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# Comparison of fluorescence optical respirometry and microbroth dilution methods for testing antimicrobial compounds

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ABSTRACT

An analysis of the usefulness of the fluorescence optical respirometry test method to study several antimicrobials was performed. An oxygen-sensitive sensor: ruthenium-tris(4,7-diphenyl-1,10-phenanthroline) dichloride ($Ru(DPP)_3Cl_2$), the phosphorescence of which is quenched by molecular oxygen, was synthesised according to a method modified by us and then applied. A prototype sensitive measurement system was designed and constructed. Analyses of the impact of various antimicrobial chemical factors were performed: ampicillin, co-trimoxazole, nystatin, and newly synthesised compounds. It was shown that optical respirometry allows for analysis of the culture growth kinetics of bacteria and fungi and determination of cell growth parameters. It was shown also that MIC values obtained by fluorescence optical respirometry are consistent with the results of the MIC determinations made by serial dilution method (traditional MIC testing using CLSI). The method allows the time to obtain results to be significantly reduced (from 24–48 h to 5–7 h for bacteria and 24 yeasts) and allows the effect of concentrations below the MIC for the metabolic activity of microorganisms to be monitored. The sensitivity of the method allowed the volume of the tested samples to be lessened from 160 μl to 50 μl. Fluorescence optical respirometry allows for the rapid detection and evaluation of the action of various chemical compounds on the metabolic activity of microorganisms in real-time measurement of fluorescence intensity.



## 1. Introduction

The rapid development of various fields of science and technology has resulted in the introduction of new analytical methods and technologies that affect people's quality of life and work. New technologies allow the production of new generations of therapeutic agents but also the ability to cope with old and new threats. In particular this applies to the problem of the increasing resistance of microorganisms to chemotherapeutic agents and disinfectants, environmental contamination or bioterrorism. Consequently there is a search for new rapid and low-cost methods of analysis of antimicrobial and anticancer compounds. The development of the analysis methodology for testing the susceptibility of bacteria to antibiotics and other antimicrobial compounds has a long history, from analyses based on phenotypic properties of microorganisms using the dilution method, by numerous modifications of diffusion methods, to the technology for genotyping (Wheat, 2001). The introduction of new molecular methods for routine analysis of susceptibility must be fully tested and standardised based on parallel analysis of the growth of microorganisms carried in the presence of antimicrobial agents. Therefore, growth analysis of microorganisms remains still the most effective method to analyse the impact of chemicals on bacteria and fungi. Significant technological advances in the automation of the identification process of microorganisms and evaluation of sensitivity to antimicrobial compounds increase the importance of these analyses (Hobson et al., 1996; Felmingham and Brown, 2001; O'Hara, 2005; Strager and Davis, 1992). Traditional test methodologies encounter difficulties, especially when the number of samples is large, and when the physicochemical properties can have an influence on the test results. One of the major disadvantages of these methods is also that the obtained information is limited. This applies primarily to the impact on microorganism concentrations lower than the minimum inhibitory concentrations of microorganisms. In recent years, a number of new methods for quantitative analysis of microorganisms have been introduced that utilise physical, biochemical or bio-electrochemical analysis techniques. However, there is no single universal method that gives satisfactory results of quantitative assessment of the number of microorganisms in the analysed samples. Such a method should be accurate and fast, should retain sufficient sensitivity, should have wide application, should allow the differentiation of live cells from dead, should have a short preparation time for the sample and should use the minimum amount of reagents. The available

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methods have several shortcomings: they are complicated and require complex and time-consuming processing of the sample prior to testing, and some of them use expensive equipment and reagents. Existing automatic systems allow identification of the presence of the organism in the sample, or serve to identify it. They are not suitable for real-time analysis of the behaviour of the culture under the influence of various physical and chemical factors that affect the rate of metabolism of the microorganism. An interesting method of growth analysis of microorganisms in cultures using the so-called optical respirometry has been proposed (Wodnicka et al., 2000). This method is based on analysis of the fluorescence of an oxygen-sensitive sensor, the fluorescence of which is dependent on the amount of oxygen in the tested sample because molecular oxygen quenches the fluorescence. Microorganisms growing in culture consume the oxygen, thus affecting the fluorescence intensity of the sample. By analysing variations in the intensity of the fluorescence in the cultures one can track the metabolic activity of microorganisms.

In this work the usefulness of respirometry based on a fluorescence-quenching oxygen-sensitive sensor for the optimisation of culture conditions and in studies of the effects of chemotherapeutic agents and toxic agents to bacteria and eukaryotic cells is presented. The measuring platform using a single photon counting system emitted by the fluorescent oxygen-sensitive sensor molecules was designed and constructed. A biosensor, the action of which is based on the utilisation of fluorescence quenching by molecular oxygen was synthesised. Q6 Characterisation of the action of the measuring system was carried out and its suitability for the analysis of microbial growth and the effect of various compounds on microorganisms were assessed.

## 2. Materials and methods

### 2.1. Materials

Oxygen-sensitive sensor: Ruthenium-tris(4,7-diphenyl-1,10-phenanthroline) dichloride ($Ru(DPP)_3Cl_2$) sensor prepared according to the modified method of Watts and Crosby (1971). Mineral oil (Sigma-Aldrich), Silica gel, Davisil™, grade 633, 200–425 mesh, 60A, 90%, (Sigma-Aldrich), 4,7-diphenyl-1,10-phenanthroline, $C_{24}H_{16}N_2$ (Sigma- Aldrich), ampicilin, 0.5 g (Polfa-Tarchomin S.A.), co-trimoxazole (trimethoprim/sulfamethoxazole) at a concentration of 16/80 mg/ml (Polfa-Tarchomin S.A.), and ruthenium chloride (III) (Sigma-Aldrich). Newly synthesised compounds: 3,5,6,7-tetrahydrospiro[imidazo[2,1-c][1,2,4]triazole-2,4′-morpholin]-2-ium 2-hydroxyliminoimidazolidine-O-sulfonate (this relationship is known as TCA-laboratory designation 252) from the Department of Technology Resources Medicines, Faculty of Pharmacy GUMed, and dimethylhexadecylamine quaternised by static copolymer containing 30% group γ-chloropropyl-methylsiloxane moieties ended by $Si(CH_3)_3$ groups was obtained from the Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Engineering of Polymer Materials, Lodz, Poland.

### 2.2. Bacteria strains and growth conditions

*Escherichia coli* ATCC 8739 ampicillin and trimethoprim/sulfamethoxazole-resistant strain; *E. coli* 6002 ampicillin and trimethoprim/sulfamethoxazole-sensitive strain; (clinical isolate); *Candida albicans* ATCC 1023; and *Saccharomyces cerevisiae* (Department of Pharmaceutical Microbiology collection).

*E. coli* ATCC 8739 and *E. coli* 6002 strains were grown in Mueller–Hinton broth (MH cation-adjusted, Becton Dickinson) in an aerobic atmosphere at 37 °C for 48 h. *C. albicans* and *S. cerevisiae* were grown in Sabouraud broth (Becton Dickinson) at 37 °C for 48 h. In order to determine the bacterial viability, MH and Sabouraud agar plates were used. Overnight bacterial or fungal culture was diluted in geometric progression with Mueller–Hinton broth or Sabouraud broth, respectively. Then 100 μL of each dilution was inoculated in agar plates and incubated at 37 °C for 24 h (bacteria) or 48 h (fungi). After incubation, colonies of bacteria and fungi were counted and the CFU/ml (CFU-Colony Forming Units) was determined.

### 2.3. Synthesis of ruthenium-tris(4,7-diphenyl-1,10-phenantroline) dichloride ($Ru(DPP)_3Cl_2$) biosensor

$RuCl_3$ (104 mg) and water (0.25 ml) were mixed with 3 ml ethylene glycol. This mixture was heated to 120 °C until the salt dissolved. Subsequently 4,7-diphenyl-1,10-phenanthroline (500 mg) was added and subjected to irradiation in a microwave reactor for 5 min. After cooling to room temperature the reaction product was mixed with chloroform (30 ml) and washed with a saturated solution of sodium chloride (40 ml). The organic layer was separated, evaporated to dryness, and the residue was recrystallised from ethanol:water (2:1) mixture. The expected compound was obtained (475 mg).

*Anal. Calcd* for $C_{72}H_{48}Cl_2N_6Ru \times 5\ H_2O$: C, 68,67; H, 4,64; N, 6,67. Found: C,65,90; H, 4,25; N, 6,10.

### 2.4. Coating the walls of the 96-well microtitre plates with $Ru(DPP)_3Cl_2$ biosensor

$Ru(DPP)_3Cl_2$ was synthesised (Methods and methods—section 2.3) Q8 and adsorbed on Davisil™ silica gel by evaporation (0.9 mg $Ru(DPP)_3Cl_2$/g silica gel). In order to avoid direct contact of microorganisms with the sensor adsorbed on the silica gel, it was embedded in the silicone rubber Lactite NuvaSil® 5091 in a proportion Q9 of 2% w/w and directly applied to the wells of the microtitre plates (MedLabor Greiner Bio-One Company). Then the plate was placed in a moist chamber for 2–3 days at 37 °C and used for tests. Silicone prevents direct contact of microorganisms with the sensor but also allows the penetration of oxygen molecules.

### 2.5. Determination the antimicrobial properties of compounds using the microbroth dilution method

The cultures of microorganisms for the experiments were prepared by transferring cells from stock cultures to the tubes with adequate broth, as described above. They were incubated without agitations for 24 h at 37 °C. The cultures were diluted with adequate broth to achieve an optical density corresponding to $10^5$ colony-forming units per ml (CFU/ml) for bacteria and $10^3$ CFU/ml for *C. albicans* and *S. cerevisiae*.

The minimum inhibitory concentration (MIC) was determined by microbroth dilution technique using 96-well plates. After filling each well with 100 μl of broth, dry test samples were dissolved in water to the final concentration for: ampicillin — 256 μg/ml, trimethoprim/sulfamethoxazole — 128/640 μg/ml, and newly synthesised compounds: 3,5,6,7-tetrahydrospiro[imidazo[2,1-c][1,2,4]triazole-2,4′-morpholin]-2-ium2-hydroxyliminoimidazolidine-O-sulfonate (this compound is known as TCA-laboratory designation 252) — 0.54% and dimethyl hexadecylamine quaternised by static copolymer containing 30% group γ-chloropropyl-methylsiloxane moieties ended by $Si(CH3)_3$ groups — 2% (we called it as homopolymer); and for yeast nystatin — 256 μg/ml. These solutions (volume 100 μl) were added to the first well of each microtitre line. Dilution in geometric progression was done by transferring the mixture/dilution (100 μl) from the first to the twelfth well. An aliquot (100 μl) was discarded from the twelfth well. The microbial suspension (100 μl), at $10^5$ CFU/ml for bacteria species or $10^3$ CFU/ml for yeast, was added to each well. The final concentration of the compounds used to the testing of antimicrobial activity ranged as follows: ampicillin — from 64 to 0.031 μg/ml, trimethoprim/sulfamethoxazole — from 32/160 to 0.0156/0.78 μg/ml; newly synthesised compounds: TCA252 from 0.135 to 0.00007%, homopolymer from 0.25 to 0.000125% and for yeast nystatin — from 64 to 0.03125 μg/ml. Assays were incubated



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