Utilization of laser induced fluorescence for the discrimination between two bacterial strains

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A R T I C L E   I N F O

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- Laser induced fluorescence
- Discrimination of bacteria
- Confocal laser scanning microscopy

A B S T R A C T

The present work reports on the evaluation of laser induced fluorescence (LIF) for the discrimination between different microbial strains. Pseudomonas aeruginosa and Staphylococcus aureus are important pathogenic bacteria for which therapeutic options are lacking nowadays. These microbial strains were selected due to their medical relevance as they are commonly found in human diseases infections. LIF is a spectrochemical analytical technique that was used in the present study to obtain bacteria spectral fingerprints in the liquid phase. Two laser wavelengths, 266 nm (UV) and 405 nm (violet), have been used as excitation light sources delivering output power 5 mW and 100 mW, respectively. The results of LIF analysis showed that the differences in fluorescence bands intensity can be used as a fingerprint for each bacterial species. In addition, the fluorescence emission intensities of the two strains were exponentially related to the concentration of the bacteria. Confocal laser scanning microscopy was used successfully to visualize the fluorescence emission of the cells in comparison with the LIF measurements. The obtained results demonstrate the potential of LIF as a fast, noninvasive, and easy technique for bacterial discrimination. The technique can be also used for the determination of bacteria concentration after performing proper calibration.

1. Introduction

Bacteriologically, Gram-positive and negative bacteria are generally identified in two groups via staining techniques, without identifying the species. Nowadays, it is possible to isolate and identify about 20,000 bacterial species relevant to human pathology using chromogenic media, metabolic tests and sequencing technology. However, it should be taken into consideration that such standard chemical techniques even with automated equipment are still time consuming for bacterial identification since they take 18–24 h in a single run. These lengthy procedures for bacterial species isolation and identification, may be life threatening for the patients under chemical treatment due to the probable emergence of antibiotic resistant bacterial species, for example P. aeruginosa.

On the other hand, validation methodologies are also too lengthy and it may take more than four days to fully identify a single microbe. In addition, most of the molecular techniques are generally expensive and technically complicated. To design the proper therapeutic protocol and arrange for infection control system, it is essential to detect for example P. aeruginosa in a well-equipped medical laboratory. The polymerase chain reaction, disk diffusion or dilution methods, broth microdilution, and enzyme block test, are the conventionally used standard microbiological assays for the detection of P. aeruginosa in pathological samples. There is no doubt that such techniques are costly, time consuming and need numerous preparation procedures before performing the measurements. It is clear that techniques that are faster, cost effective and easier for detection and characterization of P. aeruginosa strains are required desperately. Optical fluorescence is a sensitive approach that is often used for the detection and identification of a wide range of chemical compounds and biological species, both in the laboratory and in the field. Normally it offers increased sensitivity over other techniques. Often, these measurements are conducted using a commercial spectrofluorometer device with a conventional xenon or halogen lamp as the light source and fluorescence emission channels or a scanning spectrometer. Studies have shown that considerable improvement in sensitivity can be achieved with the use of a UV laser as the excitation source.

It is assumed that the auto-fluorescence or intrinsic fluorescence of cells and tissues is basically due to fluorophores in the biomolecules. Some fluorophores are considered ideal targets for excitation as they...
2. Materials and methods

2.1. Bacterial strains and culture conditions

The microbial strains used in this study were *Pseudomonas aeruginosa* (ATCC 9027) and *Staphylococcus aureus* (ATCC 25923) provided by the microbiology laboratory at.cache University Research Park (CURP). Each bacterial strain was inoculated in 50 mL trypticase soy broth medium in a 100 mL glass Erlenmeyer flask, incubated at 37 °C without stirring for about 20 h (to minimize the lag time for new growth) [18]. For sample preparation bacterial cells were harvested using centrifugation (3000 rpm, 10 min), media (supernatant) was disposed and cells resuspended in 50 mL 0.95% normal saline. Washing and re-suspension procedure was repeated twice, and then final suspension was adjusted to 1.5 optical density at 600 nm for all the following measurements.

A standard curve was prepared for each bacterial species to determine a relation between CFU/mL vs. dilution, by preparing 10 decimal dilutions for each bacterial strain then plating 100 µL of each dilution on agar (Tryptic soy agar) and incubated for direct count of colonies. Experiment was repeated three times on three consecutive days for each species with three replicates for each dilution.

2.2. Laser induced fluorescence measurements

Five decimal serial dilutions from each bacterial suspension were prepared and 1 mL of each dilution was placed in a 3 mL quartz cuvette (with four transparent sides) for laser induced fluorescence measurements. Two different excitation laser sources were used, the first was a continuous wave DPSS (diode pumped solid state) laser (Changchun new industries optoelectronics tech Co., Ltd., CHN) with an average laser output power of 100 mW at a wavelength \( \lambda = 405 \) nm and the other excitation source was also a continuous wave DPSS laser (Changchun new industries optoelectronics tech Co., Ltd., CHN) with average output power of 5 mW at wavelength \( \lambda = 266 \) nm. The use of 266 nm was mainly to know whether or not a shorter excitation wavelength can lead to a better spectral differentiation, i.e. additional fluorescence peaks may show up in the spectral range below 405 nm. The laser beam was focused into one end of an optical fiber while the
other end of the fiber delivered the beam onto one side of the quartz cuvette containing the bacterial sample solution. The emitted fluorescence was collected perpendicularly via another fiber used to deliver the fluorescent light to the spectrometer (USB2000 FLG, Ocean Optics, USA). The experiment was repeated separately on 30 samples for each bacterial strain with both laser excitation wavelengths using the same growth conditions and starting concentrations to ensure the stability of the resulted fluorescence and the reproducibility of the results.

Acquisition and analysis of the spectra obtained from the spectroscopic system is accomplished using the commercial SpectraSuit software (Ocean Optics, USA) as well as Origin 9 software. The experimental setup of the equipment used throughout the present work for LIF measurements is described in details elsewhere [14].

2.3. Confocal laser scanning microscopy

A loop full of each bacterial suspension was applied to the surface of a wipe-clean glass slide then covered with a slide cover. A Confocal Laser Scanning Microscope (CLSM) (LSM 710, Carl Zeiss, Jena, Germany) was used to visualize the fluorescence emission of the cells, with software version ZEN 2009 for image analysis. The samples were excited via a diode laser at $\lambda_{\text{ex}} = 405$ nm and $30 \text{ mW}$ power, where a 418–718 nm band-pass filter was used for scanning the emissions.

Fig. 2. Correlation between fluorescence intensity at emission peaks related to bacterial cell concentration for *P. aeruginosa* (a) and *S. aureus* (b) at $\lambda_{\text{ex}} = 266$ nm. The error bars represent the standard deviation of the experimental data of 30 samples.

Fig. 3. Correlation between fluorescence intensity at emission peaks related to bacterial cell concentration for *P. aeruginosa* (a) and *S. aureus* (b) at $\lambda_{\text{ex}} = 405$ nm. The error bars represent the standard deviation of the experimental data of 30 samples.

<table>
<thead>
<tr>
<th>Chromophore</th>
<th>Absorption (nm)</th>
<th>Fluorescence (nm)</th>
</tr>
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<tbody>
<tr>
<td>Tryptophan</td>
<td>220, 280, 288</td>
<td>320–350</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>220, 275</td>
<td>305</td>
</tr>
<tr>
<td>NADH/NADPH</td>
<td>260, 340</td>
<td>470</td>
</tr>
<tr>
<td>Flavins</td>
<td>260, 370, 450</td>
<td>530</td>
</tr>
<tr>
<td>ATP</td>
<td>260</td>
<td>300–500</td>
</tr>
<tr>
<td>Coproporphyrin</td>
<td>398, 497, 531, 565, 620</td>
<td>622</td>
</tr>
<tr>
<td>Protoporphyrin</td>
<td>406, 505, 540, 575, 630</td>
<td>633</td>
</tr>
</tbody>
</table>

Table 1

List of intrinsic fluorophores with relatively high fluorescence quantum efficiency along with their absorption and emission maxima [20].

3. Results and discussion

3.1. Laser induced fluorescence analysis

LIF spectra of undiluted samples of *P. aeruginosa* and *S. aureus* bacteria using (a) 266 nm and (b) 405 nm as excitation laser sources are shown in Fig. 1. The spectra are the average of the fluorescence from 30 samples for each species. *S. aureus* samples showed slightly higher fluorescence intensities than those of *P. aeruginosa* with a common fluorescence band around 490 nm and another low intensity band appeared around 635 nm only in case of *P. aeruginosa* spectrum. At 266 nm excitation wavelength, other shoulders appeared for both bacterial spectra indicating the presence of possible overlapped peaks.
at 438, 525 nm and another small peak at 420 nm appeared only in case of *S. aureus*.

Using the 405 nm laser as excitation source, Fig. 1(b) shows the fluorescence spectra for both *P. aeruginosa* and *S. aureus* at the highest sample concentration. It was found that both bacteria have a common emission band with maximum intensity around 490 nm, with *P. aeruginosa* showing much higher fluorescence intensity than *S. aureus* which is almost the double. At 635 nm *P. aeruginosa* showed a sharp moderately intense peak of fluorescence that isn’t present in the spectrum of *S. aureus* which solely showed very small peaks at 566 nm and 650 nm. The difference in fluorescence spectra supported the previous findings reporting that each bacterial species has its own fingerprint-like excitation-emission spectrum. That is mainly due to the difference in membrane constituents and endogenous fluorophores both quantitatively and qualitatively [12,13,19].

It can be seen that the emission upon excitation with 405 nm was more intense than that of 266 nm and that may be due to the high power density of the exciting laser. *P. aeruginosa* has more intense emission than *S. aureus* at the highest dilution and subsequent measured dilutions. While, in Fig. 1(a) it shows less intensity than *S. aureus* however, in the subsequent measured dilutions it showed higher intensity compared with corresponding *S. aureus* concentrations. This can be explained by the phenomenon of inner filter effect. Where, in high concentrations of fluorophores the absorbance of the exciting intensity
increases as well as the loss of excitation light as it passes through the cuvette. This effect is most often encountered with right-angled fluorosence instruments -which we followed- where emission slits are set to monitor the center of the sample cell and absorbance of excitation light is greater at the front surface of the cuvette. Such drawback has been overcome by using higher intensity excitation source.

The maxima of the fluorescence bands were determined (using Origin software) to obtain the intensity value for every sample at different dilutions. Fig. 2 demonstrates the correlation between the fluorescence intensity of emission maxima, at 490 and 635 nm, related to bacterial cell concentration for *P. aeruginosa* (a) and *S. aureus* (b) at $\lambda_{ex} = 266$ nm. The intensity of each fluorescence peak was plotted against cell count (log$_{10}$ CFU/mL) for each bacterial sample. The error bars represent the standard deviation of the experimental measurements on 30 samples for each point. The same trend is shown in Fig. 3 in case of 405 nm excitation, both species showed quasi-exponentially increasing fluorescence emission intensity with increasing bacterial concentration at both laser excitation wavelengths. The highest concentration has the highest intensity value which decreases sharply with dilution and levelled off for the lower concentrations. Also the low intensity emission peak at 635 nm tend to disappear after the third or fourth decimal dilution.

It has been previously reported in many studies that different endogenous fluorophores have different excitation-emission bands. The work of Koenig and Schneckenburger [20], provided some information about the absorption and emission maxima of the most important chromophores present in bacterial cells via in-vitro study of each chromophore in aqueous solution (see Table 1). The excitation-emission
wavelength for each chromophore is sensitive to the surrounding conditions (local environment) as pH, temperature, surrounding molecules and concentrations of certain ions, etc. [21]. So, the values listed in Table 1 are not considered as absolute values because in the present study we are dealing with chromophores in at-vivo conditions.

The detected fluorescence spectra show emission in the blue–green region (around 490 nm) which is related to the presence of coenzymes and in the yellow-red region (around 635 nm) relevant to porphyrins [21–23]. Metabolic coenzymes (NADH and FAD) fluorescence intensity is closely related to the metabolic state of the cell giving a reliable indication on the viability of the cell. While the aromatic amino acids and the nucleic acids, which fluoresce in shorter wavelengths, are unaffected by cell death that allows the detection of presence of cells as bacterial infections and contamination [23]. The higher fluorescence intensity in case of P. aeruginosa compared to that from S. aureus, may be due to the presence of more endogenous chromophores in P. aeruginosa cells compared to S. aureus, or due to the cell wall constituents and the clustering property of S. aureus cells that could impair laser penetration of cells [24].

3.2. Confocal laser scanning microscope imaging

CLSM has been used in order to visualize the fluorescence emission of the cells. Fig. 4(a) shows lambda scan of P. aeruginosa cells, represented in 18 out of 31 spectral images acquired in a sequential bandwidth of 10 nm, ranging between 418–588 nm at λex = 405 nm, using objective: Plan-Apochromat 63x/1.4 oil DIC M27, zoom = 0.6. Fluorescence emission starts in the blue region (438 nm) and peaks in the blue–green region then fades in the green region. The corresponding LIF signal is shown in Fig. 4(b) together with an image (inset) that depicts a merge of all spectral emission, where emission at different wavelengths are presented as different colors i.e. blue, blue green and green.

For S. aureus cells, the emission recorded by the lambda scan mode of confocal imaging showed that fluorescence emission has started in the blue–green region (478 nm) and faded in the green region as shown in Fig. 5(a) represented by 18 out of 31 spectral images acquired in a sequential bandwidth of 10 nm, ranging between 418 and 588 nm at λex = 405 nm using objective: EC PLAN neofluor 40x/1.3 Oil DIC M27 and scan zoom = 2.0. Emission of S. aureus showed much less intensity than P. aeruginosa cells which can be clearly seen in Fig. 5(b) displaying the corresponding LIF signal together with an inset image that depicts the merge of all spectral emission at different wavelengths represented as colors i.e. blue-green and green. The CLSM results greatly supported the findings of the LIF experiment. CLSM visualized fluorescence of P. aeruginosa from 438 nm to 528 nm which corresponds to the large peak in the LIF spectra, while the fluorescence at 635 nm that appeared in LIF spectra for this bacterial strain did not show up in CLSM images and this may be attributed to the low power of CLSM excitation laser which was 30 mW compared to the 100 mW for LIF.

4. Conclusion

In this study, it has been demonstrated that LIF as a spectrochemical analytical technique has the potential of discrimination between different bacterial strains. Fingerprint LIF spectra have been obtained for the two studied types of bacteria, namely P. aeruginosa and S. aureus. The obtained spectral differences can be interpreted in view of the difference in endogenous chromophores. Also fluorescence intensity could be affected by cell wall constituents and clustering properties of the cells. Imaging via confocal laser scanning microscopy greatly supported the present findings. Resulted fluorescence was visualized in terms of colors representing emitted fluorescence at different wavelengths. Using 266 nm as an excitation wavelength resulted in more fluorescence peaks, although not intense and distinctive enough (due to the low power of the laser) but it could differentiate between both bacteria.

The proposed technique for the discrimination between different bacterial species adopting LIF is reliable, easy, fast and cost effective compared to other conventional laboratory techniques. Besides, with further adjustment to the system LIF can be exploited for bacterial identification in pure cultures. After calibration of fluorescence intensity and bacterial count it is possible to use LIF as an easy and fast technique for determining bacterial concentration in pure cultures of the same strain.

References


