A method and an apparatus for detecting and quantifying bacterial spores on a surface. In accordance with the method: a matrix including lanthanide ions is provided on the surface containing the bacterial spores; functionalized aromatic molecules are released from the bacterial spores on the surface; a complex of the lanthanide ion and the aromatic molecule is formed on the surface; the complex of the lanthanide ion and the aromatic molecule is excited to generate a characteristic luminescence of the complex on the surface; and the bacterial spores exhibiting the luminescence of the complex on the surface are detected and quantified.

31 Claims, 6 Drawing Sheets


* cited by examiner
Absorption → Energy Transfer → Emission

DPA → Tb$^{3+}$

FIG. 1B
FIG. 1C
FIG. 4
METHOD AND APPARATUS FOR DETECTING AND QUANTIFYING BACTERIAL SPORES ON A SURFACE

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Ser. No. 60/519,851 filed on Nov. 13, 2003, which is incorporated herein by reference in its entirety. This application also claims the benefit of U.S. Provisional Patent Application Ser. No. 60/624,068 filed Nov. 1, 2004.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The present invention was made with support from the United States Government under Grant number NAS7-1407 awarded by NASA. The United States Government has certain rights in the invention.

BACKGROUND

1 Field

The present disclosure relates to the field of chemical detection. In particular, a method and apparatus for detecting and quantifying bacterial spores on a surface is disclosed.

2. Description of Related Art

Lanthanide complexes, particularly those of Tb\textsuperscript{3+} and Eu\textsuperscript{3+}, exhibit luminescence properties for the detection of aromatic biomolecules. The detection scheme is based on the absorption–energy transfer–emission mechanism, which is triggered by the binding of aromatic ligands to lanthanide complexes under UV excitation. Recent efforts have been focused on the detection of dipicolinic acid DPA (2,6-pyridinedicarboxylic acid), which is a unique constituent of bacterial spores present at high concentrations (up to 1 M). Dipicolinic acid is also a commercially available product having the following characteristics: CAS #: 499-83-2, Synonyms: 2,6 Pyridine Dicarboxylic Acid, Molecular Formula: C\textsubscript{6}H\textsubscript{5}NO\textsubscript{4}, Molecular Weight: 167.12, Description: White crystalline powder, Sulphated Ash: 0.3% max, Moisture Content: 0.5% max, Melting Point: 242.0 to 245.0°C, Assay: 99.0% min.

U.S. Pub. App. No. 2003-0138876 for “Method bacterial endospore quantification using lanthanide dipicolinate luminescence” to Adrian Ponce discloses a lanthanide that is combined with a medium to be tested for endospores. Dipicolinic acid released from the endospores binds the lanthanides, which have distinctive emission (i.e., luminescence) spectra, and are detected using photoluminescence. The concentration of spores is determined by preparing a calibration curve that relates emission intensities to spore concentrations for test samples with known spore concentrations. A lanthanide complex is used as the analysis reagent, and is comprised of lanthanide ions bound to multidentate ligands that increase the dipicolinic acid binding constant through a cooperative binding effect with respect to lanthanide chloride. The resulting combined effect of increasing the binding constant and eliminating coordinated water and multiple equilibria increases the sensitivity of the endospore assay by an estimated three to four orders of magnitude over prior art of endospore detection based on lanthanide luminescence.

U.S. Publication application No. 2004-0014154 for “Methods and apparatus for assays of bacterial spores” to Adrian Ponce discloses a sample of unknown bacterial spores which is added to a test strip. The sample of unknown bacte-
Preferably, the step of collecting bacterial spores carried in
the air comprises continuously sampling the air and the step
detecting the luminescence to determine the presence of
the bacterial spores comprises continuously monitoring the
luminescence.

When the step of releasing DPA from the bacterial spores
comprises microwaving the bacterial spores to heat the
solution, the step of combining the terbium ions with the DPA in
solution comprises cooling the heated solution to increase the
fraction of bound Tb-DPA complex.

Currently, bioburden levels are determined using the cul-
ture-dependent methods, with which bacterial spores are
quantified in terms of colony forming units (CFU’s) that
become visible on growth plates after incubation. There are
several limitations for culture-dependent methods. First, this
process requires 3-5 days to complete. Second, a large num-
ber of bacterial spores can aggregate on individual particu-
lates giving rise to a single CFU, and thus a large underesti-
mation of the bioburden. Third, colony-counting methods
only account for cultivable spore-forming species, which
constitute less than 1% in environmental samples.

It is desirable to provide a very sensitive method and appa-
ratus for counting bacterial spores after a short time.

SUMMARY

According to a first aspect, a method for detecting and
quantifying bacterial spores on a surface is disclosed, com-
prising: a) providing a matrix including lanthanide ions on the
surface containing the bacterial spores; b) releasing function-
alized aromatic molecules from the bacterial spores on the
surface; c) forming a complex of the lanthanide ion and the
aromatic molecule on the surface; d) exciting the complex of
the lanthanide ion and the aromatic molecule to generate a
characteristic luminescence of the complex on the surface;
and e) detecting and quantifying the bacterial spores exhib-
ting the luminescence of the complex on the surface.

According to a second aspect, a method for detecting and
quantifying bacterial spores on a surface is disclosed, com-
prising: a) transferring the bacterial spores from the surface
containing bacterial spores to a test surface; b) providing a
matrix including lanthanide ions on the test surface; c) releas-
ing functionalized aromatic molecules from the bacterial
spores on the test surface; d) forming complexes of the lan-
thanide ions and the aromatic molecules on the test surface;
e) exciting the complexes of the lanthanide ions and the aro-
natic molecules to generate a characteristic luminescence of the
complexes on the test surface; and f) detecting and quanti-
fying the bacterial spores exhibiting the luminescence of the
complexes on the test surface.

The disclosure also provides an apparatus for detecting and
quantifying bacterial spores on a surface including lanthanide
ions and aromatic molecules released from the bacterial
spores on the surface comprising: an UV light radiation
device for exciting a complex of a lanthanide ion and an
aromatic molecule to generate a characteristic luminescence of
the complex on a surface; a microscope for detecting and
quantifying bacterial spores exhibiting the luminescence of
the complex on the surface; and an imaging devise for imag-
ing bacterial spores exhibiting the luminescence.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a microscopic image of a spore (about 1 µm in
diameter) highlighting a DPA rich spore core.

FIG. 1B is a diagram of a Tb³⁺ ion (shaded ball) which by
itself has a low absorption cross section (<10 M⁻¹cm⁻¹) and
consequently has low luminescence intensity. The Tb³⁺ ion
binds very efficiently with DPA (absorption cross section
>10² M⁻¹cm⁻¹) originating from the spore. DPA binding gives rise
to bright Tb luminescence.

FIG. 1C is a diagram of the photophysical scheme for DPA
sensitized luminescence of the Tb complex (absorption-
energy transfer-emission, AETE).

FIG. 2 depicts a photograph of a backlight illuminated
agar plate with Tb-DPA microspheres (1-µm) on fluorescent
plates that require 3-5 days before results are available. The
method and apparatus according to the present disclosure will
yield results within minutes for obtaining total bacterial spore
counts on surfaces.

FIG. 3 depicts Eu³⁺ microspheres (1-µm) on fluorescent
paper imaged with an ImageX-TiTi gated CCD camera
mounted on a Carl Zeiss fluorescence microscope with 40x
objective, excited with a 300-Hz Perkin Elmer flashlamp.
Images are obtained (A) without gating, (B) with gating (100-
µs delay, 2.7-ns gate), and (C) 100-µm reference graticule to
estimate spatial resolution.

FIG. 4 depicts a schematic apparatus for imaging quanti-
fying and counting of bacterial spores.

FIG. 5 depicts two lifetime gated photographs showing
bacterial spores on R2A agar before germination (left portion
of the figure) and after germination (right portion of the figure).

DETAILED DESCRIPTION

Bacterial spores are generally accepted to be indicator
species for validating sterility since they are the most resilient
form of life towards sterilization regimens. Sterility testing of
surfaces is traditionally performed with RODAC growth
plates that require 3-5 days before results are available. The
method and apparatus according to the present disclosure will
yield results within minutes for obtaining total bacterial spore
counts on surfaces.

Dipicolinic acid (DPA, 2,6 pyridinedicarboxylic acid) is
present in high concentrations (about 1 molar or about 15% of
by weight) in the core of bacterial spores as a 1:1 complex
with Ca²⁺ as shown in FIG. 1A. For all known lifeforms, DPA
is unique to bacterial spores and is released into bulk solution
upon germination, which is the process of spore-to-vegetative
cell transformation. Thus, DPA is an indicator molecule for
the presence of bacterial spores. DPA is also a classic inor-
ganic chemistry ligand that binds metal ions with high affini-
ty. DPA binding to terbium ions (or other luminescent lan-
thanide or transition metal ions) triggers intense green
luminescence under UV excitation as shown in FIGS. 1B and
1C. The green luminescence turn-on signal indicates the pres-
ence of bacterial spores. The intensity of the luminescence
can be correlated to the number of bacterial spores per milli-
liter.

The Tb-DPA luminescence assay can be employed to
detect bacterial spores on surfaces, including the surfaces of
air filters, water membrane filters, and adhesive polymers or
agar used to collect bacterial spores from surfaces to be tested.
In this disclosure, surfaces to be analyzed with the Tb-DPA
assay are called “test surfaces”. For example, the Tb-DPA
luminescence assay can be combined with an optically trans-
parent, adhesive polymer or agar to collect bacterial spores from
surfaces to be tested. Once the bacterial spores are
located on the test surface, they can be induced to release their
DPA content by germination or physical lysis, for example by
autoclaving or microwaving. The highly concentrated DPA
from the spores spills into the surrounding area, generating a
high concentration region around the spore body. The
reagents used for detection and induction of germination, if
that is the chosen method for DPA release, can be added into the matrix before or after the spores are sampled. The Tb-DPA luminescence arising from the region around the spore body is then imaged onto a camera. The bacterial spore regions manifest themselves as bright spots which can be counted. Due to the long-lived excited state of luminescent lanthanides, lifetime-gated detection enables any fluorescent background from interferents to be eliminated. Lifetime gating drastically reduces the background and enables much greater contrast between the Tb-DPA luminescence regions and the background.

One example of an adhesive polymer for the Tb-DPA luminescence assay for bacterial spores on surfaces is polydimethylsiloxane (PDMS) doped with TbCl₃ and L-alanine. The L-alanine induces germination to release the DPA from the core of the spore to the immediate surroundings. The TbCl₃ binds the DPA, which triggers green luminescence (543.5 nm) under UV excitation (250-300 nm) that can be quantified with a photodetector. Specifically, imaging individual germinating spores within a microscope field of view using a lifetime-gated camera will be used as an example.

From the perspective of our sensor design, we treat the bacterial spore essentially as a ~1-µm sphere containing ~10⁹ molecules of DPA. In our previous experiments, we collected spores from surfaces using the standard cotton swabbing method, resuspended the spores into water, and then released the DPA contents into bulk solution by germination or physical lysing and subsequently performed the Tb-DPA luminescence assay. This approach led to very dilute DPA solutions (e.g., 1 spore per ml of solution yields [DPA]=1 pM), which ultimately limits the sensitivity.

Instead of diluting the DPA into bulk solution, we immobilize the bacterial spores onto an adhesive polymer (e.g., PDMS), and then induce germination or physically lysis in the spore population on the polymer to generate local high DPA concentrations (i.e., the DPA remains in the immediate surroundings of the spore body). To obtain viable counts, germination will be induced by doped L-alanine (or other germination inducing agents) into the polymer matrix; TbCl₃, also doped into the polymer, report the presence of bacterial spores by triggering luminescence in the presence of DPA. To obtain total counts, the bacterial spores immobilized on the TbCl₃ containing polymer will be physically lysed (e.g., by heat, microwaving, or autoclaving) leads to DPA release and luminescence turn-on.

The present disclosure also includes a method and apparatus to measure the fraction of viable bacterial spores that remain on a surface. The method combines dipicolinic acid triggered terbium luminescence and dipicolinic acid release from (1) viable bacterial spore through germination, and (2) all viable and nonviable bacterial spores by autoclaving, sonication, or microwaving. The ratio of the results from steps (1) and (2) yield the fraction of bacterial spores that are alive.

The traditional culture based assays require 3 days for colonies to grow and be counted. However, a significant fraction of bacterial spores can undergo stage-1 germination, during which DPA (i.e., the chemical marker that is unique to bacterial spores) is released, in less than 40 minutes. See FIG. 2. A DPA-triggered Tb luminescence with Tb-doped agar was investigated. The samples were prepared by adding ~100 µl of agar doped with 1 mM TbCl₃ onto a quartz slide and allowing it to solidify. On top of the agar, we added 10 µl of 10⁴ spores/ml *Bacillus subtilis* spores (i.e., 10⁹ spores), and then added a drop of 10 µl of 1-mM L-alanine to induce germination.

Under UV (blacklight) illumination, the luminescence of the embedded Tb increased dramatically upon germination within 40 minutes of the bacterial spores, while the embedded Tb luminescence in the control sample that had no exposure to L-alanine remained weak. See FIG. 2. An agar control sample without Tb that was covered with bacterial spores also did not yield detectable luminescence. Note that the bright edges of the spots are artifacts of drying due to refraction from accumulated material, which would not appear in a lifetime-gated image.

The pictures in FIG. 2 were taken without magnification, and thus the individual spores cannot be enumerated as they germinate. However, in the proposed effort, germinating bacterial spores will be imaged with a lifetime-gated microscope. As the spores germinate, DPA is released from the core to generate local high DPA concentrations, which will show up as bright green luminescent halos surrounding the spore body. These results demonstrate that viable bacterial spores on surfaces can be enumerated using a lifetime-gated microscope.

Lifetime-gated images of Eu³⁺ microspheres on highly fluorescent paper were obtained with a lifetime-gated camera (Photonic Research Systems Ltd, United Kingdom). See FIG. 3. Eu³⁺ microspheres were employed because they are commercially available and have analogous photophysical properties. The ImageX system effectively rejected all of the background fluorescence when a delay time of 100 µs was used. It is striking that the microspheres exhibiting weak, long-lived luminescence immobilized on a highly fluorescent matrix are imaged with high contrast against a silent background when gating is applied.

Another example of the invention is illustrated in FIG. 5, where bacterial spores were added onto the surface of R2A agar doped with 10 mM L-alanine to induce germination and 100 uM TbCl₃ to generate bright luminescent spots around the spore body as they germinated and released DPA. A Xe-flash lamp firing at 300 Hz with a 275 nm interference filter provided excitation for the Tb-DPA complex, and the corresponding bright spots from the bacterial spore-Tb-DPA luminescent halos where imaged with a lifetime-gated camera set at a delay time of 100 µs and an integration time of 2 ms. The individual bacterial spores become clearly visible as countable spots after they germinated. The images shown in FIG. 5 can be obtained by an apparatus as shown in FIG. 4, which contains a Xenon flash lamp, a microscope objective, a microscope, and a lifetime gated camera mounted on the microscope.

**EXAMPLES**


Aerosolized bacterial spores were captured with an aerosol biosampler. The biosampler was filled with 20 ml of 10 µM TbCl₃ glycerol solution, which has a 95% transfer efficiency for microbe-containing aerosols. Once bacterial spores were suspended in the biosampler collection vessel, DPA was
released by microwave into the bulk solution within 8 minutes. The resulting free DPA then bounded Tb in bulk solution, giving rise to luminescence turn-on under UV excitation. A fiber optic probe immersed in the sample solution transmitted the luminescence to a spectrometer.

Approximately 10,000 bacterial spores per 1 ml solution produced enough DPA to obtain sufficient amount of DPA-Tb complexes to provide enough luminescence turned-on under UV excitation to be detected by a spectrometer.


Comparative Example 2 was performed like Comparative Example 1. A fiber optic probe immersed in the sample solution transmitted the luminescence to a fluorimeter.

Approximately 1,000,000 bacterial spores per 1 ml solution produced enough DPA to obtain sufficient amount of DPA-Tb complexes to provide enough luminescence turned-on under UV excitation to be detected by a spectrometer.

Example 1

Bacteria spores were immobilized onto a test sample surface of thin, flexible, clear, adhesive polymer polydimethylsiloxan (PDMS). PDMS was doped with L-alanine to induce germination and generate local high concentration of DPA. Tbc13 was also doped into the PDMS sample. The bacterial spores immobilized on the L-alanine and Tbc13-containing polymer was physically lysed by microwave irradiation, wherein DPA was released and luminescence was turned on. The detection of bacterial spores on the PDMS adhesive polymer was manifested itself as a bright green luminescence that was imaged with a lifetime gated microscope. The green dots within the microscope field of view were counted to determine the concentration of viable spores found on the surfaces that was sampled. Therefore, every bacterial spore releasing luminescence can be individually counted. A concentration of 10,000 bacterial spores per 1 ml as in comparative example 1 or 1,000,000 bacterial spores per 1 ml in comparative example 2 is not required in example 1. As a consequence, the method according to the disclosure can be carried out even with an extremely low concentration of bacterial spores, even a single bacterial spore.

Another embodiment of the present invention is an apparatus for detecting and quantifying bacterial spores on a surface including lanthanide ions and aromatic molecules released from the bacterial spores on the surface. See FIG. 4. The apparatus comprises an UV-light radiation device for exciting a complex of a lanthanide ion and an aromatic molecule to generate a characteristic luminescence of the complex on a surface. The source for the UV-light is preferably a Xenon flash lamp, which is approximately 5 cm away the test surface. Between the Xenon flash lamp and the test surface are two C-amount elliptical lenses. The Xenon flash lamp and the test substrate are positioned in an angle of 45° to each other. The area of irradiation by the Xenon flash lamp is observed by a microscope objective with a red bandpass filter suitable for Eu3+ for detecting and quantifying bacterial spores exhibiting the luminescence of the complex on the surface. The image is transferred from the microscope to the imaging device for imaging bacterial spores exhibiting the luminescence, preferably an imageX nanoCCD camera.

The method and apparatus of the present disclosure provide the imaging of the spherical resolution of the high concentrating region of DPA around each spore body, which has been lysed. The present method makes it possible to detect and quantify extremely low concentrations of bacterial spores in very short time.

Bioburden testing is an assessment of the numbers and types of microorganisms present on a product, and may be used to support sterilization validations. Sterility determination for surfaces are required by the pharmaceutical, health care, and food preparation industries for compliance with bioburden standards as outlined by USP, FDA, PDA, and AAMI.

What I claim is:

1. A method for detecting and quantifying individual bacterial spores on a test surface comprising:
   providing a matrix including one or more lanthanide ions on the test surface containing the bacterial spores;
   releasing functionalized aromatic molecules from the bacterial spores onto the surface;
   forming a complex of the lanthanide ion and the aromatic molecule on the test surface;
   exciting the complex of the lanthanide ion and the aromatic molecule to generate a characteristic luminescence of the complex on the test surface;
   detecting and quantifying, through lifetime-gated imaging, the individual bacterial spores exhibiting the luminescence of the complex on the test surface.

2. The method according to claim 1, wherein one or more lanthanide ions are provided to the matrix prior to the bacterial spores, after the bacterial spores.

3. The method according to claim 1, wherein the provided lanthanide ions are terbium or europium ions or a mixture thereof.

4. The method according to claim 1, wherein the released aromatic molecules are dipicolinic acid (DPA) and/or derivatives thereof.

5. The method according to claim 1, wherein the aromatic molecules are released from the bacterial spores by micro-waving the bacterial spores.

6. The method according to claim 1, wherein the aromatic molecules are released from the bacterial spores by germinating the bacterial spores with L-alanine, inosine and mixtures thereof.

7. The method according to claim 1, wherein the aromatic molecules are released from the bacterial spores by sonicating the bacterial spores with microspheres.

8. The method according to claim 1, wherein the aromatic molecules are released from the bacterial spores by autoclaving.

9. The method according to claim 1, wherein the complex of the lanthanide ion and the aromatic molecule is excited by UV light.

10. The method according to claim 1, wherein the lifetime-gated imaging comprises counting bright spots due to DPA triggered luminescence around the bacterial spores shown in images obtained by a camera, preferably mounted on top of a microscope.

11. The method according to claim 10, wherein the imaging shows the specific resolution of the concentration of dipicolinic acid around the individual bacterial spores, which release dipicolinic acid.

12. A method for detecting and quantifying individual bacterial spores on a surface comprising:
transferring the bacterial spores from the surface containing bacterial spores to a test surface;
providing a matrix including lanthanide ions on the test surface;
releasing functionalized aromatic molecules from the bacterial spores on the test surface;
forming complexes of the lanthanide ions and the aromatic molecules on the test surface;
exciting the complexes of the lanthanide ions and the aromatic molecules to generate a characteristic luminescence of the complexes on the test surface; and
detecting and quantifying through lifetime-gated imaging, the individual bacterial spores exhibiting the luminescence of the complexes on the test surface.

13. The method according to claim 12, wherein the test surface contains at least one polymer.

14. The method according to claim 12, wherein the test surface contains at least one adhesive polymer.

15. The method according to claim 12, wherein the test surface contains at least one adhesive polymer, which is transparent until about 250 nm, thereby providing a transparent test surface.

16. The method according to claim 12, wherein the test surface contains at least one lanthanide ion and L-alanine doped polymer.

17. The method according to claim 12, wherein the test surface contains at least one lanthanide ion and L-alanine doped polydimethylsiloxane polymer.

18. The method according to claim 12, wherein the test surface contains at least one agar.

19. The method according to claim 12, wherein the provided lanthanide ions are terbium or europium ions or a mixture thereof.

20. The method according to claim 12, wherein the released aromatic molecules are dipicolinic acid (DPA).

21. The method according to claim 12, wherein the aromatic molecules are released from the bacterial spores by microwaving the bacterial spores.

22. The method according to claim 12, wherein the aromatic molecules are released from the bacterial spores by germinating the bacterial spores with L-alanine.

23. The method according to claim 12, wherein the aromatic molecule are released from the bacterial spores by sonication of the bacterial spores with microspheres.

24. The method according to claim 12, wherein the complex of the lanthanide ion and the aromatic molecule is excited by UV light.

25. The method according to claim 12, wherein the imaging shows the specific resolution of the concentration of dipicolinic acid around bacterial spores, which release dipicolinic acid.

26. The method according to claim 1, wherein the matrix containing bacterial spores comprises at least one air filter.

27. The method according to claim 12, wherein the matrix containing bacterial spores comprises at least one air filter.

28. The method according to claim 1, wherein the matrix containing bacterial spores comprises at least one water filter.

29. The method according to claim 12, wherein the matrix containing bacterial spores comprises at least one water filter.

30. An apparatus for detecting and quantifying individual bacterial spores on a surface including lanthanide ions and aromatic molecules released from the bacterial spores on the surface comprising:
a test surface having thereon bacterial spores, lanthanide ions and aromatic molecules released from the bacterial spores, wherein the aromatic molecules have been released on the test surface by germination of bacterial spores by the addition of L-alanine, inosine or mixtures thereof or the lysis of bacterial spores by means of one selected from autoclaving, microwaving, heating and sonication;
a UV-light radiation device for exciting a complex of lanthanide ion and aromatic molecule to generate a characteristic luminescence of the complex on the test surface; a microscope for detecting and quantifying bacterial spores exhibiting the luminescence of the complex on the test surface; and
a lifetime-gated imaging device for imaging bacterial spores exhibiting the luminescence.

31. The method according to claim 1, wherein one or more lanthanide ions are provided as a mixture with the bacterial spores.