ENHANCED CAPTURE OF BACTERIAL SPORES ON AN IMMUNOSENSOR, USING A FLOW-THROUGH ULTRASONIC STANDING WAVE SYSTEM

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Abstract

A technique for the concentration of bacterial spores on an immunosensor surface is described. The glass reflector of an ultrasonic standing wave chamber of acoustic pathlength of the order of ¼ wavelength was coated with immobilised anti-BG (*Bacillus subtilis* var. *niger*) spore antibodies. The spores were driven by radiation force towards a pressure node at the glass surface. Significant ultrasound enhancement of spore capture was detected by a fluorescence microscopy technique at concentrations of 10^4 per ml.

Introduction

The potential use of biological warfare agents is widely recognised. The best defence against such materials relies on the integration of a wide range of technologies. Timely warning and identification of the presence of such agents is key to this approach. A critical aspect, therefore, of improving protection against these materials is the development of faster and more reliable detection systems. There are a number of potential biological warfare agents, for example, *Bacillus anthracis*, *Yersinia pestis*, and variola virus, which are the pathogenic agents of anthrax, plague and smallpox, respectively.

Bacillus subtilis var. *niger* is used here as a non-pathogenic simulant for *Bacillus anthracis*. The methods which have been tested to detect *Bacillus subtilis* var. *niger* (BG) spores, include biochips and microarrays [1-2], Surface Plasmon Resonance [3] and PCR [4]. All methods mentioned, apart from the PCR, utilise antibodies to identify and capture the spores from suspension.

Procedures that enhance molecular [5] or bacterial approach towards and interaction with a defined immobilised matrix on solid surfaces are of particular relevance in optimising biosensor performance. A 1.5 MHz Flexural Plate Wave (FPW) device has been used as part of an immunoassay for the detection of breast cancer antigens, where the FPW served as a source of ultrasonic agitation or mixing, during the antibody incubation steps [6]. The antibody binding increased by a factor of three in comparison to the same assay in the absence of ultrasonic agitation.

Ultrasound at 3 MHz has been used to manipulate the movement and concentration of bacteria to remove them from suspension by concentrating them within the pressure node of the ultrasonic wave [7]. The movement to the pressure node is due to the radiation force on the particle. The radiation force (F_r) required to move the bacterial cell to the pressure node a distance of z, is proportion to the cells volume (V_c) compressibility (β_c) and density (ρ_c) [8], as is demonstrated by the equation below

$$F_r = -(0.5\pi P_0^2 V_c \beta_w \lambda^{-1}) \cdot \phi(\beta,\rho) \cdot Sin(4\pi z/\lambda) \qquad (1)$$

where P_0 is the peak acoustic pressure amplitude and λ is the wavelength of sound in the aqueous suspending phase, which has a compressibility β_w. The function of $\phi(\beta,\rho)$ equals

$$[(5\rho_c - 2\rho_w)/(2\rho_c + \rho_w) - (\beta_c/\beta_w)] \qquad (2)$$

where ρ_w is the density of the suspending phase.

An approach has recently been described in which a quarter wavelength ultrasonic standing wave chamber has been employed to deposit spores from flow onto a solid surface [9]. A 200 fold increase in capture was obtained at a suspension concentration of 6.6 x 10^6 spores/ml. In the present work spore capture has been examined as a function of acoustic pressure amplitude and flow rate through the chamber. The significant consequences for spore capture at 1 x 10^4 spores/ml are reported.

Materials & Methods

Materials

Phosphate buffer (PBS) tablets, Protein A, 3-aminopropyltriethoxysilane (APTS), Tween 20 and Auramine O were obtained from Sigma (Poole, UK), and glutaraldehyde from BDH. Anti-*Bacillus subtillis* var. *niger* (BG) spore antibody was supplied by Dstl (Porton Down, Wiltshire, UK). Decon 90 was purchased from Decon Laboratories Ltd (Hove, E.Sussex, UK).

Ultrasonic technique.

A flow chamber was prepared which consisted of three basic elements, a 3MHz ultrasonic transducer, a steel coupling plate and a glass reflector (a 1 mm thick conventional glass microsope slide), as described previously [10]. The transducer was separated from the suspending layer by means of a 3 mm (1½ λ in steel at 3 MHz) thick steel coupling plate, which was glued to the transducer. The transducer was a 30 x 30 mm, PZ 26 transducer from Ferroperm (Krisgard, Denmark). The back electrode was etched [11] to give a central 20 x 10 mm active transducer area. The glass reflector was held in place by a brass top-plate secured with screws, which had a 14 x 64 mm window to allow microscopic observation. The distance between the steel coupling plate and the glass reflector, and hence the depth of the suspending layer was 165 ± 5 μm. A gasket (internal dimensions 10 x 60 mm) was prepared from *Sylgard*® 182 silicone elastomer (Farnell, Leeds) and this surrounded the chamber cavity to maintain an air and water tight seal between the reflector and coupling plate. Spore suspensions were drawn through the chamber by a peristaltic pump (Gilson minipuls 3) at various flow rates, while washing at 6 ml/min.

Generation [10] and control [11] of the frequency and voltage applied to the transducer were as previously described.

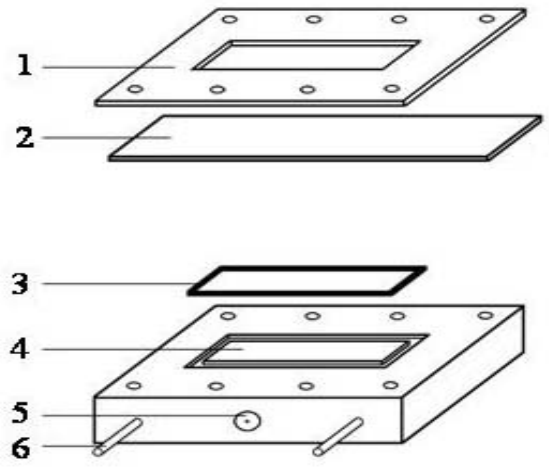


Figure 1: The chamber used, where 1 = the brass top-plate, 2 = the glass slide, 3 = the gasket, 4 = the coupling plate, 5 = BNC connector, 6 = in- and out-flow.

Preparation of slides.

The slides were immersed in a 2 % decon 90 solution prepared using warm water, for 20 min, to clean them before use. They were dried using a nitrogen gas stream, to evaporate the water of the slide. The surface was then modified using a 2 % solution of APTS in acetone, and left for 20 min, after which the slides were rinsed three times with acetone, and allowed to dry. Using an ImmEdge™ Pen (from Vector Labs Inc., Burlingame, CA, USA), a rectangle (15 x 10 mm) was outlined in the centre of the glass slide. Once this had dried, 100μl of a 1 % glutaraldehyde solution (prepared in deionised water) was pipetted within the rectangle, and left for 1 h in a petri dish, before being washed several times with PBS, and dried using N_2 gas. A petri dish was used at this stage, with protein A and the Ab incubation to control the evaporation of the respective solutions. At this point, the slides were incubated with 100 μl of 1 mg/ml Protein A in PBS, which was pipetted onto the selected area within the rectangle, for 1 h. The slides were then washed with PBS and dried using N_2 gas as previously described. After this, the slides were placed in a 0.05 % Tween 20 (in PBS) solution for 20 min to block any unbound aldehyde groups, and then washed with PBS and dried using N_2 gas. Following this, a 100 μl of 100 μg/ml solution of the anti-BG Ab was pipetted onto the area outlined by the rectangle drawn previously, and left overnight. The modified slide was then washed with PBS containing 0.05 % Tween 20 and left to dry, before use. The slides were stored in a dry state at 4 °C, until use. The immobilised antibody remained active for at least 3 days.

Assay method.

The slide was installed into the chamber. A BG spore suspension was pumped through the chamber at a constant flow rate, while an ultrasonic standing wave was produced at the predetermined frequency. The spore suspension was exposed for the periods described, allowing the capture of the spores by the immobilised antibody on the reflector surface. The chamber was then emptied. A solution of the fluorescent dye, Auramine O at 2 % (w/v) in PBS was added for 2 min. The chamber was then washed for 2 min with distilled water at a flow rate of 6 ml/min. Images of the fluorescently labelled captured spores were taken *in situ* using an Olympus BX41M microscope with a F-view camera, and analysed using Analysis® software (Soft Imaging System, Münster, Germany).

Results & Discussion

Optimisation of flow rate at a fixed pressure

A range of flow rates from 0 to 0.4 ml/min was used, with the BG spores concentration at 1 $x10^7$ /ml,and an exposure time of 5 min. At this concentration of spores, the maximum capture was at 0.2 ml/min (see fig. 2), where 7500 spores were observed per mm^2. The capture was 1900 spores per

mm^2 at the lower flow rate of 0.1 ml/min. The difference is even more significant in the absence of flow through the chamber, where only 220 spores per mm^2 were captured. The observation that a low flow rate will lead to lower capture is attributed to the lower number of spores passing through the chamber in a given time. The spores also tended to form clumps at the lower flow rate. At flow rates above the optimal, the residence time in the field is reduced and the yield falls. In all further experiments, the flow rate of 0.2 ml/min was used.

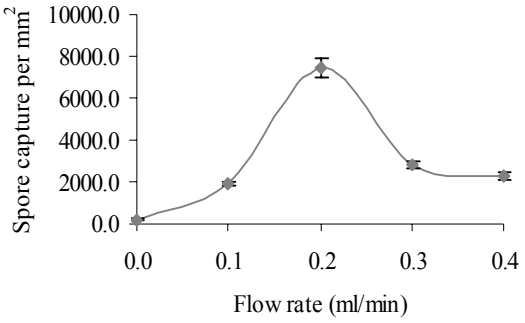


Figure 2: The effect of flow rate through the chamber upon the capture of BG spore (at 1 x10^7 spores/ml) at the reflector surface. Error bars indicate the standard error of the mean (n=3).

Optimisation of acoustic pressure at fixed flow rate.

The acoustic pressure amplitude in the standing wave is linearly proportional to the driving voltage applied to the chamber and can be controlled by varying that voltage. The radiation force driving the spores to the node is proportional to the pressure amplitude squared. If the pressure is too low, then the BG spores will not reach the node before they are carried away by the flow through the chamber. If the pressure is too high, the spores will form aggregates or clumps, which reduce the possibility of spore capture at the reflector surface. In our experiments, we found that the optimal pressure was 232 kPa (950 spores captured). At pressures below the maximum (150 kPa 25 spores captured) many spores were not reaching the reflector and at greater pressures (e.g. 312 kPa 200 spores captured), the spores were forming clumps (see fig 3). In all further experiments, an acoustic pressure of 232 kPa was used.

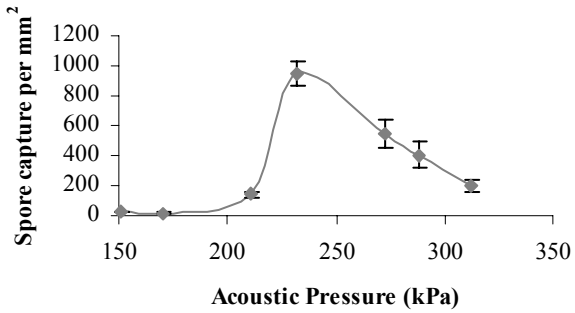


Figure 3: The influence of acoustic pressure upon the capture of BG spores (at 1 x 10^6 /ml and an exposure time of 1 min) at the reflector surface. Each value is the mean ± standard error of the mean (n=3).

Capture of BG spores at different concentrations.

The concentrations studied ranged from 1 x 10^6 to 1 x 10^4 spores/ml. At all concentrations, BG spores were captured at the reflector surface (see fig 4). The capture of BG spores between the concentration of 1 x 10^6 (2024 spores per mm^2) and 1 x 10^4 (225 spores per mm^2) was significantly greater than the background (101 and 55 spores/mm^2 respectively). At lower concentrations (less than 1 x 10^5 spores/ml), The spores tended to clump together, and move in a stream to another clump normal to the flow direction. The clumps formed at a distance of approximately 40 µm below the reflector. This clumping may explain the poor capture levels at these concentrations.

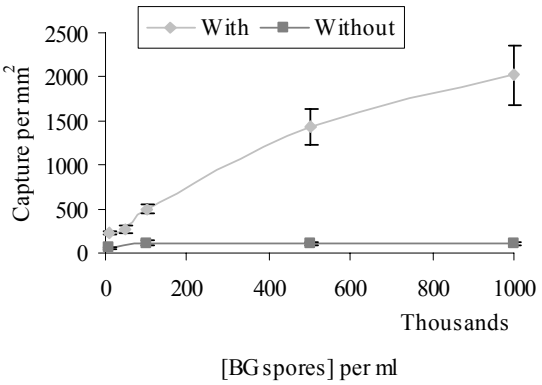
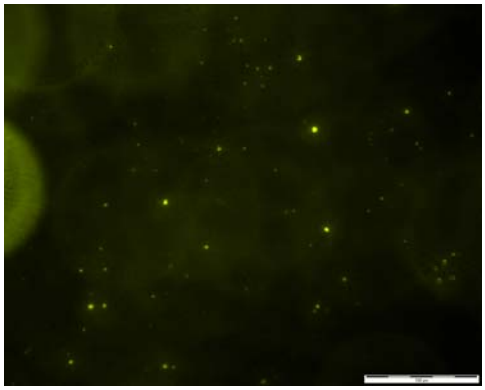


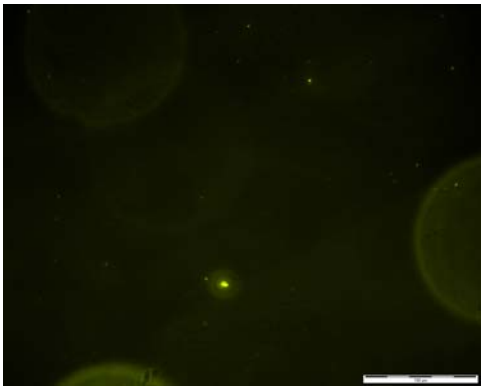
Figure 4: The capture of BG spores over the range of 1 x 10^6 to 1 x 10^4 /ml (1 min exposure time), using the optimised flow rate and acoustic pressure. Error bars indicate the standard error of the mean (n=3).

Table 1: Comparison of the ultrasound technique with other published methods for detection of *Bacillus subtilis* var. *niger* (LOD = lower limit of detection).

Method	Time (min)	LOD (spores/ml)	Ref
Fluorescence with ultrasound	6	10^4	Present work
ELISA	14	10^5	[1]
Fluorescence microarray	15	6.2 x 10^4	[2]
SPR	60	10^7	[3]



(a)



(b)

Figure 4: Images of the captured BG spore fluorescently labelled with Auramine O, with (a.) at 1 x 10^4 spores/ml and ultrasound applied, and (b.) at the same concentration, but with no ultrasound.

Conclusions

In the present work, the flow rate through the chamber and the acoustic pressure were optimised at 0.2 ml/min and 232 kPa, respectively, to maximise capture as a first step towards optimised capture. Ultrasound enhanced the capture of BG spores between the concentrations of 1 x 10^6 and 1 x 10^4 spores /ml, ranging from a 20- and 4-fold increase respectively. The level of detection reported here compares favourable with other published results (table 1).

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