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Rapid Spectrophotometric Method for Determining Surface Free Energy of Microalgal Cells

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Abstract

Microalgae are one of the most promising renewable energy sources with environmental sustainability. The surface free energy of microalgal cells determines their biofouling and bioflocculation behavior and hence plays an important role in microalgae cultivation and harvesting. To date, the surface energetic properties of microalgal cells are still rarely studied. We developed a novel spectrophotometric method for directly determining the surface free energy of microalgal cells. The principles of this method are based on analyzing colloidal stability of microalgae suspensions. We have shown that this method can effectively differentiate the surface free energy of four microalgal strains, i.e., marine *Chlorella* sp., marine *Nannochloris oculata*, freshwater autotrophic *Chlorella* sp., and freshwater heterotrophic *Chlorella* sp. With advantages of high-throughput and simplicity, this new spectrophotometric method has the potential to evolve into a standard method for measuring the surface free energy of cells and abiotic particles.

Graphical abstract

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Notes

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Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Microalgae are one of the most promising renewable energy sources due to their fast growth rate, low nutrient requirement, and ability to accumulate high lipid and carbohydrate contents.^{1,2} Moreover, microalgae cultivation has beneficial environmental impacts, including fast carbon dioxide mitigation and effective wastewater treatment.³ To optimize microalgae cultivation and harvesting, there is an urgent need to understand the physicochemical and rheological properties of microalgal cells and their culturing medium, such as their colloidal viscosity, thermophysical properties, and surface properties.^{4,5}

The surface free energy of microalgal cells plays a crucial role in their cultivation and harvesting. First, the surface free energy determines the formation and development of microalgal biofilms.^{5,6} While cultivating in a photobioreactor, microalgal cells tend to form highly productive biofilms over solid surfaces. This microalgal biofouling can significantly lower the performance of the photobioreactor and hinder cell proliferation in the culture medium.^{6,7} Second, the surface free energy of microalgal cells determines their self-aggregation and sedimentation, thus directly influencing microalgae harvesting.^{5,8,9} Hence, understanding the surface free energy of microalgal cells can have a profound impact on the design of more effective photo-bioreactors for large-scale algal cultivation and on the optimization of algal harvesting and downstream processing techniques toward a more cost-effective production of biofuels.

Despite its importance, the surface free energy of microalgal cells is rarely reported.^{5,10} One reason is the lack of easy-to-use methods for determining the surface free energy of live cells. To date, nearly all quantitative determinations of surface free energy of particles or cells (mainly bacterial cells) rely on the contact angle method.^{11–15} In this method, contact angles are measured on filtrated cell lawns using the sessile drop technique, followed by theoretical interpretation that indirectly determines the surface free energy of cell layers, which is in turn assumed to be identical to the surface free energy of individual cells. Although it is a plausible method, it is well-known that contact angle measurement on live cells and its energetic interpretation are not trivial tasks, which can introduce significant methodological complications for nonexperts.^{16–19}

In this paper, we have developed a novel easy-to-use spectrophotometric method for directly determining surface free energy of microalgae cells. We first verified the accuracy of this method by studying polystyrene microbeads with known surface free energy. Subsequently, we used this method to measure the surface free energy of four widely used microalgal

strains in biofuel production, i.e., marine *Chlorella* sp., marine *Nannochloris oculata*, freshwater autotrophic *Chlorella* sp., and freshwater heterotrophic *Chlorella* sp. We showed that this spectrophotometric method is highly sensitive in differentiating the surface free energy of various microalgal strains. With combined advantages of high-throughput and simplicity, this spectrophotometric method has the potential to evolve into a standard method for measuring surface free energy of cells and abiotic particles.

EXPERIMENTAL SECTION

Principles of Measurement

Fundamental principles of this spectrophotometric method follow the sedimentation volume method developed by Neumann and co-workers in 1980s.^{20–22} Without loss of generality, as demonstrated in Figure 1, the colloidal stability of a particle suspension is determined by the balance between the electrostatic repulsion and the van der Waals attraction, as predicted by the DLVO theory.²³ The thermodynamic free energy for particle coagulation can be expressed as

$$\Delta E = E_{11} + E_{22} - 2E_{12} \quad (1)$$

where 1 denotes the suspending medium and 2 denotes the particles.

The contribution of van der Waals interactions in this coagulation process can be attributed to the determination of the interaction Hamaker constant A_{212}

$$A_{212} = A_{11} + A_{22} - 2A_{12} \quad (2)$$

Applying the geometrical mean combining rule, $A_{12} = (A_{11}A_{22})^{1/2}$, the Hamaker constant of two particles interacting across the suspending medium is

$$A_{212} = (\sqrt{A_{11}} - \sqrt{A_{22}})^2 \quad (3)$$

It should be noted that this geometrical mean combining rule is derived from the Lifshitz theory and has been widely used in solution theory to calculate heterogeneous interactions.²³ However, it is applicable only when dispersion forces dominate the interactions but breaks down when applied to media with high dielectric constants.^{23,24}

Relating the Hamaker constant to surface tensions (γ) using $A = 24\pi D^2 \gamma$,^{23,24} it yields

$$A_{212} = 24\pi D^2 (\sqrt{\gamma_1} - \sqrt{\gamma_2})^2 \quad (4)$$

where D is the minimum separation distance between two surfaces.

Equation 4 implies that when the surface tension of the suspending medium (denoted by 1) and the surface free energy of the dispersed particles (denoted by 2) are equal to each other, the van der Waals attractions between particles (across the medium) are reduced to a

minimum. Consequently, the particles are expected to be largely dispersed in the medium without significant aggregation, thus resulting in a minimum sedimentation.

To implement this principle, one can disperse particles in a series of liquids with known surface tension values that cover the possible surface free energy of the particles. When measuring the sedimentation volume or mass of the particles, as in the original method developed by Neumann and co-workers,^{20–22} a minimum is expected at which the surface tension of the liquid is equal to the surface free energy of the particles (see Figure 1). However, the measurement of sedimentation volume/mass in a series of suspending liquids is a time-consuming procedure that requires careful handling of individual samples with highly sensitive balances. These difficulties have rendered the sedimentation volume/mass method unpopular in the past three decades.

To circumvent these difficulties, here we propose a novel approach of implementing the sedimentation principles. Instead of analyzing the sediment, we focus on the supernatant and measure the absorbance of transmitted light through the supernatant after particle sedimentation. Since the amount of sedimentation should be inversely related to the amount of free particles suspended in the supernatant, we hypothesize that a maximum optical density (OD) should coincide with the minimum sedimentation volume/mass (see Figure 1). This spectrophotometric method allows us to take advantage of the highly sensitive and high throughput capacity of a microplate reader to quickly determine the surface free energy of particles.

Algal Cultivation and Sample Preparation

The algal strains used in this study were marine *Chlorella* sp., marine *Nannochloris oculata*, freshwater autotrophic *Chlorella* sp., and freshwater heterotrophic *Chlorella* sp. The marine *Chlorella* sp. and marine *Nannochloris oculata* were both cultivated in the f/2 medium. The freshwater autotrophic *Chlorella* sp. was cultivated in Bold's Basal medium. The freshwater heterotrophic *Chlorella* sp. was cultivated in the Fitzgerald medium. All culture media were prepared on the basis of the procedures described by Andersen.²⁵ All microalgal strains were grown in 500 mL flasks containing 100 mL of culture medium at 25 ± 1 °C under continuous irradiance of $100 \mu\text{mol}/\text{m}^2\cdot\text{s}$. The suspensions were cultured for about 7 days before cells were harvested.

Samples used in this study were prepared from actively growing cultures during their exponential growth phase. After harvesting, the cultivated microalgal cell suspensions were centrifuged at 4000 rpm for 3 min at room temperature to remove cell debris, followed by washing with phosphate buffer solution. After three rounds of centrifugation and washing, the microalgal cells were resuspended in phosphate buffer solution to prepare the concentrated samples used in our experiments. Before surface free energy measurements, the concentrated microalgae suspensions were ultrasonically vibrated for 2 min to break aggregates and to remove air bubbles.

Algal Cell Characterization

Before determining their surface free energies, the microalgal cells were characterized with their colloidal properties, including primary size, hydrodynamic size, and surface charge.

The primary size was determined by scanning electron microscopy (SEM) with the particle size analyzed by ImageJ (NIH). The hydrodynamic size was determined by a laser particle sizer (S3500SI, Microtrac Inc., Largo, FL). The surface charge was measured by a zeta potential analyzer (Nanotracer wave, Microtrac Inc.).

Implementation of the Spectrophotometric Method

A series of suspending liquids was prepared with binary mixtures of ethanol and ultrapure water at predefined mixing ratios. Surface tensions of these binary liquids range from ~ 22 mJ/m² (for pure ethanol) to ~ 73 mJ/m² (for pure water), which were determined at room temperature with a Wilhelmy plate surface tensiometer (K100, Kruüss GmbH, Germany). (Density and viscosity of these liquids were also determined and listed in Table S1 of the Supporting Information.) This liquid surface tension range covers the surface free energy of most polymeric particles and biological cells.^{12,24} When studying unknown particles/cells, coarse measurements with suspending liquids spanning a large surface tension range were performed followed by fine measurements with liquids in a narrower range of surface tensions.

Specifically, a trace amount of condensed microalgal cells ($5 \mu\text{L}$ at $50\text{--}100$ g/L) was added to a series of 10 to 12 clearly labeled suspending liquids, each at 1 mL, and fully mixed. To accelerate the sedimentation process, the cell-containing suspensions were centrifuged at 1000 rpm for 1 min. After centrifugation, $200 \mu\text{L}$ of supernatant of each suspending liquid was transferred from centrifuge tubes to a 96-well microplate. Optical densities of these supernatants were measured with a microplate reader (Epoch, BioTek, Winooski, VT) at a characteristic wavelength of 690 nm, determined in a separate experiment. (See Figure S1 of the Supporting Information.) The measured OD_{690} was plotted against the surface tensions of the suspending liquids. A maximum was determined by peak fitting experimental points with extreme function in statistics using OriginLab.²⁶ Each measurement was repeated for at least 5 times and averaged. For the purpose of comparison, we conducted parallel measurements of the sedimentation mass using the analytical balance associated with the Kruüss K100 surface tensiometer.

RESULTS AND DISCUSSION

Characterization of Microalgal Cells

The four microalgal cells were characterized with their primary size, hydrodynamic size, and surface charge. As shown in Table 1, all four microalgal cells have a nearly spherical shape with a mean primary diameter (D_p) close to $3 \mu\text{m}$, determined by SEM. The mean hydrodynamic diameter (D_H) of these microalgal cells, determined with the particle sizer, ranges from $3.8 \pm 0.8 \mu\text{m}$ for freshwater autotrophic *Chlorella* sp. to $5.1 \pm 0.9 \mu\text{m}$ for freshwater heterotrophic *Chlorella* sp. All four microalgal cells are negatively charged with the zeta potential (ζ) ranges from -14.8 ± 2.1 mV for freshwater heterotrophic *Chlorella* sp. to -42.8 ± 3.8 mV for marine *Chlorella* sp. These characteristic surface properties ensure the applicability of the DLVO theory and our measurement principles to these microalgal cells.

Proof-of-Principle: Measuring Surface Free Energy of Polystyrene Microbeads

Prior to measuring microalgal cells, we first validated our method by studying polystyrene microbeads with the similar size and surface charge as our microalgal cells. The studied polystyrene microbeads (Aladdin Reagents Co., Shanghai, China) have a mean diameter of $\sim 3 \mu\text{m}$ and a zeta potential of approximately -30 mV , both comparable to our microalgal cells (Table 1). The surface free energy of polystyrene has been well studied and reported from 30 to 33 mJ/m^2 in the literature.^{27,28}

Figure 2 illustrates the experimental results pertinent to measuring the surface free energy of polystyrene microbeads. Figure 2a shows the change of the sedimentation mass within the first 7 min after adding the microbeads into three representative suspending liquids whose surface tensions are 28.0 , 33.6 , and 39.6 mJ/m^2 , respectively. It should be noted that, in our experiments, a series of 10 to 12 liquids with a range of surface tensions from ~ 22 to $\sim 50 \text{ mJ/m}^2$ was used as suspending liquids (see Table S1, Supporting Information, and Figure 2d). However, for the sake of clarity, only experimental results of three representative liquids are shown in Figure 2a–c. Surface tensions of these three suspending liquids are lower than, close to, and higher than the expected surface free energy of the polystyrene microbeads.

Figure 2a clearly shows that the sedimentation mass increases steeply within the first minute after adding the microbeads into the suspending liquids. After 1 min, sedimentation reaches equilibrium. The sedimentation mass in the liquid with the intermediate surface tension (i.e., 33.6 mJ/m^2) was significantly lower than that in the other two suspending liquids. A similar trend was found with OD measurements. As shown in Figure 2b, the OD value levels off after 1 min of centrifugation. The equilibrium OD value of the 33.6 mJ/m^2 suspending liquid is higher than that of the other two liquids.

To verify that the different behaviors in sedimentation and light absorbance (Figure 2a,b) are indeed due to the different particle aggregations as predicted by the theory shown in Figure 1, we directly measured the particle size distribution in these three suspending liquids using a particle sizer. As shown in Figure 2c, more than 50% of particles in the liquids of surface tension 28.0 and 39.6 mJ/m^2 has a mean diameter larger than $1000 \mu\text{m}$, indicating significant particle aggregation/agglomeration. The additional 40% of particles in these two liquids is free particles with a size of about $3 \mu\text{m}$, plus a small portion ($\sim 10\%$) minor agglomeration at $\sim 20 \mu\text{m}$. However, when dispersing particles in a liquid of surface tension of 33.6 mJ/m^2 , i.e., a liquid surface tension close to the surface free energy of the polystyrene microbeads, a majority of the particles (87%) remains dispersed at $\sim 3 \mu\text{m}$, with only a small portion (13%) of moderately aggregated particles at $\sim 20 \mu\text{m}$. This direct characterization of particle size distributions validates our principles of measurement and explains the different behaviors of sedimentation and light absorbance in these three representative suspending liquids (Figure 2a,b).

Figure 2d shows the sedimentation mass and OD in a range of suspending liquids of different surface tensions, measured at 5 min after adding the polystyrene microbeads (i.e., at the equilibrium sedimentation). As predicted by theory (Figure 1), a minimum appears for the sedimentation mass which coincides with a maximum OD. Using peak fitting, the minimum sedimentation mass is located at 32.4 mJ/m^2 and the maximum OD is located at

32.7 mJ/m². With one-way ANOVA analysis, these two measurements were not statistically significant ($p > 0.05$). This indicates that the spectrophotometric method has a comparable accuracy as the sedimentation mass method. Both methods determine the surface free energy of polystyrene microbeads to be around 32–33 mJ/m², which is in good agreement with the literature values.

It should be noted that, although it has a comparable accuracy, the spectrophotometric method is much quicker and more sensitive than the sedimentation mass method. Taking advantage of the high throughput processing of a microplate reader, the spectrophotometric method can simultaneously implement OD measurements in all suspending liquids. Hence, the entire measurement can be completed within 10 min. Moreover, since the OD measurement is much more sensitive than measuring sedimentation mass or volume, the spectrophotometric method only uses less than 1% of the sample required for the sedimentation measurement.

Measuring Surface Free Energy of Microalgal Cells

Figure 3 shows the experimental results for determining the surface free energy of four commonly used microalgal strains, marine *Chlorella* sp. (a), marine *Nannochloris oculata* (b), freshwater autotrophic *Chlorella* sp. (c), and freshwater heterotrophic *Chlorella* sp. (d). It is clear that both the minimum sedimentation mass and the maximum OD consistently indicate the surface free energy of these microalgal cells to be around 35, 41, 43, and 53 mJ/m², respectively.

With very scarce literature data and abundant microalgae species, we can only conduct a limited comparison between our measurements and those reported by others. Using contact angle measurement in conjunction with the surface tension components theory, the surface free energy of freshwater *Chlorella vulgaris* was reported to be 42.9 mJ/m² by Ozkan and Berberoglu⁵ and 40.4 mJ/m² by Procházková et al.¹⁰ The surface free energy of freshwater autotrophic *Chlorella* sp. determined by our spectrophotometric method (i.e., 43 mJ/m²), albeit not directly comparable, is found to be very close to these literature data.

The surface free energy reflects the phytochemical properties of microalgal cells and their extracellular matrix due to the use of different culture media and conditions.^{29,30} All four microalgal strains studied here are green algae (*Chlorophyta*). The cell wall of all green algae generally consists of similar chemical components of cellulose, glucan, proteins, and lipids.³¹ However, compared to their autotrophic counterparts, which only require an inorganic culture medium, the heterotrophic microalgae are nonphotosynthetic and hence require an external source of organic components and nutrients to supply energy. This difference explains the relatively high surface free energy of freshwater heterotrophic *Chlorella* sp. (i.e., 53 mJ/m²), which was found to have a higher polysaccharide content at its surface compared to autotrophic algal strains.^{32–34} Adsorption or binding of the extracellular matrix on the surface of freshwater heterotrophic *Chlorella* sp. is also evident from its relatively large hydrodynamic size as shown in Table 1.

It should be noted that the use of ethanol/water mixtures as suspending liquids should not significantly affect viability of microalgal cells. We have measured the viability of the

microalgal cells recovered from the suspending liquid of which the surface tension is equivalent to the surface free energy of the cells. The cell viability was measured using the CellQuanti-Blue Cell Viability Assay Kits (BioAssay Systems, Hayward, CA), which determined cell viability by measuring cell metabolism. As shown in Table S2 of the Supporting Information, the viability of marine *Chlorella* sp., marine *Nannochloris oculata*, freshwater autotrophic *Chlorella* sp., and freshwater heterotrophic *Chlorella* sp. is 71%, 91%, 91%, and 98%, respectively. The relatively low cell viability of marine *Chlorella* sp. (i.e., 71%) is likely due to the exposure to a relatively high ethanol content of approximately 30 v/v% in the suspending liquid (Table S1, Supporting Information).

Implications in Microalgae Cultivation and Harvesting

Despite the microscale heterogeneity of cell surfaces,³⁵ surface free energy has been used as a physicochemical property that measures the contribution of surface molecular structures on the overall cellular surface properties such as the cell surface hydrophobicity.³⁶ Specifically for microalgal cells, surface free energy has a profound impact on their biofouling and bioflocculation behaviors. It has been shown that microalgal strains with a lower surface free energy (i.e., more hydrophobic) had a higher adhesion density and strength on solid surfaces, thus causing more severe biofouling and biocorrosion of the photobioreactor used for algae cultivation.⁶ Surface free energy also plays a significant role in algae harvesting that is the most energy-intensive process in current industrial microalgae biomass production.¹ Although there are no standard harvesting techniques, algae flocculation is required prior to most harvesting methods such as filtration, floatation, or sedimentation.¹ It has been shown that microalgal strains with a high surface free energy (i.e., hydrophilic) tend to form a planktonic state without flocculation due to the difficulty of excluding water between algal cells.⁶ Most of the current methods use the addition of flocculants such as multivalent cations or cationic polymers (e.g., chitosan) to neutralize the negative charge of microalgae, thus promoting flocculation.¹ Our present study demonstrates that algae flocculation can also be induced by increasing van der Waals attractions (see Figure 1 for schematic), albeit to a lesser degree of commercial feasibility compared to the approach of reducing the electrostatic repulsion. Nevertheless, understanding the surface free energy of microalgal cells can help predict and manipulate the flocculation and sedimentation behavior of microalgal cells, thus optimizing microalgae cultivation and harvesting techniques, reducing energy cost, and increasing environmental sustainability of microalgae production.

CONCLUSION

We have developed a novel easy-to-use spectrophotometric method for directly determining the surface free energy of microalgal cells. This method is a modified sedimentation technique based on the DLVO theory of colloidal stability, aggregation, and sedimentation. In comparison with the original sedimentation method, the spectrophotometric method is much quicker, more sensitive, and comparably accurate. We have shown that this spectrophotometric method can effectively differentiate the surface free energy of various microalgal strains. With combined advantages of high-throughput and simplicity, this

method has the potential to evolve into a standard method for measuring surface free energy of cells and abiotic particles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic of the experimental principles of the sedimentation method. Colloidal stability of a particle suspension is determined by the balance between the electrostatic repulsion and van der Waals attraction, as predicted by the DLVO theory. Dispersing particles in a liquid of surface tension similar to the surface free energy of the particles minimizes the van der Waals attraction between particles across the suspending liquid, thus resulting in a minimum amount of particle sedimentation. Since the amount of sedimentation is inversely related to the amount of free particles suspended in the supernatant, a maximum light absorbance should coincide with the minimum sedimentation volume/mass. Therefore, the surface tension of the liquid that results in a minimum sedimentation volume/mass or a maximum optical density (OD) should be close to the surface free energy of the particles.

Figure 2.

Proof-of-principle: determination of the surface free energy of polystyrene microbeads. (a) Sedimentation mass as a function of time after adding the polystyrene microbeads into suspending liquids of surface tensions 28.0, 33.6, and 39.6 mJ/m^2 , respectively. (b) Optical density (OD) as a function of time in these three suspending liquids. (c) Particle size distributions in these three suspending liquids. (d) Sedimentation mass and OD as a function of the surface tensions of a series of suspending liquids. After peak fitting, both the locations of the minimum sedimentation mass and the maximum OD consistently indicate that the surface free energy of the polystyrene microbeads is around 32–33 mJ/m^2 , which is in good agreement with the literature values.

Figure 3.

Determination of the surface free energy of various microalgal strains using both the spectrophotometric and the sedimentation mass methods: (a) marine *Chlorella* sp.; (b) marine *Nannochloris oculata*; (c) freshwater autotrophic *Chlorella* sp.; and (d) freshwater heterotrophic *Chlorella* sp. Both methods consistently determine the surface free energy of these four microalgal cells to be approximately 35, 41, 43, and 53 mJ/m², respectively.

Table 1

Characterization of Microalgal Cells with Their Primary Diameter (D_P) Determined by SEM, Hydrodynamic Diameter (D_H) Determined by a Particle Sizer, and Zeta Potential (ζ)

Algal strain	Marine <i>Chlorella</i> sp.	Marine <i>Nannochloris oculata</i>	Freshwater autotrophic <i>Chlorella</i> sp.	Freshwater heterotrophic <i>Chlorella</i> sp.
SEM				
D_P (μm)	3.2 ± 0.8	3.1 ± 0.9	2.8 ± 0.7	2.9 ± 0.8
D_H (μm)	4.3 ± 1.0	4.5 ± 0.9	3.8 ± 0.8	5.1 ± 0.9
ζ (mV)	-42.8 ± 3.8	-32.8 ± 4.1	-15.2 ± 1.3	-14.8 ± 2.1