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Observing bacterial activity interferometrically

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Abstract It is shown that bacterial activity, even of slowly growing species, can be detected by precise interferometric measurements of refractive index changes of the culture medium. The bacteria-containing sample is kept in an isothermal block together with a reference liquid without bacteria. The biological activity is obtained from the difference of the index changes of these samples. Experiments were performed with Bacilo Calmette-Guérin. The order of magnitude of the observed total refractive index change was compatible with theoretical estimates based on the amount of available oxygen. An unexpected positive index change during the lag phase was observed, which might permit fast diagnostics in medical applications. This technique may provide cheap and quick tests of bacterial susceptibility with respect to antibiotics.

Keywords Bacilo Calmette-Guérin · Bacterial susceptibility · Interferometry · Refractive index Tuberculosis

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Introduction

Despite the enormous advances due to the invent of antibiotics and inoculation, the battle against bacteria is far from its end. For instance, it is estimated that three million people die every year from tuberculosis (Raviglione et al. 1995; Saunders and Cooper 2000). Pauperism, lack of sanitary education and principally inadequate use of antibiotics have helped to develop new resistant breeds of tuberculosis (Hart et al. 1996; Saunders and Cooper 2000). Acquired immunodeficiency also contributed to revival of the bacterial problem (Osborn 1983; Gupta et al. 2001). Experiments looking for antibiotic activity against bacteria are usually performed in vitro with the disc antibiogram method or in vivo through accounting of colony forming units (CFU) of the chosen organ (Ascenzi et al. 1987). These investigation methods consist of the observation of bacterial colonies formed from single cells. Both methods are time consuming and do not allow observation of the immediate proliferation of bacteria in real time. For example, observation of first colonies in culture of *Mycobacterium tuberculosis* through the CFU method takes typically 5–16 days (Ascenzi et al. 1987). Another method, which permits observation of bacterial activity in real time, is the well-known radiometric method (BACTEC 460 TB), which uses ^{14}C marking. However, the use of radioactive nutrients limits this method to a few research laboratories (Middlebrook et al. 1977; Inderlied et al. 1987; Murray et al. 1995). In the present work, a cheap method to monitor the metabolic activity of micro-organisms in real time is described.

Materials and methods

Measuring principle

The measuring principle consists of an interferometric measurement of small changes of the refractive index of the nutrient solution due to the metabolism of the micro-organisms. The sample is

kept in a stainless steel vessel of 46.55 mm length that can be sealed hermetically and that has two entrance glass windows. This sealed sample is put into an interferometer, which could be of Michelson, Mach-Zehnder or Fabry-Perot type. In our experiment a Fabry-Perot interferometer was used, where the two interfering beams are generated at air/glass interface reflections. The primary light source was a polarized low-power He-Ne laser. In our experiments the changes of refractive index due to bacterial metabolism are typically of the order of 10^{-5}. This value is so small that temperature changes as small as 0.01 K would seriously interfere with the measurement. In principle it would not be too difficult to control the temperature with such precision. However, it turned out to be of great help to permit small temperature variations, which create additional interference oscillations, and to subtract the corresponding index change. This way a small index change caused by metabolism, which would result only in a single oscillation, may be monitored with many intermediate data points taken with high precision from the maxima and minima of the additional oscillations caused by the temperature change.

In order to be able to perform a reliable subtraction of the temperature effect and other possible index changes, such as chemically induced ones, the sample holder is equipped with a second compartment that contains the pure nutrient solution without bacteria. A second identical interferometric measurement is performed simultaneously with this reference liquid, giving rise to a reference signal. In order to be able to subtract the reference phase change with the correct sign, the temperature is monitored during the whole experiment.

The sample holder is kept in a water bath inside thermal insulation. The stainless steel sample holder acts as an isothermal block. Two incandescent lamps are used for heating the bath. In initial experiments the temperature was controlled manually; later a computer-controlled regulator was used. Figure 1 shows a schematic view of the interferometer.

Results

The experiments performed in this work used Bacilo Calmette-Guérin (BCG) (strain Pasteur), which is a non-virulent mycobacterium obtained from sequenced cultures of *Mycobacterium bovis* (Osborn 1983). The BCG is a strictly aerobic species, which floats on the sample surface (Moore and James 1982). The probing laser beam passes a few millimeters beneath the bacteria-containing surface so that the changes of concentration due to metabolism are brought to the measuring beam by diffusion with relatively little delay. The culture media used were Bacto Middlebrook 7H9 Broth with OADC enrichment.

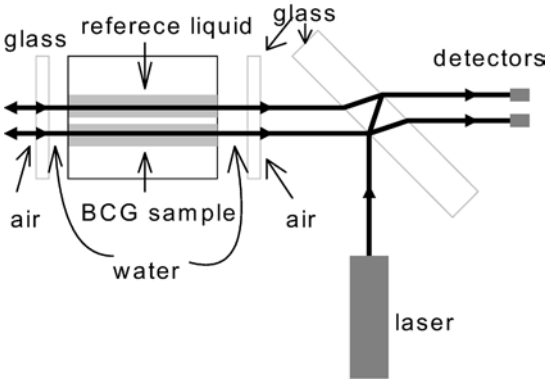

Fig. 1 Experimental setup. The interfering beams are generated by glass/air interfaces as indicated by *triangles*

Figure 2 shows a typical signal of the light intensities registered from the signal and reference beams, as well as the recorded temperature. The temporal position of the maxima and minima of the light intensity are determined by means of quadratic fittings around the extrema. From these times and the information on the sign of the temperature change, two curves of light phase versus time are constructed. Changes of the light phase, $\delta\Phi$, are related to changes of refractive index δn as follows:

$$\delta n = \delta\Phi \frac{\lambda}{2\pi \times 2l} \tag{1}$$

where λ is the vacuum wavelength of the laser, and l is the length of the sample holder. Figure 3 shows curves of the refractive index of the sample and reference liquids constructed this way. Finally, the index change due

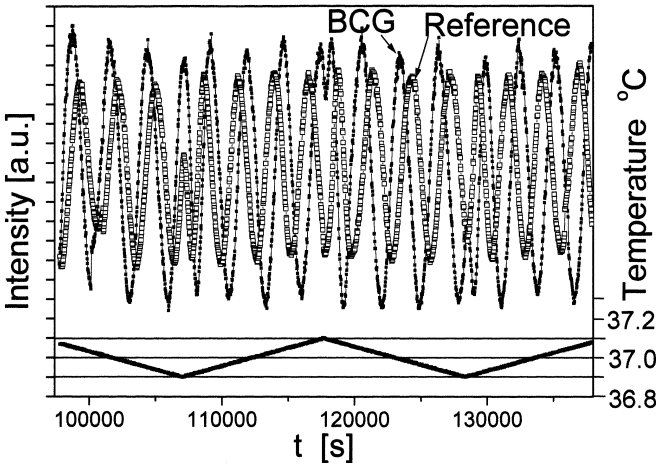

Fig. 2 Typical signal from the interferometer. Two light intensities and the temperature (*lower curve*) of the sample are shown. A change of relative phase is clearly visible: compare, for instance, the minima around $t_1 = 110{,}000$ s and $t_2 = 135{,}000$ s

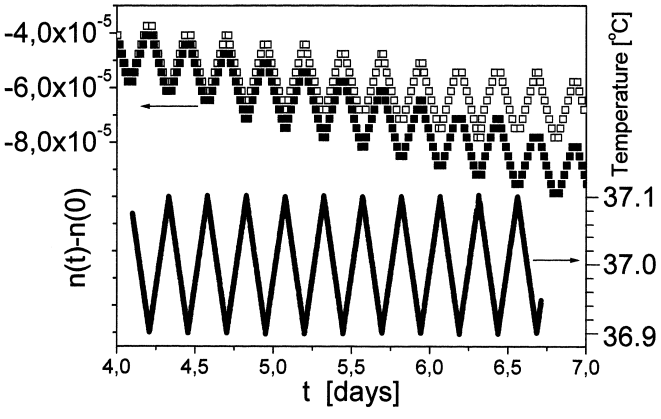

Fig. 3 Refractive index change of reference liquid (*open squares*) and BCG sample (*full squares*). The triangular oscillations are caused by the imposed temperature changes (*solid line*). The final biological signal is obtained from the deviation of the two upper curves

to bacterial activity is obtained by subtracting the latter curves. This subtraction involves calculated interpolation points because, in general, the data points of the two curves have different time values. Figure 4 shows the final result of such an experiment. As can be seen from Fig. 3, the average refractive index changes in time even in the reference liquid at a rate of -7.45×10^{-6} per day. This change is due to chemical changes in the nutrient solution and it shows that the use of a reference liquid is necessary even if the temperature was constant.

In the experiment of Figs. 2, 3, 4, each compartment of the sample holder contained (2.5 ± 0.1) mL of nutrient solution and the sample contained 4×10^6 bacteria at the time when the compartment was sealed. This number was chosen to be very high in comparison with conventional experiments in order to make the onset of bacterial activity clearly visible. The compartment with the bacteria-containing liquid contained (3.7 ± 0.2) mL ambient air above the liquid. At the time when the sample was prepared, a probing glass with the culture medium was inoculated and kept at 37 °C. After 8 days this liquid showed a very slight opacity. At the end of the experiment, two plates with the solid culture medium were wetted with the liquids from the two compartments of the sample holder. After 14 days the reference liquid showed no growth of any species but the BCG sample showed BCG colonies.

The metabolism of BCG is expected to decrease the refractive index of the nutrient solution. Indeed, as can be seen from Fig. 4, after 3 days, when the log phase is expected to start, the refractive index decreases, roughly following an exponential growth law with a time constant of about 2 days. After 13 days the exponential growth turns over into an exponential decay law [time constant 4.55 days and final index change $\Delta n(\infty)=(-2.155\pm0.002)\times10^{-4}$]. This saturation is probably due to depletion of oxygen in the sample compartment, as can be seen from the following estimate.

From the gas volume and atmospheric conditions at the time the sample was sealed, it was estimated that the amount of O_2 present in the sample was $(2.9\pm0.2)\times10^{-5}$ mol. This includes oxygen dissolved in the nutrient solution. Admitting an overall reaction of the type:

$$C_6H_{12}O_6 + 6O_2 \;\rightarrow\; 6CO_2 + 6H_2O \qquad (2)$$

one concludes that the total amount of glucose that could be processed by the micro-organisms is $(0.48\pm0.04)\times10^{-5}$ mol. The dependence of the refractive index of the nutrient solution on the glucose concentration was determined with an independent measurement and found to be $\partial n/\partial c=(32\pm6)$ mL/mol. This way the expected decrease of refractive index due to glucose consumption is[1]:

$$\begin{aligned}\Delta n_{\text{glucose}} &= \frac{\partial n}{\partial c}\Delta c \\ &= (32\pm6)\,\frac{\text{mL}}{\text{mol}}\,\frac{(0.48\pm0.04)\times10^{-5}\ \text{mol}}{(2.5\pm0.1)\ \text{mL}} \\ &= (0.6\pm0.2)\times10^{-4}\end{aligned} \qquad (3)$$

The observed index change is approximately 2×10^{-4}, which is of the same order of magnitude. As the above calculation does not take into account absorption of any other chemical species, it is expected that the observed index change is larger than the pure glucose contribution. However, the right order of magnitude corroborates the hypothesis that, in our experiment, bacterial life was limited by the oxygen rather than by nutrients.

It is interesting to note that the refractive index shows a slight increase during the first 3 days. This increase was found in all experiments performed with BCG. One might speculate whether this index growth is due to release of cytosol from dying bacteria (Russell 2001) or due to some specific product of metabolism during the lag phase. Figure 5 shows an amplification of the initial part of Fig. 4, together with the trace of another BCG experiment where the nutrient solution was prepared without catalase. In the incomplete culture medium, no bacterial growth was observed during 13 days. Therefore one might suppose that in this case the observed index change is due to release of cytosol. The growth of the refractive index in the complete culture medium is of the same order of magnitude, but it is definitely larger. Both experiments used the same amount of liquid and the same number of bacteria. This seems to indicate that the small index changes during the first 3 days may already give relevant information about bacterial activity. This point will be investigated in future work.

As the refractive index depends on temperature, one might think that heat production from biological activity could contribute to the observed index change. In order to estimate this contribution, we introduced an electrical heating element into one of the compartments

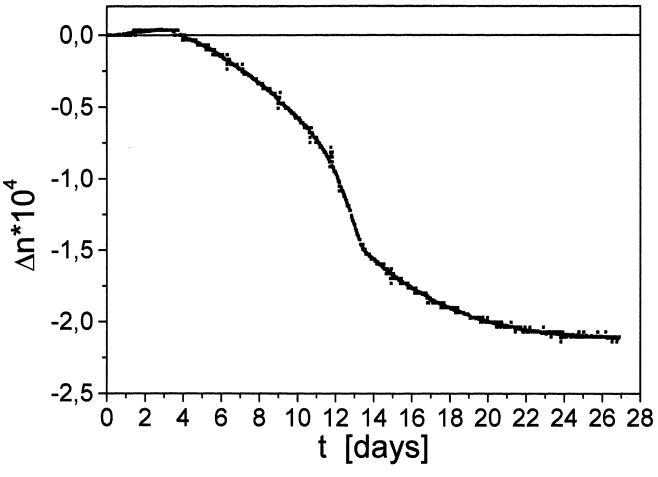

Fig. 4 Index change of a BCG sample caused by biological activity

[1] The effect of dilution due to water production by reaction (2) can be neglected

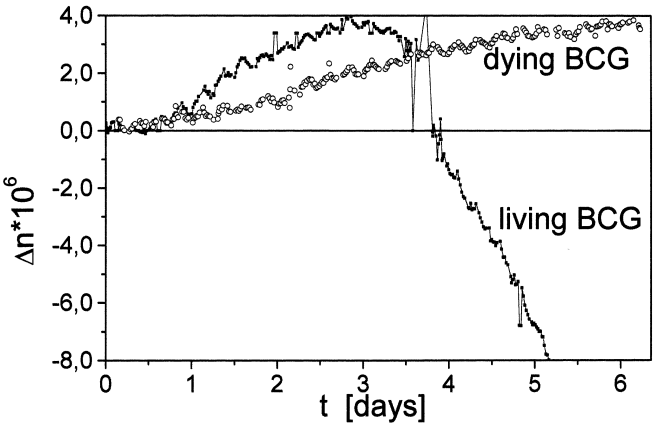

Fig. 5 Biological index change of two BCG samples. The *continuous line* shows the same experiment as Fig. 4; the *scattered open circles* show a dying BCG sample

of the sample holder and injected a thermal power of 0.1 W, which caused a temperature difference between the liquids of the two compartments of 0.05 K. The energy production of reaction (2) is 2808 kJ/mol. With a consumption of 0.48×10^{-5} mol of glucose during 10 days the thermal power produced by biological activity is certainly smaller than 1.6×10^{-8} W. Correspondingly, one expects that the temperature difference between the compartments caused by biological heat production is smaller than 8×10^{-9} K. With a temperature coefficient of the refractive index of $\partial n/\partial T\approx10^{-4}$ K^{-1}, one obtains a corresponding index change of 8×10^{-13}, which is completely negligible.

Conclusions

Summarizing, it was shown that bacterial activity of slowly growing species such as BCG can be observed by interferometric measurements of the refractive index of the nutrient solution.

Instead of measurements of refractive index, one might also measure changes of optical activity of the nutrient solution. However, optical rotatory power provides a method that is by orders of magnitude less sensitive. In the present experiments, changes of refractive index of $\delta n=1\times10^{-6}$ are clearly resolved. In terms of glucose concentration, such index changes correspond to 6×10^{-6} g cm^{-3}. With a specific rotation angle of $[\alpha]_D^{20}=$ 113 degree cm^3 g^{-1} dm^{-1} and supposing a sample holder of 0.5 dm length, this corresponds to a rotation angle of only 3×10^{-4} degrees! The present sensitivity of the interferometric technique can surely be improved by at least a factor of 10.

In principle, the proposed measuring technique does not impose any limitations to attach safety equipment such as UV illumination. Therefore the same technique could be applied to tuberculosis and other dangerous species. It is planned to enhance the resolution of the method at least by a factor of 100 in order to be able to detect signals from much fewer bacteria. Index changes as small as 10^{-8} can be measured interferometrically (Barnes et al. 1991). It is also planned to build an interferometer that permits measurement of several samples simultaneously. Finally, the unexpected positive initial index change, which was observed with the BCG samples, will be investigated with high-precision interferometers.

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References

Ascenzi JM, Ezzel RJ, Wendt TM (1987). A more accurate method measurement of tuberculocidal activity of disinfectants. Appl Environ Microbiol 53:2189–2192

Barnes HT, Matsumoto K, Eiju T, Matsuda Ooyama KN (1991) Grating interferometer with extremely high stability suitable for measuring small refractive index changes. Appl Opt 30:745–751

Gupta R, Kim JY, Espinal MA, Coudron JM, Pecoul B, Famer PE, Raviglione MC (2001) Public health. Responding to market failures in tuberculosis control. Science 293:1049–1051

Hart CA, Beeching MJ, Duerde BI (1996) Tuberculosis into the next century. J Med Microbiol 44:1–00

Inderlied CB, Young LS, Yamada JK (1987) Determination of in vitro susceptibility of mycobacterium-avium complex isolates to antimycobacterial agents by various methods. Antimicrob Ag Chemother 31:1697–1702

Middlebrook G, Reggiardo Z, Tigert WD (1977) Automatable radiometric detection of growth of *Mycobacterium tuberculosis* in selective media. Am Rev Respir Dis 115:1066–1069

Moore DF, James AM (1982) Growth studies on *Mycobacterium BCG*: oxygen preference. Microbios 35:151–159

Murray PR, Baron EJ, Pfaller MA, Yolken FC (1995) Manual of clinical microbiology, 6th edn. American Society for Microbiology, Washington, pp 00

Osborn TW (1983) Changes in BCG strains. Tubercle 64:1–13

Raviglione MC, Snider DEJ, Kochi A (1995) Global epidemiology of tuberculosis: morbidity and mortality of a worldwide epidemic. J Am Med Assoc 273:220–226

Russell DG (2001) *Mycobacterium tuberculosis*: here today and here tomorrow. Nat Mol Cell Biol 2:569–586

Saunders BM, Cooper AM (2000) Restraining mycobacteria: role of granulomas in mycobacterial infections. Immunol Cell Biol 78:334–341