Understanding the Growth and Attachment of Algae on Nanocomposites

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Abstract
The goal of this research is to understand the attachment and growth of algae on nanocomposites that are made from polylactic acid (PLA) and cellulosic materials. In nature, multiple types of algae can grow together on rocks and other surfaces, but the details governing algae attachment and growth are not fully understood. Commercially, cultivated algae can be used for fuel, nutrient supplements, and for pollutant removal. However, different types of algae are preferred for different applications. By better understanding the growth and attachment of algae on different materials, specific substrata could be designed for increasing the yield of specific types of algae. Therefore, a better understanding of the algae attachment and growth would improve the economic viability of algae cultivation for specific applications.

Introduction
Due to the increased media attention on climate change and the negative impacts some fossil fuel processes may have on the environment, many petrochemical companies are investigating alternatives to fossil fuels. For example, the United States is shifting its focus toward the use of biofuels, and plans to replace 20% of fossil fuels with biofuels by 2030.[1] One reason biofuels are of interest for replacing fossil fuels are their lower CO_2 emissions. Algae can have high oil content, making them a promising alternative biofuel that can potentially yield more fuel per unit area than other biofuels.[2] An additional advantage of algae-derived biofuels is that algae can grow rapidly in extreme environments and require less total crop area than is currently used by other biofuel crops.[3]

The high oil content of algae makes it a beneficial nutrient source as well. Fish oil is a popular dietary supplement known for having beneficial omega-3 fatty acids, but current overfishing problems have companies looking for an alternative.[4] Some algae are known to contain those same omega-3 fatty acids in addition to omega-6, vitamin C, vitamin K, and absorbed marine minerals such as iodine, potassium, calcium, and magnesium.[5] Also, the higher concentration of omega-3's in algae means the recommended dosage is less than that of fish oils.

However, the potential benefits of algae for biofuels and nutraceuticals are not being fully realized because of high production costs. The current cost of algae as a biofuel is higher than other biofuel crops. This is largely because of the high cost of harvesting and separating suspended algae from the water in which they grow; this can account for up to 21% of capital costs.[5] Alternative methods of cultivation, where algae is cultivated attached to material surfaces, promises to lower harvesting and separation costs because of operational advantages.[6] In addition, chemical design of material surfaces may allow advantageous selection for desirable algal species, potentially increasing overall yield of bioproducts.[7]

This research explored the attachment of a model planktonic algae, *Scenedesmus dimorphus*, to bio-derived nanocomposites made from polylactic acid (PLA), sulfonated cellulose nanocrystals (SA-CNC), and lignin-coated cellulose nanocrystals (L-CNC). *Scenedesmus dimorphus* was chosen because even though it prefers to be free floating, it can attach to surfaces and is commonly used in investigations of algae attachment on substrates and in studies of biofilm formation.[1,8] PLA was chosen because it is bioderived and biodegradable, and the two types of CNC were chosen because they are also bioderived and have markedly different surface chemistries. The algae were cultivated in flasks in a laboratory, and a method for measuring algae concentration based on UV-vis spectroscopy was developed. Next, the effects of the surface composition on contact angle and algae attachment were measured. The results of this research provided insights into the attachment of *Scenedesmus dimorphus* on the nanocomposites and established protocols that can be applied to more complex types and mixtures of algae.

Materials and Methods

Algal Culture: The *Scenedesmus dimorphus* culture was contained in a closed 1000 mL Erlenmeyer flask and kept in constant motion on a stir plate at ambient conditions. The algae culture was fed Bold Modified Basal Freshwater Nutrient Solution 50 x (Sigma-Aldrich) once every other week. The attachment testing requires a measured concentration of algae, so the concentration of the overall culture must be known throughout the experiment. The concentration of algae was initially determined using a hemocytometer. A micropipette was used to take 10 µL of the algae culture and inject it into one side of the hemocytometer. A Nikon Eclipse 80i microscope was used to observe the hemocytometer, and the individual algae cells were counted and recorded. This was repeated on the other side of the hemocytometer; then the counted algae cells were used to calculate the concentration of the algae culture. The hemocytometer method requires cells to be individually counted via microscopy, and becomes more inaccurate the larger the algae concentration. Therefore, a UV-vis spectroscopy calibration curve was developed for calculating the algae concentration. A culture with 5.6 x 10^6 cells/mL based on the average of five hemocytometer counts was diluted to 1:1, 1:1.5, 1:2, 1:2.5, 1:3, 1:3.5, 1:4, 1:4.5, and 1:5 algae to water ratios. The diluted samples were put into 10-mm pathway cuvettes and the absorbance of each sample was obtained at a wavelength of 600 nm on a ThermoFisher NanoDrop spectrophotometer.

Substrate Preparation: Nanocomposite substrates were produced using a previously established procedure.[9] A 3 wt% cellulose nanocrystal (CNC) dispersion was created by dispersing either SA-CNC (University of Maine, Orono, Maine) or lignin-coated CNC (Amercian Process Inc., Atlanta, Georgia) into deionized water. The dispersion was then sprayed over liquid nitrogen and freeze-dried to create powdered CNC. This powder was then placed in a ThermoFisher Haake MiniLab compounder along with PLA pellets and allowed to mix at 190 °C and 60 rpm for 20 min to make 8 wt% composites of CNC and PLA. The composite was then extruded into 2-mm filaments. The filaments were then heat pressed into films using a home use electric heat press (Brentwood Appliances) with nonstick surface at medium to high heat. For the plain PLA samples, the PLA pellets were directly heat pressed to create the samples.

Contact Angle Measurements: After the samples were created, their surface energy properties were investigated using a standard goniometer (ramé-hart Instrument Co., Mountain Lakes, New Jersey) and three probe fluids (water, ethylene glycol, and hexadecane). The surface of the material was first cleaned with ethanol and then rinsed using deionized water. Then a drop of the probe fluid was placed on the surface and the contact angle was measured every half second for 15 seconds for a total of 30 measurements per composite sample.

Attachment studies: An attachment test was developed based on modifications to the procedure used in previous literature.[3] A 1000-mL beaker was filled with a 750-mL algae solution with a concentration approximately 100,000 cells/mL. The composites and plain PLA were cut into 1.5 cm x 1.5 cm samples. These samples were each placed in the bottom of the 1000-mL algae-solution beaker, using one sample per type. The beaker was then placed in a dark environment for 24 hr to allow the cells to settle on the surface. The samples were then removed from the beaker and washed on an orbital mixer to remove any unattached or loosely attached cells. The test was repeated for four times. Each composite was analyzed by taking 10 images across the sample using reflected microscopy and a 20X/0.45 LU Plan Fluor objective using a Nikon 80i microscope with Nikon Elements software. The number of algae cells in each 85,300 µm² image were counted; a total of 40 images per substrate were acquired for analysis (with the exception of PLA/L-CNC for which 29 images were analyzed).

Results and Discussion

Figure 1 shows that the absorbance of the algae was linearly related to concentration in accordance with the Beer-Lambert Law, $A= \varepsilon bc$, where A is absorbance, ε is the extinction coefficient, b is the path length, and c is concentration. Based on the slope of Figure 1, $\varepsilon = 3 \times 10^{-7}$ mL (number of cells)$^{-1}$ cm^{-1} for *Scenedesmus dimorphus*. The development of this spectroscopy-based method for concentration enabled faster determination of the algae concentration than counting with a hemocytometer.

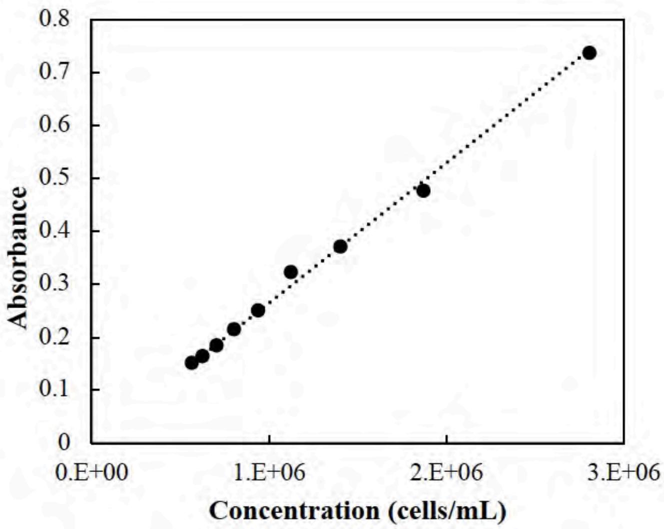

Figure 1. Plot of absorbance versus algae concentration. Equation: $y = 3.0 \times 10^{-7}$; $R^2 = 1.0$

Contact angle measurements were used to obtain information needed for use in the extended Derjaguin-Landau-Verwey-Overbeek (xDLVO) thermodynamic model for predicting the interactions of the substrate and algae cells. This model is commonly used in colloid science to understand how particles interact with each other or surfaces as they approach one another. It has also been used to understand the interaction of *Scenedesmus dimorphus* with some polymeric surfaces[10] but not the nanocomposites used in this research. As described in the literature,[10] the model considers the total Gibbs free energy of interaction as the sum of three components:

$$G^{Tot} = G^{vdW} + G^{AB} + G^{EL}$$

where G^{vdW} is the interaction energy due to van der Waals forces, G^{AB} is the energy due to acid-base interactions, and G^{EL} is the energy due to electrostatic interactions.

Measurements of the contact angle show different values for different materials, across all probe liquids (Table 1). Compared to plain PLA, the contact angles changed significantly with the addition of different types of CNC, where PLA/SA-CNC had a lower contact angle and PLA/L-CNC had a higher contact angle (Table 1). These changes suggest that the incorporation of SA-CNC and L-CNC changes the surface chemistry of the PLA. For example, the measurements using water showed that adding the SA-CNC into PLA made the substrates slightly more hydrophilic, whereas adding the lignin-coated CNC (L-CNC) made the samples more hydrophobic. It is expected that these changes could affect surface thermodynamics that affect algae deposition and attachment.

Values from the contact-angle measurements were used in the xDLVO model to predict the interaction energy between *Scenedesmus dimorphus* and the nanocomposite substrates (Figure 2). A negative G_{Total} value indicates attraction, suggesting the attachment of algal cells to the material. Although the model did not display any primary minima which would indicate strong attraction, the energy profiles showed shallow

Table 1. Contact angle measurements of samples with three probe liquids (errors are standard deviation n=30).

	Water	Ethylene Glycol	Hexadecane
PLA	78.5° +/- 0.1°	56.0° +/- 0.1°	18.6° +/- 0.1°
PLA/SA-CNC	77.3° +/- 0.1°	52.7° +/- 0.1°	17.1° +/- 0.2°
PLA/L-CNC	82.5° +/- 0.1°	58.8° +/- 2.3°	22.0° +/- 1.8°

secondary minima with negative G_{Total} values. These minima are highlighted in Figure 2b; in accordance with colloid thermodynamic theory, they are indicative of attraction between the algae and surface which could result in loosely attached algae on the surface.

The results of the model were compared to the experimental attachment tests. Figure 3a shows the average number of cells per area for each composite resulting from attachment tests. These results show differences in number of attached algal cells among the materials, with average values of 313 cells for PLA, 265 cells for PLA/SA-CNC, and 246 for PLA/L-CNC per 85,300 μm² of surface area. These results were obtained by repeating the attachment experiment four times and analyzing the total number of 40 microscope images per material (with the exception of PLA/L-CNC, for which 29 images were analyzed). The error bars displayed on plot 3a represent the standard deviation value. Additional statistical tests show that differences in the number of cells attached were significantly lower for composites with CNC compared to plain PLA (Figure 3b). This is in agreement with the model results, which also suggested that PLA was more favorable for attachment than PLA/L-CNC. However, the model predicted only negligible difference between the PLA and PLA/SA-CNC. This discrepancy may be due to the significant error in the measurements. One source of error was that the color and opacity of the composites made it difficult to visualize the algae and precluded the use of standard image analysis software. Therefore, the images had to be manually counted; this introduced human error. Another source of error was uneven settling of algae onto the surfaces during the attachment tests. These sources of error are expected to be mitigated by refinement of methods in future experiments.

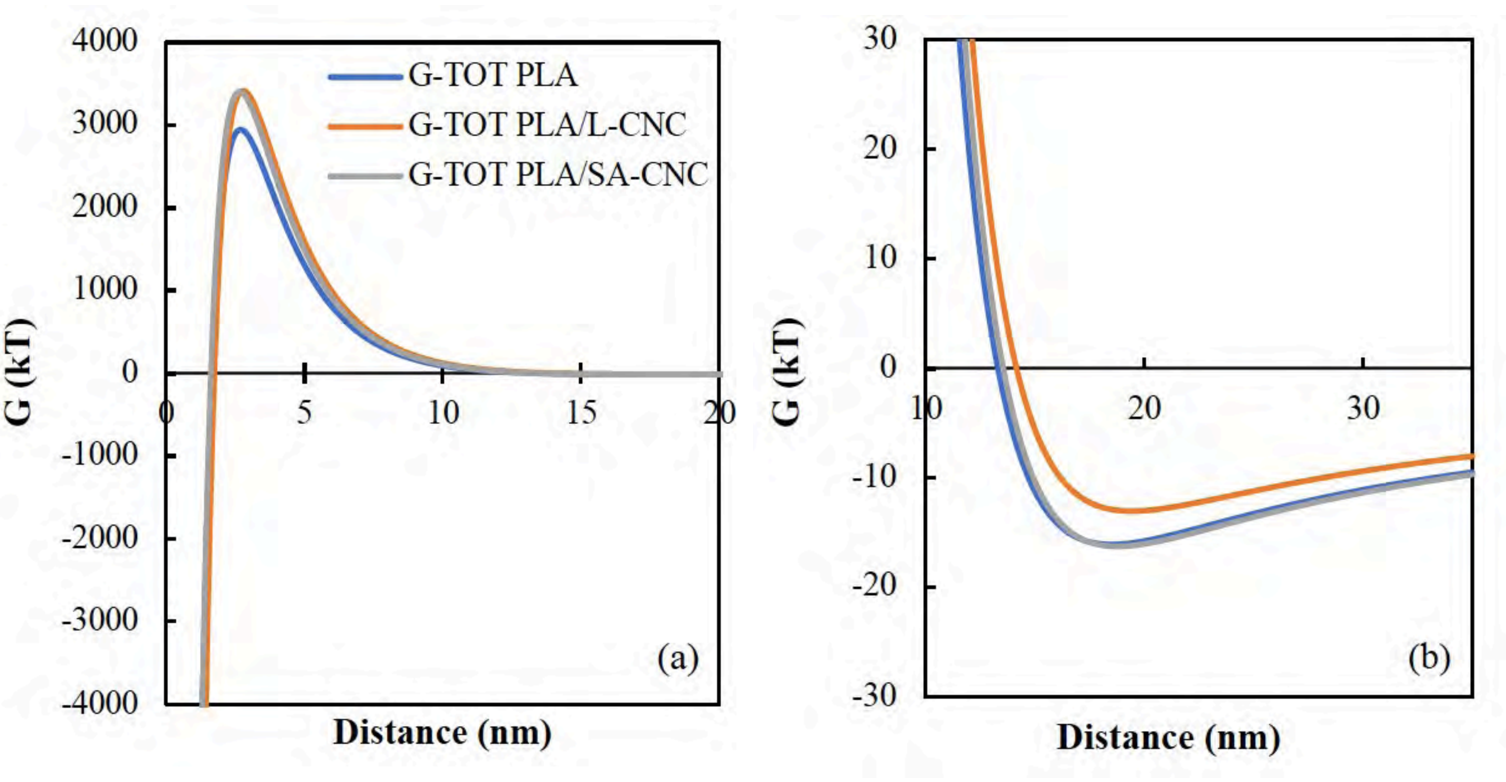

Figure 2. (a)Total interaction energy of samples. (b) Secondary energy minimum from Figure 2a.

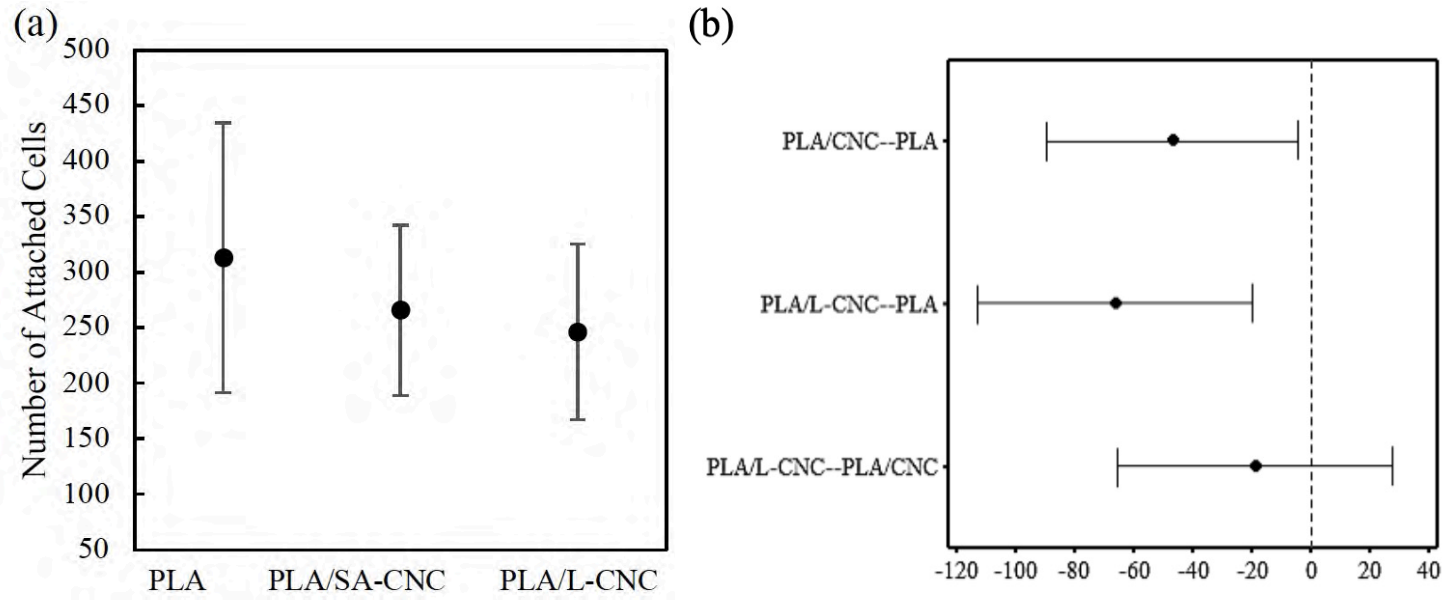

Figure 3. (a) Number of algae cells per image for composites; error bars represent standard deviation.(b) Fisher's statistical significance test for sample pairs; if an interval does not contain zero, the corresponding means are significantly different.

Conclusions

This research developed a rapid spectroscopy-based method for determining the concentration of *Scendesmus dimorphus*, a commonly studied planktonic algae in growing cultures. It also showed that the surface chemistry of a bioderived polymer, PLA, could be modified by the incorporation of hydrophilic SA-CNC or hydrophobic L-CNC. Both thermodynamic modeling and experimental results showed that differences in surface chemistry through CNC addition resulted in differences in the attachment of the model planktonic algae. The protocols established in this l research can be further refined to study substrates with a greater range of surface chemistries, such as additiona PLA nanocomposites or other materials. They can also be extended to the investigation of other algae including industrially relevant attached filamentous algae.

Acknowledgments

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