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# FTIR and Raman spectroscopic studies of selenium nanoparticles synthesised by the bacterium *Azospirillum thiophilum*

Anna V. Tugarova, Polina V. Mamchenkova, Yulia A. Dyatlova, 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

Vibrational (Fourier transform infrared (FTIR) and Raman) spectroscopic techniques can provide unique molecular-level information on the structural and compositional characteristics of complicated biological objects. Thus, their applications in microbiology and related fields are steadily increasing. In this communication, biogenic selenium nanoparticles (Se NPs) were obtained via selenite ($SeO_3^{2-}$) reduction by the bacterium *Azospirillum thiophilum* (strain VKM B-2513) for the first time, using an original methodology for obtaining extracellular NPs. Dynamic light scattering (DLS) and transmission electron microscopy (TEM) showed the Se NPs to have average diameters within 160–250 nm; their zeta potential was measured to be minus 18.5 mV. Transmission FTIR spectra of the Se NPs separated from bacterial cells showed typical proteinacious, polysaccharide and lipid-related bands, in line with TEM data showing a thin layer covering the Se NPs surface. Raman spectra of dried Se NPs layer in the low-frequency region (under 500 $cm^{-1}$ down to 150 $cm^{-1}$) showed a single very strong band with a maximum at 250 $cm^{-1}$ which, in line with its increased width (ca. 30 $cm^{-1}$ at half intensity), can be attributed to amorphous elementary Se. Thus, a combination of FTIR and Raman spectroscopic approaches is highly informative in non-destructive analysis of structural and compositional properties of biogenic Se NPs.



## 1. Introduction

Vibrational (Fourier transform infrared (FTIR) and Raman) spectroscopic techniques represent a well-established complementary and highly informative analytical arsenal, which has been successfully used in biology, particularly in microbiology and related fields, for studying samples of various complexity, from simple organic and biological molecules to supramolecular biosystems, cells and tissues (see, e.g. representative reports [1–11] and references cited therein). In our previous studies [12–19], FTIR and Raman spectroscopic techniques were used to monitor molecular-level changes in the structure and composition of cellular macrocomponents that accompanied metabolic responses of different strains of the ubiquitous rhizobacterium *Azospirillum brasilense* [20,21] (which showed different adaptation capabilities and often different ecological behaviour) to various stress conditions.

As was concluded in our recent review [22], vibrational spectroscopic techniques can also be useful in studying microbially synthesised selenium nanoparticles (Se NPs), FTIR spectroscopy being indispensible for analysing the bioorganic surface-associated components whereas Raman spectroscopy being sensitive to differences in various allotropic modifications and crystallinity of selenium in Se NPs.

The genus *Azospirillum* includes more than 15 species of bacteria which occupy different ecological niches [20,21]. For the first time for azospirilla, it has been shown [23,24] that *A. brasilense* (strains Sp245 and Sp7), a ubiquitous and most widely studied phytostimulating rhizobacterium of the genus *Azospirillum*, can reduce selenite ($SeO_3^{2-}$) to elementary selenium ($Se^0$) in the form of Se NPs (nanospheres, 50–400 nm in size). The ability of bacteria to reduce selenium oxyanions to elementary $Se^0$, besides possible nanobiotechnological applications of the biogenic Se NPs, can be promising for bioremediation of excessively selenium-rich soils and aquifers [22–26].

Using strain *A. brasilense* Sp7, a scheme has recently been developed for the synthesis of extracellular Se NPs more homogeneous in size (around 90 nm in diameter); their zeta potentials were determined, and the Se NPs obtained were characterised by FTIR spectroscopy [27]. In particular, Se NPs separated from bacterial cells showed FTIR bands of proteins, polysaccharides and lipids associated with the particles (in line with their TEM images which showed a thin layer over the NPs), in addition to strong carboxylate bands, which evidently stabilised the NP structure and morphology [27].

In this study, biogenic selenium nanoparticles (Se NPs) were obtained via selenite ($SeO_3^{2-}$) reduction by another species of *Azospirillum* (isolated from a sulphide-containing spring), *A. thiophilum* (strain

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

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VKM B-2513) [28], for the first time, using the original methodology [27] for obtaining extracellular NPs. The resulting Se NPs isolated from the bacterial cells were characterised by dynamic light scattering (DLS) and transmission electron microscopy (TEM), as well as by FTIR and Raman spectroscopic techniques.

## 2. Materials and Methods

### 2.1. Bacterial Strain and Growth Conditions

Strain *Azospirillum thiophilum* VKM B-2513 [28] was taken from The Collection of Rhizosphere Microorganisms [WDCM 1021], according to the World Federation of Culture Collections, http://www.wfcc.info/ccinfo/collection/by_id/1021 maintained at the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov, Russia. Bacteria were cultivated in a liquid modified malate salt medium (MSM) [29] which contained the following salts ($g \cdot l^{-1}$): $K_2HPO_4$, 3.0; $KH_2PO_4$, 2.0; $NH_4Cl$, 0.5; NaCl, 0.1; $FeSO_4 \cdot 7H_2O$, 0.02 (added as chelate with nitrilotriacetic acid); $CaCl_2$, 0.02; $MgSO_4 \cdot 7H_2O$, 0.2; $Na_2MoO_4 \cdot 2H_2O$, 0.002; sodium malate, 5.0 (obtained by mixing 3.76 g of malic acid with 2.24 g NaOH per litre), yeast extract, 0.1; final pH 6.8–7.0.

The culture (volume 100 ml in 250-ml Erlenmeyer flasks) was grown under aerobic conditions on a shaker (180 rpm) for up to 18–20 h at 28° C. Cell growth was monitored at $\lambda = 595$ nm (Spekol 221, Germany); the optical density ($A_{595}$) values of the resulting culture suspensions were about 0.7–0.8 (optical thickness 1 cm).

### 2.2. Bacterial Synthesis of Se NPs and Their Purification

Se NPs were synthesised by the strain *A. thiophilum* VKM B-2513 using the scheme reproduced for this bacterium species from our earlier work [27], in the presence of 5 mM $Na_2SeO_3$. All the next steps were performed under sterile conditions. Bacterial cells were harvested by centrifugation in 2-ml Eppendorf tubes (Minispin centrifuge; 15 min, 7000*g*) and washed three times with sterile saline solution (0.85% NaCl aqueous solution) to remove the culture medium components and extracellular components or bacterial exudates. The resulting wet biomass pellet was resuspended in half of the initial volume of sterile saline solution. Sodium selenite ($Na_2SeO_3 \cdot 5H_2O$, "Merck") as a 0.5 M stock aqueous solution was added to the suspensions up to a final concentration of 5 mM. Suspensions containing the cells (washed as above) and selenite were placed in a thermostat (at 31–32 °C). The culture of the same cell density without sodium selenite was used as a control. The process of $SeO_3^{2-}$ reduction was monitored by colour change of the bacterial suspension from colourless to reddish [24]. The Se NPs formed therein and their localisation were monitored by transmission electron microscopy (TEM; see below).

After 24 h, the bacterial cells were removed from the suspension by 'soft' centrifugation (1400*g*, 5 min); the supernatant with Se NPs was collected and filtered through a 0.22 or 0.44 μm PVDF filter to remove occasional bacterial cells. The suspensions of Se NPs were further centrifuged at 12000*g* for 30 min, and the collected precipitate pellet was resuspended in a minimum volume of MilliQ water. The Se NPs obtained were characterised by TEM as well as by FTIR and Raman spectroscopic techniques (see below).

### 2.3. Transmission Electron Microscopy (TEM)

Samples of bacterial cells (after cultivation for 24 h with 5 mM selenite before centrifugation) or Se NPs (obtained and isolated as described above) were placed onto nickel or copper grids coated with formvar (1% formvar in 1,2-dichloroethane). TEM images were registered on a Libra-120 transmission electron microscope (Carl Zeiss, Jena, Germany).

### 2.4. Dynamic Light Scattering (DLS) and Zeta Potentials of Se NPs

The sizes of the bacterially synthesised Se NPs (using DLS) and their zeta potentials were measured on a Zetasizer Nano-ZS particle sizer and zeta potential analyzer (Malvern Instruments Ltd., Malvern, UK).

### 2.5. Fourier Transform Infrared (FTIR) Spectroscopy

For transmission FTIR measurements, the aqueous suspensions of Se NPs (isolated and purified as described above) were placed as thin films on clean flat ZnSe discs (CVD-ZnSe, "R'AIN Optics", Dzerzhinsk, Russia; diameter 2.5 cm, thickness 0.2 cm) and dried in a thermostatted desiccator at 45 °C.

Infrared spectroscopic measurements were performed on a Nicolet 6700 FTIR spectrometer (Thermo Electron Corporation, USA; DTGS detector; KBr beamsplitter). Spectra were collected in the transmission mode with a total of 64 scans (resolution 2 $cm^{-1}$) against the ZnSe disc background and manipulated using the OMNIC (version 8.2.0.387) software supplied by the manufacturer of the spectrometer. All spectra were smoothed using the standard "automatic smooth" function of the software which uses the Savitsky-Golay algorithm (95-point moving second-degree polynomial), and then the baseline was corrected using the "automatic baseline correct" function. All the FTIR spectroscopic measurements were repeated two or three times for each sample and were well reproducible.

### 2.6. Raman Spectroscopy

For Raman spectroscopic measurements, the aqueous suspensions of Se NPs (obtained as described in Subsection 2.2) were placed as thin films on small pieces of aluminium foil and dried in air at ambient temperature. Normal Raman spectra were acquired with a Peak Seeker Pro 785 Raman spectrometer (Ocean Optics) using 785 nm excitation (30 mW; spectral range 150–2100 $cm^{-1}$). The acquisition interval was 10 s, and all spectra were averaged over 10 independent runs. The acquired digital experimental spectroscopic data were processed and plotted by using Microsoft Excel 2010.

## 3. Results and Discussion

### 3.1. General Considerations

For *Azospirillum thiophilum* (strain VKM B-2513), we utilised the original scheme developed earlier for the synthesis of extracellular Se NPs [27]. Thus, in this work, extracellular Se NPs were synthesised for the first time by the *A. thiophilum* species in the presence of 5 mM $Na_2SeO_3$. The recently described bacterium *A. thiophilum* had been isolated from a sulphur-containing bacterial mat collected from a sulphide spring [28]. Thus, one of the aims in this study was to check whether this species can produce pure $Se^0$ in the form of Se NPs in the course of selenite reduction (vide infra). Note that there were indications in the literature that biogenic Se NPs produced, e.g. by methane-oxidising bacteria contained trace admixtures of sulphur, and the elemental maps of selenium and sulphur visualised by energy-dispersive X-ray spectroscopy (EDXS) for those Se NPs overlapped [30]. In addition, sulphur and selenium were reported to co-precipitate in the presence of sulphate-reducing bacteria [31] and in sulphur-accumulating bacteria [32].

### 3.2. TEM and DLS Studies

The Se NPs obtained in this work were separated including the removal of bacterial cells by centrifugation and filtration, purified by triple washing in Milli-Q water and concentrated. Typical transmission electron microscopic (TEM) images of the bacterial cells (incubated in saline solution for 24 h), after 24 h with 5 mM $SeO_3^{2-}$ (before centrifugation),