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(54) Title: A METHOD FOR DETECTING AND/OR REMOVING A PROTEIN COMPRISING A CROSS-ß STRUCTURE FROM A PHARMACEUTICAL COMPOSITION

(57) Abstract: The invention relates to the field of compositions comprising a protein, more specifically to pharmaceutical compositions. More specifically, the invention relates to the detection and/or removal of conformationally altered proteins and/or molecules comprising a cross-ß structure from a pharmaceutical composition or any of its constituents comprising a protein. The present invention discloses that unwanted and/or toxic side effects of pharmaceuticals are caused by proteins present in said pharmaceutical and adopting a cross-b structure conformation. The invention further discloses a method for detecting a protein and/or peptide comprising a cross-ß structure in a pharmaceutical composition or any of its constituents comprising a protein, said method comprising, contacting said pharmaceutical composition or any of its constituents comprising a protein with at least one cross-ß structure-binding compound resulting in a bound protein and/or peptide comprising a cross-ß structure and; detecting whether bound protein and/or peptide comprising a cross-ß structure are present in said pharmaceutical composition or any of its constituents comprising a protein. The invention further disclose methods for removing cross-ß structures from a pharmaceutical composition and controlling manufacturing a pharmaceutical composition.

Title: A method for detecting and/or removing a protein comprising a cross-β structure from a pharmaceutical composition.

The invention relates to the field of compositions comprising a protein, more specifically to pharmaceutical compositions. More specifically, the invention relates to the detection and/or removal of conformationally altered proteins and/or molecules comprising a cross-β structure from a pharmaceutical composition or any of its constituents comprising a protein.

Pharmaceutical compositions are in general suitable for administration to a subject, said subject being an animal or a human. Many pharmaceutical compositions are available that are either manufactured or purified by processes in which proteins or peptides are involved, or are based on protein and/or polypeptide and/or peptide or amino-acid compositions, including compositions with amino-acid derivatives. Important categories of nowadays pharmaceutical compositions comprising a protein or a proteinaceous compound as an active substance include, but are not limited to hormones, enzymes, vaccines and antigens, cytokines and antibodies. In addition to the above-mentioned proteinaceous pharmaceutical compositions, a large number of pharmaceutical compositions are manufactured with the help of a production and/or purification step comprising proteins. For example, many pharmaceutical compositions comprise one or more proteins as a stabilizing agent.

Safety aspects are of great concern with any pharmaceutical composition. Drug stability during production and storage, and after administering to the body, attracts much effort during development of new active compounds, and thereafter . Market withdrawals of initially successful pharmaceutical compositions are sometimes necessary because of the occurrence of unforeseen and undesired side effects. For example: plasma, erythropoietin, insulin, antibodies, aprotinin, albumin, thrombopoietin, interferon α, factor VIII, have all caused unwanted side effects after

administration in individuals. These examples underline that continuous improvement of the current safety testing methodologies is necessary to reduce the risk for unforeseen, unwanted and/or deleterious side effects after administering pharmaceutical compositions to a human or animal.

Health problems related to the use of pharmaceutical compositions are for example related to the fields of haematology, fibrinolysis and immunology. An incomplete list of observed side-effects after administration of pharmaceutical compositions comprises for example fever, anaphylactic responses, (auto)immune responses, disturbance of haemostasis, inflammation, fibrinolytic problems, including sepsis and disseminated intravascular coagulation (DIC), which can be fatal. Said side effects can be caused by either an alteration of a protein or a proteinaceous compound present in said pharmaceutical composition, or by added diluents or carrier substances of said pharmaceutical composition. A proteinaceous compound in this specification means any compound which comprises a peptide, polypeptide, or protein, and/or altered or degraded forms thereof. Alteration of the proteinaceous compound of a pharmaceutical composition comprises for example denaturation, multimerization, proteolysis, acetylation, glycation, oxidation or unfolding of proteins.

An increasing body of evidence shows that unfolding of initially properly folded native proteins leads to the formation of toxic structures in said proteins.

The present invention discloses that said toxic structures are cross-ß structures, The present invention further discloses methods and means for detecting cross-ß structures in pharmaceutical composition and/or any of its constituents comprising a protein.

In this specification, the terms " cross-ß structure conformation" and "cross-ß structure" are synonymous and are interchangeably used herein.

A cross-ß structure is defined as a part of a protein or peptide, or a part of an assembly of peptides and/or proteins, which comprises an ordered group of ß-strands, typically a group of ß-strands arranged in a ß-sheet, in particular a group of stacked or layered ß-sheets also referred to as "amyloid". A typical form of stacked ß-sheets is in a fibril-like structure in which the ß-sheets may be stacked in either the direction of the axis of the fibril or perpendicular to the direction of the axis of the fibril. The term peptide is intended to include oligopeptides as well as polypeptides, and the term protein includes proteins with and without post-translational modifications, such as glycosylation. It also includes lipoproteins and complexes comprising proteins, such as protein-nucleic acid complexes (RNA and/or DNA), membrane-protein complexes. Different fluorescent light scattering profiles of amyloid dyes, such as for example Congo red or Thioflavin T in staining various amyloid-like aggregates indicate that different cross-β structures occur. Said cross-β structures are for example found in glycated proteins and in fibrils[1]. Such fibrillar aggregates accumulate in various tissue types and are associated with a variety of degenerative diseases. The term "amyloid" is being used to describe fibrillar deposits (or plaques)[2]. In literature, an amyloid fibril is preferably defined as an aggregate that is stained by Congo red and/or Thioflavin T, that appears as fibrils under an electron microscope, and that contains an increased amount of ß-sheet secondary structure[2]. Additionally, the presence of ß-sheet rich structures can be defined with X-ray fibre diffraction techniques and/or Fourier transform infrared spectroscopy. A common denominator of amyloid-like structures is the presence of the cross-ß structure structural element. Peptides or proteins with amyloid-like structures are cytotoxic to cells [3-6]. Diseases characterized by amyloid are referred to as conformational diseases or amyloidoses and include for example Alzheimer's disease (AD), light-chain amyloidosis, type II diabetes and spongiform encephalopathies like for example Bovine Spongiform Encephalopathy (BSE) and Creutzfeldt-Jakob's disease.

In addition, deleterious effects of aggregated proteins are not solely mediated by said amyloid fibrillar depositions of proteins, but also by soluble oligomers of aggregates with amyloid-like properties and by diffuse amorphous aggregates [3,5]. The recent finding that toxicity is an inherent property of misfolded proteins implies a common mechanism for said conformational diseases [1,3,6].

We showed that tissue-type plasminogen activator (tPA) and factor XII (FXII) are specifically activated by many polypeptides, once they have adopted the cross-β structure conformation[7]. This led us to recognize that a 'cross-β structure pathway' exists that regulates the recognition and clearance of unwanted proteins[1]. Polypeptides can refold spontaneously, at the end of their life cycle, or refolding can be induced by environmental factors such as pH, glycation, oxidative stress, heat, irradiation, mechanical stress, proteolysis or contact with denaturing surfaces or compounds, such as negatively charged lipids, plastics or biomaterials. At least part of the polypeptide refolds and adopts the amyloid-like cross-β structure conformation. This cross-β structure containing conformation is then the signal that triggers a cascade of events that induces clearance and breakdown of the particle. When clearance is inadequate unwanted polypeptides can aggregate and form toxic structures ranging from soluble oligomers up to precipitating fibrils and amorphous plaques. Such cross-β structure containing structures underlie various diseases, depending on the polypeptide that accumulates and on the part of the body where accumulation occurs.

The presence of cross-β structures in proteins triggers multiple responses. As mentioned, cross-β structure comprising proteins can activate tPA and FXII, thereby initiating the fibrinolytic system and the contact system of haemostasis. Besides activation of the coagulation system through FXII, the cross-β structure conformation may induce coagulation, platelet aggregation and blood clotting via direct platelet activation and/or the release of tissue factor (Tf) by activated endothelial cells. In addition, the complement system is

another example of a proteolytic cascade that is activated by cross-β structures. This system can be activated by the amyloid-β peptide associated with Alzheimer's Disease or by zirconium or aluminum or titanium. The latter being compounds that can induce cross-β structure conformation in proteins. The innate and adaptive immune systems are yet another example. Amyloid-β activates the innate and adaptive immune response[8]. β2-glycoprotein I is an auto-immune antigen only upon contact with a negatively charged lipid surface, such as cardiolipin[9]. We have now shown that cardiolipin induces cross-β structure conformation in β2-glycoprotein I (described in more detail elsewhere). Moreover, we have shown that ligands for Toll-like receptors that are implicated in the regulation of immunity induce cross-β structure conformation in proteins. These ligands include lipopolysaccharide and CpG oligodeoxynucleotides (ODN) (described in more detail elsewhere).

The β2-glycoprotein I protein (ß2GPI), together with IgM antibodies, C1q and likely other proteins are all also acting in another way in the proposed cross-β structure pathway. It is assumed that a set of cross-β structure binding proteins bind specifically to sites of 'danger', e.g. negatively charged phospholipids, amyloid plaques, sites of ischemic injury, necrotic areas, all with its own specificity. Upon binding, the 'dangerous' condition is neutralized and for example excessive coagulation at negatively charged lipid surfaces will not occur. Secondly, the proteins bound to the 'dangerous' site undergo a conformational change resulting in the formation of the cross-β structure conformation. This fold then acts as a signal for cross-β structure binding proteins that are part of the 'cross-β structure pathway', leading to the clearance of the bound protein or protein fragment and removal of the 'danger'.

The cross-ß structure pathway also acts in yet another way. Proteins that circulate in complex with other proteins may comprise a shielded cross-ß structure conformation. Once the protein is released from the accompanying protein, the cross-ß structure becomes exposed, creating a binding site for cross-ß structure binding proteins of the cross-β structure pathway. This then

results in breakdown or clearance of the released protein. An example is factor VIII, which circulates in complex with von Willebrand factor (vWF). In this complex, factor VIII is prevented from clearance, so vWF covers the clearance signal that becomes exposed after the complex is dissociated. This clearance signal is the cross-ß structure. Treatment of hemophilia patients with recombinant factor VIII (FVIII) may induce inhibitors (anti-FVIII autoantibodies) because the patients lack sufficient vWF to protect the clearance signal comprising the cross-β structure conformation. Excess exposure of FVIII comprising cross-β structure conformation may induce activation of the immune system and generation of anti-FVIII antibodies similar to the generation of anti-β2GPI autoimmune antibodies by β2GPI bound to negatively charged phospholipids and possibly autoimmune responses.

The compounds listed in Table 1 and the proteins listed in Table 2 all bind to polypeptides with a non-native fold. In literature, this non-native fold has been designated as protein aggregates, amorphous aggregates, amorphous deposit, tangles, (senile) plaques, amyloid, amyloid-like protein, amyloid oligomers, amyloidogenic deposits, cross-ß structure, ß-pleated sheet, cross-ß spine, denatured protein, cross-ß sheet, ß-structure rich aggregates, infective aggregating form of a protein, unfolded protein, amyloid-like fold/conformation and perhaps alternatively. The common theme amongst all polypeptides with an amyloid-like fold, that are ligands for one or more of the compounds listed in Table 1 and 2, is the presence of a cross-ß structure.

The compounds listed in Table 1 and 2 are considered to be only an example of compounds known to day to bind to amyloid-like protein conformations. The lists are thus non-limiting. More compounds are known today that bind to amyloid-like protein conformation. For example, in patent AU2003214375 it is described that aggregates of prion protein, amyloid, and tau bind selectively to polyionic binding agents such as dextran sulphate or pentosan (anionic), or to polyamine compounds such as poly

(Diallyldimethylammonium Chloride) (cationic). Compounds with specificity for amyloid-like folds of proteins listed in this patent and elsewhere are equally suitable for methods and devices disclosed in this patent application. Moreover, also any compound or protein related to the ones listed in Table 1 and 2 are covered by the claims. For example, point mutants, fragments, recombinantly produced combinations of cross-β structure binding domains and deletion- and insertion mutants are part of the set of compounds as long as they are capable of binding to protein with cross-β structure conformation (i.e. as long as they are functional equivalents). Even more, also any newly discovered small molecule or protein that exhibits affinity for a protein and/or peptide with the cross-β structure conformation can be used in any one of the methods and applications disclosed here.

The compounds listed in Table 3 are also considered to be part of the '*Cross-β structure pathway*', and this consideration is based on literature data that indicates interactions of the listed molecules with compounds that likely comprise the cross-β structure conformation but that have not been disclosed as such.

Generally, for the production of a proteinaceous pharmaceutical composition, a protein or proteinaceous molecule or compound is isolated from an animal or plant or is synthesized *in vitro*. Said protein or proteinaceous molecule or compound is subjected to a number of processes like for example a purifying or isolating process from an animal or plant source, or asynthesis process, such as for example a peptide synthesis process, or a synthesis in a plant cell, a yeast cell or a bacteria, or a synthesis in a eukaryotic cell, and/or a manufacturing process, like for example the coupling of chemical molecules to a peptide or protein, and/or an isolation procedure or a purification procedure, and/or concentrating process, like for example the isolation of recombinant protein from a bacterial production cell, or purification by a physical, or a chemical, or an immunological isolation method, and/or a formulation and/or a

storage process, including for example a lyophilization process and/or the addition of a suitable stabilizer, a diluent and/or an adjuvant.

Any one of these processes affects the folding of a protein or a proteinaceous compound. Quality control in a manufacturing process preferably aims at identifying and/or minimizing the deleterious effects of each process step for said pharmaceutical composition, thereby increasing the activity of the composition in the final composition and/or decreasing the undesired side effects of the composition.

Alteration of a protein or proteinaceous composition is generally detected by measuring a specific binding site or a specific activity of said protein or proteinaceous composition, or an increase in size or multimerization state of said protein or proteinaceous composition, or a decrease in therapeutic activity of said proteinaceous composition.

As to the first of said methods, a partially unfolded or misfolded protein can still expose a specific binding site. Therefore, testing the quality of a pharmaceutical composition by only testing for a specific binding site is not always a reliable method, because the partial unfolding or degradation of said protein is not detected.

The second of said methods, the size-related detection method is based on the concept that denaturation leads to aggregation of proteins, thereby increasing the size of the proteinaceous molecule. One of several methods for detecting an increase in size of proteins is called size exclusion chromatography. Nowadays, size exclusion chromatography is widespread used as a method to analyse the contents of a protein drug. This technique is generally accepted for the testing of protein drug stability (http://etd.utmem.edu/WORLD_ACCESS/ymi/reviewofanalyticmethod.htm).

Because said detection method only detects the size of proteinaceous molecules, it cannot detect misfolded proteins or proteins with increased content of cross-β structure conformation that have not aggregated or increased in size. Therefore, quality control based on the above-described

method of detecting an increase in size of the proteinaceous molecules, does not prevent undesired side effects caused by conformational changes such as for example cross-β structure conformation formed upon denaturation, proteolysis, chemical modification, or unfolding of proteins, in the absence of increased molecular size. Moreover, nowadays guidelines that determine the acceptable amounts of aggregates in proteinaceous drug solutions are based on technical limitations of the available purification methods, rather than on knowledge about expected undesired side effects of the aggregated proteins. Therefore a better quality control method is highly needed by scientists involved in development of proteinaceous compositions and/or pharmaceutically active compounds and formulations and for manufacturers of proteins or proteinaceous compositions and/or vaccines and/or pharmaceutical compositions and constituents thereof, comprising a protein.

The present invention discloses that unfolded and/or misfolded proteins or proteinaceous molecules like for example molecules that are proteolysed, denatured, unfolded, glycated, oxidized, acetylated, multimerized or otherwise structurally altered, adopt a cross-β structure conformation. Furthermore, the present invention discloses that unwanted and/or toxic side effects of pharmaceuticals are caused by proteins present in said pharmaceutical and adopting a cross-β structure conformation.

The invention provides methods to detect the presence of cross-β structure conformation. The invention provides also methods for the removal of proteins or peptides from pharmaceutical compositions comprising a cross-β structure conformation, thereby reducing the toxicity and unwanted side effects and increasing the specific activity per gram protein of said compositions. Therefore, the methods of the invention provide a person skilled in the art with a method for monitoring and optimising the production methods and storing conditions of a pharmaceutical composition.

In one embodiment, the present invention provides a method for detecting a protein and/or peptide comprising a cross-ß structure conformation in a pharmaceutical composition or any of its constituents comprising a protein, said method comprising: contacting said pharmaceutical composition or any of its constituents comprising a protein comprising at least one cross-ß structure-binding compound, preferably selected from Tables 1-3 or functional equivalents thereof, resulting in a bound cross-ß structure, detecting whether bound cross-ß structures are present in said pharmaceutical composition or any of its constituents comprising proteins.

The invention discloses that various binding molecules or binding compounds, as described in Table 1, 2 and/or 3 of the application, alone or in combination with other binding compounds, are capable of binding to a protein and/or peptide comprising a cross-ß structure conformation. Binding of one or more of the cross-ß structure-binding compounds of Table 1, 2 and/or 3 or others to a protein and/or peptide comprising across-ß structure conformation is detected by means of a visualization reaction as for example by fluorescent staining or an enzymatic or colorimetric detection, or by any other visualization system available to a skilled person. Therefore, the invention provides a method of the invention, wherein said cross-ß structure-binding compound is a compound according to Table 1, or Table 2, or Table 3 or a functional equivalent of any of said compounds and/or a combination of any of said compounds.

In Table 1, 2 and/or 3, various different binding compounds are described that bind to compounds with cross-ß structure conformation. For example, Table 1 comprises among other, dyes like Thioflavin T, Thioflavin S, and Congo Red, that are used for staining amyloid molecules in histological sections or in solution. Table 2 comprises bioactive compounds binding to compounds comprising cross-ß structure conformations such as tissue-type plasminogen activator, factor XII, fibronectin, and others.

In Table 3, proteins are disclosed that are involved in the cross-ß structure pathway, like for example, antibodies, heat shock proteins and receptors. In another embodiment, the invention provides a protein specific way of detecting and removing compounds with cross-ß structure conformations, by combining the protein specific binding of an antibody or functional part thereof (i.e. a part of an antibody that specifically binds to a protein), with the compound with cross-ß structure conformations binding of a cross-ß structure binding compound. Therefore, the invention also provides molecular recognition units binding to compounds with cross-ß structure conformations, single chains of antibodies, or recombinant binding molecules. The invention also provides bi-specific binding molecules for example comprising the binding portion of tPA and an antibody, or the binding portion of a bioactive compound binding to proteins with cross-ß structure conformations with the binding portion of an antibody.

A constituent of a pharmaceutical composition is any substance that is present in or added to a proteinaceous molecule to produce a pharmaceutical composition. The invention also relates to any component that has come into contact with the pharmaceutical composition during the manufacturing process and storage. Because cross-ß structure conformations generally develop in a protein or a proteinaceous compound, a constituent comprising a protein is a constituent that may contain a cross-ß structure conformation.

The term: " constituent of a pharmaceutical composition" comprises any substance suitable for administering a proteinaceous composition to a body of a human or animal. Said constituent comprises for example carrier substances and conserving substances, fluids for injection or ingestion, mannitol and cellulose, and the usual excipients for parenteral, enteral, ocular, otic, and transdermal administration.

In a preferred embodiment of the invention detection of a cross-ß structure is in a soluble state. In this embodiment, a cross-ß structure binding

compound is added to a pharmaceutical composition or to a constituent of said composition, said constituent comprising a protein, resulting in binding of said protein and/or peptide comprising a cross-ß structure conformation with said binding compound. Said bound protein and/or peptide comprising across-ß structure conformation is then detected by physical or chemical or enzymatic detection methods. In another preferred embodiment of the invention, a compound of Table 1, and/or 2, and/or 3 is attached to a solid surface or solid phase, either by chemical or physical means or by another binding molecule. Detection of a proteinaceous compound such as a protein, a peptide, or a with cross-ß structure results from contacting said pharmaceutical composition or any of its constituents with said cross-ß structure-binding compound derived from the group depicted in Table 1, and/or 2, and/or 3, or a functional equivalent thereof, more preferably with a solid phase comprising a cross-ß structure-binding compound derived from the group depicted in Table 1, and/or 2, and/or 3 or a functional equivalent thereof, and measuring or detecting the protein and/or peptide comprising across-ß structure bound to the solid phase. In yet another embodiment, a cross-ß structure-binding compound is attached to a solid phase after binding a protein and/or peptide comprising across-ß structure. As a solid phase, many materials are suitable for binding a cross-ß structure-binding compound, such as for example, glass, silica, polystyrene, polyethylene, nylon, vinyl, sepharose beads, beads containing iron or other metals and so on. In one embodiment of the invention, said solid phase has the physical form of beads. In another embodiment said solid phase has the shape of a tube or a plate or a well in, for instance an ELISA plate, or a dipstick. Numerous binding techniques are available for coupling the cross-ß structure-binding compounds to said solid phase, like for example, CyanogenBromide (CnBr), NHS, Aldehyde, epoxy, Azlactone, biotin/streptavidin, and many others. The amount of bound protein and/or peptide comprising cross-ß structures is measured for example by staining said protein and/or peptide comprising cross-ß structures and is a measure for the quality of the proteins

in said pharmaceutical composition. In another embodiment, the cross-ß structure binding compound is bound to another compound which in turn is bound to another compound and so on. This indirect binding is suitable for increasing the efficiency of the detection and removal of protein and/or peptide comprising across-ß structure in a pharmaceutical composition and any of its constituents comprising a protein.

The compounds of Tables 1 , 2 and 3 are various in chemical size and structure. A common characteristic of all compounds of Tables 1 and 2 is their propensity to bind to protein and/or peptide comprising across-ß structure. Compounds that comprise a function which is similar or equivalent to the compounds of Table 1, like the compounds in Table 2 or 3, have been detected by direct binding experiments as disclosed in the invention, in literature and in European patent application no. 02077797.5. A functional equivalent of a binding compound of the invention is a substance that exerts a similar function as said compound i.e. a substance that binds to a compound with cross-ß structure conformation.

Therefore, the present invention discloses a method for detecting a protein and/or peptide comprising across-ß structure wherein said cross-ß structure-binding compound is a compound according to Table 1, or Table 2, or Table 3 or a functional equivalent of any of said compounds, or a combination of any of said compounds. The methods of the invention are useful for controlling the different stages of a manufacturing process of a pharmaceutical composition. In general, the specification of a process for manufacturing a pharmaceutical composition is described in a handbook according to good manufacturing practice (GMP) and good laboratory practice (GLP). GLP and GMP quality control is a valuable tool for manufacturers of pharmaceutical compositions and for manufacturers of proteinaceous constituents for said pharmaceutical compositions and it helps and enables them to produce products of a steady quality and to increase the quality by monitoring the

manufacturing and storage process. The present invention discloses methods that help manufacturers to detect compounds with cross-β structures in a pharmaceutical product and/or in its constituents. A qualitative difference is thus made between products with cross-β structures or products without cross-β structures, or with low levels of cross-β structures. By monitoring the processes with methods of the invention, manufacturers are capable of omitting processes or chemicals or physical conditions or circumstances that induce the formation of cross-β structures, and it enables them to select processes or chemicals or circumstances that do not induce cross-β structure conformations and/or raise the level of cross-β structure conformations in a pharmaceutical composition or any of its constituents and/or excipients comprising a protein.

In one preferred embodiment, the present invention discloses a method for detecting and/or measuring a cross-β structure-inducing ability of a solid surface, by contacting said surface with a protein and detecting denatured protein by subsequently contacting said surface with a cross-β structure-binding compound. With said method of the invention, a person skilled in the art is capable of selecting materials for a recombinant protein container. Said container comprising a reaction vessel, a production vessel, a storage vessel and/or a tube connecting said vessels. The above-described method is also suitable for detecting and/or measuring a cross-β structure-inducing ability of a molecule, for example of a salt, or a dye, or an enzyme, or a chemical compound such as for example alcohol or formaldehyde or glucose. Therefore, the present invention discloses in another embodiment a method for detecting and/or measuring a cross-β structure-inducing ability of a molecule, by contacting said molecule with a protein and detecting denatured protein by subsequently contacting said molecule and/or said protein with a cross-β structure binding compound. Molecules that have the ability to induce a cross-β structure are then removed or avoided in the production, purification and storage of a recombinant protein and/or a pharmaceutical composition.

Therefore, the present invention enables a person skilled in the art to avoid the use of material comprising said molecule as a part of the wall of a container for production, purification, or storage of said proteinaceous molecule. In another embodiment, the invention teaches the person skilled in the art to avoid molecules inducing cross-β structure in the preparation of a proteinaceous solution or a pharmaceutical composition. Therefore, the present invention provides a method for selecting molecules for production and/or dilution, and/or preservation of a recombinant proteinaceous composition.

In yet another embodiment, the present invention discloses a method for detecting and/or measuring a cross-β structure-inducing ability of a physical condition such as for example, pH, pressure, stirring, shaking, temperature, salt concentration and/or protein concentration. A recombinant proteinaceous composition is subjected to various physical conditions and the increase of the amount of cross-β structure conformations is measured by contacting said proteinaceous composition with a cross-β structure-binding compound according to a method of the invention. Binding of a protein with cross-β structure conformation from said proteinaceous composition with a cross-β structure-binding compound is detected using the methods of the invention. The above-described method is a valuable tool for detecting cross-β structure-inducing circumstances during production, purification, and storage. Therefore, the present invention discloses a process to improve production, purification and storage of recombinant proteinaceous material.

Therefore, the present invention discloses a method according to the invention for controlling a manufacturing process, and/or storage process of a pharmaceutical composition or any of its constituents comprising a protein, said method comprising contacting said pharmaceutical composition or any of its constituents comprising a protein with at least one cross-β structure-binding compound resulting in a bound protein and/or peptide comprising a cross-β structure, detecting whether a bound protein and/or peptide comprising a cross-β structure is present in said pharmaceutical composition or any of its

constituents comprising a protein at various stages of said manufacturing and/or storage process.

In another embodiment of the invention, protein and/or peptide comprising a cross-ß structure bound to a binding molecule are separated from the pharmaceutical composition or any of its constituents comprising a protein, for example by collecting the solid phase comprising said cross-ß structure-binding compound, bound to protein and/or peptide comprising cross-ß structures. Separation of said solid phase is for example performed by g-forces like for example by gravity, or by centrifugation, or by magnetic forces, or by filtration. Said separation is performed in a continuous mode or batch-wise, or with a combination of a batch-wise and a continuous mode. Therefore, the present invention discloses a method for removing a protein and/or peptide comprising a cross-ß structure from a pharmaceutical composition or any of its constituents, said method comprising contacting said pharmaceutical composition or any of its constituents comprising a protein with at least one cross-ß structure-binding compound, allowing binding of said protein and/or peptide comprising a cross-ß structure to said cross-ß structure-binding compound, and, separating said bound protein and/or peptide comprising a cross-ß structure from said pharmaceutical composition or any of its constituents comprising a protein.

A non-limiting number of compounds capable of binding to protein and/or peptide comprising a cross-ß structure is disclosed in Tables 1, 2, and 3. Therefore, the present invention discloses a method according to the invention, wherein said cross-ß structure-binding compound is a compound according to Table 1, or Table 2, or Table 3 or a functional equivalent of any of said compounds.

For efficient removal of bound proteins and/or peptides comprising a cross-ß structure, a cross-ß structure-binding compound is attached to another binding compound or to a solid phase by chemical or physical methods.

As a solid phase, many materials are suitable for binding a cross-β structure-binding compound, such as for example, glass, silica, polystyrene, polyethylene, nylon, vinyl, sepharose beads, beads containing iron or other metals and so on. In one embodiment of the invention, said solid phase has the physical form of beads. In another embodiment said solid phase has the shape of a tube or a plate or a well in, for instance an ELISA plate, or a dipstick. Numerous binding techniques are available for coupling the cross-β structure-binding compounds to said solid phase, like for example, CyanogenBromide (CnBr), NHS, Aldehyde, epoxy, Azlactone, biotin/streptavidin, and many others.

As described above, it generally depends on the chemical attachment method that is selected how and when the cross-β structure-binding compound is attached to another molecule or compound. For example, a preferred binding of said compound of Table 1 to another compound occurs before binding a compound with cross-β structure conformation, or more preferred during the process of said binding of a compound with cross-β structure conformation, or most preferred after binding of a compound with cross-β structure conformation. Therefore, the present invention discloses a method according to the invention, wherein said cross-β structure-binding compound is bound to a second compound before, during or after the binding of said cross-β structure-binding compound to a compound with cross-β structure conformation.

As described above, it depends on the attachment method and on the type of solid phase how and when the cross-β structure-binding compound and/or its second binding compound is attached to a solid phase. In one embodiment, the compound of Table 1 is attached to a solid phase, and in another embodiment of the invention, said compound of Table 1, 2, or 3 or an equivalent thereof is first attached to a second binding compound, which in its turn is attached to a solid phase. Therefore, the present invention discloses a method according to the invention, wherein said second compound is bound to a solid face. For example said second compound comprises an antibody directed

against part of a compound of Table 1, 2, or 3, or comprises a chemical linker that is capable of binding a compound of Table 1, 2, or 3. Although in many cases it will be enough to contact a compound with cross-ß structure conformation with a cross-ß structure-binding compound , or said complex with a second binding compound, it of course also in the present invention that the second binding compound is also capable of binding to a third binding compound or even to a fourth or fifth and so on. Therefore, the present invention in another embodiment discloses a method of the invention, wherein said cross-ß binding compound, bound to a second compound is further bound to a third or fourth or further binding compound before, during or after the binding of said cross-ß binding compound to a compound with cross-ß structure conformation. In a preferred embodiment a third or fourth or further binding compounds is bound to a solid phase. Therefore, the present invention also discloses a method, wherein said second, third, or fourth compound is bound to a solid phase In another embodiment of the invention, said continued binding of more binding molecules induces the formation of aggregates that do not need a further solid phase to be separated from the pharmaceutical composition or any of its constituents comprising a protein.

The presence of bound cross-ß structures is in another embodiment detected by an enzymatic assay.

As an example of an enzymatic assay the specification provides tPA and plasminogen and plasmin substrate S-2251 (Chromogenix Spa, Milan, Italy) in a suitable buffer. Preferably the buffer is HBS (10 mM HEPES, 4 mM KCl, 137 mM NaCl, pH 7.3). Standard curve is made with a control with cross-ß structure conformation. Titration curves are made with a sample before and after a treatment/exposure to a putatively denaturing condition. Alternatively the detection of bound proteins or peptides comprising cross-ß structures is achieved by a test wherein factor XII with activated factor XII substrate S-2222 or S-2302 is present in a suitable buffer. Preferably, the buffer is 50 mM, 1 mM EDTA, 0.001% v/v Triton-X100. Standard curves are made with known

cross-ß structure rich activators of factor XII; preferably DXS500k with a protein; preferably the protein is endostatin or albumin; preferably with glycated haemoglobin, Aß, amyloid fibrin peptide NH_2-148KRLEVDIDIGIRS160-COOH with K157G mutation. In yet another embodiment, the presence of bound proteins or peptides comprising cross-ß structures is detected by a test comprising factor XII with prekallikrein and high molecular weight kininogen and either substrate Chromozym-PK for kallikrein or a substrate for activated factor XII in a suitable buffer; preferably HBS. Standard curves are made with known cross-ß structure rich activators of factor XII; preferably DXS500k or kaolin with a protein; preferably the protein is endostatin or albumin; preferably with glycated haemoglobin, Aß, amyloid fibrin peptide NH_2-148KRLEVDIDIGIRS160-COOH with K157G mutation.

The present invention discloses a method for both the detection and the removal of protein and/or peptide comprising a cross-ß structures from a pharmaceutical composition and/or any of its constituents. Because protein and/or peptide comprising cross-ß structures are also capable of inducing the unfolding and degeneration of proteins, the presence of protein and/or peptide comprising a cross-ß structure is deleterious for the protein in a pharmaceutical composition. By removing protein and/or peptide comprising a cross-ß structure from a pharmaceutical composition, the specific activity per gram protein of said pharmaceutical composition is preferably retained. Because protein and/or peptide comprising a cross-ß structure are toxic and induce undesired side effects after administration in a human or animal, removal of said protein and/or peptide comprising a cross-ß structure at least diminishes said undesired side effects upon administration. In a preferred embodiment, said undesired side effects are even prevented. Therefore, the present invention discloses a method for decreasing and/or preventing undesired side effects of a pharmaceutical composition and/or increasing the specific activity per gram protein, said method comprising detecting and

removing any unfolded protein or peptide and/or aggregated protein or peptide and/or multimerized protein or peptide comprising a cross-β structure from said pharmaceutical composition or any of its constituents comprising a protein.

A pharmaceutical composition, which is processed according to any one of the methods of the present invention, comprises less protein and/or peptide comprising a cross-β structure, and is therefore less toxic, thrombogenic, immunogenic, inflammatory or harmful for a mammal including a human after administration of said pharmaceutical composition. Furthermore, because of the decreased presence of protein and/or peptide comprising a cross-β structure conformations in said pharmaceutical composition, the purity and the biological activity of said pharmaceutical composition is preferably higher per gram protein present in said pharmaceutical composition, and therefore, more pharmaceutical composition can be made from an amount of protein and still achieve the same pharmacological effect. A pharmaceutical composition that is purified by any of the methods of the invention is therefore of higher quality, and exerts less side effects than a pharmaceutical composition that is not purified. The difference between a pharmaceutical composition according to the invention and another pharmaceutical composition is in the amount of compounds with cross-β structure conformations detectable in said pharmaceutical composition according to any of the methods of the invention.

Therefore, the present invention in another embodiment discloses a pharmaceutical composition or any of its constituents comprising a protein, said composition obtainable by a method according to a method of the invention.

In another embodiment, the specification provides a kit of parts, comprising for example one or more cross-β structure binding compounds as depicted in Table 1, or 2, or possibly 3, and optionally one or more compounds binding said cross-β structure binding compound, and a means for detecting

bound cross-ß structure as described elsewhere in this specification, thereby making the kit suitable for carrying out a method according to the invention such as for example detecting compounds with cross-ß structure conformations, and or removing compounds with cross-ß structure conformations from a pharmaceutical composition or any of its constituents comprising a protein. The specification provides in one embodiment of a kit for example a filter-like element binding compounds with cross-ß structure or binding cross-ß structure binding compounds. Said filter is placed in or on a syringe through which a pharmaceutical composition is passed before inoculation or administration to a mammal. In another embodiment, said filter is used in the production or packaging of a pharmaceutical composition or any of its constituents. In another embodiment, the kit of the specification provides an ELISA plate, or a dipstick for detecting compounds with cross-ß structure in a pharmaceutical composition or any of its constituents or a filtration device for removing compounds with cross-ß structure conformations of a pharmaceutical composition or any of its constituents.

After removal of the cross-ß structure from a pharmaceutical composition or any of its constituents, the resulting pharmaceutical composition or any of its constituents is tested again to control whether the amount of cross-ß structures in said composition or any of its constituents has actually decreased. The decrease in cross-ß structures, and therefore, the decrease in toxicity is tested by conventional methods known in the art such as in vitro or in *in vivo* tests for toxicity and/or thrombogenicity and/or immunogenicity of said pharmaceutical composition or any of its constituents.

Our observations indicate that the presence of cross-ß structures, or the potential that the cross-ß structure conformation can be formed in these therapeutics, as well as those cross-ß structures that may be present in all other protein therapeutics or constituents thereof is potentially harmful with respect to the induction of unwanted side-effects during treatment with said therapeutic. Such undesirable side effects include, but are not limited to

thrombosis, bleeding, disseminated intravascular coagulation (DIC), septic shock, multi organ dysfunction syndrome (MODS), anaphylactic shock, an inflammatory reaction and/or the development of an adaptive immune response with antibodies against the drug and/or the endogenous protein. A person skilled in the art is now able to use a method included in the invention to determine the content of cross-ß structure in any solution containing a protein, preferably a protein therapeutic, or a protein or protein therapeutic in solution that is being produced or stored during the production process of said protein or protein therapeutic. A person skilled in the art is also able to use a method of the invention to determine the content of cross-ß structure in the circulation of a human or mammal suffering from any of the aforementioned diseases, preferably associated with the use of said protein therapeutic by said human or mammal. A person skilled in the art is now also able to deplete any cross-ß structure comprising protein, preferably protein therapeutic from a solution. After depletion, using any of the methods included in the present invention, said person is able to determine the amount of cross-ß structure that is being left in the solution. Moreover a person skilled in the art can determine the consequence of removal of the cross-ß structure on any of the possible unwanted side effects, such as described above, that said protein comprising cross-ß structure may induce. For example a solution containing a protein therapeutic, preferably interferon α, factor VIII, erythropoietin, thrombopietin, glucagons, GH or Etanercept can be analyzed by any of the methods provided in the present invention. For experimental purpose, said protein therapeutic may be treated to induce an additional amount of cross-ß structure to enhance the strength of the method. Said treatment may comprise, but is not limited to heating, glycation, oxidation, acetylation. Subsequently, said solution can be depleted cross-ß structure by a method of the invention. Subsequently, the effect of said method of depletion on the side effect, preferably immunogenicity, of said protein can be tested. Preferably the effect on the generation of antibodies is determined. Preferably said effect is being determined in serum

obtained, after administration of said solution, before and after depletion, to a mouse , preferably a mouse transgenic for said protein or a human. Preferably said determination is analyzed by an ELISA in which said protein is being immobilized on a microtiter plate. Subsequently, serial dilutions of serum are being added. Binding of antibodies is subsequently determined by standard procedures using preferably peroxidase-conjugated antibodies. Alternatively, said effect of the method can be analyzed in vitro. For example the effect of said depletion method on the induction of inflammatory cytokines by cells, preferably cells of the innate immune system, preferably dendritic cells or macrophages. Preferably said cytokine to be determined is TNFα. Preferably said cytokine is determined by ELISA or rtPCR. Alternatively the effect of said method of depletion on the activation of inflammatory cells, preferably dendritic cells or macrophages can be tested by FACS analysis. Preferably the levels of so-called co-stimulatory molecules, such as B7.1, B7.2, MHC class II, CD40 are determined on preferably CD11c positive cells. Alternatively any of the experiments described above or a modification thereof can be used as long as they are used to test an unwanted side effect of said cross-β structure comprising protein.

In another embodiment, the present invention discloses a method for influencing the immunogenicity of a protein, comprising influencing the formation of at least one cross-β structure in said protein. Of course, it is clear that avoiding the formation of a cross-β structure in said protein renders the protein less immunogenic, and enhancing the formation of a cross-β structure in said protein enhances the immunogenicity.

The invention is further explained in the examples, without being limited by them.

Table 1: cross-β structure binding compounds		
Congo red	Chrysamine G	Thioflavin T
2-(4'-(methylamino)phenyl)-6-methylbenzothiaziole	Any other amyloid-binding dye/chemical	Glycosaminoglycans
Thioflavin S	Styryl dyes	BTA-1
Poly(thiophene acetic acid)	conjugated polyelectrolyte PTAA-Li	

Table 2: proteins that bind to and/or interact with misfolded proteins and/or with proteins comprising cross-β structure

Tissue-type plasminogen activator	Finger domain(s) of tPA, factor XII, fibronectin, HGFA	Apolipoprotein E
Factor XII	Plasmin(ogen)	Matrix metalloprotease-1
Fibronectin	75kD-neurotrophin receptor (p75NTR)	Matrix metalloprotease-2
Hepatocyte growth factor activator	α2-macroglobulin	Matrix metalloprotease-3
Serum amyloid P component	High molecular weight kininogen	Monoclonal antibody 2C11(F8A6)‡
C1q	Cathepsin K	Monoclonal antibody 4A6(A7) ‡
CD36	Matrix metalloprotease 9	Monoclonal antibody 2E2(B3) ‡
Receptor for advanced glycation endproducts	Haem oxygenase-1	Monoclonal antibody 7H1(C6) ‡
Scavenger receptor-A	low-density lipoprotein receptor-related protein (LRP, CD91)	Monoclonal antibody 7H2(H2) ‡
Scavenger receptor-B	DnaK	Monoclonal antibody 7H9(B9) ‡
ER chaperone Erp57	GroEL	Monoclonal antibody 8F2(G7) ‡
Calreticulin	VEGF165	Monoclonal antibody 4F4‡
Monoclonal conformational antibody WO1 (ref. (O'Nuallain and Wetzel, 2002))	Monoclonal conformational antibody WO2 (ref. (O'Nuallain and Wetzel, 2002))	Amyloid oligomer specific antibody (ref. (Kayed et al., 2003))
formyl peptide receptor-like 1	α(6)β(1)-integrin	CD47
Rabbit anti-albumin-AGE antibody, Aß-purified[a)]	CD40	apo A-I belonging to small high-density lipoproteins
apoJ/clusterin	10 times molar excess PPACK, 10 mM εACA, (100 pM – 500 nM) tPA[2)]	CD40-ligand
macrophage scavenger receptor CD163	broad spectrum (human) immunoglobulin G (IgG) antibodies (IgIV, IVIg)	BiP/grp78
Erdj3	haptoglobin	

‡ Monoclonal antibodies developed in collaboration with the ABC-Hybridoma Facility, Utrecht University, Utrecht, The Netherlands.
a) Antigen albumin-AGE and ligand Aß were send in to Davids Biotechnologie (Regensburg, Germany); a rabbit was immunized with albumin-AGE, antibodies against a structural epitope were affinity purified using a column with immobilized Aß.
2) PPACK is Phe-Pro-Arg-chloromethylketone (SEQ-ID 8), εACA is ε-amino caproic acid, tPA is tissue-type plasminogen activator

Table 3: Proteins likely to be able to interact with misfolded protein comprising crossbeta structure

Monoclonal antibody 4B5	Heat shock protein 27	Heat shock protein 40
Monoclonal antibody 3H7‡	Nod2 (= CARD15)	Heat shock protein 70
FEEL-1	Pentraxin-3	HDT1
LOX-1	Serum amyloid A proteins	GroES
MD2	Stabilin-1	Heat shock protein 90
FEEL-2	Stabilin-2	CD36 and LIMPII analogous-I (CLA-1)
Low Density Lipoprotein	LPS binding protein	CD14
C reactive protein	CD45	Orosomucoid
Integrins	alpha-1 antitrypsin	apo A-IV-Transthyretin complex
Albumin	Alpha-1 acid glycoprotein	β2-glycoprotein I
Lysozyme	Lactoferrin	Megalin
Tamm-Horsfall protein	Apolipoprotein E3	Apolipoprotein E4
Toll-like receptors	Complement receptor CD11b/CD18 (Mac-1, CR3)	CD11d/CD18 (subunit aD)
CD11b2	CD11a/CD18 (LFA-1, subunit aL)	CD11c/CD18 (CR4, subunit aX)
Von Willebrand factor	Myosin	Agrin
Perlecan	Chaperone60	b2 integrin subunit
proteins that act in the unfolded protein response (UPR) pathway of the endoplasmic reticulum (ER) of prokaryotic and eukaryotic cells	proteins that act in the endoplasmic reticulum stress response (ESR) pathway of prokaryotic and eukaryotic cells	Macrophage receptor with collagenous structure (MARCO)
20S	CHAPERONE16 family members	HSC73
HSC70	translocation channel protein Sec61p	26S proteasome
19S cap of the proteasome (PA700)	UDP-glucose:glycoprotein glucosyl transferase (UGGT)	carboxy-terminus of CHAPERONE70-interacting protein (CHIP)
Pattern Recognition Receptors	Derlin-1	Calnexin
Bcl-2 asociated athanogene (Bag-1)	GRP94	Endoplasmic reticulum p72
(broad spectrum) (human) immunoglobulin M (IgM) antibodies	proteins that act in the endoplasmic reticulum associated degradation system (ERAD)	The (very) low density lipoprotein receptor family
Fc receptor		

‡ Monoclonal antibodies developed in collaboration with the ABC-Hybridoma Facility, Utrecht University, Utrecht, The Netherlands.

5

Examples

Materials & Methods

Preparation of cross-β structure conformation rich compounds

For preparation of advanced glycation end-product (AGE) modified bovine serum albumin, 100 mg ml^{-1} of albumin was incubated with phosphate buffered saline pH 7.3 (PBS) containing 1 M of glucose-6-phosphate (g6p) and 0.05% m/v NaN_3, at 37°C in the dark. Glycation was prolonged up to 23 weeks[1]. To prepare glycated haemoglobin (Hb-AGE), human haemoglobin (Hb, Sigma-Aldrich, H7379) at 5 mg ml^{-1} was incubated for 32 weeks at 37°C with PBS containing 1 M of g6p and 0.05% m/v of NaN_3. In control solutions, g6p was omitted. After incubations, solutions were extensively dialyzed against distilled H_2O and, subsequently, stored at 4°C. Protein concentrations were determined with advanced protein-assay reagent ADV01 (Cytoskeleton, Denver, CO, USA). Glycation and formation of AGE was confirmed by measuring intrinsic fluorescent signals from AGE; excitation wavelength 380 nm, emission wavelength 435 nm. In addition, binding of AGE-specific antibodies was determined. Presence of cross-ß structure conformation in albumin-AGE was confirmed by enhancement of Congo red fluorescence, enhancement of Thioflavin T (ThT) fluorescence, the presence of ß-sheet secondary structure, as observed with circular dichroism spectropolarimetry (CD) analyses, and by X-ray fiber diffraction experiments[1]. Presence of cross-ß structure conformation in Hb-AGE was confirmed by tPA binding, CD analyses, transmission electron microscopy (TEM) imaging of fibrillar structures and by Congo red fluorescence measurements. Amyloid preparations of human γ-globulins were made as follows. Lyophilized γ-globulins (G4386, Sigma-Aldrich) were dissolved in a 1(:)1 volume ratio of 1,1,1,3,3,3-hexafluoro-2-propanol and trifluoroacetic acid and subsequently dried under an air stream. Dried γ-globulins were dissolved in H_2O to a final concentration of 1 mg ml^{-1} and kept at room temperature for at least three

days, or kept at 37°C for three days and subsequently at -20°C. Aliquots were stored at -20°C and analyzed for the presence of cross-β structure conformation. Fluorescence of Congo red and ThT was assessed. In addition tPA binding was analyzed in an ELISA and tPA activating properties in a chromogenic plasminogen (Plg) activation assay. In addition, the macroscopic appearance of denatured γ-globulins was analyzed with TEM imaging. Human amyloid-ß (Aβ) (1-40) Dutch type (DAEFRHDSGYEVHHQKLVFFAQDVGSNKGAIIGLMVGGVV,) and human fibrin α-chain(148-160) amyloid fragment with Lys157Gly mutation (FP13, KRLEVDIDIGIRS) (BB, unpublished and [7]) were disaggregated in a 1:1 (v/v) mixture of 1,1,1,3,3,3-hexafluoro-2-isopropyl alcohol and trifluoroacetic acid, air-dried and dissolved in H_2O (Aß: 10 mg ml^{-1}, FP13: 2 mg ml^{-1}). After three days at 37°C, the peptide was kept at room temperature for two weeks, before storage at 4°C. Aß solutions were tested for the presence of amyloid conformation by ThT or Congo red fluorescence and by TEM imaging. Negative control for cross-ß structure detection assays was non-amyloid fragment FP10 of human fibrin α-chain(148-157) (KRLEVDIDIK)[7,10]. FP10 was dissolved at a concentration of 1 mg ml^{-1} in H_2O and stored at 4°C. This solution was used as a negative control for ThT fluorescence assays.

Cloning and expression of recombinant fibronectin type I domains

F4-5 domains and the F domain of tPA with a carboxy-terminal His_6-tag were also expressed in *Saccharomyces cerevisiae*. The cDNA constructs were prepared following standard procedures known to a person skilled in the art, by the Biotechnology Application Center (BAC-Vlaardingen/Naarden, The Netherlands). Domain boundaries of Fn F4-5 and tPA F were taken from the human Fn and human tPA entries in the Swiss-Prot database (P02751 for Fn, P00750 for tPA) and comprised amino-acids NH_2 – I182-V276 – COOH of Fn F4-5 and NH_2 – G33-S85 – COOH of tPA. Affinity purification of the expressed proteins was performed using His_6-tag – Ni^{2+} interaction and a desalting step.

Constructs were stored at -20°C in PBS pH 7.0. The molecular size of the constructs was checked on a Coomassie brilliant blue-stained SDS-PAGE gel.

Totally chemical synthesis of fibronectin type I domains

Totally chemical synthesis of the F domains of hepatocyte growth factor activator (HGFA, SwissProt entry Q04756) and tPA (SwissProt entry P00750) was performed in the laboratory of Dr. T.M. Hackeng (Academic Hospital Maastricht, The Netherlands), according to standard procedures known to a person skilled in the art. Both domains were synthesized as two separate peptides that were subsequently ligated using native chemical ligation. The tPA F domain was completed with a carboxy-terminal acetylated lysine residue or biotinylated lysine residue. The HGFA F domain was supplied with an acetylated lysine residue. Products were analyzed on a reversed phase HPLC column and with mass spectrometry.

Cloning, expression and purification of the soluble extracellular domains of receptor for advanced glycation endproducts

The soluble extracellular part, of the receptor for AGE (sRAGE) was cloned, expressed and purified as follows (Q.-H. Zeng, Prof. P. Gros, Dept. of Crystal- & Structural Chemistry, Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, the Netherlands). Human cDNA of RAGE was purchased from RZPD (clone IRALp962E1737Q2, RZPD, Berlin, Germany). For PCRs, the gagatctGCTCAAAACATCACAGCCCGG forward primer was used comprising a BglII site, and the gcggccgcCTCGCCTGGTTCGATGATGC reverse primer with a NotI site. The soluble extracellular part of RAGE comprises three domains spanning amino-acid residues 23-325. The PCR product was cloned into a pTT3 vector, containing an amino-terminal His-tag and a thrombin cleavage site. The sRAGE was expressed in 293E hamster embryonic kidney cells at the ABC-protein expression facility (Utrecht University, Utrecht, the Netherlands). Concentrated cell culture medium was applied to a Hi-trap

Chelating HP Ni^{2+}-NTA column (Amersham Biosciences Europe, Roosendaal, The Netherlands). The running buffer was 25 mM Tris-HCl, 500 mM NaCl, pH 8.0. The protein was eluted by using a step gradient of 0 to 500 mM imidazole. Purity of the His-sRAGE was depicted from Coomassie stained SDS-PAGE gels. After concentration, the buffer was exchanged to 20 mM Tris-HCl, 200 mM NaCl, 100 µM phenylmethylsulfonyl fluoride (PMSF), pH 8.0. Various stocks at 1, 5 and 20 mg ml^{-1} were first kept at 4°C for several weeks and then stored at -20°C. In this way, the PMSF will be sufficiently inactivated at 4°C.

Plasminogen-activation assay and factor XII activation assay.

Plasmin (Pls) activity was assayed as described[7]. Peptides and proteins that were tested for their stimulatory ability were regularly used at 100 µg ml^{-1}. The tPA and plasminogen (Plg) concentrations were 200 pM and 1.1 µM, respectively, unless stated differently. Chromogenic substrate S-2251 (Chromogenix, Instrumentation Laboratory SpA, Milano, Italy) was used to measure Pls activity. Conversion of zymogen factor XII (#233490, Calbiochem, EMD Biosciences, Inc., San Diego, CA) to proteolytically active factor XII (factor XIIa) was assayed by measurement of the conversion of chromogenic substrate Chromozym-PK (Roche Diagnostics, Almere, The Netherlands) by kallikrein. Chromozym-PK was used at a concentration of 0.3 mM. Factor XII, human plasma prekallikrein (#529583, Calbiochem) and human plasma cofactor high-molecular weight kininogen (#422686, Calbiochem) were used at concentrations of 1 µg ml^{-1}. The assay buffer contained HBS (10 mM HEPES, 4 mM KCl, 137 mM NaCl, 5 µM $ZnCl_2$, 0.1% m/v albumin (A7906, Sigma, St. Louis, MO, USA), pH 7.2). Assays were performed using microtiter plates (Costar, Cambridge, MA, USA). Peptides and proteins were tested for their ability to activate factor XII. 150 µg ml^{-1} kaolin, an established activator of factor XII was used as positive control and solvent (H_2O) as negative control. The conversion of Chromozym-PK was recorded kinetically at 37° C for at least 60 minutes. Assays were done in duplicate. In control wells factor XII was

omitted from the assay solutions and no conversion of Chromozym-PK was detected. In some assays albumin was omitted from the reaction mixture. Alternatively, chromogenic substrate S-2222 (Chromogenix) was used to follow the activity of factor XII itself. With S-2222, activation of factor XII in plasma was measured, using 60% v/v plasma, diluted with substrate and H_2O with or without potential cofactor. Furthermore, auto-activation of factor XII was measured by incubating 53 µg ml^{-1} purified factor XII in 50 mM Tris-HCl buffer pH 7.5 with 1 mM EDTA and 0.001% v/v Triton-X100, with S-2222 and H_2O with or without potential cofactor.

Surface plasmon resonance studies

Binding of cross-ß structure conformation containing peptides/proteins was studied using surface plasmon resonance technology with a Biacore 2000 apparatus (Biacore AB, Uppsala, Sweden). A standardized amine coupling procedure was used to couple proteins with F domains to a CM5 chip (Biacore AB, Uppsala, Sweden). First, the dextran surface of the chips was activated by a 35 µl injection with a 1:1 mixture of 0.1 M N-hydroxysuccinimide (NHS) and 0.4 M N-ethyl-N'-(dimethylaminopropyl)carbodiimide (EDC) at a flow rate of 5 µl $min.^{-1}$. Then, the proteins were covalently coupled to the activated dextran surface. Remaining activated groups in each of the four flow channels were blocked by injection of 35 µl of 1 M ethanolamine hydrochloride pH 8.5. EDC, NHS and ethanolamine hydrochloride were obtained from Biacore. On one chip, on channels 1 to 4, buffer (reference channel), the soluble extracellular part of receptor for advanced glycation endproducts (sRAGE), tPA and K2P-tPA were immobilized. The immobilization buffer for the reference channel, channel 2 (sRAGE), channel 3 (tPA) and channel 4 (K2P-tPA) was 10 mM acetate pH 3.75. In channel 2, 2000 response units (RU) sRAGE was immobilized, 2700 RU and 2400 RU tPA and K2P-tPA are immobilized, respectively. The flow rate was 10 µl $min.^{-1}$, the injection time was 120". The running buffer during immobilization was 10 mM HEPES pH 7.4, 140 mM

NaCl. Buffers were filtrated on a 0.22 µm filter (white GSWP, 47 mm, Millipore) and degassed at room temperature. For subsequent binding experiments, the running buffer was 10 mM HEPES pH 7.4, 140 mM NaCl, 1.5 mM $CaCl_2$, 10 mM εACA, 0.005% Tween-20. Binding of albumin-AGE was determined with a solution of 3.9 µg ml^{-1} albumin-AGE in running buffer. albumin-AGE was filtered on a Millex-GV 0.22µm filter unit (Millipore). Binding of filtered Hb-AGE was tested at 32 µg ml^{-1}. Binding of amyloid γ-globulins were tested at 62.5 µg ml^{-1}. After each injection of protein, the chip was regenerated with 0.1 M H_3PO_4 pH 1.0. After injections with albumin-AGE and Hb-AGE this regeneration step was successful and sufficient, after injection with amyloid γ-globulins, the bound protein could not be released, not even after injection with more harsh regeneration buffers (HCl, NaOH). Binding of Hb-AGE was also tested after centrifugation for 10 min. at 16,000*g alternative to filtration. tPA activation before and after filtration was assessed with a Plg-activation assay. Also amyloid γ-globulins and amyloid endostatin (EntreMed, Inc., Rockville, MD, USA) were tested before and after centrifugation.

On a second chip, buffer, chemically synthesized HGFA F domain, chemically synthesized tPA F domain and Fn F4-5-His6, expressed in *S. cerevisiae*, were immobilized. HGFA F was immobilized in 10 mM acetate buffer pH 4.0, 190 RU. tPA F was immobilized in 5 mM maleate pH 5.5, 395 RU, Fn F4-5 in 5 mM maleate pH 6.0, 1080 RU. Now, the running buffer was 10 mM HEPES pH 7.4, 140 mM NaCl, 1.5 mM $CaCl_2$, 10 mM εACA, 0.05% Tween-20. Regeneration buffer was running buffer supplemented with 1 M NaCl. Binding was tested with endostatin at 0-800 nM, Hb-AGE at 0-25 nM, recombinant β2-glycoprotein I (β2GPI) at 0-300 nM and 25 nM native Hb. For the Fn F4-5 channel, the maximum binding expressed in RU was plotted against the concentrations.

For both chips, channel 1 was used for reference purposes. The signal obtained with this channel was subtracted from the signals obtained with the channels with immobilized proteins.

Thioflavin T fluorescence

Fluorescence of ThT – protein/peptide adducts was measured as follows. Solutions of 25 μg ml^{-1} of protein or peptide preparations were prepared in 50 mM glycine buffer pH 9.0 with 25 μM ThT. Fluorescence was measured at 485 nm upon excitation at 435 nm. Background signals from buffer, buffer with ThT and protein/peptide solution without ThT were subtracted from corresponding measurements with protein solution incubated with ThT. Regularly, fluorescence of Aβ was used as a positive control, and fluorescence of FP10, a non-amyloid fibrin fragment[7], was used as a negative control. Fluorescence was measured in triplicate on a Hitachi F-4500 fluorescence spectrophotometer (Ltd., Tokyo, Japan).

Congo red fluorescence

Solutions of 25 μg ml^{-1} protein/peptide were incubated with 25 μM Congo red in PBS and fluorescence was measured at 590 nm upon excitation at 550 nm. Background signals from buffer, buffer with Congo red and protein/peptide solution without Congo red were subtracted from corresponding measurements with protein solution incubated with Congo red. Fluorescence was measured in triplicate on a Hitachi F-4500 fluorescence spectrophotometer (Ltd., Tokyo, Japan).

Transmission electron microscopy imaging

For TEM analysis of protein en peptide solutions grids were prepared according to standard procedures. Samples were applied to 100-mesh copper grids with carbon coated Formvar (Merck, Germany), and subsequently washed with PBS and H_2O. Grids were applied to droplets of 2% (m/v)

methylcellulose with 0.4% (m/v) uranylacetate pH 4. After a 2'-minutes incubation grids were dried on a filter. Micrographs were recorded at 80 kV, at suitable magnifications on a JEM-1200EX electron microscope (JEOL, Japan).

Structural analysis of formulated protein therapeutics

Formulated protein therapeutics were obtained from the local hospital pharmacy and were used as supplied by the manufacturers. The following protein therapeutics were purchased: 1) human growth hormone (GH) (Genotropin, batch 52344B51, 5 mg ml^{-1} KabiQuick, Pharmacia B.V., Woerden, The Netherlands), 2) recombinant human Zn^{2+}-chelated insulin (Monotard, batch NS61694, 100 IE ml^{-1}, Novo Nordisk, Bagsvaerd, Denmark), 3) human albumin (Cealb, batch NS61694, 200 mg ml^{-1}, Sanquin-CLB, Amsterdam, The Netherlands), 4) human modified gelatin (Gelofusine, batch 030606H4, 40 mg ml^{-1}, Braun Medical BV, Oss, The Netherlands), 5) rapid acting human insulin analogue (NovoRapid Flexpen, batch PH70008, 10 U ml^{-1}, Novo Nordisk), 6) blood cell growth factor filgrastim (Neupogen Singleject, batch N0693AD, 960 µg ml^{-1}, Amgen Europe, Breda, The Netherlands), 7) human-murine chimeric monoclonal antibody (Remicade-infliximab, batch 03D06H120A, 10 mg ml^{-1}, Centocor, Leiden, The Netherlands), 8) abciximab, an inhibitor of blood platelet aggregation (ReoPro, 2 mg ml^{-1}, Centocor, Leiden, The Netherlands) and 9) human coagulation factor VIII (FVIII) isolated from healthy volunteers (Aafact, lot 02L046250A, 3.6 mg ml^{-1}, Sanquin-CLB, Amsterdam, The Netherlands). Lyophilized therapeutics were dissolved according to the manufacturers recommendations. GH, zinc-insulin, Cealb and gelatin were stored at -20, 4, room temperature, 37 and 65°C. Other protein therapeutics were only kept at 4°C, and assayed for the presence of cross-β structure conformation at shown time points. Enhancement in fluorescence of ThT and Congo red was measured with all formulated protein therapeutics. For this purpose, proteins were diluted to the indicated concentrations. In addition, tPA binding to the protein therapeutics was analyzed by ELISA and activation

of tPA was tested using the Plg-activation assay. Zinc-insulin was diluted tenfold in the activation assay, GH was diluted to a final concentration of 500 µg ml^{-1}. Activation of factor XII and prekallikrein by the therapeutics was tested in the chromogenic factor XII assay (see above). For tPA ELISAs, 5 µg ml^{-1} of the protein therapeutics were coated onto Greiner high-binding Microlon plates (#655092, Greiner Bio-One, Alphen a/d Rijn, The Netherlands). After coating, plates were blocked with Blocking Reagent (Roche Diagnostics, Almere, The Netherlands). A concentration series of tPA or K2P-tPA in PBS with 0.1% v/v Tween-20 and 10 mM ε-amino caproic acid was applied and the plates were incubated for 1 h at room temperature with constant swirling. Binding of tPA was assessed with monoclonal antibody 374b that binds to the protease domain of both tPA and K2P-tPA (American Diagnostica, Tebu-Bio, The Netherlands), peroxidase-conjugated rabbit anti-mouse immunoglobulins (RAMPO, P0260, DAKOCytomation, Glostrup, Denmark), and stained with 3'3'5'5'-tetramethylbezidine (TMB, catalogue number 4501103, buffer, catalogue number 4501401, Biosource Int., Camarillo, CA, USA).

Activation of tPA by β_2-glycoprotein I, binding of factor XII and tPA to β_2-glycoprotein I, and ThT and TEM analysis of β_2-glycoprotein I

Purification of β_2-glycoprotein I (β_2GPI) was performed according to established methods[11,12]. Recombinant human β_2GPI was made using insect cells and purified as described[11]. Plasma derived β_2GPI as used in a factor XII ELISA, the chromogenic Plg-activation assay and in the anti-phospholipid syndrome antibody ELISA (see below), was purified from fresh human plasma as described[12]. Alternatively, β_2GPI was purified from, either fresh human plasma, or frozen plasma (-20°C) on an anti-β_2GPI antibody affinity column[13]. Activation of tPA (Actilyse, Boehringer-Ingelheim) by β_2GPI preparations was tested in the Plg-activation assay (see above). Hundred µg ml^{-1} plasma β_2GPI or recombinant β_2GPI were tested for their stimulatory cofactor activity in the

tPA-mediated conversion of Plg to Pls, and were compared to the stimulatory activity of peptide FP13 (ref. [7]).

Binding of purified human factor XII from plasma (Calbiochem) or of purified recombinant human tPA to β_2GPI purified from human plasma, or to recombinant human β_2GPI was tested in an ELISA. Ten µg of factor XII or tPA in PBS was coated onto wells of a Costar 2595 ELISA plate (Cambridge, USA) and incubated with concentration series of the two β_2GPI preparations. Binding of β_2GPI was assessed with monoclonal antibody 2B2[13].

Binding of factor XII to ß2GPI was also tested using immunoblotting. $ß_2$GPI (33 µg) purified either from fresh plasma or from frozen plasma was brought onto a 7.5% SDS-PAGE gel. After blotting to a nitrocellulose membrane, the blot was incubated with 1000x diluted rabbit polyclonal anti-human factor XII antibody (#233504, Calbiochem) and after washing with 3000x diluted peroxidase-conjugated swine anti-rabbit immunoglobulins (SWARPO, #P0399, DAKOCytomation, Glostrup, Denmark).

ThT fluorescence of $ß_2$GPI was measured as follows. Purified $ß_2$GPI from human plasma (400 µg ml^{-1} final concentration) was incubated with or without 100 µM cardiolipin (CL) vesicles or 250 µg ml^{-1} of the factor XII activator dextran sulphate 500k (DXS500k, Pharmacia, Uppsala, Sweden), in 25 mM Tris-HCl, 150 mM NaCl, pH 7.3. CL vesicles were prepared according to an established procedure. Briefly, CL was dried under a stream of nitrogen. The lipids were resuspended to a concentration of 10 mg ml^{-1} in 25 mM Tris-HCl, pH 7.3, 150 mM NaCl by vigorous agitation, using a vortex. In the ThT fluorescence assay, fluorescence of $ß_2$GPI in buffer, of CL or DXS500k in buffer, of buffer and ThT alone, and of $ß_2$GPI-CL adducts and $ß_2$GPI-DXS500k adducts, with or without ThT, was recorded as described above (section ThT fluorescence). In addition, TEM images were recorded with CL, ß2GPI from human plasma, with or without CL, and with recombinant ß2GPI, as described[1].

Interference with binding of anti-β_2GPI autoantibodies from antiphospholipid syndrome auto-immune patients to immobilized β_2GPI by recombinant β_2GPI and not by plasma derived β_2GPI

When plasma derived β_2GPI is coated onto hydrophilic ELISA plates, anti-β_2GPI auto-antibodies isolated from plasma of antiphospholipid syndrome auto-immune patients can bind[14]. To study the influence of co-incubations of the coated β_2GPI with the antibodies together with plasma β_2GPI or recombinant β_2GPI, concentration series of β_2GPI were added to the patient antibodies. Subsequently, binding of the antibodies to coated β_2GPI was determined.

Activation of U937 monocytic cells by LPS and cross-β structure conformation comprising polypeptides

U937 monocytes were cultured in six-wells plates. Cells were stimulated with buffer (negative control), 1 μg ml^{-1} LPS (positive control), 100 μg ml^{-1} amyloid endostatin[1,7], 260 μg ml^{-1} Hb-AGE and 260 μg ml^{-1} control Hb. After 1 h of stimulation, cells were put on ice. After washing RNA was isolated and quantified spectrophotometrically. Normalized amounts of RNA were used for 26 cycli of RT-PCR with human TNFα primer and 18 cycli of RT-PCR with ribosomal 18S primer for normalization purposes. DNA was analyzed on a 2% agarose gel.

Structural analysis with CpG-ODN-protein and LPS-protein mixtures

CpG oligodeoxynucleotides (ODN) (Coley Pharmaceutical Group, MA, USA) at a concentration of 10.7, 21.4 and 42.8 μg ml^{-1} was incubated for 30 min. at room temperature or o/n at 4°C, on a roller with 1 mg ml^{-1} lysozyme or endostatin. Enhancement of ThT fluorescence was measured similarly as described above.

Alternatively, CpG-ODN at 21.4 μg ml^{-1} was mixed with 1 mg ml^{-1} of chicken egg-white lysozyme (Fluka, #62971), albumin (ICN, #160069, fraction V),

endostatin (Entremed, Inc, Rockville, MD), human γ-globulins, plasma human β2-GPI (see above) and recombinant human β2-GPI (see above), and incubated o/n on a roller at 4°C, before ThT fluorescence measurements. For this purpose, protein solutions at 2 mg ml^{-1} were ultracentrifuged for 1 h at 100,000*g before use, and subsequently diluted 1:1 in buffer with 42.9 µg ml^{-1} CpG-ODN.

Lipopolysaccharide (LPS) binds to lysozyme, which can prevent biological activities of LPS, and LPS activates factor XII . We tested whether binding of lysozyme is accompanied by a conformational change in the protein with introduction of amyloid-like structure. For this purpose 0, 10, 25, 100, 200, 600 and 1200 µg ml^{-1} LPS (from Escherichia coli serotype 011:B4, #L2630, lot 104K4109, Sigma-Aldrich) was incubated overnight at 4°C or for 30 min. at room temperature on a roller with 1 mg ml^{-1} lysozyme (ICN, 100831) in HBS. Subsequently, the ability to enhance ThT fluorescence was determined with 40x diluted solution, as described above.

Alternatively, similarly as described above for CPG-ODN, LPS at 600 µg ml^{-1} was mixed with 1 mg ml^{-1} of lysozyme, albumin, endostatin, γ-globulins, plasma β2GPI and recombinant β2-GPI, and incubated o/n on a roller at 4°C, before ThT fluorescence measurements. Again, protein solutions at 2 mg ml^{-1} were ultracentrifuged for 1 h at 100,000*g before use, and subsequently diluted 1:1 in buffer with 1200 µg ml^{-1} LPS.

Preparation of amyloid-like ovalbumin, human glucagon, Etanercept and murine serum albumin

To prepare structurally altered ovalbumin (OVA) with amyloid cross-β structure conformation, purified OVA (Sigma, A-7641, lot 071k7094) was heated to 85°C. One mg ml^{-1} OVA in 67 mM NaP_i buffer pH 7.0, 100 mM NaCl, was heated for two cycles in PCR cups in a PTC-200 thermal cycler (MJ Research, Inc., Waltham, MA, USA). In each cycle, OVA was heated from 30 to 85°C at a rate of 5°C/min. Native OVA (nOVA) and heat-denatured OVA

(dOVA) were tested in the ThT fluorescence assay and in the Plg-activation assay. In the fluorescence assay and in the Plg-activation assay, 25 and 100 µg ml^{-1} nOVA and dOVA were tested, respectively. TEM images of nOVA and dOVA were taken to check for the presence of large aggregates.

Modified murine serum albumin (MSA) was obtained by reducing and alkylation. MSA (#126674, Calbiochem) was dissolved in 8 M urea, 100 mM Tris-HCl pH 8.2, at 10 mg ml^{-1} final concentration. Dithiothreitol (DTT) was added to a final concentration of 10 mM. Air was replaced by N_2 and the solution was incubated for 2 h at room temperature. Then, the solution was transferred to ice and iodoacetamide was added from a 1 M stock to a final concentration of 20 mM. After a 15 min. incubation on ice, reduced-alkylated MSA (alkyl-MSA) was diluted to 1 mg ml^{-1} by adding H_2O. Alkyl-MSA was dialyzed against H_2O before use. Native MSA (nMSA) and alkyl-MSA were tested in the ThT fluorescence assay and in the Plg-activation assay. In the ThT-fluorescence assay 25 µg ml^{-1} nMSA and alkyl-MSA were tested, and in the Plg-activation assay 100 µg ml^{-1} was tested. The presence of aggregates or fibrils was analyzed using TEM.

Amyloid-like properties in human glucagon (Glucagen, #PW60126, Novo Nordisk, Copenhagen, Denmark) were introduced as follows. Lyophilized sterile glucagon was dissolved at 1 mg ml^{-1} in H_2O with 10 mM HCl. The solution was subsequently kept at 37°C for 24 h, at 4°C for 14 days and again at 37°C for 9 days. ThT fluorescence was determined as described above, and compared with freshly dissolved glucagon. tPA-activating properties of both heat-denatured glucagon and freshly dissolved glucagon was tested at 50 µg ml^{-1}. TEM analysis was performed to assess the presence of large multimeric structures.

Immunization of Balb/c mice with ovalbumin and amyloid-like ovalbumin

Eight to ten weeks old female Balb/c mice are immunized with OVA according to two immunization regimes (Central Animal Laboratories, Utrecht University, The Netherlands). Pre-immune serum was collected prior to the immunizations. In one regime two groups of five mice were subcutaneously injected five consecutive days per week, for three consecutive weeks. Doses comprised 10 µg native OVA or heat-denatured OVA for each injection. Alternatively, according to the second protocol, three groups of five mice were injected once intraperitoneally with doses comprising 5 µg nOVA, 5 µg OVA or 5 µg native OVA mixed 1:1 with complete Freund's adjuvant (CFA). Each week, blood was taken. After three weeks, a second dose was given. Incomplete Freund's adjuvant (IFA) was used instead of CFA. Blood was taken after one week after the start of the immunization. Antibody titers in sera were determined and sera were analyzed for the presence of cross-β structure conformation specific antibodies. For this purpose, nOVA was coated onto wells of 96-wells ELISA plates and incubated with dilution series of sera. Sera of the groups of five mice were pooled prior to the analyses. Plates were washed and subsequently incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulins (RAMPO, P0260, DAKOCytomation, Glostrup, Denmark). Plates were subsequently developed with tetramethylbenzidine (TMB) substrate. The reaction was terminated with H_2SO_4.

Example 1

Protein assemblies with cross-β structure conformation bind to immobilized fibronectin type I domains in a Biacore surface plasmon resonance set-up

We used a surface plasmon resonance set-up of Biacore to test whether immobilized proteins with affinity for cross-ß structure conformation can capture amyloid-like polypeptides from solution under flow. This set up also allows to test suitable elution buffers to disrupt the interaction. In this way insight into suitable methods to deplete proteins with cross-ß structure conformation from solutions is obtained, as well as insight into how to compete for the interaction of cross-ß structure conformation binders, which are for example immobilized on beads in a column, with proteins comprising cross-ß structure conformation.

On one chip we immobilized sRAGE, tPA and K2P-tPA. One channel was left empty for reference purposes. Protein solutions were centrifuged for 10' at 16,000*g before the solutions were applied to the Biacore chip. Centrifugation had no effect on the stimulatory effect of Hb-AGE and amyloid γ-globulins on tPA-mediated activation of Plg (Fig. 1A). Moreover, we filtrated all protein solutions before they were applied to the Biacore to exclude the presence of large aggregates with a density equal to buffer. For Hb-AGE similar response units were obtained after centrifugation or filtration (not shown). Subsequent experiments showed that Hb-AGE, albumin-AGE and amyloid γ-globulins bind to immobilized tPA and sRAGE (Fig. 1B-D). The interaction of tPA and sRAGE with Hb-AGE and albumin-AGE could be disrupted with 0.1 M H_3PO_4 buffer pH 1.0. Amyloid γ-globulins, however, were not removed by this buffer. After trying several more harsh regeneration buffers, the binding capacity of the chip was lost.

One a second chip, chemically synthesized HGFA F and tPA F, and Fn F4-5-His expressed in *S. cerevisiae* were immobilized. None of the polypeptides with

cross-ß structure conformation bound to the two single F domain constructs. Hb-AGE, endostatin and recombinant ß2GPI bound, however, to the Fn F4-5 doublet, whereas native Hb did hardly bind (Fig. 1E-H). Affinities of the three proteins for Fn F4-5, expressed as the concentration of ligand that results in half maximum binding, ranges from 8 nM for Hb-AGE, via 165 nM for recombinant ß2GPI to up to 800 nM for endostatin. In fact, based on the absence of tPA activating properties in 100 µg ml^{-1} endostatin (Fig. 1A), we did not expect any binding at all. Putatively, the surface plasmon resonance is more sensitive for the cross-ß structure conformation under the conditions used. We observed that when a stock solution of endostatin at 7.9 mg ml^{-1} in the buffer as supplied by the manufacturer, is kept at ice or at room temperature, readily aggregates. Perhaps, during the course of our experiments, part of the endostatin molecules start to denature, giving rise to the observed binding to Fn F4-5. With this chip, interaction between Fn F4-5 and the protein ligands could be abolished simply by increasing the NaCl concentration from 140 mM to 1 M. This shows that the interaction was primarily based on charge interactions.

Our surface plasmon resonance data show that F domains expressed in *S. cerevisiae* can bind to polypeptides with the cross-ß structure conformation. Furthermore, the data show that both 0.1 M H_3PO_4 buffer pH 1.0 and 10 mM HEPES pH 7.4, 1 M NaCl, 1.5 mM $CaCl_2$, 10 mM εACA, 0.05% Tween-20 are suitable buffers to release polypeptides with cross-ß structure conformation from cross-ß structure binding compounds. These buffers are also suitable to release cross-ß structure binding compounds and proteins that are bound to a ligand with cross-ß structure conformation. These data are helpful during the design of a method to deplete solutions from cross-ß structure conformation rich compounds by using cross-ß structure binding polypeptides that are immobilized on a suitable supporting material.

Activation of factor XII and tPA by protein aggregates with amyloid-like cross-β structure conformation

Contacting factor XII to artificial negatively charged surfaces results in its activation, as measured by the conversion of prekallikrein to kallikrein, which can convert chromogenic substrate Chromozym-PK (Fig. 2). Now, we demonstrate that also peptide aggregates with cross-ß structure conformation, the protein conformation found in amyloid, also stimulate factor XII activation (Fig 2). Moreover, we demonstrate that kaolin is able to stimulate factor XII activation only when a protein cofactor, e.g. albumin or endostatin, is present at 1 mg ml^{-1} in the assay buffer (Fig. 2C, D). Similar results were obtained when DXS500k surface was used as the factor XII activator; again DXS500k only activates factor XII when albumin or endostatin are added to the reaction mixture (Fig. 2E, F). Contacting DXS500k with various proteins, including lysozyme, γ-globulins, whole plasma and factor XII itself, results in the introduction of amyloid-like properties in the proteins, e.g. activation of tPA (Fig. 2G), enhanced fluorescence of ThT (Fig. 2H-J) and binding of tPA (Fig. 2K-N), indicative for the formation of cross-ß structure conformation in the protein aggregates after exposure to the negatively charged surface. We also tested the ability of protein aggregates with cross-ß structure conformation to induce auto-activation of factor XII. For this purpose, purified factor XII was incubated with substrate S-2222 and either buffer, or 1 µg ml^{-1} DXS500k, 100 µg ml^{-1} FP13 K157G, 10 µg ml^{-1} Aß(1-40) E22Q and 10 µg ml^{-1} Hb-AGE. All three amyloid-like aggregates are able to induce factor XII auto-activation (Fig. 2P). FP13 K157G and Hb-AGE have a potency to induce auto-activation that was similar to the established surface activator DXS500k, whereas the potency of the Aß(1-40) E22Q was somewhat lower. Freshly dissolved native Hb, ultracentrifuged for 1 h at 100,000*g, and freshly dissolved FP13 K157G did not or hardly auto-activate factor XII (not shown).

Factor XII, tPA, Fn and their recombinant Fn type I, or finger domains interact with aggregates comprising cross-β structure conformation

Like tPA, factor XII, Fn, tPA F domain, factor XII F domain and Fn F4-5 domains bind to peptide aggregates with cross-ß structure conformation. In addition, the Fn F10-12 domains and the HGFA F domain bind to amyloid-like cross-ß structure conformation rich aggregates (B. Bouma, data not shown). Moreover, like tPA[1,7], factor XII becomes activated by amyloid-like aggregates (Fig. 2). This has not only been established in an indirect way by measuring activated kallikrein from prekalikrein upon activation of factor XII, but also in a direct way by measuring auto-activation of factor XII upon exposure to amyloid-like protein aggregates (see Fig. 2O). Our data also show that several negatively charged surfaces, that are well known for their ability to activate factor XII, i.e. kaolin and DXS500k, need a protein cofactor to gain stimulatory capacities (Fig. 2C-F). Binding of ThT and tPA after exposure of proteins to DXS500k shows that the protein aggregate cofactors adopt the cross-ß structure conformation, that are essential for both the factor XII activation and the tPA activation. In addition, our data show that recombinantly expressed F domains as well as a totally chemical synthesized F domains can bind to polypeptides with cross-ß structure conformation.

Our data show that both the fibrinolytic cascade and the contact system of blood coagulation become activated by activation of tPA and factor XII via protein aggregates with amyloid-like cross-ß structure conformation. This predicts that presence of amyloid-like protein conformation in the circulation or elsewhere in the body is a risk factor for inducing pathological activation of the fibrinolytic cascade and/or the contact activation system. Indeed, we found elevated levels of activated FXII as well as elevated levels of plasmin-α2-antiplasmin (PAP) complexes in plasma obtained from patients suffering from systemic amyloidosis. Thus, it can be predicted that excessive systemic activation of the contact activation system and the fibrinolytic system by

proteins comprising cross-β structure may also lead to undesirable complications, including, but not limited to thrombosis, bleeding, disseminated intravascular coagulation (DIC), septic shock, multi organ dysfunction syndrome (MODS) and/or anaphylactic shock. With the present invention it is now disclosed that such effects may be triggered by protein therapeutics or their constituents/excipients comprising cross-β structure or by protein therapeutics or their constituents/excipients that induce cross-β structure formation before, during or after administration into a subject.

Our data on factor XII activation open avenues that allow further analysis of the role of the cross-β structure conformation in factor XII activation. The influence of cross-β structure binding proteins and compounds on the activation of factor XII in the presence of cross-β structure conformation can be studied. Our observation that both tPA and factor XII become activated by proteins that are contacted to DXS500k further show that the fibrinolytic cascade and the contact activation cascade of the haemostatic system are activated by a common mechanism, in which protein aggregates comprising amyloid-like cross-β structure conformation play an key role. Considering HGFA, similar cross-β structure-mediated activating mechanisms are predicted.

Our surface plasmon resonance data show that F domains expressed in *S. cerevisiae* can bind to polypeptides with the cross-β structure conformation. Furthermore, the data show that both 0.1 M H_3PO_4 buffer pH 1.0 and 10 mM HEPES pH 7.4, 1 M NaCl, 1.5 mM $CaCl_2$, 10 mM εACA, 0.05% Tween-20 are suitable buffers to release polypeptides with cross-β structure conformation from cross-β structure binding compounds. These buffers are also suitable to release cross-β structure binding compounds and proteins that are bound to a ligand with cross-β structure conformation. These data are helpful during the design of a method to deplete solutions from cross-β structure conformation rich compounds by using cross-β structure binding polypeptides that are immobilized on a suitable supporting material.

Example 2

Formulated protein therapeutics for human use contain protein aggregates with cross-ß structure conformation.

Structural analysis of formulated protein therapeutics

Formulated protein therapeutics for human use were obtained from the local hospital pharmacy. The therapeutics were analyzed for the presence of cross-ß structure protein conformation. All analyses were performed before the expiring dates were reached. As controls, the therapeutics were stored as recommended by the manufacturers. Therapeutics were also stored at -20°C, room temp., 37°C and 65°C. Fluorescence of Congo red and ThT in the presence or absence of the therapeutics was analyzed, as well as tPA binding, tPA activation and factor XII activation. For fluorescence assays, 10 µg ml^{-1} Aß(1-40) E22Q amyloid was used as a positive control and gave typical values of approximately 1250 and 1800 A.U., respectively. Furthermore, TEM images were recorded to get insight whether amorphous aggregates are formed or fibrillar like structures. Gelatin, Cealb, FVIII and to some extent GH, stored at the recommended storage temperature of 4°C, enhanced the fluorescence of Congo red (Fig. 3A). In addition, Cealb, GH and FVIII enhance fluorescence of ThT (Fig. 3B). GH also induced tPA activation (Fig. 3C). Insulin activated tPA to a lesser extent, but still significantly (Fig. 3C). Both insulin and zinc-chelated insulin activate the factor XII/prekallikrein contact system (Fig. 3D). Gelatinous collagen fragments stored at 4°C and 37°C displayed enhanced Congo red fluorescence in a storage temperature dependent manner (Fig. 3E). Only gelatin kept at 37°C activated factor XII (Fig. 3F). In an ELISA set-up, binding of tPA was established for Cealb, a therapeutic antibody, gelatin, zinc-chelated insulin (Fig. 3G) and GH (Fig. 3H), all stored at the recommended temperature of 4°C. For both ELISAs, Hb-AGE was coated as a positive control

(not shown for clarity). In the ELISA depicted in Fig. 3G, truncated K2P-tPA, which lacks the amyloid-binding F domain, was also tested for binding to the immobilized protein therapeutics. K2P-tPA did not bind to any of the therapeutics tested (not shown). On TEM images various condensed aggregates are seen with modified gelatin (Fig. 3I). GH appeared on TEM images as linear, branched and condense particles, all apparently composed of spherical particles (Fig. 3J). Zinc-chelated insulin appears on TEM images as thin linear unbranched fibrils with varying length (Fig. 3K). FVIII and the antibody did not appear as visible particles under the electron microscope. Cealb and insulin appeared as visible aggregates with no sign of a fibrillar nature (Fig. 3L, M). Reopro displays storage temperature dependent ThT fluorescence enhancement properties and tPA activating properties (Fig. 3N, O). Only after storage at 65°C ReoPro enhanced ThT fluorescence and induced Pls activity. Apparently, only at 65°C ReoPro adopts the amyloid-like cross-ß structure conformation. A TEM image of ReoPro that was stored at the recommended temperature of 4°C revealed that some non-fibrillar aggregates were present, that apparently do not have ThT fluorescence enhancing or tPA activating properties under the conditions tested.

Discussion: Formulated protein therapeutics for human use display amyloid-like characteristics

Based on the observed binding of Congo red, ThT and tPA, based on the appearance on TEM images, and based on the observed activating properties towards tPA and factor XII, the tested protein therapeutics Cealb, gelatin, insulin, zinc-insulin, GH, antibody and FVIII displayed amyloid-like properties, when stored under recommended conditions. For human Cealb, binding of tPA, Congo red and ThT is indicative for the presence of cross-ß structure conformation. Binding of Congo red and activation of factor XII shows the presence of cross-ß structure conformation in gelatin. Binding of ThT and tPA, and activation of tPA by GH are indicative for amyloid-like

properties in this formulated therapeutic. Finally, both activation of tPA and factor XII by insulin/zinc-insulin show the presence of cross-ß structure conformation. Hence, taken together our observations show the presence of protein or peptide aggregates with amyloid-like properties or the potential that the cross-ß structure can be formed upon storage in these formulated protein therapeutics.

Structural analysis of protein therapeutics can be expanded using techniques and assays such as X-ray diffraction experiments, Fourier transform infrared spectroscopy, size exclusion HPLC, CD spectropolarimetry and binding assays using amyloid binding proteins, and can be expanded by introducing new protein therapeutics in the series of analyses.

Example 3

Cross-ß structure and immunogenicity

Incubation of cultured U937 monocytes with proteins comprising cross-ß structure conformation results in upregulation of tissue necrosis factor-α mRNA levels, and the immunopotentiators LPS and CPG-ODN induce formation of amyloid-like structures in proteins.

Cross-β structure rich compounds induce expression of TNFα RNA in monocytes

After exposure of U937 monocytes to LPS or cross-β structure rich amyloid endostatin or Hb-AGE, TNFα DNA was obtained after RT-PCR with isolated RNA (Fig. 4A). Control Hb did induce TNFα RNA upregulation only to a minor extent, which did not exceed approximately 30% of the values obtained after stimulation with amyloid endostatin or Hb-AGE. Amounts of TNFα DNA obtained after RT-PCR with monocyte RNA are normalized for the amounts of ribosomal 18S DNA present in the corresponding samples.

LPS and CPG-ODN act as a denaturants and induces cross-β structure conformation

After exposure of 1 mg ml^{-1} lysozyme to 10, 25, 100, 200, 600 and 1200 µg ml^{-1} LPS in solution, ThT fluorescence was enhanced 1.1, 1.3, 1.6, 2.3, 5.7 and 13.1 times respectively when compared to lysozyme incubated in buffer only, indicative for the formation of amyloid-like conformation with cross-ß structure (Fig. 4B). After exposure of lysozyme and endostatin to 200, 400 and 600 µg ml-1 LPS, ThT fluorescence was enhanced approximately 5, 11 and 18 times and 8, 20 and 26 times, respectively (Fig. 4D, E). Similarly to what was observed with CPG-ODN (Fig. 4F), when 1 mg ml^{-1} lysozyme, albumin, γ-globulins, endostatin, plasma ß2GPI or recombinant ß2GPI were exposed to 600 µg ml-1 LPS, ThT fluorescence was enhanced approximately 10, 3, 2, 10, 2 and 4 times, respectively (Fig. 4C). Furthermore, CPG-ODN at 10.7, 21.4 and 42.8 µg ml^{-1} incubated overnight with 1 mg ml^{-1} lysozyme enhanced ThT fluorescence with a factor 1.1, 1.2 and 1.4, respectively, further show the cross-ß structure inducing capacity of CPG-ODN (not shown). In addition, when 10.4 or 21.7 µg ml^{-1} CPG-ODN was incubated with 1 mg ml^{-1} lysozyme or endostatin for 30 min. at room temperature, an increase in ThT fluorescence of approximately 8 to 7 times for lysozyme and 39 to 56 times for endostatin was observed, respectively (Fig. 4D, E). In addition, exposure of 1 mg ml^{-1} albumin, endostatin, plasma ß2GPI or rec. ß2GPI to 21.4 µg ml^{-1} CPG-ODN results in increased ThT fluorescence with approximately a factor 3, 10, 2 and 5, respectively (Fig. 4F). Additional TEM imaging could shed further light on whether the LPS and CPG-ODN exposed proteins have rearranged their conformation into amyloid like fibrils or into other visible aggregates. The ThT fluorescence enhancement data show that LPS and CPG-ODN act as denaturants that convert initially globular proteins into an amyloid-like polypeptide. Previously, it has already been demonstrated that lysozyme can bind to purified LPS and to complete Freund's adjuvant, comprising bacterial cell wall fragments with LPS, accompanied by structural changes in the

protein[15,16]. Furthermore, Morrison & Cochrane[17] showed that LPS can potently activate factor XII, which adds to our finding that LPS acts as compound capable of inducing cross-β structure, which in turn is responsible for the activation of factor XII. Thus, our results now disclose that LPS binding induces cross-β structure conformation and that LPS activation of factor XII is mediated by protein with cross-β structure conformation, providing an explanation for these previously reported observations.

Similar to LPS, cross-β structure rich proteins induce TNFα upregulation in monocytes, and LPS induces amyloid cross-β structure conformation in lysozyme

Stimulation of U937 monocytes with proteins that comprise cross-β structure conformation as part of their tertiary/quarternary fold results in expression of TNFα RNA, similar to the upregulation of TNFα RNA by LPS. The observation that control Hb did influence TNFα RNA levels only to some extent shows that the presence of cross-β structure conformation is an important factor for the observed upregulation. Since we here show that LPS acts as a cross-β structure conformation-inducing agent we conclude that the activation of cells, including cells of the immune system, by LPS is induced, at least in part, by a conformationally altered protein comprising cross-β structure conformation. Thus, LPS acts as a denaturing surface or adjuvant that induces cross-β structure conformation formation in a protein that is present on the cell surface or in the cell environment, similar to our observation that LPS introduces amyloid-like cross-β structure conformation in lysozyme. The formed cross-β structure conformation is then a stimulator of the immune response. Our results and conclusions are supported by the observations in literature that the endotoxic activity of LPS is enhanced in the presence of albumin or Hb. Moreover, LPS induces formation of β-sheets in albumin, a structural element that is absent in the albumin native fold and which suggests that cross-β structure conformation is formed[18]. Similar

responses of microglial cells towards LPS and aggregated Aß are reported[19]. Our observations give a rationale to these and recent additional observations that the LPS receptor CD14 is involved in Aß phagocytosis[20,21]. In the light of our results CD14 perhaps interacts with a denatured protein associated with LPS and with Aß via a similar non-native protein conformation in the ligands. This would suggest that CD14 is a possible member of the class of amyloid-like cross-ß structure binding proteins[1]. Blocking experiments using cross-ß structure binding compounds and proteins, e.g. ThT, Congo red, Thioflavin S (ThS), tPA and fragments thereof, factor XII and fragments thereof, anti-cross-ß structure hybridomas, can provide further evidence for the role of the cross-ß structure element in the activation of the immune system. Furthermore, cellular assays can be used to study which appearance of the cross-ß structure conformation bears the immunogenic nature, i.e. soluble oligomers, fibrils, or other appearances.

Our results show that the potentiating effects of LPS, when it is used as an adjuvant in immunization experiments, are attributed at least in part by the introduction of immunogenic cross-ß structure conformation in the administered antigen, in a co-administered or in an endogenous protein or set of endogenous proteins.

A person skilled in the art can now further assess whether a protein with cross-ß structure conformation is activating cells of the immune system is by use of a 'whole blood' assay. For this purpose, at day 1 freshly drawn human EDTA-blood should be added in a 1:1 ratio to RPMI-1640 medium (HEPES buffered, with L-glutamine, Gibco, Invitrogen, Breda, The Netherlands), that is pre-warmed at 37°C. Subsequently, proteins comprising cross-ß structure conformation can be added. Preferably a positive control is included, preferably LPS. An inhibitor that can be used for LPS is Polymyxin B at a final concentration of 5 µg ml^{-1}. Standard cross-ß structure conformation rich polypeptides that can be tested are Aß, amyloid γ-globulins, glycated proteins, FP13, heat-denatured OVA and others. Negative controls are native γ-

globulins, native albumin, native Hb, freshly dissolved Aß or FP13, nOVA. As a control, all protein samples can be tested in the absence or presence of 5 µg ml^{-1} Polymyxin B to exclude effects seen due to endotoxin contaminations. The blood and the medium should be mixed carefully and incubated overnight in a CO_2 incubator with lids that allow for the entrance of CO_2. At day 2 the medium should be collected after 10' centrifugation at 1,000*g, at room temperature. The cell pellet can be frozen and stored. The medium should be again be centrifuged for 20' at 2,000*g, at room temperature. Supernatant can be analyzed using ELISAs for concentrations of markers of an immune response, e.g. tissue necrosis factor-α (TNF-α) or cytokine When positive and negative controls are established as well as a reliable titration curve, any solution can be tested for the cross-ß structure load with respect to concentrations of markers for immunogenicity. Furthermore, putative inhibitors of the immune response can be tested. For example, F domains, ThT, Congo red, sRAGE and tPA may prevent an immune response upon addition to protein therapeutic solutions comprising aggregates. Alternatively the effect of proteins comprising cross-ß structure on the induction of inflammatory cytokines, including but not limited to TNFα, are tested using cultured cells in vitro. For example monocytic cells such as U937 or THP-1 monocytes care used stimulated with proteins comprising cross-ß structure. ELISA's are used to determine the release of cytokines by these cells. Alternatively, RT-PCR is used.

Example 4

Relationship between the structure of $ß_2$-glycoprotein I, the key antigen in patients with the antiphospholipid syndrome, and antigenicity.

The anti-phospholipid syndrome and conformationally altered $ß_2$-glycoprotein I

The anti-phospholipid syndrome (APS) is an autoimmune disease characterized by the presence of anti-ß2-glycoprotein I autoantibodies. Two of the major clinical concerns of the APS are the propensity of autoantibodies to induce thrombosis and the risk for fetal resorption. Little is known about the onset of the autoimmune disease. Recent work has demonstrated the need for conformational alterations in the main antigen in APS, ß2-glycoprotein I (ß2GPI), before the initially hidden epitope for autoantibodies is exposed [22]. Binding of native ß2GPI to certain types of ELISA plates mimicks the exposure of the cryptic epitopes that are apparently present in APS patients[22]. It has been demonstrated that anti-ß2GPI autoantibodies do not bind to globular ß2GPI in solution, but only when ß2GPI has been immobilized to certain types of ELISA plates[22]. The globular (native) form of the protein is not immunogenic, but requires the addition of CL, apoptotic cells or modification by oxidation[9]. Thus the generation of autoantibodies seems to be triggered by and elicited against a conformationally altered form of ß2GPI. It has previously been proposed that the induction of an adaptive immune response requires a so-called "danger" signal, which among other effects stimulates antigen presentation and cytokine release by dendritic cells[23]. The following results imply that CL induces cross-ß structure conformation in ß2GPI which than serves as a danger signal. In analogy other negatively charged phospholipids, or structures that contain negatively charged lipids, such as liposomes or apoptotic cells, or other inducers of cross-ß structure conformation, including LPS, CPG-ODN that possess cross-ß structure conformation inducing properties, may be immunogenic due to the fact, at least in part, that they induce cross-ß structure conformation.

Factor XII and tPA bind to recombinant β2GPI and to β2GPI purified from frozen plasma, but not to β2GPI purified from fresh plasma

Recombinant β2GPI, but not β2GPI purified from fresh plasma stimulate tPA-mediated conversion of Plg to Pls, as measured as the conversion of the Pls

specific chromogenic substrate S-2251 (Fig. 5A). Using an ELISA it is shown that tPA and factor XII bind recombinant ß2GPI, but not bind to β2GPI purified from fresh human plasma (Fig. 5B, C). Recombinant β2GPI binds to factor XII with a k_D of 20 nM (Fig. 5C) and to tPA with a k_D of 51 nM (Fig. 5B). In addition, β2GPI purified from plasma that was frozen at -20°C and subsequently thawed, factor XII co-elutes from the anti-β2GPI antibody affinity column, as shown on Western blot after incubation of the blot with anti-factor XII antibody (Fig. 5D). This suggest that β2GPI refolds into a conformation containing cross-ß structure upon freezing. In Figure 5E, the inhibitory effect of recombinant β2GPI on binding of anti-β2GPI autoantibodies isolated from patients with APS to immobilized β2GPI is shown. It is seen that plasma derived β2GPI in solution has hardly an effect on the antibody binding to immobilized β2GPI. Fig. 5F shows that exposure of ß2GPI to CL or DXS500k introduces an increased ThT fluorescence signal, indicative for a conformational change in ß2GPI accompanied with the formation of cross-ß structure conformation. Again, recombinant ß2GPI initially already gave a higher ThT fluorescence signal than native ß2GPI purified from plasma. In addition, exposure of plasma ß2GPI and rec. ß2GPI to adjuvants/denaturants LPS or CPG-ODN also induces an increase in ThT fluorescence, which is larger with rec. ß2GPI than with plasma ß2GPI for both adjuvants (see examples in patent P71713EP00). These data not only show that recombinant ß2GPI already comprises more cross-ß structure conformation than plasma ß2GPI, but that recombinant ß2GPI also adopts more readily this conformation when contacted to various adjuvants and surfaces, i.e. CL, DXS500k, LPS and CPG-ODN. In figure 5G it is shown that exposure of ß2GPI to CL, immobilized on the wells of an ELISA plate, renders ß2GPI with tPA binding capacity. Binding of ß2GPI directly to the ELISA plate results in less tPA binding. These observations also show that CL has a denaturing effect, thereby inducing amyloid-like conformation in ß2GPI, necessary for tPA binding. These observations, together with the observation that exposure of ß2GPI to CL

vesicles induced ThT binding capacity (Fig. 5F), show that exposure of ß2GPI to a denaturing surface induces formation of amyloid-like cross-ß structure conformation.

Epitopes for autoantibodies are specifically exposed on non-native conformations of β2GPI comprising cross-β structure conformation

Figure 5 shows that preparations of β2GPI react with amyloid cross-β structure markers ThT, tPA and factor XII. In addition, exposure of ß2GPI to CL introduces tPA binding capacity (Fig. 5G). Furthermore, large fibrillar structures are seen on TEM images of plasma ß2GPI in contact with CL (Fig. 5H, image 2 and 3). Small CL vesicles seem to be attached to the fibrillar ß2GPI. Images of plasma ß2GPI alone (Fig. 5H, image 1) or CL alone (not shown) revealed that no visible ultrastructures are present. In contrast, non-fibrillar aggregates and relatively thin curly fibrils can be seen on images of recombinant ß2GPI (Fig. 5H, image 4). These observation show that exposure of ß2GPI to CL and expression and purification of recombinant ß2GPI result in an altered multimeric structure of ß2GPI, when compared to the monomeric structure observed with X-ray crystallography[24]. The ß2GPI preparations with cross-ß structure conformation express epitopes that are recognized by anti-β2GPI auto-antibodies isolated from APS patient plasma. Furthermore, exposure of ß2GPI to CL or DXS500k induces an increased fluorescence when ThT is added, indicative for the formation of cross-ß structure conformation when ß2GPI contacts a negatively charged surface. Interestingly, it has previously been observed that exposure of ß2GPI to CL is a prerequisite for the detection of anti-ß2GPI antibodies in sera of immunized mice[9]. These combined observations point to a role for conformational changes in native β2GPI, necessary to expose new immunogenic sites. Our results show that the cross-β structure conformation is part of this epitope. We predict that the cross-ß structure conformation can be relatively easily formed by one or more of the five domains of the extended ß2GPI molecule[24]. Each domain comprises at least

one ß-sheet that may function as a seed for local refolding into cross-ß structure conformation.

A person skilled in the art is now able to test the hypothesis that the cross-ß structure conformation is essential to elicit anti-$ß_2$GPI antibodies.

Immunization studies with native β_2GPI and conformationally altered β_2GPI, with or without cross-β structure conformation, can be performed in the presence or absence of a compound, including ThT, tPA, RAGE, CD36, anti-cross-ß structure antibodies or a functional equivalent thereof, that inhibits the activity of cross-ß structure conformation. Alternatively, in vitro studies with antigen presenting cells (APC), including dendritic cells (DC) can be performed. Sources of conformationally altered $ß_2$GPI are recombinant $ß_2$GPI, or $ß_2$GPI exposed to any denaturing surface, e.g. plastics, CL, DXS500k and potentially other adjuvants. In addition, structurally altered $ß_2$GPI may be obtained by any other chemical or physical treatment, e.g. heating, pH changes, reduction-alkylation. A person skilled in the art is able to design and perform in vitro cellular assays and in vivo mouse models to obtain further evidence for the role of the cross-β structure conformation in autoimmunity (see below). To establish whether the cross-ß structure element is essential for eliciting an immune response or for antibody binding, inhibition studies can be conducted with any cross-ß structure binding compound that may compete with antibody binding or that may prevent an immune response.

Our observations show that cross-ß structure conformation is necessary for the induction of an adaptive immune response. The cross-ß structure conformation can also be part of an epitope recognized by autoimmune antibodies. Based on our studies it is expected that other diseases and complications in which autoantibodies are implicated are mediated by a protein comprising cross-ß structure conformation. In addition to the antiphospholipid syndrome such conditions include, but are not limited to systemic lupus erythematosus (SLE), type I diabetes, red cell aplasia and the formation of inhibitory antibodies in hemophilia patients treated with FVIII. A person skilled in the art is now able

to screen hemophilia patients with anti-FVIII autoantibodies for the presence of antibodies in their plasma that recognize the cross-ß structure conformation. A more detailed analysis will reveal whether putative cross-ß structure binding antibodies specifically bind (in part) to cross-ß structure conformation in the antigen, or whether the antibodies bind to cross-ß structure conformation present in any unrelated protein.

A role for the cross-ß structure element in immunological reactions upon administering protein therapeutics with cross-ß structure conformation can be addressed by a person skilled in the art (see below). Moreover, a person skilled in the art can test, for example, the immunogenicity of a protein therapeutic, including but not limited to FVIII comprising cross-ß structure conformation before and after contacting the said protein therapeutic solution with immobilized cross-ß structure binding compounds or proteins to remove proteins comprising cross-ß structure. After such contacting, the decreased amount of cross-ß structure conformation is determined and *in vivo* or *in vitro* experiments is used to determine the effect of the removal of cross-ß structure conformation (see also below).

EXAMPLE 5

Immunogenicity of denatured proteins with amyloid cross-ß structure conformation without the use of an adjuvant.

Preparation of antigens with cross-β structure conformation

The data disclosed in Example 2, showing that in various protein therapeutics signs for the presence of cross-ß structure conformation can be gathered, and the data disclosed in Example 2 and 4 of patent application P71713EP00, showing that various adjuvants used in animal and human vaccination regimes induce the cross-ß structure conformation in proteins show that immunogenicity is attributed, at least in part, to cross-ß structure comprising

proteins or polypeptides. This prompted us to set up immunization trials with cross-β structure conformation rich compounds, without addition of an adjuvant. Based on the results described above it is predicted that the presence of the immunogenic cross-β structure conformation is essential and even sufficient to induce an immune response, such as for example seen with various protein-based pharmaceuticals that lack an adjuvant. Indeed higher antibody titers were obtained when we used chicken OVA with cross-β structure conformation (dOVA) in comparison with OVA without cross-β structure conformation (nOVA) in immunization experiments (Fig. 6L). Titers were also obtained with OVA without cross-β structure conformation. Since the formation of cross-β structure in OVA can readily occur it is predicted that the generation of antibodies after immunization with nOVA is also mediated by molecules with cross-β structure conformation. In this case the cross-β structure conformation is induced during or after the subcutaneous injection. A person skilled in the art can perform similar experiments with any protein or set of proteins, for example MSA, but preferably protein therapeutics, preferably with interferon α, glucagon or Etanercept to further obtain evidence for the role of the cross-β structure in immunogenicity of proteins, preferably protein therapeutics or constituents thereof. Preferably the cross-β structure conformation is induced by heating (see below), oxidation (see below), glycation or treatment with an adjuvant, such as CPG-ODN oligodeoxynucleotides, LPS or CL. The content of cross-β structure conformation is preferably measured by ThT, Congo red, TEM, size exclusion chromatography, tPA-activating activity, and or binding of any other cross-β structure binding protein listed in Tables 1-3. For example, native and modified, preferably oxidized, forms of a protein therapeutic, preferably interferon α should be tested. Preferably, different amounts of said native and modified therapeutic should be mixed and used for immunization. Preferably mice are used for immunization and even more preferably mice transgenic for said therapeutic. These experiments will further establish that the presence of the cross-β structure conformation in a protein

can induce immunogenicity. In the case of a protein therapeutic, removing or diminishing the cross-ß structure content of the therapeutic will aid to a safer medicine.

Amyloid-like OVA was obtained by heat denaturation at 85°C (Fig. 6A, B, I, K). The presence of the cross-ß structure conformation was established with ThT fluorescence and Plg-activation assays and by TEM imaging. The fibrillar structures of at least up to 2 µm in length, seen on the TEM images are likely not the only OVA assemblies with cross-ß structure conformation present, as concluded from the observation that filtration through a 0.2 µm filter does not reduce the enhancement of ThT fluorescence. A person skilled in the art can perform similar experiments with MSA, human glucagon and Etanercept stock solutions with the cross-ß structure conformation, such as those described below (Fig. 6).

The amyloid-like protein fold was induced in MSA by heat denaturation at 85°C and by reduction and alkylation of disulphide bonds (Fig. 6A-D). We observed that also native MSA enhanced ThT fluorescence to some extent, but this was not reflected by stimulation of tPA activation. Although heat-denatured MSA and alkylated MSA enhance ThT fluorescence to a similar extent, they differ in tPA activating potential. This suggests that tPA and ThT interact with distinct aspects of the cross-ß structure conformation. Previously, we observed that Congo red, another amyloid-specific dye, can efficiently compete for tPA binding to amyloid-like aggregates in ELISAs, whereas ThT did not inhibit tPA binding at all (patent application WO2004/004698) .

Amyloid-like cross-ß structure conformation was induced in glucagon by heat-denaturation at 37°C at low pH in HCl buffer (Fig. 6E, F, J). In this way, a potent activator of tPA was obtained, that enhanced ThT fluorescence to a large extent. In addition, long and bended unbranched fibrils are formed, as visualized on TEM images (Fig. 6J). Noteworthy, at high glucagon concentration, also native glucagon has some tPA activating potential,

indicative for the presence of a certain amount of cross-ß structure conformation rich protein.

Alkylated Etanercept does not activate tPA at all, whereas heat-denatured Etanercept has similar tPA activating potential as amyloid γ-globulins (Fig. 6G). After heat denaturation, Etanercept also efficiently induces enhanced ThT fluorescence (Fig. 6H). Native Etanercept both induces some tPA activation and gave some ThT fluorescence enhancement.

For immunizations of Balb/c mice, nOVA, dOVA and nOVA with complete Freund's adjuvant were used. Similar immunizations and analyzes can be performed with n-MSA, heat-denatured MSA, alkyl-MSA, native glucagon, heat-denatured glucagon, native Etanercept, denatured Etanercept, native ß2GPI, alkyl-ß2GPI, denatured ß2GPI, recombinant ß2GPI, ß2GPI together with CPG-ODN, ß2GPI together with CL and ß2GPI together with DXS500k. Furthermore, the analysis of the various titers may point to improved immunization protocols with respect to dose, number of injections, way of injection, pre-treatment of the antigen to introduce more immunogenic cross-ß structure conformation.

For example, 25 µg Etanercept, heat-denatured Etanercept, glucagon and heat/acid-denatured glucagon will be administered subcutaneously without adjuvant at day 0 and at day 18. Blood for titer determinations will be drawn from the vena saphena at day -3, day 18 and day 25. Native ß2GPI (15 µg), reduced/alkylated ß2GPI (15 µg) and native ß2GPI (15 µg) with 1.35 µg CL will be administered intravenously at day 0, day 4, day 14 and day 18. The ß2GPI and CL will be premixed and incubated at 400 µg ml^{-1} and 25 µM final concentrations. Blood will be drawn at day -3, day 9, day 25. At first, titers will be determined with ELISA's using plates coated with the native proteins. From our analyses we conclude that ß2GPI with CL, dOVA, alkyl-MSA, heat/acid-denatured glucagon and heat-denatured Etanercept comprise the cross-ß structure conformation. The presence of the cross-ß structure conformation can be further established by circular dichroism

spectropolarimetry analyzes, X-ray fiber diffraction experiments, Fourier transform infrared spectroscopy, Congo red fluorescence/birefringence, tPA binding, factor XII activation and binding, and more.

The present invention discloses that proteins containing cross-ß structure conformation are immunogenic. For a person skilled in the art it is now evident that further evidence can be obtained that support the proposed role for the cross-ß structure conformation in immunogenicity. For example the immunogenicity of proteins, including OVA, ß2GPI and/or protein therapeutics such as interferon α, glucagon or Etanercept can be tested in vivo as described above, but also in vitro. Preferably such experiments are performed with the native state of these proteins and compared with a state in which the cross-ß structure conformation has been introduced. Preferably the cross-ß structure conformation is induced by heating, oxidation, glycation or treatment with an adjuvant, such as CPG-ODN oligodeoxynucleotides, LPS or CL. The content of cross-ß structure conformation is preferably measured by ThT, Congo red, TEM, size exclusion chromatography, tPA-activating activity, and or binding of any other cross-ß structure binding protein listed in Tables 1-3. The immunogenicity of said protein is tested preferably in vitro and in vivo. For a person skilled in the art several in vitro assays are preferable to determine the immunogenicity of said protein in vitro. Preferably, activation of antigen presenting cells (APC), preferably dendritic cells (DC) is tested following treatment with said native or cross-ß structure comprising protein. Preferably, this is performed according to established protocols. Activation of antigen presenting cells can be determined by FACS (Fluorescence Activated Cell Sorter) analysis. Preferably the levels of so-called co-stimulatory molecules, such as B7.1, B7.2, MHC class II, CD40, CD80, CD86 are determined on preferably CD11c positive cells. Alternatively, activation of NF-κB and/or expression of cytokines can be used as indicators of activation of cells involved in immunogenicity, such as APC and DC. Preferably, the following cytokines

should be quantified: TNFα, IL-1, IL-2, IL-6, or IFNγ or other. Preferably, the cytokine levels should be quantified by ELISA. Alternatively, the mRNA levels are quantified. For a person skilled in the art it is evident that function of APC and DC can be tested as well. Preferably the cross-presentation of antigen can be tested. Preferably this can be achieved using OVA, in its native conformation and conformations with cross-β structure conformation, as model protein. The ability of DC or APC to activate MHC class I-restricted or MHC class II-restricted T-cells should be analyzed. For a person skilled in the art this can be done according to established protocols. The role of proteins with cross-β structure conformation in the activation of APC and their role in antigen presentation is further addressed with these aforementioned experimental procedures using cross-β structure binding compounds in competition assays. Preferably DC activation and functional antigen presentation are tested in the presence or absence of ThT, Congo red, tPA, or any other cross-β structure binding protein, including those listed in Table 1-3 or a functional equivalent thereof.

The immunogenicity of proteins with cross-β structure conformation can also be further demonstrated in vivo. For example the induction of antibodies and the induction of cytotoxic T lymphocyte (CTL) activity upon immunization of proteins, including OVA, β2GPI and/or protein therapeutics such as interferon α, glucagons, factor VIII, erythropoietin, thrombopoietin, GH or Etanercept can be tested as described already briefly above. Preferably the immunogenicity of the native state of these proteins is compared with a state in which the cross-β structure conformation has been introduced. Preferably the cross-β structure conformation is induced by heating, oxidation, glycation or treatment with an adjuvant, such as CpG-ODN, LPS or CL. The content of cross-β structure conformation is preferably measured by ThT, Congo Red, TEM, size exclusion chromatography, tPA-activating activity, and or binding of any other cross-β structure binding protein listed in Tables 1-3. Preferably the antibody titers are measured after immunization by ELISA and the CTL

activity is measured using ^{51}Cr-release assay. Alternatively the release of cytokines, including IL-2 can be measured.

Example 6

The following example shows that with proteins or protein fragments with affinity for amyloid-like misfolded proteins, affinity matrices can be constructed that specifically extract misfolded protein from buffer or complex protein solutions, thereby depleting the protein solutions from potentially harmful cytotoxic or immunogenic obsolete molecules. Moreover, the examples demonstrate that in biopharmaceuticals that are currently on the market and that are known for their potential to induce a humoral immune response when administered to human individuals, protein molecules that have amyloid-like protein conformation, can be identified with our technology.

Preparation of misfolded protein affinity matrix

Expression of DNA constructs comprising synthetic genes of human BiP, human fibronectin finger 4,5 (Fn F4,5) fragment, and human tissue-type plasminogen activator finger EGF (tPA F-EGF) fragment.

Synthetic genes of human BiP, human fibronectin finger 4,5 (Fn F4,5) fragment, and human tissue-type plasminogen activator finger EGF (tPA F-EGF) fragment were ordered from Geneart (Regensburg, Germany). These DNA constructs were digested using BamHI and NotI, and ligated into vector pABC674 (ABC-expression facility, Utrecht University, The Netherlands), which contains a carboxy-terminal FLAG-tag – His-tag. HEK293E cells were transiently transfected with these constructs using the polyethylene-imine method, and grown for 5-6 days.

Purification of human BiP, human fibronectin finger 4,5 (Fn F4,5) fragment, and human tissue-type plasminogen activator finger EGF (tPA F-EGF) fragment.

The cells were pelleted by centrifugation and the supernatant was concentrated on a Quixstand concentrator (A/G Technology corp.), using a 30 or 5 kDa cut-off filter (GE Healthcare) for BiP and for Fn F4,5 and tPA F-EGF, respectively. A dialysis step was performed on the same concentrator, and the proteins were dialysed either against PBS+0.85 M NaCl pH 7.4 (BiP), or against 25 mM Tris pH 8.2 + 0.5 M NaCl (Fn F4,5 and tPA F-EGF). The concentrated and dialysed medium was filtered (0.45 µm, Millipore) and incubated with Ni-Sepharose beads (GE-Healthcare, catalogue number 17-5318-02) in the presence of 10-20 mM imidazole, for either 3 h at room temperature or overnight at 4°C under constant motion. A column was packed with the beads and the proteins were extracted by increasing imidazole concentration. The proteins purified in this way had a purity of 80-90%, as established by SDS-PAGE (Invitrogen, NuPage 4-12% BisTris NP0323), using MOPS buffer (Invitrogen NP0001) for BiP or MES buffer (Invitrogen NP0002) otherwise, and Coomassie stain (Fermentas PageBlue R0571).

Determination of affinity of denatured proteins to human BiP, human fibronectin finger 4,5 (Fn F4,5) fragment, and human tissue-type plasminogen activator finger EGF (tPA F-EGF) fragment.

Denatured proteins and their native controls BSA (Sigma, A7906), glycated BSA, Hb (Sigma, H7605), glycated Hb, ovalbumin (Sigma, A6741), heat-denatured misfolded ovalbumin, human γ-globulins (Sigma, G4386), heat-denatured misfolded γ-globulins, alkyl-γ-globulins, lysozyme (ICN Biochemicals, 100831) and alkyl-lysozyme were coated on ELISA plates (Greiner Microlon high-binding, 655092) in 50 mM $NaHCO_3$-buffer pH 9.6. The plates were blocked using Blocking reagent (Roche 1112589). A dilution series of the protein of interest was applied to the coated proteins and wells were

subsequently washed using TBS-T (50 mM Tris pH 7.3, 150 mM NaCl and 0.1% Tween20). Bound protein is detected by the FLAG-tag using 1:3000 anti-FLAG-HRP (Sigma A-8592) in PBS-T, or by 1:1000 Ni-NTA-HRP (Qiagen 34530) in PBS-T. The HRP reaction Is performed using the TMB substrate (Biosource 4501103 or Tebu Bio 101TMB100-500), stopped using 10% H_2SO_4 and absorbance was measured at 450 nm.

Preparation, expression and purification of sRAGE-His

The soluble extra-cellular fragment of human receptor for advanced glycation end-products (sRAGE) was cloned, expressed and purified as follows (Q.-H. Zeng, Prof. P. Gros, Dept. of Crystal- & Structural Chemistry, Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, the Netherlands, and Cor Seinen, Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, the Netherlands). Human cDNA of RAGE was purchased from RZPD (clone IRALp962E1737Q2, RZPD, Berlin, Germany). For PCRs, the gagatctGCTCAAAACATCACAGCCCGG forward primer was used comprising a BglII site, and the gcggccgcCTCGCCTGGTTCGATGATGC reverse primer with a NotI site. The soluble extracellular part of RAGE comprises three domains spanning amino-acid residues 23-325. The PCR product was cloned into a pTT3 vector, containing an amino- or carboxy-terminal His-tag. The sRAGE was expressed in 293E hamster embryonic kidney cells at the ABC-protein expression facility (Utrecht University, Utrecht, The Netherlands). Concentrated cell culture medium was applied to a Hi-trap Chelating HP Ni^{2+}-NTA column (Amersham Biosciences Europe, Roosendaal, The Netherlands). The running buffer was 25 mM Tris-HCl, 500 mM NaCl, pH 8.0. The protein was eluted by using a step gradient of 0 to 500 mM imidazole. Purity of the His-sRAGE was depicted from Coomassie stained SDS-PAGE gels. After concentration, the buffer was exchanged to 20 mM Tris-HCl, 200 mM NaCl, pH 8.0.

Matrix preparation

After His-tag based purification, pooled fractions with construct were dialysed in a 3.5 kDa cut-off membrane (Spectra/Por 132720) against their column running buffer without imidazole. Occasionally occurring precipitates were removed by centrifugation (30 minutes at 16.000*g) or filtration (0.45 µm). Ni-Sepharose beads (GE-Healthcare17-5318-02) were incubated overnight with dialysed protein at 4°C in the presence of 20 mM imidazole. After discarding the protein solution, beads were washed 5x using PBS + 0.1% Tween20 + 20 mM imidazole (PBS-TI).

Depletion experiments

BSA-AGE was diluted in PBS-TI to a concentration of 50 µg/ml. This solution was ultra-centrifuged at 100,000*g for 1 hour at 4°C. The resulting solution had a concentration of approximately 45 µg/ml. This was diluted 10-fold for the fishing experiments (working concentration: 4.5 µg/ml). Fishing experiments were performed in PBS-TI, 256-fold diluted human serum in PBS-TI or 512-fold diluted human plasma in PBS-TI, with or without 4.5 µg/ml BSA-AGE. Forty µl 50% beads suspension in PBS-TI was added to 170 µl solution with or without BSA-AGE and incubated overnight at 4°C under constant motion. Unbound material was extracted and tested for BSA-AGE content in a sandwich ELISA set-up.

BSA-AGE detection by sandwich ELISA

For BSA-AGE sandwich ELISA, anti-AGE monoclonal antibody 4B5 ([1]) was coated to an ELISA plate (Greiner Microlon high-binding, 655092), which was subsequently blocked. Solutions containing BSA-AGE were allowed to bind for 1 h at room temperature. BSA-AGE was detected using polyclonal rabbit anti-HSA (DakoCytomation, A0001; 1:1000 in PBS with 0.1% Tween20 (PBS-T)) followed by SWARPO (DakoCytomation, P0217; 1:4000 in PBS-T). The

peroxidase reaction was performed using 100 µl of OPD in phosphate citrate buffer pH 5, and stopped by 50 µl of 10% H_2SO_4 and measured at 490 nm.

Platelet aggregation assay with protein solutions depleted of amyloid-like protein

I. Coupling of tPA F-biotin to Streptavidin-Sepharose

Total chemically synthesized lyophilized tPA F-biotin (T.Hackeng,University Maastricht) is dissolved at 5 mg/ml in 20 mM HEPES, 137 mM NaCl, 4 mM KCl, pH 7.4; HBS). For preparation of affinity matrix 175 µl Streptavidin-Sepharose (Amersham Biosciences AB, SE-751 84 Uppsala Sweden, 17-5113-01) is washed 10 times with 175 µl 1x HBS. Filter tubes (Millipore Non-Sterile Ultrafree MC 5 µM filter unit, UFC30SV00 Millipore Corporation Bedford MA 01730 USA) are used to wash beads. An Eppendorf table-top centrifuge is used, 30 seconds at 500 rcf. Hundred-twenty µl tPA F-biotin is added to beads depleted from buffer by centrifugtion. Approximately 0.6 mg tPA F-biotin is added to beads. Coupling procedure was according to the guidelines of the manufacturer. Incubation of beads and tPA F-biotin is done under constant motion at room temperature for 1 hour. Beads are subsequently washed 12x. Wash buffer is analyzed for tPA F-biotin content to allow for determination of the coupling efficiency. Beads with bound tPA F-biotin are stored in HBS at 4°C. The coupling procedure is performed in parallel with control beads, omitting the tPA F-biotin. Coupling efficiency is assessed using ELISA. A concentration series of tPA F-biotin is immobilized on the well of a 96-wells plate (Greiner Microlon high-binding). The tPA F-biotin solution after contacting the Streptavidin-Sepharose beads, as well as the wash buffer after washing tPA F-biotin contacted Streptavidin-Sepharose was diluted in coat buffer, accordingly, and also coated. The plate was blocked with Blocking Reagent (cat.no. 37545, Pierce, Perbio Science Nederland B.V., Etten-Leur, The Netherlands). Detection antibody used is Streptavidin-HRP, 1:1000

dilution (cat.no. P0397, Dako, Heverlee, Belgium). TMB substrate (100 µl/well) is used for staining (cat.no. 4501103, 4501401, Biosource, Invitrogen, Breda, The Netherlands) and the stain reaction is stopped with 50 µl of 10% H_2SO_4. Absorbance is measured at 450 nm. Incubations are for 30 minutes at room temperature under constant shaking. Washes (5x times between incubation steps) and dilutions are in PBS with 0.1% Tween20.

II. Depletion of amyloid-like misfolded protein from protein solutions

See the Materials & Methods section of example 6 for the misfolded depletion experiment with tPA F-EGF, fibronectin F4,5, BiP and sRAGE, above. Similar as to those experiments, a spike of 1 µg/ml ultracentrifuged BSA-AGE was added to PBS/0.1% v/v Tween20 or to 512-fold diluted single human donor plasma in PBS/0.1% v/v Tween20. Solutions were added to either control beads or to tPA F-biotin Streptavidin-Sepharose. The solution after incubations was analyzed for the presence of remaining BSA-AGE, in a sandwich ELISA, as described above.

In a next series of experiments, diluted plasma was enriched with a 250 µg/ml BSA-AGE spike and subsequently added to tPA F-biotin – Streptavidin-Sepharose. After contacting 150 µl of the plasma with BSA-AGE spike for 2 hours under constant motion, to 15 µl of the affinity matrix for depletion of misfolded proteins, the supernatant was analyzed for its property to induce platelet activation resulting in their aggregation. Results are compared to platelet activating properties of the spiked plasma before depletion of BSA-AGE.

Freshly drawn human aspirin free blood was mixed gently with citrate buffer to avoid coagulation. Blood was spinned for 15' at 150*g at 20°C and supernatant was collected; platelet rich plasma (PRP) with an adjusted final platelet number of 200,000 platelets/µl. Platelets were kept at 37°C for at least 30', before use in the assays, to ensure that they were in the resting state. For

the aggregometric assays, 270 µl platelet solution was added to a glass tube and prewarmed to 37°C. A stirring magnet was added and rotation was set to 900 rpm, and the apparatus (Whole-blood aggregometer, Chrono-log, Havertown, PA, USA) was blanked. A final volume of 30 µl of tester solution was added, containing the agonist of interest (buffer, control, diluted plasma with BSA-AGE, before and after contacting tPA F-biotin – Streptavidin-Sepharose), prediluted in HEPES-Tyrode buffer pH 7.2. Aggregation was followed in time by measuring the absorbance of the solution, that will decrease in time upon platelet aggregation. As a positive control synthetic thrombin receptor activating peptide TRAP was used. Aggregation was recorded for 15' and expressed as the percentage of the transmitted light (0-100%).

Binding of LRP to misfolded protein.

Cloning and expression of LRP cluster IV.

Cluster IV of the low-density lipoprotein (LDL) receptor-related protein (LRP cl-IV) was cloned from complete cDNA of THP1 cells by PCR using the following forward and reverse primers: GGATCCTCCAACTGCACGGCTAGC (oLRPIVF) and GCGGCCGCGATGCTGCAGTCCTCCTC (oLRPIVR) introducing BamHI and NotI sites (underlined), respectively at the amino- and carboxy-terminus of cluster IV. This PCR fragment was cloned into TOPO TA vector (Invitrogen). The sequence was verified and the construct was subsequently cloned in the pABC-based expression vector 675 (ABC-expression facility, Utrecht University, The Netherlands) using the BamHI and NotI sites. This vector introduces an amino-terminal cystatin signal sequence to the expressed protein of interest enabling secretion into the medium. Furthermore it has a carboxy-terminal FLAG-HIS tag for purification and detection purposes.

Two and a half µg of the obtained construct was transfected into 5 ml HEK293E/S cells, using the polyethylene-imine method, and medium was harvested after one week of cell culturing by centrifugation at maximum speed for 20 seconds (performed by the ABC-expression facility). Presence of expressed LRP cl-IV was verified by analyzing a Western blot after staining with anti-FLAG-tag antibody and chemiluminescent compound. The cell culture supernatant comprising LRP cluster IV protein was used directly without further purification for the ELISA experiments (see below).

Enzyme linked immunosorbent assay for testing of LRP cluster IV binding to misfolded proteins.

Binding of LRP cl-IV to misfolded protein was determined using an enzyme linked immuno sorbent assay (ELISA) set-up. For this purpose 50 µl of a 5 µg/ml solution of BSA, BSA-AGE, Hb or Hb-AGE or coat buffer (for negative control) was coated for 1 h with motion. Proteins were diluted in coat buffer (100 mM $NaHCO_3$ pH 9.6). The BSA and Hb controls were prepared freshly by dissolving proteins at 1 mg/ml in PBS by rolling for 10 minutes on a roller bank at room temperature, 10 minutes incubation at 37°C followed by again 10 minutes incubation at the roller bank. Coat controls were performed with anti-glycated protein antibody 4B5, anti-albumin antibody or anti Hb antibody. After coating the plates were washed twice with PBS/0.1%Tween-20 (v/v) and blocked with 300 µl/well blocking reagent (Roche Diagnostics, Almere, The Netherlands) for 1 h at room temperature with motion. Plates were washed twice and incubated in duplicate with a dilution series of medium containing LRP cl-IV (5, 50 or 500 times diluted cell culture supernatant) in PBS/0.1%Tween-20 (v/v) or buffer control for 1 h at room temperature with motion. After five wash cycles, a HRP conjugated anti FLAG antibody or, for the coat controls, anti-glycated protein antibody, anti-albumin antibody or anti-Hb antibody, was added to the wells (50 µl). The anti-FLAG antibody was diluted 3000 times, the anti-glycated protein antibody, the anti-albumin

antibody and the anti-Hb antibody were diluted 1000 times, all in PBS/0.1%Tween-20 (v/v). After five washes with wash buffer binding of antibody was assessed with a secondary antibody. For the coat controls, RAMPO (3000 times diluted) was used to monitor binding of anti-glycated protein antibody, SWARPO (3000 times diluted) was used to monitor binding of anti-albumin antibody and anti-Hb antibody. No secondary antibody was needed to monitor binding of anti-FLAG antibody since HRP is conjugated to this antibody. After 5 washes with wash buffer, binding of anti-FLAG antibody and secondary antibodies was assessed with 100 µl/well TMB substrate (ready to use from Tebu Bio). The reaction was stopped by adding 50 µl/well of 2 M H_2SO_4 in H_2O. After ~2 minutes absorbance was read at 450 nm.

To test whether amyloid-like crossbeta structure binding compounds tPA, Congo red, Thioflavin T and Thioflavin S interfere with LRP cl-IV binding to BSA-AGE, concentration series of the potential inhibitory amyloid binding moieties were tested in the presence of 50 times diluted medium containing LRP cl-IV. The following inhibitors were used: tPA, Congo red, Thioflavin T (ThT) and Thioflavin S (ThS). As a control to tPA, K2P tPA, which lacks the amyloid-like misfolded protein binding finger domain, was included in the analyses. The influence of tPA and K2P tPA was tested in the presence of 10 mM ε-amino caproic acid to avoid binding of the kringle2 domain of tPA and K2P tPA to lysine- and arginine residues. Binding buffer and K2P tPA served as negative controls in these inhibition studies. The concentration series was measured in triplicate, the values averaged and standard deviations calculated. Background signals obtained with buffer-coated wells were subtracted. Signals obtained with binding of LRP cluster IV to BSA-AGE was set arbitrarily to a reference binding of 100% and signals obtained with the concentration series of misfolded protein binding moieties and K2P tPA were calculated based on this set reference.

Misfolded ß2-Glycoprotein I immunizations

Stock solutions

Stock solution of ß2-Glycoprotein I; 800 µg/ml in 1x Tris Buffered Saline, pH 7.2 (1x TBS)

Cardiolipin vesicles were prepared from a lamellar solution of cardiolipin (Sigma; C-1649) according to a protocol by Subang et al. ([2]). Twohundred µl of cardiolipin was placed into a glass tube and ethanol was evaporated by a constant stream of N_2. The dried cardiolipin was reconstituted in 104 µl of 1x TBS and vortexed thoroughly. The resulting solution contained 10 mg/mL (7.14 mM) of cardiolipin vesicles. This solution could be stored for 14 days at 4 °C, maximally. All dilutions were in TBS and after storage, the solution was vortexed before use.

Modifications: preparation of alkyl-β2gpi

ß2-GPI was reduced and alkylated as follows. Sixhundredforty µl of ß2-GPI stock was mixed with 640 µl of 8 M Urea (cooled solution) in 0.1 M Tris pH=8.2. The solution was degassed with N_2 gas for approximately 6 minutes. From a 1 M DTT stock 12.8 µl was added to the solution, mixed and incubated for 3 hours at room temperature. A 1 M Iodoacetamide (Sigma; I-6125) was prepared, of which 25.6 µl was added to the ß2-GPI reaction mixture. The solution was subsequently dialysed against PBS. Misfolding of the resulting alkyl-ß2gpi was established by the enhancement of Thioflavin T fluorescence and by the increased ability to activate tPA/plasminogen, resulting in plasmin in the chromogenic assay. The chromogenic assay is performed with 400 pM tPA, 20 µg/ml plasminogen. Signals obtained with alkyl-ß2gpi are compared with those obtained with native ß2gpi starting material.

Immunizations of mice with native β2gpi, alkyl-β2gpi and cardiolipin-β2gpi

Female BalB/C cAnNHSd (Harlan) 7-9 weeks were housed in filtertop cages in groups of 5 mice per group. After approximately one week of adjustment to the environment, pre-immune sera were drawn. On the start of the first week, mice were given either 100 µl plasma (150 µg/ml) ß2-Glycoprotein I, 100 µl alkyl- ß2-Glycoprotein I (150 µg/ml) or 100 µl of a mixture of 150 µg/ml of ß2-Glycoprotein I with 9.33 µM cardiolipin (CL-ß2gpi). This latter sample was prepared by pre-incubating 400 µg/ml of ß2-GPI with 25 µM of cardiolipin vesicles for at least 10 minutes at RT after mixing the sample by pipetting; afterwards samples were diluted to 150 µg/ml. The presence of misfolded ß2gpi in the CL-ß2gpi preparation was determined by measuring enhanced Thioflavin T fluorescence and increased potential to stimulate tPA/plasminogen activation. All dilutions were made freshly in TBS and kept on ice. Injections were given intravenously in the tail veins of the mice and given on Mondays and Fridays of the first and third week. Blood was drawn three days prior to the experiment, and on Wednesdays of week 2 and 4 by puncture of vena Saphena. Blood was collected in Easycollect tubes, with Z serum clot activator. Sera were prepared by centrifugation in a tabletop centrifuge, with a rotor diameter of 7 cm, at 3800 rpm for 10 minutes (slow start and stop) and stored at -20 °C before analysis.

Titer determinations

Sera were analyzed for antibodies against unmodified native (coated) ß2-GPI. Microlon high-binding 96-well plates (Greiner, Alphen aan den Rijn, The Netherlands) were coated with 50 µL native ß2-GPI (5 µg/mL in 100 mM $NaHCO_3$, pH 9.6, 0.05% NaN_3) per well for 1 hour. Then the wells were drained and washed twice with 300 µL Phosphate Buffered Saline, 0,1% Tween20 (PBST). After washing, wells were blocked by incubating with 200 µL Blocking Reagent (Roche, Almere, The Netherlands) in PBS for 1 hour. The

wells were drained and washed twice with 300 µL PBST. Antibody titers were determined by adding pooled sera of each experimental group (n=5) in three-fold serial dilutions (starting from 1:30, 50 ul/well) to plates coated with native human ß2gpi. The plates were washed four times with 300 µL PBST. RAMPO, diluted 1:3000 in PBST, was added to the wells and incubated for 1 hour. Plates were drained and washed four times with 300 µL PBST and twice with 300 µL PBS. The plates were stained for approximately 5 minutes using 100 µL/well of TMB substrate (Biosource Europe, Nivelles, Belgium), the reaction was stopped with 50 µL/well of 2 M H_2SO_4 and read at 450 nm on a Spectramax340 microplate reader. The absorbance values were plotted against log dilution. Curves were fitted with a sigmoidal curve (GraphPad Prism version 4.02 for Windows, Graphpad Software, CA, USA). For comparison, the dilution that yielded a residual absorbance after background subtraction of 0.1 was arbitrarily taken as the titer of the various sera.

In a similar ELISA approach, binding of 100-fold diluted sera after immunization with native human ß2gpi, alkyl-ß2gpi, CL-ß2gpi and pre-immune serum to immobilized murine ß2gpi was assessed. In this way, it was determined whether immunizations of mice with human ß2gpi elicits a humoral auto-immune response against murine ß2gpi.

Structural analyses of biopharmaceuticals

tPA binding assay with immobilized biopharmaceuticals in an ELISA

Nunc Immobilizer plates (Nalge Nunc, #436013, Rochester, NY, USA) were coated with 50 µL containing 5 µg/mL of sample protein (unless indicated otherwise) in 100 mM $NaHCO_3$, pH 9.6, 0.05% m/v NaN_3 for 1 hour at room temperature. Plates were washed twice with Tris buffered saline pH 7.2 containing 0.1% Tween20 (TBST) and blocked with PBS containing 1% Tween20 for 1 hour at room temperature. Plates were washed twice with TBST and incubated, in duplicate, with a concentration series of either tPA

(Actilyse, Alteplase; Boehringer-Ingelheim, Alkmaar, The Netherlands) or a truncated form of tPA (Reteplase; Rapilysin, Roche Diagnostics GmbH, Mannheim Germany), lacking the amyloid binding domain, diluted in PBS containing 0.1% Tween 20 (PBST). We found that the finger domain interacts with amyloid-like misfolded proteins (unpublished data). Incubations were performed for 1 hour at room temperature in the presence of 10 mM ε-amino caproic acid (εACA). εACA is a lysine analogue and is used to avoid potential binding of tPA to lysine-containing ligands via its kringle2 domain. Plates were washed five times with TBST and incubated with antibody 374b α-tPA (American Diagnostica, Instrumentation Laboratory, Breda, The Netherlands) diluted 1:1000 in PBST for 1 hour at room temperature. Plates were washed five times with TBST and incubated with peroxidase labeled anti-mouse immunoglobulins (RAMPO; DAKOCytomation, Glostrup, Denmark) diluted 1:3000 in PBST for 30 minutes at room temperature. Plates were washed five times with PBS 0.1% Tween20, and stained with 100 µL/well of tetramethyl-benzidine (TMB) substrate (Biosource Europe, Nivelles, Belgium). The reaction was terminated with 50 µL/well of 2 M H_2SO_4 and substrate conversion was read at 450 nm on a Spectramax340 microplate reader. Curves were fitted with a one-site binding model (GraphPad Prism version 4.02 for Windows, Graphpad Software, CA, USA) from which Kd and Bmax were determined.

tPA/plasminogen activation assay

Exiqon Peptide Immobilizer plates were blocked for 1 hour with PBS, 1% Tween20 and rinsed twice with distilled water. The conversion of the chromogenic substrate S-2251 (Chromogenix, Italy) by plasmin was kinetically measured at 37°C on a Spectramax340 microplate reader at a wavelength of 405 nm. The assay mixture contained 400 pM tPA, 100 µg/mL plasminogen (purified from human plasma) and 415 µM S-2251 in HEPES buffered saline (HBS) pH 7.4. Denatured γ-globulins (100 µg/ml) with amyloid-like structure was used as reference and positive control. Lyophilized γ-globulins (Sigma,

MO, USA) were dissolved in a 1(:)1 volume ratio of 1,1,1,3,3,3-hexafluoro-2-propanol and trifluoro-acetic acid and subsequently dried under air. Dried γ-globulins was dissolved in H_2O to a final concentration of 1 mg/ml and kept at room temperature for at least three days and subsequently stored at -20 °C. Maximal tPA activating capacity was determined from the linear increase seen in each activation curve and expressed as a percentage of the standardized positive control. To confirm tPA dependence of plasmin generation, all samples were assayed for their ability to convert plasminogen into plasmin in absence of tPA.

Analyses of protein therapeutics

Protein therapeutics were obtained from the local hospital pharmacy and analyzed within the expiry limits as stated by the manufacturers. Five µL of the various protein therapeutics were tested for their ability to enhance both ThT and CR fluorescence. tPA activating capacity of the protein therapeutics was determined in 1:10 diluted samples (unless indicated otherwise). tPA binding ELISA's were performed by coating protein therapeutics 1:10 in 100 mM $NaHCO_3$, pH 9.6, 0.05% m/v NaN_3.

Stability testing of biopharmaceuticals

To mimic accelerated stability testing several therapeutics were exposed to denaturing conditions and assayed for amyloid-like properties before and after treatment by tPA activation assay at 100 µg/mL protein and ThT fluorescence enhancement assay at 25 µg/mL protein. For this purpose, 5 mg/mL Glucagon (Glucagen; Novo Nordisk Farma B.V., Alphen aan de Rijn, The Netherlands) was incubated at 37 °C in 0.01 M HCl for 48 hours. One mg/mL Etanercept (Enbrel; Wyeth Pharmaceuticals B.V., Hoofddorp, The Netherlands) in 67 mM sodium phosphate buffer, 100 mM NaCl pH 7.0 was gradually heated from 30 °C to 85 °C over a period of 12 minutes and afterwards cooled to 4 °C for 5 minutes, this treatment was repeated 4 times. Abciximab (Reopro; Centocor

B.V., Leiden, The Netherlands) and Infliximab (Remicade; Schering-Plough B.V., Utrecht, The Netherlands) were incubated at 65 °C for 16 and 72 hours, respectively.

5

Results:

Protein expression and purification.

The proteins tPA F-EGF, Fn F4,5 and BiP were expressed to high final concentrations in the medium of HEK293E cells. Subsequent purification using Ni-Sepharose resin resulted in 80-90% purity, as observed on SDS-PAGE gel. Resulting protein samples were dialysed and tested for their affinity for several misfolded proteins (described below). The proteins were coupled to Ni-Sepharose beads to prepare affinity matrices that were used for misfolded protein depletion ("Fish") experiments.

Binding affinities of BiP, fibronectin F4,5 and tPA F-EGF for misfolded proteins

In a first test, binding of tPA F-EGF, BiP and Fn F4,5 to glycated BSA was analysed (Figure 7A). Next, BiP was tested for its affinity for BSA-AGE or heat-denatured BSA versus BSA, Hb-AGE versus Hb (Figure 7B, C). It was found to bind to BSA-AGE with a high affinity, but not to freshly dissolved BSA or heat-denatured BSA. It also bound Hb-AGE, but not freshly dissolved Hb.

The affinity of fibronectin F4,5 for several misfolded proteins and their native controls was tested in an ELISA setup (Fig. 7D-H). High affinities for AGEs (BSA-AGE and Hb-AGE) were found, whereas the affinity for their native controls was very low. A clear difference in binding affinity for heat-denatured OVA versus freshly dissolved OVA was observed, whereas reduced and alkylated OVA acted as its native control. Amyloid γ-globulins (denatured at 37°C) was able to bind Fn F4,5 with high affinity, whereas freshly dissolved γ-globulins and alkyl-γ-globulins had low affinity for Fn F4,5. Freshly dissolved lysozyme as well as reduced and alkylated lysozyme both showed high affinity for Fn F4,5.

Finally, tPA-F EGF, that was purified using Ni-Sepharose, binding to misfolded protein was tested. In the subsequent dialysis step, most protein precipitated. The remaining soluble protein was tested for its affinity to several misfolded proteins. High binding affinities for BSA-AGE relative to its native control was observed (Fig. 7A). Much lower binding to heat-denatured OVA relative to freshly dissolved OVA was observed (Figure 7I).

Misfolded protein extraction experiments

The purified and dialysed proteins BiP, fibronectin F4,5, tPA F-EGF and sRAGE, all with a carboxy-terminal His-tag were bound to Ni-Sepharose to obtain an affinity matrix for binding of misfolded protein. Samples with or without a 0.5 µg/ml spike of BSA-AGE were incubated with the affinity matrices. Depletion of the solutions from BSA-AGE by the affinity matrix was analysed in an ELISA (Figure 8). BSA-AGE was extracted from three solutions: PBS, 256-fold diluted serum in PBS and 512-fold diluted plasma in PBS, all in the presence of 0.1% Tween20 and 20 mM imidazole.

When comparing residual BSA-AGE content in a solution that was incubated with empty control Ni-Sepharose beads, with BSA-AGE starting solution, the control beads did not bind BSA-AGE (Figure 8). Incubation of BSA-AGE in PBS, diluted serum or diluted plasma with either of the four affinity matrices revealed that fibronectin F4,5 and sRAGE (Figure 8) were more efficient misfolded protein binding moieties for depletion of the solutions from BSA-AGE than BiP and tPA F-EGF. Both Fn F4,5-Ni Sepharose and sRAGE-Ni Sepharose beads extracted the 0.5 µg/ml BSA-AGE almost completely from the solution.

These results show that proteins and protein domains that are natural misfolded protein binding moieties and suitable for being implemented in misfolded protein depletion/isolation technology. Based on the requirements, the misfolded protein binding moieties are immobilized on a suitable solid

support of choice. Based on the application, binding conditions are adjusted. Based on the misfolded protein ligand that has to be depleted, the misfolded protein binding moiety are chosen and refined. For example, when depletion of a biopharmaceutical from misfolded constituents including misfolded biopharmaceutical itself, is required, binding conditions are driven by the excipients combination of the biopharmaceutical. Adjustable parameters are still the type or combination of types of misfolded protein binding moieties, the incubation time, the incubation technique (batch wise, (linear/circulating) flow), temperature, type of support with the binding moiety etcetera.

I. Coupling of tPA F-biotin to Streptavidin-Sepharose

To analyze whether tPA F-biotin is coupled to Streptavidin-Sepharose beads, solution after coupling and wash buffer was analyzed for the presence of tPA F-biotin in a direct ELISA with coated dilution series of solutions with tPA F-biotin and a tPA F-biotin standard. A representative curve for the dilution series of tPA F-biotin before and after contacting Streptavidin-Sepharose is shown in Figure 9A. The ELISA analysis of the tPA F-biotin coupling efficiency revealed that approximately 44% of the tPA F-biotin is coupled to Streptavidin-Sepharose. This has resulted in a tPA F-biotin density of approximately 1.5 µg/µl beads. Coupling was also verified by analyzing beads on Western blot (not shown). When comparing with a standard tPA F-biotin dilution series, it is concluded that indeed approximately 0.25-1.25 µg F-biotin is coupled per µl beads.

II. Depletion of buffer or plasma from misfolded protein upon contacting with tPA F-biotin – Streptavidin-Sepharose

Similarly to the experiments with BiP – Ni-Sepharose, tPA F-EGF – Ni-Sepharose, fibronectin F4,5 – Ni-Sepharose and sRAGE – Ni-Sepharose, diluted plasma and buffer was spiked with 1 µg/ml BSA-AGE and contacted to tPA F-biotin – Streptavidin-Sepharose, and the supernatant was subsequently analyzed for the remaining fraction of BSA-AGE. The control was unspiked buffer or plasma, and Streptavidin-Sepharose without misfolded protein affinity ligand. Figure 9B shows the results of a sandwich ELISA for detection of BSA-AGE in solution. It can be clearly seen that upon contacting buffer or diluted plasma with BSA-AGE spike, most of the BSA-AGE is specifically extracted from the solutions, when compared to starting solutions. Control beads do not exert any effect on the amount of BSA-AGE in solution.

In a next experiment, 512-fold diluted human single donor plasma was spiked with 250 µg/ml BSA-AGE and platelet activating properties of a tenfold diluted solution was analyzed (Figure 9C). Platelets readily aggregate upon contacting the misfolded protein. The diluted plasma with BSA-AGE spike was also contacted to tPA F-biotin – Streptavidin-Sepharose, which is an affinity matrix for misfolded proteins. After incubation for 2 hours, supernatant was analyzed for platelet activating potential. As seen in Figure 9C most of the platelet activating potential has been efficiently removed by the tPA F-biotin – Streptavidin-Sepharose. By removal of BSA-AGE from plasma, the pro-thrombotic activity of the solution comprising the amyloid-like misfolded protein is strongly reduced. This shows that removal of misfolded protein from solution is beneficial with respect todverse effects on cells. With the current parameters used, it is now possible to refine the depletion technology towards the required conditions for a specific application. Furthermore, depletion of plasma from misfolded proteins can be optimized by adjusting parameters like

for instance incubation buffer, time, temperature, affinity ligand, solid support/type of matrix, and more.

RESULTS: binding of LRP cluster IV to misfolded protein

Human extracellular LRP fragment cluster IV was successfully cloned from THP-1 cell DNA, and subsequently expressed in HEK 293E cells. On a Western blot, protein with the expected molecular weight was detected upon incubation of the nitrocellulose blot membrane with anti-FLAG-tag antibody (not shown).

To analyze the property of the expressed LRP cluster –IV-FLAG protein to bind to misfolded protein, binding was assessed using an ELISA set-up with coated misfolded glycated albumin and haemoglobin, and their freshly dissolved lyophilized non-glycated counterparts. As can be seen in Figure 10A, LRP cl-IV binds specifically to BSA-AGE as well as to Hb-AGE, and not to the freshly dissolved BSA and Hb.

Now that specific binding of LRP cluster IV to amyloid-like BSA-AGE was established, we wondered whether known amyloid-binding moieties tPA, ThT, ThS and Congo red influence the binding. This would further show the involvement of the amyloid-like misfolded protein conformation in binding of LRP or in inducing the LRP binding site. As can be seen in figure 10B, C, tPA, K2P tPA and ThT at the assay conditions and concentrations tested do not interfere with binding of LRP cl-IV to BSA-AGE. Congo red and ThS, however do inhibit binding of LRP cl-IV to BSA-AGE to a large extent (Figure 10D, E). This shows that amyloid-binding dyes Congo red and ThS bind to, or close to the binding site of LRP for misfolded proteins. Apparently, tPA and ThT may bind to a different feature of the misfolded BSA-AGE. This makes LRP to a valuable tool for incorporation in development programs of technology for depletion of misfolded protein from solution. Depending on the application and

the targeted misfolded protein(s), LRP is a preferred misfolded protein binding moiety, next to, as alternative for, or in combination with other identified moieties with affinity for amyloid-like misfolded proteins.

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Results after immunization of mice with misfolded β2gpi.

Exposure of human native ß2gpi to cardiolipin, or alkylation of cysteine residues in ß2gpi induces amyloid-like protein conformation (Figure 11A, B). Immunization of mice, that received four injections of 15 µg antigen, revealed that alkyl-ß2gpi and CL-ß2gpi elicited far higher humoral immune responses than native ß2gpi (Figure 11C, D). This shows that misfolding of ß2gpi accompanied by the appearance of amyloid-like characteristics, renders it with higher immunogenic potential. When antibody titers against mouse self-ß2gpi were assessed after immunizations with native human ß2gpi, alkyl-ß2gpi and CL-ß2gpi, it was clearly seen that apart from increased titers of antibodies that bind to human native ß2gpi, also auto-immune antibody titers against the mouse ß2gpi were increased when amyloid-like structure is present in human ß2gpi (Figure 11E). This shows further evidence for the insight that amyloid-like properties of proteins are a trigger for clearance and defense mechanisms within the Crossbeta Pathway for clearance of obsolete proteins. Furthermore, it is shown that tolerance is not a decisive aspect for whether a humoral immune response will occur or not. It is the amyloid-like nature of the antigen that determines whether the moiety is considered dangerous to the individual or not, and thus whether invasion of the individual with the amyloid-like moiety should be adequately conquered. Whether the underlying amino-acid sequence is of self-origin or is of non-self origin is not a primary parameter. Therefore, when a self-protein excessively adopts the amyloid-like protein conformation, auto-immunity occurs by the fact that clearance of the misfolded protein in order to free the body from toxic protein moieties is of utmost importance.

Amyloid-like protein conformation in biopharmaceuticals

Over the past decades, the use of therapeutic proteins has become common practice in medicine and as their use is very promising, many more biopharmaceuticals are under development. Unfortunately, a major drawback of protein therapeutics is the risk of antibody formation. These immunogenicity problems are for instance of concern regarding therapeutic efficacy and patient safety. For example, drug-induced neutralizing antibodies to erythropoietin (EPO) result in pure red cell aplasia (PRCA), whereas drug-induced acquired anti-factor VIII (fVIII) antibodies worsen the pathology associated with hemophilia. As more and more recombinant therapeutic proteins become licensed for marketing, the incidence of immunogenicity problems is expected to rise.

Protein misfolding is an intrinsic and problematic property of proteins, Protein misfolding is accelerated by a number of environmental factors, including protein modifications such as glycation, deamidation or oxidation, interaction of proteins with surfaces, such as for instance mica or negatively charged phospholipids or other conditions, such as heating, lyophilization, sonication, packaging materials.

We now show that misfolding of therapeutic proteins also leads to the formation of amyloid-like properties and that this underlies the triggering of antibody formation.

We first examined whether proteins with amyloid-like properties are present in marketed biopharmaceuticals. As indicators for amyloid-like properties we measured the fluorescence of Thioflavin T (ThT), Congo Red and binding and activation of tissue-type plasminogen activator (tPA), all qualitative measures for the presence of amyloid-like misfolded protein conformation in proteins in solution. As shown in Table 4, several

biopharmaceuticals showed significant potential to enhance fluorescence of Thioflavin T and/or Congo Red, indicating the presence of amyloid-like structure. These biopharmaceuticals also bound to tPA with high affinity and activated tPA-mediated plasminogen activation (Table 4). These findings demonstrate that amyloid-like properties are present in various marketed therapeutic proteins.

Table 4
The presence of protein with amyloid-like properties in various biopharmaceuticals.

	Fluorescence (a.u. +/- SD)			tPA Binding	tPA activation
Therapeutic protein	ThT	CR	Bmax (OD_{450nm})	Kd (nM)	Max. Activation (%)
Albumin *	1970 +/- 5	978 +/- 2	1.228	11.22	47.67
Somatropin	1317 +/- 10	429 +/- 2	0.9369	9.048	113.95
Insulin Zn Suspension	387 +/- 72	79 +/- 6	0.7558	105.4	17.44
Insulin Aspart	172 +/- 3	81 +/- 2	3.617	694.7	70.93
Factor VIII *	306 +/- 12	290 +/- 6	0.5398	229.8	4.22
Abciximab	8 +/- 8	25 +/- 1	0.5329	216.3	0
Epoietin Alfa	14 +/- 2	19 +/- 3	ND	ND	0
Etanercept	23 +/- 3	ND	ND	ND	0
Infliximab	19 +/- 1	67 +/- 2	ND	ND	0
γ-Globulins *	25 +/- 2	0 +/- 1	ND	ND	ND
Glucagon	48 +/- 1	ND	ND	ND	11.25

Content of protein with amyloid-like properties in biopharmaceuticals was determined by enhancement of Thioflavin T (ThT) and Congo Red (CR) fluorescence, binding of tissue-type plasminogen activator (tPA) and tPA-dependent plasminogen activation (% of standardized positive control). Biopharmaceuticals containing the highest levels of cross-ß structure are listed at the top. (* plasma purified drug products)

Most protein pharmaceuticals can be stored for prolonged periods of time without losing their bioactivity. However, some fraction of proteins gradually looses its structure and degrade. We examined the effect of storage on the level of protein with amyloid-like structure in a number of biopharmaceuticals. Figure 12 shows that the level of protein with amyloid-like properties increases when the biopharmaceuticals were examined closer to their expiration date.

During manufacturing and storage, biopharmaceuticals also become exposed to various conditions of stress that underlie the formation of amyloid-like properties. To artificially mimic stability testing we examined whether exposure of biopharmaceuticals to conditions of severe stress, such as low pH

and heat, induced amyloid-like properties. Figure 13 shows that amyloid-like properties are adopted by Etanercept, Glucagon, Abciximab, and Infliximab upon exposure to these harsh denaturing conditions. Thus, like any protein, biopharmaceuticals adopt similar amyloid-like properties and this is enhanced upon storage or under conditions of stress.

The advent of recombinant technology has enabled the large scale production of biopharmaceuticals, such as fVIII, EPO, IFN and various monoclonal antibodies. The use of these biopharmaceuticals is very promising and the number of biopharmaceuticals is expected to rise rapidly. Unfortunately, the generation of antibodies against therapeutic proteins has posed a mystifying problem for biopharmaceutical manufacturers, medical practitioners and scientists. Taken together, we show that adoption of generic amyloid-like properties, a hallmark of misfolded proteins is the basis of drug-induced immunogenicity.

Our data show that proteins with amyloid-like properties are responsible for enhanced immunogenicity of biopharmaceuticals and breaking of tolerance. We disclose a unifying mechanism by which individual immunogenic factors, such as oxidation or formulation changes, result in adoption of amyloid-like properties, ultimately leading to immune responses.

The innate immune system is activated by recognition of these amyloid-properties. Indeed, several cellular receptors for the amyloid-like protein fold have been identified: scavenger receptor A (SR-A), CD36, receptor for advanced glycation end products (RAGE), low density lipoprotein receptor like protein (LRP) and scavenger receptor B type I (SR-BI)[1]. Moreover, these receptors are expressed on dendritic cells and are able to initiate an immune response against amyloid-like proteins.

LEGENDS TO THE FIGURES

Figure 1. Binding of polypeptides with cross-β structure conformation to tPA, sRAGE and Fn type I domains, studied with Biacore surface plasmon resonance.

A. TPA activation assay showing that 10' centrifugation at 16,000*g of Hb-AGE and amyloid γ-globulins hardly influences the tPA activating properties of the supernatant when compared to uncentrifuged amyloid stocks. Also protein therapeutic endostatin is tested for tPA activating properties. Concentrations of potential activators were 100 µg ml^{-1}. **B.** Binding of 32 µg ml^{-1} Hb-AGE to tPA and sRAGE in a Biacore surface plasmon resnonance experiment. **C.** On the same chip relatively strong binding of 62.5 µg ml^{-1} to tPA and sRAGE is observed. **D.** More albumin-AGE, injected at 3.9 µg ml^{-1}, binds to tPA than to sRAGE. **E.** By testing a concentration series of Hb-AGE for binding to a Biacore CM5 chip with immobilized Fn F4-5, it is deduced that half maximum binding is obtained with 8 nM Hb-AGE (indicated with the arrow). **F.** As a control, 25 nM native Hb was tested for binding to a Biacore chip with immobilized Fn F4-5, HGFA F and tPA F. **G.** By testing a concentration series of endostatin it is revealed that half maximum binding to Fn F4-5 is obtained with 800 nM endostatin (arrow). **H.** Half maximum binding of recombinant ß2GPI to immobilized Fn F4-5 is obtained with 165 nM ß2GPI (arrow).

Figure 2. Activation of factor XII by protein aggregates with cross-β structure conformation.

A. Like kaolin, amyloid-like peptide aggregates of FP13 and Aß stimulate the activation of factor XII, as detected by the conversion of Chromozym PK, upon formation of kallikrein from prekallikrein by activated factor XII. Buffer control and non-amyloid controls FP10 and mIAPP do not activate factor XII. **B.** Like FP13 and Aß, also cross-ß structure conformation rich peptides LAM12

and TTR11 stimulate factor XII activation, to a similar extent as kaolin. **C.** In the chromogenic factor XII/kallikrein activity assay, the stimulatory activity of 150 µg ml^{-1} kaolin is strongly dependent on the presence of 1 mg ml^{-1} albumin in the assay buffer. albumin alone also shows to some extent factor XII/prekallikrein activating properties, likely due to the presence of amyloid-like aggregates in the albumin solution after dissolving it from a lyophilized stock. **D.** similar effects are seen with albumin and DXS500k. **E.** Like albumin endostatin is a requirement for kaolin-induced factor XII activation. **F.** With DXS500k and endostatin, similar effects are seen in the factor XII activation assay as with albumin and DXS500k. **G.** Contacting plasma, lysozyme and γ-globulins to DXS500k results in activation of tPA and Plg, as measured in the chromogenic tPA/Plg activation assay. DXS500k alone also results in some activation. Plasma, lysozyme or γ-globulins controls do not activate tPA and Plg. **H.** Overnight incubation at room temp. of plasma with kaolin or DXS500k results in increased fluorescence of amyloid dye ThT, when compared to incubation with buffer. **I.** Incubation of γ-globulins with kaolin or DXS500k also induces increased ThT fluorescence. **J.** Only DXS500k induces ThT fluorescence with lysozyme. Kaolin incubation results only in a small increase in ThT fluorescence, when compared to buffer. **K-N.** In an ELISA set-up tPA binds specifically to plasma proteins (**K**), γ-globulins (**L**), lysozyme (**M**) and factor XII (**M**) that were pre-incubated overnight with DXS500k, whereas tPA does not bind to buffer-incubated proteins. K2P tPA that lacks the F domain does not bind to surface-contacted proteins. **O.** In the tPA ELISA Hb-AGE with amyloid-like properties was used as a positive control for tPA binding. **P.** Auto-activation of factor XII is established by incubating purified factor XII with DXS500k or with various amyloid-like protein aggregates with cross-ß structure conformation, in the presence of chromogenic substrate S-2222.

Figure 3. Presence of amyloid cross-β structure conformation in commercially available formulated protein medicine.

A-D. With protein therapeutics stored at the recommended temperature of 4°C, influence on Congo red- (**A.**) and ThT fluorescence (**B.**) was established as well as the ability to activate tPA (**C.**) and factor XII (**D.**), as determined with chromogenic assays which record Pls and kallikrein activity, that is established upon activation of Plg by tPA and prekallikrein by factor XII, respectively. Gelatin, Cealb and FVIII clearly enhance Congo red fluorescence. Cealb, GH and FVIII enhance ThT fluorescence. GH and insulin potentiate Plm activity. Amyloid γ-globulins at 100 μg ml^{-1} was used as a positive control. Zinc-insulin and insulin activate factor XII. Kaolin at 150 μg ml^{-1} was used as a positive control. **E.** Both modified gelatin for infusion stored at 4°C and at 37°C show enhanced Congo red fluorescence comparable to the positive control, 25 μg ml^{-1} Aß. **F.** Only modified gelatin for infusion that was stored at 37°C, and not gelatin stored at 4°C, exhibits factor XII stimulatory activity, as measured in a chromogenic kallikrein activity assay. The positive control for factor XII mediated prekallikrein activation was 150 μg ml^{-1} kaolin. **G.** tPA ELISA showing the binding of tPA to immobilized protein therapeutics zinc-insulin, an antibody, FVIII and Cealb. Positive control in the ELISA was Hb-AGE, that is not shown for clarity. **H.** tPA ELISA showing the binding of tPA to immobilized formulated Cealb and GH. K_D's are 23 nM for Cealb and 72 nM for GH. **I.** TEM image of modified gelatin showing various relatively condense aggregates. The scalebar is 1 μm. **J.** TEM image of GH showing a linear, a branched and a condense particle all apparently composed of spherical particles. The scale bar is 100 nm. **K.** TEM image of zinc-insulin showing the appearance of insulin as thin unbranched fibrils with varying length. The scale bar represents 100 nm. **L.** TEM image of protein therapeutic Cealb, stored at 4°C. Scale bar: 100 nm **M.** TEM image of Novo Rapid insulin, stored at 4°C. Scale bare: 100 nm. **N.** Influence of storage temperature on ThT fluorescence enhancement by protein therapeutic Reopro. **O.** TPA activating properties are

largely dependent on the storage temperature of Reopro, as assessed in a tPA activation assay. **P.** TEM image of ReoPro anticoagulant, stored at 4°C. Scale bar: 1 µm.

Figure 4: Synthesis of TNFα RNA in monocytes after stimulation with cross-β structure conformation rich compounds and LPS, which acts as a denaturant.

A. Cultured U937 monocytes were incubated for 1 h with buffer, LPS, amyloid endostatin, amyloid Hb-AGE or native Hb. Upregulation of TNFα RNA was assessed by performing RT-PCR with RNA isolated form the monocytes and TNFα primers. Amounts of TNFα cDNA after RT-PCR were normalized for the amounts of ribosomal 18S cDNA, obtained with the same RNA samples. In monocytes incubated with buffer no TNFα RNA is detected. Endostatin and Hb-AGE induce approximately 30% of the TNFα RNA expression, when compared to LPS, whereas the TNFα RNA expression induced by native Hb is approximately threefold lower. **B.** Exposure of 1 mg ml^{-1} lysozyme to 0-1200 µg ml^{-1} LPS results in a 1.1 up to a 13.1 fold increase of ThT fluorescence with respect to lysozyme incubated with buffer only, indicative for the denaturing capacity of LPS, resulting in amyloid-like structures in lysozyme. Standard deviations were typically less than 10% (not shown). **C.** Exposure of 1 mg ml^{-1} lysozyme, albumin, endostatin, γ-globulins, plasma ß2GPI or rec. ß2GPI to 600 µg ml^{-1} LPS results in increased ThT fluorescence with approximately a factor 2 to 10. **D-F.** Exposure of 1 mg ml^{-1} lysozyme (**D**) or endostatin (**E**) to indicated concentration series of LPS or CPG-ODN induces an enhanced ThT fluorescence signal. **F.** Exposure of 1 mg ml^{-1} albumin, endostatin, plasma ß2GPI or rec. ß2GPI to 21.4 µg ml^{-1} CPG-ODN results in increased ThT fluorescence with approximately a factor 2 to 10. With these assay conditions no effect is seen with lysozyme and γ-globulins.

Figure 5: Binding of factor XII and tPA to β_2-glycoprotein I and binding of anti-β_2GPI auto-antibodies to recombinant β_2GPI.

A. Chromogenic Plg-activation assay showing the stimulatory activity of recombinant β_2GPI on the tPA-mediated conversion of Plg to Pls. The positive control was amyloid fibrin peptide FP13. **B.** In an ELISA, recombinant β_2GPI binds to immobilized tPA, whereas β_2GPI purified from plasma does not bind. The k_D is 2.3 $\mu g\, ml^{-1}$ (51 nM). **C.** In an ELISA, factor XII binds to purified recombinant human β_2GPI, and not to β_2GPI that is purified from human plasma, when purified factor XII is immobilized onto ELISA plate wells. Recombinant β_2GPI binds with a k_D of 0.9 $\mu g\ ml^{-1}$ (20 nM) to immobilized factor XII. **D.** Western blot incubated with anti-human factor XII antibody. The β_2GPI was purified either from fresh human plasma or from plasma that was frozen at -20°C and subsequently thawed before purification on a β_2GPI affinity column. Eluted fractions are analyzed on Western blot after SDS-PA electrophoresis. When comparing lanes 2-3 with 4-5, it is shown that freezing-thawing of plasma results in co-purification of factor XII together with the β_2GPI. The molecular mass of factor XII is 80 kDa. **E.** In an ELISA recombinant β_2GPI efficiently inhibits binding of anti-β_2GPI auto-antibodies to immobilized β_2GPI, whereas plasma β_2GPI has a minor effect on antibody binding. Anti-β_2GPI auto-antibodies were purified from plasma of patients with the auto-immune disease Anti-phospholipid syndrome. **F.** Exposure of 25 $\mu g\ ml^{-1}$ β_2GPI, recombinantly produced (rβ_2GPI) or purified from plasma (nβ_2GPI), to 100 μM CL vesicles or to 250 $\mu g\ ml^{-1}$ dextran sulphate 500,000 Da (DXS) induces an increased fluorescence of ThT, suggestive for an increase in the amount of cross-β structure in solution. Signals are corrected for background fluorescence of CL, DXS, ThT and buffer. **G.** Binding of tPA and K2P tPA to β_2GPI immobilized on the wells of an ELISA plate, or to β_2GPI bound to immobilized CL is assessed. B_2GPI contacted to CL binds tPA to a higher extent than β_2GPI contacted to the ELISA plate directly. K2P tPA does not bind to β_2GPI. TPA does not bind to immobilized CL. **H.** Transmission

electron microscopy images of 400 µg ml^{-1} purified plasma ß2GPI alone (1) or contacted with 100 µM CL (2, 3) and of 400 µg ml^{-1} purified recombinant ß2GPI (4).

Figure 6. Amyloid-like cross-β structure conformation in alkylated murine serum albumin and in heat-denatured ovalbumin, murine serum albumin, human glucagon and Etanercept and immogenicity of ovalbumin.

A. Plg-activation assay with Pls activity read-out using chromogenic substrate S-2251. Activating properties of reduced and alkylated MSA (alkyl-MSA) and heat-denatured OVA (dOVA) are compared with amyloid γ-globulins (positive control), buffer (negative control), and native MSA (nMSA) and OVA (nMSA, nOVA). **B.** ThT fluorescence assay with native and denatured MSA and OVA. **C.** TPA activation assay for comparison of reduced and alkylated MSA and heat-denatured MSA. **D.** ThT fluorescence assay with reduced/alkylated MSA and heat-denatured MSA. **E.** tPA activation assay with concentration series of heat/acid denatured glucagon. **F.** ThT fluorescence assay with native and heat/acid denatured glucagon. **G.** Comparison of the tPA activating properties of heat-denatured Etanercept, native Etanercept and reduced/alkylated Etanercept. **H.** ThT fluorescence of native and heat-denatured Etanercept. **I.** TEM image of dOVA. The scale bar represents 200 nm. **J.** TEM image of heat/acid-denatured glucagon. The scale bar represents 1 µM. **K.** ThT fluorescence assay showing that filtration through a 0.2 µm filter of denatured OVA does not influence the fluorescence enhancing properties. **L.** Titer determination of anti-nOVA antibodies in pooled sera of mice immunized with nOVA or dOVA. Titer is defined as the sera dilution that still gives a signal above the background value obtained with 10 times diluted pre-immune serum.

Figure 7. Binding of tPA F-EGF, fibronectin F4,5 and BiP to misfolded proteins.

A. Binding of BiP, fibronectin F4-5 and tPA F-EGF to BSA-AGE, as observed by ELISA, detected using Ni-NTA-HRP. The finger domains show high affinity binding, whereas BiP shows low affinity for BSA-AGE in this set-up. **B-C.** Affinity of BiP for misfolded proteins tested in an ELISA (detection anti-FLAG-HRP). BiP has a high affinity AGEs (BSA-AGE (**B.**) and Hb-AGE (**C.**)), but not for their freshly dissolved controls. **D.-H.** Binding of fibronectin F4,5 (Fn F4,5) to several (mis)folded proteins as observed in an ELISA set-up (detection anti-FLAG HRP). Fn F4,5 binds to most misfolded proteins with higher (AGEs, **D.**, **E.**) or lower (heat-denatured OVA (**F.**) or denatured γ-globulins (**G.**)) affinity, without recognising their native controls. **H.** Fn F4,5 recognises both native and alkyl-lysozyme with medium affinity. **I.** Binding of tPA-F EGF to heat-denatured and native OVA, as tested in an ELISA setup.

Figure 8: Extraction with misfolded protein affinity matrices of BSA-AGE from solution.

BSA-AGE at 0.5 µg/ml in PBS, 256-fold diluted serum in PBS and 512-fold diluted plasma in PBS, all in the presence of 0.1% Tween20 and 20 mM imidazole, was incubated with empty control Ni-Sepharose beads or indicated misfolded protein binding moieties tPA F-EGF, BiP, sRAGE and Fn F4,5, all bound to Ni-Sepharose. The content of BSA-AGE before and after the incubation was assessed by applying the solutions in a sandwich assay with anti-AGE antibody and anti-albumin antibody. Background signals when using PBS, serum or plasma without the BSA-AGE spike were subtracted from the depicted signals. **A.** Depletion of PBS from BSA-AGE. **B.** Depletion of diluted serum from BSA-AGE. **C.** Depletion of diluted plasma from BSA-AGE.

Figure 9. Effect of depletion of a solution from misfolded protein on activation of platelets.

A. Representative standard curve of tPA F-biotin in a direct ELISA for detection of tPA F-biotin in solution. Shown is the tPA F-biotin supernatant before and after contacting to Streptavidin-Sepharose beads for coupling purposes. **B.** Contacting buffer or diluted plasma with a 1 µg/ml BSA-AGE spike with tPA F-biotin – Streptavidin-Sepharose results in depletion of the solutions from BSA-AGE, as determined in a sandwich ELISA using coated anti-AGE antibody and anti-albumin detecting antibody. **C.** Platelet aggregation is induced by 512-fold diluted plasma with 250 µg/ml BSA-AGE spike. After contacting the diluted plasma with BSA-AGE with tPA F-biotin – Streptavidin-Sepharose, platelet aggregating properties is strongly reduced.

Figure 10. Binding of recombinant human extracellular cluster IV fragment of low density lipoprotein receptor related protein to misfolded amyloid-like glycated protein.

A. LRP cluster IV binds specifically and in a dose-dependent manner to immobilized amyloid-like misfolded glycated albumin and glycated haemoglobin. **B-E.** ELISA showing the influence of tPA and K2P tPA (**B.**), ThT (**C.**), Congo red (**D.**) anf ThS (**E.**) on binding of LRP cl-IV to immobilized amyloid-like misfolded BSA-AGE.

Figure 11. Misfolded amyloid-like β2-glycoprotein I elicits a humoral auto-immune response in mice.

A. Generation of plasmin from tPA/plasminogen is accelerated when ß2gpi is exposed to cardiolipin (CL-ß2gpi), which results in amyloid-like properties in ß2gpi. **B.** Alkylation of cysteine residues in ß2gpi induces amyloid-like protein conformation, as shown by enhanced Thioflavin T fluorescence. **C.** Immunization of five mice with cardiolipin-ß2gpi induces a 25-fold higher antibody titer then when mice are immunized with native human ß2gpi. **D.**

Alkylation of β2gpi results in a more immunogenic moiety when compared to the immune response induced in mice by native human β2gpi. **E.** Immunization of mice with amyloid-like misfolded human β2gpi (alkyl-β2gpi, CL-β2gpi) induces higher titers against mouse self-β2gpi, showing an auto-immune response when immunized with foreign amyloid-like β2gpi.

Figure 12. Amyloid-like properties of protein therapeutics increase during storage within expiry limits, under conditions as defined by manufacturer information.

Biopharmaceutical preparations were tested (at 25 μg/ml protein) twice over several months for their capacity to enhance ThT and Congo red fluorescence. Samples were measured in triplicate at each time point.

Figure 13. Various biopharmaceuticals adopt amyloid-like properties after exposure to conditions of stress.

Etanercept, Glucagon, Abciximab and Infliximab were exposed to denaturing conditions (see materials & methods) and subsequently analyzed for the presence amyloid-like properties, using ThT-fluorescence (**A.**) and tPA activation assay (**B.**; expressed as percentage of standardized positive control). N = native, D = denatured.

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Claims

1. A method for detecting a protein and/or peptide comprising a cross-β structure in a pharmaceutical composition or any of its constituents comprising a protein, said method comprising:
   a. contacting said pharmaceutical composition or any of its constituents comprising a protein with at least one cross-β structure-binding compound resulting in a bound protein and/or peptide comprising a cross-β structure,
   b. detecting whether bound protein and/or peptide comprising a cross-β structure are present in said pharmaceutical composition or any of its constituents comprising a protein.
2. A method according to claim 1, wherein said cross-β structure-binding compound is a compound according to Table 1, or Table 2, or Table 3 or a functional equivalent of any of said compounds.
3. A method for controlling a manufacturing process, and/or storage process of a pharmaceutical composition or any of its constituents comprising a protein, said method comprising:
   a. contacting said pharmaceutical composition or any of its constituents comprising a protein with at least one cross-β structure-binding compound resulting in a bound protein and/or peptide comprising a cross-β structure,
   b. detecting whether bound protein and/or peptide comprising a cross-β structure is present in said pharmaceutical composition or any of its constituents comprising a protein at various stages of said manufacturing and/or storage process.
4. A method for removing a protein and/or peptide comprising a cross-β structure from a pharmaceutical composition or any of its constituents comprising a protein, said method comprising:

a. contacting said pharmaceutical composition or any of its constituents comprising a protein with at least one cross-β structure-binding compound,

b. allowing binding of said protein and/or peptide comprising across-β structure to said cross-β structure-binding compound, and,

c. separating said bound protein and/or peptide comprising a cross-β structure from said pharmaceutical composition or any of its constituents comprising a protein.

5. A method according to claim 4, wherein said cross-β structure-binding compound is a compound according to Table 1, or Table 2, or Table 3 or a functional equivalent of any of said compounds.

6. A method according to claim 4 or 5, wherein said cross-β binding compound is bound to a second compound.

7. A method according to claim 6, wherein said second compound is bound to a solid phase.

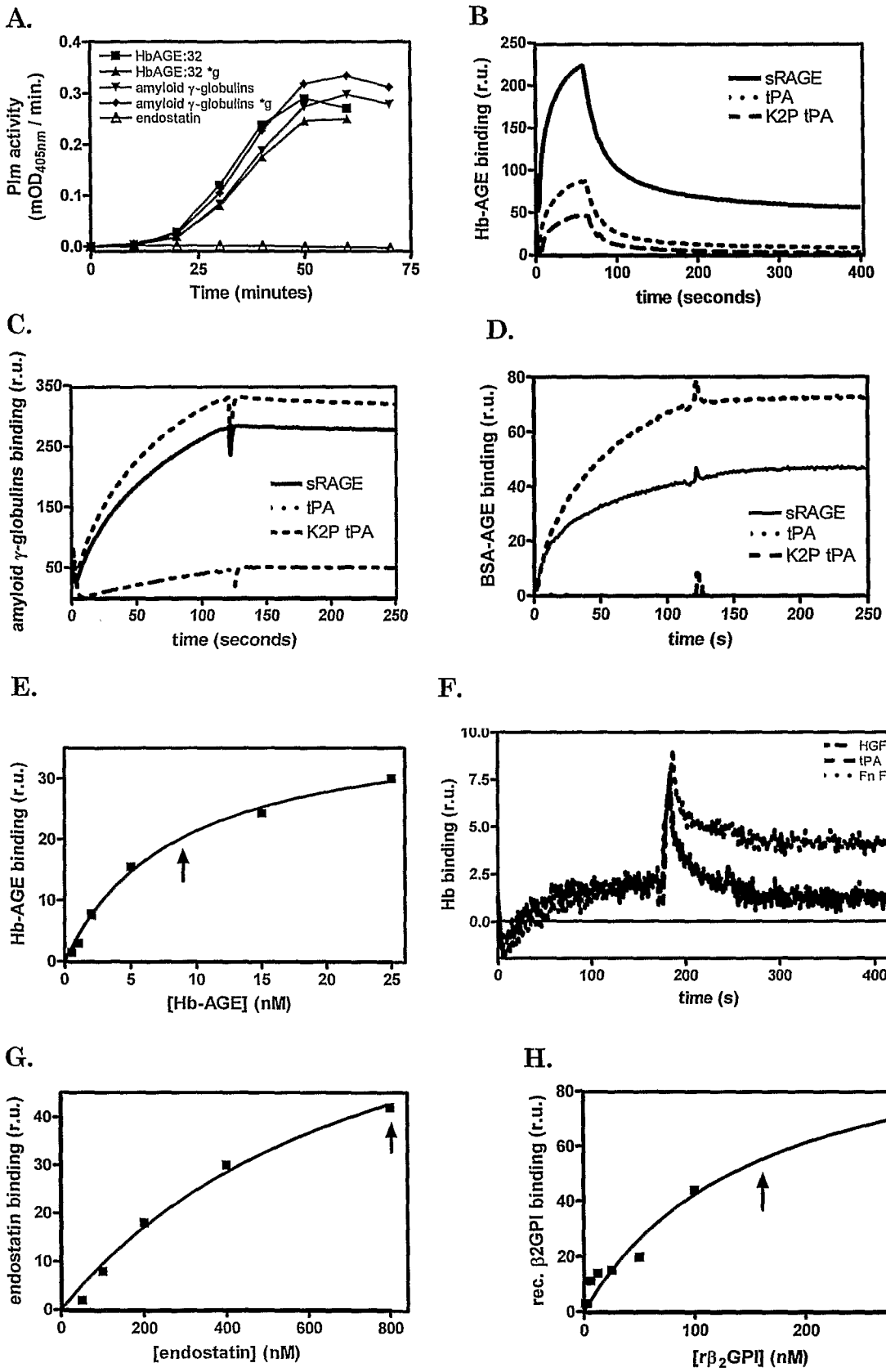
8. A method for decreasing and/or preventing undesired side effects of a pharmaceutical composition and/or increasing the specific activity per gram protein, said method comprising detecting and removing any unfolded protein or peptide and/or aggregated protein or peptide and/or multimerized protein or peptide comprising a cross-β structure from said pharmaceutical composition or any of its constituents comprising a protein.

9. A pharmaceutical composition or any of its constituents comprising a protein, obtainable by a method according to any one of claims 1-8.

10. A kit for carrying out a method according to claims 1-8, comprising all necessary means for binding a protein and/or peptide comprising a cross-β structure to a cross-β structure-binding compound, and/or removing a protein and/or peptide comprising a cross-β structure from a pharmaceutical composition or any of its constituents comprising a protein.

11. A method for influencing the immunogenicity of a protein, comprising influencing the formation of at least one cross-β structure in said protein.

Figure 1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time (seconds)
D.
BSA-AGE binding (r.u.)
sRAGE
tPA
K2P tPA
time (s)
E.
Hb-AGE binding (r.u.)
[Hb-AGE] (nM)
F.
HGFA F
tPA F
Fn F4-5
Hb binding (r.u.)
time (s)
G.
endostatin binding (r.u.)
[endostatin] (nM)
H.
rec. β2GPI binding (r.u.)
[rβ2GPI] (nM)

Figure 2

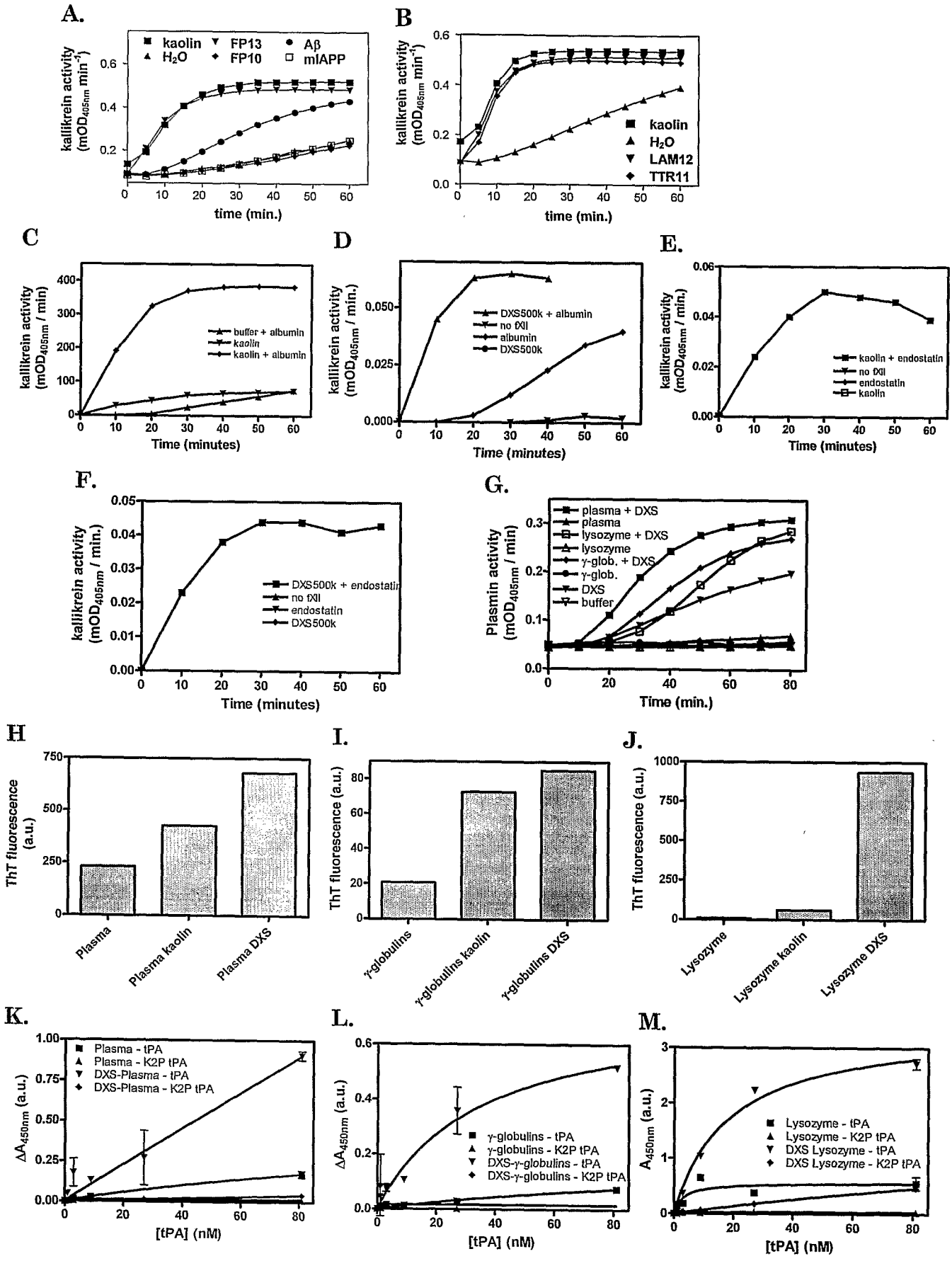
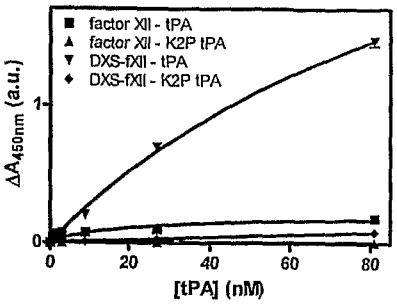
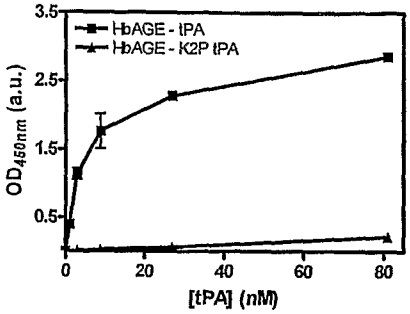

A.
kaolin
H2O
FP13
FP10
Aβ
mIAPP
kallikrein activity (mOD405nm min-1)
time (min.)
B
kaolin
H2O
LAM12
TTR11
kallikrein activity (mOD405nm min-1)
time (min.)
C
buffer + albumin
kaolin
kaolin + albumin
kallikrein activity (mOD405nm / min)
Time (minutes)
D
DXS500k + albumin
no fXII
albumin
DXS500k
kallikrein activity (mOD405nm / min.)
Time (minutes)
E.
kaolin + endostatin
no fXII
endostatin
kaolin
kallikrein activity (mOD405nm / min.)
Time (minutes)
F.
DXS500k + endostatin
no fXII
endostatin
DXS500k
kallikrein activity (mOD405nm / min.)
Time (minutes)
G.
plasma + DXS
plasma
lysozyme + DXS
lysozyme
γ-glob. + DXS
γ-glob.
DXS
buffer
Plasmin activity (mOD405nm / min)
Time (min.)
H
ThT fluorescence (a.u.)
Plasma
Plasma kaolin
Plasma DXS
I.
ThT fluorescence (a.u.)
γ-globulins
γ-globulins kaolin
γ-globulins DXS
J.
ThT fluorescence (a.u.)
Lysozyme
Lysozyme kaolin
Lysozyme DXS
K.
Plasma - tPA
Plasma - K2P tPA
DXS-Plasma - tPA
DXS-Plasma - K2P tPA
ΔA450nm (a.u.)
[tPA] (nM)
L.
γ-globulins - tPA
γ-globulins - K2P tPA
DXS-γ-globulins - tPA
DXS-γ-globulins - K2P tPA
ΔA450nm (a.u.)
[tPA] (nM)
M.
Lysozyme - tPA
Lysozyme - K2P tPA
DXS Lysozyme - tPA
DXS Lysozyme - K2P tPA
A450nm (a.u.)
[tPA] (nM)

Figure 2 (continued)

N



O



P.

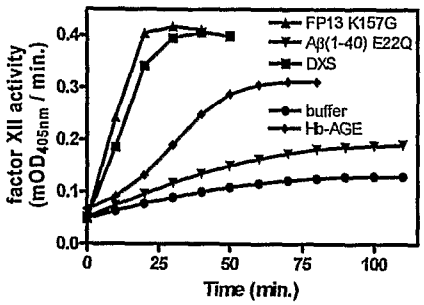


Figure 3

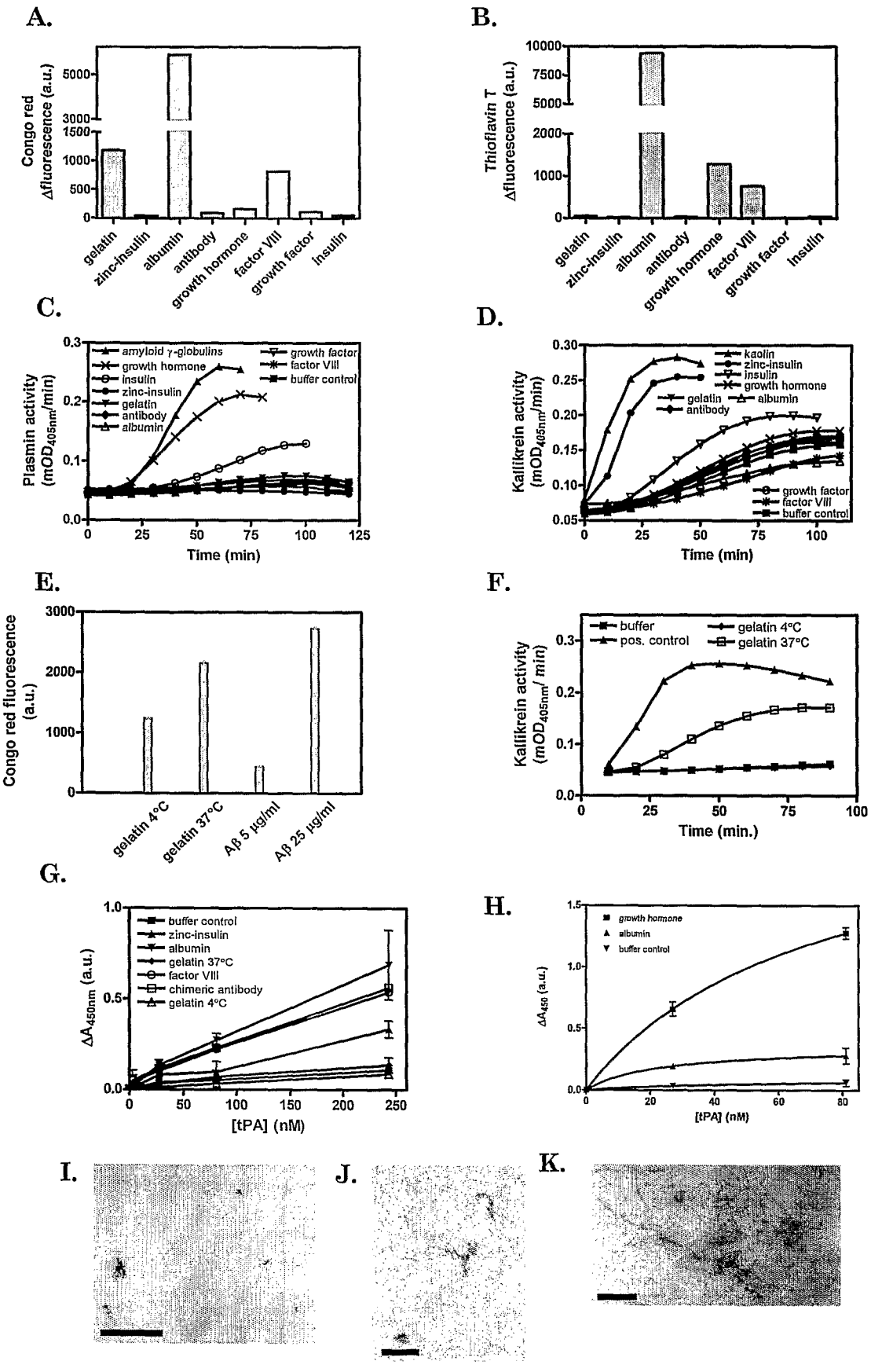

A.
Congo red Δfluorescence (a.u.)
gelatin
zinc-insulin
albumin
antibody
growth hormone
factor VIII
growth factor
insulin
B.
Thioflavin T Δfluorescence (a.u.)
C.
Plasmin activity (mOD405nm/min)
amyloid γ-globulins
growth hormone
insulin
zinc-insulin
gelatin
antibody
albumin
growth factor
factor VIII
buffer control
Time (min)
D.
Kallikrein activity (mOD405nm/min)
kaolin
zinc-insulin
insulin
growth hormone
gelatin
albumin
antibody
growth factor
factor VIII
buffer control
Time (min)
E.
Congo red fluorescence (a.u.)
gelatin 4°C
gelatin 37°C
Aβ 5 µg/ml
Aβ 25 µg/ml
F.
Kallikrein activity (mOD405nm/ min)
buffer
pos. control
gelatin 4°C
gelatin 37°C
Time (min.)
G.
ΔA450nm (a.u.)
buffer control
zinc-insulin
albumin
gelatin 37°C
factor VIII
chimeric antibody
gelatin 4°C
[tPA] (nM)
H.
ΔA450 (a.u.)
growth hormone
albumin
buffer control
[tPA] (nM)
I.
J.
K.

Figure 3 (continued)

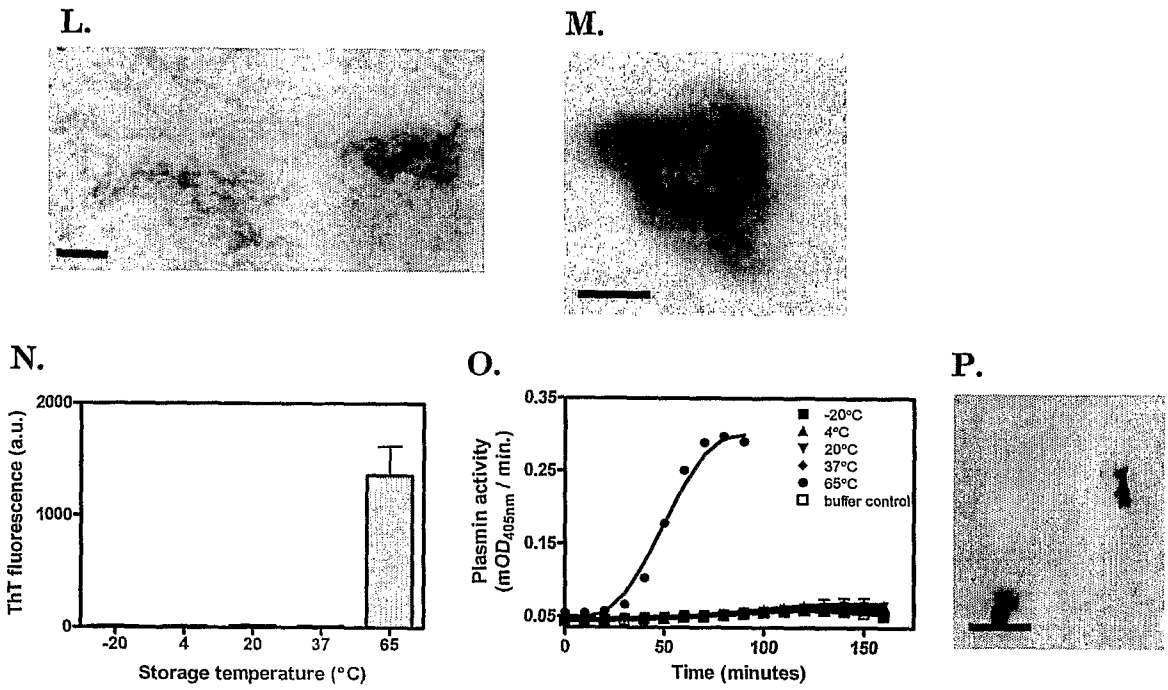


Figure 4

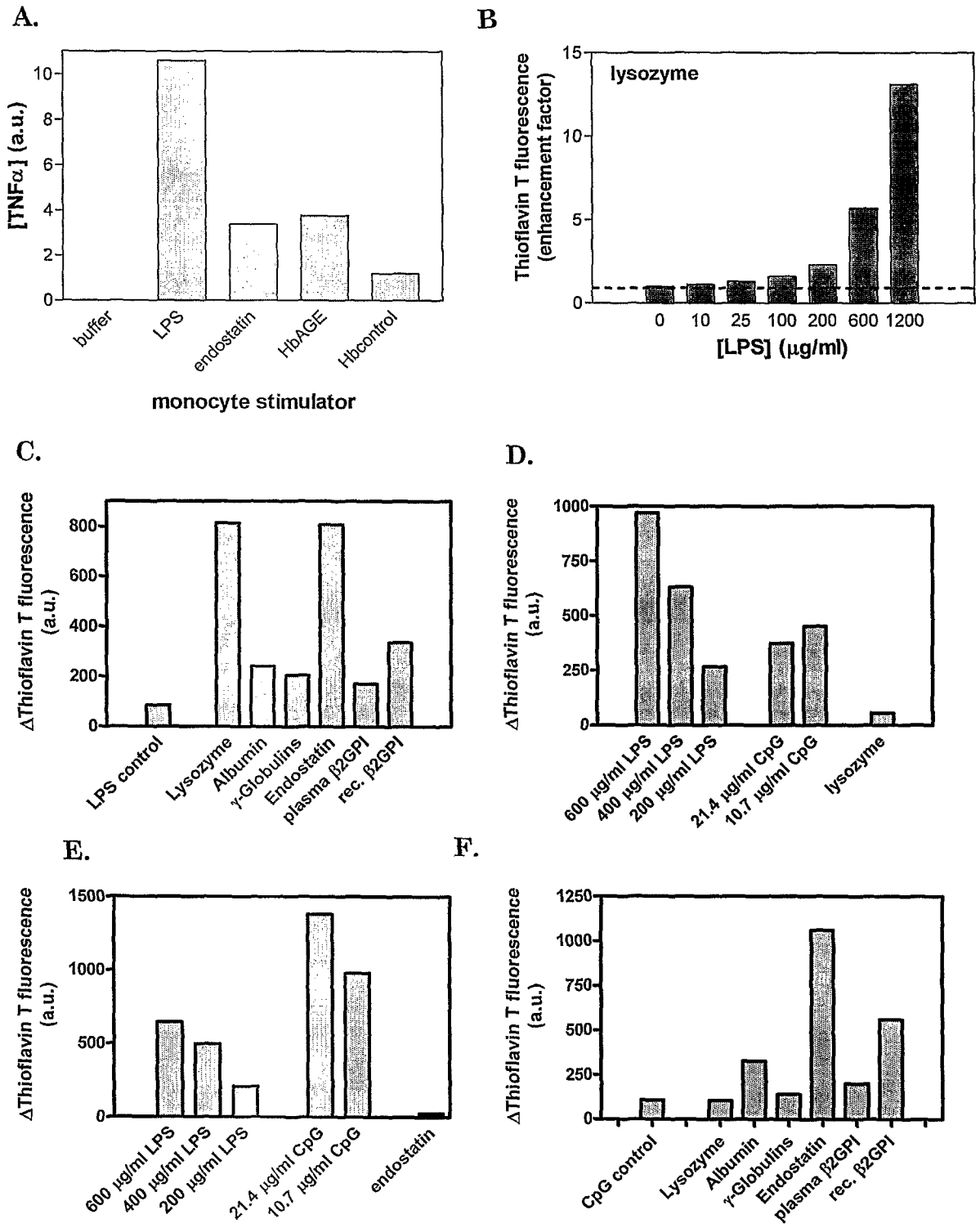

A.
[TNFα] (a.u.)
buffer
LPS
endostatin
HbAGE
Hbcontrol
monocyte stimulator
B
lysozyme
Thioflavin T fluorescence (enhancement factor)
[LPS] (μg/ml)
C.
ΔThioflavin T fluorescence (a.u.)
LPS control
Lysozyme
Albumin
γ-Globulins
Endostatin
plasma β2GPI
rec. β2GPI
D.
ΔThioflavin T fluorescence (a.u.)
600 μg/ml LPS
400 μg/ml LPS
200 μg/ml LPS
21.4 μg/ml CpG
10.7 μg/ml CpG
lysozyme
E.
ΔThioflavin T fluorescence (a.u.)
600 μg/ml LPS
400 μg/ml LPS
200 μg/ml LPS
21.4 μg/ml CpG
10.7 μg/ml CpG
endostatin
F.
ΔThioflavin T fluorescence (a.u.)
CpG control
Lysozyme
Albumin
γ-Globulins
Endostatin
plasma β2GPI
rec. β2GPI

FIGURE 5

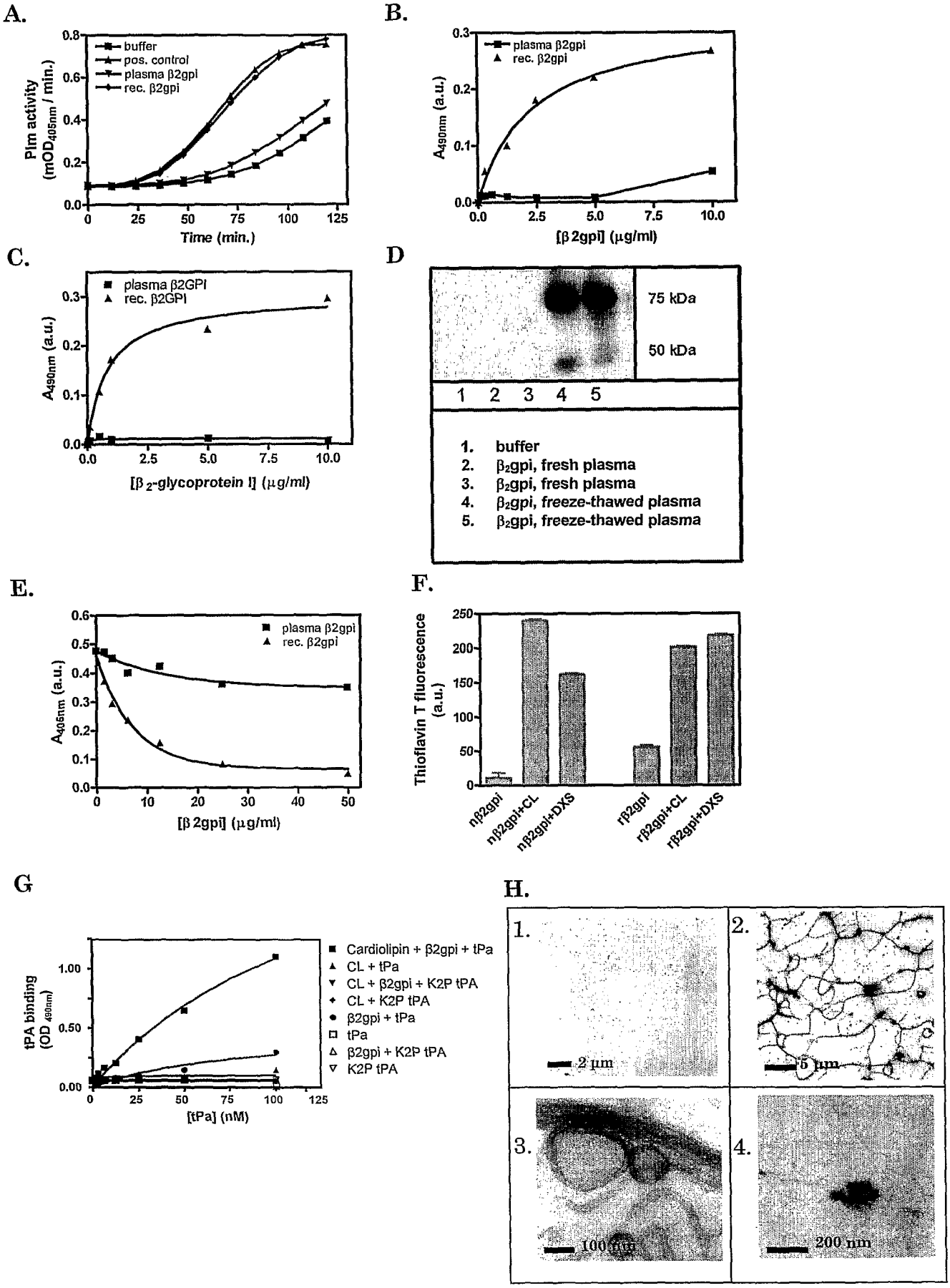

A.
buffer
pos. control
plasma β2gpi
rec. β2gpi
Plm activity (mOD405nm / min.)
Time (min.)
B.
plasma β2gpi
rec. β2gpi
A490nm (a.u.)
[β 2gpi] (μg/ml)
C.
plasma β2GPI
rec. β2GPI
A490nm (a.u.)
[β 2-glycoprotein I] (μg/ml)
D
75 kDa
50 kDa
1 2 3 4 5
1. buffer
2. β2gpi, fresh plasma
3. β2gpi, fresh plasma
4. β2gpi, freeze-thawed plasma
5. β2gpi, freeze-thawed plasma
E.
plasma β2gpi
rec. β2gpi
A405nm (a.u.)
[β 2gpi] (μg/ml)
F.
Thioflavin T fluorescence (a.u.)
nβ2gpi
nβ2gpi+CL
nβ2gpi+DXS
rβ2gpi
rβ2gpi+CL
rβ2gpi+DXS
G
tPA binding (OD 490nm)
[tPa] (nM)
Cardiolipin + β2gpi + tPa
CL + tPa
CL + β2gpi + K2P tPA
CL + K2P tPA
β2gpi + tPa
tPa
β2gpi + K2P tPA
K2P tPA
H.
1.
2 μm
2.
5 μm
3.
100 nm
4.
200 nm

Figure 6

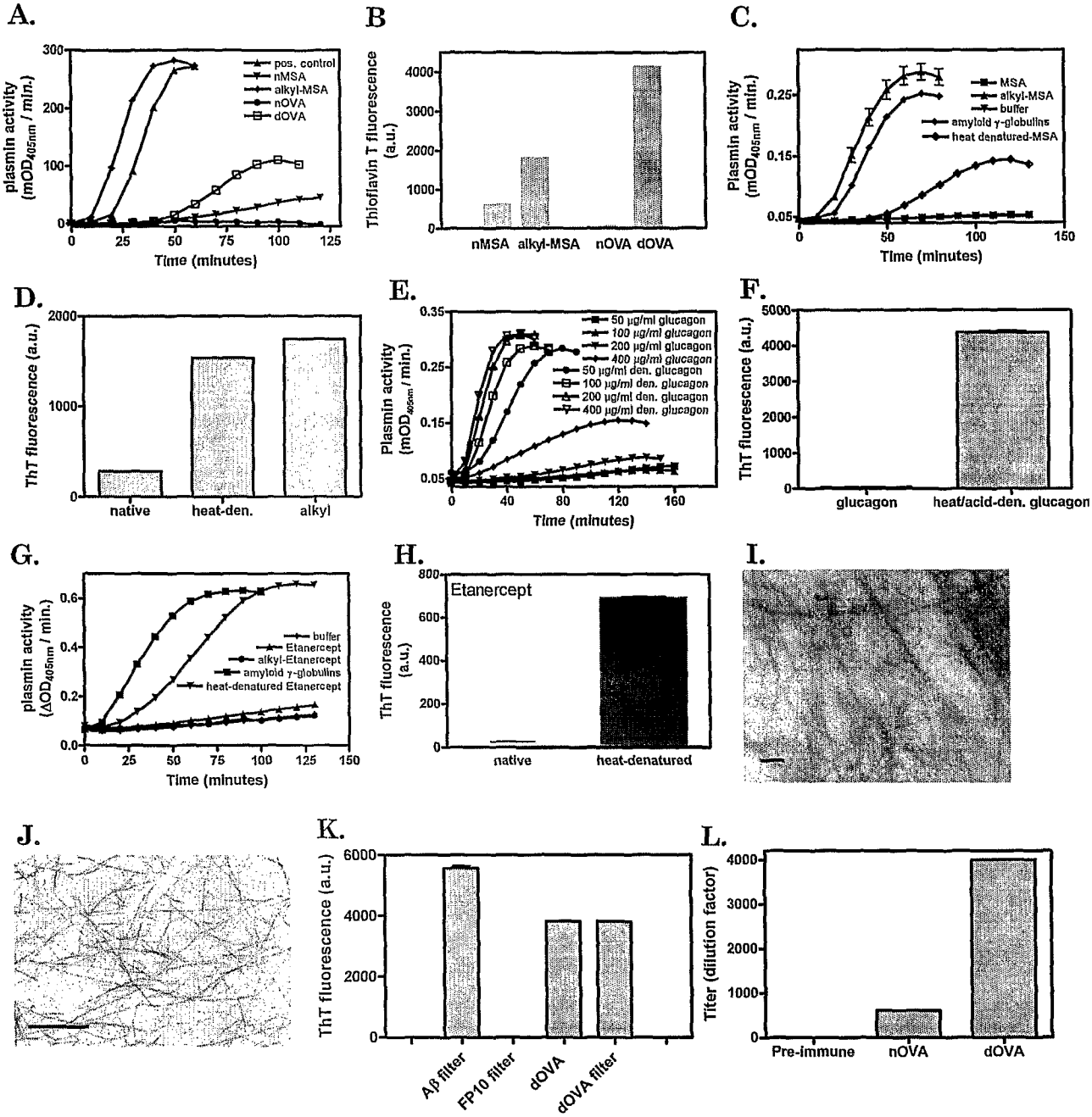

A.
plasmin activity (mOD405nm / min.)
pos. control
nMSA
alkyl-MSA
nOVA
dOVA
Time (minutes)
B
Thioflavin T fluorescence (a.u.)
nMSA alkyl-MSA nOVA dOVA
C.
Plasmin activity (mOD405nm / min.)
MSA
alkyl-MSA
buffer
amyloid γ-globulins
heat denatured-MSA
Time (minutes)
D.
ThT fluorescence (a.u.)
native
heat-den.
alkyl
E.
Plasmin activity (mOD405nm / min.)
50 µg/ml glucagon
100 µg/ml glucagon
200 µg/ml glucagon
400 µg/ml glucagon
50 µg/ml den. glucagon
100 µg/ml den. glucagon
200 µg/ml den. glucagon
400 µg/ml den. glucagon
Time (minutes)
F.
ThT fluorescence (a.u.)
glucagon
heat/acid-den. glucagon
G.
plasmin activity (ΔOD405nm / min.)
buffer
Etanercept
alkyl-Etanercept
amyloid γ-globulins
heat-denatured Etanercept
Time (minutes)
H.
ThT fluorescence (a.u.)
Etanercept
native
heat-denatured
I.
J.
K.
ThT fluorescence (a.u.)
Aβ filter
FP10 filter
dOVA
dOVA filter
L.
Titer (dilution factor)
Pre-immune
nOVA
dOVA

Figure 7

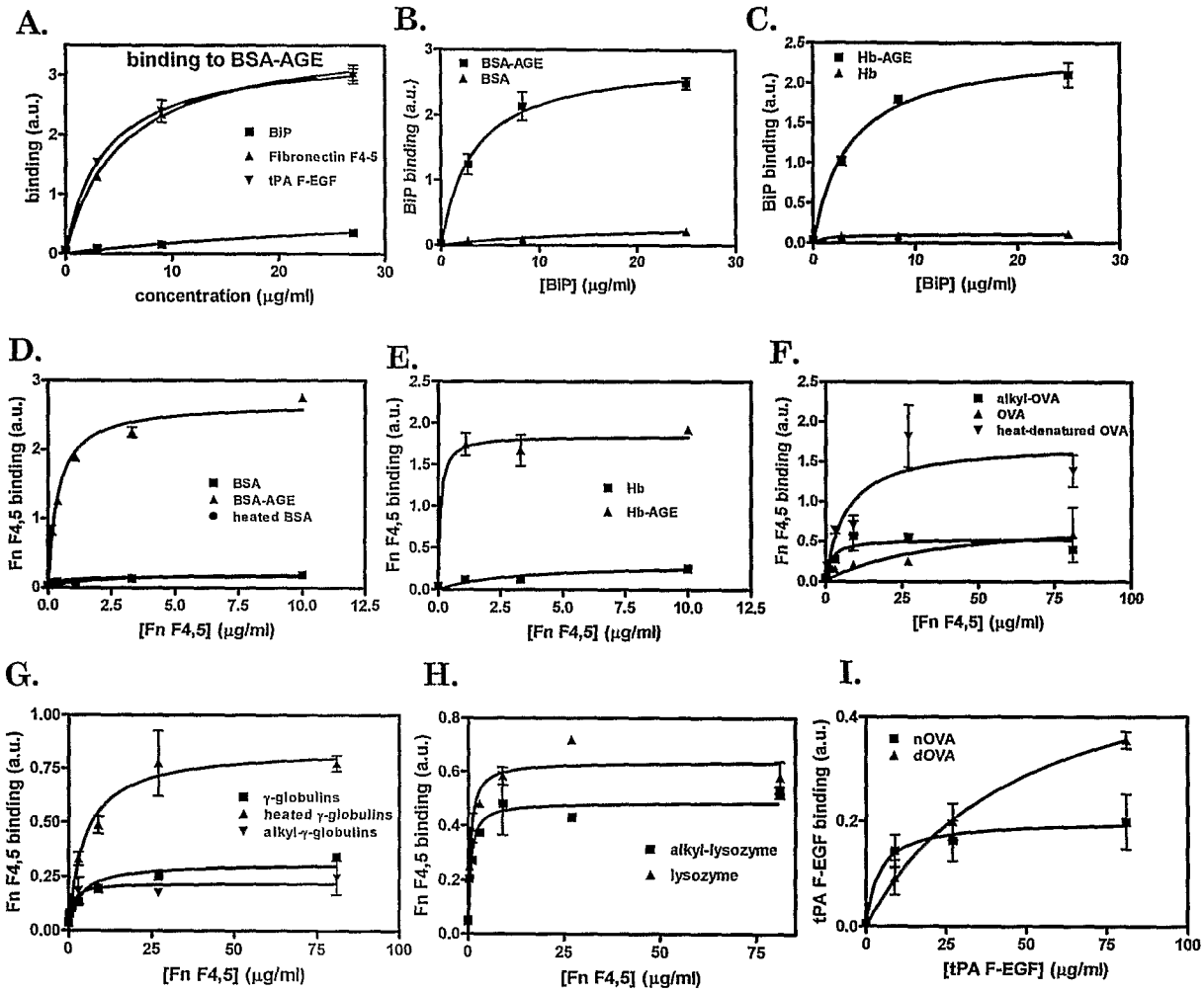

A.
binding to BSA-AGE
binding (a.u.)
BiP
Fibronectin F4-5
tPA F-EGF
concentration (µg/ml)
B.
BSA-AGE
BSA
BiP binding (a.u.)
[BiP] (µg/ml)
C.
Hb-AGE
Hb
BiP binding (a.u.)
[BiP] (µg/ml)
D.
Fn F4,5 binding (a.u.)
BSA
BSA-AGE
heated BSA
[Fn F4,5] (µg/ml)
E.
Fn F4,5 binding (a.u.)
Hb
Hb-AGE
[Fn F4,5] (µg/ml)
F.
Fn F4,5 binding (a.u.)
alkyl-OVA
OVA
heat-denatured OVA
[Fn F4,5] (µg/ml)
G.
Fn F4,5 binding (a.u.)
γ-globulins
heated γ-globulins
alkyl-γ-globulins
[Fn F4,5] (µg/ml)
H.
Fn F4,5 binding (a.u.)
alkyl-lysozyme
lysozyme
[Fn F4,5] (µg/ml)
I.
tPA F-EGF binding (a.u.)
nOVA
dOVA
[tPA F-EGF] (µg/ml)

Figure 8

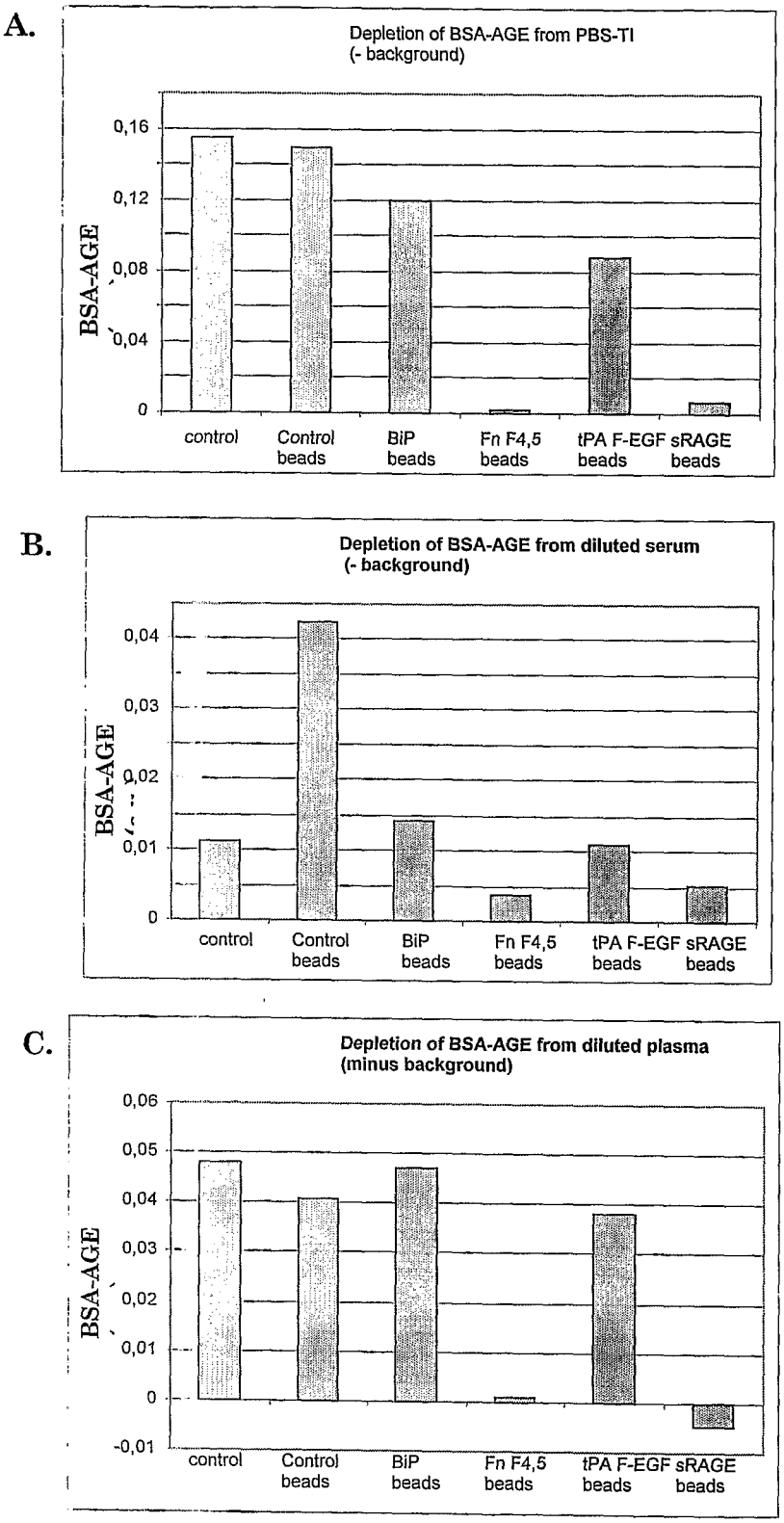

A.
Depletion of BSA-AGE from PBS-TI
(- background)
BSA-AGE
0,16
0,12
0,08
0,04
0
control
Control beads
BiP beads
Fn F4,5 beads
tPA F-EGF beads
sRAGE beads
B.
Depletion of BSA-AGE from diluted serum
(- background)
BSA-AGE
0,04
0,03
0,02
0,01
0
control
Control beads
BiP beads
Fn F4,5 beads
tPA F-EGF beads
sRAGE beads
C.
Depletion of BSA-AGE from diluted plasma
(minus background)
BSA-AGE
0,06
0,05
0,04
0,03
0,02
0,01
0
-0,01
control
Control beads
BiP beads
Fn F4,5 beads
tPA F-EGF beads
sRAGE beads

Figure 9

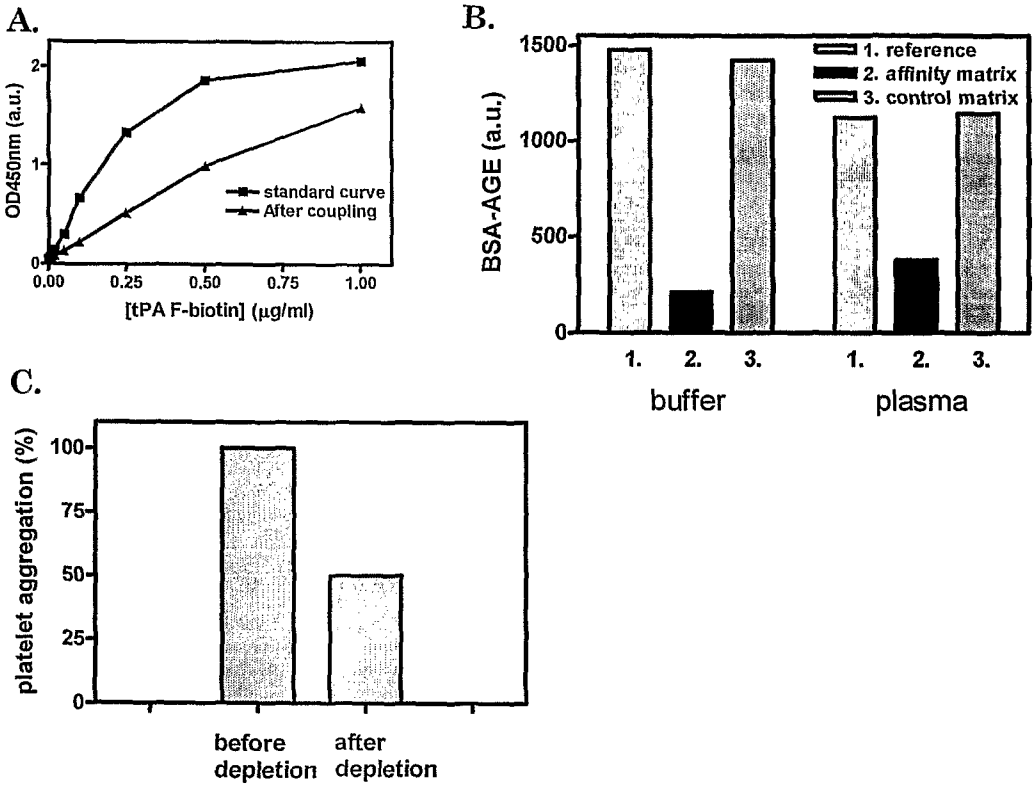

A.
OD450nm (a.u.)
standard curve
After coupling
[tPA F-biotin] (µg/ml)
B.
BSA-AGE (a.u.)
1. reference
2. affinity matrix
3. control matrix
buffer
plasma
C.
platelet aggregation (%)
before depletion
after depletion

Figure 10

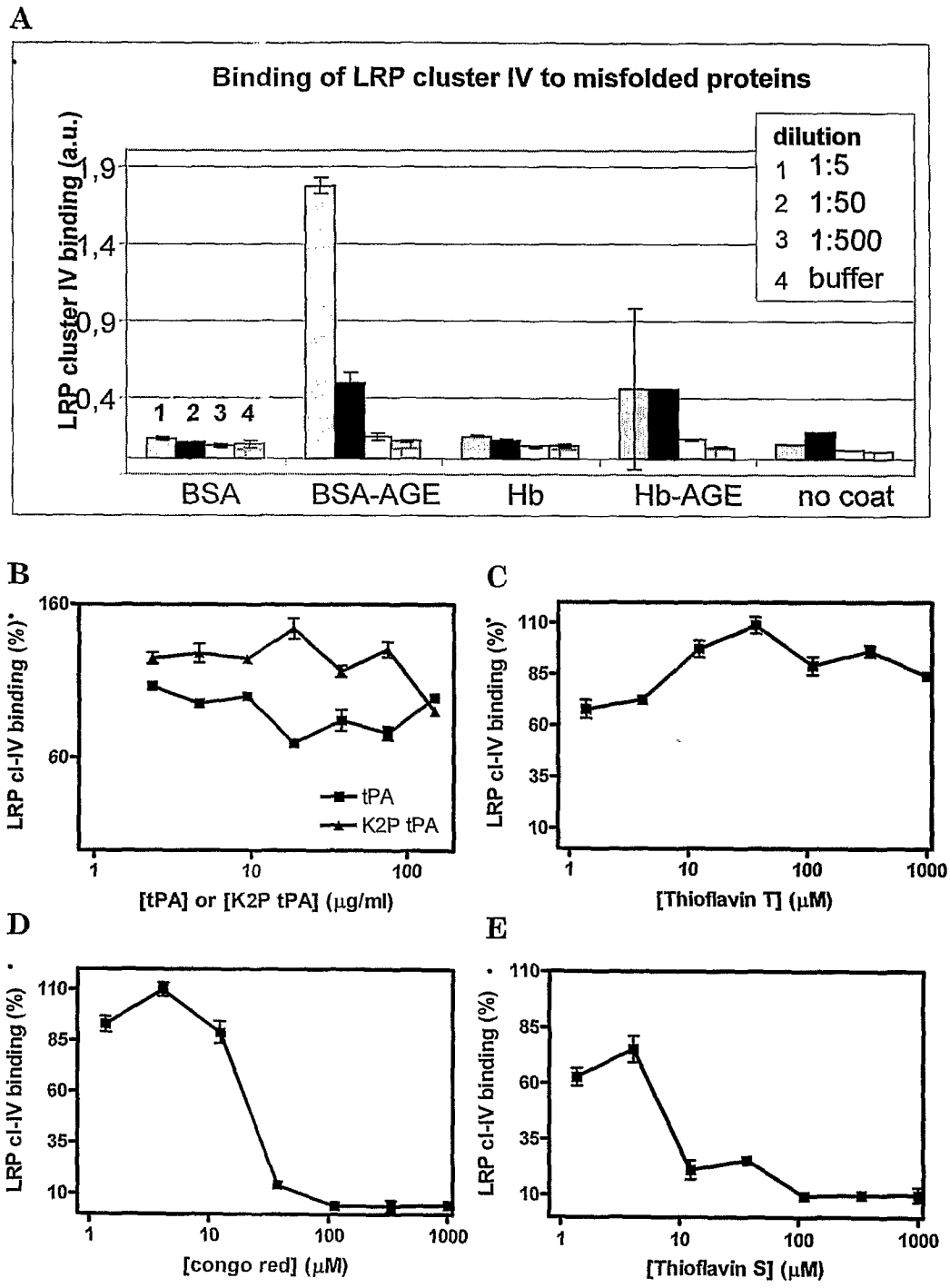

A
Binding of LRP cluster IV to misfolded proteins
LRP cluster IV binding (a.u.)
1,9
1,4
0,9
0,4
1 2 3 4
dilution
1 1:5
2 1:50
3 1:500
4 buffer
BSA
BSA-AGE
Hb
Hb-AGE
no coat
B
LRP cl-IV binding (%)
160
60
tPA
K2P tPA
1
10
100
[tPA] or [K2P tPA] (µg/ml)
C
LRP cl-IV binding (%)
110
85
60
35
10
1
10
100
1000
[Thioflavin T] (µM)
D
LRP cl-IV binding (%)
110
85
60
35
10
1
10
100
1000
[congo red] (µM)
E
LRP cl-IV binding (%)
110
85
60
35
10
1
10
100
1000
[Thioflavin S] (µM)

Figure 11

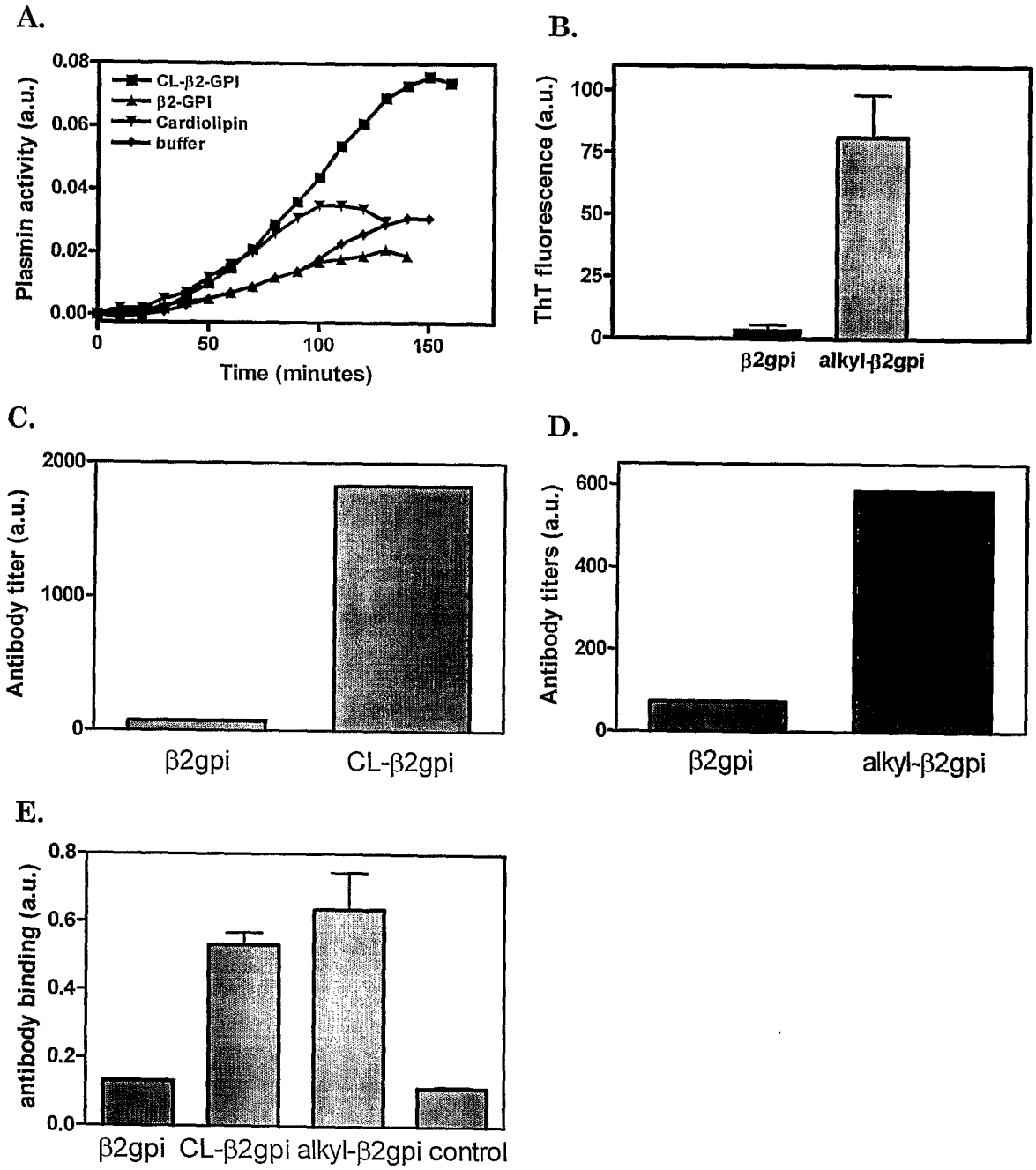

A.
Plasmin activity (a.u.)
0.08
0.06
0.04
0.02
0.00
CL-β2-GPI
β2-GPI
Cardiolipin
buffer
0
50
100
150
Time (minutes)
B.
ThT fluorescence (a.u.)
100
75
50
25
0
β2gpi
alkyl-β2gpi
C.
Antibody titer (a.u.)
2000
1000
0
β2gpi
CL-β2gpi
D.
Antibody titers (a.u.)
600
400
200
0
β2gpi
alkyl-β2gpi
E.
antibody binding (a.u.)
0.8
0.6
0.4
0.2
0.0
β2gpi
CL-β2gpi
alkyl-β2gpi
control

Figure 12

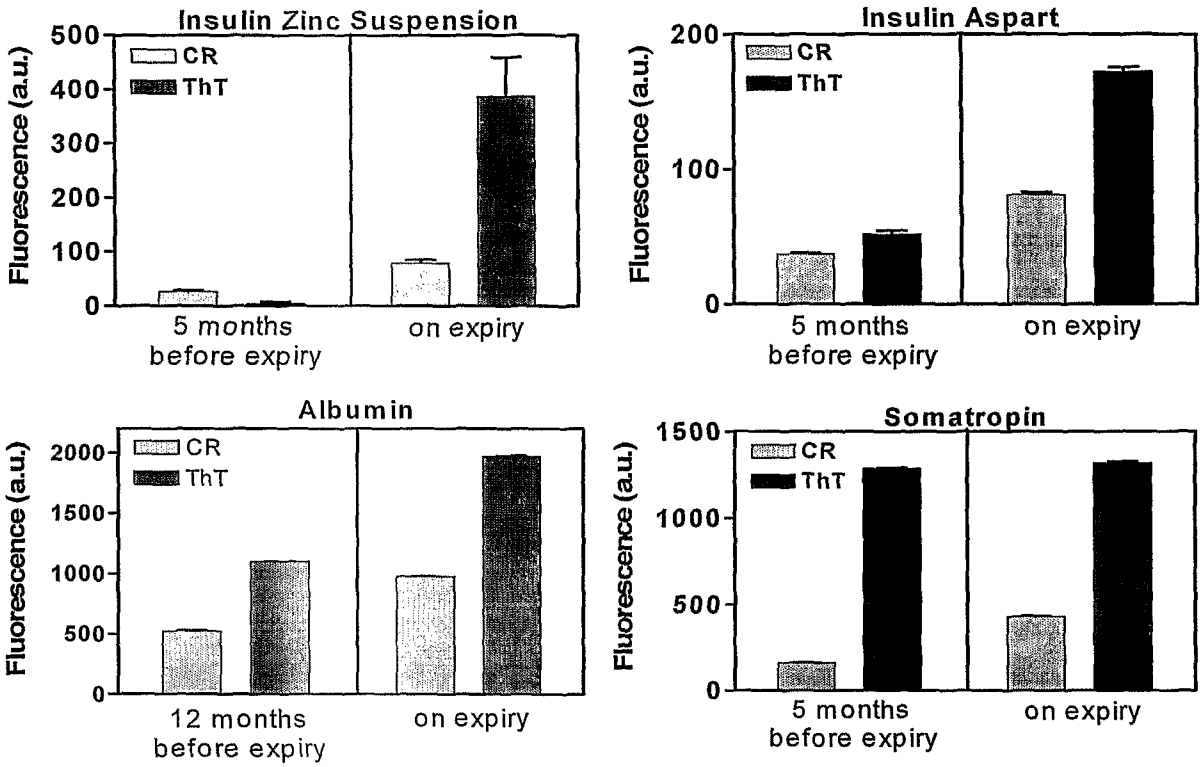
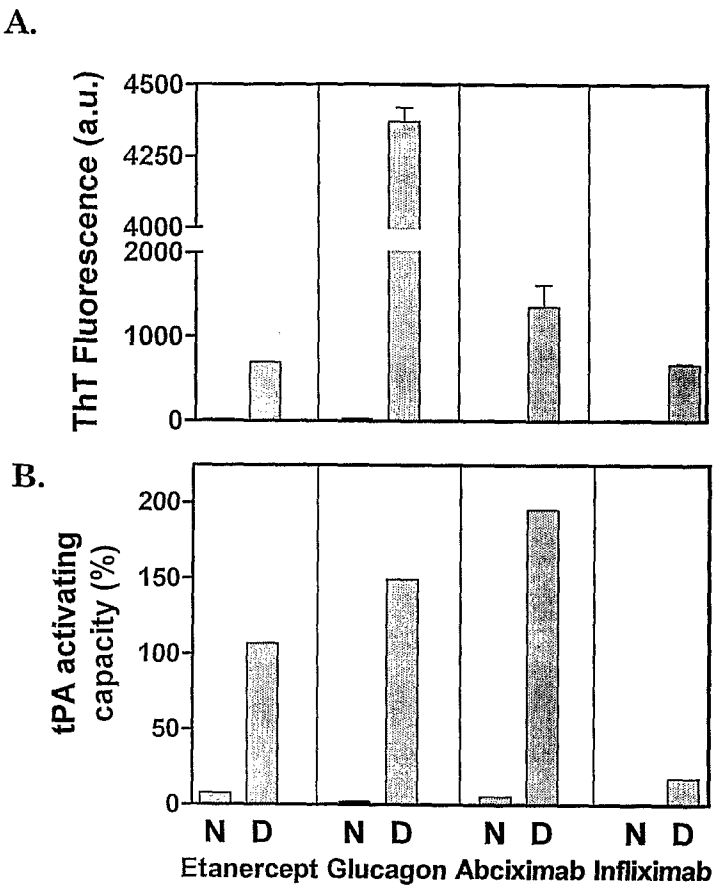

Insulin Zinc Suspension
Fluorescence (a.u.)
500
400
300
200
100
0
CR
ThT
5 months before expiry
on expiry
Insulin Aspart
Fluorescence (a.u.)
200
100
0
CR
ThT
5 months before expiry
on expiry
Albumin
Fluorescence (a.u.)
2000
1500
1000
500
0
CR
ThT
12 months before expiry
on expiry
Somatropin
Fluorescence (a.u.)
1500
1000
500
0
CR
ThT
5 months before expiry
on expiry

Figure 13


A.
ThT Fluorescence (a.u.)
4500
4250
4000
2000
1000
0
B.
tPA activating capacity (%)
200
150
100
50
0
N D N D N D N D
Etanercept Glucagon Abciximab Infliximab