ACS APPLIED MATERIALS & INTERFACES

Research Article

www.acsami.org

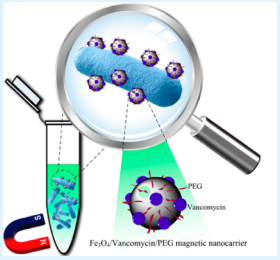
Construction of Fe_3O_4/Vancomycin/PEG Magnetic Nanocarrier for Highly Efficient Pathogen Enrichment and Gene Sensing

Minjun Zhu, Weipeng Liu, Hongxing Liu, Yuhui Liao, Jitao Wei, Xiaoming Zhou,* and Da Xing*

MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, College of Biophotonics, South China Normal University, Guangzhou 510631, China

Supporting Information

ABSTRACT: Infectious diseases, especially pathogenic bacterial infections, pose a growing threat to public health worldwide. As pathogenic bacteria usually exist in complex experimental matrixes at very low concentrations, developing a technology for rapid and biocompatible sample enrichment is essential for sensitive diagnosis. In this study, an Fe_3O_4/Vancomycin/PEG magnetic nanocarrier was constructed for efficient sample enrichment and in situ nucleic acid preparation of pathogenic bacteria for subsequent gene sensing. We attached Vancomycin, a well-known broad-spectrum antibiotic, to the surface of Fe_3O_4 nanoparticles as a universal molecular probe to target bacterial cells. Polyethylene glycol (PEG) was introduced to enhance the nanocarrier's water solubility and biocompatibility. Results show that the proposed nanocarrier achieved a 90% capture efficiency even if at a *Listeria monocytogenes* concentration of 1×10^2 cfu/mL. Contributing to the good water solubility achieved by the employment of modified PEG, highly efficient enrichment (enrichment factor 10 times higher than PEG-free nanocarrier) can be completed in 30 min. Moreover, PEG would also develop the nanoparticles' biocompatibility by passivating the positively charged unreacted amines on the magnetic nanoparticles, thus helping to release the negatively charged bacterial genome from the nanocarrier/bacteria complexes when an in situ nucleic acids extraction step was executed. The outstanding bacterial capture capability and biocompatibility of this nanocarrier enabled the implementation of a highly sensitive gene-sensing strategy of pathogens. By employing an electrochemiluminescence-based gene-sensing assay, *L. monocytogenes* can be rapidly detected with a limit of detection of 10 cfu/mL, which shows great potential for clinical applications.



KEYWORDS: *pathogenic bacterial infections, Fe_3O_4/Vancomycin/PEG magnetic nanocarrier, sample enrichment, gene sensing, electrochemiluminescence*

INTRODUCTION

Infectious diseases, especially pathogenic bacterial infections, pose a great threat to public health worldwide.[1,2] According to statistics from the World Health Organization, 25% of the global deaths are caused by infectious diseases, which accounts for more than 13 million lives annually. Pathogenic bacteria are the main reason for the wide spread of these infectious diseases.[3] Bacteria at low concentrations are hard to detect; however, the low infectious dose of bacterial pathogens in water, food, and other biological samples not only pose a widespread threat to human health but also cause serious damage.[4] Moreover, it is well-known that the majority of bacteria can double their population in 30 min.[5] Thus, rapid and sensitive detection of pathogenic bacteria to avoid and minimize the contamination of environment, food, and bacterial infections are strongly demanded.

A large focus has therefore been on the detection of such pathogenic bacteria. The most commonly used methods are culture-based assays. These assays are often composed of a series of steps including selective cultural enrichment, differential plating, and biochemical/serological testing.[6] However, these steps are all time-consuming and labor-intensive, and the specificity and sensitivity of biochemical/serological testing are also unsatisfactory. Polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) are popular methods nowadays. They are also widely used in bacterial detection.[7,8] PCR is frequently used for the amplification of nucleic acids (DNA or RNA) from samples containing small quantities, and ELISA always uses antibodies for bacterial capture on a surface followed by their subsequent detection.

Compared to antibody-based biomarkers, nucleic acids are thought to be more suitable for specific and senstive bacterial detection because of their predictable molecular behaviors and perfect compatibility with enzymatic target amplification methods.[9−11] These nucleic acid-based amplification methods could provide 1 million-fold enrichment of a single specific nucleic acid sequence in less than an hour with a extreme low detection limit of only one bacterial cell.[12] On the basis of these properties, specific nucleic acid sequence enrichments have the potential to replace the cultural enrichment with higher detection specificity and shorter detection time.

Despite these advantages, the applications of nucleic acid amplification for pathogenic bacterial detection in complex food and experimental matrixes remain in developmental stages. Several factors restrict the widespread use of PCR in complex samples: (1) large sample volumes (≥10 mL or gm),

Received: March 18, 2015
Accepted: May 25, 2015
Published: May 25, 2015

DOI: 10.1021/acsami.5b02374
ACS Appl. Mater. Interfaces 2015, 7, 12873−12881

which is far beyond the ability of PCR (10−50 μL), (2) enzymatic reaction inhibitors in residual food components, and (3) low levels of contaminating pathogenic bacterial.[13] By and large, the tedious culture-based enrichment is still necessary for providing sufficient amplification of bacteria before the nucleic acid amplification steps.[14] It has been suggested that the sensitivity of many rapid detection technologies could be developed if the bacteria were enriched from the complex sample matrixes effectively before detection.[15]

Centrifugation, filtration, and magnetic separation are commonly used for rapid bacterial enrichment. Compared to nonspecific centrifugation and filtration, specific probe-modified magnetic nanomatarials is quite suitable for bacterial enrichment.[16,17] Magnetic nanomatarials are famous for thier unique properties: convenient manipulation by a magnet, fast kinetics in solution, and high surface-to-volume ratios; thus, magnetic manipulation assays have been widely used in rapid, efficient, and specific capture and enrichment of target bacteria from the complex samples.[18,19] Biomolecules, such as antibody, carbohydrate, antibiotics, and small organic molecules, have been modified on the surface of magnetic nanoparticles (MNPs) for bacterial labeling and enrichment.[20−25] Antibiotic is a kind of natural small molecule; it could be able to bind on the receptor located on the cell wall of bacteria. In recent reports, the antibiotic was conjugated to the magnetic nanoparticles for bacterial capture and enrichment under external magnetic fields.[26−28] Compared to conventional antibodies, antibiotics used in functional MNPs has more advantages, such as broad-spectrum bacterial targeting, convenient preparation, cheap, easy to transport, and preservation. Vancomycin (Van) is a famous glycopeptide antibiotic, which is called the last weapon of doctor. It could interact with a broad range of pathogenic bacteria through a surprisingly simple five-hydrogen bond.[29,30] The interaction is quite strong with a dissociation constant (K_d) of ∼1−4 μM at pH 7, which is similar to many antibody antigen interactions (K_d = 1 mM to 1 fM).[31,32] Though Van offers less specificity and selectivity than antibodies, these results will be further developed by being combined with nucleic acid amplification-based methods.

In this work, an integrated bacterial enrichment/gene-sensing method has been reported. A Fe_3O_4/Van/PEG magnetic nanocarrier was constructed for efficient sample enrichment and in situ nucleic acid preparation of pathogenic bacteria for subsequent gene-sensing. More than 90% of *L. monocytogenes* could be enriched from artificially contaminated samples at the concentration ranging from 1×10^4 cfu/mL to 1×10^2 cfu/mL. Even at a ultralow concentration of 10 cfu/mL, effective enrichment was achieved. Benefiting from good biocompatibility of the functional nanocarrier, high-quality genomic template, which is necessary for sensitive nucleic acid detection, could be conveniently extracted from the enriched bacteria by a commercial nucleic acid extraction kit. At last, an electrochemiluminescence (ECL)-based gene-sensing strategy was performed. Compared to conventional gel electrophoresis-based nucleic acid detection, it has better sensitivity and specificity, which is more suitable for accurate pathogenic bacterial detection.

MATERIALS AND METHODS

Materials and Reagents. Amine-terminated MNPs (aminated Fe_3O_4 nanoparticles, 100−200 nm in diameter, the concentration of the amine-terminated MNPs is 5 mg/mL, the amount of the amine on the MNPs is 100 μmol/g) were purchased from Research Center for Analytical Sciences, College of Chemistry, Nankai University. *N*-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), Van, and MES were bought from Sigma-Aldrich and used without further purification. mPEG-SC (SC refers to succinimidyl carbonate, 5.0 kDa) were bought from Institute of Process Engineering, Chinese Academy of Sciences. Phosphate-buffered saline (PBS) buffer (20×) solution, NaCl, and the reagents related to electrophoresis were purchased from Shanghai Sangon Biotechnology Co. Ltd. Water (≥18.2MΩ) used throughout the experiments was generated by a Milli-Q water purification system (Millipore, Bedford, MA). Fluorescence dye, Hoechst 33258, was purchased from Beyotime Biotechnology Co. Ltd.

Bacteria Sample Preparation. The bacteria strain *L. monocytogenes* (CMCC54007) was grown at 37 °C overnight on a rotary shaker at 180 rpm in lysogeny broth. The broth includes 0.5 g of yeast extract, 1.0 g of Tryptone, and 1.0 g of NaCl in 100 mL of water, whose pH was adjusted to 7.0. Before use, the bacterial cells were centrifuged at 10 000 rpm for 2 min and isolated from the broth. Then the isolated bacterial cells were washed twice with Tris-borate buffer (50 mM, pH 7.0). A desired level of bacterial concentration was adjusted by measuring the optical density at 600 nm (OD600). All glassware in contact with bacteria was sterilized in an autoclave at 121 °C for 30 min before and after use. Considering the safety, the bacteria samples were also sterilized to kill the bacteria after use.

Determining the Exact Concentration of *Listeria monocytogenes*. The exact concentration of the initial inoculum coming from an overnight culture in lysogeny broth was found by dilution and plating in LB agar. 1×10^6-fold dilution and 1×10^7-fold dilution were tested; the table of their colony-counted numbers is shown in Supporting Information, Table S1.

Preparation of the Magnetic Nanoparticles. We synthesized the functional magnetic nanoparticles through a several-step process. First of all, we modified amine-terminated MNPs with Van using the method reported by Kell and Simard. Amine-terminated MNPs (4.14 mL) were centrifuged and redisposed in fresh 30 mM MES buffer (10 mL) three times with a magnetic separator. After washing, 30 mg of Van, 60 mg of NHS, and 80 mg of EDC were added to the redisposed amine-terminated MNPs and mixed for 6 h at room temperature. The resulting Van-modified nanoparticles were washed three times and redisposed in fresh 30 mM MES buffer (10 mL) with a magnetic separator. Second, mPEG-SC (25 mg) was added to 5 mL of Van-modified nanoparticles to react with unmodified amines on the MNPs. After they mixed overnight at room temperature, the resulting PEG/Van-modified nanoparticles were washed three times to ensure all of the unreacted mPEG-SC were washed. Following these wash steps, the modified magnetic nanoparticles were dispersed in 5 mL of 30 mM MES and stored in the fridge at 4 °C, and the concentration of these MNPs was 0.207 mg/mL.

Evaluation of the Modification of Magnetic Nanoparticles by Zeta Potential Measurement. The zeta potentials of MNPs were measured at room temperature in neutral water solution (pH 7.0) with a Zetasizer Nano ZS (Malvern). Carbodiimide chemistry on amine-terminated MNPs was validated by a significant decrease in ζ potential upon addition of Van and mPEG-SC. (Data are mean ± standard deviation. N = 3 for all groups.)

Magnetic Capture of *Listeria monocytogenes* from Complex Sample Matrixes. *L. monocytogenes* (CMCC54007) was obtained from Guangzhou Institute of Microbiology China. Standard LB broth (10 mL) was inoculated with *L. monocytogenes* using standard sterile technique. This culture was incubated at 37 °C and agitated at 180 rpm in an orbital shaker incubator overnight. The exact concentration of the initial inoculum coming from an overnight culture in LB broth was found by dilution and plating in LB agar. For magnetic capture of *L. monocytogenes*, solutions from a concentration of 1×10^6 to 1 cfu/mL in LB culture were studied. Vancomycin-modified nanoparticles (1 mL) or PEG/Van-modified nanoparticles (1mL) were added to 10 mL of the obtained bacterial suspension (the concentration of these MNPs is 0.207 mg/mL). An incubation step was performed at 25 °C and agitated at 180 rpm in an orbital shaker incubator for 30 min. After

DOI: 10.1021/acsami.5b02374
ACS Appl. Mater. Interfaces 2015, 7, 12873−12881

that, the MNPs with the attached bacteria were centrifuged with a magnetic separator and then washed three times in MES buffered water (pH = 6, [NaCl] = 100 mM). Finally, the collected modified MNPs were redisposed in 1 mL of milli-Q water.

Determining the Amounts of Vancomycin, PEG, and Unreacted Amine on the Proposed Nanocarrier. For determining the real content of Van, PEG, and unacted amine on our proposed nanocarrier, Figure S3 was added in the Supporting Information. First, the original amount of amines on our proposed nanocarrier could be determined by the known information. More detailed parameters of the commercially available amine-terminated MNPs are provided in the section of Materials and Reagents; for example, the concentration of the amine-terminated MNPs is 5 mg/mL, and the amount of the amine on the MNPs is 100 μmol/g. On the basis of the method provided in the section of Preparation of the Magnetic Nanoparticles, the original concentration of the amine on our proposed nanocarrier could be calculated; the value is 0.207 μmol/mL (100 μmol/g). As Van exhibits absorption (λmax = 220 nm) in its UV−vis spectrum, which originates from the phenyl groups, we detected the increased absorption value of the nanocarrier in 220 nm after Van modification (Figure S3A,B), and then the concentration of the modified Van on the nanocarrier could be calculated according to a calibration curve (Figure S3C,D); the value is ∼0.036 μmol/mL (∼17.4 μmol/g). However, according to Figure 1C, the ratio of molarity of the modified

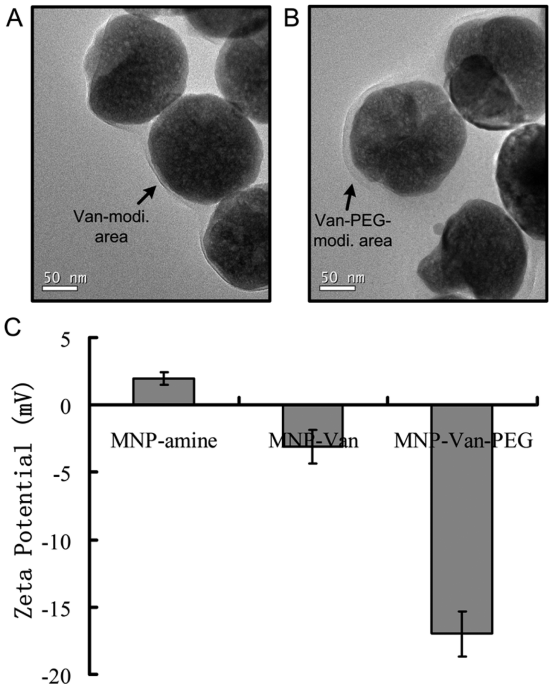


Figure 1. Different surface properties of Fe_3O_4/Van MNPs and Fe_3O_4/Van/PEG MNPs. (A) The TEM picture of Fe_3O_4/Van MNPs. (B) The TEM picture of Fe_3O_4/Van/PEG MNPs. (C) The statistical results of zeta potential.

Van and PEG is ∼0.365 [(−3.10 − 1.98)/(−17.00 + 3.10)], and the concentration of the modified Van on the nanocarrier could be calculated; the value is ∼0.098 μmol/mL (the amount is ∼47.3 μmol/g). The concentration of the unreacted amine could also be calculated by subtraction; the value is ∼0.073 μmol/mL (∼35.3 μmol/g).

Evaluation of the Magnetic Capture by Aggregation Experiment, TEM, Fluorescence Microscopic Observation Experiment, and Classical Culture Methods. We added 100 μL of PEG/Van-modified nanoparticles to 1 mL of bacterial suspension; the concentration was 1×10^8 cfu mL^{-1} in MES-buffered water (pH = 6, [NaCl] = 100 mM). An incubation step was performed at 25 °C and agitated at 180 rpm in an orbital shaker incubator for 30 min. After that, an obvious bacterial aggregation phenomenon could be observed, indicating the great interaction between the modified MNPs and bacteria. The TEM images of MNPs-bacteria conjugates were obtained on a JEM-2100HR transmission electron microscope (JEOL, Tokyo, Japan).

To study the capture efficiency of Van-modified nanoparticles and PEG/Van-modified nanoparticles, the classical culture method and the fluorescence microscopic observation experiment were carried out. Aliquots (500 μL) from 10 mL of bacterial suspension before capture of different concentration (ranged from 1×10^6 to 1 cfu/mL) were plated in LB agar and grown for 18−24 h at 37 °C, and the number of colonies was recorded as *A*. Aliquots (500 μL) from 10 mL of bacterial suspension after capture were also plated in LB agar and grown for 18−24 h at 37 °C; the number of colonies was recorded as *B*. The capture efficiency is $1 - (B/A)$ %. We found the capture efficiency of PEG/Van-modified nanoparticles was much higher than that of Van-modified nanoparticles especially at a low concentration of bacteria. The magnetic capture efficiencies presented in the article are the result of plate-counting analysis unless otherwise stated.

The process of the fluorescence microscopic observation experiment is basically the same as that of the classical culture method except the plate-counting step was replaced by glass slide observation. Briefly, 1 mL of bacterial suspension of different concentration (ranged from 1×10^8 to 1×10^3 cfu/mL) was prepared in 30 mM MES buffer (pH = 6, [NaCl] = 100 mM); then, the fluorescent dye Hoechst 33258 was added, and the final concentration of Hoechst 33258 is 2 μg/mL. After incubation in the dark for 15 min, the stained bacteria were centrifugated and washed three times for removing the unreacted fluorescence dye. After that, the bacteria were suspended to 1 mL in MES buffer for use. Before magnetic capture by the proposed nanocarrier, 20 μL of the stained bacterial suspension was dripped on a piece of glass slide. After it dried over an alcohol lamp, the piece of glass slide was observed by an Mshot MS31 higher-resolution CCD under UV excitation for evaluating the concentration of the original bacterial sample solution. Then, 100 μL of proposed nanocarrier was added into the left solution for bacterial enrichment. An incubation step was performed at 25 °C and agitated at 180 rpm in an orbital shaker incubator for 30 min. After that, the MNPs with the attached bacteria were centrifuged with a magnetic separator, and the remaining supernatant was collected for evaluating the concentration of the bacterial sample solution after magnetic enrichment. The separated nanocarrier/bacteria complexes were also observed under UV excitation and bright field to assess the enrichment capability of our proposed nanocarrier directly.

DNA-Tris-(2,2′-bipyridyl) Rutheium Labeling. The method of DNA labeling was as the same as that described in our previous work.[33] First, amino-modified DNA was dissolved in 0.1 M sodium borate buffer, whose pH was adjusted to 8.5. Then, the TBR-NHS ester was added, and the concentration is 20-fold molar excess over that of the amino-modified DNA. After an incubation step in the dark for 12 h by gentle shaking, precooled 100% ethanol was added to precipitate the TBR-labeled DNA. After incubation at −80 °C for 1 h, the mixture was centrifuged at 12 000 rpm for 20 min. Then the isolated labeled DNA was washed twice with precooled 80% ethanol. After drying in room temperature for 10 min, the TBR-labeled DNA was dissolved in pure water for further use.

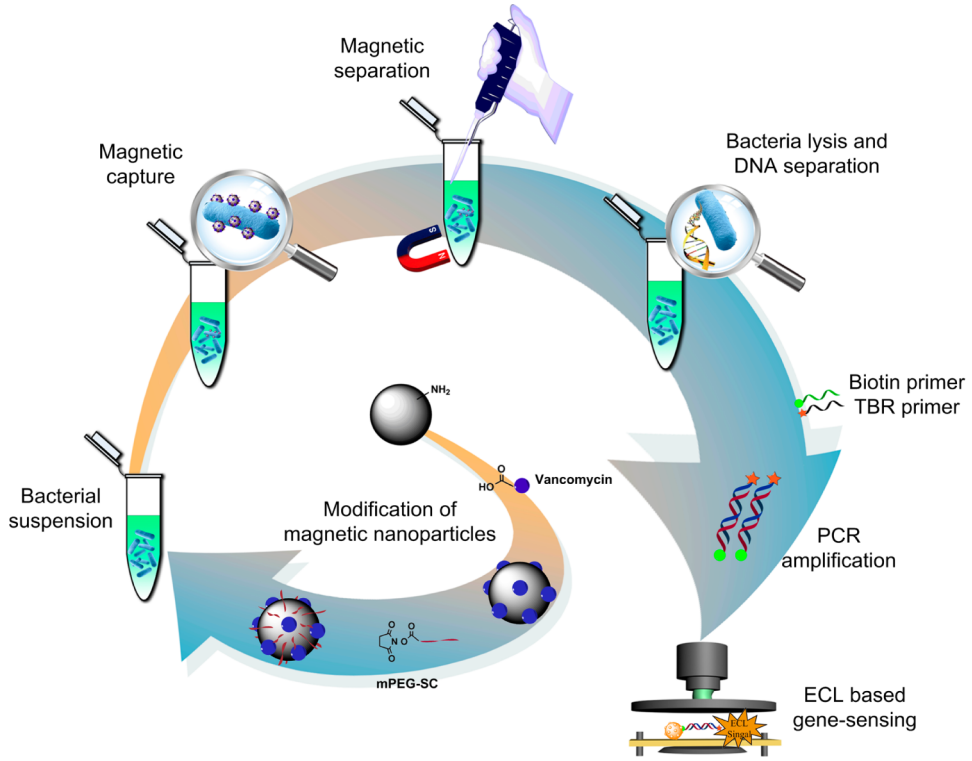
Gene Sensing of the Captured Bacteria by a TBR Primer-Based Amplification and Electrochemiluminescence. The bacterial DNA was isolated from the captured *L. monocytogenes* using the protocol and reagents from the TIANamp Bacteria DNA Kit and finally suspended in 50 μL of TE buffer. The reagents for PCR included DNA Template 5 μL, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 mM each primer, and 0.1 U/μL Taq DNA polymerase. The total reaction volume was 25 μL. The control solution (blank) contained all the PCR reagents except for the DNA template. The sequence of the primers we chose was designed based on the virulence gene *hlyA* of *L. monocytogenes*, whose target product size was 325 bp. The sequences were as follows:

Forward Primer. 5′-Bio-AGGAATGACTAATCAAGACAAT-3′

Reverse Primer. 5′-HN_2-Poly(T)$_{12}$-GGAAGGTCTTGTAGGTT-CATTA-3′

DOI: 10.1021/acsami.5b02374
ACS Appl. Mater. Interfaces 2015, 7, 12873−12881

Scheme 1. Schematic Procedure of the Integrated Bacterial Enrichment/Gene-Sensing System with Fe_3O_4/Vancomycin/PEG Nanocarrier



PCR was performed in a thermocycler and programmed with an initial step of denaturation at 95 °C for 5 min. Cycling conditions were as follows: denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and elongation at 72 °C for 40 s. In total, 35 cycles of the above program were performed. The last round of elongation was 8 min. The PCR products were analyzed by electrochemiluminescence. After the amplification, the products were captured by streptavidin magnetic beads for 30 min at 25 °C. Followed by the separation of the magnetic separator, the complexes of amplification products and streptavidin magnetic beads were redissolved in 1× PBS buffer. After the cleaning process was repeated three times, the ECL signals are detected by the Elecsys 2010 system.

Comparison of the Current Method and Other Methods. A comparison of the current method and other methods for sensitive analysis of bacteria by functional MNPs is shown in in the Supporting Information, Table S2 in terms of: functional groups modified on MNPs, LOD, incubation time, sample volume, and detection method.

RESULTS AND DISCUSSION

The Scheme of Current Assay. The schematic procedure of the integrated bacterial enrichment/gene-sensing system is outlined in Scheme 1. *L. monocytogenes* serves as a model for bacterial detection in this work. For highly efficient and rapid enrichment of bacteria, Van and PEG were immobilized on the surface of amine-terminated Fe_3O_4 nanoparticles. Van is a glycopeptide antibiotic; it was used as a bacterial probe to direct the MNPs to the bacterial cell wall. It can interact with a wide range of bacteria via strong hydrogen-bonding interactions with bacterial cells. In the process of experiment, we found that the dispersibility of the amine-terminated Fe_3O_4 nanoparticles in aqueous solution decreased after Van modification. This will have a negative impact on the enrichment effect, especially at low concentrations of bacteria. Moreover, we observed the effect of extraction of bacterial genome from bacteria/nanocarrier complexes decreased too, which will have an adverse effect on the subsequent gene sensing. We speculated that the surface charge changed after Van modification. It will weaken the repulsion force between nanoparticles, and the nanoparticles tend to gather together. In addition, the positively charged unreacted amines are thought to interact with the extracted genome from the enriched bacteria, which has a negative charge at neutral pH. To solve these problems, polyethylene glycol (PEG) was introduced in this work. PEG is a widely used chemical, which is well-known for good water solubility. We modified it on the Van functional MNPs to improve the nanoparticles' dispersibility to enhance the enrichment efficiency at low infectious dose. On the other hand, PEG will also develop the biocompatibility of the functional nanoparticles for extraction of bacterial genome. It would passivate the unreacted amines on the MNPs to help to release the extracted bacterial genome from the bacteria/nanocarrier complexes. Profiting from the nanocarrier's good capture capability and biocompatibility, high-quality genomic templates used in ECL-based gene-sensing strategy could be conveniently prepared by a commercial nucleic acid extraction kit. Compared to conventional gel electrophoresis-based detection, the ECL-based gene-sensing strategy could provide more sensitive and accurate results for the detection of pathogenic bacteria.

Synthesis of Fe_3O_4/Vancomycin/PEG Magnetic Nanocarrier for Pathogen Enrichment. A several-step process was introduced for the construction of the functional magnetic nanocarrier. Vancomycin was modified on the surface of the commercially available aminated MNPs through carbodiimide chemistry to obtain Fe_3O_4/Van MNPs. After magnetic separation and washing steps, excess MNPEG-SC (SC refers to succinimidyl carbonate) was added to react with the left amines on the MNPs to obtain Fe_3O_4/Van/PEG MNPs.

DOI: 10.1021/acsami.5b02374
ACS Appl. Mater. Interfaces 2015, 7, 12873−12881

Transmission electron microscope (TEM) observation was made to reveal the different surface properties of these two functional MNPs. Figure 1 shows the different surface properties of Fe_3O_4/Van MNPs and Fe_3O_4/Van/PEG MNPs. From the TEM picture (Figure 1A), we can see that the Fe_3O_4/Van MNPs are surrounded by a thin membranelike structure. After further modification with PEG, membranelike structure becomes thicker, as shown in Figure 1B. The molecular weight (MW) of PEG used here is 5000 Da, and we speculate that it will be formed as a hydrophilic layer on the surface of the MNPs. The hydrophilic layer will develop the dispersibility of the MNPs to help to increase the efficiency of the bacteria enrichment. The zeta potentials of MNPs were also measured at different stages of modification process. Figure 1C shows that the zeta potential of amine-terminated MNPs decreased from 1.98 to −3.10 mV when Van was added. Then after further modification with PEG, the left unreacted amines were passivated, the zeta potential decreased from −3.10 to −17.00 mV. (Data are mean ± standard deviation. $N = 3$ for all groups.) Carbodiimide chemistry on the MNPs is confirmed by a significant decrease in zeta potential, indicating that Van and PEG were modified on the MNPs successfully.

Compared to the antibody, Van is a very small molecule. Thus, a large amount of Van would be attached to a single MNP. If the bacterial capture capability of the functional MNPs is really good, the single MNP would also be attached to more than one bacteria, and the phenomenon of the functional MNPs mediated bacterial aggregation would be observed. To validate this hypothesis, the Fe_3O_4/Van/PEG MNPs were added to the bacterial suspension at a concentration of 1×10^8 cfu/mL. Figure 2A−C shows the phenomenon of bacterial aggregation. It refers to the effect of enrichment in macroscopic view. We predict that when the amount of bacteria is large enough in the water solution, the Fe_3O_4/Van/PEG MNPs will be attached to more than one bacteria. Then, the MNPs will make the dispersed bacteria gather in clusters, just like the adhesive. The TEM picture (Figure 2D) was also taken to show the MNPs mediated bacterial aggregation on the microscopic scale. Figure 2E shows the connection between MNPs and bacteria in detail.

The Enrichment Performance and Biocompatibility. To assess the enrichment effect of Fe_3O_4/Van/PEG MNPs directly, the fluorescence microscopic observation experiment was prepared. Different concentrations of bacteria were stained by fluorescence dye Hoechst 33258. Hoechst 33258 is a nonintercalating fluorescent dye that tends to interact with the AT-rich DNA in living cells, and the stained cells will emit blue light under the UV excitation. After incubation and washing steps, the stained bacteria were suspended to the original volume. A drop of the stained bacterial suspension was dripped on a piece of glass slide for fluorescence observation to evaluate the concentration of the original bacterial sample solution. Then, the Fe_3O_4/Van/PEG MNPs were added into the left solution. After enrichment and magnetic separation, a drop of the left solution was dripped on another piece of glass slide for fluorescence observation to evaluate the concentration of the left bacterial sample solution after enrichment. The significant difference between the two series of glass slides indicates the excellent bacterial capture capability of the functional nanocarrier (Figure 3A,B). The captured bacteria by modified nanocarrier was also spilled on a glass slide for observation of the nanocarrier/bacteria complexes. From the fluorescence microscopic image, the blue light was still observed at a

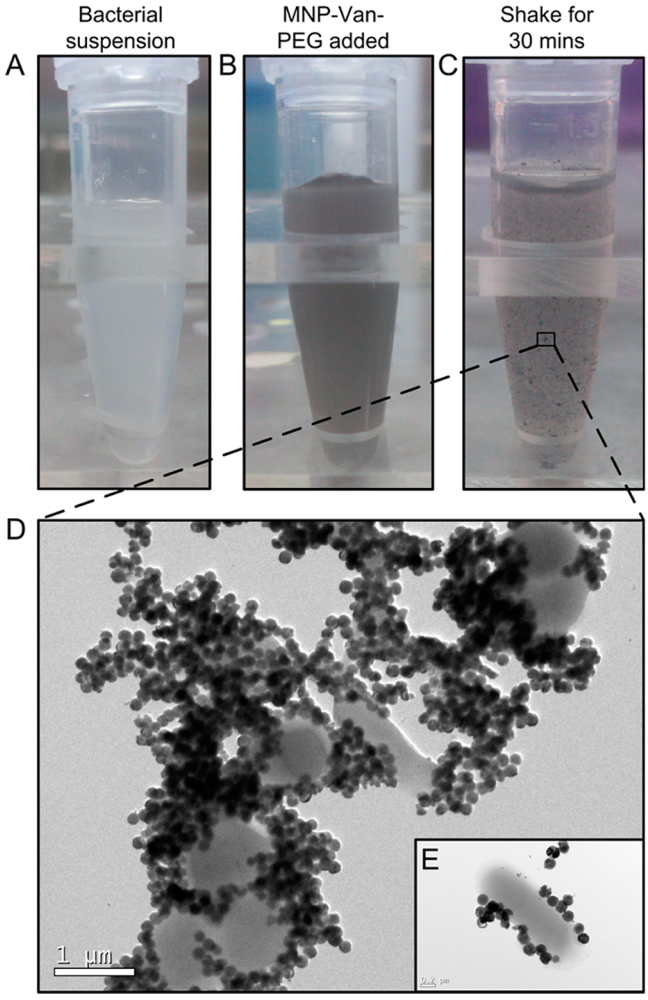


Figure 2. Phenomenon of functional MNPs mediated bacterial aggregation. (A−C) The phenomenon of bacterial aggregation in macroscopic view. (D, E) The TEM pictures of the phenomenon of bacterial aggregation in microcosmic view.

concentration of 1×10^3 cfu/mL, indicating the effective enrichment of bacteria with the functional nanocarrier could be achieved even at low infection dose. (Figure 3C,D)

Quantitative evaluation of the nanocarrier's capture capability was performed by the classical culture method. The concentration of bacterial suspension ranged from 1×10^4 cfu/mL to 1×10^1 cfu/mL. Different concentrations of the bacteria were dispersed in an MES/NaCl buffer, and Fe_3O_4/Van/PEG MNPs and Fe_3O_4/ Van MNPs were added for bacterial enrichment. The photos of plant counting are shown in Figure 4A. The plates from a1 to a4 show the counted colony number of original bacterial solution of the concentration range from 1×10^4 cfu/mL to 1×10^1 cfu/mL. The plates from b1 to b4 refer to the counted colony number of the left solution by Fe_3O_4/Van MNPs. The plates from c1 to c4 refer to the counted colony number of the left solution after the enrichment by Fe_3O_4 /Van/PEG MNPs. The difference between the original number and the left number is the amount of the bacteria enriched by the modified nanocarrier. The ratio of enriched number and original number refers to the enrichment efficiency of the MNPs. Statistical data are shown in Figure 4B (Data are mean ± standard deviation. $N = 3$ for all groups). The excellent enrichment capability of Fe_3O_4/Van/PEG MNPs was observed; the efficiency of enrichment is still

DOI: 10.1021/acsami.5b02374
ACS Appl. Mater. Interfaces 2015, 7, 12873−12881

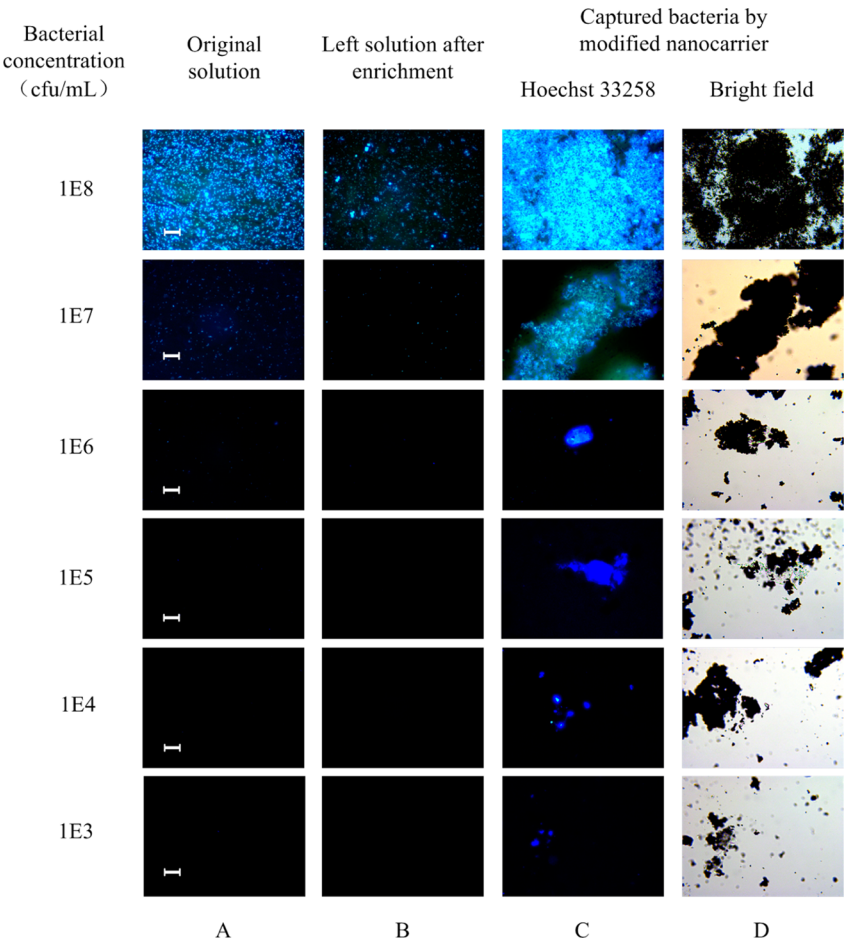


Figure 3. Fluorescence microscopic images of *L. monocytogenes* captured by modified nanocarrier and stained by fluorescence dye Hoechst 33258. The concentrations of *L. monocytogene* range from 1×10^8 cfu/mL to 1×10^3 cfu/mL. Lane A is original contaminated sample solution. Lane B is the remaining solution after enrichment by modified nanocarrier. Lanes C and D show the captured bacteria by modified nanocarrier, lane C is excited by UV, lane D is bright field. (Hoechst 33258: excitation 352 nm, emission 461 nm).

above 60% even at ultralow concentration of bacteria, 1×10^1 cfu/mL. Meanwhile, the comparison was also made with Fe_3O_4/Van MNPs, which is lack of a hydrophilic layer contributed by PEG on the surface. The enrichment efficiency of Fe_3O_4/Van MNPs decreases as the concentration of bacteria decreases, and no significant enrichment capability is observed when the concentrations of bacteria are 1×10^2 cfu/mL and 1×10^1 cfu/mL. This indicates that the hydrophilic layer contributed by PEG plays an important role in bacterial enrichment, especially in the situation of low concentration of bacteria, which is similar to the practical situation.

Excellent enrichment capability was achieved; however, it is not the end of our work. The biocompatibility for extraction of bacterial genome of the functional MNPs is what we are most interested in. Then, we tested the biocompatibility of Fe_3O_4/Van/PEG MNPs and the PEG free MNPs to investigate the contribution of PEG for the functional MNPs. First, Fe_3O_4/Van/PEG MNPs were tested; they were mixed with 10 mL of bacterial suspension, where the concentration ranged from 1×10^4 cfu/mL to 1×10^1 cfu/mL. After magnetic separation, Proteinase K was added to the complexes of bacteria and MNPs for cell lysis to release the bacterial genome. After that, a commercial nucleic acid purification kit was used for nucleic acid extraction, and the purified DNA template was kept in 50 μL of TE buffer for subsequent PCR-based nucleic acid detection. Then, we tested the biocompatibility of Fe_3O_4/Van MNPs and amine-terminated MNPs. The procedures of these assays were the same as in the Fe_3O_4/Van/PEG MNPs-based assay, and the results of the gel electrophoresis are shown in Figure 5. Results show that the sensitivity obtained from the assay using Fe_3O_4/Van/PEG MNPs is much higher than that of Fe_3O_4/Van MNPs-based assay, which is consistent with the results of classical culture method. In fact, we found that amine-terminated MNPs exhibit good bacterial enrichment capability, which can be observed from classical culture method. However, as shown in Figure 5, no bright target bands corresponding to amine-terminated MNPs were observed; the results of PCR-based nucleic acid detection tell the different stories with the classical culture method. This indicates that the bacterial genome failed to isolate from the cells enriched by amine-terminated MNPs. We speculate that the bacterial genome, which has a negative charge at neutral pH, was attached to the positively charged amine-terminated MNPs.

Highly Sensitive Gene Sensing of *Listeria monocytogenes* Assisted by Current Enrichment Technique. To assess the necessity of sample-enrichment step, we tested the sensitivity of nucleic acid detection of our integrated enrichment/gene-sensing system and the traditional enrichment-free method. The process of the test using Fe_3O_4/Van/PEG MNPs is the same as that described in Section 2.3. In the test of the traditional enrichment-free method, DNA template was prepared directly from 1 mL of bacterial suspension.

DOI: 10.1021/acsami.5b02374
ACS Appl. Mater. Interfaces 2015, 7, 12873−12881

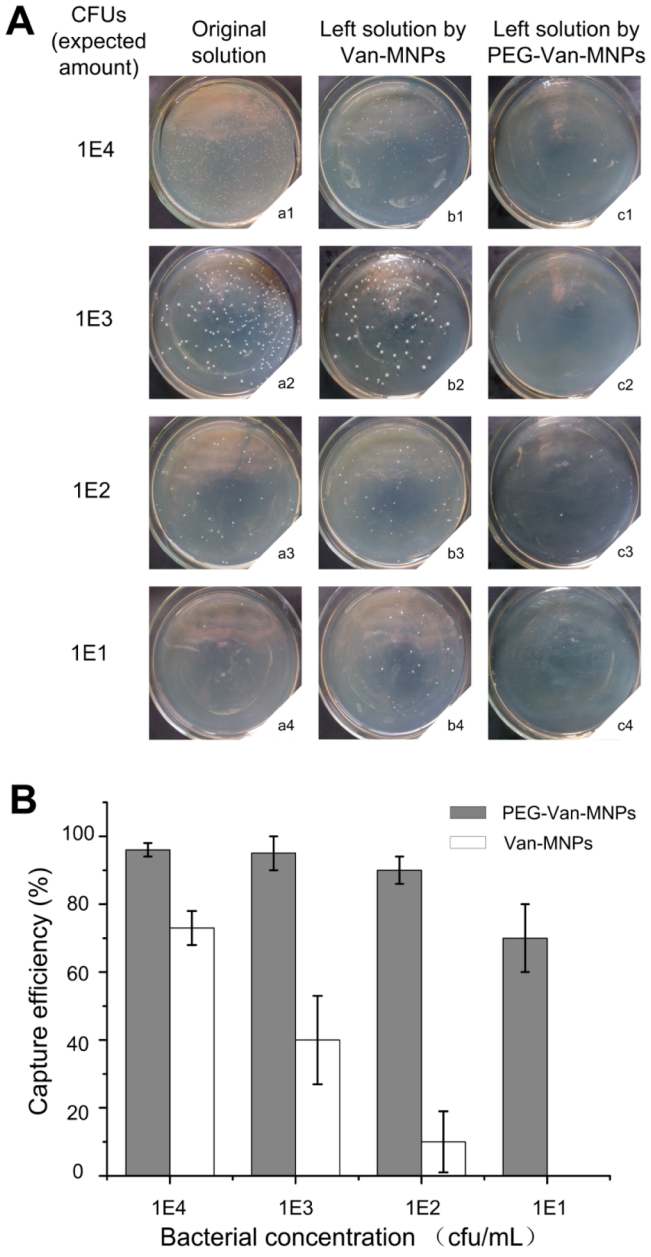


Figure 4. Comparison of enrichment capability between Fe_3O_4/Van MNPs and Fe_3O_4/Van/PEG MNPs. Evaluation by classical culture methods. (A) The photos of plate culture. (B) The statistical results. Data are mean ± standard deviation (N = 3 for all groups).

Centrifugation was operated instead of magnetic separation, then Proteinase K was added to the left bacteria for cell lysis to release the bacterial genome. After that, a commercial nucleic acid purification kit was used for nucleic acid extraction, and the purified DNA template was kept in 50 μL of TE buffer for subsequent PCR-based nucleic acid detection. The photo of the gel electrophoresis is shown in Figure 6, the sensitivity of the proposal with enrichment is nearly 10 times higher than the one without enrichment, indicating the rapid and biocompatible sample-enrichment step is essential for extending the nucleic acid-based bacterial detection strategy.

To take full advantage of the high-quality bacterial genome template, an electrochemiluminescence-based gene sensing was performed for result output of highly sensitive nucleic acid detection. To overcome the interference of false positive

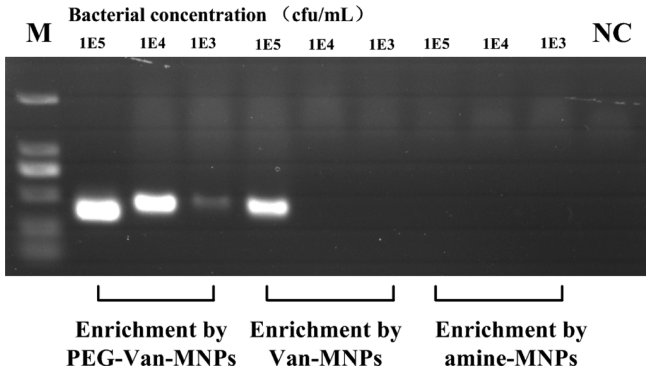


Figure 5. Comparison of the biocompatibility for extraction of bacterial genome between Fe_3O_4/Van/PEG MNPs and other MNPs. Evaluation by classical gel electrophoresis-based PCR method.

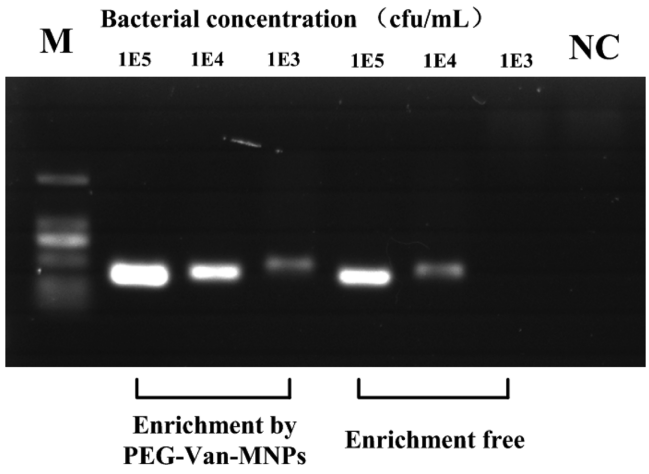


Figure 6. Comparison of the sensitivity of nucleic acid detection between Fe_3O_4/Van/PEG MNPs-based assay and enrichment-free assays. Evaluation by classical gel electrophoresis-based PCR method.

results, which comes with high sensitivity of ECL-based assay, a new pair of primers for target *hlyA* gene of *L. monocytogenes* was designed. The melting temperature of the new designed primer is 58 °C, so the production of dimers during PCR will decrease, which is a main reason for the false positive results. We also used qPCR to test the primers, the results of which are detailed in the Supporting Information, Figure S1. Then, we artificially inoculated bacteria into LB culture to test the sensitivity and specificity of our integrated assay. As the LB culture is composed of many natural ingredients, the composition is very complex; the LB culture is a good choice to simulate the actual sample. In specificity test, 10 mL of LB culture of different species of bacteria were prepared, namely, *L. monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella*. The concentration of each of them was 1×10^4 cfu/mL. The sterilized LB culture was set as a negative control. The results are shown in Supporting Information, Figure S2; as expected, only the ECL signal and bright band for *L. monocytogenes* could be obtained. The others are similar to the situation of the negative assay. These confirm the good specificity of our proposal. In sensitivity test, 10 mL aliquots of LB culture of *L. monocytogenes* of different concentrations were prepared. The concentration ranged from 1×10^6 cfu/10 mL to 1×10^0 cfu/10 mL. The sterilized LB culture was set as a negative control. The results are shown in Figure 7. We find that a significant ECL signal could be obtained even at a concentration of 10

DOI: 10.1021/acsami.5b02374
ACS Appl. Mater. Interfaces 2015, 7, 12873−12881

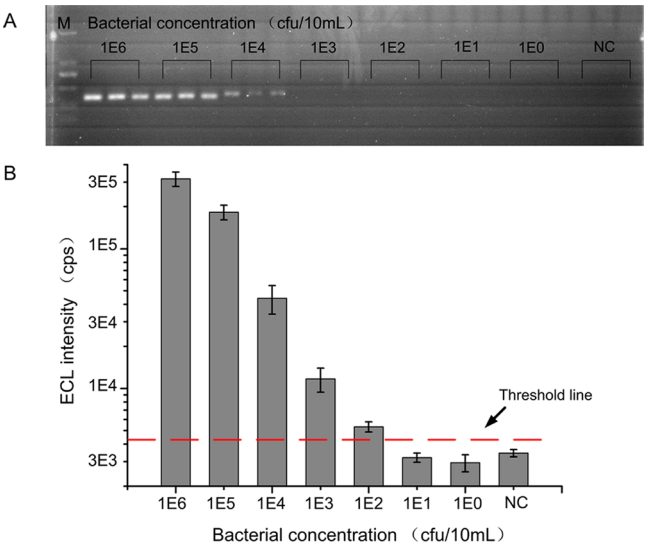


Figure 7. Sensitivity study of the integrated enrichment/gene-sensing method based on Fe_3O_4/Van/PEG MNPs in artificially inoculated LB culture samples. (A) Picture of gel electrophoresis. (B) The statistical results of electrochemiluminescence. The concentrations of *L. monocytogene* range from 1×10^6cfu/mL to 1×10^0 cfu/10 mL (Data are mean ± standard deviation. $N = 3$ for all groups).

cfu/mL, which is much stronger than that of negative control. The detection limit of our full system can reach 10 cfu/mL and the reliable result can also be applied in practical detection.

CONCLUSIONS

In conclusion, we successfully constructed an Fe_3O_4/Van/PEG magnetic nanocarrier for efficient sample enrichment and in situ nucleic acid preparation of pathogenic bacteria for sensitive gene sensing. Compared to the PEG-free nanocarrier, the PEG-modified Van Fe_3O_4 nanocarrier obtained better dispersibility and biocompatibility. With this proposed nanocarrier, perfect enrichment performance has been achieved. More than 90% *L. monocytogenes* could be efficiently captured from complex sample matrixes in 30 min. Benefiting from the nanocarrier's good biocompatibility, high-quality genomic templates for nucleic acid detection could also be conveniently prepared by a commercial nucleic acid extraction kit. An ECL-based highly sensitive gene-sensing assay is performed for result output of the integrated enrichment/gene-sensing strategy with a limit of detection of 10 cfu/mL. These properties and performance make the Fe_3O_4/Van/PEG magnetic nanocarrier a potentially useful tool for sample preparation in clinical diagnosis of pathogenic bacteria.

ASSOCIATED CONTENT

Supporting Information

Standard curve of Fe_3O_4/Van/PEG magnetic nanocarrier-based pathogens enrichment and gene sensing by qPCR, specificity study of the integrated enrichment/gene-sensing method based on Fe_3O_4/Van/PEG MNPs, determining the amount of Van on the proposed nanocarrier, a table for comparison of the current method and other methods for sensitive analysis of bacteria by functional MNPs, and a table for determining the exact concentration of *L. monocytogenes* at OD_{600} 1.0. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.5b02374.

AUTHOR INFORMATION

Corresponding Authors

*Phone: (+86-20) 8521-0089. Fax: (+86-20) 8521-6052. E-mail: zhouxm@scnu.edu.cn. (X.Z.)

*E-mail: xingda@scnu.edu.cn. (D.X.)

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was supported by the National Basic Research Program of China (2010CB732602), the National Natural Science Foundation of China (21475048), the National Science Fund for Distinguished Young Scholars of Guangdong Province (2014A030306008), and the Program of the Pearl River Young Talents of Science and Technology in Guangzhou, China (2013J2200021).

REFERENCES

(1) Shuman, E. K. Global Climate Change and Infectious Diseases. *N. Engl. J. Med.* **2010**, *362*, 1061−1063.

(2) Lin, M.; Pei, H.; Yang, F.; Fan, C.; Zuo, X. Applications of Gold Nanoparticles in the Detection and Identification of Infectious Diseases and Biothreats. *Adv. Mater.* **2013**, *25*, 3490−3496.

(3) Singh, A.; Arutyunov, D.; Szymanski, C. M.; Evoy, S. Bacteriophage Based Probes for Pathogen Detection. *Analyst* **2012**, *137*, 3405−3421.

(4) Fauci, A. S.; Morens, D. M. The Perpetual Challenge of Infectious Diseases. *N. Engl. J. Med.* **2012**, *366*, 454−461.

(5) Crameri, A.; Whitehorn, E. A.; Tate, E.; Stemmer, W. P.; Crameri, A.; Kitts, P. A.; Kitts, P. A. Improved Green Fluorescent Protein by Mol- ecular Evolution Using. *Nat. Biotechnol.* **1996**, *14*, 315−319.

(6) Braga, P. A. C.; Tata, A.; Gonçalves dos Santos, V.; Barreiro, J. R.; Schwab, N. V.; Veiga dos Santos, M.; Eberlin, M. N.; Ferreira, C. R. Bacterial Identification: from the Agar Plate to the Mass Spectrometer. *RSC Adv.* **2013**, *3*, 994−1008.

(7) Toze, S. PCR and the Detection of Microbial Pathogens in Water and Wastewater. *Water Res.* **1999**, *33*, 3545−3556.

(8) Muldrew, K. L. Molecular Diagnostics of Infectious Diseases. *Curr. Opin. Pediatr.* **2009**, *21*, 102−111.

(9) Hartman, M. R.; Ruiz, R. C.; Hamada, S.; Xu, C.; Yancey, K. G.; Yu, Y.; Han, W.; Luo, D. Point-of-Care Nucleic Acid Detection Using Nanotechnology. *Nanoscale* **2013**, *5*, 10141−10154.

(10) Leung, C. H.; Chan, D. S.; He, H. Z.; Cheng, Z.; Yang, H.; Ma, D. L. Luminescent Detection of DNA-Binding Proteins. *Nucleic Acids Res.* **2012**, *40*, 941−955.

(11) He, H. Z.; Leung, K. H.; Yang, H.; Chan, D. S.; Leung, C. H.; Zhou, J.; Bourdoncle, A.; Mergny, J. L.; Ma, D. L. Label-Free Detection of Sub-Nanomolar Lead(II) Ions in Aqueous Solution Using a Metal-Based Luminescent Switch-On Probe. *Biosens. Bioelectron.* **2013**, *41*, 871−874.

(12) Erlich, H. A.; Gelfand, D.; Sninsky, J. J. Recent Advances in the Polymerase Chain Reaction. *Science* **1991**, *252*, 1643−1651.

(13) Jones, D. D.; Bej, A. K. In *PCR Technology: Current Innovations;* CRC Press: Boca Raton, FL, 1994; pp 341−365.

(14) Long, Y.; Zhou, X.; Xing, D. An Isothermal and Sensitive Nucleic Acids Assay by Target Sequence Recycled Rolling Circle Amplification. *Biosens. Bioelectron.* **2013**, *46*, 102−107.

(15) Stevens, K. A.; Jaykus, L.-A. Bacterial Separation and Concentration from Complex Sample Matrices: a Review. *Crit. Rev. Microbiol.* **2004**, *30*, 7−24.

(16) Liebana, S.; Spricigo, D. A.; Cortes, M. P.; Barbe, J.; Llagostera, M.; Alegret, S.; Pividori, M. I. Phagomagnetic Separation and Electrochemical Magneto-Genosensing of Pathogenic Bacteria. *Anal. Chem.* **2013**, *85*, 3079−3086.

(17) Jyoti, A.; Vajpayee, P.; Singh, G.; Patel, C. B.; Gupta, K. C.; Shanker, R. Identification of Environmental Reservoirs of Non-

DOI: 10.1021/acsami.5b02374
ACS Appl. Mater. Interfaces 2015, 7, 12873−12881

typhoidal Salmonellosis: Aptamer-Assisted Bioconcentration and Subsequent Detection of Salmonella Typhimurium by Quantitative Polymerase Chain Reaction. *Environ. Sci. Technol.* **2011**, *45*, 8996−9002.
(18) Yang, H.; Li, H.; Jiang, X. Detection of Foodborne Pathogens Using Bioconjugated Nanomaterials. *Microfluid. Nanofluid.* **2008**, *5*, 571−583.
(19) El-Boubbou, K.; Gruden, C.; Huang, X. Magnetic Glyco-Nanoparticles: a Unique Tool for Rapid Pathogen Detection, Decontamination, and Strain Differentiation. *J. Am. Chem. Soc.* **2007**, *129*, 13392−13393.
(20) Shepherd, J.; Sarker, P.; Rimmer, S.; Swanson, L.; MacNeil, S.; Douglas, I. Hyperbranched Poly(NIPAM) Polymers Modified with Antibiotics for the Reduction of Bacterial Burden in Infected Human Tissue Engineered Skin. *Biomaterials* **2011**, *32*, 258−267.
(21) Gu, H.; Ho, P. L.; Tong, E.; Wang, L.; Xu, B. Presenting Vancomycin on Nanoparticles to Enhance Antimicrobial Activities. *Nano Lett.* **2003**, *3*, 1261−1263.
(22) Huang, Y. F.; Wang, Y. F.; Yan, X. P. Amine-Functionalized Magnetic Nanoparticles for Rapid Capture and Removal of Bacterial Pathogens. *Environ. Sci. Technol.* **2010**, *44*, 7908−7913.
(23) Wen, C. Y.; Hu, J.; Zhang, Z. L.; Tian, Z. Q.; Ou, G. P.; Liao, Y. L.; Li, Y.; Xie, M.; Sun, Z. Y.; Pang, D. W. One-Step Sensitive Detection of Salmonella Typhimurium by Coupling Magnetic Capture and Fluorescence Identification with Functional Nanospheres. *Anal. Chem.* **2013**, *85*, 1223−1230.
(24) Lee, J. J.; Jeong, K. J.; Hashimoto, M.; Kwon, A. H.; Rwei, A.; Shankarappa, S. A.; Tsui, J. H.; Kohane, D. S. Synthetic Ligand-Coated Magnetic Nanoparticles for Microfluidic Bacterial Separation from Blood. *Nano Lett.* **2014**, *14*, 1−5.
(25) Jin, Y.; Liu, F.; Shan, C.; Tong, M.; Hou, Y. Efficient Bacterial Capture with Amino Acid Modified Magnetic Nanoparticles. *Water Res.* **2014**, *50*, 124−134.
(26) Wang, C. H.; Chang, C. J.; Wu, J. J.; Lee, G. B. An Integrated Microfluidic Device Utilizing Vancomycin Conjugated Magnetic Beads and Nanogold-Labeled Specific Nucleotide Probes for Rapid Pathogen Diagnosis. *Nanomedicine* **2014**, *10*, 809−818.
(27) Kell, A. J.; Stewart, G.; Ryan, S.; Peytavi, R.; Boissinot, M.; Huletsky, A.; Bergeron, M. G.; Simard, B. Vancomycin-Modified Nanoparticles for Efficient Targeting and Preconcentration of Gram-Positive and Gram-Negative Bacteria. *ACS Nano* **2008**, *2*, 1777−1788.
(28) Qi, G.; Li, L.; Yu, F.; Wang, H. Vancomycin-Modified Mesoporous Silica Nanoparticles for Selective Recognition and Killing of Pathogenic Gram-Positive Bacteria over Macrophage-Like Cells. *ACS Appl. Mater. Interfaces* **2013**, *5*, 10874−10881.
(29) Gu, H.; Ho, P. L.; Tsang, K. W.; Wang, L.; Xu, B. Using Biofunctional Magnetic Nanoparticles to Capture Vancomycin-Resistant Enterococci and Other Gram-Positive Bacteria at Ultralow Con- centration. *J. Am. Chem. Soc.* **2003**, *125*, 15702−15703.
(30) Walsh, C. Deconstructing Vancomycin. *Science* **1999**, *284*, 442−443.
(31) Rao, J.; Colton, I. J.; Whitesides, G. M. Using Capillary Electrophoresis to Study the Electrostatic Interactions Involved in the Association of D-Ala-D-Ala with Vancomycin. *J. Am. Chem. Soc.* **1997**, *119*, 9336−9340.
(32) Sundram, U. N.; Griffin, J. H.; Nicas, T. I. Novel Vancomycin Dimers with Activity against Vancomycin-Resistant Enterococci. *J. Am. Chem. Soc.* **1996**, *118*, 13107−13108.
(33) Zhou, X.; Zhu, D.; Liao, Y.; Liu, W.; Liu, H.; Ma, Z.; Xing, D. Synthesis, Labeling and Bioanalytical Applications of a Tris(2,2′-bipyridyl)ruthenium(II)-Based Electrochemiluminescence Probe. *Nat. Protoc.* **2014**, *9*, 1146−1159.

DOI: 10.1021/acsami.5b02374
ACS Appl. Mater. Interfaces 2015, 7, 12873−12881