The Medusa strain

An unknown life form is hardening your arteries and turning your kidneys to stone. That's if it exists at all, says **Bijal Trivedi**

THEY are minuscule egg-shaped structures mere billionths of a metre across, dwarfed by the tiniest living cell and smaller than many viruses. They have a hard bony shell, replicate like a living organism and are wiped out by antibiotics and radiation, yet seem to lack DNA. Some say they are infectious microbes, possibly even an unknown form of life, able to cause diseases ranging from Alzheimer's to atherosclerosis. Others say they are simply harmless crystals.

Welcome to the topsy-turvy world of nanobacteria. Once described as the "cold fusion of microbiology", the very existence of these microbes has been denied or ridiculed by mainstream opinion for nearly a decade and their proponents branded mavericks. Just like cold fusion, though, nanobacteria have refused to go away and now – under the new guise of "calcifying nanoparticles" – they are making a renewed bid for scientific respectability. The stakes are high. If diseases long thought incurable are actually caused by nanobacteria, they could be prevented with vaccines, or treated with antibiotics.

The story began in the early 1990s when Olavi Kajander, a biochemist at the University of Kuopio in Finland, was carrying out some important, if unexciting, work on cell-culture contaminants. In 1993 he reported finding a hitherto unknown contaminant in cow serum: tiny, self-replicating spheres which he tentatively called "nanobacteria". He and a colleague, Neva Çiftçioglu, went on to find the same particles in the blood of cows and humans, as well as in supposedly sterile blood products. In 1997, the duo claimed to have sequenced a small piece of DNA extracted from the particles which proved they were a new type of bacterium.

In 1998 the rumpus kicked off. The trigger was a paper in which Kajander and Çiftçioglu claimed not only that nanobacteria were alive, but also that they were the cause of kidney stones (*Proceedings of the National Academy of Sciences*, vol 95, p 8274). The pair had noticed that their nanobacteria often cocooned themselves in thick coats of calcium phosphate – the material in some types of kidney stone. So they examined 30 human kidney stones and found that all of them contained signs of nanobacteria. Putting two and two together, they proposed that these microbes were the cause of the stones.

To say this went against the grain is an understatement. Kidney stones are an example of "pathological calcification", in which tissue becomes clogged with deposits of calcium phosphate. The root cause of this has long been a mystery, but it is widely considered to be a purely chemical process. Now here was a team arguing that it was caused by infection with a class of organisms that many doubted was even real.

That wasn't all. Calcification is also linked with other chronic and often fatal diseases, including atherosclerosis (hardening of the arteries), cancer, dementia and arthritis. If nanobacteria could cause kidney stones, why not these diseases too? In a commentary in the same issue (p 7846), Dennis Carson of the University of California at San Diego proposed exactly that.

These ideas sparked an immediate uproar. Critics quickly pointed out that these supposed bacteria weren't big enough to be alive. At between 50 and 100 nanometres across, they are widely considered too small to house the machinery a living organism needs.

Researchers began trying to replicate Kajander and Çiftçioglu's research, with scant success. The most convincing rebuttal came from a group led by microbiologist John Cisar at the National Institutes of Health in Bethesda, Maryland. He successfully isolated nanobacteria-like particles from human saliva and propagated them in the lab. However, he was unable to detect any proteins or genetic material associated with them. What is more, the DNA sequence Kajander used to classify nanobacteria wasn't new but belonged to a common contaminant of cell cultures. Cisar concluded that the calcification attributed to so-called nanobacteria was nothing more than crystallisation triggered by non-living particles (*Proceedings of the National Academy of Sciences*, vol 97, p 11511).

As far as most researchers were concerned, that was that. Kajander and Çiftçioglu were branded mavericks and their funding slashed. But interest in nanobacteria did not diminish – it grew.

In the past few years, the tide has started turning in Kajander and Çiftçioglu's favour. Other researchers, most notably a team from the Mayo Clinic in Rochester, Minnesota, have replicated and extended many of the pair's findings. Nanobacteria-like particles have been isolated from numerous diseased tissues, and several groups now share the view that they are the cause of calcification. There is even preliminary evidence that drugs designed to knock out nanobacteria can cure a disease associated with calcification.

The turnaround began in 2004, when the Mayo team, led by nephrologist John Lieske and physiologist Virginia Miller, published a report supporting many of Kajander and Çiftçioglu's early findings. They collected diseased arteries and heart valves from surgical waste and found that many contained spheres matching the description of nanobacteria. They went on to show that these spheres replicated in culture and stained positive for DNA (*American Journal of Physiology – Heart and Circulatory Physiology*, vol 287, p H1115).

The Mayo group also tried another method to interrogate the nanoparticles for signs of life. Called spectromicroscopy, this technique relies on generating an optical signature for microbes according to how they absorb and ▶

reflect light. The Mayo team compared the signature of the nanoparticles with those from two types of bacteria known to build calcium phosphate shells. They found clear similarities but, crucially, the nanoparticles lacked a DNA signature, though Miller says this is probably an artefact of the set-up. "This was our first experiment," she says.

Perhaps unsurprisingly, this research failed to convince the hard-core sceptics. But it helped the atmosphere thaw a little. By 2006, nanobacteria were considered respectable enough for the organisers of the annual Experimental Biology meeting to invite Kajander, Çiftçioglu and the Mayo team to San Francisco to discuss their research.

Alive without DNA?

At that meeting the Mayo team reported yet more evidence. They had cultivated nanobacteria from kidney stones and extracted DNA, RNA and four proteins that closely resemble other bacterial proteins. "I think that [the proteins] would be evidence supporting that they are a microorganism," says Lieske cautiously, "but one can still argue that it is a contaminant, so it is not definitive proof." And the DNA? "Still inconclusive," says Lieske. The quantity they collected was minuscule, providing little material for further analysis. Lieske and his colleagues now aim to collect more DNA and sequence it. By culturing nanobacteria in different nutrient solutions, they think they can grow them without their bony shells, making it easier to extract any DNA that might be there.

Another unexpected boost came from the discovery of nanoscale microbes in Richmond mine, California. They were archaea rather than bacteria, but were smaller than the theoretical minimum size for living cells (*Science*, vol 314, p 1933).

Despite these advances, many observers remain unconvinced. One sceptic is David Relman, a microbiologist who heads the infectious diseases section at Stanford University. He says that none of Lieske's assays are definitive tests for life, and that their positive results can all be explained by non-living processes.

So what would be definitive proof? "DNA or RNA," says Fredric Coe, a nephrologist at the University of Chicago. "With modern genomics, if you have really got important DNA, you are going to track it down. That's what I think happens when you are onto something real." Coe also points out that the Richmond mine nanoarchaea were quickly sequenced, proving that size doesn't matter when it comes to plucking out a genome. "Those are really small and they yielded immediately to modern science," he says.

In the absence of a genome, the argument over whether nanobacteria are living or non-living will rumble on. Curiously, however, Kajander and Çiftçioglu have washed their hands of the debate – at least for now. They have stopped using the word "nanobacteria" in favour of the more ambiguous term "calcifying nanoparticles" or CNPs.

"We are aware that the name 'nanobacteria' is wrong," concedes Çiftçioglu, now the science director of Nanobac Pharmaceuticals in Tampa, Florida, which she and Kajander set up in 2003. "We don't know if this is final but it seems like they don't have genomic DNA."

Kajander stresses that the new name doesn't mean they have abandoned the idea that the spheres are alive. "I think that we are talking about a new form of primordial life," he says. "It can't be classified with the existing groups of life." Even so, he now considers the debate to be something of a sideshow. The real issue, he says, is whether the nanoparticles have a role in disease.

"I think that we are talking about

To that end, the past few years have seen a series of suggestive findings. Scientists in China, India, Turkey, Germany and the US, some of whom have ties to Nanobac, have found CNPs in gallstones, kidney stones, diseased coronary arteries and certain tumours. There is also some early, though controversial, evidence that pathological calcification can be cured with antibiotics.

In 2003, Kajander was carrying out an early clinical trial involving 13 patients with a range of afflictions including coronary artery disease, rheumatoid arthritis and chronic fatigue. All had tested positive for CNP markers, and the trial aimed to find out whether a drug cocktail based on the antibiotic tetracycline could reduce these markers.

A few of the patients had a condition called chronic pelvic pain syndrome, which is caused by inflammation of the prostate. CPPS is hard to treat but, surprisingly, the drug cocktail seemed to help. This led Kajander to suggest that CPPS was related to calcification of the prostate, caused by nanobacteria. The data has not been published in a peer-review journal, but is included in a 2006 patent application.

He took the idea to urologist Daniel Shoskes, a CPPS specialist at the Cleveland Clinic Florida in Weston. Prostate calcification is often seen in patients with CPPS but is considered irrelevant to the disease, so Shoskes was doubtful. "A lot of older men have calcification," he says. "It's always considered a normal consequence of ageing and doesn't have any role in the symptoms." Even so, Shoskes agreed to test Nanobac's drug. He gave it to 15 CPPS patients who had failed all other therapies and found that after three months, 12 of them had improved significantly (*Journal of Urology*, vol 173, p 474). Shoskes describes the result as "an interesting observation" but says a full-scale clinical trial is needed. Nanobac is moving in this direction: in January the company met the FDA to set the wheels in motion.

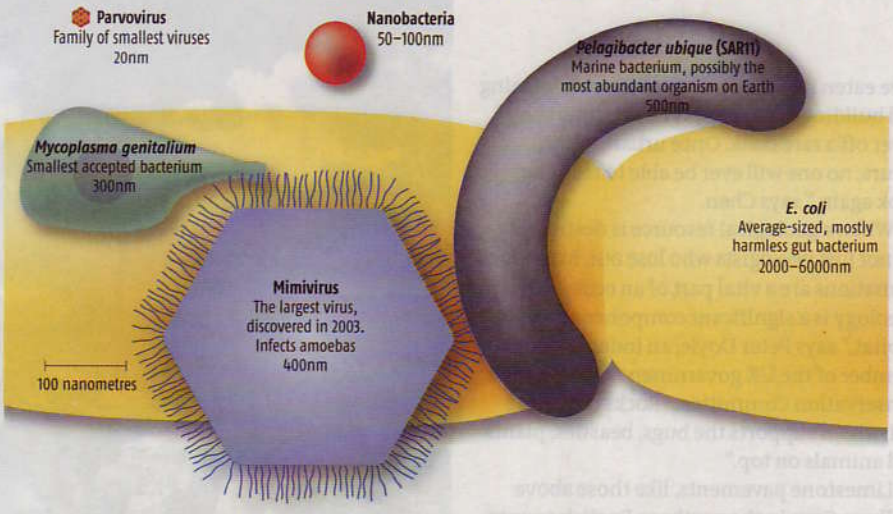
Shoskes, now at the Glickman Urological Institute in Cleveland, Ohio, has since joined Nanobac's medical advisory board and is still using the drug. "It doesn't help everyone but continues to help a lot of people," he says. He is also studying prostate calcification to see whether it is associated with CPPS.

So far so good, but this still doesn't answer a key question: are CNPs involved?

To find out, the Mayo team has embarked on another round of experiments, this time funded by Nanobac. In April, they reported

TOO SMALL TO BE ALIVE?

Nanobacteria, also known as calcifying nanoparticles, may not be big enough to house the machinery an organism needs



SHUTS

a new form of primordial life"

preliminary results at the Experimental Biology 2007 meeting in Washington DC.

Team member Maria Kraemer harvested nanoparticles from human aneurysms and injected them into adult male rabbits whose carotid arteries had been stripped of their cellular lining to simulate the damage seen in early atherosclerosis. After 35 days the rabbits' carotid arteries were badly calcified and had significantly narrowed compared with controls. This is at least preliminary evidence that CNPs contribute to atherosclerosis, says Kraemer. Lieske is also looking for signs of kidney stone formation in those same rabbits.

Research into CNPs has come a long way since 1998. Even arch-critics are now prepared to accept that the spheres exist and can replicate. Even so, supporters have more to do.

One unanswered criticism is that CNPs are so common in human tissue that you could blame them for almost everything. "It would be like saying that oxygen is the cause of disease," says nephrologist David Goldfarb at the New York University Medical Center. In 2004, Goldfarb reviewed the evidence for the role of nanobacteria in kidney stone formation (*Nephron Physiology*, vol 98, p 48). "The ubiquity also allows a sweeping, amazingly broad theory that this is the cause of calcification in every organ," he says.

Cisar, too, is sticking to his guns. Not only is he convinced that CNPs are not alive, he doesn't see any new evidence that they cause disease. He believes that calcification is not the cause of diseases such as atherosclerosis, but an effect of the underlying disease process.

Coe agrees. He studies kidney calcification with transmission electron microscopes and says he has never seen any sign of CNP involvement. "You see gorgeous structures – the more you magnify, the stranger and more beautiful they are – but we know exactly what they are: they are crystallisation," he says.

Cisar is also openly suspicious of Nanobac. "One has to acknowledge that there are commercial interests here," he says. "These people are selling a product." Çiftçioglu retorts that going into the private sector was the only way to continue the research. "When the only funding comes from investors who believe in your science, you take this opportunity," she says. "Who wouldn't?"

Perhaps the greatest barrier to acceptance is a set of rules laid down in the 19th century by German physician Robert Koch. Known as Koch's postulates, they remain the gold standard for proving a link between an infectious agent and a disease. To satisfy Koch's postulates, a putative disease-causing agent must pass four tests. First, the organism must be found in animals suffering from the disease. Then it must be possible to isolate and grow it in the lab. The lab-grown microbe must produce the same disease when inoculated into healthy animals, and finally, the organism must be isolated once more from the sick animal.

When it comes to CNPs, Koch's postulates have not been fully satisfied, though Lieske and Miller are arguably close. "We have identified CNPs in diseased tissues, we can cultivate them from diseased tissue and the recent studies have shown that CNPs localise to diseased arteries and promote disease," says Miller. All that is missing is to recover nanoparticles from the newly infected rabbits.

Also, as Kajander and Çiftçioglu are quick to point out, prions fail to satisfy Koch's postulates despite now being widely accepted as the cause of scrapie, BSE, CJD and several other brain diseases. It took decades for the prion hypothesis to be accepted by mainstream scientific opinion. Perhaps CNPs will be the same.

The view is gradually gaining ground. "This is not a case of the emperor's new clothes," says Goldfarb. "There is something there, but exactly what it is isn't clear to me." ●

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