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Development of a new oxygen consumption rate assay in cultures of *Acanthamoeba* (Protozoa: Lobosea) and its application to evaluate viability and amoebicidal activity *in vitro*

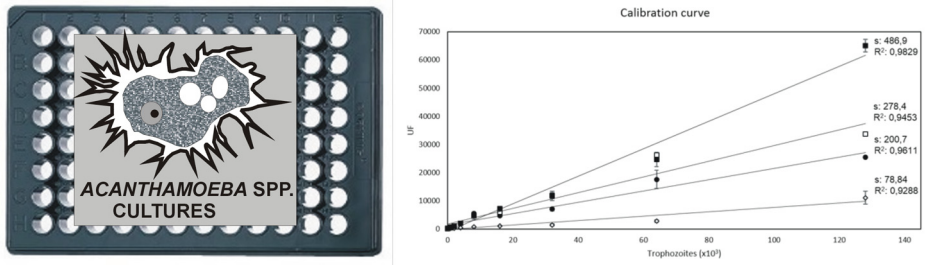
I. Heredero-Bermejo [a,*], A. Criado-Fornelio [a], J. Soliveri [a], J.A. Díaz-Martín [b], J. Matilla-Fuentes [b], J.A. Sánchez-Arias [b], J.L. Copa-Patiño [a], J. Pérez-Serrano [a]

[a] Departamento de Biomedicina y Biotecnología, Facultad de Farmacia, Universidad de Alcalá, 28871 Alcalá de Henares, Madrid, Spain
[b] Oxoprobics Biosciences S.L., Francisco Giralte 2, Desp.12, 28002 Madrid, Spain

HIGHLIGHTS

- A new real time microplate assay for measuring OCR in *Acanthamoeba* has been set up.
- Useful for amoebicidal viability tests and analysis of drug toxicity *in vitro*.
- This assay is easy-to-use, non-toxic, sensitive and medium cost.

GRAPHICAL ABSTRACT



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ABSTRACT

A new fluorometric method has been developed for measuring the oxygen consumption rate (OCR) of *Acanthamoeba* cultures in microplates and for screening molecules with amoebicidal activity against this microorganism. The use of a biofunctional matrix (containing an oxygen-sensitive fluorogenic probe) attached to the microplate wells allowed continuous measurement of OCR in the medium, hence assessment of amoebic growth. The new OCR method applied to cell viability yielded a linear relationship and monitoring was much quicker than with indirect viability assays previously used. In addition, two drugs were tested in a cytotoxicity assay monitored by the new OCR viability test. With this procedure, the standard amoebicidal drug chlorhexidine digluconate showed an IC_{50} of 3.53 + 1.3 mg/l against *Acanthamoeba polyphaga* and 3.19 + 1.2 mg/l against *Acanthamoeba castellanii*, whereas a cationic dendrimer [G1Si(NMe3+)4] showed an IC50 of 6.42 + 1.3 mg/l against A. polyphaga. These data agree with previous studies conducted in our laboratory. Therefore, the new OCR method has proven powerful and quick for amoebicidal drug screening and is likely to be applied in biochemical studies concerning protozoa respiration and metabolism.

* Corresponding author. Fax: +34 91 885 4663.
E-mail address: irene.heredero@edu.uah.es (I. Heredero-Bermejo).

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1. Introduction

Acanthamoeba species are free-living protozoa, albeit some of them are capable of infecting animal tissues under certain conditions becoming opportunistic pathogens. Interest in amphizoic amoeba research has steadily grown over the past 50 years due to the increasing number of reported cases of human keratitis (Siddiqui and Khan, 2012). Treatment of the disease is complicated by the fact that these amoebae produce cysts with a wall that is not freely permeable and the toxic effects of drugs on the target organisms are either absent or dramatically reduced (Khan, 2009). For this reason, the search for new and effective treatments for amoebic keratitis is of paramount importance. Initial evaluation of the efficacy of amoebicidal drugs is usually performed *in vitro* first. For such purpose, the assessment of population density and viability in *Acanthamoeba* cultures mainly relies on direct-counting of trophozoites or, alternatively, indirect dye degradation assays (such as MTT or resazurin sodium salt), which measure mitochondrial viability (Lück et al., 1998; McBride et al., 2005; Mosmann, 1983; Zhao et al., 2003). Either procedures show drawbacks. Trophozoite count is cumbersome and indirect assays may not accurately reflect loss of cell viability due to metabolic alterations that may occur *in vitro* (Heredero-Bermejo et al., 2013). Furthermore, these assays may be altered by different factors such as culture medium constituents, cell density, pH and chemical interactions (Rampersad, 2012).

The measurement of the oxygen consumption rate (OCR) is indeed a good indication of cell status and viability, but a continuous monitoring system of OCR has not been readily available so far. In fact, OCR studies have been conducted to study metabolic conditions in *A. castellanii* (Trocha and Stobienia, 2007) and *Toxoplasma gondii* (Vercesi et al., 1998), however, using a Clark-type electrode fitted to an oxygraph, which cannot be easily adapted to the microplate format. Alternative microplate readers exist for measuring OCR in cell (Cerveira et al., 2014) and bacteria cultures (Hutter and John, 2004), but these are more expensive than standard instruments. A new method for determining OCR in human cell cultures has been recently developed (J.A. Díaz. J. Matilla, J.A. Sánchez, Spanish Patent Application P201430899). The assay is based on an oxygen-sensitive fluorogenic probe anchored to a biocompatible and biofunctional matrix placed in the bottom of each well of a standard microplate, which is obtained through the use of organically modified silicates (ORMOSILs, Pang et al., 2007). Aerobic respiration, as well as other enzymatic oxygen consumption processes, rapidly depletes the surrounding medium of oxygen, thereby allowing the probe to fluoresce, which can be detected by any commercial time-resolved fluorescence (TRF) plate reader. This procedure has been tested and validated for *Acanthamoeba* viability tests in the present contribution, in an attempt to improve currently available methods for indirect analysis of amoeba population dynamics and cytotoxicity assays for new drugs.

2. Materials and methods

2.1. Acanthamoeba *strain and culture conditions*

A. polyphaga 2961 (a clinical isolate kindly supplied by Dr. E. Hadas, Poznan University of Medical Sciences, Poland) and *A. castellanii* ATCC® 30234 were chosen for this study. Amoebae were cultured in 25-cm^2 flasks containing 5 ml proteose peptone/yeast extract/glucose (PYG medium supplemented with 2% Bactocasitone medium, abbreviated as PYG-B medium) according to Heredero-Bermejo et al. (2012) and incubated at 32 °C or 25 °C, respectively.

2.2. Amoeba cell-based calibration curve

In order to validate the new OCR test as a model system for evaluating amoeba viability, calibration curves were constructed.

Plates for OCR analysis (Oxygen-sensitive probe, plate type OCR-LT96) were purchased from Oxoprobics Biosciences (Madrid, Spain). Log-phase *A. polyphaga* trophozoites cultured in PYG-B medium were centrifuged, washed twice in PBS and finally resuspended in fresh PBS. After counting, 100 µl of trophozoite solution was inoculated in triplicate into ninety-six-well microtiter plates (NUNC™), in a range of 1 to 128×10^3 amoebae per well. A standard sodium sulfite (anhydrous Na_2SO_3/Sigma-Aldrich, St. Louis MO, USA) calibration solution (0.1 g/ml) was employed to obtain a zero-oxygen calibration point.

The assay started with the incubation of *A. polyphaga* trophozoites in plates for 1 h at 32 °C (adhesion step). Subsequently, positive control wells were loaded with 10 µl of starter reagent (calibration solution) and mineral oil was added (100 µl per well) to seal all the wells. The microplate was covered with its lid to avoid contamination and then placed in a pre-warmed (32 °C) plate reader (VICTOR® - Perkin Elmer, Waltham, MA, USA), which was programmed to obtain two readings per well and hour (up to 24 hours). The plates were sealed with Parafilm® to minimize the interference from atmospheric oxygen.

For comparative purposes, a MTT assay was performed as well, employing the same trophozoite loads as in the OCR assay. The MTT assay was carried out as described by Heredero-Bermejo et al. (2013). Ten µl of MTT reagent (a 5 mg/ml stock solution in PBS) was added to 100 µl of *A. polyphaga* trophozoite suspension and incubated at 32 °C for 4 or 24 h.

2.3. *The cytotoxic effect of drugs on* A. polyphaga *and* A. castellanii *as determined by OCR assays*

Chlorhexidine digluconate was provided by Sigma and used as reference antiamoebic chemical agent against *A. polyphaga* and *A. castellanii*. In addition, a cationic dendrimer [$G_1Si(NMe_3^+)_4$], synthesized at the Universidad de Alcalá (Alcalá de Henares, Spain) as described by Heredero-Bermejo et al., 2015, was tested in OCR assays performed with 100×10^3 amoebae per well and different drug concentrations. Chlorhexidine was tested at concentrations ranging from 0.5 to 40 mg/L, whereas dendrimer [$G_1Si(NMe_3^+)_4$] was assayed at concentrations ranging from 2 to 64 mg/L. A negative (blank well with no amoebae) and a positive control were always included in the cytotoxicity tests.

The experiments on cytotoxicity were designed as follows: 50 µl of amoebae suspension was aliquoted into each microplate well in triplicate. After one hour of incubation at 32 °C or 25 °C (time required for amoebae adhesion to well), 50 µl of drug solution at the required concentration was added. In positive controls, the volume was 90 µl. Microplates were incubated for 23 h at 32 °C or 25 °C and then 10 µl of calibration solution was added to positive controls. Wells were subsequently covered with 100 µl of sterile mineral oil. Plates were then introduced in the microplate reader (set at either 32 °C or 25 °C) and fluorescence was measured one hour later. Finally, the toxic effect of drugs was assessed after a total incubation time of 24 h.

The IC_{50} values were calculated considering that fluorescence in untreated wells corresponded to 100% viability. Inhibitory concentration at 50% inhibition was obtained by comparison of fluorescence between treated and control wells.

2.4. Manual counting

Toxic effects of chlorhexidine digluconate on *A. polyphaga* were likewise determined by manual counting, in order to compare

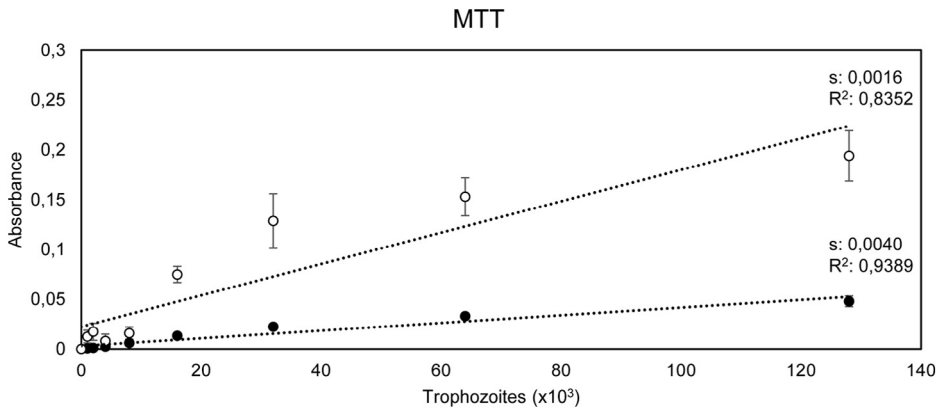

Fig. 1. MTT cytotoxicity assay graph. Increase in absorbance caused by mitochondrial activity was assessed by incubating *A. polyphaga* trophozoites for 4 h (solid circles) and 24 h (hollow circles) at 32 °C. Results are expressed as means for triplicate cultures ± SD of at least two independent assays. R^2: determination coefficient, s: slope.

the performance of manual counting and viability assays. Trophozoites were detached using cold shock and counted in a Fuchs-Rosenthal chamber using 0.2% Congo red (Heredero-Bermejo et al., 2013).

For drug cytotoxicity experiments, sterile 48-well microtiter plates (NUNCTM®) were seeded with trophozoites. Amoebae from log-phase cultures were resuspended in PYG-B medium at a density of 5×10^5 trophozoites/ml. Two hundred microliters of the calibrated trophozoite suspension was added to each well. Two-hundred µL of the drug under assay was added to the trophozoite suspension in treated wells, whereas control wells received 200 µL of distilled water instead. Plates were sealed with Parafilm® and incubated at 32 °C for 24 h. Assays were performed in triplicate and were repeated at least twice.

2.5. Statistical analysis

Each experimental condition was done in triplicate and results are given as mean ± SD of data obtained from at least two independent experiments. The significance of the differences was determined using *t* test independent by groups using Statistica 10.0 (StatSoft). The statistical significance was defined as $p < 0.05$.

The half maximal inhibitory concentration (IC_{50}) values were calculated by linear regression analysis using GraphPad Prism 5® with a 95% confidence limit.

3. Results and discussion

Initially, *Acanthamoeba* viability tests were carried out using PYG–B medium. However, high background signal (produced by culture medium) was observed in both MTT and OCR assays. In order to reduce signal noise, PYG-B medium was used in MTT assays at 1/4 dilution, whereas PBS was employed instead of PYG-B in OCR assays. Under such conditions, no decrease in amoebae viability was observed in control experiments.

At the amoebae concentrations tested, the standard MTT viability assay produced a linear response after incubating for 4 h (Fig. 1). However, differences in absorbance between wells containing different number of amoebae were very low. In comparison, the OCR assay (Fig. 2) showed noticeable differences in fluorescence between wells with different amoeba loads, along with a good reproducibility and linearity with incubation periods from 1 h. The fluorescence registered in positive wells with calibration solution was always over 250.000 UF, which was not reached by wells containing trophozoites. When comparing slopes, values yielded by OCR assay (Fig. 2) were significantly much higher than those obtained with MTT (Fig. 1).

The OCR assay allowed to assess the toxic effect of different molecules in the respiration rate of amoebae. In this sense, the standard drug chlorhexidine digluconate produced (as expected) toxic effects in *A. polyphaga* (Fig. 3A). The IC_{50} obtained with OCR assay for this

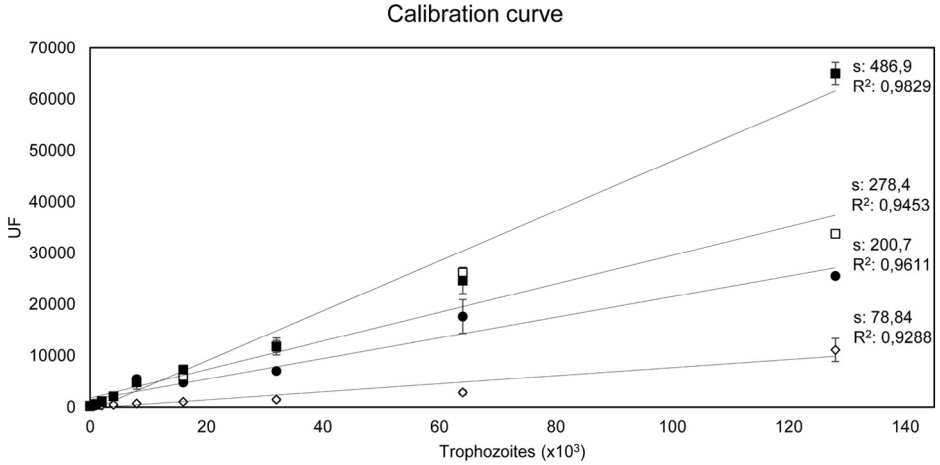

Fig. 2. Fluorescence increase by oxygen depletion in *A. polyphaga* cultures using different plating densities in 100 µl of PBS over 1 (hollow rhombs), 6 (solid circles), 12 (hollow squares) and 24 h (solid squares). Results are expressed as means for triplicate cultures ± SD of at least two independent assays. Fluorescence and *Acanthamoeba* cell number yielded a linear relationship. R^2: determination coefficient, s: slope.

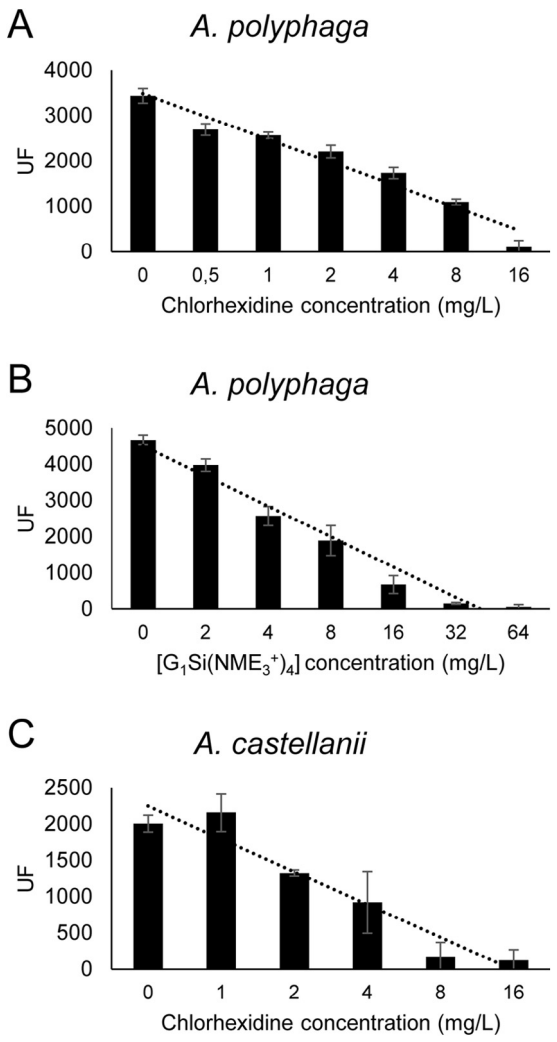

Fig. 3. Fluorescence decrease caused by toxic effects of drugs on *A. polyphaga* (Fig. 3A chlorhexidine digluconate; Fig. 3B, dendrimer) and *A. castellanii* cultures (Fig. 3C, chlorhexidine digluconate) after 24 h of incubation. Results are expressed as means for triplicate cultures ± SD of at least two independent assays.

compound at 24 h was 3.53 mg/L ± 1.3, which is comparable to a value of 3.2 mg/L ± 0.3 obtained by manual counting. Besides, chlorhexidine digluconate was slightly more toxic for *A. castellanii* than for *A. polyphaga*, because the IC_{50} value was 3.19 ± 1.2 mg/L (Fig. 3C). For the cationic dendrimer $[G_1Si(NME_3^+)_4]$, the IC_{50} value using OCR assay for *A. polyphaga* was 6.42 ± 1.3 mg/L (Fig. 3B). This means that the cationic dendrimer is less toxic than chlorhexidine digluconate.

The performance of currently available indirect viability/cytotoxicity assays may be affected by diverse factors, such as fluorescent interference from compounds being tested, the species or strains employed therein, the media used for culture or the reagents added in order to obtain fluorescent or colorimetric signal, which many times show cytotoxic effects (Heredero-Bermejo et al., 2013; Rampersad, 2012). Thus, it seemed essential to explore alternative experimental models. A comparison of current available methods employed for *in vitro* viability/cytotoxicity tests in *Acanthamoeba* spp. and the new OCR assay (as described in the present contribution) is shown in Table 1. Previously published methods such as manual counting (Buck and Rosenthal, 1996; Narasimhan and Madhavan, 2002) are costly and labor intensive. Alternative methods based on mitochondrial-enzyme activity (Henriquez et al., 2015; Jha

Table 1
Comparison of some published methods for determination of viability or cytotoxicity in *Acanthamoeba* spp. and other protozoa.

Publication	Protozoa/Assay	Seeding requirements (trophozoites)	Time required to measure viability/cytotoxicity	Contamination likelihood (due to consecutive addition of reagents)	Cost	Assay protocol
Buck and Rosenthal (1996)	*Acanthamoeba castellanii* / Manual counting	From 1000 - 10^5 amoebae	NA/2–15 days	NA	HC	IL
McBride et al. (2005)	*A.castellanii* and *A. polyphaga* / Alamar Blue (final time)	From 1250 amoebae (viability) to 5×10^4 (cytotoxicity)	24 h/96 h	Yes	MC	ML
Heredero-Bermejo et al. (2013)	*A.castellanii* and *A. polyphaga* / MTT, Resazurin, Presto Blue® and Cell Titer® (final time)	From 2×10^4 amoebae	6 h/24 h	Yes	MC	ML
Present work	*A. polyphaga* / MTT (final time)	From 1000 amoebae	4 h*/ND	Yes	MC	ML
Present work	*A. castellanii* and *A. polyphaga* / OCR (real time)	From 1000 amoebae	1 h/24 h	No	MC	LL
Furtado et al. (2012)	*Trypanosoma cruzi* / OCR (real time)	5×10^7 trypanosomes	1 h/24 h	No	HC**	LL

Note: NA = Not applicable; HC = High cost; MC = Moderate cost; IL = Intensive labor; ML = Moderate labor; LL = little labor; * = Minimal differences in absorbance between wells at 4 h of incubation (best results obtained at 24 h) and ** = Available for use only in Seahorse® instruments with its proprietary product microplates.

et al., 2015; Martín-Navarro et al., 2010; McBride et al., 2005; Zhao et al., 2003) are endpoint assays, which show also evident disadvantages. As mentioned above, they may also be influenced by the cultured organism and other factors. In addition, a substrate must be added when incubation has ended to evaluate cell viability. Therefore, when different incubation periods are used for different wells, the microplate cover must be removed several times during the experiment in order to evaluate amoebae growth, thus increasing chances of culture contamination. Seeding requirements are in general higher for final time assays, since low cell loads imply long incubation periods, which makes the test long-lasting (such as in the assay by McBride et al., 2005). In comparison, the new OCR method has low seeding requirements and small volumes of culture medium may be employed (there are 384-well OCR plates commercially available) with noticeable economy of reagents. In other respects, the Seahorse® plate reader and its proprietary microplates have been employed to measure OCR in trypanosomes (Furtado et al., 2012), but high protozoa loads are required in order to obtain results equivalent to those reported here. Moreover, both instrument and reagents are high-prized. Also, a microplate method based on optical oxygen sensors was described for yeast cultures (Pang et al., 2007), but up to now it has not been applied for use with protozoa cultures. Finally, there is a minor drawback for the OCR system described here, such as the need to add mineral oil to wells. Such step is inherent to OCR assays (otherwise oxygen in the medium would be continuously replenished by oxygen from the air) but unnecessary in other fluorometric or colorimetric methods currently employed in protozoa cytotoxicity assays.

The IC_{50} values for chlorhexidine digluconate obtained with the new OCR cytotoxicity assay in the present study are comparable to those reported by McBride et al. (2005), based on the Alamar Blue method, and toxicity values found by direct counting (Alizadeh et al., 2009; Heredero-Bermejo et al., 2015; Shirai et al., 2013). Slight differences in toxicity between these former works and the data reported here may be easily explained in terms of differences in assay methods, laboratory conditions, protozoa isolate susceptibility, and technical performance. Moreover, the IC_{50} for dendrimer $[G_1Si(NME_3^+)_4]$, determined by the OCR assay, was very close to that previously found by Heredero-Bermejo et al. (2015) by manual counting. Therefore, the measurement of oxygen concentration in trophozoite cultures is useful for drug screening and toxicity, since it leads to reproducible results in the determination of IC_{50} values on this *Acanthamoeba* model.

In summary, the current work has shown that the OCR system is a promising tool for screening synthetic compounds, since it is fast, easy-to-use, non-toxic and sensitive. Such features are of paramount importance due to the urgent need for testing new effective antimicrobials.

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