Candidate's Answer

Immunological Assay

The present invention relates to an immunological assays for detecting salmonella bacteria, and, more specifically a method for detecting salmonella bacteria and labelled antibodies for use therein.

Salmonella bacteria are one of the most common causes of food borne intestinal infections in the developed world, with over 40,000 cases reported in the United States each year. Up to 1,000 of these infections are fatal.

Salmonella bacteria are often difficult to detect. A food sample with a suspected salmonella contamination needs to be cultured for 2-3 days. A skilled microbiologist can then identify Salmonella bacteria in the culture. This test is however very time consuming and when the sample only contains low bacterial counts often provides false negative results.

A number of groups have recently been attempting to provide a faster and more reliable test for salmonella bacteria. One approach that has shown considerable promise is the use of tests using labelled antibodies for the detection of the bacteria. An antibody useful in the invention is an immunoglobulin protein having a specific binding site for a bacterium. Antibodies with binding sites for salmonella bacterial will selectively bind to these bacteria. The label on the antibody is a compound or group that can be detected by chemical or spectroscopic means. The test thus involves contacting the salmonella bacteria with the labelled antibody, allowing the antibody to bind to the bacteria and detecting the resulting labelled antibody-bacteria complexes. This method is very selective since the antibody will only bind to the bacteria of interest. These tests are however still often not very sensitive requiring a high concentration of bacteria in the sample being tested to provide a positive result.

Document 1 (D1) discloses a method for detecting salmonella bacteria in a culture a contaminated food sample. In the method disclosed in D1, antibodies are labelled w chemiluminescent, a chemiluminescent agent (or label), to measure the number of bacteria present. The chemiluminescent agent used in D1 is an acridinium derivative, a preferred acridinium derivative being a substituted phenyl ester of 10-methylacridinium-9-carboxylic acid. However, the level of detection of the acridinium derivative could be improved, i.e. the sensitivity could be improved, since if a sample tested using this method containing salmonella, but this is not detected using the acridinium derivative, then a false negative result would be obtained. It would be desirable to produce a test which can detect bacteria present in a sample at a concentration lower than 100 cells/ml, the current known limit of detection.

D2 discloses osmium complexes, in particular unsubstituted or carboxylic acid substituted tris(2,2'-bipyridine) osmium complexes. These are organic light-emitting compounds used in the field of LEDS. However, it has been surprising found by the inventors of the present invention that such complexes would not in themselves be suitable for use in a immunological assay since they do not bind to antibodies - an essential requirement for use in an immunological method of detection. The compounds are, however, known to be very effective in emitting light and as such it would be desirable to incorporate such compounds into an immunological test method.

It is an object of the present invention to overcome or at least mitigate the problems presented by the prior art. The present invention provides a tris (2,2'-bipyridine) osmium complexes as defined in claim 1.

Although, osmium complexes were well known chemiluminescent agents (e.g. see D2) the known osmium complexes did not bind well, if at all, with antibodies. However, the present inventors have surprisingly found that by attaching a linking group to an osmium complex, the complex will bind with an antibody. The linking group must be an ester of a carboxylic acid having 3 to 8 carbons and either N-hydroxysuccinimide or N-hydroxyphthalimide.

A particularly preferred group of compounds is defined in claim 2. The present inv further provides a method (as defined in claim 3) for preparing a tris (2,2'-bipyridine) osmium complex as defined in claim 1. The starting complex may *be prepared by first reacting a dichloro bis(2,2' bipyridine) osmium with a carboxylic acid substituted 2,2' bipyridine (this first step of the reaction scheme used to make the label has been published in the J. Complex Chem. Vol. 66, (1953), 44), to obtain ~~a~~* the C_3 to C_8 *carboxylic acid substituted tris (2,2' bipyridine) complex.*

The preferred tris (2,2' bipyridine) osmium complex defined in claim 2 may be prepared according to the method defined in claim 4, in which a tris (2,2'-bipyridine) complex substituted with a C3 to C4 carboxylic acid is reacted with N-hydroxysuccinimide to form the complex defined in claim 2.

The present invention further provides an antibody labelled with a chemiluminescent label, as defined in claim 5. If the antibody is to be used in an immunological method for detecting salmonella bacteria, the antibody should be an antibody having a specific binding site for salmonella bacteria to provide a useful assay.

The preferred antibody is a monoclonal antibody belonging to the class of proteins called immunoglobulins gamma (IgG) derived from rabbits. The antibody may be made by conventional techniques known per se and is commercially available.

The antibody may be labelled according to the process defined in claim 7 or claim 8.

The present invention further provides an immunological method as defined in claim 9 for detecting salmonella bacteria present in a bacteria culture or in a contaminated food sample. By using an antibody with binding sites for salmonella, which has been labelled with a osmium complex substituted with a linking group as described above, it is possible to detect salmonella in concentrations lower than 100 cells/ml, thus reducing the number of false negative results.

The method of the present invention preferably uses in step a) a solid phase in the of a bead, a tube or a microtiter plate well, which is made of polystyrene, polyvinyl chloride, nylon or agarose. It is particularly preferred that the solid phase is a microtiter plate well made of polystyrene.

~~A~~ In the process above, *first incubation time of between 15 minute and 3 hours, and preferably between 20 minutes and 2 hours 30 minutes is normally used. The temperature during the first incubation is preferably maintained in the range of 20ºC to 40ºC. Generally the incubation is performed in the presence of a buffer which is preferably a saline buffer giving a pH between 8.0 and 9.5.*

Generally, the wash steps described under points b) and d) are each performed by using a buffer and preferably a saline buffer also giving a pH between 8.0 and 9.5.

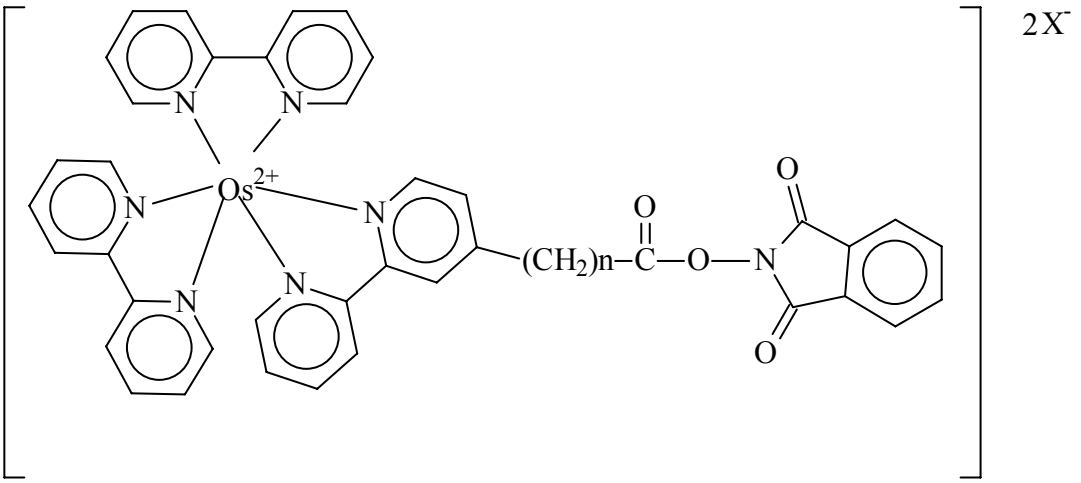
The second incubation time used in step c) of our process (exposure of the labelled antibody to the bacteria immobilized upon or inside the structure of the solid phase) generally varies between 15 minutes and 3 hours, and preferably between 20 minutes and 2 hours and 30 minutes. The temperature used is preferably in the range of 20ºC to 40ºC. This step is generally performed in the presence of a buffer which is preferably a saline buffer giving a pH of 8.0 to 9.5.

In the final step e) of the method of the invention the labelled antibody bound to the bacteria to be detected undergoes a light emitting reaction. An activating solution is contacted with the labelled antibodies bound to the bacteria. This causes the osmium complex to emit light. The activating solution is typically an aqueous solution of a sodium oxalate (0.05 M) and hydrogen peroxide (0.02 M). The intensity of the light emitted is proportional to the concentration of osmium complex and thus the number of bacteria present. The intensity of the light emitted can be measured using a luminometer. The number of bacteria in the sample is proportional to the relative light unites (RLU) registered on the luminometer. A sample highly contaminated by salmonella bacteria will give a high RLU count, whereas a negative sample will give a very low RLU count.

The present invention further provides a kit as defined in claim 14 for testing salm bacteria in a culture or in a contaminated food sample. Preferably, the antibody is a monoclonal antibody of the IgG type, labelled with a tris(2,2' -bipyridine) osmium complex as defined in claim 1.

1. A tris(2,2 -bipyridine) osmium complex substituted with an ester of (i) a carboxylic acid containing 3 to 8 carbon atoms and (ii) N-hydroxysuccinimide or N-hydroxyphthalimide.

2. A tris(2,2' -bipyridine) osmium complex as claimed in claim 1 having the structure:



wherein n is 3 or 4.

3. A method for preparing a tris(2,2' -bipyridine) osmium complex as defined in claim 1, the method comprising reacting (i) a starting complex comprising a tris(2,2' -bipyridine) complex substituted with a C_3 to C_8 carboxylic acid with a (ii) a N-hydroxysuccinimide or N-hydroxyphthalimide to form the tris(2,2' -bipyridine) osmium complex defined in claim 1.

4. A method as claimed in claim 3, wherein in the method, the starting complex is a tris(2,2' -bipyridine) complex substituted with a C_3 to C_4 carboxylic acid, and this starting complex is reacted with N-hydroxysuccinimide to form the complex defined in claim 2.

5. An antibody labelled with a chemiluminescent label comprising a tris(2,2' -bipyridine) osmium complex as defined in claim 1 or claim 2.

6. An antibody as claimed in claim 5, wherein the antibody is a monoclonal antibody belonging to the immunoglobulins gamma class of proteins derived from rabbits (a monoclonal antibody of the IgG type).

7. A process for labelling an antibody, the process comprising:

(i) dissolving an antibody in a saline buffer at a pH of 8.0 to 9.5 to form an antibody solution;

(ii) adding the antibody solution to a label comprising a tris(2,2' -bipyridine) osmium complex as defined in claim 1 or claim 2.

(iii) stirring the antibody solution for a period of 5 to 30 minutes to allow the antibody to bind to the label.

8. A process for labelling an antibody as claimed in claim 7, wherein, after step (iii), the labelled antibody solution is purified.

9. An immunological method for detecting salmonella bacteria present in a bacterial culture or in a contaminated food sample, which comprises the following steps:

(a) bringing the sample to be tested into contact with a solid phase, during a first incubation time sufficient to allow the bacteria to be immobilised upon or inside the structure of the solid phase,

(b) subjecting the solid phase with the immobilized bacteria to a first wash step to remove the unbound bacteria,

(c) bringing the washed solid phase into contact with an antibody labelled with a chemiluminescent label comprising an osmium complex as defined in claim 1 or claim 2, wherein said antibody has binding sites for salmonella bacteria,

(d) subjecting the solid phase from step (c) to a second wash step to remove a unbound labelled antibody, and

(e) placing the solid phase obtained in step (d) under conditions whereby light may be produced, and the luminescent activity produced may be quantatively measured with the help of an appropriate device.

10. An immunological method as claimed in claim 10, wherein in step (a), the first incubation time is from 20 minutes to 2 hours 30 minutes.

11. An immunological method as claimed in claim 9 or claim 10, wherein the wash steps are each performed using a saline buffer having a pH of from 8.0 to 9.5.

12. An immunological method as claimed in any one of claims 9 to 11, wherein the incubation time in step (c) is from 20 minutes to 2 hours 30 minutes.

13. An immunological method as claimed in any one of claims 9 to 12, wherein the incubation step is performed in the presence of buffer at a pH of 8.0 to 9.5.

14. A kit for testing salmonella bacteria in a culture or in a contaminated food sample, the kit comprising:

(i) a solid phase

(ii) an antibody, labelled with a chemiluminescent label, as defined in claim 5, and wherein said antibody has binding sites for salmonella bacteria and

(iii) an aqueous solution of sodium oxalate (0.05 M) and of hydrogen peroxide (0.02 M).

15. A kit as claimed in claim 14, wherein the antibody is a monoclonal antibody of the IgG type.

16. A kit as claimed in claim 14 or claim 15, wherein the solid phase is in the form of a microtiter plate well.