Green engineering of silicon and titanium dioxide architectures and realizing downstream applications

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Green Engineering of Silicon and Titanium Dioxide Architectures and Realizing Downstream Applications

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Biomineralization has long been a source of inspiration and frustration for researchers in a wide variety of disciplines from ecologists and dental practitioners to materials scientists. An amazing variety of organisms have the capacity to produce inorganic mineral complexes through biomineralization. In this context, different organisms use proteins, peptides, and polysaccharides as templates to control the nucleation, growth, and morphology of structures containing minerals and metals. Due to lack of clarity in the field, distinctions are provided between the various biomineralization processes as Type I, II, and III biomineralization. Synthetic biomineralization is an emerging field in which these processes are applied to unnatural substrates to create useful inorganic materials with applications in a variety of fields. A comprehensive overview of silica and titanium oxide biomineralization is given, covering the major achievements this sub-field has attained since its emergence. The ground-breaking discoveries are focused based on the templating agent used and the mechanisms that are proposed in the field are discussed. Synthetic biomineralization are led, which are more recently demonstrated to have feasible applications in energy, electronics, construction, and biotechnology. These possibilities are discussed alongside prospects based on the current trend of research in the field.

1. Introduction

Biomineralization is a process by which living organisms and produce inorganic mineral complexes. It is a widespread phenomenon observed in a broad range of organisms across the bacteria, algae, plant, and animal kingdoms. In biomineralization, living organisms control the nucleation, growth, and morphology of minerals and metals using organic molecules, such as proteins and polysaccharides, as templates. Many organisms have been identified that are capable of biomineralization, with a variety of target metals and minerals having been found in their processes.[1] In this review, we broadly refer to any mineralization process involving enzymes, proteins, or peptides as a "natural biomineralization." In natural biomineralization, three distinct biomineralization processes have been identified. Type I: "Controlled biomineralization" is an enzymatic process with a nucleation site.[2] Type II: "Induced biomineralization," is a proteinaceous templating agent, and not an enzymatic process Type III: "Processive biomineralization" is a normal enzymatic process.

In Type I and III biomineralization, the organism has complete control over the formation of the mineral. This is accomplished by an enzyme where the organism can closely regulate all aspects of the mineral formation process. In Type I the enzyme is precipitated with the mineral, in Type III it is not. Type II biomineralization involves establishing a favorable environment using a proteinaceous templating agent that allows for the formation of minerals (e.g., an organism creates a nucleation site to allow mineral formation based off nucleation site).

The biomineralization of unnatural substrates is an emerging field in materials science that explores the use of

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biological molecules as templating reagents for synthesizing inorganic materials on surfaces or substrates that are not typically found in biological systems.[3] This type of (synthetic) biomineralization has broad applications in fields such as energy, electronics, construction,[4] and biotechnology, including the development of bio-compatible coatings, biomimetic sensors, and novel electronic devices.[5] Despite being a relatively new field, there have been several successful examples of biomineralization on non-natural substrates, such as the formation of titanium dioxide.[6,7] Titania thin films, consisting of titanium dioxide (TiO_2), are widely used due to their unique properties, such as being an excellent transparent n-type semiconductor and maintaining high chemical and thermal stability. Based on these properties, titania thin films are a suitable material for a variety of applications in biotechnology, agriculture, food, medicine, and energy.[8] Specifically, within the energy field, titania thin films have been utilized in solar cells[6] and lithium-ion cells.[9] In addition, titania layers are also commonly used as surface coatings due to their high refractive index and photocatalytic activity. These coatings can be used in various applications, including biomedical implants and self-cleaning surfaces. Multiple methods have been developed for the coating of surfaces with titania thin films, including sol-gel, chemical vapor deposition (CVD), atomic layer deposition (ALD), electrochemical deposition (ECD), spraying, and dip-coating; all of which have their own unique limitations.

To circumvent these, a new method to engineer a titanium dioxide layer was initially proposed by Bawazer et al.[7] and solidified by Van Amelrooij et al.[6] This new method is a combination of dip-coating with biomineralization. Titanium dioxide formed using biomineralization will be hence referred to as biogenic TiO_2. The usage of enzymatic mineralization allows the formation of different biological structures through the creation of (meso-)porous materials.

2. Silica Biomineralization

Since the first analysis of complex architectures in sea sponges, the notion that enzymes could be employed to construct complex inorganic mineral architectures has been a source of inspiration. In nature, two distinct methods of silica mineralization have been found and described.[10–12] First, silicatein that is found in glass sea sponges can drive the mineralization of silica (**Figure 1**A,B). Second, silaffins that are found in diatoms are used as templates for the formation of silica structures.[13]

2.1. Silicatein

In 1998 Shizumi achieved the first isolation of silicatein in their seminal research, deriving the term from a combination of silica and protein. Initial homology alignments of silicatein revealed a striking similarity to cathepsin proteases, i.e., proteins that degrade other proteins.[14] A typical member of this family is papain, a cysteine protease derived from *Carica papaya* (Papaya). A surface charge model of silicatein is depicted in Figure 1C with detailed positioning of a catalytic triad of active site residues composed of a serine residue at position 26, a histidine at 165 and an asparagine at position 185 (Ser26, His165 and Asn185 respectively, see Figure 1C inset).

However, despite such similarity, using enzymes such as papain, trypsin, and BSA showed poor results for silica mineralization, which led to the proposal of a mechanism by Cha et al. and Zhou et al.[10,11] The mechanism consists of a serine (Ser26) performing a nucleophilic attack on silica, thereby forming a protein-silica intermediate. Hydrolysis is followed by condensation leads to the formation of amorphous silica. The nucleophilic serine is activated by a nearby histidine, which forms a hydrogen bond with the serine protonDD. After deprotonation, this histidine can activate a hydroxyl group of the silica, which in turn can perform a nucleophilic attack on the silica already bound to the protein. This results in the formation of di-silicic acid. During this process, the hydrolysis of silica is stimulated through activation of a water molecule by the histidine (Figure 1D). However, this mechanism only explains the creation of di-silicic acid and does not describe the cyclization that is present in amorphous silica and observed in the skeletons created by microorganisms. In 2008, Fairhead et al.[18] obtained the crystal structures (Figure 1C) of several human α-cathepsin and L-chimera silicatein proteins and proposed a different mechanism.

In contrast with the mechanism proposed by Cha et al. and Zhou et al.[10,11] the researchers do not describe the formation of a protein-silica intermediate, instead proposing an acid-base activation mechanism, which would suggest a Type I rather than Type III enzymatic process. However, the cyclization is still lacking. In 2011, Wang et al. proposed a third mechanism that explains cyclization as well as polycondensation.[12] This mechanism is similar to the mechanism proposed by Cha and Zhou et al. Wang et al. proposed a full participation of the catalytic triad (see Figure 1C, inset; Ser26, His165 and Asn185) is responsible for the reaction. First, Asn185 together with His165 stabilizes the deprotonation of the serine, a common mechanism in serine proteases. The deprotonated Ser26 performs a nucleophilic attack on the δ^+-Si, which initializes condensation with the proton originally bound to Ser26. This binds the silica to the protein at Ser26. The proton of the silanol is stabilized by His165 of the enzyme, which in turn increases the negative moment of the oxygen from the silanol hydroxyl group. This oxygen can perform a nucleophilic attack on a different silica molecule, causing condensation and the evolution of di-silicic acid. Continuing in a similar manner, a tri-silicic acid is made which is still bound to the enzyme. The paper also states that the tri-silicic acid is condensed to ortho-silicic acid, however, this was not clearly shown. Finally, the silica is released. The same mechanism is explained in Schröder et al.[19] However, in this mechanism, the emphasis is mainly on the Ser26- His165 pair since it was shown that mutation of the Asn185 resulted in an activity decrease of 60%, but otherwise retention of enzyme function. This critical observation suggests that while as in a Type I biomineralization process, there is a "chemical environment requirement", the participation of other residues reducing activity indicates further catalytic events to indicate processivity making it Type III biomineralization.

2.2. Silaffins

Silaffins are proteins that are created and used by diatoms, a type of single-celled eukaryotic algae. These algae can mineralize silica to render complex silicon oxide nanostructures. In 2000, Kröger et al. isolated and characterized silaffins from *Rotheca*

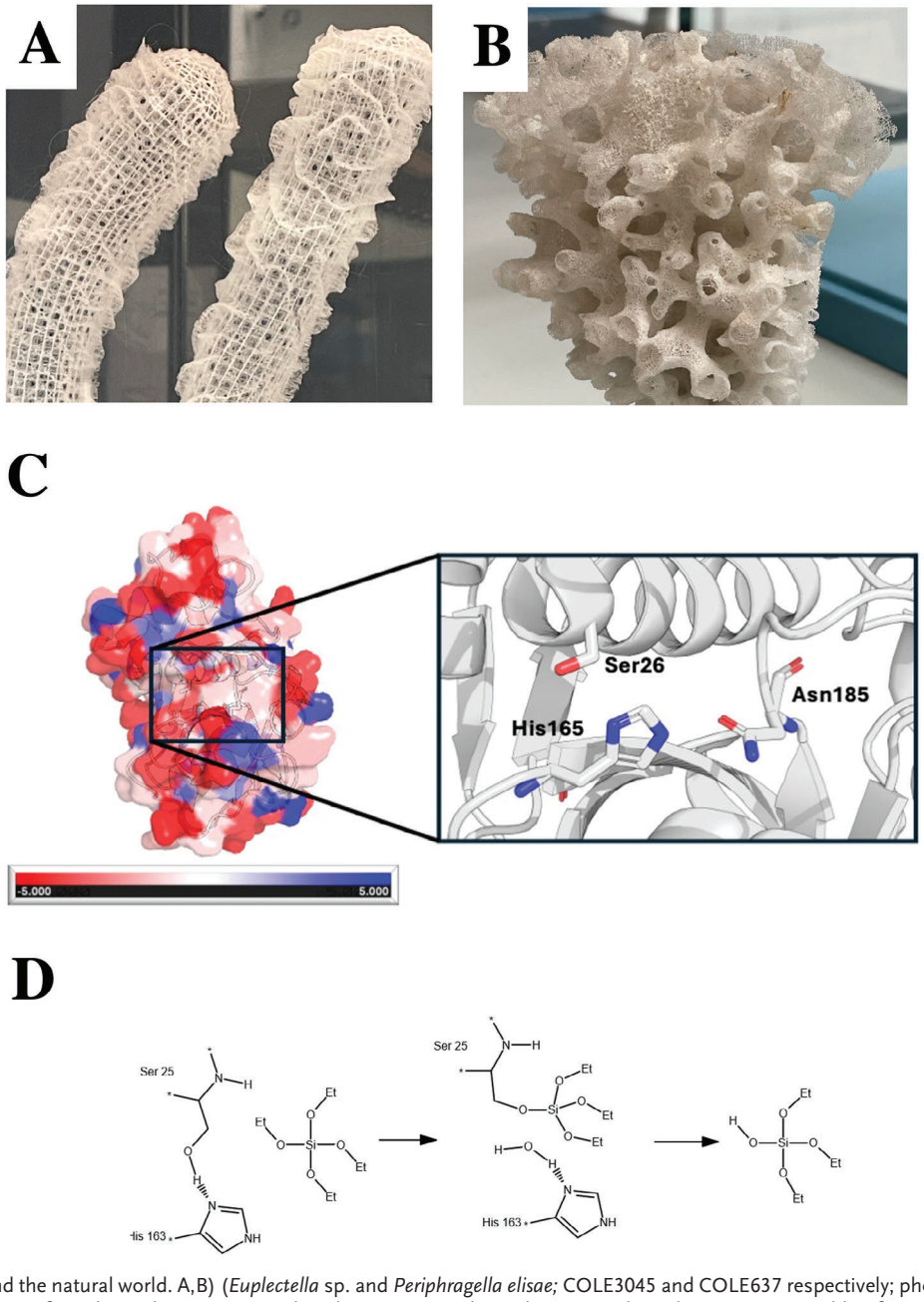


Figure 1. Silicateins and the natural world. A,B) (*Euplectella* sp. and *Periphragella elisae;* COLE3045 and COLE637 respectively; photos: D.G.G. McMillan Cole Museum, University of Reading) show two examples glass sponges, the architectures that silicateins are capable of engineering in the natural world. C) A surface charge representation of the crystal structure of silicatein (PDB: 6ZQ3).[15] Blue (positive) and red (negative) reveal the surface with the electric potential calculated using the method of Dolinsky et al.[16] The zoomed inset shows the catalytic triad (Ser26, His165, Asn185) represented using a cartoon model for the broader structure and a stick representation for residues. Rendering was performed with Pymol v2.6.[17] D) The proposed mechanism by Cha et al. and Zhou et al.[10,11]

fusiformis.[20] Three distinct proteins were identified, ranging in mass from 4 kDa (Silaffin-1A (S1A) and Silaffin-1B (S1B)) to 17 kDa (Silaffin-2 (S2)). S1A and S1B are homologous, as indicated by the name, whereas S2 showed a larger difference. All three Silaffins were capable of rapid mineralization of silica, which was determined to be 10^6 fold faster than the synthetic methods available at the time. For S1A alone it was found that the ratio of silica/S1A was around 12, creating spherical silica particles of 500 to 700 nm in diameter. However, a mixture of the proteins showed a more aggregated structure with a diameter of ≈50 nm, indicating coordinated control over particle size. Later research by Kröger et al. revealed that S1A contained even more post-translational modifications which were not previously detected due to the isolation method that was utilized, which in turn resulted in increasing the determined mass to 6.5 kDa.[21] Several peptides have been created from S2A, which are commonly referred to as R1 through R7 in literature. Another diatom, *Thalassiosira pseudonana*, was found to contain

five different silaffins (tpSil1H, -1L, -2H, -2L, and -3).[22] It was found that there as no sequence homology between the silaffins isolated from *Rotheca fusiformis* and *Thalassiosira pseudonana*. However, many similarities were found in terms of amino acid composition (e.g., lysines) and corresponding post-translational modifications. Although the mineralization of silicon oxides is clearly the output, the mechanism here appears to a function of "chemical environment" suggesting that silaffins are inducing silicon oxide deposition and are a proteinaceous templating agent, only capable of a Type II biomineralization process.

3. TiBALDH

In most TiO_2 biomineralization reactions titanium (IV) bis-ammoniumlactatodihydride (TiBALDH) has been used as a substrate, since it is stable under ambient conditions. TiBALDH is a stable water-soluble titanium salt, which is commonly represented as a Ti^{4+} ion bound to two bidentate lactate molecules and two hydroxy groups. However, the presence of these OH-ligands is not fully confirmed, since both ligands have not been observed at the same titanium ion.[23] Crystallization of the substrate performed by Seisenbaeva et al. in 2013 showed the creation of $(NH_4^+)_8Ti_4O(lactate)_8.4H_2O$.[23] Another salt structure was found in the form of $(NH_4^+)Ti(lactate)_3$, where the hydroxy groups are replaced with an additional lactate, which has been stripped off a different TiBALDH molecule. This reaction was found to be in equilibrium. Polar solvents appear to push the equilibrium to the $Ti[lactate]_3$ form, while additions of more solvating agents push the equilibrium to the $(NH_4^+)_8Ti_4O(lactate)_8.4H_2O$ complex. In 2010 the reaction mechanism to produce crystalline TiO_2 was investigated and in this study TiBALDH was represented with $[Ti(lactate)_2(OH)_2]$.[24] It was shown that the pH had an influence on the formed crystal structure, due to a difference in coordination chemistry. In biomineralization studies, TiBALDH should be handled with care, since spontaneous deposition can occur in the presence of phosphate groups. Even phosphate groups as part of DNA can mineralize titania using TiBALDH as a substrate.[25] Therefore, any investigation into biomineralization of TiO_2 using TiBALDH should keep in mind that phosphorylated sites of enzymes, proteins and even phosphorylated amino acids could lead to false positive results if one was to consider this as an *enzymatic mechanism*. We note that in any area of biocatalysis using non-natural substrates, without mutational or inhibition studies, one cannot confirm an *enzymatic* catalysis and rule out abiotic mechanisms. Furthermore, it has been suggested that TiBALDH in aqueous solution consists of an equilibrium of molecular structures, containing $[Ti[(lactate)OH]_2]^{2-}$, $[Ti_4O_4(lactate)_8]^{8-}$, $[Ti(lactate)_3]^{2-}$ and TiO_2.[20,26–28] This equilibrium can be influenced by both acidic and basic environments.[25]

In nature, TiO_2 can be found in a variety of crystalline forms, most notably anatase, rutile, brookite, and akaogiite, although several other natural and synthetic crystalline forms have been discovered.[29] Rutile (tetragonal) is thermodynamically the most stable form[30] and is used in many different applications including energy storage and glass production. Brookite (orthorhombic) is a far less studied form with some photo- and electrochemical applications that is gathering more attention in recent years.[31] Akaogiite (monoclinic) is optically similar to rutile and has seen very little research since its recent discovery.[32] Finally, anatase (tetragonal) is the kinetically favored product[33] and is by far the most investigated polymorph of TiO_2, mainly due to its applications in photovoltaics and energy storage.[34,35] Aside from anatase, rutile can be used for a variety of other applications including possible pathogen inactivation and eliciting plant defense mechanisms.[36]

Using methods such as annealing and calcination, the crystal structure of TiO_2 can be altered.[37] Most commonly the crystal structure matures from amorphous → anatase → anatase/rutile → rutile, through subjection to time under increased temperature.[38,39] However, the exact composition of the minerals plays an important role in the phase change temperature. The most important factor for the phase change from anatase to rutile is the presence of brookite, which decreases the phase change temperature.[40] Furthermore, the larger the crystal size, the fewer crystal defects are found, thus lowering the phase change energy.

3.1. A Proposed Abiotic Reaction Mechanism of TiBALDH to TiO_2 Precipitation

While no definitive mechanism has been elucidated for the enzymatic conversion of TiBALDH to TiO_2, there have been several suggestions. To this end, a mechanism was proposed for the conversion in a basic environment.[26] The researchers describe employing UV–Vis to observe the reaction, where it was found that the turbidity of TiO_2 scales linearly with its concentration, and the extinction coefficient scales exponentially with the diameter of formed particles.

A model was constructed which shows that TiBALDH was rapidly converted toward a monomeric product, after which a slower process of condensation causes the polymerization of the product to a gel-like structure. In **Figure 2** a schematic representation of the hydrolysis of TiBALDH and the condensation of $Ti(OH)_6$ is shown.

4. Titanium Dioxide Biomineralization

Enzymatic biomineralization of titanium dioxide has been shown on several occasions, including by silicatein,[27,41] trypsin,[42] lysozyme,[43] protamine,[44] and papain.[6,7,42]

4.1. Silicatein

The first step taken towards the discovery of enzymatic titanium dioxide deposition was through the characterization of silicatein. In 1998, Shimizu et al.[14] were able to isolate the protein(s) responsible for the biomineralization of silica. In this process, it was discovered that silicatein-α, a silica-forming enzyme, belonged to the cathepsin L subfamily of papain-like cysteine proteases. However, one significant difference between the two is an alteration in the catalytic triad, a key substitution of serine to a cysteine residue. Sumerel et al. also achieved mineralization of titania using silicatein, whereas Curnow et al. managed to mineralize titanium phosphates using silicatein, showing at least some

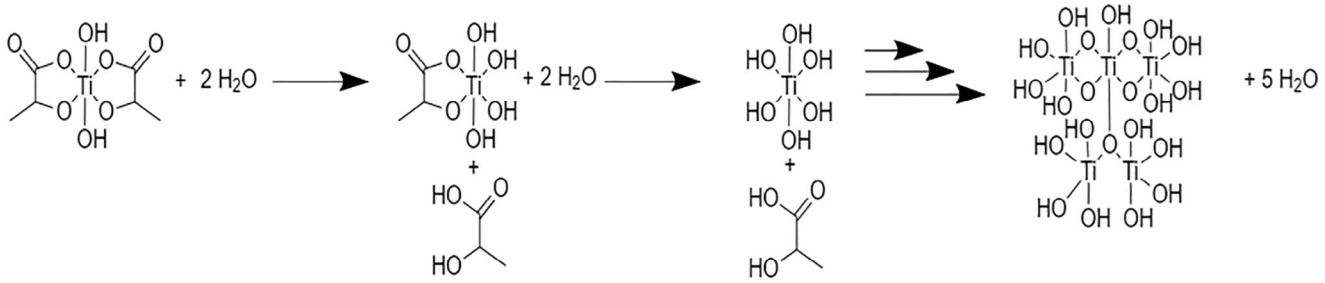


Figure 2. A schematic representation of the formation of anatase TiO_2 aggregates. Here, TiBALDH is first hydrolyzed to form $Ti(C_3H_6O_3)(OH)_4$. The next reaction is the condensation of the leftover lactic acid ligand. Following this, $Ti(OH)_6$ auto-condensates. It is noted that condensation could possibly already occur after dissociation of the first lactic acid ligand.

possible substrate versatility.[27,41] Furthermore, it was suggested that the addition of favorable surfaces would allow the formation of more homogenous nucleation.

Experimental enzymatic biomineralization of titanium dioxide has also been demonstrated on three occasions.[27,41,45] In all cases, TiBALDH was used as a substrate. Interestingly, biomineralized titania, when compared to chemical acid-base catalyzed titania revealed that the structure using silicatein was more distinctly layered, The most predominant form of the materialized titania was amorphous, however, Sumerel et al.[27] showed that some anatase titania was also present while the acid-base method showed more spherical structuring.[45] For this reason, it has been suggested that silicatein may act as a nucleation site, which lowers the surface roughness. The authors suggest that this is a similar mechanism to the precipitation of silica. In **Figure 3** a mechanism of the hydrolysis of TiBALDH with silicatein is shown, based on the mechanisms proposed for the mineralization of silica. While it is yet to be fully confirmed what Type of biomineralization process this is, the evidence to date suggests silicatein has a Type I mechanism.

4.2. Silaffin Derivatives

4.2.1. R5

In 2001 Kröger et al. characterized silaffins originating from *Cylindrotheca fusiformis*, a membrane protein that promotes the mineralization of silica.[13] They then expanded the substrate scope, demonstrating they were also able to precipitate titania using several recombinant silaffins.[46] This method used TiBALDH as substrate showed instant precipitation upon contact with the silaffins. In **Figure 4** several SEM images are shown from this work. The nanoparticles were found to be cracked in most cases. Thermogravimetric analyses showed that 50 wt% and 56 wt% of the rSilC- and rSil1L-titania was organic material, respectively.

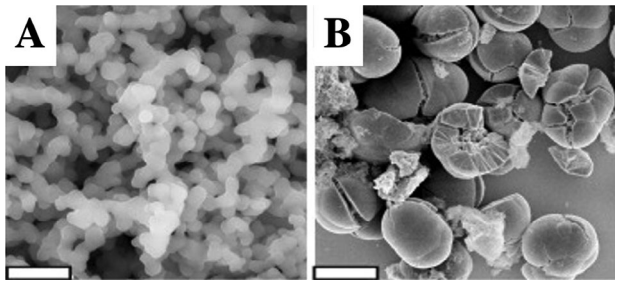


Figure 4. SEM images of titanium dioxide nanoparticles precipitated with rSil1L and rSilC. A) An aggregate of titanium dioxide nanoparticles precipitated with rSil1L with a scale bar of 1 µm. B) Titanium nanoparticles precipitated with rSilC in sodium phosphate/citrate buffer (50×10^{-3} M, pH 7), with a size of roughly 20 to 50 µm. This image originates from Kröger et al. and Reproduced with permission.[46]

Multiple post-translationally modified lysines were discovered in the protein. Further research was conducted on the R5 peptide (H2N SSKKSGSY SGSKGSKRRIL CO2H), a component of this protein. Mineralization of silica using this R5 peptide was found to be possible.[47] Multiple mutations were introduced that provided important insights into the deposition mechanism. It was found that the first eight amino acids in the sequence had almost no effect on the activity, and no activity on their own. This is surprising since even relatively simple primary and polyamines can precipitate silica.[48] However, it was discovered

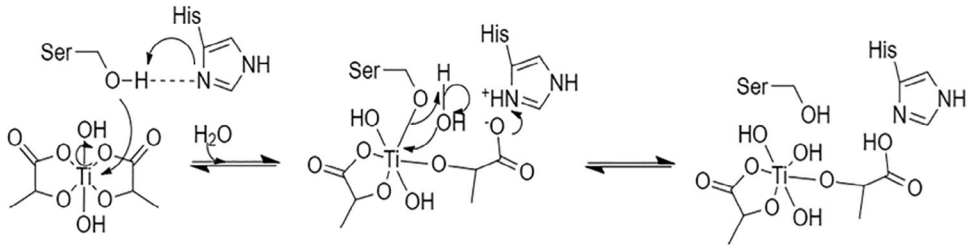


Figure 3. The reaction mechanism of silicatein with TiBALDH. The mechanism is based on the reaction mechanism of silicatein with tetraethoxysilane suggested by Cha et al.[10]

that with the addition of RRIL to the first eight amino acids on either side (C or N terminus), the activity is almost restored. In two separate studies, further investigations led to the conclusion that TiBALDH can be used as a substrate by this peptide to create amorphous titania nanoparticles.[38,49] It was confirmed that the peptide expressed no activity in the absence of the RRIL motif. This suggests that the mechanism is like the mechanism of silica precipitation with this peptide: acting as an acid-base catalyst protonating the lactate ligand and priming the precursor.[20,38]

Furthermore, Sewell et al. suggested that the RRIL domain causes the self-assembled structure which is vital for the activity. It is thought that the RRIL motif may mediate the assembly of a highly cationic complex which allows the interaction of multiple anionic TiBALDH molecules. Using the R5 peptide, Bregnhøj et al. showed the creation of titanium nanosheets at the water–air interface.[50] The 3D structure was determined, and it was found that the last two amino acids (IL) are located at the water-air interface, due to their hydrophobicity. Overall, it is not exactly clear how the RRIL motive is folded. It was suggested that it folds through a micelle-like self-assembly process,[47] however, more recent results suggest the involvement of salt bridges.[51,52] These mutational studies support the notion that silaffins are a templating reagent, supporting a Type II biomineralizing process.

4.2.2. PPL

Poly peptide poly(L-lysine) is a synthetic peptide inspired by the R5 peptide from silaffins, consisting solely of lysine residues. This peptide has been shown by Sewell and Wright[38] to have the ability to precipitate titania from TiBALDH, although it was found to be less active than R5. Three lysines were determined to be the minimum amount for titania precipitation activity, where adding more lysines showed an increase in activity relative to the number of lysines, up to eight tested monomers.[53] Activity analysis of several peptides showed that the distance between two lysines is the most important aspect of the catalysis; too far and TiBALDH will not interact (1.67 nm) with other TiBALDH molecules, too close and there is not enough room for more than one instance of TiBALDH.[54] This result could explain the importance of the RRIL motif in R5, which likely allows the necessary self-assembly of R5, thereby causing the lysines to be at the correct distance from each other to allow titania precipitation. These studies further support the notion that peptides in general can be used as templating reagents, supporting a Type II biomineralizing process, however; data also strongly suggests that there may be co-operativity between different molecules of substrate. This indicates that engineering is possible when considering this type of biocatalysis.

4.2.3. Peptides and Amino Acids

At present, the simplest biomimetic method of titania precipitation and deposition is through usage of a short peptide such as R5.[47] In the literature, mineralization has been shown with a variety of organic structures, including peptides, viral capsids, amino acids, and metabolites.[3,43,49] These methods most often result in the creation of amorphous titania. Most commonly the peptide acts as nucleation site, although it can also act as an acid-base catalyst in a Type II biomineralization process.

Attempting to mineralize titania from $TiCl_4$, Durupthy et al. tested Gly, Glu, Asp, Ser, His, Pro, Lys, Arg at different pH values to determine their ability to influence the mineralization.[55] Interestingly, Glu, Asp, Ser, and His achieved the mineralization of pure anatase crystals of 6 to 10 nm, in a pH range of 1–6. It was suggested that this was due to the amino acids binding to the formed titania, thereby blocking the formation of rutile and brookite. A similar result was obtained by a different research group using acetic acid.[56]

Other ionic compounds have been shown to be able to change and influence the mineralization, some through electrostatic interaction, others through ligand binding; which could be the case here.[57,58] The rest of the tested amino acids also showed mineralization of predominantly anatase, however, also brookite and/or rutile was found to be present. At pH $\geq$ 8, all amino acids showed formation of amorphous titania. Overall, it is thought that the mineralization of titania using $TiCl_4$ as substrate more easily results in the formation of crystalline material, since all of the ligands are easily replaced with water. This makes it more likely that full hydrolysis occurs, thus preventing steric hindrance and a non-crystalline structure, which would result from condensation. However, it is suggested that since $TiCl_4$ more easily undergoes hydrolysis, it is more difficult to control the mineralization.

Nonoyama et al. were able to mineralize titania by using a β-sheet peptide nanofiber template that was synthesized through polymerization of Fmoc-amino acids in order of the desired sequence.[59] By subsequently incubating these β-sheet peptides with TiBALDH in a solution of NaOH at pH 11, it was observed that crystalline TiO_2 could be obtained in either rutile or an anatase/rutile mixture upon heating at 400 °C or 700 °C, respectively. While incredibly interesting, particularly since the formed TiO_2 phases seem to correlate in shape and structure with the β-sheet peptides, suggesting that the peptides act as a template for the TiO_2 structure, the question remains whether it is in fact the peptides that are initiating the mineralization. Since these reactions were performed in aqueous NaOH at pH 11 and TiO_2 mineralization can be achieved at high pH using NaOH,[25] perhaps it is the NaOH that is initiating the mineralization, while the β-sheet peptides act as a template for the structure.

4.3. Protamine

Protamine, a highly cationic protein derived from sperm nuclei, has also been shown to be able to deposit titania.[44,60] Protamine is widely used in the medical field and is produced at a large scale, thus readily obtainable.[61] Dickerson et al. demonstrated that the high abundance of arginine residues present in protamine has been critical for the titania deposition observed.[62] Protamine is capable of a Type II biomineralization process. Firstly, it acts as a nucleation site, and with its positive charge can undergo electrostatic interactions with TiBALDH. The addition of ions (e.g., inorganic phosphate and sodium[63] reduces the mineralization capability, which is caused by Na^+ blocking the charges of the protein. Secondly, the protein should be regarded as an acid-base catalyst, since it is thought to be responsible for the protonation of the lactate and the priming of the hydrolyses. Since protamine

is a nucleation site, the protein co-precipitates with the titania and creates amorphous titania with (in some cases) traces of anatase crystals. Jiang et al. showed layer-by-layer assembly of titania microcapsules of up to five layers which resulted in a 200 nm titania surface. These capsules have been shown to be capable of immobilizing and stabilizing the enzymes which increased the thermal and pH stability.[60] Using this technique Sun et al. created "pseudo cell factories" which were three enzymes encapsulated with titanium dioxide that were able to convert CO_2 to methanol.[64]

4.4. Glucose Oxidase, Catalase, and Lysozyme

In 2010, Chen et al. demonstrated biomineralization of titania with glucose oxidase by using titanium sulfate as a substrate.[9] Interestingly, the resulting titania showed a rutile crystal structure. However, the near complete absence of organic material in the resulting TiO_2 layer suggests that this enzyme uses a distinctly different mechanism, potentially one where the protein has no lasting direct contact with the titanium substrate. This suggests that the enzyme does not function as a nucleation site, as it would have precipitated with the titania, revealing that it seems to have a Type III biomineralization mechanism. When titania was followed during the reaction it was found that it was first formed as very small (1.8nm) anatase crystals, after which a rutile phase together with an anatase phase was observed, which disappeared leaving only rutile. This corresponds to the "dissolution-recrystallization" mechanism already posited, which was previously only been favored in strongly acidic environments (pH < 1) and high temperatures.[65,40,66] To date, glucose oxidase remains the only enzyme found in literature that is capable of synthesizing rutile titania. Normally, rutile titania is only created with extremely acidic pH and high temperatures.[40,66] The general evolution of titania crystal formation starts with amorphous (non-crystalline) to anatase, then mixed anatase and rutile, and finally only rutile. This makes direct formation of rutile crystalline titania especially energy intensive.[38,39,66] The final rutile crystals were found to be 3.8 nm (in [110] direction) by 7.1 nm (in [001] direction), smaller than most reported rutile nanostructures.

In the same research by Chen et al., it was also shown that catalase is capable of mineralizing anatase titania. As with catalase, titanium sulfate was used as a substrate, thereby releasing sulfate. It is suggested that the sulfate could act as a capping agent able to influence the formation of the different crystalline states. Mineralization of titania using lysozyme was also found to be possible, although here TiBALDH was used as a substrate. Lysozyme was able to precipitate titania, albeit in amorphous form. Upon inspection of the nanoparticles using XPS, it was found that for the reactions using catalase and lysozyme, the final nanostructures contained organic material (most notably N), suggesting that the enzymes are stabilized by TiO_2 and that the enzymes co-precipitate with the titania. This demonstrates that both enzymes have a Type I biomineralization mechanism.

Lastly, in this vein it should be noted that the formation of rutile can also be achieved using glycolic acid or citric acid together with titanium powder in a solution of hydrogen peroxide and ammonium hydroxide, as shown by Kobayashi et al.[67] Interestingly, they were able to control not only the crystal structure on a nanoscale, but also the overall shape of the crystal on a macroscale.

4.5. Papain

As previously described, silicatein is homologous to papain,[14] a cysteine endoprotease which is industrially used as a meat tenderizer, in the leather industry (**Figure 5A**), and for cosmetics, textiles, and detergents.[24] Papain has high stability and is readily available due to it being a waste product of the *Carica papaya* (papaya; Figure 5B) horticultural industry. Insights into enzyme kinetics were first observed in the hydrolysis of casein by papain, a more biologically relevant substrate. In these assays a low level of substrate inhibition was observed. A surface charge model of papain is depicted in Figure 5C with detailed positioning of the active site residues in Figure 5C inset. The biological mechanism of papain is represented as suggested by Harrison et al., is shown in Figure 5D.[68] Aside from its use in industry, papain has also found applications in biomineralization due to its ability to facilitate precipitation with substrates such as TiBALDH, tetramethoxysilane (TMOS) and $RuCl_3$.[42] In this way, it has been viewed as a promising substitute for silicatein for applied biogenics, since heterologous expression and reconstruction of silicatein has been shown to be difficult and often leads to the formation of inclusion bodies and aggregates.[41]

Smith et al. demonstrated the first use of papain to precipitate of titania in a solution-phase assay.[42] In this work, the researchers investigated multiple enzymes on their biomineralization properties. For papain, it was found that the TiBALDH concentration had a significant effect on the formation of titania nanoparticles. Unfortunately, they also observed a lack of processivity at higher concentrations of TiBALDH, thus limiting applications. They suggested that this was caused by a limitation of the measurement due to the formation of small nanoparticles that were unable to be pelleted. The biomineralization of TiO_2 with papain was later further investigated by Bawazer et al.[7] and Amelrooij,[6] where the focus was on the mineralization of TiO_2 on surfaces, thereby creating titania thin films which allowed use in applications such as solar cells (dye-sensitized and perovskite).[70,71]

It was found that the titania surface contained both anatase and rutile crystalline formation (3.5:1). Bawazer et al. achieved an important proof of principle, inactivating the active site of papain through covalent binding of biotin to Cys25 (see Figure 5C), which resulted in a complete lack of enzyme activity toward titania mineralization. This demonstrated that titania deposition is indeed an enzymatic process and strongly supported the suggestion that the active site plays an essential role in the mineralization process.[7] However, it was observed that there are two specific rates during the deposition process, and papain precipitated with the titania indicating a Type I biomineralization process.

Alongside thermal stability, another advantage of papain is its high pH stability. The optimal pH for papain was determined by Foda et al. to be 6.4 while the thermal optimum was found to be 60 °C.[72] Although the activity at room temperature was not determined, at 30 °C papain showed an activity of roughly 50% of the activity at 60 °C. While the rates at different substrate concentrations were tested, they are not discussed.

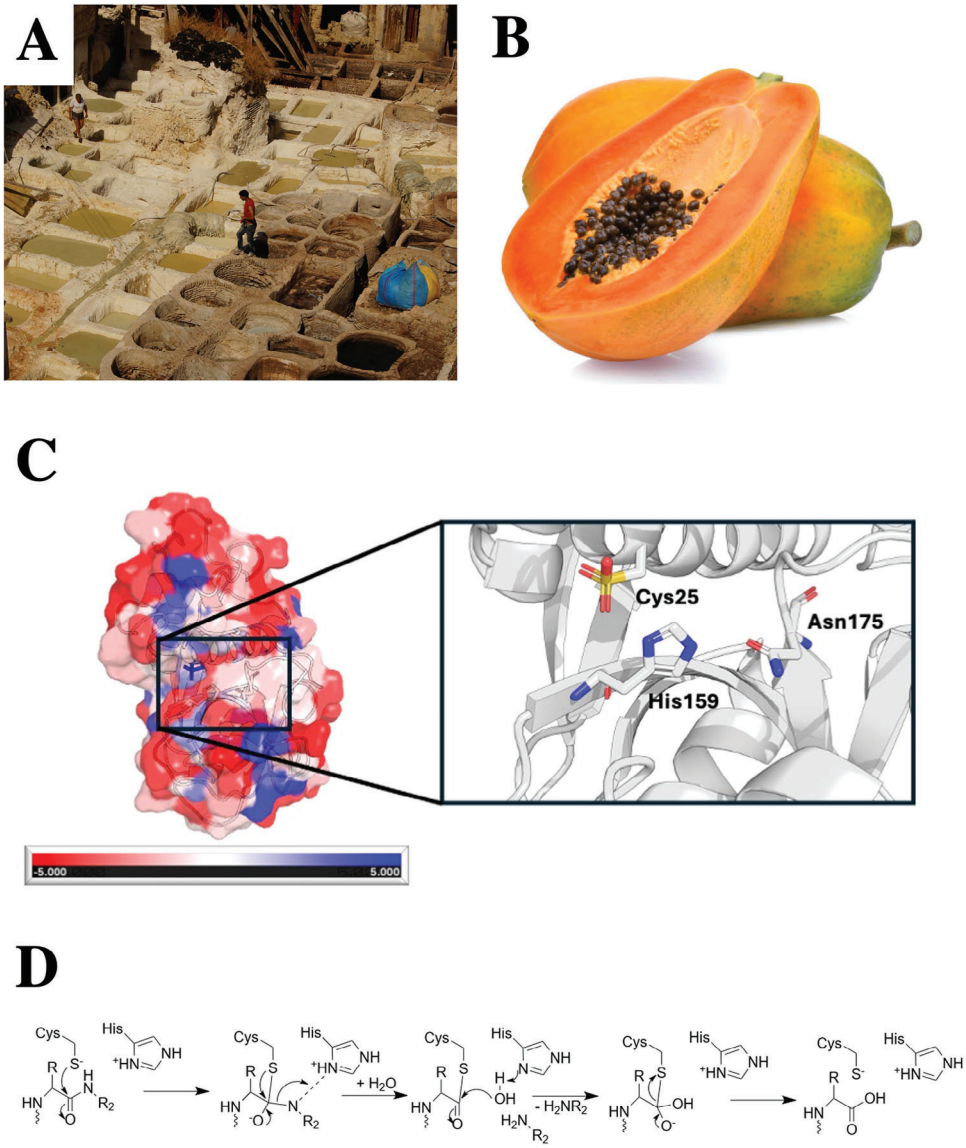


Figure 5. Papain industrial use, structure, and mechanism. A) An example of industrial use of papain is in the leather processing and textile industry (photo: D.G.G. McMillan Fes, Morocco). B) The source of papain is from Papaya latex and is found in the unripe papaya (not ripe as pictured). C) The mechanism and crystal structure of papain. C) Shows the surface of the papain crystal structure 9PAP which has been colored according to it electrostatic potential.[16,70] The catalytic triad (Cys25, His159, Asn175) has been presented with a stick representation in the inset. D) Shows the natural activity of papain where the sulfur group attacks the amide bond of a polypeptide as suggested by Harrison et al.[68]

Sathish and co-workers tested the inhibitory properties of Cd^{2+} and Zn^{2+} ions on papain and found that they both showed significant enzyme inhibition with K_i values of 8×10^5 and 5×10^5, respectively.[73] At low concentrations, the inhibition appears to be competitive; however, it is believed that at higher concentrations, the Zn ions may cause conformational changes that permanently inhibit the enzyme. Conversely, it was found that in the presence of Mg^{2+} and Ca^{2+} ions, the activity of papain increases.

Similarly, Hg^{2+} and Cu^{2+} have also been discovered to inhibit papain activity.[74,75] The biological compounds spermine, protamine, spermidine, polybrene, poly(L-lysine), and polyvinylpyrrolidone have been found to also inhibit papain at low concentrations.[76]

4.6. Deposition of TiO_2 onto a Surface

In contemporary literature, almost all attempts at biomineralization have been performed in solution-phase experiments. However, Bawazer et al. recently demonstrated by that mineralization of titania on a surface using papain is not only possible, but feasible.[7] In this study, the researchers utilized a quartz crystal microbalance with dissipation to reveal that

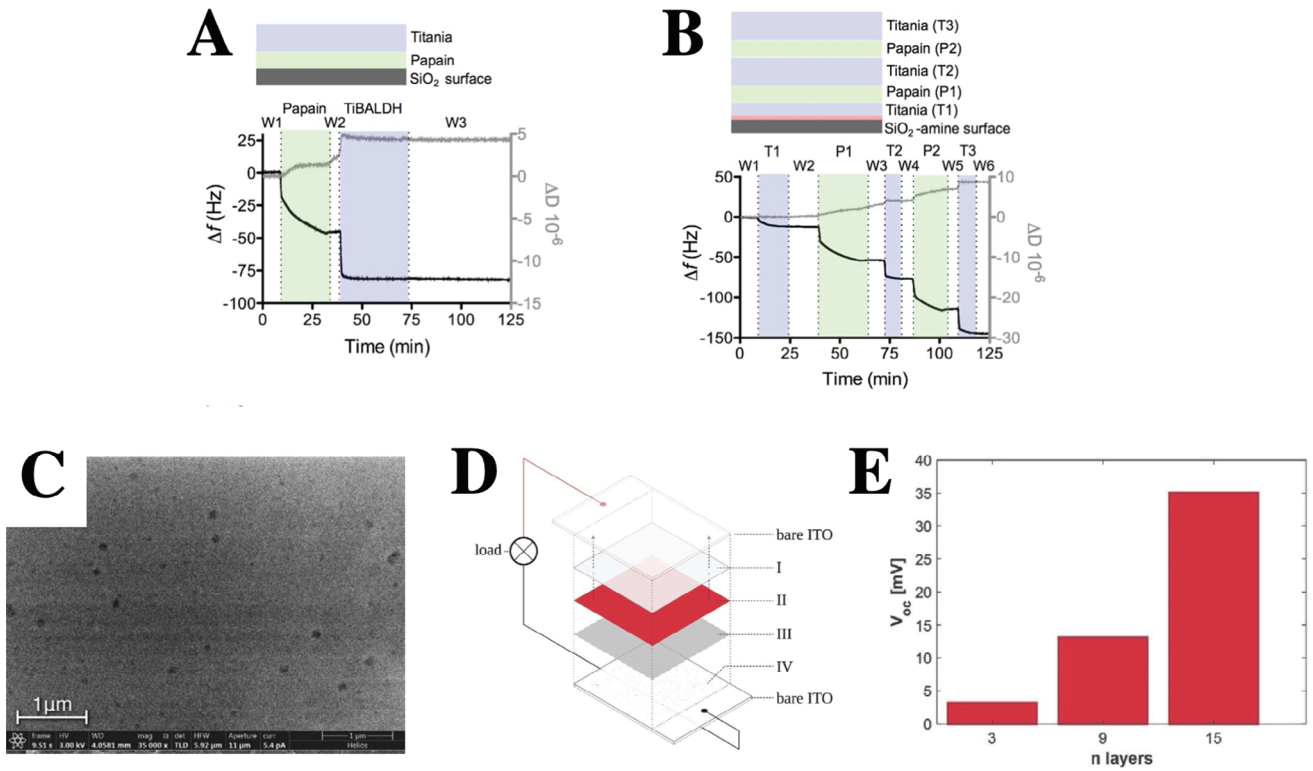


Figure 6. Surface biomineralization of titania and engineering a primitive device. A,B) QCM-D analysis of papain-mediated titania synthesis, where the associated schematics show the thin film structure. Frequency (black line, left axis) and dissipation (gray line, right axis) are shown, with changes in solution flows are indicated. "W" indicates wash buffer (20×10^{-3} M MES buffer pH 7), "Papain" indicates 10×10^{-6} M papain in 20×10^{-3} M MES buffer at pH 7, and "TiBALDH" represents 20×10^{-3} M TiBALDH + 24×10^{-6} M coumarin in water. A) Single-layer mineralized film synthesized on an SiO_2 surface. B) Triple-layer mineralized titania film synthesized on an amine-modified SiO_2 surface. A,B) Originate from Bawazer et al. and are reproduced with permission.[7] C) SEM image of an annealed papain/titania layer, showing porosity generated by papain destruction. D) Exploded representation of the different layers in the van Amelrooij et al. dye-sensitized solar cell (DSSC) test device using papain-mediated biomineralized titania.[6] I) Indium tin oxide (ITO) glass with ITO coating facing inward, II) the biomineralized and annealed titania layer, III) immobilized dye layer from pomegranate juice, IV) liquid electrolyte (I^-/I_3^-). E) Maximum determined open circuit voltage of DSSCs with different stacks of annealed papain/titania layers. Voltages were measured with a multimeter. C–E) Originate from van Amelrooij et al. and are reproduced with permission.[6]

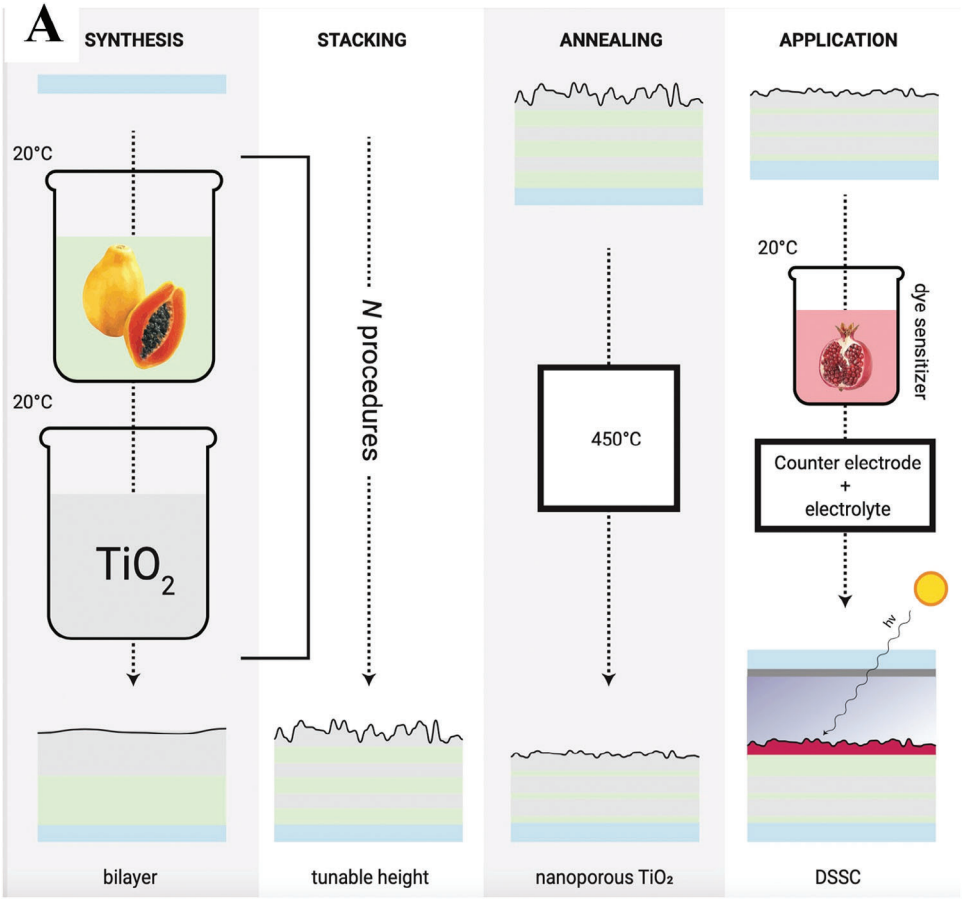
papain is capable of self-assembly on amine and oxide surfaces (**Figure** 6A,B), where it can then drive the formation of continuous amorphous titania/ protein films which can be refined to 93% anatase post-annealing.

It is unsurprising an amine surface is capable of mineralizing titania from TiBALDH considering both peptides R5 and PPL are both capable using a Type II biomineralization process.[38,47] This examination also revealed that this activity relies upon the cys25 residue of the catalytic triad (see Figure 5C), solidifying that the activity versus TiBALDH is indeed catalytic in nature, albeit a Type I process. After this initial finding, the researchers were able to drive layer-by-layer assembly, allowing a flexible route to achieving bioengineered titania heterostructures. In a follow-up study, van Amelrooij et al. showed that a simple dip coating procedure could be used, demonstrating that this process could be repeated for at least 50 layers while maintaining stability of the stack of successive layers.[6] These layers were then further characterized using positron annihilation Doppler broadening and lifetime spectroscopy, scanning electron microscopy, and X-ray diffraction. This revealed the thin titania films have low densities $\rho \approx 0.6$ g cm^{-3} and contained small micropores and proteins (Figure 6C). They also revealed that annealing at temperatures of 300 °C or higher leads to the destruction and evaporation of most of the organic material, resulting in a purer titania film with increased density and an increase in micropore size. With practical applications in mind, van Amelrooij showed that the TiO_2 layer resulting from deposition with papain can in fact be applied to dye-sensitized solar cells (DSSC).[6] It was demonstrated that the titania layer is capable of charge transfer, since an increase in voltage was observed under exposure to sunlight (Figure 6D,E). Encouragingly, successive layer addition resulted in increased current density, suggesting the microporosity was a property that could be exploited further.

5. Outlook

5.1. Protein Mechanism and a View to Protein Engineering

Biomineralization of metals—specifically titania—has garnered significant attention in recent decades as a viable green alternative to energy-intensive deposition methods classically used for this purpose.[77] Additionally, agricultural waste products have been proven to be a feasible and reliable source of biomineralization agents,[6,7,77] which when applied to the development



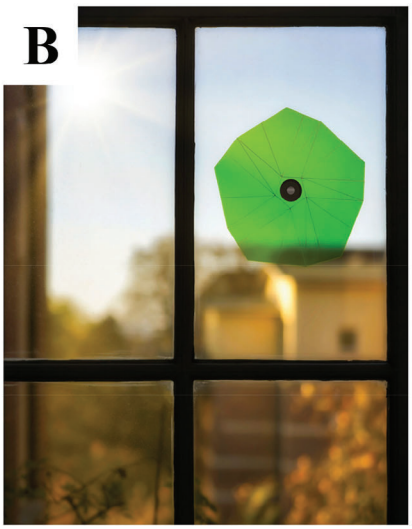


Figure 7. The process from biomineralization to product development. A) Depicts the 4 stages of papain-driven titania biomineralization. Synthesis of titania using papain; the ability to stack the layers to derive increased efficiency; the annealing process; and the assembly to form a DSSC as an application. B,C) A concept product in which the mineralization process is currently being used to design a fold-out DSSC lamp where B) the photon-accepting state and C) the light emitting state (The lamp design "Sun Catcher" is property of Teresa van Dongen and Applied Biogenics Ltd).

of energy generation aligns with sustainable development goals (SDG) 7 & 12 as set by the UN.[78,79] As discussed, by using enzymes (silicatein, papain, glucose oxidase), and templating proteins (silaffins) and peptides, nature's power can be harnessed for this purpose.

In this review we draw clear distinctions between the types of processes that there are in biomineralization. Type I, where an enzyme is catalytic, but is eventually trapped within its own processivity and co-precipitates with the mineral formed. Type II, which is non-enzymatic, but templates the mineral. Type III, which is purely enzymatic. We have also identified that there is scope to potentially engineer enzymes into taking the kind of process desired for downstream application. However, to date, there are no phylogenetic comparisons in plants and microbes which allow comparison or prediction of biomineralizing enzymes, the field is currently dependent on ecological observation and attempts to mimic the chemistry observed, limiting the intelligence of the investigation. Such a database would also aid in redesign of enzymes for artificial substrates such as titania species.

In this same vein, while advances have been made into the function of the catalytic triad of the silicateins, there are clearly other amino acids involved meaning further mutational studies should be conducted. Such analysis would not only aid in understanding of mineralization mechanism in silicateins, but also in cysteine proteases such as papain. To date, the mineralization mechanism between silicateins and papain appears similar. Both have almost identical active site clefts, with the major difference being the substitution of a serine residue (position 26 silicateins) for a cysteine residue (position 25 in papain; compare Figures 1C and 5C). We note that the Asn185 mutation in silicateins directly implied that the Ser26-His165 pair are most critical for function,[19] which is also supported by the finding in the Bawazer et al.[7] that covalent modification of Cys25 eliminated function. The Ser26-His165 pairing mechanism is also supported by studies in the synthetic peptide PPL, where the distance between two lysines was the critical factor for TiBALDH mineralization.[54] Collectively, these studies suggest active site shape is a matter to be further explored with a view to engineer for enhancing efficiency.

5.2. Potential Applications

As described above, a variety of biological agents have been found to be capable of mineralizing different metal oxides, wherein TiO_2 is likely the most investigated due to its many different applications.[80–82] As a result of being a relatively cheap semiconductor with photoactive and light scattering capabilities, TiO_2 is often applied within the field of photovoltaics, especially for dye-sensitized and perovskite solar cells.[83] While industrial deposition of titania is well-researched and developed,[84] advancements can be made using biomineralization to reduce the environmental impact of these processes. A relative unexplored possibility is the possibility of orientation of enzyme on the surface, where the active site face towards the solution phase, rather than the physical support structural surface is likely to be an important factor for enzymatic catalytic longevity.[85–87]

In addition, doping of semiconductors is a common practice in photovoltaics in order to tune the semiconductor properties. In particular, Mg-doping has been achieved using various techniques other than biomineralization, including sol-gel, hydrothermal, and co-precipitation methods, among others.[88–91] The choice of doping method and conditions can affect the final properties of the material. For instance, Zhang et al. reported that sol-gel doping of titania with Mg improved the photocatalytic activity of the material.[92] Here, $TiCl_4$ was combined with $MgCl_2$ to produce a Mg-doped titania layer. The degradation of methyl orange using a Mg-doped titania layer has also been shown by Avasarala et al.[93] By adding $[Ti(O\text{-}Bu)_4]$ to ethanol, followed by the addition of water and acetic acid, a sol-gel was produced. Interestingly, the Mg was simply incorporated into the titania layer post-annealing without any molecular alterations. Furthermore, Mg-doped TiO_2 has been explored as an anode material in Li-ion batteries due to its high theoretical capacity and good cycling stability.[94] Finally, Mg-doping has also been shown to improve the performance of titania-based photovoltaic devices, such as dye-sensitized solar cells.[95]

Whereas semiconductor doping is a common practice within photovoltaics as a means of tuning photovoltaic properties,[96,97] even researched more frequently now in organic semiconductors,[98] doping within biomineralization of metal oxides has been touched upon, but not investigated thoroughly.[99,100] Further research into doped biomineralization is therefore warranted and could potentially improve the efficiency of dye-sensitized solar cells. With a view on a sustainable future, further investigation into the use of biomineralization in energy generation is warranted. To take this beyond the level of research into industry, production of energy devices (e.g., photovoltaics) that itself are produced using sustainable, circular resources are being developed (**Figure 7**). In doing so, the field of biomineralization and photovoltaics can contribute a significant and important part to the energy transition.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

A.D. and L.M. contributed equally to this work. Conceptualization and resources: D.G.G.M.; writing—original draft preparation. Original draft preparation, writing, review, and editing: A.D., L.M., T.G., E.B.Q., M.Al-Q., T.vD., and D.G.G.M. Supervision, project administration, funding acquisition: D.G.G.M. All authors have read and agreed to the published version of the manuscript.

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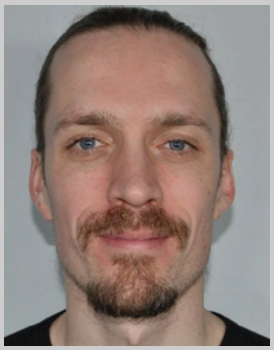

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