

[54] **DIRECT AGGLUTINATION
IMMUNOLOGICAL REAGENT**

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[58] Field of Search **424/8, 11, 12**

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[57] **ABSTRACT**

Immunological reagents capable of direct agglutination with antigens are prepared by pre-polymerizing the antibody to form an aggregate and then coupling this aggregate to a microbial cell carrier particle with a chemical coupling agent. The pre-polymerization of the antibody can be accomplished by the use of the same or another coupling agent. The use of the microbial cell carrier particle allows the reagents formed to be used as immunological indicator particles in slide tests which permit a quick read-out of test results. The reagent formed may be prepared and shipped in dry form and then reconstituted with liquid for use.

6 Claims, No Drawings

DIRECT AGGLUTINATION IMMUNOLOGICAL REAGENT

BACKGROUND OF THE INVENTION

This invention relates to immunological reagents which are in the form of indicator particles which are capable of direct agglutination with antigens. More particularly, it relates to immunological indicators wherein a pre-polymerized aggregate of the antibody to an antigen selected for detection is bound to a microbial cell carrier particle through a coupling agent.

The prior art indicates many difficulties in making immunological reagents in the form of indicator particles which are capable of direct agglutination with antigens. One difficulty has been that the antibodies necessary to sensitize the carrier particles have become inactivated when chemically coupled to said carrier particles. This effect was apparently due to the high level of reactivity which most reactive carrier particles exhibit prior to the chemical coupling with the antibody and to steric hindrance once the antibody has been coupled. In those reagents made by physical adsorption of the antibody onto the carrier particle a continuing problem has exhibited itself in that the antibody leached from the surface of the carrier particle during testing and combined with the antigen being detected and, by preferentially reacting with such antigen thereby lower the observed concentration of the antigen. Both of these difficulties led to inaccurate test results.

Another problem which the prior art presents is in the continuing use of erythrocytes of red blood cells as carrier particles. The problem which red cells incur in that there are wide variations in the nature of different batches of red blood cells, even when withdrawn from the same test animal. It has been found that the surface characteristics, size, and suitability of the red blood cells as carrier particles all vary with respect to the condition, age, medication and previous past history of the test animals from which the red blood cells are withdrawn. This causes difficulty in arriving at standardized immunological reagent indicator particles. An inherent difficulty in the use of red blood cells is that their large size and their surface characteristics limit the type of testing in which they can be used to generally those techniques in which the reaction is run in a test tube, which can only be properly read after a long period of time. Moreover, since red blood cells cannot be used in a slide-type test, the reaction time cannot be shortened by employing such a test. The size and surface characteristics also have been found to limit the sensitivity of the resulting test. Olovikov, A.M.: POLIKONDENSIROVANNYI SUSPENZIONNYI ANTITELO IMMUNOSORBENT I EGO ISPOLZOVANIE V REAKTSII AGGLUTINATSII DLYA OPREDELENIYA SODERZHANIYA ANTIGENOV (Poly-condensed Antibody Immunosorbent Suspension And Its Use In The Agglutination Reaction For The Determination Of The Antigen Content): Dokl. Adad. Nauk SSSR 1964 158 (5) 1202 - 5 mentions the problem of the disadvantage of the use of erythrocytes as carrier particles.

In review, red blood cells in the forms used in the prior art do not lend themselves to the construction of sensitive slide-type agglutination tests due to their fragility, size, weight, and surface characteristics as well as the difficulty in obtaining such cells.

It has now been discovered that immunological reagents capable of direct agglutination with antigens can be prepared in a reproducible and highly active form by pre-polymerizing an antibody and then chemically coupling this polymerized aggregate to a microbial cell carrier particle. The pre-polymerization step forms an aggregate in which at least some of the active sites of the antibody are exposed at the external surfaces thereof. The use of the microbial cell as a carrier particle provides a uniformly sized particle which is of size and weight characteristics capable of forming an immunological reagent which will allow slide agglutination test results to be determined in a short time period.

It is therefore an object of the present invention to provide an immunological indicator reagent capable of direct agglutination with an antigen wherein a pre-polymerized aggregate is chemically attached to a microbial cell carrier particle.

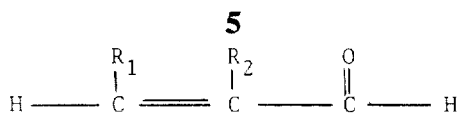
Yet another object of the present invention is to provide a process for making an immunological indicator reagent capable of direct agglutination with an antigen.

SUMMARY OF THE INVENTION

The immunological indicator reagent of the present invention is formed by polymerizing an antibody with a coupling agent to form an aggregate of said antibody and then reacting this aggregate with a reactive microbial cell carrier particle. The antibody is obtained in the form of the gamma globulin fraction of immune serum from animals injected with various antigens. The microbial cell is rendered reactive by reacting the same with a polyfunctional coupling agent. One of the functional groups of the coupling agent reacts with the surface of the carrier particle, leaving a nonbounded reactive group on the exterior surface of said carrier particle. The pre-polymerized aggregate of said antibody is then reacted with the reactive carrier particle in order to form a covalent chemical bond therebetween. These reactions are generally carried out in a fluid such as buffered liquid which can be maintained at optimum conditions.

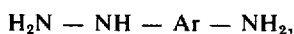
After the immunological indicator is formed it can be reduced to a dry form, if desired, by separating the fluid medium therefrom. This dry indicator may be later reconstituted with a liquid or the test sample for use in testing.

The immunological indicator particles made by the above process are than comprised of a microbial cell carrier particle having bound to at least a portion of the external surface thereof a pre-polymerized aggregate of the antibody. The aggregate is bound to the surface through covalent bonds formed by the reaction with the residues of coupling agent molecules which were first reacted with said carrier particles. As an alternative the coupling agent can be added to a mixture of the carrier particles and the pre-polymerized aggregate so that it contacts both simultaneously. These indicator particles have sizes of about from 0.2 to 1.5 microns in the smallest dimension and up to about 5 microns in the largest dimension. The microbial cell carrier particles can be rendered stable by reaction with a preservative such as formaldehyde prior to contact with the coupling agent. In a like manner the indicator particles can be rendered more visually distinguishable from any given background by staining the microbial cells with a suitable dye.

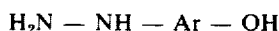


wherein any one of R_1 or R_2 can be hydrogen or a methyl group. Representative of this aldehyde are: acrolein, methacrolein, and 2-butenal crotonaldehyde. Of these acrolein is preferred.

It is also possible to convert the aldehyde reactivity remaining on the surfaces of the cells after treatment with dialdehydes or the unsaturated aldehydes to amino groups by further treating the cells with compounds having one of the following formulas:



or



where Ar is selected from the group of phenyl, biphenyl, stabilized heterocyclic ring, polynuclear hydrocarbon structures, polynuclear heterocyclic rings, triphenylmethane or substituted groups and mixtures thereof. Generally all of the polyfunctional compounds so defined have at least one diazotizable amino group and at least one amino, hydroxyl, or hydrazino group. The diazotizable group can be activated by nitrous acid for ultimate use. When desired, many of these polyfunctional converting compounds can be used in their water soluble forms by using the acid salts thereof. Sulfonic acid salts and hydrochloride salts are of utility in this regard. The sulfonic acid groups can be activated by phosphorus pentachloride.

Examples of the polyfunctional compounds are the following: 2,7-diaminofluorene; 2,5-diaminofluorene; benzidine · HCl; 3,3'-diamino benzidine; o-tolidine; hydrazine · HCl; 3,3'-dimethoxybenzidine dihydrochloride; o-dianisidine; p-rosaniline chloride and acetate; thionin; basic fuchsin; safranin-O, amino group substituted triphenyl methane dyes, p-hydrazine benzene sulfonic acid. Some of these compounds have only two functional groups while others have a greater number of functional groups, in which case there are two or more reactive functional groups available on the surface of the particles for reaction with the subsequently added biological materials. In the above formulas the reactive groups in excess of the two required groups are included in the substituted Ar groups.

The term "antibodies" as used herein denotes immunological materials produced as a result of antigenic stimulation in animals. The term "immunological counterpart" as used herein denotes either an antigen or an antibody which reacts specifically with the corresponding antibody or antigen, respectively.

As mentioned above the microbial cells can be colored by a dye or stain in order to improve the visual distinction of the final immunological reagents from the surrounding background. Stains such as hematoxylin, fuchsin and crystal violet can be used for this purpose. Another optimal preparation treatment for the microbial cells is washing with an organic solvent such as alcohol, ether, etc. to remove any polysaccharide or wax layers which may be present and which might interfere with the reaction between the microbial cells and the coupling agents.

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The immunological reagent indicator particles produced according to the present invention are capable of direct agglutination with the antigen which is the immunological counterpart to the antibody used in making the indicator particles. The indicator particles are mixed with a fluid sample containing such antigen, and the particles are then observed to determine whether or not an agglutination or a nonagglutination pattern results. The occurrence of an agglutination pattern denotes the presence of the antigen tested for, while the absence of an agglutination pattern denotes the absence of that antigen.

While the primary capability of the immunological reagent of the present invention is to allow direct testing for antigens, this same reagent can be used for detecting the presence of the antibody itself in a fluid sample. For example, when testing for the presence of an antibody, a quantity of the antigen thereto can be added to the test medium prior to or along with the indicator particles of the present invention which consist of the microbial cells coupled to the pre-polymerized antibody aggregate. If the fluid sample contains the antibody, the antigen added to the testing medium will preferentially react with this antibody and the antigen will thus be unable to agglutinate with the immunological reagent. Hence, the agglutination which would otherwise occur is inhibited, and the pattern which appears is said to be a nonagglutination pattern.

The agglutination testing and the inhibition of agglutination testing above referred to are preferably carried out by a slide agglutination method in which the test reagents are mixed with a fluid sample on a flat glass surface and the resulting pattern observed after a short time period. A smooth milk-like consistency is an indication of a nonagglutination pattern whereas an agglutination pattern is denoted by a number of clumps or floccules. If desired, microbial cells large enough in size and weight characteristics can be employed so that the agglutination reaction can be carried out according to a micro-titrator method wherein the immunological reagent is placed in each of a series of wells formed in a row in the upper surface of a plastic or other suitable plate. The linear arrangement of the wells allows serial dilution of the fluid sample used in the testing.

The serial dilution is carried out by first placing one drop of a diluent in each of the wells in the row and then adding a one drop volume of the fluid sample having an initial dilution of, for example, 1:5 to the first well using a loop or spiral calibrated to hold one drop of fluid. The loop is submerged in the first well, mixed and one drop of the fluid is withdrawn and is then mixed with the diluent in the second well. The dilution of the first well is then 1:10 and the dilution of the second well is 1:20. This process of serial dilution is repeated until all of the wells in the row have been treated, thus producing a series of antigen dilutions in each well differing by a factor of one-half from the adjacent earlier treated well.

To perform an inhibition of agglutination test, serial dilutions of a test sample are made in each of the wells in a row, and fixed amounts of the antibody added to each of the wells, followed by one drop of a suspension of the immunological reagent of the present invention. In such inhibition of agglutination testing the dilutions of the test sample are controlled so that the concentration of the antigen therein lies in the mid-portion of the serial dilutions made. This procedure allows an identifi-

cation of the concentration of the antigen in the fluid sample and hence a semi-quantitative determination thereof. The same semi-quantitative determination can be made by using a direct agglutination procedure with the micro-titrator method.

The process for making the immunological indicator of the present invention involves three major steps, each of which can be carried out in any of a number of buffered liquid media. The first and last steps are preferably carried out at room temperature, and the second is preferably carried out at 4°C. The first step is to form a polymerized aggregate of the antibody to the antigen which is to be detected, and this is accomplished by reacting a coupling agent with the antibody. Next, the microbial cell is made reactive to the polymerized aggregate of the antibody by reacting the cell with a coupling agent which can, but need not necessarily, be the same coupling agent employed in the first step.

In the second step the microbial cells are dispersed in a fluid medium at a 1 to 10 percent suspension concentration. Short times of less than 1 hour can be used for this step when conducted at room temperature (20° to 25°C.).

When a preservative agent is employed, the microbial cells are pre-treated therewith prior to carrying out the second step. As aforementioned the preservative agent used is preferably formaldehyde. In a like manner, when a stain is employed, the microbial cells are treated therewith prior to reacting the coupling agent with the cells in the second step.

In the third step the polymerized aggregate of the antibody is placed in contact with the reactive carrier particles for a time period sufficient to allow a coupling reaction to take place therebetween. Upon completion of the third step the immunological indicator formed can be separated from the suspending liquid and dried. This separation and drying is preferably accomplished by a lyophilization method in which volumes of several drops of a suspension of the immunological indicator are placed in containers and cooled to -40°C. under a vacuum. This method can be carried out in standard commercial lyophilizers in a short time. The dried reagent can then be packaged under a low humidity condition and later reconstituted for use.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Bovine serum albumin (BSA) is injected into a rabbit to produce anti-BSA globulin. This antibody-containing globulin is separated from the other serum proteins by DEAE-cellulose (diethylaminoethyl cellulose) chromatography.

To the separated globulins 1 ml. of a 1:80 dilution of 0.024 M bis-diazobenzidine (BDB) in 0.15 M phosphate buffer, pH 7.3 is added to form the pre-polymerized antibody aggregate. The reaction can be carried out at room temperature (22°C) with occasional shaking until a faint turbidity develops.

E. coli cells are cultured in advance, separated from the growth medium and formalinized in order to preserve the same. Then BDB is coupled to the formalinized *E. coli* cells at 4°C. and these cells are then washed with saline in order to remove all unreacted BDB. The pre-polymerized antibody aggregate is then added to the washed BDB cells and the mixture is incubated at 22°C. for thirty minutes, after which it is washed with saline. The sensitized cells are then resus-

ended in 1 percent normal rabbit serum (NRS) to protect the cells during a subsequent lyophilization. The cells as thus constituted are usable for testing in the manner described. Preferably, the concentration of the immunological indicator is adjusted to the optimum level for each particular test situation.

The above objects and description are further detailed in the examples which are to be construed as illustrative only and are not to be limitative of the invention.

EXAMPLE 1

An immunological indicator reagent was made by pre-polymerizing the globulin fraction from a rabbit which had been injected with BSA. This antibody aggregate was then coupled to reactive microbial cell carrier particles in order to form an immunological reagent for detecting BSA.

DEAE-cellulose chromatography was employed in the standard manner to separate the antibody containing globulin fraction from the other serum proteins of rabbit anti-BSA serum. Two and one-half mg. of the purified globulins were then dissolved in 1 ml. of 0.15 M sodium phosphate-potassium phosphate buffer, pH 7.3 which was made up by adding 808 ml. of 0.15 M $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ to 192 ml. of 0.15 M KH_2PO_4 . To this mixture was added 1 ml. of a 1:80 dilution of 0.024 M BDB in the 0.15 M phosphate buffer, pH 7.3 (resulting in a 0.0003 M BDB concentration). The resulting mixture was incubated at 22°C. for 30 minutes with occasional shaking at which time a faint turbidity was noticed, indicating the presence of the pre-polymerized antibody aggregate.

This antibody aggregate was coupled to carrier particles prepared in the following manner. Five ml. of a 2.5 percent suspension of formalinized *E. coli*, previously cultured and preserved, were washed 3 times with 10 ml. of physiological saline solution and then resuspended in 3 ml. of the 0.5 M phosphate buffer, pH 7.3 at 4°C. To this mixture was added 10 ml. of 0.0012 M BDB in the 0.15 M phosphate buffer. The resulting mixture was agitated for 30 minutes at 4°C. after which it was washed twice with cold saline at 4°C. After the last wash the reactive carrier particles were resuspended in 3 ml. of the 0.15 M phosphate buffer at room temperature and the above pre-polymerized aggregate dispersion added thereto. The coupling reaction was allowed to proceed for 30 minutes at room temperature with occasional mixing, after which time the preparation was centrifuged and the immunological indicator recovered and washed three times with physiological saline, after which it was adjusted to a 4 percent indicator concentration in saline.

The immunological indicator thus prepared was tested by a direct slide agglutination method by employing the following concentrations of BSA: 0.1, 1, 10, and 100 micrograms per ml. Agglutination patterns were observed for the last three BSA concentrations. A negative control in which saline alone was employed gave no agglutination pattern. In an inhibition test which was conducted using a 10 microgram per ml. solution of BSA in saline pretreated with a 1:50 dilution of anti-BSA in saline, inhibition of agglutination was observed.

In a corresponding experiment the same procedure as above set out was followed except that the antibody was not pre-polymerized. When the immunological in-

indicator prepared in this manner was reacted with BSA, no agglutination was seen at any of the concentrations employed above. However, the thus prepared indicator did react with a 1:200 dilution of anti-rabbit globulin in saline to provide a strong agglutination pattern. This showed that although the antibody molecules had indeed coupled to the microbial cells, their antibody properties had been denatured or in some manner impaired, but their antigenic properties due to the protein nature of the globulin were still active. This would indicate that the antibody sites of the gamma globulin molecules are inactive when the gamma globulin molecules are reacted directly with carrier particles without the pre-polymerization step.

EXAMPLE 2

An immunological indicator capable of detecting diphtheria toxoid was prepared in the following manner. One ml. of diphtheria antitoxin, containing 100 AU (antitoxin units International Standard) in the above 0.15 M phosphate buffer, pH 7.3 was polymerized with 1 ml. of 0.0006 M BDB in the 0.15 M phosphate buffer. The subsequent reaction for the preparation of the pre-polymerized antibody aggregate and the coupling of this aggregate to reactive *E. coli* cells was then carried out in the manner of Example 1 above.

This immunological indicator was tested with the following concentrations of diphtheria toxoid: 0.01, 0.1, 1 and 10 LF units/ml. (based on the International AU Standard). Agglutination patterns were seen with the last three diphtheria toxoid concentrations while a saline control showed no agglutination.

EXAMPLE 3

An immunological indicator capable of detecting tetanus toxoid was prepared in the following manner. One ml. of tetanus antitoxin containing 80 antitoxin units (International Standard AU) in the above 0.15 M phosphate buffer, pH 7.3 was polymerized with 1 ml. of 0.0006 M BDB in the 0.15 M phosphate buffer. This aggregate was then allowed to react in the same manner as for Example 1 and was then coupled to the active *E. coli* carrier particles in the same manner as in that example. It was then tested in the same manner as for the indicator reagent of Example 2 using the same concentrations of tetanus toxoid instead of the diphtheria toxoid. Agglutination patterns were observed in the last two concentrations of the tetanus toxoid and a saline control showed no agglutination.

EXAMPLE 4

An immunological indicator capable of detecting C-reactive protein (CRP) by agglutinating therewith was made following the process generally as in Example 1.

The antibody to the CRP was produced in a goat by injecting an adjuvant-CRP mixture into the animal and then bleeding the animal after a sufficient length of time. The antibody containing globulin was then separated from the other serum proteins by DEAE-cellulose chromatography. Three mg. of the separated globulins were dissolved in 1 ml. of the 0.15 M phosphate buffer, pH 7.3 and this solution then reacted with a 1 ml. of 0.0006 M BDB in the same buffer for 25 minutes at room temperature. A turbidity developed at approximately this time and the antibody aggregates were then reacted with BDB treated *E. coli* cells in the same manner as described in Example 1.

The immunological indicator thus prepared was tested against the following dilutions of commercially available CRP in saline: 1:5, 1:10, 1:100 and 1:1000. Agglutination patterns were observed in the first two dilutions while a saline control did not show agglutination. The immunological indicator also agglutinated with a 1:100 dilution of anti-goat serum proteins in saline showing that the gamma globulin derived antigenic properties of the antibody are in fact active.

An inhibition test was also performed using both 1:10 and 1:50 dilutions of CRP in saline and employing for the inhibition 0.1 ml. of CRP antibody suspended in saline. Inhibition of the agglutination patterns was observed.

EXAMPLE 5

An immunological indicator was prepared to detect BSA by agglutinating therewith. The pre-polymerized anti-BSA aggregate was coupled to microbial cells in a 4-stage coupling procedure in which acrolein was first used to coat the microbial cells with active aldehyde groups and then o-dianisidine was used to convert the aldehyde reactivity to an aminogroup activity, after which the amino groups were reacted with BDB to provide the final reactive carrier particles. The pre-polymerized antibody aggregate was then coupled to said reactive particles. It is also possible to employ a one stage coupling procedure using only the acrolein, since the aldehyde groups directly couple to the pre-polymerized antibody aggregate.

More specifically, *E. coli* was deantigenated by extracting the cells with a 1:20 dilution of phosphate buffered saline, pH 7.2 in a 100°C. water bath for 2.5 to 3 hours. The concentration of the cells was then adjusted to 2.5 percent in saline, and 2 volumes of a 4 percent saline solution of freshly distilled acrolein were added thereto. This mixture was incubated at 22°C. for 72 hours with occasional shaking. The reactive cells were then washed three times with 250 ml. of saline and the cell concentration adjusted to 2.5 percent prior to the addition of an equal volume of 0.25 percent o-dianisidine dihydrochloride in water, after which the mixture was incubated under the same conditions as above.

The prepared cells were then washed 5 times with saline and coupled to BDB in the manner described in Example 1.

The anti-BSA pre-polymerized aggregate was prepared in the manner set out in Example 1 and coupled to the reactive carrier particle as set out in Example 1.

A BSA concentration of 1 microgram per ml. agglutinated this immunological reagent and a saline control did not.

EXAMPLE 6

The procedure of Example 5 was followed except that 2,7-diaminofluorene dihydrochloride was substituted for the o-dianisidine dihydrochloride. The same test results were obtained.

EXAMPLE 7

It is possible to vary the size and surface characteristics of the carrier particles by coating them with one or more layers of a proteinaceous material as mentioned above. In this example a microbial cell is first coated with BSA in order to increase the size and weight of the base carrier particle.

BSA was used to coat *E. coli* by the following treatment. Five ml. of 2.5 percent formalized *E. coli* were washed 3 times and then resuspended in 3 ml. of the 0.15 M phosphate buffer, pH 7.3. Next 10 mg. of BSA dissolved in 2.5 ml. of saline was added together with 10 ml. of 0.0024 M BDB in the 0.15 M phosphate buffer. The mixture then placed on a rotary shaker for 30 minutes at room temperature, centrifuged and washed twice with 10 ml. of saline prior to resuspending the cells in 10 ml. of a 0.6 percent BSA solution in saline. After letting the carrier particles stand 30 minutes they were washed again with saline three times and resuspended to a 2 percent concentration in saline.

An anti-BSA pre-polymerized aggregate was prepared and coupled to these BSA coated carrier particles by following the procedure of Example 1. When tested with BSA, agglutination was seen down to a concentration of 1 microgram per ml. while a saline control showed no agglutination. Also a strong agglutination pattern was observed when the immunological indicator was mixed with a 1:100 dilution of anti-rabbit gamma globulin in saline showing that the antigenic activity of the pre-polymerized aggregate was not impaired.

A test was also made to determine whether or not the coating layer of BSA had been effectively covered with the pre-polymerized antibody aggregate. This check was carried out by testing the indicator with a 1:100 dilution of anti-BSA in saline, with the result that no agglutination was found.

What is claimed is:

1. An immunological indicator reagent capable of detecting an antigen selected from the group consisting of bovine serum albumin, diphtheria toxoid, tetanus toxoid, and C-reactive protein, comprising a pre-polymerized

aggregate of an antibody coupled to a bacterial cell with a coupling agent said; bacterial cell being of uniform shape and size selected from the group consisting of *E. coli*, *B. subtilis*, *L. leichmannii*, *B. pumilus* and *P. fragi* said coupling agent being selected from the group consisting of bis diazobenzidine, bis diazobenzidine disulfonic acid, acrolein, difluorodinitrobenzene, dicyclohexylcarbodiimide, cyanuric chloride, 2,7-diaminofluorene, toluene diisocyanate, dichlorotriazine, difluorodinitrodiphenyl sulfone, tetrazo-phenylenediamine, glutaraldehyde and N-5-butyl-5-methylisoxazolium perchlorate.

2. A reagent as in claim 1 capable of detecting bovine serum albumin comprising the pre-polymerized aggregate of an antibody covalently bound to *E. coli* with acrolein.

3. A reagent as in claim 1 capable of detecting diphtheria toxoid comprising the pre-polymerized aggregate of an antibody covalently bound to *E. coli* with bis diazobenzidine.

4. A reagent as in claim 1 capable of detecting tetanus toxoid comprising the pre-polymerized aggregate of an antibody covalently bound to *E. coli* with bis diazobenzidine.

5. A reagent as in claim 1 capable of detecting C-reactive protein comprising the capable of detecting C-reactive protein comprising the pre-polymerized aggregate of an antibody covalently bound to *E. coli* with bis diazobenzidine.

6. A reagent as in claim 1 capable of detecting bovine serum albumin comprising the pre-polymerized aggregate of an antibody covalently bound to *E. coli* with 2,7-diaminofluorene.

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