NANOBACTERIA: FACT OR FICTION?

CHARACTERISTICS, DETECTION AND MEDICAL IMPORTANCE OF NOVEL SELF-REPLICATING, CALCIFYING NANOPARTICLES

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What do we Know About Biomineralization/Calcification?

Including humans, many multicellular organisms produce similar hard tissues such as bones, teeth, shells, skeletal units, and spicules. These hard tissues are biocomposites and incorporate both structural macromolecules (lipids, proteins, and polysaccharides) and inorganic minerals.,**1** We do not fully understand the control mechanism of biomineralization either in primitive or in developed organisms. The mineral phase of hard tissue is sometimes called biological apatite, i.e. a non-stochiometric hydroxyl-apatite. Pure hydroxyapatite has the formula $Ca_{10}(PO_4)_6(OH)_2$. In contrast, a biological apatite (like in bone) is non-stoichiometric and contains several other ions, mainly carbonate and other elements in traces like Mg^{2+}, Na^{+}, Fe^{2+}, HPO_4^{2-}, F^{-}, Cl^{-}. Consequently, a more appropriate structural formula for the composition of bone is $(Ca,X)_{10}(PO_4,CO_3,Y)_6(OH,Z)_2$ with X substituting cations and Y and Z substituting anions (with the indices 10, 6 and 2 changing according to stoichiometry)., **2**

There is a paradox in medicine. While some researchers have been discussing the cytotoxic effect of apatite *in vitro*, **3,4** others have been announcing the safety of *in vivo* apatite applications., **5-8** Although these disagreements have not been completely resolved, both biogenic and non-biogenic apatite materials have been continuously used in drug delivery and transplantation., **6,9** We know that when apatite is found in soft tissue, it is considered to be pathological calcification., **10** Causes of apatite-deposit formations in soft tissue have been discussed for decades, but still remain speculative. For example, calcification in the coronary arteries has been widely regarded as an uncommon, end-stage, insignificant, passive, degenerative process of aging--a notion that has paralyzed research in this area for decades., **11** Interestingly, these same terms were once used to describe atherosclerosis., **11** Today, we know that coronary artery calcification occurs, almost exclusively, at sites of atherosclerotic lesions., **12** Calcification in the development of these plaques is a complicated, actively regulated process of mineralization that is similar to bone formation and remodeling., **13,14** Mineralogists explain that all that is needed for crystal formation/biomineralization to start is nidi (nucleus) and an environment of available dissolved components at or near saturation concentrations, along with the absence of inhibitors for crystal formation., **15** Bacteria or other agents producing such nidi, if present in blood and in urine, are very likely candidates to launch and accelerate pathologic calcification *in vivo*., **16,17** This is clinically important since blood contains phosphate near its saturation level., **18**

"Nanobacteria": Potential Nidi for Calcification, a Good Model for Studying Calcification Mechanism

What is known about microbial infections is based on the study of well known microbes. There exists a poorly known, blood-born agent (discovered and tentatively termed "nanobacteria" (NB) by our team) **19** that behaves as a microbe and appears to show a correlation with such diverse calcification-related health problems as arterial heart disease, **20-22** Alzheimer's disease, **23** kidney stone formation, **24-28** polycystic kidney disease (PKD), **29,30** gall stones and gallbladder inflammation, **31** prostatitis, **32,33** calciphylaxis, **34,35** and cancer., **36,37** Furthermore, this agent has unique properties

including an extremely small size (0.1-0.5 μm) as seen in Fig 1. Although the biological characterization of NB is yet to be fully understood, the precipitation and growth of calcium phosphate readily occurs in systems containing trace amounts of NB, but not in identical control systems lacking NB., **19** The exact mechanism(s) by which apatite is nucleated and formed around NB is unknown. When the serum concentration in the medium is reduced (≤ 5%) in the NB culture conditions, NB start to mineralize and grow bigger in size due to calcium and phosphate deposition on their surface (Fig 2). DIC microscopy revealed a several-micrometer thick age-ring-like mineral layers forming around NB in these cultures (see Fig 2). Chemical analysis using energy-dispersive X-ray microanalysis (EDX) of these mineral layers shows Ca and P peaks., **19** The effectiveness of NB biomineralization is remarkable: apatite formation *in vitro* stopped only when the calcium level decreased by 50% from 1.8 to 0.9 mM and the phosphate levels fell to near zero., **24** Our results indicate that the NB calcium phosphate phase can be formed at pH 7.4 consistent with human physiological phosphate and calcium concentrations., **19,24,38,39** This can be also monitored by ^{85}Sr incorporation and provides a unique model for *in vitro* studies on calcification., **24** NB-induced apatitic biofilm formation is dependent on the presence of oxygen, **19, 40** and can be prevented with several antibiotics and anti-metabolites, and by high gamma irradiation at sterilizing doses., **39,41** The apatite produced by NB is biogenic because it is formed in a carbon-containing biomatrix, forms small spherical units of apatite in nanoscale crystal size, (that are very resistant to acid hydrolysis), and can be formed at non-saturating concentrations of calcium and phosphate., **19** Such spherical units were identified in most human kidney stones examined (see Fig. 3)., **24,42,43** Non-biogenic apatite has larger crystals that are easily dissolved in acidic solution. Fourier Transform Infrared Spectroscopy (FTIR) of NB revealed the mineral as almost identical to bone mineral (Fig. 4). Models for bone formation, which use metastable concentrations of calcium and phosphate, involve gels that include nidi, such as matrix vesicles, apoptotic vesicles or collagen, but exclude the known proteinaceous inhibitors for crystal formation., **44** Such systems have not been tested with nanobacteria. Vali et al. have shown that nanoforms contain apatite-protein complexes and immuno electron microscopy reveals protein antigens in proximity to apatite suggesting a novel form of protein-associated mineralization., **45**

In our earlier studies, we examined NB cultures in High Aspect Rotating Vessels (HARVs) designed at the NASA's Johnson Space Center, which are designed to simulate some aspects of microgravity., **25** NB cultured in HARVs multiplied 4.6 times faster than under stationary conditions and 3.2 times faster than in shaker flask incubation. Interestingly, the results demonstrated that the degree of apatite crystal formation on NB (biomineralization) and the properties of the apatite are strongly affected by the gravity and other specific culture conditions used., **25** Although some researchers believe that microgravity does not affect crystal formation and biomineralization, **46** it has been shown that long periods in a microgravity environment does cause loss of bone, and enhance kidney stone formation-like biomineralization disorders in astronauts., **47-49**

In summary, NB is a perfect model for studying biogenic mineralization/calcification because NB a) are self-replicating particles and have less complicated metabolic pathways b) accumulate calcium and phosphate under physiological conditions, c)

produce a calcium phosphate mineral similar to bone, d) exist in physical conditions (pH, gravity, temperature, etc) that are easy to manipulate, and which can be replicated for the physiological model.

Controversy

The first debate about NB revolved around whether these minute particles are alive or not. To this day, critics argue that a particle just 50 to 200 nanometers in diameter can't possibly harbor the components necessary to sustain life. Maniloff's work suggests that to contain the DNA and proteins needed to function, a cell must be at least 140 nanometers across., **50** However, recently it has been shown that a genome constructed to encode 387 protein-coding and 43 structural RNA genes could sustain a viable synthetic cell, a *Mycoplasma laboratorium* which can shrink its size below that limitation., **51** NB are also incredibly resistant to heat and other methods that would normally kill bacteria, which makes some scientists wonder if they might be an unusual form of crystal rather than organisms. Cisar et al, presented an alternative theory for the experimental findings of NB scientist. They stated that biomineralization previously attributed to NB may be initiated by nonliving macromolecules and transferred on "subculture" by self-propagating microcrystalline apatite., **52**

Detection Methods for NB

Methods to diagnose NB in biologicals, cells, tissues, blood and urine include immunodetection with NB-specific monoclonal antibodies, electron microscopy and culture techniques., **39,53** Because NB pass through 0.22 μm pore size filters, which exclude most common microbes, filtration is often used to clean up fluid specimens before culture for NB., **53** Replication can be measured by particle counting and optical density at 650 nm., **41** It has been also shown that growth of the NB could be detected by specific methods, such as ELISA, turbidity, SDS-PAGE or methionine and uridine incorporation., **19,20** Susceptibility tests can be used to test effects of antibiotics and other chemotherapeutics., **19** Growth could be prevented with tetracycline, high doses of aminoglycoside antibiotics, EDTA, cytosine arabinoside, 5-FU and gamma-irradiation., **19**

Are NB a Living Entity?

Isolation of any kind of nucleic acid from NB has been difficult, in part because of the mineral surface that they produced during their culture period. They could not be lysed with lysozyme, proteinase K, several other proteinases, lipases, amylases, alkali, ultrasound, X-press, detergents or solvents., **40** We needed to use acid or EDTA like chelators before the analysis, which were the factors for structural change in nucleic acid. It has been our experience that NB actually inhibit the amplification of added exogeneous classical bacterial DNA by polymerase chain reaction methods.

In a preliminary research, NB cultures incorporated increasing amounts of radiolabel S35 into macromolecules separable by gel electrophoresis during the 18-day incubation

period. Protein pattern detected with Coomassie stain of SDS-PAGE transferred to an Immobilon membrane and autoradiography showed methionine incorporation into several proteins (Fig. 5). Miller et al showed that autoradiographs of isolated NB nucleic acid indicated incorporation of uridine into several "nucleic acid" bands., **20** The negative control, media containing gamma-irradiated serum (*ie.*, inactivated NB), did not yield radioactive bands after exposure to these radiolabeled DNA/RNA and protein precursors**.** In order to define the mechanism of incorporation, and systematic location of NB, further molecular research is needed.

Are Nanobacteria Cytotoxic?

Calcified and non-calcified forms of NB have been observed as free, cell-attached and internalized particles in mammalian cell cultures *in vitro*., **19,53** In our experiments we have chosen 6 different fibroblast lines as experimental models, because they are the most ubiquitous cells in the animal body and might be most accessible in wound tissue for invading pathogens with the exception of professional phagocytic leucocytes., **53** We have shown that NB were bound as clusters on the cell surfaces within 15 minutes. It is concluded that NB are internalized either by receptor-mediated endocytosis or by a closely related pathway within 12 hours., **53** Internalization seems to be necessary for cytotoxicity. We showed that cytotoxicity was dependent on NB concentration and exposure time. Dying cells always contained numerous ingested NB., **53** Hybridomas and many lymphocytes were found to be affected by NB, but considerably higher doses were needed., **40,53** The findings that NB are cytotoxic are of interest to nanoparticle toxicity mechanisms in general: similar mechanisms could be used by other nanoparticles for entering cells and causing cytotoxicity., **54**

***In vivo* Effects of NB**

Akerman et al. reported that radiolabeled (^{99m}Tc) viable NB accumulated in the kidney and appeared in urine after 15 minutes of their intravenous injection into rabbits. This could be due to the fact that kidneys are the preferred sides for this agent, unlike other known nanoparticles, and the presence of injured epithelium or a nucleus in the kidney/urinary tract provides a preferable niche for NB to adhere and grow, resulting in biocrystallization., **55** A control study in rabbit was performed administering a similar dose of ^{99m}Tc-labelled albumin cross-linked particles or tin-technetium nanoparticles as nanocolloids. Nanocolloids were not targeted to kidneys as NB were., **55** Shiekh et al too observed that NB, when injected intravenously into rats, were localized in kidneys., **28** In their research they observed regions of chronic inflammation infiltrated in the cortex and medulla, which could be due to damage induced by NB. The research team has also shown NB adhering to the surface of the epithelium and their penetration into the epithelial cells.

NB and Connections to Calcification Related Diseases

Calcium is the most important intracellular regulator of physiological responses and is a messenger for hormonal actions and as well regulates cell death and multiplication.

Additionally, calcium regulates inflammation, blood clotting, immunological responses and neural transmitting and muscle contraction. In these actions calcium-binding proteins are the main players. Recently, a large number of calcium-binding proteins have been mapped to be bound on NB., **56** NB may thus participate in activation – inhibition processes regulating a large number of responses inside and outside cells. Thus, NB could have multiple pathological actions in the body.

The life-long prevalence of kidney stones appears to have increased throughout the whole 20th century and occurs in up to 15% of the population., **57** Treatment of kidney stones is now estimated to cost over $3000 per patient per year. The incidence of new cases and recurrences may continue to rise. Thus, new approaches in treatment and prevention could have a huge economic effect apart from benefits in terms of reduced morbidity. Electron microscopic observations show that apatite units produced in serum-free NB cultures are very similar to human apatitic kidney stones (Figs. 2 and 3). Both grow as layers of mineral (Fig. 2) and matrix. Chemical analysis, EDX, revealed that the composition of this solid mineral formation was similar to that of most extraskeletal tissue calcification and stones., **19** The crystalline components of kidney stones are calcium oxalate, calcium phosphate, struvite, purines, or cystine., **58** Fermentor model studies have shown that in kidney stones calcium phosphate nidi are always formed initially and may subsequently become coated with oxalate or other components., **59,60** Apparently, apatite may play a key role in the formation of all kidney stones. Our hypothesis underlines the role of active nidi: even supersaturated urine needs nidi for crystallization to appear, and active nidi could make the process more thermo-dynamically favoured, so that it could happen apparently outside equilibrium. We have proposed that NB may be active nidi that attach to, invade and damage urinary epithelium of collecting ducts and papilla forming calcium phosphate center(s)., 19,24-27,30 We found NB in 70 out of 72 human kidney stones studied in Finland., **24** Khullar et al demonstrated 62% culture positivity of NB in stones collected from an Indian population., **61** Additionally, they showed DNA and distinct protein bands of NB. However, one group has been unsuccessful at replicating NB culture from stones although they have observed nanoparticles morphologically similar to NB in SEM analysis., **62**

Another urinary disease, PKD, is the most common autosomal dominant lethal disease in humans. There are reports of endotoxin, NB, and fungal antigens and antibodies in human kidney cyst fluids. Interestingly, a higher prevalence of kidney calcifications is observed in PKD than in the normal population., **63** It has been proposed that the currently known cellular toxicities, tissue distribution, and pharmacology of NB are plausibly related to the known pathology and pharmacology of PKD., **29** Hjelle et al evaluated 13 PKD cyst fluids and detected NB antigen positivity in each sample as well as in liver cystic fluid from affected individuals., **29**

Biopsies, urine, and prostatic secretion cultures fail to demonstrate bacterial pathogens more frequently than in asymptomatic controls, yet inflammation or at least inflammatory markers are often detected in chronic prostatitis., **64** Likewise, the presence of prostatic calculi in younger men is associated with both inflammation and symptoms of chronic

pelvic pain syndrome., **65, 66** The core of prostatic calculi is typically calcium apatite**, 67** which is the hallmark of NB action. Wood et al, proposed that there is a potential role of NB in chronic prostatitis., **32** Recent clinical research targeting these agents has proven effective in treating some patients with refractory category III prostatitis (chronic pelvic pain syndrome)., **33** In that research, NB antigen or antibody was found in 60% of serum and 40% of urine samples. In 10 patients who underwent transrectal ultrasound after therapy, prostatic stones were decreased in size or resolved in 50%., **33**

Mechanisms mediating vascular calcification remain incompletely understood. Some have hypothesized the potential role of NB in arterial calcification., **20,21,35** Miller et al, cultured nanosized particles from calcified but not from noncalcified aneurysms. These particles were stained with Nb-specific monoclonal antibodies, recognized by a DNA-specific dye and incorporated radiolabeled uridine, and, after decalcification, they appeared via electron microscopy to contain cell walls., **20** Therefore, nanometer-scale particles similar to those described as nanobacteria isolated from geological specimens and human kidney stones can be visualized in and cultured from calcified human cardiovascular tissue., **20** Puskas et al, propagated NB- like spherical particles from 26 of 42 sclerotic aorta and carotid samples and confirmed their nature by dot immunoblot by using Nb-specific monoclonal antibodies, light microscopy and TEM. [3H]L-aspartic acid was incorporated into high molecular weight compounds of demineralized particles. PCR amplification of 16S rDNA sequences from the particles was unsuccessful using traditional protocols., **21** Identification of NB-like particles at the lesion supports, but does not by itself prove the hypothesis that these agents contribute to the pathogenesis of atherosclerosis, especially vascular calcifications. Specific therapies targeting these particles has demonstrated reduced plaque formation, regression of plaques, and improved lipid profiles., **68** The potential of anti-NB treatments are controversial and await larger clinical trials. Epidemiological studies have implicated antibodies made by the body against NB to be a strong independent risk factor for coronary artery calcification. The risk appears to be comparable to that of diabetes in the two preliminary studies., **69,70** The importance of this is that coronary artery calcification is an excellent predictor of future coronary events and death.

Originally, Sedivy and Battistutti reported that NB promoted crystallization of psammoma bodies in ovarian cancer., **71** Hudelist et al. soon verified the 100% concordance between the expression of NB and the presence of psammoma bodies in malignant ovarian tumours. In their research, several lines of evidence suggest the involvement of these organisms in the process of biomineralization. Therefore they have concluded that NB infection of malignant ovarian tissue contributes to mechanisms leading to the formation of calcified deposits known as psammoma bodies., **36**

Additionally, NB have been shown to be detected at higher rates in serum of patients with gallstone disease, **31** and mitral valve calcification., **34** Others have suggested that NB may contribute to the development of peripheral neutropathy in HIV positive patients, **72,73** and periodontal problems, **22,74** and even osteoporosis., **75** All these hypothetical approaches require further investigation.

Koch's Postulates

In a small study, Garcia Cuerpo et al found that translumbar, percutaneous intrarenal injection of NB (isolated from kidney stones) into rats resulted in kidney stone formation., **76** Additionally, Shiekh et al has examined NB's role in biocrystallization and *in vivo* effects on kidney pathology. Calcium oxalate monohydrate assay (COM) was carried out in the presence of NB to study biocrystallization. Wistar rats were given an intravenous injection of NB and the kidneys were examined for pathological changes. The COM assay showed accelerated biocrystallization of (14)C-oxalate in the presence of NB, indicating them to be efficient candidates for biomineralization. Histopathological studies revealed bacteria induced renal tubular calcifications and various manifestations of infection., **28** Their studies confirm that NB may be involved in the pathogenesis of renal tubular calcification. Such findings are required to prove Koch's postulates linking NB to other pathological calcification related diseases.

Conclusion

Whether NB themselves serve as the nucleus for crystal formation, or whether the NB are simply able to lower the activation energy barrier and thus allow precipitation and growth of crystals under much lower supersaturation conditions is yet to be determined. However, it is immaterial whether or not they are bacteria, viruses, or other living or nonliving forms; their properties of promoting ready crystallization and growth of Ca minerals are well established. These self-replicating particles may induce calcification and stone formation *in vivo* because: NB a) have been detected in human blood, b) are transported from blood into urine and bile as living organisms, c) are renotropic, d) cause apoptotic cell death, e) are present in human stone-isolates, and tissues with calcification, f) cause kidney stone formation in rats within one month when injected in an intra-renal route.

Since NB have been detected in blood and blood products, they should be of interest to the biopharmaceutical industry. For example recently, NB have been isolated and cultured from the cultured nasopharyngeal carcinoma epithelia HNE1 cell supernatant., **77** Safety of vaccines produced in cell culture by using (bovine) serum or serum-derived materials in culture of the cells, sterilized by filtration is an issue needing thorough risk analysis and method validation., **78,79**

Only continued research will reveal the nature of NB and their impact on health and disease. NB are a good model system to use in developing drugs to alter the likely diverse pathways involved in tissue calcification. While the controversy of whether they are living or nonliving entities will continue until new definitive data is collected, this controversy should not overshadow the critical medical importance of understanding the already demonstrated effects of NB on pathologic calcification in the human body and on research into countermeasures to reverse or eliminate these effects.

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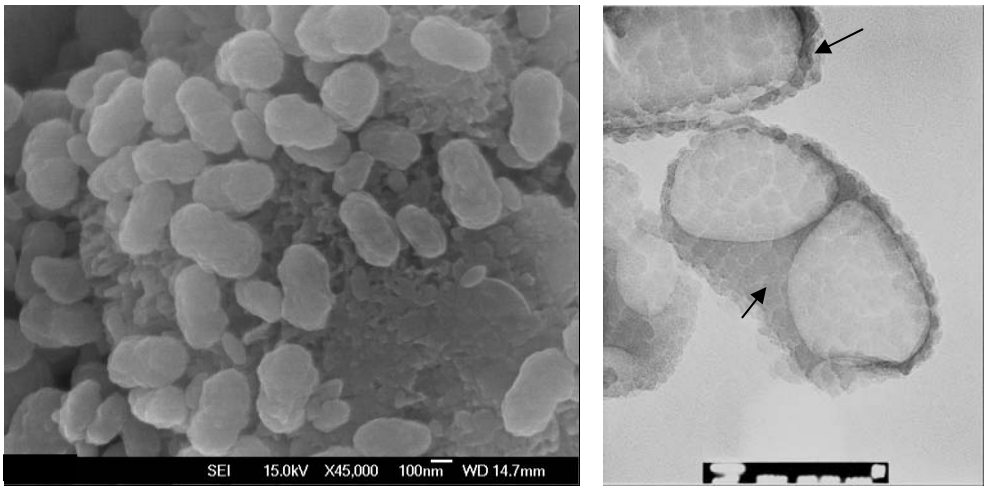

Figure 1. Scanning Electron Microscopic (left), and Transmission Electron Microscopic (right) images of cultured nanobacteria (NB). Arrows on the right show apatite envelop of apparently multiplying NB. Bars; 100nm left, 50nm right

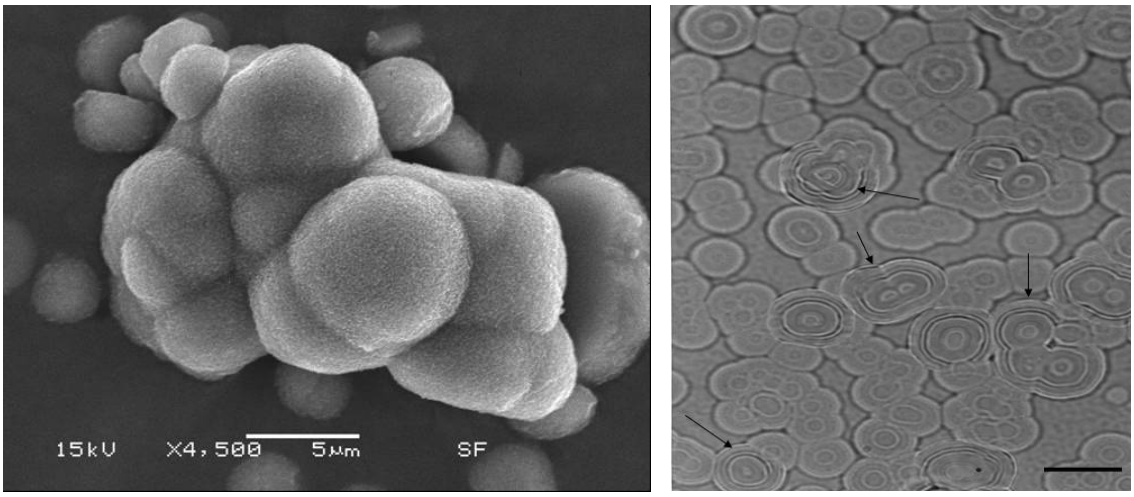

Figure 2. SEM (left), and DIC (right) image of an apatite clusters formed by NB under serum-free culture conditions. Arrows on the right show age-ring like apatite layers on around NB "colonies". Bars, 5 micrometers.

Figure 3. SEM image of spherical apatite formations in an apatite kidney stone. Bar 10 micrometers.

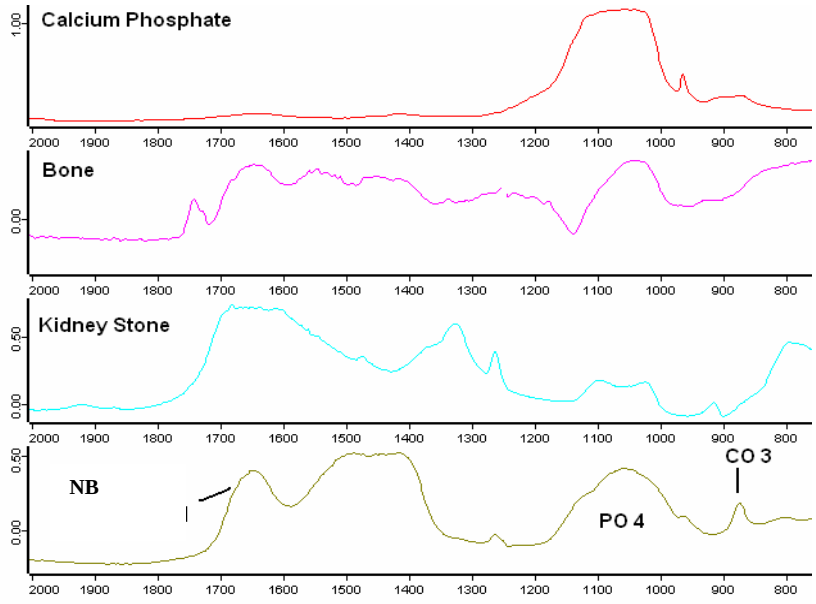

Figure. 4. FTIR results showing the distinct phosphate absorption signature at 1000 to 1200 cm-1 is seen in all four spectra, with inorganic apatite, bone, kidney stones and NB.

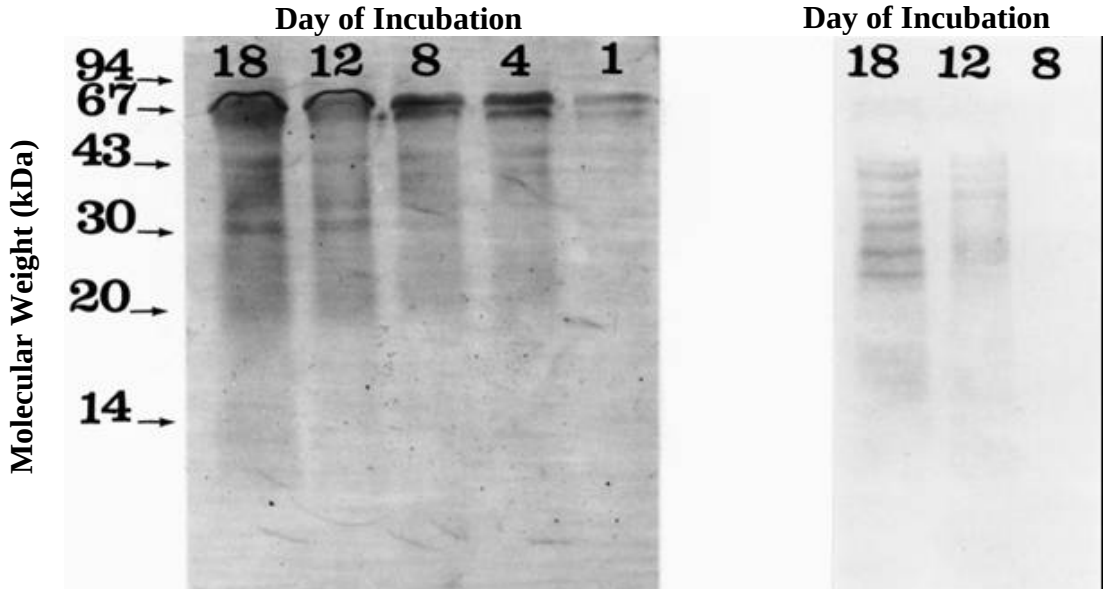

Figure 5. Metabolic labeling of NB. Images showing incorporation of radiolabeled precursor molecule into macromolecules.