Report as of FY2006 for 2006ND98B: "Rapid and Sensitive Determination of Bacteria in Water Using Nanoparticles"

Publications

- Conference Proceedings:
  - Jin, Y., X. Zhao, M. Wu, 2006, "Determination of bacteria using luminescent nanoparticles", Pittcon 2006, Orlando, FL.
  - Jin, Y., J. Parisian, M. Wu, X. Zhao, 2007, "Simultaneous Detection of Multiple Bacterial Cells Using Fluorescent Nanoparticles", Pittcon 2007 Chicago.
  - Jin, Y., Jenna Parisien, M. Wu, and Julia Zhao. 2007, "Sensitive Determination of Bacterial Cells Using Fluorescent Nanoparticles". Third International Water Conference, International Water Institute, Grand Forks, March 13-15.

Report Follows

RAPID AND SENSITIVE DETERMINATION OF BACTERIA IN WATER USING NANOPARTICLES

SIGNIFICANCE OF THE PROPOSED RESEARCH TO THE STATE OF NORTH DAKOTA

Sensitive and Rapid Detection of Bacteria in Drinking Water

Bacteria can grow or re-grow in distribution systems of drinking water. In fact, potable water is a major source of some bacteria colonization,[7,8] for example *L. pneumophila* and *E coli.* etc. The *L. pneumophila* in potable water can replicate rapidly and increase in virulence.[9] The British Communicable Disease Surveillance Center reported that 19 of 20 hospital outbreaks of Legionnaires' Disease in the United Kingdom from 1980 to 1992 were attributed to the *Legionella*-contaminated potable water.[10] Given the low infectious dose of pathogenic bacteria, the presence of even a single bacterium in potable water may pose a serious health risk. Therefore, sensitive and rapid detection of bacteria in water is critical.

However, the current definitive method for the detection of bacteria is the culture of the organism, which requires about 24 hours for bacterial growth.[11] The method is too slow to meet the public need. The PCR-based method can detect bacteria within six hours; but the method requires pre-enrichment of the target bacteria. The proposed method will be able to specifically identify target pathogenic bacteria at a single bacterium level within 30 minutes in water samples. The method will be accurate, rapid and sensitive to meet the public need.

Application of Nanotechnology in North Dakota

Nanotechnology has been a rapidly developing area in recent years and is becoming a hot topic worldwide. However, it is an area that is underdeveloped in North Dakota. In the University of North Dakota, Dr. Zhao is the first faculty member working in the nanotechnology area. Dr. Zhao's group is the only nanotechnology research group at UND. There is a great need to develop emerging nanotechnology in North Dakota to advance the economic and educational development of the state. Four students in Dr. Zhao's group will participate in the proposed project: a postdoctoral research associate, a Ph.D. student, an American Indian undergraduate student, and a high school student. The proposal will undoubtedly benefit the development of North Dakota, both in terms of economy and education.

BACKGROUND OF THE APPLICANT'S RESEARCH GROUP

The proposed work will build upon Dr. Julia Zhao's previous successes in synthesizing fluorescent nanoparticles and identifying bacteria. In these fields, Dr. Zhao holds three patents, has authored three book chapters, 25 conference presentations and 41 journal articles. Her previous work on bacteria determination using nanotechnology has been published in ***Proc. Natl. Acad. Sci. USA (PNAS).***[12] This paper was among the top 100 articles accessed in 2004 in ***PNAS***. This work was also featured in a ***Nature*** research highlight section on October 2004.[12] So far, Dr. Zhao has developed several methods for the synthesis of different types of silica nanoparticles.[13-

[19] in 2005, Dr. Zhao received four research grants for development of functionalized silica nanoparticles. One of these grants, the Society of Analytical Chemists of Pittsburgh Start Award, is highly competitive and is only offered to **one national awardee each year**. Currently, Dr. Zhao's group has four Ph.D. students, one postdoctoral research associate, two undergraduate students, and one high school student working on five research projects in bionanotechnology.

The co-advisor, Dr. Min Wu in the Department of Biochemistry and Molecular Biology in the School of Medicine and Health Sciences at UND, has abundant experience in the field of bacteriology. His earlier significant work on genetically engineered macrophages as cell therapy to increase immune function against bacteria was published in ***Proc. Natl. Acad. Sci. USA*** [20] and received media coverage including ***Nat. Rev. Immun.***[20] He has also worked on cell signaling and host-pathogen interaction with bacterial infection (*Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) for many years.[21-23]. Dr. Wu has also worked on mechanisms of DNA damage/repair and cell death in lung cells, indicated by his significant work published in high impact journals such as ***Cell Death and Differentiation, Breast Cancer Research*** etc.[24,25] Dr. Wu's recent work on new chemical conjugates containing omega-3 fatty acid for cancer therapy has attracted a broad media attention including BBC and Fox59 Channel (June 2005).

Dr. Zhao and Dr. Wu have have successfully co-advised the applicant on a project of the study of toxicity of the luminescent nanomaterials to living systems; [2,5,6] In this project, Dr. Zhao will advise the applicant on the development of nanoparticles and the techniques of conjugation of the antibodies to the nanoparticles. Dr. Wu will advise the applicant on bacteria culture and determination. So far, they have obtained some promising results.

PROJECT DESCRIPTION AND OBJECTIVES

The objective of this proposal is to develop a rapid and ultrasensitive method for the specific identification and quantitative determination of pathogenic bacteria in water. The major feature of the proposed method is the employment of fluorescent nanoparticles as target bacteria identifiers that could emit strong fluorescent signals. The method will consist of three major steps. First, the nanoparticles will react with a specific antibody to form a conjugate of nanoparticle-antibody (NP-Ab). Second, based on the antibody-antigen reaction, the NP-Ab conjugates will identify target bacteria cells from a sample by attaching the NP-Ab to the bacteria surface antigen. Third, target bacteria will be qualitatively and quantitatively determined by measuring the fluorescence intensity and wavelength (Figure 1).

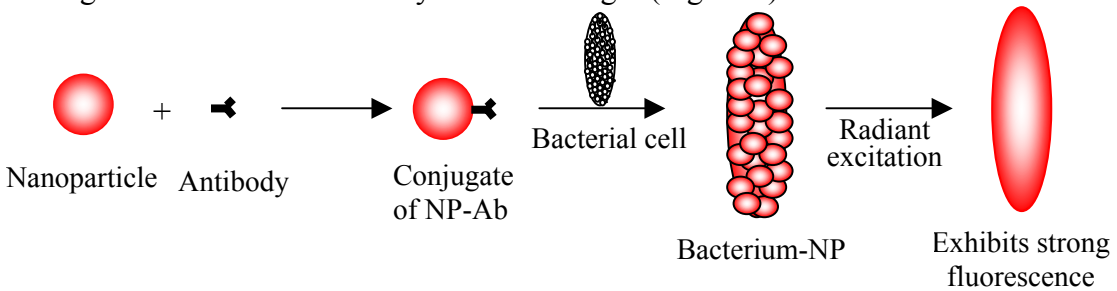

Figure 1 Schematic diagram of determination of bacteria using fluorescent nanoparticles

The method will have three major advantages.

(A) High sensitivity. The nanoparticles will be synthesized using organic dye molecules. Thousands of dye molecules will be encapsulated within a single nanoparticle. Traditional dye labeling method for the determination of bacteria only links one dye molecule to an antibody, which reacts with a single antigen. The proposed method links one nanoparticle, rather than one dye molecule, to each antibody. The advantage is that a single nanoparticle contains thousands of dye molecules, resulting in a highly amplified fluorescent signal. Furthermore, because a bacterial surface contains a number of antigens, a single bacteria cell can be linked with multiple highly fluorescent nanoparticles, making the determination of single bacteria possible.

(B) High specificity and accuracy. The high specificity and accuracy will come from two factors. (1) The identification of the target bacteria will be based on the antibody-antigen reaction. The selected antibody will only specifically recognize a target bacterial antigen. (2) Due to the strong fluorescent signals provided by nanoparticles, the signal difference between target and non-target cells is significant even in the presence of only a single target bacterium. Thus, the false positive reading will be reduced dramatically.

(C) Rapid determination. The size of the nanoparticles is adjustable in the range of 10 nm to 60 nm in diameter. The small size makes nanoparticles highly mobile, which enables them to easily reach target bacteria in a matrix. According to our previous study, the assay will take less than 30 minutes to complete the sample preparation and the determination of bacteria. Compared to the traditional bacteria detection method, plate counting, which takes about 24 hours, the proposed assay will significantly reduce the bacteria determination time.

This proposal will focus on following two specific goals.

Goal 1: Development of Quantum Dot-like Highly Fluorescent Nanoparticles

Two quantum dot-like fluorescent nanoparticles have been developed in Dr. Zhao's group (see preliminary results below). In this proposal, more such nanoparticles will be developed. The nanoparticles will emit fluorescent signals at different wavelengths when a single excitation is used. In this aspect, the nanoparticles will have a similar property as quantum dots. On the other hand, the nanoparticles will contain a large number of dye molecules. Therefore, the fluorescence intensities will be much stronger than those of quantum dots. The principle of the synthesis of the nanoparticles will be based on the fluorescence resonance energy transfer.

Goal 2: Specific, Rapid and Sensitive Identification of Target Bacteria

The proposal will focus on the method development. Targets can be any interested bacteria. Since the specific identification of target bacteria is based on antibody-antigen reaction, as long as a specific antibody to target bacteria is available, the bacteria would be identified. Primarily, *Legionella pneumophila*, *E coli.,* and *Klepsiella pneumoniae* will be our target bacteria. The reason of selecting *Legionella pneumophila* (*L. pneumophila*) as one of the targets is that *L. pneumophila* can grow easily in potable water. Meanwhile, it is among the top three microbial causes of community-acquired pneumonia. The research has demonstrated that *L. pneumophila* causes more severe disease than most common bacterial pathogens associated with community-acquired pneumonia. However, accurate diagnosis of the disease has been a significant challenge since sensitive determination of *L. pneumophila* is difficult. The principle discovered in this study could be extended to the rapid and sensitive determination of various bacteria.

RESEARCH PLAN, EXPECTED RESULTS AND FACILITIES

Development of Quantum Dot-like Fluorescent Nanoparticles

Quantum dot (QD) is a novel nanomaterial that has attracted researchers' great interest due to its unique advantage, which is simultaneously giving different emission wavelengths based on varying sizes when a single excitation source is used.[26] However, quantum dots are restricted by their low quantum yield that results in limited signals when they are used as luminescent labeling materials. Meanwhile, most of QDs are toxic to living systems which limits their applications in biological and medical fields. The designed nanoparticles will possess the advantage of quantum dots and overcome their disadvantages. Due to very high quantum yields of the organic dye molecules used, the nanoparticles will emit much stronger fluorescence signals than QDs. Meanwhile, the silica based fluorescent nanoparticles are not toxic to living systems according to current research results.[2] This nanoparticle is expected to be of great interest as a novel labeling nanomaterial for simultaneous analysis of multiple biomolecules, especially for labeling cells.

Principle of the method: The key idea of this design is to use energy transfer of fluorescence molecules to produce various dye-doped nanoparticles with different colors using a single excitation source. For instance, as shown in Figure 2, the emission wavelength of fluorescence molecule A is near the absorbance wavelength of fluorescence molecule B. As molecule A's excitation light is applied to a solution containing molecule A only, molecule A will emit at 514 nm. Subsequently, molecule B is added to the molecule A solution and thus form a mixture of A and B (with a proper ratio of A to B), the emission energy of molecule A is absorbed by molecule B. So, the emission of molecule B is observed and molecule A is quenched.

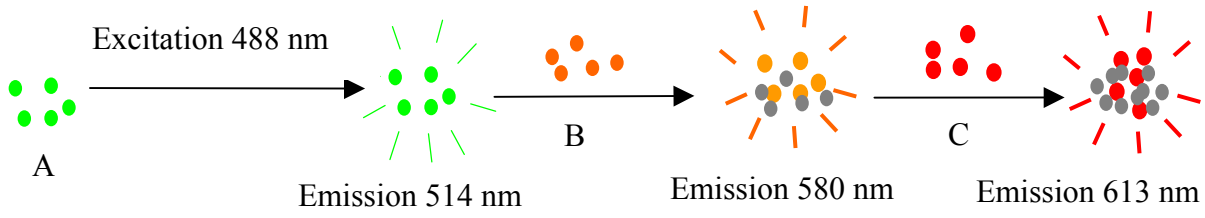

Figure 2 Multiple fluorescence signals using a single excitation source based on fluorescence resonance energy transfer

Furthermore, molecule C is added to the mixture of A and B, the emission energy of molecule B will be absorbed by molecule C. Then, the mixture will emit C's spectrum and A and B are all quenched. Therefore, different mixtures will simultaneously emit different colors using a single excitation source. By doping a mixture of dyes into a silica matrix, quantum dots-like nanoparticles will be produced.

Synthesis approach: The reverse microemulsion method will be used for synthesis of dye-doped silica nanoparticles. Silica matrix can be formed by polymerizing tetraethylorthosilicate in the microemulsion. Several dye molecules will be chosen, such as fluorescein, tetramethylrhodamine, and Alexa Fluor dyes. The desgined nanoparticles are expected to emit strong fluorescent signals at different wavelengths.

SPECIFIC AND RAPID IDENTIFICATION OF TARGET BACTERIA

Conjugation of the antibody onto nanoparticles. The research plan will be described using *L. pneumophila.* The other target bacteria will be determined using the same method. Currently**,** more than 14 serogroups of *L. pneumophila* have been identified. Among them, serogroup 1 accounts for more than 80 percent of the reported cases of legionellosis caused by *L. pneumophila*. One phenotypic difference between avirulent and virulent *L. pneumophila* is the presence of flagella; isogeneic avirulent strains obtained by passage lose their flagella. A surface antigen of *L. pneumophila* serogroup 1 that is recognized by one particular monoclonal antibody is associated with virulence. The monoclonal antibody is commercially available. The antibody will be immobilized onto the nanoparticle surface. The conjugated conditions will be optimized, including ratio of the nanoparticle to antibody, reaction temperature, reaction time, and pH.

Specific identification of *L. pneumophila* from a mixture of bacteria. The NP-Ab conjugates will be used to identify target bacteria from a mixture of bacteria. Based on a specific antibody-antigen reaction, the conjugates will only react with the antigen of the serogroup 1 of *L. pneumophila*. Thus, the target bacteria will be labeled with certain colored nanoparticles. A slightly physical adsorption of NP-Ab to the non-target cells may occur. The effective washing using PBS buffer will minimize this problem.

Quantitative determination of target bacteria. To accurately determine the amount of target bacteria, three types of instruments to measure fluorescence will be used. The first one is a spectrofluorometer to determine the fluorescence intensity of bacterial cells in solution. The fluorescence intensity will be proportional to the amount of target bacteria in the solution. The fluorescence intensities of unknown samples will be measured, and consequently, the amounts of target bacteria will be determined based on the calibration curve. The second instrument is a plate reader fluorometer. The instrument can perform high throughput determination of multiple samples. Currently, 384-well plate and 96-well plate can be used in our plate reader fluorometer. Thus, up to 384 samples can be analyzed simultaneously. The third type of instrument will be fluorescence microscopes. Each bacterial cell will show a fluorescence spot on the image. By counting fluorescence spots on the image, the number of the target bacteria can be obtained.

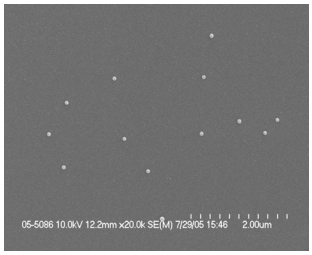

Figure 3 SEM image of the nanoparticles

Facilities. Olympus Fluorescence Microscope, JY HOBRIBA Fluorolog-3 Spectrofluorometer, Fluorescence Microplate Reader. Siemens P-4 single crystal X-ray diffractometer, FT-IR spectrometers, ICP emission spectrometers. Scanning Electron Microscope, Transmission Electron Microscope, Confocal Fluorescence Microscope. EACI horizontal/vertical laminar flow hood, NuAir CO_2 incubator, and IEC clinical centrifuge required for performing tissue culture.

PRELIMINARY RESULTS

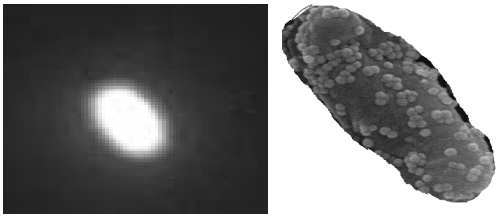

Figure 4 Fluorescence image and SEM image of a *Salmonella* cell conjugated with fluorescent nanoparticles

Synthesis of Multiple Colored Fluorescent Nanoparticles. Most recently, several multiple colored fluorescent silica nanoparticles have been developed in Dr. Zhao's group. These nanoparticles emit fluorescence at different wavelengths with high intensities. The fluorescence intensity of one nanoparticle is about 10,000 times higher than one dye molecule. The size of the nanoparticles is very uniform with a diameter of 50 nm (Figure 3, scanning electron microscopy (SEM) image).

Immobilization of Antibodies onto the Nanoparticle Surface. The specific antibodies were immobilized onto the nanoparticle surface for identification of target cells. The immobilization of antibodies was based on the reaction of amine groups on the antibody with carboxyl groups on the nanoparticles. Before the immobilization, the surfaces of the nanoparticles were activated using 5 mL of 100 mg/mL of EDC (1-ethyl-3-3(3-dimethylamino-propyl) carbodimide hydrochloride) and 5 mL of 100 mg/mL of NHS (N-hydroxy-succinimide) in an MES (morpholineethane-sulfonic acid) buffer (pH 6.8), for 25 minutes at room temperature with continuous stirring. Water-washed nanoparticles were dispersed in 10 mL of 0.1 M PBS (pH 7.3). To covalently immobilize monoclonal antibodies onto the nanoparticle surface, 5 mL of 0.1 mg/mL nanoparticles were reacted with 2 mL of 5 μg/mL antibody for 2-4 hours at room temperature with continuous stirring, followed by washing with a PBS buffer. Dr. Zhao has previously detected *Salmonella* using the nanoparticles (Figure 4).

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