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Original research paper

Hemoglobin aggregates studied under static and dynamic conditions involving the formation of nanobacteria-like structures

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Laser light scattering and scanning electron microscopy (SEM) are used to study hemoglobin in the aqueous phase. The impact that salts [NaCl, $Ca_3(PO_4)_2$] and iron oxide nanoparticles have on the hemoglobin size are also studied. The first set of experiments examined hemoglobin aggregates in the aqueous phases in the presence of salts and nanoparticles. Aqueous phase samples were then dehydrated and examined using SEM. The resulting structures resemble those observed in nanobacteria studies conducted in other labs. This study demonstrates that aggregates of hemoglobin and various salts found in a physiological environment can produce structures that resemble nanobacteria.

Keywords: nanobacteria, hemoglobin aggregates

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Nanobacteria are considered by some to be cellular microorganisms with a diameter on the order of tens of nanometers, much smaller than what is considered the lower limit for microorganisms with a cell wall. They have been implicated in the formation of minerals such as dolomite, travertine, sulfides and iron oxides in geological samples (1–3).

Nanobacteria have been correlated with a number of human diseases (4–12). For example, high levels of nanobacteria have been identified in the synovial fluid of patients suffering from osteoarthritis (4). Nanobacteria have been suggested as the cause of treatment-resistant kidney stones and other urinary tract diseases (5). A study by Candemir *et al*. (6) suggests that nanobacteria may play a major part in the pathogenesis of mitral annular calcification. Nanobacteria have also been suggested to play a role in ovarian cancer, where they were correlated with the formation of psammoma bodies or calcified deposits in papillary tips (7). Sommer (8) suggested that nanobacteria surrounding the perineurium in the spinal cord could interrupt signaling processes and restrict natural elasticity. Nanobacteria in the heart have been attributed to shifts in an individual's

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blood chemistry (9); they appear to grow in size and shape in serum with the addition of carbon dioxide and sodium bicarbonate (10). In a separate study, synovial fluids from diseased knee joints were allowed to sit for two months and nanobacteria propagated with most having a spherical shape less than 2 micrometers in diameter (11). A study done at autopsy of an individual with atherosclerosis supports, but does not prove, that nanobacteria contributed to its pathogenesis (12). It has also been reported that nanobacteria anchor themselves in aqueous environments in the body by slime and produce a nano-crystalline apatite shell. This was suggested to be a chemical polymer slime link between individual nanobacteria (13).

With the growing number of illnesses supposedly associated with nanobacteria, some researchers believe pharmaceutical agents can help combat nanobacteria buildup (14, 15). Biosensors are being developed that can distinguish biocolloids and nanobacteria biogenic particles on the basis of ring formation of polystyrene nanospheres in water drops (16).

Nanobacteria have not been without controversy in the scientific community. The existence of nanobacteria has been openly questioned by a number of scientists (17–19). In effect, some scientists argue that nanobacteria are not living entities but are composed of salts and other physiologically based organic compounds. This study addresses this controversy by focusing on the formation of aggregates of hemoglobin (Hb) that resemble nanobacteria images commonly observed in mammals. It is demonstrated that hemoglobin forms aggregates in the aqueous phase and this aggregation process is shaped by the matrix (*i.e.*, salt content). These aggregates have the appearance of some nanobacteria reported in the literature when dried and studied by scanning electron microscopy.

MATERIALS

The hemoglobin (M_r = 64.5 kDa) was purchared from MP Biomedical (USA). The stock concentration was 0.020 g per 100 mL of purified (RO, DI) water. This solution was diluted for different experiments run at 20 °C and 1 atm (laser diffraction of aqueous phase). Sodium chloride salt (Fisher Chemicals, USA) and iron oxide nanoparticles (Nano Tec, USA) were purchased commercially. Calcium phosphate was made by mixing calcium chloride and sodium phosphate and collecting, washing and drying the precipitate. Hemoglobin-salt solutions were diluted up to 300-fold for the aqueous phase laser diffraction measurements. A Laser Diffraction Particle Size Analyzer (Model SALD-3001, Shimadzu, Japan) was used to measure the diameter and relative concentration aggregates in water. This instrument is used on a circulating system and runs 300 mL of solution for any measurement. Samples for SEM were dried under vacuum, mounted on aluminum stubs and sputter-coated with gold-palladium. Samples were viewed with a JEOL JSM 6480LV scanning electron microscope operating at 20 kV. The mass spec samples were analyzed on a Bruker (USA) Autoflex MALDI-TOF mass spectrometer using reflectron mode. 2,5-Dihydroxybenzoic acid or sinapic acid were dissolved in 50 : 50 acetonitrile/water with 0.1 % trifluoroacetic acid to form the MALDI matrix.

RESULTS AND DISCUSSION

Results are divided into dynamic (laser diffraction particle size analysis) and static (SEM imaging) systems. Few, if any, studies have attempted to study nanobacteria in the aqueous phase by any method of light scattering. These techniques can provide information about the size and population density for a large sample (> 5 mL). There is no reason to believe that these aggregates in the aqueous phase formed a specific shape (*i.e.* spheres, rods, *etc.*) but the data did indicate that there was a mixture of relatively small (< 0.5 nm) and large (> 10 nm) sized aggregates in equilibrium. This equilibrium is easily perturbed by chemical and physical (vibration, temperature) conditions and does not result in the type of reproducible equilibrium constant (*K*) regularly applied to acid/ base, metal/ligand systems. Instead, we simply measure the ratio of small to large aggregates (see Fig. 1)

$$K = [\text{small aggregates}] \; / \; [\text{large aggregates}]$$

The lack of a reproducible equilibrium constant also results from the fact that hemoglobin aggregates have a fairly large size distribution that can vary from a single hemoglobin molecule up to a large aggregate that is heavy/dense enough to precipitate from solution. The first set of experiments focused on establishing a concentration range in which the hemoglobin aggregates would stay suspended for several hours and be detectable by the laser scattering instrument. Fig. 2 illustrates how the concentration of hemoglobin affects the diameter of the aggregates until it reaches a maximum size sustainable in the aqueous phase. Once this plateau is reached, the hemoglobin begins to precipitate. This experiment was conducted at 20 °C with a pH of 7.2. If dispersed at extremely low concentrations, individual hemoglobin molecules would eventually precipitate; concentrations were sought that the instrument would easily detect. The hemoglobin aggregates were measured here by size. Measurements were conducted in two modes. The first mode is by size and shows that the bulk of the hemoglobin is found in relatively few larger aggregates. The second mode is by number and shows that there may be relatively large numbers of smaller sized aggregates compared to the larger ones al-

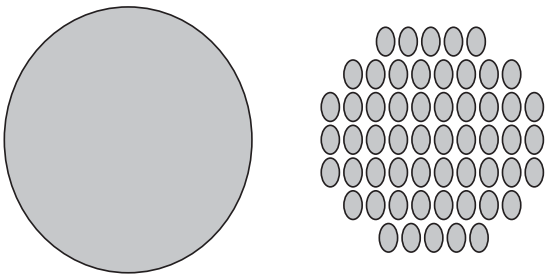

Fig. 1. A single large sphere (left) occupies more volume than many smaller spheres (right). If measured by volume, there is more hemoglobin in a small number of large particles. If measured by number, there are more small particles.

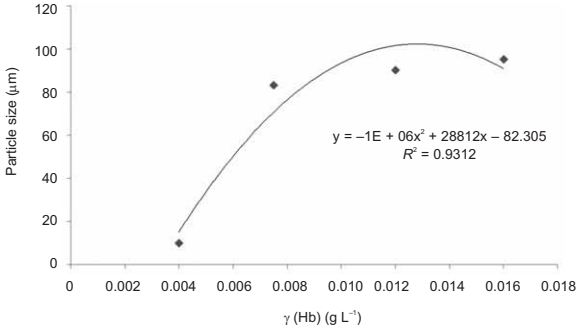

Fig. 2. The first experiment in water with high Hb concentrations resulted in large aggregates that precipitated from solution. This set of experiments was used to define a precipitation point on Hb concentration.

though their total mass or volume (second mode) is much smaller than the total mass or volume of the larger aggregates (first mode) (Fig. 1).

Fig. 3 and 4 show graphs of aggregation in different matrices of 1×10^{-5} g L^{-1} of substrates (sodium chloride, iron oxide nanoparticles, calcium phosphate). The static analysis of samples taken from the experiments producing graphs is analyzed under SEM in images shown in Figs. 5 through 7.

Fig. 3 shows the change in hemoglobin aggregation in RO water, NaCl, $Ca_3(PO_4)_2$ and FeO nanoparticles. Both the FeO nanoparticles and $Ca_3(PO_4)_2$ limit the size of the hemoglobin aggregates while the NaCl does not limit the formation of larger aggregates. This is attributed to the fact that NaCl is a strong electrolyte that increases the viscosity of the solution and can form ion-dipole bonds with the hemoglobin structure, increasing its water solubility. Both $Ca_3(PO_4)_2$ and FeO nanoparticles are salts of low solubility in solution. These structures are tens of nanometers in size and are intercalated in the hemoglobin aggregates. Fig. 3 provides the size by number (number of aggregates present

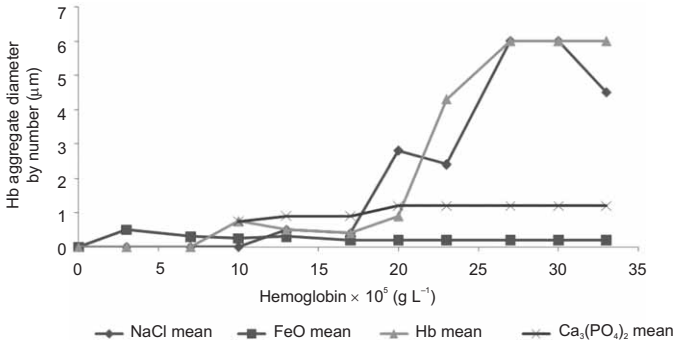

Fig. 3. The average diameter of Hb aggregates measured by number changes/grows with Hb concentration.

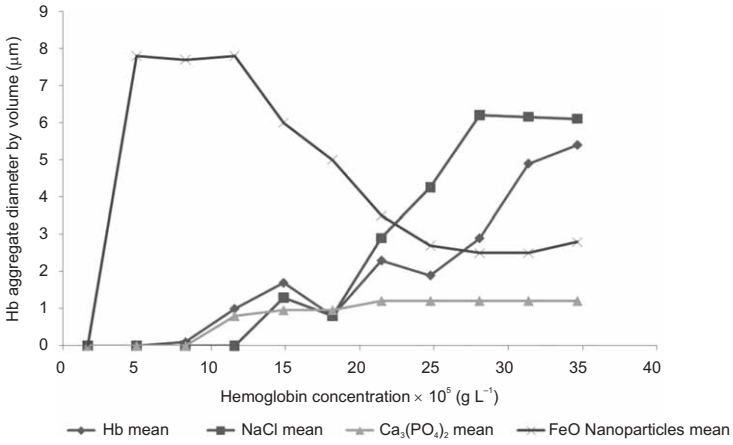

Fig. 4. The size of Hb aggregates as measured by volume varies with changes in Hb concentration.

vs. hemoglobin concentration) while Fig. 4 provides data by volume (volume of aggregates *vs.* hemoglobin concentration). Fig. 4 also shows that a few larger aggregates can form at lower hemoglobin concentrations due to FeO nanoparticles. Hemoglobin structure has an affinity for iron and pulling these nanoparticles in may cause tightening or contraction of the structure.

Figs. 5 through 7 show the hemoglobin aggregates dried and prepared using common techniques used by many nanobacteria researchers for SEM studies.

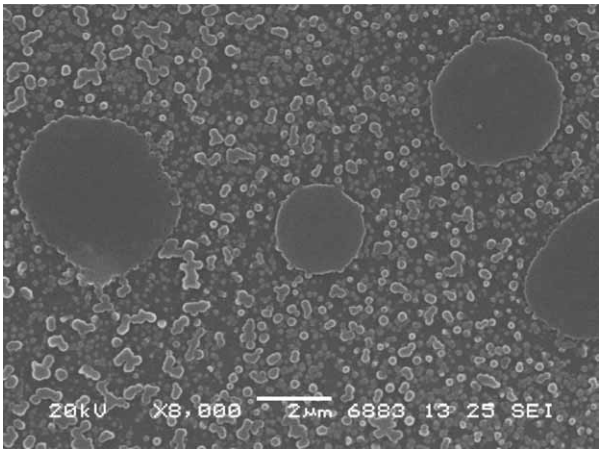

Fig. 5. Large and small aggregates from a solution of hemoglobin and calcium phosphate. These inert aggregates have the small size and shape of some forms of nanobacteria.

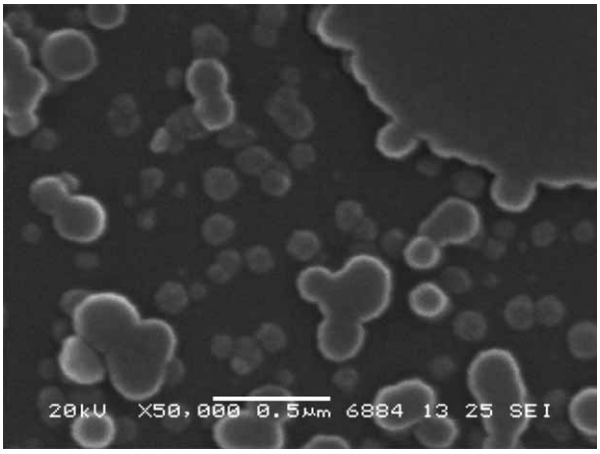

Fig. 6. Hemoglobin aggregates, once dried and coated, are 100–200 nanometers across, similar in size to nanobacteria.

In solution, aggregates have a dynamic equilibrium between smaller (< 0.4 μm) and larger (5–100 μm) particles. These aggregates contact the solvent (water) and change their shape and size instantly. Once dried and coated, these hemoglobin and hemoglobin-salt compounds take on a homogeneous shape and size that are consistent with the appearance other researchers have claimed to be nanobacteria.

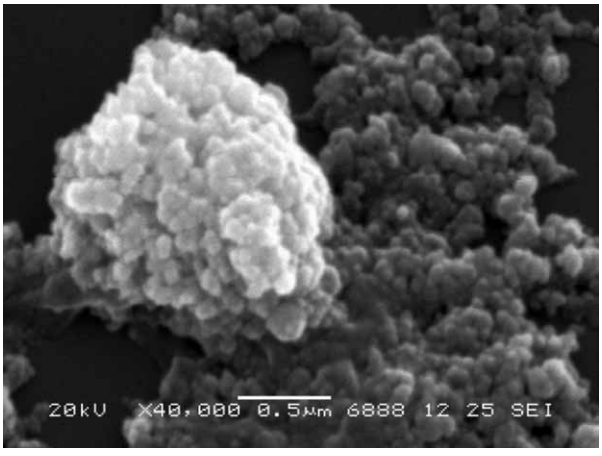

Fig. 7. Side view of hemoglobin with iron oxide nanoparticles (angle of tilt 30°) confirms the structural height of aggregates coming off the stub and showing that large aggregates are denser in nature compared to the other matrices tested.

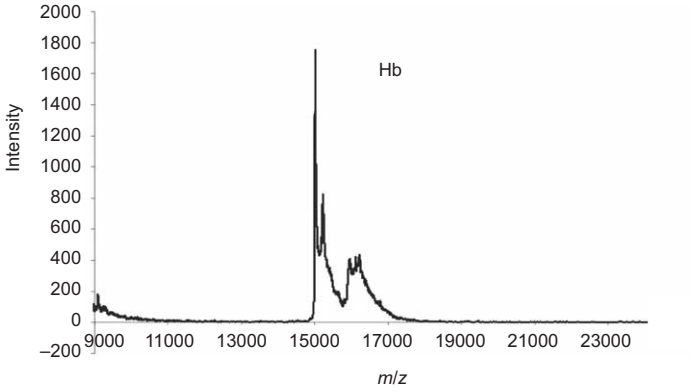

Fig. 8. Mass spectrometry is used tostudy hemoglobin aggregates.

The measurements taken in dynamic settings show those hemoglobin aggregates in larger pieces that have a critical mass before they become too large and precipitate. At this point, pieces fall off and reform. Mass spectrometry studies we conducted (Fig. 8) show that hemoglobin maintained its molar mass throughout the procedure. Changes in aqueous conditions affect the size and number of aggregations as well as their critical mass size. When a strong electrolyte such as sodium chloride dissociates completely, the aggregates reach their critical mass in volume and number at a higher concentration of hemoglobin. However, a weak electrolyte or one that does not dissolve significantly in solution, like calcium phosphate, remains relatively low in the aqueous phase. When iron oxide nanoparticles are added to the solution, they act in a manner similar to that of calcium phosphate with regard to hemoglobin aggregate. The static SEM images show that these salts and nanoparticles, when combined with hemoglobin, resemble nanobacteria in size and shape. Regardless of the aqueous phase matrix, the aggregates of hemoglobin, once dried, coated and studied by SEM, all had the same appearance. These inert structures resemble the images of nanobacteria found in diseased hearts, kidneys, and the urine of HIV patients, as well as in cancer tissue (1, 3, 4, 11–13, 15).

CONCLUSIONS

Some studies cited pointed out that nucleic acids were identified in the formations identified as nanobacteria. Our studies use only hemogloblin, but once an aggregate of this nature is in a physiological environment, it can adsorb a range of molecular fragments found in the blood stream that are in the process of being flushed from the body, including genetic material, cell membranes and other proteins.

The goal of this study is to show that relying on the results of one technique alone can potentially lead to false interpretation. Comparison of SEM images of nanobacteria with the data of the four matrixes with hemoglobin may suggest that the nanobacteria

present in tissue fluids may be dried aggregates. The SEM controls for this study (*i.e.*, FeO nanoparticles, NaCl and calcium phosphate) do not resemble hemoglobin aggregates. We believe that a host of proteins will also dry and form aggregates with similar appearance.

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S A Ž E T A K

Agregati hemoglobina proučavani pod statičkim i dinamičkim uvjetima uključujući i nastajanje struktura poput nanobakterija

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Metoda dinamičkog rasapa svjetlosti i pretražna elektronska mikroskopija (SEM) korištene su za proučavanje hemoglobina u vodenoj fazi. Nadalje, ispitivan je utjecaj soli [NaCl, $Ca_3(PO_4)_2$] i nanočestica oksida željeza na veličinu hemoglobina. U prvom setu pokusa proučavani su agregati hemoglobina u vodenoj fazi u prisutnosti soli i nanočestica. Vodena faza je dehidrirana i ostatak analiziran pomoću pretražne elektronske mikroskopije. Agregati hemoglobina i različitih soli pronađeni u fiziološkim uvjetima čine strukture slične nanobakterijama.

Ključne riječi: nanobakterije, agregati hemoglobina

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