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Short communication

Determination of sulphate-reducing bacteria based on vancomycin-functionalised magnetic nanoparticles using a modification-free quartz crystal microbalance

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ABSTRACT

A fast, sensitive and reliable quartz crystal microbalance (QCM) biosensor is described for the selective detection of the marine pathogenic sulphate-reducing bacterium (SRB), *D. desulfotomaculum*. Based on the amplification of the response of vancomycin-functionalised magnetic nanoparticles (Van-mNPs), under an external magnetic field, the bacteria-mNPs conjugates attach to the surface of an Au electrode. The QCM biosensor gave a distinct response to the vancomycin-sensitive, *D. desulfotomaculum*, but had no obvious response to the vancomycin-resistant bacterium, *Vibrio anguillarum*. The effects of the optimization conditions such as the incubation time and pH on the detection were also investigated, respectively. Optimised assays showed that the biosensor could obtain the best response with a 30 min incubation of the bacteria with the Van-mNPs. A linear relationship between the QCM response and the logarithm of the bacterial concentration was observed in the range of 1.8×10^4 to 1.8×10^7 cfu/ml. The sensor system has a potential for further applications and provides a facile and sample method for detection of pathogenic bacteria.

1. Introduction

Rapid and sensitive approaches for pathogen detection are essential for applications such as food safety, environmental monitoring, and clinical diagnosis, to allow faster decisions in dealing with public health issues such as food poisoning, water pollution or disease outbreaks. Various methods have been developed for the detection of pathogens, including plating and culturing (Swaminathan and Feng, 1994; Wain et al., 1998), biochemical tests (Louis et al., 1998), flow cytometry (Karo et al., 2008) and polymerase chain reaction (Xu et al., 2004). Although these techniques for pathogen detection are sufficiently sensitive and selective, most have several disadvantages including being time-consuming (e.g., sample and culture plating procedures), cost-intensive (e.g., specific enzyme-labelled antibodies), or technically complex (e.g., DNA analysis). In this paper, we present an alternative method, the quartz crystal microbalance (QCM) biosensor, which can be used for simple and rapid pathogen detection.

The QCM technique, a nonlabelled biosensor, offers promising advantages over conventional detection techniques, from the viewpoints of system miniaturisation, cost effectiveness and simplicity of operation. Following the theory of Sauerbrey, the QCM is a very sensitive tool for detecting changes in weight and thus is a helpful method for sensing binding events between an analyte and a sensing layer (Sauerbrey, 1959). A variety of methods for pathogen detection, based on the piezoelectric crystal device, have been developed over the last few decades (Schofield et al., 2007; Wu et al., 2007; Hong et al., 2009; Poitras et al., 2009). Classical QCM biosensor design for the detection of pathogens typically involves the use of a Au electrode modified with either an antibody that specifically traps the targeted bacteria or a cell-selective polymer film that recognises the bacterium via a molecular imprinting technique. Wang et al. recently introduced a novel application of QCM for the immunophenotyping of acute leukaemias by immunomagnetic separation. Unlike conventional QCM, signal reception and enhancement occurred via an external magnetic field rather than by receptor–ligand recognition (Wang et al., 2006).

The potential use of magnetic nanoparticles (mNPs) has been recognised in a variety of fields, including magnetic fluids, catalysis, biomedicine, magnetic resonance imaging, data storage, and environmental remediation (Lu et al., 2007; Guntupalli et al., 2007). Recently, the development of mNPs that can be conjugated to biological/chemical molecules has opened up a whole new arena of applications for biosensor systems (Kaittanis et al., 2007; Varshney and Li, 2007; Ravindranath et al., 2009).

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Vancomycin is a glycopeptide antibiotic used in the prophylaxis and treatment of infections caused by Gram-positive bacteria. It acts by inhibiting cell wall synthesis in bacteria, as the antibiotic molecule is able to form a five-point hydrogen bond that recognises the terminal D-Ala-D-Ala moieties, preventing the incorporation of the N-acetylmuramic acid- and N-acetylglucosamine-peptide subunits into the peptidoglycan matrix (Williams and Butcher, 1981; Molinari et al., 1990; Walsh, 1999). As these five-point hydrogen bond interactions play an important role in the stability and rigidity between vancomycin and D-Ala-D-Ala moieties, it seemed logical to use the D-Ala-D-Ala structural domain as a receptor element for detection of Gram-positive pathogens. Gu et al. reported that biofunctional FePt mNPs could be used to capture vancomycin-resistant enterococci and other Gram-positive at ultralow concentration (Gu et al., 2003a, 2003b). Lin et al. used matrix-assisted laser desorption ionisation mass spectrometry as a method to characterise isolated bacteria based on vancomycin-modified nanoparticles (Lin et al., 2005). Kell et al. developed a series of vancomycin-modified nanoparticles to isolate a variety of pathogens from a complicated matrix. They found that only one orientation/architecture of vancomycin on the surface of the nanoparticles was critical for mediating fast and effective interactions between the functionalised nanoparticles and the pathogen cell wall surface (Kell et al., 2008).

In the present paper, we introduce a nanoparticle-based immunoassay that combined vancomycin-functionalised mNPs (Van-mNPs) with QCM for detection of sulphate-reducing bacteria (SRB). We employed vancomycin-functionalised mNPs as probes to selectively recognise and isolate pathogens from a sample matrix under an external magnetic field. The isolated bacteria could be quantified using a QCM sensor. The signals of QCM detection could be amplified by magnetising the mNP/bacteria conjugate onto the surface of an Au electrode by application of an external magnetic force.

2. Materials and methods

2.1. Instrumentation

QCM measurements were performed in a custom-built cell setup equipped with a Ti-gold plated AT-cut quartz crystal (CH Instruments, Inc.) 5 mm in diameter with a resolution frequency of 7.995 MHz. The frequency output from the oscillator was measured with a CHI 430C (CH Instruments, Inc.).

2.2. Chemicals and solutions

Ferrous sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), ferric trichloride hexahydrate ($FeCl_3{\cdot}6H_2O$), ammonium hydroxide, ethanol and sodium hydroxide (NaOH) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Tetraethyl orthosilicate (TEOS), (3-aminopropyl)trimethoxysilane (APTMS), suberic acid bis(N-hydroxysuccinimide ester) (DSS), and vancomycin hydrochloride (Van) were obtained from Sigma (St. Louis, MO). All other chemicals were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and Milli-Q water (TGI Pure Water Systems, USA) was used throughout.

2.3. Preparation of biofunctional magnetic nanoparticles

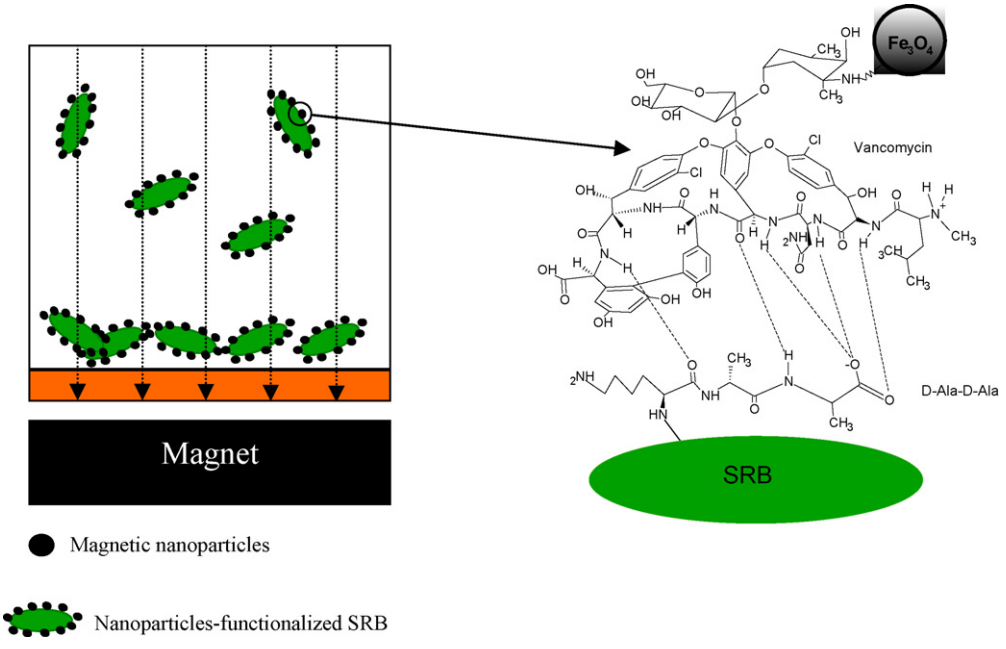
Van-mNPs were designed for capturing pathogenic bacteria based on two kinds of interactions including magnetic dipole interaction that aggregate the magnetic nanopaticles under external magnetic field and specific multiple ligand-receptor interaction (see this section in the supplementary material).

2.4. Bacterial cultivation

The SRB seed was isolated from marine mud collected from the Bohai Sea, China. The viable cell number was determined by the Most Probable Number (MPN) method, according to the American Society of Testing Materials Standard D4412-84 (see this section in the supplementary material). The vancomycin-resistant bacterium, *Vibrio anguillarum*, was a gift from Dr. Zhaolan Mo and was used as a control organism.

2.5. Detection of SRB

Scheme 1 shows the determination of SRB based on Van-mNPs using QCM. The Van-mNPs were incubated with a bacterial sample for 1 h. The bacterium-mNPS conjugates were isolated



Scheme 1. Schematic presentation of sulphate-reducing bacteria (SRB) detection based on the signal amplification of magnetic nanoparticles using QCM.

through magnetic separation, rinsed three times with PBS, and then resuspended in PBS (1 ml). A suspension (250 μl) containing the bacterium-mNPS conjugates was added to the QCM cell to determine the concentration of SRB by measuring the frequency change after 10 min under a magnetic field. To prevent the interfering response of sensor from external magnetic field, the measurements were performed until the bacteria-mNPs conjugates on the surface of gold crystal adsorbed completely. Functionalised mNPs were deposited on the surface of gold electrode under external magnetic field, which was result in background signal (50 ± 7 Hz). The background signal should be deduct in all data of detection of bacteria using modification-free QCM. The MPN method was also used as a control experiment to determine the concentration of SRB. The error bars represent standard deviation calculated from three parallel measurements ($n = 3$).

3. Results and discussion

3.1. Preparation and characterisation of Van-mNPs

The superparamagnetic NPs were synthesised as reported by Kang et al. To protect the magnetic cores and to develop more stable nanoparticle bioconjugates, a thin layer of silica was first coated onto the bare mNPs, followed by modification with another layer of amino groups using TEOS and APTMS. As a result of the cross-linking of the bifunctional reagent, DSS, with amino groups, vancomycin molecules were covalently conjugated to the aminosilane-modified mNPs (see this section in the supplementary material).

3.2. Response amplification of biofunctionalised mNPs

The detection of bacteria based on the amplification of a nanoparticle signal has attracted increasing interest over the last decade (Liu et al., 2007). Nanoparticles can be used to amplify a detection signal, improve the electron transduction and reduce the limitations of detection by a biosensor. The frequency response of 1.8×10^7 cfu/ml SRB coated with Van-mNPs shifted rapidly toward stability, in contrast to the gradually decreasing process seen for SRB without conjugated Van-mNPs. The blank response (0 ± 6 Hz) of SRB without conjugation to the Van-mNPs was distinctly low, as compared with that obtained with conjugation to Van-mNPs (-275 ± 21 Hz). The response signal of the sample coated with Van-mNPs was higher than the background response under the same assay conditions. To summarise, the approach had a short response time and yielded a maximum response signal to the same target bacteria. Therefore, Van-mNPs might be useful for detection of vancomycin-sensitive bacteria.

3.3. Specificity of the vancomycin-functionalised mNPs to SRB

Vancomycin is a well-known glycopeptide antibiotic capable of strongly interacting with vancomycin-sensitive bacteria through a five-point hydrogen bond between the peptide backbone of vancomycin and the terminal D-Ala-D-Ala moiety of peptidoglycan precursors with an association constant (K_a) of 4.4×10^5 M^{-1} (McComas et al., 2003). Therefore, vancomycin is very attractive as a ligand, allowing affinity capture of a broad of range of vancomycin-sensitive bacteria when functionalised onto nanoparticles. Nevertheless, vancomycin has a lower specificity than a monoclonal antibody. The specificity of the biosensor was investigated in relation to other bacteria such as the Gram-negative bacteria, *V. anguillarum*, another marine pathogenic bacterium. Fig. 1 shows a comparison of the signal response of QCM after exposure of 1.8×10^7 cfu/ml *D. desulfotomaculum* or *V. anguillarum*

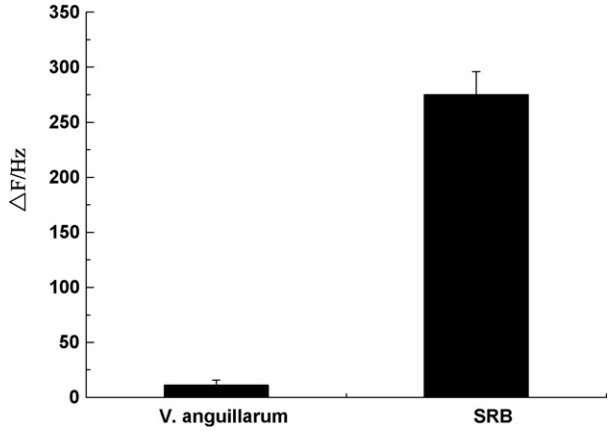


Fig. 1. A comparison of response of QCM biosensor based on signal amplification of vancomycin-functionalised magnetic nanoparticles to 1.8×10^7 cfu/ml sulphate-reducing bacteria (SRB) (a) or *V. anguillarum* (b).

of incubated with Van-mNPs for 1 h. The frequency shift corresponding to SRB reached a rapid maximum of 275 ± 21 Hz, in ca. 100 s. However no obvious response was seen to *V. anguillarum* (11 ± 5 Hz). These results clearly indicate that Van-mNPs as probes are capable of specifically recognising the vancomycin-sensitive bacteria, SRB, rather than the Gram-negative bacterium, *V. anguillarum*. This was due to the unique e structure of the outer cell wall of Gram-negative bacteria, which consists of a complex lipopolysaccharide. The thick outer cell wall is responsible for the vancomycin resistance due as it is highly impenetrable to vancomycin, binding to the terminal D-Ala-D-Ala moiety.

3.4. Optimisation of assay conditions

The Van-mNPs were attached to the surface of targeted bacteria via vancomycin, which was capable of specifically recognising the D-Ala-D-Ala moiety in the outer cell wall. The conjugation of bacterial mNPs plays a key role in the detection of *D. desulfotomaculum* using QCM. The assay conditions were optimised as a function of two parameters; namely, the incubation time and the effect of pH on the ligand–receptor interaction between SRB and Van-mNPs.

The incubation time between SRB and Van-mNPs was studied over a range of times between 0 and 1.5 h. These results (see Fig. 2S in the supplementary material) illustrate the dependence of the incubation time and frequency response when a fixed SRB concentration (1.8×10^7 cfu/ml) was maintained. As the incubation time increased, the increase in frequency response was rapid and almost linear at first, but then levelled off until a stable platform (c.a. 275 ± 21 Hz) was reached after 30 min. An incubation time of 60 min was selected to effectively bind the bacteria, since no distinct changes in the QCM signal were observed at longer incubation times.

The pH could also contribute to the frequency change. The influence of pH on the frequency response was investigated in 0.2 M PBS to optimise the assay conditions. As seen in Fig. 3S in the supplementary material, the frequency response increased when the pH increased from 4.0 to 7.0 but then decreased at pH > 7.0. This is because the formation of the five-point hydrogen bonds between vancomycin and the D-Ala-D-Ala moiety is influenced by pH. This extreme sensitivity to pH is due to the ionisation/deionisation of carboxyl or amino groups under different pH conditions, which may generate some electrostatic double-layer repulsion that would subsequently eliminate hydrogen bonding (Wood, 1974; Tareste et al., 2007). This implies that the number of Van-mNPs attached to the surface of SRB under pH conditions other than pH 7.0 will be lower. Therefore, compared with the pH 7.0 condition, the bacteria-

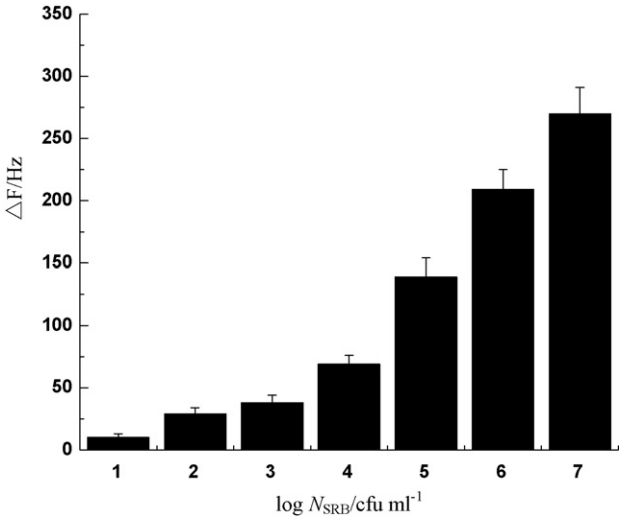


Fig. 2. The relationship between the QCM responses and concentration of sulphate reducing bacteria (SRB).

mNPs attaching to the surface of the Au crystal of the QCM in the same magnetic field will show only a relatively minor frequency response. Therefore, the optimised pH level of 7.0 was used in our study for the detection of SRB.

3.5. Detection of SRB

Seven SRB concentrations from 1.8×10^1 to 1.8×10^7 cfu/ml were prepared by serial dilution in PBS. The Van-mNPs were added to the bacterial suspension to form bacteria-mNPs conjugates by interaction of vancomycin and D-Ala-D-Ala. The bacteria-mNPs, when exposed to an external magnetic field, were adsorbed onto the surface of the Au film and induced significant frequency changes. As seen in Fig. 2, the concentration of bacteria and the frequency response were highly correlated. A linear relationship between QCM response and the logarithm of the bacterial concentration was obtained for the concentration range from 1.8×10^4 to 1.8×10^7 cfu/ml, giving a slope of 68.31 and a correlation coefficient of 0.999 ($\Delta F = 68.31 \log N_{SRB} - 203.8$, $R^2 = 0.999$).

Several QCM biosensors (Su and Li, 2005; Shen et al., 2007; Hao et al., 2009; Poitras and Tufenkji, 2009) have been reported for detection of various pathogenic bacteria. These QCM immunosensors were fabricated using protein A, glutaraldehyde, or MPA for antibody or lectin immobilisation, to recognise and detect specific pathogenic bacteria. Compared with these QCM biosensors, the approach used in the present paper for the detection of SRB based on amplification of Van-mNPs using QCM is faster and accurate, due to the combination of three effects. First, the bacteria-mNPs conjugates are adsorbed quickly onto the surface of the Au electrode through the strong magnetic force, resulting in a frequency response that quickly reaches a stationary phase (ca. 100 s). In contrast, achieving steady-state results for a classic QCM biosensor based on molecular recognition takes at least 60 min. Second, the majority of the bacteria-mNPs conjugates in the QCM cell are capable of attaching to the Au electrode under an external magnetic field, which results in an amplification of the response, whereas the conventional QCM biosensors, theoretically speaking, are merely able to integrate a monolayer of bacterial cells onto the surface of a Au electrode. Third, before use, the Au crystals of typical QCMs require extensive and prolonged modification processes (more than 24 h), unlike the Au electrode of the present QCM approach, which can be used directly to detect bacterial concentrations.

4. Conclusion

We have described a faster and reliable method for SRB detection based on the amplification of response of Van-mNPs, using QCM under an external magnetic field. A linear relationship between QCM response and the logarithm of the bacterial concentration was observed in the range of 1.8×10^4 to 1.8×10^7 cfu/ml. The selectivity of the modification-free QCM will be further developed in our future work. The sensor system has a potential for further applications and provides the basis for incorporating the method into an integrated system for rapid pathogen detection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.12.028.

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