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Proteins in microbial synthesis of selenium nanoparticles

Anna V. Tugarova, Alexander A. Kamnev*

Laboratory of Biochemistry, Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, 13 Prosp. Entuziastov, 410049 Saratov, Russia

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ABSTRACT

Biogenic formation of nano-sized particles composed of various materials (in particular, selenium) by live microorganisms is widespread in nature. This phenomenon has been increasingly attracting the attention of researchers over the last decade not only owing to a range of diverse applications of such nanoparticles (NPs) in nanobiotechnology, but also because of the specificity of methodologies and mechanisms of NPs formation related to “green synthesis”. In this mini-review, recent data are discussed on the multifaceted role of proteins in the processes of microbial reduction of selenium oxyanions and the formation of Se NPs. Besides the involvement of proteins in reducing selenites and selenates, their participation in the microbially driven growth and stabilisation of Se NPs is analysed, which results in the formation of unique nanostructured materials differing from those obtained chemically. This mini-review is thus focussed on proteins involved in microbial synthesis of Se NPs and on instrumental analysis of these processes and their products (biogenic nanostructured selenium particles functionalised by a surface-capping layer of various biomacromolecules).

1. Introduction

Nanoparticles (NPs) have unusual physical, chemical and optoelectronic properties, which make them different from the bulk-like materials they are composed of. The properties of nanoparticles are determined by their greater surface-to-volume ratio, greater surface energy and geometrical constraints. Hence NPs serve as building blocks in new-generation optoelectronic electronic chemical and biochemical sensors [1] and have found applications in biology, medicine and environmental remediation [2–5].

Microbial synthesis of NPs, as compared with chemical methods, belongs to "green chemistry", a special area in chemistry that aims to minimise negative effects on the environment caused by the synthesis of chemicals. Thus, the involvement of biological objects (microorganisms including bacteria, fungi and viruses; plants, animals and/or enzymes obtained thereof) in biotechnological methods for obtaining NPs of various elements is an ecologically non-hazardous alternative to chemical syntheses and is currently in active development [6–10].

Biogenic formation of various nanostructured materials, including Se NPs, by live microorganisms, which is widespread in nature, has been increasingly attracting the attention of researchers over the last decade [5–7,9,11–13]. Besides a range of diverse applications of such NPs in nanotechnology [1,14], this is due to the specificity of methodologies and mechanisms of nanoparticle formation via “green synthesis” [11,12,15], considering also some specific properties of biogenic NPs which differ from those obtained chemically [16].

Moreover, detailed knowledge of the related microbially driven processes of selenium biomineralisation can provide biotechnological bases in developing bioremediation (phytoremediation) strategies for cleanup of Se-polluted soils or aquifers involving rhizospheric and soil or marine bacteria [5,13,17–19]. Besides that, nanostructures of elementary selenium have shown great potential for versatile biotechnological and medical applications [3,20–25].

As mentioned above, over the last years a few reviews have appeared on the production of NPs by various eukaryotic systems [15] and microorganisms (including marine microbiota) [7,8,26–29], etc., which is a direct evidence of the fast-growing attention to this topic. Note that the interest to bacteria capable of reducing selenium oxyanions has been growing over the last 20 years. Related studies addressed mechanisms of this phenomenon; efforts were made to identify proteins involved in the reduction process in various bacteria, such as *Thauera selenatis* [30–34], *E. coli* as a classical bacterial object [35], different aerobes and rhizobacteria: *Bacillus mycoides* [36] and *Stenotrophomonas maltophilia* [12] (*vide infra*).

2. Occurrence of microbial synthesis of selenium nanostructures in nature

As is now well established, numerous microorganisms are capable of reducing selenate ($Se^{VI}O_4^{2-}$) and selenite ($Se^{IV}O_3^{2-}$) oxyanions to

* Corresponding author.
E-mail addresses: tugarova_anna@mail.ru (A.V. Tugarova), aakamnev@ibppm.ru, a.a.kamnev@mail.ru (A.A. Kamnev).

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Se^0 as an elementary substance (largely, but not always, in different nanostructured forms). These include bacteria isolated from areas contaminated with various pollutants, including selenium compounds (*S. maltophilia* [37], *Bacillus* sp. [38], *Ralstonia metallidurans* [39], *Dechloromonas* sp., and *Thauera* sp. [40]); anaerobes that can utilise selenites and selenates in their respiratory chain as electron acceptors, often along with sulphites and sulphates (*Desulfomicrobium* sp. [41], *Desulfurispirillum indicum* [42], and hyperthermophilic archaea [43]); as well as some rhizosphere microorganisms (*Rhizobium* [44,45], *Pseudomonas* sp. [46] and *Azospirillum brasilense* [47,48]). As the topic is of multifaceted interest from both basic and applied aspects, new reports appear on microbial reduction of selenium oxyanions and Se NPs formation [49,50].

All these microorganisms can transform the toxic selenite and/or selenate anions to the insoluble (and hence nontoxic) Se^0. Most of published studies have reported the transformation of selenates or selenites to the amorphous red modification, a form of Se^0. The formation of spherical extracellular or intracellular deposits (Se NPs) have also been documented in [37,39,47,48] and other publications and reviewed in [33,51].

The mechanisms used by bacteria to reduce selenites and selenates are diverse and may include one or several metabolic pathways and enzymes as well as other proteins. It has been shown for several microorganisms that nitrate and nitrite reductases, which are responsible for denitrification, are involved in the reduction of Se^{IV} compounds. Selenite reduction to Se^0 may occur under the action of either nitrate reductase (e.g. in *E. coli* [52]) or nitrite reductase (e.g. in *Rhizobium* [44]). Thus, the ability to reduce selenites, selenates and tellurites is often linked to denitrification [53].

In *T. selenatis,* a selenate reductase was found that is involved in selenate reduction [30]. Selenite reduction also involves thiols (RSH): glutathione (GSH), most widespread among eukaryotic cells, the cyanobacteria, and the α-, β- and γ-groups of the proteobacteria, and its recently discovered functional analogue – bacillithiol (that differs from glutathione in chemical structure [36]).

3. Mechanisms of bacterial synthesis of selenium nanoparticles

We now consider in greater detail the currently known mechanisms of bacterial reduction of selenite and selenate with the formation of Se NPs and proteins involved in these processes.

The process of Se NP synthesis can be arbitrarily divided into four stages: (1) transport of Se oxyanions (in)to the cell; (2) the redox reactions as such; (3) export of elementary Se^0 nuclei out of the cell and (4) assembly of elementary Se^0 into NPs at the nuclei.

In this scheme, stages (2) and (4) are always present, whereas the presence of stages (1) and (3) depends on the localisation of stages (2) and (4): extracellular, in the periplasmic or intracellular space. Let us now consider each of the stages.

3.1. Transport of selenium oxyanions into the cell

The first step in Se metabolism, the transport of selenates and selenites into bacterial cells (and, in particular, the intracellular reduction of these oxyanions) has been little documented.

The selenate oxyanion (as well as the simple mononuclear molybdate, $Mo^{VI}O_4^{2-}$, and chromate $Cr^{VI}O_4^{2-}$) is structurally related to sulphate. Uptake of selenate oxyanions occurs mainly through sulphate permeases [54]. Thus, sulphate transport by the SulP-type Rv1739c permease of *M. tuberculosis*, expressed in *E. coli*, was inhibited by selenate [55], which suggests that this oxyanion can also be taken up through this permease. In contrast, there are no specific uptake systems for selenate and chromate; instead, these are taken up by transporters of structurally related oxyanions, such as sulphate or molybdate [54]. Besides that, there is a non-specific low-efficiency anion transport system that requires high molybdate concentrations, and which also transports sulphate, selenate and selenite; this transporter, however, has not been further characterised [54].

Uptake of selenite in *E. coli* is effected by the sulphate transport complex ABC, which is encoded by the cysAWTP operon. The complex is composed of two CysA ATP-binding proteins and two transmembrane proteins (CysT and CysP). In *E. coli*, selenite is also transported by sulphate permease, althought significant uptake has been observed after the repression of this transporter as well, indicating that there exists at least one more selenite uptake system [56]. A more rapid uptake of selenite after several hours of its slow uptake in the microorganism *Ralstonia metallidurans* may be explained by a slow activation of an unknown selenite transport system; thus, selenite accumulates in the cell by two competing routes, assimilation and detoxification (a slower route). These routes were shown to have similar kinetics [57]. Using biochemical and genetic approaches, it was shown that in *Rhodobacter sphaeroides*, transfer of selenite to the cytoplasm is presumably effected through a polyol transporter [58]. No specific transporters of selenite have yet been found in microorganisms. As the reduction of Se oxyanions does not always occur intracellularly, this stage is nonobligatory.

3.2. Bacterial mechanisms of reduction of selenium oxyanions

The second and the best studied stage is the direct reduction of Se oxyanions in redox reactions. One reduction mechanism is the dissimilatory (respiratory) reduction of selenate and selenite to Se^0 in reaction catalysed by anaerobic microorganisms living in oxygen-free sediments. About 16 diverse bacterial and archaeal species have been described that grow anaerobically and in which the oxidation of organic substrates or H_2 is associated with the dissimilatory reduction of Se oxyanions [16]. The end products of these reactions are the red amorphous or monoclinic allotropic modifications of Se^0, which accumulate in the medium and result from the microbial reduction of selenates or selenites to Se^0.

Bacterial reduction of selenate follows a sequential series of reductive steps, ultimately yielding Se^0 [34]. Eqs. (1) and (2) summarise the overall reactions:

$$Se^{VI}O_4^{2-} + 2e^- + 2H^+ \leftrightarrows Se^{IV}O_3^{2-} + H_2O; \quad (1)$$

$$Se^{IV}O_3^{2-} + 4e^- + 6H^+ \leftrightarrows Se^0 + 3H_2O. \quad (2)$$

The use of Se oxyanions as alternative electron acceptors is energetically advantageous. The free energies, when coupled with the energy obtained from H_2 oxidation, are significant, with values of 15.53 kcal mol^{-1} for SeO_4^{2-} and 8.93 kcal mol^{-1} for $HSeO_3^-$ [33]. The respiratory reductases for Se oxyanions contain molybdenum and are associated with the plasmic membrane.

Oremland et al. [16] investigated three physiologically and phylogenetically distinct species of selenate- and selenite-respiring bacteria: *Sulfurospirillum barnesii*, *Bacillus selenitireducens* and *Selenihalanaerobacter shriftii*. When grown with Se oxyanions as the electron acceptor, all these organisms formed extracellular granules composed of stable, uniform nanospheres (with diameters around 300 nm) of Se^0 having monoclinic crystalline structures. Se^0 also accumulated intracellularly. The intracellular accumulation of Se^0 could be reduced by first growing cells with nitrate as the electron acceptor and then adding selenite ions to washed cells. This resulted in the formation of primarily extracellular Se nanospheres. The purified extracellular nanospheres produced by the three species exhibited large differences in optical properties (UV–vis absorption and Raman spectra). In turn, the spectral properties differed substantially from those of amorphous Se^0 formed by chemical oxidation of H_2Se and of black, vitreous Se^0 formed chemically by reduction of selenite with ascorbite. Microbially synthesised Se nanospheres are unique, complex

arrangements of Se atoms. The differences in the synthesised NPs reflect a diversity of enzymes involved in dissimilatory reduction in different microorganisms. As pointed out by Oremland et al. [16], these conditions cannot be achieved by the presently available methods of chemical synthesis.

The mechanism of selenate reduction used by *T. selenatis* in the process of anaerobic respiration has been best explored [30–33]. In this bacterium, a respiratory selenate reductase (Ser) responsible for selenate reduction has been found and thoroughly studied. It is a heterotrimer consisting of three subunits (SerA, SerB and SerC), with a native molecular weight of 180 kDa. It has a high affinity for selenate with a *Km* of 16 μM and does not use selenite, nitrate, nitrite, sulphate, arsenate or arsenite as substrates [33]. The resultant product from SerABC is selenite (SeO_3^{2-}). No respiratory selenite reductase has been found thus far. Possible mechanisms of Se NP formation in *T. selenatis* have been described by Debieux et al. [34] and Butler et al. [51].

In particular, Butler et al. [51] considered in detail how *T. selenatis* can utilise selenate as a respiratory substrate and dispose of the Se by stabilising and secreting Se NPs. The reductive pathway starts by the reduction of selenate to selenite in the periplasm by the Ser. The reaction is bioenergetic, drawing electrons from the Q-pool via a dihaem *c*-type cytochrome. The electron transfer, if mediated by a quinol-cytochrome *c* oxidoreductase, could generate $2q^+/2e^-$ of proton electrochemical gradient. The resultant selenite is thought to be taken across the membrane into the cytoplasm by a currently unknown transporter. Once in the cytoplasm, the reduction of selenite leads to the accumulation of Se deposits as Se NPs that are ultimately secreted into the surrounding medium. The protein SefA is associated with Se biomineralisation in *T. selenatis* and possibly plays an active role in Se NPs assembly (*vide infra*). Analysis of the available genome sequences shows that SefA is among a distinct (although niche) family of proteins (the mineralins) that might function to facilitate biomineralisation and possibly export of minerals from bacteria [51]. Stolz et al. [33] demonstrated that this respiratory selenate reductase is part of the DMSO reductase family and is most closely related, on the basis of sequence alignment, to the chlorate reductase (ClrA, 84% identity and 96% similarity) from *Ideonella dechloratans*, dimethyl sulphidedehydrogenase (DdhA, 43% and 62%) from *Rhodovulum sulfidophilum*, and ethylbenzene dehydrogenase (EbdA, 31% and 47%) from *Azoarcus* sp. EB 1 [33].

In *T. selenatis*, the reduction of selenite does not support growth, and selenite is not a respiratory substrate. There has been much debate regarding the mechanisms by which selenite is reduced to Se in bacterial cells. In the recent years, there has been an increasing discussion of the involvement of intracellular thiols in selenite reduction. In *Enterobacter cloacae* and *E. coli*, selenite reduction may occur through a pathway involving glutathione (the tripeptide γ-glutamylcysteinylglycine) and organoselenium intermediates [59,60], and in *T. selenatis*, it involves bacillithiol (Cys-GlcN-mal; L-cysteinyl-D-glucosamine conjugated with malic acid as a thiol compound**)** [34]. Such reduction is accompanied by release of O_2 and creates oxidative stress. In the presence of selenite, *R. metallidurans* CH3 overexpresses Fe-contaning superoxide dismutase, an enzyme associated with oxidative stress [61]. This might confirm the hypothesis that in this bacterium also, glutathione is involved in Se reduction. Kessi and Hanselmann [62] examined the possible role of the glutathione/glutathione reductase system in the formation of Se^0 from SeO_3^{2-}. Using the phototrophic proteobacterium *Rhodospirillum rubrum,* they showed that the rate of selenite reduction decreased when the bacterium synthesised smaller than normal quantities of glutathione, whereas in *R. sphaeroides* and *E. coli*, the reduction of SeO_3^{2-} induced glutathione reductase activity. Garbisu et al. [63] also observed considerable induction of thioredoxin and thioredoxin reductase in *B. subtilis* grown with a millimolar concentration of selenite. This mechanism was subsequently confirmed: for *B. selenitireducens*, peroxiredoxins were identified that contained catalytic cysteine thiols [64]. The mechanism of Se reduction in *Bacillus* that was proposed by Lampis et al. [36] also presupposes the involvement of bacillithiol – a recently found analogue of glutathione [65] that differs from it in chemical structure (*vide supra*).

Kessi and Hanselmann [62], while investigating the possible involvement of the Painter-type reaction (3) in selenite reduction to Se^0 in *R. rubrum* and *E. coli*, suggested that, at first, there occurs quick formation of a Se-digluthathione intermediate, which is followed by Se^0 production. The Painter-type reaction was so called after the investigator who had observed the high reactivity of selenite with thiol groups. He was the first to demonstrate the formation of selenodisulphides (RS-Se-SR) [66]. Currently, the Painter-type reaction (3) is considered one of the basic reactions in the microbial detoxification of oxyanions:

$$4RSH+H_2SeO_3 \rightarrow RS\text{-}Se\text{-}SR+RSSR+3H_2O. \quad (3)$$

There were also indications on the formation of reactive oxygen species (ROS; e.g. $O_2^{\bullet -}$) in the Painter-type reaction involving glutathione (GSH) [67]; however, the full reaction was not presented.

Glutathione functions as an electron donor in the reduction. The first intermediary product is selenodiglutathione (GS-Se-SG), a substrate for glutathione reductase and also for bacterial thioredoxin. The oxidised thioredoxin is regenerated by thioredoxin reductase. The reduction of selenodiglutathione by glutathione reductase (GR) or thioredoxin reductase (TR) yields glutathione selenopersulphide (GS-Se^-), which dismutates into reduced glutathione (GSH) and Se^0. In the biological reaction, superoxide degradation is catalysed by the enzymes induced under oxidative stress. The electron source for the regeneration of glutathione is NADPH.

Despite the fact that GSH is regarded as the main candidate for reducing selenite (and its involvement in this process has been documented in various bacteria), as discussed above, in some bacteria there is an alternative way. Thus, for *Azoarcus* sp., a physiologically versatile group of beta-proteobacteria of great environmental relevance, the main molecular mechanism of selenite resistance for cells growing under anaerobic conditions was proposed based on the existence of an uncharacterised energy-dependent exporter of selenite out of the cell, that is functional only at the exponential phase of growth [68]. The main source of reduced glutathione in the cell is glutathione reductase (GOR), but any *gor* gene orthologue in the genome of the strain studied was not identified. Moreover, the addition of 3 mM buthionine sulphoximide (BSO, a compound that had been shown to decrease the intracellular GSH concentration) to the *Azoarcus* cells, growing anaerobically in the presence of 1 mM selenite, did not decrease the formation of SeNPs as compared with that observed when the cells grew in the absence of BSO. These results suggest that selenite reduction in *Azoarcus* sp. does not depend on levels of intracellular glutathione.

There is also a fairly large literature on the involvement of nitrate reductases in selenite reduction. One can speculate that in this case, an important role may be played by the structural similarity between selenite and nitrate ions. It is known that structurally similar ions (oxyanions) can often replace each other in biochemical reactions [69]; in this case, nitrate (NO_3^-) is replaced by selenite (SeO_3^{2-}). It can be assumed that bacteria, which are capable of nitrate reduction and have nitrate reductases, will be able to reduce selenite but not selenate (SeO_4^{2-}), the structural formula of which differs largely from those of nitrate and selenite. This is what was observed, for example, in the case of reduction of Se oxyanions by the rhizobacterium *Azospirillum brasilense* (having well documented denitrification traits), which indeed can reduce selenite but not selenate [48].

This assumption is also supported by the data reported by Watts et al. [70] and Ridley et al. [59] that the reduction of nitrate and selenate in *E. cloacae* is performed by two different membrane-bound enzymes, each of which catalyses the reduction of either nitrate or selenate, but not both of them. Purification of nitrate reductase from

solubilised membranes of *E. cloacae* was followed using SDS-PAGE, nondenaturing PAGE and activity assays. The nitrate reductase enzyme was purified to homogeneity by using two sequential anion-exchange columns; SDS-PAGE of the purified nitrate reductase showed two peptides resolved at molecular masses of 140 kDa and 58 kDa featuring its α and β subunits [59]. Nondenaturing polyacrylamide gel electrophoresis (PAGE) gels stained for selenate and nitrate reductase activity revealed that two distinct membrane-bound enzymes catalyse the reduction of either selenate or nitrate [70].

It has recently been reported that *Salmonella enterica* (serovar *Typhimurium*), a Gram-negative bacterium with a flexible respiratory capability, under anaerobic conditions can utilise a range of terminal electron acceptors, including selenate, to sustain respiratory electron transport [71]. Selenate reductase from this bacterium was shown to be a membrane-bound enzyme encoded by the *ynfEFGH-dmsD* operon; the active enzyme was predicted to comprise at least three subunits where YnfE is a molybdenum-containing catalytic subunit.

However, despite the aforementioned arguments on structural similarity, yet there have been reports on the reduction of selenate by nitrate reductases [53]. It has also been mentioned that selenate reductase (SER) from *T. selenatis* is a member of a distinct class of the TAT-translocated type II molybdoenzymes and is closely related to a group of thermostable nitrate reductases (pNAR) found in hyperthermophilic archaea [72]. Thus, further research is necessary in order to elucidate the mechanisms of reduction of selenites/selenates involving nitrate/nitrite reductases.

In *R. sphaeroides*, selenite reduction supposedly involves some chaperones, elongation factors, and some enzymes responsible for oxidative stress reactions [58]. Selenite reduction by *Shewanella oneidensis* is mediated by fumarate reductase in the periplasm [73].

A genetic approach was undertaken by Guymer et al. [74] to determine factors required for selenate reductase activity in *S. enterica* serovar *Typhimurium* and *E. coli*. It has been reported for both biological systems that an active Tat translocase (the twin-arginine transport (Tat) system is dedicated to the translocation of folded proteins across the bacterial cytoplasmic membrane) and a TorD-like chaperone (DmsD) are required for complete *in vivo* reduction of selenate to elementary red selenium. Further mutagenesis and *in vitro* biophysical experiments implicate the *Salmonella ynfE* gene product, and the *E. coli* YnfE and YnfF proteins, as putative Tat-targeted selenite reductases.

Kuroda et al. [75] also used a genetic approach to identify the genes involved in selenate reduction in the selenate- and arsenate-reducing bacterium *Bacillus selenatarsenatis*. The product had typical features of membrane-bound and molybdopterin-containing oxidoreductases. Those authors proposed that the *srdBCA* operon encoded a respiratory selenate reductase complex, featuring the first report of genes encoding selenate reductase in Gram-positive bacteria.

To summarise, selenite can be reduced to Se^0 through reactions involving nitrate/nitrite reductases, as well as thiol groups of peptides or proteins (in Painter-type reactions). Other proteins, including molecular chaperones and elongation factors, may also have a role in the reduction of selenites and selenates to Se^0. Some other details of the reduction processes and Se^0 formation can be found in recent related reviews [11,76]. Despite the evident existence of multiple possible mechanisms of Se oxyanion reduction as well as enzymes and peptides involved in these processes, there are still debatable questions on the possibility of their parallel functioning and on the conditions for their realisation, which requires further investigations.

3.3. Assembly of selenium nanoparticles of bacterial origin

In this section, stages (3) and (4) are considered (export of Se^0 nuclei out of the cell through the membrane and assembly of Se^0 NPs, respectively; *vide supra*). Evidently, stage (3) is not obligatory if both the reduction of Se oxyanions and SeNPs assembly occur either extra- or intracellularly. It should take place only in the case of the intracellular reduction and extracellular assembly. The information on how this transmembrane 'carry-over' of Se^0 nuclei occurs is so far insufficient. There are several hypotheses of such transfer.

For *Thauera selenatis* it has been supposed that, once in the cytoplasm, selenite is reduced, and the resultant Se^0 binds to SefA (a specific ca. 95-kDa "Se factor A" protein up-regulated and secreted in response to increasing selenite concentrations), forming a Se nanosphere prior to its export from the cell [34,76]. However, the process by which SefA–Se^0 is exported from the cell remains unknown. It has been shown by *in vitro* assays that SefA has a role in stabilising Se nanospheres during selenite reduction by GSH [34]. Lampis et al. [12] propose a different mechanism for *Stenotrophomonas maltophilia*: reduced Se NPs seeds are formed intracellularly and then released outside the cells. One mechanism for secretion of both soluble and insoluble material is the process of outer membrane vesiculation: in response to stress, a section of the outer membrane forms a distinct spherical vesicle composed of a lipid bilayer and encloses material exclusively from the periplasm. However, Lampis et al. [12] conclude that "at this point it is not clear if there is an active system for SeNP secretion or if it is a result of some cells bursting".

Let us consider in more detail the next stage (4), the assembly of the reduced Se into Se (including NP) formations. This stage has been understudied, and it is not entirely clear how exactly the assembly of Se NPs occurs after the reduction of Se oxyanions. Some authors suggested that the formation of NPs might involve an Ostwald ripening mechanism [36]. Kessi and Hanselmann [62] suggested that larger-sized Se NPs could form by aggregation of small ones: the small sizes of Se^0 particles (35–45 nm) present suggested that the smallest particles purified from the culture medium represented the original size of biologically produced Se^0 particles. The larger particles (250–300 nm), which sedimented during centrifugation of the cells, were assumed to be produced by aggregation of the 35–45-nm particles in cells of *R. rubrum* grown in the presence of selenite. As distinct from the NPs synthesised chemically, microbiologically produced Se NPs contain proteins [34,64] (among other bioorganic constituents; *vide infra*). These proteins are associated with Se NPs and are thought to have a stabilising role, in addition to the classical mechanisms of NP assembly.

4. Analytical studies of microbially produced Se NPs

Characterisation of biogenic Se NPs, including their bioorganic (particularly, proteinaceous) surface-associated constituents, involves a range of conventional modern analytical and biochemical techniques (in line with those that are commonly used for studying nanoparticles, i.e. UV–visible and vibrational (mainly FTIR and Raman) spectroscopies, X-ray diffraction (XRD), scanning (SEM) and transmission electron microscopy (TEM), dynamic light scattering (DLS) and some others). Thus, in Se NPs (also within cell biomass samples) the presence of selenium can be confirmed by X-ray fluorescence analysis (XFA) and electron energy-loss spectroscopy (EELS) (see, e.g. [48]), whereas its oxidation state can be determined by using X-ray absorption techniques (XANES and EXAFS) [39,57,77,78]. Special consideration has to be given to determining in which allotropic modification(s) elementary selenium is present in NPs.

Note that, in contrast to crystalline solids where the order is governed by the laws of dynamics and thermodynamics, a lack of long-range periodicity in amorphous solids is responsible for several anomalies. Although the relation between those anomalies and the 'bulk structure' is generally clear, the surface structure and the corresponding vibrational spectra of amorphous solids are insufficiently studied, particularly for amorphous selenium [79]. Resolving the structure of amorphous surfaces is of special importance for explaining (and, especially, prognosing) the properties of nanostructured particles in which the surface-to-mass ratio is significant. It is

important that the use of Raman spectroscopy allows different modifications of elementary Se^0 (including those in microbially produced NPs) to be distinguished owing to differences in main vibration modes [80–82].

As mentioned above, bacterially synthesised Se NPs may be covered by various surface-associated bio(macro)molecules, including proteins, polysaccharides and lipids [12,83–85]. In this case, Fourier transform infrared (FTIR) spectroscopy can provide useful semiquantitative information on the presence of bioorganic components of nanoparticles' capping layers and their relative contents featured by vibration modes of their typical functional groups [82,86]. Particularly for proteins of the Se NPs 'capping layer', their secondary structure can differ from that of natural cellular proteins, as featured by the positions of various spectral components of the amide I band (typically within the region 1620–1680 cm^{-1}) (see, e.g. [86] and references cited therein). Surface-associated bio(macro)molecules of the Se NPs capping layers were reported to be responsible for negative values of zeta potentials owing to the presence of negatively charged functional groups (e.g. ionised carboxyl moieties) [83,86]. These similarly charged groups evidently serve as an additional factor stabilising Se NPs in aqueous suspensions and their morphology in the course of their biogenesis. Nevertheless, while FTIR spectroscopy is sensitive to the presence of various types of bio(macro)molecules and their structural modifications (which is particularly important for proteins), it cannot provide their individual characteristics, which requires additional methods of classical biochemistry (such as sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) for the detection and analysis of proteinaceous or polysaccharide components of the Se NPs capping layers, as well as proteomic methods for the analysis of the related proteins).

In the comprehensive study by Lenz et al. [64], high-affinity proteins associated with selenium nanoparticles (synthesised by *S. barnesii)* were isolated using density-based centrifugation in sodium polytungstate solution (SPTS), with their further separation by SDS-PAGE and capillary liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) analysis (the MS/MS spectra were searched against the NCBI data bank). It has been demonstrated that Se NPs can be associated with a great variety of high-affinity proteins. The protein modification on the Se NPs is expected to change the physicochemical properties of the Se nanostructured solid (at least, to some extent) and, as a consequence, influence the environmental fate of Se, as was shown for other bionanominerals. The high-affinity proteins identified by Lenz et al. [64] might be used to design specific probes for immunofluorescence or immunoelectron microscopy to further study and visualise the mechanisms of NP formation. It is also important to note that identification of proteins associated with Se NPs critically depends on the successful isolation of proteins associated intrinsically from adventitiously associated proteins that are potentially copurified in the density-based centrifugation in SPTS.

On the one hand, owing to the high importance of Se as a micronutrient for animal and human health, there is a clear need to further study the association of the protein fractions with NPs, e.g. by proteomic analysis focusing on expression of the proteins/enzymes found. On the other hand, future studies should also consider the dissociation or biodegradation of the protein fractions on Se (or other bionanominerals), hence opening a fascinating yet challenging future field of interdisciplinary biogeochemical research.

As mentioned above for *T. selenatis,* it has been shown that biogenic Se nanospheres are associated with a protein of approximately 95 kDa, called Se factor A (SefA; homologues of known function have not been reported previously) [34]. Experiments to investigate the expression and secretion profile of this protein have demonstrated that it is up-regulated and secreted in response to increasing selenite concentrations. The protein was purified from Se nanospheres, and peptide fragments from a tryptic digest were used to identify the gene in the draft *T. selenatis* genome. A matched open reading frame was located, encoding a protein with a calculated mass of 94.5 kDa. N-terminal sequence analysis of the mature protein revealed no cleavable signal peptide, suggesting that the protein is exported directly from the cytoplasm.

The *sefA* gene was cloned and expressed in *E. coli*, and the recombinant His-tagged SefA was purified. *In vivo* experiments demonstrated that SefA formed larger (~ 300-nm) Se nanospheres in *E. coli* when treated with selenite, and these were retained within the cell. *In vitro* assays demonstrated that the formation of Se nanospheres upon the reduction of selenite by glutathione was stabilised by the presence of SefA. Thus, the role of SefA in Se nanosphere assembly has the potential for use in bionanomaterial fabrication. The identification of a protein that can stabilise Se nanospheres secreted from *T. selenatis* could, through molecular engineering, enable particles to be produced with structural arrangements that are not only unique but also have yet to be reproduced by conventional chemical synthesis. The extracellular secretion of such nanoparticles from *T. selenatis* also has distinct benefits when compared to those bacterial systems that display intracellular accumulation [34]. Amino acid sequence of SefA revealed no cysteine residues, indicating that the Se–SefA interaction does not involve thiol moieties. This apparently means that the proteins and/or enzymes involved in selenium oxyanion reduction differ from those involved in Se NPs assembly.

Dobias et al. [35] investigated the role of proteins in controlling selenium nanoparticle size. A proteomic study was carried out, and proteins associated with biogenic Se NPs produced by *E. coli* were compared to chemically synthesised Se NPs and magnetite nanoparticles. Those authors identified four proteins with different functions: propanol-preferring alcohol dehydrogenase (AdhP), isocitrate dehydrogenase [NADP] (Idh), Outer membrane protein C precursor (Porin ompC, OmpC) and isocitrate lyase (AceA) that bound specifically to SeNPs. A narrower size distribution as well as more spherical morphology were observed when the particles were synthesised chemically in the presence of the proteins. A more detailed study of AdhP confirmed the strong affinity of this protein for the SeNP surface and revealed that this protein controlled the size distribution of the SeNPs and yielded a narrow size distribution with a three-fold decrease in the median size. These results suggest that proteins may become an important tool in the industrial-scale synthesis of SeNPs of uniform sizes and properties.

Proteomic analysis performed for *S. maltophilia* also showed the presence of an alcohol dehydrogenase (AdH) homologue, possibly associated with the biogenic synthesis of Se NPs. This result was confirmed by the identification of the gene encoding for alcohol dehydrogenase in the draft genome sequence of *S. maltophilia* SeITE02 [12].

A selenium-binding protein (SeBP; 42 kDa) from *Methanococcus vannielii* was identified by Self et al. [87], and the gene encoding a SeBP monomer was isolated and overexpressed in *E. coli*, giving a recombinant rSeBP protein which consists of identical 8.8-kDa subunits, each containing a single cysteine residue. When isolated in the absence of reducing agents, rSeBP contained oxidised cysteine (89%) and very little bound selenium (≤ 0.05 equivalents per subunit). Further studies showed [88] that, with selenite as the selenium source and the isolated protein (reduced with tris(2-carboxyethyl)phosphine) as a sole reductant, binding of one Se per tetramer under anaerobic conditions required four cysteine thiol groups, one at each subunit. In the corresponding Painter-type reaction with reduced glutathione (GSH), equimolar amounts of selenodiglutathione (GS-Se-SG) and glutathione disulphide (GS-SG) were formed from selenite and 4 GSH. At GSH-to-selenite ratios over 4:1, GS-Se-SG was reported to convert to a perselenide derivative, $GS\text{-}Se^-$. However, with the reduced rSeBP as a sole electron donor in the reaction with selenite, further conversion of the RS-Se-SR product was not observed. It is thus logical that prior alkylation of the cysteine thiol groups in reduced rSeBP was

found to prevent selenite reduction and selenium binding under comparable conditions [88].

To conclude, a range of proteins have been reported, both already known and newly identified, associated with selenium in microbially produced Se NPs. However, this information is still scarce. Besides that, the following questions remain open: how the assembly of such NPs occurs, what the role(s) of proteins is (are) and which proteins are involved; whether they serve as a matrix at which Se "seeds" are assembled, or they cover already formed small (primary) NPs (or both variants are in action). In any case, it is clear that microbial synthesis of Se NPs cannot be regarded as a purely chemical process (such as "Ostwald ripening" which is characteristic of nanoparticles obtained by classical "wet" methods, or the Gibbs-Thomson law (see, e.g. [89,90] and references therein)). Using conventional biochemical (gel electrophoresis, etc.) and modern proteomic methods and bioinformatics (e.g., LC-ESI-MS/MS and protein identification in databases), together with modern instrumental (spectroscopic) techniques allows for moving forward in studying structural and compositional properties of biogenic Se NPs.

5. Drawbacks and limitations vs. advantages and perspectives of microbial synthesis of selenium nanoparticles

From the viewpoint of nanobiotechnology, there are a number of problems yet to be solved in microbial synthesis of Se NPs, the main of which are: 1) heterogeneity (particularly, size heterogeneity) of biogenic NPs; 2) relatively large duration of synthesis (up to several days). Along with these drawbacks, it is now possible to reveal some advantages of biogenic syntheses as compared with classical chemical methods: a combination of several processes (NPs biosynthesis, their functionalisation and stabilisation) [85], biological compatibility and lower toxicity [91], unique spectroscopic (optical) characteristics [16]. As pointed out by Oremland et al. [16], these conditions cannot be achieved by the presently available methods of chemical synthesis. Microbial cells work as microscopic "factories" producing already biofunctionalised Se NPs (capped with various biomacromolecules). It is important that Se NPs synthesised by different bacteria include bioorganic molecules with different biological activity. As is often the case, some of the drawbacks of microbial synthesis of Se NPs are "the other side" of their advantages: this diversity of the biofunctionalised Se NPs in each case requires additional thorough analyses and studies of the biomacromolecules in the capping layers. For applications of biogenic Se NPs in animal husbandry and human nutrition, additional toxicity tests are required (*vide infra*).

It is also of interest from a practical viewpoint that Se NPs produced by Gram-negative *S. maltophilia* and Gram-positive *B. mycoides* both achieved much stronger antimicrobial effects than synthetic selenium nanoparticles [92]. This phenomenon also needs comprehensive scientific explanation and justification.

On the other hand, the phenomenon of microbial formation of Se NPs is interesting and important *per se*, being an intrinsic component of the biogeochemical cycle of selenium [76,78], and requires further studies for a more complete understanding of selenium transformation and redox processes in the environment.

Despite actively developing studies of selenium oxyanion reduction (particularly with the formation of nanostructured products) and different suggested mechanisms, details and characteristics of these microbially driven process are yet incompletely understood [12,68,76]. Hopefully in time, when the molecular mechanisms of such synthesis appear to be known in more detail, along with relationships and regulators allowing microbial synthesis of Se NPs to be controlled, together with genetic and proteomic approaches involved, this phenomenon will find its place in the field of "green chemistry". The latter is in active development nowadays and presents a more ecologically friendly alternative to the classical chemical synthesis [93]. In particular, such knowledge may be utilised for creating and development of biosystems involving enzymes and other proteins (not necessarily with the use of live bacteria) for nanobiotechnology.

It is also evident that even now, the information accumulated so far is sufficient to be applicable for developing phytoremediation strategies to clean up highly seleniferous environments. These approaches may involve the use of natural or transgenic Se-accumulating plants [94–96] and various phytostimulating soil microorganisms [96,97], including plant-growth-promoting rhizobacteria (PGPR, e.g. in the form of plant-microbe associations), such as PGPR of the genus *Azospirillum* [48,86]. On the other hand, Se-enriched plant biomass obtained from phytoremediation could be used as green fertiliser (especially where Se is in deficiency) or as selenium-fortified fodder or foodstuff [96]. Among possible limitations of these approaches, the main are: the levels of microbial and plant tolerance to various selenium species at their critical concentrations in soils and aquifers; bioavailability of selenium compounds in the environments (which may be affected by soil organic matter [98]); compatibility of phytostimulating microorganisms with plant species applied in phytoremediation; the level of Se accumulation in plant tissues.

The development of Se-fortified food or fodder additives, including those containing Se NPs, with the use of lactic acid bacteria or bifidobacteria (see, e.g. review [99]) may be advantageous in comparison with those produced by other means, as the former products are likely to be more compatible with living organisms. Nevertheless, the assessment of selenium and selenium species bioavailability in such foodstuff is of special concern, especially in the context of human nutrition [100], since selenium, depending on the dose (in a relatively narrow range) and bioavailability, may equally be "a source of aliment and ailment" [101].

6. Conclusions and outlook

Detailed understanding of the mechanisms involved in Se NPs biosynthesis and, particularly, the multiple roles of proteins in these processes, is of undoubted importance from both basic and applied aspects. This may serve as a basis for verifying biogeochemical origins of mineral deposits or creating biotechnological strategies for bioremediation (phytoremediation) of polluted soils or aquifers by using soil and rhizospheric microorganisms, including phytostimulating bacteria, possibly in associations with Se-accumulating plants.

Concerning those proteins (including enzymes) which participate in the processes of Se oxyanion reduction, knowledge of their roles could show ways to control those processes, ultimately resulting in methods of obtaining Se NPs of controlled sizes and morphology. On the other hand, proteins which have been reported among the bioorganic constituents capping the assembled biogenic Se NPs, play a significant role in fabrication of biofunctionalised nanostructured Se^0 particles with a variety of possible applications.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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