A basic protein, N25, from a mollusk modifies calcium carbonate morphology and shell biomineralization

Dong Yang¹, Yi Yan¹, Xue Yang¹, Jun Liu¹, Guilan Zheng¹, Liping Xie¹*, Rongqing Zhang¹, ²*

¹, Institute of Marine Biotechnology, Collaborative Innovation Center of Deep Sea Biology, School of Life Sciences, Tsinghua University, Beijing 100084, China
², Department of Biotechnology and Biomedicine, Yangtze Delta Region Institute of Tsinghua University, Jiaxing, Zhejiang Province 314006, China

Running title: Functions of N25 on CaCO₃ morphology

* Correspondence should be addressed: Rongqing Zhang: rqzhang@mail.tsinghua.edu.cn; Liping Xie: lpxie@mail.tsinghua.edu.cn; Tel.: +86 010 62776230; fax: +86 010 62772899.

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Abstract

Biomineralization is a widespread biological process in the formation of shells, teeth, or bones. Matrix proteins in biominerals have been widely investigated for their roles in directing biomineralization processes such as crystal morphologies, polymorphs, and orientations. Here, we characterized a basic matrix protein, named mantle protein N25 (N25), identified previously in the Akoya pearl oyster (Pinctada fucata). Unlike some known acidic matrix proteins containing Asp or Glu as possible Ca²⁺-binding residues, we found that N25 is rich in Pro (12.4%), Ser (12.8%), and Lys (8.8%), suggesting it may perform a different function. We used the recombinant protein purified by refolding from inclusion bodies in a Ca(HCO₃)₂ supersaturation system and found that it specifically affects calcite morphologies. An X-ray powder diffraction (XRD) assay revealed that N25 could help delay the transformation of vaterites (a metastable calcium carbonate polymorph) to calcite. We also used fluorescence super-resolution imaging to map the distribution of N25 in CaCO₃ crystals and transfected a recombinant N25-EGFP vector into HEK-293T cells to mimic the native process in which N25 is secreted by mantle epithelial cells and integrated into mineral structures. Our observations suggest N25 specifically affects crystal morphologies and provide evidence that basic proteins lacking acidic groups can also direct biomineralization. We propose that the attachment of N25 to specific sites on CaCO₃ crystals may inhibit some crystal polymorphs or morphological transformation.

Introduction

Biomineralization is a crucial and widespread process among various species from prokaryotic magnetic bacterium to advanced eukaryotic organisms. Typical structures like magnetosome, spicule, shell, teeth, and bone are the products of these processes. These biominerals, which mainly consist of inorganic materials, offer organisms the functions of hunting, navigating and defending.(1,2) Calcium carbonate is one of the most abundant biomineralization materials utilized by plenty of creatures since Ca²⁺ and CO₃²⁻ are common metabolic substance both inside and outside cells and CaCO₃ crystals possesses excellent mechanical properties and plasticity.(3,4) CaCO₃
mainly deposits in three anhydrous crystalline polymorphs, namely calcite, aragonite and vaterite. Amorphous calcium carbonate (ACC) is considered as the precursor of different polymorphs of CaCO$_3$ and exists indeed in several mineral structures, although it is unstable and likely to turn to vaterites before ultimately transforming to other polymorphs in ambient conditions.(1,5)

One of the most concerned themes in biomineralization is the molecular mechanism that regulates the ordered deposition of inorganic ions into solid phases. It has been shown that the organic macromolecules, mainly containing proteins, chitin and other components, may play substantial roles during these processes, though the content of macromolecules is less than 5% of the dry weights of the biominerals.(6,7) Bivalve shell of P. fucata is well investigated in previous researches as a model for pearl formation as well as shell biogenesis.(8,9) The typical structure of the shell is constituted by three primary parts: the periostracum, the prismatic layer mainly composed of calcite and the nacreous layer mainly made of aragonite, also identical to the pearl both in components and structure.(10) In common with other biominerals, shell formation is considered to be under the control of organic materials. A typical model was proposed that the insoluble chitin and silk fibroin built the frame inside which calcites or aragonites were regulated by matrix proteins and deposited into organized structures. (8,11)

Over 50 different kinds of matrix proteins from P. fucata have been reported and some of them were well investigated.(9,10) Protein Pif97 and Pif80 were believed to regulate the lamellar sheets of nacreous layer and during this process, Pif97 functioned as a chitin binding protein and probably recruited Pif80, maybe together with N16, to induce the deposition of aragonite on chitin membrane in specific c-axis orientation.(12-14) Protein Nacrein, isolated from the nacre layer, was a carbonic anhydrase with potential calcium binding sites that may induce the local Ca$^{2+}$ supersaturation followed by nucleating with HCO$_3^-$ generated by its anhydrase activity.(15,16) ACCBP was a specific ACC-binding protein that inhibited undesired growth of aragonite and could assemble to a decamer as a regulator for ACC’s maintenance.(17,18) Other proteins such as Aspein(19,20), MSI60(21), N19(22), PfN23(23), PfN44(24), Prisimalin14(25) and PfY2(26), plus N16 family(12,27), KRMP family(28) and Shematrin family(29), were also thoroughly characterized and reported, however, the functional information about the rest of other components was still poorly understood.

In previous research(30), several candidate matrix proteins genes with tandem repeat sequences(10) were discovered by microarray analysis between different developmental stages of P. fucata from fertilized egg to juvenile stage(5,31,32). These candidate genes were upregulated at least 20 times at the juvenile stage and were thought to be closely related to biomineralization since the typical shell structures begin to form in this period. One of the candidates, protein PfY2, was identified as a novel matrix protein and has been reported as an inhibitory regulator of CaCO$_3$ precipitation(26), while the other candidates are still rarely understood. A deeper investigation was conducted in this study and a new matrix protein-coding gene N25 was cloned and this protein was further identified as a matrix protein exhibiting calcite binding and morphological modifying abilities.

**Results**

**Expression and distribution pattern of N25.**

After cloning the full-length cDNA of N25, it was necessary to figure out the sites where N25 was synthesized and expressed and the position...
where N25 protein located. The RT-PCR assay was conducted in different tissues including foot, viscus, gonad, mantle edge, mantle pallial, gill and adductor muscle, to reveal the tissue-specific expression pattern. The result showed that the N25 mRNA was narrowly synthesized in the mantle tissue, which was thought to be the most substantial organ directing the shell formation, with a much higher amount than other tissues (Figure 1D). Correspondingly, N25 protein was also detected in both the extracts from the decalcified prismatic insoluble matrix (PISM) and nacreous insoluble matrix (NISM) while the signal from PISM was stronger than that of NISM (Figure 1C). These results implied that N25 was mainly expressed in mantle tissue and finally integrated into shell structure as a part of the insoluble frame.

Synthesis and secretion of N25 in HEK-293T.

It is believed that the mantle tissue mainly controls shell formation, during which cells facing the inner side of the shell are responsible for the secretion of matrix proteins and other components. However, it is unavailable to observe the process of how N25 was synthesized and secreted by the mantle epithelial cells due to the lack of both appropriate bivalve cell line and efficient gene transduction system. As a compromise, the eukaryotic 293T cell was chosen as a substitute. N25-EGFP with secretion signal peptide (Figure S1) was expressed initially and distributed uniformly in the cytoplasm, and the subsequent appearance of the tiny fluorescence spots represented the local regions with highly condensed recombinant protein. We speculated these spots were vesicles. What’s more, we found that the average fluorescence intensity of N25-EGFP decreased after imaging the cells with a time-lapse method. The absolute intensity of the region of interest o (ROIo) declined significantly from 0 min to 42 min (Figure 2E) and correspondingly, the amounts of spots in ROIo deceased compared to 0 min (Figure 2A, 2B). The region of interest c (ROIc) was chosen as a control to eliminate and minimize the possibility that the decreased fluorescent intensity induced by nonspecific photobleaching. The relative intensity of ROIo, which also dropped at 42 min (Figure 2F), was represented by the intensity ratio of ROIo to ROIc to subtract the influence of the decline of the base intensity. The motion image was available in Movie S1. To further investigate the exact secretion process of a single vesicle we performed a TIRF imaging of the cells and only these vesicles that located near or even anchored to the bottom membrane were observed. Figure 2G to 2J indicated a secretion process, during which a spot disappeared by rapid diffusion between 130 s and 130.3 s, as indicated with the white arrows. The dynamic image was available in Movie S2. It could be concluded from these observations that the predicted signal peptide of N25 worked in the eukaryotic cell and involved the synthetic proteins into a secretion pathway, which could mimic the similar events in mantle cells.

Expression and purification of N25.

Several methods have been applied to explore the expression output of protein in E.coli, however, it was fairly tough to get condensed and relatively pure protein N25 because most of the protein existed in inclusion bodies except a tiny amount in soluble form. The inclusion bodies were washed with 1% Triton X-100 and dissolved in denaturant followed by purification with affinitive chromatography. Denatured N25 was further linearized by reducing disulfide bonds with DTT and dropped into a refolding environment with no denaturant, leading to an instant dilution of N25 into a low enough concentration that prevented the unfolded N25 from aggregating, then the protein molecule
would refold spontaneously to a stable native packed conformation. After affinity chromatography with Ni-NTA, the relatively pure N25 was harvested and condensed by ultrafiltration before analyzing by SDS-PAGE (Figure 1A). The apparent molecular weight of N25 was about 35 kD, consistent with the bands in Figure 1C, which nevertheless was different from the calculated value of 25 kD. To further identify the purified protein, the band in SDS-PAGE was analyzed and confirmed by mass spectrum (Figure S5). The post-translational modifications analysis (Table S1) and practical MW measurement (Figure S6) were also conducted by LC-MS and QTOF-MS respectively. The measured MW of N25 was 25776 Da with extra 124 Da larger than the predicted value of 25652 Da. It could be speculated that the molecule structure or shape probably contributed more to the migration alteration.

**Binding of N25 to CaCO₃ and chitin.**

CaCO₃ and chitin binding assay was performed to explore the affinity properties and interaction between N25 and different crystal polymorphs since the attachment of proteins might play critical roles in regulating the crystal characteristic. Protein N25 was mixed with calcite, aragonite, and chitin respectively and incubated for 2 hours at 4 °C. 1% Triton X-100 was applied to wash away the nonspecifically absorbent proteins before analyzing the samples by SDS-PAGE. Components remained after washing were thought to have a relative strong interaction with the solid materials. As was shown in Figure 1B, protein N25 showed the ability to bind all of these three materials while the calcite binding was a bit stronger than that of aragonite, both of which were obviously higher than chitin. As a contrast, the maltose binding protein (MBP), set as a negative control, showed no affinity for any of the materials. These data demonstrated that N25 attached specifically to calcite, aragonite, and chitin, implying its natural integration into the shell components.

**N25 stabilized the vaterite during ACC transformation assay.**

The influence of N25 on the transformation of ACC to stable crystalline was estimated by the ACC transition assay. ACC formed immediately at the moment Ca²⁺ was mixed with CO₃²⁻ and transformed to calcite rapidly in less than 15 min (Figure 3, buffer group). The compositions of buffer groups all changed into calcites at 15 min, 35 min and 55 min (Figure 3). MBP proteins present in the reaction solution would slightly delay the transition to calcites from vaterites (Figure 3, MBP group), a kind of crystal form that was considered as a metastable polymorph of CaCO₃ and would finally change to calcites long enough the time. At 15 min, there was 29.9% percentage of vaterites remained in the MBP group and the proportion declined to 22.7% at 35 min and finally to 16.7% at 55 min. As a contrast, vaterites in N25 groups maintained for a longer period with a percentage of 51.3% at 15 min and 51.8% at 55 min, while at 35 min the proportion was 39.8%, which was relatively lower compared with the other time points but still significantly higher than those of the MBP groups. These observations and results indicated that N25 protein might help to stabilize the vaterites and delay its transformation into calcites.

**In vitro calcium carbonate crystallization assays.**

The effects of N25 on the morphology and polymorph of CaCO₃ crystals were explored by its addition to the reaction system containing saturated Ca(HCO₃)₂ solution, constituted by mixing Ca²⁺ and HCO₃⁻ solution to an ultimate Ca²⁺ concentration of about 8 mM. Compared to the typical rhombohedral calcite crystals in
control group supplied with storage buffer (Figure 4A,4A1), the crystals in the MBP group that contained 200 μg/mL MBP protein showed no obvious morphology alteration (Figure 4B,4B1) while the crystals in N25 group showed significant changes in crystal morphologies (Figure 4C,4C1). The degree of the morphology alteration increased with elevated concentrations of N25 from 4 μg/mL to 40 μg/mL and 200 μg/mL respectively (Figure 4D-4F). We noticed that the rhombohedral faces began to shrink at the edges and corners while some extra growth structures also arose randomly and irregularly on the faces of crystal particles. At the 4th day, Figure 4G showed an extreme status of the abnormal morphology that might be caused by a complicated cooperative effect contributed by extra over-growing on each faces together with the inhibition of the corners and edges. Raman spectrum was introduced to identify the crystal forms of these particles and indicated that most of the particles in each group were calcites with specific peaks at around 283, 713 and 1088 cm⁻¹, except these spherical particles indicated with black arrows in Figure 4C and 4G, whose characteristic peaks were 301, 620, 1075 and 1091 cm⁻¹, illustrating its vaterite property (Figure 4H). These data showed that N25 may play critical roles in regulating calcite morphology.

Distribution of N25 in calcite crystals and the computational simulation for morphology.

In order to get deeper understandings on how N25 affected the calcite crystallization, the distribution mapping assays were conducted by labeling N25 with fluorescent dye Cy5-NHS ester before adding to the calcite crystallization reaction. The super-resolution images were harvested with Z-axis stacks at a Z resolution of 0.1 μm and reconstructed to a 3D image by a Nikon structured illumination microscopy (N-SIM) at an excitation wavelength of 645 nm. It could be noticed that the fluorescent signal showed inhomogenous distribution from the front view (Figure 5A1) and the side view (Figure 5A2), moreover, protein N25 seemed to encircle the crystal and reside on the surfaces. To further confirm this observation, the fluorescent intensity was analyzed for each section slice and the 2D histograms were presented for slice 23, 53 and 83 respectively (Figure B1-B3, C1-C3), in which most of the signal pixels distributed in a circular pattern without fluorescent signals inside the crystals. A surface simulation was performed to further clarify the N25 distribution pattern by replacing the red fluorescent signals with white granules. These white granules were shown to attach to the main faces and the shrunk borders of calcite crystal habit (Figure 5D2), which could be correlated to the contractive edges in Figure 5E2. A smaller signal ring, indicated by the white arrow in Figure 5D1, arose inside the large fluorescent circle near the bottom of the glass plate and was corresponded to the irregularly sinking cavities presented and indicated in Figure 5E1 with a white arrow. We then mimicked the theoretic morphology and crystal habit in normal condition and specific situation with modified morphological parameters. The growth equilibrium theory was proposed to predict the normal morphology according to the structure of calcite and a hexahedron shaped crystal habit was generated (Figure 5F1), which was consistent with the typical crystal habit of calcite in ambient condition. Several growth faces were listed with different growth rates while the {1 0 4}, {0 1 8}, {1 1 0}, {0 1 2} and {0 0 6} were the five most possible growth faces with relatively low growth rates. The final habit of calcite showed only one face {1 0 4} because this face showed the lowest growth rate and constituted the main crystal habit. In a modified condition (Table 1), the distances of other faces were decreased to 5920.8, 6720.8,
7740.6 and 6640.6 respectively and induced these faces to emerge (Figure 5F2). When further decreasing the distances to 4920.8, 5720.8, 6030.6 and 5040.6 respectively, the area of these faces expanded while the \{1 0 4\} face area declined correspondingly (Figure 5F3). Parameters that were utilized for calculation were listed in Table 1. We could infer from these observations that N25 bound to specific crystal surfaces of calcites firstly and then modified the morphology potentially through increasing the attachment energy and decelerating the growth of these crystal faces.

**Discussion**

Matrix proteins have been the extensively researched components as one of the three main ingredients of shell biominerals besides CaCO$_3$ and chitin frame. They are also thought to be critical for regulating the structure constitution.(10) The matrix proteins profiles of prismatic layer differed from that of nacreous layer though some kinds of proteins existed in both structures.(37-39) The difference in protein distribution may contribute to the prior precipitation of prismatic calcite followed by the growth of nacreous tablets.(40,41) Proteins from both of the layers existed in two main states: soluble or insoluble proteins in EDTA solution. The EDTA-soluble matrix proteins were thought to regulate the precipitation rate, crystal polymorph and/or morphology, while the EDTA-insoluble proteins mainly worked as a part of the frame of the shell and were very likely to function in regulating the crystallization nucleation sites and crystal orientation.(40,42)

In this study, a novel matrix protein N25 was identified as a matrix protein and its properties in CaCO$_3$ crystallization were explored from different levels. The $N25$ gene expression pattern during several tissues showed its specific location in the mantle tissue whose edge region expressed more N25 proteins than the pallial regions, while much weaker signals were detected in other tissues (Figure 1D). Based on previous reports, proteins in mantle edge were mainly responsible for controlling of the prismatic layer while proteins in mantle pallial were thought to regulate nacre formation.(43,44) Protein N25 could be detected in both layers by Western blot, nevertheless, the protein level in the prismatic layer was markedly higher than that of the nacreous layer (Figure 1C), which was corresponding to the results of RT-PCR.

The knowledge about the secretion of matrix proteins was insufficient because it was not convenient enough to monitor the protein behaviors inside mantle cells since no bivalve cell line was available for transfection.(45) In this study, we utilized the mammalian 293T cells instead and successfully observed this process (Figure 2). The analysis of the primary amino sequence of N25 demonstrated that there was a typical signal peptide in the N-terminal (Figure S1), implying the possibility of secretory simulation by 293T cells. (46,47) During the longtime imaging with Delta vision, we observed the decrease of the average fluorescence intensity probably caused by the releasing of the hypothetical secretion vesicles from the membrane, which was a rapid diffusion process accomplished within 300 ms. In the actual mantle cells of *P. fucata*, there might be a similar routine from the synthesis of N25 or other matrix proteins to their secretion, after which these proteins could bind to particular sites of the chitin frame or crystals, as indicated by the binding assays *in vitro* (Figure 1B). It could be considered that N25-EGFP underwent secretory post-translation processing that mimicked the natural event happening in mantle epithelia and the signal peptide prediction principle was likely to work for matrix proteins of bivalve.

Based on the primary sequence, the
molecular weight of N25 was theoretically calculated to be 25 kD and the predicted PI was 9.21, indicating that N25 was a basic protein and unlike the common known acidic matrix proteins.(48) Acidic carboxyl of glutamic or aspartic acid residue was considered as substantial and potential calcium-binding groups that could recruit and induce a local calcium saturated site, leading to the initial nucleation of CaCO$_3$.(20,49) However, N25 was rich in Ser (13.9%) and Pro (13.5%) but insufficient in acidic residues (6.9%). However, it could be noticed there were considerable amounts of Asn (7.8%), Gln (5.7%) and Lys (9.1%) residues, all of which have a free amino reside at the terminal of the side chain. Consequently, we inferred that both the carboxyl groups and the hydrogen bonding amino groups of N25 were able to interact with the certain crystal surfaces of CaCO$_3$ since carboxyl was able to interact with Ca$^{2+}$ while amino group probably contributed to the interaction with HCO$_3^-$.(50,51) The prediction results of N25 protein secondary (Figure S2) and tertiary structure (Figure S3) demonstrated that N25 was likely to take a disordered and highly flexible configuration that might assist N25 with attaching to the crystal surfaces.(52) We assumed N25 might possess the binding affinity for free Ca$^{2+}$ or CO$_3^{2-}$ as an extension of CaCO$_3$ binding capability, however, the isothermal titration calorimetry (ITC) assays showed a negative result, namely the titrations with Ca$^{2+}$, CO$_3^{2-}$ or HCO$_3^-$ caused no obvious exothermic or endothermic effects (Figure S4). It seemed to be contradictory, but it could be interpreted that N25 may interact with specific ions arrays on certain surfaces through its flexible configuration, even the molecule itself showed little ability to combine free ions in solution.

N25 also exhibited the characteristic in modifying the calcite crystal morphology. When N25 was introduced to the precipitation reaction, the borders around the main crystal surfaces of the typical rhombohedral calcite started to depauperate and shrink (Figure 4C,4E1), besides some additional structures also formed on the main surfaces (Figure 4F, 4G1). Based on these observations, it could be postulated that both the border shrinking and extra structures growing contributed to the morphology alteration. In the N25 groups with a concentration of 200 μg/mL, we observed considerable amounts of spherical vaterite forming and distributing around other calcite particles randomly, however, the diameter of these vaterites declined when deposition time extended to 4 or 5 days, as shown in Figure 4C and 4G. Consistent with our in vitro crystallization results, the ACC transforming assay showed a similar effect that during the early precipitation period, there was a relative long vaterite phase in both MBP and N25 groups before transition to calcite phase, yet the vaterite in N25 group maintained more stable than that of the MBP group (Figure 3). Most of the proteins show a trend to attach on solid phase because of the weak interaction contributed by the charge on molecule surface. Mild detergent Triton-X100 could disrupt these weak and nonspecific interactions between protein and the crystal without obstructing the specific interactions, thus it was the specific attachment of N25 to CaCO$_3$ that made it unable to be washed away in the binding assay (Figure 1B). We supposed that the molecules specifically binding to the surface of vaterite might suppress the subsequent transformation to other stable types of polymorphs, which led to the longer existence of vaterite in the N25 ambiance.

It has been reported that matrix proteins might distribute around the surfaces of CaCO$_3$ or be integrated inside the crystals in vivo during the formation of biomineral.(54) In this research, N25 seemed to take the former way. The Cy5-conjugated N25 involved in the crystallization
assay illustrated the distribution pattern that signal was only detected on the crystal surfaces, including the main faces of calcite and the edges of each face, while almost no N25-Cy5 fluorescence was present inside the crystal (Figure 5C1-C3). It could be supposed the effect of N25’s adhesion to CaCO₃ changed the crystal morphologies. Considering the PBC theory and the attachment energy (E-att) theory,(55,56) crystal morphology is controlled by a series of bonds between the crystal lattice while the attachment energy is the energy released when a newly formed layer slice is added to a certain crystal face. Theoretically, crystal consists of countless faces and each face has a specific E-att since the bonds and interactions within the lattice of the face differ from each other. The relative growth rate of a certain face is proportion to the E-att so that faces with larger E-att absolute values grow faster than those with lower E-att.(57) As a result, the most slowly growing faces encircle a volume and construct the crystal habit or morphology because fast growth would lead to the disappearance of a face. Taking these principles into account, we performed a calculated simulation with a computer to predict the morphology and elucidate the habit alteration. The calcite structure cell was constructed and the possible growth face list was generated, followed by calculating the E-att and relative distance, which represented the distance from the face to the growth center. The distance was proportion to the growth rate, therefore it could be defined equal to the absolute value of E-att. The common rhombohedral shaped calcite habit was presented according to the setting parameters (Figure 5F1) with only one kind of face {1 0 4} whose distance was 4960.4. Faces listed in Table 1 were the most probable faces because of their relative lower E-att values, however, faces {1 0 4} were the lowest and the most important faces for normal calcite morphology while other faces were absent in most situations. When the E-att and the distances of other faces were modified to 5920.8, 6720.8, 7740.6 and 6640.6 respectively (Table 1), the habit changed with the edges of each face to shrink, caused by the altered distances which were shortened enough for these hidden faces to emerge. If further shifting the E-att and distances to 4920, 5720, 6030 and 5040, these faces areas expanded, leading to the apparently increased intensity of the border shrink (Figure 5F3). These predicted habits could be correlated to the actual calcite crystal in Figure 5E2, the exposed faces indicated with a yellow arrow in Figure E3 might represent the face {0 1 8} or face {1 1 0}. Based on these results, we deduced that N25 bound to certain CaCO₃ growth faces first and then induced the attachment energies to decline, followed by slowing down the growth rates of some crystal faces and ultimately altered the morphology of CaCO₃.

A schematic diagram was summarized in Figure 6 to explain the process from protein secretion to calcite binding and morphology alteration. Because of its narrow expression pattern, gene N25 should be upregulated by a specific pathway, although unknown at present, then locally expressed and secreted by the mantle cells facing the inner side of shell in a manner of vesicle-membrane fusion. The released protein N25, probably working synergistically with other matrix proteins, then bound to vaterite and prolonged its existence. Vaterite may transform to calcite in two ways simultaneously, that is by directly inner transformation of the lattice of vaterite or by the Ostwald ripening method,(56) in which the Ca²⁺ and CO₃²⁻ in vaterite will redissolve into free ions and re-deposit to a larger and more stable particle, since the larger crystal had lower surface area to volume ratio, more stable bonds, and thus lowered total energy. Both of the two ways might be delayed as a result of the attachment of N25, leading to crystal to inhibit...
the lattice rearrangement and the releasing of ions into solution. On the other side, calcite crystals could also grow directly from an initial nucleation core followed by free ions deposition onto their surfaces. According to the predicted attachment energy, the face \{1 0 4\} has the lowest attachment energy, making it the most important face for the calcite morphology in normal aqueous environment. N25 affected calcite morphology through the interaction between protein molecule and immobilized ions of calcite, which might block the sites for newly-formed crystal layer, thus increased the energy cost for deposition and decreased the growth rates of some faces. And probably due to the difference in bounding intensity, the relative growth rates of several faces such as \{1 1 0\} and \{0 1 8\} declined more rapidly than that of \{1 0 4\}, causing the regular edges and corners to shrink and the \{1 0 4\} faces to externally grow with the help of redissolve of small vaterite particles. The exact physiological meaning of morphology modification has not been fully understood, we speculate that morphology alteration might be crucial for the formation of some specific structures, or might be just a concomitant phenomenon of inhibitory regulation of the crystal growth. Since there have been several reports on the inhibitory effects of matrix proteins, it could be suggested that N25 might integrally and subtly work together with other matrix proteins to regulate the crystallization.

**Conclusion**

In this work, the novel matrix protein N25 functions as a morphology regulator of CaCO$_3$ through the attachment to the crystal surfaces. A computer simulation implies N25 protein may induce the decrease of certain face attachment energy and finally alter calcite morphology. This study extends the understanding of biomineralization regulation by matrix proteins and provides a possible aspect of exploring the mechanism by combining simulative method and experimental observation.

**Experimental Procedures.**

**Ethics statement:** All animal studies were approved by the Animal Ethics Committee of Tsinghua University, Beijing, China.

**Animals:** Pearl oysters, *P. fucata*, were from the Pearl Farm in Zhan Jiang of China and were cultivated in artificial seawater. These animals were fed with yeast or spirulina powder dissolved in seawater every 3 days.

**Gene cloning and sequence analysis:** Total RNA was extracted from mantle tissue with the standard protocol of TRIzol reagent (Life Technology, Thermo, USA) for general cloning of the genes. The RNA concentration was measured with NanoDrop2000 at 260 nm (Life Technology, Thermo, USA). The quality of RNA was evaluated by agarose gel and OD$_{260/280}$ plus OD$_{260/230}$. For RACE PCR, the cDNA was reverse transcribed by SMARTer RACE cDNA Amplification Kit (Clontech, Takara, Japan). For Realtime-PCR (RT-PCR), RNAs from mantle edge, mantle pallial, adductor muscle, gill, foot, gonad, and viscus were extracted the same way and each cDNA template was synthesized from 600 ng total RNA with PrimeScript™ RT Master Mix (Takara, Japan) respectively. Primers N25-SP1-F, N25-SP1-F, N25-SP2-F, N25-SP3-R were used in 3’RACE together with the combination of primers UPMlong, UPMshort and NUP. Primers N25-SP1-R, N25-SP2-R, N25-SP3-R were used in 5’RACE the same way, following the standard procedures. The whole sequence of N25 was confirmed with primers N25-F and N25-R. Putative protein sequence was predicted online with the ExPASy Translate tool (https://web.expasy.org/translate/) and the signal
peptide was analyzed by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/).

**Plasmids construction:** Gene sequence without signal peptide was amplified by PCR with primers N25NOSIG-28NCOL and N25NOSIG-28RXHOI, then inserted into the prokaryotic expression vector pET-28a(+), giving the recombinant plasmid pET28a-N25C with an 8 X His tail at the C terminal of N25 protein. Gene sequence with the native signal peptide was magnified with primers N25EGFPN1-XHOL and N25EGFPN1-KPNR followed by inserting into the eukaryotic mammalian expression vector pEGFP-N1, generating the recombinant vector pEGFPN1-N25, which produced the corresponding fusion protein N25-EGFP with EGFP at the C terminal of N25.

**N25 expression specificity analysis with RT-PCR:** cDNA templates from seven main tissues were applied to detect the mRNA abundance of N25 by RT-PCR in the LightCycler 480II (Roche) with gene Actin as an internal control. Primers RT-25F, RT-25R, RT-ACTIN-F, and RT-ACTIN-R were used with SYBR Premix Ex Taq™ (Tli RNase H Plus) kit (Takara, Japan) accordingly. All primers are listed in Table 2.

**Distribution of N25 in different shell extracts:** The nacreous layer was separated from and prismatic layer by manually polishing, then they were decalcified by EDTA to extract the EDTA-soluble matrix (SM) and EDTA-insoluble matrix (ISM), respectively. NSM and PSM were obtained after dialyzing and condensing the EDTA solution with ultrafiltration, while NISM and PISM were harvested by boiling the insoluble pellet of the shell in the protein loading buffer for SDS-PAGE. Protein N25 was detected by Western blot with polyclonal antibody from rabbit.

**Protein expression and purification:** Purified plasmid pET28a-N25C was used to transform *Escherichia coli* strain Transsetta (DE3) (Transgene, China) for expression. Transformed *E.coli* was cultured in LB medium at 37°C 200 rpm and induced with 0.6 mM IPTG (isopropyl 1-thio-β-D-galactopyranoside, SIGMA, USA) when OD600 reached 0.7-0.8, then cultured at 37°C 200 rpm for another 12 h. *E.coli* cell pellet was collected after centrifugation at 6000 g for 5 min, 4 °C and then suspended in lysis buffer (50 mM Tris, 100 mM NaCl, 5% glycerol, 1 mM DTT, 1 mM EDTA, pH=8.0), after which the ultrasonic homogeniser was applied to disrupt the bacteria cells. After centrifuging at 15000 g for 10 min, the pellet was resuspended and washed in lysis buffer with 1% Triton-X100 (Promega, USA) to isolate the inclusion bodies of N25. The washed inclusion bodies were dispersed and dissolved in lysis buffer with 6 M urea (binding buffer) and the filtered supernatant was applied to 1 ml Ni-NTA resin (Sangon Biotech, China) column. The resin was washed with 30 mM imidazole in the binding buffer before eluting with 500 mM imidazole, which was also prepared with binding buffer containing 6 M urea. The fractions were collected, boiled and loaded to SDS-PAGE and the corresponding bands were cut and utilized to immunize the rabbit.

**Protein mass spectrometry (MS) analysis:** For identification of the peptides coverage and post-translational modifications (PTM), N25 protein was subjected to SDS-PAGE and the band was excised. The protein sample in the gel band was then treated by the method described in the literature before LC-MS analyzing with OrbiTrap Fusion LUMOS (Thermo Fisher, USA). Trypsin was used to digest the protein and the missed cleavage site value was set to 2 when searching the sequence. The PTMs results were described in Table S1. For measuring relatively
precise molecule weight (MW), purified N25 protein was dialyzed against the sample buffer (2mM Tris, 30mM NaCl, pH=7.8), then the sample was analyzed by QTOF in a SYNAPTTM G2-Si HDMS system (Waters, USA) and results were presented in Figure S6. All of the tests and data analyses were conducted in the Center of Biomedical Analysis, Tsinghua University.

**Protein refolding:** Resembling the method mentioned above, the inclusion bodies were dissolved in lysis buffer, however, with 6 M Guanidine HCl instead of urea and 1 mM DTT for 1 h at room temperature, then centrifuged and filtered with 0.45 μm filter. The concentration of this stock solution should be adjusted to 1-2 mg/ml. 3 mL of the stock solution was added drop by drop into 200 mL of the rapidly stirred refolding buffer (20 mM Tris, 500 mM NaCl, pH=8.0) at the 1/2 of the vortex cone within 10 min.(34) After the dilution, the solution was kept still for 1 h then filtered with 0.45 μm filter before loading to the balanced Ni-NTA resin. The resin was washed with 30 mM imidazole and ultimately eluted with 500 mM imidazole, both of which were prepared with refolding buffer. N25 was dialyzed to storage buffer (20 mM Tris, 500 mM NaCl, pH=7.8) and the concentration was determined by NanoDrop2000 (Thermo) at A280nm and detected by SDS-PAGE.

**Calcium carbonate and chitin binding assay:** 0.1 g calcite, aragonite (Alfa Aesar) or chitin (Biodee, China) was washed and supplemented with 1 mL storage buffer respectively. Each material was mixed with 30 μg of protein N25, while the control group was supplied with equal maltose binding protein (MBP) that was purified following the instruction of pMAL-c5x (NEB). The mixtures were rolled and incubated at 4°C for 2 h, then centrifuged to remove the supernatant. The pellets were suspended with 1 ml storage buffer 3 times, followed by suspending in storage buffer containing 1% TritonX-100 for 6 times, ending with washing again with storage buffer for 3 times. The washed materials were ultimately treated with loading buffer and analyzed by SDS-PAGE.

**The synthesis and secretion of N25-EGFP in the eukaryotic cell:** HEK-293T cells (China Infrastructure of Cell Line Resources, China) was thawed and cultured in DMEM medium (Gibco, USA) with 10% FBS (fetal bovine serum) at 37 ºC with 5% CO2. Plasmid pEGFPN1-N25 was transfected into the HEK-293T cells via Vigofect (Vigorous, China) according to the manufacturer instruction. 0.5 μg vector was transfected into cells cultured in a 35 mm confocal dish and 12 h after transfection the medium was replaced with fresh complete DMEM, then the cells were cultivated for another 36 h. These cells were imaged directly by Delta Vision time-lapse system (GE, USA) by recording every 2 minutes for about 3 h and the results were processed with ImageJ software. For observing the secretion process by Ti microscope TIRF(35) system (Nikon, Japan), cells cultivated for 36 h were digested with trypsin and diluted to separated individual cells, then plated to a new confocal dish. Six hours later, the medium was replaced with fresh DMEM without FBS and cultured for another 12 h before imaging.

**In vitro CaCO₃ crystallization:** The saturated calcium bicarbonate solution was prepared by mixing 100 mM NaHCO₃ and 50 mM CaCl₂ to a final Ca²⁺ concentration of 8 mM(18,36) in Milli-Q water immediately before mixing with proteins. 15 μL saturated solution was mixed respectively with 5 μL protein samples whose concentration were uniformly modified to 4 μg/mL, 40 μg/mL and 200 μg/mL, respectively. MBP was set as a control with a final concentration of 200 μg/mL.
The mixtures were loaded on a silicified cover plate and incubated in an enclosed box supplied with water at room temperature for 48 h. Crystals were washed gently with Milli-Q water and dried in air, then a LabRAM HR Evolution system was applied to analyze the crystal polymorphs with an extinction wavelength at 514 nm and scanning range from 100 to 1500 cm\(^{-1}\). FEI Quanta 200 SEM was introduced to image the crystals.

**ACC transformation assay**: N25 was diluted into 7.5 mL of 50 mM CaCl\(_2\) solution to a final concentration of 130 μg/mL, then 7.5 ml of 50 mM Na\(_2\)CO\(_3\) was added into this solution. Turn the tube upside down several times immediately. Three replicates were repeated and the reactions were terminated at 15 min, 35 min and 50 min respectively by centrifuging and washing the pellets with ethanol three times. The pellets were dried in air and analyzed via the powder XRD.

**Cy5 labeling and SIM imaging**: N25 protein was dialyzed to the labeling buffer (0.1 M phosphate, 500 mM NaCl, pH=8.3) and the concentration was estimated by A280. 1 mg Cy5 NHS ester dye (AAT Bioquest, USA) was dissolved in 100 μL DMSO, then diluted to 0.1 μg/μL with DMSO and formed the dye solution. The amount of dye (mg) used was calculated via an empirical formula: 0.01 \times 855 \times \text{weight of protein (mg)/25000}. Then the dye solution was added into the dialyzed N25 and mixed quickly, followed by putting the solution at a dark box for 30 min. The labeled N25 was desalted into the storage buffer (20 mM Tris-HCl, 500 mM NaCl, pH=7.8 by HiTrap TM Desalting (GE, USA) in AKTA system (GE, USA)). The labeled N25 was further used in *in vitro* CaCO\(_3\) crystallization assay and after incubating for 48 h, the crystals were washed and air dried before imaging with a super-resolution Ti NSIM system (structured illumination microscopy, Nikon, Japan).

**Image processing and editing**: All images of this research were processed in Photoshop CC2017, figures generated from Origin2018, the surface render was dealt with and processed by iMaris, and the morphology simulation was calculated with Materials Studio following the manual instruction.
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Author contributions
DY, LPX, and RQZ conceived and planned the project. DY carried out the experiments and data collection. DY, YY performed the data analysis. YY, XY, JL, GLZ contributed to materials, reagents and scientific discussion. DY, RQZ wrote the manuscript.

Conflict of interest
The authors declare there exist no competing interests.
Functions of N25 on CaCO₃ morphology

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FOOTNOTES
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The abbreviations: ACC, Amorphous calcium carbonate; ACCBP, ACC-binding protein; ISM, EDTA-insoluble matrix; MBP, maltose binding protein; E-att, attachment energy.

| hkl   | Multiplicity | E-att       | Distance | Modified E-att A | Modified Distance A | Modified E-att B | Modified Distance B |
|-------|--------------|-------------|----------|------------------|---------------------|------------------|---------------------|
| {1 0 4} | 6            | -4960.4     | 4960.4   | -4960.4          | 4960.4              | -4960.4          | 4960.4              |
| {0 1 8} | 6            | -9920.8     | 9920.8   | -5920.8          | 5920.8              | -4920.8          | 4920.8              |
| {1 1 0} | 6            | -9920.8     | 9920.8   | -6720.8          | 6720.8              | -5720.8          | 5720.8              |
| {0 1 2} | 6            | -8940.6     | 8940.6   | -7740.6          | 7740.6              | -6030.6          | 6030.6              |
| {0 0 6} | 2            | -7940.6     | 7940.6   | -6640.6          | 6640.6              | -5040.6          | 5040.6              |

Table 1 lists the parameters used in simulating the morphology of calcite in this work. The hkl indicated the miller indices of the crystal growth face. Multiplicity indicated the number of faces with equivalent indices. E-att was the attachment energy, usually a negative value. The distance was proportional to |E-att| and in this case the coefficient was defined as 1, so that the distance was identical to |E-att|.
Table 2 Primer summary

| Primer category          | Sequence                        |
|--------------------------|---------------------------------|
| General Primers          |                                 |
| RV-M                     | GAGCGGATAACAATTTCAACAGAG        |
| M13-47                   | CGCCAGGGTTTTCCCAGTCAGCAG       |
| T7                       | TAATACGACTCACTATAGGG           |
| T7TER                    | TGCTAGTTATAGCTCAGCGG           |
| PEGFPN5                  | TGGGAGGTCTATATAAGCAGAG         |
| PEGFPN3                  | CGTCGCGGTCCAGCTCGACCAG         |
| Primers for pET-28a and pEGFP-N1 |                 |
| N25EGFPN1-XHOL           | TACCGGAATCTACAGTCGCCACCATGAAACGGATTATGTTTC |
| N25EGFPN1-KPNR           | ATCCCGGGGCCCGGGATTGGCCTGTGATCTTG |
| N25NOSIG-28NCOL          | TAAGAAGGAGATATACCATGCAAAAGAAATCCAAAGATAA |
| N25NOSIG-28RXH01         | TCAGTGGGTGTCGATGTCGATGCTGATCTTG |
| Primers for RT-PCR       |                                 |
| RT-25F                   | CCTCCAACATCACGTAATACGGCAAC     |
| RT-25R                   | CCCTGGTTTTTCATTCGGCATGTTTC     |
| RT-ACTIN-F               | CTGGTGGTTCTACCTACATGCTCCAGG    |
| RT-ACTIN-R               | GATGGACCGGACCTCGATCCTGATCTCTG  |
| Primers for RACE         |                                 |
| N25-SP1-F                | GATCAAGGATACAGAATTCGGGACC      |
| N25-SP2-F                | ATGCCAATGGGCGGACCACCACCGG      |
| N25-SP3-F                | CCATCATCTACAGAATATGAACATGCCG   |
| N25-SP1-R                | CTAGTTTTTCATTTTCGCTGGACC       |
| N25-SP2-R                | CAGGAGACTTGGCCTTTGGCCCGCG      |
| N25-SP3-R                | GGATTTCTTTTTCGCTCCCGC          |
| UPMlong                  | CTAATACGACTCATAAGGCAAGCAGTGGATCAACGCAGATG |
| UPMshort                 | CTAATACGACTCATAAGGGA           |
| NUP                      | AAGCAGTGATCAACGCAGAGT          |
| N25-F                    | CGGAAATACATACGTAGACCTTTAG      |
| N25-R                    | TGGCAGTAAAATATGTGTTAATG        |
Figure 1. Binding assay and the distribution of protein N25. A, a denaturation-renaturation method was applied to the purification of N25 followed by detecting with SDS-PAGE; B, Purified N25 was mixed with calcite, aragonite and chitin respectively; MBP was set as a control and all materials were washed and boiled before SDS-PAGE. MBP had a calculated molecular mass of 45 kD, N25 protein was near 35 kD. C, N25 protein localization in four matrix extracts from nacreous layer and prismatic layer was analyzed by Western Blotting; D, gene expression pattern of N25 in various tissues was decided by RT-PCR. NSM, nacreous EDTA-soluble matrix; NISM, nacreous EDTA-insoluble matrix; PSM, prismatic EDTA-soluble matrix; PISM, prismatic EDTA-insoluble matrix.
Figure 2. Observation of protein N25-EGFP expression and its secretion from HEK-293T via Delta-vision and TIRFM. In one time-lapse observation from 0 min to 156 min (A-C), the intensity of region c showed little decrease (D) while region o declined obviously (E). F, relative intensity of region o to c. G-J, representative time points of the continuous imaging with TIRFM, the white arrows indicated a secretary vesicle. Scale bars in C, J were 10 μm.
Figure 3. Powder XRD assays of ACC transforming. Three groups of 15 min, 35 min and 55 min were shown. Buf, buffer group; MBP, maltose binding protein group; N25, N25 group; v, vaterites; c, calcites.
Functions of N25 on CaCO\textsubscript{3} morphology

**Figure 4.** Crystallization of calcium carbonate with protein additives. A was buffer group, B and C were MBP and N25 group respectively, with a concentration of 200 μg/mL. A1-C1 were the magnified photos of the corresponding regions of white frames, the black arrow in C indicated a partially dissolved vaterite and the magnified image was presented in upper right. D-F, N25 groups with the concentration of 4 μg/mL, 40 μg/mL and 200 μg/mL respectively. G indicated another case of N25 group with a concentration of 200 μg/mL. D1-G1 were the amplified images of the white frames in the left photos. The Raman shift results were summarized in H for each group plus the vaterite indicated in C with black arrows. Scale bars in A-C, 200 μm; in A1-C1, 10 μm; in D-G, 100 μm; in D1-G1, 10 μm.
Figure 5. Distribution of N25-cy5 in calcites crystallization experiment. A1, view from bottom; A2, view from side. B1-B3, Z axis slices 23, 53 and 83 of A1; C1-C3, the corresponding fluorescent 2D intensity histograms of slices from B1-B3. D1-D3, bottom view, side view and slant view of the surface rendering of N25-cy5 in the crystal respectively. E1, SEM image of one of the crystals from a crystallization experiment to show the cavities in the main face indicated with white arrows. E2, SEM image of one of the crystals to show the newly emerging crystal faces and the shrunk edges, while E3 was the magnified photo of E2. F1, the habit simulation in control group with \{1 0 4\} as the main faces; F2, habit simulation with decreased E-att of \{0 0 6\}, \{0 1 2\}, \{0 1 8\} and \{1 1 0\}; F3, habit simulation with further decreased E-att of the same faces as F2. Scale bars were about 5 μm.
Figure 6. Schematic diagram of the hypothetical process from the secretion to the functional location of N25. The figure inside the dashed line box showed a theoretical interaction of protein N25 with the ions within the surfaces of calcites. Binding of N25 may cover some sites on crystal surfaces and block the entry of free ions. The blue balls indicated Ca$^{2+}$, red balls indicated CO$_3^{2-}$, and the green granules represented N25 molecules.
A basic protein, N25, from a mollusk modifies calcium carbonate morphology and shell biomineralization

Dong Yang, Yi Yan, Xue Yang, Jun Liu, Guilan Zheng, Liping Xie and Rongqing Zhang

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