Part IV

Selenium Nanoparticles and Quantum Dots

Chapter 10

Se nanoparticle manufacturing for medical applications

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10.1 INTRODUCTION

The use of biological material or various life forms to produce nanomaterials is routed in the idea that their use will be more eco-friendly than chemically synthesized materials. The present chapter focuses on the current knowledge of how biological organisms and their associated biomolecules or biomass reduce selenium (Se) oxyanions to Se^0 atoms (Figure 10.1). The Se atoms then subsequently assemble on the nanoscale, thus producing 'biogenic' Se nanoparticles (BioSeNPs).

The world of 'very small materials' implies the manipulation of matter at the molecular or atomic level (Horikoshi & Serpone, 2013), a field known as nanotechnology. Materials scaled down to the nano range (1–100 nm) are defined as nanomaterials (NMs), and possess unique physical-chemical properties arising from their high surface-to-volume ratio, large surface energy, and high spatial confinement (Cao, 2004; Yuwen & Wang, 2013). NMs exist in various sizes and shapes including nanoparticles (NPs), nanorods (NRs), quantum dots (QDs), nanowires (NWs), and nanotubes (NTs) (Rao *et al.*, 2004). These materials have enhanced chemical, catalytic, mechanical, electrical and opto-magnetic properties (Appenzeller, 1991; Yuwen & Wang, 2013). Hence, NMs can be applied in a vast range of applications such as: biomedicine and biotechnologies, energy production, food and chemical industries, environmental engineering, mechanics,

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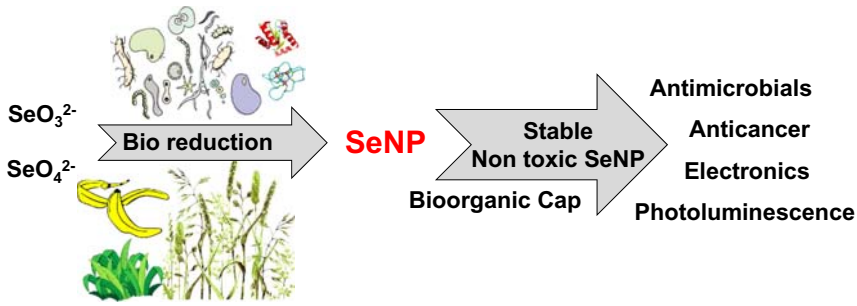

Figure 10.1 Illustration of catalytic sources for the biological applications in SeNP manufacturing.

sensors, optics, and material science (Ahmed *et al.*, 2016; Cao, 2004; Horikoshi & Serpone, 2013; Sharma *et al.*, 2015). The research interest around this field has grown exponentially in the past decade, reflected in the high number of scientific reports for the fabrication of novel nanomaterials through chemical (chemogenic) or biological (biogenic) means.

SeNPs can be produced through a variety of physical (laser ablation, UV radiation, and hydrothermal techniques), and/or chemical (precipitation, acid decomposition, catalytic reduction using a variety of reducing agents) methods. However, these approaches require high temperatures and/or harsh/hazardous chemicals and pH (Dwivedi *et al.*, 2011; Quintana *et al.*, 2002; Zhang *et al.*, 2010a). Thus, biosynthesis approaches have been explored that turn out to be safer, less expensive and utilize more eco-friendly materials and operational conditions.

Selenium is an essential trace element in humans and many microorganisms as an important cofactor in biochemical processes (see Chapter 3). Indeed, the 21st amino acid, selenocysteine, is an important active center of at least 25 different selenoproteins (Rayman, 2012), and its deficiency can cause diverse diseases in humans (Brigelius-Flochè & Maiorino, 2013; Morenoreyes *et al.*, 1998; Zhang *et al.*, 2010b). Despite this, high concentrations of selenium compounds (i.e., the highly soluble oxyanions selenate [SeO_4^{2-}] and selenite [SeO_3^{2-}]) in the environment can be toxic at relatively low concentrations (Presser & Ohlendorf, 1987; Weres *et al.*, 1989). This is due to the oxyanions' mobility through the trophic chain and its tendency to bioaccumulate. Yet, there are now many organisms identified that are highly tolerant to excessive selenium loads. Bacteria resistant to and/or able to respire selenium oxyanions started to be identified in the late 1980s and through the 1990s (Stolz & Oremland, 1999; Stolz *et al.*, 2006). However, it was not recognized until the late 2000s that this process led to potentially valuable technologies in bioremediation and nanomaterial manufacturing (Gadd, 2010; Nancharaiah & Lens, 2015).

This chapter overviews biological sources as reducing agents of Se oxyanions, either as whole organisms/cells or biomass components. This chapter does not go into the engineering of the reaction process, which would in most cases be batch reactors and fermenter systems scaled to match reagent availability and production needs (see Chapter 6). Since 2015, there have been a number of reviews published in the area of Se nanomaterial production and their subsequent (bio)technological uses, both potential and realized. For general information on SeNPs see: Khurana *et al.* (2019); Hosnedlova *et al.* (2018); Menon *et al.* (2018); Kielczykowska *et al.* (2018); Guan *et al.* (2018); Sharma *et al.* (2017); and Verma (2015). For reviews specifically on biogenic SeNPs, see Tugarova and Kamnev (2017); Wadhwani *et al.* (2016); and Tan *et al.* (2016).

10.2 BIOLOGICAL SYNTHESIS OF SELENIUM NANOPARTICLES

Research on Se polluted sites has led the exploration of diverse organisms that can tolerate high Se loads. Some plant species have been found growing in soils and aquatic systems that were highly impacted by Se pollutants, and several Se oxyanion resistant bacteria were isolated associated with these plants (Wu, 2004). Utilizing the microorganisms capable of transforming Se toxicants became a strategy to attenuate the presence of these compounds in the environment. Their study led to the observation of an orange-red coloration, that is now known to be the result of the formation of biogenic SeNPs. It was also found that certain anaerobic bacteria could respire selenium oxyanions, which often resulted in the accumulation of elemental selenium (Se^0).

The spectral properties of BioSeNPs differ considerably from those of amorphous Se^0, formed by chemical oxidation of hydrogen selenide (H_2Se), and of black, vitreous Se^0 formed chemically by reduction of selenite with ascorbate (Pettine *et al.*, 2013). The microbial synthesis of Se^0 nanospheres results in a unique, complex, and compact nano structural arrangement of Se atoms. Furthermore, it turns out that the natural organic material supplied by the organism that is found to 'cap' the SeNPs leads to a more stable SeNP than that found through chemical/physical methods (Piacenza *et al.*, 2018a; Wadhwani *et al.*, 2016). Yet, their shape and size vary considerably depending on the biogenic system used. This probably reflects the wide diversity of enzymes involved in the dissimilatory reduction that differ in various microorganisms. To date, these conditions cannot be achieved by current chemical/physical synthesis methods (Hosnedlova *et al.*, 2018; Oremland *et al.*, 2004). It is now recognized that using biological systems to produce SeNPs is an eco-friendly approach to manufacture high quality and stable SeNPs with unique properties (Ingale & Chaudhari, 2013; Wadhwani *et al.*, 2016).

10.2.1 Bacteria mediated selenium nanomaterial production

For quite some time, it was known that microorganisms had an important role in the transformation of selenium compounds from one oxidation state to another (Favre-Bonte *et al.*, 2005; Herbel *et al.*, 2003; Stolz *et al.*, 2006). These transformations are key to the selenium biogeochemical cycle. For example, studies reported on the ability of various soil bacteria to reduce SeO_4^{2-} and SeO_3^{2-} (Dowdle & Oremland 1998; Sarathchandra & Watkinson, 1981), while some other microbes generate volatile organo-selenium species such as hydrogen selenide (H_2S) or methylated selenium species (e.g., $HSeCH_3$; $Se(CH_3)_2$) (Favre-Bonte *et al.*, 2005; McCarty *et al.*, 1993; Stolz *et al.*, 2006). The biotic process behind the reduction of selenium oxyanions elicited by bacteria either under oxic (Antonioli *et al.*, 2007; Bajaj *et al.*, 2012; Hunter & Kuykendall, 2007; Hunter & Manter, 2009; Piacenza *et al.*, 2018b, 2019; Presentato *et al.*, 2018a) or anoxic (DeMoll-Decker & Macy, 1993; Hunter & Kuykendall, 2006; Kessi, 2006) conditions most often results in the formation of red-colored selenium deposits scaled at the nano range in the form of spheres with a diameter ranging from 50 to 500 nm (Jain *et al.*, 2014; Oremland *et al.*, 2004). An example of cell and culture coloration is shown in Figure 10.2. There is now a

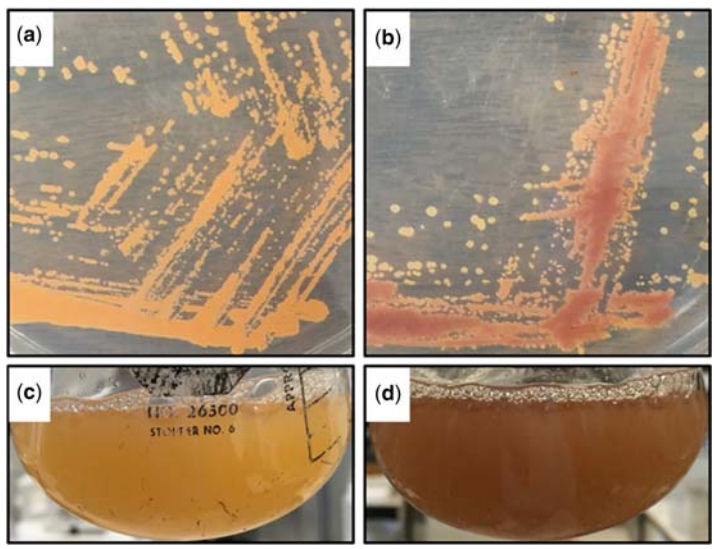

Figure 10.2 *Rhodococcus aetherivorans* BCP1 grown onto solid (A and B) and liquid (C and D) Luria–Bertani rich medium. B and D highlight the typical color change – from yellow to dark red – of the bacterial culture upon addition of 2 mM sodium selenite to the cultivation medium, due to the formation of Se^0 nanomaterial.

wide diversity of microorganisms that have been identified to respire different species of Se (Maltman *et al.*, 2016; Nancharaiah & Lens, 2015). However, the biochemical mechanism(s) of reduction of the selenium oxyanions have not yet been fully elucidated in all systems.

It is difficult to define a common mechanism for the assembly of biogenic SeNPs in selenium tolerant microorganisms, as their accumulation has been observed to be intracellular, membrane-bound, and extracellular. Some aspects of Se biochemistry are common between bacteria, likely related to the metabolic pathways laid out in *Escherichia coli* (Turner *et al.*, 1998). However, differences in genomics and physiology would add up to unique mechanisms in distant strains. This aspect is of interest, as unrelated bacteria, from a phylogenetic perspective, can give rise to diverse SeNMs in terms of structure and properties. Therefore, most biogenic SeNP synthesis outcomes are not predictable, nor reproducible, by the measure of conventional chemical procedures (Oremland *et al.*, 2004). This is because there is a collective knowledge gap regarding the mechanism exploited by bacteria to synthesize SeNMs; particularly, it is not clear how a bacterial cell factory controls the parameters of size, shape, and polydispersity of the final nanomaterial product. By deciphering the biochemical route used for NM synthesis, the fabrication of novel and unique nanomaterials may be honed using either living biological catalysts or specific macromolecules derived from them. Though, at this time, the best production conditions are still empirically determined, leading to biotechnological challenges towards scale-up.

Thauera selenatis, formerly defined as a *Pseudomonas* sp. strain (Macy *et al.*, 1989), was the first bacterial strain cultured in axenic conditions where its ability to respire selenate for energetic purposes was established (DeMoll-Decker & Macy, 1993; Macy *et al.*, 1993; Schröder *et al.*, 1997; Rech & Macy, 1992). Whatever the source of selenium precursor, the main phenotype was always represented by the color of the *T. selenatis* culture turning red, highlighting how the biotic reduction process led to the accumulation of selenium deposits, which occurred as extracellular SeNPs in this strain. It was speculated that *T. selenatis* could utilize an intracellular reductant to further detoxify selenite to Se^0 during selenate respiration, leading inevitably to the accumulation of Se^0 atoms within the cells. This observation raised critical questions: (i) How do elemental selenium atoms assemble forming NPs? (ii) How is the latter secreted to the extracellular environment? (iii) Is the process reversed where atoms are exported and then extracellularly assembled? (iv) Do NPs form both intracellularly and extracellularly with no Se or SeNP transport? The evidence is still ambiguous as experimental observations do not resolve the mechanism. *T. selenatis* cells entering the stationary phase during anaerobic growth in the presence of acetate as a carbon source and selenate as a terminal electron acceptor showed both intra- and extracellular selenium particles (Macy *et al.*, 1993). However, there was no evidence of cell lysis or distortion of the membrane suggesting two independent processes.

The use of microorganisms to produce SeNMs has other advantages over the chemical procedures, beyond being eco-friendly. The parameters of choice of the cell factory to perform a biogenic synthesis including growth temperature (Wang *et al.*, 2010), pH, type of media (Piacenza *et al.*, 2019), bacterial cell physiology (i.e., actively growing or resting cells (Presentato *et al.*, 2016, 2018b; Srivastava & Mukhopadhyay, 2013), culture incubation time (Ahmad *et al.*, 2015; Zhang *et al.*, 2011), and concentration of the metal(loid) precursor supplied (Piacenza *et al.*, 2019) can all be optimized to drive the production of a certain NM type(s). Above all, it is important to consider the metabolic potential of a given bacterial strain investigated. Strains belonging to either *Rhodococcus* or *Ochrobactrum* genera have high metabolic versatility (Martínková *et al.*, 2009) that allow them to survive harsh conditions. For example, *Rhodococcus aetherivorans* BCP1 is capable of simultaneously producing SeNPs and SeNRs when cultured in the presence of selenite under oxic conditions (Presentato *et al.*, 2018a). Examples of these nanomaterials are shown in Figure 10.3. This was also reported for *Ochrobactrum* sp. MPV1 cultivated under metabolically controlled growth conditions or in complex medium amended with very high concentrations (10 mM) of SeO_3^{2-}, producing high yields of SeNPs (Piacenza *et al.*, 2018b, 2019).

The stress on bacteria from Se oxyanions can represent a trigger to produce energetically valuable hydrophobic storage compounds to keep them thriving when the cells are experiencing an oligotrophic environment (Alvarez *et al.*, 1996). Surfactant-like molecules (i.e., biosurfactants) are produced to absorb and attenuate the stress derived from the carbon source exploited for energy production (Kumar *et al.*, 2014; Piacenza *et al.*, 2018b) or the toxicity exerted by a high metal(loid) load (Piacenza *et al.*, 2019). The presence of amphiphilic compounds, that act like surfactants, was identified in the biogenic SeNM extract. This was determined through fluorescence spectroscopy. A hydrophobic

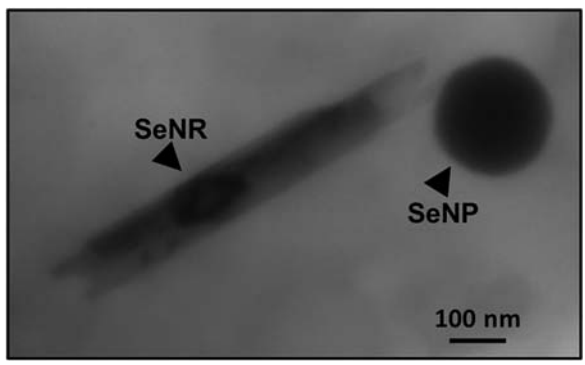

Figure 10.3 Transmission electron micrographs of extracted selenium nanomaterials in the form of nanorod (SeNR) and particle (SeNP) produced by *Rhodococcus aetherivorans* BCP1.

fluorescent dye [$DiOC_{18}(3)$] was used to label either biogenic NM extract or a sample containing liposomes, unveiling that the fluorescence signals were very much alike (Piacenza *et al.*, 2018b; Presentato *et al.*, 2018b). The presence of these amphiphilic molecules in the biogenic NM extract can have multiple roles, acting either as a stabilizing agent of the NM, as a driving force for the nucleation, or for the one-dimensional growth of nanomaterials (i.e., NRs), or even as a mediating component of the NM functional properties. This is inferred from chemical synthetic approaches, where the addition of surfactant compounds to the reaction mixture leads to NRs as products (Eastoe & Tabor, 2014; Evans & Wennerstrom, 1994). Finally, the nanostructures can exist in either amorphous or crystalline configurations, where the latter gives higher thermodynamic stability in suspension, which is a trait typically found for NRs (Jeong *et al.*, 2006). It are the biosurfactants in nanorod synthesis that lead to the stable crystalline rods (Presentato *et al.*, 2018b).

10.2.2 Fungi mediated selenium nanomaterial production

The high sensitivity of most fungi to Se compounds (Hanson *et al.*, 2004) has led to their limited exploitation for SeNM production as compared to bacteria. Regardless, fungal species have several advantages over other microorganisms, such as their ease and cost-effectiveness in culturing, scaling up and downstream processing (issues reviewed in – Piacenza *et al.*, 2018a), as well as their ability to adsorb and accumulate metals (Thakkar *et al.*, 2010). To date, the tolerance of fungi towards Se seems to be linked to long and/or constant exposure of these microorganisms to Se-containing compounds in their natural habitats, such as the rhizosphere of Se-hyperaccumulator plants (Shoeibi *et al.*, 2017). In this respect, fungal strains belonging to *Alternaria*, *Curvularia*, *Cladosporium, Pleurotus*, *Aspergillus*, *Chaetomium*, *Trichoderma*, *Aureobasidium*, *Mortierella*, *Phoma*, and *Agaricarius* feature higher tolerance towards Se (Liang *et al.*, 2019; Sarkar *et al.*, 2011; Vetchinkina *et al.*, 2019; Wangeline *et al.*, 2011).

Sarkar and coworkers (2011) were among the first groups to report the exploitation of fungi, using filtered fungal-free spent media of *Alternaria alternata*, for SeNP biosynthesis. These biogenic SeNPs showed high polydispersity, ranging in size between 30 and 150 nm, and an amorphous nature (Sarkar *et al.*, 2011). They appeared to be coated by a protein layer, which in turn made the SeNPs stable over several months (Sarkar *et al.*, 2011). Similarly, *Aspergillus terreus* displayed a good proficiency in bioconverting SeO_3^{2-}, producing relatively stable extracellular SeNPs with an average diameter of 48 nm (Zare *et al.*, 2013). Selenite was also the precursor for the extracellular SeNP formation by the basidiomycete *Lentinula edodes*. This species was also capable of biotically transforming the organo-Se compound 1,5-diphenyl-3-selenopentanedione (DAPS-25), forming nanosized Se products with an average size of 180 nm (Vetchinkina *et al.*, 2013).

The effect of using actively growing fungal cultures or extracts for the biosynthesis of SeNPs was investigated by Vetchinkina *et al.* (2019). Strains of *L. edodes* and *Pleurotus ostreatus* produced polydisperse (50–150 nm) SeNPs with uniform shape, while those obtained from the mycelium extracts of the same species were less regular in shape. Similar results were obtained by exploiting *Grifola frondosa*, *Ganoderma lucidum*, *Agaricarius bisporus*, and *A. arvensis*: small (20–50 nm) and homogeneous SeNPs were detected using the intracellular extracts of their mycelium (Vetchinkina *et al.*, 2019). *Aureobasidium pullulans*, *Mortierella humilis*, and *Phoma glomerata* generated extracellular, granular and amorphous SeNPs between 40 and 60 nm. Se oxide (SeO_2) NPs that were heterogeneous in shape and size have been detected on the *Trichoderma harzianum* cells' outer surface (Liang *et al.*, 2019).

Besides myceliating fungi, the single-celled yeasts have also been explored in SeNM production. Yeasts accumulate high amounts of Se during their growth (Marinescu *et al.*, 2011). This has been observed for *Saccharomyces cerevisiae*, whose bioaccumulation capacity was found to be regulated by several external factors, such as temperature, fermentation time, pH, shaking speed, and Se concentration (Esmaeili *et al.*, 2012). Similarly, the aerobic bioconversion of SeO_3^{2-} oxyanions carried out by the same yeast strain led to the biosynthesis of 30–100 nm SeNPs, which demonstrated good antimicrobial properties against Gram-positive and Gram-negative pathogens (Hariharan *et al.*, 2012).

At this time, the synthetic manufacturing of SeNPs by fungi is still in its infancy. The mechanistic information is still sparce. Studies only suggest a potential role played by glutathione, as well as cysteine residues present in the hypha cell walls, for the bioconversion of Se precursors to Se^0 on the mycelium through Painter-type reactions (Poluboyarinov *et al.*, 2009).

10.2.3 Plants and selenium nanomaterials production

Besides bacteria and fungi, other biological systems that have been investigated for SeNM production include plants (and plant material) in part due to their resistance towards a wide range of toxic compounds. Although a broad spectrum of plants has been explored for a breadth of metal NM generation, particularly silver (Shoeibi *et al.*, 2017), their use in SeNM production is currently limited. Trials with plant leaves (Alam *et al.*, 2019; Fardasadegh *et al.*, 2018; Li *et al.*, 2007; Sownadarya *et al.*, 2017), fruit, and their plant wastes/extracts have been explored.

Capsicum annuum leaf extract was used to biotically reduce SeO_3^{2-}, simultaneously forming amorphous SeNPs surrounded by a protein layer, which controlled the nucleation and growth of the NPs (Li *et al.*, 2007). Additionally, the SeNP size and structure was affected by the concentration of the leaf extract as well as the pH of the medium (Li *et al.*, 2007). Selenious acid was used as a Se-precursor for the generation of SeNPs by *Trigonellafoeum graecum* seed extract, by exploiting ascorbic acid as an initiator of the reduction process.

The resulting SeNPs featured high polydispersity, between 50 and 150 nm in size, and a good crystalline structure, which seemed to be mediated by the presence of bioactive compounds of the plant extracts containing C═C, NH_2, COOH, and C═O functional groups (Ramamurthy *et al.*, 2013). These associated biomolecules were considered responsible for the slight cytotoxicity observed under a prolonged exposure of human breast cancer cells to increasing concentrations of the biogenic SeNPs (Ramamurthy *et al.*, 2013). On the contrary, SeNPs recovered from *Allium sativum* extract were not cytotoxic towards human kidney epithelial Vero cells, a feature that the authors ascribed to the presence of biocompatible molecules containing N═O as stabilizing agents (Anu *et al.*, 2017). The size of these SeNPs ranged from 40 to 100 nm, being homogeneous in shape and relatively monodispersed in solution (Anu *et al.*, 2017).

Trigonal SeNPs of ca. 8 nm were synthesized by the dried fruit extract of *Vitis vinifera*, whose lignin molecules appeared to be associated with the NPs in solution. These lignin molecules conferred these SeNPs' high thermodynamic stability (Sharma *et al.*, 2015). Larger SeNPs (46–78 nm) were obtained from *Clausena dentata* leaf extract with proteins found as NP capping agents. These SeNPs had good insecticidal activity towards mosquito larvae (Sownadarya *et al.*, 2017). Microwave irradiation was exploited by Fardasadegh *et al.* (2018) to obtain 50 nm SeNPs from *Pelargonium zonale* leaf extract through SeO_3^{2-} bioreduction. This approach was considered to be mediated by tannins, flavonoids and other metabolites containing highly reactive -OH groups, while proteins were responsible for the stabilization of the formed SeNPs. Furthermore, these biogenic SeNPs showed good antimicrobial properties towards both bacterial and fungal pathogens, having a higher efficacy against the bacteria (Fardasadegh *et al.*, 2018). Similar results were reported for SeNPs of ca. 10–20 nm in size obtained from *Psidium guajava* leaf extract, where ascorbic acid residues and phenolic compounds were responsible for the bioconversion of SeO_3^{2-} (Alam *et al.*, 2019).

10.3 ROLE OF BIOMOLECULES IN THE SYNTHESIS OF SELENIUM NANOPARTICLES

Several biochemical reactions can catalyze the reduction of selenate or selenite generating the seed atoms of the nanomaterials, Se^0. The most widely reported reaction is the Painter-like reaction with thiols. However, one can have other reductive biochemical reactions with a variety of metabolites (such as plant flavonoids and lignin). Key biochemical electron mediators (cytochromes and quinones) can also catalyze the reduction reaction as demonstrated with the quinone analogue lawsone to produce SeNPs with *E. coli* (Wang *et al.*, 2011) or with *Rhodobacter capsulatus* (Borghese *et al.*, 2014). There is also the potential for direct enzymatic reactions with the oxyanion as the substrate. In this context, some early studies reported evidence that the catalytic enzyme may in fact

nucleate the crystal growth of SeNPs (DeMoll-Decker & Macy, 1993; Rech & Macy, 1992).

10.3.1 Proteins involved in selenium nanoparticle synthesis

Tugarova and Kamnev reviewed proteins involved in SeNP synthesis in 2017. This review highlights that although some enzymes have been implicated in Se oxyanion reduction and NP formation, their role is found mostly in NP size modulation and capping, where they provide thermodynamic stability to the NP. Below follows a brief description of enzymes involved in the reduction reactions followed by key studies finding proteins associated with the caps of the SeNPs.

The research in this field mostly recognizes that the biomolecular reduction of selenite occurs through Painter-like reactions (Painter, 1941) with glutathione (GSH) molecules or similar cysteine peptides (cysteine, bacillithiol, and mycothiol). The reactions (10.1) to (10.3) follow the process of reduction with glutathione and subsequent reduction to Se^0 mediated by a NADP(H) dependent reductase:

$$3GSH + 6SeO_3^{2-} + 4H^+ \rightarrow 3GSSeSG + 2O_2^- + 5H_2O \quad (10.1)$$

$$GSSeSG + NADP(H) \rightarrow GSSe^- + GSH + NAD(P)^+ \quad (10.2)$$

$$GSSe^- + H^+ \rightarrow GSH + Se^0 \quad (10.3)$$

The reaction with GSHs leads to a seleno diglutathione (GS-Se-SG) intermediate. This GSSeSG intermediate can be further acted on by the enzyme glutathione reductase to generate GSH and Se^0. Alternatively, the GSSeSG can be acted on by thioredoxin with further reductive cycling by the enzyme thioredoxin reductase leading to glutathione seleno persulfide (GS-Se^-), which then rapidly dismutates into GSH and Se^0. Both of these enzyme-catalyzed reactions utilize NADPH as a source of electrons (Ganther, 1971; Turner *et al.*, 1998).

Within the group of selenate respiring bacteria, several complex iron-sulfur molybdoenzymes (CISM) have been demonstrated to use selenium oxyanions as substrates, leading to red selenium particles (reviewed by Nancharaiah & Lens, 2015; Zannoni *et al.*, 2008). Early reports observed the CISM nitrate reductases of *E. coli* to have this catalytic activity, using selenate as a substrate (Avazeri *et al.*, 1997). This NarG or NarZ nitrate reductase dependent reduction was later confirmed in other bacteria (Sabaty *et al.*, 2001), with shared mechanisms and thus demonstrating cross-reactivities between microbial selenate and nitrate enzymatic reduction (Watts *et al.*, 2005). Another CISM enzyme encoded by the *ynfEFGH* operon in *Salmonella enterica* was also shown to be responsible for the respiration of selenate to selenite, and subsequently converting the latter to SeNMs (Connelly *et al.*, 2016). A cartoon description of a CISM enzyme process

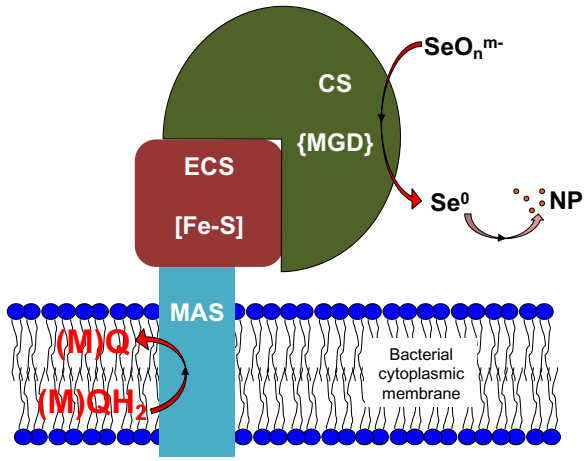

Figure 10.4 Description of a complex iron-sulfur molybdoenzymes (CISM) involvement in generating SeNPs. CS = catalytic subunit; ECS = electron conduit subunit; MAS = membrane anchor subunit, may or may not contain cytochromes; (M)Q = (menaquinone) or quinone; [Fe-S] = iron sulfur clusters, different atom number clusters are present from [4Fe-4S], [3Fe-4S] to [2Fe-2S]; the CS may or may not contain an Fe-S or cytochrome as well; MGD = Bis molybdopterin guanine dinucleotide; in some organisms, Mo may be replaced with W; NP = nanoparticles.

is shown in Figure 10.4. Unfortunately, purification of pure CISMs protein complexes is extremely difficult, and thus, they would be difficult to use directly as a catalyst in SeNP manufacturing. However, the knowledge of these enzyme systems allows research to focus on the genetic regulation of these systems for application in biotechnological processes for selenium bioremediation and nanomaterial production.

T. selenatis is one of the most studied selenate/selenite-respiring species (Butler *et al.*, 2012). Beyond the CISM related SerABC enzyme for its respiration of selenate (Schroder *et al.*, 1997), *T. selenatis* anaerobic respiration of selenite finds a single protein associated with SeNPs. Cell-free spent medium from cultures of *T. selenatis* containing SeNPs was analyzed for its protein content, and a single 95 kDa protein, named selenium factor A (SefA), was found. This protein was detected when *T. selenatis* cells were grown in the presence of nitrate (electron acceptor) during anaerobic growth in complex media and in the presence of selenate or selenite. The absence of either selenate or selenite led to undetectable levels of SefA, highlighting that the selenium oxyanions likely induced the expression of *sefA*. Bioinformatic analysis revealed that, although this protein was somehow associated with extracellular SeNPs, it does not possess a signal peptide responsible for its secretion. Moreover, the heterologous expression of

the *sefA* gene in *E. coli* revealed the presence of the corresponding protein in both the soluble fraction and in the extracellular environment. *E. coli* cells expressing the *sefA* gene accumulated large SeNPs in the cytoplasm, while these NPs were not observed outside the cells, suggesting that (i) SefA might be a substrate for protein export, (ii) its secretion was not dependent on the binding with Se^0 atoms, and (iii) there may exist a specific export system for SeNPs in *T. selenatis,* since *E. coli* cells expressing *sefA* did not reveal any trace of extracellular SeNPs.

SefA presents a possible tool for SeNP manufacturing. When SefA was added to the reaction mixture containing glutathione and selenite in a 4:1 molar ratio, stable SeNPs were generated. However, in the absence of SefA only vitreous Se deposits were detected, underlining that SefA functions to bind and stabilize Se^0. This process occurs naturally in the cytoplasm of *T. selenatis* cells, and the SeNPs are subsequently exported to the extracellular environment once the spheres reach a particular size (ca. 150 nm in diameter). The whole mechanism by which *T. selenatis* can secrete these NPs through the inner membrane, periplasm, and outer membrane remains unknown (Dobieux *et al.*, 2011). Although this evidence suggests SefA has a role in the assembly of biogenic SeNPs under anaerobic conditions, it is not easy to find equivalent genetic determinants for a similar function in aerobic bacterial strains (Gonzalez-Gil *et al.*, 2016; Lenz *et al.*, 2011). It is also speculated that protein binding to SeNPs leads to a gain in thermodynamic stability. Hence, the SefA helps the NPs avoid aggregation. Such stabilization was also displayed by Bovine Serum Albumin bound to SeNPs *in vitro*, thus acting as a capping agent likely generating a sort of protein shell (Bücking *et al.*, 2010).

Dobias and colleagues (2011) tried to unravel the role of proteins in biomineralized selenium, with the aim of gaining the advantage of avoiding the maintenance of live cultures for NPs synthesis. *E. coli* derived SeNPs and associated proteins were purified by performing a sucrose gradient fractionation, whose fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Among the proteins associated with SeNPs, some remained bound to NPs despite washing steps of the NM extract with increasing concentrations of denaturing solutions or after boiling in 10% w/v SDS solution. A similar observation was made for biochemicals associated with SeNPs from a variety of Gram negative and positive environmental isolates (Bulgarini *et al.*, 2021). This highlights the strong interaction of certain proteins to SeNPs providing the hypothesis that proteins involved in NP formation could have covalent bonds to the metal atoms of the NP. A similar finding was reported for SeNPs produced by *Lysinibacillus* sp. ZYM-1, where hexane treatments used in extracting and purifying the NPs did not successfully remove all the biomolecules present in the biogenic colloidal suspension, and thermogravimetric analysis revealed the presence of proteins in the coating (Che *et al.*, 2017). The *in vitro* synthesis of biogenic and chemically synthesized SeNPs in the presence of *E. coli* cell-free extract revealed that none of the proteins associated with SeNPs

are known to be involved in selenium metabolism. Instead, the proteins found were involved in carbohydrate or fatty acid metabolism. Only two proteins (elongation factor Tu and 3-oxoacyl synthase) were present in all *in vitro* conditions tested, which suggested a non-specific mode of binding.

Comamonas testosteroni S44 grown under aerobic conditions in the presence of selenite had several proteins bound to SeNPs resulting from physical-chemical interactions (Xu *et al.*, 2018). Dobias *et al.* (2011) defined four proteins of varying size and isoelectric point (isocitrate lyase [AceA], isocitrate dehydrogenase [Idh], outer membrane protein C precursor [OmpC], and alcohol dehydrogenase [AdhP]) were found bound to the colloidal Se. Among these proteins, AdhP was selected for further studies aimed towards the formation of SeNPs by *in vitro* synthesis or using *E. coli* cell-free extract. These studies revealed that AdhP binding to SeNPs gave rise to larger NPs (122 nm average diameter) as compared to those produced in the presence of the cell-free extract (ranging from 10–90 nm). Yet, no effect of different proteins on the structure and crystallinity of the SeNPs was found (Dobias *et al.*, 2011). Other proteins like Mop and CysB from *C. testosteroni* S44, as well as PhoB1 and PhoB2 from *Agrobacterium* strain GW4, utilized to synthesize SeNPs *in vitro*, were also found to bind to the final SeNP product.

An explanation put forward for the protein-SeNP interaction focuses on the charged amino acids (i.e., Asp, Glu, Arg, and Lys) content of the associated proteins. Several proteins that are of low cell abundance and have a high charged amino acid content are found adsorbed onto the BioSeNPs surface; as reported in the case of SeNPs synthesized by an actinobacteril (Ramya *et al.*, 2019). However, the zinc-dependent alcohol dehydrogenase (AdH), which contains a low charged amino acid content, is also frequently found on the surface of SeNPs (Dobias *et al.*, 2011; Kessi *et al.*, 1999; Lampis *et al.*, 2017; Lenz *et al.*, 2011; Xu *et al.*, 2018). Thus, the diversity of proteins associated with the SeNPs to date suggests a non-specific mode of interaction of proteins with NPs, yet their robustness of association to the particles suggest a covalent bonding style (Xu *et al.*, 2018).

Having proteins, particularly large bulky proteins like AdH, likely provides stability to the SeNPs through steric hindrance inhibiting the Se cores of the NPs from interacting with each other, thus preventing their aggregation. This supports the observations that proteins are important in governing the size of NMs and contributing to generate a more monodisperse size population. This provides stability from a thermodynamic perspective, as this biogenic nanomaterial has a low tendency to form aggregates. This is likely because of repulsive interactions derived from charged proteins (Srivastava & Mukhopadhyay, 2013) and the steric hindrance effect exerted by proteins (Piacenza *et al.*, 2018a). Indeed, the thermodynamic stability of a colloidal suspension is a feature that is as fundamental and important as the eco-friendliness of the synthetic procedure adopted, when it comes to a potential application of nanomaterials.

10.3.2 Other biomolecules involved in selenium nanoparticle synthesis

Besides proteins, it has been hypothesized that bacterially derived extracellular polymeric substance (EPS) might act as a potential capping agent driving the assembly of SeNPs. This is thought to occur mostly in those bacterial strains for which the synthesis localization is either near the membrane or in the extracellular environment. The complex macromolecular composition of EPS, which contains proteins, polysaccharides, metabolites, lipid vesicles, humic-like molecules, and extracellular DNA (eDNA) (More *et al.*, 2014; Sheng *et al.*, 2010) offers many chemical functional groups capable of interacting with SeNMs. Moreover, EPS has been found to influence the surface properties, size, and shape of SeNPs (Jain *et al.*, 2014). Since many bacterial species have demonstrated either extracellular or periplasmic located reduction of selenite to elemental selenium (Jiang *et al.*, 2012; Li *et al.*, 2014), it is reasonable to suggest a possible involvement of EPS in the nanomaterial production extracellularly. The *in vitro* reduction of selenite with glutathione in the presence or absence of extracted EPS material, respectively, led to the generation of either stable spheres (NPs) or wires (NWs). This indicates that EPS might act as a template and/or capping agent and can tune the NM morphology (Zhang *et al.*, 2010a). This is similar to the polymers polyvinyl alcohol or polyethylene glycol used in chemogenic synthesis protocols (Shah *et al.*, 2010; Zheng *et al.*, 2012).

The complexity of the EPS associated with BioSeNP extracts was investigated by Jain and colleagues (2015) through Fourier transformed infrared spectroscopy (FTIR), which unveiled the presence of proteins, carbohydrates, and humic-like compounds. Since it is expected that sugar residues show more hydroxyl moieties as compared to proteins, it is reasonable to hypothesize interactions occur between these hydroxyl moieties and Se^0. Since only very small amounts of DNA were found in the SeNP extracts, its role as a capping agent derived during cell lysis events as a mechanism for SeNP release, was dismissed. Bulgarini *et al.* (2021) quantified the carbohydrate, protein and lipid levels of SeNPs from five different environmental isolates including *B. mycoides* SeITE01 and *S. maltophilia* SeITE02. The amount and content ratios varied remarkably between strains, yet the lipids were dominant for all isolates. It is presently unknown if these biochemicals are involved in the reduction or just dictating size and structure. Clearly the bioorganic capping layer is typically very complex, yet it is responsible for the SeNPs surface charge, which laid within the theoretical stability ($\zeta > |25$ mV|) range for colloidal suspensions, as a function of the pH and ionic strength of the dispersing medium (Jain *et al.*, 2015).

The capability of several plants and plant extracts to bioconvert a broad spectrum of Se-containing compounds into Se^0 is considered to originate mostly by Painter-type thiol reactions (Jablonski & Anderson, 1982). Nevertheless, secondary metabolites of plant extracts, such as alkaloids, flavonoids, proteins,

polysaccharides, glycosides, and saponins were reported to mediate *in vitro* reduction of SeO_4^{2-} (Li *et al.*, 2007; Ramamurthy *et al.*, 2013). Gallic acid (GA), a common plant polyphenol, was described for its ability to interact with selenite forming antioxidant GA-Se nanofibers, which could potentially be used in anticancer treatments (Barnaby *et al.*, 2011).

Overall, the *in vitro* production of BioSeNPs using various extracted biomolecules is still in its infancy. Although the use of plant and microbial extracts has been successful, there are still issues of reproducibility. Thus, producing BioSeNM with live microbial cultures of selected strains with tuned physiology presently has the most promise.

10.3.3 Approaches to monitor microbial manufactured selenium nanoparticles

Although the chemical and biophysical characterization of SeNPs is beyond the context of this chapter, a brief commentary on the methods used to characterize the nanomaterial is worthwhile. Beyond the microbiology and plant biology of BioSeNP manufacturing, to understand the biological process, information acquired from various analytical methods needs to be employed in BioSeNM characterization and quality control, because a serious limitation of biogenic synthesis is the relatively poor reproducibility. Adequate characterization of the bioNMs is an overlooked crucial step prior to evaluating the BioSeNM functional properties. A variety of well-established physical-chemical methods routinely used for the characterization of macromolecular structures and biomolecules are available to the study of BioSeNPs.

Dynamic light scattering (DLS) for size and dispersion as well as Zeta surface potential measurements are standard to the nanotechnology field for an impression of the material stability and quality of NMs. Scanning and transmission electron microscopy (SEM or TEM) coupled with energy dispersive X-ray (EDX) spectroscopy provide accurate information regarding the actual size, shape and elemental compositional of the NMs under study. X-ray diffraction (XRD) pattern analysis is also becoming a standard in the nanomaterial field.

Evaluating the composition of the biomolecular organic material associated with the biogenic nanomaterial is still a challenging task due to biology's intrinsic complexity. Nevertheless, techniques such as UV-visible absorption and vibrational (Fourier Transmission Infrared (FTIR) or Raman) spectroscopies can be used to identify the biomolecular classes present within the organic material. Nuclear magnetic resonance (NMR) and routine protein chemistry approaches are also available. Of course, the modern system biology approaches of genomics, metabolomics, proteomics and lipidomics can also be applied to evaluate the presence of specific nucleic acids, metabolites, proteins and lipids, respectively. The metal composition can be determined by atomic absorption or inductively coupled plasma (ICP) methods. The oxidation state and coordination can be

inferred through X-ray synchrotron techniques such as X-ray absorption near edge spectroscopy (XANES). The overall quality of the Se core can also be inferred from its fluorescence, whereas electron diffraction or XRD patterns will reveal the crystalline nature/configuration of the Se within the nanomaterials.

A good first step towards repeatable characterization for the BioSeNP area is to investigate the types and ratios of various biomolecules associated with BioSeNPs. Bulgarini *et al.* (2021) suggest a facile approach to establish trends from various biogenic processes. Next, a set of key physical-chemical approaches should be applied in order to effectively compare SeNPs produced by different biological processes. This has been suggested for the antimicrobial activity of biogenic silver nanoparticles giving a standard characterization protocol that includes DLS, Zeta, XRD, absorption spectra, and TEM analysis (Duran *et al.*, 2016). However, such a mandate has not yet been proposed for the SeNM field.

10.4 TOXICITY OF SELENIUM-BASED NANOMATERIALS

As with any metal(loid), the toxicity of Se depends on its concentration, speciation, and other compounds that may influence toxicity through antagonistic or synergistic mechanisms. Thus, fundamentally, the toxicity of SeNMs will come from three components: (i) the selenium and any composite heteroatoms; (ii) properties of the NP itself, such as size, thermodynamic stability, and its amorphous/crystalline nature; and (iii) the capping material, which can influence the SeNMs bioavailability and could also have toxicity properties of its own. Differences between chemogenic and biogenically synthesized NMs lie in subtle differences in crystallinity and size distributions, but more so in the capping material. The stabilizing and capping agents of chemogenic SeNMs have defined and well-known physical-chemical properties, which could make them biologically active, whereas the heterogeneous composition and chemical behavior of the organic material associated with biogenic SeNMs are still not elucidated. Nevertheless, since the organic material features biomolecules, it can play an active role in influencing the bioavailability and toxicity of BioSeNMs.

10.4.1 Toxicity of selenium

Before considering the toxicity of SeNPs, a brief assessment of the biochemistry and toxicity of selenium is worthwhile. Since selenium is an essential trace element for most lifeforms, its biochemistry and physiology has similar issues as other trace elements: that is, how does one get enough, yet not too much? For most of these elements, carefully regulated import and export transporters as well as specific binding proteins that act as holding reservoirs exist in cells. Under physiological conditions, the most toxic species of Se is the highly reduced selenide (S^{2-}, II-), which is highly reactive, yet readily oxidized to the less bioavailable Se^0. Of the oxyanion forms, selenite (SeO_3^{2-}, IV) is far more toxic than selenate (SeO_4^{2-}, VI). These oxyanions typically get into cells through either phosphate or

sulfate uptake systems, where they are used to form either selenomethionine or selenocysteine via specific biochemistry. Some of this chemistry is mediated by thiol (RSH) redox homeostasis peptides and proteins (e.g., thioredoxin and glutaredoxin) through Painter-like reactions (Turner *et al.*, 1998). In eukaryotes and Gram-negative bacteria, the most common peptide exploited for this bioprocess is glutathione, while for Gram-positives this peptide is in carbohydrate-modified version either as mycothiols (MSHs; typical of actinobacteria) or bacillithiols (BSHs; typical of *Bacillus* spp.), which show a greater redox stability as compared to GSH molecules (Presentato *et al.*, 2016). These peptides are found at concentrations in the order of 10 mM. The product of the RSH-mediated reduction of selenite is oxidized thiols and reduced selenium either as a Se-amino acid or as Se^0 atoms which may assemble into SeNMs.

The mechanism(s) of how metal nanomaterials enter a cell is unclear. Yet once inside the cell, the metal NP may decompose providing a localized high concentration pulse of the antimicrobial metal. Any membrane disruption mediated by the NP during passage could uncouple the electron transfer chain which would produce reactive oxygen species (ROS) (Wang *et al.*, 2017). Additionally, ROS are produced if the metal is redox active. The toxicity of selenite/selenate is founded on the basis of cellular thiol redox homeostasis in that some biochemical reactions produce a lot of reactive oxygen species (ROS) and there must be enough metabolic reducing potential to recover the oxidized peptides and proteins from damage. With this in mind, the Se oxyanions react with RSH containing biomolecules in the cell. In this way, the Se oxyanions consume the reducing equivalents that would normally be used to buffer naturally produced ROS. Thus, one observes increased ROS from selenite exposure because the redox buffering has been unbalanced. The ROS measured is from the electron transport chain and not directly catalyzed by the Se atoms. However, some of the Se metabolism steps can lead to ROS species due to imbalanced redox reactions (Turner *et al.*, 1998).

A consideration of SeNPs toxicity is that they can often be synthesized with other metals present (heteroatoms). An example of this is Se-based quantum dots (QDs) which contain cadmium (Cd). Although they are primarily synthesized chemically, approaches towards their biogenic production are underway (Siresj, 2014; Suresh, 2014). SeCd QDs have not been found to have toxicity *per se* as they are capped for high stability. Indeed, no signs of necropsy in mice or in tissue cultures were found if there was no QD decomposition. However, if decomposition of SeCd QDs occurs, a cellular response with a dose-dependent pattern like that of molecular Cd is observed (see review Farre *et al.*, 2009). Thus, the heteroatom toxicity can overwhelm any effect of an increased Se load. The nature of the polymer coating on the SeCd QDs thus has a large influence on toxicity via governing their decomposition rate in the environment (Sharma *et al.*, 2017).

It has been well discussed how organic molecules involved in the capping of the Se atom core lead to size, shape, and thermodynamic stability characteristics

(Piacenza *et al.*, 2018a). However, it is important to acknowledge the source of this biogenic material and biomolecules from the organism that manufactured it. The examples in this chapter provide evidence that the biogenic material has different and enhanced activities compared to chemically synthesized nanomaterials. These properties are in part due to the biomolecules making up the organic material. Thus, to properly investigate the toxicity of BioSeNMs, it is imperative to consider the content of the biogenic extracts, that is, both the organic material and the Se^0 atoms.

10.4.2 Selenium nanoparticle toxicity to Prokaryotes

We are now in a time often referred to as the Antibiotic Resistance Era, where antimicrobial resistance (AMR) is rampant in most pathogens. In response, there has been increased exploration towards novel antimicrobial agents and antibiotic stewardship. One response to this call is metal and metalloid based antimicrobials (MBAs) (Turner, 2017). The application of metal salts used alone or mixed with other antimicrobials is used for wound treatment, in device coatings and for high touch surfaces to limit contamination (Monych *et al.*, 2019). In the last decade, metal and antimicrobial conjugated nanomaterials have been explored with significant promise to become the next generation of antimicrobial agents (see reviews: Huh & Kwon, 2011; Rudramurthy *et al.*, 2016; Sanchez-Lopez *et al.*, 2020; Vimbela *et al.*, 2017; Want *et al.*, 2017; Yah & Simate, 2015). At this time, silver nanoparticles AgNP are leading with many formulations and applications now out to market. Nevertheless, nanomaterials of gold (Au), zinc (Zn), nickel (Ni), titanium (Ti), copper (Cu), and iron (Fe) are all promising. Alternative technologies include antimicrobial-conjugated silica NPs (Bernardos *et al.*, 2019), other organic nanomaterials (reviewed in Raghunath & Perumal 2017), and even carbon nanomaterials either alone or as antibiotic carriers (Maas, 2016). The strong movement towards the exploitation of metal(loid) NMs as antimicrobials derives from the assumption that NPs will be able to overcome the common mechanisms of resistance towards traditional antibiotics. This is based on observations that MBAs have broad biochemical targets mediating their antimicrobial effects (Lemire *et al.*, 2013). Thus, spontaneous mutations can give rise to resistance towards an antibiotic that has a single cell target. However, it is highly unlikely that multiple mutations will occur to protect against all antimicrobial mechanisms of MBAs (Wang *et al.*, 2017). A challenge is, however, that the toxicity of a metal(loid) is correlated to its speciation.

To date, a number of general reviews have been published evaluating the antimicrobial mechanisms of metal nanomaterials and biogenic nanoparticles, however, few mention SeNMs. Yet, a few studies are finding that biogenically produced SeNPs have good antimicrobial activities. Unfortunately, what is often overlooked when evaluating efficacy of metal-based antimicrobials is that growth media and bacterial strain differences can lead to a large range of antimicrobial

inhibition effects, which makes it difficult to compare efficacies between studies. Furthermore, most studies miss the difference between the chemogenic and biogenic NM capping material, and this cap may play a significant role in the NMs antimicrobial efficacy.

To date, the mechanisms of metal NP antimicrobial activities include: (i) photocatalytic production of ROS or direct Fenton style reactions; (ii) cell membrane and wall damage with or without direct release of the metal(loid) ions; (iii) disruption of energy production (uncoupling the proton motive force or inhibiting specific bioenergetics enzymes); and (iv) direct inhibition of specific enzyme activity and central biology processes (Bernardos *et al.*, 2019; Huh & Kwon, 2011; Rudramurthy *et al.*, 2016; Wang *et al.*, 2017). It is likely that SeNPs could affect prokaryotes in similar ways.

The bacterial response to selenium exposure has been reviewed previously (Zannoni *et al.*, 2008), and would be applicable to SeNPs if at some point the SeNPs would decompose (naturally or via biological actions) and expose a bacterium to a local dose of oxidized selenium. In order to make the Se toxic, the biochemical processing from the microbe would need to change the Se(0) species by reducing the Se to the minus II state or oxidizing it to plus IV. From here the selenite ions could cause cell damage in two major ways within bacterial cells. 1. Reduction: The toxicity originates from the oxidation of cellular components or stealing electrons from the bioenergetic enzymes and electron transfer chain. This oxidation can lead to reactive oxygen species production, which would lead to further biomolecule damage. 2. Specific chemistry: Methylation reactions seem to be quite common (Chasteen & Bentley, 2003), where a variety of methylated species can be produced, including but not limited to: CH_3SeH, CH_3SeCH_3, CH_3SeSCH_3, and $CH_3SeSeCH_3$. If released these compounds are volatile and lost, however, if they are trapped within the cell, particularly through thiol based biochemical reactions, they can be damaging by inhibiting enzymes. These end point reactions could be the alkyl capping of active site functional -SH groups, such as in cysteines and cofactors such as coenzyme-A and lipoamide. These reactions result in the irreversible inhibition of the subsequent biochemistry and related metabolism.

The AMR issue is amplified by the capability and preference of most bacteria to grow as a surface attached community, which is referred to as a biofilm, showing remarkable antimicrobial tolerance. Human infections see 65–80% caused by the formation and proliferation of pathogens as biofilms (Bryers, 2008; NIH, 2002). BioSeNPs produced by *Stenotrophomona smaltophilia* SeITE02 and *Ochrobactrum* sp. MPV1 showed excellent efficacy against *E. coli*, *P. aeruginosa,* and *S. aureus* both planktonically and as a biofilm (Zonaro *et al.*, 2015). Similar results were observed by Piacenza *et al.* (2017), who used *Bacillus mycoides* SelTE01 to produce BioSeNPs with good efficacy against planktonic growth of *P. aeruginosa* and *S. aureus.* In these studies, the BioSeNPs had greater antimicrobial efficacy than the chemogenic SeNPs. Incorporating the

BioSeNPs on hydroxyapatite coated surfaces led to excelled antibiofilm activity. In these studies, it appears that some of the antimicrobial activity originates from the biochemical organic capping of their SeNMs, as efficacy appears to be lost when this organic cap is extracted (Cremonini *et al.*, 2018).

10.4.3 Selenium nanoparticles toxicity towards Eukaryotes

The cytotoxic effects of nanomaterials on various body tissues and their interaction with various cell types is still poorly understood. The characteristics of the NMs including size, shape, quantity, charge and surface structure along with the target cell type and incubation conditions influence their effect on a vast array of biological properties (Beyth *et al.*, 2015). One sees positive effects on cell growth and proliferation as well as vasodilation, whereas negative effects can be genotoxicity, carcinogenesis, apoptosis, and inhibition of cell proliferation. As noted below, some of these negative effects are exploited therapeutically.

There does not seem to be much cytotoxicity associated with BioSeNMs produced by bacteria towards normal eukaryotic cells. Antibacterial BioSeNMs showed only minor effects on human dendritic cells and fibroblasts (Cremonini *et al.*, 2016). Their lack of cytotoxicity is an exciting aspect of BioSeNMs as antimicrobials. Fluorescence microscopy performed on both Gram-positive and Gram-negative pathogens revealed the ability of BioSeNMs produced from plants to disrupt the bacterial cell structure, yet no cytotoxic effect was detected on human cell lines, indicating their biocompatibility for future applications (reviewed by Alam *et al.*, 2019). Overall, chemogenic SeNMs show more toxicity than Se oxyanions in aquatic toxicity studies (fish), whereas for mammals the oxyanions tend to be more toxic than the chemogenic SeNMs.

Beyond the toxicity towards humans, there are eukaryotic pathogens where BioSeNMs may be useful for their control, even though this aspect has been far less explored. Indeed, BioSeNMs have been explored for topical treatment of lesion infections from the protozoan *Leishmania* with an IC_{50} of 25 µg/ml (Soflaei *et al.*, 2014). Similarly, selenium supplementation helps control Trypanosomal infections (da Silva *et al.*, 2014), which provides another possible application of BioSeNMs.

10.5 MEDICAL APPLICATIONS OF SeNP FOR HUMAN HEALTH

10.5.1 Benefits of selenium for human health

Selenium is not only an important essential trace element, but over the past 30 years its role in protection of oxidative stress-induced DNA damage and oncogenic-induced DNA adduct formation has been elucidated. This protection occurs primarily through modulating glutathione peroxidases and thioredoxin

reductase. Selenium can also induce apoptosis in transformed and cancer cells. The mechanism for this apoptosis is considered to be through p53, mitogen-activated protein kinases (MAPK), and other cell signaling pathways, triggering redox-dependent apoptosis. Selenium has more recently shown promise as an anti-diabetic and anti-inflammatory agent, mostly through its antioxidant ROS scavenging abilities (Khurana *et al.*, 2019). In another context, selenium supplementation has been shown to ameliorate the toxicity of other heavy metals such as mercury (Watanabe, 2002) and arsenics (Gailer *et al.*, 2002). Such observations endorse selenium as a key nutritional element for humans, primarily for its antioxidant properties (Kielczykowska *et al.*, 2018), but is also noted for its antitumor, endocrine regulation, enhancing immune responses, and cognitive abilities (Guan *et al.*, 2018).

The efficacy of Se use for human applications for the above functions was noted to be enhanced and far more effective in the NM form (Guan *et al.*, 2018). There is considerable interest in the use of BioSeNMs, which have higher bioavailability and lower toxicity than inorganic or organoselenium nutritional supplements (Hosnedlova *et al.*, 2018). The inorganic ions are more quickly cleared by the body and nonspecific, whereas SeNPs are less toxic, provide a more even release as a nutritional supplement, and are more specific to tumors, thus leading to significant medical application potential (Guan *et al.*, 2018). The SeNPs for nutritional delivery are often capped with chitosan**.** In the past decade, Se delivered in the form of SeNPs has seen increased interest for medical and therapeutic use (Soumya *et al.*, 2018).

Although it is recognized that biogenically produced SeNPs are eco-friendly (Ingale & Chaudhari, 2013), few defined studies have compared the therapeutic differences between chemogenic and biogenically produced SeNMs. One can only project that due to the nature of the biochemical capping material, BioSeNMs would behave differently as compared to their chemogenic counter parts. Regardless, few studies to date exist where the cytotoxicity of bacterial produced SeNMs were evaluated (Cremonini *et al.*, 2016). From these studies one can only presume that the BioSeNMs can be used in the same biomedical applications as the chemogenic SeNMs. However, significant caution must be taken as there may be allergic and immunogenic responses to the biological derived molecules of the NP caps. As indicated above, these biochemical caps are composed mostly of uncharacterized biomolecules. Therefore, more research is required into BioSeNMs cytotoxicity before they are used in nutrition supplements in agriculture or for human health.

10.5.2 Biological synthesis of selenium nanoparticles for medical applications

Most of the synthetic procedures to produce metal(loid) nanomaterials rely on dangerous operational conditions and the use of toxic reagents, posing serious concerns regarding the disposal of the generated waste (Zhang *et al.*, 2006). This aspect also represents a disadvantage in terms of the clinical applications

of synthetic NMs (Jeevanandam *et al.*, 2018). On the other hand, the important role played by biological catalysts (e.g., bacteria and fungi) in reducing various sources of bulk metal(loid) precursors into the less bioavailable and less toxic nanoscale forms is now widely accepted and recognized as a valid, eco-friendly, and cost-effective strategy to produce NMs (Bhainsa & D'Souza, 2006; Song & Kim, 2009; Suresh *et al.*, 2004; Vetchinkina *et al.*, 2019). This has led the biotechnology search for improved processes, but these have problems as well.

10.5.3 Limitations of biological SeNM synthesis

As promising as the biosynthesis of SeNMs can be, there are still major drawbacks for the implementation of these procedures, such as the need to elucidate details of the biological processes behind NM production and the physical-chemical characteristics of the biogenic extracts. Indeed, the parameters influencing SeNM production, the biochemical processes governing their biogenic synthesis, their extensive physical-chemical characterization and their potential biotechnological applications are still the black holes of this emerging scientific field. All these slow down the implementation of biological systems as cell factories for SeNM synthesis. Making a parallel with the workflow behind chemical synthetic procedures, key aspects influencing the biogenic production of SeNMs are the choice of reducing agents, precursor-to-reducing agent ratio, reaction time, temperature and pH of the system. Additionally, the choice of microorganisms to exploit for SeNM production and their physiological states are among the most important parameters to consider, directly implying the need to deeply understand the bacterial genetic and metabolic backgrounds. For instance, the use of microorganisms highly tolerant towards Se oxyanions assure the use of higher concentrations of Se oxyanions which should provide higher yields. With more tolerant organisms, a greater concentration range can be used to control the biosynthesized nanomorphologies. Se precursor concentration and incubation time are fundamental parameters observed in all studies that need to be taken into account to optimize NM biosynthesis. This appears to be as complex as the biological system itself, bringing to light the necessity to further investigate the bacterial response to selenium species.

A major drawback of the biogenic SeNMs synthesis is represented by their recovery from the biomass used as a catalyst. Anecdotal evidence suggests only a small fraction of NMs are extracted out of the total amount produced, although this is hard to ascertain as in most studies yields and mass balances are not reported. Poor SeNM recovery from the biomass strongly discourages the implementation of large-scale manufacturing of NMs, making it imperative to develop defined recovery strategies. In this regard, the localization of biogenic SeNMs plays a fundamental role in terms of the feasibility and ease of their recovery. Extracellular production is preferred, as cell disruption by means of

expensive or non-*eco-friendly* procedures (e.g., sonication or chemical lysis) can limit the amount of product obtained. On the other hand, stable and crystalline SeNRs are generally obtained through bacterial intracellular processes, likely due to the high local concentrations of Se^0 atoms available for their deposition and growth as well as the availability of reductive processes (Presentato *et al.*, 2018a). Thus, depending on the desired final product, different cell factories could be used for SeNM biosynthesis, leading to the necessity to optimize multiple procedures for their recovery. Nevertheless, the identification and purification of bacterial enzymes responsible for Se precursor bioconversion may lead to the development of *in vitro* synthesis approaches that allow manufacturers to overcome issues encountered when working with live microorganisms.

Although biogenic SeNMs are highly valuable from a physical-chemical point of view (Piacenza *et al.*, 2018a), their polydispersity in size represents another disadvantage for applications, as several properties of material confined in the nano-range directly depend on a uniform size and shape. Thus, focusing the research on obtaining the most monodisperse NM population possible is of importance for many uses. This will be directly linked to increasing the knowledge around the microbial cell factory exploited and the consequences of varying their growth or exposure conditions to the metalloid precursors. In this regard, it has been suggested that the use of bacterial cells under resting mode or metabolically controlled conditions can limit the number of NS nucleation events occurring within the cells, decreasing the chances to obtain polydisperse populations (Piacenza *et al.*, 2018b; Presentato *et al.*, 2018b). Yet, using a low bacterial cell density results in decreased oxyanion bioconversion, leading to the necessity to adjust the precursor concentration to obtain a high amount of NMs produced (Piacenza *et al.*, 2019).

The complex organic material generally present within the BioSeNM extracts also requires more investigations aimed at elucidating the composition and concentration of the biomolecules present. Issues remain in characterizing the associated organic material, as the limit of detection of most techniques used to date to identify or quantify these biomolecules is too low for the amount of material normally recovered in lab bench-scale experiments. As indicated in Section 10.3.3, various techniques are used to identify the biomolecules associated with the BioSeNPs, although reports systematically evaluating the identification, concentration and ratios of these molecules are still missing in the literature. Additionally, the inherent complexity of biological systems of active growing cells in complex media makes it unlikely to always obtain the same concentration of the different biomolecules in the organic capping material. A solution could be found in using bacteria under defined growth conditions, which may represent more controllable work systems to standardize production procedures.

10.6 PERSPECTIVES OF THE BIOGENIC MANUFACTURING OF SELENIUM NANOMATERIALS

10.6.1 Medical applications

The field of BioSeNPs is progressing forward from its adolescence and it is time to move from the general discovery that SeNMs can be made with various microorganisms towards focused studies to control biogenic synthesis of SeNMs with specific sizes and/or shapes. Additionally, biogenic production research should also be tuning the BioSeNPs towards specific applications. Here we propose directions for the field:

- A recognized problem in the field of using BioSeNPs therapeutically, particularly in the use as an anticancer agent, is that studies have been poorly designed (as noted in reviews by Hosnedlova *et al.*, 2018; Khurana *et al.*, 2019). The issues are highlighted around missing appropriate comparisons to other Se sources, and from our point of view, missing thought toward the influences from the subtle differences in NP properties (size and shape) or consideration of the role of the biochemical capping material in their application and roles in toxicity or lack thereof.
- There is significant potential in the use of BioSeNPs to fight AMR, both as an antiseptic in pathogen control in topical treatments or for touch surfaces in pathogen transfer. However, their use as an oral antibiotic is yet to be properly explored and it will take longer for such a product to pass to the market. Additionally, when groups are evaluating their BioSeNPs for antimicrobial efficacy, defined pathogen indicator strains from strain depositories should be used, with universal robust antimicrobial testing protocols in order to provide effective and useful comparisons.
- There are still gaps to be filled in the microbial nanotechnology field. There is still a lot of fundamental research to be done. A complete understanding of the biochemical and biological processes behind selenate/selenite conversion to elemental selenium (Se^0), as well as the route exploited by microorganisms towards the assembly of these Se^0 atoms in nanostructures (NSs) is still lacking. Directed systematic studies are required to enhance our understanding of these processes.
- Characterization of the BioSeNPs needs to be standardized. The full chemical and biophysical characterization of the BioSeNP is not always carried out and also the assessment of the cap's biomolecule character and composition needs to be more routine. Defined purification and quantifying yields need to be developed and implemented to industrial scales if we are to see biogenic NMs used routinely.

10.6.2 Non-medical applications

Given the similarities between chemogenic and biogenic SeNMs in terms of physical-chemical features, the investigation regarding BioSeNMs potential

applications is still in its infancy as compared to those chemically synthesized. At this time, we see applications of BioSeNPs being almost entirely focused on biomedical applications. However, the particular features of Se at the nanoscale as well as the necessity to develop new 'low-carbon technologies' must push us to explore new properties of BioSeNMs beyond biomedical ones. Making a parallel with applications already evaluated for chemogenic NMs can support the development of innovative applications for BioSeNMs. Applications to consider are novel catalysts, nanobiosensors, environmental pollutant capture, photo and electrical devices, and others suggested in the review by Wadhwani *et al.* (2016). Here, we suggest the strength of the strategy to use microbes in selenium pollution remediation is not only to detoxify an environment, but also for the bioconversion of Se into useful SeNMs, allowing remediation biotechnologies to link to bioconversions for a value added outcome.

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