In Vivo Shaping of Inorganic Functional Devices using Microalgae

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The usage of biomineralization processes performed by living microalgae to create 3D nanostructured materials are advantageous compared to conventional synthesis routes. Exploitation of in vivo shaping using living cells leads to inorganic intricate biominerals, produced with low environmental impact. Since biomineralization processes are genetically controlled, the formation of nanostructured materials is highly reproducible. The shells of microalgae, like coccoliths, are particularly of great interest. This study shows the generation of mesoporous highly structured functional materials with induced optoelectronic properties using in vivo processes of the microalga species Emiliania huxleyi. It demonstrates the metabolically driven incorporation of the lanthanide terbium into the coccoliths of E. huxleyi as a route for the synthesis of finely patterned photoluminescent particles by feeding the microalgae with this luminescent element. The resulting green luminescent particles have hierarchical ordered pores on the nano- and microscale and may act as powerful tools for many applications; they may serve as imaging probes for biomedical applications, or in microoptics. The luminescent coccoliths combine a unique hierarchical structure with a characteristic luminescence pattern, which make them superior to conventional produced Tb doted material. With this study, the possibility of the further exploitation of coccoliths as advanced functional materials for nanotechnological applications is given.

1. Introduction

3D nanostructured materials generated by living organisms have many advantages compared to chemical or physical syntheses routes: inorganic intricate biominerals are produced with low environmental impact and production costs, are complex-structured, multifunctional, and biocompatible. Additionally, since they are generated under genetic control, their formation and thus their specific structure is highly reproducible. A consequence, to use the formation processes of living organisms instead of chemical or physical syntheses routes enhance the complexity of shapes and structures of the produced materials. This leads to an expansion of the spectrum of structures and multifunctional materials are created. In this regard, especially the shells of microalgae, like the frustules of diatoms or the coccoliths of coccolithophores are of great interest.[1–3]

Coccolithophores like Emiliania huxleyi are unicellular marine microalgae, which cover themselves with elaborated finely-patterned calcite (CaCO₃) disks, called coccoliths (Figure 1). Coccoliths are mesoporous, 3D sieve-like structures of about 3 µm in diameter with pore diameters ranging from micrometer to nanometer scale and possess a total pore volume of 0.05 cm³ g⁻¹.[2,4] The overall geometry has a specific surface area of 19 m² g⁻¹.[2] Coccoliths are produced inside the cell under genetic control in special organelles, the coccolith vesicles (cv).[5,6] The cell-controlled growth allows for an accurate replication of the coccoliths with monodisperse size distribution. When a coccolith is completed, it is extruded and arranged outside the cell. Each E. huxleyi cell is surrounded by 10–15 coccoliths, but the coccoliths are easily detached and new coccoliths are constantly built. The coccoliths can be obtained in high yields of around 5 g L⁻¹ d⁻¹.[2]

The nanoporosity of the coccoliths with special slit patterns suggests applications as micro/nano optical devices or fillers in adhesives.[4,7] They may have also very interesting applications in the field of nanofluidics, as their nanopores and nanochannels can be used, for example, as sieving structures for the separation of particles or molecules.[8] Since coccoliths consist of nontoxic CaCO₃, which can be dissolved by manipulating the pH, they could also be used for drug delivery or other applications inside the human body.[3] The high specific surface area and porosity of these mesoporous particles are of particular importance for their application, because these properties offer high absorption capacity.[9] In addition, their intracellular generation under genetic control leads to identical particles with reproducible nanostructures. Such mesoporous calcite materials with hierarchical structures cannot be achieved by chemical/physical syntheses.

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routes. However, concrete applications of the nanostructured coccoliths as functional materials were not reported so far.

The calcite coccoliths of *E. huxleyi* naturally contain other elements additionally to Ca\(^{2+}\): strontium (Sr\(^{2+}\)), barium (Ba\(^{2+}\)), magnesium (Mg\(^{2+}\)), and boron (B\(^{3+}\)) are taken up by the microalgae out of the surrounding environment and incorporated in trace amounts in the calcite lattice.\([10-14]\) All these ions are micronutrients for the microalgae, that is, are essential in small amounts for their growth. They occur naturally in seawater, where *E. huxleyi* lives. It is suggested that Sr\(^{2+}\) and Ba\(^{2+}\) are transported via the same biologically controlled pathway like Ca\(^{2+}\) into the coccolith vesicle, the other ions obviously take other uptake routes.\([12-14]\) We previously also reported the incorporation of zinc ions (Zn\(^{2+}\)) into coccoliths of *E. huxleyi*.\([15]\) Zn\(^{2+}\) is also a micronutrient for microalgae, but can be toxic in high amounts. We used elevated, but nontoxic Zn\(^{2+}\) concentrations for feeding *E. huxleyi* with Zn\(^{2+}\) via the growth medium.

Lanthanides (Ln\(^{3+}\)) like terbium Tb\(^{3+}\) show photoluminescence, this means that they emit photons (light) at characteristic wavelengths upon the absorption of photons. For Tb\(^{3+}\) the main emission band is located around 540 nm, respectively in the green region of the visible spectra. This feature is also true when incorporated in different materials, where only trace amounts of the Ln\(^{3+}\) are needed to receive luminescence of the doped material. These luminescence properties make Ln\(^{3+}\) interesting candidates for the usage as luminescent stains in many materials: their emission bands are very sharp and they exhibit long luminescence lifetimes in the range of several milliseconds, which make them superior to organic dyes.\([16,17]\) Ln\(^{3+}\)-doped micro- and nanoparticles are powerful tools for many applications; they may serve as tracking assays, imaging probes for biomedical applications, or in microoptics.\([18-23]\) Ln\(^{3+}\), including Tb\(^{3+}\), have the same ionic radii as calcium ions (Ca\(^{2+}\)), thus they can replace the Ca\(^{2+}\) and might be incorporated in calcite, which then functions as host-lattice.\([16,24]\) Several studies deal with the incorporation of the lanthanide europium (Eu\(^{3+}\)) or Tb\(^{3+}\) into chemically produced calcite, leading to red and green luminescent materials, respectively.\([24-29]\) Using these chemical production routes, only CaCO\(_3\):Tb\(^{3+}\) and CaCO\(_3\):Eu\(^{3+}\) powders with nonuniform grains of wide ranging dimensions in shape and size have been synthesized.

In contrast, to use the biomineralization processes of *E. huxleyi* for the in vivo shaping of Tb\(^{3+}\)-doped coccoliths would lead to luminescent nanostructured 3D particles of identical sizes and morphologies. Functionalizing the micro-patterned calcite coccoliths with this photoluminescent element would offer more potential applications and has further advantages: the coccoliths would be visualized and better trackable when used in nanofluidic devices or as biological assays and in drug delivery. They could be further used as material in light-emitting diodes and microoptical applications or as staining for special paintings. Due to their sophisticated, inimitable 3D micro- and nanostructure, they could label documents or bank notes, making them fraud-resistant or label items against theft with an invisible but unique pattern.

Even though lanthanides are nonessential elements, they are taken up by microalgae and can have stimulatory effects.\([30]\) They can replace Ca\(^{2+}\) in the cells and it was suggested that Ca\(^{2+}\) transporters play a role in the internalization of these elements into microalgae.\([30,31]\) Different lanthanides accumulate in different parts of the cells.\([32]\) We also showed that the microalgae species *Chlamydomonas reinhardtii*, which does not produce coccoliths, takes up Tb\(^{3+}\) and accumulates them as nanoparticles inside the cells.\([33]\) The accumulation of lanthanides in coccoliths were not investigated so far.

Our approach to create chemically modified coccoliths with luminescent properties is based on the in vivo incorporation of the Tb\(^{3+}\) into the calcite lattice by using biomineralization mechanisms of the living *E. huxleyi*. The great advantage of incorporating the Tb\(^{3+}\) into the coccoliths by biomineralization processes and not just by labeling them is the permanent alternation of the coccoliths calcite. To the best of our knowledge, the accumulation of lanthanides in *E. huxleyi* in particular, the intracellular incorporation into their coccoliths

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**Figure 1.** Coccoliths of *E. huxleyi*. a) Coccoliths without Tb\(^{3+}\), b) single coccolith without Tb\(^{3+}\), c) coccoliths with Tb/Ca atom ratio of 0.0007, and d) coccoliths with Tb/Ca atom ratio of 0.0013.
via biomineralization processes leading to green luminescent nanostructured coccoliths has not been reported so far.

2. Results and Discussion

2.1. Feeding the E. Huxleyi with Tb\(^{3+}\) and Tb/Ca Ratios of the Built Coccoliths

According to our successful incorporation of Zn\(^{2+}\) into E. huxleyi coccoliths,\(^{15}\) we applied Tb\(^{3+}\) via the nutrition medium. The aim was that the microalgae take up the Tb\(^{3+}\) out of the medium into the cell and subsequently incorporate them into their coccoliths via biomineralization processes. Since the biomineralization of the coccoliths is intracellular and only takes place in living cells, it is crucial that the Tb\(^{3+}\) concentration is not toxic for the microalgae. Thus, we conducted prior rapid tests to establish the appropriate Tb\(^{3+}\) amount in the experimental media. Shortly, we used a modified ESAW medium containing 1.8 or 3.6 mg L\(^{-1}\) Tb\(^{3+}\), chelated equimolar with ethylenediaminetetraacetic acid (EDTA). The ligand was used to prevent precipitation of the Tb\(^{3+}\), and thus to maximize its uptake by the microalgae.\(^{15}\) In the first step, we removed the coccoliths from the E. huxleyi cells grown in the nutrition medium and subsequently inoculated the bare cells in the Tb\(^{3+}\)-enriched media. We used microalgae with removed coccoliths “forcing” them to use the ions applied by the experimental media, including Tb\(^{3+}\) to build new coccoliths. The microalgae proliferated in the Tb\(^{3+}\)-enriched media and produced new coccoliths. The newly grown coccoliths and as the reference, coccoliths grown in media without Tb\(^{3+}\), were collected and bleached in a sodium hypochlorite (NaOCl) solution, before they were further investigated. The bleaching step is crucial to remove organic material, and also possibly attached ions on the coccoliths surface, and thus to investigate only ions incorporated in the calcite lattice of the coccolith.\(^{10,13,15,35}\) Then their Tb\(^{3+}\), their strontium (Sr\(^{2+}\)) and Ca\(^{2+}\) contents were chemically analyzed via inductively plasma optical spectrometry (ICP-OES; Spectro CIROS). Using the results of the ICP-OES analyses, we calculated the Sr/Ca and the Tb/Ca weight and atom ratios (Table 1). Both the Sr/Ca weight ratios and respective Sr/Ca atom ratios of the coccoliths grown in both media, that is, with and without Tb\(^{3+}\), were all very similar (0.007 for the weight ratio and 0.003 for the atom ratio). The Sr/Ca ratio of the coccoliths found within the present work are similar to Sr/Ca ratios of coccoliths found in previous work that was carried out in the growth media with comparable Sr/Ca ratios.\(^{11}\) The coccoliths grown in the Tb\(^{3+}\)-enriched media additionally contained Tb\(^{3+}\) in different amounts; the respective Tb/Ca ratios were positively correlated to the Tb/Ca ratios in the applied media (Table 1). This suggests that the incorporation of Tb\(^{3+}\) into the coccolith is not directly replacing Sr\(^{2+}\), but added in addition. This means that the incorporation of these two metabolically inert trace metals is due to two different and co-occurring mechanisms. It is suggested in the literature that Sr\(^{2+}\) is transported through the same pathways as Ca\(^{2+}\) into the cell, but their concentration in

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Table 1. Sr/Ca and Tb/Ca weight and atom ratios of the different media and corresponding coccoliths.

| Media          | ESAW | 1   | 2   |
|----------------|------|-----|-----|
| Amount         |      |     |     |
| Tb [mg L\(^{-1}\)] | —    | 1.8 | 3.6 |
| Sr [mg L\(^{-1}\)] | 7.2  | 7.2 | 7.2 |
| Ca [mg L\(^{-1}\)] | 360  | 360 | 360 |
| Weight ratios  |      |     |     |
| Sr/Ca          | 0.020| 0.020| 0.020|
| Tb/Ca          | —    | 0.005| 0.010|
| Atom ratios    |      |     |     |
| Sr/Ca          | 0.009| 0.009| 0.009|
| Tb/Ca          | —    | 0.001| 0.002|

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Coccoliths

| Media          | ESAW | 1   | 2   |
|----------------|------|-----|-----|
| Weight ratios  |      |     |     |
| Sr/Ca          | 0.0075| 0.0073| 0.0072|
| SD             | 0.0000| 0.0002| 0.0003|
| Tb/Ca          | —    | 0.0027| 0.0050|
| SD             | 0.0006|         | 0.0010|
| Atom ratios    |      |     |     |
| Sr/Ca          | 0.0034| 0.0033| 0.0032|
| SD             | 0.0000| 0.0002| 0.0002|
| Tb/Ca          | —    | 0.0007| 0.0013|
| SD             | 0.0001|         | 0.0002|
the cv and thus in the coccolith underlies two different mechanisms: Ca\(^{2+}\) is pumped into the cv to keep its level always close to saturation for precipitation of calcite, in contrast, Sr\(^{2+}\) is transported into the cv according to its value at the cell surface and thus in the medium.\(^{[10,11]}\) Metal ions principally enter microalgae cells by first binding onto the cell surface, afterward they are actively transported into the cell.\(^{[36]}\) Since lanthanides have the same ionic radii as calcium ions, and it was shown that they can replace Ca\(^{2+}\) in its functions in microalgae,\(^{[30]}\) we suggest that the Tb\(^{3+}\) used in our studies also are transported to the coccolith vesicle and incorporated into the coccoliths via the Ca\(^{2+}\) pathway. This Tb-transport mechanism seems to be independent from the Sr transport; consequently, the Sr\(^{2+}\) in the coccoliths are not replaced by Tb\(^{3+}\), resulting in constant Sr/Ca ratios (independent of the Tb\(^{3+}\) content). The Tb/Ca ratios are instead correlated to the Tb/Ca ratios applied by the media.

2.2. Structure of the Coccoliths with Incorporated Tb\(^{3+}\)

We further investigated the crystal structure of the Tb-doped coccoliths via powder X-ray diffraction (XRD) measurements. As the reference, we analyzed coccoliths, which were biomineralized in nutrition media without Tb\(^{3+}\). The results showed that calcite is the only calcium carbonate modification in the coccoliths without and also with incorporated Tb\(^{3+}\) (Figure 2).

Next, we investigated the microstructure of the coccoliths with incorporated Tb\(^{3+}\) using scanning electron microscopy (SEM). The SEM images revealed that their unique micropattern is not altered compared to undoped coccoliths (Figure 1). This nanoporosity allows their further use as functional devices for example, nanotechnological applications or for labeling documents to make them fraud-resistant.

2.3. Cathodoluminescence Analyses

To further investigate the incorporation of the Tb\(^{3+}\) ions into the coccoliths, especially to visualize the site of accumulation, we performed cathodoluminescence (CL) measurements. CL depends on the presence of activators like lanthanides in a lattice structure, these are stimulated to emit light when bombarded by electrons.\(^{[29]}\) For lanthanides the CL emission wavelengths are characteristic for the respective ion,\(^{[37]}\) that is, a lanthanide element as terbium can be identified by its specific emission peaks, mostly independent from its incorporation in any host material.\(^{[38,39]}\) The spectral analysis of the CL emissions combined with spectral filtered photomultiplier (PMT) imaging allows for the detection of lanthanides below the detection limit of other microanalytical methods, such as energy-dispersive X-ray spectroscopy.\(^{[37]}\) Thus, CL spectrometry is a valuable tool to visualize the Tb\(^{3+}\) incorporated in the coccoliths, although
the Tb/Ca atomic ratio is as low as 0.0007–0.0013, as shown by our ICP-OES measurements (Table 1).

Coccoliths with Tb/Ca atomic ratios of 0.0013 showed four CL emission peaks characteristic for Tb³⁺ ([Figure 3](#)). The main peak appears at 545 nm, thus the coccoliths are green luminescent. As compared to the luminescence behavior of other Tb-doped host matrices; the luminescence pattern of our coccoliths with incorporated Tb is almost indistinguishable. In contrast to the Tb³⁺ containing coccoliths, the reference coccoliths and the substrate did not show these characteristic peaks.

To further investigate the light emitted by CL from the Tb³⁺-doped coccoliths, the light was guided through a band pass filter with a passband of 525–575 nm, followed by an intensity measurement using a PMT tube ([Figure 3](#)). This setup allows for the collection of CL-intensity maps of specific regions on the sample. Moreover, this setup allows imaging of the Tb³⁺ specific emission lines only. In this case the Tb³⁺ D₂⁷F₅ transition was selected due to its high relative intensity.

Next, we investigated thin sections of the Tb-doped coccoliths to measure the CL emission from the cross section of the coccoliths. These measurements should show how deep into the coccolith the Tb³⁺ is incorporated and how it is distributed inside the calcite lattice of the coccoliths. For this investigation electron transparent samples (TEM samples) have been prepared by means of a focused ion beam system (FIB), leading to slices of 100 nm thickness. The investigation of TEM samples has been carried out in a low energy scanning transmission electron microscope (low-kV STEM) ([Figure 4](#)). CL intensity maps were collected from the cross section of the coccoliths, showing a strong CL signal across the whole cross section. The CL investigations revealed that Tb³⁺ is deeply incorporated into the coccoliths and distributed through the whole coccolith structure.

To further investigate, if the Tb³⁺ ions are incorporated into the coccoliths calcite lattice or only adsorbed onto them, we conducted a reference experiment. Pure coccoliths without algae, produced in normal medium without added Tb³⁺, were incubated in deionized water containing 3.6 mg Tb L⁻¹. Afterward,
they were collected, bleached with NaOCl, and finally investigated by CL. In contrast to the coccoliths, which were grown and biomineralized in media containing Tb$^{3+}$, the CL signal of these coccoliths was under the detection limit (data not shown). This result undoubtedly confirms that the Tb$^{3+}$ cations are not only adsorbed onto the coccoliths but incorporated into their calcite lattice. It also shows that the incorporation of the Tb$^{3+}$ into the coccoliths can only be achieved if the living *E. huxleyi* cells undertake the biomineralization process under accurate genetic control.

The underlying biomineralization process to build the elaborated coccolith calcite nanostructures, in particular the Ca$^{2+}$ pathway from the surrounding medium into the coccolith vesicle, has been investigated by many scientists, but is still not fully understood.\[6,35,40,41\] Very recently, a vacuole-like compartment was identified in *E. huxleyi*, where Ca$^{2+}$ and Sr$^{2+}$ are stored before being dispatched to build the coccoliths in the coccolith vesicle.\[14,42\] If the terbium ions used in our study also take this pathway into the coccolith is speculative, but seems likely since the Tb$^{3+}$ are regarded as substitutes for Ca$^{2+}$ in the metabolism of microalgae as previously reported.\[16,30\] Further investigations on the underlying biomineralization process in *E. huxleyi* for generating calcite coccoliths with incorporated Tb$^{3+}$ are needed.

### 3. Conclusion

To summarize, we proved for the first time the in vivo incorporation of a lanthanide into the calcite coccoliths of *E. huxleyi* using biomineralization processes of the living microalgae. By this doping of the coccoliths with Tb$^{3+}$, which emit light in the green range upon excitation, we produced green luminescent 3D mesoporous microstructures, demonstrating the metabolically driven incorporation of Tb$^{3+}$ as a route for materials synthesis. The Tb/Ca ratios in the coccoliths are positively correlated to the Tb/Ca ratios in the applied media, meaning that the amount of Tb$^{3+}$ in the coccoliths reflects the adsorption rate on the cell surface. Obviously, the cells took up the Tb$^{3+}$ adsorbed on their surface and transported them into their interior and into the cv, the production site of the coccoliths. Then the Tb$^{3+}$ ions were incorporated into the calcite lattice at the Ca$^{2+}$ sites, since these ions have the same radii, leading to an intracellular incorporation of the Tb$^{3+}$ into the coccoliths. Further studies are needed and planned by us to confirm these assumptions.

Former studies concerning the incorporation of lanthanides including Tb$^{3+}$ into synthetic calcites report a Ln$^{3+}$/Ca$^{2+}$ ratio of one magnitude lower than in our study, where the incorporation was metabolically influenced by the cells.\[24,29\] In addition, these chemically produced Ln$^{3+}$-doped calcites were not nanostructured. Compared to conventional produced terbium-doped material, our coccoliths with incorporated Tb combine luminescence properties with a unique hierarchical structure in one material. This makes them superior to conventional produced Tb doped material.

With this study we would like to promote the use of coccoliths as advanced functional materials for nanotechnological applications. Our biologically produced luminescent 3D microstructures may have the following applications as functional devices: since the coccoliths consist of biocompatible calcite, they could be used in bioassays or as drug delivery systems in the human body, where they can easily be tracked due to their luminescent properties. Luminescent coccoliths represent also an ideal candidate for applications in nanofluidic studies, due to their fine-patterned nanostructured feature. Moreover, since this nano-pattern is unique for *E. huxleyi* coccoliths and the calcite is easily embedded into papers and other materials, our luminescent coccoliths could for example be embedded in important documents or bank notes, where their specific luminescence is only visible when irradiated by light of specific wavelength, thus making the document fraud-resistant. Another example is an invisible labeling of precious materials, components, and products against theft.

Due to the incorporation of the luminescent Tb$^{3+}$ into the coccoliths and not just their absorbance onto them, their luminescent properties will not weaken. The emission bands of lanthanides are very sharp and exhibit long luminescence lifetimes, thus they are superior to organic dyes.\[16,17\] Furthermore, the biologically controlled production of the nanostructured material is environmentally friendly, cheap, and highly reproducible. The microalgae species *E. huxleyi* is easily cultivated in the lab and the coccoliths can be produced in large amounts of around 5 g L$^{-1}$ d$^{-1}.\[8\] Our here presented protocol to produce green luminescent coccoliths may also be used to generate coccoliths, which show luminescence in other colors by feeding the microalgae with the appropriate lanthanides like europium (red) or thulium (blue).

### 4. Experimental Section

All used vials were rinsed with hydrochloric acid (HCl) for 24 h before use to remove possibly adherent terbium ions.\[6\]

**Growing of *E. Huxleyi* Cultures:** The experiments were conducted with living *E. huxleyi* cells (strain 920/9), which were cultured since several years in the laboratory at the Institute for Materials Science, University of Stuttgart, originally provided by the culture collection of algae and protozoa CCAP, Oban, UK. The cells were incubated in artificial ESAR medium (enriched sea water artificial medium)\[43\] at pH 8.2 with added 1.8 or 3.6 mg L$^{-1}$ Tb$^{3+}$, respectively, chelated equimolar with EDTA. The ligand was used to prevent precipitation of the Tb$^{3+}$ and thus maximize its uptake by the cells.\[43\] The original ESAR recipe was modified by adding only 75% Na$_2$HPO$_4$ and omitting Na$_2$SiO$_3$, since prior experiments had shown that these two salts caused precipitation of the Tb$^{3+}$. The amount of Ca$^{2+}$ and Sr$^{2+}$ was not altered. The resulting experimental media with added Tb$^{3+}$ were sterile-filtered to remove any precipitation before the microalgae were added.

Preliminary experiments showed that the algae proliferated in these media and produced coccoliths. All experiments were run in triplicate. The reference was cultivated in the modified ESAR medium without added Tb$^{3+}$. The microalgae were cultivated at room temperature and under a 12:12 light:dark cycle (Philips MASTER TL-D 58W/840 Super 80 Weiss) in 250 mL Erlenmeyer flasks, sealed with a permeable membrane to prevent contamination but allow ventilation.

Before the inoculation of *E. huxleyi* cells to these experimental media, the algae were decalcified, to ensure that all new coccoliths were formed exclusively in the cultivation medium with added Tb$^{3+}$. The cells were decalcified by adding them to 0.02 mol L$^{-1}$ HCl in Ca$^{2+}$-free ESAR medium and gently mixed. After 15 min, the cells had been settled on the bottom of the flask, and the HCl–ESAR medium was removed by using a pipette. After this decalcification the cells were carefully washed by adding Ca$^{2+}$-free ESAR medium, and afterward inoculated...
into the experimental media containing Tb$^{3+}$. They further proliferated and built new coccoliths, that showed that the decalcification process was not harmful for the cells. It had been already used successfully this decalcification method to gain E. huxleyi coccoliths with incorporated zinc.\[13] The final amount of algae in the experimental media was $8 \times 10^4$ cells mL$^{-1}$. The microalgae were cultured for seven days in the media containing Tb$^{3+}$, afterward the coccoliths were further analyzed.

**Sample Preparation of the Coccoliths:** After seven days of cultivation, the coccoliths newly grown in media containing 1.8 and 3.6 mg L$^{-1}$ Tb$^{3+}$, respectively and in the reference without Tb$^{3+}$ were collected and chemically investigated via ICP-OES. Due to the different mass density of the calcium carbonate and the organic cells, it was possible to mostly separate the coccoliths and the cells via centrifugation. The cell cultures were centrifuged at 4000 rpm for 10 min, then the algal cells (brown) and beneath them the coccoliths (white) were deposited at the bottom of the vials. By this procedure, the coccoliths were mostly separated from the cells. To obtain pure coccoliths without organics, they were washed in 10% NaOCl for 24 h.\[19] By this, all organics and possibly adhering ions would be removed. Afterward, the samples were centrifuged (4000 rpm, 10 min) to remove the oxidizing solution and the pellet was resuspended in deionized water at pH 9 (adjusted with NaOH, to prevent solution of the CaCO$_3$ coccoliths). After removing the water, new water was added and this washing procedure was repeated six times to obtain clean coccoliths. In the end, the cleaned coccoliths were dried in a drying box at 37 °C. This method was successfully used before to analyze the incorporation of Zn$^{2+}$ into coccoliths.\[19]

**Chemical Investigations via ICP-OES:** The cleaned and dried coccoliths were dissolved in 0.1 M HCl and chemically analyzed by ICP-OES (Spectro CIROS). Three different analyses per culture were conducted. The amount of Tb, Ca, and Sr in the coccoliths in the experimental media was measured and calculated the Tb/Ca and Sr/Ca weight and atomic ratios including SD.

**Powder X-Ray Diffractionometry:** XRD measurements of the coccoliths with and without incorporated Tb$^{3+}$ were performed to investigate their crystal structure. The XRD measurements were conducted with a PANalytical X’Pert MPD with Bragg-Brentano geometry equipped with a sealed Cu tube, a Ge Johannsson monochromator in the primary beam, and an X’Celerator detector in the diffracted beam. This setting allowed use of the Cu K$_{\alpha1}$ radiation ($\lambda = 1.54056$ Å). The diffraction patterns were recorded in the range 20 $= 10^\circ$–60$^\circ$.

**Cathodoluminescence Analyses:** Different preparation and measurement routines were applied for the CL measurements of the bulk and of cross-sectional coccolith samples measured in transmission, as described in the following.

The coccolith solution samples (coccoliths in deionized water) were drop casted each on a 1 x 1 cm piece of highly p-doped silicon wafer. The silicon wafer substrate was cleaned previously in a 1:1 mixture of acetone and isopropanol in an ultrasonic bath for 5 min. The substrates were dried under a stream of dry nitrogen. The samples used for the preparation of TEM lamellae were coated with a platinum layer of $\approx$50 nm thickness using a sputter coater (Leica ACE 200).

The SEM/CL experiments on the bulk coccolith samples were carried out on a FEI Helios 660 G3 UC FIB/SEM-System. SEM images were collected by centrifugation and the pellet was suspended in 10% NaOCl and afterward of 5 µm (Whatman Nucleopore membranes). Since the cells were bigger than coccoliths, the latter could be collected in the filtrate. Then the filtered coccoliths were incubated in deionized water (pH 8.2) containing 3.6 mg Tb L$^{-1}$ for 7 days, the reference experiment were pure coccoliths in deionized water without Tb$^{3+}$. Afterward, both types of coccoliths were collected by centrifugation and the pellet was suspended in 10% NaOCl for 24 h to remove all organics. After frequent washing to remove the oxidizing solution, the remaining coccoliths were analyzed by CL.

**Statistical Analysis—Chemical Investigations of the Coccoliths:** Three different ICP-OES analyses per culture were conducted. The Tb/Ca and Sr/Ca mean weight and atomic ratios and the SD were calculated using Excel.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

biomineralization, cathodoluminescence, coccoliths, microalgae

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