Available online at www.sciencedirect.com

SciVerse ScienceDirect

Procedia Chemistry 6 (2012) 125 – 131

Procedia Chemistry

2nd International Conference on Bio-Sensing Technology

Discriminating Bacteria With Functionalised Nanoporous Xerogels

Sabine Crunaire[a], Pierre R. Marcoux[b], Laure-Hélène Guillemot[a,b], Khanh-Quyen Ngo[a], Frédéric Mallard[c], Jean-Pierre Moy[b], Thu-Hoa Tran-Thi[a,*]

[a] *Laboratoire Francis Perrin, CEA/DSM/IRAMIS/SPAM-CNRS URA 2453 CEA-Saclay, bât. 522, Gif-sur-Yvette,F- 91191, France*
[b] *Department of Technology for Biology and Health, CEA-LETI-MINATEC, 17 avenue des Martyrs, Grenoble, F-38054, France*
c bioMérieux, 17 avenue des Martyrs, Grenoble, F-38054, France

Abstract

A new colorimetric and easy-to-use solid sensor for the detection of indole-producing bacteria is described. The sensor is a nanoporous matrix with tailored pores doped with a reactant, DMACA. The sensor turns rapidly from transparent to green-blue when exposed to metabolic indole coming from bacteria cultures, both in liquid and gas phases. An example of the discrimination between indole-positive (*Eschericia coli*) and indole-negative bacteria (*Hafnia alvei*), two important contaminants in food industry is given.

Keywords: indole ; bacteria detection; bacteria identification; microbial volatile organic compound; colorinetric sensor; xerogel; nanoporous materials; hybrid organic-inorganic; gas detection

1. Introduction

Microbial contamination is an important problem in medicine, food and the pharmaceutical industry where a rapid identification of pathogenic microorganisms is crucial for proper treatment or safety procedures. Bacteria can be detected and identified by standard microbiological methods based on their phenotypes: shapes, biochemical characteristics, and virulence properties. The use of such methods is limited by the complexity of protocols which remain long and time-consuming. An alternative approach consists in discriminating the microorganisms by detecting the specific volatile organic compounds (VOCs) that they emit. Indeed, according to their phenotype, bacteria produce a different set of VOCs

* Corresponding author. Tel.: +0033(0)1-69-08-49-33; fax: +0-000-000-0000 .
E-mail address: thu-hoa.tran-thi@cea.fr.

doi:10.1016/j.proche.2012.10.138

that can be considered as a metabolic fingerprint. This is an interesting method since it allows non-invasive investigations.

Recent investigations carried out for the identification of bacterial metabolic fingerprint use very sensitive techniques such as gas chromatography (GC),[1] GC coupled with mass spectrometry (GC-MS),[2] proton-transfer reaction mass spectrometry (PTR-MS)[3] and selected ion flow tube coupled with mass spectrometry (SIFT-MS).[4] However, they are too expensive and request high technical expertise. Indeed, there is a need for fast, cheap, easy-to-use detection devices.

Artificial noses have emerged as alternative – potentially portable – testing systems. For instance, inexpensive and simple methods based on colorimetric sensors have recently been developed: the "colorimetric nose"[5] is a cross-responsive array of 36 sensor elements, each sensor being a spot of colorimetric chemoreactant.. Sensors of the array are combined in order to cover a wide range of families of chemicals. This approach is attractive but has some drawbacks: detection and identification of bacteria in complex samples (complexity of chemical composition and of bacterial ecology) may be impossible, due to the overlapping of VOC profiles. An alternative solution to overcome these problems is the use of selective probes: McDonald et al. report the identification of microorganisms by using probe molecules able to react selectively with a targeted volatile compound giving a detectable coloured product. [6] This identification method based on colorimetric VOC sensors is very simple and may allow cheap and easy test devices.

This study addresses the detection of indole, a widespread metabolite produced by a large variety of both Gram-positive and Gram-negative bacteria, including many pathogens, [7] such as Escherichia coli. 4-(dimethylamino)cinnamaldehyde (DMACA) was chosen as the selective probe molecule giving a green blue product in presence of indole. The present work demonstrates how indole was detected either in the gas phase above a bacterial culture, or in a liquid culture medium, with a nanoporous xerogel pastille functionalised with DMACA.

2. Experimental

2.1. Synthesis of nanoporous xerogel pastilles doped with DMACA

The pastilles were prepared using the sol-gel process. Silane precursors such as TMOS and APTES were purchased at the reagent grade. Deionized water and concentrated hydrochloric acid were used. DMACA was dissolved in methanol. The reagents were mixed according the following molar ratio (TMOS/APTES)/MeOH/H2O/HCl/DMACA: (0.97/0.03)/5/4/140/3.4. 80µL of this sol were then poured in 6 mm diameter wells to obtain final pastilles of diameter = (3.0±0.1) mm, thickness= 800 µm. Drying was operated slowly at 22°C and with 55% relative humidity during 3 days with an adhesive microporous film (ABGene Gas permeable adhesive seals). Such pastilles are stored under nitrogen at 4°C for a shelf-life of 6 months.

2.2. Characterisation of the xerogel pastilles

The porosity properties (surface area and pore size distribution) of the pastilles were determined with a porosimeter Autosorb-1 Quantachrome, by establishing the isotherms of adsorption and desorption of nitrogen at 77K and using both BET (Brunauer, Emmett and Taylor) and DFT analytical methods. The matrices display a high adsorption surface area, 610±80 m2.g-1, and a distribution of pore sizes ranging from 10 to 60 Å, with a maximum peaking at 30 Å. They are optically transparent over the 300-800 nm domain.

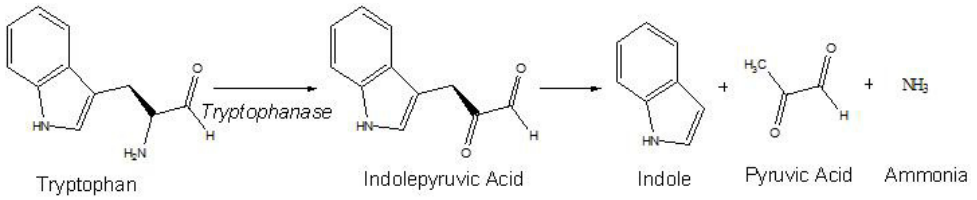
2.3. Pastilles exposure to bacterial cultures

Lysogeny Broth (LB Lennox, Q-Biogene) is used as liquid nutrient medium. The corresponding agar medium is obtained by adding 15 g/L of agar (Fluka 05039). *Escherichia coli* ATCC 11775 and *Hafnia alvei* ATCC 13337 are employed as tryptophanase-positive and tryptophanase-negative strains, respectively. Their indole-producing ability is confirmed with API 20E strips (bioMérieux). For liquid-phase experiments, non-inoculated LB and LB inoculated at 5×10^6 cfu/mL at *t=0* are incubated at 37°C in the same conditions. A measurement is operated by dropping 20 µL of non-filtrated bacterial culture onto the pastille. Each pastille is used only once. The analysing light coming from a Deuterium-Halogen Light Source (Melles Griot, 215-2000 nm) is focused on the pastille via an optical fiber coupled with lenses and the transmitted light is collected with an optical fiber and transmitted to a spectrometer (Ocean Optics). For gas phase detection experiments, 100 µL mL of bacterial suspension is spread at *t=0* on LB agar plate and incubated at 37°C.

3. Detection method

Bacteria that produce the enzyme tryptophanase emit indole. In fact, this enzyme splits the aminoacid tryptophan into pyruvic acid, ammonia and indole (Fig. 1a). The detection of indole is achieved thanks to its selective condensation reaction with DMACA under acid conditions which gives a strongly green product (Fig. 1b). This azafulvenium chloride salt absorbs in the visible at λmax= 624 nm and displays a high extinction coefficient value (97000±13000 mol-1.L.cm-1 (this work)).

a)



b)

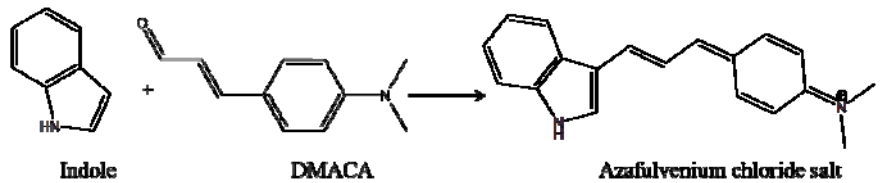


Figure 1: a) Tryptophanase enzyme splits tryptophan aminoacid into pyruvic acid, indole and ammonia. b) Condensation reaction between indole and DMACA (4-dimethylaminocinnamaldehyde).

DMACA can react with tryptophan in solution, leading to a red-violet product absorbing at 563 nm. [8] In culture media containing tryptophan and indole, both condensation reactions can occur and the absorption bands of the two products overlap. This interference is here overcome with the hybrid nanoporous matrix, whose role is fourfold: 1) the pores sizes are tailored to selectively trap indole and discard tryptophan; 2) due to its high adsorption surface area, the matrix acts as a sponge to concentrate the targeted analyte; 3) the sensor can be heavily doped to display a high sensitivity; and 4) each nanopore is a nanoreactor in which the targeted reaction is enhanced due to the reactants confinement.

As the pastille is optically transparent, the formation kinetics of the azafulvenium chloride salt can therefore be monitored spectroscopically by recording its absorption spectrum as a function of time.

In this work, the behaviour of our DMACA-doped sensor is followed when exposed to different sources of indole: i) with commercial indole diluted in aqueous solutions, ii) with bacterial indole generated by bacteria in a liquid nutrient medium, and iii) with bacterial indole in the gas phase above bacteria growing on an agar nutrient medium.

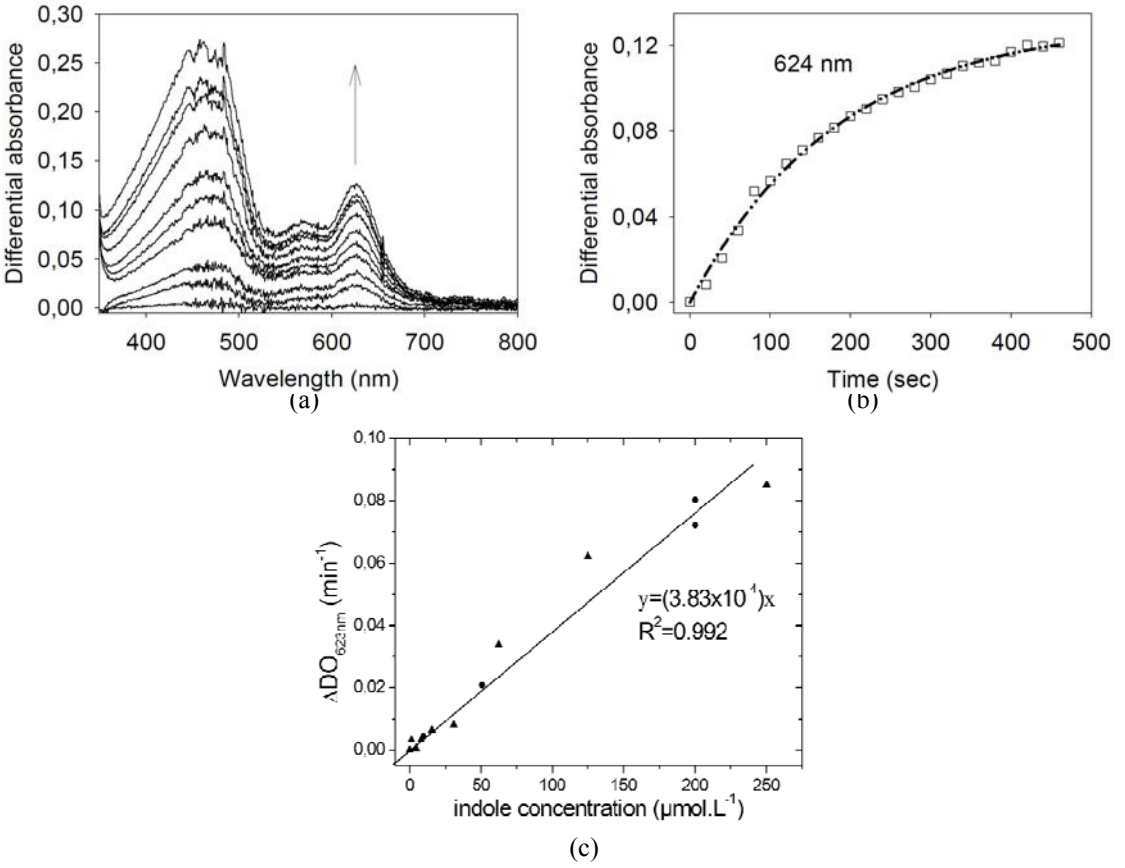


Figure 2: (a) Spectral variation of the pastille doped with DMACA as a function of time after the addition of 20 µL of an aqueous solution of indole (63 µM) to a pastille doped with DMACA. (b) Kinetics of formation of the azafulvenium salt at λ= 624 nm, fitted with an exponential rise with a plateau. The formation rate of azafulvenium salt corresponds to the slope at time t=0. (c) Calibration curve for indole detection with a nanoporous sensor doped with DMACA: Azafulvenium salt formation rate versus indole concentration, from 10^{-6} to 2.5×10^{-4} mol.L^{-1}.

4. Results and discussion

4.1. Detection of indole in solution

Doped-DMACA pastilles were exposed to indole aqueous solutions with increasing concentrations (from 1 to 250µM) in order to establish a calibration curve.

For each concentration, a small aliquot (20 µL) was added to a pastille placed in a transparent well. The spectral change of the pastille is followed as a function of time. The appearance of an absorption band peaking at 624 nm reveals that indole reacts with DMACA leading to the formation of the azafulvenium salt in the xerogel pastille (Fig. 2a). The absorption band peaking at 460nm nm corresponds to the absorbance of deprotonated DMACA molecules, due to the intrapore pH change..

The kinetics of formation of the azafulvenium salt is followed via the absorbance variation at 624nm with time. The kinetics curve can be fitted with an exponential rise with a plateau (Fig. 2b). The reaction rate, extracted from the linear part of the slope, is proportional to the indole concentration. The calibration curve is established over a wide range of indole concentration, from 0.4 to 100 µmol.L^{-1}, the absorbance variation at 624 nm, per unit of time and of indole concentration, is found to be equal to 6.4 ± 0.1 s^{-1}.L.mol^{-1}. This calibration curve is valid over the 0.02 to 5 nanomoles range (Fig. 2c).

4.2. Detection of bacterial indole in the liquid phase

DMACA-doped pastilles were exposed to indole released by bacteria growing in a liquid LB Lennox nutrient medium. In this set of experiments, the objective is to show the absence of chemical interference with the azafulvenium formation, in the presence of tryptone, a mixture of amino acids and short polypeptides that contain tryptophan moieties. The experiment was done using *Escherichia coli,* an indole-producing bacterium and *Hafnia alvei*, an indole-negative bacterium, used as a negative control. Each culture medium was sampled during bacterial growth over a large incubation period (29 h): at various incubation times, a 20 µL aliquot was taken from the culture medium and added to a pastille. The growth of absorbance of the azafulvenium chloride salt is then plotted at 624 nm. From the calibration curve previously established, the concentration of indole released in the nutrient medium was determined and plotted as a function of the incubation time (Fig. 3). It appears that a significant amount of indole (100 µmol.L^{-1}) is released in *E. coli* culture after only 5h of incubation.

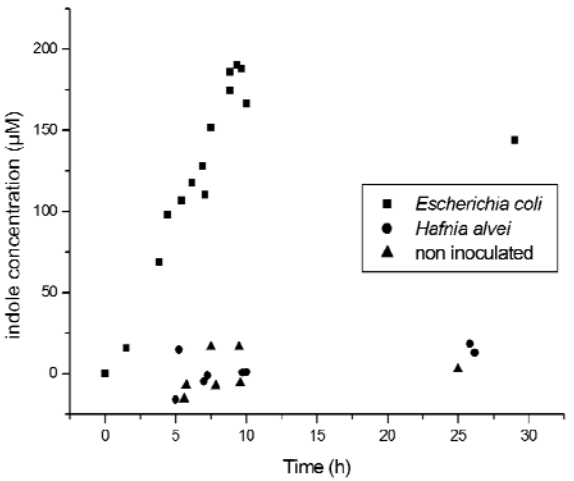


Figure 3: Detection of indole released by bacteria in LB medium at 37°C for *E. coli*, *H. alvei* and a blank sample versus incubation time.

Interestingly, no significant amount of indole was detected at *t=0* in cultures of *E. coli*, nor throughout the whole experiment in cultures of *H. alvei*, and in non-inoculated LB samples. These results demonstrate that *H. alvei* bacteria do not show any tryptophanase activity and that LB Lennox does not contain any significant amount of indole. More importantly, this result shows that the sensor is insensitive to the uncontrolled variety of organic molecules present in the culture medium. In particular, tryptophan does not interfere with indole due to a size exclusion effect linked to the finely tuned pore size of the xerogel.

4.3. Detection of bacterial indole in gas phase

DMACA-doped pastilles were exposed to gaseous indole above bacterial cultures on agar medium. The sensor is placed on a plastic knob in the middle of a small dish containing a desiccant, $CaCl_2$. This container is placed in the centre of a standard Petri dish containing an agar nutrient medium that is later inoculated with *E. coli* cells (Fig. 4a). There is no contact between the sensor and the bacterial culture. Two other Petri dishes filled with the same nutrient medium free of bacteria or inoculated with indole-negative bacteria, *H. alvei*, were used as control experiments. The Petri dishes are covered and incubated at 37°C during 24h. The formation of the azafulvenium chloride salt is then monitored by taking a photo every 15 minutes.

On a densely inoculated agar plate (10^8 to 10^9 cells), the colour change, from colourless to green, can be detected after 7 to 8 hours of incubation at 37°C *i.e.* before the colonies get visible to naked-eye. Starting from a much lighter inoculum (92 cells on the whole plate), the colour change gets eye-visible after 17 hours. After 24 h of incubation, the sensor is dark green with a too large absorbance to be measured. With the two control Petri dishes without bacteria and with *H. alvei*, the sensors turn from colourless to dark orange (Fig 4b). The absence of green colour witnesses the absence of indole. The orange colour is due to the absorbance of deprotonated DMACA, as explained above.

(a) (b)

Figure 4: Detection of gaseous indole above an *E. coli* culture on LB agar. (a) Experimental set-up: the sensor is placed on the central dish filled with $CaCl_2$ and surrounded by the bacterial culture in the Petri dish. (b) Coloration of the sensors after 24h: dark green for *E. coli* (indole-positive) and dark orange for the two control tests: *H. alvei* (indole-negative) and blank sample (bacteria-free).

5. Conclusion

We produced a non-invasive and low-cost indole sensor that discriminates indole-positive from indole-negative bacteria. Its sensitivity, selectivity and rapidity are achieved.via the choice of a matrix with tailored pores size and a selective reactant. This simple-to-use sensor could be easily used, both in the gas and liquid phase, for the detection of pathogen bacteria in food industries or in hospitals.

Acknowledgements

The authors acknowledge Dr Sylvain Orenga and Dr Victoria Girard from bioMérieux for helpful discussions, Charles Rivron from Laboratoire Francis Perrin for the BET measurements. The authors are grateful to bioMérieux, CNRS and CEA for their financial support.

References

[1] E. Holst and L. Larsson, *Eur. J. Clin. Microbiol*., 1987, 6, 724-728.

[2] L. Larsson, P. A. Mardh and G. Odham, *J. Clin. Microbiol*., 1978, 7, 23– 27.

[3] M. Bunge; N. Araghipour, T. Mikoviny, J. Dunkl, R. Schnitzhofer, A. Hansel, F. Schinner, A. Wisthaler, R. Margesin and T. D. Märk, *Appl. Environ. Microbiol*., 2008, 74, 2179-2186.

[4] R. A. Allardyce, A. L. Hill and D. R. Murdoch, *Diagn. Microbiol. Infect. Dis*., 2006, 55, 255-261; R. A. Allardyce, V. S. Langford, A. L. Hill and D. R. Murdoch, *J. Microbiol. Methods*, 2006, 65, 361-365; J. M. Scotter, V. S. Langford, P. F. Wilson, M. J. McEwan and S. T. Chambers, *J. Microbiol. Methods*, 2005, 63, 127-134; J. M. Scotter, R. A. Allardyce, V. S. Langford, A. Hill and D. R. Murdoch, *J. Microbiol. Methods*, 2006, 65, 628-631.

[5] E. Holst and L. Larsson, *Eur. J. Clin. Microbiol.*, 1987, **6**, 724-728. L. Larsson, P. A. Mardh and G. Odham, *J. Clin. Microbiol.*, 1978, **7**, 23– 27. M. Bunge; N. Araghipour, T. Mikoviny, J. Dunkl, R. Schnitzhofer, A. Hansel, F. Schinner, A. Wisthaler, R. Margesin and T. D. Märk, *Appl. Environ. Microbiol.*, 2008, **74**, 2179-2186. R. A. Allardyce, A. L. Hill and D. R. Murdoch, *Diagn. Microbiol. Infect. Dis.*, 2006, **55**, 255-261; R. A. Allardyce, V. S. Langford, A. L. Hill and D. R. Murdoch, *J. Microbiol. Methods*, 2006, **65**, 361-365; J. M. Scotter, V. S. Langford, P. F. Wilson, M. J. McEwan and S. T. Chambers, *J. Microbiol. Methods*, 2005, **63**, 127-134; J. M. Scotter, R. A. Allardyce, V. S. Langford, A. Hill and D. R. Murdoch, *J. Microbiol. Methods*, 2006, **65**, 628-631.

[6] J. G. McDonald and R. A. Borders, *US Pat.*, 2006, US 2006/0223052 A1.

[7] J.-H. Lee and J. Lee, *FEMS Microbiol. Rev.*, 2010, **34**, 426-44; H. H. Lee, M. N. Molla, C. R. Cantor and J. J. Collins, *Nature*, 2010, **467**, 82-86; P. Di Martino, R. Fursy, L. Bret, B. Sundararaju and R. S. Phillips, *Can. J. Microbiol*., 2003, **49**, 443-449; T. H. Han, J.-H. Lee, M. H. Cho, T. K. Wood and J. Lee, *Res. Microbiol.*, 2011, **162**, 108-116.

[8] B. L. Lowrance, P. Reich and W. H. Traub, *Appl. Microbiol.*, 1969, **17**, 923-924.