polymer film. The obtained enzyme electrodes show a dinitro-2,5-dione; see Scheme 1) instead of the bithiophene mono-thiophene derivative, we have been able to prepare analogous glucose active ester groups available to react with the enzyme. Immobilization of the enzyme is of course equivalent and independent from the specific needs for the formation of the polymer film. The obtained enzyme electrodes show a slightly lower response as those obtained with the functionalized poly(bithiophene) films, despite there are double active ester groups available to react with the enzyme. Probably, due to the size of an individual enzyme molecule the increased number of binding sites at the electrode surface does not concomitantly increase the immobilized enzyme activity.

To conclude, we have shown that, despite the high oxidation potential of thiophene and its derivatives, polythiophene films can be advantageously used for the entrapment or covalent immobilization of enzymes. This was realized by entrapment of glucose oxidase using dimeric oxidation potential of thiophene and its derivatives, polythiophene films can be advantageously used for the polymerization process in CH3CN/water mixtures or, according to a two-step procedure, based on functionalized polythiophene or poly(bithiophene) films and covalently derivatization of thiophene in the 3-position and the synthesis of oligomers is much easier than for pyrrole. Second, due to the higher redox potential of the polymer film itself, deterioration e.g. by enzymatically generated H2O2 is less probable. This is even more important for a next sensor generation, in which the conducting polymer is expected to act as molecular wire between the active site of the enzyme and the electrode surface. Possibly, the improved stability of polythiophene against oxidation in ambient environments will allow the construction of biosensors with improved sensor characteristics and life-time.

Stabilization of Amorphous Calcium Carbonate by Specialized Macromolecules in Biological and Synthetic Precipitates**

By Joanna Aizenberg,* Gretchen Lambert, Lia Addadi,* and Stephen Weiner*

Some organisms form stable minerals that would never precipitate under ambient conditions in an inorganic environment, or, if formed, are unstable precursors of more stable compounds.[1,2] One of the most fascinating examples is biogenic amorphous calcium carbonate, because its transformation into crystalline polymorphs is not only thermodynamically favored, but also kinetically fast.[3] We report here an example of a single skeletal element, spicules from the calcareous sponge Clathrina, composed of crystalline calcite in one layer and stable amorphous CaCO3 in another. Differential dissolution of the amorphous phase of these spicules released macromolecules with proteins rich in glutamic acid (and/or glutamine), serine, glycine and polysaccharides. Similar macromolecules are present in spicules of the ascidian Pyura pachyderma which are composed entirely of amorphous CaCO3. We show that the presence of small amounts of these macromolecules in saturated solutions of CaCO3 results in the formation in [1] W. Schuhmann, Mikrochim. Acta, 1995, 121, 1.

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Communications
Fig. 1. Scanning electron micrographs of: a) Intact triradiate spicule from the calcereous sponge Clathrina sp. b) Heated Clathrina spicule, showing two different CaCO₃ phases; an outer amorphous layer and a stable calcitic core. Note the separated calcite core indicated by an arrow. c) Partially KOH etched Clathrina spicule reveals a thin, apparently crystalline, outer sheath. d) Antler spicule from the ascidian Pyura pachydermatina. The entire spicule is composed of amorphous CaCO₃. e) Synthetic amorphous calcium carbonate precipitated in the presence of 2.0 μg/ml proteins extracted from the amorphous phase of Clathrina spicules. Insert: electron diffraction pattern of the precipitate embedded in vitrified ice, showing its amorphous character. f) Synthetic calcite crystal grown in the presence of 2.0 μg/ml proteins extracted from the calcitic core of the Clathrina spicules. Note that the acidic proteins interfere with calcite growth in the c axis direction, causing the development of the new (001) face. Insert: Synthetic calcite crystal of regular morphology grown in the absence of additives.

Fig. 2. Synchrotron powder X-ray diffraction analyses of different CaCO₃ phases. a) Geological calcite. b) Clathrina spicules. Note the presence of the sharp calcite reflections and the base-line rise due to the amorphous material. From the comparison of the intensities of the different crystallographic peaks with those of an equal volume of pure calcite, the amount of crystalline material in the Clathrina spicules was found to be about 20%. c) P. pachydermatina spicules.

vitro of stable amorphous CaCO₃. In contrast, the proteins extracted from the crystalline calcitic phase are rich in aspartic acid (and/or asparagine) and induce calcite formation.

The formation of composite structures in which amorphous and crystalline phases coexist apparently endows the biogenic material with advantageous properties, that may be interesting to investigate. The ability to control the formation of stable amorphous phases may also have application to different synthetic materials.

The calcareous sponges (Porifera) form a wide variety of spicules, each of which is generally thought to be composed of a single crystal of Mg-bearing calcite. Significant differences have, however, been observed between the spicule mineral and inorganically formed calcite crystals. Most of the sponge spicules have conchoidal rather than smooth cleavage surfaces and lower specific gravities. Some tend to disintegrate when heated, presumably due to loss of water. However, polarized light microscopy, etching, overgrowth, and X-ray diffraction studies all support the notion that the spicules are composed of calcite. The sponge Clathrina forms regular triradiate spicules (Fig. 1a) that were observed to form from non-birefringent material deposited in three separate vesicles, which upon fusion becomes completely crystalline.

Our attention was drawn to the fact that an additional amorphous mineral phase may be present, by the observation that the intensities of the diffracted beam from single Clathrina spicules accounted for only about 20% of the expected value of an equivalent volume of a pure single calcite crystal. A high-resolution powder X-ray diffraction pattern of Clathrina spicules using synchrotron radiation revealed, in addition to the sharp calcite reflections (Fig. 2a), an intense but broad peak around 2θ = 15° (Fig. 2b) indicative of the presence of an amorphous phase. An infrared spectrum of the intact spicules (Fig. 3c)
Infrared spectra of calcium carbonates of different origin. a) Geological calcite. b) Separated calcitic cores from the Clathrina spicules. c) Intact Clathrina spicules. Note the atypical broadening of the calcitic peaks and the additional absorption at 1080 cm\(^{-1}\). d) P. pachydermina spicules. e) Synthetic amorphous CaCO\(_3\) grown in the presence of the macromolecules extracted from the amorphous layer of Clathrina spicules.

Fig. 3. shows a broadening of all the characteristic calcitic peaks (Fig. 3a), as well as additional broad peaks around 3430 and 1080 cm\(^{-1}\), typical of hydrated amorphous calcium carbonate.\(^{[12]}\)

Upon heating (~100 °C) in a drop of water, the spicules decompose into two separate domains, with a smooth stable inner core (~8–15 vol.-%) and a heavily corroded outer layer (Fig. 1b). Under mild acidic conditions (pH ~ 5) well defined triangular etch figures indicative of crystalline calcite are formed on the surface of the central part of the spicules, in contrast to the irregular shapeless etch patterns of the ray extremities. The outer layer can be solubilized in dilute (< 5 mM) CaCl\(_2\) solution or doubly distilled water, while the core remains intact.

The infrared spectrum of the separated core material contains only calcitic absorption bands (Fig. 3b). One puzzling observation is that freshly extracted un-etched spicules show a clear birefringence.\(^{[5]}\) This implies that the outer layer must possess at least a thin exterior crystalline sheath. KOH etching (pH ~ 9), which does not affect pure calcite,\(^{[5]}\) does reveal a thin outer sheath (Fig. 1c). On the basis of the above observations, we deduce that the spicule is composed of a core of calcite embedded in a thick amorphous calcium carbonate layer and is apparently covered by a thin calcitic sheath. We can infer from Minchin’s observations\(^{[13]}\) that the initial mineral deposited is amorphous and stable, and that the deposition of the crystalline core material and of the external sheath occurs in separate domains as a later event.

Specialized macromolecules are found within the calcite crystals of many organisms.\(^{[13,14]}\) Their presence is known to affect the mechanical properties of calcite,\(^{[15]}\) and in the case of calcareous sponge spicules to be involved in shaping their morphology during growth.\(^{[9,11]}\) The protein content of Clathrina spicules is 0.12 wt.-%. In order to study their possible effects on the observed phase stabilization, the macromolecules associated with the two different mineral phases were extracted by differential dissolution. The amino acid compositions of the two protein assemblies are quite different (Table 1). The amorphous material contains GIX-, Ser- and Gly-rich proteins, whereas those in the crystalline core are AsX-rich. The latter composition is typical of the proteins associated with many other calcitric and aragonitic mineralized tissues.\(^{[10]}\) Infrared spectra of the two macromolecular fractions show that the amorphous phase macromolecules are also rich in polysaccharides, while the main constituent of the core fraction is not.

To further investigate the ability of organisms to stabilize a metastable amorphous phase, we studied the ascidian Pyura pachydermata (Chordata), which forms antler-shaped and dog bone-shaped spicules. The antler-shaped spicules (Fig. 1d) were found to be composed entirely of amorphous CaCO\(_3\) (Fig. 2c, 3d). Spicules composed of

Table 1. Amino acid compositions of the proteins extracted from ascidian antler spicules and triradiate sponge spicules.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Pyura pachydermata</th>
<th>Clathrina triacta spicules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx*</td>
<td>7.8</td>
<td>7.2</td>
</tr>
<tr>
<td>Thr</td>
<td>4.6</td>
<td>4.3</td>
</tr>
<tr>
<td>Ser</td>
<td>18.0</td>
<td>19.2</td>
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<tr>
<td>Glx**</td>
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<td>Pro</td>
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<tr>
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</tr>
<tr>
<td>Ile</td>
<td>2.4</td>
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</tr>
<tr>
<td>Leu</td>
<td>3.5</td>
<td>2.3</td>
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<tr>
<td>Tyr</td>
<td>1.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Phe</td>
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<td>1.0</td>
</tr>
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<td>His</td>
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</tr>
<tr>
<td>Arg</td>
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</tr>
</tbody>
</table>

*Asx - combination of Asp and Asn.  
**Glx - combination of Glu and Gln.
amorphous CaCO$_3$, as well as of a variety of other minerals, are known to be formed by the *Pyuridae*. Amino acid and infrared analyses of the macromolecules within the antler spicules (0.09 wt.-% protein) showed that they are also polysaccharide-rich proteins with amino acid compositions that are remarkably similar to those from the amorphous layer of the *Clathrina* sponge spicules (Table 1).

We added small amounts of the macromolecules from within the *Clathrina* spicule amorphous phase to solutions saturated with respect to CaCO$_3$. This resulted in complete inhibition of crystallization at protein concentrations of 1.0 $\mu$g/ml. A similar phenomenon occurred with the macromolecules extracted from the *Pyura* spicules, but at protein concentrations of 0.5 $\mu$g/ml. A few irregular crystals were formed when the solutions were doped with lower concentrations of proteins. Precipitation of a non-birefringent CaCO$_3$ phase occurred with further increases in protein and calcium concentrations (2 $\mu$g/ml and 25 mM, respectively). The yield of synthetic amorphous CaCO$_3$ in this experimental setup using *Clathrina* proteins was estimated by atomic absorption (i) from the difference in the calcium contents between the solutions before and after precipitation. These were found to be 1000 and 690 $\mu$g/ml Ca, respectively. (ii) Directly from the yield of dissolved precipitate which was 300 $\mu$g/ml Ca. The yield of precipitate is thus 30.0–31.0 wt.-%. This corresponds to the formation of 775 $\mu$g/ml of CaCO$_3$. The maximum possible fraction of protein in the final solid is 0.26 wt.-%, assuming all 2 $\mu$g/ml protein is incorporated. We therefore conclude that the precipitate is composed of $>99.5$ % CaCO$_3$ and water.

Figure 1e shows the spherical particles formed in the presence of 2.0 $\mu$g/ml proteins from the amorphous layer of *Clathrina* spicules. A material of the same appearance was obtained with the addition of 1.0 $\mu$g/ml soluble proteins from the *Pyura* antler spicules. The size of the spherules is about 50–70 nm. The amorphous character of this material was unequivocally confirmed by infrared spectroscopy (Fig. 3e), as well as by the absence of a discernible electron diffraction pattern from the precipitate embedded in vitrified ice (Fig. 1e). No transformation of the air-dried synthetic amorphous CaCO$_3$ into the favored crystalline forms was observed during three months. If the precipitate is kept in the mother liquor, some calcite crystals are formed in addition to the amorphous material after three to four days. In contrast, under the same experimental conditions numerous calcite crystals with well-developed (001) crystallographic faces were obtained in the presence of 0.5–4.0 $\mu$g/ml of the AsX-rich macromolecules from the calcitic cores of the *Clathrina* spicules (Fig. 1f). In the latter case, macromolecules do not inhibit crystallization, but modulate crystal morphology by interfering with calcite growth in the direction of the c-axis.

These results suggest that the specialized sugar-, GIIX-, Gly- and Ser-rich macromolecules associated with the amorphous calcium carbonate phases in the ascidians and the sponges are responsible for inhibition of crystallization, the precipitation of an amorphous metastable phase, and its stabilization. Following the observation that low concentrations of these proteins in solution drastically reduce the amount of calcite crystals formed, we suggest that the macromolecules poison the growth of embryonic nuclei of crystallization. Suppression of calcite nucleation thus presumably allows the concentration of the ions in solution to increase to the point where the amorphous phase becomes supersaturated. This mechanism is analogous to that proposed for the stabilization of amorphous calcium phosphate by Mg$^{2+}$ and ATP. In vitro precipitation of almost exclusively amorphous calcium carbonate with higher concentrations of protein and in the absence of any other additives indicates that it is sufficient to add only macromolecules from biogenic amorphous phases to maintain metastable amorphous CaCO$_3$ in a stable state. The manner in which the macromolecules stabilize the amorphous phase is still to be understood.

Stable biologically produced amorphous CaCO$_3$ is known to be formed by members of the plant kingdom, Cyanobacteria, Mollusca, Arthropoda and Chordata. The difficulties involved in detecting amorphous CaCO$_3$ when it is associated with one or more of the crystalline polymorphs in the same skeletal element probably account for the fact that this phase remained undetected in *Clathrina* spicules for more than 100 years. We therefore suspect that biologically formed amorphous calcium carbonate may well be a lot more widespread than commonly supposed. It is thought to function as temporary storage sites in some organisms, as a precursor of crystalline CaCO$_3$ polymorphs in others, and also as a functional material in skeletal elements where it is present as a stable entity.

Amorphous materials have the advantage of being isotropic and less brittle. On the other hand, their crystalline counterparts are harder and less soluble. The combination of the two phases, as in *Clathrina* spicules, may offer some advantages that we do not yet fully appreciate. If the mechanisms by which the different glycoproteins control the formation and stabilization of the different phases could be elucidated, this knowledge may well have practical applications in the field of materials science.

**Experimental**

*Materials*: Individual *Clathrina* spicules (Autil, Israel) were isolated and cleaned as described previously. *Pyura* spicules suspended in a drop of doubly distilled water were placed on an aluminum SEM stub and heated until all the water evaporated. The volume fraction of the remaining crystalline parts in the entire spicules was measured from the micrographs. The evaluated crystalline fraction is 11±5%. Antler spicules from *Pyura* (Otago Harbour, Portobello, New Zealand) branchial sac tissue were isolated as described previously, except that fresh unfixed tissue was used. The samples were examined in a JEOL 6400 SEM after gold coating.

*Extraction procedure*: Cleaned *Clathrina* spicules were placed on a rocking table for 3 days in doubly distilled water. The unsolved calcitic cores were separated by low speed centrifugation. Extensive dialysis of the solution (Spectrapor 3 dialysis tubing), followed by lyophilization, yielded the macromolecules from the amorphous phase. The calcitic cores were dissolved separately as described previously to yield an additional protein fraction. Macromolecules from within the *Pyura* antler spicules were extracted and analyzed as described for the sponge spicule amorphous phase. Amino acid
Compositions were determined using ninhydrin detection (Dionex BIOLC) after hydrolysis in 6 N HCl.

Precipitation of CaCO₃: In vitro CaCO₃ precipitation was performed by slow diffusion of ammonium carbonate vapor in a 25 mM calcium chloride solution doped with aliquots of the proteins extracted from the biogenic amorphous CaCO₃ phases and the calcitic cores of sponge spicules. Protein concentrations were determined from amino acid composition analyses.

Atomic absorption measurements: After precipitation, the mother liquor (0.75 ml) was quantitatively separated and diluted to 25 ml. The precipitate that underwent precipitation and in the dissolved precipitate, using atomic absorption (Perkin Elmer 5100 GFAAS).

The amounts of calcium were measured in the initial solution, in the solution obtained was then diluted to the same volume as the mother liquor.


10. E. A. Minchin, Q. J. Microsc. Sci. 1898, 40, 469 (see Addendum).

Donor–Acceptor Substituted Tetraethynylethenes**

By Rik R. Tykwinski, Martin Schreiber, Volker Gramlich, Paul Seiler, and François Diederich*

Derivatives of tetraethynylethene (TEE, 3,4-diethynylhex-3-ene-1,5-diyne)[11] constitute a versatile “molecular construction kit” which provide access to conjugated acetylenic acyclic[12] and macrocyclic[13] molecular scaffolds with unusual electronic and structural properties as well as linearly-conjugated polymers with the polytriacetylene (PTA) backbone.[4]

Tetrakis(phenylethynyl)ethene[5] has been shown to form highly ordered charge transfer complexes with π-acceptors in the solid state and in solution.[6] Substituents in all TEE derivatives reported to date were either silyl, alkyl, alkynyl, phenyl, and alkylphenyl groups, or halogens. To enhance the interest in TEEs for potential applications as conjugated materials for electronics and photonics,[7] we became interested in preparing derivatives with electron donor and/or acceptor groups. By varying the attachment of p-donor and p-acceptor substituted phenyl rings to the planar TEE chromophore, different donor–acceptor conjugation paths are generated (Fig. 1). Trans- and cis-orientations of the substituents provide one-dimensional linear donor–acceptor conjugation paths (1 and 2), geminal substitution affords one-dimensional cross-conjugation (3), and substitution at all four terminal alkynes gives full, two-dimen-

Fig. 1. Schematic representations of possible conjugation paths in donor–acceptor substituted tetraethynylethenes (TEEs). D = donor. A = acceptor. R = Si(Ph)₃ or SiMe₃.