

The invention concerns a method for carrying out a homogeneous immunoassay based on agglutination in which a conjugate of a Fab' fragment and a component K is used.

Very many substances occur in body fluids and tissues which are capable of binding to a specific binding partner and which serve as parameter for certain diseases or the state of health of the human body. These include on the one hand immunologically active proteins which have binding sites on their surface such as e.g. tumour markers, hormones or viral proteins and on the other hand, DNA fragments. Since these substances often only occur in very small amounts, methods based on immunoassays are used for their detection with which these substances can be determined very specifically and exactly. There are many variants for this. The various immunological methods of determination may be classified into homogeneous and heterogeneous methods. A solid phase reaction always forms part of the heterogeneous method in order to immobilize complexes which contain the substance to be detected and a labelled component and thus to separate them from unbound components. In the homogeneous method variant there is no separation of bound label and unbound label so that bound and unbound label have to be differentiated by other methods.

There are different methods for this.

A method for the detection of proteins is known from DE-OS 27 49 956 which is based on the evaluation of an

agglutination reaction. In this method antibodies against the substance to be detected are bound directly to the agglutinable particles. However, the reactivity of the antibodies can be impaired by this binding. In addition such a method of determination is susceptible to interference by rheumatoid factors.

A homogeneous method of detection is described in EP-A 0 356 964 which already overcomes the disadvantages of the previously known methods. In this case the sample solution is incubated with at least two receptors which are capable of binding to one another and of which one can bind to the substance to be detected. In this method an agglutination can only take place when the substance to be detected binds to both receptors but not, however, when the receptors bind to one another or the substance to be detected binds to only one of the two receptors. Conjugates consisting of one partner of a pair which specifically bind to one another and a component K capable of specific binding to the substance to be detected are used as the receptors capable of binding to the substance to be detected. If the method described here is used for the detection of proteins which have several identical epitopes then a conjugate has to be used as the receptor capable of binding to the substance to be detected which only has a single binding site for the substance to be detected in order to prevent an unspecific agglutination.

The production of papain-free $F(ab')_2$ antibody fragments which are obtained from a complete IgG antibody cleaved with papain is described in EP-A-0 307 754. Fab' antibody fragments with free sulfhydryl groups in the hinge region are obtained from the $F(ab')_2$ antibody fragments.

The object of the present invention was to provide a method for carrying out homogeneous immunoassays in which unspecific agglutinations are avoided as far as possible in order that exact and reproducible results are obtained.

This object is achieved by a method for carrying out a homogeneous immunoassay based on agglutination in which a conjugate of a Fab' fragment and a component K is used, which is characterized in that a conjugate is used which is obtained by subjecting a $F(ab')_2$ fragment of an antibody of the immunoglobulin G class to reducing conditions and subsequently reacting it with the component K which either has a functional group suitable for the binding or was derivatized in a suitable manner by introduction of a functional group whereby the component K is bound via the functional group to the free SH group of the Fab' fragment formed during the reduction and agglutinable particles are used which carry a substance capable of binding to K.

Surprisingly it was found that unspecific agglutinations can be avoided to a large extent by using these conjugates. Methods based on agglutination immunoassays could be further improved with regard to accuracy and reproducibility by using the monovalent binding partners.

Conjugates consisting of a Fab' fragment of an IgG antibody which is capable of specifically binding to the substance to be detected and a component K are used for the method according to the present invention. Such conjugates can be produced by treating the antibody which is to be used in each case with pepsin in a known

way. In this process fragments denoted $F(ab')_2$ are formed which have two paratopes and in which the two heavy chains are held together by disulfide bridges. The $F(ab')_2$ fragments are then subjected to mild reducing conditions so that the intramolecular disulfides are preferentially cleaved in the hinge region but not, however, the disulfide bridges between the light and heavy chain. Therefore, the reduction is preferably carried out with a mild reagent. Cysteamine, cysteine, mercaptoethanol or boron hydride are e.g. suitable for this. In this way Fab' fragments are obtained which have 1 to 3 free SH groups. All free SH groups are available for binding to the components used. It has, however, turned out that, because of steric hindrance, either one component is bound or only one of two or more bound components retains its activity.

A component K which is a partner of a specific binding pair is bound to these Fab' fragments. The component K can be bound to the Fab' fragment either via a functional group which is already present or which is introduced or which is activated, if desired. The binding either takes place directly between SH groups of the Fab' fragment and a functional group of the component K or via a spacer.

The binding of the Fab' fragment and component K can take place directly via the functional groups present in each case. In this form of the method the proportion of unspecific linkages is particularly small. It is also possible to use a spacer for the binding. The length of the spacer depends in this case on the position of the free SH groups which is in turn dependent on the subclass of the IgG antibody used. It was found that up to three component molecules can be bound but that

because of steric hindrance only one of them is capable of binding to the partner of the specific binding pair.

Bifunctional compounds which have a functional group which is capable of binding to the SH group and a second functional group which can be the same or different and which can covalently bind to the component are suitable as the spacer. The length of the spacer depends on the position of the SH bonds and thus on the subclass of the antibody from which the fragment is derived. If the spacer is too long it has the effect that even when several SH groups are relatively close together, a binding to all components is possible since the steric hindrance is overcome. The length of the spacer is therefore dependent on the position of the SH bonds and is thus defined by the Fab' fragment used and is in the range of 4 to 16 atoms preferably 4 to 8 atoms for Fab' fragments of the IgG subclass I, and in the range 4 to 10 atoms, preferably 4 to 6 atoms for Fab' fragments of antibodies of the subclass IgG II. Maleimidobutyllysine is preferably used for the production of conjugates consisting of a component K and a Fab' fragment of an IgG antibody of the subclass I and maleimidoethylamine is preferably used for conjugates containing Fab' fragments of the IgG subclass II.

The conjugate of Fab' fragment and component K is used in a known manner as the receptor when carrying out a homogeneous immunoassay. In the method according to the present invention agglutinable particles which carry a substance capable of binding to the component K are used as an additional receptor. In a preferred embodiment agglutinable particles are used to which a substance capable of binding to the component K is covalently bound by means of a bifunctional photoreactive spacer.

Such agglutinable particles can be produced by first reacting the substance capable of binding to the component K with a heterobifunctional reagent which has an arylazide group on one side and a functional group which is reactive with amino groups of the binding partner on the other side. In this process this group which is reactive with amino groups reacts with the bindable substance. After removing non-reacted reagent, the biological material is brought into contact with the inert carrier material which can for example be a polystyrene latex with a particle size below 100 nm, for a pre-determined period and then the photoreaction is triggered by the action of light whereby the binding to the inert carrier takes place via formation of a reactive nitrene from the arylazide group. Subsequently non-covalently bound material is effectively detached by washing with detergent and removed by centrifugation procedures or dialysis on membranes. The surface properties of such particles can be specifically modified by e.g. applying an immunologically inert protein such as e.g. BSA onto these particles by pre-, co- or recoating. The stability of the reagent is also increased by this means. The proportion of specifically bindable substance per particle is defined by the amount of protein as well as the latex concentration used for the coating and can be adjusted reproducibly. In addition a surface with a defined specific bindability can be regulated by selection of the heterobifunctional reagent or its chain length. An advantage of this process is that the specifically bindable substance can be bound to any type of solid phase i.e. it does not rely on solid phase particles with functional groups. Homopolymers and copolymers of styrene and methylstyrene, of acrylic acid and its esters, of methacrylic acid and its derivatives such as

acrylonitrile or acrylamide and of dienes such as butadiene, chloroprene or isoprene and vinyl chloride are particularly suitable as the solid phase. Undesired latex-latex linkages virtually do not arise because of the type of linkage but rather only the covalent linkage of latex and protein occurs as a result of the unspecific reaction of the azido group and to a slight extent a linkage of protein with protein.

The substance bound to the agglutinable particles and the component K are capable of binding to one another. All pairs which specifically bind to one another are suitable for this. Streptavidin or avidin and biotin are preferably used as component K and thus as the bindable substance.

When the method is carried out, i.e. when the sample solution is incubated with the conjugate and with the agglutinable particles, on the one hand the substance to be detected binds to the Fab' fragment of the conjugate and on the other hand the component K of the conjugate binds to the agglutinable particles. An agglutination can only be triggered by these linkages. Since the conjugate is monovalent with respect to binding to the substance to be detected and with respect to binding to the agglutinable particles, an agglutination cannot take place via the receptors per se. The more substance to be detected present in the sample solution, the greater is the agglutination. The amount of agglutination, which can be detected according to known methods, is therefore a direct measure of the content of substance to be detected.

The method according to the invention is particularly

suitable for embodiments as described in EP-A 349 988 or EP-A 356 964. They describe homogeneous immunoassays in which a sample solution containing the substance to be detected is incubated with at least two receptors, one of which is a conjugate of a partner of a specific binding pair and a component which binds specifically to the substance to be determined and the other receptor has at least two binding sites for the specific binding partner. Incubation of the sample solution with the two receptors results in binding of the conjugate to the substance to be detected and to the other receptor which causes an agglutination. Only those complexes in which the substance to be detected and both receptors are bound can agglutinate. In order to increase the accuracy, it is preferable to use a conjugate with monovalent components with respect to the binding.

The invention is elucidated by the following examples:

Example 1

Preparation of streptavidin-latex

Streptavidin (Boehringer Mannheim) was dissolved at a concentration of 10 mg/ml in 50 mM K_2HPO_4 , pH 8.0. Subsequently a solution of N-hydroxysuccinimidyl-4-azidobenzoate (HSAB) in DMSO was added in the dark such that a molar ratio of 10:1 (HSAB:streptavidin) results. It was incubated at 25°C for two hours in the dark. Subsequently excess HSAB was removed by dialysis in the dark.

In order to coat the latices with streptavidin, a 1 % suspension of polystyrene particles (Dow < 100 nm) was

incubated overnight in the dark with the activated streptavidin (1 mg/ml, production see above). Subsequently the particles were irradiated with UV light (340 nm). The streptavidin lattices were washed twice with 50 mM glycine, pH 7.5 and then incubated for 16 hours with a recoating solution (0.1 % BSA). After a further washing step with 50 mM glycine, pH 7.5 the streptavidin lattices were stored in glycine buffer at 4°C.

Example 2

Production of Fab'-biotin conjugates

Monoclonal antibodies of the immunoglobulin class G I and G IIa, which were obtained in the usual way by immunization of mice, were converted into Fab'-biotin conjugates according to the method described in the following:

50 mg mouse IgG was dissolved in 100 mM sodium citrate, pH 3.6 to a protein concentration of 10 mg/ml. A pepsin solution (1 mg/ml in 100 mM sodium citrate, pH 3.6) was added in a ratio 1:1000 to the solution which was heated to 37°C. After stirring for 2.5 hours at 37°C the pH value of the reaction mixture was adjusted to 8.3 by addition of Tris. Subsequently it was dialyzed against 20 mM Tris/HCl, pH 7.8. The separation of undigested IgG and Fc fragments from F(ab')₂ was carried out by chromatography on DEAE Sepharose® fast flow and Sephacryl® S-200. The F(ab')₂ fragments were collected and lyophilized.

10 mg F(ab')₂ was dissolved in 100 mM KH₂PO₄, 1 mM EDTA,

pH 6.3 at a concentration of 10 mg/ml. Subsequently 20 μ l of a solution of 500 mM sodium arsenite, 1 mM EDTA, 100 mM KH_2PO_4 , pH 6.8 as well as 25 μ l of a solution of 200 mM mercaptoethylamine, 1 mM EDTA, 100 mM KH_2PO_4 , pH 6.8 were added. It was incubated at 37°C for 2 hours while stirring. Subsequently the Fab' pool was separated from lower molecular components by chromatography on a Sephadex® G-25 column.

Maleimidoethylamine biotin and maleimidobutyryllysine-biotin were each dissolved at a concentration of 1 mg/ml in DMSO for the biotinylation. In separate preparations maleimidoethylamine biotin or maleimidobutyryllysine-biotin were added to a solution of the Fab' fragments in such a way that a molar ratio of 10:1 (biotin derivative: Fab') resulted. After incubating for one hour at 25°C cysteine was first added to a final concentration of 1 mM and after a further 30 minutes iodoacetamide was added to a final concentration of 5 mM. After a further incubation of 30 minutes it was dialyzed against 2 mM KH_2PO_4 , pH 7.5. The Fab'-biotin conjugates obtained were stored at -20°C.

Example 3

It was investigated to what extent the unspecific agglutination is reduced by using the conjugates according to the invention in a test using latex particles coated with streptavidin and conjugates of anti-TSH antibody fragments and biotin.

A monoclonal anti-TSH antibody of the immunoglobulin class G I (ECACC 87122202) was used. For comparison a complete antibodies was biotinylated via the amino

groups of the antibody according to JACS 100 (1978), 3585-3590 by reaction with N-hydroxysuccinimide-X-biotin in a ratio of 7:1 (biotin:IgG).

The Fab'-biotin conjugate according to the invention was produced from this antibody using maleimidobutyryl-lysine-biotin as described in Example 2.

In order to determine the agglutination, 60 μ l 1 % streptavidin-latex (production see Example 1), 800 μ l 100 mM Tris/HCl, pH 8.0 and 20 μ l anti-TSH-IgG-biotin conjugate (comparison) or 20 μ l anti-TSH-Fab'-biotin conjugate (according to the invention) were pipetted into cuvettes of a Perkin-Elmer photometer. The protein concentration of the conjugates was in each case 10 μ g/ml.

The increase in absorbance at 405 nm was measured between t=1 min and t=10 min.

The result is shown in Table 1. It showed that the unspecific agglutination could be reduced to less than 1/5 by using the Fab'-biotin conjugate according to the present invention of the reference value (IgG-biotin conjugate).

Table 1

Antibody conjugate	Increase in absorbance (mA)
IgG-biotin (comparison)	663
Fab'-biotin (according to the present invention)	119

Example 4

The unspecific agglutination of conjugates produced according to the invention was examined as described in Example 3.

Two different monoclonal anti-myoglobin antibodies, which both belong to the immunoglobulin class IgG I, were converted into Fab'-biotin conjugates as described in Example 2. In order to investigate the influence of the spacer length on the unspecific agglutination, both Fab' fragments were reacted with biotin using maleimidoethylamine (MEA) and maleimidobutyryllysine (MBL) as spacer.

The results are summarized in Table 2. It is clear that the unspecific agglutination can be reduced even further by using the shorter MEA spacer.

Table 2

Antibody conjugate (Fab'-biotin)	Increase in absorbance (mA)	
	MBL	MEA
Anti-myoglobin-Ab 1	267	88
Anti-myoglobin-Ab 2	228	72

Example 5

Two different monoclonal anti-AFP antibodies, one of which belongs to the immunoglobulin class G I (Mab LJ738) and the other to the class G IIa (Mab Tu11, ECACC 87041002), were converted into Fab'-biotin conjugates as described in Example 2. In this process both Fab' fragments were biotinylated with biotin using MEA or MBL as spacer.

In order to determine the unspecific agglutination, 20 μ l of each of the different conjugates (10 μ g/ml), 800 μ l 100 mM Tris/HCl, 2 % PEG 40000, 2 % Pluronic[®] F68, pH 7.0 and 20 μ l 1 % streptavidin-latex (production see Example 1) were pipetted into the cuvettes of a Perkin-Elmer photometer. The increase in absorbance was measured at 405 nm between t=1 min and t=10 min.

The results are shown in Table 3.

As already measured for the other monoclonal antibodies of the immunoglobulin class I (cf. Examples 3 and 4), a low unspecific agglutination was also obtained with the anti-AFP-Fab'-biotin from IgG I which could be suppressed even further by using the shorter MEA spacer.

In contrast the anti-AFP-Fab'-biotin conjugate from the IgG IIa resulted in a relatively high unspecific agglutination with the MBL spacer. However, more favourable results could be obtained by using the shorter MEA spacer.

Table 3

Antibody conjugate (Fab'-biotin)	Increase in absorbance (mA)	
	MBL	MEA
Anti-AFP-Mab-LJ738 (IgG I)	42	3
Anti-AFP-Mab-Tu11 (IgG IIa)	434	212

Example 6

A homogeneous immunoassay based on streptavidin-latex was carried out with the conjugates Mab-Tu11-Fab'-MEA-biotin and Mab-LJ738-Fab'-MBL-biotin:

50 μ l of a solution of both conjugates (10 μ g/ml, ratio of the antibody conjugates of 1:1), 800 μ l 100 mM Tris/HCl, 2 % PEG 40000, 2 % Pluronic[®] F68, pH 7.0 and 20 μ l 1 % streptavidin-latex as well as 50 μ l in each case of an AFP standard (0, 50, 100, 1000 and 2000 ng/ml) were pipetted into cuvettes. The increase in the agglutination was measured at 405 nm in a Perkin-Elmer photometer between t=1 min and t=10 min.

The result of the immunoassay is shown in Table 4.

	Standard concentration (ng/ml)					
	0	50	100	500	1000	2000
Absorbance (mean)	44	51	60	151	299	547
CV (%)	17.6	12.8	12.2	8.4	4.5	1.9
Standard 0 + 3s range	67	-	-	-	-	-



C l a i m s

1. Method for carrying out a homogeneous immunoassay based on agglutination in which a conjugate of a Fab' fragment and a component K is used,
w h e r e i n
a conjugate is used which is obtained by subjecting a $F(ab')_2$ fragment of an antibody of the immunoglobulin G class to reducing conditions and subsequently reacting it with the component K which either has a functional group suitable for the binding or was derivatized in a suitable manner by introduction of a functional group whereby the component K is bound via the functional group to the free SH group of the Fab' fragment formed during the reduction and agglutinable particles are used which carry a substance capable of binding to K.
2. Method as claimed in claim 1,
w h e r e i n
a hapten is used as the component K.
3. Method as claimed in claim 2,
w h e r e i n
biotin is used as the hapten.
4. Method as claimed in one of the previous claims,
w h e r e i n
the Fab' fragment and the component K are linked via a spacer.



5. Method as claimed in one of the previous claims,
w h e r e i n
a Fab' fragment of an antibody of the IgG subclass
I is used and a spacer is employed with a chain
length of 4 to 16 atoms.
6. Method as claimed in one of the claims 1 to 4,
w h e r e i n
Fab' fragments of an IgG antibody of the subclass
II are used and a spacer is employed with a length
of 4 to 10 C-atoms.
7. Method as claimed in one of the previous claims,
w h e r e i n
latex particles are used as the agglutinable
particles to which a substance capable of binding
to the component K is bound via a bifunctional
compound.
8. Method as claimed in claim 7,
w h e r e i n
the agglutinable particle is produced by reacting a
substance capable of binding to the component K
with a bifunctional compound which has a N-hydroxy-
succinimide ester group and an arylazide group
under weak alkaline conditions in an aqueous
solution, excess bifunctional compound is
subsequently removed, a latex is added to this
solution and then it is irradiated with photons
which results in binding to the latex.
9. Conjugates composed of a Fab' fragment of an IgG
antibody and a component K which are obtained by
subjecting a $F(ab')_2$ fragment of an antibody of the



immunoglobulin G class to reducing conditions and subsequently reacting it with the component K which either has a functional group suitable for the binding or was derivatized in a suitable manner by introduction of a functional group whereby the component K is bound via the functional group to the free SH group of the Fab' fragment formed during the reduction.

10. Conjugates as claimed in claim 9,
w h e r e i n
the binding between the Fab' fragment and the component K either takes place directly between SH groups of the Fab' fragment and a functional group of the component K or via a spacer.
11. Conjugates as claimed in claim 10,
w h e r e i n
the spacer is a bifunctional compound in which a functional group is capable of binding to a SH group of the Fab' fragment and the second functional group which can be the same or different can covalently bind to the component K.
12. Conjugates as claimed in claim 10 or 11,
w h e r e i n
the Fab' fragment is derived from an antibody of the IgG subclass I and the spacer has a chain length of 4 to 10 C atoms.



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13. Conjugates as claimed in claim 10 or 11,
w h e r e i n
the Fab' fragment is derived from an antibody of
the IgG subclass II and the spacer has a chain
length of 4 to 10 C atoms.
14. Conjugates as claimed in one of the claims 9-13,
w h e r e i n
the component K is a hapten.
15. Conjugates as claimed in one of the claims 9-14,
w h e r e i n
the component K is biotin.
16. Use of conjugates as claimed in one of the claims
9-15 in a homogeneous immunoassay in which there is
no separation of bound and unbound label.