

Chapter 2

Biom mineralization and the Sequence: Function Effects on the Peptide

Abstract The biomineralization peptide is chiefly responsible for regulating the process involved in nanostructure formation. Thus, understanding the intricate details relating to the peptide is of utmost importance in order to use biomineralization in crafting highly functional nanomaterials. In this chapter, recent findings regarding how variations in the sequence of the peptide influence biomineralization are presented and discussed.

Keywords Biomimetic material synthesis • Peptide-dependent factors • Biomineralization peptide post-translational modification • Conformational analysis

2.1 Introduction

2.1.1 Biomineralization as a Natural Phenomenon

Biomineralization refers to the process of inorganic structure formation in biological systems (Mann 2001). These inorganic structures are very important for organisms since these structures serve a variety of purposes from the simple unicellular bacteria to human beings which are essential for survival. For instance, a certain class of bacteria called magnetotactic bacteria uses iron oxide-based nanostructures as sensors to orient themselves to the external magnetic field (Yan et al. 2012). These bacteria possess a special organelle called magnetosomes in which these specialized inorganic structures are formed. The eukaryotic unicellular diatoms mineralize silica into micro-sized shells with amazing shapes and a high degree of symmetry (Hildebrand 2008). Plants also utilize biominerals composed of amorphous carbonate and silica called cystoliths as light scatterers to help in photosynthesis (Gal et al. 2012). The leaves of the sandpaper fig (*Ficus sp.*) have a very rough surface due to the presence of micron-sized biominerals. On a larger dimension, the bones, shells, and teeth of higher forms of organisms are similarly

essential inorganic structures. These structures provide mechanical support to the organism, help in nutrition-related functions, and also provide defensive mechanisms. In addition to their aforementioned roles, these structures also have taxonomical significance. Mollusks possess species-specific shells that can be used as tools for their identification and proper classification (Weiss 2012). This diversity in which biomineralization proceeds also manifests itself at the protein level which highlights the complexity of the process. For example, the shell matrix proteins of oysters that are highly involved in the regulation of shell and pearl formation were characterized wherein 66 out of the 80 proteins were deemed unique (Marie et al. 2012). In spite of this seemingly complex process, a general model of biomineralization has been described by Mann (2001). The model demonstrates two possible sites of biomineralization wherein it can occur either within or outside the cell. For extracellular biomineralization, ions are encapsulated in a vesicle and shuttled out of the cell where it is deposited onto a polymeric support. In the case of intracellular biomineralization, the ions start nucleation within the cell. Small units of the precipitated mineral are then combined with the extracellular organic matrix. In any case, both types of biomineralization share the common feature of being boundary organized and the use of an organic matrix to direct the process. From these features, the different forms of regulation are achieved such as chemical, spatial, structural, morphological, and constructional control. Alienating the process within confined spaces facilitates concentration control of the different components that influences the overall property of the mineral. For instance, the shape of the spicules of the sea urchin was found to be highly dependent on the concentration of its endothelial growth factor (Knapp et al. 2012). Moreover, this feature has important implications on the manner in which the organism responds to its environment that also has an impact on biomineralization. It has been shown that silification is pH-dependent wherein the diatom *Thalassioria weissflogii* modifies its intracellular silica content in response to the pH of the environment (Herve et al. 2012). Boundary organization also controls the size of the mineralized structure since the size of the micro-compartment can dictate the overall dimensions of the biogenic mineral. Constricting the growth of the materials in confined spaces will therefore result to nanomaterials with size and shape directly dependent on the micro-compartment. On the other hand, the use of an organic matrix to induce biomineralization affects the nucleation and arrangement of the ions (Mann 1988), as in the case of collagen and bone formation (Wang et al. 2012).

2.1.2 Biomineralization as a Synthetic Route Towards Nanomaterial Synthesis

The materials produced from nature boast of efficiency and high-performance (Bhusan, 2009). These outstanding properties of natural materials are linked to

the ability of biomineralization to precisely regulate numerous parameters during the formation process. All of these have led to the application of biomineralization for the preparation of synthetic materials (Nudelman and Sommerdijk 2012). Bio-inspired approaches toward the preparation of materials showcase a relatively benign and simple reaction conditions in contrast to the other known synthetic methods (Kwon and Hyeon 2008). In addition, it serves as an excellent alternative in the preparation of nanostructures that highlights an innovative approach integrating two disciplines together. A common method of applying biomineralization for nanostructure preparation involves the utilization of bacteria (Butler et al. 2012) and fungi (Das et al. 2012). Both systems exploit the ability of the microorganism to direct mineralization after which the produced materials are harvested. Another method involves the utilization of peptides and proteins as templates to guide inorganic structure growth (Schulz et al. 2011). The utilization of peptides was conceived out of the principal tenet of biomineralization which uses organic matrices. While different kinds and classes of organic templates have been used (van Bommel et al. 2003), peptides have received special attention due to their several attractive properties. These properties include the following:

1. **Self-assembly.** Peptide self-assembly can lead to a preorganized scaffold from which structural and morphological controls during biomineralization can be attained. Moreover, the sequence can be varied in such a way so that a specific secondary structure can be formed.
2. **Metal coordination.** Peptides possess metal coordination and chelating properties due to the terminal amino and carboxylate groups (Sovago et al. 2012). Aside from these moieties, several amino acids have side chains that can also chelate with metals. A good example is the amino acid histidine wherein the imidazole side chain can chelate and coordinate with metals.
3. **Crystallographic facet recognition and binding.** Peptides can recognize and bind to specific facets of the growing inorganic material which leads to shape and size control (Chiu et al. 2011). This is an important feature of biomineralization peptides since this regulates the size, shape, and crystallinity of the formed nanomaterials. This process called capping refers to the attachment of the biomineralization peptide on the surface of the growing inorganic material which prevents further growth in that region. The peptide sequence, length, secondary structure, and position of a specific amino acid can influence the facet recognition capabilities of biomineralization peptides. From a functional perspective, peptide templates have also been shown to have an effect on the catalytic activity of metal nanostructures. The bound peptide onto the nanostructure surface can modulate the reactivity of the nanocatalysts (Li et al. 2014).

The diversity of biomineralization peptides makes peptide-mediated biomineralization a powerful tool for nanomaterial synthesis. Different biomineralization peptides are available for different inorganic materials (Table 2.1). In addition, different types of nanostructure can be formed by using different sequences for the same inorganic material. Through this method of nanomaterial synthesis, various

Table 2.1 Biomineralization peptide sequences and the type of inorganic nanostructure they form

Peptide sequences	Inorganic materials
NPSSLFRYLPSD	Ag nanoparticles
AHHAHHAAD	Au nanoparticles
NNPMHQN	ZnS nanoparticles
SLKMPHWPHELLP	GeO ₂ nanoparticles
FDFDFDFD	CaCO ₃

These peptide sequences have been summarized in the review by Chen and Rosi (2010)

forms of highly active materials have been developed. Biomineralized nanostructures have been created as catalysts (Pacardo et al. 2009), plasmonic materials (Song et al. 2013), and biomolecular recognition platforms (Slocik et al. 2005). These are just a few examples of numerous applications where biomineralized nanostructures have been highly relevant.

2.1.3 Biomineralization Peptide Discovery

Biomineralization peptides are usually discovered through peptide libraries. Phage peptide display is a powerful method in isolating peptides exhibiting binding affinity toward metal surfaces (Douglas and Young 2006). Phage peptide display has led to the identification of numerous peptide sequences that are able to support the growth of nanostructured silver, cobalt, palladium, titanium, germanium, and even calcium molybdate (Ahmad et al. 2006), among others. This method involves a phage library wherein peptide sequences are expressed as a fusion protein of the virus coat. The phage library is incubated with the materials of choice such as crystals and particles. After incubation, phage-displaying peptides that exhibit little or no affinity toward the chosen material will be removed during the wash step. Only phage-displaying peptide sequences that favor binding to the material will remain and be eluted. The collected phages will be digested and its genetic material will be amplified through PCR. This procedure is repeated for several cycles and the final step involves the determination of the peptide sequence based on the DNA sequence (Naik et al. 2004). The affinity of a target peptide towards a given inorganic surface provides the possibility that the peptide can support the growth and the stabilization of nanostructures. It should be noted, however, that not all peptides that exhibit binding affinity to inorganic surfaces are effective templates and capping agents for nanomaterial synthesis. This is due to the possibility of ineffective elution of peptides bound to the surface during the wash step. Such was the case in a study of Naik and co-workers wherein only one silver-binding peptide out of three was able to support nanoparticle synthesis (Naik et al. 2002). Moreover, the danger of false positive results is always present due to several factors such as contaminations, and propagation advantage (Vodnik et al. 2011). The combinatorial system of Split-and-Mix library is another viable approach in

discovering biomineralization peptides (Lam et al. 1991). Belser et al. (2009) used this method to isolate and characterize silver mineralizing peptides. In their work, the Split-and-Mix system of generating a library starts by having seven equal proportions of amino acid-loaded Tentagel resins. Each proportion is encoded with an inert, unique tag, or a unique combination of tag for identification and tracking of reaction history. A linker is then coupled with the on-resin amino acid followed by the addition of another set of unique tags. The third and final round involves the coupling of amino acids and tag addition. After the library has been created, the beads were then incubated with a solution of silver nitrate. Reduction with UV irradiation or ascorbic acid will lead to bead coloration if the peptide present in the bead has affinity for silver. The colored beads can then be easily picked followed by peptide identification.

It is evident that the biomineralization peptide occupies a central role in this biomimetic technique of nanomaterial formation. The peptide is involved in all three stages of biomineralization, namely (1) complex formation, (2) reduction, and (3) stabilization and capping. The first stage of biomineralization involves the complex formation between the peptide and the metal ion. The ability of the biomineralization peptide to form complexes with the metal is dependent on the presence of certain amino acids that can initiate complexation, such as histidine. The reduction of the metal ions into their zero-valent state can also be regulated by the biomineralization peptide as long as reducing amino acids are also present in the sequence, such as tyrosine. The final stage of biomineralization involves the binding of the peptide at the surface of the growing nanoparticle after reduction. In this phase, the sequence, charge, and conformation of the biomineralization peptide dictates the manner in which it will bind with the material. This, in turn, determines the resultant morphology of the material. Thus, it is very evident that understanding how peptide-dependent parameters govern biomineralization is very critical in the success of the synthesis. Moreover, a strong knowledge on these principles can lead to a tailorable preparation of the materials. In this chapter, most of these ideas are discussed in-depth, wherein we explore how the sequence and composition of the biomineralization peptide influence its function and nanostructure formation.

2.2 Peptide-dependent Factors Influencing Biomineralization

2.2.1 Amino Acid Composition and Sequence

The primary structure refers to the amino acid sequence that makes up the peptides. It is one of the tunable parameters in biomineralization since subtle substitutions in the amino acid composition of the biomineralization peptide can have wide and far-reaching effects in the nanomaterial synthesis. Each residue within

the peptide sequence has a role to play. Histidine and cysteine are residues commonly associated with binding and chelation, whereas tyrosine is known to induce reduction of metal ions into their zero-valent state. Acidic and basic residues are responsible for the overall charge of the biomineralization peptide, and the hydrophobic amino acids are implicated in the conformation of the peptide especially in an aqueous environment. Most of the properties of metal-binding peptides that are critical in regulating biomineralization are consequences of the synergism that exists among the different amino acids. Some of these properties are the binding affinity with the metal substrate, the proper conformation during capping, and the net charge which all have an effect on the kinetics and thermodynamics of biomineralization.

One of the earlier works that examined the peptide sequence effects on biomineralization was conducted by Coppage et al. (2010). In their work, the palladium-binding peptide named Pd4 was used. This 12-mer peptide was discovered through a phage display assay and has the sequence TSNVHPTLRHL (Pacardo et al. 2009). The goal of the study was to evaluate the effects of replacing the histidine residues which are essential for peptide binding onto the surface of the material. The histidine residues were substituted with alanine and the resulting morphology of the palladium nanoparticles was evaluated. The results showed that replacing both the histidine with alanine residues led to marginally larger nanoparticles in contrast when only one histidine was replaced. Moreover, varying turnover frequency for the Stille coupling reactions was obtained for the different variants wherein the sequence that had both histidine replaced showed the least catalytic activity. The reason for the observed variations in the nanoparticle size and catalytic activity was attributed to the changes in the manner in which the peptide binds with the material. These changes were associated with removing the histidine residues with a weaker chelating amino acid. A related study published by the same group in 2013 expanded the scope of the study by including cysteine substitutions to the aforementioned histidine residues of the Pd4 palladium biomineralization peptide (Coppage et al. 2013). In this paper, two kinds of amino acids replaced the histidine residues, alanine, and cysteine. The reason for including cysteine was to be able to gauge the effect of amino acid-binding strength on the outcome of the nanomaterial. Cysteine is regarded as a strong binder, whereas alanine is a weak binder and histidine is a moderate binder. It was anticipated that alanine substitutions will decrease the affinity of the biomineralization peptide with the palladium surface. However, quartz crystal micro-balance (QCM) experiments revealed otherwise. No correlation can be made between the measured affinity of the peptides and the binding strength based on amino acid composition. A similar scenario was observed when the sizes and morphologies of the resulting nanoparticles were evaluated. All biomineralization peptide variants yielded nanoparticles with negligible differences with respect to size. However, the turnover frequency of the nanomaterials was significantly different from each other. It was reasoned that probably, conformational changes associated with the substitutions outweighed the contributions of the binding strength of the residues in determining the affinity of the peptides with

palladium surface. The conclusions brought forward by the paper were supported by circular dichroism measurements which showed significant changes in the ellipticity of the spectra of the peptides indicating variations in the conformations and flexibility. The atomic-scale interactions imparted by these substitutions on the Pd4 biomineralization peptide were further probed by Bedford et al. (2015). In this contribution, extensive characterization was conducted on the nanomaterials using the variants of the Pd4-substituted biomineralization peptide. The analyses included computational simulation in conjunction with atomic pair distribution function (PDF), high-energy X-ray diffraction (HE-XRD), and X-ray absorption fine structure spectroscopy (XAFS). The paper revealed that cysteine substitution led to a stronger attachment of the Pd4 peptide variant on the palladium surface. The strong attachment of the peptide resulted in a relatively rigid conformation. Moreover, the strong binding of the peptide blocks the surface of the nanoparticle, thereby decreasing the effective area for catalysis. The strong peptide adsorption also hinders the abstraction of the palladium atoms from the nanoparticle surface which is also another route in which the reaction is catalyzed. This is far from the observed results for the alanine Pd4 variants which exhibited weaker binding to the surface of the material. This weaker binding resulted in a more flexible conformation which does not permanently occupy the catalytic sites of the nanoparticle.

The thermodynamics associated with biomineralization was recently studied by Limo and Perry (2015) by using a series of zinc oxide biomineralization peptides and related alanine mutants. The thermodynamic variables and binding constants related to the interaction between the biomineralization peptides and the zinc oxide substrate were analyzed through isothermal titration calorimetry (ITC). This form of calorimetry allows the direct measurement of thermodynamic variables associated with biomolecular interaction by measuring the corresponding heat changes during the gradual addition of adsorbate with the substrate (Leavitt and Freire 2001) or, in this case, the biomineralization peptide with the zinc oxide substrate. The study found that single alanine substitution altered the thermodynamics of the binding process. However, these changes were not significant enough since the calculated Gibb's free energy for binding remained negative for both the wild-type ZnO biomineralization peptide and the related alanine mutants. A negative Gibb's free energy indicates the binding of the peptide with the substrate is spontaneous. The study also determined that the binding between the peptide and the substrate is biphasic. The first phase involves an endothermic process which was attributed to the peptide conformational changes and the expulsion of the solvent molecules that were adsorbed to the surface of the substrate in exchange for peptide binding. The second phase was deemed to be exothermic in nature which can be accounted for by both peptide-peptide interactions and peptide-substrate interactions. The study concluded that changes brought about by variations in the peptide sequence are confined locally to the conformation of the peptide. These findings were consistent with the aforementioned findings by the study of Coppage and colleagues and by Bedford et al. Thus, it can be said that minor sequence changes, such as single amino acid substitution, can affect

the biomineralization process although its impact on the thermodynamics of the process is not that great. Peptide sequence effects also influence the electronic properties of the biomineralization peptide which is crucial for the formation of the nanostructures. It is well-known that cationic peptides are needed to induce the biomineralization of the TiO_2 . However, Zhao et al. (2012) found that TiO_2 -binding peptides with identical length and charges possessed varying mineralization activities. The differences in the biomineralization activity are due to the type of cationic amino acids present in the sequence. According to the paper, the presence of lysine imparted the strongest biomineralization activity to the peptide followed by arginine, then histidine. Furthermore, the inclusion of glutamic acid in the sequence can inhibit the Titania biomineralization activity of the peptide.

2.2.2 Conformation

The previous section highlighted how minor sequence variations lead to peptide conformational changes which is the main reason for the observed morphological variations of the resulting nanostructures. The next question that should be addressed is what is the effect on biomineralization if the metal-binding peptides had very different conformations? For example: What will be the effect if one peptide has a linear structure, whereas the other peptide exhibits a globular conformation? This question was answered by the work of Choi et al. (2012) wherein they evaluated the effects on the nanostructures produced by linear and cyclic biomineralization peptides. A TiO_2 biomineralization peptide was constrained in order to adopt a cyclic structure by forcing the formation of a disulfide bond between the terminal cysteine residues. The binding affinity of both the linear and the cyclic peptides was determined using the Langmuir adsorption isotherm model and it was found that both peptides had similar binding affinities. However, the TiO_2 nanostructures produced from both peptides were very different from each other wherein the linear peptide formed micro-sized structures. Moreover, the cyclic biomineralization peptide was far more effective over the linear peptide in terms of biomineralization yield as measured by the amount of precipitated TiO_2 . The reason for the observed differences lies in the peptide rigidity imposed by a cyclic structure. It was reasoned that a rigid peptide template favors TiO_2 mineralization by being an effective nucleus to initiate and spur the growth of the nanostructures. In a related study, the relationship between the spatial proximity of positively charged amino acids and the mineralizing activity of the peptides was assessed by Park et al. (2013). In their work, repeating units of lysine was used as the biomineralization peptide since it was already established that a highly positive peptide is ideal for mineralizing TiO_2 . The spatial proximity of the positively charged side chain of lysine was controlled by inserting glycine residues in between the lysine repeats. Glycine introduces a turn or facilitates twisting of the peptide owing to its simple structure. The number of glycine residues introduced in the lysine repeats determines the spatial

proximity of the positively charged lysine side chains and was used to make the side chains closer. From there, it was concluded that if the side chains were close enough with initiating electrostatic repulsion, then the biomineralization activity of the peptide can be enhanced. It appears that a cyclic and constrained peptide motif favors strong binding to the surface. For example, a constrained CuO₂-binding peptide exhibited stronger surface binding compared to a linear variant (Choe et al. 2007). The same observation was also recorded by Seker et al. (2007) concerning the binding properties of platinum biomineralization peptides. Surface plasmon resonance (SPR) spectroscopy was used to measure the binding affinities of the cyclic and linear peptides. The cyclic peptides exhibited larger adsorption rate constants compared to their linear counterparts suggesting that a looped peptide favors binding to the platinum surface. However, caution must be exercised whenever generalizing observations since a universal trend between peptide conformation and binding is most likely nonexistent and such a relationship should be analyzed for every system.

From the examples given and discussion presented, it is very evident that controlling biomineralization peptide local structures and conformation is an effective way in controlling nanostructure formation. This approach was explored and utilized by Kuno et al., wherein two types of silica biomineralization peptides were used: a linear, sheet-like peptide and an alpha-helical peptide (Kuno et al. 2012). The objective of the study was to evaluate the influence of peptide secondary structures on the biomineralization of silica. The alpha-helical peptide yielded wrinkled paper-like silica nanostructures, whereas the beta-sheet peptide yielded silica nanotubes. These results clearly demonstrate that the biomineralization peptide conformation is reflected and passed on the morphology of the resulting nanostructures produced. Another approach in modulating the conformation of the biomineralization peptide is by defining the chain length. Uper et al. (2012) demonstrated that the size of the silver nanoparticles can be controlled if the length of the silver-binding peptide will be defined.

2.2.3 Post-translational Modification

Post-translational modification refers to the chemical modification on peptides and proteins after synthesis in living systems. Post-translational modification is important because the type of chemical modification dictates the role of the synthesized protein in the signal transduction pathway. For example, incorporation of ubiquitin to a protein may indicate that the tagged protein will undergo degradation. As such, several studies have explored incorporating post-translational modifications onto biomineralization peptides. This is a viable approach in designing peptide sequences used in biomineralization since this technique of nanomaterial synthesis is founded on the biosynthesis of inorganic materials by organisms. Addison et al. (2010) examined the effect of serine phosphorylation on the biomineralization activity of an apatite-binding peptide discovered through phage

display. Protein phosphorylation plays a major role in cellular regulatory mechanisms and it is also regarded as a major currency in signal transduction (Hunter 1995). The paper reported that phosphorylation of the twelve-residue calcium-binding peptide led to an enhancement of the adsorption capability of the biomineralization peptide. Computational methods revealed that the binding enhancement is indeed brought about by serine phosphorylation wherein the phosphate moiety anchors the peptide to the apatite surface. In contrast, the unphosphorylated biomineralization peptide utilizes several amino acids to bind to the surface which results into a weaker binding. In addition, the phosphorylated biomineralization peptides inhibited *in vitro* osteogenesis since the peptides are able to immediately sequester calcium ions that are needed for bone formation. A similar rationale was used by Peltier et al. (2012) wherein they also used phosphorylated peptide sequences to control the growth of iron oxide nanoparticles. The biomineralization peptide used was not specifically for iron, but rather an anti-freeze peptide extracted from an Antarctic fish. The fourteen-residue peptide is composed of repeating units of alanine–alanine–threonine where the phosphorylation is incorporated at the threonine residue. The results of the experiment suggest that phosphorylation of biomineralization peptide can influence nanoparticle formation. The basis for this conclusion was the smaller iron oxide core-shell nanoparticles produced when phosphorylated sequences was used as opposed to the larger nanoparticles produced by non-phosphorylated peptides. However, correlation between particle size and number of phosphorylated threonine cannot be established. The underlying reason for the observed size diminution in the nanostructure produced by phosphorylated sequences can be attributed to the phosphate group. As stated earlier, phosphate groups can interact strongly with metal surfaces and this strong interaction leads to effective capping of the growing nanomaterial. Once the phosphorylated peptide is adsorbed to the surface of the material, it arrests further growth of the nanoparticle. Aside from phosphorylation, C-terminal amidation is also a form of post-translational modification and its effect on hydroxyapatite formation was evaluated by Hosseini et al. (2013). The peptide used was derived from the first thirteen residues from the helix of osteocalcin, a non-collagenous protein of bone tissues released by osteoblasts and osteocytes. The paper referred to this peptide as OSC, whereas the C-terminal amidated variant was referred to as OSN. The morphology of the calcium phosphate nanostructures produced from both biomineralization peptides during the first 60 minutes of mineralization was similar as characterized by globular and chain-like appearance. However, after 60 minutes and up to the 120th minutes of mineralization, the structure formed in the presence of OSN appeared to have plate-like morphologies. On the other hand, the structures produced by OSC were still the globular. Another striking difference between the materials produced from both peptides was that OSN facilitated the formation of crystalline hydroxyapatite, in contrast to the amorphous calcium phosphate produced by OSC. High-resolution TEM showed that the fringe lattices of the OSN produced materials were consistent with the (211) surface of crystalline hydroxyapatite. In order to account for these changes brought about by C-terminal amidation, both the OSC and the OSN

sequences were subjected to circular dichroism (CD) spectroscopy, dynamic light scattering (DLS) spectroscopy, and zeta-potential measurements. The conformation of the OSN peptide drastically changes in the presence of calcium phosphate compared to OSC. Another point of difference between the two peptides is the surface charge wherein OSN has lower negative charge compared to OSC. The more positive nature of OSN is believed to be the reason why OSN disperses in the presence of both calcium and phosphate as revealed by DLS. The exact mechanism behind the improved mineralization of OSN over OSC was not elucidated but the results clearly conclude the C-terminal amidation has a significant positive effect on hydroxyapatite mineralization.

2.3 Case Study

2.3.1 *Uncovering Sequence-based Relationships Among Biomineralization Peptides*

Numerous biomineralization peptides have been reported in literature wherein most of these peptides were discovered through the phage display assay which expresses various combinatorial peptide libraries. These randomly sequenced peptides which exhibit a certain affinity to the substrate used have been studied and analyzed in order to design improved biomineralization peptides. For example, it is now common knowledge that at least, histidine, cysteine, or serine should be present in the sequence since these residues are essential for binding. In the same manner, the presence of tyrosine immediately implies that this phenolic amino acid is involved in the reduction of the metal ions. To date, biomineralization peptides are conveniently grouped together based on their substrate. However, this is unreliable given that several peptides are capable of regulating the nanostructure growth of several other metals. For example, the R5 sequence was discovered as a silica-precipitating peptide (Knecht and Wright. 2003) but it has been used to form several other nanostructures such as gold (Bhandari and Knecht. 2012), titanium (Sewell and Wright. 2006), and palladium (Jakhmola et al. 2010). Thus, gaining a deeper understanding about the commonality or differences among the numerous known biomineralization peptides will be helpful in tailoring the peptide sequences to create better materials. In addition, unraveling relationships among biomineralization peptides on the basis of common and dissimilar features will aid in the development of a general understanding regarding the properties of these peptides.

Recently, Janairo et al. (2015) attempted to statistically analyze the primary structures of various biomineralization peptides reported in literature. The use of statistical-based methods is a reliable way in order to determine connections that may or may not exist among the various biomineralization peptides. In order to realize this objective, a two-cluster solution using *k*-means method was

Table 2.2 Biomineralization peptides used in this study including their sequences and references

Name	Sequence	Substrate	Reference
HG12	HGGGHGHGGGGHG	Cu	Banerjee et al. (2003)
HRE	AHHAHHAAD	Au, Cu, Pd	Slocik et al. (2002)
R5	SSKKSGSYSGKSGKRRL	Silica	Knecht and Wright (2003)
A3	AYSSGAPPMPFF	Au, Pd, Pt	Slocik et al. (2005)
Ag4	NPSSLFRYLPSD	Ag	Naik et al. (2002)
AgP35	WSWRSPTHVVV	Ag	Naik et al. (2004)
Col-P10	HYPTLPLGSSTY	Co	Naik et al. (2004)
P7A	TLHVSSY	Pt	Li et al. (2009)
Flg	DYKDDDDK	Pd, Pt	Slocik et al. (2005)
Pd4	TSNAVHPTLRHL	Pd	Pacardo et al. (2009)
Pd2	NFMSLPRLGHMH	Pd	Pacardo et al. (2009)
AuBP1	WAGAKRLVLRGE	Au	Hnilova et al. (2008)
AuBP2	WALRRSIRRQSY	Au	Hnilova et al. (2008)
GBP1	MHGKTQATSGTIQS	Au	Kulp et al. (2004)
Midas2	TGTSVLATPYV	Au	Kim et al. (2010)
Z1	KHKHWHW	Au	Peelle et al. (2005)
Z2	RMRMKMK	Au	Peelle et al. (2005)
AgBP1	TGIFKSARAMRN	Ag	Hnilova et al. (2012)
AgBP2	EQLGVRKELRGV	Ag	Sedlak et al. (2012)
Ag5	SLATQPPTPPV	Ag	Naik et al. (2004)
Col-P2	KLHSSPHTLPVQ	Co	Naik et al. (2004)
Col-P1	HSVRWLLPGAHP	Co	Naik et al. (2004)
Col-P15	QYKHHPQKAAHI	Co	Naik et al. (2004)
q7	QQSWPIS	Pd	Chiu et al. (2010)
B7	CTTCGCG	Ni	Chung et al. (2008)
LSTB1	AHKKPSKSA	TiO ₂	Choi et al. (2012)
Pt-1	YQPWKTQRELSV	Pt	Forbes et al. (2010)

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constructed based on sequence-dependent clustering variables from a sample of 27 randomly chosen biomineralization peptides (Table 2.2).

From these sequences, the clustering variables were calculated as summarized in Table 2.3.

The molecular weight and isoelectronic point were determined through expasy (http://web.expasy.org/compute_pi/) while the other clustering variables were calculated by counting the number of residues that correspond to each category, and this number was divided by the length of the peptide. From these variables, the significant clustering variables were identified. A significant clustering variable means that this variable is able to differentiate one peptide from another. The significant clustering variables were determined through analysis of variance (ANOVA).

Table 2.3 Short description of the clustering variables

Clustering variable	Description
Length	The number of amino acids presents in the peptide
Molecular weight	The average molecular weight of the peptide
Isoelectric point (pI)	The pH in which the peptide exists in a charge-neutral state
% Heterogeneity	The percentage of non-repeating amino acids within the peptide
% Aliphatic residues	The percentage of aliphatic or non-polar amino acids within the peptide
% Aromatic residues	The percentage of aromatic amino acids within the peptide
% Polar residues	The percentage of polar amino acids within the peptide
% Acidic residues	The percentage of acidic amino acids within the peptide
% Basic residues	The percentage of basic amino acids within the peptide
% Sulfur-containing residues	The percentage of cysteine or methionine present within the peptide

Table 2.4 Significant clustering variables

Variable	Significant p -value
Length	0.000001
Molecular weight	0.000000
% Heterogeneity	0.006701
% Aliphatic residues	0.034096

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A variable that exhibits the highest p -value that is greater than the confidence level was discarded and clustering was repeated using the remaining variables. This iterative process was done until the remaining variables all had p -values lower than 0.05. All statistical analyses were conducted at a 5 % confidence level.

From the ten sequence-dependent clustering variables, only four were judged as significant after a step-wise elimination of insignificant variables (Table 2.4). This indicated that only the length, molecular weight, % heterogeneity, and % aliphatic residues are the only variables that can differentiate and cluster together the biomineralization peptides into two groups. Furthermore, molecular weight carries the most weight in terms of significance in clustering the peptides since it has the lowest p -value.

Using the significant clustering variables, the 27 biomineralization peptides were grouped into two clusters which can be seen in Table 2.5.

The descriptive statistics of each group revealed that cluster 1 has a longer sequence, higher molecular weight, and more diverse with respect to the amino acid composition and contains more aliphatic amino acids relative to cluster 2 (Table 2.6).

Table 2.5 Cluster memberships of the biomineralization peptides

Cluster 1	Cluster 2
R5	HG12
A3	HRE
Ag4	P7A
AgP35	Flg
Col-P10	Z1
Pd4	Z2
Pd2	q7
AuBP1	B7
AuBP2	LSTB1
GBP1	
Midas2	
AgBP1	
AgBP2	
Ag5	
Col-P2	
Col-P1	
Col-P15	
Pt-1	

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Table 2.6 Descriptive statistics for the members of cluster 1 and cluster 2

Variable	Cluster 1	Cluster 2
Length	12.556 ± 1.6881	8.0000 ± 1.7321
Molecular weight	1426.633 ± 177.7227	908.1178 ± 127.8418
% Unique residues	67.833 ± 11.9127	49.0000 ± 21.4301
% Aliphatic	40.667 ± 12.3860	25.6667 ± 22.6605

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Taken together, the results of the cluster analysis showed that biomineralization peptides can be differentiated and grouped together on the basis of length, molecular weight, percent unique residues, and percent aliphatic residues. Moreover, the results also advocate carrying out truncation studies wherein a long biomineralization peptide sequence can be systematically reduced into a shorter sequence while maintaining its ability to direct nanostructure growth. For example, Sano and Shiba (2003) discovered a titanium-binding peptide that is twelve residues long. A systematic analysis of the roles of each residue in binding deduced that only the N-terminus hexapeptide was important in titanium binding. Generally, using shorter biomineralization peptides such as those belonging to cluster 2 will further broaden the scope and applicability of biomineralization as a tool for nanomaterial synthesis. The utilization of shorter and less heterogeneous peptides is more practical since the synthesis is more straightforward and more cost-effective.

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