Towards Understanding the Influence of Physico-Chemical Environment on Biological Synthesis of Inorganic Materials

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Applied Biology and Biotechnology)

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Rajesh Ramanathan
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This thesis marks my journey through the most exciting and unforgettable period in obtaining my PhD. Throughout this journey, I have made close associations with many people without whom I would not be the person I am. Today, I take this opportunity to extend my sincere gratitude and appreciation to all who were involved, for their guidance, help, friendship, patience, and constructive criticism.

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Rajesh Ramanathan
This thesis is dedicated to,

my parents, Ramanathan Rajamani and Shanthi Ramanathan

and in sweet memories of my brother Rohit
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Abstract

Mother Nature is replete with mechanisms that enable self-assembly at the nanoscale to produce a myriad of inorganic materials with precise dimensions, controlled morphology and high complexity from the assemblage of many smaller and simpler components, which has triggered multidisciplinary research at the threshold where biology meets chemistry and physics. The formulation of these nanostructures appears simple, yet is achieved using complex biochemical pathways. Elucidating the complexity of Nature’s artwork has long been a source of inspiration for materials scientists. The attempt at designing strategies to create inorganic nanomaterials has led to the development of biological ‘Green’ synthesis routes over the past decade or so. This approach typically encompasses biosynthesis (use of live organisms) and biomimetic (use of biomacromolecules) routes and has revolutionised the aspect of nanoparticle synthesis. The challenge now lies in understanding the underlying principles/mechanisms employed by nature towards the synthesis and assembly of these technologically important nanomaterials.

Although biological routes have been well-studied, the role of physico-chemical environment during the synthesis of nanomaterials is still a niche wherein there is limited information. The main objective of this research is to understand the role of physico-chemical environment during biological synthesis of materials that would help elucidate the complex supramolecular chemistry involved in the formation of the intricate and ornate morphologies. This research would, in a nutshell, provide information on some of the critical underlying fundamental principles employed by nature during the synthesis of bioinorganic nanomaterials. The research work outlined herein is split into two distinct aspects involving biological synthesis viz. biosynthesis (metallic silver and copper) and biomimetic synthesis (silica). Although the two aspects concentrate on different systems, essentially the common theme of the important role of physico-chemical environments in understanding the morphological control of nanomaterials links the different chapters.

Firstly (Chapter III), the vital role of a silver resistant bacterial species (*Morganella psychrotolerans*) in controlling the morphology of silver nanoparticles by controlling the bacterial growth kinetics is elucidated. Earlier observations of extracellular nanoparticle
synthesis were challenged and conclusive evidence that the bacterial cell and bacterial physiology can also be truly involved in the reduction process of metal ions is established using electrochemical measurements. This study, for the first time, revealed the important role of physico-chemical environment in controlling the shape of the nanoparticles during biosynthesis approach. Furthermore, it was established that the ability for silver nanoparticles synthesis was a genus-wide phenotypic characteristic and not just adaptive physiology for the Genus *Morganella*. The significance of an entire genus to be able to reduce silver ions was attributed to the presence of silver binding protein SilE (sil gene cluster/silver resistance mechanism) present in the periplasmic space of the bacterial cell. In addition to establishing the presence of silver resistant mechanism and given its similarities to other metal resistance machineries (copper ions), this allowed the synthesis of difficult to stabilize copper nanoparticles using a ‘Green’ biological route. In a nutshell, these studies established the importance of biosynthesis routes and their potential ability to compete with the current chemical synthesis methods.

Secondly, Chapter IV deals with biomimetic silicification that leads to the formation of complex, ornate and hierarchical morphologies, a common occurrence in marine diatoms and sponges. Although the biochemical macromolecules involved in the biosilicification process have been identified with extensive studies utilizing these molecules to hydrolyse silica precursors, understanding the underlying processes involved in the formation of ornate and hierarchical morphologies still remains an open challenge. This chapter outlines the additional and critical role of physico-chemical environment on the formation of 3D ornate structures wherein replacing the commonly employed aqueous solvent with ionic liquids that may mimic natural biosilicification systems has momentous effect in controlling the morphology of biosilica. In particular, this study highlights the role of cationic amino acids in the formation of complex silica morphologies. This chapter further outlines the effect of different amino acids at different concentrations in fabricating unique silica structures, and tries to explain the diversity observed in diatom morphologies. This study takes us one step closer to- and lays the foundation for future insights into understanding the formation of ornate 3D structures resulting from biosilicification.
This chapter provides an overview of the field of nanotechnology, providing a brief history, followed by the importance of shape control and the techniques employed to study this phenomenon including the importance of ‘Green Nanosciences’. The motivations behind this research project revolve around the synthesis of nanomaterials using methods inspired by nature. The rationale for developing this new green route for nanomaterials synthesis is also discussed. Finally, a chapter wise summary of the thesis has been presented.
Part of the work presented in this chapter has been published:

**Refereed Journal Review Articles**


**Articles under preparation**

- **Ramanathan, R.**; Bhargava, S. K.; Bansal, V. “Bacteria as microbial cell factories towards ‘Green’ synthesis of technologically important metal nanoparticles: Past, Present and Future.” to be communicated.
1.1 Nanotechnology: The Science of Miniaturization

The world has witnessed some startling discoveries since the early ages that still remain an inspiration for the future of science. To name a few, Sir Isaac Newton’s monograph “Philosophiae Naturalis Principia Mathematica”; Albert Einstein’s “Theory of Relativity” and Watson and Crick’s “A structure for Deoxyribose Nucleic Acid”. In modern day science, we can relate to the seminal work of Dr. Kim Eric Drexler’s “Engine of Creation: The coming era of nanotechnology” in which he discusses Richard Feynman’s famous talk “There is plenty of room at the bottom” that set the wheels in motion for the establishment of the field of nanotechnology. In the past twenty five years the premises of nanotechnology have been confirmed and we are starting to apply this modern technology in a wide variety of applications from fabricating extremely small devices that can be used for sensing to the development of new generation tools for identifying and targeting infected cells. In the next few decades, it is possible to observe the full realization of Drexler’s concept of the development of an easy and inexpensive way of synthesizing nano-sized materials which may enable us to solve some of the world’s major problems concerning health, the environment and energy that have plagued mankind for eons.

![Figure 1.1: Cartoon representing the scale of nanotechnology showing relative size range of various naturally occurring and man-made objects. (Courtesy: Encyclopaedia Britannica, Inc.)](image-url)
Cutting edge research in nanotechnology has seen the amalgamation of the fields of physics, chemistry, engineering and biology leading to the development and refinement of technology to fabricate and examine materials in the nanoworld. The prefix ‘nano’ is actually derived from the Greek word ‘νάνος’ which means ‘dwarf’ and is commonly used to quantify one billionth of the base unit, which in this case refers to one nanometer (≈10^{-9} m). The term ‘Nanotechnology’ itself was coined back in 1974 by Norio Taniguchi in the work titled “On the basic concepts of nanotechnology” where he describes the fabrication of materials with nano-dimensions. The development in nanotechnology has seen researchers routinely fabricating miniaturized structures of macro- and nano- dimensions (Figure 1.1) with excellent control over morphological characteristics of materials. Interestingly, Nature through its sheer persistence, elegance and ingenious ways has evolved numerous functional assemblages of proteins, nucleic acids, and other macromolecules that can be synthesized and replicated with high precision (Figure 1.1), however replicating the complexity and precision observed in nature is still a distant goal.

\[\text{Figure 1.2: Cartoon representing the two main approaches employed in nanoparticle synthesis.}\]

To achieve this goal of creating miniaturized materials, researchers commonly employ one of two approaches viz. ‘top-down’ or ‘bottom-up’ as outlined in Figure 1.2. Among the two approaches, the top-down approach can be considered as that with which the human race first learnt the art of fabricating materials and in due course, perfected this art to formulate structures at the nanoscale. The top-down approach, in general, involves first fabricating the bulk material before gradually reducing the size of the fabricated material through externally controlled engineering tools. Some examples of this approach include ball-milling, where steel balls are used to grind the
bulk material into nano dimensions; atomisation:\textsuperscript{24-25} where liquid is converted to gas by forcing the liquid through a nozzle at high pressure; and nanolithography:\textsuperscript{26-27} where parts of a bulk substrate are selectively removed to fabricate nano-meter scale structures.

Alternatively, in the bottom-up approach, nanomaterials or nano-structures are fabricated from the build-up of atoms or molecules in a controlled manner that is largely regulated by thermodynamic means such as self-assembly. Illustrations of this approach are very commonly observed in natural processes like protein synthesis, DNA replication etc.\textsuperscript{28} In fact the most commonly used methods for the synthesis of metal nanoparticles using sodium borohydride reduction or citrate reduction are few of the classic examples of the bottom-up approach.\textsuperscript{29-33}

In this journey of creating nanomaterials, much of what is known about bulk properties of these materials breaks down at these length scales.\textsuperscript{13,15-16} For example, semiconductor and metal nanomaterials have physical properties that are significantly different from their bulk counterparts.\textsuperscript{34} Therefore, such technologies potentially generate new opportunities and applications. One such property that we come across frequently is seen in the ‘Lycurgus Cup’ that dates back to 4\textsuperscript{th} century AD, where the optical property of gold nanoparticles was exploited in making stained glass that changed colour depending on the light emanating from the surface (transmission and reflectance).\textsuperscript{35} The advent of technological advances combined with scientific inquisitiveness, has seen scientists developing protocols for fabricating nanoscale materials and explore their potential towards a wide range of applications including but not limited to molecular diagnostics,\textsuperscript{2} catalysis,\textsuperscript{36-39} electronics,\textsuperscript{40} drug delivery,\textsuperscript{5} sensing,\textsuperscript{3-4,6} solar cells,\textsuperscript{41-42} fuel cells,\textsuperscript{43} non-linear optical devices,\textsuperscript{44-45} and surface-enhanced Raman scattering.\textsuperscript{3} Today, the realization of the potential of nanotechnology is only limited by the imagination.

The great deal of potential applications of nanostructured materials is derived from their unique physico-chemical, magnetic, electronic, and optical properties that can be tailored by modifying the shape, size, composition, molecular environment, and the proximity of the nanoparticle to other nanoparticles.\textsuperscript{14-16,46-54} The unusual properties of nanosized materials stem from the fact that small dimensions contain fewer constituent atoms or ions. Therefore there are fewer wave functions in superposition, and thus the
smoothing out of bulk properties resulting from the superposition of many wave functions does not occur. Instead resolution between peaks in the wave functions may result which gives properties which vary considerably from point-to-point in space when compared to those of a bulk material. The small number of atoms or ions therefore results in a smaller number of possible electronic states, and therefore electronic bands of the material, which again may exhibit resolution between them as the number of electrons decreases. This can endow nanosized materials with unusual and very useful properties.\textsuperscript{46,53-54} Hence it is of utmost importance to understand the influence of shape and size of nanoparticles. Another readily distinguishable property is the colour imparted by metal nanoparticles. Dimensions in the nanometer range may impose constraints on the plasma oscillation of electrons on the surfaces of particles, leading to resonances at frequencies specific to the constraints, providing a simple method of detecting modifications to particle dimensions or surface properties. This is more commonly known as surface plasmon resonance (SPR).\textsuperscript{46,48-50}

One of the most important factors that can have a significant influence on the structure and dynamics of nanoparticles (nucleation, growth and aggregation) is the physico-chemical environment.\textsuperscript{55-56} The common processes involved in the synthesis of nanoparticles are through the reduction of metal salts, which almost always stabilizes the particles in a spherical shape that represents its thermodynamically stable form in an isotropic medium.\textsuperscript{55,57} A great deal of work has been performed in order to understand this phenomenon and it is best explained by the classical LaMer theory of nucleation that was proposed in the early 1950s. Although, LaMer studied the crystallization behaviour of sulfur in ethanol, this concept can still be applied to understand the formation of colloids or nanoparticles from a homogeneous medium. This theory suggests that the growth of nanoparticles involves two main processes viz. nucleation and growth.\textsuperscript{58} LaMer’s mechanism suggested that the synthesis of nanoparticles occurs in a way such that there is a rapid increase in concentration of reactants that rises briefly above the saturation concentration. This initiates an initial burst of growth that result in the formation of a large number of small nuclei. This formation of nuclei reduces the concentration of the reaction medium to a point where no further nucleation occurs and further growth of particles occurs through a slow diffusion limited process (Figure 1.3a).\textsuperscript{55-56} This suggests
the rapid nature of the process at initial time points steadily slows down with increase in time. Furthermore it was determined that a short nucleation burst followed by long growth periods is desired for the synthesis of monodispersed particles.\textsuperscript{55,59}

The further understanding of the growth process of these nuclei was given by a diffusion limited Ostwald ripening process, given the extremely high surface area to volume ratios of systems of small particles.\textsuperscript{60} In the case of recently formed extremely small nuclei, the surface excess energy becomes very important in comparison to the total energy of the system. Considering that the system is initially not at thermodynamic equilibrium, a diffusion limited growth process occurs where the formation of larger particles at the expense of smaller particles occurs via Ostwald ripening to reduce the surface area to volume ratio of the system, which in turn reduces the surface excess energy.\textsuperscript{55,58} This effectively lowers the total energy, promoting the growth of larger nanostructures at the expense of smaller particles instead of the formation of new nuclei.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure13.png}
\caption{Schematic representations of (a) the conditions of nucleation proposed by LaMer; (b) mean particle size through a diffusion or reactant limited process.}
\end{figure}

The growth of nanoparticles in solution is governed by the concentration of reactants, and by extension, their diffusion (Figure 1.3b).\textsuperscript{61} E.g. when the rate of the reaction at the surface of the nanoparticle is a great deal slower than the diffusion of reactants through the solution (the rate of reaction is limited by reactants). In this process, the size distribution of the nanoparticles formed when the reaction is completed will be much broader and more symmetrical than those which are governed by a diffusion
limited process (a process where the diffusion of the reactants through the solution is much slower than the rate at which the reactants are consumed).\textsuperscript{61} It is through a complex interplay between differences in the local reactant concentration and the propensity of the reactants to diffuse through the solute that nanoparticles are formed. Another important concept pertaining to crystal growth is the process of ‘self-organization’ that is often used to explain spontaneous ordering forming a hierarchical assembly of nanocrystals to superstructures. The final morphology then formed is essentially affected by the driving force of crystallization (Figure 1.4).\textsuperscript{61}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure14.png}
\caption{The process of self-assembly and self-organization on the morphological variation in crystal growth. Reprinted with permission from ref 61.}
\end{figure}

In the case where the crystal growth occurs near equilibrium conditions, polyhedral crystals are formed and are categorized as self-assembly (This process can also occur from re-organisation where some species surface atoms are highly mobile, and will diffuse into potential energy minima, creating space-filling structure). With increase in driving forces, the crystal growth is governed by mass diffusion, resulting in dendritic forms. In the case where driving forces reach extremes, a diffusion-limited aggregation (DLA) process is observed leading to superstructure formation.\textsuperscript{11,62} This process of superstructure formation is one of the crucial factors proposed for the control of morphologies in the biomineralization process. In later chapters of this thesis, this concept of diffusion and reactant limited processes that lead to different shapes and sizes
of nanomaterials is discussed in detail with particular emphasis on diffusion limited processes that lead to the formation of complex and hierarchical structures.

As outlined previously, due to the focus on energy minimization (reduction in the surface energy of the system), nanoparticles almost always tend to adopt the simplest spherical or quasi-spherical shape. As the shapes and sizes of nanoparticles are in direct correlation with their physico-chemical properties, several other geometric shapes like nanocubes, nanoprisms, nanorods, nanowires, nanodiscs, and nanoplates can also be synthesized by controlling the parameters and kinetics of the reaction.

![Figure 1.5: Schematic outlining the various approaches for the synthesis of nanoparticles.](image)

Given the importance and potential applications of nanomaterials and with the knowledge of nucleation and growth to control the shape anisotropy of these particles, several different routes for the synthesis of nanoparticles (Figure 1.5), especially metal nanoparticles were demonstrated. During initial phase of research in the area, physical routes like vapour condensation, spray pyrolysis, photo-irradiation and thermal decomposition were routinely used for the synthesis of nanomaterials. With advancements in the field, wet chemical methods for the synthesis of nanomaterials were developed that have typically received a wider acceptance than physical methods. With increasing interest and application base of nanomaterials, chemical methods are still the most commonly used route for the synthesis of nanoparticles with reports on excellent control over shape and size of the particles.
The chemical process involves the reduction or oxidation of metal ions with the control over size, shape, stability and assembly, which is achieved by incorporating different capping agents, solvents and templates. Several reports and excellent reviews are available outlining the processes involved in chemical synthesis of nanoparticles.\textsuperscript{65-66,68-70,75}

With the growing importance of nanomaterials, the scope for new synthesis routes is increasing constantly with innovative contributions. Although chemical and physical routes for nanomaterials synthesis of different shape and size have principally dominated the ‘nanosphere’, concern over a negative impact on the environment is also growing. For example, some of the chemical procedures involved in the synthesis of nanomaterials use toxic solvents that could potentially generate hazardous by-products, and often involve high energy consumption (not to mention the unsolved issue of the potential toxicity of certain nanomaterials).\textsuperscript{11,63,74,76-81}

Therefore, the recent past has seen the addition of biological synthesis (also known as green synthesis), which is being seen as one of the most important additions to the repertoire of nanoparticle synthesis routes. This technology includes production of nanomaterials without harming the environment (i.e. environmental friendly fabrication processes for synthesis of nanomaterials which includes reducing chemical wastes, minimizing and/or eliminating use of toxic chemicals, employing methods that do not involve use of extreme temperatures and pressures) and production of nanomaterials that provides facile solutions to environmental challenges (the use of these nanomaterials to reduce pollution and provide efficient alternate energy source).\textsuperscript{74,76-81}

In the following sections of this chapter, the majority of the focus is on biological routes towards nanomaterials synthesis with brief introductions to:

1) Motivation behind this thesis
2) Bio-based approach towards synthesis of nanomaterials
3) Biosynthesis
4) Biomineralization and Biomimetic biomineralization
1.2 Nature: An Inspiration to Design Novel Nanostructures

Mother Nature that engineers materials at the nanoscale is probably one of the most efficient architects known to us. During evolution, Mother Nature has ingeniously created ways to tailor an impressive array of inorganic crystals that almost defies description. All of this is achieved in an environment nurturing manner that has precise dimensions, controlled morphology, is highly reproducible, and shows specific biological functions. These functional nanostructured materials have long been a source of fascination for scientists and engineers and have lured nanotechnologists to explore biological systems to learn and improve skills for precise fabrication of nanomaterials. While Nature is enthralled in formulating nanostructured biomaterials using complicated yet noble biochemical pathways; biochemists, materials scientists and engineers struggle to elucidate the complexity of Nature’s artwork in their respective laboratories. The question that is posed here is can we learn to fabricate materials, The Nature’s Way? This thesis is an attempt, in this relatively new niche and largely unexplored area, to synthesize nanomaterials using methods which mimic those of the Natural World and explain some of the underlying principles employed by Nature.

1.3 Bio-Based Approach Towards Synthesis of Nanomaterials

The knowledge gained from the biological world for materials synthesis has led to relentless attempts to create functional miniaturized structures the nature’s way. In this quest to synthesize materials, two predominant approaches are being explored (i) Biomimetics approach, where biomacromolecules or molecules that imitate naturally occurring biomolecules are employed to create nanomaterials with tuneable properties (e.g. include amino acids, polyamines, peptides, etc.) and (ii) Biological approach or biosynthesis where whole living organisms are employed for the synthesis of nanomaterials (e.g. algae, fungi, bacteria, plants, plant extracts, etc.).

1.3.1 Biosynthesis of Nanomaterials

Biosynthesis approach typically employs whole living organisms for the synthesis of bio-inorganic materials. Although solution-based chemical methods enjoy a long history dating back to the pioneering work of Faraday on the synthesis of aqueous gold
colloids, biosynthesis is still largely in the ‘discovery phase’ wherein different nanomaterials are synthesized using micro-organisms like fungi, bacteria, algae, plants, etc. Micro-organisms have been employed for an eon of time towards remediation of toxic metals due to their inherent capability to withstand high concentrations of heavy metal ions through specific resistance mechanisms, but the possibility of exploring these organisms for nanomaterials synthesis is a relatively recent phenomenon. A few early reports in this area encompassed organisms that are known to create specific functional materials in natural habitats e.g. silica in diatoms, gold in algal and bacterial cells, CdS in bacteria and yeast, ZnS in sulphate reducing bacteria, and magnetite in magnetotactic bacteria. Although using diatoms or magnetotactic bacteria to synthesize nanomaterials in our laboratories might sound interesting, this is not necessarily highly appealing from a fundamental perspective since these organisms are already known to create these specific inorganic materials during their natural growth. An important and even more interesting aspect of biological synthesis is the use of living organisms for the synthesis of those inorganic materials, which these organisms are not known to encounter during their natural growth environments (e.g. gold, silver, oxide nanomaterials, etc.). These observations were the source of inspiration for using micro-organisms for deliberate synthesis of a range of nanomaterials (intracellularly or extracellularly), including bacteria for the synthesis of Au, Ag, Pd, Au-Ag alloy, CdS, ZnS, iron sulphide, and magnetite, algae for the synthesis of gold and silver, fungi for the synthesis of gold, silver, silica, titania, zirconia, barium titanate, CdS and Au-Ag alloy nanoparticles.

One important aspect outlined in chemical synthesis routes is the ability to control shape and size, which confer unique properties to these particles. In an effort to compete with chemical methods, monodispersed gold nanoparticles were synthesized using an extremophilic actinomycete, Thermonospora sp (Figure 1.6a). Furthermore, the extension of this field was triggered by expanding the horizon from micro-organisms to plants and plant extracts, wherein in one of the pioneering works by Sastry et al., the first ever report on shape controlled bio-based approach involving the use of lemon grass extract towards controlling the shape of nanoparticles to form Au nanoprisms (Figure
1.6b) was presented.\textsuperscript{119} Similarly, gold and silver nanoplates were synthesized using extracts of unicellular alga \textit{Chlorella vulgaris} at room temperature.\textsuperscript{88}

![Figure 1.6: Transmission electron micrographs of (a) monodisperse Au nanoparticles synthesized using an actinomycetes \textit{Thermonospora} sp.; (b) Au nanoprisms synthesized using lemon grass plant extract. Reprinted with permission from ref 118, 119.](image)

In the following sections, a broad overview in the area of biosynthesis encompassing eukaryotic and prokaryotic based synthesis is presented. As the work presented in this thesis describes bacterial mediated synthesis of metal nanoparticles (prokaryotic system), only a brief outline of the eukaryotic system is given in the following section with emphasis only on metal nanoparticle synthesis.

### 1.3.1.1 Biosynthesis of Metal Nanoparticles in Eukaryotic Systems

Synthesis of metal nanoparticles, especially those of noble metals, is of enormous importance due to their optical, electronic and chemical properties. Interestingly, highly evolved eukaryotic system including plants, algae, diatoms, as well as other cellular components were recently shown to possess the potential to reduce inorganic metal ions to metal nanoparticles. Of the different eukaryotic systems, plants and fungi have been the most popular choice for the synthesis of metal nanoparticles. Plants are reported to accumulate heavy metal ions and convert them to their non-toxic forms, a property commonly used in the recovery of noble metals from mines. Different plant parts have also been explored for the synthesis of noble metal nanoparticles including silver,\textsuperscript{120-121} gold,\textsuperscript{120,122} and copper.\textsuperscript{123} Though this synthesis route is interesting, additional processing steps are required to recover the particles via this intracellular synthesis. This issue was addressed with the use of plant leaf extracts, wherein these extracts showed the ability to
reduce metal ions and synthesize metallic gold and silver nanoparticles with excellent shape control.\textsuperscript{119,121} Similarly, algal extracts were shown to synthesize nanoparticles with shape control.\textsuperscript{124} In most of these cases where particles were synthesized using extracts, ketones, aldehydes and certain amino acids were claimed to be responsible for the shape control.\textsuperscript{119,124}

On a similar note, fungi like \textit{Fusarium oxysporum}, \textit{Colletotrichum} sp., \textit{Trichothecium} sp. \textit{Penicillium} sp. and many others were also shown to synthesize metal nanoparticles either intracellularly or extracellularly. As biosynthesis of metal nanoparticles using eukaryotic systems is a vast field and is not the main focus of this thesis, this part is only discussed in brief, highlighting only the most relevant studies. Some excellent reviews in the area have been published and the reader is referred to these reviews for further information.\textsuperscript{76,78,125-126}

\subsection{1.3.1.2 Biosynthesis of Metal Nanoparticles Using Prokaryotic Systems}

Microbes (bacteria), like eukaryotic systems, are also known to interact with metals and have been explored for biotechnological application such as ore leaching and metal recovery.\textsuperscript{82-84} However, only in the recent past, have microbes gained significant attention and been explored for the deliberate synthesis of metal nanoparticles. Bacteria isolated from different habitats and nutritional modes have been employed for the synthesis of metal nanoparticles either intracellularly or extracellularly. Among the different biological entities employed for the biosynthesis of nanoparticles, bacteria have received the most attention and are preferred due to their ability to withstand high concentrations of heavy metal ions, ease of culturing, manipulation of genetic make-up and downstream processing.\textsuperscript{63,74,78-79,81}

Most of the research in the field of bacteria mediated biosynthesis has been concentrated on the synthesis of noble metal nanoparticles that dates back to the 1970’s where \textit{Pedomicrobium}-like budding bacteria was shown to accumulate lace-like gold decorated structures and it was postulated that in the near future the ‘Midas-gene’ could be isolated, cloned and over-expressed for fast synthesis of gold nanoparticles.\textsuperscript{127-128} Today, after about two decades of research in the area, a repertoire of bacterial species isolated from different families in the bacterial classification system have been reported
for the synthesis of gold nanoparticles. This ability of bacteria to synthesize nanoparticles was attributed to the presence of potential anionic sites on the cell wall which includes teichoic phosphodiester groups, free carboxylic groups of the peptidoglycan layer, and the sugar hydroxyl groups from wall polymers and amide groups of the peptide chains that bind and reduce gold ions to their Au\(^{0}\) forms.\(^{129-131}\) Of most of the bacterial species isolated for the synthesis of gold nanoparticles, most of the reports are on isotropic spherical or quasi-spherical nanoparticles. But bacterial isolates like *Escherichia coli* (Figure 1.7),\(^ {132-133}\) *Lactobacilli*,\(^ {95}\) *Rhodopseudomonas capsulata*,\(^ {134-135}\) *Bacillus licheniformis* (Figure 1.8c),\(^ {136}\) and *Shewanella algae*\(^ {137}\) have been reported for the synthesis of anisotropic particles ranging from triangular or hexagonal plates to nanowires and nanocubes.

![Figure 1.7: TEM images of (a) Au and (b) Ag nanoparticles biosynthesized using recombinant E. coli by incubating cells with Au ions (1.25 mM) and Ag ions (5 mM) respectively. Reprinted with permission from ref 133.](image)

In addition to gold nanoparticles, the ability of bacterial systems to synthesize nanoparticles from the platinum group of metals (PGM) including palladium and platinum nanoparticles has also been reported. Although the reports for this group are scarce, this shows the potential of bacterial species to synthesize a range of metal nanoparticles with a wide size range.\(^ {138-139}\) In addition to PGM, bacteria have also been shown to survive in high concentrations of tellurium and selenium ions and have evolved cellular mechanisms to convert the highly toxic ionic states of these metalloids to their zerovalent forms. Interestingly, the cellular mechanism for the bioremediation of these metalloids has been well studied with reports of reductase enzymes present in metalloid resistant bacteria mediating the reduction of selenate/selenite and tellurate/tellurite.\(^ {140-141}\) Even more interesting is the fact that some of the organisms reducing these ions have shown the
ability to control the nanoparticle shapes that includes e.g. selenium nanorods by *Pseudomonas alcaliphila*,\textsuperscript{142} and nano-rosettes of tellurium by *Bacillus beveridgei* (Figure 1.8d and 1.8e).\textsuperscript{143} Recently, an even more interesting ability of *Enterobacter* sp. was shown towards the synthesis of mercury nanoparticles. This ability of bacteria to stabilize mercury nanoparticles is important because elemental mercury is known to be volatile in nature.\textsuperscript{144}

![Figure 1.8](image)

*Figure 1.8*: (a & b) shows silver nanoparticles of different sizes and shapes accumulated in *Pseudomonas stutzeri* AG259; (c) SEM of gold nanocubes biosynthesized by *Bacillus licheniformis*; SEM images of (d) Te nanoparticles; and (e) Te nano-rosettes synthesized by *Bacillus beveridgei*. The inset shows the corresponding electron dispersive X-ray spectra. Reprinted with permission from ref 96,136,143.

In addition to the biosynthesis of a range of metals and metalloids like gold, platinum, selenium and tellurium nanoparticles; biosynthesis of silver nanoparticles encompasses a large population of bacterial species with the first reports only dating back to 1992 where silver resistant *Pseudomonas stutzeri* was shown to accumulate silver nanoparticles.\textsuperscript{145} Unlike gold, where the resistance mechanism was only recently reported, ionic silver is known to be toxic to bacterial cells and genes conferring silver resistance have been studied and reported for bacterial survival in high silver
concentration environments.\(^{63,79,81,145-147}\) Although silver biosynthesis is well studied, most studies report isotropic nanoparticles with the exception of \textit{Pseudomonas stutzeri} AG259 where a few triangular plates were found to accumulate in the periplasmic space of the bacterium (Figure 1.8a and 1.8b).\(^9^6\) Although the field of biosynthesis has been much explored, achieving shape control is still one of the biggest challenges with very few reports entailing shape control.\(^6^3\) Even in the case where anisotropic shapes have been achieved, it only reports the outcomes of exposure to heavy metal ions to bacteria, without making any deliberate efforts to control the bacterial growth kinetics to achieve shape control. Another interesting aspect is that, although a wide range of Genuses have been reported (Appendix B-Table B.1) for biosynthesis of metal nanoparticles, in all cases typically only a few species of those particular Genuses have shown the ability to biosynthesize nanoparticles. While this is interesting, if the whole Genus is shown to synthesize nanoparticles, it would be even more interesting. In further chapters of this thesis pertaining to biosynthesis, some of these issues have been addressed and the ability of all the species belonging to a particular single Genus to synthesize silver nanoparticles with excellent control over the shape by controlling the bacterial growth kinetics in one of the species has been elucidated.

### 1.3.2 Biomimetic Synthesis of Nanomaterials

Another important aspect in biological synthesis of nanomaterials is the biomimetic approach (as outlined in Figure 1.5). The term ‘Biomimetics’ was first coined by Otto H. Schmitt and the term itself is derived from \textit{bios} meaning life, and \textit{mimesis}, meaning to imitate.\(^{14^8}\) This relatively new science encompasses the study involving copying, imitating, and learning from biology or nature. Many of the technologies currently in use today are indeed truly inspired by nature. E.g. robotic control systems is inspired by natural neural networks,\(^{14^9}\) artificial intelligence algorithms are inspired by ant and bird behaviour,\(^{15^0}\) aircrafts are inspired from birds,\(^{15^1}\) hydrophobic and hydrophilic surfaces are inspired by gecko feet\(^{15^2}\) and the list continues. Moreover, in addition to mimicking the properties, the way some of the materials are made in nature is also interesting. Some of these materials made in nature contain multifaceted features that are fabricated to form complex nanostructures.\(^{14^8,15^0,15^3}\) The study of these minerals is
broadly known as biomineralization and mimicking these structures represents the area of biomimetic biomineralization that is discussed in the following sections.

1.3.2.1 Biomineralization

Biomineralization is a field that refers to the processes by which organisms form minerals. This typically involves the intersection of many scientific disciplines including biochemistry, molecular biology, geology, crystallography, material sciences, and condensed matter physics. Although this process is not exhibited by all organisms, the phenomenon is still extremely widespread among the five kingdoms which exhibit the capability to synthesize around 60 different minerals of various shapes and sizes. These minerals exhibit a variety of biological functions and typically rely on hierarchical structuring of bioinorganic-organic composites on several length scales ranging from a few Angstroms to centimetre levels. The 60 different biominerals that are currently known to us incorporate metal carbonates, phosphates, halides, sulfates, silicates, oxides, or oxalates.

The complex nature of inorganic materials formed in association with living organisms is not only expressed at the macro-scale but resides in the mesoscopic, microscopic and nanoscopic organization of these biomineralized structures. Indeed, at these different scales, it brings into operation different intermolecular forces and hence the hierarchical orders of construction. Despite these complicated hierarchical structures, one finds it most interesting to observe that the smallest building blocks in such materials are generally of the nanometer length scale. For instance, the bone structure consists of mineral crystal platelets of thickness around few nanometres, embedded in a collagen matrix.

Some well-known biominerals are outlined in Table 1.1. It is clear from the table that minerals, macromolecules and water are the major components of biominerals that are formed under controlled conditions. These biominerals can broadly be classified into three major groups depending on the organisation of mineral constituents. (i) Type 1 encompasses multicrystalline arrays wherein individual crystals are at least arranged in one dimension and more often in all three dimensions. The more common examples include bones, teeth and shells; (ii) Type 2 includes single crystals or finite arrays of...
large crystals. Echinoderms are the best example, (iii) Type 3 incorporates amorphous minerals with the most common example including silica structures of diatoms and sponges of various sizes and shapes depending on the species. The main difference between synthetic minerals and biominerals is the intimate association of various biomacromolecules with biominerals that are responsible for the high level hierarchical ordering and specific functioning in these structures. Some of these biomacromolecules have been studied and constitute proteins, polysaccharide moieties, peptides, and amino acids.

Table 1.1: Examples of the diversity of biominerals seen in nature

<table>
<thead>
<tr>
<th>Biominerals / biogenic minerals</th>
<th>Formula</th>
<th>Biological organism</th>
<th>Biological location</th>
<th>Biological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbonates (calcite, vaterite, aragonite)</td>
<td>CaCO$_3$, CaCO$_3$·nH$_2$O</td>
<td>marine organisms, aves, plants, mammals</td>
<td>shell, eye lens, crab cuticle, eggshells, leaves, inner ear</td>
<td>exoskeleton, optical, mechanical, strength, protection, Ca stone, buoyancy, receptor</td>
</tr>
<tr>
<td>Phosphates (hydroxyapatite, dahlrite, octacalcium phosphate)</td>
<td>Ca$_5$(PO$_4$)$_3$(OH)$_2$, Ca$_3$(PO$_4$)$_2$(OH)$_2$, CaH$_2$(PO$_4$)$_2$</td>
<td>vertebrates, mammals, fish, bivalves</td>
<td>bone, teeth, scales, gills, mitochondria, gizzard plates</td>
<td>endoskeleton, ion store, cutting/grinding, protection, precursor</td>
</tr>
<tr>
<td>Oxalates (whewellite, whewellite)</td>
<td>CaC$_2$O$_4$·H$_2$O, CaC$_2$O$_4$·2H$_2$O</td>
<td>plants, fungi, mammals</td>
<td>leaves, hyphae, renal stone</td>
<td>protection/deterrent, Ca storage/removal, pathological</td>
</tr>
<tr>
<td>Oxides (magnetite, goethite, ferrhydrate)</td>
<td>Fe$_3$O$_4$, α-FeOOH, γ-FeOOH, SFe$_2$O$_3$·9H$_2$O</td>
<td>bacteria, chilons, tuna/salmon, mammals</td>
<td>Intracellular, teeth, head, filaments, ferritin protein</td>
<td>magnetotaxis, magnetic orientation, mechanical strength, iron storage</td>
</tr>
<tr>
<td>Sulfates (gypsum, celestite, barite)</td>
<td>CaSO$_4$·2H$_2$O, SrSO$_4$, BaSO$_4$</td>
<td>jellyfish, acantharia, locusts, chara</td>
<td>statoconia, cellular, intracellular statoliths</td>
<td>gravity receptor, skeleton gravity device/receptor</td>
</tr>
<tr>
<td>Halides (fluorite, herarite)</td>
<td>CaF$_2$</td>
<td>mollusc, crustacean</td>
<td>gizzard plate, statocyst</td>
<td>crushing gravity perception</td>
</tr>
<tr>
<td>Sulfides (pyrite, greigite, wurtzite, sphalerite)</td>
<td>FeS$_2$, ZnS, PbS, Fe$_3$S$_8$</td>
<td>thiopneutes</td>
<td>cell wall</td>
<td>sulfate reduction/ion removal</td>
</tr>
<tr>
<td>Silicates (silica)</td>
<td>SiO$_2$·nH$_2$O (amorphous)</td>
<td>diatoms, plants, radiolaria etc.</td>
<td>cell wall, cellular leaves</td>
<td>exoskeleton, skeleton protection</td>
</tr>
</tbody>
</table>

Of the different biominerals, silica in particular is technologically an important material that is extensively used in a wide range of applications that is not limited to catalyst supports, molecular sieves, resins, polymer supports, and biomedicine. Additionally, it has been estimated that the global market for silica is about two billion dollars per year with the current industrial capacity for silica production standing about $10^6$ tonnes per annum. Commercial silica synthesis is well established, but often requires high temperatures, pressure, and pH. In contrast, silica syntheses in living organisms
like diatoms, sponges, radiolarian, and cyanobacteria proceeds under mild physiological conditions that result in complex and hierarchical silica nanostructural frameworks with exquisite morphologies (Figure 1.9). \textsuperscript{156,174-177} This process of silica formation in living organisms is termed biosilicification. Only recently, an appropriate definition of the biosilicification process has been coined and is given by “The movement of silicic acid from environments where its concentration does not exceed its solubility to intracellular compartments in which it is accumulated for subsequent deposition as amorphous hydrated silica”. \textsuperscript{173}

![Figure 1.9: SEM images of biogenic silica obtained from diatoms and radiolarians.](image)

To elucidate the process of biosilicification, a straightforward systematic approach was employed where the biomolecules associated with the inorganic phase deposition were isolated and studied in detail. \textsuperscript{11,85,169,173,175,178-196} In the process, several proteins and peptides were isolated from different species of diatoms and sponges that were analysed and used \textit{in vitro} for silica synthesis. Table 1.2 provides a brief summary of the proteins considered to be involved in the biosilicification process.
Table 1.2: Biodiversity, characteristics, and proposed role of silica associated proteins.\textsuperscript{169,196}

<table>
<thead>
<tr>
<th>Peptides/proteins</th>
<th>Biological Source</th>
<th>Domains</th>
<th>Reported modifications</th>
<th>Proposed role in biosilicification</th>
</tr>
</thead>
<tbody>
<tr>
<td>pleuralins</td>
<td>diatoms</td>
<td>enriched in Pro, Ser, Cys, Asp</td>
<td>n.d.</td>
<td>theca formation, does not induce silica formation</td>
</tr>
<tr>
<td>silaffins</td>
<td>diatoms</td>
<td>rich in Ser, Lys and Pro</td>
<td>long chain polypeptides, lysine units phosphorylation, glycolylation, and sulfated</td>
<td>formation of spherical particles and particle networks \textit{in vitro}</td>
</tr>
<tr>
<td>silicateins</td>
<td>sponges</td>
<td>cathepsin-like: catalytic triad of His, Asn, and Ser</td>
<td>n.d.</td>
<td>catalysis of silica polymerization \textit{in vitro}</td>
</tr>
<tr>
<td>polyamines</td>
<td>diatoms, sponges</td>
<td>methylated amines N=up to 20</td>
<td></td>
<td>formation of spherical particles \textit{in vitro}, size depends on the chain length</td>
</tr>
</tbody>
</table>

1.3.2.2 Biomimetic Nano-Engineered Silicification: The Art of Imitating Nature

With billions of years of experience and availability of the most all-embracing laboratory at its disposal, we can consider Nature to be the world's principal architect, and it conducts research incorporating every branch of science known to mankind.\textsuperscript{153} The applications of these intricate and ornate structures are many, but one of the major limitation in using diatoms and sponges is that harvesting them from their natural habitats directly would endanger the environmental niche and balance of nature. Additionally the process to artificially grow diatoms in the laboratory is not a simple process. To address these issues, scientists have taken the daunting task of understanding, replicating, and modelling Nature's strategy to build materials from nano- to macro-scale structures.\textsuperscript{197-198} During the past several years, with much research, several proteins and peptides have been isolated from biosilica synthesizing organisms. Recently, biomimetic synthesis approaches using these biomacromolecules and molecules mimicking these proteins and peptides have been utilized to understand the silicification process under ambient conditions.\textsuperscript{11,85,169,173,175,178-181,184-195,199-208}

During the process of biosilification under natural marine conditions, diatoms and sponges take up silica in the form of silicic acid from sea water, and catalyze its polymerization through diffusion-limited precipitation to form well-defined, exquisite, intricate, and most often hierarchical and symmetric patterns of amorphous biosilica nanostructures, which are generally species-specific, and genetically controlled.\textsuperscript{11,172} During this process, it is believed that cationic proteins like silicatein and silaffins promote...
the hydrolysis of silicic acid and act as templates/scaffolds in the formation of the silica structures. These proteins along with synthetic polymers, and molecules that resemble these biomolecules (outlined in Figure 1.10) were employed under laboratory conditions to make silica nanostructures in order to better understand the biosilicification process.11,85,169,173,175,178-181,184-195,199-210

Figure 1.10: Selected examples of biological, bioderived, bioinspired, and synthetic additives used in silica formation in vitro. The five major groups shown are polypeptides, polysaccharides, peptides/proteins, small molecules and synthetic polymers. Reprinted with permission from ref 173.

Inspired by the availability of organic extracts from marine organisms, extracted proteins like peluralins, silicateins, and silaffins were employed for in vitro synthesis of silica-based materials. Under ambient conditions and in the presence of phosphate ions, a mixture of different proteins catalyses in vitro silica formation, but minimal morphological control was observed. It was believed that due to the complex nature of bioextracts, and issues of their availability and purity, investigating their properties could prove a challenging task. Therefore, analogues of bioextracts allowing access to simple or model
biomolecules, non-biological additives or in certain cases even commercially available molecules were employed to study the biosilicification process.

A few selected examples of the several molecules used in the process are summarized in Table 1.3. In the process, a significant amount of understanding was gained and some of the valuable conclusions drawn from these studies are summarized below.

- The use of phosphate ions increased the polymerization process of silica revealing the importance of post-translational modifications for silica polycondensation.
- Although proteins promote silica formation, they do not allow for reproducing the complex nanostructures formed in nature suggesting the involvement of other peptides/proteins or other unknown parameters like physico-chemical environment, salt concentrations etc.

### Table 1.3: Proposed role of various proteins/peptides and synthetic molecules in bioinspired silicification

<table>
<thead>
<tr>
<th>Templating systems</th>
<th>Conditions</th>
<th>Particle size (nm)</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>purified silaffins</td>
<td>mixed and pure silaffins</td>
<td>&lt; 50, 500-700</td>
<td>spherical and aggregates of spherical particles</td>
</tr>
<tr>
<td>native silaffins</td>
<td>different ratios of natSII/natSII2</td>
<td>50-1000 nm</td>
<td>spherical, but leads to controlled silica morphology with medium ratio of natSII2/natSII1</td>
</tr>
<tr>
<td>polyamines</td>
<td>600-1250 Da, pH dependent</td>
<td>100-1000 nm depends on size and pH</td>
<td>spherical</td>
</tr>
<tr>
<td>p(lys)</td>
<td>stirring/shaking, concentration dependent</td>
<td>50-100/500-1000</td>
<td>spherical, hexagonal plates, ladder like structures</td>
</tr>
<tr>
<td>p(arg)</td>
<td>concentration dependent</td>
<td>100-500 depending on concentration</td>
<td>spherical</td>
</tr>
<tr>
<td>p(his)</td>
<td>phosphate-citrate buffer</td>
<td>50-200</td>
<td>spherical and partially fused</td>
</tr>
<tr>
<td>p(lys)</td>
<td>electric field/shear stress/N2 gas</td>
<td>400-700/100-300/500</td>
<td>fused-spherical/fiber like/patterned arch like</td>
</tr>
<tr>
<td>PAH</td>
<td>shaking/stirring</td>
<td>500-1500/100-200</td>
<td>spherical and elongated silica particles</td>
</tr>
<tr>
<td>p(l-amino acid), b- (l-lysine)2</td>
<td>N2 and air</td>
<td>variable</td>
<td>non-ordered silica, spherical, elongated globules, and columns depending on the amino acid</td>
</tr>
<tr>
<td>polyethyleneimine</td>
<td>no special condition</td>
<td>200-800</td>
<td>nearly spherical and fused particles</td>
</tr>
<tr>
<td>lysozyme</td>
<td>stirring, sonication, pH</td>
<td>20-200</td>
<td>poly disperse particles</td>
</tr>
<tr>
<td>bovine serum albumin protein</td>
<td>presence of phosphates and alkylated</td>
<td>100 nm</td>
<td>gel like</td>
</tr>
<tr>
<td>sugars and PEG</td>
<td>different buffers and molecular weight</td>
<td>200-1000</td>
<td>meso- and micro-porous silica depending on weight</td>
</tr>
<tr>
<td>amino acids</td>
<td>no special condition</td>
<td>variable</td>
<td>spherical (larger in presence of N containing, smaller in hydroxyl containing)</td>
</tr>
</tbody>
</table>
• Oligomeric amines show significant activation of silica especially when three amine groups are present.
• In the case of poly cationic amino acids, it shows that L-arginine is more efficient in promoting silica formation than L-lysine, indicating that either hydrogen bonding or nucleophilic activity of the three amines of guanidinium group may promote silica formation.
• In addition to natural polyamines and proteins, block copolypeptides of general formula poly (amino acid<sub>n</sub>-lysine<sub>m</sub>), polyethylene imine (PEI), and polypropylene imine (PPI) also showed ability to promote silica formation in vitro.
• Cationic proteins (silicateins/silaffins) and model proteins (BSA and lysozyme) show enhanced activity in the presence of phosphates emphasising the importance and the role of post-translational modifications in silicification.
• Most biomimetic studies mainly include the use of cationic biomacromolecules emphasizing the importance and requirement of cationic nature.
• The silica precipitation in all these cases leads to an amorphous material that is similar to the biosilica formed in nature.
• Chemical influences (like hydroxyl ion and cation concentrations) and physical influences (like electric fields, hydrodynamic fields) affect the morphology of in vitro polycationic peptide-mediated silica formation into 2D and 3D networks.
• To understand the role of proteins, it was found to be essential to understand the influence of amino acids that form their backbone. Studies involving amino acids were also carried out that yielded spherical silica nanoparticles with no or minimal anisotropic particles/ superstructure formation.

Although bioinspired silica research provides fruitful information that has determined some aspects of biosilicification, structure-directing properties resulted either from the intrinsic self-assembly properties of molecules or through the application of external forces. A few selected examples of nanostructured materials obtained during biomimetic silicification studies are outlined in Figure 1.11. Yet, the controlled assembly of these particles in architectures that would resemble diatom frustules or sponge spicules is far from being achieved. We can convectively say that the
first step towards understanding and elucidating the biosilicification nanostructure formation has been achieved.

![Monodispersed particles](image1.png)

**Figure 1.11**: Selected examples of structures and morphologies of silica that could be produced using bioinspired chemistry. These include monodisperse silica particles from (a) phosphorylates silaffins, (b) cyclic amines, and (c) diblock copolymers; structure controlled complex silica particles from (d) mixture of silaffin proteins, (e) and (f) chimeric silk proteins, (g) and (h) hexagonal plates using PLL under different conditions. Reprinted with permission from ref 85,183,206,211-214.

The next challenge is the possibility of assembling these silica particles into three dimensional hierarchical networks in a highly controlled manner in a process that is similar to nature. From the preliminary data from a few previous studies, it has been postulated that this phenomenon occurs when the complexity (broadly includes multi-mixture components, diffusion controlled reaction, and external forces) of the biomimetic system is increased. Hence, in the near future our aim is to study these aspects and elucidate the mechanism for the growth of the 3D ornate and ordered silica structures.

### 1.4 Nanomaterials Synthesis: A Complex Interplay of Organic and Inorganic Moieties

The conviction that Nature has evolved the best processes for synthesis of inorganic materials in natural habitats (*in situ* synthesis), coupled with the increasing
pressure to develop green chemistry routes for nanomaterials’ synthesis, has encouraged bio-materials scientists to imitate Nature by various biosynthesis and biomimetic approaches (in vitro synthesis). However, in vitro approaches still have some voids in comparison with the in situ materials syntheses. For instance, the major differences between natural and biomimetic silica formation can be understood if we compare the following aspects of mineral formation – precursor concentration, pH at which biosilica polymerization occurs, temperature, time required for biosilica deposition, control over the process and product, involvement of other molecules (ions, organic molecules, membranes, etc.), surrounding conditions where the silicification process takes place, and reproducibility.\textsuperscript{196} Biosilicifying organisms typically start with an under-saturated solution of silicic acid; on the other hand, the precipitation of silica in vitro from an under-saturated solution of silicic acid (≤ 1 mM) is yet to be demonstrated. Preparation of particulate silica synthetically requires moderately high pH; biosilicification in contrast, occurs at mildly acidic to neutral pH.\textsuperscript{172} Biological silicification imposes great control over silica formation process as well as on the form of biosilica, while there is less control under in vitro synthesis conditions. Other possible regulating factors present in biological systems include microtubules, filaments and cell organelles, which are absent under in vitro conditions. Another major difference between biological and bioinspired silicification is that the former takes place in a genetically controlled environment and under high physical pressure environment.\textsuperscript{169,215} In the light of these facts, it can be argued that if in vivo materials synthesis routes (involving whole cell or organism) could be evolved to work in silico (under laboratory condition), this might be able to fill in the gap between in situ and in vitro materials synthesis.

Since biosilicification by living organisms mostly employs aqueous environments in their natural habitats, water has so far been considered as the most obvious medium of choice to study biomimetic silicification under laboratory conditions. Hence, most of the biomimetic silicification studies reported thus far in the literature have employed aqueous solutions. Despite significant efforts towards understanding the biosilicification processes, as far as the control over silica morphology is concerned, most of the previous studies have hitherto not been able to produce 3D ornate silica morphologies through biomimetic silicification, similar to those produced by diatoms and sponges in their
natural habitats. In the recent past, it has been recognized that in addition to biomolecules involved in biosilicification, the physico-chemical environment in which this process happens in these organisms may also play a major role towards dictating the shape of nanostructured silica in these organisms.\textsuperscript{169,210,215} The role of these physico-chemical parameters such as how silicic acid is taken up from the surrounding marine environment of diatoms, transported to silica deposition vesicles (SDVs), deposited in 1000-fold higher than environmental amount in SDVs, and further condensed into ornate silica structures still remains unclear.\textsuperscript{169} Similarly, the effects of high salt concentrations, various physico-chemical forces, and high pressure levels including shear stress under deep marine conditions where these organisms grow, is also a mystery.\textsuperscript{169} Notably, in one of the previous studies, when external forces such as electrostatic field or shear stress was applied during biomimetic synthesis of silica in water, fused silica nanoparticles in the form of fibers, platelets and dendritic silica could be obtained.\textsuperscript{210} However, the possibility of achieving ornate hierarchical nanostructures that resemble diatom frustules or sponge spicules is still out of reach. This also suggests that the previous biomimetic studies, which were thus far explored in aqueous solvents, do not necessarily mimic the natural biosilicification environment. In further chapters of this thesis pertaining biosilicification, we have addressed some these issues and shown the ability of different biomolecules to control the assembly of silica structures and form 3D nanostructural framework.

1.5 Motivation

Biological synthesis encompassing biosynthesis and biomimetics is still in the discovery phase wherein different materials are synthesized by using biological entities and biomimetics. The best processes adopted by nature to fabricate nanomaterials with precision offers a great source of inspiration to explore and adopt nature’s methods. Although much work has been done on exploring the different possibilities using biological entities and biomimetics, morphological control is a niche area that requires much work and understanding to compete with chemical synthesis routes. In the work presented in this thesis, the important role of the physico-chemical environment in controlling the morphology of nanoparticles during biological synthesis (encompassing biosynthesis and biomimetics) is explained. A brief outline of the work presented in this thesis is given in the next (section 1.6), with elaborate discussions in separate chapters.
1.6 Thesis Outline

The work presented in this thesis describes biological (biosynthesis and biomimetic) approaches towards synthesis of nanomaterials with particular emphasis on the role of the physico-chemical environment in controlling the morphology of nanomaterials and understanding the underlying mechanism involved in these processes. The results obtained in this thesis is divided in two main parts viz. (i) biosynthesis of metal (silver and copper) nanoparticles; and (ii) biomimetic silicification studies in ionic liquids with emphasis on understanding the formation of 3-D nanostructures formed in diatoms in their natural habitats.

The chapter breakdown of the thesis is as follows:

Chapter II describes the range of instruments used in this body of work for the characterization of the materials synthesized. The metal and metal oxide nanoparticles synthesized using a bio-based approach were characterized using UV-visible absorbance spectroscopy (UV-vis spectroscopy), vibrational spectroscopy including Fourier-transform infrared spectroscopy (FT-IR) and Raman spectroscopy, X-ray diffraction (XRD), electron microscopic techniques like transmission electron microscopy (TEM), high-resolution transmission electron microscopy (HR-TEM), and scanning electron microscopy (SEM), elemental analysis technique like energy dispersive X-ray analysis (EDX) and X-ray photoemission spectroscopy (XPS) and Thermogravimetric analysis (TGA).

Chapter III provides details of the work performed addressing the biosynthesis aspect of biological synthesis. This chapter mainly discusses the biosynthesis of silver and copper nanoparticles using the Genus Morganella. As this chapter encompasses two different metallic nanoparticles employing a biosynthesis route, this chapter is split in to two sub-chapters. Chapter III A discusses the ability of the Genus Morganella (Family: Enterobacteriaceae) towards the synthesis of silver nanoparticles. Furthermore, the unique ability of a psychrotolerant species belonging to the same Genus was shown to control the shape anisotropy of the silver nanoparticles by controlling the bacterial-growth kinetics at different temperatures (physico-chemical environment). Although the synthesis of silver nanoparticles was found to be extracellular, electrochemical evidence suggested that the actual synthesis of silver nanoparticles happens within the bacterial
cell. This work advocates the complex nature of biosynthesis that still requires significant understanding. **Chapter III B** is an extension of the silver work where the ability of silver resistant *Morganella morganii* RP42 to synthesize and stabilize metallic copper nanoparticles is discussed. This ability was attributed to the similarities observed in the silver and copper resistance machinery that was validated using bioinformatics sequence alignment tools.

**Chapter IV** deals with biomimetic silicification to understand the formation of 3D nanostructural framework in diatoms. In order to understand the role of proteins/peptides in the biosilicification process, it was essential to understand the role of each amino acid separately, followed by increasing the complexity of the system by introducing polyamines, peptides and finally proteins. In this chapter, we demonstrate that by changing the choice of medium from water (which was considered as the medium of choice as diatoms are found in aqueous medium) to ionic liquids (molten salts at room temperature), biomacromolecules that only demonstrated the ability to synthesize particulate silica, now showed the ability to arrange these silica particles in nanostructural, exquisite frameworks that somewhat resemble diatom structures found in nature (physico-chemical environment). As outlined earlier, cationic proteins and peptides play a major role in silicification process, hence the first part of the chapter explains the biomimetic silicification process with particular emphasis on the role of cationic amino acids. This study was further extended to understand the role of other amino acids correlating the structure of amino acids to the silica morphology obtained. Interestingly, the resulting structure obtained from each amino acid showed unique morphology suggesting that a permutation and combination of these amino acids might be responsible in determining the complex nature of structures formed in nature.

**Chapter V** provides a summary of the research completed during this PhD candidature and provides a scope for future possible research in the areas studied.

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Chapter II

Characterization Techniques

The operating principles of the different experimental characterization techniques used during the course of the present work are discussed in this chapter.
2.1 Introduction

Biological and biomimetic synthesis of metal and metal oxide nanoparticles under ambient conditions and an understanding of the underlying mechanism involved in the synthetic routes is the central theme of this thesis. In this quest, a range of spectroscopic, microscopic, and other biological techniques were used to probe the nanomaterials and their synthesis mechanisms. This chapter provides an overview of the basic principles governing these techniques used in characterization of the nanomaterials synthesized.

2.2 UV-Visible Absorption Spectroscopy (UV-vis spectroscopy)

Electronic transitions within molecules are often induced by excitation with electromagnetic radiation, whereby a photon interacts with the molecule to promote an electron from a bonding or non-bonding orbital to an anti-bonding orbital. The quantum of energy of a photon that can be exchanged with the molecule is therefore a measure of the separation of energy levels of the transition and is dependent on the frequency of the incident radiation, which can be explained by ν (in s\(^{-1}\)) as per the following equation:

\[
\Delta E = h \nu = \frac{hc}{\lambda}
\]

where ΔE gives the change in energy of the molecule in an electronic transition, \(h\) is Planck’s constant (6.626 × 10\(^{-34}\) J.s.), \(c\) is the speed of light (3 × 10\(^{8}\) ms\(^{-1}\)), and \(\lambda\) is the wavelength of incident photon.

The excitation of one photon simply follows the Beer-Lambert law and relates the intensity of incident photon (\(I_0\)) to the intensity of transmitted photon through the sample (\(I\)), via the following equation:

\[
\log_{10} \frac{I_0}{I} = \varepsilon cl = A
\]

where \(\varepsilon\) is the molar extinction coefficient of the sample, \(c\) is the molar concentration, \(l\) is the pathlength of sample, and \(\log_{10} \frac{I_0}{I}\) the absorbance or optical density that is simplified to \(A\).

UV-vis spectroscopy is an important and powerful tool in the characterization of materials on the nanoscale. In the case of nanomaterials, the photon absorbance is due
to the surface plasmon resonance (SPR) that has an effect of masking the electronic transitions.\textsuperscript{3} As the SPR is the result of collective oscillations of the surface electrons, the number and position of the absorbance bands observed in a UV-vis spectrum are in correlation with the axis along which the electrons can resonate.\textsuperscript{3,5} For instance, spherical particles have continuous rotational symmetry and electrons generally oscillate at a same length along all axes giving rise to a single absorbance peak. Conversely, anisotropic particles possess multiple axes along which the electrons can oscillate and result in multiple absorption bands (Figure 2.1) each corresponding to a linear dimension of the particle.\textsuperscript{3,5} By studying the changes in absorption maxima using this technique, the changes in the SPR features can be observed and the interpretation of this can be related to the nanoparticle shape, size and composition.\textsuperscript{5} In the case of particles with two-dimensional anisotropy (e.g. rods, triangles), the two SPR absorption bands are the function of the aspect ratio of nanorods or thickness of the triangles.\textsuperscript{6-8}

\textbf{Figure 2.1:} SPR features of anisotropic particles showing the correlation between the shape of particles and the absorption bands. Reprinted with permission from ref 6,8.

In the work presented in this thesis, UV-vis absorption spectroscopy has been used to monitor the shape and composition of metal nanoparticles synthesized using a bacterial mediated approach. These measurements were performed on Cary 50 Bio Spectrophotometer operating at a resolution of 1 nm over a wavelength range of 200-1000 nm.
2.3 Vibrational Spectroscopy

Vibrational spectroscopy is an important and definitive technique to identify molecules and surface species by observing the atomic vibrations of molecules on exposure to electromagnetic radiation of wavelengths typically of 2.5 – 25 µm, which is equivalent to 400-4000 cm\(^{-1}\) (typically the wavelength is larger than that used in UV-visible spectroscopy). Spectroscopic techniques can be employed to study both organic as well as inorganic materials with the exception of metallic materials due to their ability to strongly reflect electromagnetic waves below the plasma frequency.\(^9\) Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy, the two most commonly used characterization techniques employed in materials science assist the study of molecular interaction by providing a fingerprint of the absorbed species and provide vital information for deducing molecular bonding orientations on the material surfaces.\(^10\)

In molecular vibration, each atom executes a simple harmonic oscillation about its equilibrium position. An atom in excited state can possess different stretching vibrations along the x, y and z directions. Generally, for molecules with N atoms, there are 3N-5 for a linear molecules, while 3N-6 possible modes of vibration for a non-linear molecule.\(^11\) The vibrational states can be probed in a variety of ways. The most direct approach is through infrared spectroscopy where energy is transferred from incident radiation (a photon) to the molecule resulting in infrared absorption while when the photon is inelastically scattered, it results in weak bands shifted toward either side of the infra-red absorption, a phenomenon more commonly known as Raman scattering.\(^10\)

2.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

The atoms in a molecule are in a constant state of vibration about their mean positions at non-zero temperatures. Due to this vibrational motion, if there is a periodic alternation in the dipole moment, such modes of vibration are infrared (IR) active.\(^12\) The vibrating molecules absorb energy only from that radiation, with which it can coherently interact \(i.e.\) the radiations of its own oscillation frequency. This endows each functional group with specific vibrational frequencies, which are very sensitive to its chemical environment and the neighbouring species.\(^9\)\(^-\)\(^12\) The appearance or non-appearance of certain vibrational frequencies in the IR spectrum of any molecule gives valuable
information about the structure and orientation of that particular molecule. The vibrational frequency can be calculated using the following equation:

\[ \nu = \frac{1}{2\pi} \sqrt{\frac{k}{\mu}} \]

where \( k \) is the force constant of the associated chemical bond and \( \mu \) is the reduced mass.

In addition to infrared spectrum, employing the mathematical operation of Fourier transformation enables researchers to dramatically increase the signal to noise (S/N) ratio of a spectrum by reducing the time required to obtain a scan and hence increasing the total number of scans that can be performed. This can be achieved by illuminating the sample with a beam of light of multiple frequencies at once and measuring the intensity, rather than illuminating the sample with a monochromatic beam of light which can take a great deal of time to analyse all frequencies. A Fourier transformation is then employed to convert the time domain (intensity vs. time) signal to a frequency domain signal (intensity vs. frequency) that can then be deciphered by the researcher.\(^{14-15}\)

FTIR has been used to characterize the metal and metal oxide nanoparticles synthesized using a biological/biomimetic approach. All FTIR measurements presented in this thesis were performed using a Perkin Elmer Spectrum 100 instrument equipped with micro-ATR (diamond ATR crystal), in the range of 650-4000 cm\(^{-1}\) region with a resolution of 2 cm\(^{-1}\). Each spectrum represents an average of 10 scans. The background spectra consisted of the base diamond crystal under the same experimental conditions, which have been deducted from the respective signals before presenting in this thesis.

### 2.3.2 Raman Spectroscopy

Complimentary to infrared spectroscopy, Raman spectroscopy is sensitive to changes in the vibrational states of molecules which alter their polarizability in contrast to changes in dipole moment observed in infrared spectroscopy.\(^{11,16}\) When a photon interacts with a molecule and is in-elastically scattered there occurs a net effect where the frequency of the scattered electron is altered slightly, this change in frequency can be measured and this forms the basis of the Raman spectroscopy technique.\(^{17}\) If the
molecule absorbs energy from this interaction, the scattered photon will be red-shifted (i.e. it will have a lower energy with respect to the incident photon) in order to keep the total energy of the system balanced. This is referred to as a Stokes shift (outlined in Figure 2.2). Whereas if the molecule loses energy through interaction with a photon, the scattered photon will be blue-shifted (the photon will possess greater energy than the incident photon), this is referred to as an anti-Stokes shift.\textsuperscript{11}

![Figure 2.2: The different forms of scattering](image)

Samples for Raman measurements were prepared by (liquid) drop-casting the particles or in the case of powders, the samples were directly placed on a silicon substrate deposited with 100 nm gold by electron evaporation technique and all measurements were performed using a Perkin-Elmer Raman station 200F (785 nm laser, spot size 100 µm) with an exposure of 1s and 20 acquisitions with a resolution of 2 cm\textsuperscript{-1}.

2.4 X-ray Photoemission Spectroscopy (XPS)

XPS, a highly surface sensitive technique, provides useful information about the composition and electronic transitional state of the surface region. Due to the ability of this technique to also provide a quantitative measurement, it is also referred by an alternative acronym, Electron Spectroscopy for Chemical Analysis (ESCA). XPS is primarily based on the photoelectric effect (single photon in/electron out process) first explained by Albert Einstein. In XPS, a monochromatic source of x-rays is directed at the sample (photons of fixed energy given by $E = h\nu$) that interacts and is absorbed by the sample leading to ionization of the atom (A) and emission of core electrons (inner level) termed as photoelectrons from the top layer of the sample surface (Figure 2.3). The kinetic
energy distribution of the emitted photoelectrons is measured and a photoelectron spectrum is obtained. This photoionization process can be given as:

\[ A + h\nu = A^+ + e^- \]

Conservation of energy requires:

\[ E(A) + h\nu = E(A^+) + E(e^-) \]

Since the electron that is ejected will be observed to possess kinetic energy (KE), the above expression can be rearranged in terms of kinetic energy as:

\[ KE = h\nu - [E(A^+) - E(A)] \]

This difference in the energies of the ionized and neutral atom (the final term in parentheses) is generally referred to as binding energy (BE) that leads to the following commonly quoted equation:

\[ KE = h\nu - BE \]

Also, the binding energy of electrons in various energy levels are conventionally measured with respect to the Fermi level rather than vacuum level, leading to a small correction to the above equation in order to account for the work function (\(\phi\)) of the solid:

\[ KE = h\nu - BE - \phi \]

---

**Figure 2.3:** (a) Path flow of electrons when x-rays of fixed photon energy is bombarded on a target surface in XPS. (b) The flow of electrons from lower layers leading to collisions.

Through a simple algebraic rearrangement, if photons of fixed energy are employed and the KE and work function of the samples are measured, the BE of the
electron can be calculated. As each element has a unique electronic configuration, the BE is a characteristic of the element and will therefore result in different XPS spectra.

The exact BE of an electron not only depends upon the level from which the photoemission is occurring, but also on the formal oxidation state of the atom and the chemical and physical environment. A subtle change in either of these gives rise to shifts in peak positions in the spectrum, commonly referred to as chemical shifts. This makes XPS one of the most important characterization techniques for determining the chemical states and environment of each element in a sample.

For the work presented in this thesis, the XPS spectra of various core levels were recorded and their BE were aligned with respect to C 1s BE of 285 eV. The samples (liquid dispersed) were prepared by drop-casting the particles onto a silicon substrate deposited with a 150 nm gold layer by electron evaporation or in the case of powder samples were directly analysed using a modified plate holder (CuO) designed specifically for powder samples. XPS measurements were carried out using a Thermo Scientific K-Alpha X-ray Photoelectron Spectrophotometer instrument at a pressure lower than $1 \times 10^{-9}$ Torr (1 Torr = $1.333 \times 10^2$ Pa). All scans were recorded with un-monochromatized Mg Kα radiation (photon energy of 1253.6 eV) at pass energy of 20 eV and an electron take off angle of 90°. The overall resolution for all XPS measurements was 0.1 eV. The core level spectra were background corrected using the Shirley algorithm and chemically distinct species were resolved using a nonlinear least squares fitting procedure.18

2.5 X-ray Diffraction (XRD)

X-ray diffraction is a powerful tool to probe the crystalline structure of a material by bombarding the target with x-rays and observing the interference patterns as the incident x-rays are scattered.19-21 The x-rays reflected from the surface will have travelled a shorter distance than those that are reflected from an internal plane of the crystal structure (Figure 2.4).
For a constructive interference at a particular angle, x-rays that penetrate and reflect from an internal crystal plane of a material must travel an added distance equal to an integer number \((n)\) of wavelength \((\lambda)\). This distance is dependent on the spacing between atomic layers \((d)\) and the incident angle of the electron beam \((\theta)\). This was first recognised by W. L. Bragg and is given by Bragg’s equation as follows:

\[
n\lambda = \frac{\overline{ABC}}{d} = 2d \sin \theta
\]

In the case where the angle of incidence and the wavelength of x-rays are known, the Bragg equation can be rearranged to allow the interplanar spacing to be calculated that can be given by:

\[
d = \frac{n\lambda}{2 \sin \theta}
\]

By calculating the interplanar spacing, structural information about the crystal planes of the synthesized materials can be determined.\(^{19-21}\)

All XRD patterns presented in this thesis were obtained using a Bruker AXS D8 Discover with a General Area Detector Diffraction System (GADDS) micro diffraction instrument operating at 40 kV and 40 mA over an angular – \((2\theta)\) range of 15-90°. Samples for XRD were prepared by drop-casting the particles on a freshly etched Si (100) substrate (for metal nanoparticles) or by drop-casting the particles on a 100 nm evaporated thin film coated gold substrates (in the case of oxide nanoparticles).

### 2.6 Electron Microscopy (EM)

Although some structural features and morphological information can be obtained from XRD and UV-vis spectroscopy respectively, direct imaging of particles in the nano
dimensions is only possible using electron microscopy. Although the basic principle for light microscopy and electron microscopy is similar, in light microscopes the resolution of image depends on the wavelength of light, whereas electron microscopes use beam of electrons with de Broglie wavelengths that are inversely proportional to the voltage used to accelerate them (typically shorter than visible light). This helps EM to achieve images of resolution up to sub-nanometer scale that is several orders of magnitude greater than light microscopes.

2.6.1 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) refers to a technique where a beam of electrons, typically emitted from a tungsten or lanthanum hexaboride (LaB$_6$) filament, or a field emission gun (FEG) cathode using accelerating voltages typically in the order of 100 – 400 kV, are directed through the use of magnetic condenser lenses to interact with an ultrathin sample. The accelerated electrons from the cathode that interact with the sample can have one of the several fates; (i) electrons pass through the sample undeflected, (ii) electrons are elastically scattered (iii) or electrons are in-elastically scattered. When all the three types of interaction contribute towards imaging, the resultant image would have no contrast. The insertion of different apertures alters the interaction of electrons with the sample leading to the formation of a clear image that is in direct correlation to the thickness and type of material under the microscope. E.g. heavy and dense elements scatter more electrons hence appear dark, while materials of different thickness will also deflect more electrons making them appear dark.

Samples for TEM were prepared by drop-casting the solution on to a carbon-coated copper grid followed by TEM measurements using a JEOL 1010 TEM instrument operated at an accelerating voltage of 100 kV. Samples for high resolution-TEM were also prepared by drop-casting technique on to a holey carbon support film followed by TEM measurement using a JEOL 2100F TEM instrument operated at an accelerating voltage of 200 kV.

2.6.1.1 High Resolution TEM (HR-TEM)

Contrast formation is a major mode of operation in TEMs with complex techniques utilizing the unique ability of lenses. HR-TEM is one such mode that allows
imaging of the sample at an atomic scale. This mode of operation is also called phase contrast mode, wherein the images formed are due to differences in the phase of electron waves caused by specimen interaction rather than the absorption of electrons by the sample.

### 2.6.1.2 Selected Area Electron Diffraction (SAED)

SAED is a crystallographic experimental technique performed in conjunction with TEM and HR-TEM. An ultrathin crystalline material when subjected to a parallel beam of high energy electrons results in a diffraction pattern. These patterns are observed because the wavelength of high energy electrons is a few thousandths of a nanometre while the spacing between atoms is 10-100 times higher that leads to atoms acting as a diffraction grating, leading to constructive interference at particular angles and thus a diffraction pattern.

Because the user can select the area from which the diffraction pattern is obtained, it is commonly known as selected area electron diffraction. E.g. a crystalline sample in a particular area would display diffraction spots but if the same sample is tilted, depending on the symmetry, aligning the optical axis with a different zone axis will result in a different diffraction pattern. In addition, different volumes of the same sample can be irradiated and their diffraction patterns can be compared to probe homogeneity. Similar to XRD, this technique can be used to identify crystal structures and crystal defects, however the region from which the diffraction pattern is obtained in this technique is typically a few hundred nanometres in diameter in comparison to a few millimetres as observed by XRD.

### 2.6.2 Scanning Electron Microscopy (SEM)

Similar to TEM, SEM also uses a beam of electrons to image sample surface but instead of recording the electrons passing through the sample, SEM records the secondary electrons released from the sample as a result of interaction with the electron beam resulting in a pseudo 3-D image of the sample. As in certain TEMs, SEMs also generate electrons using a field emission gun (FEG) or Tungsten or LaB₆ filament. Additionally, the observed image is only a pseudo 3-D image reconstructed from the
signal emitted from interactions of the beam of electrons with atoms at or near the surface of the sample.\textsuperscript{25}

The interaction of electron beam and the sample results in two basic types of signals viz. secondary electrons (SEs) and backscattered electrons (BSEs). For the purpose of this body of work we have not utilized BSEs and hence they are not discussed here in detail. Scattered electrons on the other hand, are ejected from the valence or conduction band that escapes from a relatively shallow depth of the sample resulting in high resolution images of the sample surface. In SEM images, the topographic features appear brighter as scattered electrons are readily available to escape from the surface, which is a contributing factor to the 3-D imaging ability. Image contrast is based on the intensity of electrons emitted from each spot on the sample.\textsuperscript{24-26}

Samples for SEM were prepared by drop-casting the samples on to an aluminium stub and coated with 40 Å of platinum prior to imaging to minimize surface charging. SEM images were obtained using FEI Nova NanoSEM instrument operated at an accelerating voltage of 30 kV, 4 nm spot size and 5 mm working distance.

\section*{2.7 Energy Dispersive X-ray Analysis (EDX)}

EDX is a microanalysis technique that detects x-rays emitted from the sample during bombardment by an electron beam and is often used in parallel to SEM analysis. The electrons generated in the SEM interact with the sample and generate high enough energy that forces the ejection of electrons to the conduction band from the innermost orbitals of the atoms, as depicted in Figure 2.5. In the event where an electron is ejected, a momentary vacancy is created and an electron from a higher energy shell (relative to the ejected electron) fills the resulting vacancy. As this process involves two shells possessing different energy states, the excess energy is released as a quantum of energy that corresponds to the x-ray region of the spectrum.\textsuperscript{27}

The photon generated by this electronic transition is largely dependent on the shell from which the electron is ejected. E.g. if an electron is ejected from the K shell and replaced by an electron from the L shell, the photon generated corresponds to Kα,
whereas if an electron from the M shell falls into the vacancy the photon generated will be designated as Kβ and so on (these phenomena are shown in Figure 2.5).\textsuperscript{25,27}

The unique electronic configurations possessed by different elements generate unique X-ray spectra. Therefore, the intrinsic elements present within a sample can easily be qualitatively determined using EDX analysis. Additionally, the collected X-rays can also be used to semi-quantitatively determine the abundance of elements within a composite material through comparison with internal standards. However, an error of about 15% can be expected for lighter elements.\textsuperscript{24-25,28}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2_5.png}
\caption{The mechanism for EDX where the ejection of electron to valence or conduction band leads to various possible X-rays detected in EDX analysis.}
\end{figure}

EDX analyses were performed on a FEI Nova NanoSEM instrument coupled with an EDX Si (Li) X-ray detector. The accelerating voltage and spot size was 30 kV and 3 nm respectively, while the working distance was 5 mm.

\section{2.8 Thermogravimetric Analysis (TGA)}

Thermal analysis is another important technique in which the physical property of the material is measured as a function of temperature. In TGA, in addition to determining the changes in chemical or physical properties of the material, information about phase
transformations as a function of temperature can also be obtained. The principle behind TGA is the measurement of weight loss as a function of temperature, where the TGA curve provides information on the thermal stability of the initial material, intermediate compounds and the residues, if any are formed during the process. Additionally, the weight losses observed in TGA can also be quantified to predict the pathway of degradation or to obtain compositional information. The experimental data offers more sophisticated understanding of reactions occurring when a material heating occurs.\textsuperscript{29} This ability to obtain measurements at higher temperatures is highly useful for the inorganic materials used in this thesis.

TGA was performed to find out the contribution of biomolecules in the silica–amino acid hybrid nanostructures. TG analysis of the silica powders was performed on a TGA–7 Perkin Elmer instrument at a scan rate of 10 °C per min.

\section*{2.9 References}

Chapter III

Biological Synthesis of Metal Nanoparticles: A Link Between Bacterial Heavy Metal Resistance and Nanoparticle Synthesis

This chapter discusses the use of a silver resistant bacterium *Morganella* for the biosynthesis of metal nanoparticles. An interesting proposition to control the shape of nanoparticles by a psychrotolerant species from Genus *Morganella* is described. More interesting is the fact that although the nanoparticles were found extracellularly, the process of metal ion reduction was discovered as an intracellular phenomenon, indicating the complexity of the overall process. Furthermore, all the members of Genus *Morganella* were also investigated for its ability to extracellularly biosynthesize silver nanoparticles. The similarities in the resistance machinery of silver and copper ions led us to further explore the same Genus to synthesize metallic copper nanoparticles. The synthesis route was found to be very similar to that observed in the case of silver nanoparticles suggesting the similarities can be extrapolated for the reduction of other metal ions.
Part of the work presented in this chapter has been published:

**Refereed Journal Articles**


**Refereed Conference articles**


**Refereed Conference Extended Abstracts**


**Articles under preparation**

3.1 Introduction

As nanotechnology emerges as an interdisciplinary field with potential to influence various aspects of human life through a myriad of applications; biological synthesis of nanomaterials is gaining particular attention as a rapidly growing discipline of Bio-nanotechnology with an enormous application potential in the coming future. As outlined in Chapter I, the potential of a wide array of biological entities including bacteria, fungi, plants, algae, and yeast has been explored towards the biosynthesis of metal, metal oxide, and metal sulfide nanoparticles. The beauty lies in the fact that these processes occur at near ambient temperatures, pressures and neutral pH. From a materials perspective, the formation of nanoparticles by various biological entities in the natural way is highly inspirational. Even more fascinating is the fact that there are organisms that do not encounter many of the heavy metal ions in their natural habitat, yet show capability to efficiently synthesize nanomaterials.

Among the different biological entities, bacteria are the preferred choice for nanoparticle biosynthesis due to extracellular nanoparticle production and ease of culturing, manipulation and downstream processing. Additionally, the ability to study metal ion transport and detoxification with relative ease makes bacteria ideal candidates for nanoparticle biosynthesis. A repertoire of species that span the bacterial classification table has been identified for the synthesis of different metal nanoparticles (Appendix B – Table B.1). In order to exploit the full potential of bacteria for biological synthesis of nanoparticles, it is essential to understand the plausible biochemical mechanism of nanoparticle synthesis.

Another important aspect to be addressed is that, to date, the majority of research has been focused on synthesis of isotropic (i.e. spherical or quasi-spherical) metal nanoparticles, with the development of several methods to achieve excellent control over size distribution. The importance of nanoparticle shape anisotropy has been realized recently, with an emphasis on exploring the correlation between different shapes and their physico-chemical properties. Importantly, most of the biosynthesis efforts exploring microorganisms for nanoparticles synthesis have hitherto led to formation of isotropic nanoparticles, with only limited reports on shape controlled...
biosynthesis of Au nanoprisms using plant extracts such as Aloe vera\textsuperscript{14} and Lemon-grass.\textsuperscript{15} More so, in these reports on plant-based synthesis of Au nanoprisms, plant extracts (and not the whole plant as such) were used, wherein chemical species such as ketones and aldehydes were identified to be responsible for shape control.\textsuperscript{15} Therefore, if plant extract-based nanomaterials synthesis is considered as pseudo-biological; there is per se no report on shape-directed biological synthesis of anisotropic metal nanoparticles by directly involving a microorganism. The propensity of microorganisms to almost always synthesize spherical nanomaterials in the presence of metal ions is most probably due to their focus on energy minimization, as it is easiest to create a spherical shape.\textsuperscript{1,3,6}

One of the pioneering works in the field of bioremediation is the discovery of prokaryotes capable of gaining energy from the oxidation/reduction of metal ions. This phenomenon in turn led to the identification of specific genes that confer bacteria with resistance to certain toxic metal ions like Hg\textsuperscript{2+}, Cd\textsuperscript{2+}, Cu\textsuperscript{2+}, Ag\textsuperscript{+}, TeO\textsubscript{3}\textsuperscript{2-}, AsO\textsubscript{4}\textsuperscript{3-} and many more.\textsuperscript{16-18} Genomic and proteomic analyses have provided a more comprehensive picture of the gene clusters (operons) involved in conferring resistance. Interestingly, although this aspect of research enjoys a long history, the reductase enzymes for only a handful of heavy metals like mercury, selenate, and arsenate have been reported.\textsuperscript{16-20} Moreover, the link between bacterial heavy metal ion resistance and nanoparticle synthesis has not been postulated.

In this chapter, the unique ability of silver resistant bacterial Genus *Morganella* to synthesize isotropic and anisotropic metal nanoparticles (silver and copper nanoparticles) is deliberated. This study was further extended to understand the link between bacterial heavy metal resistance and nanoparticle synthesis.

This chapter is sub-divided into two parts that are as follows:

**Chapter III A – Biosynthesis of Silver Nanoparticles**

In this section, the ability of bacteria belonging to *Genus Morganella* to promote anisotropic growth of silver nanoparticles is discussed. A control over the growth kinetics of the bacterium *Morganella psychrotolerans* showed the ability to synthesize anisotropic silver nanoplates. A plausible mechanism and the possibility of silver ion reductase
enzyme were postulated. We further validated that the synthesis of silver nanoparticles is a Genus wide phenomenon (Genus: *Morganella*).

- Bacterial Kinetics-Controlled Shape Directed Biosynthesis of Silver Nanoplates Using *Morganella psychrotolerans*

- Genus-Wide Physicochemical Evidence of Extracellular Crystalline Silver Nanoparticles Biosynthesis by *Morganella* spp.

**Chapter III B – Biosynthesis of Copper Nanoparticles**

In this section, the ability of bacterium *Morganella morganii* RP42 to synthesize and stabilize pure metallic copper nanoparticles is discussed. The similarity in the protein sequences between the copper and silver ions resistance mechanisms was one of the main factors attributed for its unique ability to synthesize copper nanoparticles.

- Aqueous phase biosynthesis of water stable metallic copper nanoparticles
Chapter III A

Biosynthesis of Silver Nanoparticles
3.2 Introduction

The importance of biological synthesis of nanomaterials has been identified with an enormous application potential in the future. Among the synthesis of different nanoparticles by various microorganisms, bacterial synthesis of silver nanoparticles (AgNPs) is particularly attractive from a microbiological perspective due to existence of well-known silver resistance machinery in few silver resistant bacterial species, thus making their study important for biomedical applications. Moreover, silver nanoparticles have remained an attractive choice among nanomaterials because of their ability to encompass a broad application area from electronics to medicine to food technology.

Notably, in the particular context of Ag nanoparticles biosynthesis by bacteria, ionic silver (Ag\(^+\)) is well-known to be toxic to bacteria.  Previously, a silver resistant bacterium *Pseudomonas stutzeri* AG259 was discovered from silver mines, which had the unique capability to reduce Ag\(^+\) ions into Ag nanoparticles and accumulate them within its cell to minimize the Ag\(^+\) ion toxicity. Following this, there have been a few efforts that utilized different bacteria for the synthesis of Ag nanoparticles. Surprisingly none of these reports have hitherto been able to achieve shape-control to achieve anisotropic nanostructures. This is most possibly attributed to the fact that most previous studies report the outcomes of exposing Ag\(^+\) ions to bacteria without making any deliberate efforts to control the shape. It is notable that controlling reaction kinetics and altering reaction parameters via photochemical, microwave, and ultrasound assisted techniques are known to promote anisotropic growth in conventional chemical synthesis. However, contributing factors for biological syntheses are not well understood.

In the first section of this chapter (Chapter III A) the contributing factors responsible for shape control during biological synthesis are addressed. Herein, the ability of bacterium *Morganella psychrotolerans* to synthesize anisotropic silver nanoparticles is explained, which was achieved by controlling the kinetics of this bacterium by growing it at different temperatures.

With this report, two species belonging to Genus *Morganella* were reported for their ability to synthesize AgNPs extracellularly and the phenotypic and genotypic
characters of putative silver resistant machinery in both the strains have also been explored. However, it remains an established fact that microbial physiology tends to evolve rapidly to environmental changes to increase the chances of survival. To understand the AgNPs synthesis by *Morganella* in a clearer way, it was found to be of significant importance to evaluate whether the AgNPs synthesis was an adaptive physiology of *Morganella morganii* and *Morganella psychrotolerans* as a result of environmental factors or was it independent of those factors, and rather a phenotypic attribute.

In the second section of this chapter the ability of the Genus *Morganella* (isolated from different environments) for the synthesis of silver nanoparticles is validated, which further strengthens our understanding of silver nanoparticles biosynthesis by this Genus. In addition, the presence of gene homologue of putative gene of the silver binding protein (SilE) from all strains of *Morganella* spp. was investigated, confirming the link between the presence of silver resistance and AgNPs synthesis.

### 3.3 Bacterial Kinetics-Controlled Shape Directed Synthesis of Silver Nanoplates Using *Morganella psychrotolerans*

#### 3.3.1 Rationale

It is well known that bacterial growth temperature has a significant effect on the microbial physiology especially on total protein levels, and more particularly on the activity of transcriptional and translational enzymes.\(^{25-26}\) Proteins play an important role in reducing metal ions and also act as a capping agent in biological synthesis. Hence growing bacteria at different temperatures would either result in differential protein expression or have significant effect on the activity of proteins which, in turn, would provide a facile tool to control the rate of reduction of metal ions in bacterial systems.

Controlling reaction kinetics and altering reaction parameters via photochemical, microwave, and ultrasound assisted techniques are known to promote anisotropic growth in conventional chemical synthesis. To understand and elucidate the effect of these factors for biological synthesis, it was envisioned that a control over bacterial kinetics by
controlling bacterial growth temperature might provide a facile tool to control the shape of nanomaterials synthesized using biological approaches.

To demonstrate this proof-of-concept, in this body of work, *Morganella psychrotolerans* (a psychrophilic bacterium) was chosen as a model organism for controlling the shape anisotropy of Ag nanoparticles due to its ability to grow at a wider temperature range (typically 0-30 °C with 20 °C being its optimum temperature), and its specificity towards withstanding high concentrations of toxic silver ions. This unique approach of growing bacteria at temperatures significantly different from its optimum growth temperature resulted in the formation of anisotropic Ag nanoplates.

### 3.3.2 Experimental

#### 3.3.2.1 Materials

Peptone and Yeast extract were purchased from USB Biological; silver nitrate (AgNO₃) was purchased from Sigma-Aldrich. All chemicals were used without further treatment or purifications. The bacterium *Morganella psychrotolerans* (LMG 23374 = DSM 17886) used in this experiment was obtained from Dr. Shouche’s Laboratory (National Centre for Cell Sciences, Pune, India).

#### 3.3.2.2 Synthesis of Silver Nanoplates

The bacterium *Morganella psychrotolerans* was grown in Luria-Bertani (LB) broth without added NaCl [1% peptone, 0.5% yeast extract, and pH 6.8 in deionized water] at 15, 20, and 25 °C for 24 h and 4 °C for 5 days under shaking conditions followed by the addition of 5 mM equivalent of AgNO₃. Following the addition of AgNO₃, the reactions were incubated in the dark for up to 20 h, 24 h, 5 days, and 14 days at 25, 20, 15, and 4 °C respectively, under shaking conditions. After the reaction, bacteria were removed by centrifugation, and the coloured supernatant containing the homogenous suspension of nanoparticles was collected at different time points for further analysis. The bacterial cells were washed in phosphate buffered saline (PBS) and fixed using glutaraldehyde for further analysis. In control experiments, 5 mM AgNO₃ solution was added to LB broth (bacterial growth medium) and incubated in the dark at different temperatures as
performed in all test experiments. No colour change was observed in the case of control experiments, thereby negating the possibility of Ag\(^+\) ion reduction by the growth medium.

To validate the importance of growth kinetics and reaction temperature, in another set of experiments, the same bacterium (*Morganella psychrotolerans*) was grown in LB broth without added NaCl at 4 °C for 5 days under shaking conditions followed by the addition of 5 mM equivalent of AgNO\(_3\). The reactions were further incubated in the dark for up to 20 h, 24 h, 48 h, and 14 days at 25, 20, 15, 4 °C respectively, under shaking conditions. The coloured supernatant containing the homogenous suspension of nanoparticles was collected at different time points and treated similar to the protocol used for the test reactions.

### 3.3.2.3 Characterization

The coloured supernatant containing the suspension of nanoparticles were characterized by UV-vis absorption spectroscopy, TEM, XRD and electrochemical measurements.

### 3.3.2.4 Electrochemical Measurements

Linear sweep voltammetry (LSV) experiments were conducted at (20 ± 2) °C using a CH Instruments (CHI 760C) electrochemical analyser in a three electrode configuration. A 3 mm glassy carbon GC (BAS) electrode was used as a working electrode, which was polished with aqueous 0.3 μm alumina slurry on a polishing cloth (Microcloth, Buehler), sonicated in deionized water for 5 mins, and dried with a flow of nitrogen gas prior to use. An Ag/AgCl (3M KCl) electrode as reference and Pt wire as a counter electrode were also used. All electrochemical measurements were commenced after degassing the electrolyte solution with nitrogen for 10 mins prior to any measurement. The GC working electrode was modified with 5 μL of solution containing equal number of bacteria *Morganella psychrotolerans* that had been collected at different time points (0-72h) after exposure to Ag\(^+\) ions.

### 3.3.3 Results and Discussion

The optimum temperature for the growth and physiological activities of the bacterium *Morganella psychrotolerans* is 20 °C.\(^{27}\) Although deviation of its growth
environment from 20 °C to higher temperatures (e.g. 25 °C) might lead to faster bacterial growth and replication, such higher temperatures are expected to adversely affect its physiological activities. Conversely, deviation of \textit{M. psychrotolerans} growth environment towards lower temperatures (e.g. 15 and 4 °C) will result in reduced bacterial growth as well as alteration of its physiological activities. To understand the influence of bacterial growth and is physiological activity on Ag biosynthesis capability of \textit{M. psychrotolerans}, bacteria were grown at 4 different temperatures (25, 20, 15, and 4 °C), followed by incubation with 5 mM AgNO$_3$ in LB broth without added NaCl in the absence of light. All four test reactions changed in colour from pale yellow (colour of the growth medium) to muddy green over a period of time, thus indicating the synthesis of silver nanoparticles at all temperatures. Furthermore, to validate the influence of temperature, the same bacterium was grown at 4 °C followed by incubation with 5 mM AgNO$_3$ at different temperatures (25, 20, 15 and 4 °C). Similar to the earlier experiments, all reaction mixtures changed colour from pale yellow to muddy green over a period of time.

In a typical chemical synthesis route for the shape controlled synthesis of nanoparticles, it has been suggested that the reaction conditions viz. temperature and concentration of precursor salt greatly influences the final morphology. As previously demonstrated by Xia et al., it is essential to determine the concentration where there was a steady increase in the synthesis of seed particles which could then grow and stabilize to form anisotropic nanoparticles. Hence production optimization of AgNPs synthesis was performed with respect to concentration of Ag$^+$ ions, which ranged from 0.1 to 25 mM (0.1, 1, 5, 10, and 25 mM). Since all studies involving bacterial based synthesis report the production of nanoparticles at optimum temperature that more often yields isotropic nanoparticles, a temperature of 25 °C, which is 5 °C above the optimum growth temperature was chosen to optimize the concentration of precursor salt.

UV-vis absorption spectra recorded as a function of time of solutions collected from different concentrations showed the appearance of a surface plasmon resonance (SPR) band at ca. 350-550 nm as early as 1 hour in the case of 10 and 25 mM Ag$^+$ concentration (Figure 3.1). In contrast, this feature was observed only after 3 hours of
reaction in the case of 1 and 5 mM precursor salt concentrations. With increasing time, there is an increase in the intensity of SPR peak at all concentrations except 0.1 mM which does not display this SPR feature even after 20 hours of reaction. Interestingly, the intensity of the peak increases significantly faster, but we do not observe the appearance of any anisotropic feature at 10 mM and 25 mM concentrations. On the other hand, at 5 mM concentration, we observe an additional SPR feature (ca. 650-950 nm) in the near-infrared region (NIR) after 4 hours that increases in intensity with time suggesting that this concentration is optimum for the synthesis and stabilization of anisotropic silver nanoparticles. This was probably because of the dominance of the deposition growth stage over the nucleation stage at lower concentrations.\textsuperscript{30} Hence, in all further biosynthesis experiments performed with \textit{Morganella psychrotolerans}, a precursor concentration of 5 mM was employed.

\textbf{Figure 3.1}: UV-vis absorbance spectra demonstrating the time-dependent kinetics of Ag nanoparticles biosynthesis by \textit{Morganella psychrotolerans} grown and reacted at 25 °C using different precursor salt concentrations.

UV-vis absorbance spectroscopy was employed to understand the time-dependent kinetics of Ag nanoparticle biosynthesis by \textit{M. psychrotolerans} at different temperatures (Figure 3.2). As shown in Figure 3.2b, at an optimum growth temperature of 20 °C, a broad surface plasmon resonance (SPR) band apparent at ca. 350-530 nm
appears within 2 hours of reaction, which increases in intensity with time for at least up to 24 h. The SPR feature in this range is typical of Ag nanoparticles,\textsuperscript{23} and its increased intensity over a 24 h period can be assigned to the continuous biosynthesis of Ag nanoparticles by the bacterium. The SPR feature shows similar trend even when the bacterium was grown at 4 °C and reacted with Ag\textsuperscript{+} ions at 20 °C.

![UV-vis absorbance spectra demonstrating the time-dependent kinetics of biogenic Ag nanoparticles biosynthesis by Morganella psychrotolerans grown and reacted at (a) 25 °C, (b) 20 °C, (c) 15 °C and (d) 4 °C.](image)

When \textit{M. psychrotolerans} was grown at higher temperature (25 °C) with respect to optimum temperature, Ag SPR signature at ca. 350-530 nm was observed as early as 3 hours, followed by an increase in intensity of this SPR feature with time (Figure 3.2a). Interestingly, at 25 °C, an additional SPR feature at ca. 650-950 nm was observed that extends well into the near-infrared (NIR) region of the spectra and increases in intensity with the reaction time. Such NIR SPR features are characteristic of either aggregation of metal nanoparticles with time,\textsuperscript{31} or the formation of anisotropic nanoparticles in the solution,\textsuperscript{10} or a combination of both.\textsuperscript{15} In the case where the bacterium was grown at 4 °C, the SPR is significantly different to the previous case where the SPR feature observed
at ca. 650-950 nm diminishes completely and we only observe a broad SPR feature at ca. 350-750 nm. Similar to the previous case, this broad absorbance might be due to anisotropic nanoparticles, aggregation or a combination of both.\textsuperscript{10,15,31} Interestingly, in both the cases, the biosynthesis reaches maximum within 20 hours of reaction time.

To understand the effect of lower temperatures than optimum growth temperature on Ag nanoparticles synthesis, when \textit{M. psychrotolerans} was grown at 15 °C, the bacterial activity toward Ag nanoparticles biosynthesis was found to be considerably reduced, as is evident from a weak yet detectable Ag SPR feature at ca. 350-530 nm observed only after 12h of reaction and a prominent SPR feature seen after 5 days of reaction (Figure 3.2c). With the reaction time, an increase in intensity of the Ag SPR signature accompanied by the development of a prominent SPR feature in the NIR region was observed. This is similar to the reaction at 25 °C, wherein deviation from optimal growth temperature of 20 °C leads to the development of NIR features in the spectra. The time dependent UV-vis spectra in the case where the bacterium \textit{M. psychrotolerans} was grown at 4 °C and reacted with Ag\textsuperscript{+} ions at 15 °C proceeds significantly faster (reaches peak maxima within 48 h) than when the same bacterium was grown and reacted at 15 °C (peak maxima attained only after 5 days). Although the reaction proceeds faster, similar to the reaction at 25 °C, there is no development of the NIR absorbance, instead a broad absorbance at ca. 350-650 nm is observed.

A further reduction in reaction temperature to 4 °C considerably slowed down the bacterial growth as well as the rate of Ag nanoparticles biosynthesis, thereby leading to the appearance of a prominent Ag SPR feature only after 14 days of reaction (Figure 3.2d). At 4 °C, the very broad nature of the UV–vis spectrum after 14 days of reaction along with a significantly higher absorbance in the NIR region in comparison with that in the visible region is particularly notable. In fact, comparison of highest time point spectra at reactions performed at 25, 15, and 4 °C (compare highest intensity curves in Figure 3.2a, 3.2c, 3.2d) suggests that the relative intensity of the NIR SPR features with respect to visible SPR features follow a temperature-dependent trend, wherein NIR absorbance is most predominant at 4 °C and the least at 25 °C. A comparison of time-dependent kinetics of Ag nanoparticles synthesis by \textit{M. psychrotolerans} at four different temperatures also clearly suggests that the rate of Ag nanoparticle biosynthesis follows a
temperature-dependent trend, with fastest Ag$^+$ ions reduction observed at the highest temperature (25 °C; Figure 3.2a) and the slowest at the lowest temperature employed in this study (4 °C; Figure 3.2d). This is expected as bacterial physiological activity, growth, and multiplication will be considerably slowed down at lower temperatures; thus, a lower number of bacterial biosynthesis “nanofactories” will be available to reduce Ag$^+$ ions into Ag$^0$ nanoparticles.$^{32}$

**Figure 3.3**: UV-vis absorbance spectra demonstrating the time-dependent kinetics of biogenic Ag nanoparticles biosynthesis by Morganella psychrotolerans grown at 4 °C and reacted at (a) 25 °C, (b) 20 °C, (c) 15 °C and (d) 4 °C.

To establish a baseline, the AgNPs synthesized by *M. psychrotolerans* that was grown at 4 °C and reacted with Ag$^+$ ions at different temperatures were also analysed using UV-vis spectra that showed a broad absorbance at all reaction temperatures (Figure 3.3). No significant shape control was observed with only quasi-spherical aggregates typically in the size range of 10-70 nm were formed (Figure 3.4e-3.4h) with the exception of a very few anisotropic particles. This result corroborates well with the broad UV-vis spectra observed in the case of 25, 20, and 15 °C (Figure 3.3), suggesting the strong
influence of bacterial growth temperature in controlling the shape anisotropy of nanoparticles.

Furthermore, the Ag nanoparticles biosynthesized using *M. psychrotolerans* at different temperatures were also characterized using transmission electron microscopy (TEM), as shown in Figure 3.4. At the optimum growth temperature of 20 °C, predominantly spherical Ag nanoparticles of ca. 2−5 nm diameter along with relatively few nanoplates of 100−150 nm edge length were observed during TEM imaging (Figure 3.4b). The synthesis of spherical Ag nanoparticles at the optimum growth temperature of bacteria corroborates well with previous biosynthesis studies, wherein the use of different microorganisms has hitherto been reported to result in spherical Ag nanoparticles.6

**Figure 3.4**: TEM images of biogenic AgNPs synthesized by Morganella psychrotolerans grown and reacted at (a) 25, (b) 20, (c) 15, and (d) 4 °C after 20 h, 24 h, 5 days and 14 days of reaction respectively; grown at 4 °C and reacted at (e) 25, (f) 20, (g) 15, and (h) 4 °C after 20 h, 24 h, 48 h and 14 days of reaction respectively.

However, in contrast to the previous bacteria mediated biosynthesis studies, when bacterium *M. psychrotolerans* was employed for biosynthesis of Ag nanoparticles at temperatures different from its optimum growth temperature, the formation of Ag nanoplates were observed (Figure 3.4a, 3.4c, 3.4d). For instance, at 25 °C, which is 5 °C higher than the optimum growth temperature of bacteria, a mixture of triangular and
hexagonal nanoplates along with spherical nanoparticles was obtained (Figure 3.4a). Similarly, at 15 °C, which is 5 °C lower than the optimum growth temperature, again a mixture of nanoplates and spherical particles was obtained (Figure 3.4c). Further reduction in bacterial physiological activity and growth by reducing its growth temperature to 4 °C results in a significant increase in the number of nanoplates, whereas only a relatively smaller proportion of spherical nanoparticles were formed (Figure 3.4d). It is however notable that although the proportion of spherical Ag particles formed at 4 °C is lower than that observed at other temperatures; the spherical particles formed at 4 °C are larger in size (ca. 70–100 nm). This can also be attributed to the reduction in nucleation that would result in fewer nuclei and hence the particles would grow bigger as they share the remaining metal species between them.\textsuperscript{30} This indicates that in addition to the NIR absorbance from Ag nanoplates, scattering from larger size particles formed at 4 °C might have also contributed toward the broad SPR feature obtained at 4 °C (Figure 3.2d). A higher magnification TEM image of nanoplates synthesized by bacteria at 4 °C revealed stacking and buckling faults that are typical of thin nanoplates (inset, Figure 3.4d).\textsuperscript{33} The biosynthesis of Ag nanoplates by \textit{M. psychrotolerans} at temperatures away from its optimum growth temperature is particularly interesting as there have so far been no reports on tailoring nanoparticle shape by controlling bacterial growth kinetics. Typically, \textit{M. psychrotolerans} was found to synthesize Ag nanoplates with 50–150 nm edge length at 25 and 15 °C; however, biosynthesis at 4 °C resulted in larger nanoplates with 150–450 nm edge length. The nanoplates obtained through biosynthesis often showed truncated edges similar to that observed in Ag nanoprisms synthesized by a photo-induced method.\textsuperscript{34}

X-ray diffraction (XRD) patterns of the drop-coated films of biogenic Ag nanoparticles synthesized by \textit{M. psychrotolerans} at different temperatures were similar and showed well-defined Bragg reflections corresponding to (111), (200), (220), (311), and (222) planes, which could be indexed based on the face-centered cubic (fcc) lattice structure of crystalline Ag (Figure 3.5).\textsuperscript{23} Since, it is well-known that the relative growth of the (111) plane over other crystalline planes is higher in the case of nanoplates, the ratios of the intensity of (111) to (200) peaks were plotted as a function of different temperatures at which bacterial synthesis of Ag nanoparticles was performed (insets,
Figure 3.5). It is clear from the insets in Figure 3.5 that Ag nanoparticles formed by bacteria at its optimum growth temperature of 20 °C displays the lowest intensity ratio of (111) over (200). Conversely, the relative intensity of the (111) peak increases while increasing or decreasing the growth temperature from the optimum value, with the highest intensity observed at 4 °C (when grown at different temperatures). On the contrary, in the case when the bacterium was grown at 4 °C the relative intensity of the (111) shows a similar trend, wherein 20 °C shows the least intensity, with the highest intensity observed at 4 °C. XRD analysis therefore corroborates well with UV−vis spectroscopy and TEM results, which exhibit similar trends.

![Figure 3.5](image.png)

**Figure 3.5**: XRD patterns of biogenic AgNPs synthesized by Morganella psychrotolerans grown (a) and reacted at 25, 20, 15, and 4 °C respectively (b) at 4 °C and reacted at 25, 20, 15, and 4 °C respectively. Inset shows the intensity ratio of (111) to (200) Bragg reflections for Ag nanoparticles formed by bacteria at different temperatures.

To gain an understanding of the mechanism for Ag$^+$ ion reduction by the bacteria *M. psychrotolerans*, a systematic time dependence study on Ag nanoparticles biosynthesis was performed at 25 °C using linear sweep voltammetry (LSV). To ascertain that LSV experiments provide selective information about Ag species associated only with bacteria (and not about free Ag species in solution), prior to LSV experiments any residual free Ag$^+$ ions outside bacteria were removed by centrifugation and washing bacterial cells after their growth in the presence of Ag$^+$ ions at various points in time. Figure 3.6 shows
the LSV performed on a Glassy Carbon (GC) electrode immobilized with *M. psychrotolerans* that had been exposed to Ag\(^+\) ions for various time periods (0–72 h). It should be noted that upon holding the potential at −0.20 V vs. Ag/AgCl the electro-reduction of Ag\(^+\) ions to Ag\(^0\) occurs. It is evident from Figure 3.6 that at initial time points (up to 4 h) only one oxidation peak associated with the oxidation of metallic Ag occurs. This can be attributed to the electro-reduction of Ag\(^+\) ions taken up by bacteria at the initial time points. At later stages of bacterial incubation with Ag\(^+\) ions, it is predicted that bacteria will start forming Ag nanoparticles from previously taken up Ag\(^+\) ions. When LSV is performed on bacterial samples from 12 h onward, two signatures corresponding to Ag oxidation can be clearly seen. In the LSVs after 12 h, the peak at the less positive potential can be assigned to the oxidation of metallic silver generated through the electro-reduction of Ag\(^+\) ions within the bacteria, while the peak toward more positive potential can be attributed to the oxidation of Ag nanoparticles formed within bacteria.

![Figure 3.6](image)

**Figure 3.6**: Linear Sweep voltammograms by the bacteria Morganella psychrotolerans after their exposure to Ag\(^+\) ions at 25 °C for up to 72 h.

To ascertain the absence of these processes in pristine bacterium, a control experiment using GC electrode modified with bacteria unexposed to Ag\(^+\) ions was performed. This control did not show any Faradaic process within the potential range confirming that the observed peaks were obtained only when this bacterium was exposed
to Ag\(^+\) ions (Figure 3.7a). Furthermore, for the 72 h sample, the open circuit potential was measured from which the potential was scanned in the positive direction (Figure 3.7b) where it can be seen that only one oxidation peak is recorded which is due to the oxidation of Ag\(^0\) within the bacterial film. If the same process is carried out but the potential is scanned in the negative direction (Figure 3.7c), a clear reduction process is observed due to the reduction of Ag\(^+\) cations within the bacterial film to Ag\(^0\). Upon reversing the potential the forward scan now shows two distinct oxidation peaks due to the oxidation of Ag\(^0\) from (i) Ag\(^0\) already present within the film as in (b), and (ii) Ag\(^0\) formed by reduction of Ag\(^+\) ions present in the bacteria during negative sweep.

![Cyclic voltammograms (CVs) obtained for a GC electrode modified with Morganella psychrotolerans recorded in phosphate buffer at a sweep rate of 0.2 V s\(^{-1}\) for (a) pristine bacteria, and (b and c) those exposed to Ag\(^+\) ions for 72 hours.](image)

**Figure 3.7:** Cyclic voltammograms (CVs) obtained for a GC electrode modified with *Morganella psychrotolerans* recorded in phosphate buffer at a sweep rate of 0.2 V s\(^{-1}\) for (a) pristine bacteria, and (b and c) those exposed to Ag\(^+\) ions for 72 hours.

Notably, it has been previously reported that the difference in the size of Ag nanoparticles can also result in different peak potentials in voltametric measurements. However, the possibility of Ag nanoparticles of different sizes being the origin of the two peaks in the current study can be discounted based on the control CV experiments (comparing CVs in Figure 3.7b and 3.7c). Moreover, in the work of Ivanova *et al.*, it was
found that silver oxidation was size dependent under the conditions of planar diffusion, wherein shifts in oxidation potential were only observed over the size range if 12-45 nm, following which the oxidation peak potential remained constant. In this particular study of Ag biosynthesis using Morganella psychrotolerans, since most of the nanoparticles formed were larger than 45 nm in diameter, major contribution of nanoparticle size dependencies towards the origin of two oxidation peaks can be further negated. Thus the electrochemical analysis indicates that silver ions from the solution are initially uptaken by the bacterial cells, which after association with bacteria may get bound to proteins and/or other biomacromolecules within the bacteria wherein they undergo reduction, and later released out of the cells extracellularly.

![Figure 3.8: UV-vis spectra of Ag nanoparticles synthesized by exposing 5 mM aqueous AgNO₃ solution to extracellular proteins secreted by M. psychrotolerans at different temperatures.](image)

The final outcome observed from the exposure of bacteria M. psychrotolerans to Ag⁺ ions is the extracellular appearance of Ag nanoparticles, and it is also ascertained that bacteria are somehow involved in the biosynthesis process. However, it remains an open and challenging question whether Ag nanoparticles are produced extracellularly (in solution, outside the bacteria) by some of the proteins secreted by bacteria in solution or Ag⁺ ions are initially taken up by the bacteria, followed by their reduction to Ag⁰ nanoparticles, before they are released out of bacteria in the growth medium. A previous study involving bacteria from the same Genus Morganella had predicted that Ag nanoparticles are most probably produced extracellularly during biosynthesis. In the
current study, when 5 mM Ag$^+$ ions was exposed to extracellular proteins secreted by *M. psychrotolerans* for 24 h at different temperatures, the extracellular proteins were found to reduce Ag$^+$ ions to form Ag nanoparticles (Figure 3.8); however, the rate of nanoparticles formation was significantly slower (evident from the absorbance intensity on the y-axis in Figure 3.8), and only 1–2 nm Ag nanospheres without any shape control could be obtained (absence of NIR feature in Figure 3.8). This clearly suggests that in addition to extracellular proteins, bacterial physiology also plays a significant role in the synthesis and shape control of Ag nanoparticles. Additionally, LSV studies performed in this study clearly indicate that biosynthesis of Ag nanoparticles by bacteria is not as simple as previously predicted, and a mere direct role of extracellular bacterial proteins in reduction of Ag$^+$ ions, without involving bacterial machinery is rather questionable. It is also important to note that biosynthesis studies in the past have not hitherto explored the potential of electrochemistry to study metal ions uptake and their reduction by microorganisms, as well as to understand bacterial heavy metal resistance. The interesting outcomes of the LSV experiments obtained from this study suggest that detailed electrochemistry experiments in the future might provide some vital information regarding mechanisms of metal ion trafficking and its association with metal ion resistance in microorganisms.

*Scheme 3.1: Schematic representation of the potential mechanism for extracellular AgNPs biosynthesis by silver-resistant bacterium Morganella psychrotolerans.*
Notably, *M. psychrotolerans* is in fact a silver-resistant bacterium, which was confirmed by investigating the presence of a gene homologue of the putative silver binding gene (SilE) in this bacterium (PCR data shown in later section Figure 3.14). The SilE gene is known to encode a periplasmic silver binding protein (SilE) that plays a major role in Ag\(^+\) ion uptake by providing histidine sites as primary candidates for Ag\(^+\) ions binding in silver-resistant bacteria.\(^{35}\) A potential mechanism for extracellular Ag nanoparticle biosynthesis by *M. psychrotolerans* is presented in Scheme 3.1. We believe that on exposure to Ag\(^+\) ions, the SilE protein-based silver-binding machinery of the bacterium gets activated, thereby leading to the cellular uptake of Ag\(^+\) ions (step 1), as was suggested previously in the case of another silver-resistant bacterium *Salmonella*.\(^{35}\) What happens after uptake of Ag\(^+\) ions by *M. psychrotolerans* is rather interesting (steps 2–6), as it has previously been arguably postulated that in most of the heavy-metal-resistant bacteria, metal resistance is achieved via initial uptake of metal ions followed by energy-dependent “pumping out” of these ions using membrane proteins.\(^{17-18}\) Conversely, this is not the case in mercury-resistant bacteria, wherein the presence of a mercuric reductase enzyme has been established to reduce Hg\(^{2+}\) ions to Hg\(^0\). However, the presence of a silver ion reductase enzyme in silver-resistant bacteria has not yet been established. LSV studies strongly indicate the reduction of Ag\(^+\) ions to Ag\(^0\) nanoparticles within bacteria, and therefore a unique possibility of the existence of a silver ion reductase enzyme in biological systems cannot be completely ignored. At this stage, it can be assumed that after uptake of Ag\(^+\) ions by bacteria they are presented to the Ag reduction machinery in bacteria (step 2), wherein biomolecules (silver ion reductase?) synthesized by silver reduction machinery bind to Ag\(^+\) ions (step 3), thereby reducing Ag\(^+\) ions to Ag\(^0\) nuclei or seed nanoparticles (step 4). The seed particles undergo growth and assembly within the bacterial cell leading to spherical or plate-like Ag nanoparticles (step 5). These nanoparticles/nanoplates are further released from the cell using a cellular efflux system (step 6). The bacterial efflux system involved in extracellular release of Ag nanoparticles is most possibly similar to the ATPases and chemiosmotic membrane potential-dependent membrane cation/proton antiporter proteins, as was previously reported for the efflux of metal ions.\(^{17-18}\)
Although interesting, it is not absolutely clear at this stage how a shift from optimal growth temperature of *M. psychrotolerans* led to increased formation of anisotropic Ag nanoplates in comparison to Ag nanospheres. It was, however, suggested in a recent review by Xia et al. that for seed nanoparticles to achieve a higher energy structure (such as nanoplates) a kinetically controlled growth pathway is almost essential during chemical synthesis.\(^{11}\) Notably, to achieve a kinetically controlled growth pathway, it was found important to substantially reduce the rate of precursor reduction, which could be achieved by using weak reducing agents. In this case, a similar kinetically-controlled mechanism involving slow reduction of metal ions by the bacterium *M. psychrotolerans* takes place when the reaction is deviated from the optimal bacterial growth temperature of 20 °C. A shift away from optimum growth conditions will slow down the bacterial physiological processes (including metal ions uptake and reduction capability), which is probably the most important factor that directs the growth toward shape anisotropy. Moreover, proteins and other biomolecules expected to be involved in the Ag nanoparticles formation process will act as weak reducing agents in comparison to chemical reducing agents, thus facilitating anisotropic growth of Ag nanoplates.

### 3.4 Genus-wide Physicochemical Evidence of Crystalline Silver Nanoparticles Biosynthesis by *Morganella* spp.

#### 3.4.1 Rationale

Two species belonging to the Genus *Morganella* (*M. morganii* RP42 and *M. psychrotolerans*) were thus far utilized for the biosynthesis of silver nanoparticles and the phenotypic and genotypic characteristics of the putative silver resistant machinery were explored.\(^5,23\) Microbial physiology tends to evolve rapidly to the environmental changes especially to increase the chances of survival under extreme conditions. Hence it remains significant to evaluate if silver biosynthesis was an adaptive physiology or a Genus wide phenomenon.

To demonstrate this proof-of-concept, in this body of work, all sub-species belonging to the Genus *Morganella* were employed for the biosynthesis of silver nanoparticles to validate that this phenomenon is a Genus wide phenotypic characteristic
Chapter 3

3.4.2 Experimental

3.4.2.1 Materials

Peptone and Yeast extract were purchased from USB Biological; silver nitrate (AgNO₃) was purchased from Sigma-Aldrich. All chemicals were used without further treatment or purifications. All ten strains of Morganella were obtained with thanks from National Centre for Cell Sciences (NCCS), Pune, India.

3.4.2.2 Growth and Identification of Morganella Strains

The Genus Morganella comprises of two main species viz. Morganella psychrotolerans and Morganella morganii, the latter of which encompass two sub-species morganii and sibonii. On the basis of biochemical and taxonomic profiling, the two sub-species morganii and sibonii have been further divided into a total of eight biogroups (outlined in Table 3.1). The identity of all strains of Morganella was confirmed by 16S rRNA gene sequencing and sequences were submitted to GenBank with accession numbers HM122047, HM122048, HM122049, HM122050, HM122051, HM122052, HM122053, HM122054, and HM122055 by National Centre for Cell Sciences.

Table 3.1: List of the existing members of Genus Morganella

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<th>S no.</th>
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<th>Sub-species</th>
<th>Biogroup</th>
<th>Strain no.</th>
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<td>morganii</td>
<td>A</td>
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<td>Morganella morganii</td>
<td>morganii</td>
<td>B</td>
<td>CDC 1939-76</td>
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<td>morganii</td>
<td>C</td>
<td>CDC 1427-73</td>
</tr>
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<td>4</td>
<td>Morganella morganii</td>
<td>morganii</td>
<td>D</td>
<td>CDC 2866-78</td>
</tr>
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<td>sibonii</td>
<td>E</td>
<td>CDC 8293-1</td>
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<td>F</td>
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<td>-</td>
<td>LMG 23374 = DSM 17886</td>
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<td>morganii</td>
<td>RP-42</td>
<td>Isolated from insect gut (reference strain)</td>
</tr>
</tbody>
</table>
3.4.2.3 Synthesis of Silver Nanoparticles

All reported type strains of *Morganella* were initially grown at 37 °C for 24 h in a 500 ml Erlenmeyer flask that contained 100 mL of LB broth without added NaCl in a shaker incubator set at 200 rpm, except for *Morganella psychrotolerans*, wherein the bacteria was grown at 20 °C, while maintaining all other synthesis conditions similar to those for other *Morganella* strains. The growth condition (temperature) for *Morganella psychrotolerans* was different, because this particular species is a psychrotolerant stain with 20 °C being the optimum growth temperature. It is important to note that this strain could not be grown at 37 °C even after 5 days of incubation. Following bacterial growth, all culture suspensions were incubated with aqueous 5 mM solutions of AgNO\(_3\) at 37 °C in the dark for over a period of 120 h where samples were collected at regular intervals to monitor the progress in the synthesis of silver nanoparticles. The extracellular manifestations of silver nanoparticles were initially detected by visual inspection of the culture flask for change in medium colour from pale yellow to brown/green. Extracellularly formed silver nanoparticles were separated from bacterial cells by centrifuging aliquots of culture supernatants at 3000 rpm for 6 mins at 25 °C. This brown/green supernatant (obtained at different time points) containing silver nanoparticles was used for further characterization using UV-vis absorbance spectroscopy, TEM, SAED and XRD.

3.4.3 Results and Discussion

On exposure to 5 mM colourless AgNO\(_3\) solutions, all *Morganella* biogroups formed dark brown coloured solutions within 20 h of reaction, except for M. *psychrotolerans* that formed greenish brown colloidal solution, indicating formation of extracellular AgNPs by all the biogroups. The colour of the solutions did not significantly change from that point onward (except in intensity), even after continuing the reaction for up to 5 days. The AgNPs solutions remained stable for at least up to 8 weeks without any visible aggregation or precipitation. To understand the nature of nanoparticles, detailed physico-chemical characterization of extracellular AgNPs formed by all *Morganella* strains was carried out using UV-Vis absorbance spectroscopy,
transmission electron microscopy (TEM), and X-ray diffraction (XRD) studies as described in the materials and method section.\textsuperscript{23}

Figure 3.9 shows the UV-vis absorption kinetics of colloidal solutions obtained after reaction of all \textit{Morganella} biogroups with 5 mM AgNO\textsubscript{3} for 1, 3, 8, 16, 20, 48 and 120 h. The presence of a characteristic Ag surface plasmon resonance (SPR) feature between 400 and 500 nm is clearly evident in all the samples, thus confirming the formation of extracellular AgNPs by all \textit{Morganella} biogroups.\textsuperscript{23} The differences in the position of absorbance maxima of SPR features of AgNPs synthesized by different biogroups is notable, which is most likely due to the difference in the size and/or shape of Ag nanocrystals synthesized by these biogroups.\textsuperscript{5,12}

\textbf{Figure 3.9:} Time-dependent UV-vis absorbance spectra of culture supernatants from different biogroups of \textit{Morganella} showing the extracellular synthesis of AgNPs.
It is also interesting to note that most of the *Morganella* biogroups started synthesizing AgNPs as early as within 1 h of reaction and the yield of AgNPs by different biogroups increased as the reaction progressed over a period of time. However, the amount of AgNPs produced by different biogroups reached to a saturation state somewhere between 20 h and 120 h of reaction, which varied from one biogroup to another. This suggests that although all *Morganella* biogroups have the capability to reduce Ag$^+$ ions to form AgNPs (Ag$^0$), the rate of AgNPs formation may vary among them.

To compare the rate of AgNPs formation by different biogroups, the maximum absorbance intensity ($A_{\text{max}}$) of the Ag SPR feature of different biogroups was plotted with respect to different time points of the biosynthesis reaction (Figure 3.10). It is clearly evident from Figure 3.10 that different biogroups indeed followed different reaction kinetics in terms of AgNPs formation, among which *M. psychrotolerans* showed the fastest activity towards AgNPs biosynthesis, followed by *M. morganii* strain RP-42 (compare absorbance intensities at 1 h). However, after 8 h of reaction, AgNPs production by *M. morganii* strain RP-42 superseded that from *M. psychrotolerans*, thus RP-42 strain showing largest overall AgNPs production capability within a 120 h time frame. In a control experiment, SPR signatures corresponding to Ag nanoparticles were found absent in the media control wherein no bacteria were inoculated, thus ruling out the possibility of a direct role of media components on AgNPs synthesis, and affirming that AgNPs synthesis resulted as a whole function of micro-environment created by the different strains of *Morganella*. 
It is also interesting to note that AgNPs production by most of the *Morganella* biogroups (except biogroups RP-42 and A) reached a saturation state within 20 h of reaction, after which no further increase in AgNPs synthesis was observed. Therefore, in this study, although UV-Vis analysis was performed up to 5 days to follow the reaction kinetic, we performed TEM and XRD analysis on AgNPs obtained after 20 h of reaction. It can be said that the 20 h time point provides a better representation than 120 h time point for comparison between AgNPs synthesized by different biogroups, predominantly because at 20 h time point AgNPs biosynthesis is in its log (growth) phase, which enables investigation of the state of the as-formed particles, rather than a possibility of their further modification while AgNPs stay in the bacterial growth media for up to 120 h. It should also be noted that when a detailed precursor concentration-dependent experiment on *M. morganii* strain RP-42 and *M. psychrotolerans* was performed (as discussed in the previous section, Figure 3.1), the rate of AgNPs formation was found to be maximum at 5 mM AgNO₃ concentration, and was reduced by increasing the precursor concentration. This motivated us to perform all of the experiments reported in the current study at 5 mM AgNO₃ concentration. However, considering the
differences in the rate of AgNPs biosynthesis by different Morganella biogroups, it is likely that the optimal precursor concentration for maximum AgNPs synthesis rate may vary from one biogroup to another. This will require separate detailed investigations concerning each of the biogroups in the future, wherein the influence of various parameters such as precursor concentration, solution pH, reaction time and temperature may be studied in detail to obtain greater insight about each system.

Figure 3.11: TEM images of extracellular AgNPs formed by different biogroups of Morganella. Biogroup A (a and b), Biogroup B (c and d), Biogroup C (e and f), Biogroup D (g and h), Biogroup E (i and j), Biogroup F (k and l), Biogroup G1 (m and n), Biogroup G2 (o and p), M morganii strain RP-42 (q and r), and M psychrotolerans (s and t). Scale bar in all images correspond to 100 nm.
To understand the morphology of AgNPs formed by different biogroups of *Morganella*, the TEM analysis of AgNPs synthesized by all biogroups was performed after 20 h of biosynthesis (Figure 3.11). It is evident from TEM images that AgNPs formed by all biogroups were quasi-spherical in shape, ranging 10–50 nm in diameter (Figure 3.11a-3.11r). The particle size distribution of AgNPs formed by different *Morganella* biogroups was assessed that is presented in Figure 3.12.

![Figure 3.12: Histograms showing the particle size distribution of AgNPs formed by different biogroups of Morganella spp. Biogroup A (panel a), Biogroup B (panel b), Biogroup C (panel c), Biogroup D (panel d), Biogroup E (panel e), Biogroup F (panel f), Biogroup G1 (panel g), Biogroup G2 (panel h), M. morganii strain RP-42 (panel i), and M. psychrotolerans (panel j).](image)

The particle size distribution for at least 200 particles in each biogroup revealed that the average particle diameters with the standard error of mean of quasi-spherical AgNPs synthesized by different biogroups were 39.9 ± 1.1 nm (A), 19 ± 1 nm (B), 12.3 ± 0.7 nm (C), 10.2 ± 0.2 nm (D), 32.9 ± 1.3 nm (E), 15.1 ± 0.9 nm (F), 8.3 ± 1.1 nm (G1), 14.8 ± 0.8 nm (G2), 32.8 ± 1 nm (RP-42), and 46.3 ± 1.2 nm (M. psychrotolerans).

The crystalline nature of AgNPs formed by different biogroups of *Morganella* after 20 h of reaction was investigated by XRD. As is evident from XRD patterns in Figure 3.13a, extracellular AgNPs synthesized by the entire biogroups exhibit well defined (111), (200), (220) and (311) Bragg reflections of the face centered cubic (fcc) form of crystalline silver. XRD analysis thus provided a clear indication of formation of high quality crystalline AgNPs using a *Morganella* mediated biosynthesis process by all its strain types.
Additionally, the crystallinity of AgNPs was further confirmed by performing SAED analysis of AgNPs formed by *M. morganii* strain RP-42 and *M. psychrotolerans* during TEM imaging (Figure 3.13b). The SAED patterns from both the samples revealed well-defined diffraction planes in the form of rings, which are indicative of polycrystalline silver.

![Figure 3.13: a) XRD of extracellular AgNPs formed by different biogroups of Morganella; b) SAED pattern observed from AgNPs synthesized by (1) M. morganii RP-42 and (2) M. psychrotolerans.](image)

The UV-vis, TEM, XRD and SAED results presented in this study clearly demonstrate that formation of AgNPs is a Genus-wide characteristic phenotype of all reported type strains of *Morganella* to date. Further experiments were performed to explore whether AgNPs formation is a characteristic phenotype restricted to Genus *Morganella*, or whether other taxonomically related genera of Enterobacteriaceae family also show this feature. To obtain this insight, when comparative analysis of AgNPs synthesis using laboratory strains of *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Serratia marcescens* was performed in the presence of 5 mM AgNO₃, no AgNPs formation was observed in any of these closely related organisms. Additionally, distant taxonomic relatives of *Morganella* such as *Firmicutes* and *Actinobacteria* did not...
lead to formation of detectable AgNPs in solutions. This strongly suggests that AgNPs synthesis in the presence of Ag⁺ ions is a phenotypic character that is uniquely associated with Genus *Morganella*.

**Figure 3.14**: 1% Ethidium bromide stained agarose gel showing PCR products of SilE gene homologue from all biogroups of *Morganella*. (1) biogroup A, (2) biogroup B, (3) biogroup C, (4) biogroup D, (5) biogroup E, (6) biogroup F, (7) biogroup G1, (8) biogroup G2, (9) *M. morganii* RP42, (10) *M. psychrotolerans*, (11) negative control, (M) molecular DNA marker.

The UV-vis, TEM, XRD and SAED results presented in this study clearly demonstrate that formation of AgNPs is a Genus-wide characteristic phenotype of all reported type strains of *Morganella* to date. Further experiments were performed to explore whether AgNPs formation is a characteristic phenotype restricted to Genus *Morganella*, or whether other taxonomically related genera of Enterobacteriaceae family also show this feature. To obtain this insight, when comparative analysis of AgNPs synthesis using laboratory strains of *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Serratia marcescens* was performed in the presence of 5 mM AgNO₃, no AgNPs formation was observed in any of these closely related organisms. Additionally, distant taxonomic relatives of *Morganella* such as *Firmicutes* and *Actinobacteria* did not lead to formation of detectable AgNPs in solutions. This strongly suggests that AgNPs synthesis in the presence of Ag⁺ ions is a phenotypic character that is uniquely associated with Genus *Morganella*.

It has been previously established that in silver resistant bacteria, silver resistance mechanism involves the gene (SilE) which encodes a periplasmic silver binding protein (SilE). This macromolecule plays a major role in highly specific uptake of Ag⁺ ions from the surrounding environment by providing histidine sites as primary candidates for Ag⁺ ion binding. Similarly, in our previous study, it was established that silver resistance machinery in *Morganella morganii* RP-42 is associated with AgNPs synthesis capability of
this particular strain. Since in the current study, all the reported strains of Genus *Morganella* were found to exhibit the phenotype of AgNPs synthesis in the presence of Ag^+^ ions, it was found important to associate the presence of silver resistance (SilE gene) in all these strains with their AgNPs synthesis capability. Therefore, to determine whether all strains of *Morganella* spp. exhibit silver resistance, further efforts were made to identify the gene homologue of SilE in all the members of Genus *Morganella*. As can be seen from Figure 3.14, all *Morganella* strains showed the presence of the SilE gene homologue. This observation further strengthens the probable role of silver resistance genes and gene products in AgNPs synthesis in *Morganella*. In the current study, it has been demonstrated that the phenotype of extracellular AgNPs synthesis in *Morganella* is not just restricted to an isolate pertaining to one environment, but it is indeed a unique biochemical character associated with all the members of this Genus isolated from different environments. This clearly establishes that AgNPs synthesis by Genus *Morganella* is a phenotype independent of environmental influence. Although AgNPs synthesis has been previously reported by other microorganisms, this is for the first time that extracellular synthesis of AgNPs by all the members a particular Genus (*Morganella*) has been established, and their AgNPs synthesis capability has been followed in a time-dependent manner.

### 3.5 Conclusion

In conclusion, it has been demonstrated that all species belonging to silver resistant Genus *Morganella* show the ability to synthesize silver nanoparticles. The phenotypic characteristic of extracellular AgNPs synthesis in *Morganella* is not just restricted to isolates from a single environment but it is indeed a unique biochemical characteristic associated with all members of this Genus isolated from different environments. Although other members of the family *Enterobacteriaceae* show the ability to reduce ionic silver, to date, the Genus *Morganella* is the only Genus showing a unique phenotypic characteristic to reduce silver ions.

The observation that the AgNPs synthesis is a unique phenotypic character of Genus *Morganella* might, in future, not only provide a complementary tool for easy detection and purification of *Morganella* in the presence of other members
of Enterobacteriaceae family, but might also establish new evolutionary links between
different microorganisms based on metal resistance, and metal ion reducing capabilities.

Furthermore, for biosynthesis to compete with chemical synthesis, this study was
further extended to demonstrate that by controlling the growth kinetics of *Morganella
psychrotolerans* at different temperatures, shape anisotropy of Ag nanoparticles can be
controlled. Additionally, it was also shown that, although the nanoparticles manifest
themselves extracellularly, the process of reduction is a result of a complex process that
involves the Ag ions being taken up by the cell, reduced to form Ag\(^0\) nuclei followed by
growth and assembly and further released from the bacterial cell using a cellular efflux
system. This proof-of-concept can in future be further extended to other nanomaterials,
wherein a combination of reaction parameters influencing microbial growth kinetics will
be utilized to achieve a higher degree of shape control during biological synthesis.
Chapter III B

Biosynthesis of Copper Nanoparticles
3.6 Aqueous Phase Biosynthesis of Water Stable Metallic Copper Nanoparticles

3.6.1 Introduction

Among the synthesis of different nanoparticles by various microorganisms, gold and silver nanoparticles biosynthesis has been studied extensively (Appendix B – Table B.1), while the biosynthesis of copper nanoparticles is relatively a new concept. Copper nanoparticles (CuNPs) are biocompatible and show surface-enhanced Raman scattering (SERS) properties, thereby making them a cheaper alternative to be used as a potential target in various applications. Unlike noble metals such as Au and Ag, the synthesis of pure metallic CuNPs under aqueous conditions is not straightforward, as CuNPs obtained in aqueous solutions by direct reduction of copper salts have the propensity of surface oxidation, leading to Cu core-oxide shell nanostructures, which have limited usability for various applications. Therefore, synthesis of CuNPs typically involves the use of non-aqueous solvents under controlled conditions such as low precursor concentrations, and under inert atmosphere to prevent oxidation, which generally hinders the mass production of these technologically important nanomaterials. There have also been limited reports on the synthesis of pure metallic CuNPs under aqueous environments; however these typically involve a laborious process including the use of toxic chemicals, strong capping agents, harsh experimental conditions and elaborate procedures. Therefore there is a demand for an environment friendly, economically viable approach towards aqueous phase synthesis of metallic copper nanoparticles.

As previously discussed, the significance of bacteria as an important biological entity in the repertoire of biological organisms has been realised and several bacterial species have been employed for the synthesis of nanomaterials. The most intriguing ability of bacteria to withstand high concentrations of toxic metal ions and the ability of these organisms to process these toxic ions into their corresponding non-toxic forms using an ATPase-dependent reduction pathway is extensively studied and is known to increase their chance of survival in natural environment. Incidentally, in the previous
studies outlined in this chapter (Chapter III A), a strong correlation between Ag\(^+\) ions resistance mechanism and the ability of Genus *Morganella* to synthesize Ag nanoparticles has been established.\(^2,^5\)

Notably, in particular context of the current study, copper is considered as an essential trace element that is required by the bacterial cells for the synthesis of metalloproteins, however the ionic form (Cu\(^{2+}\)) is considered toxic only at higher concentrations.\(^46-^47\) Therefore, it is of utmost importance for the bacterial cell to regulate these ions and maintain an essential supply of copper for its biosynthetic machinery while protecting the cell from high, toxic conditions.\(^47\) Copper resistant bacteria have been isolated in the past and extensive research to understand the biochemical pathways that maintain and regulate copper ions at toxic levels has been deliberated. These studies suggest that the remediation of copper ions involves energy dependent copper efflux mechanism which is either encoded within a plasmid or within the genome that essentially contains seven open reading frames encoded within the gene cluster involved in the resistance machinery.\(^48-^49\) Interestingly, the proteins believed to be involved for silver ion regulation in bacteria show high degree of similarity with the metal binding proteins involved in copper resistance system.\(^35,^47,^49\) For instance, the periplasmic silver binding protein (SilE) that binds to Ag\(^+\) ions was found to be homologous to the copper binding proteins (PcoE, CusF) involved in regulation of copper ions in microorganisms. Notably, the potential of silver resistant bacteria (*Morganella* spp.) to synthesize silver nanoparticles, wherein the involvement of the Sil gene clusters in nanoparticle synthesis was established.\(^2,^5,^23\)

### 3.6.2 Rationale

The potential applications of copper nanoparticles are many and hence, it is of obvious interest to synthesize pure metallic copper nanoparticles using a biological approach. Given the presence of SilE protein in *Morganella* spp. and its similarity with other well-known copper binding proteins such as PcoE and CusF, it was envisaged that challenging the silver resistant *Morganella* with excess copper ions might provide a simplistic approach for the synthesis of copper nanoparticles. In an effort to synthesize copper nanoparticles via a green biological route, herein it has been demonstrated for the
first time that by exposing the silver resistant bacteria *Morganella morganii* RP42 to Cu$^{+2}$ ions, pure metallic copper nanoparticles can be obtained. This is particularly important considering that when in the past, two bacteria viz. *E. coli* and *Serratia* sp., which do not have an established Ag$^+$ ion resistance mechanism, were utilised for the biosynthesis of CuNPs, only a mix phase of copper and copper oxide nanoparticles could be obtained. It is quite evident that none of the previous studies have hitherto been able to synthesize pure metallic copper nanoparticles using a biological approach under aqueous conditions. In the current study, we have also performed linear sweep voltammetry (LSV) experiments on bacteria that provide interesting insights on the mechanistic aspect of copper nanoparticle synthesis as well as strong evidence showing the link between silver resistance and copper resistance machinery in bacterial cells.

### 3.6.3 Experimental

#### 3.6.3.1 Materials

Peptone and Yeast extract were purchased from USB Biological; analytical grade copper sulfate (CuSO$_4$.2H$_2$O) was purchased from Sigma-Aldrich. All chemicals were used without further treatment or purifications.

#### 3.6.3.2 Synthesis of copper nanoparticles

The bacteria *Morganella morganii* RP42 was grown in LB broth without added NaCl at 37 °C for 24 h under shaking conditions followed by the addition of 5 mM equivalent of CuSO$_4$. Following the addition of CuSO$_4$, the reaction mixtures were incubated for up to 20 h under shaking conditions. After the reaction, bacteria were removed by centrifugation, and the bluish-black coloured supernatant containing the homogenous suspension of nanoparticles was collected at different time points for further analysis. In addition, the bacterial cells collected were washed in phosphate buffered saline (PBS) and fixed using glutaraldehyde for further analysis. In control experiments, 5 mM CuSO$_4$ solution was added to LB broth (bacterial growth medium) and incubated in the conditions similar to the test experiments. No colour change was observed in the case of control experiments, thereby negating the possibility of Cu$^{2+}$ ion reduction by the growth media.
3.6.3.3 Characterization

The coloured supernatant containing the suspension of nanoparticles were characterized by UV-vis absorbance spectroscopy, TEM, HR-TEM, SAED, and XPS.

3.6.3.4 Accession Numbers

The GenBank accession codes for the sil gene cluster involved in silver resistance and Cus / Pco gene cluster involved in copper resistance are as follows:


3.6.3.5 Electrochemical Measurements

Linear sweep voltammetry experiments were conducted at 20 ± 2 °C using a CH Instruments (CHI 760C) electrochemical analyzer in a three electrode configuration. A 3 mm glassy carbon GC (BAS) electrode was used as a working electrode, which was polished with aqueous 0.3 µm alumina slurry on a polishing cloth (Microcloth, Buehler), sonicated in deionized water for 5 min, and dried with a flow of nitrogen gas prior to use. An Ag/AgCl (3M KCl) electrode as the reference and Pt wire as a counter electrode were also used. All electrochemical measurements were commenced after degassing the electrolyte solution with nitrogen for 10 min prior to any measurement. The GC working electrode was modified with 5 µL of solution containing equal number of bacteria *Morganella psychrotolerans* that had been collected at different time points (0-20 h) after exposure to Cu^{2+} ions.
3.6.4 Results and Discussion

To investigate the ability of silver resistance bacterium *Morganella morganii* RP42 to biosynthesize CuNPs, the bacteria were grown at 37 °C, followed by incubation with 5 mM CuSO$_4$ in Luria-Bertani (LB) broth. Following the reactions, the solutions changed colour from pale yellow to bluish-black over the period of time suggesting the formation of nanoparticles. In parallel control experiments, 5 mM CuSO$_4$ was incubated in LB broth in the absence of bacteria. No significant colour change was observed in growth medium suggesting that the reduction of Cu$^{+2}$ ions to CuNPs occurs only in the presence of bacteria in the growth medium.

![UV-vis absorbance spectra demonstrating the time-dependent kinetics of metallic copper nanoparticles biosynthesized by *Morganella morganii* RP42.](image)

*Figure 3.15: UV-vis absorbance spectra demonstrating the time-dependent kinetics of metallic copper nanoparticles biosynthesized by Morganella morganii RP42.*

Since CuNPs are known to show surface plasmon response (SPR) features, UV-vis absorbance spectroscopy was employed to understand the kinetics of nanoparticles synthesis by *M. morganii* RP42 over a period of 20 h. UV-vis spectra recorded as a function of time of the reaction from bacterial solutions incubated with 5 mM CuSO$_4$ at 37 °C (Figure 3.15) show the appearance of an SPR band at ca. 550 - 570 nm, which appears just after 1 h of reaction and increases in intensity over the period of time up to
20 hours. The SPR feature at this wavelength range is typical for metallic CuNPs, and the increase in SPR intensity can be attributed to the increase in nanoparticle yield due to continuous biosynthesis by the bacteria. The absence of a well-defined SPR peak is typical of small sized CuNPs, as metallic Cu does not produce strong SPR features in contrast to those typically observed for metallic Au and Ag nanoparticles.

![Figure 3.16](image)

**Figure 3.16**: (a) TEM images of biogenic CuNPs; (b) SAED pattern obtained from CuNPs observed in figure (a); (c) HR-TEM micrograph showing lattice fringes of 0.2 nm typical of the Cu (111) plane with the inset showing the live FFT.

Transmission electron microscopy (TEM) analysis of CuNPs biosynthesized by *M. morganii* after 20 h of reaction revealed that the CuNPs were quasi-spherical in morphology, typically ranging from 15-20 nm in diameter, with an average particle diameter of 19.2 nm ± 1.5 nm (Figure 3.16a). Selected area electron diffraction pattern (SAED) showed the highly crystalline nature of biogenic CuNPs, wherein the four distinct ring patterns correspond to the interplanar spacing of 0.21, 0.181, 0.128 and 0.112 nm were observed, which are in agreement with the planes of zerovalent copper (PCPDF number: 85-1326) (Figure 3.16b). Furthermore, the crystallography of CuNPs was investigated using high resolution TEM, which showed well-ordered structure with clear lattice fringes along with a very thin passivating surface layer of an amorphous material covering these particles (Figure 3.16c). The 0.2 nm spacing between the lattice fringes in the crystalline particle corresponds to the (111) plane of face-centered cubic Cu (PCPDF number: 85-1326), which is also evident from the fast Fourier transform (FFT) image from this particle, as shown in the inset of Figure 3.16c. The high resolution TEM micrograph clearly negates the possibility of a copper oxide layer covering these nanoparticles. The outer amorphous layer around the particle can be attributed to the
proteins/peptides that play the role of reducing agent during CuNPs biosynthesis and likely to act as capping agent to stabilize these particles.\textsuperscript{2,5,23,57-58}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{xps_spectra.png}
\caption{XPS spectra showing C 1s, N 1s, O 1s, and Cu 2p core level binding energies arising from CuNPs biosynthesized using Morganella morganii RP42.}
\end{figure}

To further negate the possibility of copper oxide formation, biogenic CuNPs were analysed by X-ray photoelectron spectroscopy (XPS), which is regarded as a highly surface sensitive technique. Figure 3.17 shows C 1s, N 1s, O 1s and Cu 2p core level XPS spectra obtained from the CuNPs synthesized using \textit{Morganella morganii} after 20 h of reaction. All of the XPS spectra have been background corrected using the Shirley algorithm, and their respective binding energies (BEs) have been aligned with adventitious C 1s BE of 285 eV.\textsuperscript{57-58} C 1s spectra could be deconvoluted into three major components with the adventitious carbon component at 285 eV, a higher BE component at ca. 286.7 eV corresponding to $\alpha$-carbon and the highest BE component at ca. 288.4 eV, which can be attributed to carbonyl carbon from the protein/peptides bound to the nanoparticles.\textsuperscript{57,59} N 1s and O 1s core level XPS spectra showed BE maxima at 400.1 eV and 532.4 eV.
respectively, that corroborate well with previously reported values of amide bonds in the case of nitrogen\textsuperscript{60} and chemically equivalent oxygen of the neutral carboxylic group (COOH) from the proteins\textsuperscript{61} present on the surface of the CuNPs. The Cu 2p core level showed two characteristic 2p\textsubscript{3/2} and 2p\textsubscript{1/2} splitting components (spin-orbit splitting \textasciitilde 19.7 eV) with sharp peaks arising at BEs of ca. 933.2 and 952.9 eV respectively, which correspond to zerovalent copper.\textsuperscript{39,62} Additionally, no significant signature corresponding to shake-up satellites was observed,\textsuperscript{62-63} strongly suggesting the absence of CuO in the biogenic CuNPs. Shake-up peaks refer to non-adiabatic relaxation of the electron cloud upon photoionization where the energy is used to promote another valence electron to an unoccupied state giving rise to shake up transitions or extra peaks in the XPS spectrum. Since the shake-up satellites are mainly 3d to 4s transitions (which are forbidden in metals but present in transition metal oxides), the presence of Cu in its metallic state in biogenic CuNPs results in complete absence of shake-up satellites.\textsuperscript{64} Additionally, the expected O 1s BE in the presence of Cu\textsuperscript{+} and Cu\textsuperscript{2+} is approximately 1.4-2 eV lower than the O 1s BE obtained from the proteins/peptide in this study, which further nullifies the possibility of the presence of any oxide species.\textsuperscript{62-63}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{linear_sweep_voltammogram.png}
\caption{Linear sweep voltammogram of bacteria Morganella morganii RP42 after their exposure to Cu\textsuperscript{2+} ions for up to 20 h.}
\end{figure}
To understand the mechanistic aspect of Cu\(^{2+}\) ion reduction by bacteria *M. morganii*, and to provide further evidence for the presence of Cu\(^0\) species in CuNPs, a systematic time dependence study on CuNPs biosynthesis was performed using linear sweep voltammetry (LSV). To establish that the LSV experiments would only provide information on copper ions associated with the bacteria (and not free Cu species in the solution), prior to performing these experiments, the bacteria were washed and centrifuged several times at each time point before drop casting onto a glassy carbon (GC) electrode. Figure 3.18 shows the LSV performed on a GC electrode immobilized with *M. morganii* that had been exposed to Cu\(^{2+}\) ions for a time period of 0-20 h. It should be noted that upon holding the potential at −0.99 V vs. Ag/AgCl, the electro-reduction of Cu\(^{2+}\) ions to Cu\(^0\) occurs. It is evident from Figure 3.18 that at initial time point (up to 1 h) only one oxidation peak associated with the oxidation of metallic Cu occurs. This can be attributed to the electro-reduction of Cu\(^{2+}\) ions taken up by bacteria at the initial time points. At later stages of bacterial incubation with Cu\(^{2+}\) ions, it is predicted that bacteria will start forming Cu nanoparticles from previously uptaken Cu\(^{2+}\) ions. When LSV is performed on bacterial samples from 4 h onward, two signatures corresponding to Cu oxidation can be clearly seen. In the LSVs after 4 h, the peak at the less positive potential can be assigned to the oxidation of metallic copper generated through the electro-reduction of Cu\(^{2+}\) ions within the bacteria, while the peak toward more positive potential can be attributed to the oxidation of Cu nanoparticles formed within bacteria.

To further ascertain the absence of an oxide layer, an open circuit potential for the 1 h and 20 h samples were measured (Figure 3.19). To achieve this, when the potential was scanned in the positive direction, it was observed that only one oxidation peak is recorded which is due to the oxidation of Cu\(^0\) within the bacterial film. The concomitant increase in the relative area under of the peak towards more positive potential with an increase in the reaction time is also notable. This indicates that copper ions from the solution are initially taken up by the bacterial cells, which after association with bacteria may get bound to proteins and/or other biomacromolecules within the bacteria wherein they undergo reduction, and later released out of the cells extracellularly. LSV experiments therefore indicate a similar mechanism of CuNPs biosynthesis by *Morganella*.
morganii as we previously reported for Ag nanoparticles biosynthesis by Morganella psychrotolerans.\(^5\)

![Cyclic voltammogram (CVs) obtained for GC electrode modified with Morganella morganii recorded in phosphate buffer at a sweep rate of 0.2 V s\(^{-1}\) for bacteria exposed to Cu\(^{+2}\) ions for 1 h and 20 h.](image)

**Figure 3.19:** Cyclic voltammogram (CVs) obtained for GC electrode modified with Morganella morganii recorded in phosphate buffer at a sweep rate of 0.2 V s\(^{-1}\) for bacteria exposed to Cu\(^{+2}\) ions for 1 h and 20 h.

The final outcomes observed from the exposure of Cu\(^{+2}\) ions to Morganella morganii RP42 is the extracellular manifestation of pure metallic CuNPs and electrochemistry experiments also ascertain that the bacterial mechanism is somehow involved in the biosynthesis process. However, it remains an open and challenging question whether the biochemical processes (proteins/peptides) involved in silver resistance machinery could also bind and reduce a divalent copper system to synthesize CuNPs. In the previous study involving a bacterium from the same Genus (Morganella) indicated that in addition to extracellular proteins secreted by this organism, the bacterial physiology also plays a critical role in synthesizing Ag nanoparticles.\(^5\) Based on the similarity between the LSV results from Cu system in the current study with that of Ag system, it can be inferred that the mechanism of CuNPs biosynthesis by Morganella morganii may be quite similar to the previously reported Ag nanoparticles biosynthesis by Morganella psychrotolerans (compare Figure 3.6 and Figure 3.18).\(^5\) Therefore, the results
shown here may assist in providing some vital information in regards to the mechanisms of metal ion trafficking associated with the metal ion resistance system in *Morganella* spp.

**Figure 3.20:** Comparison of amino acid sequences of SilE from *Morganella morganii* RP42 (silver resistance) to that of PcoE and CusF from *Escherichia coli* (copper resistance) using BLAST.

Since the previous investigations confirmed that the presence of a silver-binding gene is a Genus-wide feature of *Morganella* spp., to assess the ability of this Ag⁺ ion binding protein (SilE) to bind and uptake Cu²⁺ ions, the homology of SilE with PcoE and CusF, the two previously-proposed Cu²⁺ binding proteins from the plasmid copper-resistant system, was checked. This showed 47% homology in the protein sequences (Figure 3.20), thus suggesting that SilE present in the silver resistant *Morganella* might be capable of taking up Cu²⁺ ions. It is known that cysteines act as primary metal cation ligands in most of the metal-binding proteins, conversely SilE is rich in histidines but lack cysteines. Interestingly, it has also been established that peptides with high amount of histidine residues have strong binding capability with divalent copper ions. Therefore, the richness of SilE in histidine content suggests that SilE might also be capable of binding with divalent copper ions, in addition to its normal activity of binding monovalent silver ions. This provides *M. morganii* an ability to synthesize both Ag and Cu nanoparticles on exposure to respective metal ions.
These findings raise more questions. To begin with, in the case of silver, if the periplasmic SilE protein is responsible for binding to Ag\(^+\) ions and considering, in the case of copper, if the same protein can also bind to Cu\(^{2+}\) ions, then are all or most of the proteins in copper resistance and silver resistance somewhat similar? If they are similar, can the role of each protein be extrapolated while taking into account that the copper system is well studied (than silver resistance machinery) and the function of the proteins has been determined? Most metal-binding proteins use cysteines as primary metal cation ligands however the silver binding protein (SilE) lacks cysteines, but instead it has 10 histidine residues which are primary candidates for metal-binding.\(^{35}\) Can the presence of histidine influence the uptake of copper ions within the bacteria? How is it that the copper nanoparticles synthesized by *Morganella morganii* stabilized in the metallic form rather than oxide form? To get greater insight into these questions it is important to use bioinformatics resources such as BLAST searches and sequence alignment.\(^{66}\)

![Scheme 3.2: Schematic representing the potential similarities between silver and copper resistance systems that enables *M. morganii* RP42 to biosynthesize and stabilize copper nanoparticles.](image)

A BLAST search was performed using the sequences from the Sil gene cluster of *Morganella morganii* RP42 obtained from the NCBI nucleotide databank. The results showed high similarities with other Sil proteins that belonged to different bacterial
species as well as proteins involved in copper resistance. Of particular interest, we used sequences from the best studied copper resistance system from *Escherichia coli* (Cus) and performed a sequence similarity search against all sequences from the sil cluster (Appendix C – Figure C.1-C.7). The closest homologs of SilA, SilB and SilC are CusA, CusB and CusC, of which CusA and CusC are reported to be playing the function of effluxing metal cations. Now given the similarity in sequences, we can infer that the function of SilA and SilC in *Morganella morganii* could be similar to the role played by CusA and CusC in *Escherichia coli*. This also suggests that SilA and SilC would have the ability to efflux the copper cation present in the solution into the bacterial cell. On another note, SilS showed high homology with CusS which plays the role of a sensor kinase in *Escherichia coli*, also suggesting the role that SilS might be of this very nature and would also help in sensing copper ions. Based on these observations, the role of different proteins in the silver and copper resistance machinery can be extrapolated and a schematic representing the similarities has been proposed (Scheme 3.2).

![Figure 3.21](image.png)

*Figure 3.21*: XPS spectra showing S 2p core level binding energy arising from CuNPs biosynthesized using *Morganella morganii* RP42.

Interestingly, of all the proteins involved in the Sil gene cluster, only the SilP sequence does not show any direct similarity to the Cus copper resistance system. But a BLAST search does show similarity of SilP to P-type ATPases from *E coli* as well as other P-
type ATPases that are involved in copper and silver transporting and exporting from other bacterial organisms. Considering the similarities in the protein sequences of the two resistance systems, we can postulate that *Morganella morganii* RP42 a silver resistant organism can also have tolerance towards copper but due to subtle differences in the sequences, the synthesis can be slower. Also, it has been previously demonstrated that histidine residues have affinity towards divalent copper ions, suggesting the binding and uptake of copper ions by *Morganella* spp is very much a possibility. Although interesting, it is not absolutely clear at this stage as to how a silver resistant organism can synthesize copper nanoparticles which remain stable against oxidation in an aqueous medium even after 4-5 weeks. It was however suggested in a few reports that, copper nanoparticles can be stabilized with the amino acid cysteine. Notably, in XPS analysis of the biosynthesized copper nanoparticles, a sulphur signal was also very prominent (Figure 3.21). Hence, it can be postulated that proteins/peptides with cysteine groups might be stabilizing these nanoparticles preventing their oxidation in aqueous solutions and air, thereby facilitating the stability of nanoparticles over extended periods of time.

### 3.6.5 Conclusion

In conclusion, we have demonstrated for the first time that a silver resistant organism *Morganella morganii* RP42 is capable of synthesizing stable metallic copper nanoparticles. The possibility to achieve metallic copper by using a ‘green’, eco-friendly biosynthesis approach would open up exciting avenues towards large scale synthesis of these technologically important nanomaterials. This proof-of-concept linking nanoparticle formation to the resistance mechanism can then be extrapolated to other metal nanoparticles by exploring the similarities in protein sequence and structure using bioinformatics tools. Further identification of the biomacromolecules involved in the process of formation of nanoparticles are currently under investigation, which will advance our knowledge towards the formation of nanoparticles as well as understanding the link in the fundamental aspect of silver and copper resistance.

### 3.7 Summary

In summary, this chapter provides a brief overview on the ability of bacterial species belonging to Genus *Morganella* to synthesize metallic silver and copper
nanoparticles. The ability to control biogenic silver nanoparticles shape anisotropy by controlling the growth kinetics of *Morganella psychrotolerans* is elucidated. The importance and similarities of the Sil gene cluster (silver resistance) to the Pco and Cus (copper resistance) gene cluster was further exploited to synthesize and stabilize metallic copper nanoparticles. Additionally, an important observation on the mechanistic aspect was explained using electrochemistry wherein it was shown that the synthesis of nanoparticles was an intracellular phenomenon indicating the complexity of the reduction process that was previously thought to occur extracellularly.

These observations provides conclusive evidence towards the importance of microorganisms that can act as ‘bionano-factories’ towards the synthesis of technologically important metal nanomaterials. The exciting possibility to synthesize nanomaterials using a ‘green’ biological route opens up avenues for large scale synthesis.

### 3.8 References


Chapter IV

Biomimetic Silicification in Ionic Liquid: A Quest to Understand the Formation of 3D Silica Nanostructures in Diatoms

The biochemical factors responsible for silica formation in diatoms have been extensively studied, but the formation of 3D structures similar to that found in nature is still an open challenge. This has led to an interesting debate that the physico-chemical environment surrounding diatoms might play an additional critical role towards the control of diatom morphologies. This chapter discusses the role of physio-chemical factors on formation of 3D ornate structures by replacing the commonly used aqueous solvents by ionic liquids a solvent that closely mimics natural systems. This proof-of-concept biomimetic study outlines the effect of amino acids, especially cationic amino acids towards attaining diatom like silica morphologies under biomimetics conditions in ionic liquids.
Part of the work presented in this chapter has been published:

**Refereed Journal Articles**


**Refereed Conference Articles**


**Refereed Conference Extended Abstracts**


**Articles Under Preparation/Communication**

- **Ramanathan, R.;** Bhargava, S. K.; Bansal, V. “Towards understanding the role of amino acids in the formation of 3D ornate silica structures during biosilicification: A new emergent paradigm.”

• **Ramanathan, R.**; Kandjani, A. E.; Selvakannan, P. R.; Bhargava, S. K.; Bansal, V. “Spectroscopic analysis of commonly occurring L-amino acids in nature.”

• **Ramanathan, R.**; Selvakannan, P. R.; Bhargava, S. K.; Bansal, V. “Biomimetic silicification using cationic amino acids in Ionic Liquids – Understanding the complex interplay between organic-inorganic moieties.”
## 4.1 Introduction

### 4.1.1 Ionic Liquids

Solvents are ubiquitous in chemistry that functions as facilitating media for molecules, ions etc. to interact. One of the most common solvation mediums used in chemical reactions and materials synthesis is water.\(^1\) Water is a convenient choice as it is readily available, non-toxic, and environmentally benign.\(^2\) Recent technological advances have led to the development of another important class of solvents viz. room temperature ionic liquids (RTILs). RTILs are more commonly referred to as ionic liquids (henceforth ILs) and have been a topic of fascination for researchers in various fields.\(^3\) Unlike conventional aqueous solvents, IL is a liquid electrolyte that is completely composed of ions and is generally a liquid at or close to room temperature.\(^3\) These compounds of high interest are often identified as the green and high-tech media of the future and this stems from their near-zero vapour pressure, thermal stability, wide electrochemical window, and their wide ranges of tuneable properties such as polarity, hydrophobicity, and solvent miscibility.\(^3\) Based on these properties, ILs have been used in a range of applications which includes organic synthesis, catalysis, inorganic materials synthesis, chromatography, as well as in biological systems.\(^1,7\)

![Figure 4.1: Structures of some 1-R-3-methylimidazolium tetrafluoroborate salts, where R corresponds to different alkyl groups.](image)

As the name suggests, ILs consist entirely of ionic species and are composed of two main components viz. the cation and the anion that exist dissociated as individual
cations and anions rather than intact molecules due to their molten nature at room temperature.\textsuperscript{5-6} What makes ionic liquids particularly interesting is the ability to tailor their properties for a particular purpose by modifying one or all of the constituents. Properties such as the density, viscosity, molar conductivity and miscibility with water can be easily modified by changing the length of the alkyl chain, for example.\textsuperscript{3-6} The cation, which in general is an organic molecule, helps prevent the formation of a stable crystal lattice.\textsuperscript{3,5} Some of the commonly used imidazolium based ILs is outlined in Figure 4.1

As there have been no reports on a universal ‘liquefying’ anion, the cation, that has a low degree of symmetry and poor localized charge, plays an important part in the synthesis of ILs. Many different classes of cations including imidazolium, pyridinium, pyrrolidinium, phosphonium and ammonium have been demonstrated to form stable RTILs with a range of anions such as halides, \([\text{BF}_4^-]\) and \([\text{PF}_6^-]\). In particular, dialkylimidazolium salts are synthesised by alkylation (Friedel-Crafts reaction) of N-methylimidazole that is commercially available, with an appropriate alkyl halide to give the corresponding 1-alkyl-3-methylimidazolium halide. Different anions can then be introduced through a process of anion exchange. Similarly, variations in the cation or anion can have profound effects on the chemical and physical properties of the IL.\textsuperscript{8-9} A simple example of how the cation and anion substitution can vary the melting point has been outlined in Table 4.1.

\textit{Table 4.1: The effect of cation and anion on the melting point of ionic liquids.}

<table>
<thead>
<tr>
<th>Imidazolium tetrafluoroborate salt</th>
<th>Mp (°C)</th>
<th>Anion</th>
<th>Mp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-methyl-3-methylimidazolium tetrafluoroborate</td>
<td>103</td>
<td>[Cl]^-</td>
<td>65</td>
</tr>
<tr>
<td>1-ethyl-3-methylimidazolium tetrafluoroborate</td>
<td>6</td>
<td>[I]^-</td>
<td>-72</td>
</tr>
<tr>
<td>1-butyl-3-methylimidazolium tetrafluoroborate</td>
<td>-81</td>
<td>[BF_4]^-</td>
<td>-81</td>
</tr>
<tr>
<td>1-hexyl-3-methylimidazolium tetrafluoroborate</td>
<td>-82</td>
<td>[PF_6]^-</td>
<td>-61</td>
</tr>
<tr>
<td>1-decyl-3-methylimidazolium tetrafluoroborate</td>
<td>-4</td>
<td>[TFSI]^-</td>
<td>-4</td>
</tr>
</tbody>
</table>

Another important property of ILs is their viscosity that is most often 10-100 times greater than aqueous or organic solvents as a result of strong electrostatic forces. The viscosity of an IL is not always the same and changes with respect to water content, temperature, pressure, impurity, and starting materials. ILs, especially where the cation is
imidazolium based (as discussed above in Table 1.1), tend to show decreasing viscosity through $[PF_6^-] > [BF_4^-] > [TFSI]^-$. In addition to these properties, the vapour pressure of ionic liquids is essentially zero under ambient conditions making them important green solvents for synthesis of inorganic materials.  

Like most other common materials, ILs can also decompose at elevated temperatures. The thermal decomposition temperature for an IL is mainly dependent on the structure of the ionic components and in comparison to aqueous or organic solvents, the temperature at which an IL remains stable can be much greater. While remarkable differences in the thermal decomposition temperature of an IL can be observed by altering the anion, only slight changes can be observed upon modification of the alkyl chain length of the cation. ILs with anions such as $[PF_6^-]$, $[BF_4^-]$ and $[TFSI]^-$ have been demonstrated to have a thermal decomposition temperature of up to 100 °C higher than halogens such as Cl and I.

In addition to the aforementioned advantages, one of the most significant and notable advantage of ILs is their ability to form extended hydrogen-bonded networks in their liquid states. This is similar to the role that water plays in protein folding, structure and stability arise due to its contribution from hydrophobicity and hydrogen bonds. The solvent self-structuration and supramolecular effects are extremely important especially when reactions are conducted with high concentrations of inorganic reactant. Interestingly, the self-organization process can also be supported by using amphiphilic species with longer hydrophobic tail. Due to the combination of hydrogen-bonding and amphiphilicity, well organized structures can still be obtained for pure ILs and their mixtures with water and other solvents.

The properties of ILs are many but to summarize some of the important outlying properties that make them ideal solvents for the synthesis of inorganic nanostructures include:

- ILs have low interface tensions that can result in high nucleation rates and the process of Ostwald ripening only proceeds weakly.
- Properties such as melting point and viscosity of ILs can be tailored by modifying the cation and the anion.
• The vapour pressure of ILs is negligible (essentially zero), making them ideal and easy to contain. Hence, these solvents are characterized as ‘Green solvents of the Future.’

• ILs possess good thermal stability and do not break down over a large range of temperatures. This enables the study of reactions that occur at high temperatures that would otherwise be impossible to monitor in aqueous or organic solvents.

• ILs generally do not react with metal ions however stabilization of metal complexes has been reported.

• One of the most important properties is the ability of ILs to form extended hydrogen-bonded systems in the liquid state and is therefore highly structured. As solvent structuring is the molecular basis of most self-organization processes, ILs can lead to spontaneous, well-defined, and extended ordering of materials at the nanoscale.

Given the wide range of properties, ILs have been extensively used in the synthesis of inorganic nanostructures, but only in the recent past have, scientists started exploring these solvents in biosciences especially with applications in enzyme stabilization, protein crystallization, and biofuel cells.³

4.1.2 Biomimetic Silicification: A Brief Introduction

The importance of silica as an essential inorganic material has long been established with potential applications including but not limited to catalysis, food and drug technology, biomedical applications, and as an adsorbent material.¹²⁻¹³ Chemical synthesis of silica-based materials is also well established (with production of ≈ 1 x 10⁶ tonnes per annum),¹⁴ but often requires high temperatures, pressures, and pH.¹³ In contrast, biosilicification in living organisms such as cyanobacteria, diatoms, sponges, and plants proceeds under mild physiological conditions that result in complex and hierarchical silica nanostructural frameworks with exquisite morphologies. This process proceeds at a scale that is several orders of magnitude higher than industrial scale (production of ≈ 6.7 x 10⁹ tonnes per annum).¹⁴⁻¹⁸ With billions of years of experience and availability of the most all-embracing laboratory at its disposal, biology has identified simple, yet elegant and clever routes to produce these ornate structures, and an
understanding of these processes can unveil a range of materials with novel applications or new technologies for nanomaterials production (outlined in Figure 4.2).

**Figure 4.2:** Schematic representation of the journey of research on biosilica to potential applications arising, with the arrows representing the flow of knowledge. Reprinted with permission from ref 14.

Biological silica formation, a process that is more commonly termed ‘biosilicification’ can be defined as “the movement of silicic acid from environments in which its concentration does not exceed its solubility (<2 mmol/L) to intracellular or systemic compartments in which it is accumulated in significantly higher concentrations for subsequent deposition as amorphous hydrated silica.” In simple terms, biosilicification process involves marine organisms like diatoms and sponges, that take up soluble silicon in the form of silicic acid from sea water, and catalyze its polymerization through diffusion-limited precipitation to form well-defined ornate biogenic silica structures in their skeletons under ambient conditions (mild acidic to neutral pH and ambient temperatures). It is noteworthy that biosilicification in these living organisms leads to formation of exquisite, intricate, and most often hierarchical and symmetric patterns of
amorphous biosilica nanostructures, which are generally species-specific, and genetically controlled.\textsuperscript{21} Notably, apart from the aesthetics of biogenic silica constructions in nature, a control over silica morphology under laboratory conditions is desirable, as it will enable us to understand this complicated biological phenomenon, and in turn will provide a pathway for large scale fabrication of these biomaterials for various applications.\textsuperscript{22-24}

\textbf{Figure 4.3:} Selected examples of morphologies of silica produced using biomimetic synthesis. These include silica synthesis using amino acids (a-e),\textsuperscript{25} poly-l-lysine (f)\textsuperscript{26}, p-\((\text{lys})_{20}\)\textsuperscript{27} and R5 peptide (I and j);\textsuperscript{27} in comparison to silica structures formed in natural silicifying organisms like sponge (k)\textsuperscript{28}, diatom (l)\textsuperscript{20}, and radiolarian (m and n). Reprinted with permission from ref 25-28.

Given the importance of the biosilification process, biochemical processes in these organisms have been extensively studied, and the process was hitherto believed to be triggered via hydrolysis of silicic acid by cationic proteins such as silicateins and polycationic peptides such as silaffins. These biomolecules are believed to play a multifunctional role as catalysts/templates/scaffolds during biosilica formation. \textit{In vitro}
studies using these biomacromolecules (silicateins, silaffins, cationic polypeptides, and amino acids) have demonstrated biosilica formation under biomimetic laboratory conditions (Figure 4.3a-4.3j). Although these molecules promoted silica hydrolysis, they failed to achieve the complex ornate morphologies observed in diatom and sponge morphologies witnessed under natural marine conditions (Figure 4.3k-4.3n).

The biosilicification process typically employs aqueous environments. Hence, water has so far been the most obvious medium of choice considered in most biomimetic silicification studies under laboratory conditions (biomimetics). Despite significant efforts towards understanding the biosilicification processes, as far as the control over silica morphology is concerned, to the best of our knowledge, most of the previous studies have hitherto not been able to produce 3D ornate silica morphologies through biomimetic silicification, similar to those produced by diatoms and sponges in their natural habitats. Recently, it has been recognized that in addition to the role of biomolecules, the physico-chemical environment in which this process happens in these organisms may also play a major role towards dictating the shape of nanostructured silica in these organisms. The role of these physico-chemical parameters such as how silicic acid is taken up from the surrounding marine environment of diatoms, transported to silica deposition vesicles (SDVs), concentrated to levels 1000-fold higher than the external environment in SDVs, and further condensed into ornate silica structures still remains unclear. Similarly, the effects of high salt concentrations, various physico-chemical forces, and high pressure levels including shear stress under deep marine conditions where these organisms grow, is also a mystery. Notably, in one of the previous studies, when external forces such as an electrostatic field or a shear stress was applied during biomimetic synthesis of silica in water, fused silica nanoparticles in the form of fibers, platelets and dendritic silica could be obtained. However, the possibility of achieving ornate hierarchical nanostructures that resemble diatom frustules or sponge spicules is still out of reach. This also suggests that the previous biomimetic studies, which were performed in aqueous solvents, do not necessarily mimic the natural biosilicification environment.
4.1.3 Rationale

Unlike other commonly used solvents, ILs can dissociate into individual cations and anions rather than existing as intact molecules, and due to their molten salt nature at room temperatures, ILs can in principle also mimic supersaturated salt solutions under marine conditions where biosilification process takes place. Additionally, ILs can also promote non-equilibrium large-order assembly of metal and metal oxide nanoparticles through a diffusion-limited aggregation (DLA) process, a mechanism that is believed to be involved during diatom biosilica formation. Additional properties of ILs such as their ability to form hydrogen bonded networks can also promote self-organization processes that are believed to play a critical role during the natural biosilification process. These properties suggest that replacing conventional non-ionic solvents (aqueous) with ionic solvents such as ILs may play a significant role in providing morphological control over biomimetic silification.

To demonstrate proof-of-concept, in this body of work, the potential of ILs as designer solvents for performing biomimetic reactions, especially in the context of biosilification is explored. Since cationic proteins/peptides/amino acids are believed to be the major driving force in the biosilification process, herein the important role of cationic amino acids towards controlling the morphology of biomimetic biosilica is elucidated. This study was further extended to understanding the role of other commonly occurring amino acids. This in vitro biomimetic study involving amino acids thus provides exciting evidence of the critical role of physico-chemical environment in addition to the important role of biomacromolecules during the biosilification process.

4.2 Experimental

4.2.1 Materials

1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF₄]) was purchased from Ionic Liquid Technologies (IoLiTec). All amino acids (L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamic acid, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-
tryptophan, L-tyrosine, and L-valine), and the precursor material tetraethylorthosilicate (TEOS) was purchased from Sigma-Aldrich and used as received.

### 4.2.2 Biosilica Synthesis

In the typical synthesis of silica structures, 1 M, 100 mM and 20 mM stock solutions of all 20 amino acids were separately prepared in MilliQ deionized water (in the case where the amino acids did not dissolve to the desired concentration, appropriate amount of HCl or NaOH were added). 10 µL of respective amino acid stock solutions were added to 490 µL of IL [BMIM][BF$_4$]. To these 500 µL amino acids in IL, 500 µL of ionic liquid containing 2 mM tetraethylorthosilicate (TEOS) was added. This resulted in 1 mL reactions containing a final TEOS concentration of 1 mM and final amino acids concentrations of 10 mM, 1 mM and 0.2 mM for the respective amino acids. Therefore, the final 1 mL reaction volume contained only 10 µL water and 1 µL TEOS with the remaining 989 µL IL. Water and TEOS volumes were kept to the minimum possible levels to avoid a potential change in the properties of the ILs with admixtures. In a control experiment, 10 µL of deionized water (without amino acid) was used in a 1 mL reaction containing 1 mM TEOS. In another control experiment, amino acids were replaced with liquid ammonia while maintaining the final ammonia concentration as 10 mM. The reactions were pursued for 16 h at 25 ± 0.1 °C under non-stirring conditions, during which all of the reactions involving amino acids or ammonia became turbid. However, no turbidity was observed in the control reaction containing water, thereby negating the possibility of water-mediated hydrolysis of TEOS in [BMIM][BF$_4$] and suggesting the important role of amino acids in TEOS hydrolysis in [BMIM][BF$_4$]. The reaction products in IL were further centrifuged at 8,000 rpm for 10 min and thoroughly washed with acetonitrile to remove viscous IL for further characterization.

### 4.2.3 Characterization

The precipitates containing the mixture of biomolecule-silica structures were characterized by scanning electron microscopy (SEM), X-ray photoemission spectroscopy (XPS), infra-red spectroscopy (FT-IR), selected area electron diffraction (SAED) and thermogravimetric analysis (TGA).
4.3 The Case of Cationic Amino Acids

Biosilicification has been extensively studied and cationic proteins/peptides/amino acids are believed to play a very important role in the overall process.\textsuperscript{15,30,33} As the process of biosilicification under natural marine conditions proceeds in an aqueous phase, most studies involved in biomimetics employed water as the medium of choice. It is however interesting that the previous studies in water found that cationic amino acids were incapable of promoting TEOS hydrolysis but in contrast they were able to induce tetramethylorthosilicate (TMOS) hydrolysis, however only granular silica nanoparticles without any formation of silica superstructures were obtained (Figure 4.4). In the current study, different categories of amino acids (cationic, anionic, polar and non-polar) were employed in IL [BMIM][BF\(_4\)] as the solvent to study the specificity of each amino acid in controlling the morphology of silica during biomimetic silicification.

4.3.1 Lysine Mediated Biomimetic Silicification Study

Figure 4.5 shows representative SEM images of silica structures obtained in [BMIM][BF\(_4\)] while employing TEOS to lysine molar ratios of 1:10 (a–b), 1:1 (c) and 1:0.2 (d) respectively. At higher lysine concentration (10 mM), well-defined silica microdiscs of 75–150 \(\mu\text{m}\) in diameter with brush-like appearance were formed (Figure 4.5a–4.5b). On closer observation, one of the microdiscs lying tilted on the surface of SEM substrate reveals that these silica microdiscs are about 10 \(\mu\text{m}\) in thickness (Figure 4.5b). A higher magnification of the image (inset, Figure 4.5b) further indicates that these microdisc-like
structures are formed in IL in the presence of lysine via assembly of 75–150 µm long and 50–200 nm thick silica nanorods. It is notable that although cationic amino acids are believed to be involved in silicic acid hydrolysis during biosilicification, these amino acids alone were not found to hydrolyse TEOS in water under biomimetic conditions.\textsuperscript{26} This is predominantly because polymers of these cationic amino acids such as polycationic peptides and proteins are generally considered to be essential for promoting TEOS hydrolysis in aqueous solutions.\textsuperscript{36} TEOS hydrolysis using lysine in IL \([\text{BMIM}][\text{BF}_4]\) is therefore rather interesting and suggests that IL creates a similar physical environment to that of polycationic amines through the self-assembly/organization of lysine molecules, thus promoting TEOS hydrolysis. Even more intriguing is the observation that silica structures formed by lysine-mediated hydrolysis in IL seem to have some resemblance to the overall complex silica matrices formed by some diatom species (e.g. \textit{Isthimia nervosa})\textsuperscript{20} during biosilicification in nature (Figure 4.6).

\textit{Figure 4.5}: SEM images of silica structures synthesized using lysine in IL \([\text{BMIM}][\text{BF}_4]\) involving TEOS to lysine molar ratios of (a and b) 1:10, (c) 1:1, and (d) 1:0.2 respectively. The insets show higher magnification images of structures shown in corresponding main figures.
Notably, as diatoms are biological entities, they have intricate biosilica architectures in terms of well-defined overall morphology, shape, size, surface patterns and hierarchical porosity, which result from a complex interplay of a range of genetic and environmental factors within an organic matrix. Therefore, it will probably be one of the most difficult research endeavours to mimic their forms completely. It must also be noted that the silica superstructures formed by lysine-mediated TEOS hydrolysis in IL do not entirely resemble with the diatom species *I. nervosa* in all the aforementioned parameters, but seems to be a resemblance in the overall sizes and shapes of these structures. This is the first time that ensembles of silica nanoparticles formed via biomimetic silicification have led to superstructures with a degree of resemblance to those formed by diatoms in natural habitats, which is indeed very exciting. To further investigate the role of the cationic amino acid lysine towards formation of these ornate structures, the lysine concentration was reduced to 1 mM while maintaining the ratio of TEOS-to-lysine molecules at 1:1 and 1:0.2 respectively in two separate experiments. It is evident from Figure 4.5c that although silica rods are still formed at a 1:1 TEOS-to-lysine ratio, these structures are less well-defined, and they do not assemble to form silica micro-discs. A further shift in the TEOS-to-lysine ratio of 1:0.2 results in the complete loss of morphology control, thereby mostly leading to silica spheres of ca. 700 nm diameter (Figure 4.5d). This provides a clear evidence that the concentration of amino acid lysine provides a shape-directing effect during biomimetic silicification in IL [BMIM][BF₄].

![SEM image of diatom Isthima nervosa found in nature that have some resemblance to silica structures synthesized by cationic amino acid lysine in IL [BMIM][BF₄]. Reprinted with permission from ref 20.](image)

**Figure 4.6:** SEM image of diatom *Isthima nervosa* found in nature that have some resemblance to silica structures synthesized by cationic amino acid lysine in IL [BMIM][BF₄]. Reprinted with permission from ref 20.
Previous work on biomimetic synthesis in which cationic amino acids (lysine, arginine, and histidine) were employed in water, resulted only in the hydrolysis of tetramethylothsilicate (TMOS) to yield granular silica nanoparticles without the formation of silica superstructures. These observations point towards the important role of solvents (ILs in the current study) in biomimetic reactions by means of controlling the physico-chemical growth environment via promoting self-assembly/self-organization processes. To understand and elucidate this multifarious interplay of a number of competing intra- and intermolecular forces that provides a high degree of complexity to the structures formed in biomimetic synthesis using ILs, a surface chemical analysis of structures formed using lysine was performed using x-ray photoelectron spectroscopy (XPS), which is a highly surface sensitive technique (approximate penetration depth of $\approx 10$ nm).

In order to determine the role of amino acid lysine in the formation of biomimetic silica structures, it is first essential to deconvolute the core level binding energies of the amino acid into its individual components. Figure 4.7 shows C 1s, N 1s, and O 1s core level XPS spectra of lysine and structures obtained from hydrolysis of 1 mM TEOS using 10 mM of lysine. All of the XPS spectra have been background corrected using the Shirley algorithm, and their respective binding energy (BEs) have been charge corrected with respect to the C 1s BE of 285 eV. The C 1s core levels in the case of pristine lysine was deconvoluted into three major components with the lower BE component at ca. 285 eV corresponding to alkyl carbon (C-C, C-H) while the other two BE components at 286.2 eV and 288.1 eV corresponds to the carbon bearing the amine group and the carboxylic group respectively. It is well known that amino acids are zwitterionic in nature and their net charge is dependent on the pH of the local environment (medium). In the current study, the amino acid lysine was used in the solid state for XPS (i.e. in the absence of solvent molecules) and has been previously recognized to exist in zwitterionic form. This phenomenon is particularly evident from the N 1s and O 1s core level binding energies, which on deconvolution mainly exhibits two components. In the case of the N 1s core level, the lower BE component at ca. 400.1 eV and the predominant higher BE component at ca. 401.4 eV can be assigned to the non-protonated and protonated amine group respectively. Similarly, in the case of the O 1s core level, the two BE components at ca.
531 eV and 532.7 eV can be assigned to the carboxylate anion and uncharged carboxylic group, respectively and these values corroborate well with the previously reported values for amino acid lysine.  

![Figure 4.7](image)

**Figure 4.7**: XPS spectra showing C 1s, N 1s, and O 1s core level arising from amino acid lysine and SiO$_2$ structures synthesized using 10 mM of lysine.

Interestingly, the core level XPS spectra arising from siliceous structures obtained after hydrolysis of TEOS were significantly different in terms of both peak intensities and chemical shifts. The C 1s core level binding energy could be deconvoluted to three components viz. the lower binding energy component at 285 eV that corresponds to alkyl carbon (C-C, C-H), while the two other components were observed at ca. 286.5 eV and 287.5 eV. Compared to the pristine lysine spectra, the lower BE component at ca. 286.5 eV shows a minor chemical shift of about ≈0.4 eV with substantial increase in peak intensity. This shift in the peak position can be attributed to the chemical environment that is due to the presence of SiO$_2$ (O being an electronegative element) that is in close vicinity to the charged ammonium group, while the change in peak intensity can be due to the incorporation of [BMIM] cation that also contributes to the C-N peak intensity. The peak at ca. 286.5 eV has also been previously reported to exist in a [BMIM] based IL and
corroborates well with the present scenario. The component at ca. 287.5 eV shows a shift of ≈ 0.6 eV that can be a result of (i) interaction of the carboxylate anion with [BMIM] cation and (ii) imidazolium N-C-N that is part of the final scaffold/template. The resolution limit of the instrument does not allow us to further resolve these peaks to their individual components. The N 1s core level binding energy can be deconvoluted to two major components with the lower BE component at ca. 400.2 eV and a predominant higher BE component at ca. 401.9 eV. The lower BE component is expected and is due to the presence of lysine embedded within the silica structure while the more predominant component at 401.9 eV that shows a chemical shift in comparison to lysine, corresponds to the nitrogen component of [BMIM] and suggests the presence of an electronegative element (possibly silica) in close proximity of the protonated ammonium group. Similarly, the O 1s core level could be deconvoluted into two major components with a lower BE component at ca. 531.2 eV and a higher BE component at ca. 532.6 eV. The predominant O 1s core level signature at 532.6 eV is assigned to the O atoms bonded in the Si-O-Si network of SiO₂ particles, which one would expect from a predominant SiO₂ based system. The presence of a minor O 1s low energy BE component at 531.2 eV is typical of amino acids that can be assigned to free –COO groups present in the amino acid lysine. The presence of –COO, [BMIM] and NH₃⁺ signals in the SiO₂ structures not only indicate the direct involvement of amino acid lysine but also the solvent (IL) towards formation of these unique silica morphologies. It is not surprising to note the role of ILs in the formation of the unique morphologies, in addition to the role played by amino acids, especially because the imidazolium moiety of ILs has been shown to interact with nanoparticles as well as play an important role in forming monolithic mesoporous silica structures.

In addition to C 1s, O 1s, and N 1s core levels, the Si 2p core level in the lysine mediated silica (Figure 4.8) could be deconvoluted into two major components with the major lower BE component at ca. 103.2 eV and a higher BE component at ca. 104.3 eV, which corroborate well with previously reported values of SiO₂ system in an Si-O-Si and Si-OH environment. In the case of diatomaceous silica structures, it has been identified that the conformation and orientation of cationically charged macromolecules in a matrix formed due to supersaturation and ionicity of the reaction can be crucial in the formation
of templates for the nucleation and growth of silica structures.\textsuperscript{24} XPS analysis in the current IL lysine silica system therefore strongly suggests that these novel amorphous silica morphologies (SAED showing the amorphous nature is shown in Appendix D-Figure D.1) obtained through a biomimetic route are in fact hybrid organic-inorganic composite biosilica materials similar to that seen in the case of diatoms and sponges in nature.

![Figure 4.8: XPS spectrum showing Si 2p core level arising from biomimetic SiO\textsubscript{2} structures synthesized using 10 mM of lysine.](image)

Figure 4.8: XPS spectrum showing Si 2p core level arising from biomimetic SiO\textsubscript{2} structures synthesized using 10 mM of lysine.

It is evident from XPS analysis that in addition to the role of cationic amino acid lysine, the ionic liquid [BMIM][BF\textsubscript{4}] also plays an important role in the formation of the scaffold/template necessary for the synthesis of complex morphologies observed in the SEM images (Figure 4.5). More interesting was the fact that boron and fluorine signatures from the IL were not observed during XPS analysis. This was also confirmed using EDX analysis on the silica structures where signatures only from carbon, nitrogen, oxygen, silicon and platinum (samples were coated with platinum to minimize charging) were observed (Figure 4.9). The IL [BMIM][BF\textsubscript{4}] used in the current study only comprises of a short alkyl chain (organic molecule) and it has been postulated that this solvent does not preferentially self-assemble into ordered structures by rearrangement of hydrophobic and hydrophilic chains, in contrast to a common occurrence observed in the case of long-chain alkyl groups containing ILs, surfactants and amphiphilic block-copolymers.\textsuperscript{5-6,10}
Based on XPS analysis it can be postulated that there exists a complex interplay between the IL and lysine molecules to promote the assembly of 3D hierarchical silica structures. Although this short chain IL is not known to preferentially self-assemble into ordered structures, in the current case, it can be confidently interpreted that the IL is in fact involved in forming a π-π stack especially between the imidazolium rings and the packing of alkyl groups during the formation of silica structures. Hence to further elucidate the π-π stacking templated mechanism and the important role of ILs and amino acids towards the formation of silica superstructures, infrared spectroscopy was employed as it is a powerful method to probe the molecular states and is sensitive to the environment and intermolecular interactions especially π-π stacking.

![Figure 4.9: EDX analysis of silica structures obtained following hydrolysis of TEOS using cationic amino acid lysine.](image)

Figure 4.9 shows the comparison of the IR spectra obtained from neat IL [BMIM][BF₄], TEOS, amino acid lysine, and IL-lysine templated silica. To elucidate the proposed stacked template, the π-π interaction of the imidazolium rings from the IL can be examined by the change in the peak position of the C-N stretching vibration ($\nu_{C-N}$) mode of the ring structure at 1000 cm⁻¹ in the Lys-SiO₂ spectrum. In the case when stacking occurs, this mode of vibration ($\nu_{C-N}$) normally shifts towards lower wavenumbers. Unfortunately, in the present study, it is quite difficult to observe this
shift due to the overlap from the broad Si-O stretching vibration (ν_{Si-O}) located around 1100-1000 cm\(^{-1}\) (marked by black star in Figure 4.10b).\(^{47,49}\) Nonetheless, the stacking of imidazolium rings can also be monitored by the change in aromatic C-H stretching vibration (ν_{C-H}) mode between 3200 – 3000 cm\(^{-1}\) (marked by a black dot in Figure 4.10a).

**Figure 4.10:** ATR FT-IR spectra (a) showing the overall vibrational modes of TEOS, IL [BMIM][BF\(_4\)], lysine, lysine-IL mediated silica and (b) showing the expanded region (black box) outlined in (a) for amino acid lysine and lysine-IL mediated silica.

On comparing the spectra of IL and Lys-SiO\(_2\) (Figure 4.10), it can be seen that the peak marked with the solid dot corresponds to one of the C-H stretching vibration that is found to be substantially broadened due to the decrease in electron density of ring C-H bonds, leading to a shift of the absorbance band to lower wavenumbers originating from the π-π stacking of the positively charged imidazolium rings.\(^{44,47}\) Additionally, the C-H stretching vibration observed around 2950-2850 cm\(^{-1}\) from the butyl group of [BMIM] is also influenced considerably by the packing.\(^{44}\) This observation further confirms the suggested possibility of the role of IL [BMIM][BF\(_4\)] in forming a template. Furthermore, the interaction of amino acid lysine with IL that forms the final template/scaffold is evident by observing the shift in the stretching vibrations of C=C and C=N of the imidazolium ring at about 1600 cm\(^{-1}\) (marked by red star in Figure 4.10b) due to the
electrostatic interaction of the negatively charged carboxylic group of lysine. Interestingly, these vibrational modes are stronger in intensity due to the packing of the alkyl chain from the butyl group of the IL and lysine chains. This phenomenon is also confirmed by the decrease in wavenumbers of the -COO symmetrical stretching vibration from 1421 cm\(^{-1}\) to 1407 cm\(^{-1}\) (marked by red star in Figure 4.10b).\(^{50-51}\) Importantly, XPS analysis suggests that the biomimetic silica formed by cationic lysine lies close to the positively charged amine groups. This electrostatic interaction was further confirmed by an increase in the wavenumbers observed in the NH\(_3^+\) vibrations at 1500 cm\(^{-1}\) (marked by purple star in Figure 4.10b).\(^{50}\) These experimental observations provides indicate that the proposed IL-amino acid electrostatic charge matching \(\pi-\pi\) stacked templated mechanism is responsible for the formation of 3D structures that resemble diatom like structures.

![Figure 4.11: TGA profile obtained from cationic amino acid lysine, ionic liquid [BMIM][BF\(_4\)] and amino acid templated silica.](image)

To further confirm the interaction of IL and lysine and their role in the formation of the final template/scaffold, thermogravimetric analysis (TGA) was performed on IL, amino acid lysine and the lysine-IL-templated silica structures over a temperature range of 50-750 °C under \(N_2\), with a heating rate of 10 °C min\(^{-1}\). Figure 4.11 shows the TGA curve of lysine under nitrogen flow and reveals two distinct weight loss regions viz. a gradual weight loss of 90 % between 250-500 °C and the remaining 10 % beyond 700 °C. The
weight loss observed over this temperature range corresponds to the thermal decomposition of organic compounds, resulting from the release of CO\textsubscript{2}, NH\textsubscript{3}, H\textsubscript{2}O and CH\textsubscript{4} products.\textsuperscript{24} The IL, similar to lysine, does not show any weight loss below the temperature of 250 °C and only shows a small weight loss of about 3-5 % up to 300 °C. No further degradation was observed until a temperature of about 350 °C is reached, further to which point a progressive degradation and mass loss was observed between 350-500 °C. This weight loss corroborates well with previous studies performed to study thermal stability of ILs.\textsuperscript{52}

The lysine-IL templated silica structures also undergo thermal degradation as elevated temperatures would decompose organic entities that form the underlying scaffold/template in these silica structures. In comparison to the precursor materials (IL and lysine), there are four distinct weight loss regions in the case of silica structures that typically starts beyond 200 °C. The first weight loss region (approximately 40 % loss) lies between 200-350 °C and could be attributed to the decomposition of lysine units. Beyond 350 °C, a progressive but slow degradation and weight loss of 30 % is observed between 350-650 °C, wherein a stabilization of weight occurs at 500 °C. A weight loss at these temperatures was also observed in the case of pristine lysine and IL, which is attributed to the loss of precursor materials. The weight stabilization at 500 °C is most likely due to the extended Si-O network array and the complex ionic interactions between amino acid-IL and silica that confers thermal stability and robustness to these materials. This phenomenon is similar to that observed in the case of amino acid backbone based nanoporous materials wherein the extended Ni-N and Ni-O bonds contributed to the thermal stability of the structures.\textsuperscript{53} In total, the silica materials showed a total weight loss of approximately 80 % that clearly suggests that these biomimetic silica structures obtained from lysine mediated hydrolysis typically contained high amounts of organic material. This phenomenon is similar to the biosilica found in diatoms wherein there exists a complex organic matrix that is believed to play an important role of structure control. Furthermore, the calcined product was analysed using FT-IR spectroscopy (Figure 4.12) that predominantly shows the stretching vibrations from only silica material (Si-OH at approximately 930 cm\textsuperscript{-1}, Si-O-Si\textsubscript{sym} at 800 cm\textsuperscript{-1}, and Si-O-Si\textsubscript{asym} at approximately 1100 cm\textsuperscript{-1}). The vibrations from the organic components that play a role of template/scaffold
previously observed from the as-synthesized biomimetic silica (Figure 4.12) are no longer observed.

![Figure 4.12: ATR FT-IR spectra obtained from lysine mediated silica material obtained before and after calcination.](image)

These results also point towards an important aspect of the biosilicification process wherein it is postulated that there exist strong interactions between the organic and inorganic components. During natural biosilicification, the organic biomacromolecules are believed to form the basic template/scaffold for the inorganic component which is observed in the current study. Further analysis of the calcined material confirms the absence of organic moieties confirming the similarities between the natural biosilicification process and the current biomimetic silicification. This complex interaction of ionic liquid with lysine to form a template/scaffold for the assembly of 3D hierarchical structures that resemble diatoms is both intriguing and exciting. Based on the XPS, TGA, and FTIR analyses, an electrostatic charge matching π-π stacked templated synthesis mechanism is proposed that may be responsible for playing an important role in the formation of complex intricate structures. A schematic representation of the proposed mechanism is represented in Scheme 4.1.
Scheme 4.1: Schematic illustration of the proposed electrostatic charge matching \( \pi-\pi \) stacked templated mechanism during the formation of complex 3D ornate structure using cationic amino acid L-lysine\(^a\).

This self-assembly of organic molecules especially the sophisticated organisation, electrostatic charge matching, and structure directing framework of IL and amino acid via the current biomimetic synthesis is quite intriguing. Although, the short alkyl chain IL [BMIM][BF\(_4\)] was not originally believed to form hydrogen bonding networks with water, it has already been proven that this IL forms a cation-anion-water interaction in the presence of water\(^{54-56}\). It was postulated that water molecules interact via hydrogen bonds to form a symmetric ordered structure (Aʻ...H-O-H...A),\(^{56}\) thus supporting the current proposed mechanism where IL is found to be incorporated within the lysine mediated biomimetic silica structures. In previous studies, although the imidazolium cation was not believed to interact with water to form hydrogen bonds, in the current study, the presence of additional charged lysine residues (NH\(_3^+\) and COO\(^-\)) in addition to water molecules would promote Coulombic coupling interactions between the imidazolium cation and carboxylate anion that can facilitate the formation of a template outlined in Scheme 4.1. The proposed \( \pi-\pi \) stacking/packing between the aromatic imidazolium motifs and aliphatic chains can be expected to occur as this mechanism is a
common occurrence in a wide range of natural self-assembly processes.$^{57}$ Moreover, these interactions are also apparent in the XPS and FTIR analysis of the current biomimetic silica material.

Based on the analysis of biomimetic silica, it can be confirmed that in addition to the IL, the amino acid lysine also plays a critical role in the formation of the underlying template and is involved in electrostatic charge matching that provides the underlying framework. Earlier studies on amino acid lysine confirmed that the molecule (lysine) orients itself in a linear geometry in the presence of solvation media.$^{58}$ Moreover, lysine contains two terminal groups ($\text{COO}^-$ and $\text{NH}_3^+$), and due to its linear geometric orientation, if the negatively charged terminal carboxylic group ($\text{COO}^-$) interacts with the cationic imidazolium ring, the exposed terminal protonated amine will determine the final framework preceding the hydrolysis/stabilization of silica particles. Furthermore, the grouping of the organic moiety (Lys-IL-IL-Lys) as a solid sphere that only exhibits the exposed terminal protonated amine suggests a mechanism for the formation of rod morphologies. A modified schematic that explains the formation of individual rods is outlined in Scheme 4.2. Based on the shifts observed in the $\text{NH}_3^+$ stretching frequencies and the broad peak from Si-O-Si stretching vibrations it can be confirmed that the terminal positively-charged amine motifs along with the other organic components are responsible for the assembly of silica in the form of rods.

**Scheme 4.2:** A simplified cartoon schematic version of the proposed IL-lysine mediated synthesis of ornate silica structures.
Based on the experimental evidence, this study suggests that cationic amino acid lysine serves as an effective promoter for spontaneous precipitation of silica via TEOS hydrolysis in [BMIM][BF$_4$]. Although the important role of cationic amino acid lysine as a morphology-directing agent is evident but to unequivocally assign this morphology control to lysine, additional control experiments were performed wherein 1 mM TEOS was hydrolysed in IL using 10 mM equivalent of liquid ammonia. During ammonia mediated hydrolysis of TEOS in IL, there was no evidence of morphological control and only agglomerated silica particles were observed (Figure 4.13).

![Figure 4.13: SEM images of silica structures synthesized by hydrolysis of 1 mM TEOS using 10 mM equivalent of ammonia in ionic liquid [BMIM][BF$_4$].](image)

This control experiment further confirms the important role of cationic amino acid lysine in the presence of IL [BMIM][BF$_4$] towards biomimetic formation of exquisite diatom-like silica morphologies. Furthermore, the incorporation of the organic component and the complex interplay between the organic-inorganic constituents provides evidence of the sophistication of the current system and shows similarities to the natural biosilicification process.$^{24}$ To further confirm and to further understand whether all cationic amino acids are able to use their terminal protonated amine as a structure directing agent for TEOS hydrolysis, similar studies using cationic amino acids arginine and histidine were also performed.
4.3.2 Arginine Mediated Biomimetic Silicification Study

Figure 4.14: SEM images of silica structures synthesized using arginine in IL [BMIM][BF$_4$] involving TEOS to arginine molar ratio of (a and b) 1:10, (c) 1:1, and (d) 1:0.2 respectively. The insets show the higher magnification images of the structures shown in the corresponding main figures.

In addition to lysine, the other cationic amino acid arginine is also known to be involved in biosilicification processes.$^{15,30,33}$ Therefore further investigation of arginine-mediated biomimetic silicification in IL [BMIM][BF$_4$] was carried out to explore whether structural control via biomimetic silicification in IL is an amino acid-specific feature driven by the terminal protonated amine group. When arginine was employed for TEOS hydrolysis in [BMIM][BF$_4$] at 1:10 TEOS to arginine ratio, long extended porous sheet-like silica structures with 5–10 µm pore diameter were obtained (Figure 4.14a–4.14b). At higher magnification, these micropores were found to be filled with closely-packed rectangular platelets of 1–2 µm edge length (Figure 4.14b). Notably, amorphous biosilica structures (SAED showing the amorphous nature is shown in Appendix D-Figure D.1) formed by a large number of diatom species are well-known to possess hierarchical porosity within their silica architectures. For instance, the porous surface obtained by
arginine-mediated hydrolysis of TEOS in IL has some resemblance in terms of overall pore size and pore distribution pattern with that of diatom species *Cyclotella meneghiniana* (Figure 4.15). However, this similarity is still distantly far from the level of hierarchical porosity demonstrated by diatomaceous silica in natural environments. Similar to the lysine system, when the arginine concentration was reduced to 1 mM (1:1 ratio, Figure 4.14c) or 0.2 mM (1:0.2 ratio, Figure 4.14d), assembled networks of ca. 400 nm silica particles, were formed. Formation of long range ordered porous silica morphologies at higher arginine concentrations, and their absence at lower arginine concentrations clearly suggests the shape-directing effect of arginine molecules during biomimetic silicification in IL [BMIM][BF₄].

![Figure 4.15: SEM image of diatom Cyclotella meneghiniana found in nature that has some resemblance to silica structures synthesized by cationic amino acid arginine in IL [BMIM][BF₄]. Reprinted with permission from ref 20.](image)

To further understand the underlying intermolecular forces involved in arginine mediated synthesis of ornate silica structures, XPS analysis was first performed on arginine and arginine templated silica structures. Figure 4.16 shows the C 1s, N 1s and O 1s core level binding energies obtained from arginine and arginine templated silica. Similar to that observed in lysine, the C 1s core level in the case of the pristine amino acid was deconvoluted into three components viz. 285 eV, 286.1 eV and 287.9 eV that correspond to alkyl, amine bearing and carboxylic carbon respectively. The N 1s core level binding energy was also deconvoluted into two components but the observed values were approximately 1 eV lower than those observed in the case of lysine that can be attributed to the charge delocalization on the guanidine group. Charge delocalization
generally reduces the ion-dipole interaction with a decrease in binding energies. On the other hand, the core level binding energies for O 1s are similar to those observed in the case of lysine, with lower BE at ca. 530.9 eV and a higher BE at ca. 532.1 eV corresponding to the neutral and charged carboxylic groups respectively.

**Figure 4.16**: XPS spectra showing C 1s, N 1s, and O 1s core levels arising from amino acid arginine and SiO\(_2\) structures synthesized using 10 mM of arginine.

Interestingly, when silica structures obtained following TEOS hydrolysis were analysed, there were significant different from the BEs observed for blank arginine in terms of chemical shifts and additional peaks. The C 1s core level BE from silica structures could be deconvoluted into four components in comparison to three components observed in the case of the pristine amino acid. The four components were observed at BEs of 285 eV, 286.3 eV, 288.1 eV and 289.1 eV, of which the first three components were at BEs similar to those observed in the case of lysine mediated hydrolysis of the TEOS precursor. This is not surprising as the major difference between lysine and arginine is in the R group, wherein lysine displays one protonated amine group at its exposed terminal end while arginine exhibits a positively charged guanidinium group. Interestingly, the positive charge observed in the case of the guanidyl group is delocalized because of the
conjugation between the double bond and the nitrogen electron lone pairs.\textsuperscript{60} This delocalization of charge (presence of three positively charged amine groups) is believed to attract and bind to a significantly higher number of silica molecules. Hence, we can postulate that the additional peak observed at BE 289.1 eV is due to the presence of electronegative oxygen atoms of the silica framework in close association with the positively charged nitrogen (amine) atoms. This also corroborates well with the results observed in the case of the lysine mediated silica synthesis as the terminal group in that case contained only one positively charged amine, which was interacted with the negatively charged silica molecule. In contrast, in the case of arginine, the presence of three charged groups can attract and bind to more silica molecules leading to the shift in BE observed in the C 1s core level obtained from arginine mediated synthesis of silica structures. The involvement of the positively charged guanidinium group in silica binding was further confirmed by the N 1s core level BE that showed a significantly large 2 eV shift towards higher BEs in comparison to pristine arginine. Similarly, the O 1s core level could also be deconvoluted into two major Gaussian components at ca. 531.1 eV and 532.6 eV that corroborates well with the values obtained for lysine mediated biomimetic silica structures. These results point towards the direct role of amino acid arginine in dictating the silica morphologies observed in the current study.

![Figure 4.17: XPS spectrum showing Si 2p core level arising from biomimetic SiO$_2$ structures synthesized using 10 mM of arginine.](image)

\textit{Figure 4.17: XPS spectrum showing Si 2p core level arising from biomimetic SiO$_2$ structures synthesized using 10 mM of arginine.}
In addition to the C 1s, O 1s and N 1s core levels, the Si 2p core level obtained from the silica structures could be deconvoluted into two major Gaussian components with a major higher BE component at ca. 103.1 eV which corroborates well with the previously reported values of SiO$_2$ systems in an Si-O-Si environment.$^{45}$ The most interesting observation that was significantly different is the minor component at lower BE of ca. 101.6 eV that corresponds to silicon sub oxides (SiO$_x$, with x<2)$^{61}$ in comparison to lysine which showed the presence of a higher BE component corresponding to a Si-OH environment (Figure 4.17). Similar to that observed in amino acid lysine, XPS analysis of the silica structures strongly suggests the involvement of the solvent IL in the formation of the underlying template during arginine mediated biomimetic synthesis. Similar to the case of lysine, XPS analysis of arginine mediated silica materials also did not show boron and fluorine signatures. This was also confirmed using EDX analysis on the silica structures where signatures only from carbon, nitrogen, oxygen, silicon and platinum (samples were coated with platinum to minimize charging) were observed (Figure 4.18)

![Figure 4.18: EDX analysis of silica structures obtained following hydrolysis of TEOS using cationic amino acid arginine.](image)

Based on these observations it can be assumed that the mechanism observed in the case arginine mediated silica structures is primarily similar to that observed in the case of lysine mediated biomimetic silica structures, wherein the imidazolium group is
involved in π-π stacking while the terminal amine group is involved in TEOS hydrolysis and silica binding. To further confirm this π-π stack templated mechanism, infrared spectroscopy was employed.

![Figure 4.19: ATR FT-IR spectra (a) showing the overall vibrational modes of TEOS, IL [BMIM][BF₄], arginine, and arginine-IL mediated silica; (b) showing the expanded region (black box) outlined in (a) for amino acid arginine and arginine-IL mediated silica.](image)

Figure 4.19 shows the comparison of the IR spectra obtained from neat IL [BMIM][BF₄], TEOS, arginine, and IL-arginine templated silica. To elucidate the presence of the stacked template similar to that observed in lysine, the π-π interaction of the imidazolium rings from the IL was examined by the change in peak position of the C-N stretching vibration (ν_C-N) mode of the ring structure at 1000 cm⁻¹ in the Arg-SiO₂ spectrum. However, similar to the observation in lysine, the shift in this vibrational mode [ν_C-N]⁴⁷ is quite difficult to observe due to the overlap from the broad Si-O stretching vibration (ν_Si-O) located around 1100-1000 cm⁻¹ (marked by black star in Figure 4.19b).⁴⁷,⁴⁹ Therefore, the stacking of imidazolium rings was instead monitored by the change in the aromatic C-H stretching vibration (ν_C-H) mode between 3200 – 3000 cm⁻¹. This stretching vibration is significantly broadened and shifted to lower wavenumbers due to decrease in the electron density of the ring C-H bond resulting from the packing of imidazolium groups of the IL. The butyl group C-H vibrational stretching around 2950-2850 cm⁻¹ is also
significantly influenced by the packing of IL in these silica structures. The additional role of amino acid arginine in which the terminal carboxylic group interacts to the imidazolium group of the IL, can be observed by the shift in the stretching vibrations of C=C and C=N at about 1600 cm\(^{-1}\) (marked by red star). The additional decrease in wavenumbers of the -COO symmetrical stretching from 1421 to 1407 cm\(^{-1}\) further confirms the terminal carboxylate ion interaction of arginine with the imidazolium groups of ILs. Furthermore, the increase in wavenumber observed in the case of NH\(_3^+\) (marked by purple star) also confirms that the exposed terminal positively charged guanidyl group is involved in silica binding. These observations are rather comparable to those observed in the earlier case of lysine mediated biomimetic silica formation, thereby further confirming the possible resemblances in the mechanistic aspect of biosilica formation by lysine and arginine.

![Graph](image.png)

**Figure 4.20:** TGA profile obtained from cationic amino acid arginine, ionic liquid [BMIM][BF\(_4\)] and amino acid templated silica.

To further confirm the incorporation of IL and arginine within the final template/scaffold, thermogravimetric analysis (TGA) was performed on IL, pristine arginine and arginine-IL-templated silica structures over a temperature range of 50- 750 °C under N\(_2\), with a heating rate of 10 °C min\(^{-1}\). Figure 4.20 outlines the TGA curve of pristine arginine that reveals three distinct weight loss regions viz. the first minor weight
loss of 15 % up to 250 °C, which is followed by a continuing weight loss (72 %) between 250-650 °C and the residual material decomposes by 700 °C. This observation is comparable to the previous case of lysine where elevated temperatures led to the decomposition of organic compounds resulting in the release of CO₂, NH₃, H₂O and CH₄ products. The data observed in the current case also corroborates well with previous thermal studies performed on amino acid arginine. The weight loss pattern of IL has already been discussed in the case of amino acid lysine and typically degrades by 500 °C.

Figure 4.21: ATR FT-IR spectra obtained from arginine mediated silica structures before and after calcination.

The IL-arginine silica structures also undergo thermal degradation and in comparison to the precursor IL and pristine amino acid, the templated structures are thermally more stable. They typically display four distinct weight loss regions with the initial weight loss trend (up to temperature range of 250 °C) similar to that observed in the case of arginine. Beyond 250 °C, there is a slow and continuous weight loss of about 28 % before stabilization at 600 °C. The thermal stability of biomimetic silica structures can be attributed to the extended Si-O network and the strong ionic interactions between silica-IL and the amino acid. A similar trend was also observed in the case of amino acid lysine where these interactions lead to weight stabilization. The final weight loss region
between 600-700 °C is interesting as the material rapidly degrades by 40 % before attaining thermal stability at 700 °C. In total, the material showed a weight loss of 70 % that clearly suggests the incorporation of the IL and amino acid within the templated biomimetic silica structures. The observed loss in mass in the current arginine mediated silica is lower than lysine mediated silica structures that are most likely due to higher binding capability of the charged terminal guanidyl group. Furthermore these calcined structures were analysed using FTIR that predominately showed the presence of stretching vibrations from silica material (Figure 4.21) suggesting an important role of amino acids and solvent IL in the formation of complex morphologies.

**Scheme 4.3:** Schematic illustration of the proposed electrostatic charge matching π-π stacked templated mechanism during the formation of ornate 3D structures using cationic amino acid arginine.ńska

Based on XPS and FTIR analysis, there is substantial evidence that the mechanism involved in the case of arginine is quite similar to that observed in the previous case of cationic amino acid lysine wherein the solvent IL and amino acid play an important role during the formation of biomimetic silica complex morphologies. Typically, in arginine mediated synthesis, the imidazolium group from the IL [BMIM][BF₄] is involved in π-π interaction that leads to stacking of the imidazolium rings and packing of the aliphatic
(butyl) chains (outlined in Scheme 4.3). Interestingly, the amino acid arginine, in the presence of a solvation medium, orients itself into a linear geometry. This previous observation further confirms the possibility of a similar mechanism to that observed in the case of lysine mediated synthesis (with the two terminal groups viz. the negatively charged COO$^-$ and guanidine group that contains three protonated amines). Due to the linear orientation of arginine, if the negatively charged terminal carboxylic group (COO$^-$) interacts with the cationic imidazolium ring of [BMIM], the three exposed terminal protonated amine will control the final framework preceding the hydrolysis/stabilization of silica particles. Given the similarities between lysine and arginine, it is quite surprising that arginine mediated hydrolysis forms extended porous silica sheets. To further understand the formation of sheets, albeit in a simplistic manner, the organic moiety (Arg-IL-IL-Arg) was grouped as a solid sphere with only the three exposed terminal protonated amine groups shown. A modified schematic that now shows the formation of extended sheets is outlined in Scheme 4.4. Based on the shifts observed in the NH$_3^+$ stretching frequencies and the broad peak from Si-O-Si stretching vibrations it can be confirmed that the terminal positively-charged amine motifs, along with the other organic component are responsible for the assembly of silica in the form of sheets.

\[ \text{Scheme 4.4: A simplified cartoon schematic of the proposed IL-arginine templated synthesis of silica superstructures.} \]

This provides supports for the proposed role of the imidazolium ring π-π stacked mechanism and the linear geometry of the cationic amino acid arginine in the formation
of silica superstructures. Additionally, it can be confirmed that the terminal exposed amine group dictates the final silica structures formed during cationic amino acids mediated synthesis of biomimetic silica structures. To unambiguously assign the structure directing property to the important role of the exposed terminal group (one protonated amine in case of lysine and three protonated amines in case of arginine), a third cationic amino acid histidine, wherein the exposed protonated amine is an imidazolium group, was also employed for TEOS hydrolysis.

4.3.3 Histidine Mediated Biomimetic Silicification Studies

![SEM images of silica structures synthesized using histidine in IL [BMIM][BF₄] involving TEOS to histidine molar ratio of (a and b) 1:10, (c) 1:1, and (d) 1:0.2 respectively. The insets show the higher magnification images of the structures shown in the corresponding main figures.](image)

Similar to the amino acids lysine and arginine, TEOS hydrolysis in [BMIM][BF₄] using a relatively large quantity of the cationic amino acid histidine (10 mM – 1:10 TEOS to histidine ratio) resulted in control over the final silica morphology, however it led to a completely different set of unique silica structures that contained a mixture of silica
microglobules of 20–50 µm diameter and silica plates (Figure 4.22a–4.22b). Higher magnification SEM imaging of amorphous silica microglobules (SAED showing the amorphous nature is shown in Appendix D-Figure D.1) revealed a rough surface entirely comprised of sharp-edged triangular and hexagonal platelets of 1–3 µm edge length and 100–300 nm thickness (Figure 4.22b). Similar to the previous two cases (lysine and arginine), triangular platelets formed using histidine-mediated hydrolysis of TEOS showed some degree of resemblance to triangle-shaped diatom species (e.g. *Trigonium arcticum*) found in nature (Figure 4.23), in terms of their general shape. However, other typical features of diatomaceous species such as hollow interiors, size and surface patterns were not found to be replicated under the experimental conditions used in this study. At lower histidine concentration of 1 mM (1:1 ratio), a mixture of triangular/prismatic silica plates and globular nanospheres were formed (Figure 4.22c). Further reducing histidine concentration to 0.2 mM (1:0.2 ratios, Figure 4.22d) resulted in a complete loss of flat plate-like morphologies, and instead resulted in assembled network of ca. 800 nm silica spheres. Concentration dependent loss of morphological control phenomenon is very similar to that observed in the case of the amino acids lysine and arginine.

![SEM image of diatom Trigonium arcticum found in nature](image)

*Figure 4.23: SEM image of diatom Trigonium arcticum found in nature that have some resemblance to silica structures synthesized by cationic amino acid histidine in IL [BMIM][BF₄]. Reprinted with permission from ref 20.*

Histidine is peculiar in comparison to lysine and arginine in the sense that the side chain of histidine contains an imidazolium functional group that is mostly protonated at acidic pH. Notably, the positive charge on the imidazole ring is equally distributed between the two nitrogen atoms. In the case of the other two cationic amino acids...
lysine and arginine where the π-π stacking/packing between the aromatic imidazolium motifs was restricted to the IL, in the present case, two π-π stack mechanisms are likely to occur in parallel. To determine and confirm the role of histidine in the formation of complex silica structures, XPS analysis was performed. Figure 4.24 shows C 1s, N 1s, and O 1s core level XPS spectra arising from pristine histidine, and structures obtained from hydrolysis of 1 mM TEOS using 10 mM of histidine, respectively. The C 1s core level binding energy can be deconvoluted into three major Gaussian components with a lower BE component at ca. 285 eV corresponding to alkyl carbon (C-C, C-H) while the BE components at ca. 286 and 287.5 eV corresponding to the amine bearing carbon (including C in the imidazole ring) and carboxylic carbon respectively. The zwitterionic nature of the amino acid was evident from the N 1s and O 1s core level binding energies, which on deconvolution exhibited three and two Gaussian components respectively. The two Gaussian components in the O 1s core level binding energy included the lower component at 531 eV and the minor higher BE component at ca. 532.6 eV that can be assigned to carboxylate anion and an uncharged carboxylic group respectively. On the other hand, the N 1s core level shows three Gaussian components at ca. 398.8 eV, 400.5 eV, and 401.3 eV and can be assigned to imino nitrogen, amino nitrogen (including ring), and the protonated nitrogen atoms of the amino groups in the imidazolium rings respectively.65 These values corroborate well with previously reported values for amino acid histidine on Cu substrate.65
Figure 4.24: XPS spectra showing C 1s, N 1s, and O 1s core levels arising from amino acid histidine and SiO$_2$ structures synthesized using 10 mM of histidine.

Compared to pristine histidine, there were significant changes in the core level XPS spectra arising from structures obtained following hydrolysis of TEOS using histidine. Similar to the case of lysine templated silica, the C 1s core level binding energy could be deconvoluted into three major Gaussian components with a lower BE component at ca. 285 eV corresponding to alkyl carbon (C-C, C-H) that showed an increase in relative intensity suggesting the incorporation of IL within the framework. The BE component at ca. 286.5 eV showed a chemical shift of about $\approx 0.5$ eV with an increase in relative intensity in comparison to pristine histidine. This component is generally assigned to amine carbon which shows a shift due to the presence of the electronegative element O from the silica that is in close vicinity to the charged ammonium group. The increase in relative intensity of this component is due to the incorporation of the IL as observed previously in the case of lysine-IL-silica system. The BE component at ca. 287.5 eV showed a shift of $\approx 0.4$ eV which corroborates with the lysine system. The N 1s core level spectra could be deconvoluted to three Gaussian components with the lower BE component and the middle BE component showing similar binding energies as in lysine system. Interestingly, there also exists a higher BE component at 403.2 ±0.1 eV which is quite
intriguing. This component is probably more prominent due to the presence of two nitrogen atoms in the imidazolium groups that are in close proximity to silica. The O 1s and Si 2p Gaussian components are similar to the previous cases of lysine and arginine suggesting a similar mechanism (Figure 4.25). It is evident from the XPS analysis that the mode of mechanism in the case of histidine hydrolysed silica superstructure formation is similar to the previously studied system of lysine and arginine.

![Figure 4.25: XPS spectrum showing Si 2p core level arising from biomimetic SiO$_2$ structures synthesized using 10 mM of histidine.](image)

Similar to the case of lysine and arginine, the XPS analysis of histidine mediated silica materials also did not show boron and fluorine signatures. This was also confirmed using EDX analysis on these silica structures where signatures only from carbon, nitrogen, oxygen, silicon and platinum (samples were coated with platinum to minimize charging) were observed (Figure 4.26) Based on these results it can be assumed that the mechanism observed in the case histidine mediated silica structures is fundamentally similar to that observed in the case of lysine and arginine mediated biomimetic silica structures, wherein the imidazolium group is involved in π-π stacking while the terminal amine group (also an imidazolium group) is involved in TEOS hydrolysis and silica binding.
An important additional feature in the present case of histidine is the dual $\pi-\pi$ stacking/packing possibility between the imidazolium groups of IL as well as those of histidine. To further elucidate the mechanism of the formation of sharp-edged thick triangular plates, infrared spectroscopy was employed to understand the inter- and intra-molecular forces between different molecules. Histidine, in terms of its cationic nature, is quite unique due to the presence of an imidazolium group which can exist in either a protonated or a deprotonated form depending on the pH of the solution. As mentioned earlier, the reaction proceeds under acidic conditions conferring this imidazole group with a positive charge. Figure 4.27 shows the comparison of the IR spectra obtained from neat IL, pristine histidine and IL-histidine templated silica nanoplates. Unlike the previous cationic amino acids, where the conformation of the stacking of imidazolium groups by FTIR was rather a daunting task, in the present case this mechanism is clearly evident by observing the changes in the C-H stretching frequencies from 2800-3200 cm$^{-1}$ that mainly encompass stretching vibrations from both the alkyl chain as well as the ring structures (outlined in Figure 4.27a). A shift in the C-H stretching frequencies towards higher wavenumbers is clearly evident on comparing the His and His-SiO$_2$ spectra especially in the ring stretch regions. Although the alkyl C-H stretching has also shifted towards higher

**Figure 4.26:** EDX analysis of silica structures obtained following hydrolysis of TEOS using cationic amino acid histidine.
wavenumbers, there is significant broadening in this region further confirming the packing of the butyl group from the IL. As outlined in the previous two cationic amino acids, the final silica morphology is driven by the functional group (positively charged in the previous cases) that is exposed to the silica precursor. In the current case, it is evident that the positively charged imidazolium group of histidine is exposed to the silica precursor which was confirmed by the change in ring stretching frequencies at 1400-1650 cm$^{-1}$. More specifically, the frequencies at 1410 (1414), 1570 (1579) and 1585 (1605) in the case of amino acid histidine (in comparison to His-SiO$_2$) have shifted towards more positive wavenumbers due to the presence of electronegative oxygen atoms from silica (Figure 4.27b).

![Figure 4.27: ATR FT-IR spectra (a) showing the overall vibrational modes of TEOS, IL, histidine and histidine-IL mediated silica; (b) showing the expanded region (black box) outlined in (a) for amino acid histidine and histidine-IL-silica system.](image)

In addition to the shifts observed in the stretching vibrations, it is also interesting to note that all peaks pertaining to the ring vibrations are relatively stronger in intensity which is due to the presence of imidazole group towards the outer layers of these silica structures. This furthermore confirms the initial hypothesis of the positive charged group from amino acids being exposed and therefore available for hydrolysis and binding of
silica materials. Additionally, as observed in the previous cases, the vibrations that arise from \(-\text{COOH}\) shows minimal or no shift towards lower wavenumbers suggesting binding of the carboxylate group to the positively charged imidazolium group from the ionic liquid (marked by red star in Figure 4.27b). In earlier studies involving silica, it has been observed that in the case where the IR spectrum shows a broad major peak centering at 1060 cm\(^{-1}\), it is indicative of small chain siloxanes while spectra exhibiting two strong broad bands observed between 1100-1000 cm\(^{-1}\) are indicative of long-chain polysiloxanes.\(^{66}\) The most interesting and fascinating vibrational stretching in the current case is observed in the Si-O-Si\(_{\text{asym}}\) stretching, wherein the His-SiO\(_2\) spectrum shows two strong broad bands in the region between 1100-1000 cm\(^{-1}\), suggesting the presence of long-chain polysiloxanes. This is important considering that in the previous cases when cationic amino acids lysine and arginine were employed for silica precursor hydrolysis it only resulted in a broad spectrum in the silica region indicating the presence of only short chain siloxanes.

![Figure 4.28: TGA profile obtained from cationic amino acid histidine, ionic liquid [BMIM][BF\(_4\)] and amino acid-IL templated silica.](image)

To further confirm the incorporation of IL and histidine within the final template/scaffold, thermogravimetric analysis (TGA) was performed on IL, pristine
histidine and histidine-IL-templated silica structures over a temperature range of 50 - 850 °C under N₂, with a heating rate of 10 °C min⁻¹. Figure 4.28 outlines the TGA curve of pristine histidine that typically reveals three distinct weight loss regions viz. a small but sharp weight loss of 10 % between 150-200 °C, a steady weight loss (50 %) between 250-700 °C and another sharp weight loss of the remaining 40 % between 750 and 850 °C. This weight loss pattern is typical for amino acid histidine and has been reported in previous studies. The weight loss pattern of IL has already been discussed in the case of lysine and typically it degrades completely by 500 °C.

**Figure 4.29**: ATR FT-IR spectra obtained from silica material before and after calcination.

The histidine-IL templated silica structures also undergo thermal degradation as the organic moieties decompose at elevated temperatures. This phenomenon is similar to that observed in the earlier case of lysine and arginine. In comparison to the precursor materials, histidine-IL templated silica showed four distinct weight loss regions. The first minor weight loss of about 3 % between 150-200 °C followed by a sharp decrease of about 10 % in weight between 200-250 °C. This weight loss is similar to that observed in the case of pristine histidine but occurs at temperatures between 150-200 °C. Beyond 250 °C, a progressive weight loss is observed up to 600 °C, followed by a brief stabilization phase. The weight loss at these elevated temperatures can be attributed to the
decomposition of IL and histidine that forms the backbone/scaffold in these silica based structures. The thermal stability observed in these siliceous materials is similar to that observed in the previous amino acids mediated silica structures and can be attributed to the extended Si-O network array and complex ionic interactions between the amino acid, ionic liquid and silica. In total, these silica materials showed a weight loss of 60 % that is significantly lower than observed in the case of lysine (80 %) and arginine (70 %) mediated silica structures. This is most probably because the amino acid histidine itself is stable to about 850 °C conferring more stability to histidine mediated silica structures. The calcined samples were further analysed using FTIR that predominately showed stretching vibrations from silica materials (Figure 4.29). This confirms the important role of the organic components as a template/scaffold during the formation of 3D morphology during biomimetic silicification in ILs.

Scheme 4.5: Schematic illustration of the proposed electrostatic charge matching dual π-π stack templated mechanism during the formation of a complex 3D structure (triangular plates) using amino acid histidine. 

Based on the XPS, TGA and FTIR analysis, an electrostatic charge matching dual π-π stacked templated synthesis mechanism is proposed that may be responsible for controlling biomimetic silica formation in IL using cationic amino acid histidine. A
schematic representation of the proposed mechanism is represented in Scheme 4.5. Unlike cationic amino acids lysine (linear with terminal region containing one amine) and arginine (linear with terminal region containing guanidinium group), the terminal region of histidine contains a positively charged imidazolium group that participates in the hydrolysis of silica precursor as well as forms the stacked template directing the formation of triangular plates. Scheme 4.5 shows the electrostatic charge matching dual π-π stack mechanism where the complex intermolecular ionic interactions between COOH of histidine and BMIM of IL as well as the positively charged nitrogen of the imidazolium group from histidine and silica is clearly evident.

**Scheme 4.6:** A simplified cartoon schematic illustration of the proposed IL-Histidine mediated synthesis of exquisite silica structures.

In order to understand the stacking process that may be responsible for the formation of triangular plates during histidine mediated synthesis of biomimetic silica, the previous molecular schematic can be modified wherein the organic moiety is represented as a pentagon (the shape of imidazole) and silica is represented as a flat disc instead of the spheres as in the previous two cationic amino acids (lysine and arginine). Scheme 4.6 clearly shows the stacking process that is responsible for the formation of these triangular plates.
The results observed in the case of cationic amino acids lysine, arginine and histidine points toward the important role of these positively charged biomolecules in the biosilicification process. More importantly, it outlines the important role of physico-chemical factors during the biosilicification process and provides some understanding of how different cationic amino acids in IL lead to silica superstructures similar to those observed in diatom species in their natural marine habitats.

Interestingly, cationic amino acids (lysine, arginine and histidine) could not promote TEOS hydrolysis but polyamines could promote TEOS when water was used as a solvent in previous studies. Therefore, it appears that the conformation and orientation of cationically charged amino acids in IL (close packing) mimics the polyamines system and promotes silica hydrolysis in IL. It is well established that in diatoms, the amorphous hybrid bioinorganic structures are formed as a result of cationic polypeptides providing a facile template as well as playing the biocatalyst role for silicic acid hydrolysis. In the current study using cationic amino acids in ILs, a similar templating mechanism is observed confirming the similarities between the natural biosilicification and biomimetic silicification process. Moreover the structure directing ability of these cationic amino acids is attributed to the exposed terminal cationic amine that plays an important role of TEOS hydrolysis/framework for silica binding.

In conclusion, this study demonstrates the potential of cationic amino acids for controlling silica morphologies via the control of the conformation of the biomacromolecules as templates at the molecular level induced by the physico-chemical environment.

4.4 The Case of Other Amino Acids

The earlier sections established the role of cationic amino acids in attaining complex and unique 3D silica structures using an ionic liquid as a designer solvent, and more importantly determine the important role of the physico-chemical environment and diffusion-limited aggregation processes during biomimetic silicification. It is worth noting that the biomacromolecules associated with the biosilicification process in marine organisms (silaffins, silicateins, and other cationic peptides) have a range of amino acids in different concentrations that may play a significant role in the formation of complex
morphologies. Hence, to determine the role of these biomacromolecules, it is of utmost importance to methodically study the effect of each individual amino acid during biomimetic silicification. The current section outlines a systematic model to explore the role of all amino acids as catalyst/template/scaffold for biomimetic silicification when using IL as a unique solvent. For the ease of understanding the role amino acids, they are grouped into different categories based on their functional groups.

The following section is divided into the following five main categories:

1. **Aliphatic amino acids** – that encompass amino acids alanine, glycine, isoleucine, leucine, proline and valine
2. **The amino acids** – that encompass amino acids glutamine, glutamic acid, asparagine and aspartic acid
3. **Amino acids containing an alcohol group** – that encompass serine and threonine
4. **Sulfur containing amino acids** – that encompass amino acids cysteine and methionine
5. **Aromatic amino acids** – that encompass amino acids phenylalanine, tyrosine and tryptophan

### 4.4.1 Aliphatic Amino Acids

![Figure 4.30: ‘Ball and Stick’ representation of aliphatic amino acids.](image)
As the name suggests, this group encompasses amino acids where the R groups only contain an aliphatic chain (carbon and hydrogen) with the structure of glycine displaying the simplest R group in the form of a hydrogen atom. Most amino acids in this category are generally hydrophobic, and their hydrophobic nature increases with the increase in the number of carbon atoms (Figure 4.30). Of the amino acids in this category, only proline is significantly different as the side chain is bonded to both α–carbon and the amino group rendering it very important in proteins.

Figure 4.31: SEM images of silica structures synthesized using aliphatic amino acids (a and b) alanine, (c and d) glycine, (e and f) isoleucine, (g and h) leucine, (i and j) proline, and (k and l) valine in IL [BMIM][BF$_4$] involving TEOS to amino acid molar ratio of 1:10. The insets show the higher magnification images of the structures shown in the corresponding main figures.

TEOS hydrolysis in [BMIM][BF$_4$] by using relatively large quantities of aliphatic amino acids (10 mM – 1:10 TEOS to amino acid ratio) also resulted in a control over silica morphology, however it led to a completely different set of unique silica structures. Of the different amino acids, only alanine, glycine and isoleucine showed the ability to control morphology at a higher amino acid concentration. Amino acid alanine typically
contained rod like structures of 1-5 µm length and about ca. 10-60 nm in width (Figure 4.31a-4.31b). On close observation, these rod-like structures were found to be extremely flat and usually intertwined, giving these structures fungus hyphae like appearance (inset in Figure 4.31b). Glycine and isoleucine predominantly contained particles exhibiting a quasi-spherical morphology that are typically ca. 500 nm and 50 nm respectively (Figure 4.31c-4.31f). In addition to the spherical morphology, glycine also shows formation of long brush like structures (Figure 4.31c), while in the case of isoleucine these spherical particles cluster together to give a pseudo plate-like appearance (Figure 4.31e). On the other hand, amino acids leucine, proline and valine were only able to promote silica hydrolysis to form quasi-spherical morphology typically in the range of 30-50 nm (fused), 500-700 nm, and 50-100 nm (fused) respectively (Figure 4.31g-4.31l).

**Figure 4.32:** SEM images of silica structures synthesized using aliphatic amino acids (a) alanine, (b) glycine, (c) isoleucine, (d) leucine, (e) proline, and (f) valine in IL [BMIM][BF₄] involving TEOS to amino acid molar ratio of 1:1. The insets show the higher magnification images of the structures shown in the corresponding main figures.

Decreasing the amino acid concentration to 1 mM while maintaining the ratio of TEOS to amino acid molecules as 1:1, resulted in loss of morphological control, an observation that is very similar to the previous cases of cationic amino acids. At lower concentrations, most amino acids exhibit a spherical morphology typically agglomerated
with a size range of ca. 50-100 nm that do not self-assemble to form complex morphologies (Figure 4.32). Interestingly, alanine still exhibits a flat intertwined morphology as observed in the previous case of high amino acid concentration with the same amino acid (Figure 4.31a and Figure 4.32a).

![SEM images of silica structures synthesized using aliphatic amino acids](image)

**Figure 4.33**: SEM images of silica structures synthesized using aliphatic amino acids (a) alanine, (b) glycine, (c) isoleucine, (d) leucine, (e) proline, and (f) valine in IL [BMIM][BF₄] involving TEOS to amino acid molar ratio of 1:0.2. The insets show the higher magnification images of the structures shown in the corresponding main figures.

A further shift in TEOS to amino acid ratio to 1:0.2 results in complete loss of morphological control and mostly leading to assembled silica spheres of diameter typically in the range of ca. 300-400 nm (Figure 4.33). Interestingly, of all the amino acids only leucine showed a significant control over morphology with flat spheres of ca. 300 nm leading to the formation of flat surfaces. Interestingly, some of the amino acids used in the current study were employed previously under aqueous conditions and only hydrolyzed TMOS to form particulate silica with no morphological control. These observations point towards the important role of ILs in regulating the morphology of biomimetic silica by means of controlling the physicochemical environment via self-assembly process.
4.4.2 The Amino Acids

Figure 4.34: ‘Ball and Stick’ representation of the amino acids.

Amino acids under this specific category are quite peculiar in that the R group in the acid forms contains a carboxylic group (aspartic acid and glutamic acid) in contrast to a carboxamide group in the case of asparagine and glutamine. In addition to the R group, the other major difference between Asp/Asn and Glu/Gln is the presence of an extra carbon atom in the alkyl chain of the R group (Figure 4.34). Hence these amino acids have been placed in a special group and are referred to as ‘The amino acids’ in the current body of work.

TEOS hydrolysis in [BMIM][BF₄] by using relatively large quantities of amino acids (10 mM – 1:10 TEOS to amino acid ratio) resulted in a control over silica morphology, however it led to a completely different set of unique silica structures. The acid forms of these amino acids showed significant ability to control the silica morphology and typically revealed thin flake-like morphologies (Figure 4.35). These flake-like structures in the case of glutamic acid were typically about 30 nm less in thickness than those observed in the case of aspartic acid mediated hydrolysis. The overall length in the case of aspartic acid was also significantly longer (approximately by 40 µm). Interestingly, these flakes in the case of aspartic acid are arranged in a layered structure rendering them in a hierarchical
manner (Figure 4.35g-4.35h), while no such ordering is observed in the case of glutamic acid. In the case where the R group contains a carboxamide group also results in control over biomimetic silica morphology and results in a unique set of structures viz. spherical, plate-like and rod-shaped morphology (Figure 4.35a-4.35b; 4.35e-4.35f). In contrast to the structures observed in the previous carboxylic containing R group, morphologies yielded by glutamine are significantly larger in dimensions than asparagine. This is evident when a close analysis on the spherical morphology observed in the case of glutamine was shown to be approximately 200 nm larger in comparison to asparagine (glutamine spheres were 300 nm in diameter).

**Figure 4.35**: SEM images of silica structures synthesized using amino acids (a and b) asparagine, (c and d) aspartic acid, (e and f) glutamine, (g and h) glutamic acid in IL [BMIM][BF₄] involving TEOS to amino acid molar ratio of 1:10. The insets show the higher magnification images of the structures shown in the corresponding main figures.

The second set of morphologies observed in both the carboxamide group containing amino acids were significantly different wherein asparagine showed plate-like morphologies that were typically 10-50 µm in length and 300 nm thick, while glutamine showed rod-like morphologies that assembled to form a flower like appearance. A close observation of some of the rods broken from the flower like structures revealed that these structures were typically 100 µm long and about 300 nm thick resembling the rods observed in the case of cationic amino acid lysine. However there was a stark difference
in the width of these rods synthesized by lysine and glutamine wherein the later exhibited a width of 3 µm in comparison to < 1 µm width seen in the former cationic amino acid.

**Figure 4.36**: SEM images of silica structures synthesized using amino acids (a and b) asparagine, (c and d) aspartic acid, (e and f) glutamine, (g and h) glutamic acid in IL [BMIM][BF₄] involving TEOS to amino acid molar ratio of 1:1. The insets show the higher magnification images of the structures shown in the corresponding main figures.

To further investigate the role of these amino acids in the formation of complex morphologies, the amino acid concentration was reduced to 1 mM while maintaining the ration of TEOS to amino acid molecules as 1:1 and 1:0.2 respectively. It is evident from Figure 4.36 that reducing the concentration of amino acids leads to complete loss of morphological control, thereby mostly leading to silica spheres of ca. 30-50 nm diameter (Figure 4.36).
Further reducing the concentration of amino acids with TEOS to amino acid ratio of 1:0.2 resulted in a spherical morphology (Figure 4.37) as observed in the previous 1:1 ratio of TEOS to amino acid. Although the overall morphology was similar to the previous ratio, the size dimensions were considerably larger with amino acids asparagine and aspartic acid exhibiting a diameter of 400-500 nm. Though glutamine showed spherical particles in a similar size range of 400-500 nm, it also shows complex rod shaped morphologies similar to those observed in the case of high amino acid concentration. The most fascinating observation was in the case of glutamic acid wherein these spherical particles actually consist of extremely flat discs. It is intriguing and fascinating to observe these complex morphologies in the current biomimetic silica systems as when the same amino acids were employed for the hydrolysis of TMOS in water, it only resulted in particulate silica with minimal control over morphology. Notably, these amino acids were unable to hydrolyse TEOS (the precursor used in the current study) when used in water. These observations further point us towards the important role of ILs in biomimetics especially in understanding the biosilicification process in nature.
4.4.3 Amino Acids Containing an Alcohol Group

![Figure 4.38: 'Ball and Stick' representation of amino acids containing alcohol group.](image)

The amino acids serine and threonine contain a short group ending with a hydroxyl group and its important role in proteins involved in biosilicification processes has been realized wherein hydroxyl-rich polysaccharides in addition to cationic polypeptides are postulated to accelerate silica polymerization (Figure 4.38). Another important observation from previous molecular studies on silicatein points towards the important role of serine in the active site of Cathepsin L-like protein.

![Figure 4.39: SEM images of silica structures synthesized using amino acids (a and b) serine, (c and d) threonine in IL [BMIM][BF₄] involving TEOS to amino acid molar ratio of 1:10. The insets show the higher magnification images of the structures shown in the corresponding main figures.](image)
The two hydroxyl group containing amino acids were initially employed for TEOS hydrolysis in [BMIM][BF₄] where a relatively large quantity of amino acids were used (10 mM – 1:10 TEOS to amino acid ratio) and resulted in particles that predominately showed spherical morphologies (Figure 4.39). On close observation, the spherical particles in the case of threonine mediated hydrolysis were about ca. 100 nm in diameter, while serine mediated hydrolysis yielded larger particles with a size range of 500 – 1000 nm in diameter (Figure 4.39).

![SEM images of silica structures synthesized using amino acids](image)

**Figure 4.40:** SEM images of silica structures synthesized using amino acids (a and c) serine, (b and d) threonine in IL [BMIM][BF₄] involving TEOS to amino acid molar ratio of 1:1 and 1:0.2 respectively. The insets show the higher magnification images of the structures shown in the corresponding main figures.

When the amino acid concentration was reduced, while maintaining the ratio of TEOS to amino acid molecules at 1:1, the morphology exhibited by biomimetic silica was still spherical but reduced in overall size range (Figure 4.40a-4.40b). Serine exhibited particles with an average diameter of 70 nm (Figure 4.40a) while threonine exhibited particles typically 10-30 nm in diameter (Figure 4.40b). Further reduction in the amino acid concentration resulted in agglomerated spherical particles of ca. 300 nm diameter (Figure 4.40c-4.40d).
4.4.4 Sulfur Containing Amino Acids

![Cysteine and Methionine](image)

**Figure 4.41:** ‘Ball and Stick’ representation of amino acids containing sulfur group.

Amino acids cysteine and methionine are probably the most unique of all amino acids due to the presence of sulfur in addition to the commonly found carbon, hydrogen, nitrogen and oxygen (Figure 4.41). These amino acids are generally nonpolar and hydrophobic in nature with methionine being one of the most hydrophobic amino acids and commonly found in the interior of proteins, while cysteine on the other hand does ionize to form thiolate ion. Cysteine also plays an important role in ensuring the correct protein folding due to its ability to form a disulfide bond.\(^6^9\)

Similar to the previous cases, the two sulfur containing amino acids were initially employed for TEOS hydrolysis in [BMIM][BF4] where a relatively large quantity of amino acids were used (10 mM – 1:10 TEOS to amino acid ratio) and resulted in particles that predominately showed spherical morphology. On close observation, these spherical particles were about ca. 50 nm in diameter (Figure 4.42).
Figure 4.42: SEM images of silica structures synthesized using sulfur containing amino acids (a and b) cysteine, (c and d) methionine in IL [BMIM][BF₄] involving TEOS to amino acid molar ratio of 1:10. The insets show the higher magnification images of the structures shown in the corresponding main figures.

On decreasing the amino acid concentration to 1 mM while maintaining the ratio of TEOS to amino acid molecules at 1:1, resulted in spherical morphologies similar to those observed in the case of high amino acid concentrations (Figure 4.43a-4.43b). These spherical particles were typically agglomerated with a diameter of ca. 50 nm. Although cysteine did not yield complex morphologies, the spherical particles in the case of methionine were arranged in a flat, organised appearance (Figure 4.43b).
Figure 4.43: SEM images of silica structures synthesized using sulfur containing amino acids (a and c) cysteine, (b and d) methionine in IL [BMIM][BF₄] involving TEOS to amino acid molar ratio of 1:1 and 1:0.2 respectively. The insets show the higher magnification images of the structures shown in the corresponding main figures.

A further shift in TEOS to amino acid ratio to 1:0.2 results in silica spheres of diameter typically in the range of ca. 300-500 nm (Figure 4.43c-4.43d). In addition to spherical particles, an interesting morphological control was observed in the case of cysteine that resulted in the fusion of these spherical particles to form rod-like morphologies (Figure 4.43c inset). In a previous study wherein oligomers of L-cysteine were employed for silica synthesis, it was suggested that the sulfhydryl group may have the ability to initiate hydrolysis of TEOS at physiological pH. Additionally, due to the ability of cysteine groups to form disulfide crosslinks, the biomimetic silica showed packed columns. In the current study, we can conclude that a similar mechanism might promote the hydrolysis process but instead of the block copolypeptides, the additional effect of the physiochemical environment regulates the morphology of biomimetic silica by means of self-assembly processes.
### 4.4.5 Aromatic Amino Acids

![Aromatic Amino Acids](image)

*Figure 4.44: ‘Ball and Stick’ representation of amino acids containing an aromatic side chains.*

As the name suggests, the three amino acids incorporated in this category contain an aromatic R group viz. phenylalanine (benzyl group), tyrosine (phenol group) and tryptophan (indole group). Similar to the observations in the case of cationic amino acid histidine, the amino acids pertaining to this category also have the ability to promote stacked orientations through the π electrons of the aromatic ring (Figure 4.44).

When relatively large quantities of aromatic amino acids were employed for TEOS hydrolysis in [BMIM][BF₄] (10 mM – 1:10 TEOS to amino acid ratio), control over silica morphology resulted, however it led to a completely different set of unique silica structures. The benzyl R group containing phenylalanine showed significant ability to control the silica morphology and typically revealed thin flake/plate-like morphologies (Figure 4.45a-4.45b). These plate like structures were typically about 30-50 µm in diameter with close observation revealing an average thickness of 300 nm. Although such plate like morphology was observed in some of the previous amino acids (aspartic acid and glutamic acid), the plates observed in the case of phenylalanine were significantly larger in both diameter and thickness dimension. The modification of the R group from benzyl to phenol (tyrosine) provides significant control over the biomimetic silica morphology and results in a distinctive set of structures. At high amino acid concentration, tyrosine revealed a rod-like morphology that was typically intertwined, similar to that seen in a fungal hyphae network (Figure 4.45c-4.45d). This control over morphology is both exciting and intriguing as a similar set of structures were also seen in
the case of alanine (Figure 4.31a-4.31b), an amino acid that does not contain a phenolic ring in its structure. Although the rods synthesized using alanine were typically of 1-5 µm length and about ca. 10-60 nm in width (Figure 4.31a-4.31b), the rods in the case of tyrosine were much larger in size and were typically of 3-5 µm length and about ca. 50-100 nm in width (Figure 4.45c-4.45d).

Figure 4.45: SEM images of silica structures synthesized using aromatic ring containing amino acids (a and b) phenylalanine, (c and d) tyrosine, and (e and f) tryptophan in IL [BMIM][BF₄] involving TEOS to amino acid molar ratio of 1:10. The insets show the higher magnification images of the structures shown in the corresponding main figures.

Similarly when an amino acid with R group containing an indole group (six-membered benzene ring fused to five-membered nitrogen containing pyrrole ring) is employed for hydrolysis of TEOS, it also resulted in control over biomimetic silica, predominately displaying rod shaped morphology (Figure 4.45e-4.45f). These nanorods were about 200-300 µm long and approximately 500 nm thick but did not form microdiscs via the assembly of individual nanorods, as previously observed in the case of cationic
amino acid lysine. On close observation, in addition to the rod shaped morphology, a few spherical particles of 100 nm diameter were also observed. It is unclear at this time if these spherical particles act as a precursor in the formation of rod morphology.

**Figure 4.46**: SEM images of silica structures synthesized using aromatic ring containing amino acids (a and d) phenylalanine, (b and e) tyrosine, and (c and f) tryptophan in IL [BMIM][BF₄] involving TEOS to amino acid molar ratio of 1:1 and 1:0.2 respectively. The insets show the higher magnification images of the structures shown in the corresponding main figures.

As performed in the previous cases, to further investigate the role of these aromatic amino acids towards the formation of complex morphologies, the amino acid concentration was reduced to 1 mM while maintaining the ratio of TEOS to amino acid molecules at 1:1 and 1:0.2 respectively. Evidently, it can be inferred from Figure 4.46a-4.46c that reducing the ratio of amino acids to TEOS to 1:1 leads to some loss of morphological control, with phenylalanine and tryptophan leading to silica spheres of ca. 30-60 nm diameter (Figure 4.46a-4.46c), while tyrosine leads to plate like structures that also comprise 30 nm spherical particles (Figure 4.46b). Further reducing the amino acid concentration leads to larger agglomerated spherical particles typically of 300 nm diameter with amino acid tyrosine also displaying an underlying long rod-brush like
morphology (Figure 4.46d-4.46f). These observations points towards the important role of aromatic amino acid in controlling the biomimetic silica morphology.

4.5 Summary

The current study outlines the important role of physico-chemical factors in biosilicification processes and provides insight into how different amino acids in IL lead to silica superstructures similar to those formed by different diatom species in their marine habitats. It is well known that in diatoms where amorphous hybrid bioinorganic structures are produced, cationic polypeptides play a major role by way of providing a facile template as well as playing the biocatalyst role for silicic acid hydrolysis. It has also been established that the conformation and orientation of cationically charged macromolecules in an amino acid matrix formed due to supersaturation and ionicity of the reaction can act as templates for the nucleation and growth of silica structures. Also, parameters such as pH, temperature, precursor salt, external ionic strength are known to have significant effects on rate of reaction, oligomerisation of silica, and also the formation of silica structures. The studies outlining the role of different amino acids at variable concentrations in controlling silica morphologies, especially the effectiveness of morphological control in cationic amino acids, correlates well with natural biosilicification processes. In this study, amino acid solutions used at higher concentrations (10 mM) are close to high degree of super saturation, and the presence of a viscous solvent such as an ionic liquid leads to ordered and controlled aggregation of amino acids in induced self-organization. This route has been shown to produce complex and hierarchical structures following a diffusion limited aggregation (DLA) process, which is also believed to occur during diatomaceous silicification.

In addition to the amino acids, the self-assembly is also promoted by the π-π stack interaction between imidazolium groups of the IL. The electrostatic charge matching between the IL (cationic imidazolium) and terminal groups of amino acids (anionic carboxylic group) and the exposed terminal positive charge mainly promotes the structure direction. This process is confirmed in the case of cationic amino acids where the different terminal protonated amine groups govern and provide morphological control over silica structures.
Additionally, the specificity of amino acids towards attaining unique silica morphologies in this study also suggests that the diversity in biosilica morphology seen in different diatom species might possibly be due to the compositional differences in cationic polypeptides responsible for biosilification, wherein amino acids have been found to be present in different proportions. Moreover, the complex interplay between the organic and inorganic component observed in the current biomimetic silica morphologies also points towards the important role of the organic component in the formation of the underlying scaffold/template required to attain these morphologies.

Nevertheless, the formation of exquisite diatom-like structures by amino acids-mediated biomimetic silicification in ionic liquids is both intriguing and exciting, as it may lay the foundation of significant new future insights on biosilicification processes occurring in natural habitats.

4.6 References

(8) Kirchner, B.; Clare, B. Ionic Liquids; Springer: Hidelberg, Germany, 2009.


This chapter details the significant features of the work presented in this thesis and emphasizes on potential avenues for future endeavours.
5.1 Summary of accomplished work

A promising avenue of research in materials science is to follow the strategies used by Mother Nature to fabricate ornate hierarchical structures as exemplified by organisms such as diatoms, sponges and magnetotactic bacteria. For many ages, organisms have been engaged in on-the-job testing to craft structural and functional inorganic materials and have evolved extensively to create the best-known materials. Some of the strategies used by nature may well have practical implications in the world of nanomaterials. Therefore, the effort towards exploring nature’s ingenious work in designing strategies to create inorganic nanomaterials in our laboratories has led to development of biological synthesis that mainly encompasses biosynthesis and biomimetic routes. In this thesis, a niche exploring the important and critical role of physico-chemical environment in controlling particles morphology using biological synthesis (biosynthesis and biomimetics) of nanomaterials has been discussed.

The importance of bacteria as important biological organisms for synthesis of nanomaterials has been established but this thesis additionally addresses an important aspect towards controlling the shape of nanoparticles using bacterial synthesis. The ability of bacterium *Morganella psychrotolerans* to control the nanoparticle (silver) shape anisotropy by controlling the bacterial growth kinetics was established. This is particularly important as there have been no or minimal efforts to deliberately control the shape of silver nanoparticles. The underlying principle (mechanism) of nanoparticle synthesis that was initially believed to be extracellular was also challenged. Using electrochemical techniques, it was further shown that the nanoparticle synthesis process is not as simple as previously predicted, but in fact involves a complex process that involves the bacterial cell machinery. This study truly allows the use of the term ‘bacterial nano-factories’ for biosynthesis of nanomaterials. Additionally, some fundamental understanding of the silver resistance allowed for a silver resistant bacterium (*Morganella morganii* RP42) to synthesize water stable copper nanoparticles. This is both surprising and intriguing as the link between bacterial heavy metal resistance and nanoparticles synthesis will open up new exciting avenues for large-scale eco-friendly (shape-controlled and difficult to synthesize) synthesis of nanomaterials.
For over the past few decades, the formation of 3-dimensional ornate structures via biosilicification process has intrigued scientists. Although this process has been extensively studied with the proposed biochemical factors (biomolecules) isolated, the role of these molecules in the formation of 3D structures is hitherto a mystery. In the current work, the additional role of physico-chemical environment in controlling biosilica morphology has been discussed. Replacing conventional aqueous solvents with ionic liquids that closely mimic the natural environment where biosilicification occurs resulted in the formation of ornate morphologies during biomimetic biosilicification process. Potentially, this study for the first time explains the formation of complex morphology that exhibits a complex interplay between organic molecules and inorganic material similar to that found in Nature. It takes us one step closer and lays the foundation for the future studies involved in understanding the formation of hierarchical biosilica structures. Although, each amino acid showed specificity towards attaining unique silica structures, cationic amino acids showed significant role in controlling the biomimetic silica morphology. This corroborates well with previous biosilicification studies wherein cationic proteins/peptides were postulated to be a major driving force for biosilica formation. Moreover, the specificity of each amino acid towards attaining unique silica structures also explains the diversity observed in diatom morphologies. In a nutshell, this study can be expressed as ‘Simple Chemistry Meets Complex Supramolecular Chemistry to Elucidate a Subtle Biological Process.’

5.2 Potential for future work

From a scientific point of view, the potential of biological synthesis routes is yet to realize its full potential and is limited only by our imagination. Although a large proportion of efforts have explored the use of microorganisms under conditions similar to that encountered in nature, this field is still in its infancy. With the realization of the important role of physico-chemical environment on materials synthesis using a biological approach, it is now possible to expand this avenue to greater heights and achieve feats that are difficult via conventional synthesis routes.

The potential of bacteria in synthesis of nanomaterials is not unheard of. But the ability to control the morphology of nanoparticles by controlling the growth kinetics is a
significant step away from the common ‘hit or miss’ strategy employed during biosynthesis. A more in-depth understanding of the role of physico-chemical environment on bacterial systems might provide important insights in controlling mineral morphologies. This route might, in the future, be able to compete with traditional nanoparticles synthesis methods, and further enable large-scale ‘green’ biosynthesis of metal and metal oxide nanomaterials. Additionally, as outlined in the chapters of this thesis, a significant understanding of metal ion resistance mechanisms that are suggested to play an important role during the reduction of metal ions can also outline the exciting possibilities in using bacteria as ‘nano-factories’ for continuous synthesis of nanomaterials. Furthermore, with significant understanding of the biochemical processes involved in metal ion resistance mechanisms can help extrapolate fabrication strategies towards the synthesis of other technologically important metal nanomaterials.

The biomimetic silicification reactions explored in ionic liquids have as yet been restricted to a single ionic liquid ([BMIM][BF$_4$]). With the considerable number of ionic liquids being manufactured in readily available quantities, it seems logical that these reactions described herein can be extended to ionic liquids with similar structures, yet different properties (for example N-methyl-N-alkylpyrrolidinium based ILs) or ILs with similar properties yet vastly different structures. By using different ionic liquids to perform biomimetic silicification reactions, perhaps a much deeper understanding of the mechanisms through which different ionic liquids interact with different charged molecules (amino acids) can be obtained. This would significantly change the underlying template leading to unique 3D morphologies. Moreover, experiments using combination of different amino acids would provide significant understanding on their role to self-assemble in IL that could potentially lead to deeper understanding of the morphological control in natural biosilicification process. Additionally, using more complex biomacromolecules viz. polyamines, poly-amino acids, peptides and proteins would provide deeper understanding of the underlying principles governing morphological control, thereby increasing our knowledge of the natural biosilicification process. This morphological control to obtain ornate and hierarchical 3D organic-inorganic hybrid superstructures can also be extrapolated to other commonly occurring biominerals thereby increasing the potential applications of nanomaterials.
The field of biological synthesis is only constrained by our ability to explore the
many secrets of Nature. In a nutshell, a quote that can appropriately concise this field
‘Look deep into nature, and then you will understand everything better’ – Albert Einstein.
Appendices
Appendix A

Author’s Achievements

Cover Page article in Australian Journal of Chemistry (2011)
Refereed Journal Articles


Refereed Review Articles


Peer Reviewed Conference full articles


Peer Reviewed Extended Conference Abstracts


Manuscripts under preparation


22. Ramanathan, R.; Bhargava, S. K.; Bansal, V. “Towards understanding the role of amino acids in the formation of 3-D ornate silica structures during biomimetic silification: A new emergent paradigm.”


24. Ramanathan, R.; Selvakannan, P. R.; Bhargava, S. K.; Bansal, V. “Rod shaped multiple co-doped (C, N, F) titania for photocatalysis applications.”


27. Ramanathan, R.; Selvakannan, P. R.; Bhargava, S. K.; Bansal, V. “Biomimetic silification using cationic amino acids in ionic liquids – Understanding the complex interplay between organic-inorganic moieties.”


Student Awards

1. **3 Minute Thesis Competition Winner** (School of Applied Sciences Level)

2. **Higher Degree by Research Publication Award** (2012) to support publish research findings

3. The **Centre for Advanced Materials and Industrial Chemistry High Achievement Award** (2011) for being the most outstanding student for the year 2011.
4. The **Royal Australian Chemical Institute Graduate Student award** (2011) for travelling to University of Waterloo, Ontario, Canada.

5. The **Pro Vice Chancellor Research and Innovation Postgraduate Research International Conference award** (2011) – towards attending the 2\textsuperscript{nd} International Conference on Nanotechnology: Fundamentals and Applications held in Ottawa, Canada.

6. The **Pro Vice Chancellor Research and Innovation Postgraduate Research International Conference award** (2010) – towards attending the 3\textsuperscript{rd} NanoBio conference held in Zurich, Switzerland.


8. The **CNR Rao Postgraduate Research Excellence award** (2010) for being the most outstanding postgraduate research student working in the field of Materials Sciences.

**Student supervision**

During the research candidature, the author was also involved in the supervision of two work experience students from final year TAFE.
### Appendix B:

**Table B.1: The species of bacteria that have so far been reported for the synthesis of metal or metalloid nanoparticles (classified using Bergey’s Manual version 4)**

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Appendix C:

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<th>CusA Escherichia coli</th>
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<tr>
<td>MIEWIIRSSVANRLVMMGALPLSIWGTWTTIINTPVDALPDLSDVQVIK</td>
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<td>100</td>
</tr>
<tr>
<td>TSYPQAPQIVENQVTYPLTTTMLSVPGAKTVRFSQFGDSYVYVIFBDG</td>
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<td>TDPYWARSRVLEYLNQVQKLPAGVSSEIQGDPATGVWIFEBYGALVLVRSGK</td>
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Figure C.1: Comparison of amino acid sequence of SilA from Morganella morganii RP42 to that of CusA from Escherichia coli.
Figure C.2: Comparison of amino acid sequence of SilB from Morganella morganii RP42 to that of CusB from Escherichia coli.
<table>
<thead>
<tr>
<th>SilC Morganella morganii RP-42</th>
<th>MPKLKLLL SITIFILAGCVSLAPEYQRPAAPVPQQFSLRNLSLTAPVNGY 50</th>
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</thead>
<tbody>
<tr>
<td>CusC Escherichia coli</td>
<td>MPKLKLLL SITIFILAGCVSLAPEYQRPAAPVPQQFSLRNLSLTAPVNGY 50</td>
</tr>
<tr>
<td>------------------------------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>SilC Morganella morganii RP-42</td>
<td>QDTCGRNPFFVDQVTRLI TALTNRRDLRMAAALKVEEARRQPNVTADRYY 100</td>
</tr>
<tr>
<td>CusC Escherichia coli</td>
<td>QDTCGRNPFFVDQVTRLI TALTNRRDLRMAAALKVEEARRQPNVTADRYY 100</td>
</tr>
<tr>
<td>------------------------------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>SilC Morganella morganii RP-42</td>
<td>PQLNASGGTITSGGLKDGPKNPTTEQYDAGLEFYSYELDFGGKLKNMSDADROQ 150</td>
</tr>
<tr>
<td>CusC Escherichia coli</td>
<td>PQLNASGGTITSGGLKDGPKNPTTEQYDAGLEFYSYELDFGGKLKNMSDADROQ 150</td>
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<td>------------------------------</td>
<td>-------------------------------------------------------</td>
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<tr>
<td>SilC Morganella morganii RP-42</td>
<td>NYFASEHUARRAVRILLVSVSVSFSFQQQLAYEQQRIARTLTKNYQQSYAF 200</td>
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<td>CusC Escherichia coli</td>
<td>NYFASEHUARRAVRILLVSVSVSFSFQQQLAYEQQRIARTLTKNYQQSYAF 200</td>
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<td>------------------------------</td>
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<tr>
<td>SilC Morganella morganii RP-42</td>
<td>YRQVLGVSTLNGALQARSESHRIAKRGRDLALHANNQLQLGLGTYT 250</td>
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<tr>
<td>CusC Escherichia coli</td>
<td>YRQVLGVSTLNGALQARSESHRIAKRGRDLALHANNQLQLGLGTYT 250</td>
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<tr>
<td>SilC Morganella morganii RP-42</td>
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<tr>
<td>CusC Escherichia coli</td>
<td>RALPSEKGMA--------------------------------------------------------------------------------------------------- 259</td>
</tr>
<tr>
<td>------------------------------</td>
<td>-------------------------------------------------------</td>
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<tr>
<td>SilC Morganella morganii RP-42</td>
<td>ANKLAEBRQQQSVNQIESQASFDGSDTDLRLRDLSSQQCQSQQRNYD 350</td>
</tr>
<tr>
<td>CusC Escherichia coli</td>
<td>ANKLAEBRQQQSVNQIESQASFDGSDTDLRLRDLSSQQCQSQQRNYD 350</td>
</tr>
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<td>------------------------------</td>
<td>-------------------------------------------------------</td>
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<tr>
<td>SilC Morganella morganii RP-42</td>
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</tr>
<tr>
<td>CusC Escherichia coli</td>
<td>SIQTLQRARGLYASGAVYIEVLDAERSLFLATQTMQLRDLYTSRQVNEIN 450</td>
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<tr>
<td>------------------------------</td>
<td>-------------------------------------------------------</td>
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<tr>
<td>SilC Morganella morganii RP-42</td>
<td>LPTALGGGWE 461</td>
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<tr>
<td>CusC Escherichia coli</td>
<td>LPTALGGGWE 461</td>
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**Figure C.3:** Comparison of amino acid sequence of SilC from Morganella morganii RP42 to that of CusC from Escherichia coli.
**Figure C.4:** Comparison of amino acid sequence of SilE from Morganella morganii RP42 to that of SilE from Escherichia coli.
<table>
<thead>
<tr>
<th>SilP Morganella morganii RP-42</th>
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</thead>
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<tr>
<td><strong>MKNDNAVQHHNNTASQTSQPDEGHLVRLKVRDPVCMAIPDRAHSS</strong></td>
<td><strong>MKNDNAVQHHNNTASQTSQPDEGHLVHLKVRDPVCMAIPDRAHSS</strong></td>
</tr>
<tr>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td><strong>QDHQLYFCSASCESKFKAHPDRLTEDEASHHSHHHHDDHEVSPOI</strong></td>
<td><strong>QDHQLYFCSASCESKFKAHPDRLTEDEASHHSHHHHDDHEVSPOI</strong></td>
</tr>
<tr>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>HHQAEKENSEGWMHPREIRSGPSCGCPVCMAMEPLVA</strong></td>
<td><strong>HHQAEKENSEGWMHPREIRSGPSCGCPVCMAMEPLVA</strong></td>
</tr>
<tr>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td><strong>LHDMTRFWRGLVLLAPVFPLVDLPE</strong></td>
<td><strong>LHDMTRFWRGLVLLAPVFPLVDLPE</strong></td>
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<tr>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td><strong>VVLWCGWFFARAGMSLNRSLNMFT</strong></td>
<td><strong>VVLWCGWFFARAGMSLNRSLNMFT</strong></td>
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<tr>
<td>227</td>
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<tr>
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<td>300</td>
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**Figure C.5:** Comparison of amino acid sequence of SilP from Morganella morganii RP42 to that of P-type ATPases from Escherichia coli.
<table>
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<th><strong>SiR Morganella morganii RP-42</strong></th>
<th><strong>CusR Escherichia coli</strong></th>
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<tr>
<td>MKILIVEDEIKTGELSKGLTEAGFVVDHADNGLTGYHLAMTAEYDLVIL 50</td>
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</tr>
<tr>
<td><strong>SiR Morganella morganii RP-42</strong></td>
<td><strong>CusR Escherichia coli</strong></td>
</tr>
<tr>
<td>DIMLPDVNGWDIIRMLRSAKGMPVLLLTLALTIEHRVKGLELGADDYLV 100</td>
<td>DIMLPDVNGWDIVRMLRSAKGMPIILLLTLALTIEHRVKGLELGADDYLV 100</td>
</tr>
<tr>
<td>********* <strong><strong><strong><strong><strong><strong><strong><strong><strong><strong><strong>.</strong></strong></strong></strong></strong></strong></strong></strong></strong></strong></strong>**</td>
<td></td>
</tr>
<tr>
<td><strong>SiR Morganella morganii RP-42</strong></td>
<td><strong>CusR Escherichia coli</strong></td>
</tr>
<tr>
<td>KPFAPAELLARVRTLRRGNTMTESDLQVADLQDVLQSRKVSRAGNIV 150</td>
<td>KPFAPAELLARVRTLRRGAVVIEESQFQVADLMVQSRKVTGSTRIT 150</td>
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<tr>
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</tr>
<tr>
<td><strong>SiR Morganella morganii RP-42</strong></td>
<td><strong>CusR Escherichia coli</strong></td>
</tr>
<tr>
<td>LTSKEFSLLEFFIRHQCEVLPRSLIASQVWDMPSDTSNDTAIRDVAVKRLRA 200</td>
<td>LTSKEFSLLEFFIRHQCEVLPRSLIASQVWDMPSDTSNDTAIRDVAVKRLRA 200</td>
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</tr>
<tr>
<td><strong>SiR Morganella morganii RP-42</strong></td>
<td><strong>CusR Escherichia coli</strong></td>
</tr>
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<td>KIDNDYGTKLIQTVRGVYMELIPDA- 226</td>
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**Figure C.6:** Comparison of amino acid sequence of SiR from Morganella morganii RP42 to that of CusR from Escherichia coli.
### Figure C.7: Comparison of amino acid sequence of SilS from Morganella morganii RP42 to that of CusS from Escherichia coli.

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<td>MHSKPSRRPSLALRLTFFISLSTILAFIAFTWPLHSVENHPAEQDVSD</td>
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<td>LQQISTTLNRILQSPVDPNDDKIKSSIKESIASYNVALLLLNPGEVLFS</td>
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Appendix D:

Figure D.1: Selected area electron diffraction (SAED) patterns obtained from silica superstructures obtained in ionic liquid [BMIM][BF₄] by hydrolysis of 1 mM TEOS using 10 mM of cationic amino acids (a) L-lysine, (b) L-arginine, and (c) L histidine respectively.