

## MOLAR MASS DETERMINATION OF THE POLYSACCHARIDE ASSOCIATED WITH COCCOLITHS OF *EMILIANIA HUXLEYI*

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**Abstract**—*Emiliana huxleyi* is a marine alga which produces coccoliths, elaborate calcified structures consisting of  $\text{CaCO}_3$  and a water-soluble acidic polysaccharide. The polysaccharide was subjected to light scattering measurements to determine the weight-average molar mass ( $\bar{M}_w$ ) and osmometry to assess the number-average molar mass ( $\bar{M}_n$ ). Values of  $\bar{M}_w$  of  $88,600 \pm 3200$  g/mol and of  $\bar{M}_n$  of  $49,100 \pm 1500$  g/mol were found. These experiments were performed with a polysaccharide mainly in the  $\text{Na}^+$  form. Replacement of  $\text{Na}^+$  by  $\text{Ca}^{2+}$  did not significantly affect the weight-average molar mass or the second virial coefficient. The values of the refractive index increment of these salts were different  $[(dn/dc)\text{Na}^+] = 0.145 \pm 0.004$  ml/g;  $(dn/dc)\text{Ca}^{2+} = 0.090 \pm 0.002$  ml/g, indicating a possible conformational change in the molecule.

### INTRODUCTION

Cells of the unicellular alga *Emiliana huxleyi* (Lohmann) Hay and Mohler are surrounded by a shell of about 15 intertwined delicate structures of  $\text{CaCO}_3$ , called coccoliths. The coccoliths are produced in a specialized intracellular vesicle and the component crystallites are precisely defined in size, shape and crystallographic orientation [1]. Much work has been devoted to the characterization of the cellular factors which play a role in the production of these complicated biominerals [2]. One of these factors appears to be an acidic polysaccharide which is closely associated with the mineral phase [3,4]. This macromolecule binds  $\text{Ca}^{2+}$  ions preferentially in the presence of  $\text{Na}^+$  and  $\text{Mg}^{2+}$  [5]. Moreover, it has a profound influence on the *in vitro* crystallization of  $\text{Ca}^{2+}$  salts (Kok and Westbroek, unpublished) [6]. The polysaccharide is not only associated with extracellular coccoliths, but it can also be detected in the intracellular calcifying vesicle and the Golgi region (van Emberg, unpublished) [7,8].

Structural and biochemical analysis of the polysaccharide is a necessity to understand the functional role of this macromolecule in coccolith biosynthesis. A preliminary structure of the mean repeating unit has been published [9].

Here we report on the determination of the weight-average molar mass in the absence and presence of  $\text{Ca}^{2+}$  by light scattering and the number-average molar mass by osmometry.

### EXPERIMENTAL

#### Isolation of polysaccharide

The isolation of coccoliths and the associated polysaccharide from calcifying cells of *Emiliana huxleyi* (strain L, obtained from Dr E. Paasche, Oslo) has been described previously [5].

#### Measurement of the refractive index increment

The refractive index (RI) increment of the polysaccharide was determined with a Chromatix KMX-16 laser differential refractometer as described by Mandel *et al.* [10]. Measurements were performed at room temperature and values for RI were determined with respect to pure water.

#### Light scattering

For the light scattering experiments, the coccolith associated polysaccharide was dissolved in 0.1 M NaCl. All dilutions were made with a NaCl solution with the same ionic strength. Samples of the polysaccharide were filtered through 0.22  $\mu\text{m}$  millipore filters to remove dust from the solutions. During filtration a small fraction of the polysaccharide was adsorbed on the filters. Therefore the concentration of polysaccharide in the test solutions was measured afterwards with two colorimetric assays (see below). Low angle laser light scattering measurements were performed at room temperature with the Chromatix KMX-6, equipped with a Ne-He laser ( $\lambda = 633$  nm). The scattered light was measured at an angle of  $6.7^\circ$ .

#### Osmometry

Osmometry measurements were performed on a Knauer membrane osmometer with a Sartorius Ultrafilter (cellulose acetate, type SM 117-36) at  $20^\circ$ . Polysaccharide samples were dissolved in 0.1 M NaCl and filtered through Amicon YM-10 filters. Measurement of the osmotic pressure of this preparation vs 0.1 M NaCl indicated that it contained some material able to permeate the membrane of the osmometer. Therefore the polysaccharide solutions were also filtered through Amicon XM-50 filters. After this filtration step, the osmotic equilibrium was reached immediately. The filtrate contained no polysaccharide material as determined with polyacrylamide gel electrophoresis, indicating that the retarded equilibrium mentioned above was caused by the presence of additional salt molecules.

#### Analytical assays

The polysaccharide concentrations were determined with an indole test for total carbohydrate [11] and carbazole test for total uronic acid [12]. A stock solution of polysaccharide with a known concentration was used as a standard.

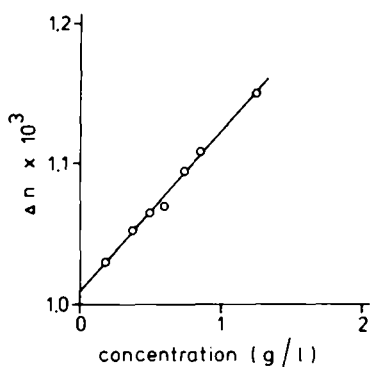


Fig. 1. Specific refractive index (RI) increment of the coccolith polysaccharide. The RI of polysaccharide solutions in 0.1 M NaCl were measured with respect to pure water at a wavelength of 633 nm at 20°. For further experimental details see Experimental section.

### RESULTS

Two physico-chemical techniques have been used to determine the molar mass of the polysaccharide, viz. low angle laser light scattering and osmometry. The weight-average molar mass ( $\bar{M}_w$ ) can be assessed using the first technique, by application of equation 1

$$\frac{Kc}{\Delta R_\theta} = \frac{1}{\bar{M}_w} + 2A_2c + 3Bc^2 + \dots \quad (1)$$

where  $c$  is the polysaccharide concentration (g/l),  $\Delta R_\theta$  is the difference in the Rayleigh numbers of solutions with and without polysaccharide,  $A_2$  is the second virial coefficient,  $B$  the third one, etc. The constant  $K$  is defined as:

$$K = \frac{2\pi^2 n}{\lambda^4 N_A} \cdot (dn/dc)^2 \cdot (1 + \cos^2 \theta).$$

Here  $n$  is the refractive index (RI) of the solvent,  $\lambda$  the wavelength of the incident light (633 nm),  $N_A$  Avogadro's number,  $\theta$  the angle under which the scattered light is measured and  $dn/dc$  is the RI increment of the polysaccharide solution. The latter was determined using differential refractometry. In Fig. 1 an example of a plot of  $n$  vs  $c$  is shown. The resulting value of  $dn/dc$  ( $0.115 \pm 0.003$  ml/g) was derived from the slope of the graph.

The result of a typical scattering experiment is shown in Fig. 2, where  $Kc/R_\theta$  is plotted vs  $c$ . The values of  $Kc/R_\theta$  obtained in the concentration range used justified a linear adaptation of equation 1.

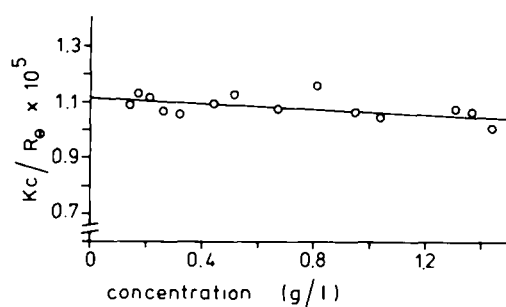


Fig. 2. Low angle laser light scattering of the coccolith polysaccharide in 0.1 M NaCl. For explanation of symbols see text and for further experimental details see Experimental section.

Consequently a molar mass of  $87,000 \pm 2700$  g/mol was calculated from the intercept of the plot. The second virial coefficient resulting from the slope was found to be  $-2.7 \pm 0.7 \times 10^{-7}$  ml mol g<sup>-2</sup>. This value (close to zero) indicates near theta conditions, conditions in which interactions between polysaccharide molecules ( $A_2$  negative) are not preferred over interactions between the polysaccharide and the solvent molecules ( $A_2$  positive) [13].

The coccolith polysaccharide has a high affinity for  $\text{Ca}^{2+}$  ions [5]. Therefore the effect of addition of  $\text{CaCl}_2$  to the polysaccharide solution on the RI increment, the second virial coefficient and the molar mass was investigated. Because the polysaccharide isolated by the standard procedure as described by de Jong *et al.* [5] contains a small amount of  $\text{Ca}^{2+}$  apart from  $\text{Na}^+$  [6], these parameters were also determined for polysaccharide extensively dialyzed against EGTA, prior to analysis. The results are summarized in Table 1. The RI increment of the polysaccharide in the presence of  $\text{Ca}^{2+}$  was significantly smaller than that of the polysaccharide isolated according to the standard procedure, whereas that of the dialyzed polysaccharide showed a remarkable increase. Addition of  $\text{Ca}^{2+}$  did not significantly affect the molar mass nor the second virial coefficient of the polysaccharide. Both parameters seemed to be slightly increased after extensive dialysis of the polysaccharide. It should be noted, however, that the standard deviations in these parameters, shown in Table 1, do not include the error of about 3% in the RI increment. Therefore, the molar masses and second virial coefficients of all polysaccharide preparations can be considered to be equal within standard deviation. The increase in molar mass due

Table 1. Physico-chemical parameters of the coccolith associated polysaccharide

Treatment	$dn/dc$ (ml/g)	Molar mass (g/mol)	$A_2$ (ml mol g <sup>-2</sup> )
None*	$0.115 \pm 0.003$	$87,000 \pm 2700$	$-2.7 \pm 0.7 \times 10^{-7}$
10 mM $\text{CaCl}_2$ †	$0.090 \pm 0.002$	$86,600 \pm 2700$	$-3.1 \pm 1.5 \times 10^{-7}$
EGTA‡	$0.145 \pm 0.004$	$92,300 \pm 2800$	$-7.3 \pm 3.0 \times 10^{-7}$

\*Isolation of polysaccharide by dissolving the  $\text{CaCO}_3$  of the coccoliths with 10% EDTA and dialysis on an Amicon YM-10 filter (standard procedure).

†Isolation of polysaccharide as in \* with addition of 10 mM  $\text{CaCl}_2$ .

‡Isolation of polysaccharide as in \* followed by extensive dialysis against EGTA to remove all  $\text{Ca}^{2+}$  ions.

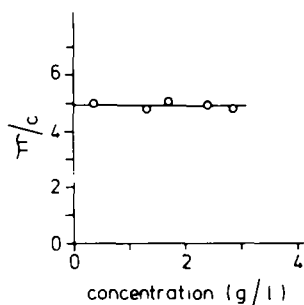


Fig. 3. Osmotic pressure data for the coccolith polysaccharide in 0.1 M NaCl. For further experimental details see Experimental section.

to replacement of  $\text{Na}^+$  by  $\text{Ca}^{2+}$  may amount to 3%, which will not be detected considering the error in the measurements.

The number-average molar mass ( $\overline{M}_n$ ) of the polysaccharide isolated according to the standard procedure was determined with osmometry. The result of a typical osmometry experiment is shown in Fig. 3, where  $\pi/c$  (the osmotic pressure in mm Hg divided by the polysaccharide concentration in g/l) is plotted vs  $c$ . In this experiment,  $\overline{M}_n$  could be calculated using a linear adaptation of equation 2,

$$\frac{\pi}{c} = R T \left( \frac{1}{\overline{M}_n} + A_2 c + B c^2 + \dots \right) \quad (2)$$

where  $A_2$  is the second virial coefficient,  $B$  the third one, etc.  $\overline{M}_n$  was found to be  $49,100 \pm 1500$  g/mol and  $A_2 = 8.4 \pm 1.0 \times 10^{-7}$  ml mol g<sup>-2</sup>, this last value in fair agreement with that found from light scattering experiments (cf. Table 1).

#### DISCUSSION

In this paper we have determined  $\overline{M}_w$  and  $\overline{M}_n$  of the polysaccharide associated with coccoliths of *Emiliania huxleyi* with light scattering and osmometry, respectively. The values of  $\overline{M}_w$  (88,600 g/mol, cf. Table 1) and  $\overline{M}_n$  (49,100 g/mol) result in a  $\overline{M}_w/\overline{M}_n$  ratio of about 1.8, indicating a "normal" distribution of molar mass for this natural polysaccharide [14]. It is generally agreed that the biosynthesis of polysaccharides is not matrix mediated like protein and nucleic acid synthesis. Consequently, the absence of specific termination signals will result in a variation in molar mass.

Addition of  $\text{Ca}^{2+}$  ions in excess to the  $\text{Ca}^{2+}$ -binding sites of the polysaccharide did not

affect the molar mass of the latter. Apparently no  $\text{Ca}^{2+}$  bridges between different polysaccharide molecules were formed at this  $\text{Ca}^{2+}$  concentration.

The marked difference in the RI increment of the  $\text{Na}^+$  and  $\text{Ca}^{2+}$  salt of the polysaccharide (0.145 and 0.090 ml/g, respectively) may indicate a conformational change of the individual macromolecules upon addition of  $\text{Ca}^{2+}$  ions. This observation is supported by preliminary sedimentation studies in our laboratory. The sedimentation rate of the  $\text{Ca}^{2+}$  salt was higher than that of the  $\text{Na}^+$  salt, indicating a more compact folding of the former (Borman, unpublished). In view of the proposed function role of the polysaccharide in  $\text{CaCO}_3$  crystallization, this phenomenon deserves more detailed investigation. With angle dependent light scattering for instance, the radius of gyration may be evaluated, permitting speculations about the conformation of the macromolecule in solution.

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