uted either to an inability to measure further reductions in ingestion under circumstances in which the initial uptake is already quite low or to alternative ligand-receptor interactions between LPS-rough *P. aeruginosa* and ΔF508 CFTR–expressing cells. Purified *O* polysaccharides from LPS-smooth wild-type strains did not inhibit bacterial ingestion by the airway epithelial cell lines.
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Control of Aragonite or Calcite Polymorphism by Mollusk Shell Macromolecules

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Many mineralizing organisms selectively form either calcite or aragonite, two polymorphs of calcium carbonate with very similar crystalline structures. Understanding how these organisms achieve this control has represented a major challenge in the field of biomineralization. Macromolecules extracted from the aragonitic shell layers of some mollusks induced aragonite formation in vitro when preadsorbed on a substrate of β-chitin and silk fibroin. Macromolecules from calcitic shell layers induced mainly calcite formation under the same conditions. The results suggest that these macromolecules are responsible for the precipitation of either aragonite or calcite in vivo.

Many organisms are able to exert remarkable control over the minerals they form. Numerous mineralized tissues contain crystals all having a preferred orientation and a mineral composition and morphology different from precipitates that form abiologically under the same environmental conditions (*1*, *2*). A striking example of biological control is the ability of many organisms to determine which polymorph of $CaCO_3$ will precipitate at a given location (*3*). In mineralized tissues a mixture of $CaCO_3$ polymorphs is almost never found at the same site. There are, however, numerous examples of species that precipitate one polymorph type at one location and a second type at an adjacent location (*1*).

The most common biologically formed $CaCO_3$ polymorphs are calcite and aragonite. Vaterite, a less stable polymorph, is not commonly formed by organisms (*1*, *4*). Calcite is thermodynamically more stable than aragonite at ambient temperatures and pressures (*5*). Calcite and aragonite have very similar crystal structures (*6*). The calcium ions are located almost in the same lattice positions in (001) layers, alternating with layers of carbonate ions. The major differences between the two polymorphs occur in the organization and orientation of the carbonate molecules. The presence of other doubly charged ions in $CaCO_3$ solutions, in particular Mg^{2+} as well as a variety of small organic molecules, favors the formation of aragonite (*7*). It has thus been widely believed that the regulation of aragonite-calcite polymorphism in organisms depends on the concentration of ions, primarily magnesium, in the precipitating solution (*8*).

A commonly used strategy in biomineralization is the elaboration of an extracellular organic matrix (*4*). The crystals are induced to form within the matrix voids. This is the strategy used by mollusks to form their shells (*9*). In the matrix sheets of nacre, the core is composed of a layer of β-chitin that is sandwiched between two layers of glycine- and alanine-rich proteins (*10*). The latter proteins have x-ray and electron diffraction patterns remarkably similar to that of silk fibroin and are predominantly in the β sheet conformation (*11*). The surfaces of the matrix are coated with hydrophilic, aspartic acid–rich macromolecules, some of them in contact with the mineral phase (*12*). These hydrophilic macromolecules specifically interact from solution with growing calcite crystals in vitro, suggesting that they are involved in the crystal formation process at the molecular level (*13*). Hare proposed that the organic matrix proteins may be responsible for the polymorph type deposited in mollusk shells (*14*). Experiments testing the ability of organic framework matrices from aragonitic and calcitic mollusk shell layers to induce nucleation of the original phase in a saturated solution met with some success, although calcite formed predominantly, rather than aragonite, irrespective of the origin of the matrix components (*15*). Furthermore, this experimental approach does not guarantee the total absence of undissolved crystal nuclei in the matrix, which could be responsible for seeding the original phase.

We developed an experimental strategy to determine whether components of the matrix, especially the hydrophilic macromolecules, have a primary role in the control of polymorphism and whether the entire matrix assembly is required. We reassembled in vitro a substrate for nucleation composed of the major mollusk shell organic matrix components: β-chitin, silk fibroin, and aspartate-rich soluble macromolecules extracted from individual mollusk shell layers (*10*). The β-chitin and silk were purified from the pen of the squid *Loligo* and the cocoons of the silkworm *Bombyx mori*, respectively (*16*, *17*), where neither are associated with any mineral phase. This approach precluded the possibility that our results were influenced by any residual undissolved mineral phase.

The *Loligo* β-chitin has a complex three-dimensional organization (*18*). The fibers are organized in an interlinked architecture forming a dense net of pores and channels. Purified soluble silk fibroin was allowed to interact with the insoluble chitin and was stabilized in the β conformation by treatment with methanol (*17*). The ensemble of macromolecules was then adsorbed from solution on the β-chitin–silk fibroin assembly. Crystallization was finally induced by incubation of the substrate complex in a saturated solution of $CaCO_3$ (*19*). The macromolecules were extracted from either calcitic or aragonitic layers of four bivalves, a cephalopod, and a gastropod (Table 1). Most of our experiments used the macromolecules from aragonitic layers, as the formation in vitro of the less stable aragonite in the absence of other additives presumably implies an active role for these macromolecules in $CaCO_3$ polymorph determination.

Crystallization on the partial or complete assemblies of chitin, silk fibroin, and mollusk shell macromolecules resulted in the formation of spherulites of $CaCO_3$ on the surface and inside the chitin scaffold (*20*). The spherulites were isolated and characterized by optical and scanning electron microscopy, and their mineralogies were determined by Fourier transform infra-

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red (FTIR) spectroscopy (Fig. 1 and Table 1). The complete assembly of chitin, silk, and soluble macromolecules induced the formation of crystals inside the chitin in six out of the eight cases investigated. When crystallization was induced, the same polymorph with which the macromolecules were originally associated always appeared as the main mineral phase; namely, when the macromolecules were extracted from an aragonitic shell layer aragonite formed in vitro, and calcite was induced to form by macromolecules from calcitic shell layers. In the absence of soluble macromolecules, no crystallization occurred inside the substrate, and only in some experiments a few vaterite spherulites formed close to the outer surface. When the substrate was composed of chitin and macromolecules without silk, vaterite spherulites formed inside the substrate if the macromolecules were of aragonitic origin, whereas calcite formed if they were of calcitic origin.

Scanning electron microscope investigations of the mineral phases produced inside the substrate (Fig. 2) showed that the chitin scaffold and the mineral were intimately associated. Each single crystalline unit appeared to be in contact with the organic matrix. Preferential orientation of the crystallites was observed locally and occasionally extended over wider regions (Fig. 2, A and C). The size, shape, and organization of the crystallites were dependent on the soluble macromolecules used, even when the same polymorph type precipitated. The aragonite crystallites did, however, tend to have elliptical shapes (Fig. 2B) with highly variable dimensions (between 100 and 300 nm), whereas the calcite crystals tended to preserve their typical rhombohedral morphology (Fig. 2D) and were much larger (~400 nm).

The results show that the macromolecules from either aragonitic or calcitic shell layers are able to specifically induce calcite or aragonite to form in vitro, provided that they are in the appropriate microenvironment. Extraneous ions such as magnesium were not required. The macromolecules must actively induce nucleation of the polymorph originally associated with them rather than inhibit the inappropriate polymorph because, in the absence of soluble macromolecules, no crystals were formed inside the substrate. In these in vitro experiments, a whole assembly of macromolecules was used. Perhaps only one or several of these are involved in nucleation. The reason why no crystals formed in the experiments involving *Nautilus* and *Tridacna* macromolecules may be that other nonnucleating macromolecules in the in vitro environment prevented the crystallization by inhibition or possibly by just reducing the concentration of the nucleating protein.

Table 1. Calcium carbonate polymorphs formed inside various substrates containing glycoproteins extracted from different mollusk shell layers. The mineral phase shown in parentheses indicates the minor component of the polymorphs formed (less than 25%). In some experiments few spherulites of calcite (less then 5%) were observed associated with aragonite spherulites. No crystal formation was induced by control substrates (β-chitin–silk fibroin or β-chitin), which lacked extracted glycoproteins.

Glycoprotein source			$CaCO_3$ polymorphs formed inside substrate	
Organism*	Shell layer	Original mineral	β-Chitin–silk fibroin + glycoproteins	β-Chitin + glycoproteins
Mytilus californianus	Nacre	Aragonite	Aragonite (vaterite)	Vaterite
	Prismatic	Calcite	Calcite (vaterite)	Calcite (vaterite)
Atrina rigida	Nacre	Aragonite	Aragonite (vaterite)	Vaterite
	Prismatic	Calcite	Calcite (vaterite)	Calcite (vaterite)
Nautilus pompilius	Nacre and prismatic	Aragonite	Void	Void
Elliptio sp.	Nacre	Aragonite	Aragonite (vaterite)	Vaterite
Strombus tricornis	Crossed lamellar	Aragonite	Aragonite (vaterite)	Vaterite (calcite)†
Tridacna sp.	Crossed lamellar	Aragonite	Void	Void

**Mytilus californianus* (Bivalvia) was obtained from Los Angeles, California; *Elliptio* sp. (Bivalvia) from central New Hampshire; *Tridacna* sp. (Bivalvia) from the Gulf of Suez, Red Sea; *A. rigida* (Bivalvia) from Venice, Florida; *S. tricornis* (gastropod) from Nuweiba, Gulf of Aqaba, Red Sea; and *N. pompilius* (cephalopod) from Palau, Italy. †A few calcite single crystals were observed.

Polymorph control is probably a function of the three-dimensional structure of the nucleation site, which includes the macromolecular conformation and the local microenvironment. Control cannot depend on the macromolecular conformation alone, because the same macromolecules when adsorbed on polystyrene with or without an adsorbed β-silk film induced calcite formation and lost the ability to form aragonite (*13*, *21*). Nor can control be due only to the chitin superstructure, because in the absence of silk, aragonite was generally not induced. In contrast, calcite, the stable polymorph, was induced by calcite-associated macromolecules even in the absence of silk.

Vaterite is formed in the chitin matrix in

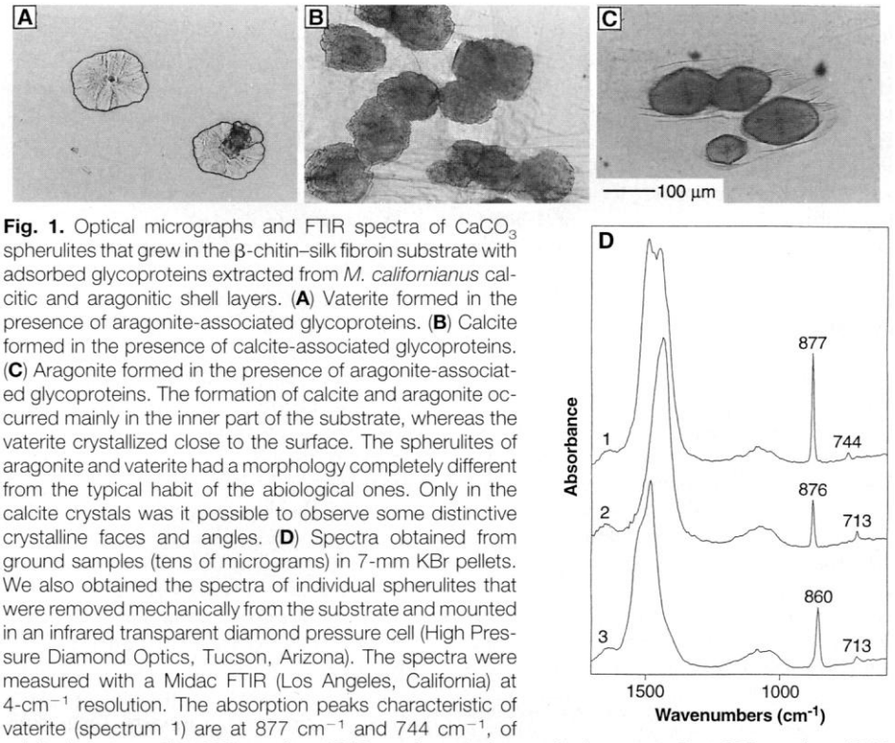

Fig. 1. Optical micrographs and FTIR spectra of $CaCO_3$ spherulites that grew in the β-chitin–silk fibroin substrate with adsorbed glycoproteins extracted from *M. californianus* calcitic and aragonitic shell layers. (**A**) Vaterite formed in the presence of aragonite-associated glycoproteins. (**B**) Calcite formed in the presence of calcite-associated glycoproteins. (**C**) Aragonite formed in the presence of aragonite-associated glycoproteins. The formation of calcite and aragonite occurred mainly in the inner part of the substrate, whereas the vaterite crystallized close to the surface. The spherulites of aragonite and vaterite had a morphology completely different from the typical habit of the abiological ones. Only in the calcite crystals was it possible to observe some distinctive crystalline faces and angles. (**D**) Spectra obtained from ground samples (tens of micrograms) in 7-mm KBr pellets. We also obtained the spectra of individual spherulites that were removed mechanically from the substrate and mounted in an infrared transparent diamond pressure cell (High Pressure Diamond Optics, Tucson, Arizona). The spectra were measured with a Midac FTIR (Los Angeles, California) at 4-cm^{-1} resolution. The absorption peaks characteristic of vaterite (spectrum 1) are at 877 cm^{-1} and 744 cm^{-1}, of calcite (spectrum 2) at 876 cm^{-1} and 713 cm^{-1}, and of aragonite (spectrum 3) at 860 cm^{-1} and 713 cm^{-1} (*24*). Additional broad peaks in the region of 1000 to 1100 cm^{-1} and 1650 cm^{-1} are probably due to the chitin and protein associated with the spherulites.

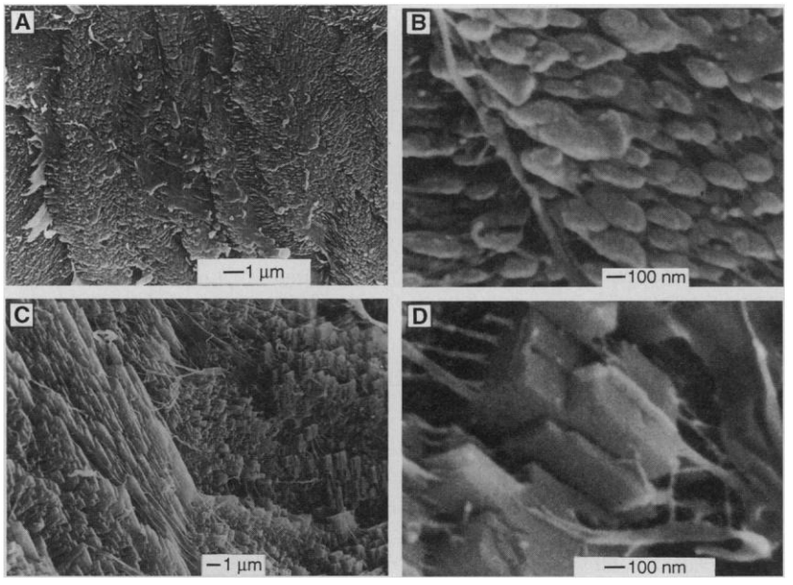

Fig. 2. Scanning electron micrographs of surfaces of the spherulites of calcite and aragonite grown inside the β-chitin–silk fibroin assembly at two different magnifications. The spherulites were mechanically extracted from the substrate, air dried, sputter coated with gold, and observed with a JEOL 6400 scanning electron microscope. (**A** and **B**) Aragonite induced by the soluble glycoproteins extracted from the aragonitic layer of the shell of *Elliptio* sp. (**C** and **D**) Calcite induced from the soluble glycoproteins extracted from the calcitic shell of *M. californianus*. Note the presence of chitin lamellae among the layers of mineral and the differences in crystal morphologies of the aragonite and calcite, as well as the local preferred orientation of the crystallites.

the presence of aragonite-associated macromolecules when the silk fibroin is absent, and it is located close to the surface, where the diffusion of ions is easier. The precipitation of vaterite preferentially occurs in highly supersaturated solutions, which are far from equilibrium (*22*). Thus, the silk fibroin may also influence ion diffusion or the accessibility to the chitin surface or both. This would be in addition to its hypothesized function (*10*) in determining the conformation of the soluble macromolecules on adsorption.

We show that, at least for mollusk shells, one or more shell macromolecules are involved in specific polymorph determination. The exact manner in which this occurs awaits isolation and characterization of these nucleating macromolecules and, in addition, requires an understanding of the roles of the β-chitin and silk fibroin.

Note added in proof: Zaremba *et al.* (*23*) have independently shown that aragonite can be induced to form in vitro on a different substrate obtained from the developing gastropod *Haliotis rufescens* in the presence of matrix macromolecules from the adult shell.

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16. The β-chitin from the pen of the squid *Loligo* sp. (Mediterranean Sea) was purified by refluxing the pen in 1 M NaOH solution by using the procedure of S. E. Darmon and K. M. Rudall [*Discuss. Faraday Soc.* **9**, 251 (1950)]. The β-chitin was then extensively washed with water and stored dry.
17. The silk fibroin was obtained from the cocoon of the silkworm *Bombyx mori*, and a purified solution was prepared by the method described by W. S. Muller, L. A. Samuelson, S. A. Fossey, and D. L. Kaplan [*Langmuir* **9**, 1857 (1993)]. The β-chitin–silk fibroin assembly was produced with the following protocol. One piece (about 1 cm^2) of pure, dry β-chitin was swollen in 10 mM $CaCl_2$ solution for at least 2 hours. The β-chitin was then overlaid with 0.35 ml of 2% silk fibroin water solution in a tissue culture dish (3.5-cm diameter). The assembly of silk and chitin was air-dried. The silk layers were then made insoluble by treatment for 1 hour with 1.5 ml of 80% methanol solution [N. Minoura, M. Tsukada, N. Nagura, *Polymer* **31**, 265 (1990)], followed by rinsing with 10 mM $CaCl_2$ solution. The control films were prepared in the same manner without the chitin.
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19. The soluble macromolecules were extracted from the shells by demineralization with a recently developed ion exchange resin technique (S. Albeck, S. Weiner, L. Addadi, *Chemistry, Eur. J.*, in press), adapted from L. B. Dotti, G. P. Paparo, B. E. Clarke, *Am. J. Clin. Pathol.* **21**, 475 (1951). The macromolecules were extensively dialyzed and centrifuged in such a manner that no nuclei of the original crystalline phase could be present. Their protein concentrations were determined by amino acid analysis after hydrolysis in 6 M HCl under vacuum for 24 hours. Some of the macromolecules were glycosylated, judged by the presence of absorption bands in the infrared spectrum between 1200 to 1000 cm^{-1}. We did not see any correlation between the saccharide/protein ratio (estimated by FTIR), the amino acid composition, and the polymorph formed in vitro. The macromolecules (1.5 ml of solution containing 100 nmol of amino acids per milliliter in 10 mM $CaCl_2$) were adsorbed from solution on the chitin substrates with or without silk (24-hour incubation at room temperature on a rocking table). The solution was removed, and the substrate was extensively rinsed with 10 mM $CaCl_2$ solution. Crystallization was induced by slow diffusion of ammonium carbonate vapor into 1.5 ml of 10 mM $CaCl_2$ solution in a closed desiccator for 2 days. All crystallization experiments were repeated four to six times. Typically, about 80 to 400 spherulites per square centimeter formed, out of which at least 10 were analyzed individually by FTIR a few hours to a few days after crystallization. The spherulites of each given polymorph have a distinctive and homogeneous morphology (Fig. 1).
20. We observed discrete centers from which nucleation occurred from locally supersaturated specific sites. We refer to these crystallization centers as "spherulites" even though their shape is not spherical.
21. Additional experiments with silk fibroin and polystyrene substrates resulted only in the formation of rhombohedral calcite crystals and a small number of vaterite crystals.
22. The empirical observation known as the Ostwald-Lussac rule of stages predicts that the initial mineral formed from a solution supersaturated with respect to more than one mineral is the one with the highest solubility, that is, vaterite. Vaterite was also observed to form under various compressed monolayers, whereas calcite formed only under Ca-stearate monolayers in stoichiometric solutions [S. Mann, B. R. Heywood, S. Rajam, J. B. A. Walker, *J. Phys. D Appl. Phys.* **24**, 154 (1991)].
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