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Selenium nanoparticle as a bright promising anti-nanobacterial agent

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ABSTRACT

The use of nanotechnology for nanobacteria (or calcifying nanoparticles) treatment is a new creative approach. Use of selenium nanoparticles (SeNPs) as anti-nanobacterial agents might be considered as a bright promising approach due to their critical role in the inhibition of crystal growth and aggregation of calcium oxalate. Hence, in this study, we investigated the probable outcome of SeNPs inhibitory effects on growth of nanobacteria. Fragments of thirty urinary tract stones were chemically analyzed by X-ray diffraction (XRD) and urinary stones Kits for calcifying nanoparticles presence. Then powder of stone fragments were resuspended in Dulbecco's modified Eagle's medium (DMEM), sterilized by filtration and cultured in presence of 1, 5, 30, 60, and 90 μmol/L SeNPs concentrations. Besides, calcifying nanoparticles growth in the culture without SeNPs was measured spectrophotometrically. Also, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analyses were used, where calcifying nanoparticles formation occurred. Results showed that in the culture without SeNPs, the positive calcifying nanoparticles detection was 60% while after adding SeNPs at 90 μmol/L, not any calcifying nanoparticles were observed. Further confirmation came out when Energy-dispersive X-ray (EDX) analysis showed calcium and phosphate peaks in the culture medium without any SeNPs while in the culture containing 90 μm/L SeNPs a decrease in calcium and other minerals was obvious. Therefore, SeNPs clearly restricted the growth of nanobacteria due to their inhibitory effects on calcium oxalate deposition.

1. Introduction

During the last two decades, nanobacteria (NB), as an amazing word, have captured the attention of many scientists in different disciplines. At the beginning of studies, the fiction or fact entity of nanobacteria was an important scientific controversy; until the worthwhile endeavor of scientists in 1998, those who demonstrated the viability of nanobacteria, caused this dream to come true [1]. Nanobacteria, also known as calcifying nanoparticles (CNPs), are capable to produce a biogenic hard shell on their membrane. These extremely tiny agents, which are 100-fold smaller than common bacteria, are protected by a crystalline carbonate apatite shell membrane. These mineralo-organic NPs or bions are just as real as any other biological entity and they may continue to reveal important insights about human health and disease [2].

Up to now, many studies have indicated that these agents act as a potential nucleus for aggregating different minerals in physiological conditions which subsequently causes widespread pathological calcification [3,4]. Furthermore, other studies have shown that these nanometer agents could be isolated from various sources [5,6]. There is a strong correlation between the presence of nanobacteria and diverse calcification-related health problems. Kidney stone formation [7–11], gallstones and gallbladder inflammation [12], dental pulp stones [13–15], prostatitis [16,17], Alzheimer's disease, polycystic kidney disease [18,19], arterial heart disease [20–22], cancer [23,24], calciphylaxis [25,26], and Human Immunodeficiency Virus are some diseases related to nanobacteria infections. Extensive efforts have been applied to inhibit the growth of nanobacteria, but due to their strong shield, conventional sterilization techniques such as heat are unable to inhibit the growth of these novel organisms. The hard apatite shell of nanobacteria has different genus. Most urinary stones are made of

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Table 1
The characteristics of patients and chemical composition of their urinary stones.

Number	Gender	Age	Oxalate (%)	Calcium (%)	Phosphate (%)	Uric acid (%)
1	Male	43	60	40	–	–
2	Male	37	80	20	–	–
3	Female	54	70	30	–	–
4	Male	33	60	40	–	–
5	Male	55	80	5	–	15
6	Female	40	80	20	–	–
7	Male	38	70	10	–	20
8	Male	67	70	20	–	10
9	Male	62	80	20	–	–
10	Male	54	70	10	–	20
11	Male	24	55	45	5	–
12	Female	49	80	20	–	–
13	Female	67	70	30	–	–
14	Female	30	60	10	–	30
15	Female	33	90	10	–	–
16	Female	42	80	20	–	–
17	Female	57	60	40	–	–
18	Male	75	60	40	–	–
19	Female	29	70	30	–	–
20	Male	33	80	10	–	10
21	Female	47	60	40	–	–
22	Female	27	70	20	–	10
23	Female	62	70	30	–	–
24	Male	43	70	30	–	–
25	Male	46	80	20	–	–
26	Female	55	80	10	–	10
27	Male	57	80	20	–	–
28	Male	46	60	30	–	10
29	Female	36	60	40	–	–
30	Male	61	80	20	–	–

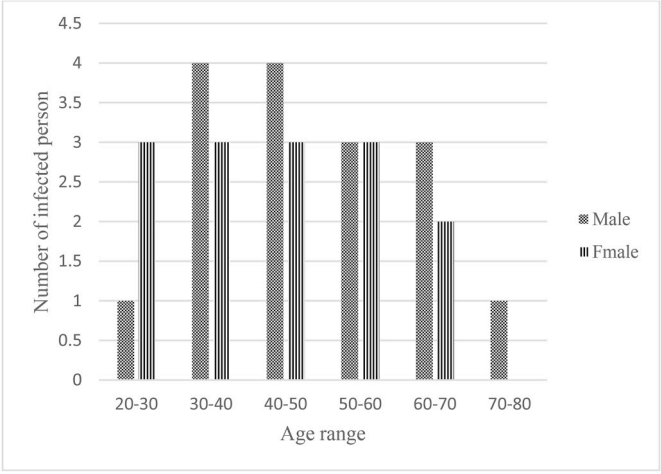

Fig. 1. Distribution of urinary stone in infected persons according to age and gender.

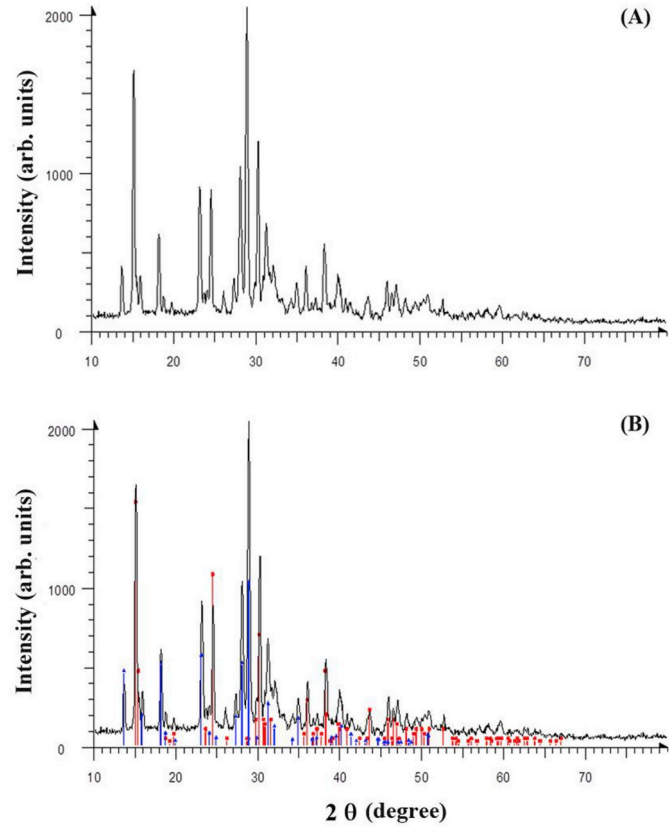

Fig. 2. X-ray diffraction (XRD) pattern of CNPs. A: Raw data. B: Red line (—) indicate calcium oxalate (CaC_2O_4) and blue line (—) indicate uric acid presence in urinary stone sample. Highest picks in 28–33 2θ angle confirm the calcium oxalate presence.

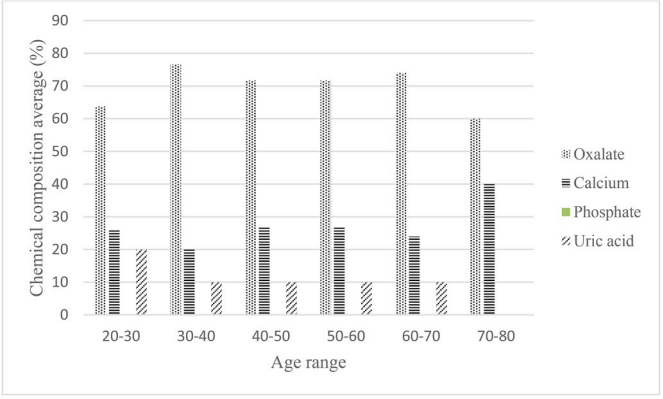

Fig. 3. Relative portion of stone component (%) according to age.

calcium oxalate. There are three different hydrate forms of calcium oxalate: Thermodynamically stable calcium oxalate monohydrate (COM) crystals, metastable calcium oxalate dihydrate (COD), and thermodynamically unstable calcium oxalate trihydrate (COT) crystals [27]. The most thermodynamically stable phase is the COM crystals. It should be mentioned that SeNPs inhibit the growth of COM crystals and induce the formation of spherical COD crystals that contain selenium, which are thermodynamically less stable and have weaker affinity to the cell membranes than COM crystals [27]. It is supposed that if we are able to prevent the precipitation of calcium oxalate on nanobacteria cell membrane, in order to prevent or reduce the formation of hard apatite shell, we might be capable to prevent nanobacteria growth. Therefore, in this study we investigated the inhibitory effects of SeNPs on nanobacteria growth which isolated from urinary stones.

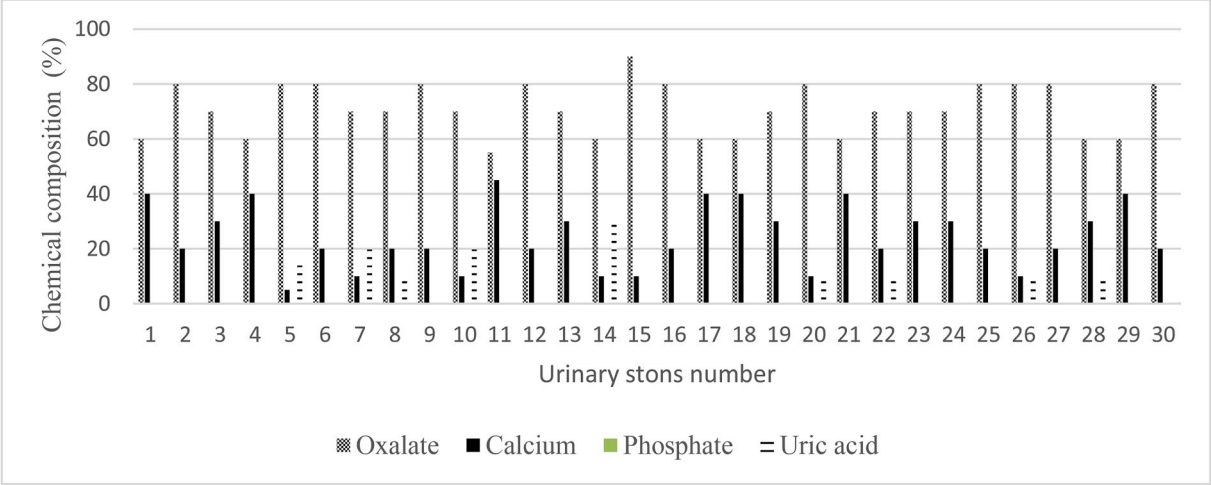

Fig. 4. Distribution of main chemical components (%) in thirty urinary stone samples.

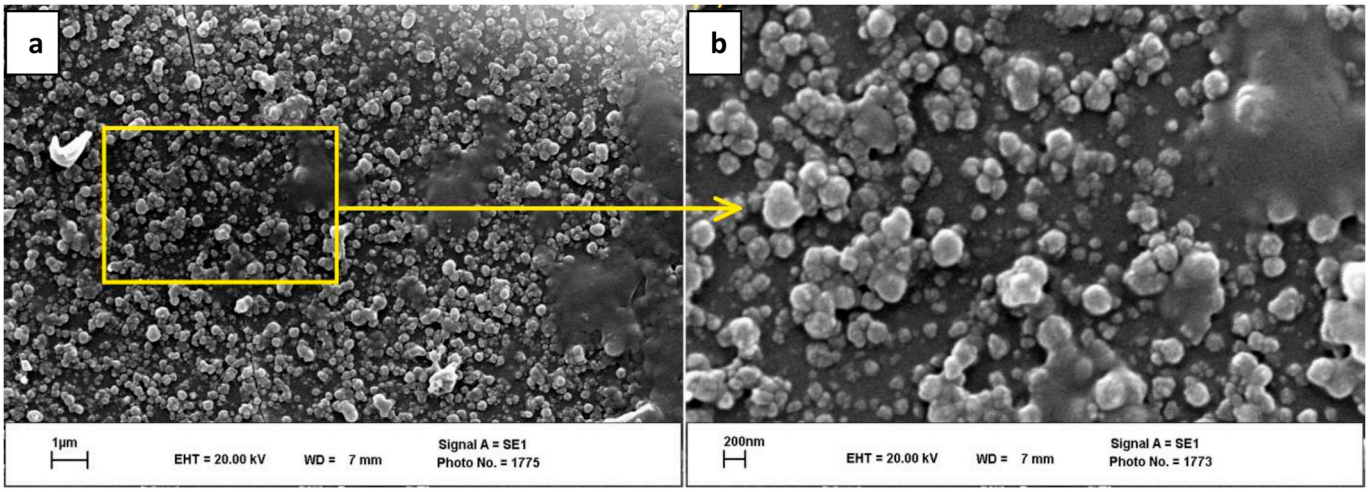

Fig. 5. SEM images of prepared urinary stone samples showing spherical shapes resembling nanobacteria. a: Nanobacteria in 1 µm scale bar; b: Nanobacteria in 200 nm scale bar (20,000× magnification).

Fig. 6. Acicular microcrystal-like structures of nanobacteria observed by invert optical microscope at 4 week (200× magnification).

2. Materials and methods

Thirty urinary tract stones were collected from Khorasan-e Razavi Province pathology laboratories. The SeNPs (Spherical shape; ~30 nm) was obtained from central laboratory in Mashhad University of Medical Sciences. The chemical analysis of positive CNPs stones was carried out by XRD technique and urinary stone commercial kit (Saba Analysis Kit, Iran). Regarding CNP isolation, 5 mg of stone fragments were manually grounded and demineralized by incubation with 1 N HCl (Merck, USA) for 10 min at room temperature and then neutralized by addition of sufficient tris buffer (pH 10.5, Sigma). After centrifugation at 20000*g* for 40 min, the precipitated pellet was suspended in Dulbecco's modified Eagle's medium (DMEM) (Gibco). The suspension was then filtered through a 0.22 µm (Orange, France) membrane filter and cultured in a six-well plate that each well contained DMEM (5 ml), SeNPs (at concentrations of 1, 5, 30, 60, 90 µmol/L) and Fetal Bovine Serum (FBS) 10% (gamma-irradiated at a dose of 30 kGy). Culturing was carried out using strict aseptic techniques without any antibacterial and antifungal agents at 37 °C in humidified CO_2 incubator (CO_2 5%, air 95%). As the control culture, nanobacteria culture medium was incubated without any SeNPs concentrations. Also, to investigate the pathogen presence in nanobacteria culture during the growth period, filtered solution was cultured in the nutrient agar culture for 72 h in 25, 30, 37 and 50 °C. In order to observe any living entity in culture medium plates, an inverted microscope (Olympus, Japan) was used. CNP growth was monitored through measuring them at 650 nm in the culture without SeNPs. After 30 days the positive plates which had the white-colored sediment on the bottom of the wells were used for SEM (LEO 1450 VP, Germany) and TEM (LEO 912 AB, Germany) analysis. In the SEM study, floating and adherent materials in culture plates were examined separately.

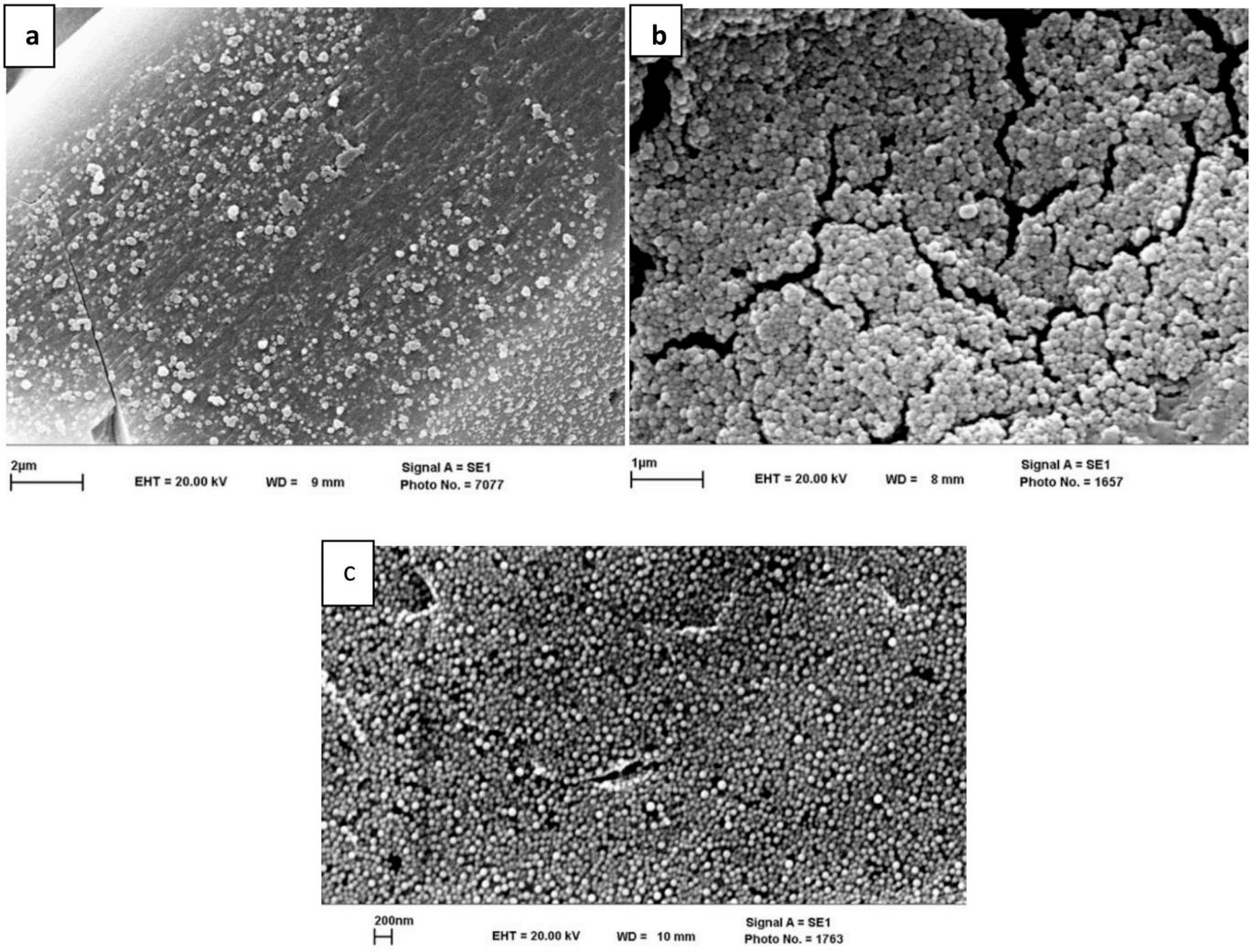

Fig. 7. SEM images of nanobacteria in control culture (without SeNPs) at 4 week; a: Nanobacteria growth on surface without biofilm formation; b; Biofilm forming growth of nanobacteria; c: Singular nanobacteria growth without biofilm formation.

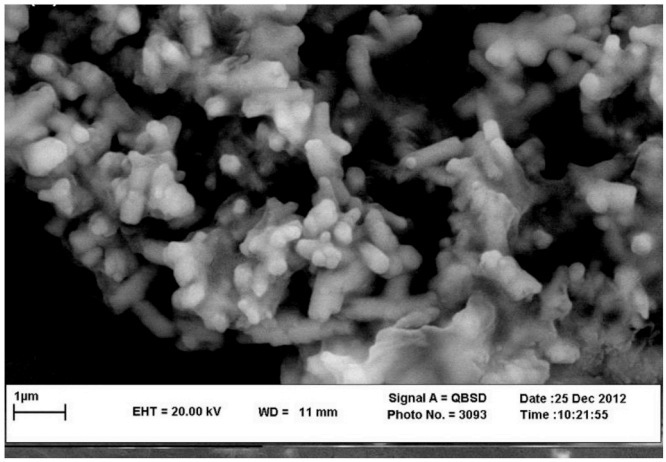

Fig. 8. SEM image of culture medium with 90 μmol/L SeNPs at 4 weeks. Spherical form similar to nanobacteria could not be observed (20,000× magnification).

3. Results

Some of the obtained data about the collected samples are summarized in Table 1. The distribution of urinary stone in infected patients ages and gender showed people with 40–50 years old were at higher risk of infection (Fig. 1). The chemical composition of CNPs was determined with XRD analysis (Fig. 2). Results showed that oxalate, calcium and uric acid were chemical components respectively in the higher amount of each 5 mg urinary stone at different ages where no phosphate were detectable (Figs. 3 and 4). CNPs size varied significantly from less than 100 nm to several micrometers. The SEM study demonstrated spherical shapes in the nanoscale (1–100 nm) (Fig. 5).

One of the striking characteristics about CNPs growth was the circular micro crystal-like structures (Fig. 6). The CNPs detected in this study were clearly similar in size and morphology to previously reported studies [10,12]. In the culture without SeNPs, the positive CNPs detection was 60% (18/30), while half of the negative detection cases (6/30) in this culture did not have any detectable spherical structures. In this study, we divided the growth modes into three groups, including (I) solitaire growth on the surface without biofilm formation; (II)

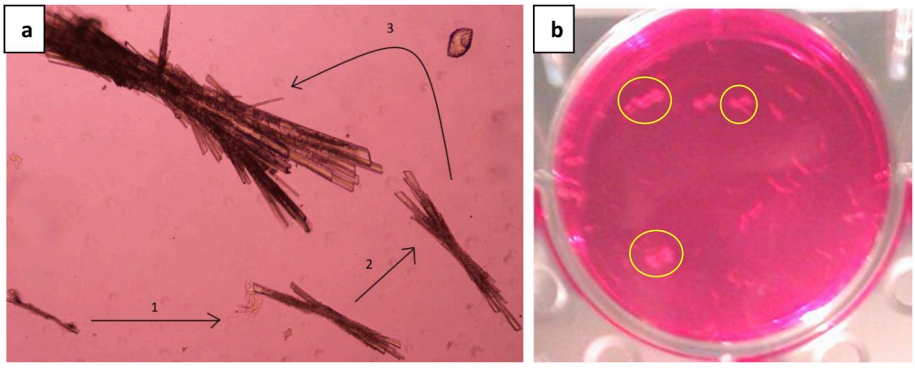

Fig. 9. Growth process of Nanobacteria; a: Crystal growth process of Nanobacteria: Cultures of Nanobacteria observed by invert optical microscope at 4 weeks (200× magnification); b: White-color sediments in bottom of the six-well plate (yellow circles) at 4 weeks.

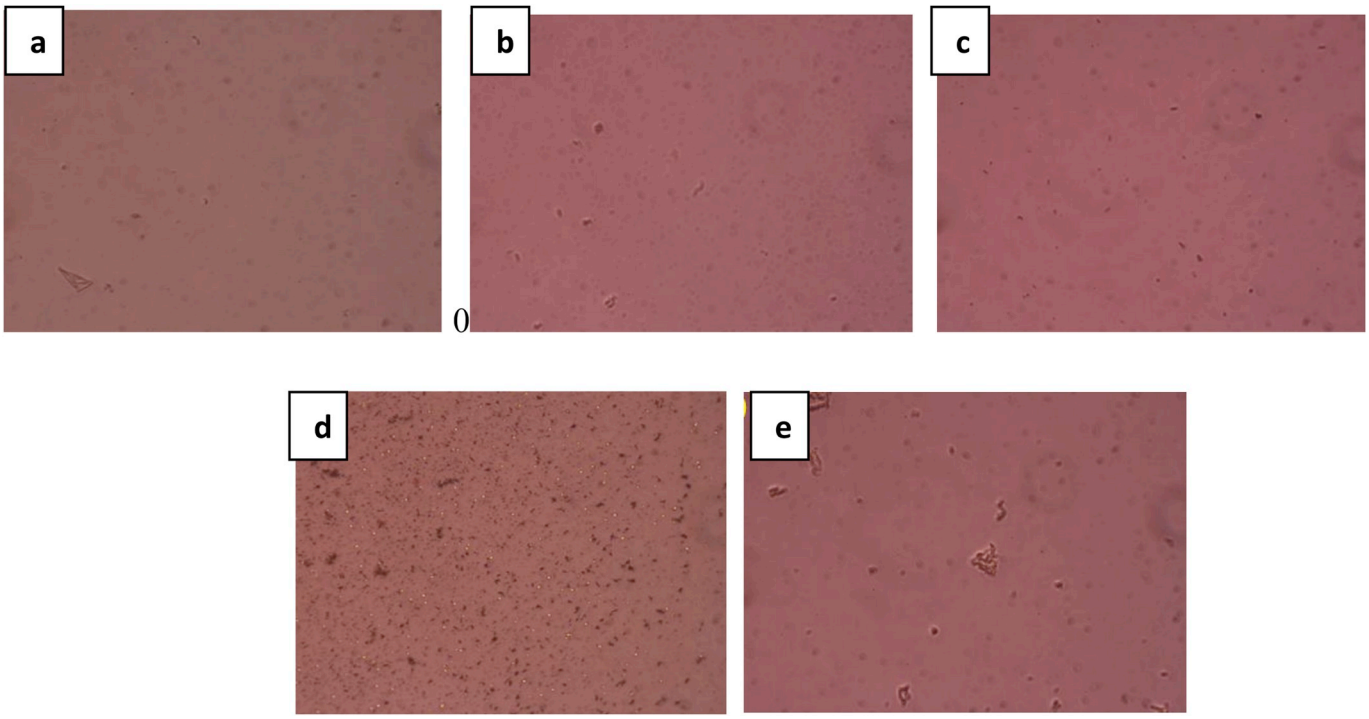

Fig. 10. Cultures of nanobacteria observed by inverted optical microscope at 4 weeks in different concentration of SeNPs. a, b, c, d and e nanobacteria culture medium with 1, 5, 30, 60 and 90 μmol/L of SeNPs respectively (200× magnification).

biofilm growth formation, and (III) solitaire floating growth without biofilm formation (Fig. 7). Some of these particles sizes are about 33 nm which are in agreement with previous studies that were carried out in this field. After adding SeNPs at 90 μmol/L concentration, spherical shape of CNP was not observed (Fig. 8).

In several micrographs that were obtained from optical invert microscopy, the process of crystal growth could be recognized (Fig. 9a). After 4 weeks of incubation, white sedimentations were found at the bottom of plates (Fig. 9b).

This sedimentation growth has a singular model. SEM analysis of these white-color sediments revealed nanoparticles in the size of 160 nm or less. During 30 days incubation periods in culture medium with 1, 5, 30, 60 and 90 μmol/L SeNPs concentration, any crystal growth were observed (Fig. 10).

Stone chemical analysis was performed by two methods, commercial kits and XRD, while both results indicated that urinary stones have calcium oxalate components. No pathogens were observed after performing suspended fluid incubation for three days at 25, 30, 37 and 50 °C temperatures. Measuring absorbance at 650 nm illustrated a slow growth of nanobacteria in the culture without SeNPs (Fig. 11). TEM results revealed a cover around the nanobacteria (Fig. 12); while EDX analysis in the culture medium without any SeNPs showed calcium and phosphate peaks rather than other minerals such as magnesium and sodium (Fig. 13). The EDX analysis in the culture that contained 90 μmol/L SeNPs concentration, showed a decrease in calcium and another minerals peak (Fig. 14).

4. Discussion

Nanobacteria are the major controversy in the modern microbiology. Nowadays, it could be argued that a particle with only 50–200 nm in diameter probably could not harbor the components that are necessary to sustain life or how a particle with 33 nm diameter,

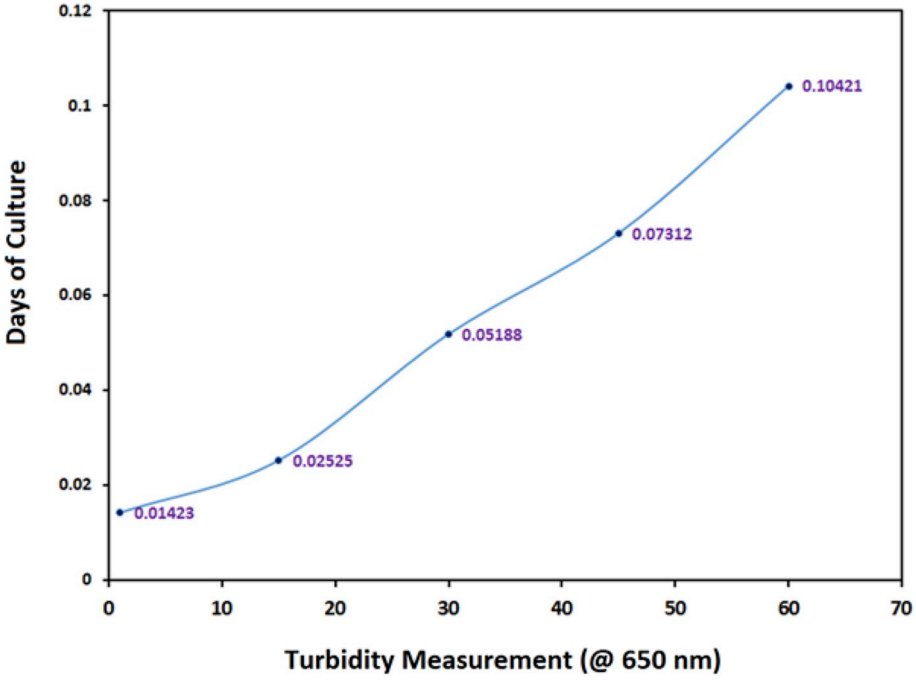

Fig. 11. The average growth of nanobacteria in the culture medium (without SeNPs) during 60 day.

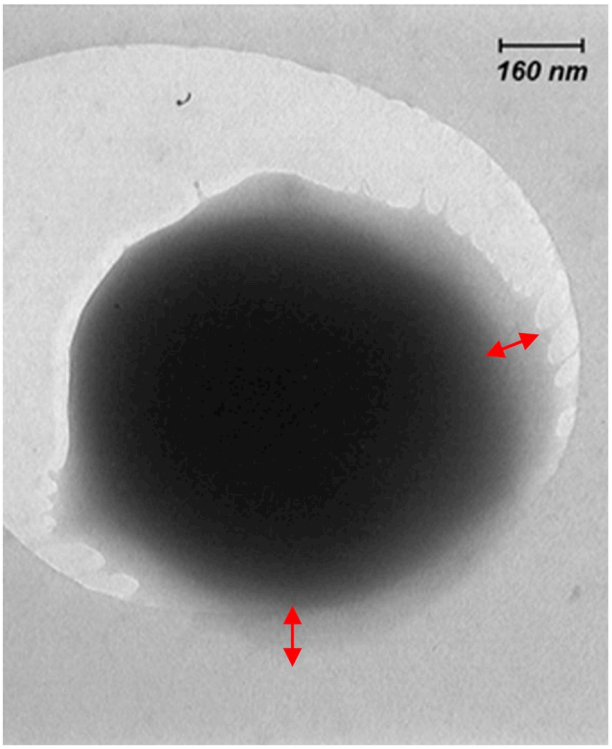

Fig. 12. The TEM image of the nanobacteria in culture medium (without SeNPs). TEM micrograph showing spherical nanoparticles (12,500× magnification) and wrap around it (indicated by red arrows).

which was observed in SEM analyses, can be alive? The ambiguity of CNPs bacteriological evidence has been leading various hypotheses about the viability of nanobacteria [28]. Some researchers are persistent on the opinion that nanobacteria are not primordial life forms and they are inert mineral fetuin complexes [29,30]. Hence, in this study, it was of great importance to ensure that nanobacteria are living entities, mainly the ones that are isolated from urinary stone samples and not from other contaminating sources. 1) All reagents and instruments were sterilized, and a rubber dam was utilized in clinical treatment. 2) The supernatants of nanobacteria cultures were filtered through a 0.22 μm Millipore filter, a generally accepted method to remove the common bacteria, fungi, and mycoplasma. 3) No other cellular entities were present as proved by microscopy and culture tests, and there was no cloudiness of color. Therefore, it could be concluded that nanobacteria were isolated and identified from urinary stones. Nanobacteria were identified in human kidney stones for the first time. One of the strict principles in achieving a pharmaceutical treatment is the equilibrium dimensions between prey and hunter. Hence, the use of nanotechnology for the treatment of nanobacteria could be an excellent suggestion. This study focuses on SeNPs inhibitory effects on the growth of nanobacteria and we used SeNPs for the main purpose: the SeNPs inhibitory effects on calcium oxalate crystal growth and aggregation.

Based on others studies and works, the main component that organizes urinary stones is calcium oxalate [31,32]. On the other hand, if SeNPs inhibit the calcium oxalate growth, we will be able to restrict nanobacteria growth after adding an appropriate concentration of SeNPs. In all SeNPs concentrations that were added to culture medium in comparison to control culture (without SeNPs), we could not observe any crystal growth. This observation indicated that as SeNPs prevented the aggregation of COM crystals and induced the formation of spherical calcium oxalate dehydrate (COD) crystals that contained selenium, which are thermodynamically less stable and have a weaker affinity to the cell membranes than COM crystals [27].

The EDX analysis of the white sediments in culture medium with 90 μmol/L SeNPs concentration, showed a decrease in calcium and phosphate due to the presence of SeNPs in stones. Other SeNPs concentrations were not analyzed. Our study provided more detailed information on the culture of NB in different growing states. First, 'concentric circles' were formed after about 4 weeks of culture; this morphology has not been reported before regarding NB cases. Second, optical inverted microscope micrographs indicated a novel form which has not been reported before for NB identifications (Fig. 15) as it is following a continuous growth pattern. Third, after 30 days of incubation in the medium that contained 90 μmol/L SeNPs, we could not find nanobacteria morphology, while the morphological changes could be observed during the culture period.

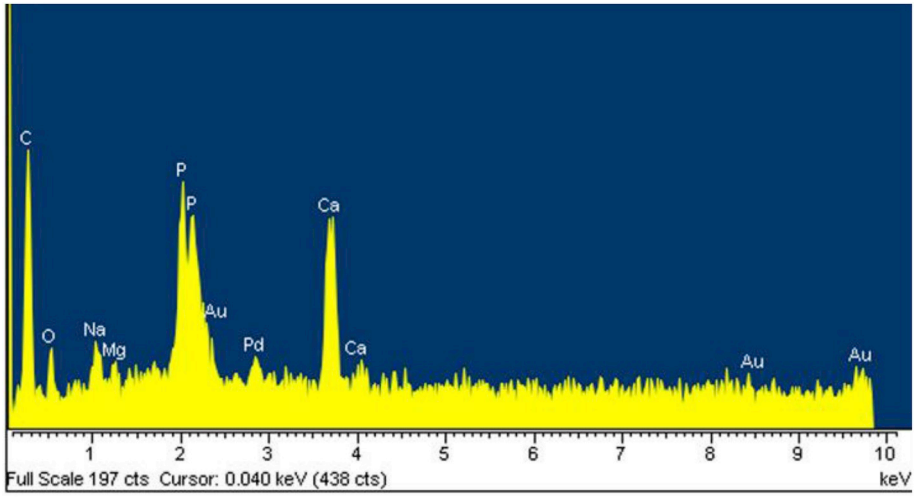

Fig. 13. The EDX analysis of nanobacteria; the topographic features on chemical composition of nanobacteria, were identified with calcium and phosphate peaks in culture medium (without SeNPs).

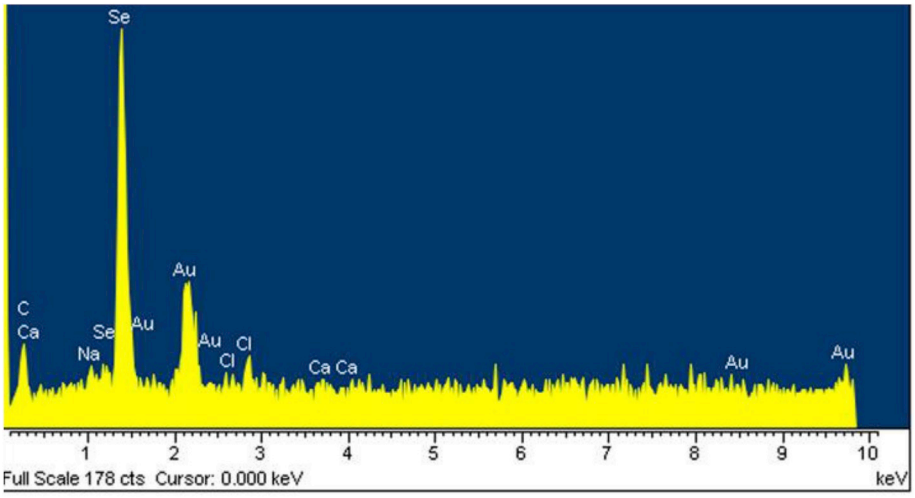

Fig. 14. The EDX analysis of nanobacteria; the topographic features on chemical composition of nanobacteria, were identified with calcium and phosphate peaks (culture medium with 90 μmol/L SeNPs).

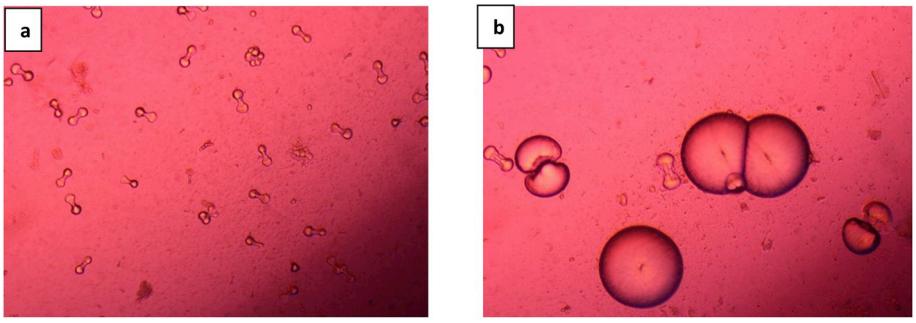

Fig. 15. Cultures of nanobacteria observed by invert optical microscope at 4 weeks.
a, b Novel ambiguous forms in culture medium (without SeNPs) (200× magnification).

5. Conclusion

Our findings indicate on the possibility of SeNPs application in inhibiting or decreasing the formation or development of NB growth due to crystal growth and calcium oxalate aggregation prevention in urinary stones. Also, present results claim a scientific approach regarding the sudden absence of nanobacteria growth evidences. This claim may be because of the SeNPs penetration into nanobacteria cell after the dissolution of the calcium oxalate protective shield via SeNPs and subsequently applying antibiotic effects. However, further studies are required to test whether CNPs are the causative agents of urinary stones. SeNPs functionalization with a monoclonal antibody could be a further nanotechnology approach for more effective NB treatments.

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