

Calcification in human arterial disease and geological specimens: The nannobacterial (nanoparticle) link

AUTHORS

Robert L. Folk¹, Brenda L. Kirkland², Jeri C. Rodgers³, and George P. Rodgers⁴

¹Jackson School of Geosciences, University of Texas at Austin, Austin, Texas 78712, U.S.A.

Email: rlfolk@mail.utexas.edu

²Department of Geosciences, Mississippi State University, Mississippi State, Mississippi 39759, U.S.A.

³Now Jeri Berlin, Atlanta, Georgia

⁴Biophysical, Austin, Texas 78759, U.S.A.

PRELUDIUM – The Back Story

In the early 1990's, Folk was using the SEM to look at all sorts of strange things for the presence/absence of nannobacteria, including stuff associated with the human body – dental plaque, beard hair, kidney stones, cataracts of the human eye, etc. (see 1998 *Alpe Adria Microbiology Jour.*). Prof. Kirkland, then at UT, was also interested in microbial subjects and asked if Folk wanted to look at some arterial plaque. They found that this material looked exactly like the nannobacterial precipitates that they had seen in so many geological occurrences. They published the first known SEM of arterial plaque (*Geology*, 1999).

Shortly thereafter, Folk gave a lecture on nannobacteria to a paleontology class, showing an SEM of the arterial plaque. In the class sat Jeri Cameron Rodgers, a Ph.D. student in vertebrate paleontology. She was married to Dr. George P. Rodgers, an Austin cardiologist, and she told him about it. He was inspired to get them some fresh samples to work on (with permission). The three of geologists took hundreds of SEM pictures and Dr. G. P. Rodgers added his medical expertise as they all studied the photographs.

At a cardiology convention, Dr. G. P. Rodgers happened to sit by accident at the same dinner table with Dr. Virginia Miller of the Mayo Clinic and found that they were also working on “nannobacteria” and arterial disease, following up on the research of Dr. Olavi Kajander, a Finnish medical researcher who had discovered “nannobacteria” in mammal blood at about the same time that Folk had discovered them

in Italian hot springs. So the four collaborated with Mayo researchers and published the first major article indicating nannobacteria as the cause of human arterial disease. (Miller, V. M., et al., 2004, *Am. Jour. Physiol., Heart and Circ. Phys.*, v. 287:H1115-1125).

At UT they had hundreds of SEMs comparing arterial plaque with precise analogues from the geological world. We wanted to publish these comparisons in a profusely illustrated article. But critics said they needed to do a lot more biology. However, they do not have the expertise, equipment, or financing to carry out the extreme microbiological research required, but they hope these photos may inspire someone to conduct further research.

ABSTRACT

The biogenicity of calcium-phosphate-precipitating nannobacteria (nannobacteria, self-replicating nanoparticles) has been supported by researchers: the tiny cells (30-70 nm in diameter) reproduce in cultures, show cell walls in TEM, and react positively for DNA and immunostain. The recently documented cells bear remarkable resemblances to those found earlier in geologic settings such as hot springs, weathered volcanic rocks, travertines, sandstones, soils, and even a Martian meteorite. In this paper we focus on the similarities of the nannobacterial morphologies found in the biologic and geologic settings, as documented by high resolution SEM. In both settings nannobacteria occur as isolated cells, as chains or sheets of phosphatized cells, finally to form hard blocky masses of hydroxyapatite.

Introduction

This study compares exceptionally high magnification (up to 100,000X) SEM photomicrographs of calcified cells found in geological specimens with similar features from human arterial tissue. These photomicrographs document the presence of small, spherical structures, which we term nannobacteria because they resemble known bacteria except for their size (Folk, 1993). Other terms include “nannobacteria” (Åkerman et al. 1993) and “propagating calcifying nanoparticles” (Kajander, 2006).

Geologists first discovered 50-200 nm nannobacteria precipitating CaCO_3 in natural hot spring deposits at Viterbo, Lazio, central Italy (Folk, 1992, 1993) at about the same time Kajander’s medical research group at the University of Kuopio, Finland was finding “nannobacteria” in bovine blood (Åkerman et al., 1993). The correspondence in shapes and sizes of these minute organisms was remarkable, even though the two groups were unaware of each other’s work. When NASA announced in 1996 that they had discovered nannobacteria-like objects in Martian meteorite ALH84001 (McKay et al., 1996) resembling those previously found in Viterbo, Italy, it established a critical mass for further research inasmuch as it linked the studies of nanobiology in geology, medicine/biology, and astronomy. If true, this discovery would point to the enormous importance of living organisms smaller than any heretofore discovered (except for viruses; see Wainwright, 1999, for a review of very small biological particles). However, most microbiologists maintained (e.g. Knoll and Osborne, 1999) that the minimal size for life was a sphere somewhere around 200 nm in diameter. Thus, although they have a considerable range in sizes, most “nannobacteria” with a cell volume of only 1/1000 that of ordinary bacteria, were considered to be much too small to be alive, under our current understanding of “Life.”

Despite these objections, work on these small, but intriguing, spheroids continued in the medical world (e.g. Kajander and Çiftçioglu, 1998; Jelic et al., 2004; Miller et al., 2004; Mulhall and Hansen, 2005; Bratos-Pérez et al., 2008); in the geological world (Vasconcelos and McKenzie, 1994; Uwins et al., 1998 and 1999; Chafetz, et al. 1998; Spark et al., 2000; Folk and Lynch, 1997b, 2001; Folk and Kirkland, 2007, Sánchez-Román et al., 2008; Folk et al., 2012) and in astrobiology (Folk and Lynch, 1997a, 1998; Folk and Taylor, 2002). Some of these workers have discovered cell walls and tiny dots resembling ribosomes in TEM sections of cells as small as ~100 nm (Folk and Kirkland, 2007), found the proper elemental components for life (C, N, O), cultured the organisms, and stained them positively for nucleic acids. Despite these findings, the battle for acceptance by microbiologists continues, and alternate origins for these small ovoids have been proposed. Nevertheless all alternatives involve biologically-associated processes (Vali et al., 2001, especially their figure 9b; Schieber and Arnott, 2003; Aloisi et al., 2006)

At the University of Texas we began looking at nannobacteria in the human body including dental plaque, beard hair coatings, gallstones, etc. (see Folk, 1998). We also worked with microbiologist Dr. R.J.C. McLean (Southwest Texas State University, San Marcos, Texas) and graduate students on nannobacteria in calf serum and kidney stones (see Prabhakaran, 1999). Kirkland obtained a single sample of arterial plaque in 1997, and found that calcified portions contained closely packed nannobacteria-like spheroids similar to those seen in natural minerals precipitated in organic-rich environments. The thoroughly calcified portion of the arterial blockage was surrounded by a fibrous web, to which were attached small (150 nm) spheres (Kirkland et al., 1999, fig 1; see also <http://www.msstate.edu/dept/geosciences/4site/nannobacteria.htm>).

The study presented here was based on acquisition from Texas patients of three more samples of calcified arterial tissue and a single sample of uncalcified mitral valve, which was meant to be a control sample. All of the samples were prepared for SEM imaging using traditional biological protocols involving ethanol and acetone dehydration with critical point drying. Once again we found that the most strongly calcified portions of the arterial tissue were composed of small (30-60 nm) spheres. We also found single isolated spheres, chains or clumps of spheres, sheets of spheres, and solid masses of spheres. The progression of morphotypes is strikingly similar to the order of silicate structures—isolated SiO_4 tetrahedra to chains to sheets to three dimensional structures (Folk et al., 2001).

We also found that the non-calcified control sample contained patches of exceptionally tiny (100-600 nm) rod-shaped objects that appear to be embedded in the tissue like bristles in skin. Because the patient was a victim of rheumatic heart disease, we suggest that these previously unrecognized rods were small bacteria that contributed to damaging the mitral valve so badly that it had to be replaced. Associated with these rods are scattered 100 nm individual balls which are similar to (but slightly larger than) the nannobacteria making up the calcified arterial plaque. Jelic et al. (2004) have recently identified nanobacteria as the cause of mitral valve calcification, confirming our work, and nanobacteria-like particles in calcified arterial plaque have also been identified by Puskas et al. (2005), Jelic et al. (2007), and Bratos-Peréz et al. (2008).

Techniques

The first tissue samples were dehydrated by Kirkland using standard critical point drying. Each individual sample was placed into a small plastic capsule with nylon netting on either end to allow for passage of fluids through the capsule. Samples were dehydrated by using a series of progressively more concentrated solutions of ethanol in water (25%, 50%, 75%, 100% and 100%). Samples were allowed to stand in each of these solutions for a minimum of 20 minutes. Samples were then immersed in progressively more concentrated solutions of EM grade acetone in ethanol (30%, 60%, 80%, 100%). Every effort was made to keep the samples wet until the final state of preparation, which was accomplished using liquid CO₂ in a critical point dryer. Samples were mounted to Al SEM stubs using commercially available carbon tape and Duco glue. Some samples were simply air-dried for comparison. Little difference was apparent in SEM images between these air-dried versus more laboriously-preserved samples, with the exception that masses of filaments tend to mat together more under air drying; however, the size of balls and filaments is the same in all techniques.

The single most important procedure in sample preparation for high-resolution study is that a gold-coating time of 1 minute or more produces tiny gold balls as artifacts which, at 20,000X or more, closely resemble “nannobacteria” (Folk and Lynch, 1997b). We gold coat for a standard time of 30 seconds only.

Our JSM-T330A (1989 Scanning Electron Microscope) at the University of Texas can obtain magnifications up to 100,000 X provided one uses (1) a very close working distance of 9 mm; (2) very low broad and dim spot size (setting of “9 o’clock” on the dial), (3) highest voltage of 30 kv, and (4) very careful stigmatism (which sometimes needs to be adjusted for each new field). To see the nannobacteria clearly, it is advisable to go up to 50,000 X, at which enlargement the average nannobacterium in human arterial plaque measures about 2 μm on the 3 x 2 cm EXP screen. On this machine, our limit of resolution at highest power is about 10-15 nm.

We examined plaque from the four Austin patients (provided by Dr. G. P. Rodgers). Each SEM field reveals a fascinating nanoworld of textures and fabrics. However, all four patients showed substantially consistent results. Undiseased portions of tissue are smooth, without any filamentous or ball-like features. Filaments, or filaments made of chains of balls, appear to herald the masses of closely-packed balls that make up the densest arterial blockage, which is composed of Ca₅(PO₄)₃OH (hydroxyapatite). Balls can form unorganized three-dimensional masses, like a bag of peas, or they may be organized into sheets made of monolayers of balls, or the balls may form stubby chains of four to eight individuals.

Biologic Work done at the Mayo Clinic

Medical scientists at the Mayo Clinic have been working on arterial plaque since 1998 under the direction of Drs. Franklin Cockerill, John Lieske and Virginia Miller; in cooperation with Dr. O. Kajander’s medical research group in Kuopio University, Finland (Folk et al., 2001). Their results began to establish, using Koch’s postulates, the infectious etiology of arterial calcifications that we studied in the SEM (Miller et al., 2004).

A critical identification of the correlation between nannobacteria/nanoparticles and arterial calcification was made by the Mayo group, who used light microscopy to identify calcification using a von Kossa stain (silver nitrate) of calcium phosphate deposits (Frondel and Prien, 1946 fig. 1). The Mayo group then identified the mineral by energy dispersive X-Ray microanalysis as hydroxyapatite, $\text{Ca}_5(\text{PO}_4)_3\text{OH}$. The adjacent microtome section of identical artery also stained positively for nannobacteria, using commercially available monoclonal antibodies (8D10) developed against bovine serum, provided by Kajander's laboratory (Nanobac Oy, Kuopio, Finland). The matching discrete area also absorbed abundant brown stain, indicating the presence of 8D10-positive structures. Other microtome sections showed some areas that stained positively with the 8D10 antibody but did not take Ca stain – suggesting the presence of nanoparticles that had not yet been calcified. Furthermore, some sections decalcified with EDTA also stained positively for nannobacteria that remained after removal of the soluble mineral.

Researchers at the Mayo Clinic also took inoculates from calcified sections of human aneurysms, cultured them for several weeks in artificial urine solution, and were able to obtain elliptical phosphate-coated nannobacterial structures that measured about $0.08 \times 0.25 \mu\text{m}$ (fig. 2); they were similar to cultures of nannobacteria from bovine serum. TEM showed elliptical organisms about 0.1×0.2 micron embedded within the phosphate (fig. 3), with cell walls measuring at 25-35 nm thick (data by current authors on images from Miller et al, 2004). At this magnification, centers of cells were lighter but no internal organelles were seen. Mayo clinic scientists also stained cultures with picogreen, which revealed the presence of DNA. The precise sequence of nucleic acids has not yet been identified, but is under investigation. All these tests provide critical information favoring the hypothesis that nannobacteria are some form of living organism.

Observations and interpretations based on high-resolution SEM microscopy (University of Texas)

The features described and illustrated here were found blocking the interior surface of arteries or occurring in lenticels within the artery wall revealed by sectioning. The majority of these observations and the photomicrographs were made at magnifications between 20,000 – 200,000 X. Fig. 4 gives a general overview of the diseased surface of an arterial wall, and we now describe the features in order of increasing complexity.

1) Smooth filaments are typically 50-80 nm in diameter, do not taper, and we do not find any evidence of branching (fig. 5). At the free, growing end a small sphere only slightly larger than the filament is present, giving the appearance of a match head. These filaments may be loose, like a spider web, or occur as closely packed parallel swaths, or form a densely felted mat. Our EDAX analysis facility is not able to detect any elements lighter than Na; these filaments do not show any patterns for heavier elements, and thus we deduce that they consist of H, C, N, and O, characteristic of non-calcified biologic tissue. They resemble fibrin (part of blood's normal clotting mechanism) and sometimes wall off hard chunks of plaque.

2) Isolated spheres are typically 25-60 nm (fig. 6 and 10) and occur scattered over the surfaces of erythrocytes and leucocytes, (red and white blood corpuscles). Individual spheres are too

small to be separately analyzed for their chemistry. These may represent solitary nannobacterial “scouts” that later develop into deleterious colonies.

3) Filaments coated with spheres, (fig. 7, 8). Filaments and spheres entangle erythrocytes (red blood cells) (fig. 7), probably as an initial stage of arterial disease. The spheres, with diameter of 35-60 nm, are generally of similar size as filament thickness (fig. 8).

4) Fluffy clumps of spheres. These irregular, very porous masses of spheres cover parts of erythrocytes and appear to ensnare them to the blood vessel wall (fig. 9A). Typically the spheres are 30-80 nm (fig. 9B), and the clumps range from 1-5 μm in overall diameter.

5) Long chains of spheres resemble rosaries, but no obvious smooth filament forms a substrate for connecting the spheres. They make extended strings similar to spider webs (fig. 10). Spheres are typically 35-80 nm.

6) Short chains of spheres are common on the exterior surface of pieces of arterial plaque “rock.” The typical sphere size is 30-50 nm, thus they are relatively small; chains appear to be 4 to 8 spheres long (fig. 11). In figure 12, the chains are longer, and the spheres appear to merge. They appear similar to altered mid-ocean ridge basalt (fig. 21).

7) Sheets usually consist of monolayer of spheres, like billiard balls on a table (fig. 13). The arrangement of spheres within the sheet is usually random, but in some examples the sheets are made of parallel chains placed tightly side by side. Spheres are typically 35-60 nm, but those on the edges of the sheets are sometimes slightly larger and brighter, resembling nannobacterial clays (Folk and Lynch, 1997b) (fig. 19).

8) Solid masses of spheres form some accumulations large enough to be visible to the naked eye. Some of these accumulations were brittle and made discernible cracking noises when broken off for mounting on the SEM stub. Others formed granule (1-3 mm) sized chunks that were literally as hard as rock. These pieces had to be broken using a craft knife as a chisel and a small hammer. In these solid masses, spheres are 30-50 nm and quite distinct, i.e., they are like peas in a bag, not connected with any obvious sort of glue-like material, though they must somehow be sticky enough to hold together (fig. 14). In some examples it can be seen that a solid mass is made of shingling monolayers of spheres (fig. 15). Structures described as (6) through (8) test out with EDAX as Ca and P, thus they are hydroxyapatite; whereas the earlier features show no pattern, thus we identified them as non-mineralized organic tissue.

9) Hollow spheres were found in one sample. These curious 1-5 μm diameter objects resemble “whiffle balls” – a hollow, irregular ball, whose wall consists of a monolayer of nanometer-sized spheroids; large holes are present in this wall (fig. 4, 6A, 16A and 16B). All the “whiffle balls” found in this study came from one of the four patients, a 72-year old hyperlipidemic (elevated concentrations of lipids in the plasma) female, a smoker with chronic pulmonary disease and renal insufficiency.

10) Finger-like projections were found in the single sample taken as a control specimen. This section of non-calcified mitral valve was removed from a trauma victim who was subsequently and unexpectedly found to have had rheumatic fever. The smooth mitral tissue displays scattered patches of minute finger-like projections about 100 nm wide and 600 nm long (fig. 17). They range from single spheres to caterpillar-like forms. They resemble microvilli, but their patchy distribution suggests they were pathologic and not part of the normal tissue.

Interpretations based on high-resolution SEM microscopy at the University of Texas

We present here a morphological sequence of a disease process that perhaps proceeds at variable rates in different arteries or in different patients. Systematic study of calcifying disease in experimental animals may help in understanding the process further.

In summary, we find (1) smooth filaments resembling fibrin that may or may not be pathologic. The “line of descent” for nanoball structures starts with (2) isolated single balls, which can rest on blood cells or (3) begin to decorate the filaments in (1); the balls very likely are pathologic, because we did not find them in the control samples. Balls then aggregate to form (4) fluffy clumps of balls. Balls then may become more organized to form (5) long chains of balls. Closely related are (6) short chains of balls, forming caterpillar-like structures. Another variant forms (7) sheets of balls: and the ultimate arterial obstruction is (8) a solid mass of balls, sometimes without any visible gross structure, at other times made of overlapping stacks of sheets.

Our training as geologists has left us either hopelessly biased or fortunately blessed. To our eyes, this sequence bears a close resemblance to the hierarchic order of silicate structures — single SiO_4 tetrahedron to chains to sheets to three-dimensional structures

The progression of morphotypes from those described in items 1-8 in human arterial disease has instructive analogues in the mineralogical world, also presumably created by the activities of nannobacteria. In numerous geologic examples we see isolated spheres and fluffy clumps of spheres (fig. 18). These geologic nannobacteria are of similar size to nannobacteria within arterial plaque. Monolayer sheets of spheres with slightly brighter and larger ones on the edges of the sheets are seen in many of the clay minerals (fig. 19). Solid masses (fig. 20) and chains of spheres also occur (fig. 21). There is a remarkable resemblance both in morphology and scale between the short, caterpillar-like chains of Ca phosphate in arterial plaque, with similar caterpillar-like chains in earthly and Martian examples (fig. 21, 22) (Folk and Taylor, 2002).

Hollow spheres and Finger-like projections

In addition to this reasonably orderly morphological sequence, several unknown entities occur. Perhaps the most curious of these are the hollow, whiffle ball-like spheres (fig. 4, 6A, 16A). We propose that the wall of spheroids originally coated some 1-5 μm object that lysed or disappeared in some way, leaving a resistant covering behind. If, as it appears from these analogues, the “whiffle balls” in our patient may have had some vanished organic object in the center, it is difficult to know what it was. The balls are over twice the diameter of most bacteria, but only about half the diameter of “live” erythrocytes or leucocytes.

Analogs to the whiffle balls occur in other situations. A dead and lysed *Staphylococcus* cell (Nilsson, 1987, p. 83), attacked by antibiotics, is about 1 μm in diameter and shows similar holes; the author stated it “looked like a skull.” Asada and Tazaki (2001) found equant hollow 2 μm balls originally covering microbial cells in Japanese hot springs. We have also seen hollow 3 X 4 μm balls in iron rust, and a sample of altered mid-ocean ridge basalt (R. T. Evans, personal collection) contains prolate ellipsoids (1-2 X 3 μm) with large holes in one end (fig. 23). We interpret these as decayed, elliptical, probably microbial bodies coated with nannobacteria. Our current thought is that the whiffle balls in

our patient are coatings on some pathologic and well-nourished brand of bacteria that perished either "naturally" or through medication. It is also possible that these are balls of lipids now disappeared, as the patient had hyperlipidemia.

A significant and unexpected discovery of this study is the documentation of finger-like projections, which were found only in the mitral valve sample selected as a control (fig. 17). Because they only appear in scattered patches we suggest that they are probably pathologic. We interpret these as rod-shaped bacteria or nannobacteria and note that these objects are very similar in appearance and in scale to the "nanobes" cultured on sandstone by P. Uwins (1998). These nanobes also show 40-100 nm spheroids grading into filaments several hundred nm long. The presence of these embedded rod-shaped bacteria is most significant, because experienced cardiologists had not seen these bacteria before. Damage to mitral valves by rheumatic heart disease has long been recognized, but the presence of embedded rod-shaped bacteria had not previously been noted. We suggest that if this is true, perhaps other pathogenic bacteria may be existing unnoticed, or at least previously unseen, in the human vascular system. A relatively new test for screening patients with heart disease, C-reactive protein, or CRP, is a measure of inflammation occurring in the body. Risk of heart disease correlates to CRP level, but the reasons why remain unclear.

This brings us back to the question what are the small (25-80 nm) spheres that form the progression of morphotypes so similar to the succession of the silicate mineral families? RLF suggests that the most likely explanation for these spheres is that they are very small bacteria, indeed nannobacteria. The case for these spheres being self-replicating entities has been argued at length based on their culturability, putative staining for DNA, immunostaining with 8D10 antibodies, absorption of uridine, and TEM evidence of cell walls (Miller et al. 2004).

Alternatively, BLK notes that similar small spheres have been described as the preserved form of polymeric substances found in microbial biofilms, which are aggregations of bacteria (and or nannobacteria) and the extracellular polymers they secrete. The solid masses of balls described in our study are similar in appearance to known biofilms (Westall et al., 2000; Fratesi et al., 2004). The polymeric substances of the biofilm may be much more abundant and visible under SEM than the bacteria that they surround (Fratesi, 2002.) The spherical forms preserved in the biofilm may in fact be the only evidence of the presence of bacteria (Westall et al., 2000).

In either case, however, these tiny spheres are evidence of bacteria, either as nannobacteria or the preserved form of polymers surrounding larger bacteria. The presence of these tiny spheres suggests that previously undocumented pathologic bacteria may indeed occur in the human vascular system and certainly merits further study. The medical community has a burgeoning interest in the role of (nano)bacteria in other human diseases (Mulhall and Hansen, 2005).

Our final suggestion, then, is that cholesterol begins forming either within the tissue of the arterial wall, or as artery-clogging clots. In some patients, nannobacteria "infect" the cholesterol, perhaps as a food source. Eventually the nannobacteria begin to precipitate calcium phosphate, which forms a replacement of their corpses – the arteries or their clots harden and the patient suffers more seriously. We propose two fruitful lines of research for medical investigators: (1) It should be important to see if

a correlation exists between patients who have nannobacterial cells in their bloodstream (work by Kajander's group) and those who have developed atherosclerosis. (2) Arteries develop calcification problems, veins do not. Could this be a matter that nannobacteria require a higher level of oxygenation in the blood?

ACKNOWLEDGMENTS

This paper would not have been possible without the contributions of Virginia Miller and her colleagues at the Mayo Institute. The first atherosclerotic tissue sample was provided by Dr. Dan Hardy from St. David's Hospital in Austin, Texas. Jennifer Roberts Rogers provided advice on the specimen processing protocol. Additional tissue specimens were provided by Dr. Stephen Dewan and Dr. Daniel Irons. John Mendenhall at the University of Texas Microscopy Core Facility provided advice on critical point drying methods. In 2013, Nicole Raney put this version on the website.

REFERENCES

- Akerman KK, Juronen I, Kajander EO (1993) Scanning electron microscopy of nanobacteria – novel biofilm producing organisms in blood. *Scanning Electron Microscopy*, 15 (Suppl. III), 90-91.
- Aloisi G, Gloter A, Kruger M, Wallman K, Guyot F, Zuddos P (2006) Nucleation of calcium carbonate on bacterial nanoglobules. *Geology*, 34, 1017-1020.
- Asada R and Tazaki K (2001) Silica biomineralization of unicellular microbes under strongly acidic conditions. *Canadian Mineralogy*, 39, 1-16.
- Bratos-Pérez MA, Sánchez PL, García de Cruz S, Villacorta E, Palacios IF, Fernández-Fernández JM, Di Stefano S, Orduña-Domingo A, Carrascal Y, Mota P, Martín-Luengo C, Bermejo J, San Roman JA, Rodríguez-Torres A, Fernández-Avilés F (2008) Association between self-replicating calcifying nanoparticles and atherosclerosis. *European Heart Journal*, 29:3, 371-376.
- Chafetz HS, Akdim B, Julia R, and Reid A (1998) Mn- and Fe- rich black travertine shrubs: bacterially (and nanobacterially) induced precipitates. *Journal of Sedimentary Research*, 68, 404-412.
- De Long E (1999) Diminutive cells in the ocean – unanswered questions. In: *Size limits of very small organisms* (eds. Knoll A, Osborne MJ, Baross J, Berg HC, Pace NR, Sogin M). Space Studies Board National Research Council, Washington, pp. 81-83.
- Folk RL (1992) Bacteria and nannobacteria revealed in hardgrounds, calcite cements, native sulfur, sulfide minerals, and travertines. *Geological Society of America Annual Meeting, Abstracts with Programs*, 24, 104.
- ___ (1993) SEM imaging of bacteria and nannobacteria in carbonate sediments and rocks, *Journal of Sedimentary Petrology*, 63, 990-999.
- ___ (1998) Nannobacteria in the natural environment and in medicine. *Alpe Adria Microbiology Journal*, 7, 87-95.
- Folk RL, Kirkland BL (2007) On the “Smallness of Life”: New TEM evidence from biofilm in hot springs, Viterbo, Italy (abs.). *Geological Society of America Abstracts with Programs*, 39:6, 421.
- Folk RL, Kirkland BL, Rodgers JC, Rodgers GP, Rassmussen TE, Lieske C, Charlesworth JE, Severson SR, Miller VM (2001) Precipitation of minerals in human arterial plaque: the potential role of nannobacteria. *Geological Society of America Abstracts with Programs*, 32, A-189.
- Folk RL, Lynch FL (1997a) Nannobacteria are alive on Earth as well as Mars. In: *Proceedings SPIE* (ed. Hoover RB). San Diego, 3111, 406-419
- ___ (1997b) The possible role of nannobacteria (dwarf bacteria) in clay-mineral diagenesis. *Journal of Sedimentary Research*, 67, 583-589.
- ___ (1998) Carbonaceous objects resembling nannobacteria in the Allende Meteorite. In: *Proceedings SPIE* (ed. Hoover RB). San Diego, 3441, 112-122

- ___ (2001) Organic matter, putative nanobacteria, and the formation of ooids and hardgrounds. *Sedimentology*, 48, 215-229.
- Folk RL, McBride EF and Yancey TE (2012). Proposed Microbial Origin of Kaolinite in Simsboro Sandstone (Upper Paleocene), Limestone County, Texas. *Trans. Gulf Coast Assoc. Geol. Soc.* 42:117-129.
- Folk RL, Taylor LA (2002) Nanobacterial alteration of pyroxenes in martian meteorite ALH84001. *Meteoritics and Planetary Science*, 37, 1057-1070.
- Fratesi SE (2002) Distribution and morphology of bacteria and their by products in microbial enhanced oil recovery operations. Unpubl. M.S. Thesis, Mississippi State University, 78 pp.
- Fratesi SE, Lynch FL, Kirkland BL, Brown LR (2004) Effects of SEM preparation techniques on the appearance of bacteria and biofilms in the Carter Sandstone. *Journal of Sedimentary Research*, 74:6, 858-867.
- Frondel C, Prien ES (1946) Deposition of calcium phosphates accompanying senile degeneration and disease. *Science*, 103, 326.
- Jelic T, Malas AM, Groves SS, Jin B, Mellen PF, Osborne G, Roque R, Rosencrance JC, Chang H-H (2004) Nanobacteria-caused mitral valve calciphylaxis in a man with a diabetic renal failure. *Southern Medical Journal*, 97, 194-198.
- Jelic T, Tomislav M, Chang HH, Roque R, Malas AM, Warren SG, Sommer AP (2007) Nanobacteria-associated calcific aortic valve stenosis. *Journal of Heart Valve Disease*, 16, 101-105.
- Kajander EO (2006) Nanobacteria – propagating calcifying nanoparticles. *Letters in Applied Microbiology*, 42, 549-552.
- Kajander EO, Ciftcioglu N (1998) Nanobacteria an alternative mechanism for pathogenic intra- and extra-cellular calcification and stone formation. *Proceedings of the National Academy of Sciences of the United States of America*, 95:14, 8274-8279.
- Kirkland BL, Lynch FL, Rahnis MA, Folk RL, Molineux IJ, McLean RJC (1999) Alternative origins for nanobacteria-like objects in calcite. *Geology*, 27, 347-350.
- Knoll A, Osborn JJ (1999) Size limits of very small microorganisms, In: *Size limits of very small organisms* (eds. Knoll A, Osborne MJ, Baross J, Berg HC, Pace NR, Sogin M). Space Studies Board National Research Council, Washington, 148 pp.
- Lynch FL (1994) The effect of depositional environments and formation water chemistry on the diagenesis of Frio Formation (Oligocene) sandstones and shales, Aransas, Nueces, and San Patricio counties, Texas. Unpublished Dissertation, University of Texas at Austin, 303 pp.
- McKay DS, Gibson EK, Thomas-Keprta KL, Vali LH, Romanek CS, Clemett SJ, Chiller ZDF, Maechling CR, Zare RN (1996) Search for past life on Mars: possible relic biogenic activity in Martian meteorite ALH84001. *Science*, 272, 924-926.
- Miller VM, Rodgers G, Charlesworth JA, Kirkland BL, Severson SR, Rasmussen TE, Yagubyan M, Rodgers JC, Cockerill FR, Folk RL, Rzewuska-Lech E, Kumar V, Farell-Baril G, Lieske JC (2004) Evidence of nanobacteria-like structures in calcified human arteries and cardiac valves. *American Journal of Physiology, Heart Circulation Physiology*, 10.1152, H1115-H1124.

- Mulhall D, Hansen K (2005) *The calcium bomb: the nanobacteria link to heart disease and cancer*. The Writers' Collective, Cranston, Rhode Island, 228 pp.
- Nilsson L (1987) *The Body Victorious*. Delacorte Press, New York, 196 pp.
- Prabhakaran S (1999) Investigation of bacterial fragmentation as a possible origin of nanobacteria. Unpublished Master's Thesis, Southwest Texas State University at San Marcos, 70 pp.
- Rasmussen TE, Kirkland BL, Charlesworth J, Rodgers G, Severson SR, Rodgers JC, Folk RL, Miller V (2002) Electron microscopic and immunological evidence of nannobacterial-like structures in calcified carotid arteries, Aortic Aneurysms and Cardiac Valves. *Journal of the American College of Cardiology*, 39, suppl. A, 206A.
- Sánchez-Román M, Vasconcelos C, Schmid T, Dittrich M, McKenzie JA, Zenobi R, Rivadeneyra MA, (2008) Aerobic microbial dolomite at the nanometer scale: Implications for the geologic record. *Geology*, 36:11, 879-882.
- Schieber J, Arnott HJ (2003) Nannobacteria as a by-product of enzyme-driven tissue decay. *Geology*, 31, 717-720.
- Spark I, Patey I, Duncan B, Hamilton A, Devine C, McGovern-Tran K (2000) The effects of indigenous and introduced microbes in deeply buried hydrocarbon reservoirs, North Sea. *Clay Minerals*, 35, 5-12.
- Uwins PJR, Webb RI, Taylor AP (1998) Novel nano-organisms from Australian sandstones. *American Mineralogist*, 83, 1541-1550.
- Uwins PJR, Taylor AP, Webb RI (1999) Nannobacteria, fiction or fact? In *Organic Matter and Mineralization* (eds. Gilkson M, Mastalerz M), Boston, Kluwer. 421-444.
- Vali H, McKee M, Çiftçioglu N, Sears SK, Plows FL, Chevet E, Ghiabi P, Plavsic M, Kajander EO, Zare RN (2001) Nanofossils: a new type of protein – associated mineralization. *Geochemica et Cosmochemica Acta*, 65, 63-74.
- Vali H, Sears SK, Çiftçioglu N, Kajander EO (1999) Nanofossils and the size limits of life. *Lunar and Planetary Science*, 18, 99.
- Vasconcelos CO, McKenzie JA (1994) Morphology of dolomite precipitating nannobacteria from bacterial culture experiments. *Eos*, 75, 377.
- Wainwright M (1999) Nanobacteria and associated elementary bodies in human disease and cancer. *Microbiology*, 145, 2623-2624.
- Westall F, Steele A, Torporski J, Walsh M, Allen C, Guidry S, McKee D, Gibson E, Chafetz H (2000) Polymeric substances and biofilms as biomarkers in terrestrial materials: Implications for extraterrestrial samples. *Journal of Geophysical Research*, 105:E10, 24, 511-24, 527.

FIGURE CAPTIONS

Figure 1. Adjacent microtome slices (5 μ m apart) from diseased sclerotic artery. Left: section stained with von Kossa stain; areas rich in calcium are dark spots. Right: section treated with specific stain for nannobacteria, which turn dark. Stars mark cholesterol crystallites. The areas of calcium and nannobacteria are in similar but not identical locations due to microtome slice thickness (Reprinted by permission from Miller et al. 2004).

Figure 2. Nannobacteria culture grown from an inoculate from a diseased artery. The elliptical bodies are encased in calcium phosphate (Reprinted by permission from Miller et al. 2004).

Figure 3. TEM slice of nannobacteria colony with phosphate. Arrows show cells with electron-dense walls and less dense interiors, as in normal bacteria (Reprinted by permission from Miller et al. 2004).

Figure 4. Low magnification view of the interior surface of a diseased arterial wall, with leucocytes (L). Three “whiffle balls” (W) are also shown. There are several fluffy clumps of nannobacteria (N).

Figure 5. Smooth filaments resembling fibrin, the product of normal clotting processes. The filaments are basically clean. Compare with Figure 8.

Figure 6. Enlargements of Figure 4. A) Leucocyte (L) and a whiffle ball (W). Fibrin is in the background, and there are a few clumps of nannobacteria (N). B) Further enlargement of the leucocyte showing isolated nannobacteria (arrows).

Figure 7. Erythrocytes (E) appear to be entangled by filaments (probably fibrin) with balls. This may be a pathologic development as the erythrocytes no longer appear to be mobile.

Figure 8. Filaments, probably fibrin, with scattered clumps of nannobacterial spheroids that may show the beginning of pathogenesis.

Figure 9. A) Erythrocyte (E) partially covered by nannobacteria (N). B) Enlargement of 9A. Compare with fluffy clump of nannobacteria in an altered basalt (Figure 18) and a denser clump in an Italian soil (Figure 20).

Figure 10. Rosary-like chain of nannobacteria on an erythrocyte. There are some isolated nannobacteria of the same size (arrows).

Figure 11. Hard “rock” of calcium phosphate plaque choking an artery. The nannobacteria appear to form chains a few cells long. Compare with silicified nannobacteria chains in an ooid (Figure 22B), iron nannobacteria from a New Mexico travertine (22C) and altered pyroxenes in a Martian meteorite (22D).

Figure 12. Somewhat longer chains of phosphatic nannobacteria in arterial “rock”; compare Figure 21, ocean bottom alteration of basalt.

Figure 13. Nannobacteria forming sheets with brighter cells on the rims, resembling clay minerals in sandstone (Figure 19). Beneath the sheets at lower left is a network of nannobacteria chains. This is from a mineralized aneurysm (Reprinted by permission from Miller et al. 2004).

Figure 14. Arterial “rock” composed of short chains of nannobacteria; chains are distinct with no cement evident between them. Note similarity to altered pyroxenes in Martian meteorite (Figure 22D).

Figure 15. Arterial “rock” made of shingling sheets of phosphatic nannobacteria. Each ball is distinct; no cementing material is visible between them.

Figure 16. A) Two “whiffle balls” in stereo. The lower one is definitely a thin hollow shell. The upper one is more solid and appears to consist of a network of nannobacteria chains. B) Enlarged view of the wall of a “whiffle ball” consisting of a curving sheet of nannobacteria plus several larger ones that rim a hole in the wall (arrows).

Figure 17. Non-calcified mitral valve from the heart of a trauma patient subsequently found to have had rheumatic fever. A) Mostly isolated spherical nannobacteria that grade to the lower left into longer filaments. B) More common finger-like filaments, some apparently made of merged balls. Compare with Uwins (1998) “nanobes” in sandstone (Reprinted by permission from Miller et al. 2004)

Figure 18. Basaltic lava flow in contact with sea water, Mt. Etna, Acireale, Sicily. Chlorite flakes with a fluffy clump of nannobacteria (see Folk and Lynch, 1998). Compare with artery in Figure 9.

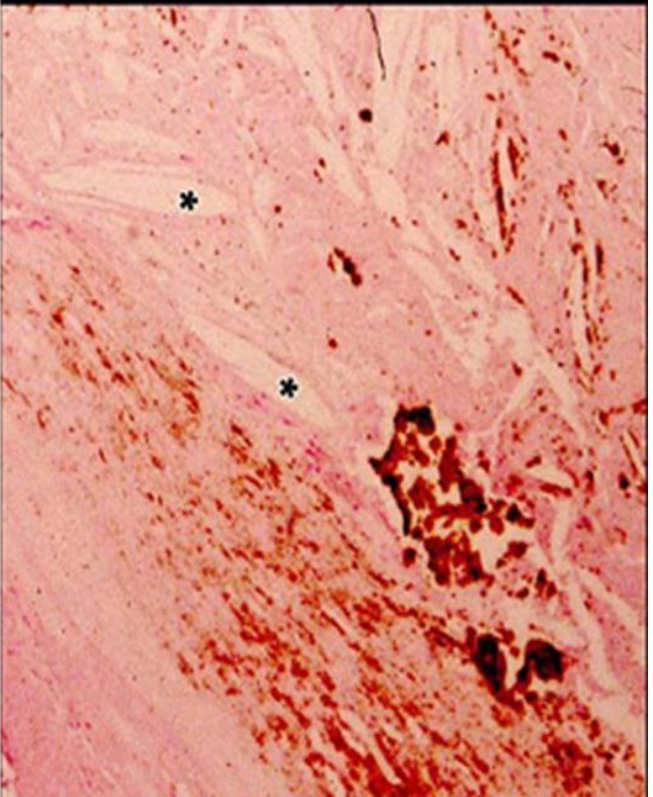
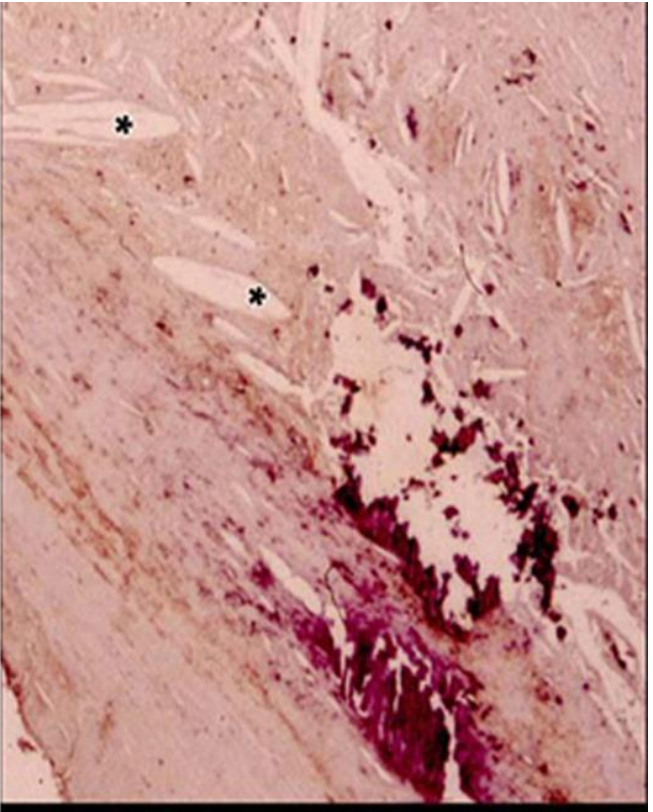
Figure 19. Chloritic shale from Oligocene Frio Sandstone, Texas; depth 11,243'. Nannobacteria form short chains of beads on the edges of clay flakes; compare with Figure 13 (Lynch, 1994, unpublished photo).

Figure 20. Soil on volcanic tuff, Viterbo, Italy showing a dense clump of nannobacteria on biotite flakes.

Figure 21. Mid-ocean ridge basalt in contact with sea water. Note long chains of nannobacteria on clay or mica flakes; compare with Figure 12.

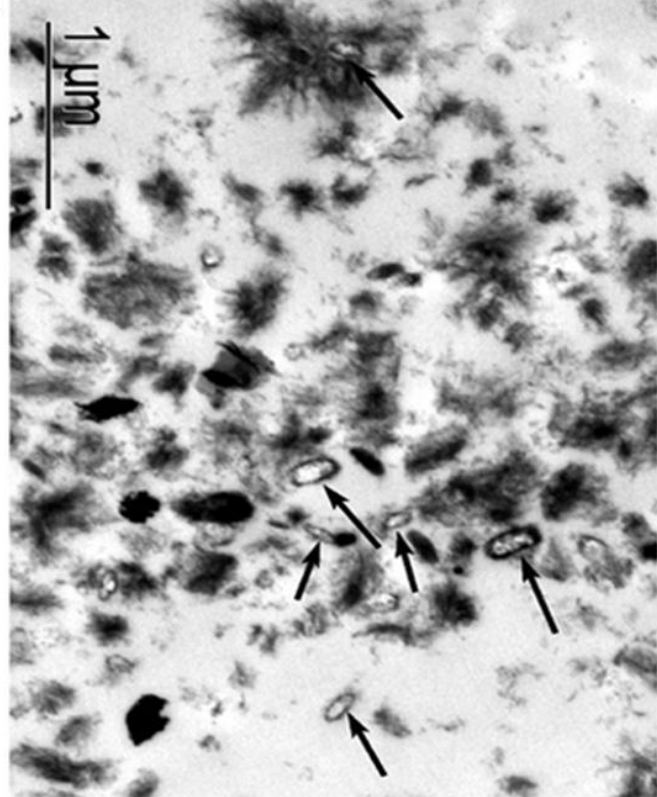
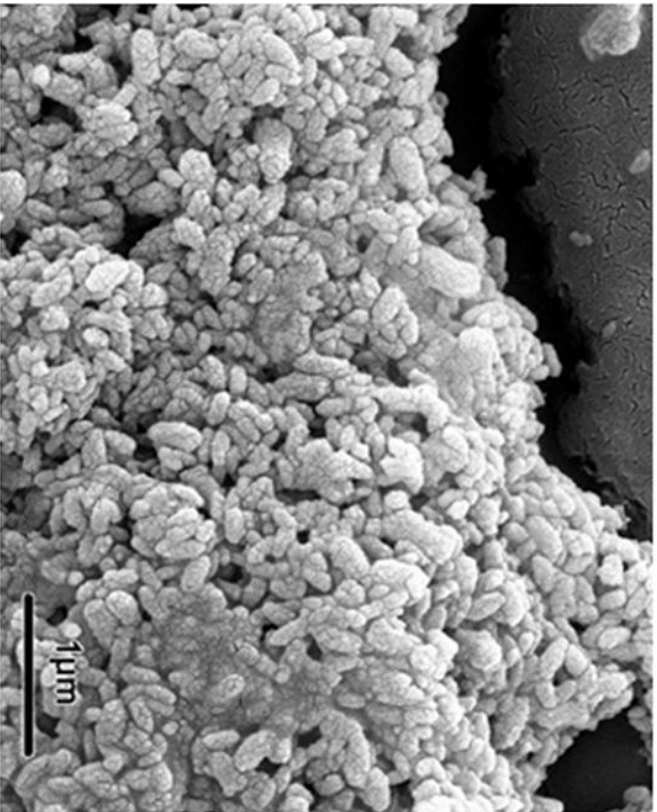
Figure 22. Comparison at approximately the same scale of human arterial plaque (A) with (B) nannobacteria chains on a quartz nucleus of an ooid, Salt Lake, Utah (C) with chains of Fe-bacteria in a New Mexico travertine; and (D) nannobacteria chains on pyroxene from the Martian meteorite ALH84001 (from Folk and Taylor 2002).

Figure 23. Mid-ocean ridge basalt, altered by exposure to sea water. A) Elliptical bacterial bodies with every gradation from complete cells (a), to those with small holes (b), with larger holes (c), those where only basal husks remain (d), to those where only the detachment scar is left (e). These may be analogues to the “whiffle balls” in diseased artery, where the bacteria cell itself is gone, leaving only the mineralized coating. B) An enlarged view, showing hollow and collapsing cells.



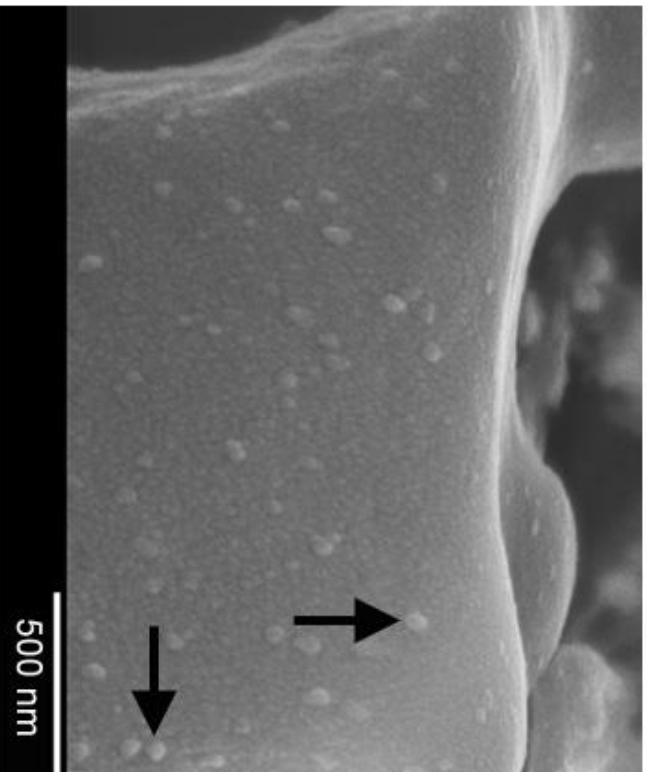
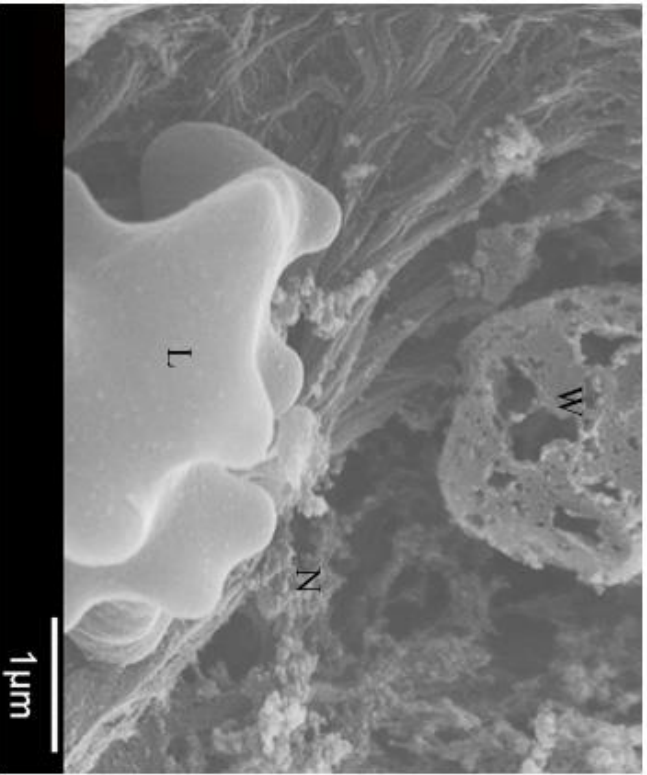
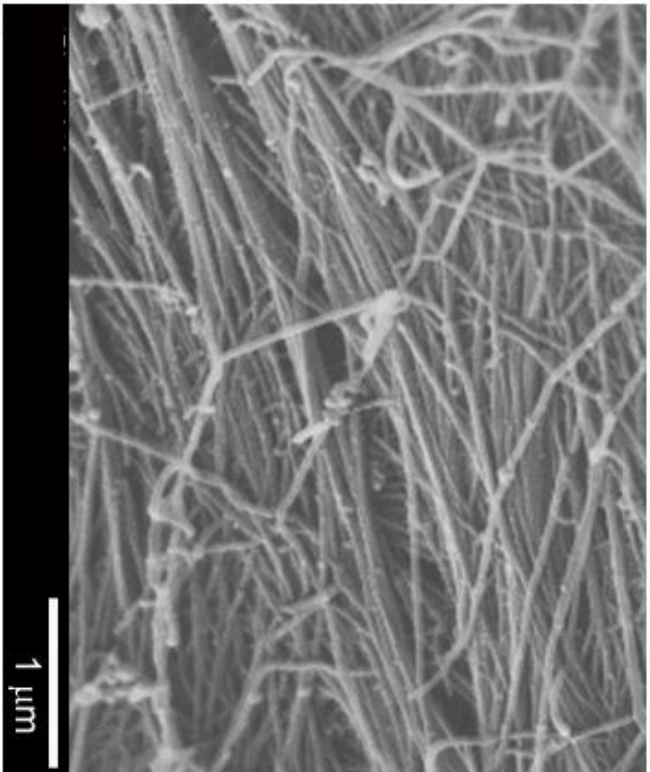
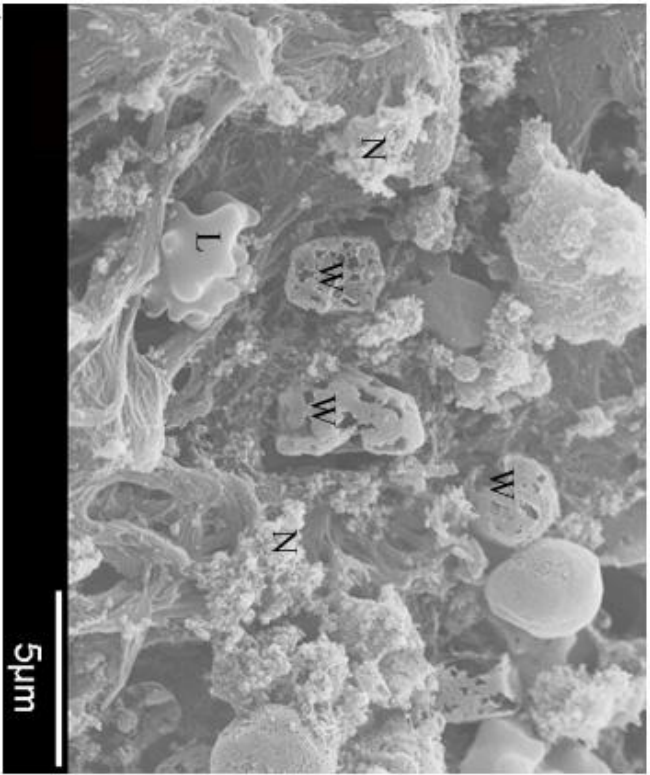
1A

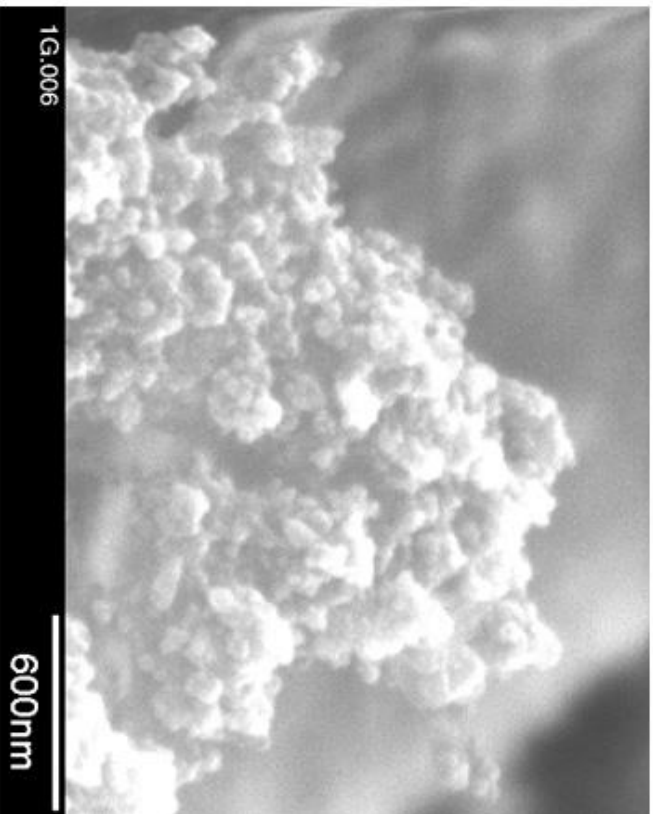
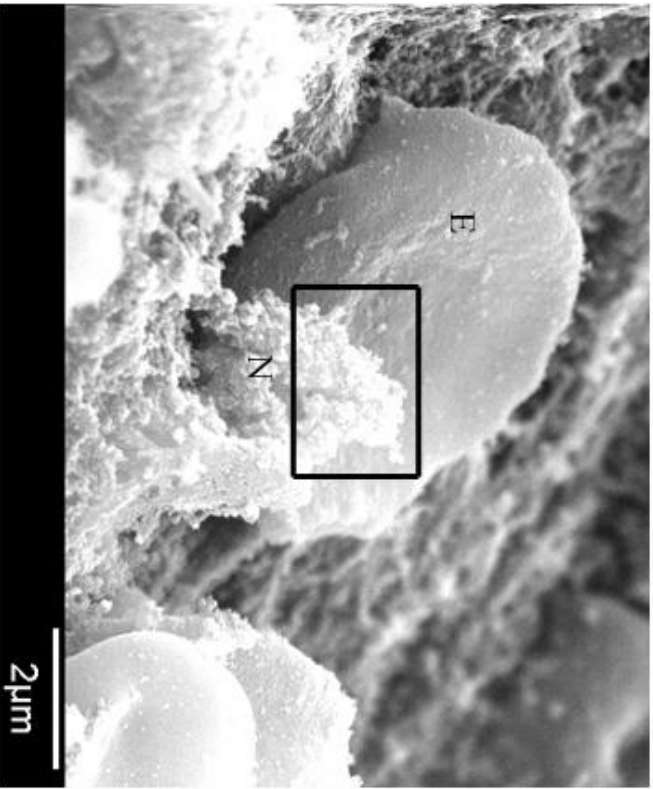
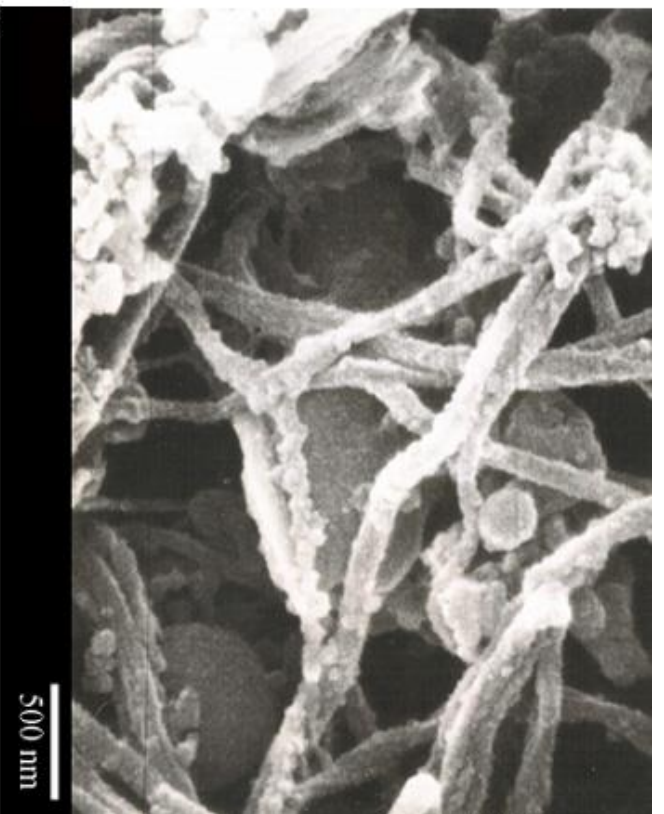
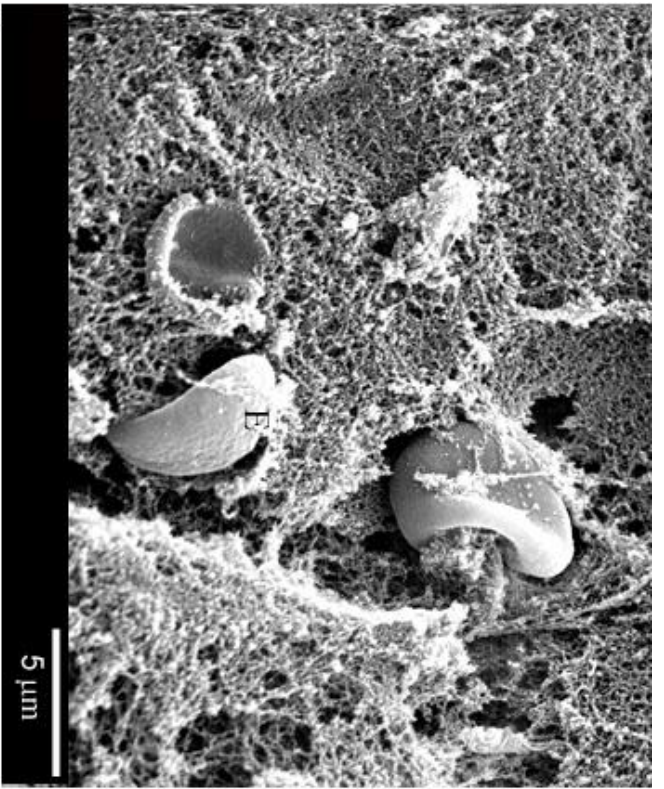
1B

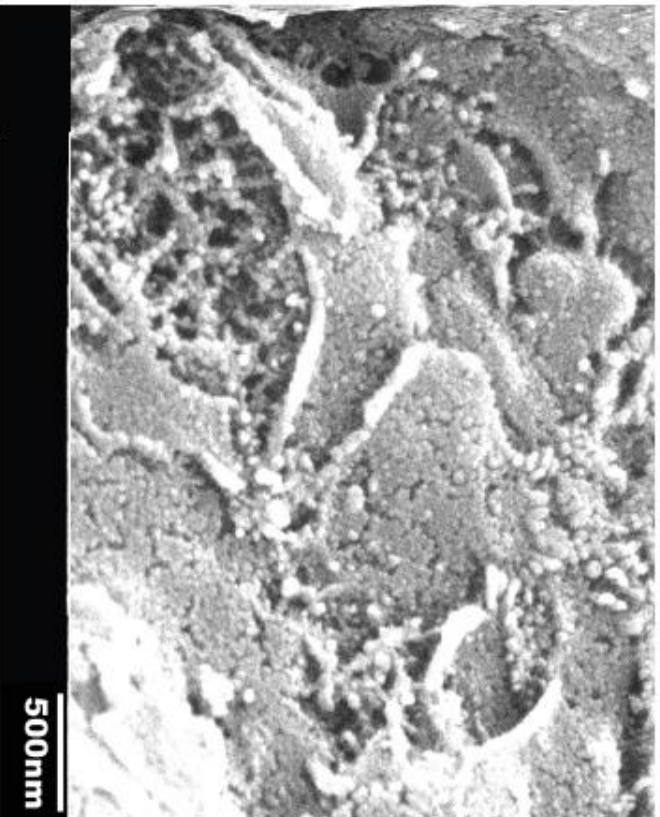
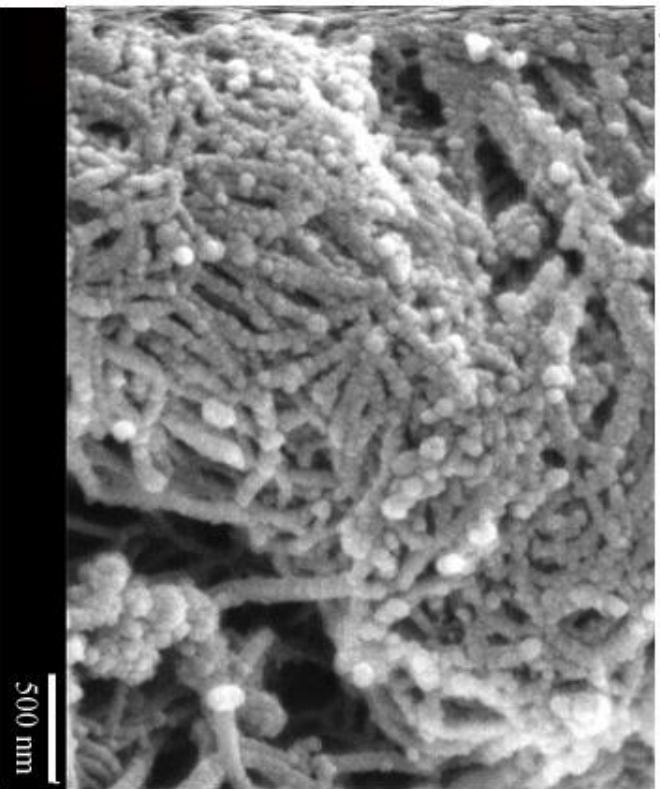
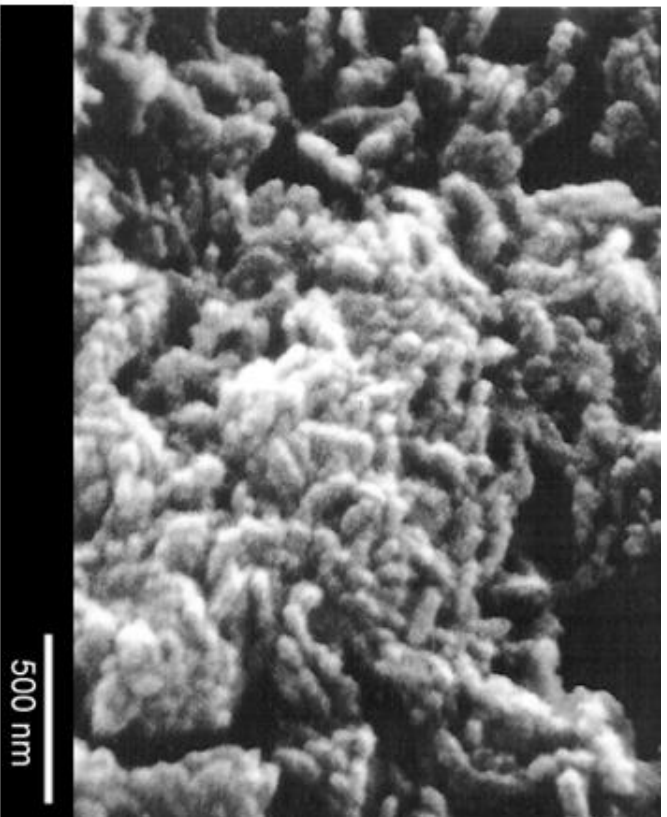
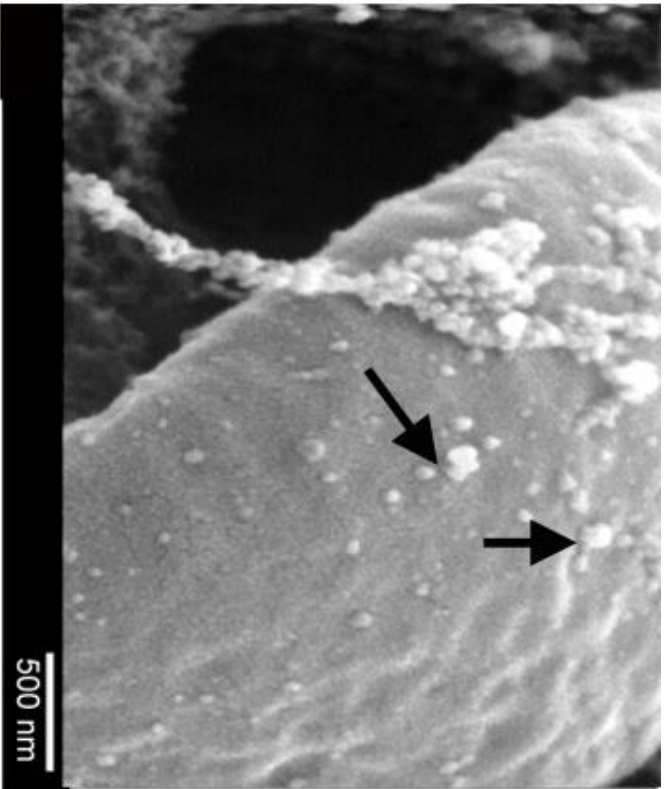


2

3

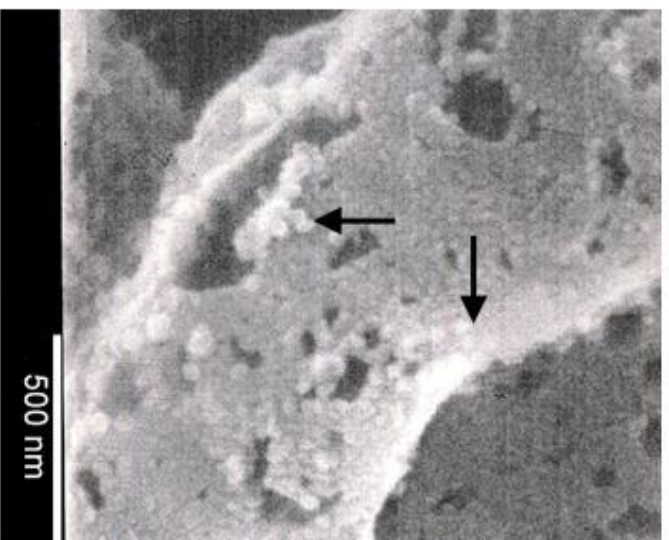
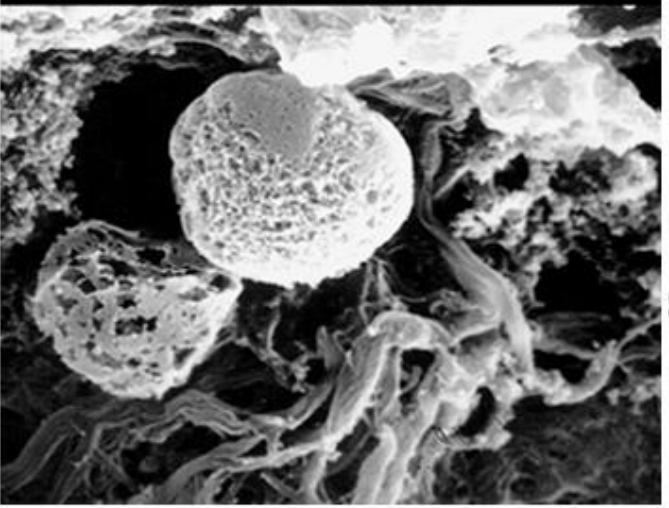
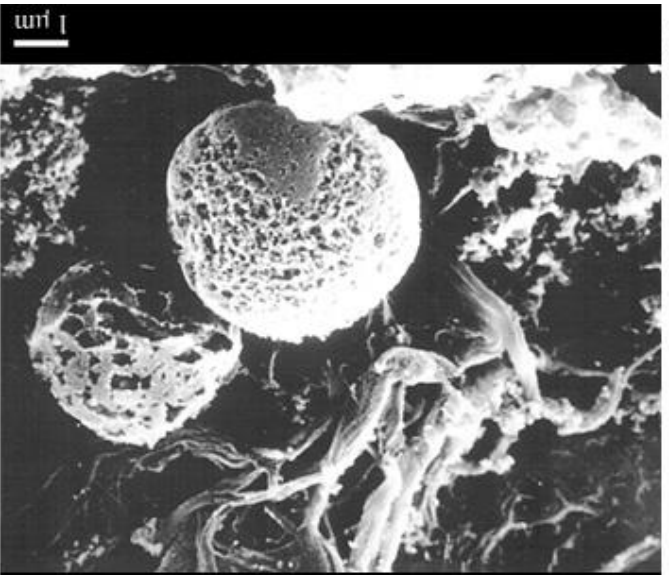
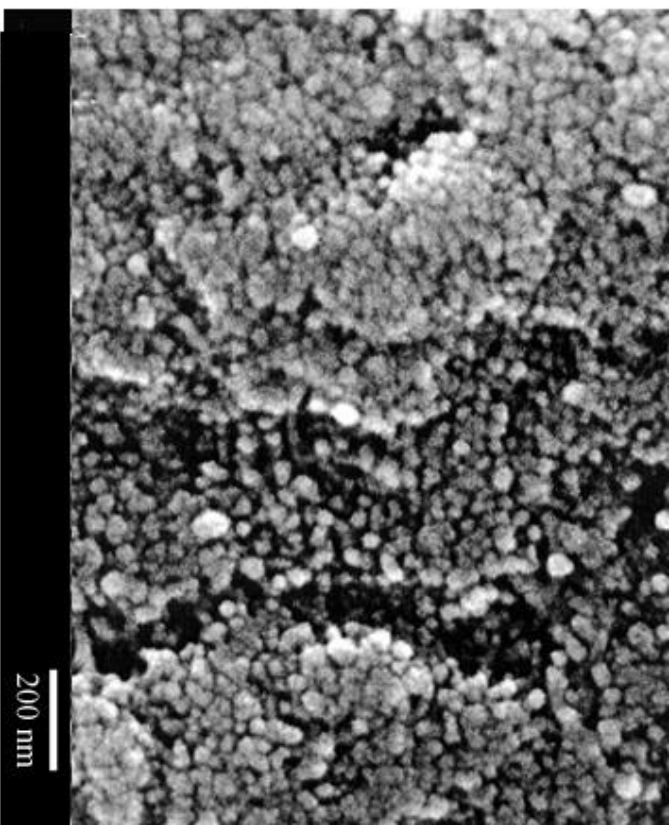
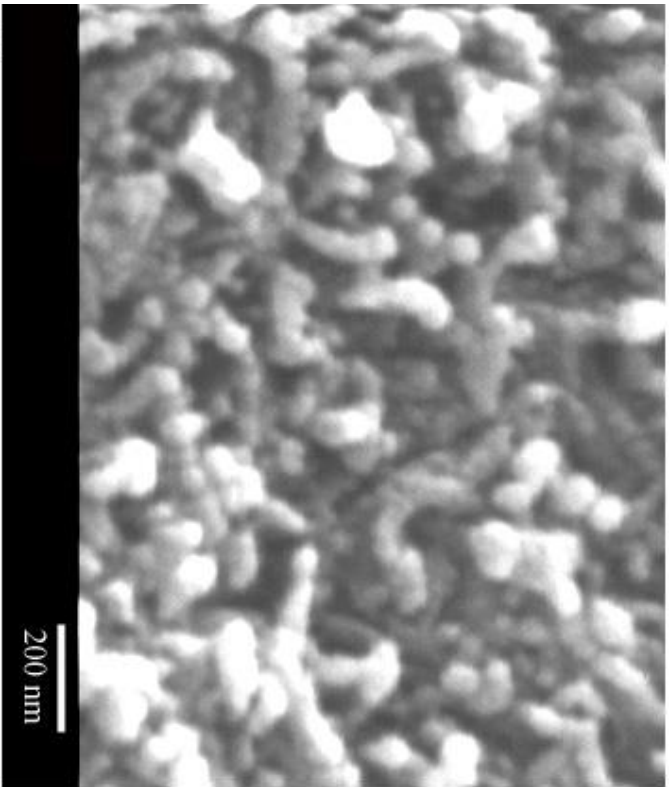


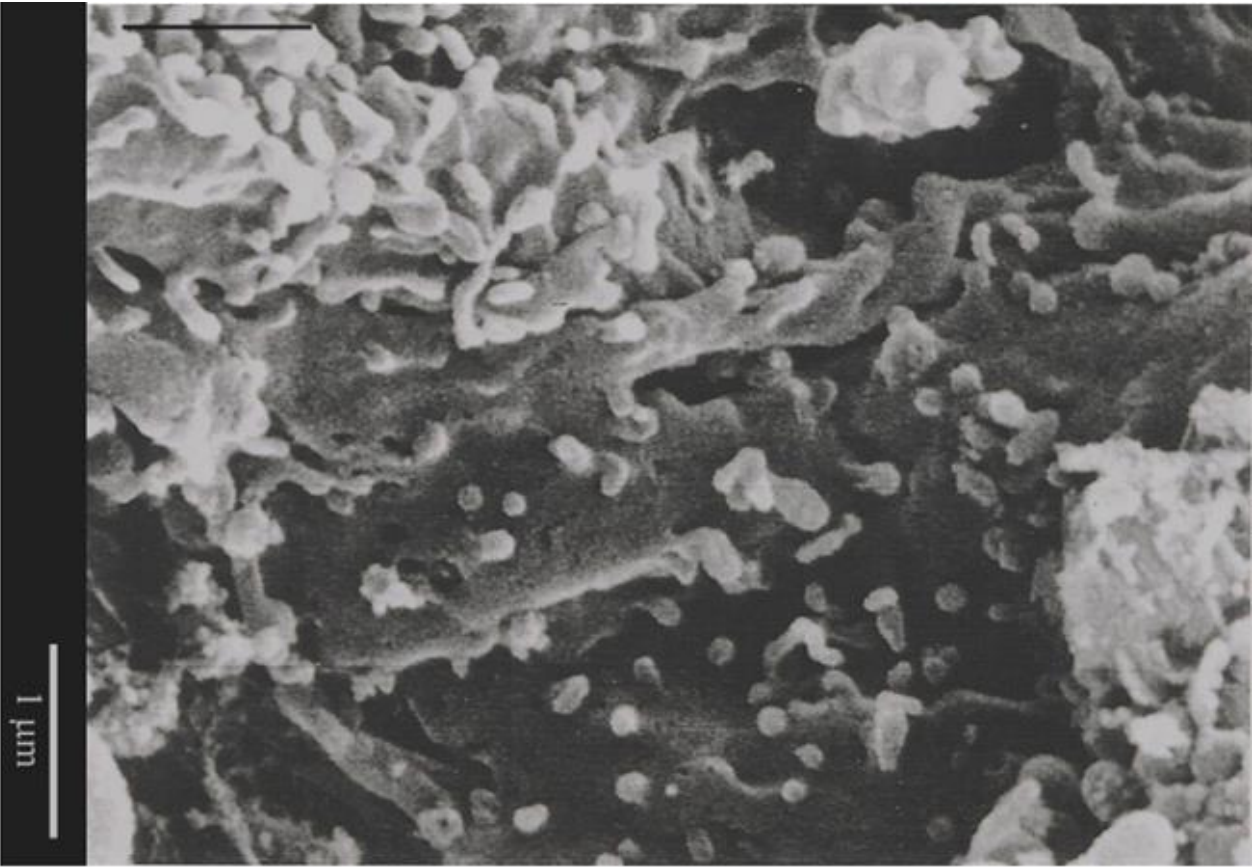




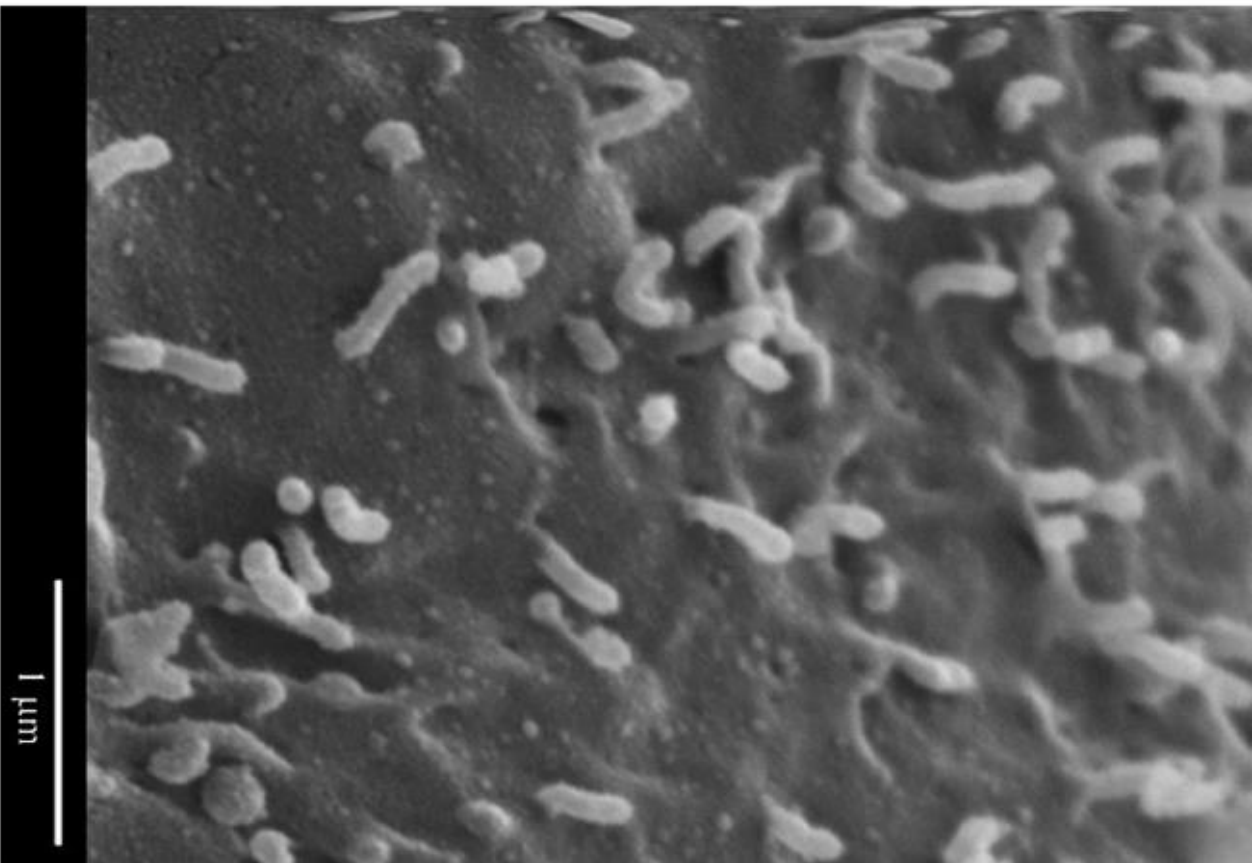
12

13

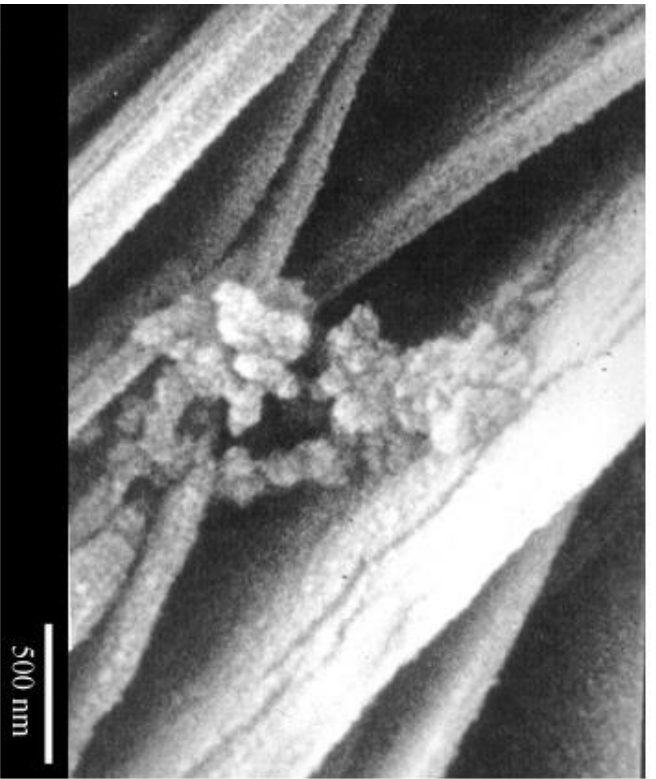




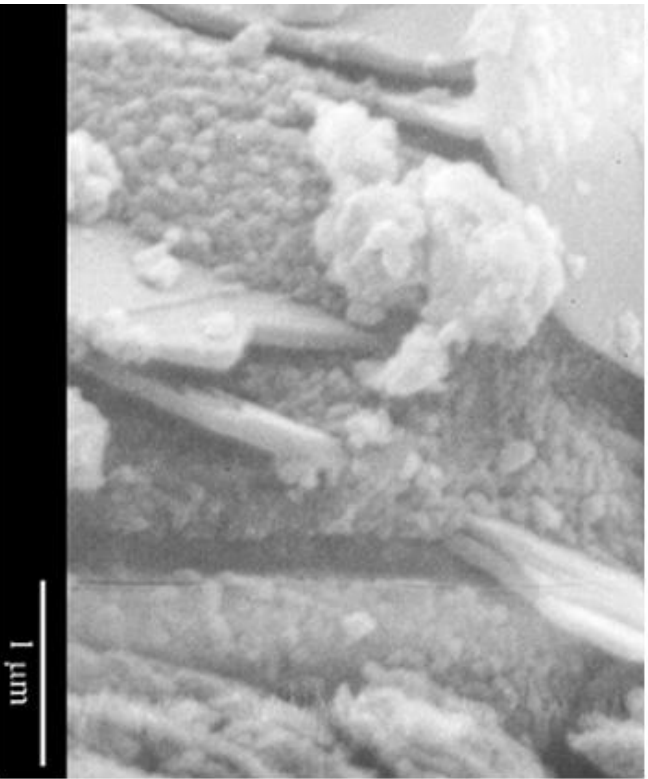
17A



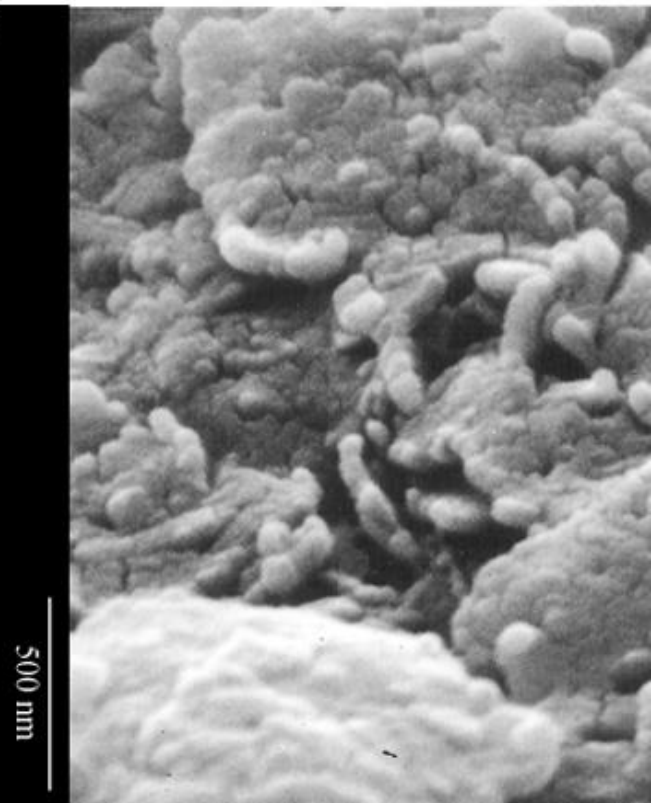
17B



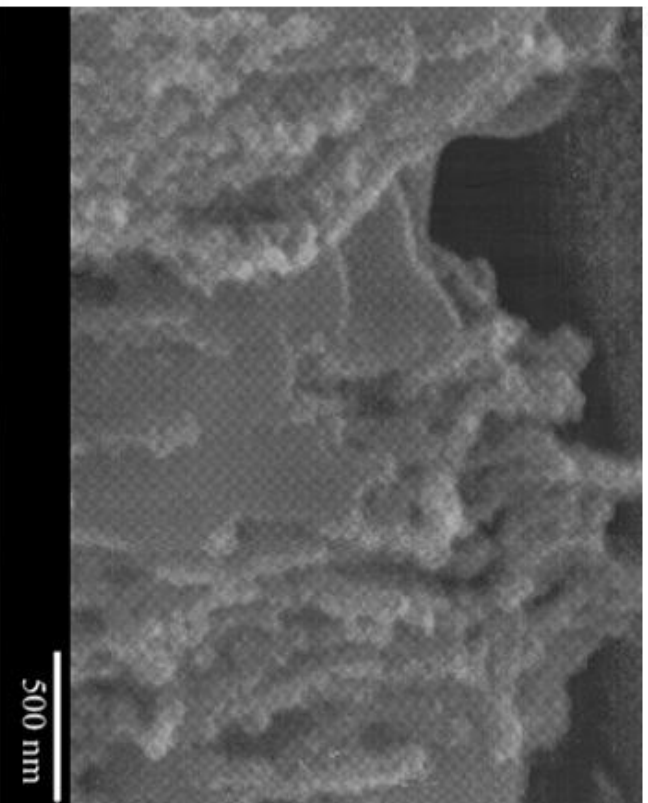
18



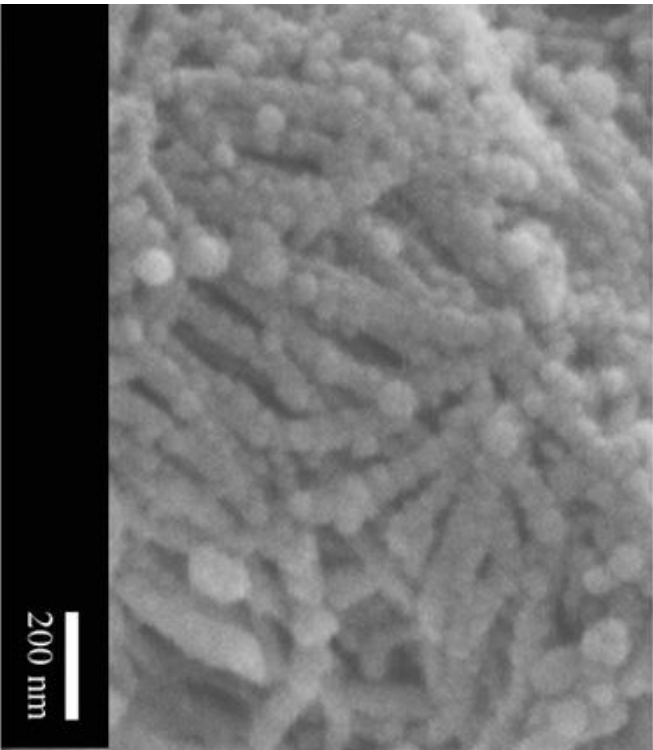
20



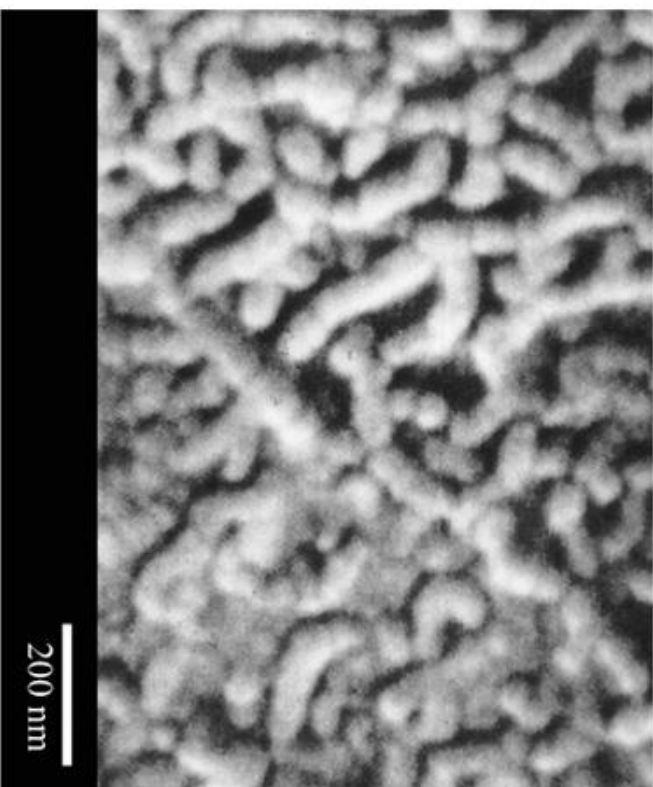
19



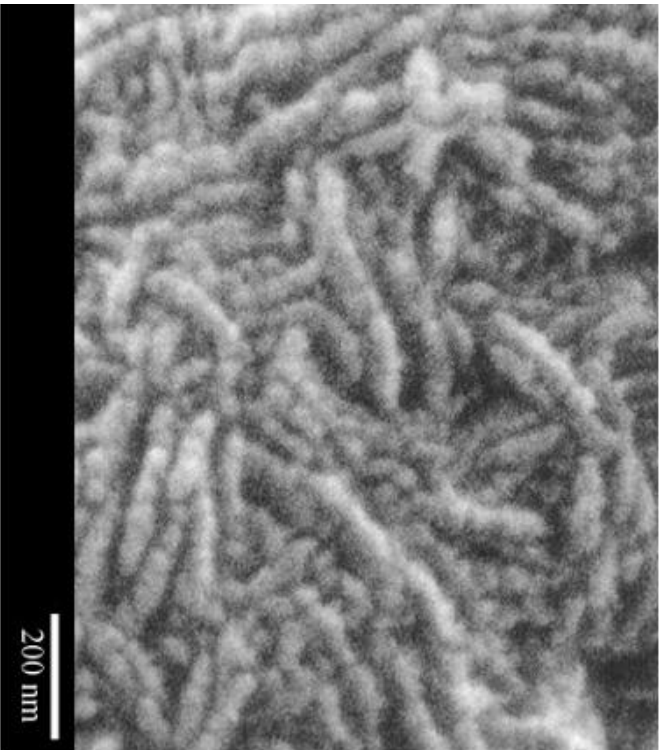
21



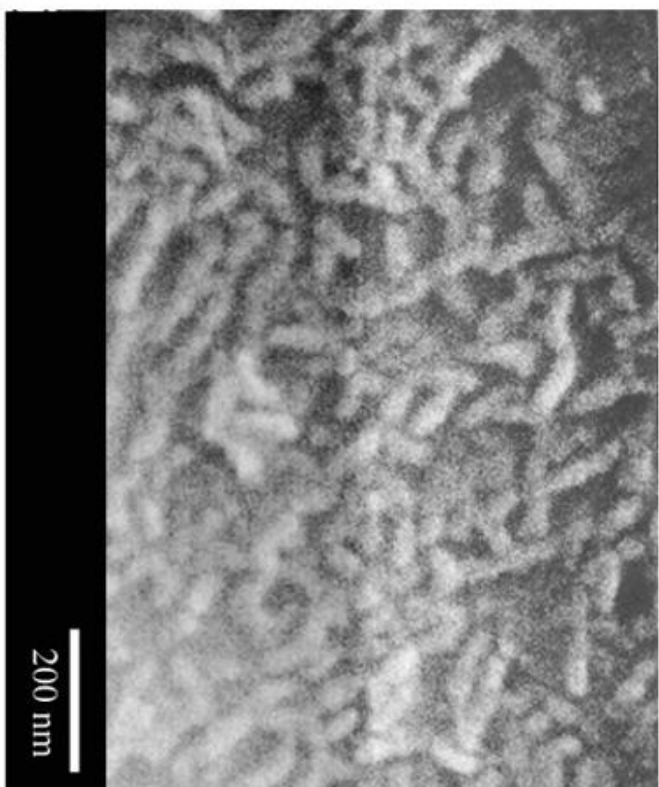
22A



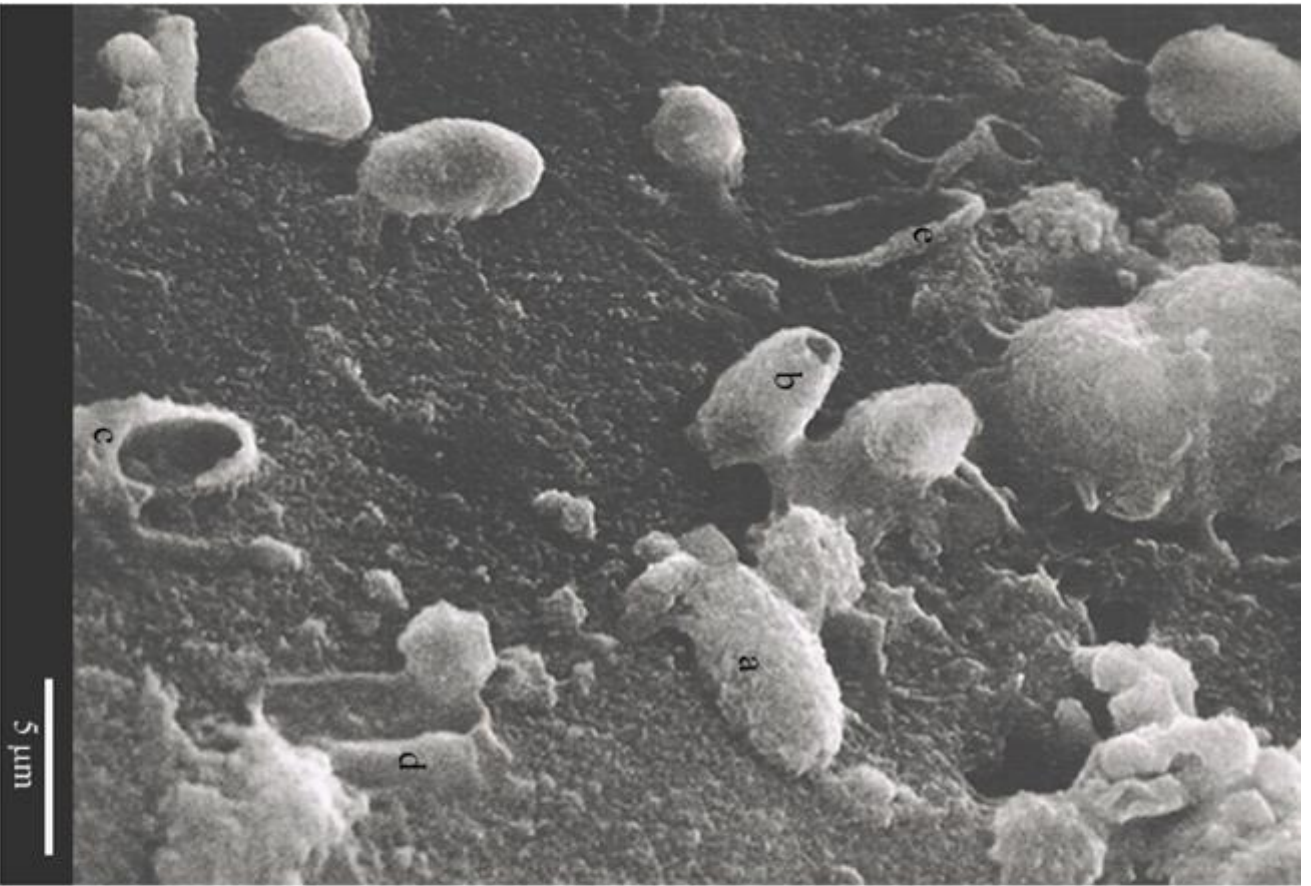
22B



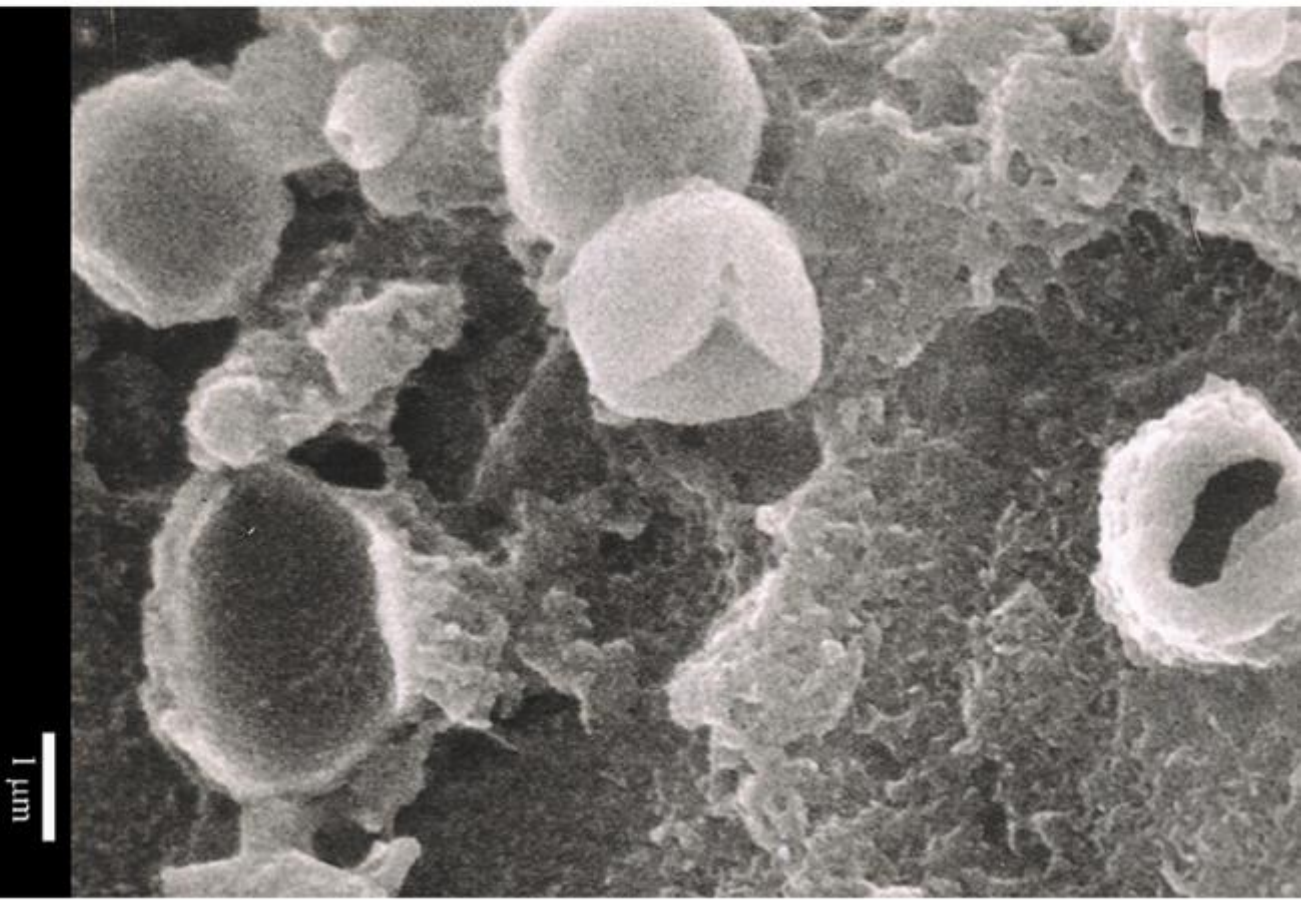
22C



22D



23A



23B