EUROPEAN QUALIFYING EXAMINATION 2005

PAPER A
CHEMISTRY

This paper comprises:

LETTER FROM THE APPLICANT

Montezuma PLC has been investigating new tests for bacteria associated with food poisoning. Our experiments have revealed some very interesting results which we believe are worth patenting. We request that you file a patent application covering as much of the work as possible.

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 bacteria 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.

We have now developed a more sensitive immunoassay for Salmonella bacteria, is based on the use of a tris (2,2'bipyridine) osmium complex as a chemiluminescent label. A chemiluminescent compound is one that will undergo a chemical reaction causing it to emit light. This light can be detected.

Specifically, we provide 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 said solid phase,

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

c) bringing the washed solid phase into contact with an antibody, which is labelled with a chemiluminescent agent comprising a tris (2,2'bipyridine) osmium complex, said contact being performed during a second incubation time sufficient to allow the labelled antibody to bind to the bacteria.

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

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

The method of the present invention preferably uses in step a) a solid phase in the form 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 first incubation time of between 15 minutes 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 20 °C to 40 °C. 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.

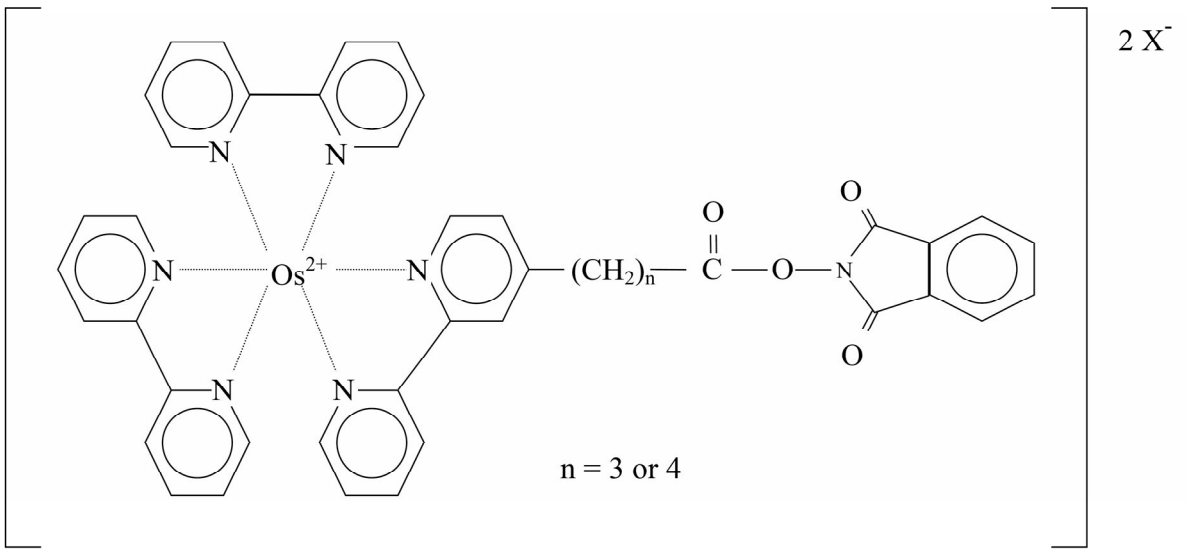
Generally, the wash steps described under points b) and d) are each performed by using a buffer and preferably a saline buffer also giving a pH between 8.0 and 9.5.

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 30 minutes. The temperature used is preferably in the range of 20 °C to 40 °C. 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 units (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 antibody used under point c) is an antibody which will bind to Salmonella bac with sufficient specificity 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 chosen antibody is labelled with a chemiluminescent agent which is a tris (2,2'-bipyridine) osmium complex. These osmium complexes are well known chemiluminescent agents. The complex as such however only binds poorly to antibodies and is therefore not a suitable label. In order for the complex to be used as a label it must be substituted with a linking group able to bind the complex to the antibody. We have found it to be essential to the invention to use an ester of a carboxylic acid containing 3 to 8 carbon atoms and N-hydroxysuccinimide or N-hydroxyphthalimide as the linking group. A tris (2,2' bipyridine) osmium complex with such a linking group may be prepared by firstly 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 carboxylic acid substituted tris (2,2' bipyridine) complex. The label is obtained by reacting this carboxylic acid substituted tris (2,2' bipyridine) complex in a second step with N-hydroxysuccinimide or N-hydroxyphthalimide to form the tris (2,2' bipyridine) complex substituted with the ester. A particularly preferred group of compounds useful as labels has the following structure:



A suitable scheme for labelling the antibody with the tris (2,2' bipyridine) osmium complex involves the following steps:

- dissolving the antibody in a saline buffer at a pH of 8.0 to 9.5;
- adding said antibody solution to the label (i.e. the osmium complex substituted with the linking group) in solution in an organic solvent, and mixing the ingredients thoroughly;
- stirring the mixture for a period of 5 minutes to 30 minutes;
- optionally purifying the labelled antibody solution.

The salmonella test may be provided as a kit for detecting Salmonella bacteria in a culture or in a contaminated food sample, which comprises:

- a solid phase, preferably in the form of a microtiter plate well;
- an antibody (preferably a monoclonal antibody of the IgG type) labelled by coupling with a tris (2,2' bipyridine) osmium complex;
- an aqueous solution of sodium oxalate (0.05 M) and of hydrogen peroxide (0.02 M).

In order to further illustrate the present invention and the advantages thereof, the followings examples are given, it being understood that they are intended only as illustrative and in no way limiting.

EXAMPLE I

Preparation of Osmium bis (2,2'-bipyridine) (2,2'-bipyridine-4-butanoic acid) bis(hexafluorophosphate) as known from J. Complex Chem. Vol. 66, (1953), 44).

Sodium bicarbonate (0.40 g), osmium dichloro bis(2,2'-bipyridine) (0.40 g), and 2,2'-bipyridine-4-butanoic acid (0.30 g) were stirred in refluxing methanol (20 ml)-water (5 ml) for 9 hours. The resulting solution was cooled in an ice bath, treated with 5 drops of concentrated sulphuric acid, and allowed to stand at ice temperature for 1.5 hours. A precipitate formed, which was separated by filtration and washed with methanol (8 ml).

The combined filtrate and wash solution were treated with a solution of sodium hexafluorophosphate (5.0 g) in water (25 ml). The resulting solution was cooled in an ice bath for 3 hours, and the resulting precipitate of red-purple crystals was collected by filtration (0.40 g).

EXAMPLE II

Preparation of N-hydroxyphthalimide ester of osmium bis(2,2'-bipyridine) (2,2'-bipyridine-4-butanoic acid) bis(hexafluorophosphate)

Dicyclohexylcarbodiimide (DCC, 0.046 g) and N-hydroxyphthalimide (0.034 g) were dissolved in dimethylformamide (DMF, 2 ml) with stirring, and cooled in an ice bath. A solution of osmium bis (2,2'-bipyridine) (2,2'-bipyridine-4-butanoic acid) bis (hexafluorophosphate) (0.101 g, prepared as in Example I) dissolved in DMF (1 ml) was added, and the mixture was stirred 5 hours at ice bath temperature. A precipitate formed and was separated by centrifugation. The supernatant containing the ester of the osmium complex was retained and is used as the label.

EXAMPLE III

Preparation of antibody

The antibody used was an anti-Salmonella IgG monoclonal antibody. This antibody was prepared, in a manner known per se, as a pure solution.

Labelling of anti-Salmonella IgG monoclonal antibody (IgG) with osmium complex

The solution of N-hydroxyphthalimide ester of osmium bis(2,2'-bipyridine) (2,2'-bipyridine-4-butanoic acid) bis(hexafluorophosphate) prepared in Example II (1 ml) was added to a stirred solution of IgG in aqueous physiologic buffered saline (PBS, 5 ml, pH 9.0; 25 mg/ml IgG). The mixture was stirred for 20 minutes, and precipitate was removed by centrifugation. The supernatant containing osmium-labeled IgG was retained.

The success of the labeling reaction was tested by dialysing the Osmium-labelled IgG solution with PBS solution. As a control, the unbound, activated osmium complex prepared in Example II was also dialysed with PBS solution. After 8 hours, the control showed no fluorescent species within the dialysis tube. The osmium-labelled IgG solution, however, showed strong fluorescence, demonstrating that the osmium complex was bound to the IgG.

EXAMPLE IV

Detection of Salmonella bacteria

The following methodology was pursued:

a) A sample containing Salmonella bacteria is added (0.1 ml/well) to the wells of a polystyrene microtiter plate used for immunoassays. This is incubated for 30 minutes at 37 °C in the presence of Tris buffer, pH 9,2;

b) Microtiter plate is then washed 5 times with PBS buffer, pH 9.0, containing 0,05% Tween 20 surfactant (polyoxyethylenesorbitan monolaurate);

c) Tris (2,2' bipyridine) osmium complex-labelled antibody (as prepared in example III) is added at 0.1 ml/well and incubated for 30 minutes at 37 °C in the presence of Tris buffer, pH 9.2;

d) Plate is washed as in step b); and

e) Luminescence measured on LB96P luminometer, which automatically adds an aqueous solution of sodium oxalate (0.05 M) and hydrogen peroxide (0.02 M) to each well of the plate.

The procedure was repeated using antibodies labelled with a number of different commercially available fluorescent labels and the sensitivity of the labelled antibodies was compared. A control well which underwent the same sequence of steps, but to which no bacteria were added was provided for each labelled antibody. A positive result is defined to be a signal having a strength in RLU at least 20 % higher than the signal obtained from the control. A sequence of samples with known bacterial counts were tested and the lowest bacterial concentration that could be detected was determined for each of the labels tested.

Label used	Detection Limit (bacteria /ml)
N-hydroxyphthalimide Ester of Osmium bis(2,2'-bipyridine) (2,2'-bipyridine-4-butanoic acid)	10
Acridinium Ester	100
Fluorescillium	700
Bacdetect	400
Glowdark	3,000

EXAMPLE V

A food sample (minced beef) was artificially contaminated with Salmonella bacteria. The beef was divided into 25 g portions, and mixed with an equal volume of Tris buffer, pH 9.2. A controlled number of bacteria were added to each sample and the samples were well shaken and cultured for 4 hours at 37 °C. The cultured samples were filtered through a membrane with 10 micrometre apertures to separate the beef from the bacteria and the liquid obtained was tested using the labelled antibody obtained in example III using the procedure of example IV.

The samples all gave positive results. All the known commercial tests require that samples are cultured for at least 24 hours and thus our test is more sensitive than known tests and can considerably reduce the time needed to test food samples.

Yours sincerely,

D. Belly,
Product development,
Montezuma PLC

DOCUMENT 1 (State of the art)

METHOD OF DETECTION OF BACTERIA

The invention relates to a new method for detecting Salmonella bacteria in a culture or in a contaminated food sample.

This invention utilises a test employing antibodies labelled with chemiluminescent molecules to measure the number of bacteria present. This invention further concerns kits for performing tests according to the method.

Salmonella bacteria exist in a range of environments but impose the greatest danger to health when found in food and feedstuffs. Poultry, meat and eggs are common sources of Salmonella. When the bacteria are consumed, they are able to establish themselves in the gut and multiply, resulting in the appearance, several days after the initial ingestion, of clinical symptoms including vomiting, diarrhoea and nausea, and in severe cases said symptoms may result in death. It is therefore highly desirable to provide test methods by means of which such dangerous bacteria may be detected. Tests for Salmonella bacteria are disclosed in the prior art. However these tests are characterized by a level of sensitivity which remains to be improved.

DESCRIPTION OF THE INVENTION

Specifically, in one aspect the present invention provides an immunological method for detecting Salmonella bacteria present in a 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 on the solid phase,

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

c) bringing the washed solid phase into contact with a specific antibody, which is labelled by coupling to a chemiluminescent agent comprising an acridinium derivative, said contact being performed during a second incubation time sufficient to allow the labelled antibody to bind to the bacteria.

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

e) placing the solid phase obtained as previously described under point d) above under conditions whereby light may be produced, and the luminescence activity produced may be quantitatively measured with the help of an appropriate device.

The present invention provides a method which is performed by using, under point a), a solid phase in the form of a bead, a tube or a microtiter plate well, which may be made of material such as, for example, polystyrene, polyvinyl chloride, nylon, agarose beads or cellulose derivatives.

It is preferred that the solid phase is a microtiter plate well made of polystyrene.

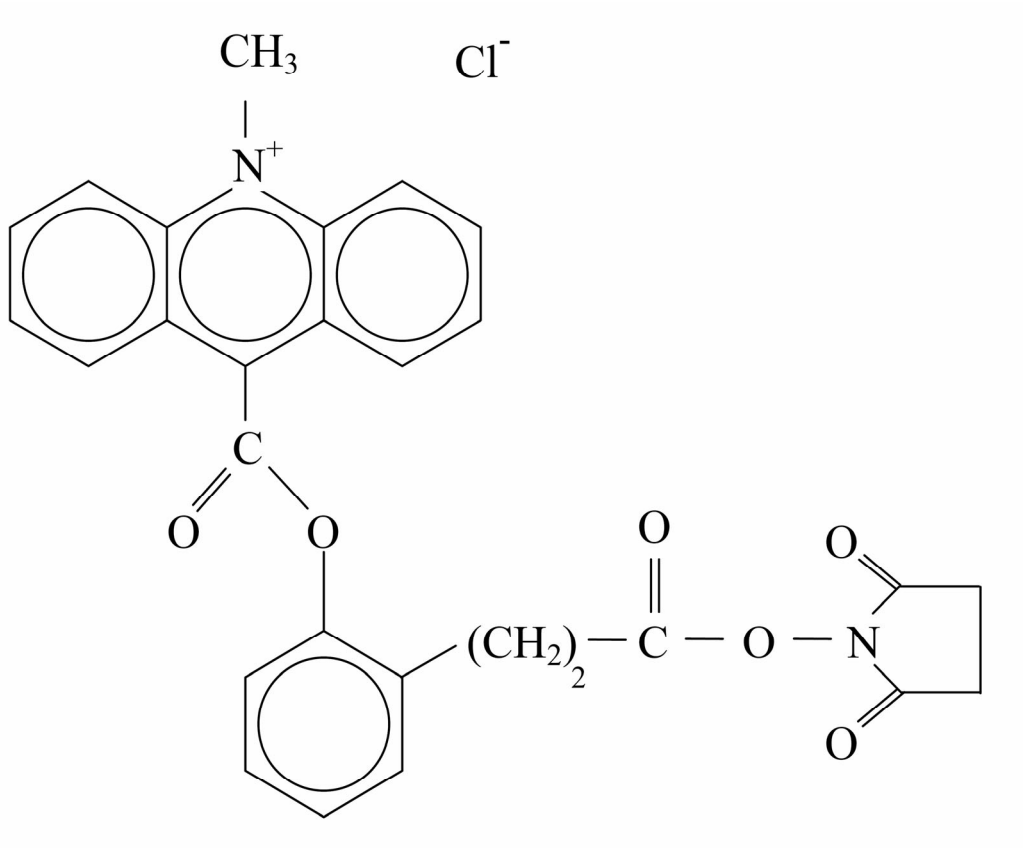
The exposure of the sample (which comprises the bacteria to detect) to the solid p
(the first incubation time) may vary between 15 minutes and 3 hours, at a temperature
the range of 20 °C to 40 °C and generally in the presence of a saline buffer giving a pH
of 8.3-9.5.

Generally, the wash steps described under points b) and d) are each performed by using a saline buffer also giving a pH of 8.3-9.5.

The exposure in step c) of the labelled antibody to the bacteria immobilized on the solid phase (the second incubation time) may vary between 15 minutes to 3 hours, at a temperature in the range of 20 °C to 40 °C and generally in the presence of a saline buffer giving a pH of 8.3-9.5.

In the final step point e) an activating solution which may be a dilute aqueous solution of a sodium oxalate (0.05 M) and hydrogen peroxide (0.02 M) is added to the labelled bacteria on the solid phase. This causes the acridinium label to emit light. The number of bacteria present can be quantified by measurement of the intensity of the emitted light by means of a luminometer. The concentration of acridinium molecules which is proportional to the number of bacteria can be determined by the relative light units (RLU) registered on the luminometer. A highly contaminated sample will give a high RLU count, whereas a negative sample will give a very low RLU count.

The antibody which is used under point c) is an antibody which will bind to Salmo bacteria with sufficient specificity to provide a useful assay. The antibody used is preferably an antibody belonging to the class of proteins called immunoglobulins gamma (molecules IgG) derived from rabbits. The antibody is made by conventional techniques known per se. The chosen antibody is labelled with a chemiluminescent agent selected from acridinium derivatives. Preferably, the labelling step is performed by using an acridinium derivative consisting of a substituted phenyl ester of 10-methylacridinium-9-carboxylic acid corresponding to formula (1) below:



The acridinium esters of formula (1) which are used to carry out the method of the present invention are known compounds, the preparation of which is described in the reference EP-A-0082 636.

A general scheme for labelling the antibody with the acridinium derivative involves the following methodology:

- dissolving the antibody in a saline buffer at a pH of 2;
- adding said antibody solution to the label (3 to 10 moles per mole of antibody protein) optionally in solution in an organic solvent, and mixing the ingredients thoroughly;
- leaving the mixture to stand in the dark at a temperature of 20-30 °C for a period of 5 minutes to 30 minutes;
- optionally followed by purifying the labelled antibody to remove the excess of labelling compound by gel permeation chromatography.

In another aspect the present invention provides a kit for detecting bacteria of the genus Salmonella present in a culture or in a contaminated food sample, which comprises:

- a solid phase, preferably in the form of either a microtiter plate well or a membrane;
- an antibody labelled by coupling with an acridinium derivative;
- an aqueous solution of sodium oxalate (0.05 M) and hydrogen peroxide (0.02 M).

In order to further illustrate the present invention and the advantages thereof, the followings examples are given, it being understood that they are intended only as illustrative and in no way limiting.

EXAMPLE I

Preparation of antibody labelled with acridinium derivative

The antibody used was an anti-Salmonella IgG antibody. This antibody was prepared, in a manner known per se, as a pure solution.

The acridinium derivative used was that of formula (1).

The acridinium labelling reaction is performed as follows:

1. Dissolve antibody (50 micrograms of IgG) in 0.2 ml of labelling buffer (0,2 M sodium phosphate, pH = 2);
2. Add antibody solution to 5 micrograms of acridinium label, and mix well;
3. Incubate mixture for 15 minutes at room temperature (25 °C) in the dark;
4. Load mixture onto a 10 ml column of Sephadex G25M and collect 15 x 1 ml fractions. A suitable elution and storage buffer is phosphate buffered saline (0.1 M, pH 6,3, with 0.15 M NaCl) containing 0,05 % (w/v) sodium azide and 0.1 % (w/v) bovine serum albumin;
5. Remove aliquot (0.01 ml) of each fraction and measure activity in luminometer;
6. Pool active fractions and store acridinium labelled antibodies at -20 °C.

EXAMPLE II

Detection of Salmonella by the method of the present invention

The following methodology was pursued:

a) Sample (of known concentration of Salmonella) is added (0.1 ml/well) to the wells of a polystyrene microtiter plate used for immunoassays. This is incubated for 30 minutes at 37 °C in the presence of Tris buffer, pH 9.2;

b) Microtiter plate is then washed 5 times with PBS buffer, pH 9.0 containing 0,05% Tween 20 surfactant (polyoxyethylenesorbitan monolaurate);

c) Labelled acridinium antibody is added to each well and incubated for 30 minutes at 37 °C in the presence of Tris buffer, pH 9.2;

d) Plate is washed as in step b); and e) Luminescence measured on LB96P luminometer, which automatically adds an aqueous solution of sodium oxalate (0.05 M) and hydrogen peroxide (0.02 M) to each well of the plate.

The present method was able to detect Salmonella bacteria at a concentration of 100 cells/ml, which is more sensitive than any commercially available test for these bacteria.

CLAIMS

1. An immunological method for detecting bacteria of the genus Salmonella present in a 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 said solid phase;

   b) Subjecting the solid phase with immobilised bacteria to a first wash step to remove unbound bacteria;

   c) Bringing the washed solid phase into contact with a specific antibody, which is labelled by coupling to a chemiluminescent agent comprising an acridinium derivative, said contact being performed during a second incubation time sufficient to allow the labelled antibody to bind to the antigen;

   d) Subjecting the solid phase from step c) to a second wash step to remove any unbound labelled antibody; and

   e) Placing the solid phase obtained in step d) above under conditions whereby light may be produced, and the luminescence activity produced may be quantitatively measured with the help of an appropriate device.

2. A kit for detecting bacteria of the genus Salmonella present, in a culture or in a contaminated food sample, which comprises:

   - a solid phase;

   - an antibody of the IgG type labelled by coupling with an acridinium derivative; and

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

DOCUMENT 2 (State of the art)

Light emitting devices (LEDs) are useful in a variety of applications. LEDs consist of a layer of a chemiluminescent compound sandwiched between two electrodes at least one of which is transparent. A current is applied to the layer from through the electrodes. This causes the compound to emit light. Chemiluminescent compounds are sought which provide a high brightness of emitted light at a low applied voltage.
Among the materials that have been studied, osmium complexes have recently attracted considerable attention.

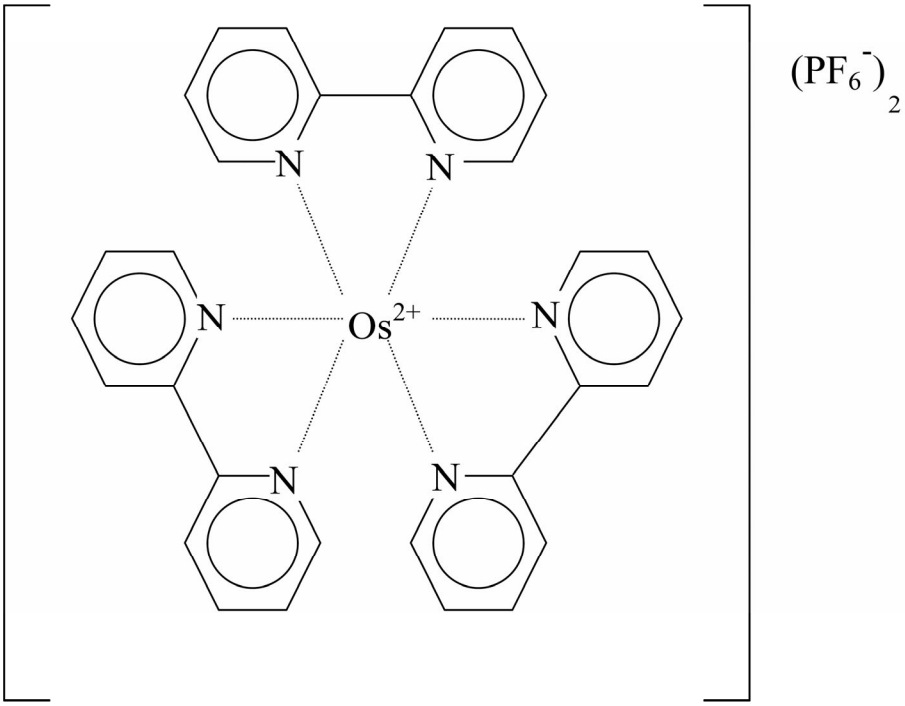
SUMMARY OF THE INVENTION

The present invention provides a method for producing LEDs with a high brightness at a low applied voltage based on a film of organic light-emitting compounds selected from unsubstituted or carboxylic acid substituted tris(2,2'-bipyridine) osmium complexes.

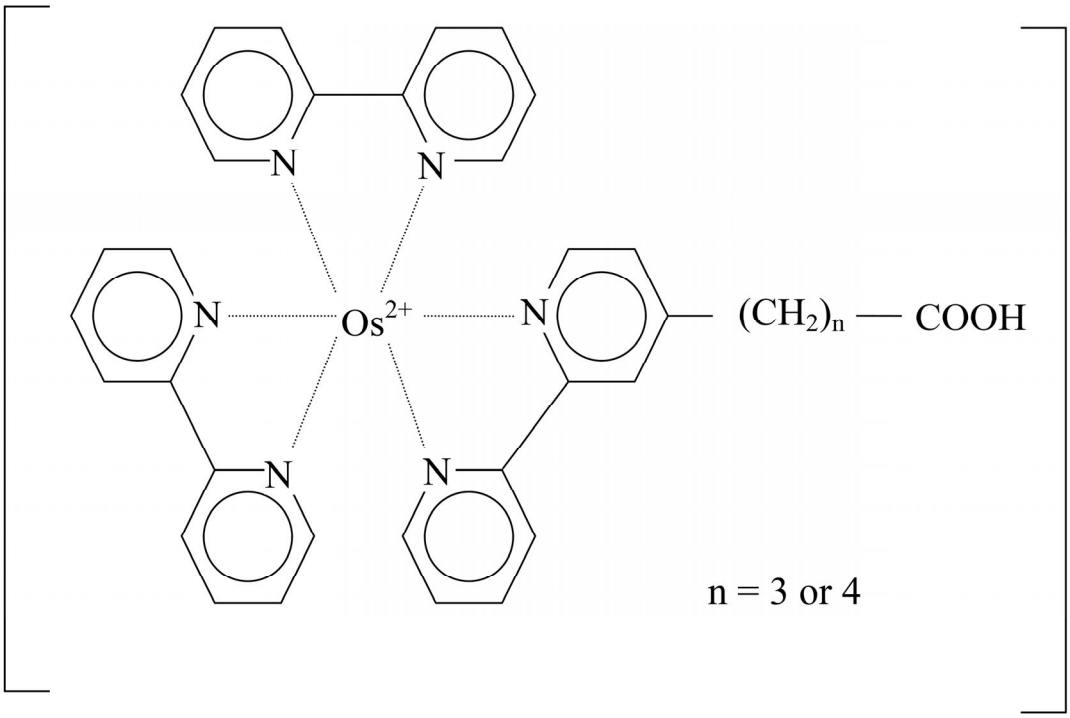
DESCRIPTION OF THE PREFERRED EMBODIMENTS

The inventors have discovered that the use of tris(2,2’-bipyridine) osmium complexes as the organic light emitting compound provides LEDs with the desired characteristics. Particularly good results have been obtained when the osmium complex used is selected from tris(2,2’-bipyridine) osmium bis(hexafluorophosphate), bis(2,2’-bipyridine)(2,2’-bipyridine-4 pentanoic acid) osmium bis(hexafluorophosphate) or bis(2,2’-bipyridine)(2,2’-bipyridine-4 butanoic acid) osmium bis(hexafluorophosphate).

The chemical structures of these osmium complexes is as follows:



$(PF_6^-)_2$



$(PF_6^-)_2$

These complexes may be synthesised using a procedure described elsewhere (J. Complex Chem. Vol. 66, (1953), 44).

Preparation of the light-emitting device:

A glass plate was sputter coated with a 5 micrometer thick layer of indium tin oxide (ITO). This layer forms a transparent anode. A thin film (about 100 nm thick) of tris(2,2'-bipyridine) osmium bis(hexafluorophosphate) was spin-coated onto the indium tin oxide coated substrates from a 4% (tris(2,2'-bipyridine) osmium bis(hexafluorophosphate) solution in acetonitrile at room temperature. The film was heated in a vacuum oven at 125 °C. for at least 8 hours. Aluminium cathodes were then printed on top of the films at room temperature. The anode and cathode were each electrically connected by soldering a thin copper wire to the electrode.

Characterisation of the light-emitting device:

The brightness-voltage characteristics of the device were measured at room temperature.

The LED manufactured as described above under a voltage of 3.0 V gave a bright red emission clearly visible in a lighted room. A similar emission was obtained for a LED in which a layer of bis(2,2'-bipyridine)(2,2'-bipyridine-4-butanoic acid) osmium bis(hexafluorophosphate) was used as the light emitting layer. These results show that the present LEDs exhibit an outstanding brightness at a very low applied voltage.

What is claimed:

1. A high brightness, low voltage thin film organic light-emitting device (LED) comprising an organic light-emitting layer consisting of unsubstituted or carboxylic acid substituted tris(2,2'-bipyridine) osmium complexes.