Coccolithophore calcification studied by single-cell impedance cytometry: Towards single-cell PIC:POC measurements

Douwe S. de Bruijn a,⁎, Paul M. ter Braak a, Dedmer B. Van de Waal b, Wouter Olthuis a, Albert van den Berg a

a BIOS Lab-on-a-Chip Group, MESA+ Institute, Max Planck Center for Complex Fluid Dynamics, University of Twente, P.O. Box 217, AE Enschede, 7500, the Netherlands
b Department of Aquatic Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Druvendaalsesteeg 10, PB Wageningen, 6708, the Netherlands

A R T I C L E   I N F O

Keywords:
Electrical impedance spectroscopy
Flow cytometry
Single-cell characterization
Algae
Calcification

A B S T R A C T

Since the industrial revolution 30% of the anthropogenic CO₂ is absorbed by oceans, resulting in ocean acidification, which is a threat to calcifying algae. As a result, there has been profound interest in the study of calcifying algae, because of their important role in the global carbon cycle. The coccolithophore Emiliania huxleyi is considered to be globally the most dominant calcifying algal species, which creates a unique exoskeleton from inorganic calcium carbonate platelets. The PIC (particulate inorganic carbon): POC (particulate organic carbon) ratio describes the relative amount of inorganic carbon in the algae and is a critical parameter in the ocean carbon cycle.

In this research we explore the use of microfluidic single-cell impedance spectroscopy in the field of calcifying algae. Microfluidic impedance spectroscopy enables us to characterize single-cell electrical properties in a non-invasive and label-free way. We use the ratio of the impedance at high frequency vs. low frequency, known as opacity, to discriminate between calcified coccolithophores and coccolithophores with a calcite exoskeleton dissolved by acidification (decalkified).

We have demonstrated that using opacity we can discriminate between calcified and decalcified coccolithophores with an accuracy of 94.1%. We have observed a correlation between the measured opacity and the cell height in the channel, which is supported by FEM simulations. The difference in cell density between calcified and decalcified cells can explain the difference in cell height and therefore the measured opacity.

1. Introduction

Since the industrial revolution 30% of the anthropogenic CO₂ is absorbed by oceans (Sabine et al., 2004), resulting in ocean acidification (Feely et al., 2004), which is a threat to calcifying algae (Meyer and Riebesell, 2015; Riebesell et al., 2000). As a result, there has been profound interest in these calcifying algae, boosted by their major role in the global carbon cycle (Armstrong et al., 2001; Bach et al., 2013; Gafar et al., 2019; Monteiro et al., 2016; Paasche, 2001; Schlüter et al., 2014). The species studied, coccolithophore Emiliania huxleyi, is considered to be globally the most dominant calcifying alga, which blooms can even be seen from outer space (Paasche, 2001). Calcifying algae create an exoskeleton from calcium carbonate platelets (coccoliths), as can be seen in Fig. 1, providing ballast which enhances the organic and inorganic carbon flux to the deep sea (Armstrong et al., 2001; Lombard et al., 2013). Organic carbon is formed by means of photosynthesis, where CO₂ is fixed and converted into organic molecules, causing removal of CO₂ from the seawater. Counterintuitively, the production of coccoliths leads to the release of CO₂ in the seawater, due to removal of carbonate from the seawater, which reduces the alkalinity and causes acidification (Rost and Riebesell, 2004). Therefore, the ratio between particulate inorganic carbon (PIC) and particulate organic carbon (POC) is an important measure for the net release or uptake of CO₂. In short, the PIC:POC ratio is a key characteristic required to understand and predict the impact of climate change on the global ocean carbon cycle (Beaufort et al., 2011; Feely et al., 2004; Gafar et al., 2019; Hutchins, 2011; Iglesias-Rodriguez et al., 2008; Schlüter et al., 2014).

In this research we explore the use of microfluidic single-cell impedance spectroscopy in the field of calcifying algae. Traditional research is done in bulk, which makes it cumbersome to vary a large

⁎ Corresponding author.
E-mail address: d.s.debruijn@utwente.nl (D.S. de Bruijn).

https://doi.org/10.1016/j.bios.2020.112808
Received 27 September 2020; Accepted 6 November 2020
Available online 10 November 2020
© 2020 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
range of parameters, whereas future challenges involve the study of evolutionary selection to “simultaneously changing environmental variables” (Hutchins, 2011). Fortunately, microfluidics allows for high-throughput, parallel and single-cell analysis under controlled conditions (Andersson and Van den Berg, 2003; Whitesides, 2006). Microfluidic impedance spectroscopy enables us to characterize single-cell electrical properties in a non-invasive and label-free way (Chen et al., 2015; De Wagenaar et al., 2016; Petchakup et al., 2017). Ultimately, single-cell PIC:POC measurements enable rapid study of phenotypic plasticity and intraspecific diversity, also in response to a number of climate change stressors including ocean acidification.

So far, microfluidic cytometry has been able to discriminate and analyze mixtures of algae using fluorescence and impedance (Benazzi et al., 2007; Hashemi et al., 2011). In other research opacity has demonstrated its power to discriminate on cell structure and properties as well (Cheung et al., 2005; Gawad et al., 2004a; Haandbæk et al., 2014).

In this paper, we show that we can measure differences between calcified coccolithophores and coccolithophores with a dissolved exoskeleton by acidification (decalcified), based on the impedance magnitude ratio at high and low frequency, known as opacity (Hoffman et al., 1981). In this case, we employ the high density of calcium carbonate to assess changes in the cellular PIC:POC ratio, reflecting the difference in calcification state between coccolithophore cells. The experimental results, supported with FEM simulations, are the first steps towards single-cell PIC:POC measurements.

2. Material & methods

An overview of the experimental setup and a flow diagram of the post-processing steps is given in Figure S1 of the supplementary information.

2.1. Device fabrication

A glass-PDMS chip was used as described by (de Wagenaar et al., 2016) The two layered chip consists of a PDMS chip with channels and a glass chip with tantalum/platinum coplanar microelectrodes as displayed in Fig. 2. The constriction channel was designed to increase the volume fraction of the cell with respect to the medium between the electrodes and thereby increase the sensitivity.

Standard well-established lithography processes were used to create a SU-8 mold for a single layered channel of 20 μm in height. PDMS chips were fabricated using a base to curing agent ratio of 10:1 v/v (Sylgard 184, Dow Corning). The PDMS was baked for at least 3 h at 60 °C, where after the chips were cut and bonded to the glass chips using 45 s of O2 plasma cleaning (Harrick PDC-001, NY, USA).

2.2. Cell culturing and preparation

The coccolithophore Emiliania huxleyi 920/9 (CCAP, UK) was cultured at 17 °C (HettCube 200 R, Hettich) in 0.2 μm filtered seawater enriched with vitamins and trace metals according to F/2 medium adopted from (Guillard and Ryther, 1962). A light intensity of 170 μmol photons/m²/s at a light:dark cycle of 16:8 h was supplied by a white LED strip. Cultures were kept in exponential growth at a rate of 0.96 d⁻¹ by weekly transfers of 60 μL in 25 mL fresh medium.

Decalcified cells were prepared by lowering the pH in a sub sample from the culture (pH < 5). This was done by introducing CO2 gas (≥ 99.7 v% CO2, carbon dioxide 2.7, Linde) in the culture medium for several minutes. The decalcified culture was used after 15 min to be sure all calcite was dissolved. This was confirmed by microscopic inspection, showing a clear difference between the calcified and decalcified cells as illustrated with Fig. 3 a and b. The same process can be accomplished by adding hydrochloric acid to the culture medium as is shown in Fig. 3 c–e.

The maximum cell densities that were reached in the culture cells were ~1 × 10⁹ cells/L, resulting in a maximum dissolution of 0.5 mM calcium and carbonate ions (Schlüter et al., 2014). The ion concentration of the seawater (1.1 M (Zeebe and Wolf-Gladrow, 2005)) is therefore orders of magnitude larger than the released ions and proton concentration at lower pH, such that no significant changes in σm, the conductivity of the medium, are expected.

A reference measurement was performed using 6 μm polystyrene
beads (Polybead® Polystyrene Yellow Dyed Microspheres, Polysciences) to characterize the system.

Before each experiment Poly (L-lysine)-grafted-poly (ethylene glycol) (PLL-g-PEG, SuSoS) was introduced in the chip for at least 15 min to create a monomolecular surface coating to prevent the cells from sticking to the channel. PLL-g-PEG was prepared at a concentration of 0.1 mg/mL in DI water.

A constant flow of approximately 0.01 μL/min was induced by creating a hydrostatic pressure difference between the inlet and outlet. The velocity of each cell is dependent on the position within the channel, however a minimum retention time between the electrodes of approximately 30 ms was observed, enabling proper detection by the lock-in amplifier as will be discussed next.

2.3. Data acquisition and analysis

The complex impedance $Z$ was recorded using a lock-in amplifier (HF2LI, Zurich Instruments, Zurich, Switzerland) in combination with a current amplifier (HF2TA, Zurich Instruments). A 0.5 and 20 MHz AC-signal was set at 0.15 VRMS. The impedance was recorded using a 4th order 99.8 Hz bandwidth filter at a sampling rate of 1800 Hz. The settling time of the filter to 99% of the signal was 6.9 ms (Instruments, 2016).

The recorded impedance data was imported in Matlab (R2017b, MathWorks) for post-processing. First, the baseline of the absolute impedance $|Z|$ was removed by subtracting a 6th order polynomial fit. Next, the `findpeaks` algorithm was used to find the temporal position of cells that induced a peak ($\Delta |Z|$) larger than 30 Ω at the 20 MHz signal. Cells that induced a smaller peak were ignored, because of their low signal-to-noise ratio.

Subsequently, the opacity of each cell was expressed (see equation (1)). The concept of opacity was introduced by Hoffman et al., 1981 as the ratio of the impedance at high frequency to the impedance at low frequency. The drop in impedance at higher frequencies can be attributed to the β-dispersion of the cell membrane (Schwan, 1957) and has been used to differentiate cells based on their internal properties ((Petchakup et al., 2017; Sun and Morgan, 2010)). In addition, the opacity decreases the error in the absolute impedance introduced by different cell sizes (Gawad et al., 2004b).

Traditionally, the low frequency is chosen such that the cell membrane is still blocking the signal, giving information about the cell volume, whereas the high frequency passes the cell membrane and probes the cell interior. For our study we chose the low frequency at 0.5 MHz and the high frequency at 20 MHz. The lowest frequency was limited by the electrode/liquid double layer capacitance and the highest frequency by the parasitic capacitance of the system.

\[
\text{Opacity} = \frac{\Delta |Z|_{20\text{MHz}}}{\Delta |Z|_{0.5\text{MHz}}} \tag{1}
\]

Note that the opacity is the ratio of the measured change in impedance at high frequency over that at low frequency. This measured number not only reflects the nature of the cell, but also the other elements of the set-up that are affected by the cell or the measurement frequency.

The measured opacity is displayed in a histogram and boxplot. Additionally, the mean, standard deviation and normal distribution of the measured opacity was calculated for each population. A two-sample t-test was performed to assess whether there was a significant difference in opacity between the calcified and decalcified cells. Normality and equality of variances of the data was confirmed using the Kolmogorov-Smirnov and the Levene's test, respectively.

Simultaneously with the impedance measurements, optical images were captured by an Olympus IX51 microscope with a FLIR Grasshopper 3 camera. The electrical detection of each cell was optically validated. Incorrect detection of multiple cells at the same time in the constriction area were discarded.

3. Results

The impedance response in the time-domain of a passing cell can be found in Figure S2. The resulting impedance difference and opacity for calcified and decalcified cells is plotted in Fig. 4a and b. These experiments were additionally repeated two times successfully and the results are displayed in Figure S4 and Figure S5. Fig. 4c and d shows the
impedance difference and opacity of subsequent measurements of only calcified cells with a time interval of 1.5 h. We have chosen to take the calcified state as control measurement, because this is the natural state of coccolithophore species. The calcified cells showed a significant higher opacity (mean opacity $= 0.52 \pm 0.06$) as compared to the decalcified cells (mean opacity $= 0.38 \pm 0.06$) ($t$-test; $P < 0.05; N = 119$), whereas we did not observe a significant effect of the 1.5 h interval between only calcified cells ($t$-test; $P = 0.46; N = 78$), in spite of a minimal opacity shift ($0.51 \pm 0.04$ vs. $0.52 \pm 0.06$) towards more calcification. This indicates that the performance of the system did not change significantly over time and between successive experiments. The decalcified cells were measured in between the calcified cells at 0 h and 1.5 h to rule out temporal deviations.

Linear regression analysis was used to improve the separation between calcified and decalcified cells as is shown in Fig. 4 e and f. We can discriminate between calcified and decalcified cells with an accuracy of 94.1%.

4. Discussion

Initially, the difference in opacity between the calcified and decalcified cells was attributed to the dielectric properties of the resistive calcium carbonate exoskeleton. However, a reference measurement with polystyrene microbeads showed predominantly lower opacity compared to the calcified cells, as shown in Fig. 5a and Fig. 5b. These results are counterintuitive for two reasons. First of all, there is a huge spread in opacity, whereas one would expect similar beads to give a constant opacity independent of the cell position in a non-homogeneous electric field ((Gawad et al., 2004a, 2001)). Secondly, the opacity of polystyrene beads is expected to be larger than those of cells, because of the pure dielectric nature of these polystyrene beads (Cheung et al., 2005), which is not the case as can be concluded from Fig. 5a.

Instead, we have found a correlation between the measured opacity and the particle height in the channel by investigating the measured opacity and the optical focus of each bead, as shown in Fig. 5 c. The beads are sorted from low to high opacity, showing a clear correlation between the measured opacity and the focus. The focus plane of the microscope is at the electrodes as illustrated in Fig. 5 d, therefore the beads in focus are close to the electrodes and have a high opacity. Unfocussed beads have a larger particle height and show lower opacity. The density of polystyrene beads ($\sim 1.05$ g/cm$^3$, Polysciences) is close to the density of seawater ($1.027$ g/cm$^3$), giving a large variation in particle height. Furthermore, the impedance response in the time domain confirms the difference in particle height by looking at the impedance response right above the electrodes as discussed in supplement Figure S3.

In short, the proposed explanation for the observed difference in measured opacity between calcified and decalcified cells is the difference in height in the channel. Calcified cells ($\rho = 1.233$ g/cm$^3$ (Bach et al., 2012)) are more dense compared to decalcified cells ($\rho = 1.09$ g/cm$^3$ (Bach et al., 2012)), due to the calcite exoskeleton ($\rho = 2.7$ g/cm$^3$ (Young and Ziveri, 2000)). The passage of calcified cells is therefore closer to the electrodes than decalcified cells, as can also be observed in Fig. 3a and b by looking at the optical focus of the cells. In general, all
Fig. 5. (a) Experimental result of Fig. 4a additionally showing $\Delta |Z|$ at 0.5 and 20 MHz for 6 $\mu$m polystyrene beads. (b) Opacity distribution of the measured polystyrene beads. (c) Optical images of beads sorted from low to high opacity. (d) Schematic of the channel geometry with the focus of the microscope on the electrodes. The particle height is relative to the electrodes.

Fig. 6. Impedance spectra for a 5 $\mu$m cell at the bottom (5 $\mu$m), middle (10 $\mu$m) and top (15 $\mu$m) of the channel relative to the electrodes (see Fig. 5d for the geometry) (a) without EDL and (b) with EDL. The electrodes are separated by 15 $\mu$m and the channel height is 20 $\mu$m. Opacity versus cell height (c) without EDL and (d) with EDL. The following parameters were used: double layer capacitance $C_{dl} = 0.2 \text{ F/m}^2$ (Bard and Faulkner, 2001), medium conductivity $\sigma_m = 4 \text{ S/m}$ (measured), medium permittivity $\epsilon_m = 72\epsilon_0$ (Meissner and Wentz, 2004), cytoplasm conductivity $\sigma_i = 1.2 \text{ S/m}$ (Bono et al., 2013), cytoplasm permittivity $\epsilon_i = 60\epsilon_0$ (Wang et al., 1997), cell membrane permittivity $\epsilon_{mem} = 11.3\epsilon_0$ (Morgan et al., 2007) and cell membrane thickness $d_{mem} = 5 \text{ nm}$ (Morgan et al., 2007).
passing calcified cells were in focus and decalcified cells were not, suggesting a structural different particle height.

We have performed 2D FEM simulations in COMSOL Multiphysics 5.5 to verify if the measured opacity correlates with the cell height. In earlier work (Gawad et al., 2001) showed that the opacity is barely influenced by the cell height, however the electrical double layer (EDL) was neglected in these simulations. These findings were repeated in Fig. 6a and c. In particular cases, like ours, the EDL has to be considered to account for a shift in the absolute impedance difference, as illustrated in Fig. 6b. This holds for systems with a non-uniform electric field, in which the cell height changes the induced absolute impedance difference and shifts the maximum absolute impedance difference in frequency and magnitude, due to interaction with the capacitive EDL.

The results in Fig. 6d show the relation between the measured opacity and cell height, which exposes the same trend as the observed experimental results: the closer to the electrodes, the higher the measured opacity. The parasitic capacitance is not included in the simulation, therefore the absolute value of the opacity is different from the experimental results. Further details on the simulations can be found in the supplementary information.

In short, we have observed multiple indications that the cell height is an important parameter in our system and can explain the difference in measured opacity between calcified and decalcified cells. Requirements for such a system are a non-uniform electric field, low flow rates and cells with different densities. At high flow rates cells will be focused due to inertial flow focusing effects (Zhou and Papautsky, 2013), therefore the throughput of this system is limited.

Furthermore, the large standard deviation of the opacity can be explained by the low signal-to-noise ratio (SNR), variations between biological cells and variations in cell height. The standard deviation of the noise floor was 5.6 Ω and 0.9 Ω for the 0.5 and 20 MHz signal, respectively (see Fig. S6). The impedance spectroscopy was performed using an easy-to-fabricate coplanar electrode setup, resulting in a non-uniform electric field, which is known to be sensitive to the cell height (Daguerre et al., 2020), however the measured opacity could help to detect the cell height.

The measurement setup can be improved by deploying a differential measurement, which cancels the baseline value and only amplifies variations in the signal (Gawad et al., 2001). Additionally, the distance between the electrodes should be increased for a more uniform electric field (Cottet et al., 2019). The throughput of the system is currently limited by the filter time constant. Improvement of the measurement setup also reduces the need of strict filter settings, which will reduce the settling time of the signal. As a result, the flow rate and the cell density could be increased to improve the throughput. High throughput measurements in a uniform electric field will therefore expose the dielectric properties of each cell instead of the cell height in the channel.

5. Conclusion

A microfluidic impedance cytometer has been used to investigate calcification of the coccolithophore Emiliania huxleyi. We have shown in our experimental work that we can discriminate between calcified and decalcified coccolithophores with an accuracy of 94.1% using the impedance magnitude at high and low frequency. We have observed multiple indications that the cell height is an important parameter in our system, such that it is a likely explanation for the difference in measured opacity between calcified and decalcified cells. The denser calcified cells are closer to the electrodes compared to the less dense decalcified cells, resulting in a difference in opacity for systems with a non-uniform electric field as supported by the simulation results.

Vice versa, a simple pair of coplanar electrodes could be deployed to measure the cell height of passing cells as a function of the measured opacity.

The presented work is a promising start for the application of single-cell impedance cytometry in the field of calcifying algae. Further development of the device and measurement setup will help to improve the signal-to-noise ratio and create a uniform electric field, which will enable rapid and high throughput assessment of the PIC:POC ratio by looking at the dielectric properties of cells. Ultimately, this may greatly support revealing the cellular variation in responses of coccolithophores to a wide range of combined climate change stressors, and thereby improving our understanding and predictive ability to the impacts of ocean acidification on coccolithophores and their feed-backs to the global carbon cycle.

CRediT authorship contribution statement

Douwe S. de Bruijn: Methodology, Investigation, Formal analysis, Writing - original draft. Paul M. ter Braak: Resources. Dedmer B. Van de Waal: Resources, Writing - review & editing. Wouter Oltius: Supervision, Writing - review & editing, Validation. Albert van den Berg: Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work is part of the research programme of the Foundation for Fundamental Research on Matter (FOM), which is part of the Dutch Research Council (NWO). We would also like to thank the Max Planck – Center for Complex Fluid Dynamics for support of this project. Furthermore, we would like to thank Darya Hadavi for the SEM image, Stella Kruit for fabrication of the SU-8 mold and Elko Westerbeek for discussions on the FEM simulations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbios.2020.112808.

References

Andersson, H., Van den Berg, A., 2003. Microfluidic devices for cellomics: a review. Sensor. Actuator. B Chem. 92, 315–325. https://doi.org/10.1016/S0925-4005(03)00266-1.
Armstrong, R.A., Lee, C., Hedges, J.J., Hanjo, S., Wakeham, S.G., 2001. A new, mechanistic model for organic carbon fluxes in the ocean based on the quantitative association of POC with ballast minerals. Deep. Res. Part II Top. Stud. Oceanogr. 49, 219–236. https://doi.org/10.1016/S0967-0645(01)00101-1.
Bach, I.T., MacKinder, L.C.M., Schulz, K.G., Wheeler, G., Schroeder, D.C., Brownlee, G., Riebesell, U., 2013. Dissecting the impact of CO2 and pH on the mechanisms of photosynthesis and calcification in the coccolithophore Emiliania huxleyi. New Phytol. 199, 121–134. https://doi.org/10.1111/nph.12229.
Bach, I.T., Riebesell, U., Sett, S., Febiri, S., Raspka, P., Schulz, K.G., 2012. An approach for particle sinking velocity measurements in the 3-400 μm size range and considerations on the effect of temperature on sinking rates. Mar. Biol. 159, 1853–1864. https://doi.org/10.1007/s00227-012-1945-2.
Bard, A.J., Faulkner, L.R., 2001. Electrochemical Methods Fundamentals and Applications, second ed. John Wiley & Sons, New Jersey, USA.
Beaufort, L., Probert, I., De Garidel-Thoron, T., Bendif, E.M., Ruiz-Pino, D., Metzl, N., Goyet, C., Buchet, N., Grelaud, M., Rost, B., Rickaby, R.E.M., De Vargas, C., 2011. Sensitivity of coccolithophores to carbonate chemistry and ocean acidification. Nature 476, 80–83. https://doi.org/10.1038/nature10295.
Benazzi, G., Holmes, D., Sun, T., Mowlem, M., Morgan, H., 2007. Discrimination and analysis of phytoplankton using a microfluidic cytometer. IET Nanobiotechnol. 1, 94–101. https://doi.org/10.1049/iet-nbt.
Bono, M.S., Ahner, B.A., Kirby, B.J., 2013. Detection of algal lipid accumulation due to nitrogen limitation via dielectric spectroscopy of Chlamydomonas reinhardtii suspensions in a coaxial transmission line sample cell. Bioreor. Technol. 143, 623–631. https://doi.org/10.1016/j.biortech.2013.06.046.
Chen, J., Xue, C., Zhao, Y., Chen, D., Wu, M.H., Wang, J., 2015. Microfluidic impedance flow cytometry enabling high-throughput single-cell electrical property characterization. Int. J. Mol. Sci. 16, 9804–9830. https://doi.org/10.3390/ijms16059804.
