Rapid detection and identification of bacteria: SEnsing of Phage-Triggered Ion Cascade (SEPTIC)

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Methods for the rapid detection and identification of bacteria are urgently needed. Here we describe a method that combines the specificity and avidity of bacteriophages with fluctuation analysis of electrical noise. The method is based on the massive transitory ion leakage that occurs at the moment of phage DNA injection into the host cell. The ion fluxes require only that the cells be physiologically viable (i.e. have energized membranes) and can occur within seconds after mixing the cells with sufficient concentrations of phage particles. To detect these fluxes, we have constructed a *nano-well*, a lateral, micrometer-sized capacitor of titanium electrodes with gap size of 150 nm, and used it to measure the electrical field fluctuations in microlitre (mm^3) samples containing phage and bacteria. In mixtures where the analyte bacteria were sensitive to the phage, large stochastic waves with various time and amplitude scales were observed, with power spectra approximately following a $1/f^2$ law from 1–10 Hz. Development of this SEPTIC (SEnsing of Phage-Triggered Ion Cascades) technology could provide rapid detection and identification of live pathogenic bacteria on the scale of minutes, with unparalleled specificity. The method has a potential ultimate sensitivity of 1 bacterium/microlitre (1 bacterium/mm^3).

Keywords: electronic noise, bacteriophage, biochip, fluctuations, nano-well, rapid bio-sensing

1. INTRODUCTION

Bacteriophages are the most numerous biological entities, estimated at 10^{31} in the biosphere, and are almost unimaginably diverse [1]. Phages exist with a wide range of host specificities, from narrow host range phages like λ, which infects only some strains of *E. coli,* to generalists like P1, which can inject its DNA into all enterobacteria and even myxobacteria [2–4]. Phages have long been used as a "low-tech" method to type bacteria in clinical microbiology environments [5]. Attempts to exploit the specificity of phages in detection and identification of pathogenic bacteria have been burdened by the requirement of culturing the target bacteria, growing the infected culture, and assaying the production of progeny virions, processes which require hours at least, and also knowledge of the culture conditions is required. However, when we consider the fundamental pathway of the phage infection process, a potential way to avoid these limitations suggests itself. The committed step in bacteriophage infection is irreversible adsorption. For double-stranded DNA (dsDNA) phages, this results from interactions between the specific adsorption apparatus, usually tail fibres, with specific receptors on the surface of the host cell [6–8]. For two of the three main morphotypes of dsDNA phages, the myophages with contractile tails and the siphophages with flexible tails, the injection of DNA into the host cell follows rapidly and involves the transitory formation of a channel through which the phage DNA passes into the target cytoplasm [9–11]. Concomitant with injection is a short-lived membrane depolarization and an efflux of ions, including a substantial fraction of the ~ 0.2 M potassium salts present in the cytoplasm, at a rate of ~ 10^6 /s per infected cell [9,12]. A poorly understood resealing process then occurs, allowing re-energization of the membrane and the commencement of the infection program [6,10, 12–15]. This phenomenon represents an ideal opportunity for bacterial diagnostics, because it not only takes advantage of the well-known specificity available in bacteriophage, but it can occur, given sufficient phage concentration, within seconds after admixture of the virions with the cells [16]. Moreover, it requires no culturing of the analyte culture but only that the target cells be physiologically viable (i.e. have energized membranes or intact membranes capable of being energized [6,9,17,18]).

2. EXPERIMENTAL TECHNIQUES

2.1. Construction of the nano-well device

The chip containing the nano-well device has a 20 nm thick Ti film on a $LiNbO_3$ substrate (Fig. 1). On the chip

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Journal of Biological Physics and Chemistry **5** (2005) 3–7
Received 21 December 2004; *accepted* 31 January 2005

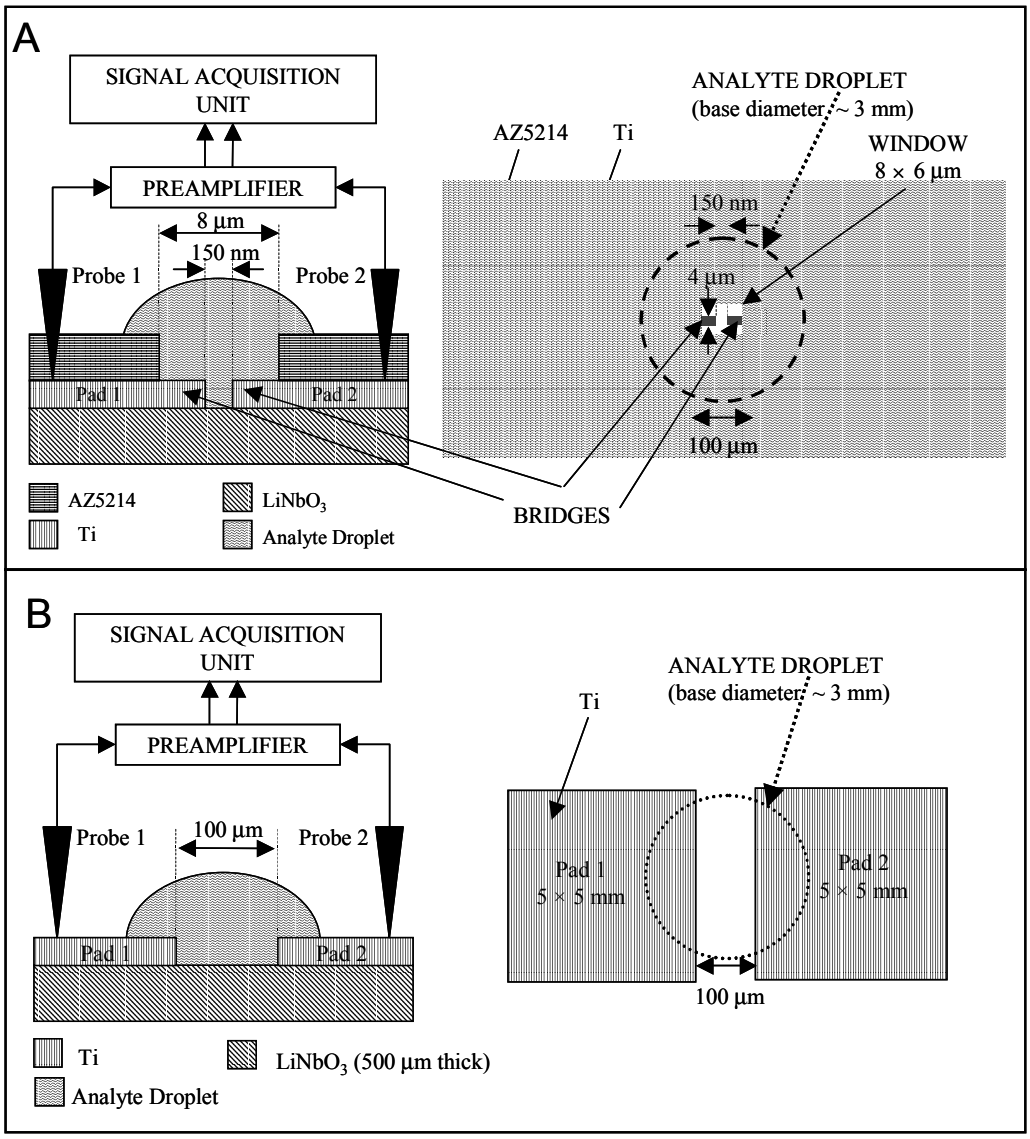

Figure 1. Nano-well and micro-well structures.

Elevations (left panels) and plans (right panels) of the nano-well (A) and micro-well (B) chips. A ML750 Powerlab/4SP signal acquisition unit and a SR560 preamplifier were used with both chips. The drawings are not to scale. (A). The nano-well is a 150 nm × 4 µm gap in the middle of the bridge window, an 8 µm × 6 µm area where the AZ5214 photoresist (1.2 µm thick) has been removed. Also shown are the titanium contact pads and bridges that connect the pads with the nano-well. (B). The micro-well is a 100 µm gap between the two titanium contact pads without the bridge structures.

there are two large Ti contact pads (5 × 5 mm) and a Ti bridge (100 × 4 µm) that connects the two pads. The pads are used for electrical connexions with the external circuitry, while the bridge connects the pads with the nano-well. The nano-well is a 150 nm wide, 4 µm long gap in the middle of the bridge, formed by electron beam lithography followed by reactive ion etching of Ti. In order to prevent current flowing directly between the two pads during on experiment, the chip was covered by AZ5214 photoresist, except for an 8 × 6 µm window, which was the only area exposed to the analyte solution. Two probes that connect with an external preamplifier penetrate the photoresist protection layer and contact the Ti pads.

2.2. Bacteria and phages

All bacteria were derivatives of *E. coli* K-12 W3110 [30]. The λ^S and λ^R strains used were W3110 Δ*fhuA* and W3110 Δ*fhuA* Δ*lamB*, respectively (both $T5^R$), and the $T5^S$ strain was W3110 *gyrA*(Nal^R) Δ*fhuB*. The phages λΔ(*stf tfa)::cat cI857 S105* lacking side tail fibres[25,31], Ur-λ (λ wt, obtained from R. Hendrix; has 4 side tail fibres), and T5 (obtained from I. Molineux).

2.3. SEPTIC measurements

Analyte bacteria were grown in LB at 37 °C to A_{550} = ~ 0.2, washed and resuspended in 5 mM $MgSO_4$.

The basic experimental protocol was to mix 10 μL of a CsCl-purified phage stock at a titre of ~ 10^{10} pfu/mL with an equal volume of the suspension of analyte cells, incubate at 37 °C for various times as indicated, apply 5 μL of the mixture to the nano-well chip and measure the voltage fluctuations over a 2 min period. The use of overnight cultures instead of mid-log phase was also tested and found to give essentially the same results (not shown). Mid-log phase cells indicate a bacterial culture with an optical density (OD) of 0.3–0.6 (measured at the wavelength of 600 nm). This also means that the cell growth is in the exponential phase of growth.

3. RESULTS

We considered that the ion flows associated with injection would cause disturbances in the local electric field that would be accessible to nanoscale fluctuation analysis. Nanoscale fluctuations have recently been used for the sensitive detection and identification of various chemicals in Fluctuation-Enhanced Sensing (FES) [19,20]. In the simplest version of FES, the power density spectrum of the chemically induced fluctuations is used to enhance the sensitivity and selectivity of the sensor [21]. To perform FES with the phage-adsorption system, we constructed a nano-well device [22], in which a gap 150 nm in width and 4 μm in length interrupts a bridge between two electrical contact pads connected to the amplifier circuitry (Fig. 1A). After incubating the analyte bacterial cells in 5 mM $MgSO_4$ with purified phage, a ~ 5 μL droplet was applied to the nano-well and the power density spectrum $S_u(f)$ measured over a 2 minute interval. Our initial experiments used two siphophages of *E. coli* with well-known outer membrane receptors: the temperate phage λ, which requires LamB, an inducible porin specific for maltodextrins; and the virulent phage T5, which requires FhuA, a porin required for ferric ion uptake [4,23]. When mixtures of either phage with sensitive bacteria were tested in the nano-well device, large, slow stochastic waves with various time and amplitude scales were observed. The voltage fluctuations had an approximately $1/f^2$ power spectrum in the frequency range 1–10 Hz (Fig. 2A). In contrast, the spectrum of voltage fluctuations in mixtures of the same phage with isogenic Δ*lamB* (λ^R) bacteria follows a $1/f$ law. This is consistent with the background voltage noise spectrum induced by the input current noise of the amplifier. This kind of background noise spectrum is proportional to the square of the impedance of the nano-well, so small variations in the conductivity of the water can cause observable changes in the noise level [24]. Much higher amplitude fluctuations were observed for the adsorption of phage T5 to sensitive cells, again with a $1/f^2$ power spectra; control mixtures with isogenic

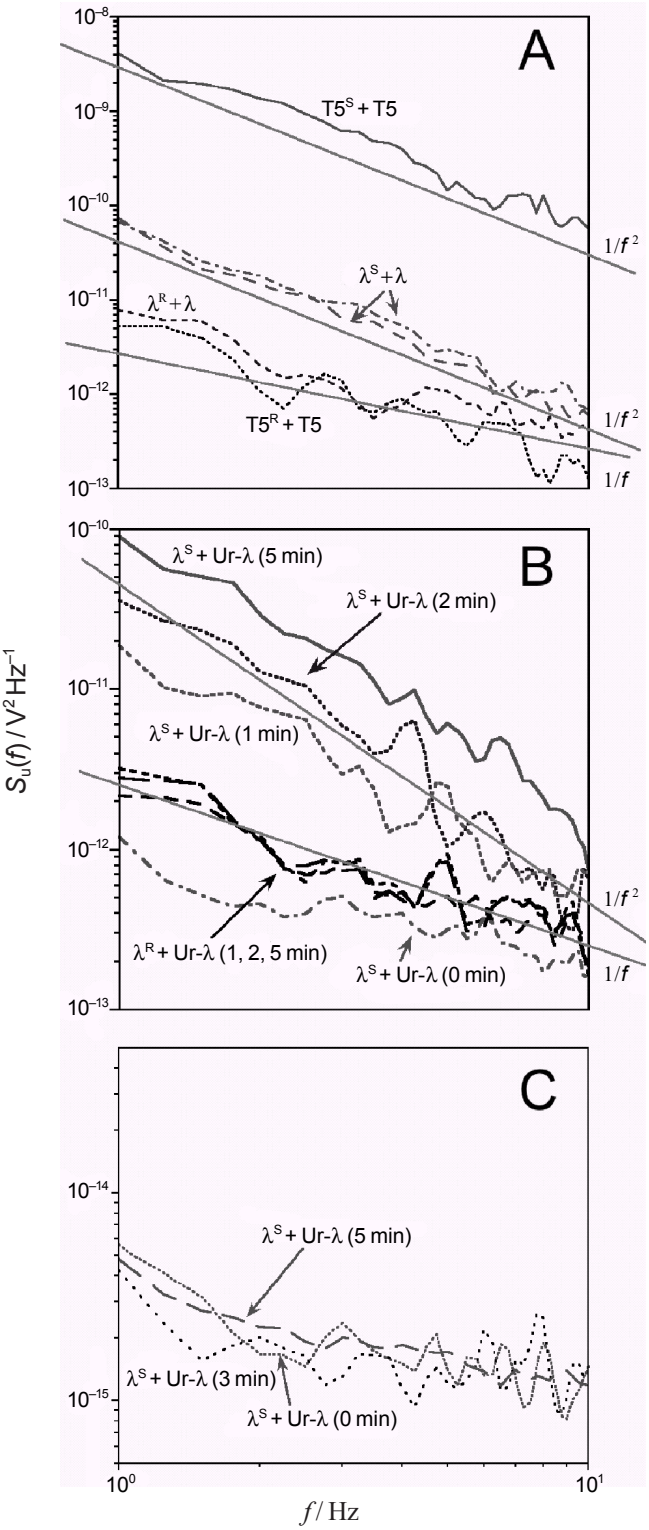

Figure 2. Power density spectra for phage-bacteria mixtures.

Power density measured for 2 min over 1 Hz bandwidths for mixtures of analyte bacteria and phages. Straight lines indicate $1/f$ or $1/f^2$ slopes. (A). Nano-well measurements. Phage λ infection of λ^S cells or λ^R cells. Phage T5 infection of $T5^S$ cells or $T5^R$ cells. (B). Nano-well measurements. Phage Ur-λ preincubated with λ^S cells for 0 min, 1 min, 2 min, or 5 min. Control experiments with phage Ur-λ and λ^R cells preincubated for 1, 2 and 5 min. (C). Micro-well measurements. Phage Ur-λ and λ^S cells preincubated for 0 min, 3 min, and 5 min.

Δ*fhuA* ($T5^R$) cells show only the 1/*f* background (Fig. 2A). The λ phage used for these experiments was derived from the standard laboratory parental strain λPaPa, constructed decades ago [25]. However, λPaPa lacks side tail fibres as a result of a frame-shift mutation in the *stf* gene and adsorbs much more slowly to sensitive cells than does Ur-λ , the original wild-type λ with an intact *stf* gene [25]. When Ur-λ was used in these experiments, much higher amplitude fluctuations with a frequency dependence approximating $1/f^2$ were observed, beginning about 1 min after mixing and increasing for about 5 min (Fig. 2B), after which they dissipated (not shown), presumably because all the phages had become adsorbed. Identical control experiments with resistant bacteria unable to adsorb Ur-λ yielded the 1/*f* background noise.

To understand the spatial nature of the fluctuations, a micro-well device was also tested. The micro-well device was formed by removing the bridge of the nano-well device so that current directly flows between the two contact pads (Fig. 1B). Note that the gap between the two pads is 100 μm. The modification yielded a characteristic device surface about 500 000 times greater than that of the nano-well device. In this case, the observed fluctuations were much smaller and the spectra of mixtures of phages with either sensitive or resistant bacteria were identical (Fig. 2C). This result indicates that the electrical field fluctuations caused by the infected bacteria are random in space and time and average out in large detectors.

4. DISCUSSION

Several technologies are available for the identification of bacteria in human, veterinary and agricultural diagnostic laboratories. Classical metabolic and metabolic profiling, diagnostic PCR, and fatty acid content are all widely used and commercialized methods [26–28]. However, each of these approaches has characteristics that limit their utility except in very well-equipped laboratory environments and make them problematic for implementation in field environments. Moreover, the time required for obtaining definitive analytical results is on the scale of hours to days; the bacteria must be isolated as colonies, grown in pure culture, and then subjected to analysis. PCR is somewhat less subject to these problems, but it requires expensive instrumentation and also cannot distinguish between living and dead bacteria. A rapid and inexpensive method for detecting and subtyping bacteria suitable for large-scale surveillance efforts, and employment in the field, is not available.

The results presented here show that nanoscale fluctuation analysis allows the use of bacteriophages for the highly specific detection of bacteria on a scale of a few minutes, without the need to culture the bacteria. This approach has the additional advantage that only living cells (i.e. with energized membranes) will produce the ion flows that underlie the voltage fluctuations. Approaches based on phage multiplication, while taking equal advantage of phage specificity and avidity, require culturing of the analyte bacteria, physiological conditions amenable to the full infective cycle, and hours or more to develop and assay the signal.

The physical basis of the fluctuations detected using the nano-well device has not been unambiguously determined but a reasonable model can be proposed. During the process of DNA injection by a siphophage or a myophage, each irreversibly-adsorbed virion opens a single channel in the cytoplasmic membrane, through which the phage DNA molecule passes. Bulk solution measurements have shown that injection is coupled to transient cellular depolarization requiring ion flows on the order of 10^8 ions per infected cell [12,18]. The emitted ions undergo rapid Brownian motion and many will be able to escape from the vicinity of the bacterium. Due to the randomness of both the timing and the spatial orientation of the ion emission, these ion leakage events are expected to generate stochastic spatiotemporal electrical field fluctuations at the micrometre or submicrometre scale, as detected in the nano-well device.

The current prototype nano-well has some limitations but they could be avoided if the nano-well and the preamplifier were integrated on a chip with junction field effect transistor (JFET) technology. The shielded cables connecting the nano-well with the preamplifier have about 1 nF capacitance, which is very large and prohibits the observation of fast fluctuations. Moreover, the preamplifier's input noise current imposes a high 1/*f* noise background. Using a system-on-chip arrangement with JFET technology, which has 4 orders of magnitude less capacitance and 3 orders of magnitude less input noise current, to detect and amplify the signal directly would increase the bandwidth by 4 decades of frequency and reduce the 1/*f* spectrum by about 4 orders of magnitude. A simple estimation based on linear response theory yields the result that the ultimate detection limit of the number of bacteria with the strongest response (with phage T5) would be about 10 bacteria in the 10 mm^3 droplet we have been using. To reach sensitivities higher than this limit of 1 bacterium/mm^3, some kind of concentration technique would be needed.

Ultimately, fluctuation analysis coupled with the unequalled specificity and avidity of bacteriophages may provide a diagnostic technology useful for clinical, veterinary and agricultural practice, as well as in applications to microbiological threat detection and reduction.

ACKNOWLEDGMENTS

Valuable discussions with Sergey Bezrukov, Bob Biard and Henry Taylor are appreciated. The authors are also grateful to Robert Atkins of the TAMU Institute of Solid-State Electronics for his valuable technical support. This work was supported in part by the TAMU Information Technology Task Force (LBK). MDK was supported in part by the U.S. Army Medical Research and Material Command Disaster Relief and Emergency Medical Services program and by a Program of Excellence award (Program of Membrane Structure and Function) from the Office of the Vice President of Research at TAMU to RY. The nano-well developments have been supported by funds from the College of Engineering to MC.

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