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Biological material free of viral and molecular pathogens and a process for the production thereof

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(54) Title: BIOLOGICAL MATERIAL FREE OF VIRAL AND MOLECULAR PATHOGENS AND A PROCESS FOR THE PRODUCTION THEREOF (54) Bezeichnung: BIOLOGISCHES MATERIAL FREI VON VIRALEN UND MOLEKULAREN PATHOGENEN UND VERFAHREN ZUR HERSTELLUNG (57) Abstract The invention relates to biological material which is free of certain pathogens, in particular of viral pathogens. It also relates to a process for depleting a biological material of viral and molecular pathogens, at least one ligand or receptor which reacts with a receptor or ligand of the particular pathogen being added to said biological material thereby producing a ligand/receptor complex, and separation of the ligand/receptor complex by a process which separates the complexed pathogen partially or completely from biological material. The invention also relates to the use of said process to produce said biological material. (57) Zusammenfassung Beschrieben wird ein biologisches Material, das frei von bestimmten Pathogenen, insbesondere von viralen Pathogenen ist, sowie ein Verfahren zur Abreicherung von viralen und molekularen Pathogenen aus einem biologischen Material, wobei ein biologisches Material mit mindestens einem Liganden oder Rezeptor, der mit einem Rezeptor oder Liganden des bestimmten Pathogens reagiert, versetzt wird, wodurch ein Ligand/Rezeptor-Komplex entsteht, und Abtrennen des Ligand/Rezeptor-Komplexes durch ein Verfahren, das die teilweise oder vollständige Abtrennung des komplexierten Pathogens vom biologischen Material erlaubt, und die Verwendung des Verfahrens zur Herstellung eines biologischen Materials.		

Biological Material, Free from Viral and
Molecular Pathogens, and Production Method

A b s t r a c t :

There is disclosed a biological material which is free from specific pathogens, in particular from viral pathogens, as well as a method of depleting viral and molecular pathogens in a biological material, wherein a biological material is admixed with at least one ligand or receptor which reacts with a receptor or ligand of the specific pathogen, whereby a ligand/receptor complex is formed, and the ligand/receptor complex is separated by a method which allows for the partial or complete separation of the complexed pathogen from the biological material, and the use of the method for preparing a biological material.

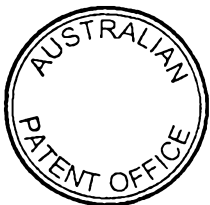


Biological Material, Free from Viral and
Molecular Pathogens, and Preparation Method

The invention relates to a biological material that is free from certain pathogens, in particular free from viral pathogens, as well as to a method of depleting viral and molecular pathogens in a biological material.

The production of therapeutical proteins and preparations by extraction from human or animal tissues or liquids, such as blood or plasma, as well as from continuously growing transformed mammalian cells frequently harbors the risk of a potential contamination by pathogens, such as viruses, virus-like particles or prions. Therefore, measures must be taken that such possibly present pathogens are not transmitted to man.

Human blood or plasma, respectively, may, e.g., contain viruses which cause diseases such as AIDS, hepatitis B or hepatitis C. In case of plasma proteins derived from plasma pools the risk of transmitting infectious agents, such as viruses, is very low due to the selection of blood or plasma donations and on the preparation method. Suitable measures, such as excluding blood donors who have an increased risk from donating blood, as well as analyses of blood or plasma donations which allow for detecting infectious donations and for excluding them from further



distribution, allow for the elimination of most of the infectious donations, yet in most cases they do not detect each and every one. Existing assaying systems for the detection of infectious viruses in biological materials do not always completely eliminate concerns regarding a potential transmission of pathogens, since with the broad range of infectious pathogens existing it is impossible to assay the starting material for all viruses or molecular pathogens which may be present in a sample. Moreover, most assays do not detect the virus, but antibodies developed against the virus, so that during the period of time of the so-called "diagnostic window", detection of a contamination cannot take place. Besides, for some groups of viruses, a reliable or sufficiently sensitive detection method does not exist. Although newly developed assaying methods, in particular nucleic acid amplification methods, such as, e.g., PCR, are very sensitive and specific, they are applicable only to pathogens whose nucleic acid sequence is known. In those cases in which the human pathogens are known, yet a sensitive detection method does not exist, there still remains the insecurity that a negative result is only obtained because the virus content is too low, i.e. a virus content lying below the sensitivity limit of the assaying system.

Therefore, specific removal and/or inactivation

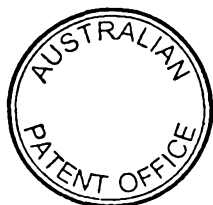


methods for the depletion of viruses have been developed for the preparation of pharmaceutical and therapeutic products so that no infectious particles are to be expected in the final product anymore.

Various inactivation methods are based on a physico-chemical treatment by heat and/or chemicals. As methods, particularly thermal treatment, pasteurizing, treatment of the protein solution with β -propiolactone and UV light, treatment with a combination of a solvent and a detergent (so-called S/D methods) or exposure of the protein solution upon addition of a photodynamic substance are being used. With these methods, a virus inactivation of up to 10^6 log steps has been achieved. The efficiency of the inactivation method may, however, vary with the type of virus. Although S/D-treated blood products are considered safe relative to the transmission of HCV, HBV or HIV, non-enveloped viruses, such as HAV or parvovirus, are not inactivated by this method (Prowse C. 1994. Vox Sang. 67:191-196).

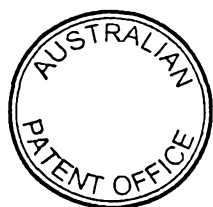
The type of inactivation method may also have an influence on the product, and thus a stabilisation has often been necessary to minimize the protein loss. Moreover, some inactivation methods must be followed by purification steps so as to remove admixed chemicals.

Methods of depleting viruses comprise in particular chromatographic methods, filtration of protein solutions over a membrane filter or adsorption of



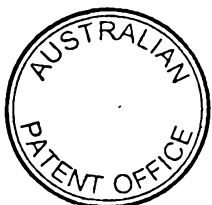
viruses to a solid phase and subsequent removal of the solid phase, as has been described in EP- 0 679 405. However, it has been found that although the treatment with a solid phase, such as, e.g. with Aerosil®, allows for a removal of HIV of up to 4 log steps from an immunoglobulin-containing solution, the loss of IgG may amount up to 42% (Gao et al., 1993, Vox Sang. 64:204-209). At such high loss rates, such a method thus is rather unsuitable for an application on a large technical scale.

With ultrafiltration, virus depletion takes place on account of an effect based on the size differences of virus particles. Bechtel et al. (1988, Biomat., Art. Cells, Art. Org. 16:123-128) showed that for viruses of different sizes (EMC: 30-50 nm, Sindbis virus: 50-70 nm, and VSV: 80-100 nm) in all cases a virus depletion of from 3.3 to 3.5 log steps is achieved and the depletion by ultrafiltration thus is independent of the virus size and is not sufficiently selective. Conventional membrane filters have the disadvantage on the basis of the exclusion size of approximately 0.1 μm to 0.2 μm that most viruses can pass the filter freely. The development of nanofilters having different nominal exclusion sizes of from 15 to 75 nm or 70 to 160 kd have made it possible to retain viruses up to a size of 28 nm (DiLeo et al. (1991). Nature 351:420-421, DiLeo et al. (1992). Bio/Technology 10:182-188, Burnouf-



Radosevich et al. (1994). Vox Sang. 67:132-138,
Hamamoto et al. (1989). Vox Sang 56:230-236).

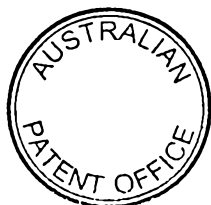
The use of nanofilters for virus depletion allows using a gentle method by which the final product is not changed and by which also viruses of smaller size can be removed from a biological material. Hoffer et al. (1995, J. Chromatography B, 669:187-196) describe a method of depleting viruses, based on the tangential flow filtration for producing a factor IX-concentrate by means of Viresolve® membranes of an exclusion size of 70 kD and they found a reduction factor of 3.5 log steps for enveloped viruses and 7.2 log steps for non-enveloped viruses. They note, however, that high-molecular products are retained by these filters. Blood factors of low molecular weight, such as factor IX or factor XI, can freely pass nanofilters having an exclusion size of between 15 and 35 nm (Burnouf-Radosevich et al. (1994). Vox Sang. 67:132-138, Feldman et al. (1995). Acta Haematol. 94:25-34). Products of higher molecular weight, such as factor VIII (350 kD), vWF (up to 2,000 kD) or immunoglobulins (IgG, 150 kD) can only pass filters of larger exclusion size. However, there is the risk that viruses of much smaller diameters are not retained by the filter and get into the filtrate. The application of nanofiltration for producing virus-safe, therapeutically usable products which are in particular free from small viruses, such



as HAV or parvovirus, is thus restricted to products containing proteins of small molecular size which can also freely pass a nanofilter of small exclusion size. Thus, only the use of nanofilters of a small exclusion size of 15 nm ensures the depletion and removal of HAV and parvovirus B19 from biological products. The disadvantage is, however, that nanofiltration with filters of this exclusion size is not applicable for the production of products containing proteins of high molecular weight, since the latter cannot pass the filter and are retained just as the viruses are.

Another potential problem for nanofiltration is that with various viruses the diameters described are not always absolute. The generally indicated size for HCV is 40-50 nm (Murphy et al. (1995). Archives of Virology 10:424). Muchmore et al. ((1993). International Symposium on Viral Hepatitis and Liver Disease. Tokyo) found, however, that a HCV-containing solution, filtered over a 35 nm nanofilter, leads to a HCV infection in chimpanzees. The possible variability of size of various viruses thus restricts the use of nanofiltration for virus depletion and for the preparation of a virus-safe product or harbours a risk factor, respectively.

Thus, there exists a demand for industrially applicable methods for the safe separation of viruses



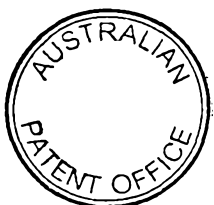
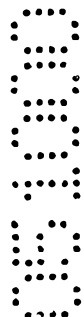
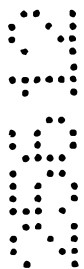
from protein solutions, so as to reduce the risk of infection for patients who are treated with pharmaceutical or therapeutical preparations of animal or human origin or with preparations produced by a genetic engineering method
5 from cell cultures.

There also exists a demand for virus-safe biological products which are free from pathogens, in which it has already been ensured by the production method that not any pathogen has been transmitted and that the method
10 is gentle enough to maintain the biological activity of the products largely unaffected.

Thus the present invention provides a biological material which optionally contains high-molecular pharmaceutically relevant proteins and which is free from
15 specific viral and molecular pathogens, as well as a method of producing such a biological material.

A further aspect of the invention is to recover a biological material which is safely virus-depleted and free from target viruses, a reduction factor of $>10^7$ being
20 attained - analogous to bacterial filters.

According to the invention, a biological material which is safely free from specific pathogens is provided, which is obtained by exposing at least one ligand or receptor contained in a biological material reacts to a
25 receptor or ligand of a specific pathogen, whereby a ligand/receptor complex



of the complexed pathogen forms. The ligand/receptor complex subsequently is removed from the biological material by a method which allows for the partial or complete separation of the complexed pathogen.

The ligand or receptor which reacts with the receptor or ligand of the pathogen may already be present in the starting material. Preferably, the biological material is, however, admixed with at least one ligand or receptor which reacts with the receptor or ligand of a specific pathogen. This makes it possible to employ a well-targeted measure for an optimal adjustment of the parameters, such as, e.g., the amount of ligands or receptors, the choice of the ligand or receptor, or mixtures of various ligands or receptors, or optionally also an addition of further components which aid the formation of the complex. Likewise, the time at which complexing is to take place can be specifically determined. Thus, e.g., if several operations or purification steps are effected prior to the separation of the pathogen, the ligand or receptor may be admixed just before the final step before the separation, in particular before the penetration of the biological material through a permeable filter. The ligand or receptor thus preferably is admixed to the biological material shortly before filtration.

The ligand or receptor admixed to the biological material thus is a component capable of binding to a



ligand or receptor and thus to a specific binding site of the pathogen, which component is able to form a complex with the pathogen, preferably a high-molecular complex. In this case, the ligand or receptor of the pathogen may be an antigen, an epitope or an antigenic determinant. According to the present invention, the reactive ligand or receptor capable of binding which is admixed to the biological material, preferably is a specific antibody or a fragment of an antibody which is still capable of binding to the ligand or receptor of the pathogen.

Preferably, the biological material of the invention is obtained in that an antibody is used as the ligand or receptor which reacts with a ligand or receptor of the pathogen, and that the ligand or receptor of the pathogen is an antigen, whereby an antibody/antigen complex forms as the ligand/receptor complex. According to the present invention, the complex subsequently is removed from the biological material.

According to a particular aspect of the invention, the biological material which is free from specific pathogens is obtained in that the ligand/receptor complex, preferably the antibody/antigen complex, is separated from the biological material by penetration of the solution through a permeable filter. Separation of the complex from the solution may also be effected



by sedimentation, preferably by density gradient centrifugation.

According to the present invention, antibodies or parts of an antibody which are still capable of binding directed against at least one infectious agent are admixed to the biological material which is suspected of possibly containing an infectious pathogen. However, also antibodies directed against several known infectious viruses, such as, e.g., HAV, HBV, HCV, HDV, HEV, HGV, HIV, CMV or parvovirus, or against molecular pathogens, such as, e.g., prions, may be admixed. The addition of the antibodies may be effected such that an immunoglobulin-containing solution is added to a protein-containing solution, the immunoglobulins possibly also being added in excess (and thus in a neutralising concentration) so that the risk of potential pathogens still being present in the biological material in free form can be excluded. However, within the scope of the present invention it has been found that also the addition of non-neutralizing concentrations of antiserum allows for a complete and full depletion of pathogens in a biological material.

Thus, preferably, the ligand or receptor, in particular an antibody, is admixed in a non-neutralizing concentration, the concentration, however, being sufficient to complex the pathogens present so that



they can be removed completely from the biological material by the separating step.

In some instances it may happen that aggregates of various sizes may form, it also being possible that small aggregates form which are still capable of freely passing the filter or whose sedimentation density is not sufficient to separate them from the biological material.

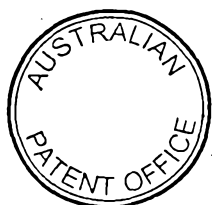
According to a further aspect of the invention, therefore, for improving the separation of the ligand/receptor complex, the aggregation of the complex is increased by adding further aggregating agents to the biological material in addition to the immunoglobulin-containing solutions, which further aggregating agents either further agglutinate free pathogens or increase the complexing of the ligand/receptor complex. This may be effected by the addition of agglutinins, such as lectins, one or more complement-components, conglutinin, rheumatoid factor, a non-toxic, water-soluble, synthetic polymer, in particular polyethylene glycol, or albumin (at least 10%, preferably 20%) or other agglutinating agents known from the prior art. The addition of the aggregating agent is effected such that preferably antigen/antibody complexes up to a size visible in the light microscope form.

The biological material according to the invention



is particularly characterised in that it is certainly free from specific viral pathogens, both from the group of lipid enveloped and of the non-enveloped viruses. Among them are particularly viruses such as HAV, HBV, HCV, HGV, HIV, HEV, HDV, CMV or parvovirus. Since the specific antibody preferably used as the ligand or receptor recognises the corresponding antigens at the surface of the respective virus and binds to the same, each virus for which antibodies are available or against which specific antibodies can be prepared or are contained in an immunoglobulin solution or can be isolated from the latter, can be complexed, and the complex can be removed according to the invention from the biological material. In this way it is possible to complex known viruses and also viruses not as yet identified, against which immunoglobulin-containing solutions are available, optionally to neutralise them completely, and to separate the antibody/antigen complex from the biological material and thus to recover safely pathogen free biological material.

Particular attention has been paid over the last years also to the transmission of potentially infectious molecular pathogens, such as prions. At present, only a few effective methods are available for reducing the causative substances of Creutzfeldt-Jakob Disease or BSE. Pocchiari et al. (1991, Horm. Res. 35:161-166) have suggested a combination of



ultrafiltration and 6 M urea for the depletion of so-called "slow viruses". However, they do not give any details as to by what extent the biological activity of a thus-treated protein is adversely affected. By treatment with high concentrations of the protein-denaturing urea, it is, however, to be assumed that proteins in a thus-treated solution are at least partially denatured and thus inactivated.

According to a further aspect, the biological material of the invention thus is also free from molecular pathogens, in particular from prions, such as the pathogens of BSE or Creutzfeldt-Jakob Disease. It is possible to produce special antibodies against these pathogens or to provide immunoglobulin-containing solutions which particularly contain anti- β -amyloid antibodies. By adding anti- β -amyloid-containing immunoglobulin solutions to a biological material, thus also potential molecular pathogens can be bound in a complex, and the complex can be removed from the biological material.

Since the separation of the ligand/receptor complex and thus of the complexed pathogen is independent of the physical-chemical properties of a virus, a virus-safe, pathogen-free biological material is obtained whose physical-chemical properties are not influenced by the depletion method. It is known that in conventional inactivation methods the addition of



chemicals or the thermal treatment have an influence on the products themselves, unless suitable measures, such as the addition of stabilizers, are taken. The depletion of the pathogens by complexing with a ligand or receptor and
5 separation of the complex from a biological material without physical-chemical treatment thus offers the great advantage that the pathogens are efficiently eliminated, while the product remains unaffected in its native form. A thus obtained biological material thus contains proteins
10 whose properties correspond to those of the native proteins in the starting material prior to the depletion step and whose activity remains unchanged. Furthermore, a possible formation of denatured proteins which have to be separated again from the protein solution in additional complex
15 purification steps, as is the case with some virus inactivation methods, is also avoided.

Upon depletion of the pathogens from the biological material, the pathogen-free material obtained may, of course, be subjected to further purification steps
20 so as to remove accompanying proteins which may not be desired in the final product. To this end, all the methods known from the prior art, such as chromatography, in particular ion exchange chromatography or affinity chromatography, or gel filtration may be carried out.

25 The separation of accompanying proteins from the



biological material of the invention, such as the separation of blood factors from a fraction destined for the preparation of an immunoglobulin-containing preparation, may also take place prior to virus depletion. Preferably, however, depletion of the pathogens from the biological material is effected before the chromatographic separation of accompanying proteins, since apart from the purification effect also an additional depletion of the complexed pathogen as well as of ligands or receptors originally admixed to the biological material and possibly still present can be attained by the chromatographic method.

The biological material obtained according to the invention is particularly characterized in that it is safely free from specific viral and molecular pathogens, the pathogens having substantially completely been removed from the biological material. "Completely" in this connection means that by this method a depletion of pathogens with a reduction factor of at least 7 log steps is attained in the biological material - analogous to bacteria-proof filters. Preferably, the separation is effected with a capacity of >7 log steps, preferably >9 log steps, and particularly preferred >10 log steps. According to the guidelines of the United States Pharmacopoeia (1995, USP 23, NF 18: 1978-1980), the retention capacity of a filter is described by the log reduction value (RF).



For instance, the retention capacity of an 0.2 μm sterile filter which can retain 10^7 microorganisms is at least 7 log steps. In this case, this amount is seen as the complete separation of the viruses by the filter.

Since an antibody-containing hyper immunoglobulin solution optionally is admixed in an excess to the biological material so as to complex any free infectious pathogens, the biological material of the invention optionally also contains non-complexed antibodies present in free form. Upon separation of the antibody/antigen complex from the biological material either by filtration or by sedimentation, the pathogen-free biological material possibly also contains anti-HAV, anti-HCV, anti-HBV or anti-parvovirus specific antibodies.

Since by an excess of ligands or receptors which are contained in the biological material or are admixed thereto, respectively, and which bind to the pathogen and complex therewith, free viruses are no longer present in the biological material and the separation of the complexed pathogen is effected either by filtration or by sedimentation, neither pathogens present in free form nor pathogens complexed with the ligand/receptor get into the filtrate. The filtrate thus obtained and containing biologically active proteins thus, according to the present invention, is



not only free from non-complexed and freely present pathogens, but also free from pathogens present in a complex with a ligand, such as an antibody.

A pathogen-free biological material obtainable according to the present invention may be a plasma fraction, an immunoglobulin-containing plasma fraction, a plasma protein-containing fraction containing blood factors, such as factor II, factor VII, factor VIII, factor IX, factor X, factor XI, protein C, protein S, vWF, a concentrate containing one of the blood factors mentioned, a supernatant of a hybridoma cell line, a cell culture supernatant from transformed or infected mammalian cells or an extract from an animal or human tissue.

According to a further aspect of the present invention, there is provided a method of depleting viral and molecular pathogens in a biological material as well as a method of recovering biological material which is safely free from viral and molecular pathogens. The method is particularly characterized in that at least one receptor or ligand contained in a biological material (with a suspected presence of a specific pathogen) reacts with a receptor or ligand of a pathogen, whereby possibly a ligand/receptor complex of a complexed pathogen forms. Preferably, a ligand or receptor which reacts with the receptor or ligand of the pathogen is admixed to the biological material. The

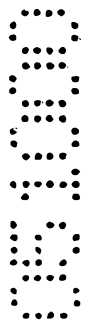
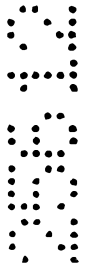


ligand/receptor complex possibly formed subsequently is removed from the biological material by a method which allows for the partial or complete separation of the complexed pathogen. By complexing a free pathogen with a
5 ligand in a complex, a complexed pathogen particle is obtained which is enlarged, having a higher density or a higher sedimentation coefficient than the free pathogen.

Moreover, the complex has a higher aggregation than the free pathogen, whereby, due to the increased
10 aggregation, the diameter of the complexed pathogen is enlarged and its ability of passing through certain membrane filters is changed.

The method of the invention for depleting pathogens and
15 recovering a pathogen-free biological material thus is effected by complexing the pathogen with a ligand/receptor so as to increase its density or sedimentation properties as well as to enlarge its diameter by increasing aggregation. Subsequently, the complexed pathogen can be
20 separated from the biological material because its properties have thus been changed.

According to a preferred embodiment of the method, separation of the ligand/receptor-pathogen-complex is effected by penetration of a permeable filter, the
25 complex being selectively retained by the filter. According to the invention, the exclusion size of the



filter is chosen such that also high-molecular proteins, such as, e.g., factor VIII, vWF or immunoglobulins, can pass the filter freely, and also if a highly concentrated protein solution is used, clogging of the filter pores is not to be expected. The exclusion size of the filter may, of course, not exceed the highest-possible aggregation size of the ligand/receptor complex, and, in particular, of the antibody/antigen (pathogen) complex, since otherwise also high-molecular, dense pathogen/antibody complexes will pass the filter and thus get into the filtrate.

Thus, nanofilters are suitable for carrying out the present invention. Particularly preferred are nanofilters having a nominal exclusion size of between 35 and 100 nm. This exclusion size allows high-molecular proteins to pass the filter freely; however, they also retain free, non-complexed pathogens having a diameter >100 nm as well as complexes of pathogens of this size. Moreover, the use of filters of mean exclusion size avoids possible clogging of the filters by higher-molecular proteins and other components possibly present in the biological material, by which the flow-through capacities of the filters would be lowered. Also when using the tangential flow filtration, a slight pressure may possibly be required to pass the liquid over the filter, whereby also the risk of the filter being adversely affected by the applied pressure



and of cracks developing is reduced. Apart from a depletion of pathogens that is efficient for the final product and gentle, the use of nanofilters having an exclusion size of ≥ 35 nm for carrying out the method of the invention thus offers the advantage of lower costs for the filters used than in case of nanofilters of small exclusion size. This makes the method interesting particularly for an application on a large technical scale.

It is, however, also possible to use filters having an exclusion size of >100 nm for carrying out the method, wherein, however, it must previously be ensured that very large pathogen-ligand complexes or antibody/antigen aggregates, respectively, are formed or that, optionally, additional agglutinating agents are utilized so that the ligand/receptor complex will be sufficiently large so that it cannot pass the filter freely. There, the conditions may, of course, also be chosen such that antigen/antibody aggregates up to a visible size will form which may even be retained by conventional sterile filters. In principle, the smaller the exclusion size of a filter, the more expensive its production.

Within the scope of the present invention it could be demonstrated that also filters of a nominal exclusion size of from $0.04 \mu\text{m}$ up to $3 \mu\text{m}$ allow for a complete separation of pathogens complexed according to



the present invention, from a biological material. Thus it has been shown that according to the present invention, ligand/receptor complexes are formed which reach a size in the visible range (e.g. in the light microscope).

According to a further embodiment, separation of the ligand/receptor complex is effected by sedimentation. Preferred is the sedimentation by density-gradient centrifugation.

According to the method of the invention, the ligand or receptor admixed to the biological material is a component capable of binding to the ligand or receptor and thus to a specific binding site of the pathogen, which component is capable of forming a complex with the pathogen, preferably a high-molecular complex. The ligand or receptor of the pathogen may be an antigen, an epitope or an antigenic determinant. The reactive ligand or receptor which is capable of binding and which is admixed to the biological material and reacts with the ligand or receptor of the pathogen preferably is an antibody or a antibody fragment which is capable of binding. The antibody may be an antibody of any sub-class, the sub-classes IgG and IgM, however, being particularly preferred. As ligands or receptors, however, all known components are considered which are capable of binding to a receptor or ligand of a pathogen and of forming a high-molecular complex with

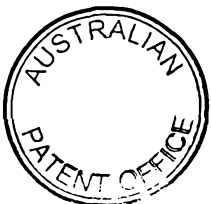


that pathogen.

By adding a receptor or ligand capable of binding to a pathogen possibly present in a biological material, a high-molecular complex is obtained from pathogen and
5 ligand/receptor, exhibiting a higher aggregation.

According to one aspect of the method of the invention, an immunoglobulin-containing solution containing specific antibodies directed against infectious human pathogens is admixed to a biological material, preferably
10 to a protein-containing solution, whereby an antibody/antigen complex will form between the pathogens possibly present in the biological material and the antibodies.

According to a further aspect of the method of
15 the invention, aggregation of the ligand/receptor-pathogen-complex is still further increased, whereby complexes of higher density and larger diameters are formed. This is effected according to the invention by adding further agglutinating agents, in particular lectins, such as
20 concanavalin A, ricin or phasin, or one or several complement-components, conglutinin, rheumatoid factor, or a synthetic polymer, such as polyethylene glykol or albumin. The aggregating agent may be admixed with the biological material either simultaneously with the ligand or receptor
25 that reacts with the pathogen, or after a predetermined span



of time after addition thereof. It is, however, preferred that the agglutinating agent is admixed with a temporal delay after the addition of the immunoglobulin-solution. By this it is ensured that the agglutinins or conglutinins do not react with the antibodies and do not aggregate or complex the latter, but aggregate merely already formed pathogen/antibody complexes to higher-molecular complexes. By this it is also ensured that a higher complex density will be attained by the further aggregation, whereby the complex can be removed more efficiently from the biological material. By the higher complexing of the antigen/antibody-aggregates it is possible to use membrane filters, even filters having a higher exclusion size of preferably ≥ 35 nm, in the separating procedure. On account of the higher density of the aggregated particles, also an improved separation by sedimentation is feasible, since all the complexed pathogens can be encompassed.

By adding immunoglobulin-containing solutions or ligands or receptors specifically reacting with the pathogens it is also possible to deplete both viral and molecular pathogens in a biological material, irrespective of their physical-chemical properties. This particularly applies to viruses or molecular pathogens which so far could not be depleted or inactivated by conventional methods. Thus, also small,



non-lipid-enveloped viruses, such as parvovirus or HAV, are encompassed by the method according to the invention, which viruses so far could not be efficiently removed or inactivated, neither by physical-chemical inactivation methods, such as S/D treatment, nor by nanofiltration, from highly concentrated protein solutions or from solutions containing high-molecular proteins (>150 kD). Therefore, this method is applicable for the depletion of all viral and molecular pathogens by which a biological material can be contaminated, in particular for the depletion of lipid-enveloped or non-lipid-enveloped viruses, such as HAV, HBV, HCV, HIV, HEV, HDV, HGV, CMV or parvorirus, but also for prions. The method according to the invention is particularly suited for the depletion of pathogens in a biological material and for the recovery of biological material which contains high-molecular proteins, e.g. immunoglobulins or vWF.

Within the scope of the present invention thus also a pathogen-free, virus-safe plasma protein-containing composition is provided, in which the plasma proteins comprise at least 80% of the activity of the starting material, the composition being obtained by the method according to the invention.

According to a special aspect of the invention, an antibody, obtained from a hyperimmunoglobulin solution



or from a supernatant of a hybridoma cell line, is used as a ligand when carrying out the method according to the invention. The immunoglobulin-containing solution may be obtained from plasma donations comprising a high titer of antibodies directed against a specific pathogen. These are particularly immunoglobulin solutions from donors containing anti-HCV, anti-HAV, anti-HBV, anti-HIV, anti-HEV, anti-HDV, anti-HGV, anti-CMV or anti-parvovirus antibodies. The antibody-containing solution may also be prepared by biotechnological methods, such as the hybridoma technique. In doing so, monoclonal antibodies directed against an antigen of a particular pathogen are secreted by a hybridoma cell line into the supernatant of the culture medium from which the antibodies can then be isolated in a high-titer solution.

Immunoglobulin-containing solutions recovered from plasma donations may possibly also contain free viruses against which the antibodies contained in the solution are directed, as well as other pathogens. This applies equally to monoclonal antibodies prepared via the hybridoma technique, for which a possible contamination with viral pathogens cannot be excluded. According to a particular embodiment of the method according to the invention, the hyperimmunoglobulin solution used for carrying out the method optionally is subjected to a virus inactivation and/or virus depletion method.



If the antibody is present at a low titer in the immunoglobulin-containing solution, the antibody optionally can be enriched and subsequently utilized in a highly concentrated solution as ligand or receptor.

According to a particular aspect, an anti- β -amyloid antibody is used as antibody for carrying out the method according to the invention. Anti- β -amyloid antibodies preferably recognize structures at the surface of prions, are able to bind thereto and form a prion/antibody complex. According to the present invention, antibodies can specifically be prepared against prions, in particular against the pathogens of the Creutzfeldt-Jakob disease, or anti- β -amyloid-containing immunoglobulin solutions can be provided and admixed to a biological material which possibly contains prions. By the attachment of anti- β -amyloid antibodies to the prions, the low-molecular prions are aggregated to a high-molecular complex which subsequently can selectively be separated from the biological material by filtration or sedimentation.

According to a further particular aspect of the invention, antibodies directed against HAV, HBV, HCV, HIV, HGV, HEV or parvovirus are utilized as ligands or receptors. To carry out the method, an immunoglobulin-containing solution containing antibodies directed against a specific virus can be added to the biological material. Yet, it is also possible to admix a mixture



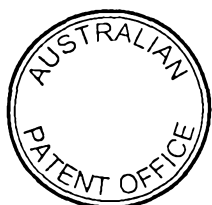
of antibodies or other ligands which are directed against different pathogens with which the biological material may possibly be contaminated. Upon addition of at least one immunoglobulin-containing solution directed against a specific pathogen, or of a mixture of antibody-containing solutions directed against various pathogens, the mixture of biological material and immunoglobulin solution is incubated for a period of time which allows complex formation between the antigen of the pathogen and the antibody, and the complex formed subsequently is separated from the biological material as described above.

The method according to the invention can be used for depleting viral and/or molecular pathogens in a biological material. The biological material may be a plasma fraction, an immunoglobulin-containing plasma fraction, a plasma protein-containing fraction containing blood factors, such as, e.g., factor II, factor VII, factor VIII, factor IX, factor X, factor XI, protein C, protein S, vWF, a concentrate containing one of the said blood factors, a supernatant of a hybridoma cell line, a cell culture supernatant from transformed or infected mammalian cells, or an extract of an animal or human tissue. The parameters for carrying out the method are each adapted to the type and nature of the biological material and of the contaminating pathogens possibly present. Thus, the



formation of the ligand/receptor complex for aggregating the pathogen is carried out under conditions which allow for optimal complexing, in particular for the binding between receptor and ligand or between antibody and antigen of the pathogen, respectively. The optimal parameters, such as pH, temperature, duration of incubation for carrying out the method according to the invention, depending on the type of pathogen, the specificity of the ligand or receptor (antibody) admixed, and on the nature of the biological material (purity of the solution, protein concentration in the solution) can be determined by any skilled artisan on the basis of his/her general knowledge. If the ligand/receptor complex is removed from the biological material by a filtration step, a filter will be used which has an exclusion size which allows for the biological material to freely pass the permeable filter and for the ligand/receptor complex to be selectively retained by the filter.

To ensure that any free pathogens present in the biological material have been complexed by antibodies, both the filtrate and the concentrate are assayed for the presence of viruses or for an excess of specific antibodies. The depletion rate of the pathogens from the biological material may be determined by virus titer determination methods or via the determination of the gene copy number or genomic



equivalents, respectively, of specific viruses, e.g. by quantitative PCR, as described by Dorner et al. (1994. 25. Hämostase-Symposium, Ed. Scharrer & Schramm, pp. 29-44), in the filtrate and in the concentrate.

According to a further aspect of the present invention, the biological material obtained by the method according to the invention is assayed for the presence (of an excess) of the ligand/receptor used, in particular of a specific antibody. Since, when admixing an excess of an antibody directed against a specific pathogen, non-bound and non-complexed antibodies have a density and a molecular size which is below that of the complexed antibody, the presence of free antibodies in the biological material is also a criterion that the concentration of antibodies in the solution has been sufficient for complexing the free pathogen. The presence of an excess of utilized ligands or receptors is determined in that a known amount of a viral or molecular pathogen which has specific ligands or receptors for the antibody is admixed to a sample of the biological material prior to and following the pathogen depletion, the thus recovered biological material containing the antibody/pathogen complex is again filtered over a permeable filter, and the residual amount of pathogen present in the filtrate is determined. In this way it can be determined whether or not the immunoglobulin solution used for the depletion



has an antibody content sufficient for complexing the pathogens, and whether or not non-complexed antibodies are present in the filtrate and, thus, in the final product.

5 The method of the invention for depleting viral and molecular pathogens and for recovering pathogen-free biological material can be combined with any known virus inactivation method, such as heat treatment, pasteurizing or the S/D method. Preferably, the inactivation methods are applied before carrying out the method according to the
10 invention, since in this manner viruses not encompassed by the inactivation method can be depleted by the method according to the invention.

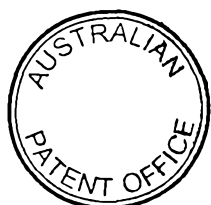
According to a further aspect, the invention provides a virus-inactivated immunoglobulin solution
15 containing specific antibodies against viral or molecular pathogens as a ligand for complexing viral or molecular pathogens in the method according to the invention.

The method according to the invention for depleting viral and/or molecular pathogens may, in
20 particular, be used for recovering and preparing a biological material which is free from specific pathogens present both in free, non-complexed form and in bound, complexed and aggregated form.

For the purposes of this specification it will be
25 clearly understood that the word "comprising" means "including but not limited to", and that the word "comprises" has a corresponding meaning.

It will be clearly understood that, although a number of prior art publications are referred to herein,
30 this reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art, in Australia or in any other country.

The invention will now be explained in more detail



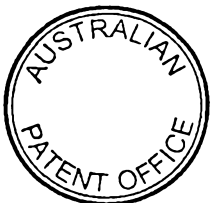
by way of the following Examples to which, however, it is not restricted.

Example 1: (At Present Considered by Applicant to be the Best Mode of Carrying out the Invention)

Depletion of HAV by HAV-Specific Antibodies and Subsequent Nanofiltration

98 ml of a 2% human serum albumin solution which was free from antibodies, and a 2% human immunoglobulin solution containing Hepatitis A virus (HAV)-specific antibodies were spiked with high-titer HAV. The virus titer was determined from aliquots of both solutions. The solutions were then subjected to a 4-hour tangential flow filtration with an initial pressure of 0.8 bar. An 0.001 m²/35 nm filter (Planova 35N, Asahi) was used. HAV titration was effected as in Barrett et al. (J. Med. Virol. (1996), Vol. 49: 1-6).

Upon serial ½ log dilution in cell culture medium, the virus titer was determined. 100 µl of each serial dilution were each put into 8 wells of a microtiter plate containing 1 x 10⁴ FRhK-4 cells per well. Subsequently the plates were incubated for 14 days at 37°C. After seven days, the medium was changed. After this incubation period, the cytopathic effect (cpe) was microscopically determined. The TCID₅₀ was determined on the basis of the number of wells of the microtiter plate which exhibited a positive CPE. The efficiency of



the process of virus depletion was expressed as reduction factor (R.F.) which was calculated according to the formula recommended by the E.C. Committee for Proprietary Medicine (Commission of the European Communities (1991): Ad hoc Working Party on Biotechnology / Pharmacology - Note for Guidance (Validation of Virus Removal and Inactivation Procedures) III/8115/89-EN):

$$10^{R.F.} = \frac{\text{Sample volume before treatment} \times \text{virus titer before treatment}}{\text{Sample volume after treatment} \times \text{virus titer after treatment}}$$

As expected on the basis of the virus size (diameter, 25-30 nm), in the absence of a HAV-specific antibody HAV was not retained by filtration over the 35 nm membrane. However, from the sample which contained immunoglobulin with HAV-specific antibodies, a complete virus removal could be achieved by filtration through the 35 nm membrane (R.F. > 5.2), with no virus being detectable in the filtrate, and the virus was retained up to 100% in the concentrate (Table 1).

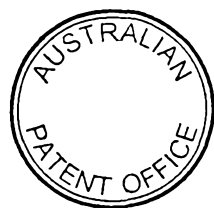


Table 1: Influence of Specific Antibodies on the
Depletion of Hepatitis A Virus by 35 nm
Nanofiltration

	Human Serum Albumin	Immunoglobulin
Virus-stock titer	$10^{7.9}$	$10^{7.9}$
Virus titer in product	$10^{6.3}$	$10^{5.3}$
Virus titer in concentrate	$10^{5.7}$	$10^{5.9}$
Virus titer in filtrat	$10^{5.7}$	$<10^{0.1}$
Reduction factor	0.6	>5.2

Example 2:

Depletion of Parvoviruses by Anti-Parvovirus-Specific Antibodies and Subsequent Nanofiltration

It was tested whether or not a rabbit antiserum against the parvovirus "minute mouse virus" (MMV, ATCC VR1346) is capable of retaining the virus at filtration over a 35 nm membrane, by forming a complex with this virus.

The rabbit antiserum was obtained by immunising the animals with concentrated, formalin-inactivated MMV preparations and complete Freund adjuvant and a subsequent booster with incomplete Freund adjuvant carried out 4 weeks after primary immunisation. Blood was obtained for the preparation of the serum 4 weeks after the secondary immunisation.



98 ml of a 2% human immunoglobulin solution were spiked with 2 ml of an MMV suspension. Upon addition of 0, 0.005, 0.05, 0.5, 5.0 ml of the anti-MMV serum, each mixture was subjected to filtration under conditions as described in Example 1.

Samples of virus-spiked starting material, concentrate (diluted to original volume) and filtrate were titrated according to the standard TCID₅₀ test, as described in Example 1, except that A9 cells (ATCC CRL6319) were used for virus propagation and an incubation period of 7 days was chosen.

As was to be expected on account of the virus size (diameter 18-24 nm), MMV without addition of antiserum was not retained by the 35 nm filter membrane, but was found again at 100% in the filtrate (R.F., -0.1). Upon addition of 5 ml of MMV-specific antiserum, the virus infectiousness could be completely neutralized so that the virus could be detected neither in the filtrate nor in the concentrate. Yet, also upon addition of non-neutralizing concentrations of the antiserum (0.5 ml), a complete removal of the virus could be attained by filtration (R.F. >6.9), no virus being detectable in the filtrate. Still lower concentrations of the MMV-specific antiserum (0.05 ml) likewise caused MMV to be largely removed by filtration (R.F., 4.4) (Table 2).

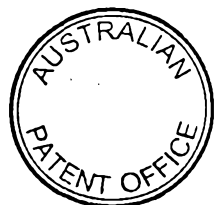


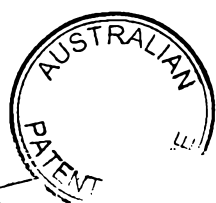
Table 2: Influence of Specific Antibodies on the
Depletion of Parvovirus by 35 nm
Nanofiltration

IgG	98 ml	98 ml	98 ml	98 ml	98 ml
MMV	2 ml	2 ml	2 ml	2 ml	2 ml
Anti-MMV serum from rabbit	0 ml	0.005ml	0.05ml	0.5ml	5 ml
Virus stock titer	$10^{8.2}$	$10^{7.6}$	$10^{7.6}$	$10^{8.1}$	$10^{7.5}$
Virus titer in product	$10^{6.0}$	$10^{5.9}$	$10^{5.3}$	$10^{7.0}$	$10^{5.5}$
Virus titer in concentrate	$10^{6.4}$	$10^{5.8}$	$10^{4.6}$	$10^{3.8}$	$<10^{0.1}$
Virus titer in filtrate	$10^{6.1}$	$10^{4.6}$	$10^{0.9}$	$<10^{0.1}$	$<10^{0.1}$
Reduction factor	-0.1	1.3	4.4	>6.9	>5.4

Example 3:

**Depletion of HCV by Anti-HCV-Antibodies and
Subsequent Nanofiltration**

The possibility to prevent virus passage of hepatitis C virus (HCV) through a 35 nm filter membrane by adding a HCV-specific antibody to the virus suspension was tested. A plasma free from anti-HCV antibody which was highly contaminated with HCV was used as the starting material for the high-titer virus stock. About 250 ml plasma were clarified in a Beckmann



centrifuge (Rotor JA10) for 20 min at 10,000 rpm. The supernatant was subjected to 2.5 hours of ultracentrifugation at 55,000 rpm in a Ti 70 rotor. After the pellet had been resuspended in PBS and pooled (about 10 ml), the HCV genomic copy number was determined in a quantitative PCR analysis, as described by Dorner et al. ((1994), 25. Häophilie-Symposium, Ed. Scharrer & Schramm, pp. 29-44).

95 ml of a 2% human immunoglobulin solution were spiked with 2.5 ml of the concentrated virus stock. 5 ml of a 10% human immunoglobulin solution containing antibodies against HCV, yet no HCV genome equivalents (which had been ensured by quantitative PCR), or 5 ml of PBS, as control, were admixed. Both samples were subjected to a tangential flow filtration under conditions as described in Example 1. In samples of the HCV-spiked starting material, the filtrate and the concentrate which was diluted to the original volume, the HCV gene copy number was determined, as described above. It could be demonstrated that upon addition of anti-HCV-specific immunoglobulin, by filtration over a 35 nm membrane, complete removal of virus from the filtrate was attained (R.F., >3.0), while in the control mixture no substantial depletion of HCV could be attained (R.F.: 1.4) (Table 3).

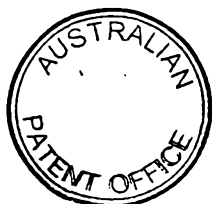


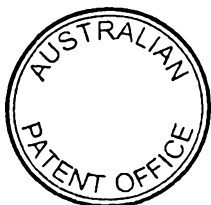
Table 3: Influence of a Specific Antibody on the
Depletion of Hepatitis C Virus by 35 nm
Nanofiltration

IgG	95 ml	95 ml
HCV	2.5 ml	2.5 ml
Anti-HCV IgG	0	5 ml
Virus stock titer (genomic copies/ml)	10^7	10^7
Virus titer in product (genomic copies/ml)	$10^{5.7}$	$10^{5.7}$
Virus titer in concentrate (genomic copies/ml)	$10^{5.3}$	$10^{5.7}$
Virus titer in filtrate (genomic copies/ml)	$10^{4.3}$	$<10^{2.7}$
Reduction factor	1.4	>3.0

Example 4:

**Depletion of HAV by Immunoglobulin Solutions Having
Different Anti-HAV-Antibody Titers**

A series of different immunoglobulin-containing solutions (lots) having HAV antibody titers of from 1.5 units/ml to 11 units/ml, and, as control, a HSA solution containing no HAV-specific antibodies, were spiked with high-titer HAV. Subsequently, the solutions were subjected to a tangential flow filtration, as described in Example 1, at a total volume of 250 ml,



and the virus titers of concentrate and filtrate were determined. The results of the HAV depletion are illustrated in Table 4.

Table 4: Depletion of Hepatitis-A Virus (HAV) by
35 N-Nanofiltration

Lot	P061960A1	P061960A1	P065960A1	Human Serum Albumin.
HAV-specific antibody titer (U/ml)	1.5	1.5	11	negative
	Virus titer (TCID ₅₀ /ml)	Virus titer (TCID ₅₀ /ml)	Virus titer (TCID ₅₀ /ml)	Virus titer (TCID ₅₀ /ml)
Virus-Stock (VS)	10 ^{8.3}	10 ^{7.6}	10 ^{7.6}	10 ^{8.0}
Virus-Stock diluted 1:40 in product	10 ^{5.8}	10 ^{6.1}	10 ^{6.4}	10 ^{6.7}
Concentrate	10 ^{5.6}	10 ^{6.0}	10 ^{6.3}	10 ^{5.7}
Filtrate	<10 ^{0.1}	<10 ^{0.1}	<10 ^{0.1}	10 ^{5.4}
Reduction factor*	>5.7	>6.0	>6.3	1.3

* The reduction factor was determined according to the CPMP guidelines (CPMP/BWP/268/95).



Also in immunoglobulin solutions of lower HAV antibody titers a reduction factor of >6 log steps is possible. With a solution that has an approximately 10-fold higher antibody titer, no improvement of the virus depletion can be attained. Hence follows that irrespective of the specific antibody titer, at least within the tested range a reproducible depletion of HAV is possible.

Example 5:

Virus Depletion Capacity of a HAV-Antibody-Containing Solution

To test the depletion capacity of the antibody-containing solution, the filtrate obtained according to Example 4 was re-spiked with HAV, and the virus depletion capacity of the antibody still present in the filtrate or the reduction factor, respectively, was determined.

In Table 5, the results of the HAV depletion in a re-spiked filtrate from low- and high-titer immunoglobulin solutions are summarized.



Table 5: Depletion of Hepatitis-A Virus (HAV) by
35 N-Nanofiltration after Re-Spiking with HAV

Lot	P061960A1	P061960A1	P065960A1
HAV-specific antibody titer (U/ml)	1.5	1.5	11
	Virus titer (TCID ₅₀ /ml)	Virus titer (TCID ₅₀ /ml)	Virus titer (TCID ₅₀ /ml)
Virus-Stock (VS)	10 ^{8.0}	10 ^{7.6}	10 ^{7.6}
VS diluted 1:40 in combined filtrate	10 ^{5.2}	10 ^{5.4}	10 ^{5.4}
Concentrate	10 ^{5.6}	10 ^{5.6}	10 ^{5.0}
Filtrate	< 10 ^{0.1}	< 10 ^{0.1}	< 10 ^{0.1}
Reduction factor*	> 5.1	> 5.3	> 5.3

* The reduction factor was determined according to the CPMP guidelines (CPMP/BWP/268/95).

It is shown that also in an immunoglobulin-containing starting material of low antibody titer (1.5 U/ml) sufficient antibody is still present to enable once more a depletion of more than 5 log steps. Compared thereto, the virus depletion capacity of a high-titer antibody solution is only slightly increased.

Summing up the depletion rates from the 1st and 2nd virus spiking, a virus depletion with a total reduction factor of at least 11 log steps can be attained.

Example 6:

Depletion of Parvovirus by Means of Anti-Parvovirus-Antibody-Containing Immunoglobulin Solution



A series of immunoglobulin preparations (lots) was tested for parvovirus-B19-specific antibodies. In the different lots, an antibody titer of from 1:400 to 1:800 was found by means of ELISA.

Two different lots having a slight B19-specific antibody titer of 1:400 were spiked with a high-titer virus stock of parvovirus B19, and the titers of both solutions were determined by means of quantitative PCR. The determination by means of quantitative PCR was effected according to the method described in EP-0 714 988 by means of the parvovirus-specific primer pair:

Parvo + 1353/FAM: 5' GGGGCAGCATGTGTTAAAGTGG 3'

bp 1353-1374*

Parvo - 1529 : 5' CCTGCTACATCATTAAATGGAAAG 3'

bp 1529-1506*

* Numbering according to the master sequence PARPVBAU (EMBL data bank)

Plasmid pParvo-wt, containing the parvovirus-specific sequences of nt 1127-1550 (according to the numbering of Shade et al. (1986), J. Virol. 58:921) was used as verifier. Plasmid pParvo-15 and plasmid pParvo+21 were used as internal standards. pParvo-15 was produced by deletion of a 35 bp fragment between nt 1455-1489 and insertion of a ds oligonucleotide generated from the primers 5' GTT CCA GTA TAT GGC ATG GTT 3' and 5' ACC CAT GCC ATA TAC TGG AAC 3'. pParvo+21 was obtained by duplication of the bp 1468-1487 between



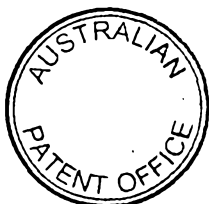
nt 1453 and 1454. After amplification with the parvovirus-specific primers, the PCR products had a respective length of 177 bp (wt), 162 bp (-15) or 198 bp (+21).

Each one of the mixtures was subjected to filtration under conditions as described in Example 1. In samples of the virus-spiked starting material, the concentrate (diluted to the original volume), and the filtrate, the titers were determined by means of a quantitative nucleic acid amplification method (PCR). The results of the titer determinations are summarized in Table 6. An excellent depletion of about 6 log steps was attained.

Table 6: Depletion of B19 Parvovirus by 35 N-Nanofiltration

Lot	P061960A1	P065960A1
B19-specific antibody titer (U/ml)	1:400	1:400
	titer (genomic copies/ml)	titer (genomic copies/ml)
Virus-Stock (VS)	$10^{11.6}$	$10^{11.0}$
VS diluted 1:40 in product	$10^{8.8}$	$10^{8.6}$
Concentrate	$10^{8.9}$	$10^{8.5}$
Filtrate	$<10^{2.7}$	$<10^{2.7}$
Reduction factor*	>6.1	>5.9

* The reduction factor was determined according to the CPMP guidelines (CPMP/BWP/268/95).



Example 7:

**Virus Depletion Capacity of the Anti-Parvovirus
B19-Antibody-Containing Solution**

To test the depletion capacity of the antibody-containing solution, the filtrate obtained according to Example 6 was re-spiked with parvovirus, and the virus depletion capacity of the antibodies still present in the filtrate or the reduction factor, respectively, was determined.

In Table 7, the results of the parvovirus depletion in a re-spiked filtrate from an immunoglobulin-containing solution having a 1:400 antibody titer are summarized.



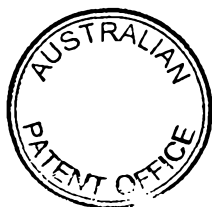
Table 7: Depletion of B19-Parvovirus by 35 N Nano-filtration and Re-Spiking with Parvovirus

Lot	P061960A1	P065960A1
B19-specific antibody titer (U/ml)	1:400	1:400
	titer (genomic copies/ml)	titer (genomic copies/ml)
VS diluted 1:40 in combined product	$10^{8.2}$	$10^{8.4}$
Concentrate	10^{10}	$10^{10.1}$
Filtrate	$10^{3.2}$	$10^{3.4}$
Reduction factor*	5.0	5.0

* The reduction factor was determined according to the CPMP guidelines (CPMP/BWP/268/95).

It has been shown that in the filtrate sufficient antibodies were still present to attain a further depletion by 5 log steps. Summing up the depletion rate and the reduction factor after the 1st and 2nd spiking, a reduction factor of at least 11 log steps is attained by the method according to the invention, which corresponds to a complete removal of pathogens from the biological material.

Hence follows that the system is suitable for industrial application and ensures that protein-containing solutions free from infectious pathogens can



be obtained.

Example 8:

**Depletion of Parvovirus with Filters of Different
Exclusion Sizes**

In Example 1 it could be demonstrated that already by adding a non-neutralizing concentration of antiserum and subsequent nanofiltration over a 35 nm membrane, a complete removal of pathogens is achieved. To test whether filters having a higher exclusion size are also suitable for the depletion of antigen-antibody complexes, a series of deep bed filters having a nominal exclusion size of between 0.04 μm and 3 μm were tested for their ability of retaining parvovirus MMV, "minute mouse virus". 196 ml of a 2% IgG solution were spiked with 4 ml of a MM virus titer preparation and subsequently admixed with 1 ml of rabbit-anti-MMV serum. A spiked gammaglobulin solution without specific anti-MMV serum was used as control. The mixtures were filtered via different deep bed filters having nominal exclusion sizes of 3 μm , 0.5 μm , 0.3 μm and 0.04 μm with a flow rate of 50 ml/min until the end. The virus titers were determined both in the starting material and in the filtrate. The results are summarized in Table 8.





Table 8: Depletion of Parvovirus MMV by Conventional Deep Bed Filtration of Gammaglobulin

	Nominal Filter Exclusion Size							
	3 μ	3 μ	0.5 μ	0.5 μ	0.3 μ	0.3 μ	0.04 μ	0.04 μ
Rabbit anti-MMV Serum	-	+	-	+	-	+	-	+
Virus Stock Titer	10 ^{8.7}	10 ^{8.3}	10 ^{8.7}	10 ^{8.0}	10 ^{8.3}	10 ^{8.7}	10 ^{8.3}	10 ^{8.1}
Virus titer in product	10 ^{7.1}	10 ^{5.5}	10 ^{7.6}	10 ^{5.4}	10 ^{7.0}	10 ^{5.3}	10 ^{7.0}	10 ^{5.2}
Virus titer in filtrate	10 ^{6.3}	<10 ^{0.1}	10 ^{5.7}	<10 ^{0.1}	10 ^{5.3}	<10 ^{0.1}	10 ^{3.7}	10 ^{0.1}
Reduction factor	0.8	>5.4	1.9	>5.3	1.7	>5.2	3.3	>5.1

It was shown that with the method according to the invention, even with filters having an exclusion size of 3 μ m a depletion by more than 5 log steps is possible, while without an addition of ligand which complexes the pathogens only with a filter of about 0.4 μ m a significant reduction factor was attained.

Example 9:

Effect of Rheumatoid Factor on the Depletion of Poliovirus

To enable assaying the influence of the addition of agglutinins, which increase the size of the complex, on the efficiency of depletion, rheumatoid factor was admixed to the spiked immunoglobulin solution as agglutinin or conglutinin. 85 ml of a 2% immunoglobulin solution were spiked with 5 ml of a high-titer poliovirus type 1-containing preparation. 10 ml of a preparation containing human rheumatoid factor with a titer of 800 U/ml were added to the mixture. 10 ml of buffer were added instead of the rheumatoid factor, as control. Both mixtures were filtered over a 35 N membrane, as described in Example 1, and the virus titers of the concentrate and of the filtrate were determined. The virus titer determination was effected by a TCID₅₀ determination on VERO cells.

The results of the determination as well as the reduction factor found with and without the addition of rheumatoid factor are illustrated in Table 9.

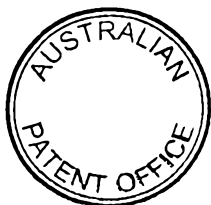


Table 9: Effect of Rheumatoid Factor on the Depletion of Poliovirus from an Immunoglobulin Solution and Nanofiltration

IgG volume (ml)	85	85
Rheumatoid Factor (ml)	-	10
Buffer (ml)	10	-
Poliovirus (ml)	5	5
Virus stock titer	$10^{10.1}$	$10^{10.4}$
Virus stock titer in product	$10^{8.3}$	$10^{8.2}$
Virus titer in concentrate	$10^{9.0}$	$10^{9.3}$
Virus titer in filtrate	$10^{5.2}$	$<10^{0.6}$
Reduction factor	3.1	>7.6

The results demonstrate that rheumatoid factor in combination with the specific anti-poliovirus antibodies complex the virus in a manner that it can be effectively retained by nanofiltration over a 35 N filter. If no further agglutinating agent (rheumatoid factor) is admixed, a virus depletion with a reduction factor of 3.1 log steps is attained, which can be increased in the presence of the rheumatoid factor to a reduction factor of >7.6 . Thus, an effective and substantially complete separation of small, non-enveloped viruses from a protein solution has been possible for the first time.

Example 10:

Effect of Agglutinins or Conglutinins, Respective-



**ly, on the Depletion of Poliovirus by means of
Conventional Deep Bed Filtration**

The ability of rheumatoid factor to influence the depletion of high-titer poliovirus when using conventional deep bed filters was determined. In doing so, a filter type was used which had proven to be ineffective in the depletion of poliovirus, even in the presence of poliovirus-specific antibodies. For this, 85 ml of a 2% gammaglobulin solution were spiked with 5 ml of a high-titer poliovirus stock solution, and 10 ml of a solution of rheumatoid factor having a titer of 800 units/ml were admixed. As control, 10 ml of buffer were admixed instead of rheumatoid factor. Both mixtures were filtered through a Seitz BKS-P deep bed filter at a flow rate of 50 ml/min. The virus titer of the starting material was determined before and after filtration. The results are summarized in Table 10. It has been shown that merely by the addition of a further aggregating agent, such as rheumatoid factor, poliovirus could be efficiently depleted by using a conventional deep bed filter. This method results in a depletion with a reduction factor of >7.7 log steps, while without the addition of rheumatoid factor, merely a reduction of approximately 1.1 log steps was achieved. This clearly demonstrates that by the method of the invention, when admixing lectins or lectin-like proteins, such as conglutinins, even when using



conventional deep bed filters, a substantially complete separation of the pathogens is achieved.

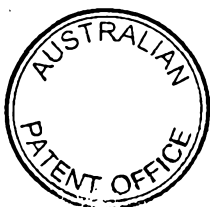
Table 10: Effect of Rheumatoid Factor on the Depletion of Poliovirus from a Gammaglobulin Solution by Conventional Deep Bed Filtration

IgG volume (ml)	85	85
Rheumatoid Factor	-	10
Buffer (ml)	10	-
Polio virus (ml)	5	5
Virus stock titer	$10^{10.6}$	$10^{10.5}$
Virus titer in product	$10^{8.0}$	$10^{8.3}$
Virus titer in filtrate	$10^{6.9}$	$<10^{0.6}$
Reduction factor	1.1	>7.7

Example 11:

Determination of the Activity of Factor VIII and vWF Before and After Virus Depletion

The activity of two plasma factors was determined before and after virus depletion with the method according to the invention. For this, 100 ml of a factor VIII/vWF-complex-containing solution were filtered through a Cuomo ZA 90 deep bed filter with a flow rate of 50 ml/min. In samples of the starting material and of the filtrate, the factor VIII activity and the vWF antigen content were determined, and a vWF multimer analysis was carried out. In a parallel



experiment, the same material was spiked with mouse minute virus (MMV), and specific anti-MMV-antiserum was admixed prior to filtration. The virus titer was determined before and after filtration in the starting material and in the filtrate. The results are summarized in Table 11 and demonstrate that under these conditions a depletion by >6.5 log steps is achieved. The factor VIII activity and the vWF antigen content, respectively, remain substantially unaffected by the method. Likewise it could be demonstrated that the vWF multimer pattern remains substantially unchanged (data not indicated).

Table 11: Determination of the Factor VIII and vWF Activities before and after Filtration

	FVIII-Activity (I.U./ml)	vWF-Antigen (μ g/ml)	log 10 MMV-Titer (TCID ₅₀ /ml)
Starting material	3.3	173.5	6.6
Filtrate	2.8	143.2	<0.1

Example 12:

Determination of the Activity of Blood Factors from a Cryo-Supernatant after Filtration

A cryo-supernatant of human plasma was spiked with a high-titer poliovirus preparation and subjected to deep bed filtration with a flow rate of 50 ml/min. The activities of factor VII, factor IX, antithrombin III (ATIII) and C1-esterase inhibitor were determined



before and after filtration. The results are summarized in Table 12 and demonstrate that with the described method a virus depletion of >7 log steps is achieved and the activity of the blood factors is not adversely affected.

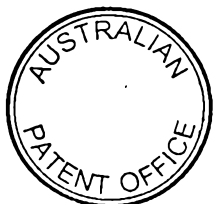
Table 12: Determination of the Activities of
Factor VII, Factor IX, ATIII and C1-Esterase
Inhibitor, before and after Filtration

Virus titer (TCID ₅₀ /ml)		Activity (IU/ml)			
		FVII	FIX	AT-III	C1-Inhibitor
Before Filtration	10 ^{7.5}	0.83	0.75	0.92	0.76
After Filtration	<10 ^{0.7}	0.80	0.76	0.95	0.76



THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A biological material free from specific pathogens, obtained by a process comprising the steps of
- 5 (a) reacting a ligand or receptor present in a biological material with a receptor or ligand of a specific pathogen in solution, whereby a ligand/receptor complex forms, and
- (b) separating the ligand/receptor complex by a
- 10 method which allows for the separation of the complexed pathogen from the biological material, and after which separation the biological material comprises antibodies in free form.
- 15 2. A biological material obtained by a process according to claim 1, wherein said process further comprises the step of admixing said biological material with at least one ligand or receptor which reacts with a receptor or ligand of the specific pathogen.
- 20 3. A biological material obtained by a process according to claim 1 or claim 2, wherein said ligand or receptor is present in an antibody, and said ligand or receptor of the pathogen is an antigen, whereby an
- 25 antibody-antigen complex is formed as a ligand-receptor complex.
4. A biological material obtained by a process according to any one of claims 1 to 3, wherein step (b) is
- 30 effected by the use of a permeable filter.
5. A biological material obtained by a process according to any one of claims 1 to 3, wherein step (b) is effected by sedimentation.
- 35 6. A biological material obtained by a process according to claim 5, wherein the sedimentation is effected by density gradient centrifugation.



7. A biological material obtained by a process according to any one of claims 1 to 6, comprising the additional step of improving the separation of the ligand/receptor complex by further aggregating the
5 ligand/receptor complex using an agent selected from the group consisting of an agglutinin, a complement component, a conglutinin, a rheumatoid factor or a non-toxic, water-soluble, synthetic polymer.

10 8. A biological material obtained by a process according to claim 7, wherein the agglutinin is a lectin.

9. A biological material obtained by a process according to claim 7, wherein the water-soluble, synthetic
15 polymer is polyethylene glycol.

10. A biological material according to any one of claims 1 to 9, which is free from viral and molecular pathogens and from aggregates or complexes of the
20 pathogens.

11. A biological material according to claims 7 to 9, wherein said pathogen is a HAV, HBV, HCV, HIV, HEV, HDV, HGV, CMV and/or parvovirus.
25

12. A biological material according to any one of claims 1 to 10, comprising one or more antibodies specific for a virus selected from the group consisting of HAV, HCV, HBV, and parvovirus.
30

13. A biological material according to any one of claims 1 to 11, comprising one or more materials selected from the group consisting of a plasma fraction, a plasma-protein-containing fraction containing blood factors, a concentrate comprising one of said blood factors, an immunoglobulin-containing plasma fraction, a supernatant of a hybridoma cell line, a cell culture supernatant of
35



transformed or infected mammalian cells, and an extract from an animal or human tissue.

14. A biological material according to claim 13,
5 wherein said blood factors are selected from the group consisting of factor II, factor VII, factor VIII, factor IX, factor X, factor XI, protein C, protein S and vWF.

15. A method of depleting viral and molecular
10 pathogens and recovering biological material which is free from pathogens, comprising the steps of

(a) reacting a receptor or ligand present in a biological material with a receptor or ligand of a pathogen in solution, whereby a ligand/receptor complex of a
15 complexed pathogen is formed, and

(b) separating the ligand/receptor complex by a method which permits the separation of the complexed pathogen from the biological material and the recovery of a pathogen-free biological material.

20

16. A method according to claim 15, further comprising the step of admixing said biological material with at least one ligand or receptor which reacts with a receptor or ligand of the specific pathogen, to form a
25 ligand/receptor complex.

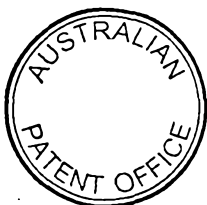
17. A method according to claim 15 or claim 16, wherein the ligand/receptor complex has a higher density or a higher sedimentation coefficient than the free pathogen.

30

18. A method according to claim 15 or claim 16, wherein the ligand/receptor complex has a higher degree of aggregation than the free pathogen.

35

19. A method according to any one of claims 15 to 18, wherein the separation of the ligand/receptor complex in step (b) is effected by filtration through a permeable



filter.

20. A method according to claim 19, wherein the
ligand/receptor complex is selectively retained by the
5 filter.

21. A method according to claim 19 or claim 20,
wherein said filter is a nanofilter.

10 22. A method according to claim 19 or claim 20,
wherein said filter is a deep bed filter.

23. A method according to any one of claims 15 to 18,
wherein the separation of the ligand/receptor complex in
15 step (b) is effected by sedimentation.

24. A method according to claim 23, wherein the
sedimentation of the complex is effected by density
gradient centrifugation.

20

25. A method according to any one of claims 15 to 24,
wherein the ligand or receptor of the pathogen is one or
more agents selected from the group consisting of an
antigen, an epitope and an antigenic determinant.

25

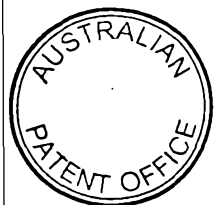
26. A method according to any one of claims 15 to 24,
wherein the receptor which reacts with the ligand or
receptor of the pathogen is selected from the group
consisting of an agglutinin, an antibody, and a fragment of
30 an antibody which is still capable of binding to the
pathogen.

30

27. A method according to any one of claims 15 to 26,
wherein the ligand/receptor complex is an antibody/antigen
35 complex.

35

28. A method according to any one of claim 15 to 27,



further comprising the step of aggregation of said complex using an agent selected from the group consisting of an agglutinating agent, a complement component, a conglutinin, a rheumatoid factor, a non-toxic, water-soluble, synthetic polymer, and albumin.

29. The method according to claim 28, wherein said agglutinating agent is a lectin.

30. The method according to claim 28 or claim 29, wherein said agglutinating agent is selected from the group consisting of Concanavalin A, Ricin and Phasin.

31. The method according to claim 28, wherein said non-toxic, water-soluble, synthetic polymer is polyethylene glycol.

32. A method according to any one of claims 28 to 31, wherein said additional step of further aggregation achieves a higher complex density, whereby the complex is removed with a higher efficiency from the biological material.

33. A method according to any one of claims 15 to 32, wherein the viral or molecular pathogen is selected from the group consisting of lipid-enveloped viruses, non-lipid-enveloped viruses and prions.

34. A method according to claim 33, wherein said pathogen is selected from the group consisting of HAV, HBV, HCV, HIV, HEV, HDV, HGV, CMV and parvovirus.

35. A method according to any one of claims 15 to 34, wherein said ligand contained in a biological material is an antibody obtained from a hyperimmunoglobulin solution or from a supernatant of a hybridoma cell line.



36. A method according to claim 35, wherein said antibody is subjected to a virus inactivation and/or virus depletion method, and the antibody is optionally enriched and used as ligand.

5

37. A method according to any one of claims 15 to 36, wherein said ligand contained in a biological material is an anti- β -amyloid antibody.

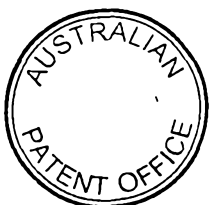
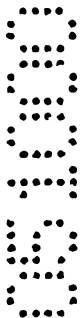
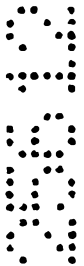
10 38. A method according to claim 37, wherein said antibody reacts with a prion and forms a complex.

39. A method according to any one of claims 15 to 38, wherein said ligand contained in a biological material is
15 an antibody selected from the group consisting of an antibody specific to HAV, HBV, HCV, HDV, CMV, HIV, HGV, HEV, and parvovirus.

40. A method according to any one of claims 15 to 39,
20 wherein said biological material is selected from the group consisting of a plasma fraction, a plasma-protein-containing fraction containing blood factors, a concentrate containing a blood factor, an immunoglobulin-containing plasma-fraction, a supernatant of a hybridoma cell line, a
25 cell culture supernatant of transformed or infected mammalian cells and an extract from an animal or human tissue.

41. A method according to claim 40, wherein said
30 blood factors are selected from the group consisting of factor II, factor VII, factor VIII, factor IX, factor X, factor XI, protein C, protein S and vWF.

42. A method according to any one of claims 15 to 41,
35 wherein said biological material comprises high-molecular proteins having a molecular weight of >150 kD.



43. A method according to any one of claims 19 to 42, wherein said biological material freely passes the permeable filter and the ligand/receptor complex is retained.

5

44. A method according to claim 43, comprising the further step of assaying said biological material obtained for the presence of a ligand.

10 45. The method of claim 44, wherein said ligand is an antibody.

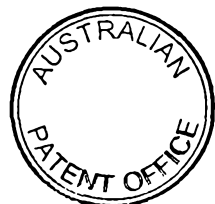
46. A method according to claim 45, comprising the further steps of admixing to the biological material a
15 known amount of a specific viral or molecular pathogen which has specific ligands or receptors for the antibody, then filtering the biological material comprising the antibody/pathogen complex again over a permeable filter, and then determining the residual amount of pathogen in the
20 filtrate.

47. A method according to any one of claims 15 to 46, wherein a virus-depleted biological material, which is free from target viruses, is recovered, wherein a reduction
25 factor of at least 7 log steps is achieved.

48. A method according to any one of claims 15 to 47, wherein the complete separation of the pathogen from the biological material is effected in combination with
30 determining the depletion rate.

49. A method according to claim 48, comprising the additional step of determining the genome equivalent of the pathogen.
35

50. The use of a virus-inactivated immunoglobulin solution as ligand in a method according to any one of



claims 15 to 48.

51. The use of a method according to any one of
claims 15 to 48, for preparing a biological material which
5 is free from specific pathogens as well as free from
aggregates or complexes of a pathogen.

52. A biological material according to claim 1,
substantially as herein described with reference to the
10 examples.

53. A method according to claim 15, substantially as
herein described with reference to the examples.

15 Dated this 5th day of October 2000

IMMUNO AKTIENGESELLSCHAFT

By their Patent Attorneys

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20 Trade Mark Attorneys of Australia

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