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Towards a better understanding of the flocculation/flotation mechanism of the marine microalgae *Phaeodactylum tricornutum* under increased pH using atomic force microscopy

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ABSTRACT

In the context of climate change, the interest for sustainable sources to produce energy is growing. One promising resource for biofuel production is microalgae, but their industrial use is limited by the lack of efficient harvesting techniques. In this study, we use a multi-scale approach to understand the magnesium hydroxide-mediated flocculation/flotation mechanism of *Phaeodactylum tricornutum*, an effective oil-producer diatom, under high pH. While flotation experiments give a population-scale quantification of the efficiency of flocculation/flotation using magnesium or calcium hydroxide, or at increased pH, AFM allows probing the mechanical properties of the cells at different pH values. Finally we develop an original strategy to functionalize AFM tips with hydroxide particles that we use in multiparametric imaging experiments to understand at the molecular scale the forces driving the adhesion of hydroxide particles to cells. Altogether, our results give a better understanding of the molecular mechanism underlying alkaline flocculation/flotation, paving the way towards the development of low-cost flocculant-free flotation harvesting processes.

1. Introduction

The group of diatoms, which comprises from 10,000 to 100,000 different species, making them the second most diverse group of photosynthetic organisms, is responsible for 40% of marine primary productivity [1, 2]. Among the diatoms that have attracted attention so far, *Phaeodactylum tricornutum* stands out because of its natural abundance in particular omega-3 eicosapentaenoic acid (EPA), pigments and antioxidants [3–5]. Today, *P. tricornutum* is mainly exploited for aquaculture and nutraceutical applications. But because of its ability to produce up to 45% of lipids, *P. tricornutum* presents a high potential in biofuel production, although for this particular purpose genetic engineering is required to modify the quantity [6] and characteristics of the produced fatty acids in regards to their usage as biofuel feedstock [7]. Moreover, the use of biofuels as an alternative to fossil fuels is at the moment, technically not feasible [5].

Indeed, while small-scale production of *P. tricornutum* to obtain high value-added molecules is efficient, the large-scale production from microalgae of molecules substituting fossil carbon resources faces a number of technical challenges that have made the current growth and development of the biofuel industry economically unviable [8]. The main limitation encountered by industrials is the harvesting of microalgae [9]. Harvesting consists in removing at a minimal cost the microalgae from their aqueous culture medium, where their concentration is low (0.3–3 g/L) [10], while keeping their cell wall intact not to lose their precious production in solution. This crucial step of harvesting and dewatering has been assumed to account for one third of the entire price of microalgal biomass production in industrial processes [11]. Several methods have been proposed for algae harvesting, including centrifugation, filtration, flocculation, sedimentation and flotation [12]. However, most of these methods present high costs and energy consumption, for low efficiency rates. Centrifugation for example consumes a large amount of electricity and causes damage to the cells because of the high shear forces, while filtration costs are increased by the clogging of membranes inevitable with unicellular small cells such as microalgae [13].

In this context, flotation that takes advantage of algae's natural characteristics of relatively low density and self-floating tendency, appears to be the most promising harvesting technique [14]. Assisted flotation consists in air or gas bubbles rising in a microalgal suspension. As a result, microalgae cells get attached to bubbles interfaces and are carried out and accumulated on the suspension surface [12]. It is a

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relatively rapid operation that needs low space, has moderate operational costs, and that could thus overcome the bottleneck of feasible microalgal biofuel production [13]. However important factors can affect microalgae flotation, such as the charge of the surface of the cells. The surface of microalgae present a negative surface charge; the surface of bubble in an aqueous medium being also negatively charged [15], the microalgal surface interaction with bubbles is then repulsive, which prevents adhesion, and therefore capture and flotation. Thus to improve flotation efficiency, chemical flocculants are added to the algal suspension to aggregate cells into large flocs that can then be easily separated from the water by further flotation [16]. However addition of these molecules might not be an ideal solution because of their potential toxicity on the algal biomass if it is used for food for example, as well as in the recycled water [17]. Therefore in many cases auto-flocculation would be a preferred alternative to improve water separation by flotation. There are several known auto-flocculation mechanisms, among which one is based on the precipitation of magnesium ions at high pH [18]. This mechanism has been described already for microalgae species such as *Dunaliella salina* [19], *Chlorella vulgaris* [20], *Nannochloropsis occulata* [20], but also for *P. tricornutum*. Indeed, in recent publications, it has been showed that flocculation of *P. tricornutum* induced by an increase of pH in marine water, was the result of the precipitation of magnesium ions into magnesium hydroxide presenting a positively charged surface and thus flocculating the cells through a charge neutralization mechanism [20, 21]. However, for the moment, there are no evidence nor characterization of the interactions between cells and magnesium hydroxide that could confirm or complement the information on this mechanism. Also, the effects of an increasing pH on cells were never investigated, as surface modification of the cells at such pH could participate in the flocculation/flotation mechanism.

It is now possible to answer such questions thanks to recent advances in atomic force microscopy (AFM) techniques. AFM, first developed in 1986 [22], is a technology particularly well suited for the study of living microorganisms, as it features high-resolution imaging capabilities and is able to operate in liquid. Furthermore, it is also a highly sensitive force machine, able to record forces as small as 20 pN in force spectroscopy mode, making it then possible for researchers to gain insights into the mechanical properties and molecular interactions of single cells [23]. Recently new force-spectroscopy based techniques were developed, such as multiparametric imaging that offers the possibility to image the surface structure of living cells, while mapping their adhesive properties at high spatial resolution [24–26]. Furthermore, AFM tips used to perform experiments can also be functionalized with particles [27] for example, and thus provide a way to understand and characterize the interaction forces between cells and these particles.

In this study, we use a multi-scale approach to understand the magnesium hydroxide-mediated flocculation/flotation mechanism of *P. tricornutum* cells under high pH. For that, we first perform flotation tests, which give a population-scale quantification of the efficiency of magnesium hydroxide as a flocculant; we also evaluate the effects of another hydroxide that can also form in marine water at high pH; calcium hydroxide. We then go down to the micro-scale and use AFM to image and probe the mechanical properties of living *P. tricornutum* cells at different pH values in order to understand the effects of increasing pH on their surface properties. We finally develop an original strategy to functionalize AFM tips with hydroxide particles that we use in multiparametric imaging experiments to understand at the molecular scale the forces driving the adhesion between these hydroxides and the cells. Altogether, our results allow giving a better understanding of the mechanisms underlying alkaline flocculation/flotation, and show that not only the precipitates are responsible for the formation of flocs and the further separation by flotation, but also the cells and the structural changes they undergo at high pH. Such information may have important impacts on the development of low-cost flocculants-free flotation processes, and thus on the further use of these processes in relevant biotechnological applications to decrease costs.

2. Material and methods

2.1. Strain and culture conditions

Phaeodactylum tricornutum strain CCMP2561 (Bigelow National Center for Marine Algae and Microbiota) was grown in synthetic Sea Salts (40 g/L, Sigma S9883) containing filtered Guillard f/2 medium [28] (Sigma G0154) without silica, at 20 °C, under agitation (100 rpm), in 75 mL non-coated culture flasks (15 mL of culture), 500 mL non-coated culture flasks (150 mL of culture), and 2 L Erlenmeyer (500 mL of culture). The incubator was equipped with white neon light tubes providing illumination of 120 μmol photons $m^{-2} s^{-1}$ with a photoperiod of 12 h light:12 h dark. All experiments in the study were performed in Sorbitol buffer 375 mM at pH = 8 or pH = 10. For that, cells were harvested by centrifugation (3000 rpm, 10 min) and washed two times in sorbitol buffer 375 mM at pH = 8 or pH = 10.

2.2. Dry weight concentration determination

Glass fiber filters (0.45 μm, Whatman GF6) were first dried at 105 °C for 24 h; their initial mass was measured after drying. *P. tricornutum* cells were harvested by centrifugation (3000 rpm, 10 min) and washed two times in sorbitol buffer 375 mM at pH = 8 or pH = 10. Then 50 mL of the cell suspension was filtered on the dried filters, which were then allowed to dry for 24 more hours at 105 °C. Filters were finally weighed again: the weight difference before and after filtration of the cell suspension corresponds to the dry mass of the filtered cells. Knowing the volume of the initial cell suspension, the concentration can be obtained.

2.3. Flotation experiments

Dissolved air flotation (DAF) experiments were achieved in a Multiplace Orchidis™ Flottatest, as described previously [19]. Algal suspensions of 500 mL, at a biomass dry concentration comprised between 0.3 and 0.4 g/L, were harvested by centrifugation (3000 rpm, 10 min), washed two times in sorbitol buffer 375 mM at pH = 8 or pH = 10, and added to flotation-test beakers. The depressurization at atmospheric pressure of sorbitol buffer 375 mM at pH = 8 or pH = 10 and saturated by air at 6 bars induced the formation of bubbles. Sorbitol buffer free of algae was pressurized for 30 min before injection into the beakers. The injection was controlled by a solenoid valve and 100 mL of pressurized sorbitol buffer was added to each beaker sample. Flotation tests were conducted in the presence or not of a flocculant; here $Mg(OH)_2$ or $Ca(OH)_2$. Tests with no flocculant were performed in sorbitol buffer at pH = 8 or pH = 10, whereas tests using $Mg(OH)_2$ or $Ca(OH)_2$ were performed in sorbitol buffer at pH = 10 to avoid dissolution of the hydroxides. In these cases, before depressurization, hydroxides were added at a final concentration of 10.5 mM for $Ca(OH)_2$ and of 5.7 mM, 14.3 mM or 57 mM for $Mg(OH)_2$ in the flotation-test beakers containing the algal suspensions in buffer. Mechanical mixing (100 rpm, 5 min) allowed homogenization of the suspension. Note that in our conditions, the hydroxide particles are bigger than if they were directly formed *in situ*, thus their specific surface is smaller, and concentrations higher than in physiological conditions are used. To evaluate the efficiency of flotation tests, KOVA counting slides were used. For each test, 10 samples of the algal suspension withdrawn in the middle of the suspension, before and 10 min after the depressurization, were used for counting and determine the cell concentration. The percentage of efficiency corresponds to the percentage of cells removed for the algal suspension after flotation.