METHOD AND APPARATUS FOR DETECTING AND QUANTIFYING BACTERIAL SPORES ON A SURFACE

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ABSTRACT
A method and an apparatus for detecting and quantifying bacterial spores on a surface. In accordance with the method: bacterial spores are transferred from a place of origin to a test surface, the test surface comprises lanthanide ions. Aromatic molecules are released from the bacterial spores; a complex of the lanthanide ions and aromatic molecules is formed on the test surface, the complex is excited to generate a characteristic luminescence on the test surface; the luminescence on the test surface is detected and quantified.

23 Claims, 12 Drawing Sheets
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Figure 1B

Absorption  Energy Transfer  Emission

DPA  Tb^{3+}
Figure 1C

$^1\pi^*$

$^3\pi^*$

Energy Transfer

$^{5}_{D_{4}}$

Absorption

Emission

$^1\pi$

DPA

$^7_{F_{j=6-0}}$

Tb$^{3+}$
Figure 2B
Figure 3
Figure 5
1. METHOD AND APPARATUS FOR DETECTING AND QUANTIFYING BACTERIAL SPORES ON A SURFACE

CROSS REFERENCE TO RELATED APPLICATIONS


STATEMENT OF GOVERNMENT SUPPORT

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 terbium (Tb+) and europium (Eu+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: C₆H₆N₂O₄, 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 degree, C., Assay: 99.0% min.

Bacterial spores are generally accepted to be indicator species for validating sterility since they are the most resilient form of life against sterilization regimens (Hindle and Hall, 1999 Analyst, 124, 1599-1604). Sterility testing of surfaces is traditionally performed by either (1) swabbing the surface with a cotton applicator, resuspending the swabbed spores, and plating the spore suspension onto growth media; or (2) using Replicate Organism Detection and Counting (RODAC) growth plates that are pressed against a surface to be analyzed. Each of these two bacterial spore assays requires 3-5 days before results are available.

As mentioned, dipicolinic acid (DPA) is present in high concentrations (about 1 mol/l or about 15% of by weight) in the core of bacterial spores (Murell, 1969, Bact. Spore 1, 216). In its deprotonated state, DPA is dipicolinate (DP) and is found in a 1:1 complex with Ca²⁺ inside the spore, as shown in FIG. 1A. For all known life-forms, DPA is unique to bacterial spores and is naturally released into bulk solution upon germination—the process of spore-to-vegetative cell transformation. DP can also be released upon lysis of the bacterial spore. Thus, DPA and/or DP are indicator molecules for the presence of bacterial spores. DPA is a classic inorganic chemistry ligand that binds metal ions with high affinity. As mentioned, DPA takes the form of dipicolinate (DP) in its deprotonated form that binds to Ca²⁺. DPA binding to terbium ions (or other luminescent lanthanide 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 presence of bacterial spores. The intensity of the luminescence can be correlated to the number of bacterial spores per milliliter.

U.S. Patent Application Publication No. 2003-0138876 for “Method bacterial endospore quantification using lanthanide dipicolinate luminescence” 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. Patent Application Publication No. 2004-0014154 for “Methods and apparatus for assays of bacterial spores” discloses a sample of unknown bacterial spores which is added to a test strip. The sample of unknown bacterial spores is drawn to a first sample region on the test strip by capillary action. Species-specific antibodies are bound to the sample when the unknown bacterial spores match the species-specific antibodies, otherwise the sample is left unbound. DPA is released from the bacterial spores in the bound sample. Terbium ions are combined with the DPA to form a Tb-DPA complex. The combined terbium ions and DPA are excited to generate a luminescence characteristic of the combined terbium ions and DPA to detect the bacterial spores. A live/dead assay is performed by a release of the DPA for live spores and a release of DPA for all spores. The detection concentrations are compared to determine the fraction of live spores. Lifetime-gated measurements of bacterial spores to eliminate any fluorescence background from organic chromophores comprise labeling the bacterial spore contents with a long-lifetime lumophore and detecting...
from dead bacterial spores in a second unit of the sample by
further discloses a method for live/dead assay for bacterial
ing DPA from the bacterial spores; combining the terbium
luminescence characteristic of the combined terbium ions
ions and DPA to detect the dead bacterial spores; generating
terbium ions with DPA in solution released from viable
spores; exciting the combined terbium ions and
DPA released from viable bacterial spores to generate a first
luminescence characteristic of the combined terbium ions
and DPA to detect the viable bacterial spores; releasing DPA
from dead bacterial spores in a second unit of the sample by
autoclaving, sonication or microwaving; combining the ter-
bium ions with the DPA in solution released from dead
bacterial spores; exciting the combined terbium ions and
DPA released from dead bacterial spores to generate a second
luminescence characteristic of the combined terbium ions
and DPA to detect the dead bacterial spores; generating
a ratio of the first to second luminescence to yield a fraction
of bacterial spores which are alive.

also discloses a method for unattended monitoring of bac-
terial spores in the air comprising the steps of: providing a solution includ-
ing terbium ions in a sample of live and dead bacterial
spores; releasing DPA from viable bacterial spores by ger-
mination from a first unit of the sample; combining the ter-
bium ions with DPA in solution released from viable
bacterial spores; exciting the combined terbium ions and
DPA from viable bacterial spores to generate a first
luminescence characteristic of the combined terbium ions
and DPA to detect the viable bacterial spores; releasing DPA
from dead bacterial spores in a second unit of the sample by
autoclaving, sonication or microwaving; combining the ter-
bium ions with the DPA in solution released from dead
bacterial spores; exciting the combined terbium ions and
DPA released from dead bacterial spores to generate a second
luminescence characteristic of the combined terbium ions
and DPA to detect the dead bacterial spores; generating
a ratio of the first to second luminescence to yield a fraction
of bacterial spores which are alive.

According to a first aspect of the present disclosure, a
method is provided for detecting and quantifying individual
bacterial spores comprising: capturing the bacterial spores;
transferring the bacterial spores to a test surface; releasing
aromatic molecules from the bacterial spores on the test
surface; forming a complex of the one or more lanthanide
ions and the aromatic molecules on the test surface; exciting
the complex to generate a characteristic luminescence of the
complex on the test surface; and detecting and quantifying
the bacterial spores exhibiting the luminescence of the
complex on the test surface.

According to a second aspect of the present disclosure, a
method is provided for quantifying viable and nonviable
bacterial spores comprising: capturing the bacterial spores;
transferring the bacterial spores to a test surface; providing
one or more lanthanide ions to the test surface; releasing
aromatic molecules from the bacterial spores by germination
of the bacterial spores on the test surface; forming a first
complex of the one or more lanthanide ions and the aromatic
molecules on the test surface; exciting the first complex to
generate a characteristic luminescence of the first complex
on the test surface; detecting and quantifying the bacterial
spores exhibiting the luminescence of the first complex on
the test surface; releasing aromatic molecules from nonger-
mated spores on the test surface by lysis; forming a second
complex of the one or more lanthanide ions and lysis-
released aromatic molecules on the test surface; exciting the
second complex to generate a characteristic luminescence of
the second complex on the test surface; and detecting and
quantifying the nonviable bacterial spores exhibiting the
luminescence of the second complex on the test surface.

According to a third aspect of the present disclosure, a
method is provided for quantifying the percent viable spores
in a mixed population of viable and nonviable bacterial
spores comprising: capturing the bacterial spores from a place
of origin to a test surface comprising one or more lanthanide
ions; inducing release of DPA/DP molecules from the trans-
ferred bacterial spores by germination; forming a first com-
plex of the one or more lanthanide ions and the DPA/DP
molecules; exciting the first complex with UV radiation;
quantifying the luminescence of the first complex; sub-
sequently inducing release of DPA/DP by lysis of non-germi-
nated bacterial spores on the test surface; forming a second
complex of the one or more lanthanide ions and lysis-
induced DPA/DP molecules; exciting the second complex
with UV radiation; quantifying the luminescence of the
second complex; and dividing the quantified luminescence
from the first complex by the sum of the luminescence of the
first and second complexes.

According to a fourth aspect of the present disclosure, an
apparatus is provided for detecting and quantifying bacterial
spores comprising: an ultraviolet light radiation device to
excite a complex of lanthanide ions and aromatic molecules
and generate a characteristic luminescence of the complex;
a microscope for detecting and quantifying bacterial spores
exhibiting the luminescence of the complex; and an imaging
device connected with the microscope for imaging the
luminescence.

SUMMARY

According to a first aspect of the present disclosure, a
method is provided for detecting and quantifying individual
bacterial spores comprising: capturing the bacterial spores;
transferring the bacterial spores to a test surface; providing
one or more lanthanide ions on the test surface; releasing
aromatic molecules from the bacterial spores on the test
surface; forming a complex of the one or more lanthanide
ions and the aromatic molecules on the test surface; exciting
the complex to generate a characteristic luminescence of the
complex on the test surface; and detecting and quantifying

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 Tb²⁺ ion (shaded ball) which by
itself has a low absorption cross section (<10 M⁻¹ cm⁻¹) and
consequently has low luminescence intensity. The Tb²⁺ ion
can bind the light harvesting DPA/DP (absorption cross
section >10⁶ M⁻¹ cm⁻¹) originating from the spore. DPA/DP
binding gives rise to bright Tb luminescence.
FIG. 1C is a diagram of a photophysical scheme for
DPA/DP sensitized luminescence of the Tb-DPA complex
(absorption-energy transfer-emission, AETE).
Transfer of Bacterial Spores from Place of Origin

The lanthanide ion-DPA/DP luminescence assay can be employed to detect individual bacterial spores from a place of origin. DPA/DP refers to DPA and/or DP. In other words, DPA/DP means at least one between DPA and DP. A place of origin includes any solid surface, water and/or air. In order to posit the bacterial spores onto a test surface, the bacterial spores are first captured from a place of origin. A place of origin can include an infinite number of possibilities. Bacterial spores on solid surfaces are transferred from the solid surface onto a cotton swab (FIG. 2A), or an adhesive polymer, such as PDMS (polydimethyl siloxane) agar or agarose (FIG. 2B). Bacterial spores in water are transferred from water onto a water filter. The spores embedded onto the membrane water filter can be induced to release DPA/DP by germination and/or physical lysis.

In one embodiment, the test surface contains an adhesive polymer. In another embodiment, the test surface contains lanthanide-DPA/DP luminescence (FIG. 3). In a second preferred embodiment, the test surface is an adhesive polymer that contains a germinating agent, contains lanthanide ions, and is transparent, allowing for detection of lanthanide-DPA luminescence (FIG. 4).

According to one embodiment of the present disclosure, bacterial spores captured from a solid surface using cotton swabs can be transferred onto a test surface by resuspending the spores on the cotton swab into water, and then plating the water suspension onto a test surface. The bacterial spores on the cotton swab could also be suspended into water followed by filtration of the water suspension through a membrane water filter. The spores embedded onto the membrane water filter are then streaked onto a test surface (Example 1, 2). Alternatively, the membrane filter is the test surface. Alternatively, the swab is not made of cotton, but is made of any suitable material.

Examples of adhesive polymers include but are not limited to: polydimethyl siloxane (PDMS). Alternatively, agar can be doped with PDMS. Similarly, agarose can be doped with PDMS. In a preferred embodiment the adhesive polymer, PDMS, used to capture the bacterial spores from the surface of origin is subsequently used as a test surface. PDMS has low chemical activity, it is hydrophobic, it is optically transparent above 250 nm and it is impermeable to water. With these characteristics, a PDMS test surface allows for induction of lanthanide-DPA/DP luminescence, detection and quantifying. In one embodiment of the present disclosure, the test surface is optically transparent greater than 250 nm. In an alternative embodiment, the test surface is partially transparent.

In a second embodiment, the spores captured from a place of origin using an adhesive polymer are subsequently transferred from the adhesive polymer. This transfer can be carried out using several methods easily envisioned by one skilled in the art. For example, the spores can be streaked onto a test surface (Example 1). In another embodiment, spores captured onto a membrane filter are transferred onto a test surface by a streaking method as disclosed in Example 2. Alternatively, spores on a membrane filter are physically lysed on the membrane filter and then pressed against an adhesive polymer such as PDMS containing lanthanide ions. Similarly, spores on a membrane filter can be streaked (Example 2) onto an adhesive polymer (e.g. PDMS, agar, agarose) that contains the test surface. The present invention provides a method of using a test surface on which bacterial spores are poised. Once the bacterial spores are located on the test surface, they can be induced to release DPA/DP by germination and/or physical lysis.
lanthanide ions and L-alanine for induction of germination. In another embodiment, the membrane filter embedded with the bacterial spores is used as the test surface.

The step of collecting bacterial spores carried in the air comprises capturing the bacterial spores with an aerosol sampler or impactor. Preferably, the step of collecting bacterial spores carried in the air comprises continuously sampling the air. In one embodiment, air is passed over quartz filter tape using an air sampler (Example 1). In one aspect the quartz filter tape is subsequently as the test surface. Alternatively, air can be passed over lanthanide-doped agar using an air sampler.

The agents used for germination and the agents used for lysing can be added to the test surface before or after the bacterial spores have been transferred onto the test surface. Alternatively, the agents used for germination agents used for lysing can be added in a mixture with the transfer of the bacterial spores. Examples of germinating agents include but are not limited to: L-alanine, L-asparagine and D-glucose. Examples of lysing methods include but are not limited to: microwaving, plasma cleaning, dry heating, autoclaving, sonicating and hydrogen chloride gassing.

When the step of releasing DPA from the bacterial spores comprises microwaving the bacterial spores to heat the solution, the step of combining the lanthanide ions with the DPA in solution comprises cooling the heated solution to increase the fraction of bound lanthanide-DPA complex. One of skill in the art can envision several methods to prepare ("dope") the test surface for germination. Likewise, one of skill in the art can envision several methods to prepare ("dope") the test surface for lysing.

Lanthanide ions can be added to the test surface before the bacterial spores have been transferred onto said test surface, or in a mixture with the bacterial spores being transferred to the test surface. Lanthanide ions can be added after or in conjunction with the induced release of DPA/DP from the bacterial spores. Examples of lanthanide ions include, but are not limited to: terbium (Tb³⁺), europium (Eu³⁺) and dysprosium. In a preferred embodiment terbium (Tb³⁺) ions are used.

Inducing the Lanthanide-DPA/DP Luminescence

A lanthanide ion-DPA/DP luminescence assay can be employed to detect individual bacterial spores on surfaces. For example, the lanthanide-DPA/DP luminescence assay can be combined with an optically transparent, adhesive polymer (PDMS, agar or agarose) 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/DP content by germination (e.g. using L-alanine) or physical lysis, for example by autoclaving or microwaving. The highly concentrated DPA/DP 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/DP release, can be added into the matrix before or after the spores are sampled. The lanthanide-DPA/DP luminescence arising from the region around the spore body is then imaged onto a camera. The bacterial spore regions manifest themselves as bright spots that can be counted. Due to the long-lived excited states of luminescent lanthanides, lifetime-gated detection enables any fluorescent background from interferences to be eliminated. Lifetime gating drastically reduces the background and enables much greater contrast between the lanthanide-DPA/DP luminescence regions and the background.

It is understood by one skilled in the art, that upon release of DPA and/or DP outside the bacterial spore, the DPA and/or DP molecules can interact with other substances in its environment, resulting in a derivative of DPA or DP.

The step of detecting the luminescence to determine the presence of the bacterial spores comprises monitoring the luminescence with a spectrometer or fluorimeter, and the step of detecting the luminescence to determine the presence of the bacterial spores comprises continuously monitoring the luminescence.

In one embodiment of the present invention, an adhesive polymer for the terbium-DPA/DP luminescence assay for bacterial spores on surfaces is polydimethyl siloxane (PDMS) doped with TbCl₃ and L-alanine. The L-alanine induces germination to release the DPA/DP from the core of the spore to the immediate surroundings. The TbCl₃ binds the DPA/DP, which triggers green luminescence (543.5 nm) under UV excitation (250-300 nm) that can be quantified with a photodetector. Individual germinating spores can be imaged within a microscope field of view using a lifetime-gated camera.

From the perspective of senior design, the bacterial spore is essentially a 1 µm sphere containing about 10⁶ molecules of DPA. In previous experiments (U.S. Patent Pub No. 2004-0014154), spores were collected from surfaces using the standard cotton swabbing method, resuspended into water, and DPA/DP was then released into a bulk solution by germination or physical lysing and a subsequent lanthanide (Tb)-DPA luminescence assay was performed. This approach led to very dilute DPA solutions (e.g., 1 spore per ml of solution yields [DPA]~1 µM), which ultimately limits the sensitivity. As disclosed in the present invention, spores collected using the cotton swab can be suspended into water, and the water suspension can then be plated onto a testing surface for subsequent DPA/DP release, lanthanide-DPA/DP complexing, excitation, lumination and quantification. Alternatively, the water suspension can be filtered through a membrane filter and the spores on the filter can be streaked onto a testing surface.

The traditional culture-based assays require 3 days for colonies to grow and be counted. This traditional culture-based assay, also known as the NASA standard assay, is reported in colony forming units (CFU), since the quantification is based on the number of colonies. 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 4 minutes. This type of quantification, is reported as germinating spore units (GSU). Experimental results shown herein (Table 1) show a comparison of the GSU calculated following the teachings disclosed in this application, versus the CFU calculation of the NASA standard assay for the same amount of starting spores (total spore units/TSU). FIG. 3 further shows an L-alanine induced germination of Bacillus subtilis spores on a TbCl₃ doped agar. The DPA/DP released upon germination luminesces when complexed with the Tb³⁺ ions. (Example 2).

Detection, Imaging and Quantification of Lanthanide-DPA/DP Luminescence

A salient feature of the present disclosure is the implementation of lifetime-gated imaging to obtain an image with good contrast of bacterial spores after germination and/or lysis. Fluorescence lifetime imaging uses special detectors and light source technology to generate images wherein the contrast is related to the fluorescence lifetime across a sample. Lifetime gating takes advantage of the fact that lanthanide ion (e.g. terbium) luminescence lifetimes are on
the order of milliseconds, while fluorescence lifetimes from impurities generally are on the order of nanoseconds. Lifetime gating drastically reduces the chance of false negatives, which could arise if the lanthanide ion luminescence is masked by background fluorescence from impurities.

More specifically, the imaging method takes advantage of the fact that a bacterial spore is essentially a 1 µm diameter bag comprising 10^6 molecules of DPA and/or DP. Releasing DPA/DP by thermal lysis or germination in the presence of lanthanide ions generates local high lanthanide-DPA/DP concentrations (in the millimolar range) with correspondingly high luminescence intensities. When the luminescence "halo" surrounding the spore body is imaged into individual lifetime-gated CCD detector elements, individual spores will be easily counted. Even when spores are clustered together, the spot counts per cluster will be proportional to the intensity arising from a cluster. Thus, the resultant "bright spots" or "halos" are counted and the number of spores per bright spot is estimated by the luminescence of the spot (i.e., the spot intensity). The lifetime gating allows imaging of the long-lived lanthanide-DPA/DP excited state in the presence of short-lived fluorescence interferences (impurities, etc.).

Under UV (blacklight) illumination, the luminescence of the embedded Tb^{3+} ions increased dramatically upon germination within 40 minutes of the bacterial spores, while the embedded Tb^{3+} luminescence in the control sample that had no exposure to L-alanine remained weak (FIG. 3). An agar control sample without Tb^{3+} 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.

An example of imaged Tb-DPA/DP complex representing spores on a PDMS test surface containing Tb^{3+} ions, which were subsequently lysed using plasma cleaning are shown in FIG. 4. Those spores that were not subject to plasma cleaning, and thus did not lyse and release DPA/DP, did not exhibit fluorescence (panel C and D of FIG. 4).

The pictures in FIG. 3 were taken without magnification, and thus the individual spores cannot be enumerated as they germinate. However, the present disclosure provides germinating bacterial spores imaged with a lifetime-gated microscope (FIG. 5, Example 3). As the spores germinate, DPA is released from the core to generate high, localized DPA/DP concentrations (i.e., DPA and/or DP remains in the immediate surroundings of the spore body). To obtain viable counts, germination is induced by doping L-alanine (or other germination inducing agents) into the polymer matrix; lanthanide ions (e.g., TbCl₃) also doped into the polymer, allow for imaging and quantification of bacterial spores by triggering luminescence in the presence of DPA/DP. To obtain total counts, the bacterial spores immobilized on the polymer test surface containing lanthanide ion are physically lysed (e.g., by dry heating, microwaving, sonication, plasma cleaning, hydrogen chloride gassing or autoclaving) and the subsequent fluorescence emitted upon excitation of the lanthanide-DPA/DP complex is imaged and quantified resulting in the total number of live and dead bacterial spores.

The present disclosure also provides a method and apparatus to measure the fraction of bacterial spores that remain viable or alive, hence a live/dead assay for bacterial spores. The method combines dipicolinic acid/dipicolinate-triggered lanthanide luminescence and DPA/DP release from (1) viable bacterial spore through germination, and (2) DPA/DP release subsequent to lysis of all viable and nonviable bacterial spores. The ratio of the results from steps (1) to the sum of steps (1) and (2) yield the fraction of bacterial spores that are alive.
In one embodiment of the present disclosure, a method is provided for quantifying the percentage of viable spores in a population mixture of viable and inviable spores. In a preferred embodiment, the method for quantifying the percent viable spores in a mixed population of viable and inviable spores comprises transferring bacterial spores from their place of origin onto a test surface containing lanthanide ions, inducing germination of DPA/DP from the transferred bacterial spores, exciting the lanthanide-DPA/DP complex with UV radiation, quantifying the luminescence associated with the lanthanide-DPA/DP of germination, subsequently lysing the non-germinated bacterial spores on the test surface, exciting the lysis-induced lanthanide-DPA/DP complex with UV radiation, and quantifying the luminescence associated with the lanthanide-DPA/DP of lysis. Using the same test surface for germination and subsequent lysis allows for an accurate calculation of the percent viable spores in any given mixed population of viable and nonviable spores. The ability to rapidly quantify the fraction of viable bacterial spores from various origins (e.g., solid surfaces, water and air) is an essential feature of the present invention.

The method and apparatus of the present disclosure provide the imaging of the spherical resolution of the high concentrating region of DPA (the "halo") around each spore body, which has been germinated or lysed. The present method makes it possible to detect and quantify extremely low concentrations of bacterial spores in very short time. The method and apparatus for bacterial spore detection and quantification according to the present disclosure yields results within minutes and requires approximately an hour for quantifying the percent viability of bacterial spores on surfaces.

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.

### Table 1

<table>
<thead>
<tr>
<th>Test Surface</th>
<th>1540 TSU/cm²</th>
<th>710 GSU/cm²</th>
<th>120 CFU/cm²</th>
<th>Ratio of GSU/CFU: 3.38</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Surface Sampling: Swab Rinse</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Water Sampling:</strong></td>
<td>5.0 x 10⁴ TSU/cm²</td>
<td>3.4 x 10⁴ GSU/cm²</td>
<td>1.2 x 10⁴ CFU/cm²</td>
<td>Ratio of GSU/CFU: 2.83</td>
</tr>
<tr>
<td><strong>Air Sampling:</strong></td>
<td>0.05 GSU/l of air</td>
<td>0.01 CFU/l of air</td>
<td>Ratio of GSU/CFU: 5.0</td>
<td></td>
</tr>
</tbody>
</table>

TSU: Total Spore Units  GSU: Germinating Spore Units  CFU: Colony Formation Units.

While several illustrative embodiments have been shown and described in the above description, numerous variations and alternative embodiments will occur to those skilled in the art. Such variations and alternative embodiments are contemplated, and can be made without departing from the scope of the invention as defined in the appended claims.
The method of claim 2, wherein the transferring is performed by way of an adhesive polymer or a swab.

5. The method of claim 3, wherein the adhesive polymer is selected from the group consisting of PDMS, agar and agarose.

6. The method of claim 5, wherein the water is passed through a water filter.

7. The method of claim 2, wherein the capturing of the at least one of the individual bacterial spores is from a place of origin selected from the group consisting of water and air.

8. The method of claim 2, wherein the transferring of the at least one of the individual bacterial spores is from a place of origin to a filter selected from the group consisting of an air filter and a water filter.

9. The method of claim 1, wherein the aromatic molecule is released by germination of its respective individual bacterial spore.

10. The method of claim 9, wherein germination is induced by contacting the at least one individual bacterial spore with L-alanine, L-asparagine, or D-glucose.

11. The method of claim 1, wherein the aromatic molecule is selected from the group consisting of dipicolinic acid, dipicolinate, and mixtures thereof.

12. The method of claim 1, wherein the aromatic molecule interacts with the test surface resulting in a derivative of the aromatic molecule.

13. The method of claim 1, wherein the aromatic molecule is released by lysis of the at least one individual bacterial spore.

14. The method of claim 13, wherein the lysis is induced by way of a method of lysis selected from the group consisting of microwaving, autoclaving, sonication, plasma cleaning, dry heating and hydrogen chloride gassing.

15. The method of claim 1, wherein the imaging comprises imaging the aromatic molecule concentration region of each of a plurality of the individual bacterial spores clustered together on the test surface.

16. The method of claim 1, wherein the individual bacterial spores are embedded in the test surface.

17. The method of claim 1, wherein the test surface is an adhesive polymer selected from the group consisting of PDMS, agar, agarose PDMS together with agar, and PDMS together with agarose.

18. The method of claim 1, wherein the lanthanide ion is selected from the group consisting of terbium ions, europium ions, or a mixture thereof.

19. The method of claim 3, wherein the adhesive polymer is a totally or partially optically transparent adhesive polymer.

20. An apparatus for detecting and quantifying individual bacterial spores, the apparatus comprising:

a test surface comprising one or more adhesive polymers and one or more lanthanide ions, the test surface including at least one bacterial spore, each said at least one bacterial spore adapted to release aromatic molecules such that the at least one bacterial spore and respective aromatic molecules are in close proximity to each other so as to render the at least one bacterial spore...
individually detectable, quantifiable, and/or imageable with a micrometric spatial resolution;
an ultraviolet light radiation device adjacent to the test surface to excite a complex made of a lanthanide ion of the one or more lanthanide ions and an aromatic molecule of the aromatic molecules and generate a characteristic luminescence of the complex; and
an imaging device for imaging the at least one bacterial spore corresponding to the luminescence, the imaging device configured to image with a micrometric spatial resolution.

21. The apparatus of claim 20, wherein the one or more adhesive polymers is a totally or partially optically transparent adhesive polymer.

22. The apparatus of claim 20, wherein a microscope is connected to the imaging device.

23. The apparatus of claim 20, wherein the test surface exhibits two conditions such that:
in a first condition, the test surface comprises the one or more adhesive polymers, the one or more lanthanide ions and the at least one bacterial spore; and
in a second condition, the test surface comprises the one or more adhesive polymers, an excited complex of one of the lanthanide ions and the aromatic molecule released from the respective at least one bacterial spore, and the respective at least one bacterial spore.