NANO LETTERS

2007
Vol. 7, No. 2
380−383

One-Step, Nanoparticle-Mediated Bacterial Detection with Magnetic Relaxation

Charalambos Kaittanis,[†,‡] Saleh A. Naser,[‡] and J. Manuel Perez*[,†,‡,§]

Nanoscience Technology Center, Burnett College of Biomedical Sciences, and Department of Chemistry, University of Central Florida, 12424 Research Parkway, Suite 400, Orlando, Florida 32826

Received October 31, 2006; Revised Manuscript Received December 6, 2006

ABSTRACT

Acknowledging the need for the development of fast and sensitive bacterial detection methods, we functionalized superparamagnetic iron oxide nanoparticles to identify *Mycobacterium avium* spp. *paratuberculosis* (MAP), through magnetic relaxation. Our results indicate that the MAP nanoprobes (1) bind specifically to MAP, (2) can quantify the bacterial target quickly in milk and blood with high sensitivity, and (3) are not susceptible to interferences caused by other bacteria. The use of the described magnetic nanosensors in the identification and quantification of bacteria in clinical and environmental samples is anticipated.

Bacterial infections are the fifth cause of mortality in the US, and their economic impact on the industrial, agricultural, and healthcare sector is significant.[1] Headlines in the press about the presence of enterrohemorrhagic *E. coli* O157:H7 in tainted produce, the identification of the extremely cytotoxic Subtilase AB_5 toxin in *E. coli* O113:H21, the aftermath of the *B. anthracis* attacks in 2001, and the recent identification of drug resistant *Mycobacterium tuberculosis* [XDR-TB] in Africa sparked public concern. Therefore, the need for developing fast, sensitive, and reliable detection methods for bacteria in food, the environment, and clinical samples is crucial in order to safeguard public health. Traditionally, the presence of a bacterium is detected microscopically, usually after growth in culture. Confirmation is based on growth patterns in various media and biochemical tests. These methods, although highly specific, have some drawbacks. First, they usually take more than 24 h and lack sensitivity due to the amount of bacteria needed, whereas not all microorganisms can grow in culture. More recently, other methods involving polymerase chain reaction (PCR) and fluorescent immunoassays have been developed. However, despite their enhanced sensitivity and specificity, these methods are costly, time-consuming, and need to be performed by trained and experienced personnel.

Nanotechnology offers an attractive alternative for the identification of molecular targets in vitro and in vivo, requiring smaller samples and less preparation time, without compromising sensitivity.[2−5] Recently, superparamagnetic iron oxide nanosensors have been designed to quantify biomolecular targets in cell lysates and tissue extracts, demonstrating high sensitivity.[6−7] The principle underlying the detection mechanism of these nanosensors is based on their ability to switch between a disperse and clustered (or assembled) state upon target interaction, with a concomitant change in the spin−spin relaxation time (T2) of the solution's water protons. On the basis of changes in T2 (ΔT2) various molecular targets such as nucleic acids (DNA and mRNA), proteins and even viruses have been detected. These targets have in common the fact that they are smaller (e.g., DNA and proteins) or of equal size (e.g., virus) to the nanoparticles. However, the use of superparamagnetic nanoparticles for the detection of larger biological targets, such as a bacterium in solution, has not been examined before. We hypothesized that the presence of multiple bacterial epitopes should compensate for the size difference between the probe and the target, promoting nanoparticle assembly on the bacterial surface and consequently resulting in a dose-dependent change in ΔT2 (Figure 1). It was also hypothesized that the lower the number of bacteria in the solution, the higher the ratio of nanoparticles available to bind and self-assemble on the surface of the bacterium, resulting in higher values of ΔT2 (Figure 1). As the number of bacteria in solution increases, it was expected that the resulting higher number of bacterial epitopes will compete with the available number of nanoparticles in solution, therefore causing the nanoparticles to bind to the bacteria in a more disperse-like state

* Correspoonding author. Ph: 407-882-2843. Fax: 407-882-2819. E-mail: jmperez@mail.ucf.edu.
† Nanoscience Technology Center.
‡ Burnett College of Biomedical Sciences.
§ Department of Chemistry.

10.1021/nl062553z CCC: $37.00
Published on Web 12/29/2006

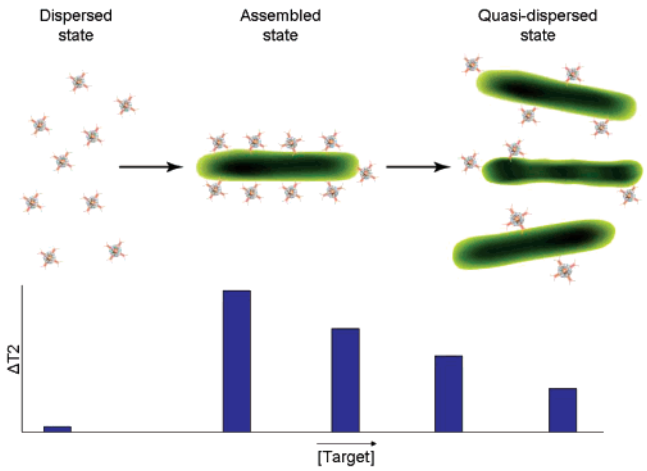

Figure 1. Proposed detection mechanism of bacterial targets using superparamagnetic nanoparticles. Disperse nanoparticles in solution self-assemble on the surface of a bacterial target inducing large changes in T2. As the number of bacteria increases, the available number of nanoparticles in solution bind in a more disperse-like state, causing minimal changes in T2.

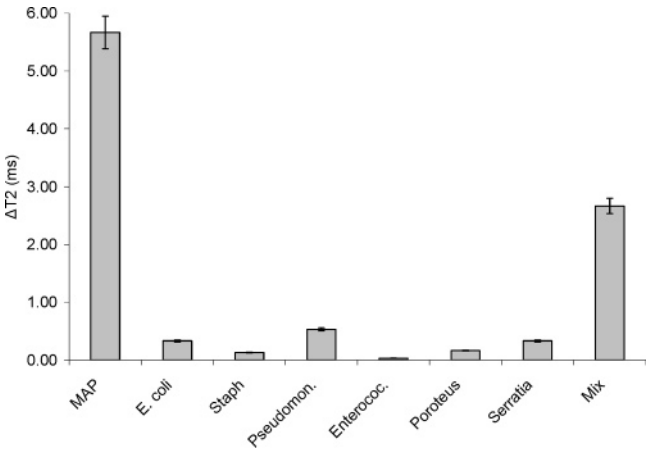

Figure 2. Specificity of MAP nanosensors. Detection of MAP (*Mycobacterium avium* spp *paratuberculosis*), and other bacteria, *E. coli* (*Escherichia coli*), Staph (*Staphylococcus aureus*), Pseudomon (*Pseudomonas aeruginosa*), Enterococ (*Enterococcus faecalis*), Proteus (*Proteus vulgaris*), and Serratia (*Serratia marcensis*) using MAP nanosensors. Detection of MAP in the presence of the other bacteria (Mix) was also achieved. Mix (MAP in the presence of the other six non-MAP species 50:50 v/v).

(Figure 1). Such a nanoparticle-based bacteria detection system would be highly sensitive, particularly at low target concentrations.

To prove our hypothesis, we used *Mycobacterum avium* spp. *paratuberculosis* (MAP), as our model organism. We selected this bacterium because its growth in culture is difficult and its identification with current methods is not easy.[8−10] In addition, MAP is the known pathogen causing Johne's disease in cattle, an economically devastating disease.[11−13] MAP-infected animals show severe intestinal inflammation, which can be degenerative, and the infection of healthy animals is high, resulting in herd's annihilation to prevent further transmission.[11−13] Apart from causing disease in cattle, there is association between MAP and Crohn's disease in humans, as the microorganism has been isolated from the blood, breast milk, and intestinal lesions of Crohn's disease patients.[8−9,14−15] Here, we report the development of MAP-specific nanosensors that can detect the bacterial target (MAP) in complex media (whole milk and blood) with high specificity and sensitivity.

The MAP nanosensors used in this study were prepared by conjugating anti-MAP antibodies[9] to superparamagnetic iron oxide nanoparticles via Protein G, as describe before.[7] These antibody-coated nanosensors were stable for months, after storage at 4 °C, with an average diameter of 70 ± 0.4 nm determined by dynamic light scattering and a transverse magnetic relaxivity (R2) of 320 s^{-1} mM^{-1} measured at 0.47 T. Preliminary studies in phosphate-buffered saline demonstrated that the nanosensors responded in a dose-dependent fashion upon addition of MAP. Formation of the bacterial-induced nanoassembly was detected upon addition of the bacteria, and this assembly was stable after a 3 h long incubation. For the first set of experiments in complex media, we compared the specificity of our nanosensors toward MAP, by adding spiked whole milk into 200 μL of MAP nanosensors (2 μg Fe/μL). Experimentally, we have found that a nanoparticle solution at a concentration of 2 μg Fe/μL works best, since lower concentrations increase the error in the measurement, while higher concentrations sacrifice on detection threshold. The sample that had MAP alone (115 Colony Forming Units [CFUs] in 10 μL) had the highest change in T2, while the samples that had other bacteria ($\sim 10^6$ CFUs in 10 μL) demonstrated minimal T2 changes (Figure 2). More importantly, the sample containing a mixture of bacteria, including MAP (77.5 CFUs in 10 μL), was identified as MAP-positive, despite the presence of interference, underlying the specificity of our nanosensors.

In addition, to verify if the observed changes in T2 were due to antibody-mediated interactions and not to nonspecific nanoparticle aggregation, the bacteria were preincubated overnight with anti-MAP antibody before performing sensing experiments with the MAP nanosensors. Results showed that the ΔT2 was abrogated in the bacterial sample preincubated with free MAP antibody (ΔT2 = 0.20 ± 0.1 ms), due to the inability of the MAP nanosensors to bind the bacterial epitopes. In contrast, a control bacterial sample that has not been preincubated with MAP antibody had significant T2 changes (ΔT2 = 8.90 ± 0.3 ms) at the same experimental conditions. Furthermore, the T2 of the MAP nanosensors (2 μg of Fe/μL) was measured at 25, 30, and 35 °C, without any temperature-dependent statistical differences. This implied that the nanoparticles' performance was steady in temperatures relevant to those at the points-of-care and that the observed changes in T2 were due to specific antibody-mediated interactions with the MAP bacterium

Detection and quantification of MAP in milk was done by incubating MAP-spiked whole milk with MAP nanosensors (2 μg of Fe/μL). We found that the change in T2 was indirectly proportional to the MAP concentration, supporting our proposed detection model. Reliable quantification of MAP from 15.5 to 775 CFUs ($R^2 = 0.93$) was achieved after a 30-min incubation at room temperature (Figure 3). In control experiments using nanoparticles with no MAP

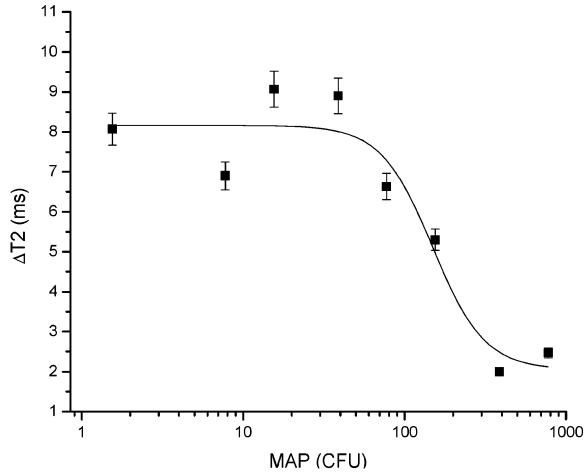

Figure 3. Concentration-dependent behavior of MAP-specific nanosensors in whole milk, at room temperature (25 °C).

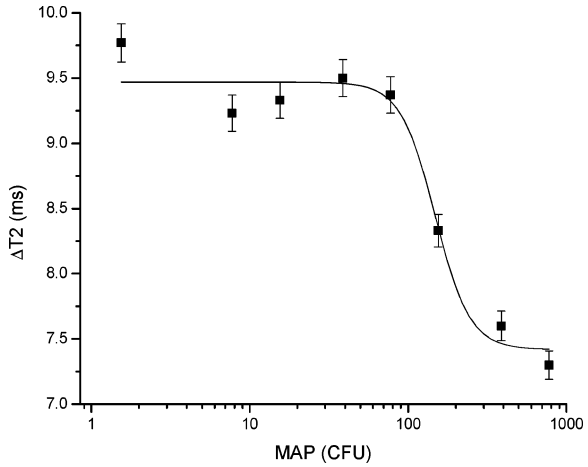

Figure 4. Quantification of MAP in whole milk using MAP-specific nanosensors, under interference conditions created by the presence of six different bacterial species.

antibody, minimal changes in T2 were observed. It is interesting to note that MAP could be identified in milk at low CFUs, even though it could not be quantified. This offers the ability to assess if a sample is MAP-positive or MAP-negative at low concentrations (<15.5 CFUs). Using the same conditions and setup, MAP was quantified with good correlation in milk at 37 °C. Notably, the described trend between ΔT2 and MAP's concentration was sustained, even after an hour-long incubation, regardless of the incubation temperature. Detection and quantification of MAP were achieved with high sensitivity in 2% milk, as well, after incubation at 37 °C.

We next investigated if the presence of other bacteria would interfere with actual quantification of MAP. In these experiments, we quantified MAP in whole milk and in the presence of six different bacterial species, similar to our previous study (Figure 2). After a 30-min incubation at 37 °C, we were able to determine if a particular sample had the bacteria (MAP positive) and even determine the actual MAP concentration, having 15.5 CFUs as the assay's detection limit under these heavy interference conditions ($R^2 = 0.86$). However, when the incubation was followed by a 45-min cool down to room temperature, the correlation improved ($R^2 = 0.96$), due to the stronger binding and hybridization of the bacterium−nanoparticle system, yet the detection limit increased to 38.75 CFUs (Figure 4).

Since MAP has been found in the blood of Crohn's disease patients[8] and bacteremia (presence of bacteria in the blood) is a critical pathological state that requires fast detection and prompt medical attention, we performed experiments in blood samples. In these experiments, we added 20 μL of blood containing increasing amount of bacteria to 200 μL of the nanosensors (2 μg of Fe/μL). Our MAP nanosensors were able to detect and quantify MAP in a concentration-dependent trend, in accordance to our hypothesis (Figure 5). The assay was sensitive enough to detect MAP even after a couple of minutes (Figure 5A) and after a 30-min incubation at 37 °C (Figure 5B), making the assay relevant to clinical sample conditions. Furthermore, blood from a healthy human subject was screened for MAP using our nanoprobes. The sample's T2 was identical to that of the negative control (rat blood with no MAP), in line with the proposed model of detection, suggesting that the individual did not have MAP bacteremia or MAP epitopes in circulation that were recognized by our nanoprobes (Table 1).

In summary, we have shown that superparamagnetic iron oxide nanoparticles can be used for the detection of bacteria in complex media, such as milk and blood, through magnetic

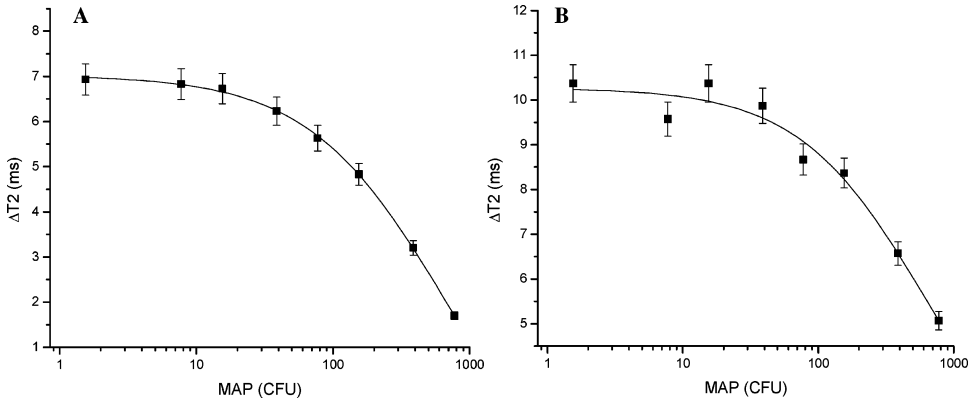

Figure 5. Quantification of MAP in rat blood using MAP-specific nanosensors: (A) upon sample preparation; (B) after a 30-min incubation at 37 °C.

Table 1. Absence of MAP Bacteremia or MAP Epitopes in the Blood of a Healthy Individual

	T2 (ms)	
	control	healthy individual
upon addition	80.67 ± 0.89	80.60 ± 0.56
30-min incubation	83.10 ± 0.78	82.10 ± 0.61

relaxation. This approach, apart from sensitive and fast, is independent of the sample's optical properties, requires minimum sample preparation, and can be used at the point-of-care. Furthermore, these nanoprobes can be easily adapted for the high throughput and expedite screening of samples from animals, humans, and food. Considering the latest advancements in NMR technology, with compact and sensitive instrumentation, the high-throughput and/or portable analysis of multiple samples in complex media should be doable, even outside of the typical laboratory setting.[16] This method provides a novel approach for microbial detection that can potentially expedite decision making in a broad range of fields including the clinical, environmental, and agricultural sectors. Additional studies are in progress to determine bacterial agents in other media and clinical samples.

Acknowledgment. The authors thank Dr. George Ghobrial for growing MAP, Alisa Tinkham for providing the non-mycobacterial organisms, and Marguerite Beckler for help in designing Figure 1. This work was supported in part by NIH grant CA101781 to J.M.P.

Supporting Information Available: Synthesis, optimization, assay pictures, sample preparation, and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Salyers, A. A.; Whitt, D. D. *Bacterial pathogenesis: a molecular approach*, 2nd ed.; ASM Press: Washington, DC, 2002; pp 3−16.
(2) Rosi, N. L.; Mirkin, C. A. *Chem. Rev.* **2005**, *105* (4), 1547−1562.
(3) Alivisatos, A. P.; Gu, W.; Larabell, C. *Annu. Rev. Biomed. Eng.* **2005**, *7*, 55−76.
(4) Bruchez, M., Jr.; Moronne, M.; Gin, P.; Weiss, S.; Alivisatos, A. P. *Science* **1998**, *281* (5385), 2013−6.
(5) Ferrari, M. *Nat. Rev. Cancer* **2005**, *5* (3), 161−71.
(6) Perez, J. M.; Josephson, L.; O'Loughlin, T.; Högemann, D.; Weissleder, R. *Nat. Biotechnol.* **2002**, *20* (8), 816−20.
(7) Perez, J. M.; Simeone, F. J.; Saeki, Y.; Josephson, L.; Weissleder, R. *J. Am. Chem. Soc.* **2003**, *125* (34), 10192−10193.
(8) Naser, S. A.; Ghobrial, G.; Romero, C.; Valentine, J. F. *Lancet* **2004**, *364* (9439), 1039−1044.
(9) Naser, S. A.; Shafran, I.; Schwartz, D.; El-Zaatari, F.; Biggerstaff, J. *Mol. Cell Probes* **2002**, *16* (1), 41−48.
(10) Vary, P. H.; Andersen, P. R.; Green, E.; Herman-Taylor, J.; McFadden, J. J. *J. Clin. Microbiol.* **1990**, *28* (5), 933−937.
(11) Cocito, C.; Gilot, P.; Coene, M.; De Kesel, M.; Poupart, P.; Vannuffel, P. *Clin. Microbiol. Rev.* **1994**, *7* (3), 328−345.
(12) Manning, E. J.; Collins, M. T. *Rev. Sci. Technol.* **2001**, *20* (1), 133−150.
(13) Shin, S. J.; Wu, C. W.; Steinberg, H.; Talaat, A. M. *Infect. Immun.* **2006**, *74* (7), 3825−3833.
(14) Hermon-Taylor, J.; Bull, T. J.; Sheridan, J. M.; Cheng, J.; Stellakis, M. L.; Sumar, N. *Can. J. Gastroenterol.* **2000**, *14* (6), 521−539.
(15) Naser, S. A.; Schwartz, D.; Shafran, I. *Am. J. Gastroenterol.* **2000**, *95* (4), 1094−1095.
(16) Perlo, J.; Demas, V.; Casanova, F.; Meriles, C. A.; Reimer, J.; Pines, A.; Blümich, B. *Science* **2005**, *308* (5726), 1279.

NL062553Z