DISCUSSION

Previous studies on molluscan radulae primarily focused on the mechanism of iron deposition (Jones *et al.* 1935, Lowenstam 1962b, Burford *et al*. 1986, St Pierre *et al.* 1986, Rinkevich 1993, Lu *et al.* 1995, Brooker and Macey 2001, Brooker *et al.* 2003), and although silica deposition was observed, the ways in which it was transported and deposited remained elusive. The data presented herein may provide hints as to how silica participates in tooth formation of the limpet, *N. schrenckii*.

Early studies on *P. vulgata* revealed that limpet teeth are initially organic structures composed of chitin and proteins rich in tyrosine (Runham 1967), and hardening of these organic skeletons possibly occurred by quinine-tanning (Runham 1961). Mineral deposition first took place in the cusps, and mineralization of tooth cusps began in the posterior region, which appeared to be under strict chemical and structural control (Mann 1985). The presence of iron within the epithelial cells encircling the radula was evident by Prussian blue staining as shown in Figures 2B, 3C, and 3D, where cells with a high level of iron content exhibited a bright blue color. In Figure 2E, the heavily stained junction zone, a region which separated the cusp from the base, implied that this vast amount of iron might be initially transferred and stored in this particular region. Despite the existence of high levels of iron content, diffraction result of junction zone showed that no subsequent crystallized structures formation occurred in this area, indicating that this region might act as a reservoir of minerals (Fig. 8), which in turn concentrated materials necessary for teeth formation to a critical amount before the onset of mineralization. A similar phenomenon was also reported in chiton (Macey and Brooker 1996, Brooker *et al*. 2003), indicating a common

mechanism in molluscan tooth formation, that is, initiation of the mineralization might begin from iron deposition which stored in junction zone. Adaptation of vacuole transport in iron transfer during tooth development in the limpet, *C. toreuma,* was reported (Lu *et al*. 1995). Vesicular transportation activities were also observed near the junction zone of *N. schrenckii* (Figs. 5C, 5D, 5E), suggesting that the minerals essential for tooth growth might be transferred to junction zone via vacuoles.

During early stages of cusp mineralization when the posterior region was aligned with organic fibrous structures, particles with diameter of about 70-150 nm were observed to associate with the organic fibrous structure at the adjacent region between cusp and base (Fig. 6). Elemental analysis showed that these particles were composed of silica (Fig. 7C). In the inset of Figure 6B, it could be clearly observed that the particles were formed with the organic fibers wrapped in the center, suggesting that the organic fibers might serve as structural scaffolds and also provide spatial constraints during the mineral deposition process. Additionally, results of the SEM EDX mapping showed that the base was primarily composed of silica (Fig. 4D). Since silica in the base did not form an ordered crystalline structure but was composed of a less-ordered structure of 'scrolls' (Mann *et al*. 1986), it was possible that the base might serve as either a temporary silica reservoir during the tooth formation process or a gateway through which silica could be transported to the cusp, which in turn suggested that the organic matrix observed in Figure 6 were located in a high-silica-content environment. The intimate association of silica particles with the organic matrix revealed in this study suggested that the organic matrix might not only serve as spatial constraints, but could play more active roles in catalyzing the nucleation, aggregation and deposition of minerals.

A large amount of silica was detected in radulae, particularly in the early stages of tooth mineralization, while the content of silica within epithelial cells surrounding the radulae showed slight alterations during different mineralization stages based on the ICP-MS analysis results (Fig. 9). This implies that the silica required for tooth growth should enter the radulae at an early stage before the mineralization process takes place. The decrease in the silica concentration in radulae during the transition from stage I to stage II shown by the ICP-MS analysis (Fig. 9B) suggests that the amorphous opal deposits along the organic filaments and forms the particles observed in Figure 6B.

In this study, silica was found not only in the base, but also encased the goethite crystals growing along the organic matrix and infiltrated the spaces not occupied by goethite crystals in *N. schrenckii*, which were consistent with the observation made in the study of Mann *et al*. 1986 on *P. vulgate*. ICP-MS analysis of the radulae also showed that the concentration of silica in radulae dramatically dropped from stage I to stage II, implying that silica might be deposited together with iron once iron influx has begun (Fig. 9B). As the teeth became more mature, silica particles observed in Figure 6B no longer present, suggesting that silica within these particles might be relocated in the cusp and deposited both on the goethite crystal surfaces as well as filled in the spaces between goethite crystals to further strengthen the tooth structure.

Based on previous studies in diatoms and the results mentioned above, it is quite likely that the biological molecules involved in silica deposition may co-precipitate within the teeth structures during limpet teeth formation. Application of HF on the cusps disturbs the mineral structure by dissolving the deposited silica within the cusps, yielded a group of low molecular weight peptides, suggesting that these peptides might co-precipitate with the silica during

teeth development. Two peptides with molecular weights of about 18 kDa and 16 kDa can be constantly identified on the SDS-PAGE gel regardless of the sources from which the peptides were extracted; however, closer examination of the gel extracted from the epithelial cells surrounding the radulae revealed that there were in fact a group of peptides with similar molecular weight ranging from 15 kDa to 20 kDa (Fig. 10). The result of Western blotting using anti-18 kDa and anti-16kDa peptide antibodies showed that both antibodies recognized several peptides within molecular weight ranging in 15 kDa to 20 kDa (Fig. 11B). This indicated the possibilities either these peptides might possess analogous epitopes, or the initial identification of the individual peptides on the gel was not precise thus resulting in mixture of 18 kDa and 16 kDa peptides. Nevertheless, the differences in molecular weights might due to different post-translational modifications or the damage caused during extraction with extreme extraction methods such as boiling in SDS and application of HF. The presence of these peptides not only in cusps but also in epithelial cells surrounding the radulae based on the Western blotting results suggested that they might originally produced in the epithelial cells and later play critical roles in the transportation of silica during teeth formation process. However, the attempt to identify the peptides in TEM sections with immunocolloidal gold staining utilizing the anti-18 kDa and anti-16 kDa antibodies was not conclusive. The gold particles were observed in a variety of regions including base, cusp and the epithelial cells. The indiscriminate distribution indicated that the antibodies were lack of specificity and further purification of the peptides will be essential. The attempt to obtain amino acid sequence of 18 kDa and 16 kDa peptides yielded no conclusive result suggested possible modification present at N-terminal, which might result from post-translational modification or from the extraction processes, and the nature of

the blocking remained to be determined. Digestion of the 18 kDa and 16 kDa peptides with proteases together with the subsequent analysis may reveal more information regarding the amino acid sequences.

Though the sequences cannot be determined, the amino acid compositions of 18 kDa and 16 kDa peptides, however, can be determined. The results were searched with Amino Acid Composition Search on the ExPASy WWW server. The proteins with the most resemblance of the amino acid compositions are mitochondrial carrier proteins and transfer proteins. Mitochondrial carrier proteins are small proteins found on the inner membrane of mitochondria, with a molecular mass ranging from 28 to 34 kDa on SDS-PAGE. Their polypeptide chains were built from three tandemly repeated sequences of about 100 amino acids. The repeated domains are folded into similar structure motifs, consisting of two transmembrane α-helices linked by an extra-membranous hydrophilic region. The majority of theses carriers transport anions such as citrate, pyruvate and ADP/ATP, while some transport cations or zwitterions such as glutamine. There are also metabolite carrier proteins and other proteins of unknown function not restricted to mitochondria identified to have similar characteristic sequence features (Palmieri 1994, Indiveri *et al.* 1997, Nelson *et al.* 1998). It is possible that the 16 kDa and 18 kDa peptides and the peptides identified on the SDS-PAGE with similar molecular weight are partial fragments of a carrier protein that was responsible for silica transportation during teeth formation.

Since the 16 kDa and 18 kDa peptides might involve in the transportation and catalysis of silica deposition, it is intriguing whether the ability to induce silica precipitation remained if the mineral layer coated over these peptides is removed.

In the silica re-deposition experiment, NH_4F was applied instead of HF to ensure a milder reaction condition (Kröger *et al.* 2002), hopefully to preserve any organic

molecules which had co-precipitated with the minerals during mineralization. The surface morphology was similar in experiment treated with NH_4F, as the treatments applied to these 2 groups were intended to remove the outermost layer of silica. In the experiment in which radulae treated with NH_4F, TMOS, and NH_4F sequentially, silica granules measuring 100-150 nm in diameter were seen (Fig. 12D). This indicated that after initial NH_4F treatment, the exposed organic surface may again induce silica precipitation upon addition of TMOS as the silica source. When subsequent treatment of NH_4F was applied after TMOS application, part of the newly deposited silica outer layer was dissolved, but a small amount of silica granules remained attached to the cusp surface. It is likely that the amount of silica deposited was not under strict biological control of the organism, resulting in uneven thickness. The granules observed in Fig. 12D might be the result of partial removal of the final NH_4F erosion step. In the experiment where silica was allowed to deposit on the exposed cusp surface without further disruption, an intriguing morphology was observed. The surface of these teeth appeared to be composed by fused granules compared to the surface morphology of the cusp in the control experiment, where organized rods were present (Fig. 12A, C). The alignment of silica granules in Fig. 12A observed with SEM appeared to be somewhat consistent with the alignment pattern observed in ultrathin sections with TEM (Fig. 6). The fused appearance of the teeth in the silica re-deposition experiment may have been the result of dissolution and re-precipitation of silica following sequential treatment with NH_4F and TMOS. Although the results in Si re-deposition suggested that the molecules within the cusp minerals may possess the ability to induce silica precipitation, yet, further confirmation is required. Since germanium (Ge) has been termed a “pseudo-isotope” of Si, and the un-discriminated uptake of Ge and Si by various

organisms had been reported (Meunier *et al.* 1999, Tallberg *et al.* 2002, Ma *et al.* 2006), substitution of Si with Ge in the deposition experiment may further confirm the involvement of the biomolecules and their affinity specificity to silica within the cusp minerals.

Degenerate DNA primers were designed based on the sequence of *SIL1* and silicatein gene; however, no PCR products can be obtained using these primers. This suggests that the silica deposition mechanism in limpets should be vastly different from that in diatoms and sponges, implying that silica deposition mechanisms may be unique in different species utilizing silica. Nevertheless, the application of antibodies available for cathepsin-L family to the limpet may still be helpful if similar molecules can be recognized in the teeth, particularly in the cusp or base region. In that case, the possibility that proteins in limpet similar to silicatein in sponge exists may further aid the attempt to reveal silica deposit mechanism in various species.

In all, it is evident that silica plays a critical role in the structural integrity of the tooth cusp and provides a cushion for limpets when feeding by producing a rather elastic tooth base. The biomolecules involved in the transport and deposition of silica were possibly related to the mitochondrial carrier family suggested the possibility that the peptides extracted in this study may belong to a subunit of a protein mediating the transportation of silica. Based on the results of the silica re-deposition experiment, the organic matrix found in the cusps may be more actively involved in silica precipitation process in addition to provide physical scaffolds and constraints. Amino acid sequences are required to further confirm whether the peptides extracted in this study actually related to the mitochondrial carrier family, therefore, how to acquire sufficient quantity of the peptides and decipher the nature of the N-terminal block will be the major tasks for the future.

Once the amino acid sequences are obtained, monoclonal antibody can be generated and the site of the protein possibly involved in silica transportation can be then identified.