger movement [49]. Our data indicate that proteins can attach to the surface of silicone oil droplets creating particles with a non-protein core and a protein surface. These data confirm previous findings by Ludwig et al. [33]. Characterization of additional properties of proteins coated on silicone oil droplets (e.g. presence of cross-ß-sheet structure or of other structural characteristics) may provide important additional information in the future.

In summary, we established a flow-cytometry-based technology for the analysis of sub-visible particles in protein formulations that facilitates the simultaneous analysis of size distribution, number and specific features of particles with a size range of 0.75–10 µm. The benefits of this technology are the relatively short time required for the analysis of each sample and the relatively small amount of required sample volume.

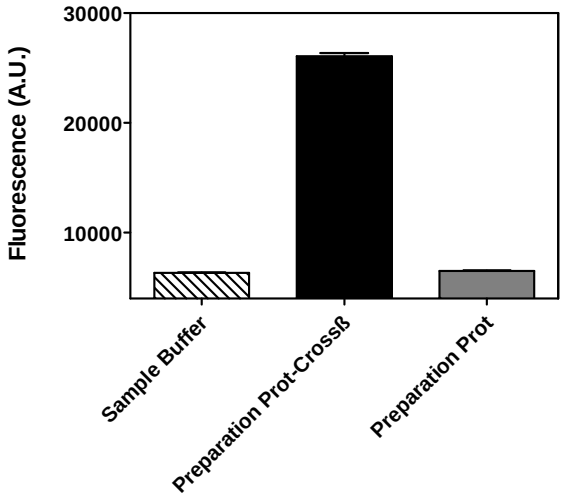
3.1.6 Acknowledgments and disclosure

The authors thank Elise Langdon-Neuner and Karima Benamara for editing the manuscript. This work was supported by Baxter Innovation GmbH. C.L., M.M., T.P., T.W., P.M., P.L.T., F.S. and B.M.R. are employees of Baxter Innovation GmbH.

3.1.7 Figures

Figure 1

A



B

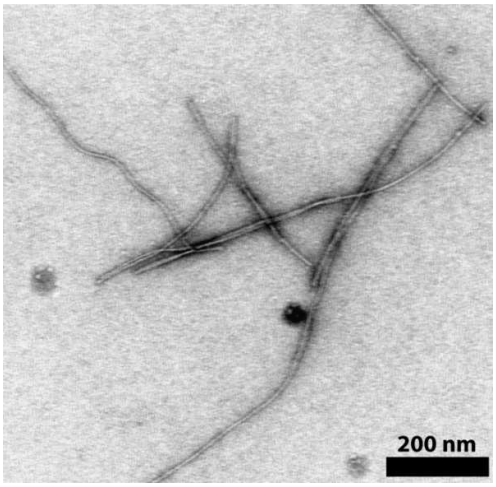


Figure 1: Confirmation of cross-β-sheet structures by ThT assay and electron microscopy

1A: Protein samples “Preparation Prot-Crossß” and “Preparation Prot” were mixed with ThT as described in the section Materials and Methods and subsequently analyzed for fluorescence using a Synergy™ 4 Hybrid Microplate Reader. “Sample Buffer” was included as negative control. Three replicates were analyzed for each sample. Results are presented as

mean + standard deviation. The high fluorescence signal for sample “Preparation Prot-Crossß” indicates the presence of cross-β-sheet structures.

1B: The morphology of cross-β-sheet containing protein aggregates in sample “Preparation Prot-Crossß” analyzed by transmission electron microscopy as described in the section Materials and Methods.

Figure 2

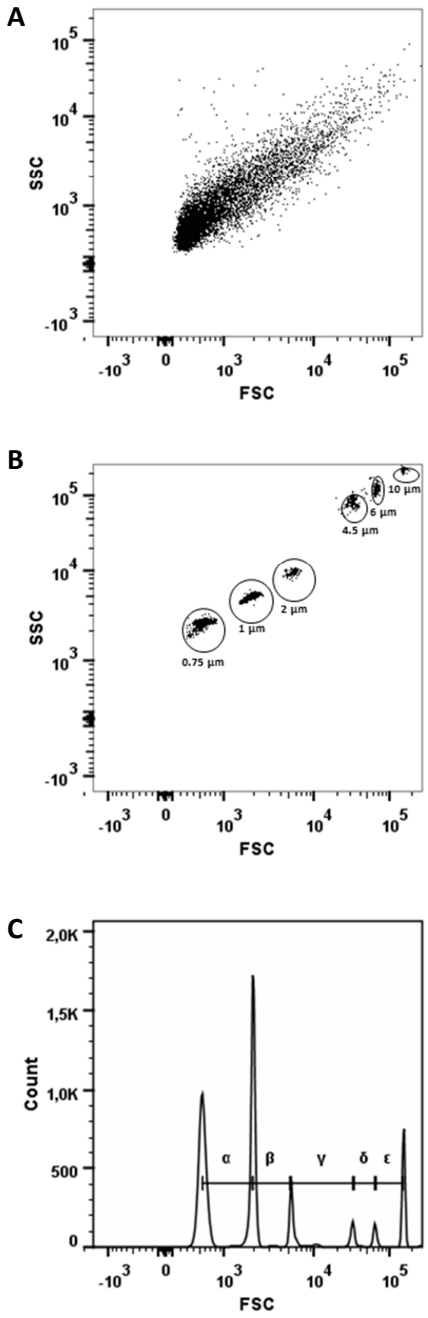


Figure 2: Detection and size distribution of sub-visible particles

A: Presented is a representative example for a dot plot of Forward Scatter (FSC) versus Side Scatter (SSC) using logarythmic scales. A sample of aggregated Aß1-40 peptide was generated and analyzed as described in the section Materials and Methods.

B: A representative example is shown for the calibration of the BD FACS Canto II using a mixture of size calibration beads with sizes of 0.75µm, 1µm, 2µm, 4.5µm, 6µm and 10µm.

C: Establishment of specific size ranges using the peak maxima for each size as determined by the size calibration beads in the FSC channel: 0.75-1 µm (α), 1-2 µm (β), 2-4.5 µm (γ), 4.5-6 µm (δ) and 6-10 µm (ε).

Figure 3

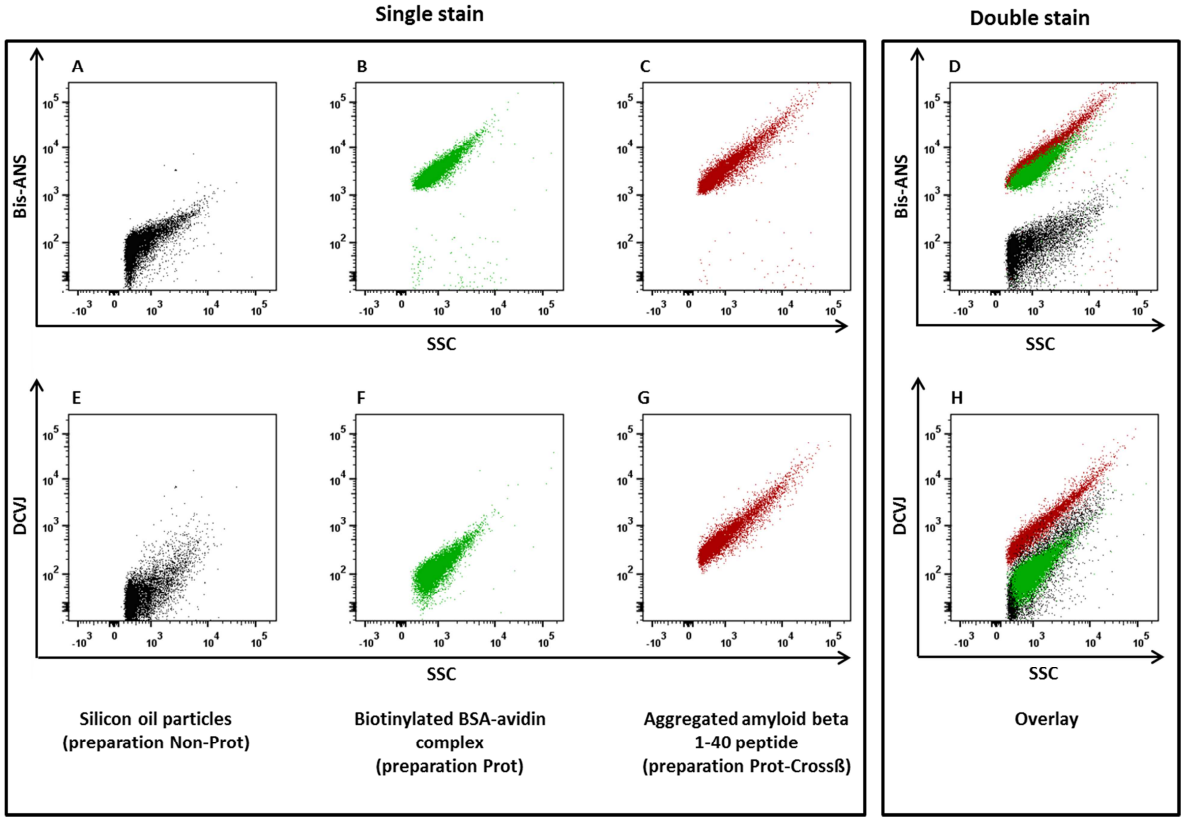


Figure 3: Characterizing the nature of sub-visible particles by staining with Bis-ANS and DCVJ

A-C: Comparison of silicon oil particles (preparation Non-Prot) with a complex of Biotinylated BSA and avidin (preparation Prot) and with a preparation of Aggregated amyloid beta 1-40 peptide (preparation Prot-Crossß) for their binding to Bis-ANS, a fluorescent dye which specifically binds to hydrophobic patches in proteins. Binding of Bis-ANS to the preparations of protein particles “Preparation Prot” and “Preparation Prot-Crossß” resulted in an increased Bis-ANS fluorescence intensity. In contrast, “Prepartion Non-Prot” containing silicon oil particles did not bind to Bis-ANS.

E-G: Comparison of silicon oil particles (preparation Non-Prot) with a complex of Biotinylated BSA and avidin (preparation Prot) and with a preparation of Aggregated amyloid beta 1-40 peptide (preparation Prot-Crossß) for their binding to DCVJ, a fluorescent dye which specifically binds to cross-β-sheets contained in protein particles. Binding of DCVJ to “Preparation Prot-Crossß” resulted in an increased DCVJ fluorescence intensity. In contrast,

“Prepartion Non-Prot” containing silicon oil particles and “Preparation Prot” containing protein particles without cross-β-sheets did not bind to DCVJ.

D, H: Double-staining of silicon oil particles (preparation Non-Prot), a complex of Biotinylated BSA and avidin (preparation Prot) and a preparation of Aggregated amyloid beta 1-40 peptide (preparation Prot-Crossß) with Bis-ANS and DCVJ. Presented are an overlay of fluorescence in the Bis-ANS channel [D] and an overlay of fluorescence in the DCVJ channel [H] for all three preparations.

Figure 4

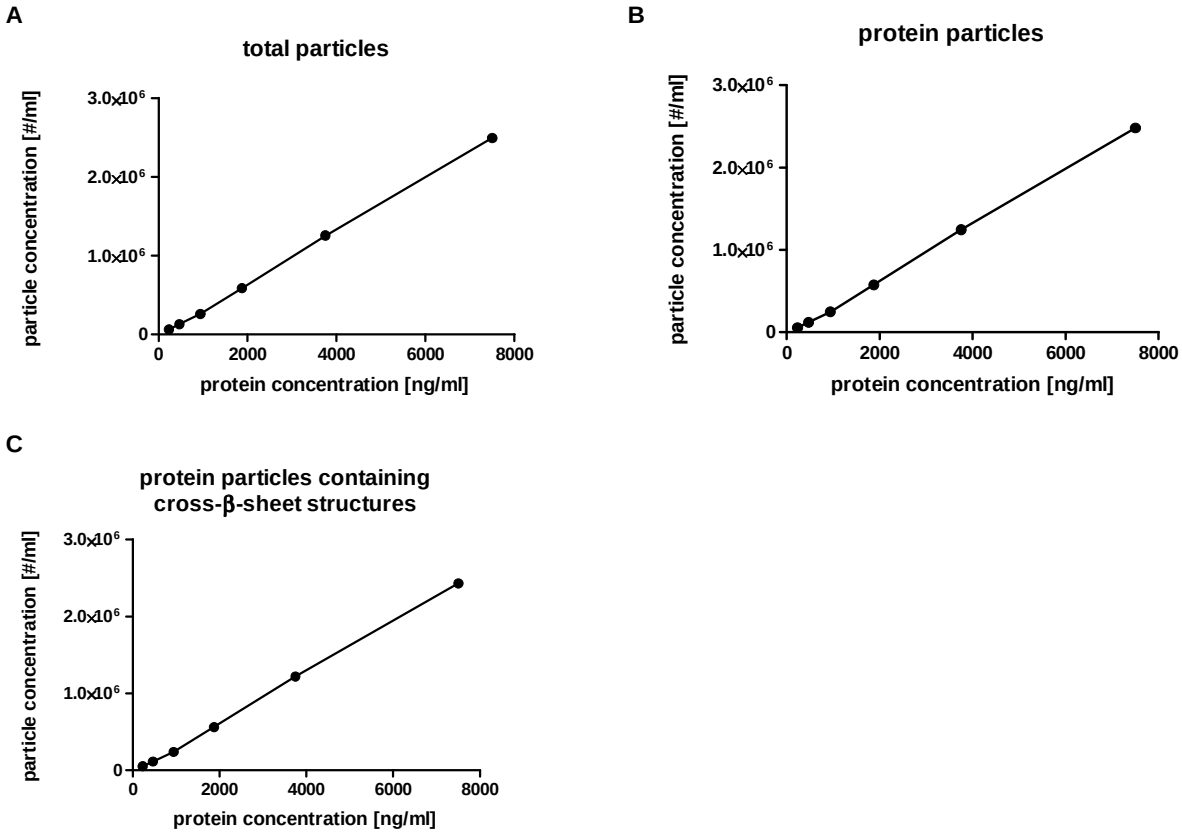
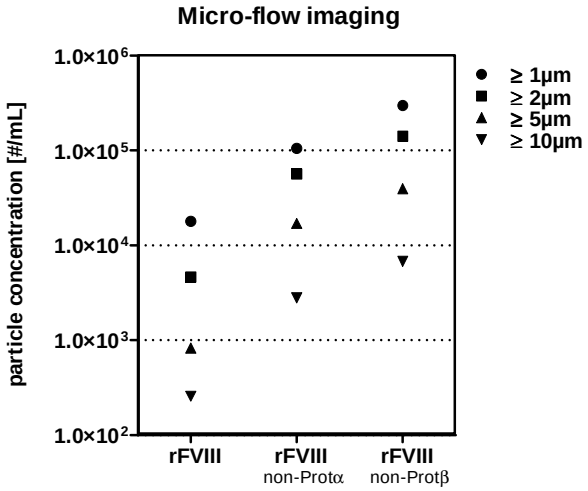


Figure 4: Linear relationship between protein concentration and detected particle count in the analysis of a preparation of protein particles using flow-cytometry

A preparation of aggregated Aß1-40 peptide (preparation Prot-Crossß), generated as described in the section Materials and Methods, was serially diluted. Each dilution was stained with Bis-ANS and DCVJ and subsequently analyzed by flow cytometry for the concentration of total particles [A], protein particles [B] and cross-β-sheet containing particles [C]. Presented are protein concentration and the concentration of sub-visible particles in the size range of 0.75-10µm. Each dilution was analyzed in 3 replicates for the concentration of sub-visible particles, mean numbers are presented.

Figure 5

A



B

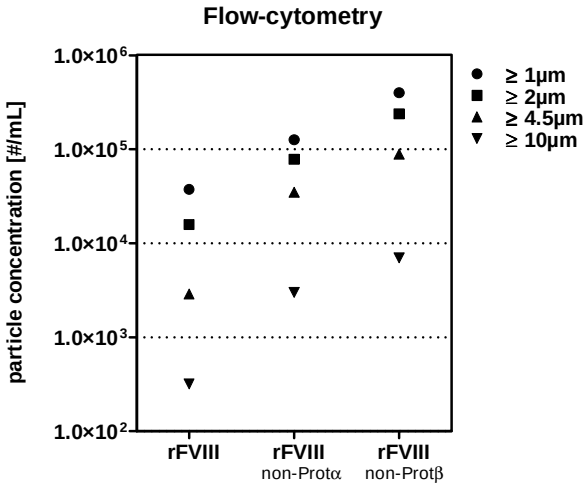


Figure 5: Comparison of flow-cytometry and micro-flow imaging for the analysis of sub-visible particles

A preparation of rFVIII was mixed with two different non-protein silicon particle preparations (rFVIII+ non-Protα; rFVIII+non-Protβ) as described in the section Materials and Methods and subsequently analyzed for the concentration of total sub-visible particles in different size ranges. For this purpose, the size ranges of the flow-cytometry-based technology were adapted to the size ranges covered by the micro-flow imaging technology,

namely ≥1, ≥2, ≥5 and ≥10µm. rFVIII without non-protein silicon particles was included as a control. Presented are the means of 3 replicates. The results obtained with the micro-flow imaging technology [A] were in good agreement with those obtained with the flow-cytometry-based technology [B]. Both technologies showed a concentration-dependent increase in sub-visible particle count after adding non-protein silicon oil particles to the preparation of rFVIII.

Figure 6

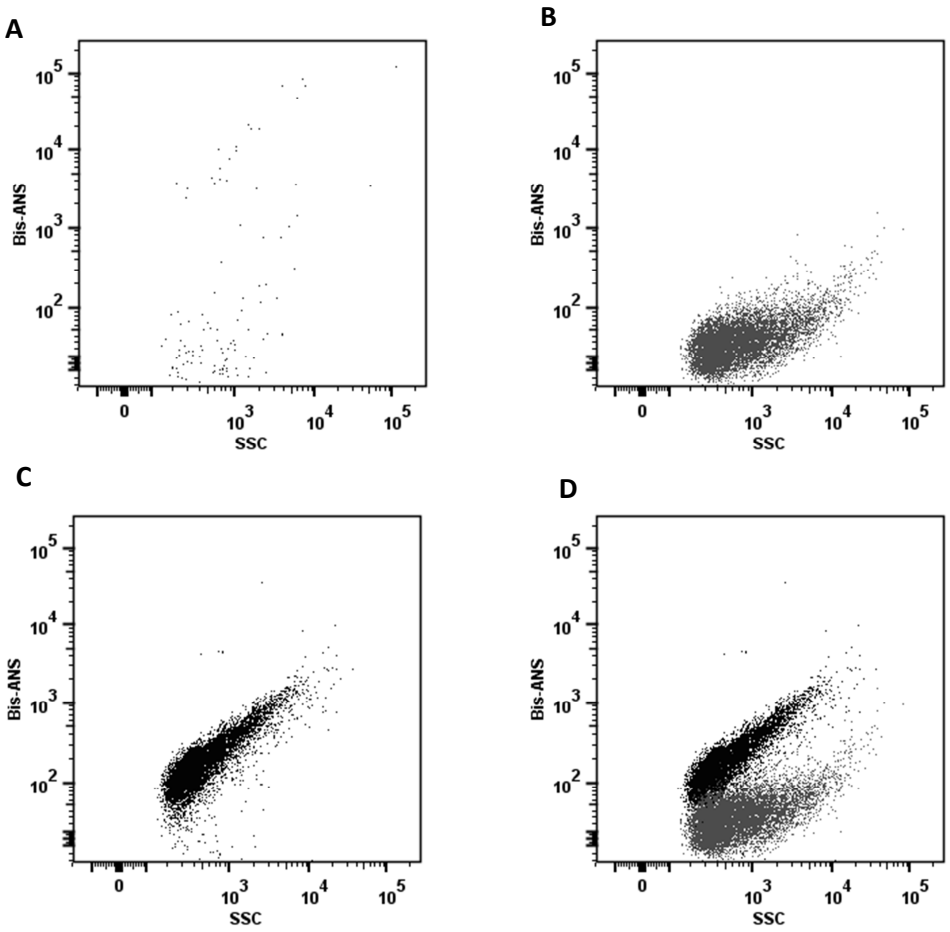
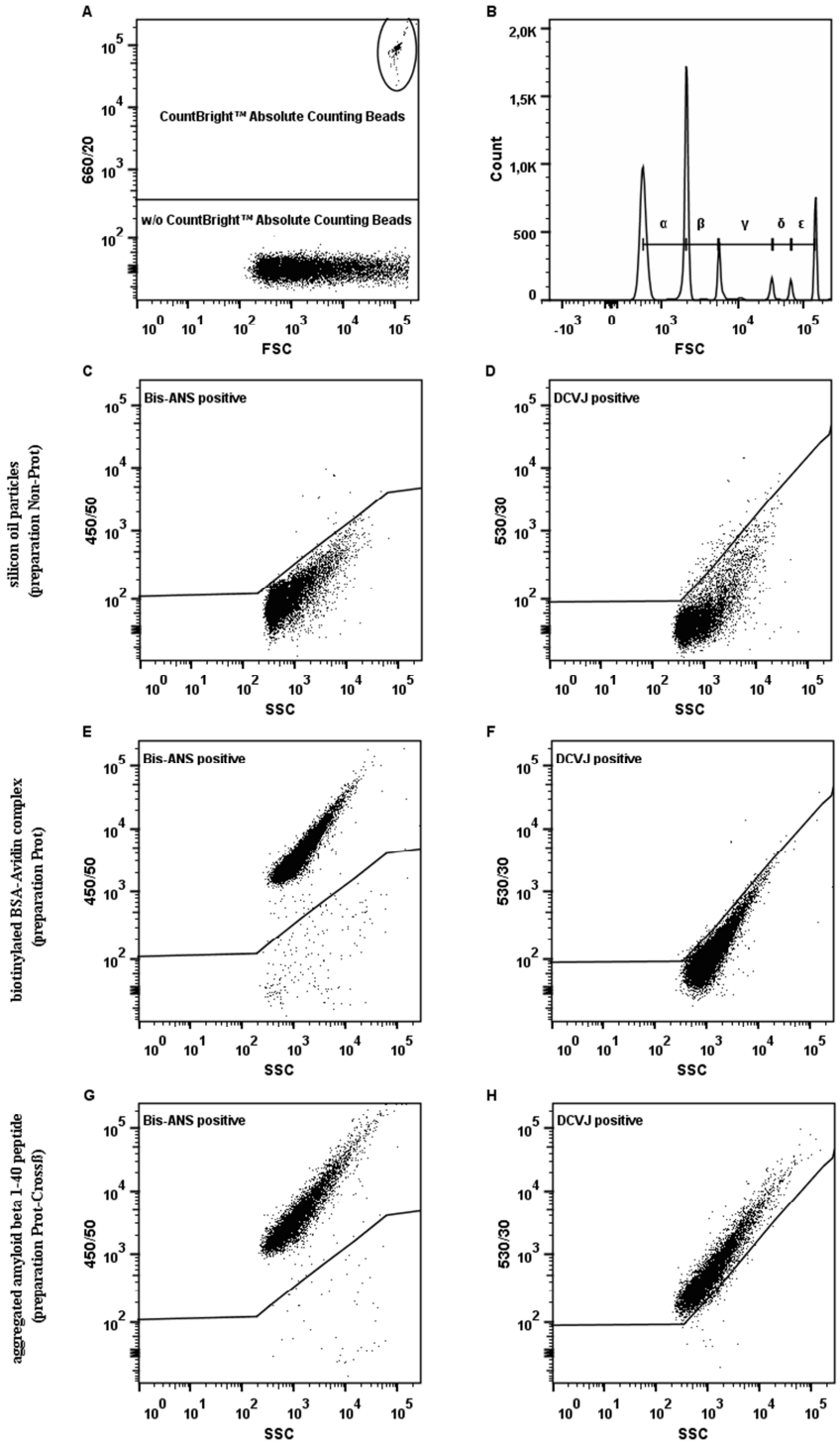


Figure 6: Detection of particles with a non-protein core and a protein surface

Preparations of rFVIII [A], silicon oil particles [B] and mixtures of rFVIII and silicon oil particles [C] were stained with Bis-ANS and subsequently analyzed for sub-visible particles by the flow-cytometry-based technology as described in the section Materials and Methods. For comparison of the Bis-ANS fluorescence intensities of silicon oil particles [B] and the mixture of rFVIII with silicon oil particles [C], an overlay of both graphs is presented in [D]. Whereas the 0.22 µm filtered rFVIII preparation presented in [A] contained few sub-visible particles, the preparation of silicon oil particles presented in [B] contained a substantial number of sub-visible particles which did not bind Bis-ANS. The mixture of rFVIII with silicon oil particles generated sub-visible particles which bound to Bis-ANS [C, D]. At the same time, particles which did not bind to Bis-ANS disappeared almost completely indicating the generation of particles with a non-protein core and a protein surface [C, D].

3.1.8 Supplement for chapter 3.1

Supplement Figure 1



Supplement Figure 1: General gating strategy

Presented is a representative gating strategy of the flow-cytometry-based technology to analyze sub-visible particles in protein-based therapeutics. Size range gates were controlled and if needed corrected using Fluoresbrite® YG Carboxylate Size Range Kit I & II [A]. CountBright™ Absolute Counting Beads were gated out using the 660/20 band-pass filter in the red detector array [B]. Gating on protein sub-visible particles in the Bis-ANS channel (violet detector array, 450/50 band-pass filter) was done by acquiring a non-protein sub-visible particle control (silicon oil particles, preparation Non-Prot) as a negative control reference [C] and protein sub-visible particle controls (biotinylated BSA-avidin complex, preparation Prot and aggregated amyloid beta 1-40 peptide, preparation Prot-Crossß) as positive controls [E and G]. Protein sub-visible particles bearing a cross-β-sheet structure were analyzed in the DCVJ channel (blue detector array, 530/30 band-pass filter) using preparation Non-Prot and preparation Prot as negative reference controls [D and F] for cross-β-sheet structures and preparation Prot-Crossß as a positive control [H]. All size range gates were applied for each staining gate.

Supplement Table 1A

run	particles/ml	CV %	protein particles/ml	CV %	amyloid-like protein particles/ml	CV %
1	2,903,406		2,889,310		2,595,874	
2	3,108,448		3,093,660		2,784,400	
3	2,350,739	**10.32 %**	2,338,488	**10.36 %**	1,765,834	**16.32 %**
4	2,688,654		2,666,654		2,495,028	
5	3,101,138		3,086,665		2,963,407	
6	2,700,258		2,690,203		2,486,555	

Supplement Table 1B

run	particles/ml	CV %	protein particles/ml	CV %	amyloid-like protein particles/ml	CV %
1	2,999,795		2,985,417		2,665,343	
2	2,862,464		2,847,235		2,607,556	
3	2,939,847	**3.92 %**	2,926,533	**3.79 %**	2,608,210	**2.05 %**
4	3,072,418		3,042,521		2,710,293	
5	2,740,746		2,725,321		2,570,223	
6	2,855,702		2,848,923		2,575,184	

Supplement Table 1C

	0.75µm-1µm		1µm-2µm		2µm-4.5µm		4.5µm-6µm		6µm-10µm	
run	particles/ml	CV %	particles/ml	CV %	particles/ml	CV %	particles/ml	CV %	particles/ml	CV %
1	892,144		263,112		160,163		20,257		10,314	
2	1,023,858		309,896		168,474		20,603		11,359	
3	704,284	**13.87 %**	208,040	**15.18 %**	124,717	**19.67 %**	14,227	**20.13 %**	7,938	**20.56 %**
4	852,891		277,445		213,051		24,287		11,561	
5	1,038,624		309,597		214,182		26,347		14,780	
6	855,401		235,712		164,945		19,461		10,014	

Supplement Tables 1A-1C: Precision and accuracy of the flow-cytometry based technology

The precision of the method was evaluated by assessing the inter- and intra-assay variations. Aggregated amyloid beta 1-40 peptide (preparation Prot-Crossß) was used as a positive control for analyzing concentrations (in particles/ml) of total sub-visible particles, protein sub-visible particles and cross-β-sheet protein particles. We analyzed preparation Prot-Crossß in 6 independent experiments for inter-assay variation [Table 1A] and in 6 parallel experiments on the same day for intra-assay variation [Table 1B]. Each sample was measured in 3 replicates in each experiment and mean numbers of sub-visible particles contained in 1 ml were calculated taking the dilution factor into account. This approach was used to calculate numbers of total sub-visible particles, protein and protein containing sub-visible particles and cross-β-sheet protein particles. All mean values for the inter-assay and for the intra-assay were used to calculate the coefficient of variation (CV %) for each property. We observed that the CV % of all the properties tested ranged between 10.32 %

and 16.32 % for the inter-assay variations and between 2.05 % and 3.92 % for the intra-assay variations. In addition, we tested the accuracy of the method regarding the size range gates by calculating the CV % of total sub-visible particle counts per ml for each size gate [Table 1C]. We met the acceptance criteria that were set to a maximum of 25 % for each CV which complies with regulatory guidelines [38, 39].

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3.2 Assessment of different recombinant FVIII preparations for the quantity of sub-visible particles and their properties

3.2.1 Introduction

BAX 855 is a PEGylated form of Baxalta's recombinant full-length FVIII product ADVATE™ for the treatment of hemophilia A. BAX 855 has been developed to increase the half-life of FVIII in the circulation of patients, making treatment more convenient [1]. Neutralizing antibodies against FVIII, so called FVIII inhibitors, are the most tremendous problem in current hemophilia A care [2;3]. The underlying immune mechanisms leading to the development of FVIII inhibitors are still poorly understood and we are still not able to predict the likelihood of a patient developing an unwanted immune response. Therefore, the immunogenic potential of BAX 855 has to be carefully evaluated.

Sub-visible particles, especially protein aggregates present in a therapeutic protein were recently linked to enhance the immunogenicity and therefor have the potential to induce unwanted immune responses [4-9]. Formation of protein aggregates is one of the possible outcomes to protein's structural alterations. It has been shown that the formulation buffer can influence the conformation of protein therapeutics [10]. Although product formulation development aim to maintain the protein molecules in their native state, protein aggregation may occur under different stress conditions like for example exposure to interfaces pH changes or light and temperature fluctuations [4,11]. In addition, non-protein particles that were previously documented to be present in protein therapeutics may provide nucleation sites and facilitate protein aggregation [12-16].

In the light of the potential impact of sub-visible particles in protein formulations on the immunogenicity of a protein therapeutic, it is essential to closely monitor und characterize novel FVIII preparations, like BAX 855 on their content of sub-visible particles. The aim of this study was to assess the total amount of sub-visible particles and their properties in the formulation of BAX 855 in comparison to ADVATE™, the base molecule of BAX 855 with a proven safety record.

3.2.2 Material and Methods

To compare the amount and nature of sub-visible particles in the formulation of BAX 855 with ADVATE™ we used three different Lots of BAX 855 and five different lots of ADVATE™ (Table 1).All lots were reconstituted with 5 mL of distilled water using a BAXJET II device and left for 15 minutes according to the manufacturer's instructions. A flow cytometry based assay was used for simultaneous detection, counting and characterization of sub-visible particles within the different lots of ADVATE™ and BAX 855 in a size range from 0.75 µm – 10 µm. The assay was performed as described in chapter 3.1.3 "Detection, Size Estimation, Characterization and Quantification of Sub-visible Particles by Flow-Cytometry". In brief, the flow cytometer was calibrated using CS&T beads (BD Bioscience) and Fluoresbrite®YG Carboxylate Size Range beads (Polyscience Inc.). Quantification of detected sub-visible particles was done using reference beads within the samples (Bright Count Beads, Invitrogen). The nature and properties of the sub-visible particles were analyzed using a unique combination of two fluorescent dyes. Bis-ANS binds to hydrophobic patches contained in proteins [17] and was used in a 20 µM concentration. Protein sub-visible particles were further characterized using 5 µM DCVJ (Sigma-Aldrich), which binds to cross-β-sheet structures present in aggregated proteins [17-19]. The prepared samples were measured subsequently after staining on a BD FACS Canto II.

3.2.3 Results

All samples were measured in triplicates and an average number of sub-visible particles was calculated taking the dilution factor into account. This approach was used to calculate total sub-visible particles, non- protein particles, protein particles and protein particles containing cross-beta-sheet structures. Results are presented in particles per mg of protein to be able to compare the different lots (Table 2 and Figure 1). An unpaired t-test was performed and showed no statistically significant difference between ADVATE™ and BAX 855 in their content of sub-visible particles. We conclude that there is no difference between different batches BAX 855 and ADVATE™ for presence of sub-visible particles using a flow cytometry based sub-visible particle detection method.

3.2.4 Figures and Tables

Table 1

rFVIII Lots	protein concentration (mg/mL)
ADVATE: Lot 1	0,114
Lot 2	0,1
Lot 3	0,0734
Lot 4	0,088
Lot 5	0,11
BAX 855: Lot 1	0,1044
Lot 2	0,0886
Lot 3	0,0882

Table 1: Protein concentration of different Lots of ADVATE™ and BAX 855 for comparison of sub-visible particles

Table 2

rFVIII Lots	total sub-visible particles (#/mg)	Non-protein particles (#/mg)	Protein particles (#/mg)	amyloid like (#/mg)
ADVATE™: Lot 1	3788895	1680693	1890947	217254
Lot 2	7237680	1498370	5436250	303060
Lot 3	6213678	584196	5197125	432357
Lot 4	11424932	614670	10474080	336182
Lot 5	5392536	506800	4381364	504373
BAX 855: Lot 1	9033611	3092519	5012730	928362
Lot 2	7269707	2111580	5050892	107235
Lot 3	6283039	1936122	4177880	169036

Table 2: Assessment of ADVATE™ and BAX 855 for the quantity of sub-visible particles and their properties using flow cytometry. All samples were measured in triplicates and an

average number of sub-visible particles was calculated taking the dilution factor into account. Values are calculate and presented on particles per mg of protein.

Figure 1

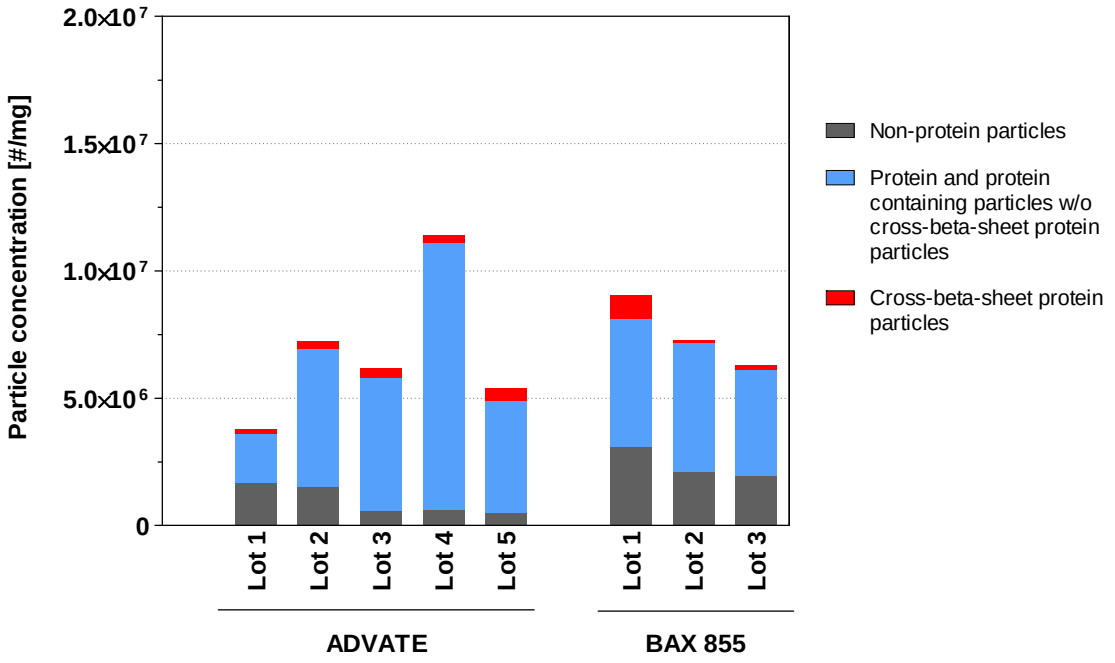

Figure 1: Different Lots of ADVATE™ and BAX 855 were analyzed using a flow cytometry based approach to quantify and characterize sub-visible particles in the range of 0.75 µm – 10 µm.

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3.3 The mystery of antibodies against polyethylene glycol (PEG) – IgM and IgG anti-PEG antibodies in healthy individuals

3.3.1 Abstract

Covalent attachment of polyethylene glycol (PEG) polymers has emerged as a successful strategy to increase the half-life and stability of protein therapeutics. Several PEGylated factor VIII and factor IX products are currently in development for the treatment of hemophilia A and B. PEG has long been considered to be a non-immunogenic moiety, however, recently published data indicate anti-PEG antibody formation in patients after treatment with PEGylated proteins. Anti-PEG antibodies in healthy individuals who have not received PEGylated therapeutics have also been reported. Some of these findings have evoked controversy due to shortcomings in the assays used for antibody analysis. Using a robust technology platform including two different analytical approaches, we generated data which confirm that a proportion of healthy individuals express anti-PEG antibodies of isotype IgM and/or IgG. The antibodies are either transient or persistent and not associated with any obvious pathology. We also provide a mechanism for anti-PEG antibody induction which supports the hapten nature of PEG. Based on these data, we conclude that the propensity of PEGylated proteins to induce anti-PEG antibodies in patients depends on the immunogenicity of the protein part. Finally, we present evidence that anti-PEG antibodies are not likely to cross-react with human tissues, which supports the benign nature of these antibodies.

3.3.2 Introduction

Protein therapeutics have become essential in the treatment of serious life-threatening and chronic diseases. However, many such compounds have short half-lives and induce unwanted immunogenicity, which limit their clinical utility [1, 2]. Several approaches to overcome these limitations have been tested, e.g. changing the amino acid sequence, attaching polymers, or fusing them to other proteins [3]. Covalent attachment of polyethylene glycol (PEG) polymers has emerged as a successful strategy to increase protein half-life and stability [4-7]. PEG is a polymer with a repetitive chemical structure [HO–(CH2–CH2–O)n–H], which is able to bind water molecules, is chemically inert and has low toxicity [1, 2, 8].

Since the end of 2014, 11 PEGylated peptide/protein therapeutics were approved in the US and Europe, including PEG-adenosine Deaminase (Adagen™) [9], PEG-asparaginase (Oncaspar™) [10], PEG-interferon-α2b (PegIntron™) [11], PEG-interferon-α2a (Pegasys™) [12], PEG-human growth hormone mutein antagonist (Somavert™) [13], PEG-anti vascular endothelial growth factor (Macugen™) [14], PEG-Granulocyte-Colony Stimulating Factor (Neulasta™) [15], PEG-erythropoietin (Mircera™) [16], PEG-anti-tumor necrosis factor Fab' (Cimzia™) [17], PEG-uricase (Pegloticase; Krystexxa™) [18], and Peginterferon beta-1 (Plegridy™) [19]. No long-term safety concerns have been identified for these therapeutics [20]. Further PEGylated compounds are in preclinical and clinical development including several PEGylated factor VIII (FVIII) and factor IX (FIX) therapeutics for the treatment of hemophilia A and B [21].

PEG has long been considered to be a non-immunogenic moiety and has been used to potentially protect proteins from the host's immune system [1, 22]. Recently, however, several reports have presented data indicating anti-PEG antibody formation after treatment with PEGylated therapeutics. In some cases, anti-PEG antibodies were associated with reduced efficacy or hypersensitivity [23-26]. PEGinesatide (Omontys), a PEGylated synthetic peptide-based erythropoiesis-stimulating agent, was recently withdrawn from the market due to severe hypersensitivity reactions which could be related to PEG [27, 28]. The FDA therefore recommends anti-PEG antibody analysis in all subjects treated with experimental PEGylated protein therapeutics, and assessment of whether these antibodies are associated with clinically significant changes in efficacy or safety [29].

Anti-PEG antibodies have also been detected in healthy individuals who have not received PEGylated protein therapeutics, suggesting that exposure to PEG polymers and PEG-like polymere structures (e.g. Tween) contained in consumer products or in pharmaceutical applications may cause anti-PEG antibody formation [30-32]. PEG polymers are used in a variety of cosmetics because of their solubility and viscosity and low toxicity [33]. PEG polymers are also contained in laxatives and used in several oral and parenteral applications in the pharmaceutical industry [34, 35]. Importantly, anti-PEG antibodies found in healthy individuals have not been associated with any health issues.

Some of the reports on anti-PEG antibody development have evoked controversy due to shortcomings in the assays used for analysis of these antibodies [36, 37]. Lack of information regarding assay sensitivity and specificity as well as the absence of assay validation have recently been highlighted as major weaknesses of these studies [36].

Here, we present comprehensive data sets, obtained in cross-sectional and longitudinal studies, on the prevalence of IgG and IgM antibodies against PEG in healthy human subjects. We established a fully validated ELISA technology platform to meet all requirements of current regulatory guidelines [38, 39] regarding sensitivity, cut-off calculation, specificity, linearity, and precision (inter- and intra-assay variability). For comparison, we also established a flow cytometry (FCM) approach which represents an optimized version of the technology described by Armstrong et al [23].

We also examine the immunological mechanisms which might be responsible for anti-PEG antibody induction. Our data indicate that PEG acts a hapten; it induces antibody responses when attached to an immunogenic protein that can provide CD4+ T cell epitopes, which are required to initiate the antibody response [40]. Our findings confirm previous hypotheses on the haptogenic properties of PEG [37, 41]. In light of these data, we discuss the potential risk of anti-PEG antibody development after treatment with PEGylated FVIII or FIX products in patients with hemophilia A or B. Furthermore, we show that anti-PEG antibodies are not likely to cross-react with human tissues, which supports the safety of PEGylated biotherapeutics.

3.3.3 Material and methods

Healthy human subjects and blood sampling

Cross-sectional studies included two cohorts of healthy individuals. Cohort 1 consisted of 710 healthy plasma donors from Austria, cohort 2 included 100 healthy plasma donors from Austria and 100 plasma donors each from Ammon (ID), Elkhart (IN), Fargo (ND), Lakeland (FL) and Laredo (TX) in the US. Thirty-eight healthy subjects from Austria were included in longitudinal monitoring of anti-PEG antibody persistence. For this purpose, blood samples were collected monthly up to 14 months.

All blood donors consented to blood sampling. Citrated plasma was obtained from each blood sample and stored at -20°C until analysis.

Detection of human anti-PEG antibodies

ELISA-based approach

A fully validated ELISA platform was established that included sensitive screening assays, competition-based assays to confirm antibody specificities, and titration assays to determine antibody titers. Details of the ELISA platform are provided in Online supplement 1. A 20 kDa branched PEG (Nektar Therapeutics) containing both ethoxy and terminal methoxy groups as well as an active N-hydroxy succinimide (NHS) ester group [42] was covalently bound to human serum-albumin (HSA) obtained from Baxalta and used as coating antigen. Importantly, no Tween 20 or Tween 80 was used in the assays. Details of the cut-off determination and the relative sensitivities of the ELISA assays are provided in Online supplement 1.

A pool of human plasma samples that tested positive for the presence of specific anti-PEG IgG antibodies was included as a positive control for IgG anti-PEG antibodies in each assay. A monoclonal rabbit IgM anti-PEG antibody (Epitomics) was used as positive control for IgM anti-PEG antibodies. Negative controls were pools of human plasma samples that tested negative for the presence of anti-PEG IgG or IgM antibodies.

All ELISA assays for the detection of IgM and IgG anti-PEG antibodies were validated regarding precision (inter-and intra-assay variability), specificity, linearity, and robustness. Details of the validation procedures are given in Online Supplement 1.

Flow Cytometry based approach

The flow cytometry method (FCM) for detection of human anti-PEG antibodies was an optimized version of the method described by Armstrong et al [23]. A multi-tiered approach that included sensitive screening assays, competition-based confirmatory assays to confirm antibody specificities, and titration assays to determine antibody titers was established. Details of the FCM technology and the cut-off determination of the FCM technology are provided in Online Supplement 2. Importantly, no Tween 20 or Tween 80 was used in the assays.

A monoclonal rabbit IgG anti-PEG antibody (clone PEG-B-47; Epitomics) was used as positive control for detection of IgG anti-PEG antibodies, and a monoclonal mouse IgM anti-PEG antibody (clone AGP3; IBMS Academia Sinica) as positive control for the IgM anti-PEG assays. Pools of human plasma samples that tested negative for the presence of anti-PEG IgG or IgM antibodies were included as negative controls.

Mouse studies

All mouse studies were approved by the local authority in Vienna, Austria, in accordance with Austrian federal law (Act BG 501/1989) regulating animal experiments, and by the institutional animal care and use committee.

Hemophilic E17 mice are characterized by a targeted disruption of exon 17 of the F8 gene [43, 44]. Hemophilic human F8 transgenic mice express a transgene of a native human F8 cDNA and are immunologically tolerant towards the human native FVIII protein [45].

Male and female mice, 8-17 weeks of age, were intravenously injected with 8 weekly doses of 200 ng (~50 IU/kg) or 1000 ng (~400 U/kg) recombinant human FVIII or PEG-modified FVIII preparations (mFVIII1 and mFVIII2) obtained from Baxalta. mFVIII1 and mFVIII2 are human full-length recombinant FVIII preparations that were chemically modified with a 20-kDa branched PEG reagent. They differ mainly in degree of PEGylation [45] (Table 1). Blood

samples for antibody analysis were taken before the first dose and 1 week after the eighth dose.

Detection of murine IgG anti-PEG antibodies

Murine IgG antibodies against PEG were detected using the FCM as described in the section "Detection of human anti-PEG antibodies". A polyclonal goat anti-mouse IgG labeled with Allophycocyanin (BioLegend) was used as the secondary antibody. The cut-off for the assay was established using a statistical approach based on background signal levels of 53 plasma samples obtained from naïve mice as described by Jaki et al and Shankar et al [46, 47]. A pool of murine plasma samples that tested positive for the presence of specific anti-PEG IgG antibodies was included as positive control, and a pool of murine plasma samples that tested negative for the presence of anti-PEG IgG antibodies was used as negative control.

Detection of murine anti-FVIII IgG antibodies

Murine IgG antibodies against human FVIII were analyzed by ELISA as previously described [48].

Statistical analysis of antibody data

The prevalence of anti-PEG antibodies in healthy human donors and corresponding two-sided 95% confidence intervals (CIs) thereof were calculated as described by Wilson et al [49].

Subject-specific covariates (age and sex) were used to model the observed anti-PEG IgG and anti-PEG IgM antibodies in healthy plasma donors using a negative binomial hurdle model to account for excess zeros and over dispersion, as suggested by Bonate et al [50] and Loeys et al [51]. A fully specified model (including covariates sex and age) was compared with the null model (trivial model with intercepts only) using the likelihood ratio test. These statistical analyses were performed with R Version 3.0.2 using R package pscl [52]. The level of statistical significance was set to 5%.

Agreement between FCM (comparative method) and ELISA (candidate method) regarding antibody response was estimated for anti-PEG IgG (n=95) and anti-PEG IgM (n=57) antibodies according to FDA guidance for industry and FDA staff [53]. Overall percent

agreement is an estimate for the percentage of samples with equal antibody response and was calculated as the percentage of double positives and double negatives from total samples. Two-sided 95% CIs were calculated according to Wilson et al [49]. Statistical analyses were done using R version 3.0.3 (R Development Core Team, 2013).

Potential cross-reactivity of anti-PEG antibodies with human tissues

The potential cross-reactivity of rabbit IgG anti-PEG monoclonal antibody (clone PEG-B-47/ KA=1,1x1011 M-1, Epitomics) with a wide range of normal human tissues (Table 4) was assessed by the CRO Asterand (UK) during a GLP-study using an optimized protocol for immunohistochemistry (IHC). The anti-PEG-B-47 antibody recognizes PEG polymers of different lengths with and without methoxy groups [54]. Details of the monoclonal antibody and the study design are outlined in Online Supplement 3.

3.3.4 Results

Prevalence and persistence of antibodies against PEG in healthy individuals

Two distinct cohorts of healthy individuals were used to study the prevalence of IgG and IgM antibodies against PEG. Values in parenthesis refer to two-sided 95% CIs. Using FCM for antibody analysis in cohort 1 identified a prevalence of 23% (20-27%) for total anti-PEG antibodies, with 9% (7-11%) for antibody titers ≥1:80 and 15% (12-17%) for titers <1:80 (Figure 1, Table 2). Thirteen percent (11-15%) of individuals had IgG anti-PEG antibodies, of which 2% (2-4) had titers ≥1:80 and 7% (5-9%) had titers <1:80 (Figure 1, Table 2). Fifteen percent (12-17%) of individuals expressed IgM anti-PEG antibodies, of which 7% (5-9%) had titers ≥1:80 and 8% (6-10%) had titers <1:80 (Figure 1, Table 2). Using the ELISA based approach in cohort 2 showed a prevalence of 24% (21-28%) for total anti-PEG antibodies, with 4% (2-5%) for antibody titers ≥1:80 and 23% (19-26%) for titers <1:80 (Figure 1, Table 2). Fourteen percent (11-17%) of individuals had IgG anti-PEG antibodies, out of which 3% (2-4%) had titers ≥1:80 and 12% (9-14%) had titers <1:80 (Figure 1, Table 2). 12% (10-15%) of individuals expressed IgM anti-PEG antibodies, of which 1% (0-2%) had titers ≥1:80 and 11% (9-14%) had titers <1:80 (Figure 1, Table 2). When comparing the prevalence among blood donors from 5 different locations in the US and Austria, the prevalence of IgM anti-PEG antibodies was similar for each location, whereas the prevalence of IgG anti-PEG antibodies varied between 0% (Laredo, TX, US) and 30% (Ammon, ID, US) (Figure 2).

Next, a head to head comparison of FCM and ELISA-based approach was run, including 95 samples for analysis of IgG anti-PEG antibodies and 57 samples for analysis of IgM antibodies. An overall percent agreement between the two technologies of 95.8% (89.7 - 98.4%) was shown for the detection of IgG anti-PEG antibodies, and of 89.5% (78.9-95.1%) for the detection of IgM anti-PEG antibodies (Online Supplement 4 Table 1). These data indicate that the analytical approaches yield similar results.

To assess whether anti-PEG antibody formation in healthy subjects is transient or persistent, 38 healthy subjects were periodically screened for IgM and IgG anti-PEG antibodies over 14 months. 20 subjects (52.6%) tested negative for IgG anti-PEG antibodies at each time point. Eleven subjects (28.9%) had confirmed positive titers ≥1:80 for at least one time point; 8 out of these 11 subjects (72.7%) showed persistent antibody expression. 7 subjects (18.4%) had

titers <1:80 for at least one time point, which were too low to be confirmed for specificity. For IgM anti-PEG antibodies, 22 subjects (57.9%) tested negative at each time point. Seven subjects (18.4%) had confirmed positive titers ≥1:80 for at least one time point; 5 out of these 7 subjects (71.4%) had persistent anti-PEG IgM antibodies. 9 subjects (23.7 %) had titers <1:80 for at least one time point, which were too low to be confirmed for specificity. Examples for a typical time course of anti-PEG antibodies are presented in figures 3A-C. One blood donor developed de novo transient IgM anti-PEG antibodies (Figure 3D).

There was no indication of any pathology associated with anti-PEG antibodies.

Specificity of antibodies against PEG

The specificity of anti-PEG antibodies was routinely confirmed by competition assays using soluble PEG 200 Da. To further investigate the specificity of anti-PEG antibodies, different PEG species for competition were used, including linear PEGs of 200, 2,000, and 20,000 Da and branched PEG of 20,000 Da. The branched 20,000 Da PEG contained terminal methoxy groups [42], the other PEGs contained none. Results presented in Table 3 indicate that anti-PEG antibodies found in healthy blood donors bind to different PEG polymers independent of the presence or absence of methoxy groups. However, the molar concentration of PEG required for 50% competition of the ELISA signal depended on the PEG molecule used. Comparison of the three linear PEG molecules which contained ethoxy groups but no methoxy groups showed the largest PEG molecule (20,000Da) to be the most efficient competitor. When comparing 20,000Da PEGs with and without methoxy groups, the branched PEG containing methoxy groups was most potent. This indicates that the anti-PEG antibodies detected in our study cohorts reacted with both ethoxy groups and terminal methoxy groups of PEG.

Correlation of donor covariates with the prevalence of anti-PEG antibodies

Assessment of the effect of sex and age on the prevalence of anti-PEG antibodies identified age as a significant covariate for the expression of IgG anti-PEG antibodies, with the highest prevalence in young adults (18-30 years old). The odds of expressing IgG anti-PEG antibodies decreased with each 10-year increase in age by a factor of 0.5682 (two-sided 95% CI: 0.5551 to 0.5817) in cohort 1 (Figures 4A, 4B), and by a factor of 0.6724 (two-sided 95% CI: 0.6570

to 0.6882) in cohort 2 (Figures 4C, 4D). Other correlations of covariates were not consistent for the two study cohorts and thus inconclusive.

PEG presents a hapten in the induction of anti-PEG antibodies

Previous studies have indicated that PEG itself is not immunogenic [55]. To evaluate whether PEG acts as a hapten that only induces anti-PEG antibodies when attached to an immunogenic protein, a PEG-modified human FVIII preparation (mFVIII1) was tested in two different mouse models. The first was a transgenic hemophilic mouse model that recognizes human FVIII as non-immunogenic self-protein, the second a conventional hemophilic mouse model that recognizes human FVIII as foreign protein. Based on the hapten hypothesis, only mice that recognize human FVIII as foreign protein should develop antibodies against PEG. A highly immunogenic positive PEG-FVIII control (mFVIII2) was also included, which was previously shown to break immune tolerance against human FVIII in the transgenic mouse model [45]. Hemophilic mice that recognize human FVIII as foreign protein developed high titers of both anti-FVIII and anti-PEG antibodies when treated with either mFVIII1 or mFVIII2 (Figure 5A). In contrast, transgenic hemophilic mice that recognize human FVIII as a self-protein only developed antibodies against PEG when treated with mFVIII2, which had previously been shown to break immune tolerance in this mouse model. Transgenic mice treated with mFVIII1 did not develop antibodies against PEG (Figure 5B), which supports the hypothesis that PEG behaves as a hapten.

Anti-PEG antibodies do not bind to human tissues

When PEG acts as a hapten, PEGylated coagulation proteins such as PEG-FVIII and PEG-FIX may induce anti-PEG antibodies in some patients whose immune systems recognize the FVIII or FIX, contained in PEGylated FVIII or FIX, as foreign protein. To assess whether anti-PEG antibodies cross-react with human tissues, which would need further safety attention, potential binding of a high-affinity anti-PEG antibody was tested against a panel of 36 different human tissues (Table 4). The results indicate that the anti-PEG antibody specifically bound to the positive control tissue (human liver tissue spiked with a PEGylated protein, Figure 6A), but not to any of the human tissues included in this study. An example is provided in Figure 6B.

3.3.5 Discussion

This study addresses several questions associated with the detection of circulating anti-PEG antibodies in both healthy individuals and patients treated with PEGylated biotherapeutics. Previous reports on anti-PEG antibody formation in healthy individuals and in patients treated with PEGylated biotherapeutics have evoked controversy due to shortcomings in the assays used for analysis of these antibodies [36, 37]. Using a robust technology platform that included two different analytical approaches, we generated data which confirmed that a proportion of healthy individuals express IgM and IgG anti-PEG antibodies which are either transient or persistent and not associated with any obvious pathology. We also provide a mechanism for the induction of anti-PEG antibodies that supports the hapten nature of PEG. Finally we present evidence that even high affinity anti-PEG antibodies are not likely to cross-react with human tissues.

Armstrong (2003) [56] reported a prevalence of >25% of anti-PEG antibody formation in 350 healthy individuals, Liu (2011) [57] reported 4.3% (n=350), and earlier, Richter (1984) [30] reported 0.2% (n=453). Our study, which included more than 1,000 healthy individuals from Austria and different locations in the US showed similar results to those of Armstrong et al [56], i.e. a prevalence of 23-24%. However, the majority of these individuals had titer antibodies (<1:80) which were too low to be confirmed for specificity, and thus could represent either specific or multi-reactive antibodies. Nevertheless, a proportion of healthy individuals (4-9%) showed anti-PEG antibody titers ≥1:80, specific for PEG, which is similar to that reported by Liu et al [57]. One explanation for the discrepancy in reported prevalence of anti-PEG antibodies in healthy individuals may be the different assay systems used in the individual studies. On the other hand, our data indicate a clear variation depending on the geographic location of the blood donor. Whereas none of the 100 donors from Laredo (TX, US) expressed anti-PEG IgG antibodies, 30 of 100 from Ammon (ID, US) did.

Competition assays showed that anti-PEG antibodies recognized different species of PEG, including PEGs of different sizes and PEGs with or without terminal methoxy groups. The competition efficiency of the different PEG species on a molar basis increased with polymer length and presence of terminal methoxy groups. Saifer [54] recently tested the specificity of various monoclonal and polyclonal anti-PEG antibodies raised against PEG polymers with or without terminal methoxy groups. The authors demonstrated that antibodies against PEG

polymers were never truly specific for methoxy PEGs or ethoxy PEGs, but recognized PEG polymers of different sizes with and without terminal methoxy groups. They concluded that the hydrophobicity of the PEG polymer rather than the presence or absence of a terminal methoxy group is the main determinant of the binding strength of PEG-specific antibodies.

So, why do healthy subjects who have never been treated with PEGylated biotherapeutics express anti-PEG antibodies and why does the prevalence of these antibodies depend on individuals' geographic location? PEG polymers and their derivatives have been widely used in cosmetics because of their solubility and viscosity and low toxicity [33]. PEG and PEG derivatives are common in toothpaste, skin lotions, deodorant sticks, shaving creams, hand creams, face makeup, cream rouge, blush, mascara, lipsticks, bath products, and hair care products [33]. Often, consumer exposure to these cosmetics is chronic and extensive, especially in young adults, which might explain the peak prevalence of anti-PEG antibodies in subjects aged 18-30 years. PEG polymers and their derivatives are also widely used in the pharmaceutical industry, as vehicles for drugs and as ointment bases, capsules, tablet and pill binders, suppositories, in liquid prescriptions, including parenteral, topical, ophthalmic, oral, and rectal preparations [33], and during manufacturing of certain vaccines [58]. An example is the use of PEG in the treatment of chronic constipation in children as young as 6 months [34, 59]. Clearly, many healthy individuals are exposed to PEG. Personal lifestyle, in particular regarding use of cosmetics, is likely to determine the extent of exposure, and could explain the observed differences in prevalence of anti-PEG antibodies according to location. The question remains whether PEG contained in cosmetics or pharmaceuticals can induce anti-PEG antibodies. We are not aware of any study which has addressed this question. However, there is evidence that smaller PEG polymers can be adsorbed after oral intake [23, 60]. If this is sufficient to induce anti-PEG antibodies remains to be shown. Our study data support the hapten character of PEG, indicating that PEG needs to be attached to immunogenic proteins or peptides to induce anti-PEG antibodies. On the other hand, PEGylated liposomes were shown to induce anti-PEG antibody responses in a T-cell independent manner [61]. Moreover, PEG polymers provide repetitive epitopes which may induce cross-linking of B-cell receptors. In the presence of concomitant innate immune activation (e.g. by triggering toll-like receptors), cross-linking of B-cell receptors could drive T-cell independent anti-PEG antibody responses as has been reported for other antigens with repetitive structures [62, 63].

Finally, we present evidence that a high-affinity anti-PEG antibody does not cross-react with human tissues. The tissue panel in our study was selected based on recommendations from regulatory authorities (FDA and EMA) [64, 65] for the use in tissue-cross reactivity studies of monoclonal antibodies. The data presented support the conclusion that persistent anti-PEG antibodies do not present a safety risk. This is in accordance to the apparent non-pathogenic character of anti-PEG antibodies observed in healthy donors

In summary, healthy individuals are shown to develop IgM and IgG antibodies against PEG without any pathology. These antibodies are temporary or persistent and are likely induced by exposure to PEG polymers present in cosmetics or pharmaceuticals. Induction of anti-PEG antibodies appears to be based on the hapten nature of PEG and requires the attachment of PEG to immunogenic peptides or proteins. According to this mechanism, patients who recognize the protein part of PEGylated FVIII or FIX products as immunogenic protein are likely to be at risk for antibody formation not only against the protein part but also against PEG. However, based on experience of anti-PEG antibody development in healthy individuals and on the negative outcome of our tissue cross-reactivity study, these anti-PEG antibodies are unlikely to be a safety concern.

3.3.6 Acknowledgment

The authors are grateful to Neriman Duman, Margit Pichler and Silvia Neppl for technical assistance. The authors also thank Karima Benamara for editing the manuscript.

3.3.7 Authorship Contribution and disclosure

C. L. designed research, performed experiments, analyzed and interpreted data, and wrote the manuscript; P.A. and F.M.H. established validated assay formats, and analyzed and interpreted data; M.d.l.R. designed and interpreted data from the tissue cross-reactivity study; A.B. performed statistical modeling of antibody titers; T.P. performed experiments and interpreted data; J.S. generated PEGylated proteins, designed and interpreted data from the prevalence study; M.W. designed, supervised, and performed the animal studies; F.S. interpreted data; B.M.R. designed research, analyzed and interpreted data, and wrote the manuscript. C. L., M.d.l.R., A.B., T.P., F.M.H., J.S., M.W., H.P.S., F.S. and B.M.R. are employees of Baxalta Innovations GmbH. This study was supported by Baxalta Innovations GmbH.

3.3.8 Tables

Table 1: Comparison of mFVIII1 and mFVIII2 with respect to PEGylation degree, specific activity, and VWF binding (adapted from van Helden et al [45])

	PEGylation degree, mol PEG/mol FVIII	Specific activity, FVIII:C/mg protein	VWF binding, KD, nM
mFVIII1	2	5773	0,82
mFVIII2	12	153	7,23

Table 2: Prevalence of anti-PEG antibodies in healthy individuals

The prevalence in % and 95% confidence intervals (95% CI) is shown. Some samples contained more than one population of antibodies of different Ig isotypes which were individually assessed. Therefore, the prevalence of IgG and IgM antibodies does not add up to the total for positive individuals.

	Study Cohort 1 (n=710)			Study Cohort 2 (n=600)		
	total % (95% CI)	IgG % (95% CI)	IgM % (95% CI)	total % (95% CI)	IgG % (95% CI)	IgM % (95% CI)
Prevalence of positive individuals	23 (20-27)	13 (11-15)	15 (12-17)	24 (21-28)	14 (11-17)	12 (10-15)
Prevalence of antibodies with titers ≥ 1:80	9 (7-11)	2 (2-4)	7 (5-9)	4 (2-5)	3 (2-4)	1 (0-2)
Prevalence of antibodies with titers < 1:80	15 (12-17)	10 (8-13)	8 (6-10)	23 (19-26)	12 (9-14)	11 (9-14)

Table 3: Human IgG anti-PEG antibodies recognize PEG polymers of different sizes and chemistries

The PEG polymer species used for competition with the ELISA signal of human anti-PEG antibodies present in representative human plasma samples are shown. Results for competition efficiency of the different PEG polymers presented in the last column of the table relate to one representative example.

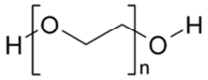
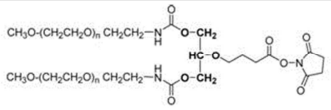
PEG polymer	Structure	Number of ethoxy monomer groups	Methoxy Group	recognition by polyclonal IgG anti-PEG antibodies	[µM] for 50 % OD reduction
linear PEG 200Da		~4	no	yes	2700
linear PEG 2kDa		~40	no	yes	1,39
linear PEG 20kDa		~450	no	yes	0,125
branched PEG 20kDa [42]		~450	yes	yes	0,00926

Table 4: Human tissue panel used in the tissue cross-reactivity study

36 different tissues were included in the tissue cross-reactivity study. For each tissue, triplicates from three different donors were analyzed.

Human tissue types			
1	Adrenal Gland	19	Parathyroid
2	Bladder	20	Parotid
3	Bone marrow	21	Peripheral nerve
4	Brain: Cerebellum	22	Pituitary
5	Brain: Cerebral cortex	23	Placenta
6	Breast	24	Prostate
7	Colon	25	Skeletal muscle
8	Eye: Retina	26	Skin
9	Fallopian tube	27	Spinal cord
10	Heart	28	Spleen
11	Ileum	29	Stomach
12	Kidney (Glomerulus, Tubule)	30	Testis
13	Liver	31	Thymus
14	Lung: Parenchyma	32	Thyroid
15	Lung: Bronchus	33	Tonsil
16	Lymph node	34	Ureter
17	Ovary	35	Uterus - Cervix
18	Pancreas	36	Uterus - Endometrium

3.3.9 Figures

Figure 1

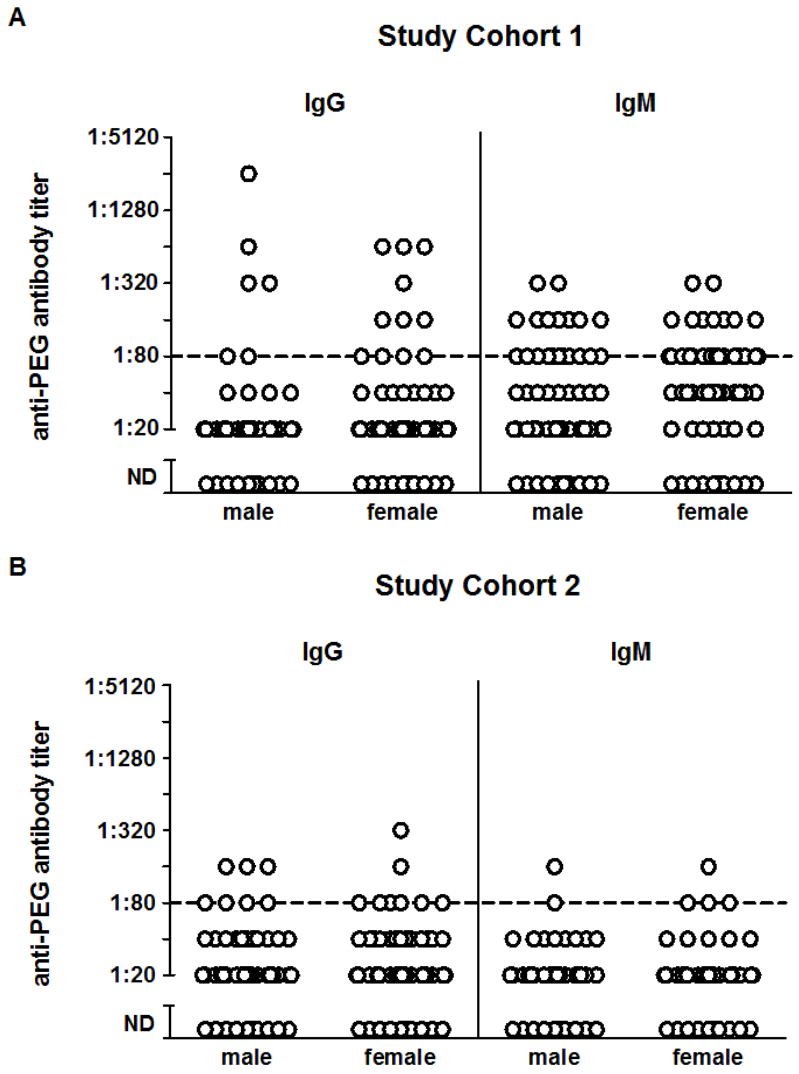


Figure 1: Anti-PEG antibodies in healthy individuals, assessed for individual IgG and IgM isotypes

Antibody titers of IgG and IgM anti-PEG antibodies as detected in study cohorts 1 and 2 are shown. Plasma samples obtained from 710 healthy subjects in cohort 1 were analyzed with FCM technology (A), plasma samples obtained from 600 healthy subjects in cohort 2 were analyzed using ELISA technology (B). An antibody titer of 1:20 was the detection limit for any anti-PEG antibody; a titer of 1:80 was the detection limit for anti-PEG antibodies with confirmed specificity (dotted line). ND: not detectable

Figure 2

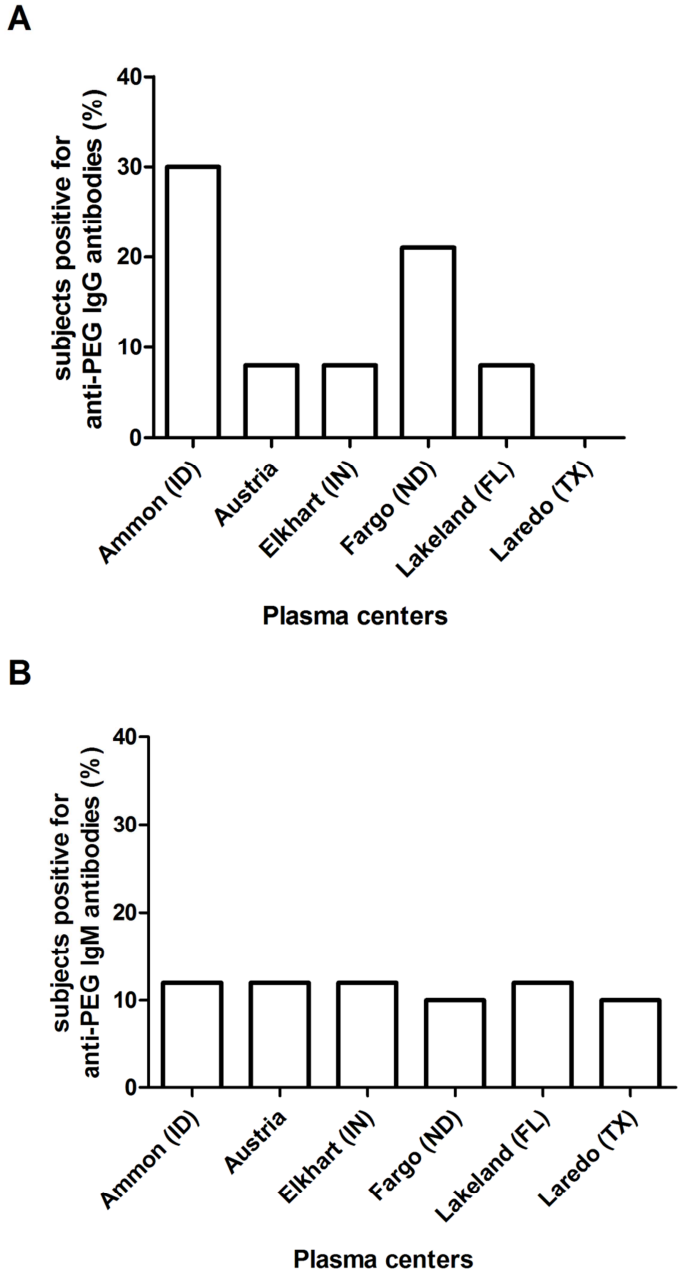


Figure 2: Prevalence of IgG and IgM anti-PEG antibodies in different plasma centers

The prevalence of IgG (A) and IgM (B) anti-PEG antibodies in plasma samples from healthy human subjects from different US locations and from Austria is shown. Plasma samples from 100 healthy individuals were analyzed from each center.

Figure 3

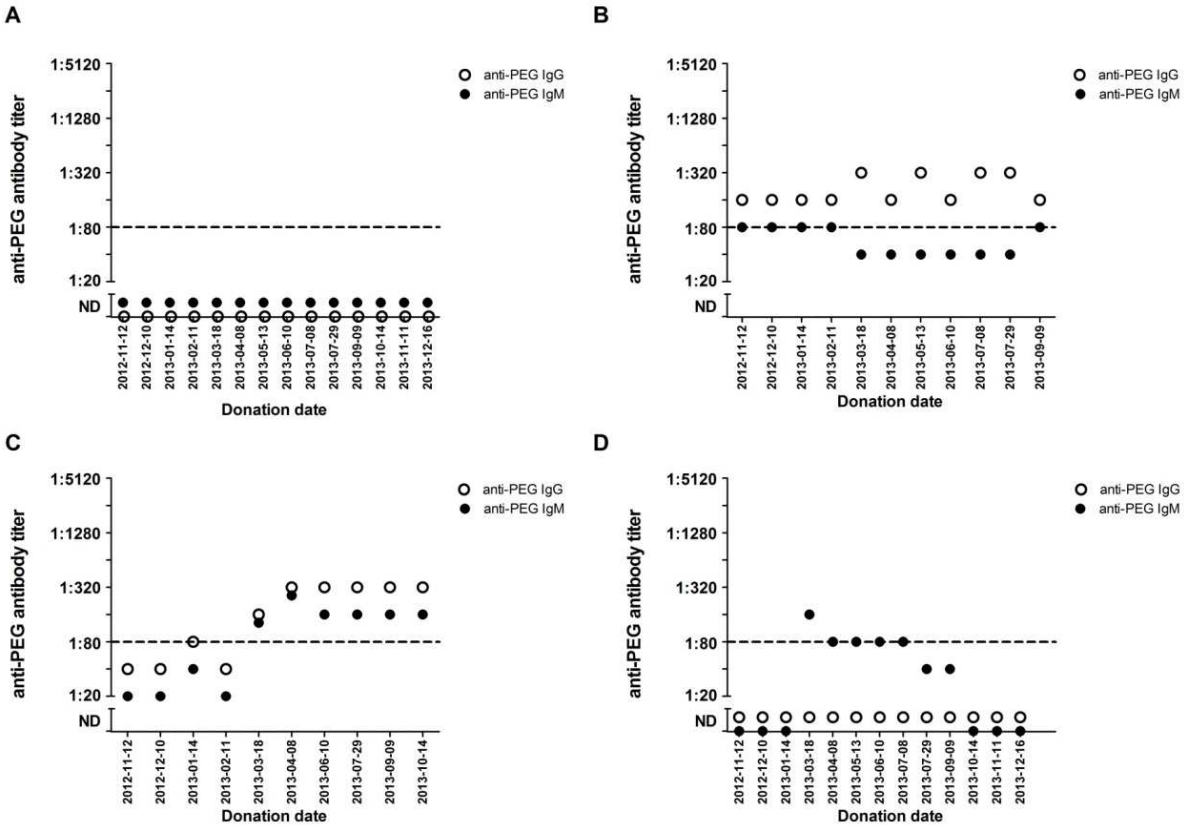


Figure 3: Persistence of IgG and IgM antibodies against PEG in healthy human subjects

Examples of different persistence patterns of anti-PEG antibodies observed in a cohort of 38 healthy subjects who were periodically screened are shown. A) Example of a subject who showed persistent negative results for anti-PEG IgG and IgM antibodies. B) Example of a subject who showed persistent positive results for anti-PEG IgG antibodies which were high enough in titer to be confirmed for specificity. This subject also expressed anti-PEG IgM antibodies which were not always high enough in titer to be confirmed for specificity. C) Example of a subject who initially expressed low titers of anti-PEG IgM and IgG antibodies which were too low to be confirmed for specificity. During the study, titers for both IgM and IgG antibodies increased to ≥1:80, which was high enough to confirm the specificity of the antibodies. D) Example of a subject who developed a de novo transient IgM anti-PEG antibody during the course of the study. IgG anti-PEG antibodies remained negative during the whole study period. A titer of 1:80 was the detection limit for anti-PEG antibodies with confirmed specificity (dotted line). ND: not detectable

Figure 4

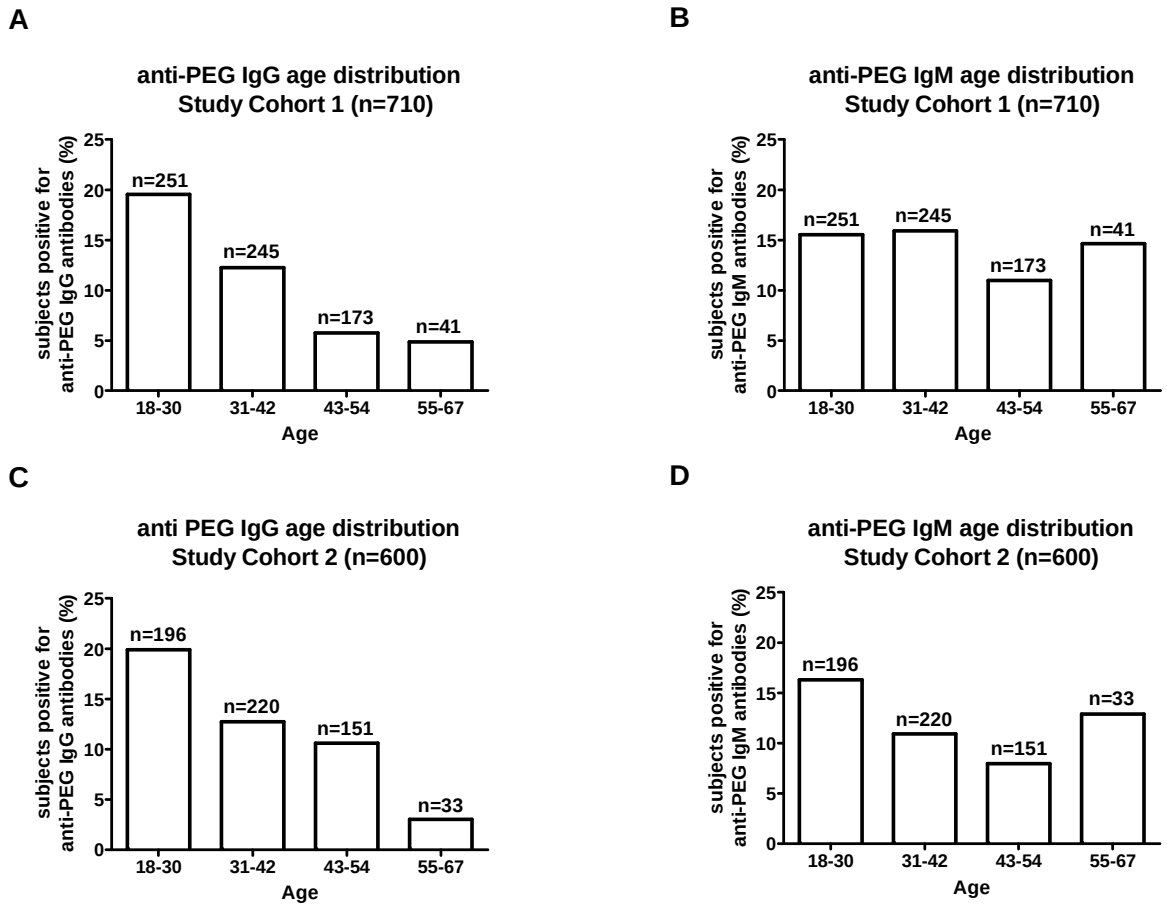
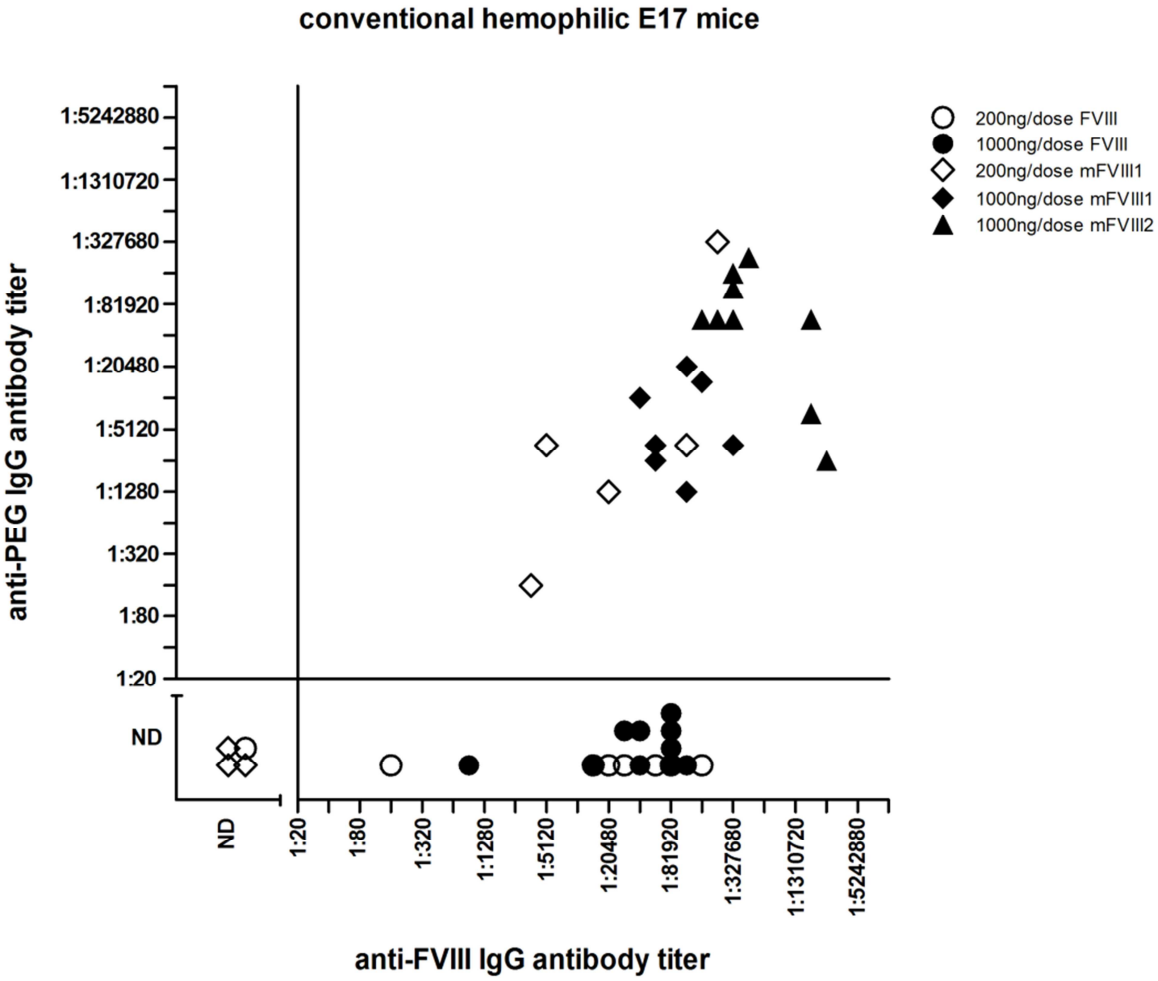


Figure 4: Impact of the age of healthy individuals on the prevalence of anti-PEG antibodies

The prevalence of IgG and IgM anti-PEG antibodies in relation to the age group of the blood donors in cohorts 1 (A, B) and 2 (C, D) is shown. The number above each column provides the total number of individuals analyzed in the respective age group.

Figure 5

A


conventional hemophilic E17 mice
anti-PEG IgG antibody titer
1:5242880
1:1310720
1:327680
1:81920
1:20480
1:5120
1:1280
1:320
1:80
1:20
ND
anti-FVIII IgG antibody titer
ND
1:20
1:80
1:320
1:1280
1:5120
1:20480
1:81920
1:327680
1:1310720
1:5242880
200ng/dose FVIII
1000ng/dose FVIII
200ng/dose mFVIII1
1000ng/dose mFVIII1
1000ng/dose mFVIII2

B

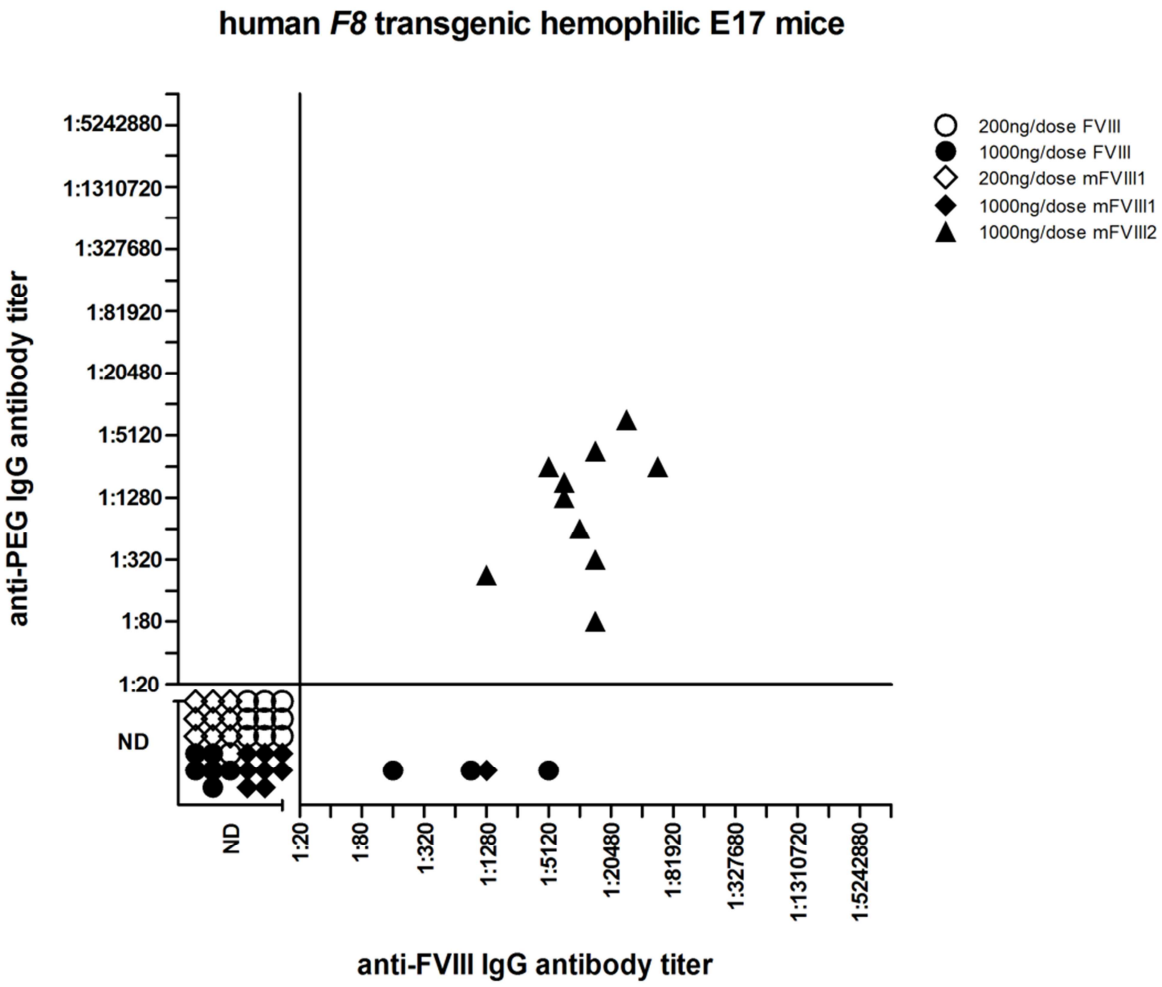
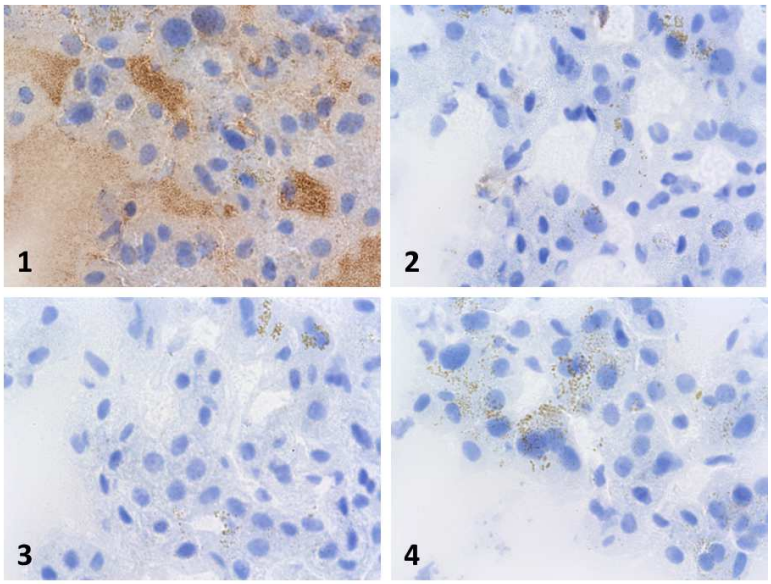


Figure 5: Development of anti-FVIII IgG antibodies and anti-PEG IgG antibodies after treatment of mice with human FVIII, mFVIII1 and mFVIII2

Two different mouse models were used to assess the immunogenicity of human FVIII and two versions of PEGylated human FVIII (mFVIII1, mFVIII2): a conventional hemophilic E17 mouse model that recognizes human FVIII as foreign protein (A), and a human *F8* transgenic mouse model that recognizes native human FVIII as self-protein (B). Mice were intravenously treated with 8 weekly doses as indicated. Antibody titers were assessed 1 week after the last dose. A titer of 1:20 was the lower limit of detection for both anti-FVIII and anti-PEG antibodies. ND: not detectable

Figure 6

A



B

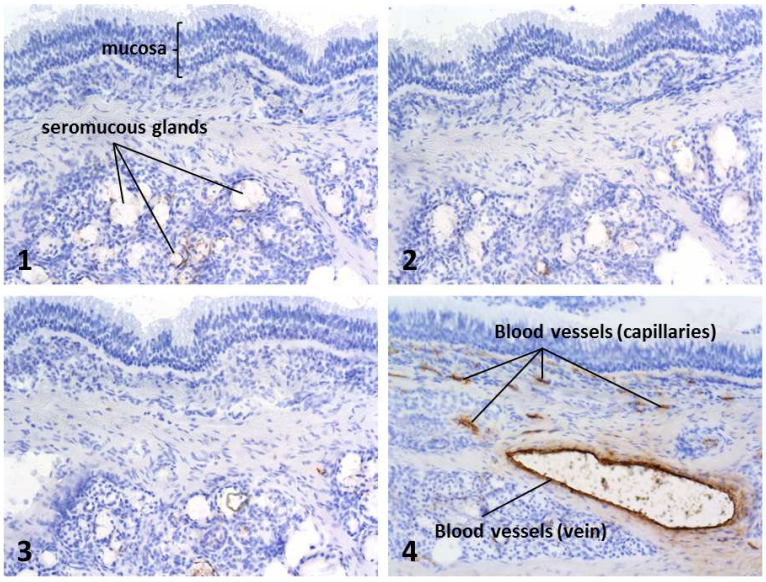


Figure 6: Anti-PEG antibody does not cross-react with human tissues

Representative results of the human tissue cross-reactivity study are presented.

A) Specificity of staining with the rabbit anti-PEG antibody (anti-PEG-47, Epitomics) used for the tissue cross-reactivity study. Sections of a PEG-spiked human liver were incubated with

the anti-PEG antibody after pre-incubation with PBS-buffer (1), soluble 200 Da PEG (2) or human PEG-FVIII (mFVIII1) (3). Normal rabbit IgG was included as negative control (4)
B) Representative example for a result of the tissue cross-reactivity study - staining of human bronchus tissue. Sections of human bronchus tissue were stained with anti-PEG antibody (1), with a normal rabbit IgG (negative control 1) (2), with the secondary anti-rabbit IgG antibody only (negative control 2) (3), or with an anti-human von Willebrand factor antibody (positive staining control) (4).

3.3.10 Supplement for chapter 3.3

Supplement 1

Detection of human anti-PEG antibodies

ELISA-based approach

The ELISA platform included a sensitive screening assay, a competition based confirmatory assay, and antibody titration assays.

Screening assays and determination of antibody titers

Screening assays were set up to exclude false negative data but allow 5% false positive data. Antibody titers of a sample were defined as the highest dilution step that gave a positive signal above the cut-off. Samples were diluted in geometric progression, starting at a dilution of 1:20 and continuing with 1:2 dilution steps. Each sample was analyzed at least twice. Based on assay validation (see below "Validation of ELISA assays for the detection of anti-PEG antibodies"), the assays showed a variability for the antibody titer of ±1 dilution steps. Therefore, a difference of 2 titer steps between the results obtained with the same sample in 2 different runs was the maximum accepted variation. Otherwise a third analysis was done. If the difference in titer was only 1 titer step, the higher titer was reported; otherwise, the median titer was reported.

Competition based confirmatory assays

Competition based confirmatory assays were established to confirm the specificity of the antibody signals found in the screening assays and to exclude false positive results. Plasma samples and controls were pre-incubated with soluble PEG 200kD (Sigma Aldrich) in a ratio of 1:8, v/v) for 1 hour at room temperature, and subsequently assessed for antibody titers as described above. Antibody specificity was confirmed when the competition assay showed an antibody titer that was at least 3 dilution steps lower than that detected without competition. Differences in antibody titers of 1 or 2 dilution steps may simply reflect the variability of the method. Thus, only plasma samples with an antibody titer ≥1:80 were evaluated for specificity. Antibody titers of 1:20 and 1:40 are too low to be confirmed for specificity and might reflect the binding of unspecific antibodies.

In some experiments, the specificity of anti-PEG antibodies was further evaluated by incubating plasma samples with different PEG species, e.g. PEG 2 and 20 kDa (Sigma Aldrich, Missouri, USA) and a branched PEG 20 kDa (Nektar Therapeutics, California, USA).

Cut-Off determination

Predetermined cut-offs were established using a statistical approach based on background signal levels of plasma samples obtained from 144 healthy plasma donors as described by Jaki et al and Shankar et al [1,2]. For detection of IgM anti-PEG antibodies, a daily floating cut-off was established. To account for daily variation, a "floating cut-point correction factor" was used as described by Shankar et al [2].

Relative Sensitivity

The relative sensitivity of the ELISA assay for detection of IgM anti-PEG antibodies as assessed using a monoclonal rabbit IgM anti-PEG antibody (clone PEG-2-128, Epitomics) was 160 ng/mL. The relative sensitivity of the ELISA assay for detection of IgG anti-PEG antibodies as assessed using a monoclonal rabbit IgG anti-PEG antibody (clone PEG-B-47; Epitomics) was 8 ng/mL.

Validation of the ELISA platform

The ELISA platform was validated for detection and titration of anti-PEG IgG and IgM antibodies in human plasma. For this purpose, inter- and intra-assay variation, specificity, linearity, and robustness were assessed to verify the method's reliability and accuracy.

The precision of the technology was defined as inter- and intra-assay variation. For inter-assay variation, positive and negative controls were analyzed on 4 independent days by three analysts. For intra-assay variation, positive and negative controls were analyzed in 10 parallels on the same plate on the same day by one analyst. Positive controls were serially diluted until the last positively assessed dilution was detected (titer endpoint). The acceptance criterion for precision of both ELISA assays was ± 1 dilution step, based on results obtained in pre-validation experiments. The mode titer of the positive controls was defined as the median titer detected during assessment of inter-assay precision. Assay validation was considered successful when the positive control did not differ more than ±1 titer step from the mode titer, and when all negative controls (diluted 1:20 and tested in 4 parallels)

tested negative. During routine analysis of plasma samples, negative and positive controls had to meet these acceptance criteria.

The specificity of the screening assay was verified using the confirmatory assay. Positive controls were serially diluted until the last positively assessed dilution was detected (titer endpoint). In addition, positive and negative controls were pre-incubated with soluble PEG 200kD (Sigma Aldrich) in a ratio of 1:8, v/v). Positive controls were subsequently serially diluted and analyzed for their titer endpoint. The specificity of the assay was considered confirmed when pre-incubation of the positive control lowered the titer endpoint for >2 titer values.

Robustness regarding repeated freezing/thaw cycles of samples was tested by analyzing a positive control sample that underwent five freeze/thaw cycles, and was considered successful when the positive control did not differ more than ± 1 titer step from the mode titer.

Linearity was defined as the relationship between antibody titer and absorbance, and was illustrated by an increase in signal resulting from an increasing amount of anti-drug antibodies using titration of the positive control.

Upon change of lot of a critical component (coating antigen, secondary conjugated antibodies, positive and negative controls), compliance of the new lot with the acceptance criteria during validation was verified by a requalification procedure.

References for supplement 1

1. Jaki T, Lawo JP, Wolfsegger MJ, Singer J, Allacher P, Horling F. A formal comparison of different methods for establishing cut points to distinguish positive and negative samples in immunoassays. *J Pharm Biomed Anal*. 2011;55(5):1148-1156.
2. Shankar G, Devanarayan V, Amaravadi L, et al. Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J Pharm Biomed Anal*. 2008;48(5):1267-1281.

Supplement 2

Detection of human anti-PEG antibodies

Flow Cytometry-based approach

A multi-tiered approach including a sensitive screening assay, a competition based confirmatory assay and the determination of antibody titers was applied for the flow cytometry platform.

The screening assay was set up to exclude false negative data but allow for 5% false positive data. The competition based confirmatory assay was set up to confirm the specificity of the antibody signals found in the screening assay and to exclude false positive results. The antibody titer of a sample was defined as the highest dilution step that still gave a positive signal above the cut-off. Samples were diluted in geometric progression, starting at a dilution of 1:20 and continuing with 1:2 dilution steps.

The specificity of anti-PEG antibodies was confirmed by competition as described for the ELISA assay (see Supplement 1)

Cut-Off determination

The cut-offs were established using a statistical approach based on background signal levels of 96 plasma samples obtained from healthy plasma donors as described by Jaki et al and Shankar et al [1,2].

References for supplement 2

1. Jaki T, Lawo JP, Wolfsegger MJ, Singer J, Allacher P, Horling F. A formal comparison of different methods for establishing cut points to distinguish positive and negative samples in immunoassays. *J Pharm Biomed Anal*. 2011;55(5):1148-1156.

2. Shankar G, Devanarayan V, Amaravadi L, et al. Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J Pharm Biomed Anal*. 2008;48(5):1267-1281.

Supplement 3

To assess potential cross-reactivity of anti-PEG antibodies with human tissues we used a anti-PEG rabbit monoclonal antibody (clone PEG-B-47/ $K_{association}$=1,1x10^{11}, Epitomics, California, USA). This antibody was induced by immunizing rabbits with a 5kDa methoxy PEG-keyhole limpet hemocyanin and was initially expected to be specific for the methoxy group of PEG polymers [1]. However, Saifer recently demonstrated that this antibody is not truly specific for methoxy PEG but also recognizes ethoxy PEGs. In fact, the authors showed that the antibody binds PEG oliglomers with more hydrophobic end-groups more tightly than a methoxy-terminated oligomer [2]. We used competition experiments as described in the main part of this manuscript, to test the specificity of this antibody and came to the conclusion that the antibody recognizes PEG polymers with and without terminal methoxy groups which confirms data presented by Saifer.

A rabbit normal IgG (Alere Inc) was used as a negative control. Both anti-PEG clone PEG-B-47 and the negative control were tested at 0.5 µg/mL which provided good staining results in set-up experiments. Tissue immunoreactivity was investigated including frozen, full-face sections from thirty-six different human tissues (Table 4 in main manuscript). Tissues from three different donors were included. The tissues were selected to meet the requirements of regulatory guidelines for tissue cross-reactivity studies of monoclonal antibodies [3,4]. A sample of frozen human liver was used to prepare positive and negative tissue control samples. For this purpose, PEG-HSA at a final concentration of 100 µg/mL (positive tissue control sample) or PBS (sham-treated negative tissue control sample) were spiked into aliquots of human liver homogenates which were subsequently transferred to pre-labelled plastic moulds and frozen on a bed of dry ice. From these samples, 12µm cryosections were prepared using the Leica cryostat and stored at ≤ -60 °C prior to further use.

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Supplement 4

Table 1: Overall agreement between FCM and ELISA

Agreement between FCM (comparative method) and ELISA (candidate method) regarding the detection of a positive signal for an anti-PEG antibody response was estimated in a head to head study for anti-PEG IgG (n=95) and anti-PEG IgM (n=57) antibodies according to FDA guidance for industry and FDA staff [50]. "0" indicates a negative result for anti-PEG antibodies, "1" indicates a positive result for anti-PEG antibodies.

Antibody	Sample number	Assay	
		ELISA	FACS
IgG	1	0	0
IgG	2	0	0
IgG	3	0	0
IgG	4	1	0
IgG	5	0	0
IgG	8	0	0
IgG	12	0	0
IgG	20	0	0
IgG	21	0	0
IgG	31	0	0
IgG	36	0	0
IgG	40	1	0
IgG	51	1	1
IgG	52	0	0
IgG	55	0	0
IgG	71	1	1
IgG	78	1	1
IgG	119	1	1
IgG	153	0	0
IgG	164	0	0
IgG	180	0	0
IgG	181	0	0
IgG	182	0	0
IgG	183	0	0
IgG	184	0	0
IgG	185	0	0
IgG	186	0	0
IgG	188	0	0
IgG	189	0	0
IgG	190	0	0
IgG	191	0	0
IgG	192	0	0
IgG	193	1	1

Antibody	Sample number	Assay	
		ELISA	FACS
IgG	194	0	0
IgG	195	0	0
IgG	196	0	0
IgG	197	0	0
IgG	199	0	0
IgG	200	0	0
IgG	202	0	0
IgG	203	1	0
IgG	204	0	0
IgG	205	0	0
IgG	206	0	0
IgG	208	0	0
IgG	209	0	0
IgG	210	1	0
IgG	211	0	0
IgG	212	0	0
IgG	213	0	0
IgG	214	0	0
IgG	216	0	0
IgG	218	0	0
IgG	219	0	0
IgG	220	0	0
IgG	222	0	0
IgG	225	0	0
IgG	227	0	0
IgG	228	0	0
IgG	228	0	0
IgG	229	0	0
IgG	229	0	0
IgG	230	0	0
IgG	231	0	0
IgG	232	0	0
IgG	233	0	0
IgG	234	0	0
IgG	237	0	0
IgG	238	0	0
IgG	240	1	1
IgG	241	0	0
IgG	242	0	0
IgG	243	0	0
IgG	244	0	0
IgG	245	0	0
IgG	246	0	0
IgG	247	0	0
IgG	249	0	0
IgG	250	0	0

Antibody	Sample number	Assay	
		ELISA	FACS
IgG	251	0	0
IgG	253	0	0
IgG	254	0	0
IgG	255	0	0
IgG	256	0	0
IgG	257	0	0
IgG	260	0	0
IgG	263	0	0
IgG	264	0	0
IgG	265	0	0
IgG	268	0	0
IgG	269	0	0
IgG	270	0	0
IgG	271	0	0
IgG	272	0	0
IgG	274	0	0
IgM	1	0	0
IgM	2	0	0
IgM	3	1	1
IgM	4	0	0
IgM	5	0	0
IgM	8	0	0
IgM	12	0	0
IgM	20	0	0
IgM	21	0	0
IgM	31	0	0
IgM	36	0	0
IgM	40	1	1
IgM	51	1	0
IgM	52	0	0
IgM	55	0	1
IgM	71	0	0
IgM	78	0	0
IgM	119	1	1
IgM	153	0	0
IgM	164	1	1
IgM	193	0	1
IgM	222	0	0
IgM	228	0	0
IgM	229	0	1
IgM	240	0	0
IgM	6391	0	0
IgM	6394	0	0
IgM	6395	0	0
IgM	6397	0	0

Antibody	Sample number	Assay	
		ELISA	FACS
IgM	6398	0	0
IgM	6402	0	0
IgM	6405	0	0
IgM	6406	0	0
IgM	6411	0	0
IgM	6414	0	0
IgM	6416	0	0
IgM	6424	0	0
IgM	6426	0	0
IgM	6428	1	1
IgM	6429	1	1
IgM	6438	0	0
IgM	6443	0	0
IgM	6444	0	0
IgM	6447	0	0
IgM	6448	0	1
IgM	6453	1	1
IgM	6465	0	1
IgM	6471	0	0
IgM	6473	0	0
IgM	6474	0	0
IgM	6475	0	0
IgM	6476	0	0
IgM	6477	0	0
IgM	6478	0	0
IgM	6479	0	0
IgM	6480	0	0
IgM	6485	1	1

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3.4 Preclinical immunogenicity assessment of Baxalta`s longer-acting FVIII candidate BAX 855 using novel preclinical models

3.4.1 Abstract

The aim of this study was to evaluate the immunogenicity of BAX855, Baxalta`s longer-acting PEGylated recombinant human factor VIII (FVIII) concentrate, in comparison to ADVATE, Baxalta`s unmodified recombinant human FVIII concentrate.

The comparative immunogenicity assessment included the potential modulation of both the innate and the adaptive immune system. The modulation of the human innate immune system was assessed by the potential of BAX855 and ADVATE to induce pro- inflammatory cytokines in human whole blood cultures and to activate human complement in human plasma samples.

The modulation of the adaptive immune system was assessed by the potential of BAX 855 and ADVATE to induce antibodies against FVIII and against polyethylene glycol (PEG) in cynomolgus monkeys and in 2 different hemophilic mouse models. Moreover, the ability of ADVATE and BAX 855 to induce FVIII-specific CD4 T-cell responses in hemophilic mice was assessed.

BAX 855 and ADAVTE induced similar low levels of cytokine release and complement activation in vitro that were not different from the buffer control groups. Furthermore, BAX 855 and ADVATE induced similar levels and incidences of antibodies against human FVIII in all animal models and similar levels of FVIII-specific CD4 T cells in hemophilic mice. Importantly, immune tolerance to human FVIII was maintained by both BAX855 and ADVATE in hemophilic mice that are immune tolerant to human native FVIII.

We conclude that BAX 855 and ADVATE express a similar FVIII immunogenicity profile in preclinical models.

3.4.2 Introduction

Patients with hemophilia A, who lack or have defective FVIII, are treated with plasma-derived or recombinant coagulation factor VIII (FVIII) concentrates to arrest or prevent bleeding episodes. As FVIII has a short half-life, patients require several infusions a week, depending on their individual pharmacokinetic profile, bleeding phenotype and life style, to maintain the replacement FVIII at an hemostatic level of 1% of normal [1].

Baxalta is developing a full-length recombinant FVIII molecule modified with polyethylene glycol (PEG) with the aim to reduce the required frequency of FVIII injections for patients while maintaining the treatment efficacy [2, 3]. The PEGylated recombinant human FVIII (BAX 855) is a full-length FVIII conjugated with 20kDa branched PEG molecules that consist of two 10kDa chains. BAX 855 is based on Baxalta`s recombinant human FVIII, ADVATE [2, 3]. Recent phase 1 and pivotal clinical studies provide evidence that BAX 855 is safe and efficacious for treating bleeding episodes and for prophylaxis administered twice weekly in patients with severe hemophilia A [4].

Therapeutic proteins potentially elicit an immune response in patients, depending on multiple patient and product related factors, e.g. the protein’s structure, formulation, contaminants or biological activity. The most serious adverse event of replacement therapy with FVIII products in hemophilia A is the development of inhibitory antibodies that neutralize the biological activity of FVIII in about 20-32% of patients with severe (plasma FVIII activities <1%), and about 3-13% with moderate (plasma FVIII activity 1-5%) and mild (plasma FVIII activity 5-25%) hemophilia A [5, 6]. Why some patients develop antibodies while others do not is unclear.

Any modification of FVIII, e.g. by PEGylation, may alter its immunogenic potential. Therefore, a comprehensive preclinical immunogenicity assessment is needed that allows evaluating potential differences in immunogenicity before initiating clinical studies. However, there are no defined strategies for preclinical immunogenicity assessments, which are beset with challenges. Firstly, animals only have a limited predictive value for the human situation because they recognize human proteins as foreign. Secondly, the mechanisms of antibody induction may differ between humans and the animal model (e.g. break of tolerance versus induction of immune response against a non-self-protein). In addition, the presentation of

immunogenic peptides by antigen-presenting cells via the MHC-class II complex that drives CD4+ T cell responses required for the development of high-affinity antibodies differs between mice and humans. Moreover, the dose, frequency and route of dosing may influence the antibody response and should therefore mirror human treatment regimens as closely as possible. Preclinical studies need to be designed taking the characteristics of the therapeutic protein, patient population and indication into consideration. Therefore a new product's immunogenicity can only be assessed by comparing the candidate's immunogenicity with a well-characterized immunogenicity profile of the parenteral marketed product. Thus, the potential immunogenicity of BAX 855 was compared with that of ADVATE, Baxalta's unmodified recombinant full-length FVIII concentrate. ADVATE has been used for treating patients for more than 10 years and has a known immunogenicity profile [7].

By comparing responses of the innate and adaptive immune system to BAX 855 and ADVATE using animal models and human in vitro assays, we show that BAX 855 has a similar FVIII immunogenicity profile to that of ADVATE. Therefore, BAX 855 can be expected to have a FVIII immunogenicity profile in patients similar to that of ADVATE.

3.4.3 Material and Methods

Animal studies

Animals

Two mouse models and cynomolgus monkeys were used to test the activation of the adaptive immune system in vivo. The first mouse model, the E17 FVIII k.o. mouse, has a disruption of exon 17 of the murine F8 gene [8]. This model mirrors the situation in humans with hemophilia A. The second mouse model, the E17 FVIII k.o. human FVIII transgenic mouse, was specifically designed to study the potential break of immune tolerance against native human FVIII. This model has a disruption of exon 17 of the murine F8 gene but expresses a human F8 cDNA as a transgene [9]. Mice do not produce antibodies to human FVIII in response to treatment unless the FVIII has increased immunogenicity or is given in high doses.

All studies were conducted in accordance with Austrian federal law (Act BG 501/1989) regulating animal experimentation and were approved by the Institutional Animal Care and Use Committee.

Study design

Male mice aged between 8 and 12 weeks were allocated into groups of ten, Cynomolgus monkeys were allocated into groups of four animals (2 male, 2 female). Each animal received 8 intravenous doses (8 or 40 µg/kg) of BAX 855, ADVATE, or buffer control. In addition, a highly immunogenic PEGylated FVIII variant known to break immune tolerance [9] against human FVIII in mice was included as a positive control in the mouse studies. A protein dose of 8 µg/kg BAX 855 and ADVATE correlates to an activity-based dose of approximately 80 IU/kg, which represents the clinically relevant dose level of ADVATE. Blood samples were taken before the first dose and after 8 doses. Lymph nodes and the spleens were harvested from E17 FVIII k.o. mice after the eighth dose for in vitro CD4+ T-cell re-stimulation.

Blood sampling

Blood samples were obtained by retro-orbital puncture (pre-dosing) or by cardiac puncture (1 week after the last dose) in mice and were collected from the vena cephalica antebrachii or vena femoralis in monkeys.

Blood samples were added at a 5:1 (v/v) ratio to 0.106 mol/L sodium citrate (4 parts blood + 1 part sodium citrate). Plasma samples were separated by centrifugation and stored ≤ -20°C until analysis.

Detection of antibodies against FVIII and PEG

Antibodies against FVIII

Enzyme linked immunosorbent assays (ELISA) were used to detect antibodies with specificity for human FVIII in mouse and monkey plasma samples. The ELISA assay for the detection of antibodies against human FVIII in mice was set up as described by Hausl et al [10]. The ELISA assay for the detection of antibodies against human FVIII in monkeys was set up in the same way with the exception that a secondary antibody was used that specifically recognized monkey IgG. Moreover, neutralizing antibodies against human FVIII were analyzed with a method based on the Nijmegen modification of the Bethesda assay[11] in monkeys.

Antibodies against PEG

Murine and monkey antibodies against PEG were detected using flow cytometry (FCM) as described by Lubich et al [12]. In brief, plasma samples were incubated with beads with PEG molecules on their surface. PEG-specific antibodies bound to PEG molecules on the surface of beads were detected using an allophycocyanin-conjugated goat anti-mouse or anti-monkey IgG antibody. The resulting complex was quantified by FCM. Plasma from mice and monkeys containing PEG-specific antibodies was used as a positive control. A pool of negative plasma samples was included as negative controls

All murine and monkey plasma samples were serially diluted starting at a minimum dilution of 1:20 and continuing with 1:2 dilution steps. The titer of a sample was defined as the highest dilution that still gave a positive signal above the cut off. Each sample was analyzed at least twice. A difference of 2 titer steps was the maximal variation accepted. If the

measured titer difference was only one titer step, the higher titer was reported. Otherwise, the median titer was reported. Log2 values of antibody titers were calculated as follows: Log2 value = [log2 of (antibody titer/20)]+1. Antibody titers of less than 1:20 were set to 0 for the log2 value.

Detection of FVIII-specific CD4+ T cells

FVIII-specific CD4+ T cells were detected by expression of CD154 after short-term in vitro restimulation with human FVIII as described by Steinitz et al [13].

Cytokine release from human whole blood cultures

200 µL fresh whole blood from eight healthy donors was diluted with 600 µL RPMI medium (Gibco). The respective test or control items were added in a volume of 200 µL to a total sample volume of 1 mL. BAX 855 and ADVATE diluted in buffer were applied at final concentrations of 5, 0.5 and 0.05 µg/mL. Concentrations of BAX 855 and ADVATE were used based on the clinical dose of ADVATE. A concentration of 0.05µg/mL in the in vitro assays correlates to an activity based dose of approximately 40 IU/kg. Concentrations up to 10- and 100-fold above the calculated clinical doses were also included (0.5 µg/mL and 5 µg/mL). ADVATE buffer was used as a negative control and LPS (500 pg/mL final concentration, correlating to an endotoxin concentration of approximately 5.0 IU/mL, diluted in ADVATE buffer) as an assay positive control. The samples were incubated for 20-22 h at 37°C/ 5% CO2. The supernatant was analyzed for cytokines (IL-1β, IL-6, IL-8, TNF-α) using a bead-based multiplex sandwich immunoassay (Bio-Plex cytokine assay, BioRad) following the manufacturer's instructions . Samples were measured using a Bio-Plex 200 Suspension Array System (BioRad). The concentration of each cytokine was quantified using a standard curve of known concentration for the cytokine. Three independent analyses were done for each sample. Mean values of n=3 analysis are reported. Single data values below the quantifiable working range were set to 0 for calculation of mean values and for graphical illustrations.

Complement activation assay

Human whole blood from eight healthy donors was centrifuged in two steps to separate plasma from whole blood: Firstly for 20min at 1100g, +2 to +8°C (setpoint), and secondly for 10min at 3990g, +2 to +8°C after transfer to the supernatant. The complement activation

was evaluated by measuring C5a as a marker. In brief, 250 µL of plasma was incubated with 250 µL of BAX 855 or ADVATE resulting in a total sample volume of 500 µL. BAX 855 or ADVATE diluted in ADVATE buffer were applied at final concentrations of 5, 0.5 and 0.05 µg/mL. Concentrations of BAX 855 and ADVATE were used based on the clinical dose of ADVATE. A concentration of 0.05 µg/mL in the in vitro assays correlates to an activity based dose of approximately 40 IU/kg. Concentrations up to 10- and 100-fold above the calculated clinical doses were also included (0.5 µg/mL and 5 µg/mL).

Advate buffer was applied as a buffer control and zymosan (10 µg/mL final concentration) as a positive assay control. The samples were incubated for 1h at 37°C and stored at ≤ -80°C until analysis using the C5a ELISA Kit II (BD OptEIA), a solid phase sandwich ELISA. The ELISA was conducted according to the manufacturer's instructions and samples were measured using Gen5 software.

The C5a concentration was quantified using the mean for a set of duplicate standard curves of known concentrations for C5a. Two independent analyses were done for each sample on one day in two independent plates. Mean values of n =2 analysis are reported.

3.4.4 Results

Induction of antibody responses against human FVIII and PEG in hemophilic mice and in Cynomolgus monkeys

The potential of BAX 855 and ADVATE to induce antibodies with specificity for human FVIII or PEG was compared in E17 FVIII k.o. and in Cynomolgus monkeys. A positive PEG-FVIII control known to break immune tolerance against native human FVIII in E17 FVIII k.o. human FVIII transgenic mice was included in this animal model.

Titers of antibodies against human FVIII observed in E17 FVIII k.o. mice were in a similar range and showed similar incidences after treatment with BAX 855 or ADVATE. Eight animals given 200 ng ADVATE (titers between 4 to 14), five given 200 ng BAX855 (titers between 8.5 to 14.5) and all given 1000 ng ADVATE (titers between 6.5 to 13.5) or BAX855 (titers between 12 and 15) were evaluated as positive for antibodies against FVIII at the end of the study (Figure 1A). All animals given 200 or 1000ng ADVATE were evaluated as negative for anti-PEG antibodies which was an expected result as ADVATE is the parenteral, non-PEGylated FVIII molecule. Five mice given 200 ng BAX 855 (titers between 4 and 15), and all given 1000 ng BAX 855 (titers between 7 and 11) were positive for anti-PEG antibodies at the end of the study (Figure 1B).

In Cynomolgus monkeys, all animals given ADVATE 8 µg/kg (titers between 9 and 13.5) or 40 µg/kg (titers between 9 and 11) and all animals given BAX 855 8 µg/kg (titers between 5 and 1) and 40 µg/kg (titers between 10 and 10.5) were evaluated as positive for anti-FVIII antibodies after 8 doses (Figure 2A). All animals given BAX 855, except one dosed 8 ug/kg, were also positive for anti- PEG antibodies after 8 doses. As expected, none of the animals given ADVATE showed any anti-PEG antibodies after 8 doses (Figure 2B), and all were evaluated as negative for antibodies against PEG at start of the study.

All animals that were positive for anti-FVIII antibodies were also evaluated positive for neutralizing antibodies against FVIII. No differences between study groups were observed.

Next we asked if BAX 855 is able to maintain immune tolerance against human FVIII in E17 FVIII k.o. human FVIII transgenic mice. All animals given 200 ng ADVATE or BAX 855 were

evaluated as negative for anti-FVIII antibodies. Two animals given 1000 ng ADVATE (titers between 4 and 9), one given 1000 ng BAX 855 (titer of 7) and all given 1000 ng PEG-FVIII control (titers between 5.5 and 11) were positive for antibodies against human FVIII (Figure 3A) The development of anti-FVIII antibodies in single animals treated with high dose human FVIII confirms previous reports [9] and possibly reflects T-cell independent antibody responses. All animals given ADVATE or BAX 855 were evaluated as negative for anti-PEG antibodies. All animals given 1000 ng of the highly immunogenic PEG-FVIII control were evaluated as positive for anti-PEG antibodies (Figure 3B).

Induction of FVIII-specific CD4 T cells in E17 FVIII k.o. mice

We assessed BAX 855 and ADVATE for their potential to activate FVIII- specific CD4+ T-cells in E17 FVIII k.o. mice after short-term in vitro re-stimulation. A highly immunogenic PEG-FVIII was included as positive control.

There was no difference in the frequency of CD154+ CD4 cells after short-term in vitro restimulation with FVIII in animals receiving BAX 855 or ADVATE indicating similar immunogenicity in this mouse model [Figure 4]. However, the frequency of CD154+ CD4 T cells after short-term in vitro restimulation was increased in the cohort of mice immunized with the immunogenic PEG-FVIII control confirming the strong immunogenic potential of this PEG-FVIII compound.

Activation of cytokine release and complement

The potential of BAX 855 and ADVATE to activate the human innate immune system was assessed in two in vitro assays using human whole blood (release of pro-inflammatory cytokines) and human plasma (activation of human complement).

The release of pro- inflammatory cytokines IL-1β, IL-6, IL-8 and TNF-α was measured after incubation of human whole blood with BAX 855, ADVATE or LPS (positive control). A robust induction of all cytokines was observed with LPS indicating the validity of the assay system (Figure 5 A-D). Levels of IL-1β, IL-6, IL-8 and TNF-α measured after BAX 855 incubation were in the same range as the respective levels observed in the buffer control group. Moreover, cytokine levels were similar to those observed after incubation of whole blood with ADVATE

(Figure 5 A-D). Two different lots of BAX 855 were included in this study and produced similar results.

Next, we evaluated the potential activation of the human complement system by assessing the generation of C5a. The complement system is activated by three distinct pathways - the alternative pathway, classical pathway and lectin pathway. All 3 pathways result in cleavage of C5 into C5a and C5b. C5a is a multifunctional pro-inflammatory mediator and therefore its generation is used as a marker for complement activation. Zymosan, a cell wall preparation from Saccharomyces cerevisiae, was included as positive control. The levels of C5a ranged between 18.90 and 36.56 ng/mL in samples incubated with the buffer control and between 128.34 and 354.06 ng/mL in samples incubated with the positive control zymosan. BAX 855 did not differ from ADVATE or buffer control in its potential to activate the human complement system. C5a levels were in the same range for all study groups. Moreover, the 2 lots of BAX 855 included in this study did not differ in their ability to activate the complement system in human plasma in vitro (Figure 6). In conclusion, neither BAX855 nor ADVATE did induce complement activation in any of the human plasma samples tested.

3.4.5 Discussion

This study was designed to assess the immunogenicity of Baxalta`s longer-acting FVIII candidate BAX 855 in preclinical models. For this purpose, BAX 855 was compared to Baxalta`s non-PEGylated recombinant human FVIII, ADVATE, for its ability to induce the innate and the adaptive immune system. The results indicate that BAX 855 and ADVATE have a similar FVIII immunogenicity in these models which gives rise to the expectation that BAX 855 will have a FVIII immunogenicity profile in patients similar to that of ADVATE.

The generation of antibodies against proteins is the result of a cascade of tightly regulated interactions between different cells of the innate and the adaptive immune system in very distinct compartments [14-16]. T-cell dependent and T-cell independent pathways may contribute to the generation of antibody responses to therapeutic proteins [16][19a, 20]. Proteins such as FVIII are generally considered to be T-cell-dependent antigens. Their immunogenicity depends on the generation of peptides by antigen presenting cells, e.g. dendritic cells (DCs), and the presentation of these peptides by MHC-class II proteins expressed on antigen presenting cells to CD4+ T cells. The activation status of DCs is an essential feature in the control of immunity and tolerance [17]. Importantly, the presence or absence of pro-inflammatory signals [18], e.g. mediated by an activation of the innate immune system, determines the activation and maturation status of DCs. While mature DC can activate CD4+ T lymphocytes which drive B cell activation and differentiation into antibody-producing plasma cells [14-16], non-activated immature DCs can induce regulatory immune mechanisms, e.g. regulatory T cells, which maintain peripheral tolerance against proteins (ref). Therefore, it is essential to assess the potential activation of both the innate and the adaptive arm of the immune response.

We used two human in vitro systems to assess the potential of BAX 855 to induce the human innate immune system. The results obtained in these studies confirm previous data indicating that native human FVIII does not activate the innate immune system [19]. Here, we can show that BAX 855 behaves similar to ADVATE which indicates that the inherent potential of BAX 855 to activate the innate immune system is low.

In another set of studies we include two preclinical mouse models of hemophilia A which mimic different populations of hemophilia A patients. The E17 FVIII k.o. mouse model is widely used in research and in preclinical product assessments [8, 20-24]. Mice recognize human FVIII as foreign protein and develop a robust immune response against the foreign protein. On the other hand, E17 FVIII k.o. human FVIII transgenic mice are immunologically tolerant to naïve human FVIII. They only develop robust antibody responses against human FVIII when treated with highly immunogenic FVIII preparations which break tolerance [9]. Data obtained in the studies presented here indicate that ADVATE and BAX 855 generate similar immune responses in both mouse models which support the conclusion that the 2 FVIII proteins express a similar inherent FVIII immunogenicity.

The anti-PEG antibody data presented here support the hypothesis that PEG attached to FVIII is a hapten and induces anti-PEG antibodies if it is attached to an immunogenic FVIII protein. Only those mice develop antibodies against PEG that also develop antibodies against FVIII. Accordingly, one would expect that hemophilia patients who recognize the protein part of PEGylated FVIII products as immunogenic protein are likely to be at risk for antibody formation not only against FVIII but also against PEG. Mitchison et al. [25] and Rock et al. [26] were the first to present evidence for the immunological principles of anti-hapten antibody responses.

Cross-linking of B cell receptors that recognize repetitive epitopes within the PEG moiety could be an alternative explanation for the development of anti-PEG antibodies. Multiple regularly spaced identical epitopes are required for this type of antibody response to allow simultaneous engagement of multiple B cell receptors with each epitope [27, 28].

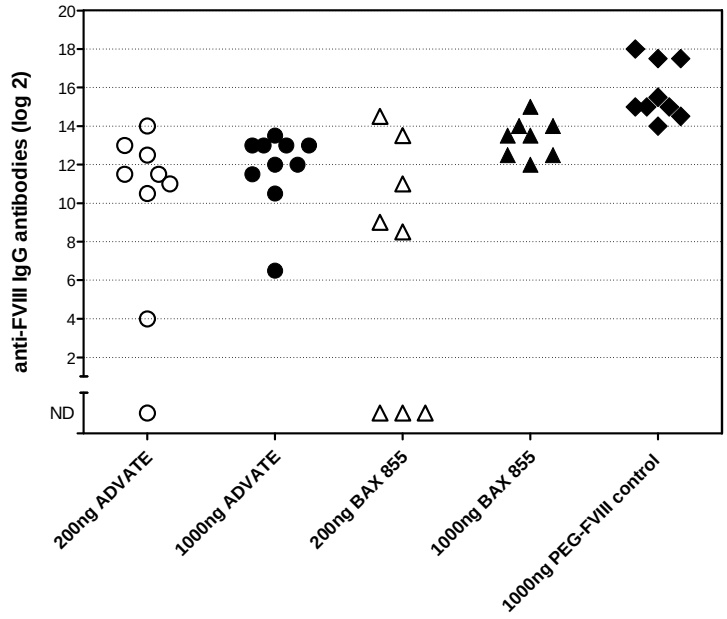
Although the results of animal studies should be used cautiously when assessing the immunogenicity of new protein therapeutics, they do provide a helpful dataset for deciding whether to progress to clinical trials. Here, we present a comprehensive preclinical assessment strategy using transgenetic partially humanized animals (i.e. mice transgenic for the human protein of interest) supplemented with ex vivo and in vitro assessments of cellular activation. We believe such a comprehensive strategy provides a greater insight into the underlying immunogenic potential of a new recombinant protein and can thus better

guide drug development than a classic animal model or a single in vitro test can do. Finally, appropriate clinical studies or post marketing studies are needed to confirm results from preclinical immunogenicity studies.

3.4.6 Figures

Figure 1

A



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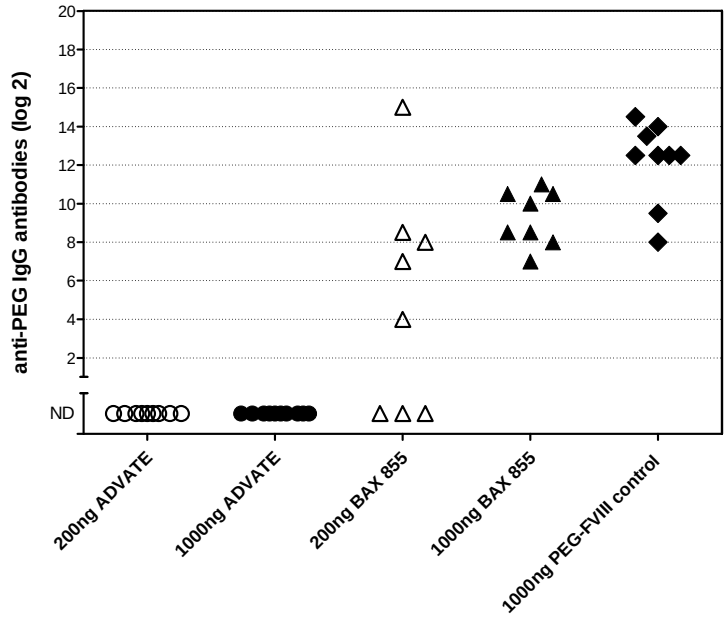
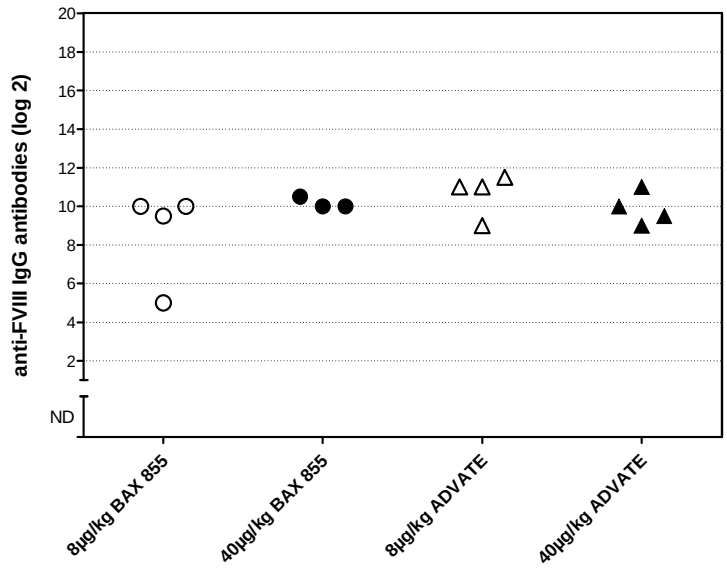


Figure 1: Development of anti- FVIII and anti-PEG antibodies in E17 FVIII k.o. mice.

Animals were dosed 8 times at weekly intervals with ADVATE, BAX 855 or a highly immunogenic PEG-FVIII positive control as indicated. Blood samples taken 1 week after the last dose were analyzed for antibodies against FVIII using ELISA (A) and PEG using FCM (B).

Figure 2

A



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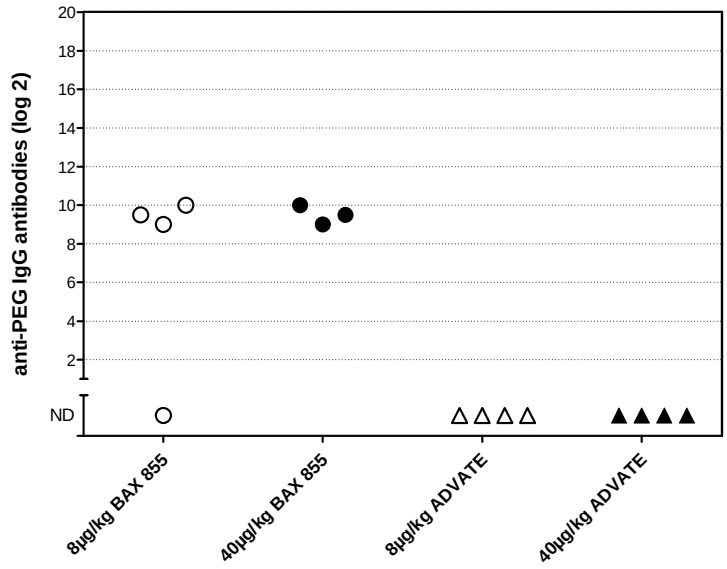
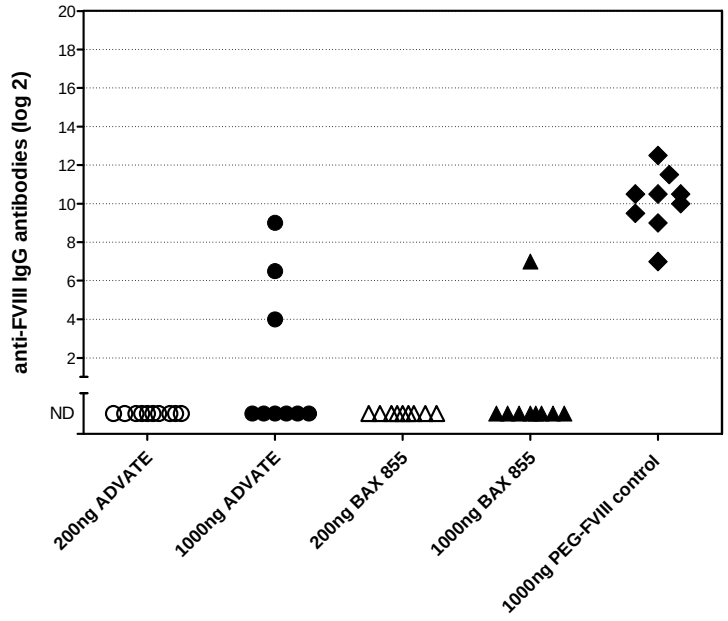


Figure 2: Development of anti- FVIII and anti-PEG antibodies in non-human primates

Cynomolgus monkeys were dosed 8 times at weekly intervals with ADVATE or BAX 855 as indicated. Blood samples taken 1 week after the last dose were analyzed for antibodies against FVIII using ELISA (A) and PEG using FCM (B).

Figure 3

A



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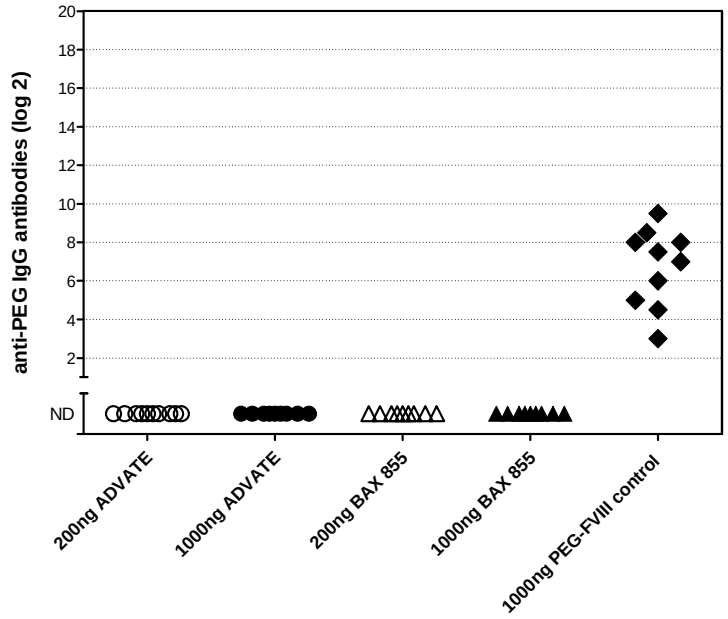
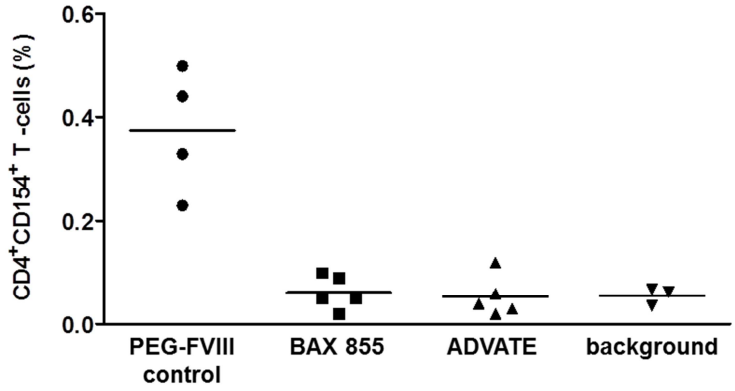


Figure 3: Development of anti- FVIII and anti-PEG antibodies in E17 FVIII k.o. human FVIII transgenic mice.

Animals were dosed 8 times at weekly intervals with ADVATE, BAX 855 or a highly immunogenic PEG-FVIII positive control as indicated. Blood samples taken 1 week after the last dose were analyzed for antibodies against FVIII using ELISA (A) and PEG using FCM (B).

Figure 4

A



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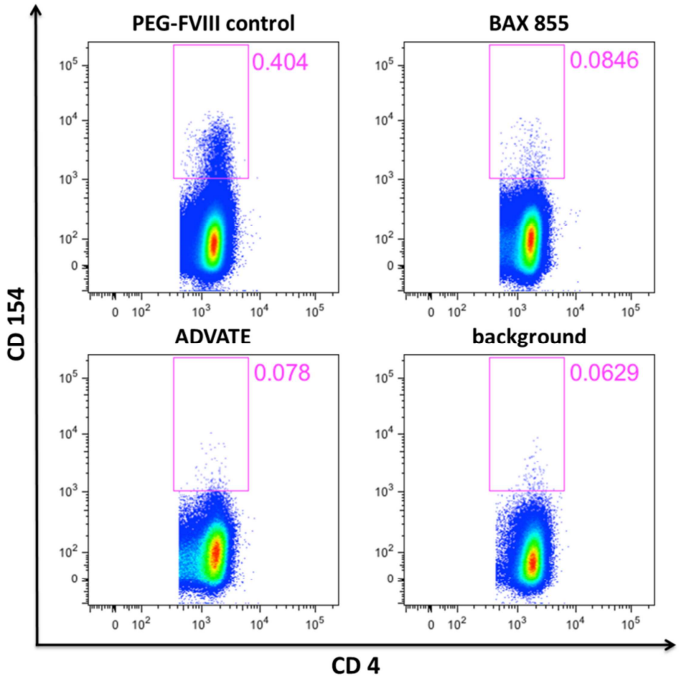
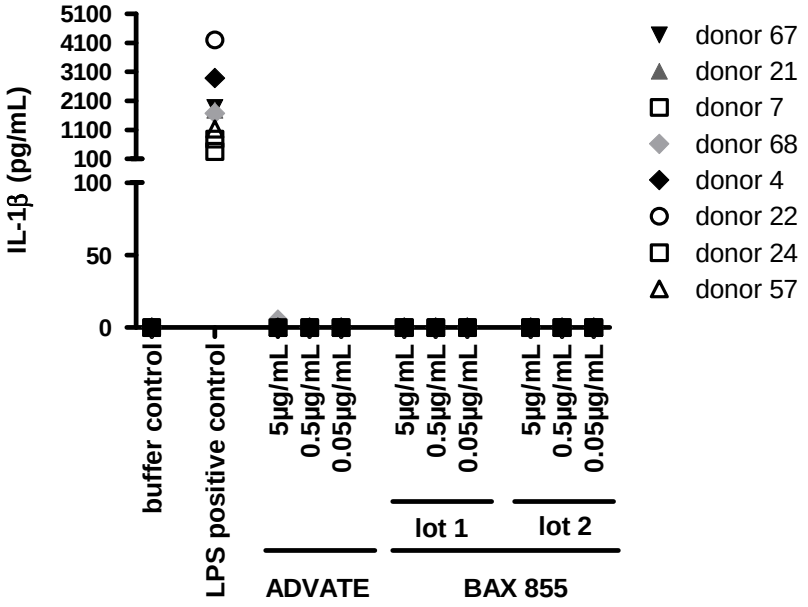


Figure 4: Detection of antigen-specific T-cells in the E17-FVIII-k.o. strain after 8 i.v. challenges

Animals were dosed 8 times at weekly intervals with medium control, ADVATE, BAX 855 or a highly immunogenic PEG-FVIII positive control. Presented are FVIII-specific $CD4^+CD154^+$ T-cells in mice after short *in vitro* restimulation of spleen cells with human FVIII (A) as described previously [13]. There was no difference in the frequency of $CD4^+CD154^+$ T-cells in animals receiving ADVATE or BAX 855 in contrast to the immunogenic PEG-FVIII control. Representative FACS Blots are shown (B).

Figure 5

A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3100
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1100
100
100
50
0
buffer control
LPS positive control
5µg/mL
0.5µg/mL
0.05µg/mL
5µg/mL
0.5µg/mL
0.05µg/mL
5µg/mL
0.5µg/mL
0.05µg/mL
lot 1
lot 2
ADVATE
BAX 855
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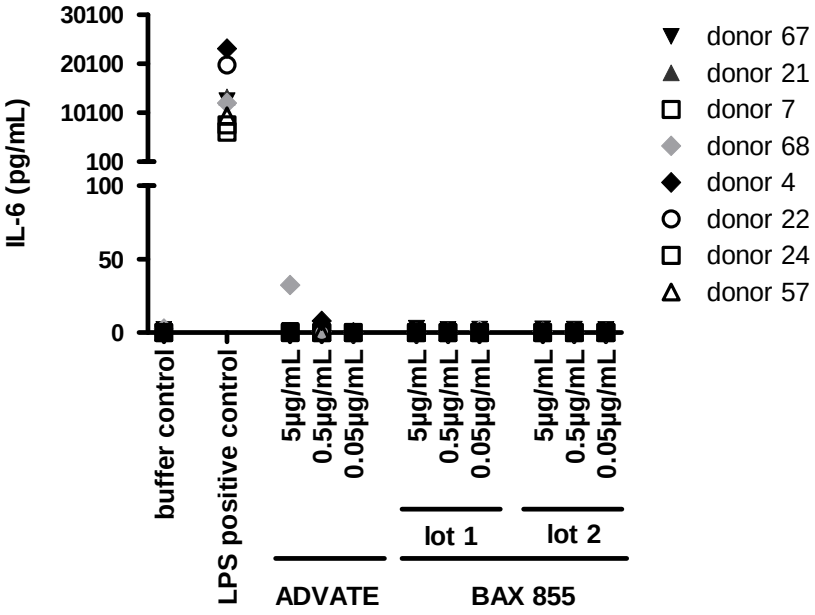
donor 21
donor 7
donor 68
donor 4
donor 22
donor 24
donor 57

B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10100
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100
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0
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LPS positive control
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lot 1
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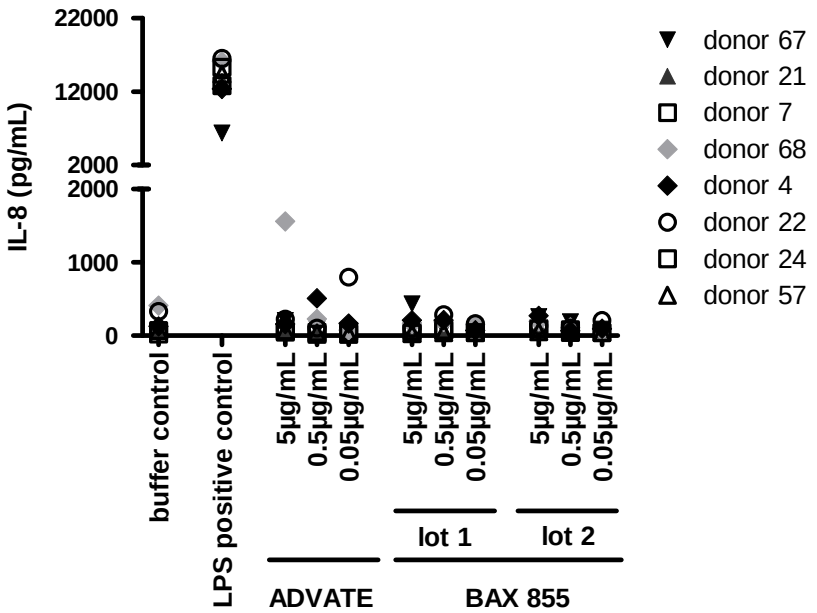
C



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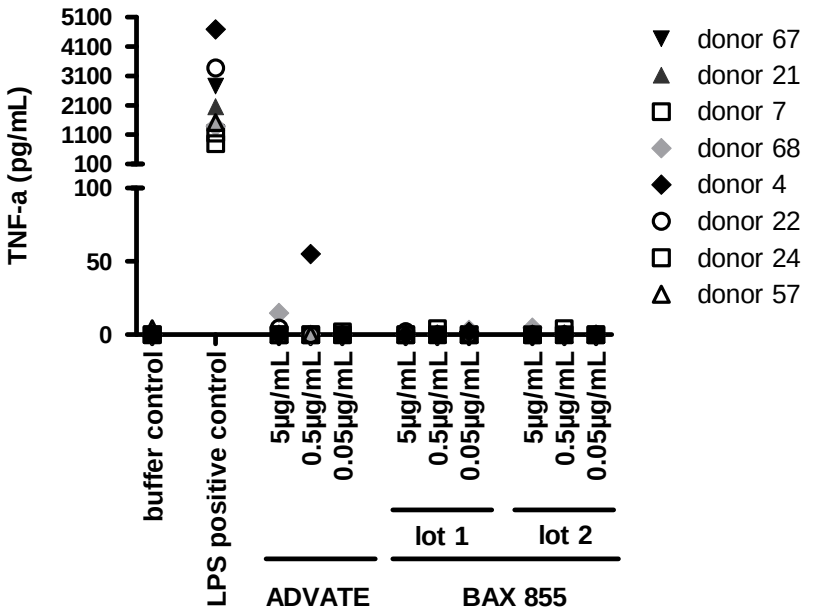

Figure 5: BAX 855 does not induce the release of pro-inflammatory cytokines

The release of pro- inflammatory cytokines IL-1β (A), IL-6 (B), IL-8 (C) and TNF-α (D) was measured after incubation of human whole blood with ADVATE, 2 lots of BAX 855 or LPS as positive control. The measured cytokine levels were similar between ADVATE, both lots of BAX 855 and the buffer control.

Figure 6

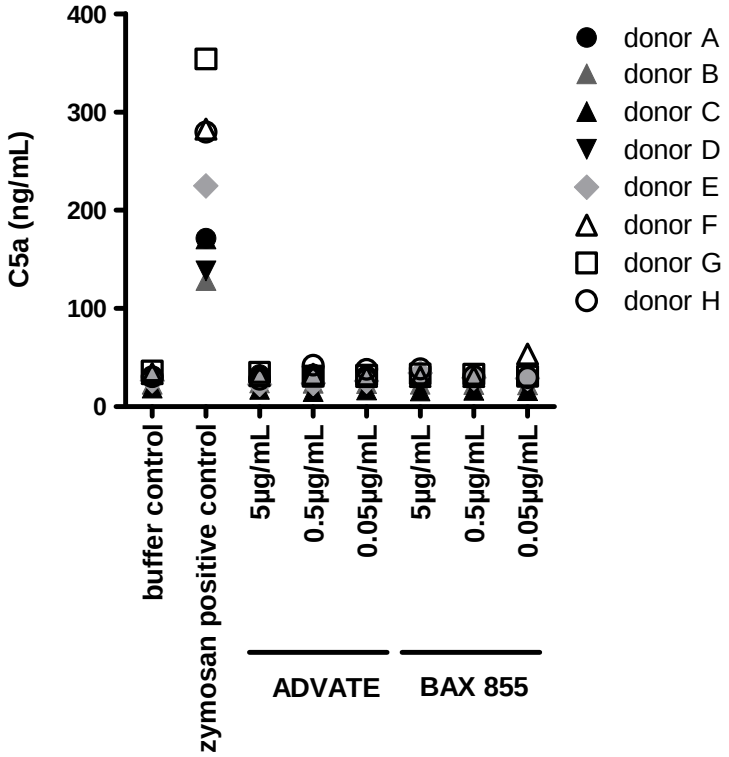

Figure 6: BAX 855 does not activate the complement system

Potential activation of the human complement system by BAX 855 was assessed by the generation of C5a. Human whole blood from eight healthy donors was incubated for 1 hrs with BAX 855 or ADVATE at final concentrations of 5, 0.5 and 0.05 µg/mL. Concentrations of BAX 855 and ADVATE were used based on the clinical dose of ADVATE. C5a was measured and quantified using ELISA. BAX 855 did not differ from ADVATE or buffer control in its potential to activate the human complement system.

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4. Discussion

The most severe complication in current standard of care in hemophilia A is the development of neutralizing antibodies against FVIII in up to 32% of patients [51]. These so called FVIII inhibitors have a huge impact on a patient's life by leaving the replacement therapy ineffective, leading to higher morbidity and a general reduced quality of life [66]. Although research has contributed a lot to understand the reasons for the formation of inhibitory antibodies and several risk factors have been already identified [81], the root cause why some patients develop inhibitors and some do not is far from understood.

BAX 855 is a newly developed FVIII product with the aim to extend the half-life of FVIII in circulation, making fewer treatments per week necessary for patients on prophylaxis. BAX 855 is a PEGylated form of Baxalta's recombinant full-length FVIII product ADVATE™ [108] and has been shown to increase the half-life by 1.5 fold [109]. Considering the high risk in hemophilia A patients to induce unwanted immune responses, the development of novel FVIII therapies is utmost challenging. Therefore, a critical assessment of the immunogenicity in newly developed FVIII therapeutics is essential before entering clinical development. The objective of this thesis was to develop and apply adequate techniques to address novel aspects that potentially influence the immunogenicity of BAX 855.

The first part of this thesis describes the development of a flow-cytometry-based approach to detect, quantify and characterize sub-visible particles within protein solutions. Sub-visible particles were recently linked to enhance the immunogenicity of protein therapeutics [96-98]. However, the underlying immunologic mechanism is yet unclear. Investigations to better understand the root cause of this phenomenon have been complicated by the wide variety of sub-visible particles which can be present in protein solutions. Although current available technologies have been proven to be useful, a comprehensive analysis of these diverse particles is only possible to a limited extent. Thus, it is necessary to develop novel methods to address this need. Flow cytometry was already used two decades ago for the analysis of amyloid plagues by Palutke and colleagues [115]. Recently, Ludwig et al [116] and Mach et al [117] suggested this technology platform to be promising for the analysis of sub-visible particles. We further explored the suitability of flow cytometry for the analysis of sub-visible particles and were able to establish a flow-cytometry-based method. Flow cytometry comprises many

advantages for the analysis of sub-visible particles in protein solutions. First, the method facilitates the simultaneous analysis of amount, size distribution and nature of sub-visible particles in protein solutions. Second, the size range of this technique was optimized to characterize particles in a range from 0.75 µm to 10 µm. This lower limit of detection could be further reduced by using specialized flow cytometers [118], which would further enhance the suitability of this technique to analyze sub-visible particles. In addition, our method is very well capable to analyze sub-visible particles in the final protein formulation. This is of utmost importance because proteins are in a thermodynamically equilibrium [119] and may change their morphology upon dilution.

Studies have shown that the content and quality of sub-visible particles within protein solutions can change upon differences in the formulation buffer [120]. Moreover, the impact of chemical modification, like PEGylation, on the stability of the protein is not known. Consequently we were interested in a comprehensive analysis of sub-visible particles in the final formulation of BAX 855 in comparison with its base molecule ADVATE™, which has an established safety and immunogenicity profile [121]. Our study revealed that both molecules have a similar content and quality of sub-visible particles. Therefore the impact of sub-visible particles on the immunogenicity of BAX 855 is expected to be comparable to ADVATE™.

The second part of this thesis was the assessment of potential antibody responses against polyethylene glycol (PEG) and the investigation of possible mechanisms on their induction. The immunogenicity of PEG is a highly controversial discussed topic. While many PEGylated biologics have been proven to be safe and non-immunogenic, reports of treatment related development of anti-PEG antibodies in patients are increasing [122-127]. Moreover, the prevalence of anti-PEG antibodies in healthy donors is not known at the moment. While Armstrong and colleagues reported an incidence of more than 25% of anti-PEG antibodies in 350 healthy donors [128,129], Richter and Akerbloom [130] as well as Liu et al [131] stated a much lower prevalence of respectively 0.2 % and 4 % positive donors. Some of these reports on anti-PEG antibodies have evoked controversial discussion because of shortcomings in the assays used for the analysis of these antibodies [132,133]. This thesis revisits the prevalence of anti-PEG antibodies in healthy subjects using validated technologies. We developed two assay formats to analyze the prevalence of anti-PEG antibodies in two cohorts of healthy human subjects (n=1310): a flow cytometry approach (FCM) and an ELISA platform validated

with respect to cut-off calculation, sensitivity, specificity, linearity, and precision (inter-assay and intra-assay variability). Our data confirms the presence of binding anti-PEG antibodies in healthy individuals. These antibodies might be induced by PEG polymers or their derivatives present in cosmetics or pharmaceuticals [134-136]. However, there was no indication of any pathology associated with these antibodies. The prevalence in our study cohort was 20-28%, with 12-26% for titers <1:80 and 2-11% for titers ≥1:80. IgG and IgM subclasses were in both cohorts equally distributed. We could identify age as significant covariate of the expression of IgG antibodies, with a decrease of the prevalence in older subjects. Competition experiments with PEG polymers of different sizes indicate that anti-PEG IgG antibodies found in healthy donors are recognizing both the methoxy group as well as the PEG-backbone.

We then assessed antibodies against PEG in the overall immunogenicity assessment of BAX 855 during non-clinical development. We observed the treatment related and dose dependent induction of anti-PEG IgG antibodies in several different animal models. We identified differences in the antibody response depending on the animal model, which seemed to correspond with the antibody responses against FVIII. Therefor we were interested in the mechanisms of the induction of anti-PEG antibodies. We hypothesized that PEG can only elicit antibody responses when attached to an immunogenic carrier protein. We used two different mouse models to test our hypothesis. The conventional hemophilic E17 mouse model recognizes human FVIII as a foreign protein. The human FVIII transgenic hemophilic mouse model recognizes human FVIII as a self-protein. We treated mice of both models with up to 8 weekly doses of two PEGylated FVIII preparations (mVIII1 and mFVIII2). We knew that mFVIII2 breaks immune tolerance to human FVIII in human FVIII transgenic hemophilic mice [137]. Both mFVIII1 and mFVIII2 induced anti-PEG and anti-FVIII antibodies in conventional hemophilic E17 mice but only mFVIII2 induced anti-FVIII and anti-PEG antibodies in human FVIII transgenic hemophilic mice. The non-PEGylated FVIII control only induced anti-FVIII antibodies in conventional hemophilic E17 mice. These results support the hypothesis that induction of anti-PEG antibodies by PEGylated FVIII requires the presence of an immunogenic carrier protein that is recognized as foreign. We believe that the immunogenic carrier protein is required to induce CD4+ T cell help to facilitate the antibody response against the hapten PEG.

Considering the possibility that treatment with PEGylated proteins could lead to the development of anti-PEG antibodies or to the boost of already present ones and that treatment with PEGylated products can be life-long, it is crucial to assess potential safety risks associated with the development of anti-PEG antibodies. In our study we therefore assessed the cross-reactivity of an IgG antibody against PEG in a panel of 36 different human tissue sections that are recommended by regulatory authorities. We could not observe any binding of this high affinity antibody against any of these tissues, which indicates that anti-PEG antibodies that are might induced during treatment with a PEGylated protein therapeutic are not very likely to bind to endogenous antigens.

In conclusion, by comparing responses of the innate and adaptive immune system to BAX 855 and ADVATE^{TM} using animal models and in vitro assays, we showed that BAX 855 has a similar immunogenicity profile to that of ADVATE^{TM}. Content and quality of sub-visible particles, which may influence the immunogenicity of protein therapeutics, are similar in both FVIII preparations and should therefore not enhance the immunogenicity of BAX 855. Although the chemical modification with PEG may lead to the development of anti-PEG antibodies, the immunogenicity against FVIII should not be increased. Despite the fact that patients who develop an antibody response against PEG may not benefit from the treatment any longer and anti-PEG antibodies should therefore be carefully monitored during clinical development, safety concerns for potential cross-reactivity with endogenous antigens are negligible. In overall conclusion, BAX 855 can be expected to have a safety profile in patients similar to that of ADVATE^{TM}.

5. Literature

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6. Appendix

6.1 Publications and Contributions

The data presented in the results section are

published:

6.1.1 A Flow-Cytometry-Based Approach to Facilitate Quantification, Size Estimation and Characterization of Sub-visible Particles in Protein Solutions.

Lubich C, Malisauskas M, Prenninger T, Wurz T, Matthiessen P, Turecek PL, Scheiflinger F, Reipert BM. Pharm Res. 2015 Sep;32(9):2863-76.

All data as summarized in Figures 1-6 as well as Supplement Figure 1 and Supplement Table 1A-C were created and contributed by C. Lubich.

not published:

6.1.2 Assessment of different recombinant FVIII preparations for the quantity of sub-visible particles and their properties

All data as summarized in Tables 1 and 2 and Figure 1 were created and contributed by C. Lubich.

submitted to the journal “Blood”:

6.1.3 The mystery of antibodies against polyethylene glycol (PEG) – IgM and IgG anti-PEG antibodies in healthy individuals

Lubich C, Allacher P, de la Rosa M, Bauer A, Prenninger T, Horling F, Siekmann J, Weiller M, Schwarz HP, Scheiflinger F and Reipert BM

All data as summarized in Tables 2 and 3 and Figures 1 to 5 were created and contributed by C. Lubich.

to be submitted:

6.1.4 Preclinical immunogenicity assessment of Baxalta`s longer-acting FVIII candidate BAX 855 using novel preclinical models

Horling F, Schwele S, **Lubich C**, de la Rosa M, Rauter I, Weiller M, Spatzenegger, Wolfsegger M, Scheiflinger F, Reipert BM

All data as summarized in Figures 1b, 2b, and 3b were created and contributed by C. Lubich.

6.2 Oral Presentations

6.2.1 Sub-visible particles in protein therapeutics – are they responsible for the induction of unwanted immune responses?

Lubich C., Malisauskas M. , Prenninger T., Lenk C., Hopfner E., Scheiflinger F., Reipert B.M.

Oral presentation at the 15th International Congress of Immunology (ICI), Milan, Italy, August 2013.

Abstract:

Sub-visible particles in protein therapeutics have received increasing attention in recent years because they are believed to play a major role in the induction of unwanted immune responses. In particular amyloid-like protein particles could be associated with an activation of the innate immune system as was demonstrated for Amyloid beta peptides. Currently available technologies such as Micro-Flow Imaging and Nanoparticle Tracking Analysis are suitable for quantification of sub-visible particles but have limited capability for more in-depth characterization of the nature of these particles.

We developed a novel flow-cytometry-based technology that combines quantification and in-depth characterization of sub-visible particles over a size range of 0.75µm- 75µm. This technology allows assessment of the amount, size and nature of sub-visible particles. The inclusion of the fluorescent dye 4-(dicyanovinyl)-julolidine (DCVJ) which binds to cross-beta-sheet structures in amyloid-like protein particles, provides important information on the nature of the particles.

We used the new technology to assess amount, size and nature of sub-visible particles in a variety of in vitro generated sub-visible particle preparations originating from different therapeutic proteins. We also included non-protein particles in the analysis. Concomitant to particle analysis we used cellular in vitro assays to assess the potential of different protein particles to trigger the innate immune system.

We believe that the combination of sub-visible particle characterization with the assessment of potential immune stimulatory activities can provide new insight into important

mechanisms regulating the induction of unwanted immune responses by protein therapeutics.

6.2.2 Why do some PEGylated biotherapeutics induce antibodies against PEG?

Lubich C., Weiller M., Siekmann J., Scheiflinger F and Reipert B.M.

Oral presentation at the 4th European Congress of Immunology (ECI), Vienna, Austria, September 2015.

Abstract:

An increasing number of biotherapeutics is conjugated with polyethylene glycol (PEG) to improve their half-life. Whereas PEG has long been considered to be non-immunogenic, recent reports indicate that treatment with PEGylated biotherapeutics can lead to the development of anti-PEG antibodies in animal models and in patients.

We assessed the potential risk of PEGylated human factor VIII (PEGFVIII) to induce anti-PEG antibodies. We hypothesized that PEG can only elicit antibody responses when attached to an immunogenic carrier protein.

We used two different mouse models to test our hypothesis. The conventional hemophilic E17 mouse model recognizes human FVIII as a foreign protein. The human FVIII transgenic hemophilic mouse model recognizes human FVIII as a self-protein. We treated mice of both models with up to 8 weekly doses of two PEGylated FVIII preparations (PEGFVIII1 and PEGFVIII2). We knew that PEGFVIII2 breaks immune tolerance to human FVIII in human FVIII transgenic hemophilic mice.

Both PEGFVIII1 and PEGFVIII2 induced anti-PEG and anti-FVIII antibodies in conventional hemophilic E17 mice but only PEGFVIII2 induced anti-FVIII and anti-PEG antibodies in human FVIII transgenic hemophilic mice. The non-PEGylated FVIII control only induced anti-FVIII antibodies in conventional hemophilic E17 mice.

Our results support the hypothesis that induction of anti-PEG antibodies by PEGylated FVIII requires the presence of an immunogenic carrier protein that is recognized as foreign. We believe that the immunogenic carrier protein is required to induce CD4+ T cell help to facilitate the antibody response against the hapten PEG.

6.3 Posters

6.3.1 Multicolor Flow Cytometry - a powerful tool to quantify and characterize sub-visible particles in protein therapeutics.

Lubich C, Malisauskas M, Prenninger T, Matthiessen P, Turecek P, Scheiflinger F and Reipert BM

Poster Presentation at the 9th ENII EFIS/ EJI Summer School on Advanced Immunology, Porto Conte, Italy, May 2014

MULTICOLOR FLOW CYTOMETRY - A POWERFUL TOOL TO QUANTIFY AND CHARACTERIZE SUB-VISIBLE PARTICLES IN PROTEIN THERAPEUTICS

Lubich C, Malisauskas M, Prenninger T, Scheiflinger F and Reipert BM
Baxter Innovations GmbH, Vienna, Austria

Baxter

INTRODUCTION AND OBJECTIVES

The number of protein therapeutics that are in different stages of preclinical or clinical development is increasing, providing more patients with effective treatments for a number of medical conditions. A major challenge in the application of these protein therapeutics is the induction of unwanted immune responses in patients. It has been suggested that sub-visible particles, i.e. protein aggregates, may enhance the immunogenicity of a protein therapeutic and negatively impact clinical performance [1,2]. The formation of such protein aggregates can be caused by physical stress factors such as elevated temperature, freeze/thaw cycles or shear stress. To better understand the underlying mechanisms, it is necessary to expand the knowledge about the nature of sub-visible particles in protein based therapeutic formulations.

Here, we introduce a two dye flow cytometry approach that combines quantification and in-depth characterization of each sub-visible particle present in protein therapeutic formulations. To evaluate the potential of this technology, we assessed the amount, size and nature of sub-visible particles in a variety of in vitro generated sub-visible particle preparations.

METHODS

Flow cytometry based particle analysis

- Size estimation by using size calibration beads
- Calculation of particle numbers by using CountBright™ absolute counting beads (Invitrogen)
- Differentiation of proteinaceous and non-proteinaceous particles by using the fluorescent dye 4,4′ Dianilino 1,1′ binaphthyl 5,5′ disulfonic acid dipotassium salt (Bis-ANS)
- Characterization of protein sub-visible particles by fluorescent dye 9-(2,2-Dicyanovinyl)julolidine (DCVJ)

Thioflavin T (ThT) assay

Protein samples were diluted with PBS to a concentration of 10 µg/mL and mixed with the ThT stock solution to obtain a final concentration of 20 µM.

Silicon oil droplets

Silicon oil particles were produced by shaking PBS in a lubricated syringe for 1-2 min.

Biotynilated Bovine Serum Albumin (BSA) – Avidin complex

Biotinylated BSA and Avidin from egg white were mixed together at concentrations of 50 µg/mL Biotin-BSA and 12 µg/mL Avidin. The mixture was incubated at room temperature for 60 minutes. The resulting Biotin-BSA – Avidin complex was diluted 1:10 with D-PBS. The absence of cross-beta sheet structures was confirmed using the ThT assay (Figure 2).

Aggregated amyloid beta 1-40 peptide

1 mg amyloid beta 1-40 peptide was dissolved in 115 µL Water For Injection (WFI) to obtain a final concentration of 2 mM. This solution was incubated at + 2-8 °C for 168 h. The resulting material was diluted with WFI to obtain a final concentration of 15 µg/mL (12 µM). The presence of cross-beta sheet structures was confirmed using the ThT assay (Figure 2).

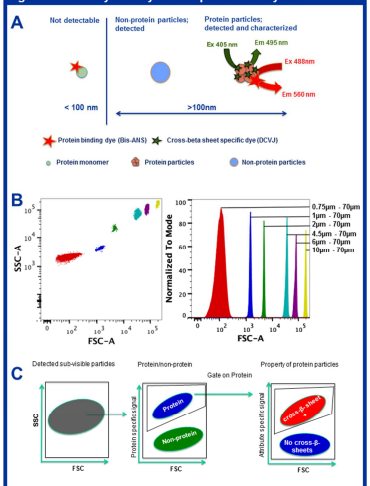
Factor VIII (FVIII) proteins

- full length rFVIII – product A
- full length rFVIII – product B
- BDD-rFVIII – product C
- BDD-rFVIII – product D

Treatment conditions

- Freshly reconstituted rFVIII products
- Clinically relevant stress:
  Agitation by hand for 10 min at room temperature and subsequently applied shear stress. The shear stress resulted from injecting the products thought "Winged infusion set with needle protection" (23G x3/4"; L=35 cm, V= 0.25 mL, Terumo Europe)

Figure 1: Flow Cytometry based particle analyses



A. the principle of flow cytometry based particles analysis

B. size calibration of flow cytometry

C. gating strategy for characterization of sub-visible particles

Figure 2: Confirmation of cross-β- sheet structures

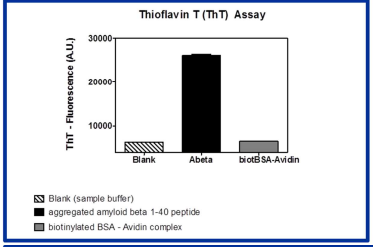


Figure 3: Characterizing the nature of sub-visible particles

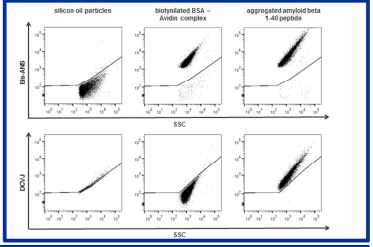
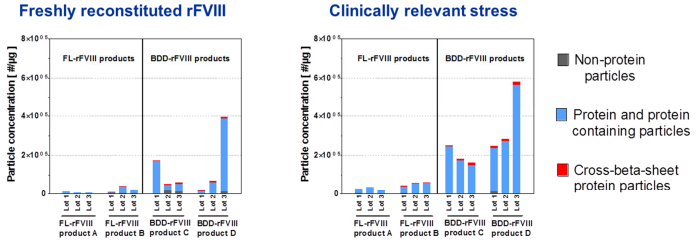


Figure 4: Sub-visible particle analyses in stressed rFVIII products



- Clinically relevant stress induced sub-visible particles in FL-rFVIII as well as in BDD-rFVIII products
- Higher concentrations of sub-visible particles were detected in BDD-rFVIII containing products in comparison to FL-rFVIII

RESULTS

Flow cytometry based particle analysis

Voltages for forward scatter (FSC) and side scatter (SSC) as well as the threshold levels for these channels were adjusted using Fluoresbrite® YG Carboxylate Size Range beads (Polyscience Inc., Pennsylvania) to establish appropriate settings for analyses of sub-visible particles. Beads with a diameter of 0.75 µm, 1 µm, 2 µm, 4.5 µm, 6 µm and 10 µm were used to define different size ranges by setting gates between the peak maxima of each bead population in a FSC histogram. These gates were used to estimate the size of each sub-visible particle and enable an assessment of the size distribution of sub-visible particles in each sample (Figure 1B).

To distinguish protein from non-protein particles we deployed the fluorescent dye Bis-ANS. Bis-ANS binds to hydrophobic surfaces and, therefore, preferentially to proteins. Binding of Bis-ANS results in an increased fluorescence intensity of protein containing samples like amyloid beta and biotynilated BSA-Avidin complex in contrast to non-protein particles like silicon oil droplets (Figure 3).

In-depth characterization of proteins was done by staining with the fluorescence dye DCVJ, which is widely used in the analyses of cross-β-sheet containing protein aggregates. The inclusion of DCVJ to the flow cytometry based particle analyses reveals proteins containing cross-β- sheet structures like amyloid beta (Figure 3).

Properties of sub-visible particles in stressed rFVIII products

Higher concentrations of sub-visible particles were detected in the BDD-rFVIII products when compared to FL-rFVIII products. Under clinically relevant stress condition, more sub-visible particles were detected in BDD-rFVIII containing products (Figure 4).

SUMMARY

We developed a novel multicolor flow cytometry based technology that enables us to simultaneously detect, quantify and characterize sub-visible particles in protein therapeutics. We used this new technology to asses different rFVIII products and revealed that BDD-rFVIII seems to be more sensitive for sub-visible particle formation when compared to FL-rFVIII. These data indicate that multicolor flow cytometry is a suitable technology to investigate the nature of sub-visible particles in protein therapeutics.

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6.3.2 Antibodies against polyethylene glycol (PEG) in healthy subjects – myth or reality?

Lubich C, Allacher P, Bauer A, Prenninger T, Horling F, Siekmann J, Scheiflinger F and Reipert BM

Poster Presentation at the ISTH 2015 Congress, Toronto, Canada, June 2015.

Poster # PO249-MON

Antibodies against polyethylene glycol (PEG) in healthy subjects – myth or reality?

Lubich C,[a] Allacher P,[b] Bauer A,[a] Prenninger T,[a] Horling F,[a] Siekmann J,[a] Scheiflinger F[a] and Reipert BM[a]

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INTRODUCTION

Polyethylene glycol (PEG) is a polymer with a broad range of applications in cosmetics, pharmaceuticals or processed foods. PEG is a polymer with a repetitive chemical structure [HO–(CH2–CH2–O)$_n$–H] which has the ability to bind water molecules. It is chemically inert and has low toxicity [1].

In the last decade the covalent attachment of PEG has emerged as successful strategy to increase the half-life of protein therapeutics. Currently, there are several PEGylated coagulation factors in clinical development with the aim to generate longer-acting FVIII and FIX products for the treatment of hemophilia A and B [2].

While many PEGylated biologics have been proven to be safe and non-immunogenic, a number of reports have presented data which indicated the development of anti-PEG antibodies following treatment with PEGylated therapeutics. In some cases, anti-PEG antibodies have been associated with reduced efficacy or hypersensitivity [3,4].

There have also been reports on the detection of anti-PEG antibodies in healthy blood donors who have not received PEGylated protein therapeutics, suggesting that exposure to PEG polymers contained in consumer products may lead to the development of anti-PEG antibodies in a subset of the population. However, some of these reports on anti-PEG antibodies have evoked controversial discussion because of shortcomings in the assays used for the analysis of these antibodies [5].

OBJECTIVES

- To establish validated methods for the detection of anti-PEG antibodies in plasma of human healthy donors
- To revisit the analysis of anti-PEG antibodies in healthy subjects using these validated technologies.

METHODS

Human healthy donors

Two cohorts of healthy individuals were included in our analysis. The first cohort comprised 710 healthy plasma donors from Austria. The second cohort included 600 healthy plasma donors from 6 different geographies - 100 individuals from Austria and 100 individuals from each of the following locations in the United States: Ammon (ID), Elkhart (IN), Fargo (ND), Lakeland (FL) and Laredo (TX). Samples of citrated human plasma were collected and stored at -20°C until analysis.

Detection of human anti-PEG antibodies

ELISA assays

The presence of human IgG and IgM antibodies against PEG was determined by validated semi-quantitative ELISA assays including sensitive screening assays and competition based confirmatory assays. The ELISA assay was validated with respect to cut-off calculation, sensitivity, specificity, linearity, and precision (inter-assay and intra-assay variability). Each assay included positive and negative controls to monitor the performance of the assays. Using monoclonal rabbit IgM or IgG anti-PEG antibodies (both Epitomics, USA) we assessed a relative sensitivity of 160ng/mL for IgM and 8ng/mL for IgG.

Flow Cytometry (FCM) assays

To detect human binding anti-PEG antibodies (IgG and IgM), TentaGel M NH2 polystyrene microspheres (Rapp Polymere, Germany) were chemically modified by attaching a 20 kDa branched PEG (Nektar Therapeutics, USA) to the amino group on the linear PEG on their surfaces. Similar to the approach taken with the ELISA assay platform, a multi-tiered approach including a sensitive screening assay and a competition based confirmatory assay was applied for all analyses.

Specificity

The specificity of the detected anti-PEG antibodies was confirmed by pre-incubating plasma samples at a dilution of 1:20 with soluble PEG 200. Based on the assay variation of ± 1 titer step, a sample needs to have an antibody titer of ≥1:80 to be confirmable for specificity. In some experiments, the specificity of anti-PEG antibodies was further evaluated by incubating plasma samples with different PEG species, e.g. PEG 2 kDa, PEG 20 kDa (both Sigma Aldrich, Missouri, USA) and a branched PEG 20 kDa (Nektar Therapeutics, California, USA), using as competition antigen a branched PEG of 20kDa, as well as linear PEGs of 2 and 20kDa with selected donors (Table 1).

RESULTS

Prevalence of PEG-binding antibodies

Prevalence

Our data confirm the presence of binding anti-PEG antibodies in healthy individuals (Figure 1). The prevalence in our study cohort was 20-28%, with 12-26% for titers <1:80 and 2-11% for titers ≥1:80. IgG and IgM subclasses were in both cohorts equally distributed (Table 2).

Correlation analysis of plasma donor covariates

Next we were interested in the potential impact of donor covariates like sex and age on the prevalence of PEG specific antibodies. Age was identified as significant covariate for the expression of IgG antibodies in both cohorts (Figure 2), with the highest prevalence observed in young adults (18-30 years old).

Figure 2. Impact of the age of the healthy individuals on the prevalence on anti-PEG antibodies

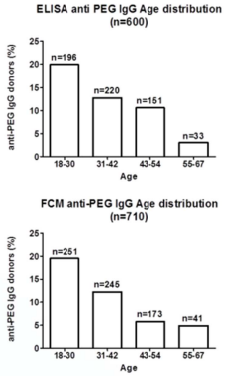


Figure 1. Titers of PEG-binding antibodies assessed for individual IgG and IgM isotypes

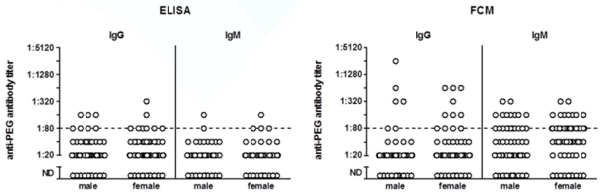


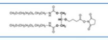
Table 2. Estimated prevalence of PEG-binding antibodies of healthy individuals

	FCM cohort analyses (n=710)			ELISA cohort analyses (n=600)		
	total % (95% CI)	IgG % (95% CI)	IgM % (95% CI)	total % (95% CI)	IgG % (95% CI)	IgM % (95% CI)
Prevalence of positive individuals	23 (20-27)	13 (11-15)	15 (12-17)	24 (21-28)	14 (11-17)	12 (10-15)
Prevalence of antibodies with titers ≥ 1:80	9 (7-11)	2 (2-4)	7 (5-9)	4 (2-5)	3 (2-4)	1 (0-2)
Prevalence of antibodies with titers < 1:80	15 (12-17)	10 (8-13)	8 (6-10)	23 (19-26)	12 (9-14)	11 (9-14)

Specificity of human IgG anti-PEG antibody

Next we investigated whether the anti-PEG antibodies are able to recognize PEG polymers of different sizes and different chemistries. We addressed this question by competitive ELISAs with plasma samples of all donors that had IgG anti-PEG antibodies with a titer of ≥1:80 . Our results shown in Table 1 indicate that anti-PEG antibodies in healthy donors bind to a variety of different PEG polymers with or without a methoxy group. Nevertheless we observed that with increasing length of the backbone of the polymers as well as using a methoxy branched PEG the amount of necessary competition antigen to reduce the OD signal in the ELISA by 50% decreases.

Table 1. Human IgG PEG-antibodies recognize PEG polymers of different sizes and chemistries

PEG polymer	Structure	Number of ethoxy monomer groups	Methoxy Group	recognition by polyclonal IgG anti-PEG antibodies	[µM] for 50 % OD reduction
linear PEG 200Da		~4	no	yes	2700
linear PEG 2kDa		~40	no	yes	1.39
linear PEG 20kDa		~450	no	yes	0.125
branched PEG 20kDa		~450	yes	yes	0.009

CONCLUSION

- In this study we present a comprehensive data set on the prevaence of IgG and IgM antibodies against PEG in healthy human subjects. For this investigation we developed two assay formats: a flow cytometry approach (FCM) and an ELISA platform validated with respect to cut-off calculation, sensitivity, specificity, linearity, and precision (inter-assay and intra-assay variability).
- We confirm that some healthy individuals express antibodies against PEG. However, there was no indication of any pathology associated with these antibodies. These antibodies might be induced by PEG polymers or their derivatives present in cosmetics or pharmaceuticals.

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DISCLOSURES

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C.L., A.B., T.P., F.M.H, J.S., F.S. and B.M.R. are employees of Baxalta Innovations GmbH.

6.3.3 Towards understanding the impact of sub-visible particles in protein therapeutics on the induction of unwanted immune responses

Lubich C, Malisauskas M, Prenninger T, Hopfner E, Scheiflinger F and Reipert BM

Poster Presentation at the ECI 2015 Congress, Vienna, Austria, September 2015.

Poster No. **P.D.24.11**

Towards understanding the impact of sub-visible particles in protein therapeutics on the induction of unwanted immune responses

Christian Lubich, Mantas Malisauskas, Thomas Prenninger, Elisabeth Hopfner, Friedrich Scheiflinger and Birgit M. Reipert

Baxalta Innovations GmbH, Vienna, Austria

Background

A major challenge in the application of protein therapeutics is the induction of unwanted immune responses in patients. It has been suggested that sub-visible particles, i.e. protein aggregates, may enhance the immunogenicity of a protein therapeutic and negatively impact clinical performance [1,2]. The formation of such protein aggregates can be caused by physical stress factors such as elevated temperature, freeze/thaw cycles or shear stress.To better understand the underlying mechanisms, it is necessary to expand the knowledge about the nature of sub-visible particles in protein based therapeutic formulations.

Here, we introduce a two dye flow cytometry approach that combines quantification and characterization of each sub-visible particle present in protein therapeutic formulations. We used the new technology in combination with cellular *in vitro* assays and suitable *in vivo* models to assess the potential of different sub-visible particle preparations to trigger the innate immune system.

Methods

Flow cytometry based particle analysis

- Size estimation by using size calibration beads
- Calculation of particle numbers by using CountBright™ absolute counting beads (Invitrogen)
- Differentiation of proteinaceous and non-proteinaceous particles by using the fluorescent dye 4,4′-Dianilino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt (Bis-ANS)
- Characterization of protein sub-visible particles by fluorescent dye 9-(2,2-Dicyanovinyl)julolidine (DCVJ)

Representative study to assess immunogenicity of sub-visible particles

Goals: Characterize sub-visible particles in test samples and assess potential risk of sub-visible particles to enhance immunogenicity of a protein therapeutic

Test samples:

- Protein 1 native
- Protein 1 aggregated
- Protein 2 aggregated (positive control for cross-β-sheet structure)

Cytokine release assay

Test samples were tested on their ability to trigger the innate immune system by incubation with a monocytic human cell line (THP-1). After 24 hours, acivation of THP-1 cells was analyzed by their release of pro-inflammatory cytokines using a human multiplex.

Immunogenicity in animal models

Protein 1 and Protein 2 aggregates were assessed on their potential to enhance the adaptive immune response against native Protein 1 in a Protein 1 knock-out mouse. Mice were treated 8 times i.v. in weekly intervals with Protein 1 native alone or in a 1:1 ratio with either Protein 1 aggregated or Protein 2 aggregated . Blood was drawn 1 week after the last immunization and analyzed for anti-native Protein 1 IgG antibodies.

Results

Flow Cytometry based particle analyses

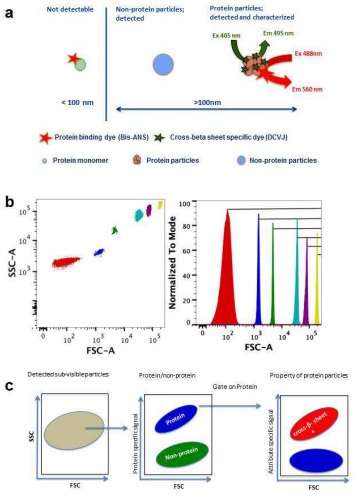

Figure 1.
a) the principle of flow cytometry based particles analysis
b) size calibration of flow cytometry
c) gating strategy for characterization of sub-visible particles

Flow cytometry based particle analysis

Voltages for forward scatter (FSC) and side scatter (SSC) as well as the threshold levels for these channels were adjusted using Fluoresbrite® YG Carboxylate Size Range beads (Polyscience Inc., Pennsylvania) to establish appropriate settings for analyses of sub-visible particles. Beads with a diameter of 0.75 µm, 1 µm, 2 µm, 4.5 µm, 6 µm and 10 µm were used to define different size ranges by setting gates between the peak maxima of each bead population in a FSC histogram. These gates were used to estimate the size of each sub-visible particle and enable an assessment of the size distribution of sub-visible particles in each sample (b).

To distinguish protein from non-protein particles we deployed the fluorescent dye Bis-ANS. Bis-ANS binds to hydrophobic surfaces and, therefore, preferentially to proteins.

In-depth characterization of proteins was done by staining with the fluorescence dye DCVJ, which is widely used in the analyses of cross-β-sheet containing protein aggregates. The inclusion of DCVJ to the flow cytometry based particle analyses reveals cross-β-sheet structures like amyloid beta (c).

Multitiered analytical approach to assess the immunogenicity of sub-visible particles

To better understand the underlying mechanism of the potential immunogenicity of sub-visible particles we combine the characterization of protein aggregates by flow cytometry with in vitro and in vivo immunogenicity assessment.

Flow cytometry analysis of the test samples in a representative study revealed, that aggregates of Protein 1 and 2 are distinct protein aggregates and only aggregated Protein 2 contains cross-beta-sheet structures (Figure 2).

Only aggregated protein 2 induces cytotoxicity (data not shown) and the release of pro-inflammatory cytokines (Figure 3).

Only aggregated protein 2 can enhance the adaptive immune responses against the co-administered native protein (Figure 4).

Multitiered analytical approach

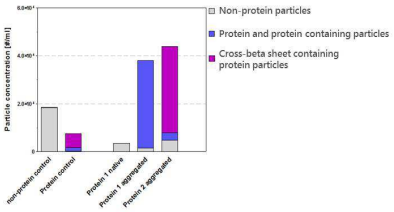

Figure 2. Sub-visible particle characterization of different protein preparations using flow cytometry.

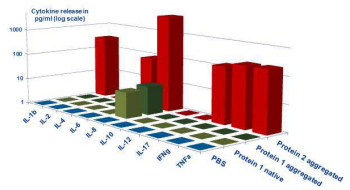

Figure 3. *In vitro* immunogenicity assessment of different protein particles to trigger the innate immune system using a cytokine release assay in THP-1 cells.

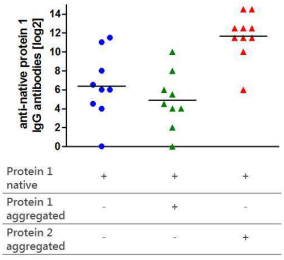

Figure 4. *In vivo* immunogenicity assessment of different protein aggregates on their ability to enhance the adaptive immune response.

Conclusion

We developed a novel multicolor flow cytometry based technology that enables us to simultaneously detect, quantify and characterize sub-visible particles in protein therapeutics [3]. Flow cytometry together with currently used standard bioanalytical methods can provide a powerful tool to investigate the nature of sub-visible particles in protein therapeutic formulations.

The combination of sub-visible particle characterization, *in vitro* immunogenicity assessment and *in vivo* immunogenicity assessment can provide new insight into mechanism regulating the induction of unwanted immune responses to protein therapeutics

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Disclosure

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C.L., M.M., T.P., E.H., F.S. and B.M.R. are employees of Baxalta Innovations GmbH.

Baxalta

6.3.4 Novel insights into antibodies against polyethylene glycol (PEG) in healthy subjects

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Novel insights into antibodies against polyethylene glycol (PEG) in healthy subjects

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Background

Polyethylene glycol (PEG) is a polymer with a broad range of applications in cosmetics, pharmaceuticals or processed foods. In the last decade the covalent attachment of PEG has emerged as successful strategy to increase the half-life of protein therapeutics [1]. While many PEGylated biologics have been proven to be safe and non-immunogenic, reports of anti-PEG antibodies in healthy subjects [2] and treatment related development of anti-PEG antibodies in patients [3] are increasing. Some of these reports on anti-PEG antibodies have evoked controversial discussion because of shortcomings in the assays used for the analysis of these antibodies [4].

Methods

Two cohorts of healthy human subjects (n=1310) were analyzed for the presence of IgG and IgM anti-PEG antibodies using two assay formats.

ELISA assays

The ELISA assay was validated with respect to cut-off calculation, sensitivity, specificity, linearity, and precision. Each assay included positive and negative controls to monitor the performance of the assays. Using monoclonal anti-PEG antibodies (both Epitomics, USA) we assessed a relative sensitivity of 160ng/mL for IgM and 8ng/mL for IgG.

Flow Cytometry (FCM) assays

A 20kDa branched PEG was attached to the surface of TentaGel M NH2 polystyrene microspheres (Rapp Polymere, Germany) to detect anti-PEG antibodies.

For both assay platforms, a multi-tiered approach including a sensitive screening assay and a competition based confirmatory assay was applied for all analyses.

Persistence of anti-PEG antibodies

We periodically screened a cohort of 38 healthy subjects for IgM and IgG anti-PEG antibodies over a period of up to 14 months using our validated ELISA.

Conclusion

We developed two assay formats to assess the prevalence of IgG and IgM antibodies against PEG: a flow cytometry approach (FCM) and a validated ELISA platform.

We confirm that some healthy individuals express antibodies against PEG. However, there was no indication of any pathology associated with these antibodies. These antibodies might be induced by PEG polymers or their derivatives present in cosmetics or pharmaceuticals

References

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Disclosure

This work was supported by Baxalta Innovations GmbH.

C.L., A.B., T.P., F.M.H., J.S., F.S. and B.M.R. are employees of Baxalta Innovations GmbH.

Results

Prevalence of PEG-binding antibodies

Our data confirm the presence of binding anti-PEG antibodies in healthy individuals (Figure 1). The prevalence in our study cohort was 20-28%, with 12-26% for titers <1:80 and 2-11% for titers ≥1:80. IgG and IgM subclasses were in both cohorts equally distributed (Table 1).

Correlation analysis of plasma donor covariates

Next we were interested in the potential impact of donor covariates like sex and age on the prevalence of PEG specific antibodies. Age was identified as significant covariate for the expression of IgG antibodies in both cohorts with the highest prevalence observed in young adults (18-30 years old).

Figure 1. Titers of PEG-binding antibodies assessed for individual IgG and IgM isotypes

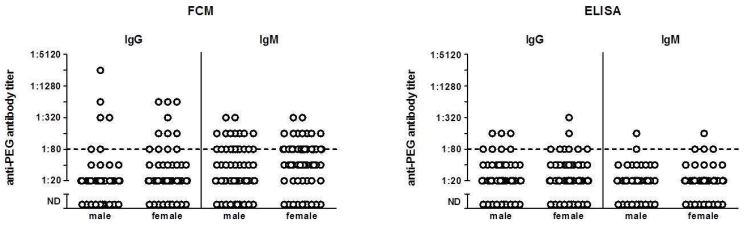


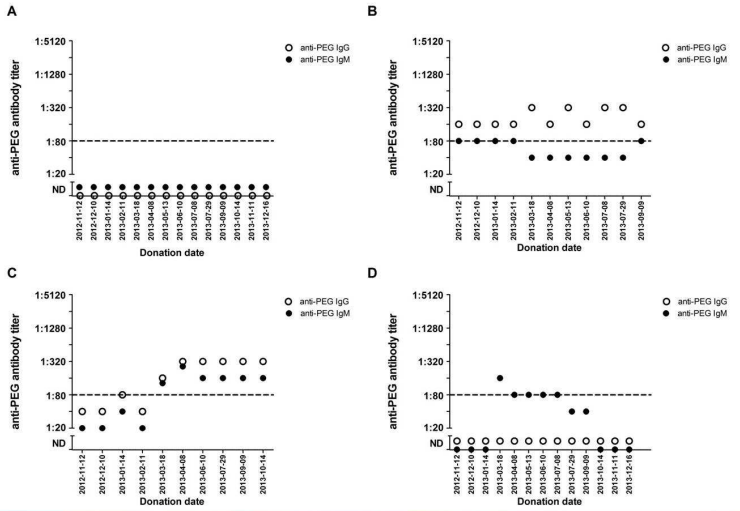
Table 1. Estimated prevalence of PEG-binding antibodies of healthy individuals

	FCM cohort analyses (n=710)			ELISA cohort analyses (n=600)		
	total % (95% CI)	IgG % (95% CI)	IgM % (95% CI)	total % (95% CI)	IgG % (95% CI)	IgM % (95% CI)
Prevalence of positive individuals	23 (20-27)	13 (11-15)	15 (12-17)	24 (21-28)	14 (11-17)	12 (10-15)
Prevalence of antibodies with titers ≥ 1:80	9 (7-11)	2 (2-4)	7 (5-9)	4 (2-5)	3 (2-4)	1 (0-2)
Prevalence of antibodies with titers < 1:80	15 (12-17)	10 (8-13)	8 (6-10)	23 (19-26)	12 (9-14)	11 (9-14)

Persistence of PEG-binding antibodies

The persistence of binding IgG and IgM antibodies against PEG was evaluated in a cohort of 38 healthy subjects using a validated ELISA method (Figure 2). Subjects were periodically screened over a period of 14 months. Shown is the course of binding anti-PEG antibodies over time in representative individual blood donors. Titers of ≥ 1:80 were confirmed for specificity to PEG. Low titers of 1:20 and 1:40 are too low to be confirmable for specificity and might represent cross-reactive antibodies.

Figure 2: Persistence of IgG and IgM antibodies against PEG in healthy human subjects



7. Curriculum vitae

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Education

2010-2015	PhD thesis at the department of Immunology at Baxalta former Baxter Innovations GmbH, Vienna, Austria
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2001-2008	Studies of Biology specialized in Genetics and Microbiology, University of Vienna, Austria
01/2006-06/2006	Exchange semester at the University of Lund, Sweden
1992-2000	Secondary school stressing Ecology, Biology and Informatics, BRG 7 (federal gymnasium), Vienna, Austria
1988-1992	Primary school, Volksschule Benedikt-Schellinger Gasse, Vienna, Austria

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2007-2015	Various positions at the department of Immunology
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