

CHARACTERIZATION OF BIOGENIC CALCITE PARTICLES FROM MICROALGAE – COCCOLITHS AS A POTENTIAL RAW MATERIAL FOR INDUSTRIAL APPLICATION

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Abstract. The coralline algae (*Coccolithophores*) *Emiliania Huxleyi* belongs to the phytoplankton and is one of the most important lime stone producers in the oceans. It produces about 2-5 μm sized calcite particles (Coccolithes), which cover the cell surface completely. Coccolithes are thanks to their structure very interesting for industrial application as lime stone micro-particles e.g. for the production of specialty papers or for medical applications. Biomineralization in algae is a complex process running in the cell interior but is influenced by many environmental factors. Rational design led to a bio-process which allowed to produce and purify larger amounts of intact Coccolithes for the first time. The challenge was to supply the cells with the necessary amounts and concentrations of calcium and inorganic carbon for calcification. This could be accomplished by pH- and pCO_2 -control and balanced Ca-feeding. First results from particle characterization will give a first glance on possible application features of these fascinating structures.

Keywords: Coccolith production, biogenic lime products, bio-inspired materials, *Emiliania huxleyi*

Introduction

Calcite (CaCO_3) particles exhibit versatile and unique properties and are therefore suitable for numerous applications. The majority of industrial calcite is directly derived from mined limestone, which is subsequently crushed (GCC = ground calcium carbonate). Besides GCC, synthetic calcite particles (PCC = precipitated calcium carbonate) are produced by controlled introduction of CO_2 into lime milk, leading to calcite precipitation. This allows for production of ultrafine calcite particles down to the nanometer scale. GCC and PCC particles differ mainly in particle shape, powder density, specific surface area and absorption properties [1]. Since PCCs are produced from high-purity source materials, they are characterized by a high degree of purity, whiteness and opacity. By varying the process parameters (temperature, pressure, concentration, reaction time) or by adding additives, it is possible to influence the crystal morphology, for example, to create cubic or acicular particles [2–5]. Calcite production from limestone deposits, originating from the remains of fossil marine organisms, follows a long tradition. However, while there is an increasing interest in innovative and inimitable products, the three-dimensional complexity of GCC and PCC products is limited. In this regard, the idea was formed to produce calcite particles *de novo* from cultured microalgae and to evaluate their potential for application.

This biogenic calcite differs from fossil calcite mainly by its exceptionally sophisticated three-dimensional structure, which is unknown for synthetically produced particles. Another interesting

difference is the presence of organic components. These components form an organic matrix, which is embedded in the calcite structure and which are known to control the crystal formation during the biomineralization process [6]. Furthermore, organic components can be found on the particle surface [7]. The best-known species which naturally produces calcite is the unicellular red alga *Emiliania huxleyi* (*E. hux.*), which belongs to the group of coccolithophores. It is found in almost every ocean, where it represents a substantial part of the phytoplankton. *E. hux.* produces calcite particles in the form of small, micro-structured platelets, so-called coccoliths, which cover the cell surface. A coccolith consists of two elliptical, disc-like, grooved shields, which are connected by a central tube [8]. 10-15 Coccoliths form a complete coccolith cover, which is referred to as coccosphere. The unique structure of *E. hux.* coccoliths derives from controlled crystal growth inside the cell and cannot be reproduced by comminution or precipitation of calcite.

It is known, that coccoliths possess certain interesting chemical and physical properties. For example, coccoliths are more robust in calcium-free saline solutions and pure water as comparable inorganic calcite particles [9]. Specialized solid state NMR technologies demonstrated the presence of P- and N-residues, which are internally integrated into the coccolith crystals [10]. Despite the incorporation of these residues into the dense crystal lattice, there is no detectable disruption of the chemical or electronic structure. The only measurable effect is a slight decrease in crystal rigidity. Illumination of coccoliths from the side leads to a dynamic change

in structural color when, at the same time, a magnetic field of 0T and 5T is applied [11]. This effect is probably caused by an alteration of the inclination angle. Furthermore, it has been suggested that ethanol can build highly organized structures on the coccolith surface [12].

Despite several studies about coccoliths properties, little is suggested about their potential applicability. However, to perform such an evaluation, an adequate quantity of homogeneous, intact coccoliths is necessary. These can be obtained from cultivation [13] but must be purified from all contaminants like cell debris and salts, prior to investigation. This article presents the results of an application-oriented study, which uses industrial standard procedures for a first assessment of the industrial potential of these highly interesting particles.

Material and Methods

Purification of coccoliths

Coccoliths of strain *E.hux.* RCC 1216 were obtained from the Institute of Process Engineering in Life-Sciences, Section III Bioprocess Engineering (Karlsruhe University), in form of culture broth in 5 L glass flasks. The coccolith concentration in the culture was approximately $1.5 \text{ g} \cdot \text{L}^{-1}$. For basic cell disruption, the culture broth was placed inside an incubator at 80°C for at least 48 hours. During incubation time, coccoliths and bigger cell fragments settled down to the vessel bottom. About 90% of the supernatant was subsequently removed by using a peristaltic pump (Ismatec, IPS-16). The remaining supernatant was suspended with the coccolith sediment, divided into 40 ml units and filled into 50 ml reaction tubes. The suspensions were centrifuged at $8.000 \cdot g$ for 10 minutes at 4°C (Hettich, Rotina 420R). The supernatant was then carefully removed with a pipette right until the 10 ml marking. The pellet was resuspended with the remaining supernatant by using a vortex. For further disruption and removal of cell fragments 3.3 mL of 12% NaOCl was mixed into the suspension and incubated for 15 minutes. The reaction tube was subsequently filled up with a 6 mM NaHCO_3 solution up to the 40 ml marking.

The washing procedure was initiated with a centrifugation step at $1.500 \cdot g$ for 6 minutes at 4°C . The supernatant was subsequently removed until the 10 ml mark. The coccoliths were resuspended and subsequently filled up with fresh 6mM NaHCO_3 solution. The washing procedure was repeated at least four times until the salinity in the suspension was decreased less than 0.2. The final suspension was checked with a light microscope (Zeiss, Axio Scope) to

ensure the absence of cell fragments and coccolith agglomerates. It was subsequently centrifuged at $1.500 \cdot g$ for 6 minutes at 4°C and the supernatant was completely removed with a pipette. The pellet was then dried at 80°C for at least 24 hours before analysis.

Coccolith analyses

The **white level determination** (compared to MgO) was conducted spectrophotometrically (Minolta, CM-3610d). The **proportion of organic material** and CaCO_3 was detected thermogravimetrically by using a simultaneous thermal analyzer (Perkin Elmer, TA 6000). The analysis was carried out under nitrogen atmosphere in the range between 40°C - 1000°C and a heating range of $20^\circ\text{C}/\text{min}$. BET-method was used for the determination of **specific surface area** (Quantachrome, Quadrasorb SI). **Pore size distribution** was measured with the same instrument by applying the nitrogen-adsorption-desorption-method (BJH method). Prior to analysis, samples were degassed for at least 24 hours at 130°C by using a flow degasser. **Sample density** was measured by using a gas pycnometer (Micromeritics Multivolume Pycnometer 1305) with helium. **Chemical composition** analyses were conducted by using X-ray fluorescence spectroscopy (Axios SST mAX, PANalytical). Melt compounds with lithiumtetraborate for measurements were prepared with a fusion machine (HAG 12/1500, Herzog). **Particle size distribution** was determined with laser diffraction (Sympatec, Laser diffraction sensor HELOS/KR). The sample was placed in an ultrasonic bath (0, 60, 120, 180 s) prior to the measure for 5 min to achieve distribution. 0.1% tetrasodium pyrophosphate decahydrate was used as the dispersion liquid. The results were evaluated by using the FREE Fraunhofer theory and a density of $2.91 \text{ g} \cdot \text{cm}^{-3}$. As a second method, sedigraphy (Micromeritics, 5100) was used to measure the **equivalence diameter** of the particles. 0.1% sodium polyphosphate solution was used for dispersion. For calculation of the particle equivalence diameter the theoretical density of calcite ($2.715 \text{ g} \cdot \text{cm}^{-3}$) was used. **Scanning electron microscopy pictures** were taken with a high voltage electron microscope (Zeiss, DSM 962) at 15kv. Samples were sputtered with a gold-palladium layer prior to measurement.

Results

Fig. 1 (A-B) shows scanning electron microscopy pictures of the purified coccoliths. Observation with lower magnification (A) shows, that the particles are

complete, homogeneous in overall size and shape and free of coccolith agglomerates. Higher magnification (B) reveals particle homogeneity regarding internal micro-structures within the upper distal shield elements and the central tube. The sophisticated crystal units in the central area of each coccolith and the double-disk form are sharp and clearly visible. The distal shield and the central tunnel element both exhibit gaps with a length of up to one micrometer. The particle edges appear smooth and undamaged, free of particle agglomerates and neither contaminants such as cell debris nor salt crystals were found in the suspension. The employed purification method was therefore able to provide efficient separation and cleaning of intact coccoliths from culture broth without causing mechanical damage.

Figure 1 (C-D) shows scalenoedrical PCC particles, which were exemplarily chosen as an example for synthetic calcite. The differences between the synthetic and biogenic particle species are evident. The chosen PCC particles exhibit a very broad size distribution, their basic form is similar, but rather simple. In comparison, coccoliths distinguish themselves by their substantially complex spatial structure, but are, at the same time, extremely uniform.

In order to confirm this visually observed uniformity given in the electron microscopy pictures, the coccoliths were further analyzed with laser diffraction and sedigraphy. Sedigraphy showed that equivalence diameter was mainly distributed between 0.7 and 1.4 μm . As can be seen in the cumulative distribution shown in table 2, 50% of the particles had a diameter smaller than 2.32 μm while 84% of the particles were equal or smaller than 4.02 μm . Figure 2 reveals that size distribution was considerably influenced by the duration of ultrasonication sample treatment. 60 seconds of ultrasound resulted in a shift towards smaller particle diameters while longer treatment time caused the opposite effect. Since the particles stayed entirely intact after treatment, it can be assumed that the shift was caused by different grades of dispersion. Efficient dispersion is a prerequisite for laser diffraction spectrometry. It was therefore assumed that ultrasonication for 60 seconds was most efficient in this case. Longer treatment time seemed to support wedging of single coccoliths, which resulted in the measurement of bigger particles.

Both methods demonstrated that coccoliths size was very narrowly distributed, although their great structural complexity. This result is not surprising, since coccolith synthesis is a strictly controlled

biological process. Variations in particle size are due to cell size, which is dependent on the cell cycle.

However, both methods fail to measure size distribution exactly. Depending on the orientation towards the laser and its point of contact (upper or lower distal shield, central tunnel), results can be influenced. In this regard, other techniques like computational image analyses are necessary to further analyze morphological particle homogeneity.

Besides the big grooves, which are part of the particle microstructure, the particle material itself exhibited porous characteristics with mesopores in the range of 4 nm and a total pore volume of 0.05 $\text{cm}^3\cdot\text{g}^{-1}$. The above values fall within the range observed for standard PCC particles, which are pictured in Fig. 1. The pore volume and the overall geometry resulted in a specific surface area of 19 $\text{m}^2\cdot\text{g}^{-1}$.

This presumption could be confirmed in the analyses of the material composition. Since coccoliths are biocomposites, the presence of organic material was anticipated before measurement, but it was not clear to which amount it would remain in and on the surface of the coccolith after purification, especially sodium hypochlorite treatment. Thermogravimetry showed that the proportion of organic material was approximately 4 %, whereas the coccolith was made up to 87 % of calcium carbonate. The remaining 9 % showed to be primarily Mg and Si residues. Other elements like Fe, Al, P, Mn and Sr were also found to a minor proportion (Table 4).

All in all, the presented results make clear that coccoliths do not simply consist of calcium carbonate and organic matter but exhibit a complex composition of various elements. This makes them unique not only on a structural but also on a chemical level.

When dried, coccoliths are a white, solid powder with a density of 2.6 $\text{g}\cdot\text{cm}^{-3}$. This is slightly lower than the density of pure calcite, which is 2.715 $\text{g}\cdot\text{cm}^{-3}$. A slight yellow shade, although hardly recognizable with the eye, was detected with color value analysis (Table 3). Both results indicate the presence of matter other than calcite in the coccolith material.

Discussion

In this study, we successfully analyzed coccoliths with standard methods from calcium carbonate particle industry for the first time. These results may give impulses for further application oriented research.

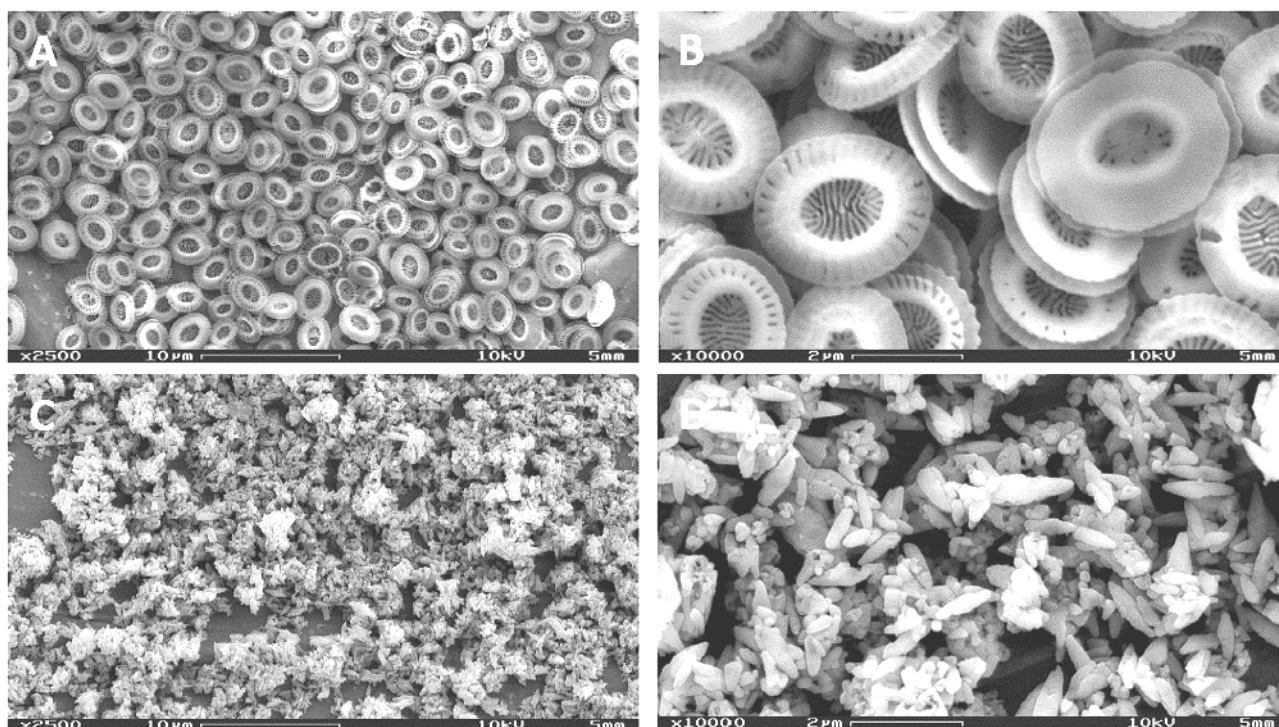


Fig. 1. A-B) Scanning electron microscopy pictures of purified coccoliths (Zeiss, DSM 962, 2500X (A) and 10000X (B)). (C-D) For comparison: synthetic scalenoedrical particles from calcite precipitation (2500X (C) and 10000X (D))

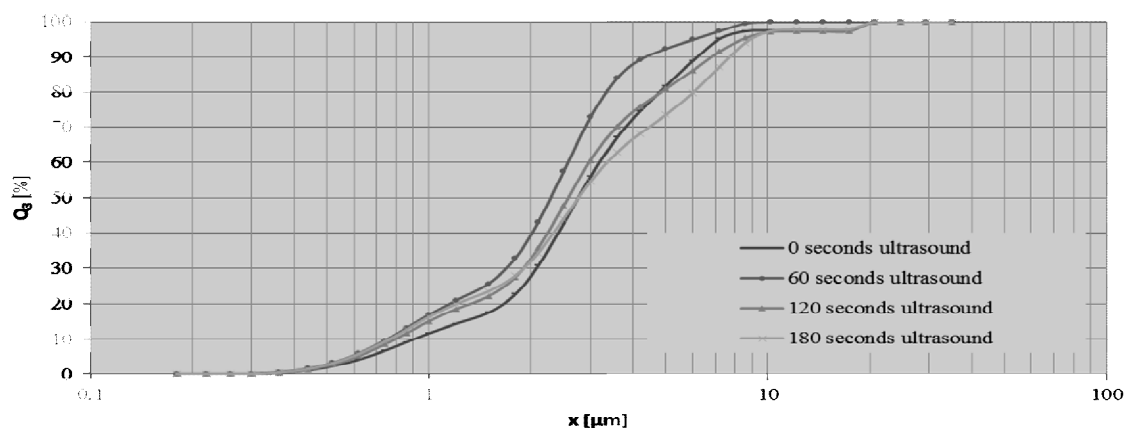


Fig. 2. Cumulative particle size distributions of coccoliths determined by laser diffraction spectrometry under different ultrasound intensities.

Table 1. Particle diameter distribution of coccoliths

Particle size diameter (laser diffraction spectrometry solvent = $\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$, 60s ultrasonication)		Equivalence particle diameter distribution (Sedigraph solvent = NPP)	
X_{84}	4.02 μm	d_{84}	1.4 μm
X_{50}	2.32 μm	d_{50}	0.9 μm
X_{16}	0.92 μm	d_{16}	0.7 μm
		<1 μm	59 %

Table 2. Summary of coccolith pore characteristics

Coccolith pore volume	
Total pore volume	0.05 cm ³ ·g ⁻¹
Most frequent pore diameter	3.7 nm
Specific surface area	19 m ² ·g ⁻¹

Table 3. Color value analysis

White-and color value analysis		
white value R ₄₅₇	relative white value related to MgO	91.1%
brightness L*	brightness	98.2
color value a*	(-)green to (+)red/brown	0.1
color value b*	(-)blue to (+)yellow	3.2
yellow value DIN 6167		6.0

Table 4. Chemical composition of coccoliths

Chemical analysis (glowed)						Thermogravimetry	
CaO	85.1%	K ₂ O	0.006%	P ₂ O ₅	0.392%	Organic material	4%
MgO	6.4%	TiO ₂	0.004%	Mn ₃ O ₄	0.038%	CaCO ₃	87%
SiO ₂	6.6%	Na ₂ O	0.222%	SO ₄	0.038%		
Fe ₂ O ₃	0.15%	SrO	0.606%	Σ	100%		
Al ₂ O ₃	0.37%	BaO	0.004%	(CaCO ₃ 86.0%)*			

*calculated value for CaCO₃.

Analyses revealed that coccoliths are porous with pores in the micrometer and nanometer range. In the future it will therefore be interesting to investigate their potential as substrate for paint- or laquer coat or filler in adhesives. A further field of use may lie in heterogeneous catalysis. Takano et al. already demonstrated the immobilization of enzymes on the coccolith surface over twenty years ago [14], but unfortunately, the idea was not further pursued.

Another topic which is very interesting is the chemical composition. In this study we demonstrated that coccoliths are more than just calcite particles. They are a composite material of organic matter, calcium carbonate and various other elements. All these elements are part of the cultivation medium, which approximates the composition of natural seawater. It is therefore comprehensible, that Si- and Mg- residues are the second most abundant elements besides calcium, because they are also present in the medium in relatively large stoichiometric quantities. On the other hand, we know that *E. hux.* can easily be cultivated in Si-free medium, with similar coccolith production rates (unpublished results). Hence, we

suggest that the elemental composition of coccoliths is influenceable by cultivation conditions. In the case of some elements like Si, this may result in a change of mechanical properties like abrasiveness. There are also elements which influence colour like iron. A reduction of iron could increase the net white value of the particles and may make them more suitable for the use as pigments in paint. Together with the birefringent properties of calcite this may lead to interesting gloss properties of the treated surfaces.

However, the presented data is a good starting point for further investigation of the structure-function potential in coccolith, which will be an exciting challenge in the future.

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