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A Study of Calcifying Nanoparticles Extracted from the Vorotilovskaya Well

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Abstract: These are the results of the further research of nanoparticles which were extracted from the Vorotilovskaya deep scientific well. The morphology and phase composition of these nanoparticles were examined using scanning and transmission electron microscopy. The phase composition of the nanoparticles and its changes during their evolution were identified and traced using the electron diffraction method. Based on these results it is concluded that the nanoparticles belong to a class of self-replicating mineral complexes known as “calcifying nanoparticles”. These nanoparticles in two months has changed their morphology and phase composition from $Ca_2Fe(PO_4)_2(OH)\cdot H_2O$ and $Mg(NH_4)_8(P_3O_{10})_2\cdot 8H_2O$ to $CaCO_3$ (aragonite).

Key words: Calcifying nanoparticles, scanning and transmission electron microscopy.

1. Introduction

During the study of pure cultures of bacteria Planomicrobium sp., isolated from the crystalline rocks (gneisses) revealed by Vorotilovskaya deep scientific well (VDSW) were detected objects, ranging in size from 50 to 400 nm (Fig. 1). These objects, reminiscent of a colony of unusually small spherical or coin-shaped bacteria, were significantly smaller than any bacteria known today, fungal spores or cells of tissues of multicellular organisms [1].

Spherical and coin-shaped particles with a diameter of 50 to 250 nm had been observed previously by researchers from different scientific disciplines (biochemistry, microbiology, geology, etc.) and, respectively, in samples of different nature and origin [2-7]. These particles have been found in deposits from mineral hot springs, sandstone, animal blood serum and in samples from the organs of individuals with common diseases such as kidney stones, arthritis, Alzheimer's, atherosclerosis, etc. [2-13]. In some cases, the researchers succeeded in isolating pure cultures of submicron particles filtered using bacteriological filters and grown on selective nutrient media. All of data on these nanoparticles, obtained through the scanning and analysis of different samples (soil, water and concrements extracted from human organs) using transmission electron microscopy, varies greatly and requires careful consideration [2-8].

Ever since submicron particles that are capable of self-replication were first described, an ongoing debate has been whether or not to regard them as living organisms [2-5, 9-10]. The size of these particles, varying in the range from 50 to 200 nm [2-7, 9-11], is in most cases too small to accommodate

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the DNA needed for reproduction[1]. As such, the question of their nature remains an open one. What is their composition? What is their minimum size? Are they able to replicate? Can we consider them alive or not? These and further questions raised in recent years have be the catalyst for the numerous theoretical and experimental studies of calcifying nanoparticles.

2. Materials and Methods

2.1 Preparation of Bacterial Cultures

As the object of the study is a pure culture of bacteria Planomicrobium sp. SM-9, isolated from the gneisses, extracted from VDSW at a depth of 2,600 m. In order to obtain samples for electron microscopy, bacteria were grown at 28 ^{o}C on agarized tenfold diluted meat-peptone broth with methanol adding. Biomass of one-day, one-week or two-weeks culture was washed from the surface of a dense medium using 0.5% NaCl and concentrated by centrifugation at 10,000 rpm for 5 minutes. After supernatant liquid removal 0.2 ml by volume of a concentrated suspension of microorganisms was applied using a sterile pipette by a thin layer to a coverslip degreased using an alcohol-ether mixture. Afterwards samples were exposed to the air and left to dry at room temperature.

2.2 Sample preparation for Electron Microscopy

The pure cultures of Planomicrobium sp. SM-9, isolated from the core of gneisses, were sowed on diluted (1:10) beveled meat-peptone agar with methanol. The sowed culture was incubated at 28 ^{o}C. For strain SM-9 cultures of different ages (1 day, week and a culture older than 2 weeks) were obtained. Coverslips were placed in an alcohol-ether mixture (1:1) for disinfection. Cultures of all ages were diluted in 5 ml of a sterile 0.5% sodium chloride solution, then centrifuged for 5 min at 10,000 rpm and drained of 4 ml of the supernatant. Using a sterile pipette, 0.2 ml of centrifugate was placed on a glass and dried at room temperature.

Subsequently the sample was placed on the stub for the electronic microscopy: a drop of sterile distilled water was dripped on the glass with the dried sample and carefully stirred. Subsequently a drop of the bacterial suspension was placed by capillary on the stub and dried at room temperature. Since the samples were non-conductive, before the samples were scanned using the electron microscope they were covered by a conductive layer of platinum of 10 nm thickness.

3. Results and Discussion

Studies were performed using a Tecnai G2 F20 U-Twin Transmission Electron Microscope (TEM) (FEI, Netherlands) and a Supra 40 Scanning Electron Microscope (SEM) (Carl Zeiss, Germany). In the initial sample of bacteria Planomicrobium, sp. SM-9 coin-shaped nanoparticles (50-400 nm in diameter) were detected. A comparison of the observed

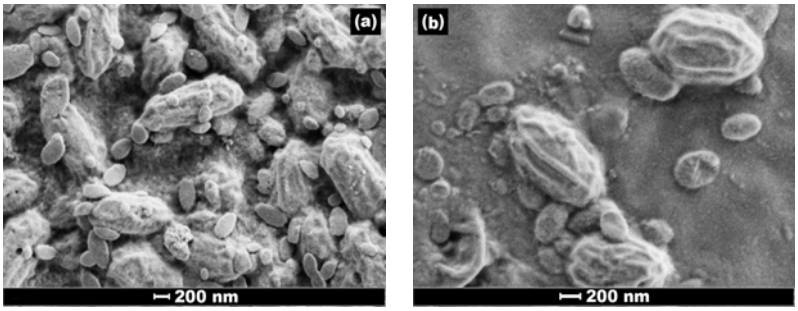

Fig.1 SEM micrographs of bacteria Planomicrobium sp. SM-9 and calcifying nanoparticles, isolated from the crystalline rocks (gneisses) revealed by Vorotilovskaya deep scientific well (VDSW). (a)obtained from place with high density of bacteria (ranging in size from 0.5 to 3 mcm) and nanoparticles (ranging in size from 50 to 400 nm), (b) obtained from the place with low density bacteria and nanoparticles.

nanoparticles of 50-200 nm in size with nanoparticles described in [2-7] suggests that they have much in common. It is natural to assume that the nanoparticles from Vorotilovskaya deep scientific well are also calcifying nanoparticles.

These nanoparticles were separated from the bacteria (0.5-3 mcm) by means of a microfilter (Schleicher & Schuell FP 030/3) with a pore diameter of 200 nm. The resulting samples were examined using transmission electron microscopy (Fig. 2) and electron diffraction (Fig. 3) on the Tecnai G2 F20 U-Twin microscope [14].

The pattern of electron diffraction on the sample, and its subsequent processing, leads to the following conclusion of structural and phase composition of the mineral component of nanoparticles: based on comparisons with the databases on the diffraction data it was concluded that the shell of nanoparticles studied most likely consists of $(Ca_2Fe(PO_4)_2(OH)\cdot H_2O$ and $Mg(NH_4)_8(P_3O_{10})_2\cdot 8H_2O$ [14].

These studies were repeated 6 weeks later. The initial suspension of nanoparticles was stored at room temperature under normal atmospheric pressure in a glass flask. The procedure for sample preparation was similar to the one previously described. Results from the repeat studies are presented in Figs. 4 and 5.

These experimental data (intensity and angles of the diffraction maxima) were compared with ICDD PDF-2 (The International Centre for Diffraction Data, powder data file, 2 ICDD) databases. The results of decoding are presented in Table 1.

Table 1 The results of decoding of electron diffraction.

this work data	$CaCO_3$ 01-071-2396
4.21-4.26	4.21216
2.45-2.50	2.484120
2.37	2.3716
2.34	2.34217
2.11-2.14	2.10608
1.98-1.99	1.97717

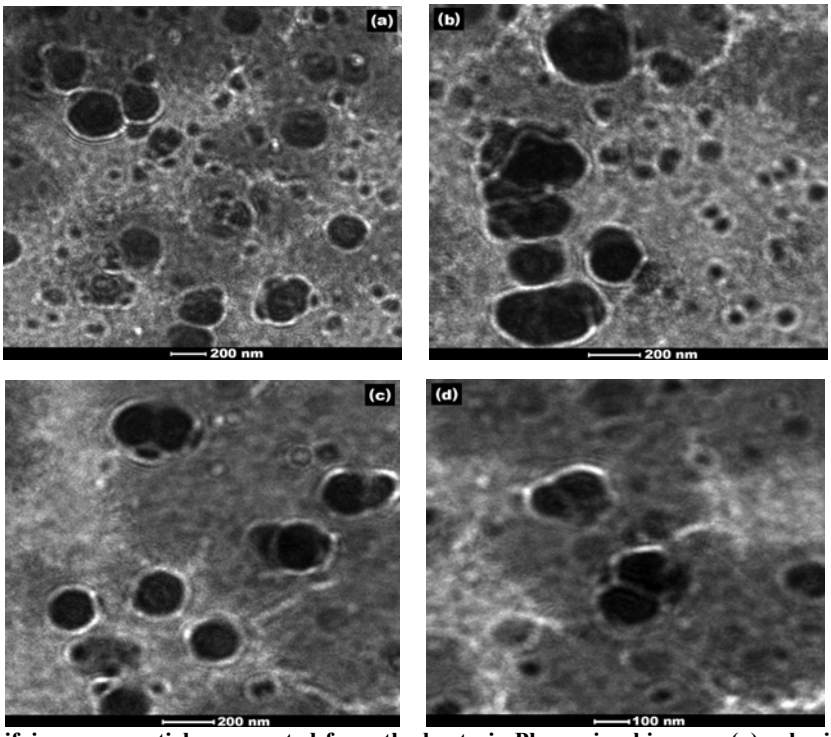

Fig. 2 Images of calcifying nanoparticles separated from the bacteria Planomicrobium sp. (a) spherical particles, the most typical image of CNP in the microscope, (b) grouped particles of irregular shape, (c) particles with bulkhead, similar to bacterial cells within division process. Particle at the top of the frame consists of three subparticles, (d) Particles with bulkhead, similar to bacterial cells within division process at magnification higher than (c).

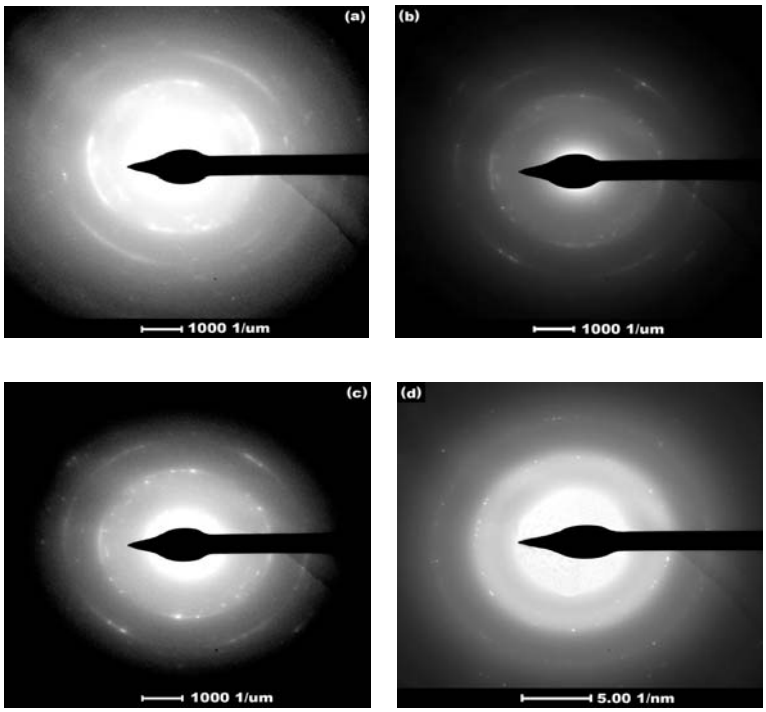

Fig. 3 Typical electron diffraction patterns of calcifying nanoparticles separated from the bacteria planomicrobium sp. Electron diffraction pattern obtained with: (a) camera length (CL)-2.1 m for large interplanar distances (ID) determination; (b) CL-2.1 m for large ID determination and the fine structure detection; (c) CL-2.1 m for large ID determination; (d) CL-0.6 m for small ID determination.

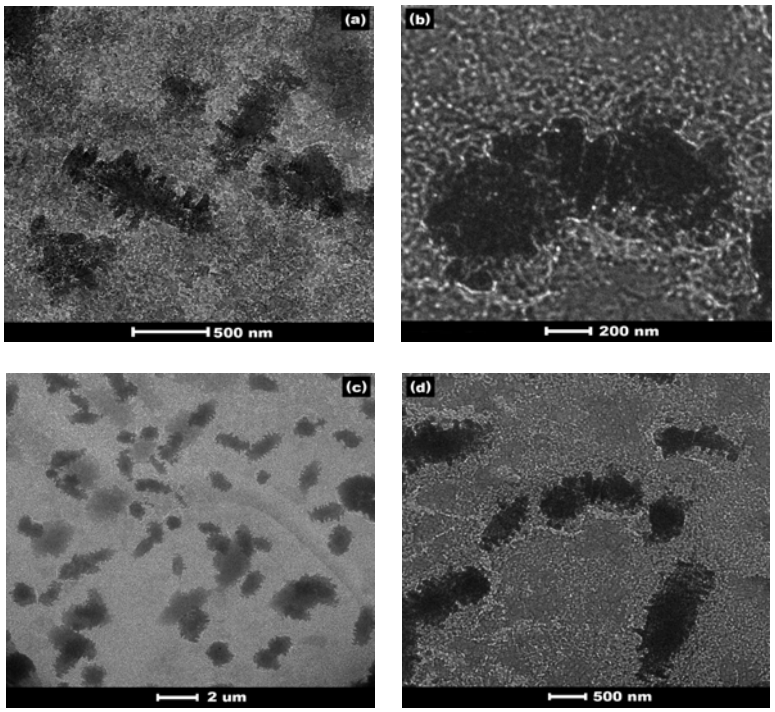

Fig. 4 Typical images of a nanoparticles extracted from pure culture Planomicrobium sp. SM-9 6 weeks later. General view of CNP sample: (a) demonstrating size and shape similarity (only) of CNP with that of bacteria within middle magnification, (b) within maximum magnification, shows the fine structure of CNP, (c) within low magnification, displays the "colony" of CNP, (d) within middle magnification, shows the similarity of the size and shape of CNP with that of bacteria.

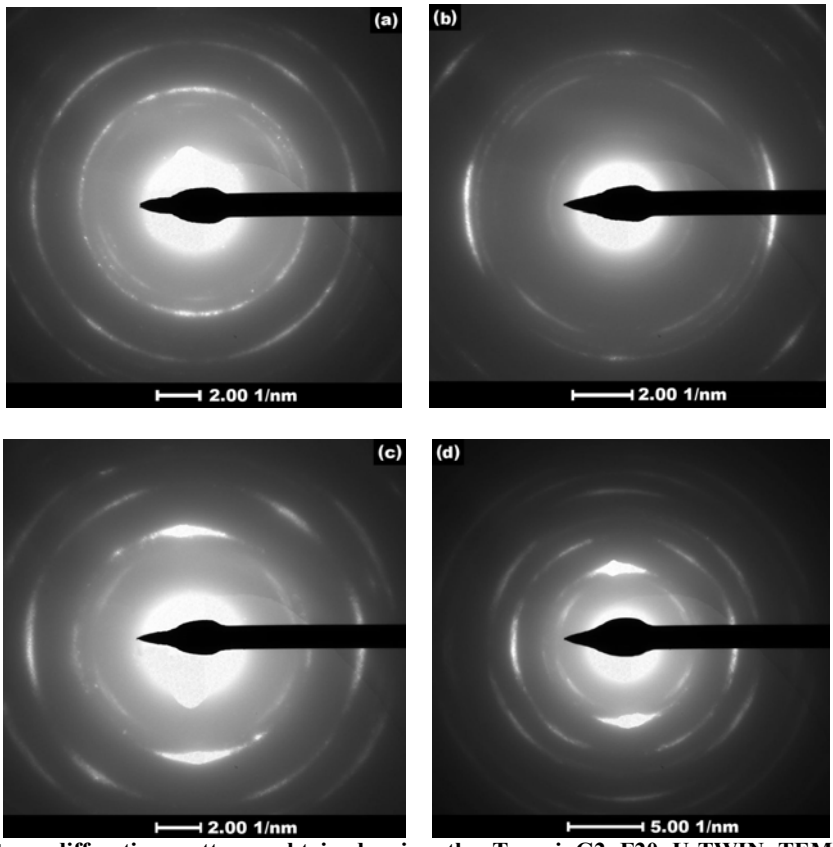

Fig. 5 Typical electron diffraction patterns obtained using the Tecnai G2 F20 U-TWIN TEM (FEI) for calcifying nanoparticles presented in Fig. 1. Electron diffraction patterns obtained with: (a) CL-1 m for middle ID determination, (b) CL-1.25 m for large ID determination, (c) CL-1 m for middle ID determination and the fine structure detection, (d) CL-0.7 m for small ID determination.

Based on the comparisons with the diffraction results database it was concluded that the shell of re-examined nanoparticles are composed of aragonite with the chemical formula $CaCO_3$.

As a result of decoding of calcifying nanoparticles electron diffraction patterns phosphates of calcium and magnesium were detected. With repeated measurements, performed two months later, in the agglomerated particles was found the aragonite, one of the natural calcium carbonate polymorphs. The question naturally arises, where the phosphates are?

We assume that they were dissolved. To test this hypothesis, studies of infrared (IR) transmission spectra of dried samples were undertaken.

The samples were examined using infrared Fourier spectroscopy (FTIR). Transmission spectra were recorded using FTIR spectrometer IFS-113v "Bruker" in the range of 4,000-400 cm^{-1} with a resolution of 4 cm^{-1} at room temperature. Evacuation of spectrometer's measuring channel enabled significant reduction of the atmospheric absorption effect on the transmission spectrum of the samples. In preparing the samples for IR measurements a drop of test material suspension in distilled water was placed onto a silicon wafer (n-Si, $\rho = 4.5\ \Omega \cdot cm$) of 0.4 mm thickness and dried at room temperature in air. The identical silicon substrate (without a dried drop of sample suspension) was placed in the reference channel for a reference spectrum recording.

In Fig. 6, a typical IR spectrum of the samples is shown. The high-frequency region (4,000-2,000 cm^{-1}) contains the well-known bands 3,500-3,000 cm^{-1} and 2,400-2,270 cm^{-1}. The first one is a broad stretching mode of OH band of the bound water; the second is the carbon dioxide (CO_2). The presence of OH (bound water) is confirmed by the existence of high-frequency

shoulder (bending vibrations of OH^-) in the region 670-600 cm^{-1} The 2,800-3,000 cm^{-1} spectral region is attributed to the CH_2 and CH_3 stretching vibrations.

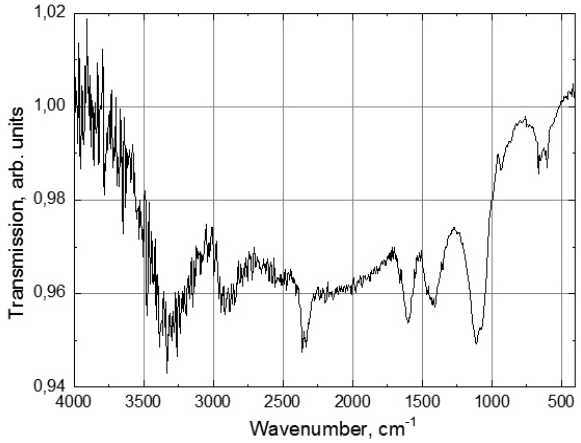

Fig. 6 Fourier infrared transmission spectra of calcifying nanoparticles obtained using IFS-113v "Bruker".

Low-frequency spectral region also contains several broad absorption bands with fine structure. The band with maximum at 1,600 cm^{-1} is associated with C=O bond. The bands in the area of 1,115 cm^{-1}, 1,076 cm^{-1}, 995 cm^{-1} are due to the absorption of phosphate compounds [15] attributed to the P-O stretching vibrations.

These results suggest that a chemical reaction, which took place in solution in the six-week period between successive registrations of electron diffraction, ions of phosphate have transferred into solution. The solid phase in the samples is a calcium carbonate in the form of aragonite.

4. Conclusions

Detected objects of 50-250 nm in size were compared with nanoparticles discovered by Finnish biochemist Olavi Kayander, geologist Robert Folk from the United States, Dr. John Lieske (USA) and a group of Australian researchers led by F.Yuvinsa of the Center for Microscopy and Microanalysis in Queensland. As a result, it can be argued that the detected objects have much in common with the nanoparticles examined by these scientists.

The feature of the detected objects is their capability to phase composition and morphology modification with time. In contrast with stable objects earlier observed by Olavi Kayander and other scientists these nanoparticles in two months has changed their morphology and phase composition from $Ca_2Fe(PO_4)_2(OH)\cdot H_2O$ and $Mg(NH_4)_8(P_3O_{10})_2\cdot 8H_2O$ [14] to $CaCO_3$.

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References

[1] B. Alberts, D. Bray, J. Lewis, M. Raff, K. Roberts. Molecular Biology of the Cell, fifth ed. Garland Science, 2007, pp. 1-20.

[2] E.O. Kajander, N. Çiftçioglu, Nanobacteria: An alternative mechanism for pathogenic intra- and extracellular calcification and stone formation, PNAS 95 (14) (1998) 8274-8279.

[3] E.O. Kajander, Nanobacteria-propagating calcifying nanoparticles, Letters in Applied Microbiology 42 (6) (2006) 549-552.

[4] E.O. Kajander, Inhibition of nanobacteria by antimicrobial drugs as measured by a modified microdilution method, Antimicrobial agents and chemotherapy 46 (7) (2002) 2077-2086.

[5] J.C. Lieske, Human-derived nanoparticles and vascular response to injury in rabbit carotid arteries proof of principle, Int. J. Nanomedicine 3 (2) (2008) 243-248.

[6] P.J.R. Uwins, R.I. Webb, A.P. Taylor, Novel nano-organisms from Australian sandstones, American Mineralogist 83 (1998) 1541-1550.

[7] R. Folk, Interaction between bacteria, nannobacteria, and mineral precipitation in hot springs of central Italy, Geographie physique et Quaternaire 48 (3) (1994) 233-246.

[8] N. Çifçioglu, R.S. Haddad, D.C. Golden, D.R. Mirrison, D.S. McKay, A potential cause for kidney stone formation during space flight: enhanced growth of nanobacteria in microgravity, Kidney Int. 67 (2) (2005) 483-491.

[9] J. Martel, J.D. Young, Purported nanobacteria in human blood as calcium carbonate nanoparticles, PNAS 105

(2008) 5549-5554.

[10] G.V. Smirnov, D.G. Smirnov, Nanobacteria-environmental factor of premature aging of human, Reports TUSUR 5 (2006) 119-123.

[11] D.R. Yarulina, O.L. Ilynskaya, N.I. Silkin, M.H. Salakhov, R.N. Hayrullin, Infectious origin of atherosclerosis. facts and hypotheses, in: Proceedings of the Kazan State University, Natural Sciences 152 (1) (2010) 19.

[12] V.M. Miller, G. Rodgers, J.A. Charlesworth, B. Kirklands, S.R. Severson, T.E. Rasmussen, et al., Evidence of nanobacterial-like structures in calcified human arteries and cardiac valves, American journal of Physiology-Heart and Circulatory Physiology 287 (3) (2004) 1115-1124.

[13] V.A. Kordium, S.P. Shpylova, E.V. Moshynets, N.I.A. Chala, D.I. Irodov, V.I. Andrienko, Biopolymers and cells in dimension of microbial community architecture, Biopolym. Cell 25 (2009) 403-423.

[14] S.V. Vasilev, N.V. Shekhovtsova, A.S. Rudy, O.Y Prikhodko, A.Y. Yagushkina, Electron diffraction studies of calcifying nanoparticles, Tentative Results Integral 3 (59) (2011) 10-12.

[15] Z. He, C.W. Honeycutt, B. Xing, R.W. McDowell, P.J. Pellechia, T. Zhang, Solid-state fourier transform infrared and ^{31}p nuclear magnetic resonance spectral features of phosphate compounds, Soil Science 172 (7) (2007) 501-515.