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# Microscopic Observation of Self-Propagation of Calcifying Nanoparticles (Nanobacteria)

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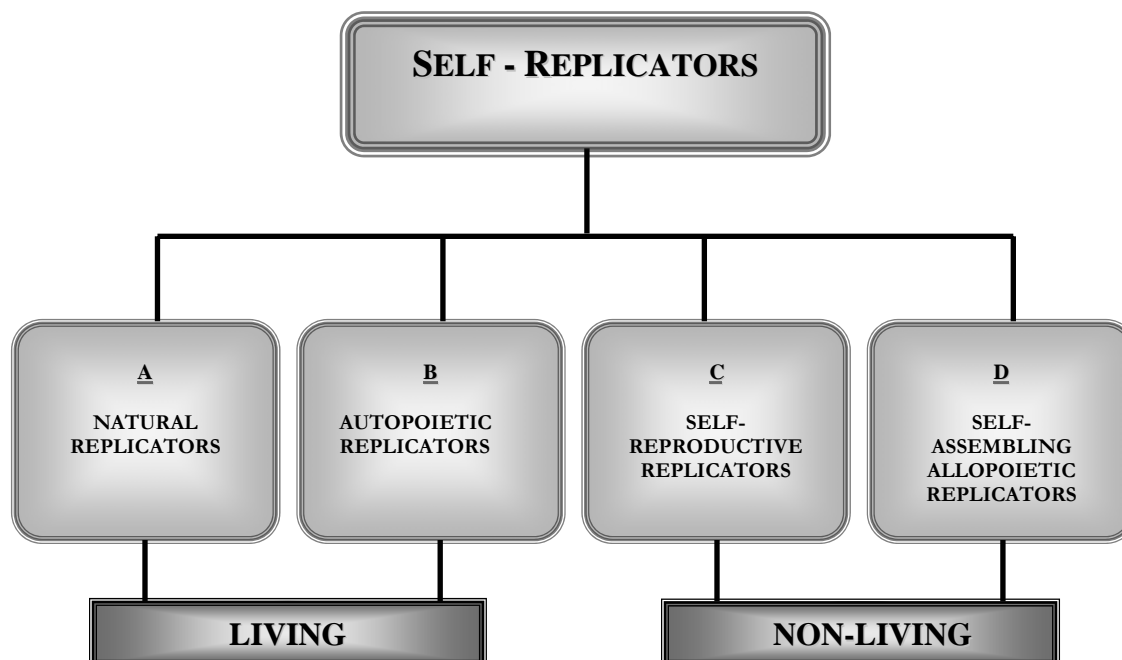
**ABSTRACT**

Biologists typically define living organisms as carbon and water-based cellular forms with “self-replication” as the fundamental trait of the life process. However, this standard dictionary definition of life does not help scientists to categorize self-replicators like viruses, prions, proteons and artificial life. CNP also named nanobacteria were discovered in early 1990s as about 100 nanometer-sized bacteria-like particles with unique apatite mineral-shells around them, and found to be associated with pathological-calcification related diseases. Although CNP have been isolated and cultured from mammalian blood and diseased calcified tissues, and their biomineralizing properties well established, their biological nature and self-replicating capability have always been severely challenged. The terms “self-replication”, “self-assembly” or “self-propagation” have been widely used for all systems including nanomachines, crystals, computer viruses and memes. In a simple taxonomy, all biological and non-biological “self replicators”, have been classified into “living” or “nonliving” based on the properties of the systems and the amount of support they require to self-replicate. To enhance our understanding about self-replicating nature of CNP, we have investigated their growth in specific culture conditions using conventional inverted light microscope and BioStation IM, Nikon’s latest time-lapse imaging system. Their morphological structure was examined using scanning (SEM) and transmission (TEM) electron microscopy. This present study, in conjunction with previous findings of metabolic activity, antibiotic sensitivity, antibody specificity, morphological aspects and infectivity, all concomitantly validate CNP as living self-replicators.

**KEYWORDS.** Nanobacteria, calcifying nanoparticles, time-lapse photography, self-replication, apatite

## INTRODUCTION

Most biologists would agree that self-replication, genetic continuity, is a fundamental trait of the life process. However, studies especially in nanoengineering field in the last couple of decades have been using terms that were originally coined for living systems to represent non living creations, which include “self replication”. Therefore, recent research<sup>1</sup> has categorized all self replicating systems based on the amount of support they require (Figure 1).



**Figure 1.** Taxonomy of self-replicators. All self-replicators can be classified into any of the 4 categories as discussed in Freitas Jr RA, Merkle RC (2004) *Kinematic self-replicating machines*. Georgetown.

Class A-Natural replicators: These are self-reproductive systems found in nature depending on only natural sources for their replication, e.g., unculturable and therefore uncharacterized microorganisms<sup>2</sup>.

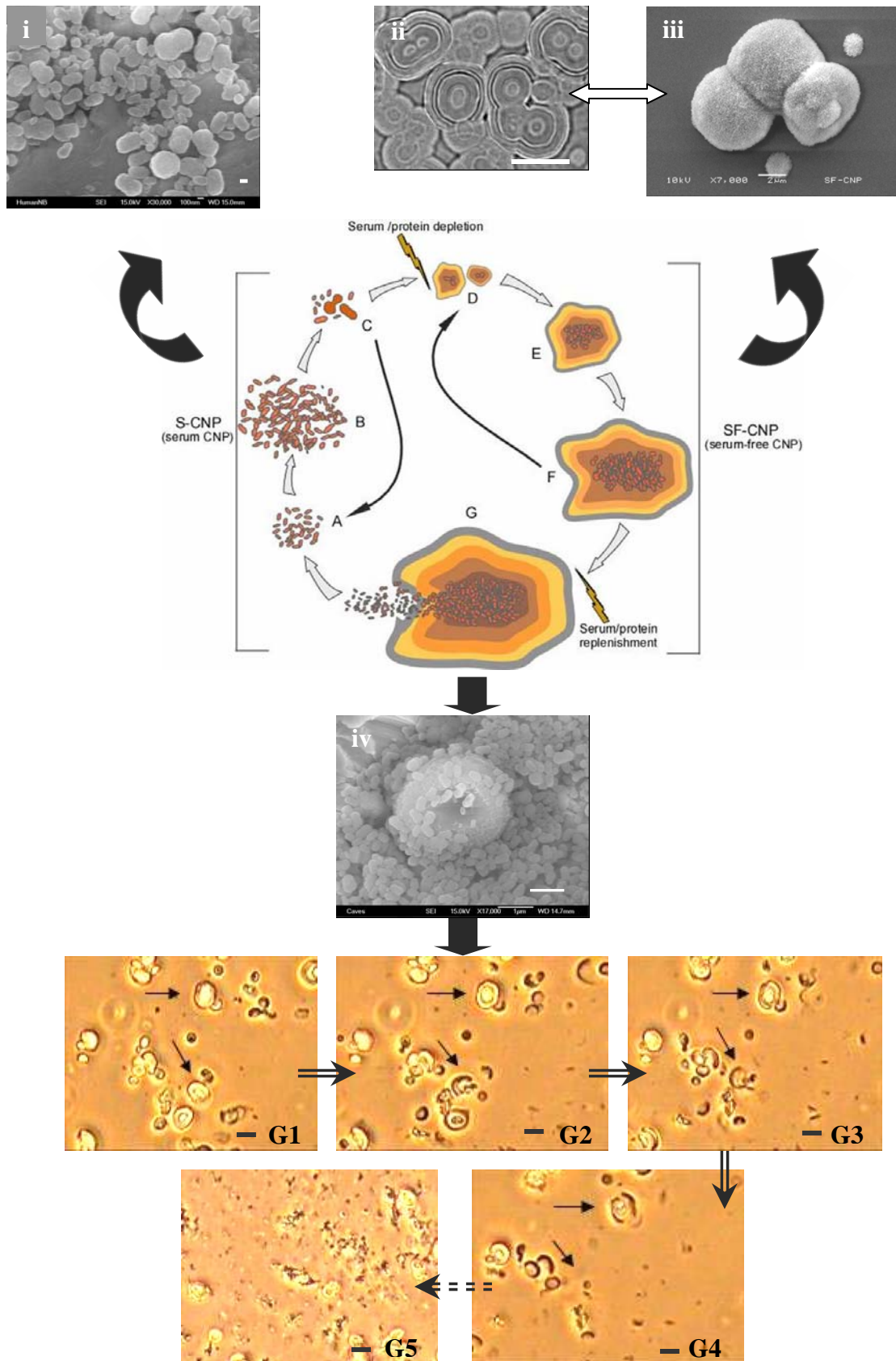
Class B-Autopoietic replicators: Self-organization is the core concept of these systems. For without a self-maintaining system, other features such as growth, development, reproduction, adaptation and metabolism cannot emerge. These systems are capable of using both natural and synthetic sources but

they require a set of instructions originating from within the system itself, e.g., bacteria, fungi, yeasts, animals and humans<sup>3</sup>. Class C-Self-reproductive replicators: These are self-reproductive systems that require an independent instruction controller not originating from within the system. These systems use synthetic, industrial/technology sources for their replication, e.g., computer viruses, crystal growth<sup>4</sup>. Class D-Self-assembling allopoietic replicators: These systems use only finished or delivered resources for their replication, e.g., an assembly line of computers, cars, toys<sup>5</sup>. The systems falling in categories C and D that do not use their self-produced constituents to maintain themselves or change their structure to meet new challenges generally would be deemed nonbiological although they can exhibit self-replication<sup>3,6,7</sup>.

CNP have been shown to be bacteria-like<sup>9</sup>, pleomorphic<sup>10</sup>, infectious particles<sup>11</sup> isolated from mammalian blood and blood products that possess unique properties including capability of passing sterilization filters because of their small size<sup>12</sup> (80-500nm), resistance to heat and  $\gamma$ -irradiation at doses typically fatal for conventional bacteria<sup>13</sup>, and formation of a calcific coating at physiologic pH and mineral concentrations<sup>9,12</sup>. CNP have been linked to pathological calcification related diseases such as arteriosclerosis<sup>20,21</sup>, kidney stone<sup>22,23</sup>, gall stone<sup>24</sup> and dental pulp stone formation<sup>25,26</sup>, prostatitis<sup>27</sup>, Alzheimer's<sup>28</sup>, polycystic kidney<sup>29,30</sup>, and cancer<sup>31,32</sup>. CNP exert cytotoxic effects on some mammalian cells *in vitro*<sup>16</sup> and on living organisms *in vivo*<sup>33</sup>. Despite their potential role in major medical health problems, CNP have not been classified in any taxonomic groups due to limited information on their biological characteristics and self-propagation capability.

In the world of microbiology, there are micro organisms which demonstrate strikingly different morphologies depending on the physiological/culture conditions to which they are exposed (e.g., rickettsias, molds, parasites)<sup>39,40,41</sup>. Similarly, CNP have morphological changes in different culture conditions. For example they form excessive amounts of biofilm when stressed with antibiotics<sup>13</sup>, calcify less when cultured in microgravity conditions<sup>42</sup> and calcify profusely when serum/protein concentration is reduced (below 5%)<sup>43</sup>. However their antigenicity remains the same and they are still

recognized by CNP-specific monoclonal antibodies (mAb)<sup>43,17</sup>. Figure 2 shows a schematic diagram of the growth phases of CNP under different culture conditions.



**Figure 2.** Schematic diagram of CNP growth phases under different culture conditions with corresponding electron and light microscopic images, and light microscopic observation of dissolution of SF-CNP to S-CNP. The drawings represent a cross-sectional view of CNP. In the presence of serum (protein), CNP are as shown in images A, B and C, and the CNP in all these phases are named as “S-CNP”. SEM image of S-CNP are shown in (i). In the absence of serum (protein), CNP are as shown in images D, E and F and the CNP in all these phases are termed as “SF-CNP”. SF-CNP have layers of apatite formed by accumulation of calcium and phosphate on their surface and can grow between 1-10 $\mu$ m in size. (ii) shows light microscopic apatite layers of SF-CNP. (iii) shows the SEM image of the same formation. When SF-CNP are exposed to serum (protein), those layers dissolve releasing the small CNP as shown in image G. (iv) shows the SEM image of phase G. A series of light microscopic images (G1-G5) show the dissolution of SF-CNP apatite layers and clumps of CNP released, with the replenishment of serum (protein). Bars: (i) = 100nm; (ii) = 5 $\mu$ m; (iii) = 2 $\mu$ m; (iv) = 1 $\mu$ m; (G1-G5) = 5 $\mu$ m.

Starting at Stage A, in a serum or protein containing medium, the tiny cell-like coccobacillar forms from the stock culture begin to acquire thin coatings of apatite crystals on their organic membrane. These forms grow slightly larger in size (Stage B) and may form dumbbell-shaped forms (Stage C). Those forms can be passaged at 1/10 dilution for years and they continue to reproduce, maintaining the same shape and narrow size range (80-400nm) as shown in Fig 2. So, the cycle from stages A to C and C to A can continue indefinitely<sup>(ref)</sup>. Those small, coccobacillar CNP are referred to as serum-CNP (S-CNP) and this type of CNP is cultured when DMEM is supplemented with 10% fetal bovine serum (FBS). However, if those minute CNP are passaged into serum (protein)-free media, the serum protein depletion cause CNP to produce biofilm-like material and they attach to the surface of the culture vessel where they develop several apatite mineral layers around them Figure. 2 ii) forming “igloos” or “shells” (Fig. 2 stages D-G, ii and iii)<sup>(ref)</sup>. The mineral around CNP has been identified as identical to bone mineral by Fourier transform infrared spectroscopy studies<sup>44</sup>. These igloo forms harbor in their interior many small CNP in a semi-dormant state which can be observed only by using electron microscopy techniques<sup>ref</sup> (Figure. 2 iv). We refer to these attached CNP igloos as serum-free CNP (SF-CNP). We have SF-CNP cultures that have been passaged 1/10 dilution monthly for over 17 years in serum and

protein-free Dulbecco's Modified Eagle's Medium (DMEM). So, the cycle from stages D to F and F to D can continue indefinitely (as schematized in Fig 2). SF-CNP mineralize and grow larger in size (1-10 $\mu$ m) when compared to S-CNP, as a result of calcium and phosphate deposition on their surface as shown in Fig. 2 D-G, and ii-iv<sup>11</sup>. The addition of the serum (protein) to the culture media brings the system back to Stage A. Many proteinaceous inhibitors of apatite crystal formation have been identified in serum, which may account for the observed dissolution of the mineral layers<sup>45</sup>. The SEM image in Figure 3-iv, and optical micrography images in Figure 3:G1-G5 show how SF-CNP, the igloos detach from the surface and the apatite layers dissolve to release the small typical coccobacillar shaped particles (50-300nm) when serum/protein replenishment takes place.

Although previous CNP studies have revealed their morphological characteristics, antibiotic and radiation resistance, antibody specificity, metabolic activity and pathological nature, until now, there was no research on capturing the growth of CNP in real-time under physiological conditions. The objective of this study was to document the propagation of both types of CNP under physiological conditions, using inverted light microscopy and the BioStation IM time-lapse imaging system. While this optical microscopic imaging may seem as a simple technology, it is the only available technique of today for viewing the CNP "alive" and behaving in a "normal" physiological manner.

## MATERIALS AND METHODS

**CNP Cultures.** In all experimental analyses we used the same CNP which was isolated from FBS (Manufacturer: Sera Lab, Lot: 901045, Country; England), and deposited in German Bank DSM no. 5819-5821. Two types of CNP (as described in Figure 2) were examined for the observation of self propagation in specific culture conditions; Subcultures of SF-CNP were conducted under strict aseptic conditions by passing a small inoculum (1/10 of a 3 week old culture) of SF-CNP into culture flasks with fresh DMEM (Invitrogen, Carlsbad, California, USA, supplemented with L-glutamine) under mammalian cell culture conditions (37<sup>0</sup>C; 5-10% CO<sub>2</sub>; 95% air at 90% humidity). For observation of S-

CNP, the culture was passaged 1/50 into FBS-free DMEM. Therefore the final serum concentration was (0.3%) which caused small CNP to attach to the culture vessel and make microscopic observations possible. As negative controls, DMEM with and without FBS (0.3%) by omitting CNP addition step were incubated under the same culture conditions and for the same culture period. All cultures were observed with microscopy for 3 weeks and were not re-fed with fresh medium for the entire duration of the experiment. At the end of experiment, cultures were passed through quality control tests checking for conventional bacterial contamination, and CNP epitope positivity, using double staining technique as described earlier<sup>14</sup>. Double staining technique is a combination technique of immunofluorescence staining (IFS) with CNP-specific mAb 8D10 (Nanobac, OY), and Hoechst (#33258) fluorochrome staining.

**Microscopy and Photography.** Two types of imaging was performed; a) using conventional inverted light microscopy (LM) (Nikon, Eclipse TE2000-U) in the phase contrast mode; b) imaging using Nikon's BioStation IM most recently developed time-lapse imaging system.

For observation of SF-CNP replication with the conventional LM, objectives with 20x and 60x magnifications, eyepiece with 10X magnification, and an intermediate optics of 1.5x magnification were used. The culture flasks were indexed with a diamond pen so as to view the same field everyday. Each time the same focus planes were located using the 2 magnifications: 300X and 900X. Images were captured digitally using Nikon's charged-coupled device camera (Digital Sight DS-L1). A few large CNP shells were marked 1-4 (with arrows) on the images (Fig. 3) in order to identify the same spots throughout the observation.

BioStation IM (Nikon Instruments Inc., Melville NY) was a time-lapse imaging system on loan to us from Nikon, so we only had time for a limited set of time-lapse experiments. This microscopy system is a novel compact cell incubation and monitoring system allowing time-lapse cell imaging without the set-up and alignment complexity of conventional time-lapse imaging systems. The system combines an incubator that maintains mammalian cell culture conditions (37°C; 5%CO<sub>2</sub>; 95%humidity), a



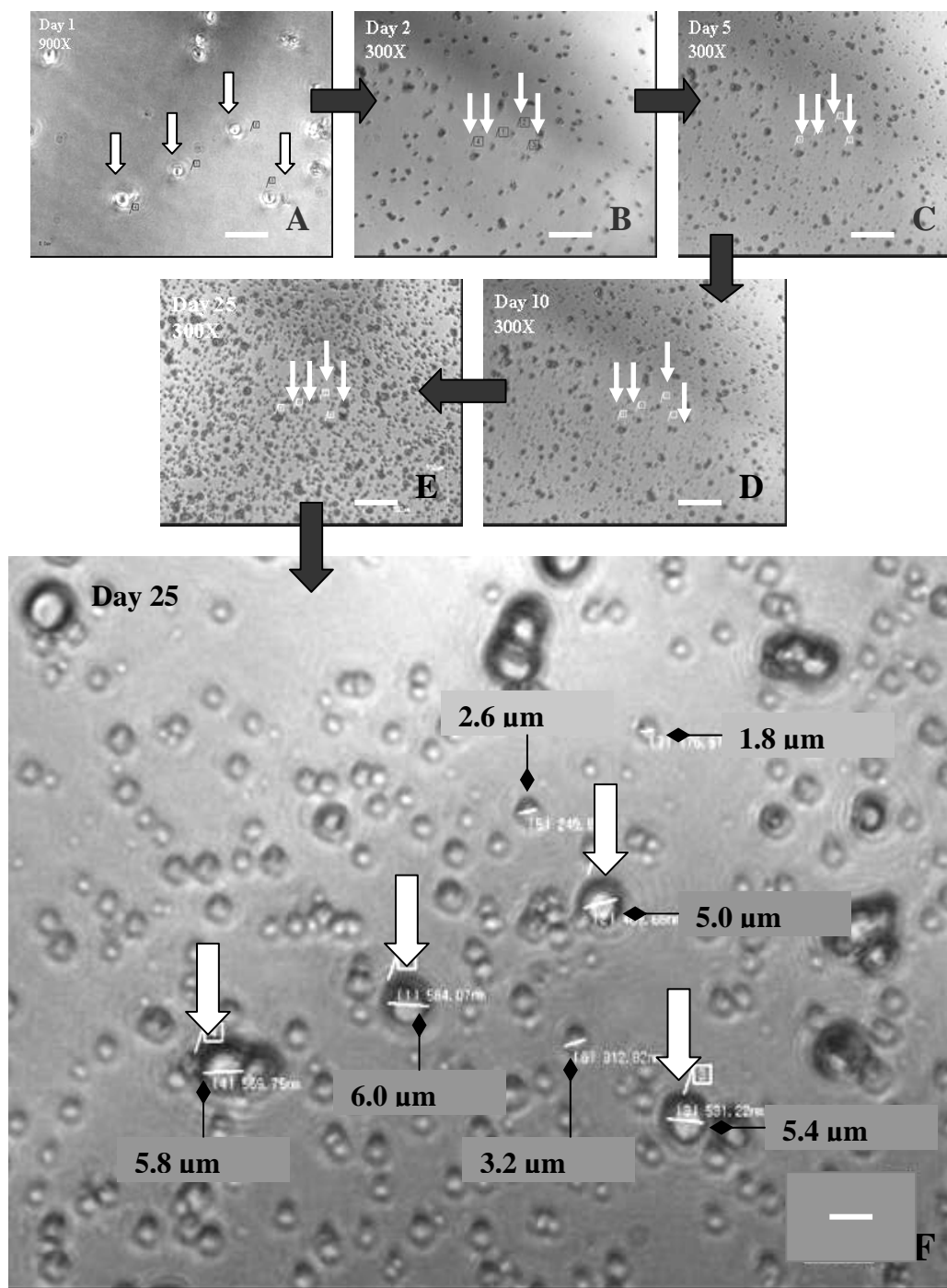
microscope with a numerical aperture of NA 0.80, delivering high resolution images in phase-contrast mode, an internal motorized stage supporting X, Y and Z dimensional movement with reduced focus-drift, and a high performance CCD digital imaging camera for capturing time-lapse image sequences (<http://www.nikonusa.com>). Both S-CNP and SF-CNP were monitored in 30mm cover-slip bottom petri-dishes under 40X magnification. Time-lapse imaging was conducted for 5 days with images taken at regular intervals. The exposure time was 1/10sec, at 1600x1200 pixel resolution. Both S-CNP and SF-CNP counts were performed using ImageJ software.

For SEM, at the end of the experiments, the cultures were either scraped with a cell culture scraper, harvested by centrifugation at 14,000 x g for 20 min, and the pellet is used as a sample, or a piece of culture vessel having attached CNP was cut with a heated scalpel and used in the sample preparation. The samples were washed twice with phosphate buffered saline, pH 7.4 (PBS) and fixed with 2% gluteraldehyde in PBS for 16 h at 4°C. The fixed samples were washed twice with PBS, dehydrated with gradually increasing ethanol concentrations, and dried with hexamethyldisilazane<sup>14</sup>. The samples were coated with gold (thickness, 20 to 40nm) prior to examination with a JEOL 5910LV SEM.

For TEM, SF-CNP cultures were harvested, fixed with formaldehyde-gluteraldehyde mixture, epoxy embedded and sectioned as described earlier<sup>9</sup>. For S-CNP cultures, negative staining is applied and observed under TEM (JEOL 2000FX; Tokyo, Japan)<sup>17,22</sup>.

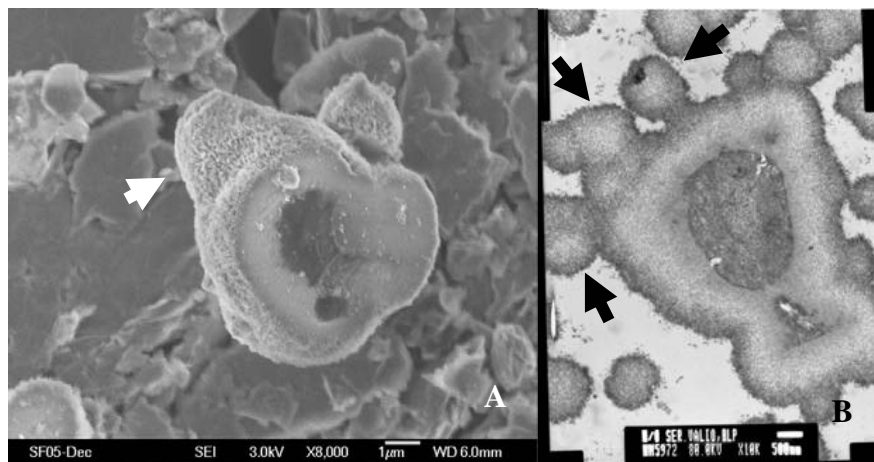
## RESULTS

For our initial set of experiments we used only SF-CNP. Figure 3 shows optical microscopic images of culture follow up of SF-CNP over a period of 25 days. Tiny ( $\leq 1\mu\text{m}$ ) coccoidal particles were observed which attached to the culture vessels by the end of first day incubation. These particles are the small CNP released from the main culture during passaging. Figure 4 show igloo-shaped SF-CNP by SEM and TEM after they are detached from the culture flask. On the first day of the SF-CNP culture, both small and larger sized, igloo-shaped formations are observed in small number.



**Figure 3.** Optical micrograph of SF-CNP showing an increase in number over a culture period of 25 days. (A) Day 1 at 900X magnification; (B-E) Days 2, 5, 10 and 25 respectively at 300X magnification. The white arrows in each image indicate the same large SF-CNP on the same spot throughout the experiment. (F) measurements of a few SF-CNP on Day 25 at 900X magnification. All particles seen in the images are the different sizes of SF-CNP. Bars: (A) = 15 $\mu$ m; (B), (C), (D) and (E) = 30 $\mu$ m; (F) = 5 $\mu$ m.

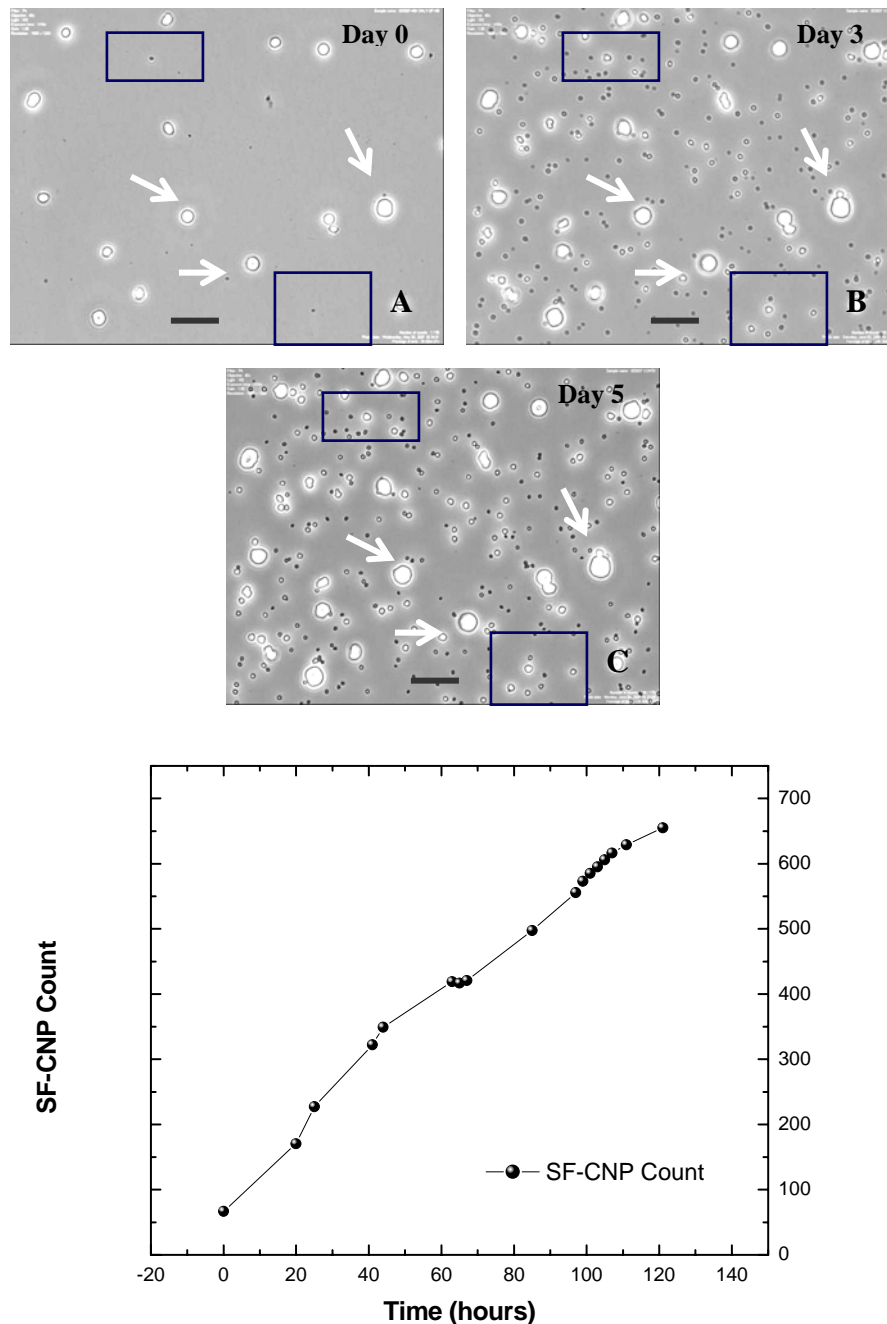
Appearance of small particles around the large coccoid cells is seen from Day 2 (Figure 3 B). Small clusters and chains of particles are also seen. During culture, the particles became more visible, optically opaque, and bigger due to calcium phosphate deposition. In this part of the experiment, we observed that CNP grow within a size range between 0.5- 6 $\mu$ m and apparently increase in number.



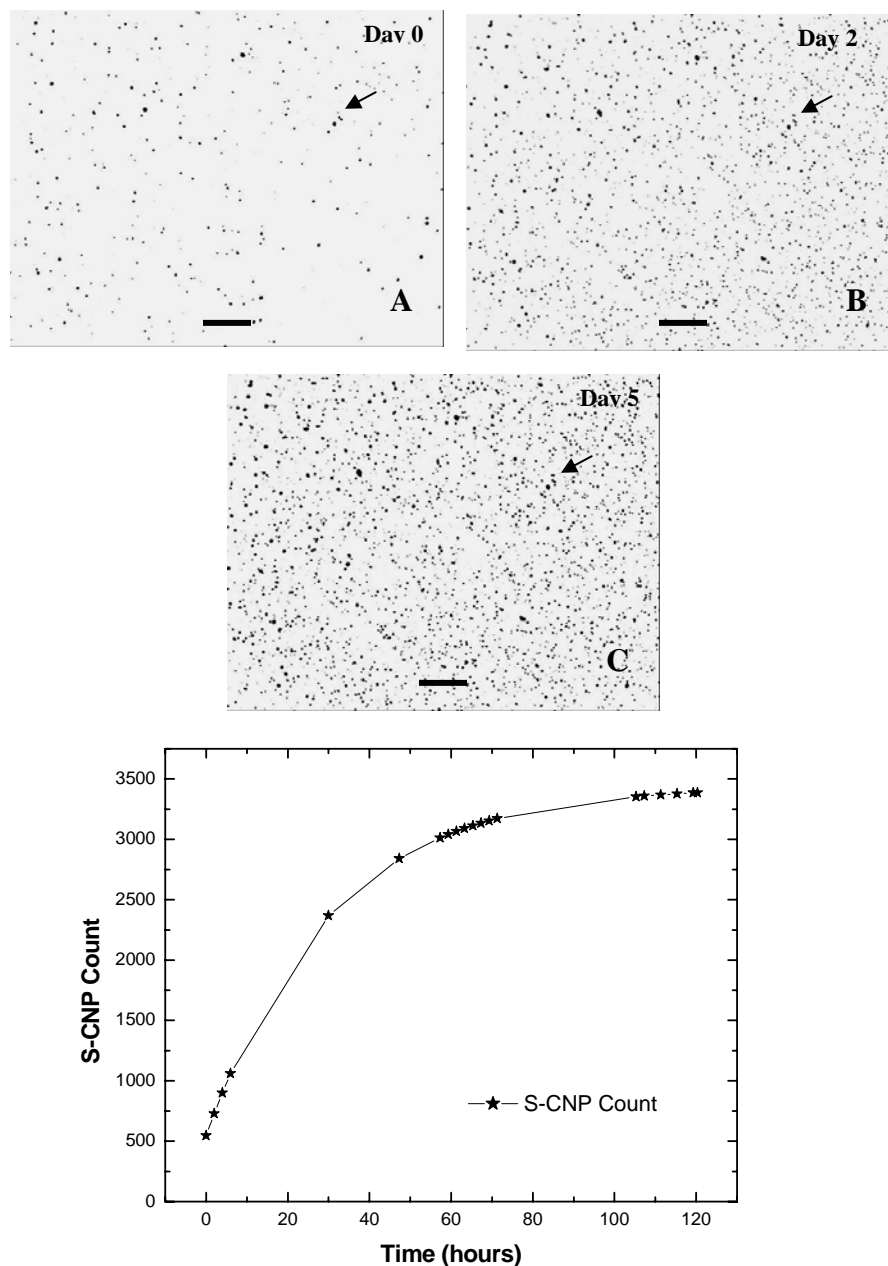
**Figure 4.** Electron microscopic images of SF-CNP. (A) SEM of an empty apatite “igloo” detached from the culture medium. (B) TEM section of a similar SF-CNP and its inner structure. Arrows point to the apparently budding side of the shell. Bars: (A) =1 $\mu$ m; (B) = 500nm.

Using Nikon’s BioStation IM imaging system, both SF and S-CNP were imaged for a period of 5 days each. Although this culture period is too short for CNP-like slow growers, we could see an increase in number in both types of CNP (Figures 5 and 6). The results obtained were comparable with that from the previous experiment using inverted LM. A graph of SF-CNP count against time was plotted (Figure 5 D). Total culture period is 120h. The graph indicates a linear increase in SF-CNP number with time.

Time-lapse imaging of S-CNP (Figure. 6) shows a gradual increase in their number over a period of 5 days.

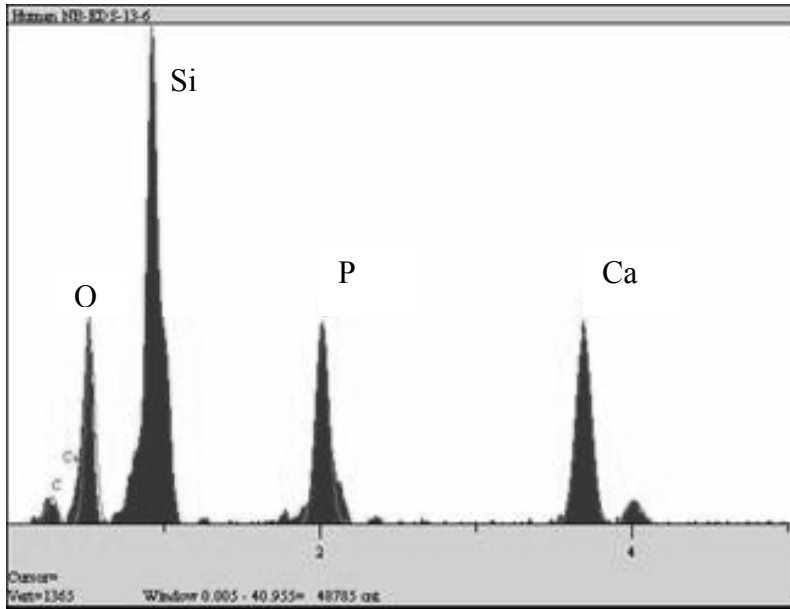


**Figure 5.** Time-lapse imaging and plot of SF- CNP from Day 0 to Day 5 using Nikon’s BioStation IM. Only a few intermediate images of SF-CNP on Days 0, 3 and 5 at 40X magnification are shown. The white arrows mark some large SF-CNP on the same spot throughout the experiment. Note the small SF-CNP within the square blocks showing an increase in size and number over time. A graph of SF-CNP count against time in hours shows a linear increase in the SF-CNP number. The images and graph together imply an increase in size and number of SF-CNP over a period of 5 days. Bars: (A), (B) and (C) = 15 $\mu$ m.



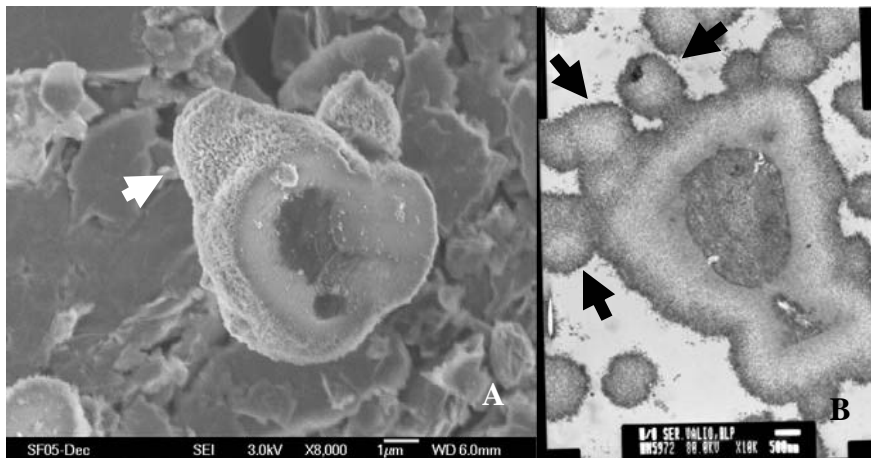
**Figure 6.** Time-lapse imaging and plot of S-CNP from Day 0 to Day 5 using Nikon's BioStation IM. Only a few intermediate images of S-CNP on Days 0, 2 and 5 at 40X magnification are shown. The black arrows point to some S-CNP on the same spot throughout the experiment. Bars: (A), (B) and (C) = 15 $\mu$ m.

For all the experiments conducted, negative controls without any CNP in DMEM with and without serum, did not show any particle formation or growth. Energy dispersive spectroscopy (EDS) showed Ca and P peaks in both forms of CNP (Figure 7). Also, negative controls with inorganic hydroxyapatite in similar media and culture conditions did not show any increase in the apatite particle number or size.



**Figure 7.** EDS analysis of CNP apatite. Si peak is because of the glass substrate on which CNP samples were placed.

The morphology of SF-CNP by SEM analysis showed spherical and semi-spherical particles with rough surface budding-like structures (Figures 2-iii and 8). In addition to the increase in number, many of the SF-CNP became slightly larger over time. While we cannot analyze individual particles directly with the optical microscope, we infer, based on SEM and TEM analyses that the better visibility under light microscope results from the apatite coating on the originally cell-like particles below the light microscope's resolution limit.



**Figure 8.** Electron microscopic images of SF-CNP. (A) SEM of an empty apatite “igloo” detached from the culture medium. (B) TEM section of a similar SF-CNP and its inner structure. Arrows point to the apparently budding side of the

shell. Bars: (A) = 1 μm; (B) = 500 nm

## DISCUSSIONS

Despite all the peer reviewed and published scientific literature related to CNP growth, antigenicity, infectivity and medical impact over the past 15 years, the precise systematic classification of CNP has not been clarified. The scientific community awaits the crucial piece of evidence, that is, the nucleic acid sequence unique to these particles. Although researchers at Mayo clinic have identified a single ~ 25 kB basepair band of DNA from CNP using a simplified protocol used for isolating DNA from archaea cultures, it has not been fully characterized<sup>36</sup>. Therefore, a significant controversy has continued to surge regarding the existence and significance of CNP. However, some replicators such as viruses and prions are distinct from bacteria, in that they both require a host cell to undergo metabolic functions and they are not affected by antibiotics. Although all virions have a nucleic acid wrapped in a protein coat, prions are infectious proteins without any nucleic acid<sup>54</sup>. After much debate they are now accepted by the scientific community as a new biological principle of infection.

Critics have proposed CNP to be protein precipitates <sup>(ref)</sup>. We conducted our study with serum-free culture media, to eliminate the possibility of protein precipitates from serum confounding the interpretation of the results. Also, the absence of serum in medium allowed the CNP to attach to the surface of the culture dish, facilitating their observance with optical microscope over a prolonged time period. In our experiments as negative controls, we used culture media with and without FBS (0.3%) and incubated them in the absence of CNP under similar culture conditions and culture period as CNP cultures. We have not observed any protein precipitation or crystal formation in those cultures.

Cisar et al 2000 have proposed that CNP are self-propagating inorganic apatite crystals. We are unaware of any report showing the nucleation and growth of inorganic apatite under physiologic conditions. Additionally, for inorganic crystallization to continue over prolonged periods of time, conditions of non-equilibrium must exist and be maintained<sup>34</sup>. TEM examination of CNP cultures have always shown a close association of apatite with submicrometer vesicles enclosed within membranous structures. We and others have always found the presence of these membranous vesicles remaining after the apatite has been chelated and dissolved away with acid or EDTA<sup>35,36</sup>.

Previous time-lapse studies of crystal growth have shown sedimentation of microcrystals onto the larger crystals with formation of defects<sup>49,50</sup>. Also, for crystal growth to occur, supersaturated conditions are required at least during preliminary stages<sup>51,52</sup>. CNP self propagates under physiological conditions.

Kahr (2007) put to test the “crystals-as-genes” hypothesis using differential interference contrast microscopy, atomic force microscopy, and luminescence labeling of hillocks in conjunction with confocal laser scanning microscopy. For crystals to resemble genes, there must be more inheritance than mutation in successive generations. However, despite the greatest of care taken to not expose the fresh crystal seeds to atmosphere, and even in the absence of cleavage, new hillocks, “mutations” proliferated<sup>53</sup>. For more than a decade, CNP have been cultured and passaged under physiological conditions similar to mammalian culture conditions, without any change in their growth characteristics and specific monoclonal antibody recognizing epitopes. Also, they have been isolated from many diseased tissues. Inorganic apatite under similar conditions did not show any growth or other characteristics as shown in Table 1.

The metabolic potential of CNP was confirmed using a tetrazolium salt detecting dehydrogenase activity<sup>ref</sup>, and S-methionine incorporation<sup>(ref)</sup>. Also,  $\beta$ -mercaptoethanol, known to enhance growth of certain microorganisms and mammalian cells, promoted CNP metabolism and growth<sup>36</sup>. Polarized light was shown to reduce CNP biofilm formation indicating a light induced metabolic process within CNP<sup>38</sup>. Apparently CNP have metabolic activity which clearly differentiates them from inorganic crystal formation.



**Table 1.** Comparison of two self-replicators; CNP and inorganic apatite crystals

| <b>Properties</b>   | <b>CNP</b>   | <b>Inorganic apatite crystals</b>                                     |
|---|--|---|
| Culture in DMEM under mammalian cell culture conditions   | Increase in number (10)  | -No increase in number (10)<br>-Small particles (up to 50nm) dissolve |
| Size  | Very narrow range (9,10)<br>S-CNP: 80-500nm<br>SF-CNP: 0.5-10 $\mu$ m  | Very wide range<br>2nm to centimeters or more                         |
| Ultramicroscopic morphology   | Always have closed membranous vesicles involved with budding-like or septa-like formations (9,10)              | No "cell like" structure  |
| Chelation with EDTA or acids  | Release and precipitation of organic matter (35,36)  | Dissolves totally without any residue (36)                            |
| Antibiotic/chemotherapeutic sensitivity   | Sensitive to tetracycline, aminoglycosides, nucleic acid synthesis inhibitors, bisphosphonates, etc (18,19,36) | Resistant to all (18,19,36)   |
| Metabolic labelling (S-methionine, uridine)   | + (9,16)   | -   |
| Recognition by monoclonal antibody (8D10)   | + (17)   | -   |
| Infectivity   | + (Increase antibody level) (11)   | - No immune response  |
| Adaptation to physiological condition (morphological changes with protein concentration, antibiotics, heat) | + (35,19,13,42)  | No effect (35,36)   |
| Stainability with pico-green, ribo-green, and Hoechst   | + (36)   | -   |
| When injected intravenously to the rabbits  | Goes to kidney (33)  | Goes to liver and spleen  |
| Polarized light treatment   | Reduces biofilm formation(38)  | No response (38,43)   |

Binary fission is the usual form of reproduction by bacteria, although a few bacteria and some eukaryotes (including yeasts) replicate by budding<sup>47</sup>. During binary fission a thin wall forms in the cell that separates a single cell into two cells. In budding, a protrusion forms from one point on the cell that enlarges and later separates from the parent cell. In environmental microbiology, it is known that there are aquatic microorganisms that use both binary division and budding mechanism while they are self-propagating<sup>48</sup>. Figures 8A and B show electron microscopic images of SF-CNP with both fission-like and budding-like divisions. At the end of the time-lapse experiment with the BioStation IM, when both S-CNP and SF-CNP were observed under SEM, it was obvious that there was more CNP beyond the light microscope resolution of the BioStation IM. Hence, it appears that CNP replicate at a much faster rate than concluded in earlier studies.

Time-lapse imaging of S-CNP shows a gradual increase in their number over a period of 5 days (Figure. 6). In previous studies, S-CNP doubling rate has been calculated as a mean value of 72 hours with a logarithmic increase in turbidity in cultures with lag and log growth phases<sup>10</sup>. In this study, since the S-CNP were passaged into SF media, they attached themselves to the culture dish. Hence, measurement of turbidometric changes for these attached CNP was not possible. It has been also reported that this growth rate is even slower in SF-CNP<sup>10</sup>. Therefore, the results obtained from a short time observation of slow growing SF-CNP may not be conclusive.

Although an increase in SF-CNP and S-CNP number alone cannot be reasoned as “living” self-replication, previous research studies confirming their capability of S-methionine incorporation<sup>9</sup>, inhibition of their propagation with antibiotics like tetracycline and metabolism inhibitors such as antimycin A, sodium azide and potassium cyanide<sup>18,19,36</sup>, adaptation to stress conditions<sup>13</sup>, monoclonal antibody specificity<sup>17</sup>, correlation with pathological calcification<sup>20-32</sup>, infection causing ability<sup>11</sup> and existence of proteins resembling prokaryotic protein fragments<sup>36</sup> together prove that these self-replicating entities are not merely self-replicating crystals or precipitates but a unique type of “life

form”. It is obvious that these CNP manifest various functions besides self-replication, using an unidentified set of instructions originating from within their system.

Despite the failure to precisely characterize any DNA or RNA in CNP, from the evidence presented here, there is no doubt that these particles are self-replicators. Moreover, according to the taxonomy of self-replicators (Figure 1), if we take into consideration all the properties of CNP, including self-replication, morphology, metabolic activity, antibiotic sensitivity, antibody specificity, adaptation to the environment, and triggering infection and pathological calcification, all functions originating from within the system itself, with self-organization as the fundamental feature, they seem to fit into the category of “living” self-replicators unlike inorganic crystals.

Nevertheless, there is truly no universally agreed definition of life. Although the theme of DNA has permeated so deeply in the scientific world, lately, there have been numerous publications questioning the concept of gene as the unit of life<sup>58,59</sup>. The theory that life could have started with very simple heterotrophic primordial cells is currently gaining recognition<sup>60</sup>. Such could be the case with CNP. It is evident that CNP involved in pathological biomineralization with their distinctive characteristics defy old standard scientific expectations and definitions of life.

## **CONCLUSIONS**

We propose that CNP most logically fall into a taxonomy based on the type of self-replication rather than based on genomics. According to our study results, CNP also known as “nanobacteria” may be classified as self-replicators of Class B category. Future studies require the application of an innovative high resolution optical microscopy-imaging system combined with fluorescence to observe in real-time the replication of CNP, and follow up of their metabolic pathways.

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