Available online at www.sciencedirect.com
ScienceDirect
Biosensors and Bioelectronics 22 (2007) 2500–2507
BIOSENSORS & BIOELECTRONICS
www.elsevier.com/locate/bios

Simple and sensitive bacterial quantification by a flow-based kinetic exclusion fluorescence immunoassay

Feng-yi Su [a,c], Yumie Endo [b], Hiroshi Saiki [b,*], Xin-Hui Xing [c,**], Naoya Ohmura [d]

[a] *National Institute of Advanced Industrial Science and Technology, Research Center of Advanced Bionics, 1404-1 Katakura, Hachioji City, Tokyo 192-0982, Japan*
[b] *Bionics Department, Tokyo University of Technology, 1404-1 Katakura, Hachioji City, Tokyo 192-0982, Japan*
[c] *Department of Chemical Engineering, Tsinghua University, Beijing 100084, China*
[d] *Department of Bioscience, Central Research Institute of the Electric Power Industry, 1646 Abiko, Abiko City, Chiba 270-1194, Japan*

Received 30 June 2006; received in revised form 20 September 2006; accepted 29 September 2006
Available online 13 November 2006

Abstract

A flow-based immunoassay system utilizing secondary-antibody coated microbeads and Cy5-secondary antibody for signal production was successfully developed to quantitate target bacteria with a kinetic exclusion assay (KinExA™ 3000 Instrument). It directly measured the concentration of unliganded antibody separated from the equilibrated mixture of antibody and bacteria through a 0.2 μm polyethersulfone membrane, enabling it to quantify the concentration of bacteria. The novel method demonstrated the qualities of rapidness, sensitivity, high accuracy and reproducibility, and ease to perform. Detection of *Pseudomonas aeruginosa* and *Staphylococcus aureus* was accomplished with low detection limits of 4.10×10^6 and 5.20×10^4 cells/mL, respectively, with an assay time of less than 15 min. The working ranges for quantification were 4.10×10^6 to 1.64×10^{10} cells/mL for *P. aeruginosa*, and 5.20×10^4 to 1.04×10^9 cells/mL for *S. aureus*. It yielded an assay with at least 10-fold greater sensitivity than ELISA and could correctly assess the concentration of predominant bacterium spiked in the mixture of *P. aeruginosa* and *S. aureus*. With this reliable platform, the average amount of antibody bound by one cell in the maximum capability could be further provided: $(1.6–2.5) \times 10^5$ antibodies for one *P. aeruginosa* cell and $(2.2–2.7) \times 10^8$ antibodies for one *S. aureus* cell. The KinExA system is flexible to determine different kinds of bacteria conveniently by using anti-mouse IgG as the same immobilizing agent. However, a higher specificity of the antibodies to the target bacteria will be required for the use of this system with higher detection sensitivity.

Keywords: Bacterial quantification; Immunoassay; KinExA; *Pseudomonas aeruginosa*; *Staphylococcus aureus*

1. Introduction

Rapid detection and quantification of target microbes and potentially pathogenic bacteria is essential for clinical diagnostics, food analysis and environmental monitoring. The conventional methods based on cultivation techniques increasingly reveal their limitations; they are time-consuming, difficult to use for treatment with non-cultivable bacteria, and highly labor-intensive. Conversely, molecular methods are able to achieve high sensitivity and specificity without the need for cultivation or additional confirmation, among which immunoassay and detection via polymerase chain reaction (PCR) are the most established and important approaches for use in bacterial identification and detection (Deisingh and Thompson, 2002; Rompré et al., 2002). Although highly sensitive and specific, PCR tests have limited capability for accurate bacterial quantification, because the cell numbers assigned to a certain amount of target genes varies by one order of magnitude depending on the growth phase and species (Amann and Ludwig, 2000; Sanz et al., 2004). Immunological methods, based on the specific recognition and high affinity between antibodies and antigens, have been developed as an efficient and practical alternative for the determination of pathogenic bacteria (Ivnitski et al., 1999; Delehanty and Ligler, 2002; Chemburu et al., 2005). Immunological methods include enzyme immunoassay (EIA), immunofluorescence assay (IFA), immunomagnetic separation

* Corresponding author. Tel.: +81 426 37 2419.
** Corresponding author. Tel.: +86 10 6279 4771; fax: +86 10 6279 4771.
E-mail addresses: saiki@bs.teu.ac.jp (H. Saiki), xhxing@tsinghua.edu.cn (X.-H. Xing).

0956-5663/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bios.2006.09.033

technique (IMS) and immunosensors (Rompré et al., 2002; Deisingh and Thompson, 2004). In recent years, much effort has been devoted to decreasing the analysis time and improving the sensitivity and selectivity through the modification or combination of various techniques (Yasui and Yoda, 1997; Zhao et al., 2004; Chemburu et al., 2005; Endo et al., 2005). However, there is still heightened interest in developing rapid and reliable methods of bacterial determination, especially with respect to real-time, simple and accurate quantification with high sensitivity and reproducibility (Rishpon and Ivnitski, 1997; Croci et al., 2001; Babacan et al., 2002).

Kinetic Exclusion Assay (KinExA), a new technique available since the 1990s, with the ability to measure true equilibrium binding affinity and kinetics in solution phase, is one of the most accurate and sensitive avenues for immunoassay. It is a computer-controlled flow fluorometer in which equilibrated solutions of antigen and antibody (Ab) complex flow over a microbead pack with immobilized antigen or other Ab capture reagents. Detection of the free (not complexed with antigen) Ab captured in the microbead pack from the equilibrated solution is accomplished with a secondary species-specific fluorescent antibody (Drake et al., 2004). KinExA has been applied for the characterization of antibody–antigen interactions; it has shown high sensitivity (Blake et al., 1999a) and has achieved the analyte theoretical limit of detectability with an antibody (Ohmura et al., 2001). The platform has the sensitivity 10–1000-fold greater than ELISA (Blake et al., 2001), and close to or even better than surface plasma resonance (SPR) Biacore (Drake et al., 2004). Although KinExA has superior characteristics in immunoassay, it has not yet been used for bacterial quantification. Since KinExA can be implemented in an exclusion assay format, it can principally quantify the bacterial cells by measuring the free cell-specific antibodies in a bacteria-mixed-antibodies system.

Among a wide range of bacterial pathogens detected in the environment, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are two typical model pathogenic bacteria. *P. aeruginosa*, an aerobic gram-negative bacillus, is ubiquitous in nature and has been one of the top three causes of opportunistic human infections during the past century (Stover et al., 2000). *S. aureus*, an aerobic gram-positive coccus, is one of the most important bacterial pathogens for humans, producing enterotoxins which are a major concern in food hygiene (Delibato et al., 2005). Quantification of these two bacteria is of great importance for human health protection. By using an Au-electrode-based immunosensor, *P. aeruginosa* has been detected in the range of 2×10^6 to 1×10^8 cells/mL, with a limit of detection (LOD) of 1×10^5 cells/mL (Bovenizer et al., 1998). Several immunological methods have been reportedly used to detect *S. aureus*, such as enzyme immunoassay (EIA), TECRA (TM) visual immunoassay, ELISA and enzyme-linked immunomagnetic electrochemical assay (ELIME) (Rishpon and Ivnitski, 1997; English et al., 1999; Fukuda et al., 2000; Delibato et al., 2005). LODs as low as 10^3 CFU/mL of *S. aureus* have been achieved (Fukuda et al., 2000; Delibato et al., 2005).

In this paper, KinExA was employed to establish a reliable and rapid method for bacterial detection and quantification, by using *P. aeruginosa* and *S. aureus* as the model microbial strains.

2. Materials and methods

2.1. Strains and cultivation

P. aeruginosa (ATCC 33358, Manassas, VA, USA) and *S. aureus* (ATCC 29740) was cultivated in trypticase soy broth (BD Diagnostic Systems 211768, Sparks, MD, USA) at 26 °C, and in nutrient broth (BD 234000) at 37 °C, respectively.

2.2. Antibodies and chemicals

Monoclonal mouse *P. aeruginosa* antibody and *S. aureus* antibody were all from Biogenesis Ltd. (7889–9007, 0200–0405, Poole, England). Cy5-conjugated $F(ab')_2$ fragment of goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories (115-176-003, West Grove, PA, USA). Goat anti-IgG (Fc) of mouse polyclonal antibody was obtained from Chemicon International Inc. (API27, Temecula, CA, USA). Anti-mouse IgG-HRP (horseradish peroxidase) antibody was purchased from R&D systems (HAF007, Minneapolis, MN, USA). DAPI (4′,6-diamidino-2-phenyl-indol) was from Sigma Chemical (St. Louis, MO, USA).

The phosphate-buffered saline solution (PBS) consisted of 140 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 1.5 mM NaN_3 (0.01%), adjusted to pH 7.40 with NaOH or HCl if needed. All these reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). The BSA–PBS buffer consisted of 1 mg/mL bovine serum albumin (BSA; Sigma Chemical) in the PBS buffer.

2.3. Detection of bacterial optical density (OD)

The OD of bacteria was read at 600 nm (as OD_{600})by a UV spectrophotometer (U0080D, Hitachi, Tokyo, Japan).

2.4. Total direct counts (TDC) of bacteria

Aliquots of bacteria were filtered onto 0.2 μm black polycarbonate membranes (Nuclepore, Whatman Inc., Clifton, NJ, USA). After reaching dryness, the cells on the membrane were stained by 1 μm/mL DAPI for 5–10 min and enumerated with a confocal laser scanning microscope (Zeiss LSM 510, Carl Zeiss, Inc., Thornwood, NY, USA). For one measurement, more than ten microscopic fields were counted, containing at least 100 cells in each field.

2.5. Sample preparation of bacteria

Aliquots of *P. aeruginosa* or *S. aureus* were collected from the cultivated broth. After being re-suspended twice in PBS buffer (pH 7.40) by repetition of centrifugation (6000 rpm for 10 min, 4 °C) and supernatant removal, the bacteria were eventually suspended and diluted in BSA–PBS buffer. Every sample was uniformly suspended by a vortex without any macroscopic flocs, and stored at 4 °C prior to use. Ab was added into every bacterial sample within 24 h after the collection. Then, Ab and bacteria were incubated by rocking at 24 °C for 17 h (*P.*

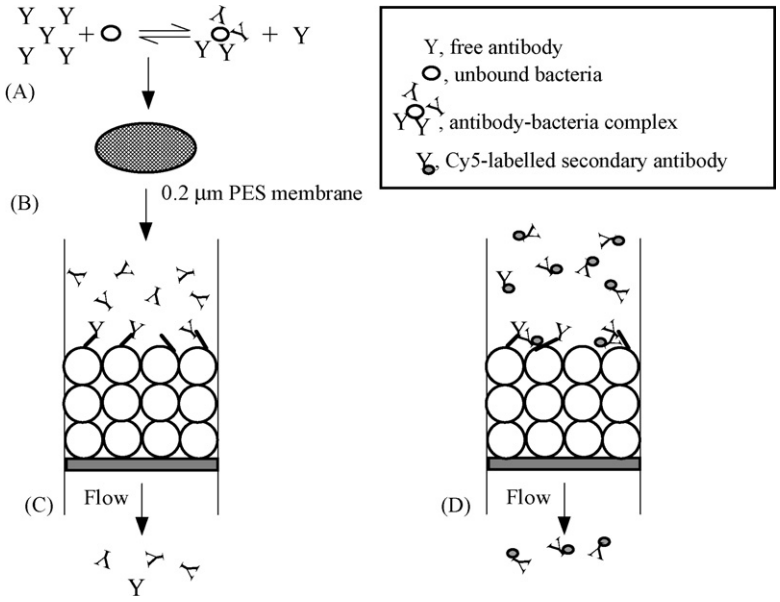

Fig. 1. Schematic illustration of KinExA immunoassay. (A) Free antibody, unbound bacteria, and antibody–bacteria complex were mixed thoroughly to reach equilibrium. (B) The equilibrated mixture was filtered by a BSA–PBS-prewetted 0.2 μm PES membrane, and the free antibody was passed through. (C) Drawn through the flow cell of KinExA, a portion of the free antibody was captured by the anti-IgG (Fc) of mouse antibody immobilized on the PMMA microbeads. Then a buffer wash step was applied to remove excess soluble free antibody. (D) Cy5-labeled anti-mouse IgG secondary antibody was sequentially passed through the observation flow cell and excess unbound fluorescent antibody was removed with two buffer wash steps.

aeruginosa) or 12 h (*S. aureus*) to reach equilibrium. Sequentially, the mixture was filtered by a 0.2 μm polyethersulfone (PES) membrane (Puradisc25AS, Whatman Inc.). The membrane was pre-wetted by BSA–PBS buffer and sample solutions were mixed thoroughly before every filtration. The filtration time of every sample was restricted to within 15 min, and the concentration of free Ab in the supernatant was detected by KinExA (Fig. 1). The loss of free Ab by filtration was less than 2%, indicating that no Ab absorption occurred by the PES membrane.

2.6. KinExA immunoassay

A KinExA™ 3000 (Sapidyne Instruments, Boise, ID, USA) instrument was utilized as the immunoassay platform. Details of the KinExA™ 3000 instrument have been described elsewhere (Blake et al., 1996, 1999a; Khosraviani et al., 2000).

The format for the KinExA-based assay employed in this study is illustrated schematically in Fig. 1. The free antibodies, separated from the antibody and bacteria mixture by a membrane, were drawn through the flow cell of the KinExA instrument. A portion of the free antibodies (mouse IgG) were captured by the mouse anti-IgG (Fc) Ab immobilized on the PMMA microbeads and labeled subsequently by the trace fluorescent Ab (Cy5-conjugated anti-mouse IgG secondary Ab). Under the same operating conditions, the generated signals were proportional to the concentrations of the free primary Abs (Ohmura et al., 2001). By this means, fixing the initial concentration of anti-bacteria Ab but serially diluting the bacteria, an immunoassay curve of signal percentage related to the bacterial concentration could be achieved. The signal percentage of the samples was estimated using Eq. (1):

$$\text{signal percentage}(\%) = \frac{\text{signal} - \text{signal}_{\text{NSB}}}{\text{signal}_{100\%} - \text{signal}_{\text{NSB}}} \times 100\% \quad (1)$$

where signal is the directly detected signal of the sample, $\text{signal}_{\text{NSB}}$ representing the non-specific binding, is the signal of only BSA–PBS buffer in the absence of free Ab, and $\text{signal}_{100\%}$ is the signal generated by the total free Ab binding sites at the initial Ab concentration without complexing with any antigen. The value of $(\text{signal}_{100\%} - \text{signal}_{\text{NSB}})$ should be nearly or more than one volt achieved by KinExA instrument, which can be optimized by adjusting the other assay parameters. Given that all the measurements were implemented after equilibrium, the curve represented the dissociation constant curve (K_{D} curve) of primary Ab and bacteria (Blake et al., 1996). According to the K_{D} curve, the bacterial concentrations could be elicited by corresponding signals.

All the experiments conducted by KinExA were operated at 24 °C.

2.6.1. Immobilization on PMMA beads

Poly(methyl methacrylate) (PMMA) beads (100-μm diameter, Sapidyne Instruments, 440107) were used as the solid phase material and were coated with goat anti-IgG (Fc) of mouse polyclonal Ab to capture the mouse IgG primary Ab. By suspending 200 mg of the beads (dry weight) in 1 mL of PBS containing 200 μg of anti-IgG (Fc) mouse Ab, the mixture was rotated at 37 °C for 2 h. After settling and removal of the supernatant solution, 10 mg/L of BSA was added (to block nonspecific binding) and the beads were rotated at 37 °C for another hour. Coated beads were stored at 4 °C and diluted to 30 mL with PBS prior to use.

2.6.2. Assay parameters

For the *P. aeruginosa* experiment, the *P. aeruginosa* Ab was fixed as 32.5 pM to mix with different concentrations of bacteria in the sample preparation. After equilibrium and filtration, the samples were perfused through the PMMA beads at 0.25 mL/min for 150 s, then followed by 30 s of PBS, 240 s of 1 μg/mL anti-mouse IgG Cy5-Ab, and finally a two-stage washing with PBS (90 s at 0.25 mL/min and 120 s at 1.0 mL/min). For the *S. aureus* experiment, the concentration of primary Ab was 168 pM. The flow time for the samples was 240 s; other parameters were the same as in the *P. aeruginosa* experiment. Bacterial immunoassay curves were constituted of the signal percentage related to the bacterial concentration.

2.6.3. Cross-reactivity evaluation

The concentrations *of P. aeruginosa* and *S. aureus* were controlled at the same optical density. Signals of the mixtures of 32.5 pM *P. aeruginosa* Ab or 168 pM *S. aureus* Ab with *P. aeruginosa* or *S. aureus* strain were measured by KinExA. The cross-reactivity was evaluated by taking the specific binding as 100% reaction efficiency. For instance, the cross-reactivity of *P. aeruginosa* Ab with *S. aureus* was calculated by Eq. (2):

$$\frac{\text{signal}_{\text{Pa–Ab}} - \text{signal}_{\text{Pa–Ab\&Sa}}}{\text{signal}_{\text{Pa–Ab}} - \text{signal}_{\text{Pa–b\&Pa}}} \times 100\% \quad (2)$$

$\text{signal}_{\text{Pa–Ab}}$ stands for the signal of 32.5 pM *P. aeruginosa* Ab alone without any bacteria; $\text{signal}_{\text{Pa–Ab\&Sa}}$ and $\text{signal}_{\text{Pa–Ab\&Pa}}$ represent the signals *of P. aeruginosa* Ab equilibrated with *S. aureus* and *P. aeruginosa*, respectively.

2.6.4. Bacterial determination in the mixture of two-species strains

Cells of *P. aeruginosa* and *S. aureus* were mixed with different OD_{600} ratios of 100:1, 1:1 and 1:10. 32.5 pM *P. aeruginosa* Ab or 168 pM *S. aureus* Ab was added into the cell mixtures respectively to determine the concentrations of *P. aeruginosa* and *S. aureus* according to the respective calibration curves. The detection results were compared with the spiked concentrations.

2.7. ELISA procedure

An amended protocol according to the original one of *P. aeruginosa* IgG ELISA Kit (Genesis Diagnostics, GD07, Cambridgeshire, UK) was used in this study, as following: (1) the bacterial samples were prepared according to the protocol described in Section 2.5, except the Ab was initially fixed as 650 pM; (2) 100 μL of each filtrate of free Ab was dispensed into the microplates pre-coated with *P. aeruginosa* lipopolysaccharides at room temperature (RT); (3) after incubating for 30 min, the well contents were decanted and the wells were washed three times using the Wash Buffer in the kit; (4) 100 μL of anti-mouse HRP Ab was dispensed into each well and incubated for another 30 min at RT; 5) after the well contents were discarded, the wells were washed four times using the Wash Buffer; (6) 100 μL of TMB (3,3′,5,5′-tetramethylbenzidine) Substrate in the kit was rapidly dispensed into each well; (7) after 10 min reaction, 100 μL of Stop Solution in the kit was added into each well and the absorbance was read at 450 nm within 10 min.

3. Results and discussion

3.1. Immunoassay curves for P. aeruginosa and S. aureus by KinExA

To investigate the effects of bacterial growth status on immunoassay, bacteria were sampled from four different growth phases to gain the respective immunoassay curves: (1) before the logarithmic growth phase (LGP), (2) in the middle of the LGP, (3) near the end of the LGP, and (4) in the stationary phase. Immunoassay curves of *P. aeruginosa* and *S. aureus* are shown in Figs. 2 and 3, respectively.

Figs. 2 and 3 clearly show that all the curves for *P. aeruginosa* or *S. aureus* sampled at different growth phases were consistent, indicating that the bacterial growth state had little effect on the immunoassay. This was probably because *P. aeruginosa* Ab is specific to the outer membrane protein *of P. aeruginosa*, and *S. aureus* Ab specifically recognizes the peptidoglycan of *S. aureus*, protein A; these components are intrinsic features of the bacterial cell wall, independent of the growth phases.

Based on one of the immunoassay curves for *P. aeruginosa* or *S. aureus* (Figs. 2 and 3), bacterial quantitative calibration curves were attained by regressing the linear range of the KinExA signal percentage with the logarithm of the bacterial OD_{600} or TDC (total direct counts). The results are shown in Fig. 4A and B. The KinExA signals and the logarithms of cell concentrations showed a very good linear relationship ($R^2 > 0.98$). Of all the immunoassay curves (Figs. 2 and 3), the standard deviations were 0.01–7.15% within single runs and 1.23–9.33% among

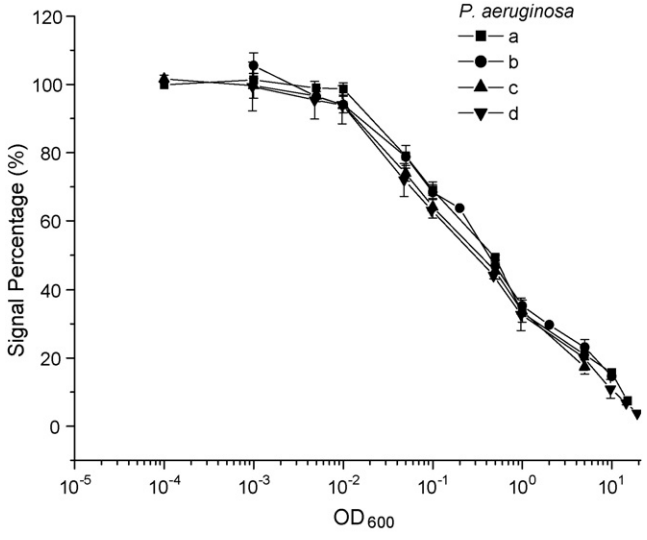

Fig. 2. KinExA immunoassay curves of *P. aeruginosa* obtained from different growth phases: (a) before the logarithmic growth phase (LGP) (2.3 h cultivation), (b) in the middle of the LGP (8.2 h cultivation), (c) near the end of the LGP (21 h cultivation), and (d) in the stationary phase (35.8 h cultivation). Each point represents the mean of duplicate measurements; error bars represent the standard deviation.

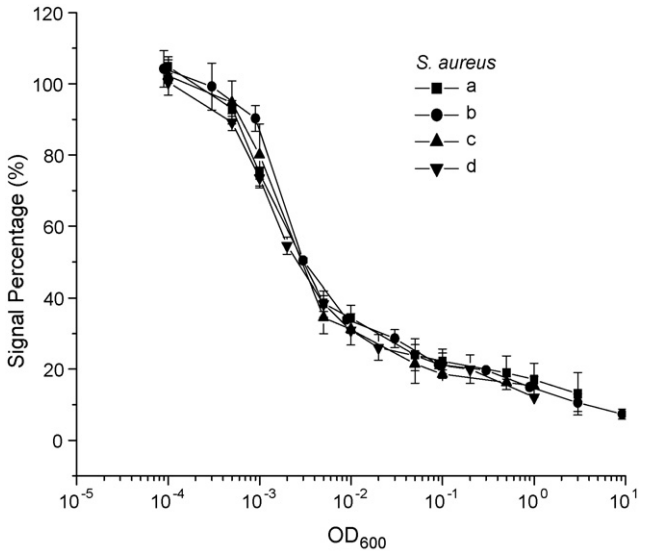

Fig. 3. KinExA immunoassay curves of *S. aureus* obtained from different growth phases: (a) before the logarithmic growth phase (LGP) (2 h cultivation), (b) in the middle of the LGP (5 h cultivation), (c) near the end of the LGP (15 h cultivation), and (d) in the stationary phase (23 h cultivation). Each point represents the mean of duplicate measurements; error bars represent the standard deviation.

four runs, respectively. These results indicated an acceptable level of reproducibility and precision of the newly developed method for bacterial quantification.

From the results shown in Figs. 2–4, the characteristics of bacterial determination by KinExA are summarized in Table 1. The limits of detection (LOD) of *P. aeruginosa* and *S. aereus* were 4.10×10^6 and 5.20×10^4 cells/mL, respectively, which was comparable with the lowest LOD by immunological methods reported elsewhere, i.e., 10^5 cells/mL for the former (Bovenizer et al., 1998) and 10^3 cells/mL for the latter (Rishpon and Ivnitski, 1997). The quantification range of *P. aeruginosa* by KinExA was 4.10×10^6 to 1.64×10^{10} cells/mL ($R^2 = 0.9903$), two magnitudes larger than the results reported by Bovenizer et al. (1998). Although there were two different slopes of the quantitative calibration curve for *S. aereus* and the critical point was around 5×10^{-3} OD_{600}, the quantitative range for *S. aereus* was actually 5×10^{-4} to 10 OD_{600}, or 5.20×10^4 to 1.04×10^9 cells/mL, with two regressive lines combined. It has been demonstrated that no disturbance of the equilibrium of Abs and bacteria will occur by the means of filtration used in this study (data not shown), and some flocs would be observed in the bottom of sample after long time settlement (>12 h). Therefore, the reason for the different slopes in different ranges of *S. aereus* concentrations was assumed to be due to the aggregation of bacterial cells at higher concentrations, which would decrease the active cellular surface area for binding with Abs.

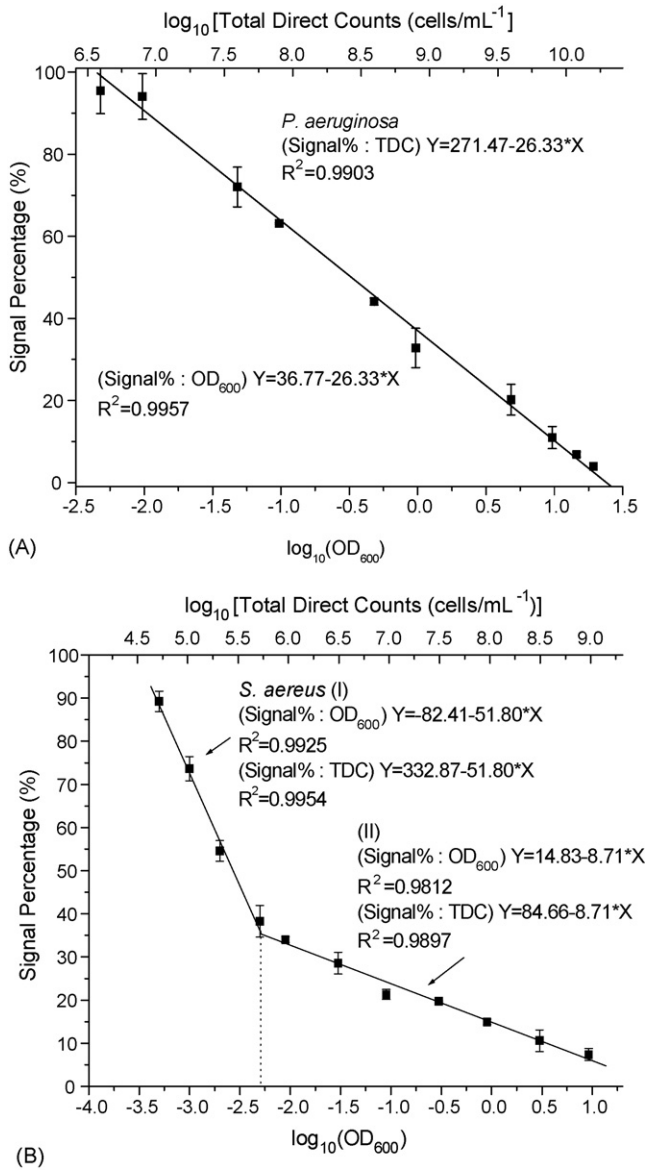

Fig. 4. Quantitative calibration curves for *P. aeruginosa* (A) and *S. aereus* (B) in terms of OD_{600} and total direct counts (TDC). Each point represents the mean of duplicate measurements; error bars represent the standard deviation.

Table 1
Characteristics of bacterial determination by KinExA

	Limit of detection		Working range for quantification	
	OD_{600}	TDC (cells/mL)	OD_{600}	TDC (cells/mL)
P. aeruginosa	5.0×10^{-3}	4.10×10^6	0.005–20	4.10×10^6 to 1.64×10^{10}
S. aereus	5.0×10^{-4}	5.20×10^4	5.0×10^{-4} to 5.0×10^{-3} 5.0×10^{-3} to 10	5.20×10^4 to 5.20×10^5 5.20×10^5 to 1.04×10^9

The detection limit is determined as the bacterial concentration which produces 95% signal value of $signal_{100\%}$.

3.2. Average amount of Ab bound to one bacterial cell

From every point in the calibration curves (Fig. 4), the amount of antibodies bound to bacterial cells could be obtained. Given that every bacterial cell had the same density of receptors, the average amount of Ab bound to one cell could be evaluated by Eq. (3):

$$\frac{[\mathrm{Ab}]_{\mathrm{initial}} \times (1 - i/100)}{[\mathrm{bacteria}]_{\mathrm{signal}i\%}} \quad (3)$$

$[\mathrm{Ab}]_{\mathrm{initial}}$ means the original concentration of primary Ab; $[\mathrm{bacteria}]_{\mathrm{signal}\,i\%}$ stands for the bacterial concentration at the signal percentage of $i\%$, and $[\mathrm{Ab}]_{\mathrm{initial}} \times (1 - i/100)$ is the total amount of Abs bound to the bacterial cells at signal $i\%$. For instance, at signal 30%, 70% of 32.5 pM *P. aeruginosa* Ab had bound to 1.50×10^9 *P. aeruginosa* cells/mL according to Fig. 4A. That meant one *P. aeruginosa* cell had bound 1.52×10^{-20} M antibodies on average, equal to about 9.15×10^3 antibodies/cell. By this means, the average amount of Ab bound to one bacterial cell could be estimated at every point within the whole quantitative calibration range, and the maximum value among those results represented the maximum capability of one cell binding with Abs on average. Through the linear range of the *P. aeruginosa* calibration curve from 0.01–20 OD_{600}, the maximum amount of Ab bound by one cell was found to be 4.20×10^{-19} M/cell at 1.1×10^{-2} OD_{600}. This meant that one *P. aeruginosa* cell could bind a maximum of 2.53×10^5 Abs. In the same way, the maximal binding capacity of Abs for *S. aereus* cells was estimated to be 2.67×10^8 Abs/cell at 8.2×10^{-4} OD_{600} within the range of 5.0×10^{-4} to 5.0×10^{-3} OD_{600}, while 1.28×10^8 Abs/cell at 5.0×10^{-3} OD_{600} within the range of 5.0×10^{-3} to 10 OD_{600}. This result supported the previous hypothesis that aggregation of *S. aereus* in high concentrations led to the reduction of the amount of Ab bound per cell. The maximal binding capacity of Ab for one *S. aereus* cell was nearly 1000 times higher than that for one *P. aeruginosa* cell, which indicated decreasing of the active cellular surface would result in a more remarkable effect on *S. aereus* binding with Abs.

Taking all the immunoassay curves for bacteria at different growth phases into account, the maximum binding capabilities of bacteria were estimated: $(1.6–2.5) \times 10^5$ Abs per *P. aeruginosa* cell and $(2.2–2.7) \times 10^8$ Abs per *S. aereus* cell, as shown in Table 2. The narrow range of the estimated values further supported the result that the characteristics of receptors on cellular surface were independent of the bacterial growth phases.

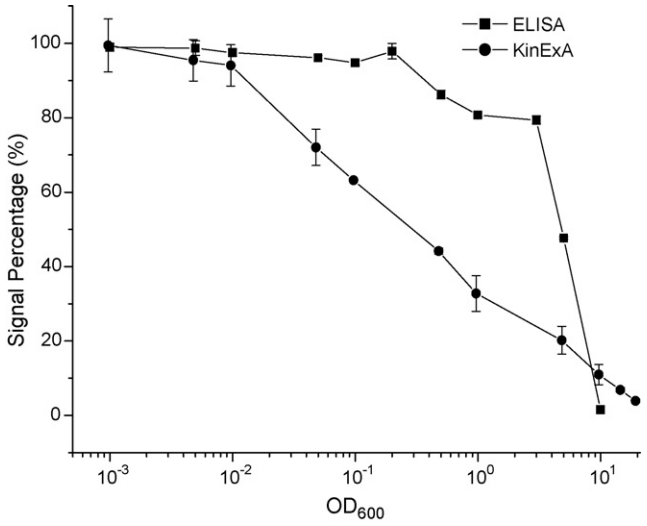

Fig. 5. Quantitative curves *of P. aeruginosa* detected by ELISA and KinExA. Each point of KinExA immunoassay curve represents the mean of duplicate measurements, while each point of ELISA curve is the average of triplicate determinations. Error bars depict the standard deviation.

3.3. Detection of P. aeruginosa by ELISA compared with KinExA

In this research, same principle was employed in the *P. aeruginosa* detection by ELISA as that used for KinExA, as described in Section 2. The intensity of luminescence, which was produced by the enzyme conjugate and substrate, was proportional to the concentration of free Abs in the samples. Accordingly, a relationship between the relative luminescence intensity and bacterial concentration could be obtained by ELISA, similar to the test conducted by KinExA (Fig. 5).

From the results of *P. aeruginosa* detected by ELISA, the LOD was 0.4 OD_{600} and the calibration range was only 0.1–10 OD_{600}, neither of which was comparable with the results of KinExA. KinExA had at least 10-fold more sensitivity than the ELISA technique, which agreed with a previous report of 10–1000-fold greater sensitivity (Blake et al., 2001).

The superior sensitivity and quantitative accuracy of KinExA compared with ELISA is a co-functional result of KinExA inherent advantages. For a primary antibody, as its concentration is decreased, the antigen concentration-response curves will be shifted to lower concentrations, and the dynamic ranges of signals broadened until becoming maximal when the primary antibody concentration equals its K_D value. After that, the sensitivity and the dynamic range of the assay will be unchanged even if the primary antibody concentration is much more lower

Table 2
Estimated maximum binding capability of bacteria

	Working range for quantification (OD_{600})	Maximum amount of Ab per cell ($cell^{-1}$)	OD_{600} range of maximum binding capability
P. aeruginosa	0.005–20	$(1.57–2.53) \times 10^5$	$(1.1–1.9) \times 10^{-2}$
S. aereus	5.0×10^{-4} to 5.0×10^{-3}	$(2.19–2.67) \times 10^8$	$(8.2–12) \times 10^{-4}$
	5.0×10^{-3} to 10	$(1.25–1.32) \times 10^8$	5.0×10^{-3}

(Ohmura et al., 2001). If an antibody has smaller K_D for the antigen, it will directly improve the sensitivity of the assay for the higher specificity. For ELISA, higher concentration of primary antibody than K_D value is usually demanded due to the insensitivity of signal detection. However, by using KinExA, a very low concentration of primary Ab near or below its K_D can still gain enough strength of signal by the accumulation from continuous sample flow. This permits the achievement of the theoretical detectable limit in quantitative determination of the assay, which is limited by the affinity of antibody and antigen, but not by the KinExA instrument. In the KinExA system, theoretical antigen concentration-response curves can be obtained as experimental results (Ohmura et al., 2001). In present study, 650 pM was the lowest Ab concentration for ELISA to get the reliable calibration curve, 20 times as that for KinExA. This limited the sensitivity of ELISA as compared with KinExA (it's indicated that 32.5 pM was closer to the K_D value of the antibody for *P. aeruginosa* than 650 pM was). In addition, the larger surface area for sample capture by bead column (approximately 260 mm^2) than does the ELISA microtiter plate (64 mm^2 per well) (Blake et al., 1999b, 2001) and arbitrarily high concentration of the effective solid phase, avoids competition with the immobilized reagent and improves the linearity of the response, leading to a quantitative response with acceptable signal-to-noise characteristics. All these factors provided KinExA performance of high sensitivity, wide dynamic range and high quantitative accuracy.

When compared to ELISA or PCR assays in terms of number of experimental steps and manipulations involved, KinExA has high simplicity and detection speed with few-to-no hands-on steps, which makes the measurements of a single sample in 2–15 min possible (Blake et al., 1999a; Ohmura et al., 2001).

In this study, the KinExA platform was employed to quantify bacteria by measuring the free anti-bacteria antibody excluded from the equilibrated solution of Ab and bacteria that contained Ab, bacteria and Ab–bacteria complex. Anti-mouse secondary antibody was immobilized on the microbeads to capture the different primary antibodies, but not antigen-like reagents such as lipopolysaccharides *of P. aeruginosa*. This enhanced the specificity range of the immobilized phase for the targets, which made the bacterial detection much simpler. Since the secondary Ab immobilized on the microbeads can capture all kinds of mouse primary antibodies, the same beads can be used for several systems to detect different kinds of bacteria.

3.4. Cross-reactivity

Cross-reactivity of monoclonal antibodies with different strains of gram-negative bacteria or several gram-negative and gram-positive bacteria is not infrequent (Hübner et al., 1992; Levasseur et al., 1992; Haralambievaa et al., 2001). Actually, this is one of the most critical drawbacks in the practical application of immunological methods for bacterial determination.

In this study, the cross-reactivity of *P. aeruginosa* Ab with *S. aureus* or *S. aureus* Ab with *P. aeruginosa* was examined (Table 3). The cross-reactivity *of P. aeruginosa* Ab with *S. aureus* was 35%, twice as high as that of *S. aureus* Ab with *P. aeruginosa.*

Table 3
Cross-reactivity of two Abs on *P. aeruginosa* or *S. aureus*

Reaction percentage (%)	*P. aeruginosa*	*S. aureus*
P. aeruginosa Ab	100	35
S. aureus Ab	17	100

Table 4
Bacterial determinations in the mixtures of *P. aeruginosa* and *S. aureus* at different ratios of cell concentration

Ratio of the detected concentration to the spiked one	OD_{600} ratio of *P. aeruginosa* to *S. aureus*		
	0.1	1	100
P. aeruginosa	7.28 ± 1.50	1.24 ± 0.33	1.09 ± 0.01
S. aureus	1.06 ± 0.01	0.93 ± 0.03	1.48 ± 0.09

3.5. Bacterial determination in a mixture of two bacterial species

P. aeruginosa and *S. aureus* were mixed with different OD_{600} ratios of 100:1, 1:1 and 1:10, and the respective signals were detected by KinExA using 32.5 pM *P. aeruginosa* Ab or 168 pM *S. aureus* Ab. From the respective calibration curves (Figs. 4A, B), the concentrations of *P. aeruginosa* and *S. aureus* were evaluated, and the ratios of the detected concentration to the spiked one under different conditions are listed in Table 4.

As indicated in Table 4, when the OD_{600} ratio of *P. aeruginosa* to *S. aureus* was below 1, a large detection deviation *of P. aeruginosa* would be produced due to the high cross-reactivity *of P. aeruginosa* Ab to *S. aureus*. However, when the OD_{600} ratio of *P. aeruginosa* to *S. aureus* was equal to or higher than 1, the detected concentration of *P. aeruginosa* was close to the spiked one, which meant that, the cross-reactivity was not significant. Similar results were found for *S. aureus*; i.e., the detected *S. aureus* concentration had good accordance with the spiked concentration when the OD_{600} of *S. aureus* was equal or greater than that of *P. aeruginosa*. Less distinct deviation was found in the determination of *S. aureus*, even when the OD_{600} of *S. aureus* was 100 times less than that of *P. aeruginosa*. This was probably because of the lower cross-reactivity of *S. aureus* Ab to *P. aeruginosa* (Table 3) and the stronger affinity of *S. aureus* Ab binding to *S. aureus* cells compared to *P. aeruginosa*.

4. Conclusions

A new method based on the KinExA format was developed and characterized, which displayed outstanding analytical characteristics for bacterial detection and quantification. It exhibited three noteworthy properties compared with other immunological methods: (1) its simplicity for manipulation due to few-to-no steps and no need for modification of the primary antibody; (2) high sensitivity and quantitative accuracy of data acquisition with low error; (3) its convenience and universality for different bacterial determination by using anti-mouse IgG as the immobilizing agent to capture all kinds of mouse primary antibodies.

For the first time, KinExA has been demonstrated as a reliable and promising alternative for bacterial determination. However, like other immunoassay methods, the achievable minimum limit of detection of this method is limited by the affinity of the antibody with antigen. By increasing the specificity of the antibody to the target bacteria, detection sensitivity would be enhanced by using KinExA, and antibodies having little-to-no cross-reactivity are potential candidates in the application for bacterial determination in complex communities.

Acknowledgements

The authors thank Hiroshi Ryu and Kayo Fujii for assistance with the bacterial cultivation. This research was financially supported by the National Institute of Advanced Industrial Science and Technology and the Central Research Institute of the Electric Power Industry.

References

Amann, R., Ludwig, W., 2000. FEMS Microbiol. Rev. 24, 555–565.
Babacan, S., Pivarnik, P., Letcher, S., Rand, A., 2002. J. Food Sci. 67, 314–320.
Blake, D.A., Chakrabarti, P., Khosraviani, M., Hatcher, F.M., Westhoff, C.M., Goebel, P., Wylie, D.E., Blake II, R.C., 1996. J. Biol. Chem. 271, 27677–27685.
Blake, D.A., Jones, R.M., Blake II, R.C., Pavlov, A.R., Darwish, I.A., Yu, H., 2001. Biosens. Bioelectron. 16, 799–809.
Blake II, R.C., Pavlov, A.R., Blake, D.A., 1999a. Anal. Biochem. 272, 123–134.
Blake II, R.C., Pavlov, A.R., Blake, D.A., 1999b. Anal. Biochem. 292, 123–134.
Bovenizer, J.S., Jacobs, M.B., O'Sullivan, C.K., Guilbault, G.G., 1998. Anal. Lett. 31, 1287–1295.
Chemburu, S., Wilkins, E., Abdel-Hamid, I., 2005. Biosens. Bioelectron. 21, 491–499.
Croci, L., Delibato, E., Volpe, G., Palleschi, G., 2001. Anal. Lett. 34, 2597–2607.
Deisingh, A.K., Thompson, M., 2002. Analyst 127, 567–581.
Deisingh, A.K., Thompson, M., 2004. Can. J. Microbiol. 50, 69–77.
Delehanty, J.B., Ligler, F.S., 2002. Anal. Chem. 74, 5681–5687.
Delibato, E., Bancone, M., Volpe, G., De Medici, D., Moscone, D., Palleschi, G., 2005. Anal. Lett. 38, 1569–1586.
Drake, A.W., Myszka, D.G., Klakamp, S.L., 2004. Anal. Biochem. 328, 35–43.
Endo, H., Hayashi, T., Ren, H., Muramatsu, H., 2005. Int. J. Environ. Anal. Chem. 85, 807–815.
English, D., Scalici, C., Hamilton, J., Destro, C., Jimenez, L., 1999. J. Rapid Meth. Autom. Microbiol. 7, 193–203.
Fukuda, S., Tatsumi, H., Igarashi, H., Igimi, S., 2000. Lett. Appl. Microbiol. 31, 134–138.
Haralambievaa, I., Iankova, I., Petrova, D., Ivanovab, R., Kamarinchev, B., Mitov, I., 2001. Diagn. Microbiol. Infect. Dis. 41, 99–106.
Hübner, I., Steinmetz, I., Obst, U., Giebel, D., Bitter-Suermann, D., 1992. Appl. Environ. Microbiol. 58, 3187–3191.
Ivnitski, D., Abdel-Hamid, I., Atanasov, P., Wilkins, E., 1999. Biosens. Bioelectron. 14, 599–624.
Khosraviani, M., Blake II, R.C., Pavlov, A.R., Lorbach, S.C., Yu, H., Delehanty, J.B., Brechbiel, M.W., Blake, D.A., 2000. Bioconjug. Chem. 11, 267–277.
Levasseur, S., Husson, M.O., Leitz, R., Merlin, F., Laurent, F., Peladan, F., Drocourt, J.L., Leclerc, H., Van Hoegaerden, M., 1992. Appl. Environ. Microbiol. 58, 1524–1529.
Ohmura, N., Lackie, S.J., Saiki, H., 2001. Anal. Chem. 73, 3392–3399.
Rishpon, J., Ivnitski, D., 1997. Biosens. Bioelectron. 12, 195–204.
Rompré, A., Servais, P., Baudart, J., de-Roubin, M.-R., Laurent, P., 2002. J. Microbiol. Meth. 49, 31–54.
Sanz, M., Lau, L., Herrera, D., Morillo, J.M., Silva, A., 2004. J. Clin. Periodontol. 31, 1034–1047.
Stover, C.K., Pham, X.Q., Erwin, A.L., Mizoguchi, S.D., Warrener, P., Hickey, M.J., Brinkman, F.S.L., Hufnagle, W.O., Kowalik, D.J., Lagrou, M., Garber, R.L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L.L., Coulter, S.N., Folger, K.R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G.K.-S., Wu, Z., Paulsenk, I.T., Reizer, J., Saier, M.H., Hancock, R.E.W., Lory, S., Olson, M.V., 2000. Nature 406, 959–964.
Yasui, T., Yoda, K., 1997. Appl. Environ. Microbiol. 63, 4528–4533.
Zhao, X., Hilliard, L.R., Mechery, S.J., Wang, Y., Bagwe, R.P., Jin, S., Tan, W., 2004. Proc. Natl. Acad. Sci. U.S.A. 101, 15027–15032.