



Modeling and measurement of a whole-cell bioluminescent biosensor based on a single photon avalanche diode

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ABSTRACT

Whole-cell biosensors are potential candidates for on-line and in situ environmental monitoring. In this work we present a new design of a whole-cell bioluminescence biosensor for water toxicity detection, based on genetically engineered *Escherichia coli* bacteria, carrying a *recA::luxCDABE* promoter–reporter fusion. Sensitive optical detection is achieved using a single photon avalanche photodiode (SPAD) working in the Geiger mode. The present work describes a simple mathematical model for the kinetic process of the bioluminescence based SOS toxin response of *E. coli* bacteria. We find that initially the bioluminescence signal depends on the time square and we show that the spectral intensity of the bioluminescence signal is inverse proportional to the frequency. We get excellent agreement between the theoretical model and the measured light signal. Furthermore, we present experimental results of the bioluminescent signal measurement using a SPAD and a photomultiplier, and demonstrate improvement of the measurement by applying a matched digital filter. Low intensity bioluminescence signals were measured after the whole-cell sensors were exposed to various toxicant concentrations (5, 15 and 20 ppm).

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1. Introduction

A whole-cell biosensor is an analyte probe consists of a biological element, such as genetically engineered bacteria, antibody or antigen, integrated with an electronic component to yield a measurable signal (Patel, 2002; Daunert et al., 2000). As an example, whole-cell biosensor can be used as a real time environmental sensor, detecting the presence of hazardous chemicals (Haugland, 1998; Asakawa et al., 2006; Popovtzer et al., 2005).

A variety of detection methods, suitable for a specific biological assay, have been developed for molecular detection. These methods include colorimetric, fluorescent, bioluminescent, and electrochemical detection (Patel, 2002). In this study we focus on the bioluminescent detection technique, which is widely used due to its high sensitivity, wide dynamic range, and relatively inexpensive instrumentation (Turner, 1985). The conventional bioluminescent detection is based on a photon multiplier (PMT), due to its low dark current (Rabner et al., 2005). However, PMT devices are expensive, relatively large and are not suitable to mobile detection systems. Several works introduced the use of sensitive silicon photodetectors, fabricated using a standard complementary metal-

oxide-semiconductor (CMOS) technology, as a low cost solution for such whole-cell biosensors, which makes it possible to integrate sensing and signal processing on the same integrated circuit (Simpson et al., 2001; Islam et al., 2007; Eltoukhy et al., 2006). Yet, the use of silicon photodiodes is challenging due to their relatively dark current.

The development of the single photon avalanche photodiode (SPAD) (Saveliev and Golovin, 2000; Bondarenko et al., 2001; Golovin and Saveliev, 2004; Saveliev, 2004) which is a silicon photon counting element with very high gain and low dark count, makes it possible to integrate the sensing and the processing units together, enabling low cost and small sample detection system.

In this work, we present a whole-cell biosensor based on an SPAD detection and we develop a mathematical model for the kinetics of the bioluminescence process. In our system, we use genetically engineered *Escherichia coli* bacteria which exhibit SOS Response to large group of toxicants (Rabner et al., 2005; Belkin, 2003; Belkin et al., 1997; Premkumar et al., 2002). In our experiments, we have used nalidixic acid (NA) as the toxin. Typically *E. coli* bacteria are used as harboring cells, in which plasmids containing the promoter–reporter gene conjugation are introduced. Promoter genes function as activators of the bio-reporter expression in the presence of a toxin. Reporters are expression genes that encode proteins or enzymes that function as light sources or electron sources for monitoring metabolic cell activity (Belkin,

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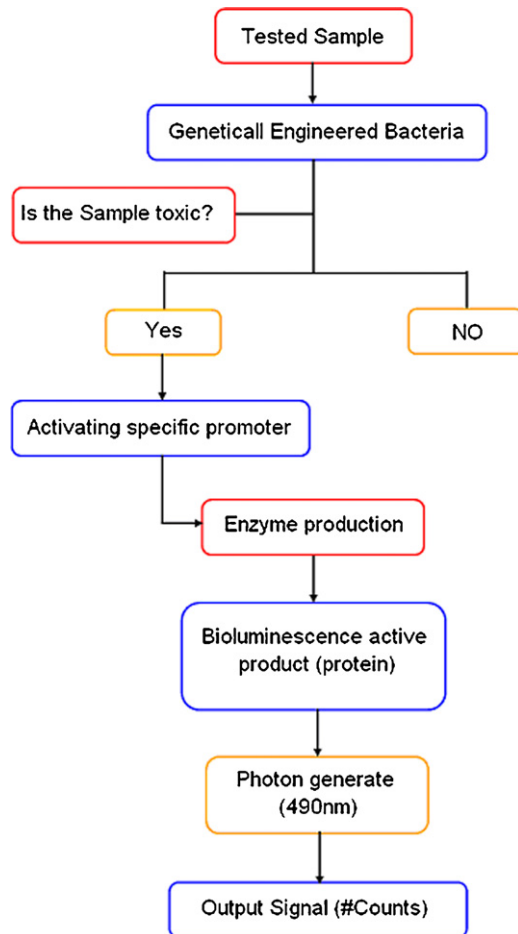


Fig. 1. Flowchart of the biosensor biochemical reactions and the generation of photon current. The toxin analyte (NA) to be tested is introduced to the genetically engineered bacteria. The bacteria react to the presence of a toxin by an SOS response by the activation of a specific promoter. This promoter induces the production of the reporter enzymes and substrate.

2003). Microbial cell sensors have been constructed by genetically binding the *Lux* gene with an inducible gene promoter for toxicity testing. Genetic promoters have been utilized to act as very precise detectors of environmental toxins (Hakkila et al., 2004). The main function of the bio-reporters is to provide a detectable signal response correlated to the magnitude of the toxin dose. The cascade mechanism by which *E. coli* respond to toxins is illustrated in Fig. 1.

Various mathematical models have been developed for the bioluminescent detection scheme (Salama et al., 2004). In this work, we will use our developed model to calculate the spectral density of the response together with its cutoff frequency and to improve the performance of the SPAD by applying a digital matched filter. In Section 2 we present the mathematical model. The experimental setup is described in Section 3. The experimental and simulation results are presented in Section 4. Finally, in Section 5 we discuss the results and conclude.

2. Theory

2.1. The kinetic model of the bioluminescent reaction

Bioluminescence is the phenomena of visible light emission in living organisms that accompanies the oxidation of organic compounds, such as luciferin, mediated by an enzyme catalyst, known as luciferase (Wilson and Hastings, 1998). The light generation

kinetics from the bioluminescence process depends on the chemical reaction kinetics, and can be described by the stoichiometric formula (1) (Salama et al., 2004):



where α_f, α_r are the association and disassociation rate constants and $[E]$, $[S]$, $[P]$ are the concentrations of the enzyme, substrate and product, respectively. If we neglect the disassociation rate and assume that the above process is a bioluminescence process with quantum yield η (Van der Meer et al., 2004), we can write the light generation rate I and the number of the photons N_{ph} emitted at an integration time ΔT (Salama et al., 2004) as

$$I = \eta V N_A \frac{d[P]}{dt} \quad (2)$$

$$N_{ph} = \int_t^{t+\Delta T} I dt'$$

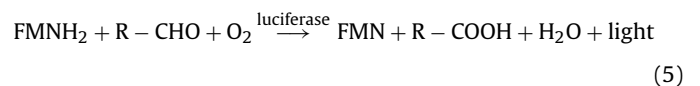
where V is the reaction chamber volume and N_A is Avogadro's number. In our measurements we measure the light for small integration time and therefore the measured signal is expressed by

$$N_{ph} = \eta V N_A \int_t^{t+\Delta T} \frac{d[P]}{dt} dt' = \eta V N_A \{ [P(t + \Delta T)] - [P(t)] \} \quad (3)$$

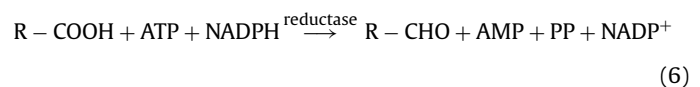
For very small integration time we can rewrite Eq. (2) as

$$I_s = \frac{N_{ph}}{\Delta T} = \frac{\eta V N_A \{ [P(t + \Delta T)] - [P(t)] \}}{\Delta T} \approx I \quad (4)$$

In our experiment we measure the photon count rate I_s . Generally, we are working with very small integration time (≤ 1 s) compared to the long time scale of the measurements (~ 200 min). The bacterial luminescent reaction, which is catalyzed by luciferase, involves the oxidation of a long-chain aliphatic aldehyde ($R-CHO$) and reduced flavin mononucleotide ($FMNH_2$) with the liberation of excess free energy in the form of light. The process is described by the following reaction (Wilson and Hastings, 1998):



where FMN is flavin mononucleotide, and $R-COOH$ is the fatty acid. The *LuxA* and *LuxB* genes encode the α and β subunits of the luciferase. The fatty acids for this reductase enzyme (gene *LuxCDE*) complex are removed from the biosynthesis pathway via the enzyme acyl-transferase (gene *LuxD*) (Wilson and Hastings, 1998). This enzyme reacts with acyl-ACP (acyl carrier protein) to release free fatty acids ($R-COOH$), which are then reduced to an aldehyde by a two-enzyme (*LuxCE*) system via the following reaction:



where ATP is adenosine triphosphate and AMP is adenosine monophosphate, both of which are molecules that act as energy carriers. $NADPH$ and $NADP^+$ are nicotinamide adenine dinucleotide phosphate molecules in reduced and oxidized forms which facilitate electron transfer, and PP is disphosphate. One enzyme, acyl-protein synthetase (gene *LuxE*) activates the fatty acid via the transition of ATP to $R-CHO-AMP$. This serves as the substrate for the final enzyme, acylreductase (gene *LuxC*) that catalyzes the $NADPH$ -dependent reduction of the activated fatty acid to an aldehyde. Fig. 2 describes the block diagram of the light generation (the reporting

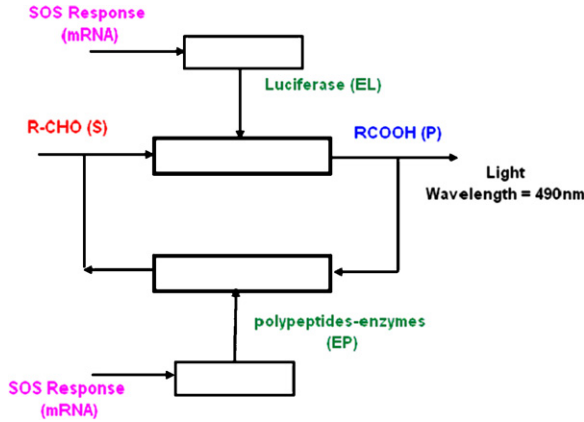


Fig. 2. Block diagram of the reporting element. The promoter induces the production of the reporter enzymes. (1) luciferase and (2) reductase enzyme complex. The luciferase enzyme is involved in the oxidation of a long-chain aliphatic aldehyde (R-CHO) and reduced flavin mononucleotide (FMNH₂) with the liberation of excess free energy in the form of light. The second enzyme, reductase, is then reducing the free fatty acids to an aldehyde.

element). If we assume that the disassociation rates are insignificant (Salama et al., 2004), we can write the reaction in (5) and (6) as

$$\frac{dP}{dt} = -\frac{dS}{dt} = \alpha_S E_L S - \alpha_P E_P P \quad (7)$$

where the product P is the concentration of the fatty acid, the substrate S is the concentration of the long-chain aliphatic aldehyde, E_L and E_P are the concentrations of the luciferase and reductase enzyme complex, respectively. α_S is the rate constant in which the luciferase combines to the substrate, given in unit of $(M \text{ min})^{-1}$, and α_P is the rate constant in which reductase enzyme complex combines to the substrate. In our model we assume that the second substrate of luciferase, FMNH₂ is being consumed at the same rate as the aldehyde substrate. Moreover, recycling of FMNH₂ (reduction of the FMN product by a parallel cycle) occurs spontaneously by the abundant enzyme Flavin oxyreductase. Therefore the availability of FMNH₂ substrate is not a limiting factor. We assume that in the beginning of the process, the initial concentration of the substrate $S(0)$ is larger than the concentration of the product $P(0)$, therefore, when combining the two equations and using the fact $S + P \approx S(0)$, we get:

$$\frac{dP}{dt} = \alpha_S S(0) E_L - (\alpha_S E_L + \alpha_P E_P) P \quad (8)$$

The concentration of the bioluminescence enzymes E_L and E_P are functions of time and are expressed and translated by the messenger ribonucleic acid mRNA. The mRNA dynamics are assumed to be very fast (Belta et al., 2001). With this assumption we can assume that the concentrations of the bioluminescence enzymes are proportional to the promoter (P_r). The luciferase enzyme is considered as a stable enzyme (Belta et al., 2001) with halftime of approximately 3 h (Thompson et al., 1991). However, the reductase enzyme complex is an unstable enzyme with small halftime τ_P of 11 min (Vrettakou and Mitropoulos, 1983). The enzymes kinetics can be described as follows (Belta et al., 2001):

$$\begin{cases} \frac{dE_L}{dt} = \alpha_L P_r \\ \frac{dE_P}{dt} = \alpha_L P_r - \frac{E_P}{\tau_P} \end{cases} \quad (9)$$

where α_L is the rate constant. A simple model would assume that the promoter production rate is constant and proportional to the

toxicant concentration till a maximum tolerated concentration. Above this maximum toxicant concentration, the cell functions deteriorate, including its bioluminescence response. Based on these assumptions, we will express the promoter kinetics as (Popovtzer et al., 2007):

$$\frac{dP_r}{dt} = \mu G_0 C_{NA} \quad (10)$$

where μ is the rate constant, G_0 is the concentration of the bacteria (M), and C_{NA} is the concentration of the NA analyte (M). Substituting Eqs. (9) into (10) and assuming that the concentrations of the luciferase enzyme and the reductase enzyme complex are zero at $t = 0$, we get:

$$\begin{cases} E_L = \frac{\gamma C_{NA}}{2} t^2 \\ E_P = \gamma C_{NA} (\tau_P t + \tau_P^2 (e^{-t/\tau_P} - 1)) \end{cases} \quad (11)$$

where $\gamma = \mu G_0 \alpha_L$. For $t \gg \tau_P$, we can neglect the exponential term and τ_P^2 in Eq. (11). For simplifying the solution we assume $\alpha_P = \alpha_S$. Then, the production concentration can be expressed as

$$P = S(0) \beta C_{NA} \frac{\int t^2 / 2 \exp(\beta C_{NA} ((t^3/6) + (\tau_P t^2/2))) dt + C_P}{\exp(\beta C_{NA} ((t^3/6) + (\tau_P t^2/2)))} \quad (12)$$

where $\beta = \alpha_S \gamma$ and C_P is defined by the initial condition $P(0) = 0$.

2.2. Noises in photon counting devices

To achieve high detection sensitivity, the system noise must be minimized while maximizing the signal to noise ratio (SNR). In this section we will model the noise sources and calculate the signal to noise ratio for photon counting devices. In photon counting mode in which randomly generated photons are detected, the number of signal pulses counted for certain period of time exhibits a temporal fluctuation that can be expressed as a Poisson distribution. For every Poisson process, the variance of the events number is equal to the average events number ($\text{var}n = \bar{n}$) (Van Der Ziel, 1910). In photon counting devices, the only noise sources are shot noise (quantum noise). The shot noise sources in photon counting include:

1. Shot noise resulting from the signal: $\delta n_{ph} = \sqrt{\bar{N}_{ph}}$, where $\bar{N}_{ph}$ is the average number of counts by signal light.
2. Shot noise resulting from dark counts (thermal generation of carriers): $\delta n_d = \sqrt{\bar{N}_d}$, where $\bar{N}_d$ is the average number of counts by dark counts.

In actual measurements, it is not possible to detect $\bar{N}_{ph}$ separately. Therefore, the total number of $N_{ph} + N_d$ is first obtained and then the dark counts are measured over the same period of time by removing the input light. Then N_{ph} is calculated by subtracting N_d from $N_{ph} + N_d$. From this, each noise component can be regards as independent factor, so the total noise is $N_{tot} = \sqrt{2\bar{N}_d + \bar{N}_{ph}}$ and the signal to noise ratio (SNR) becomes as follows:

$$SNR = \frac{\bar{N}_{ph}}{\sqrt{\bar{N}_{ph} + 2\bar{N}_d}} \quad (13)$$

If the integration time is ΔT , the SNR can be expressed by

$$SNR = \frac{\bar{n}_{ph}}{\sqrt{\bar{n}_{ph} + 2\bar{n}_d}} \sqrt{\Delta T} \quad (14)$$

where $\bar{n}_d = \bar{N}_d / \Delta T$, $\bar{n}_{ph} = \bar{N}_{ph} / \Delta T$ are the photons count rates. The signal to noise ratio can be improved as the integration time is

Table 1

Parameter specifications of the SPAD and PMT devices

Parameter	SPAD (C10507-11-50U)	PMT (H7467)
Quantum efficiency	0.4	0.2
Dark counts rate (1/s)	270×10^3	100
Effective area (cm ²)	0.01	0.502
Integration time (s)	0.1	0.1
Gain	7.5×10^5	10^6
$P_{\min}$ (#Photon/s cm ²)	$\frac{1.83 \times 10^5}{\sqrt{\Delta T}}$	$\frac{141}{\sqrt{\Delta T}}$

made longer. The minimum detectable signal (MDS) is defined for SNR = 1, and in our case $\bar{n}_d > \bar{n}_{ph}$ yielding

$$MDS = \sqrt{\frac{2 \cdot \bar{n}_d}{\Delta T}} \quad (15)$$

For a given detector quantum efficiency η_D , the minimum detectable light power is given by:

$$P_{\min} = \frac{hc}{\lambda \cdot \eta_D \cdot A_D} \sqrt{\frac{2 \cdot \bar{n}_d}{\Delta T}} \quad (16)$$

where $h = 6.626 \times 10^{-34}$ J s is Planck's constant, $c = 3 \times 10^8$ m/s is the speed of light, A_D is the detector effective area and λ is the wavelength. In our case $\lambda = 490$ nm. We used the largest integration time in order to improve the signal to noise ratio. Table 1 summarizes the parameter specifications of the SPAD and the PMT.

3. Experimental

The reporter strain (DPD2794 carrying a *recA::luxCDABE* fusion) was kept as colonies at 4 °C or in a 50% glycerol suspension at –80 °C. Prior to the experiment, the bacteria were grown overnight with shaking at 37 °C in Luria Bertani (LB) broth containing 100 mg/l of ampicillin. Overnight-grown cultures were diluted 200-fold in fresh LB broth (without ampicillin) and grown with shaking at 30 °C to the early exponential growth phase (optical density at 600 nm, 0.12). The measurements were performed with PMT (Hamamatsu H7467) and with SPAD (Hamamatsu's MultiPixel-PhotonCounting (MPPC) C10507-11-50U). The biochip was fabricated in Tel-Aviv University using Polyoxymethylene (POM), commonly known under name Delrin, as a substrate. The integration time was set to 1 s or 100 msec. The chambers were filled by solution containing LB medium with bacteria concentration of 5×10^{11} bacteria/l and different concentrations of nalidixic acid (NA). The chamber volume was 10 μ l. The following NA concentrations were used: 5, 8, 13 ppm (mg/l).

4. Simulation and experimental results

In this section we present a simulation of the bioluminescence process and compare it to the experimental results. We make the following assumptions:

1. We neglect the NA analyte transport in the medium via diffusion.
2. The growth of the bacteria is stopped after we insert the NA analyte, therefore, we assume that the concentration of the bacteria is constant during the experiment.
3. We assume that all the physiological coefficients and rate constants are independent on the NA concentration.
4. We did not consider effects from second order in our model, such as, the influence of the oxygen concentration on the system and edge effects (the influence of the chamber volume on the system).

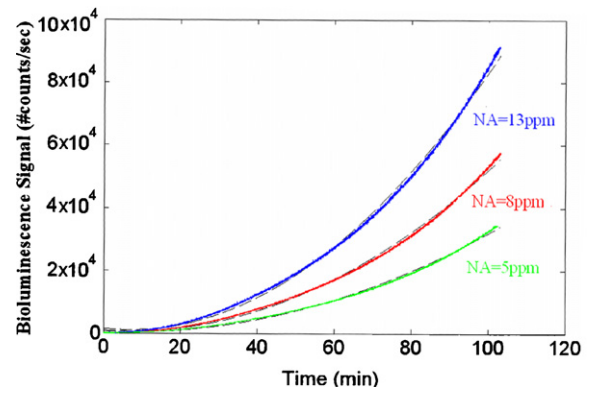


Fig. 3. Comparison between the model simulation and the experimental measurement of the light generation rate as a function of time for various NA concentrations when we used PMT as the light detector (NA concentration was 5, 8, 13 ppm, chamber volume 10 μ l, integration time 1 s).

The model is based on two equations: the production concentration Eq. (12) and the photon generation rate Eq. (2). With two unknown coefficients, $\theta \equiv S(0)\alpha_S\gamma\eta V N_A$, which is used for normalizing the signal and $\beta = \mu G_0 \alpha_L \alpha_S$, which is used as a fitting parameter. The simulation was run with simulation step of 1 s for 100 min, using a numerical method and Matlab software for solving the model equations. The fitting parameters were estimated according to root mean square algorithm. The start point ($t=0$) of the model is when the bioluminescence signal start to increase. This assumption is based on the fact that in order to get light signal we must mix the Bacteria solution with NA solution outside the reaction chamber, therefore there is a delay that must be considered in the simulation results. The DC level was defined by the measurement results during the first minutes. For all the presented figures we used the same parameters and rate constants, when the fitting was done for the measured light signal.

The simulation model and the experimental results based on PMT (photon counting) are shown in Fig. 3, which shows the fitting to experiments for three different concentrations of NA analyte (5, 8, 13 ppm). The solid black lines represent the simulation results. We can see a very good agreement between the simulation and the experimental results. According to our model, we found that the peak of the bioluminescence generation rate is far from our measurement time. This indicates that the enzyme production function (promoter activity) changes slowly compared to the kinetics of the reporter element, which means that the limiting factor in the reaction is the enzyme production rate.

In order to find the best matched filter, we need to calculate the Fourier transform and the effective bandwidth of the bioluminescence signal rate. The bioluminescence signal is measured in finite range time $0 \leq t \leq t_f$, thus, the target function which is expressed in Eqs. (2) and (12) has a Fourier transform. Fig. 4 shows the normalized spectral intensity of the bioluminescence signal for different NA analyte concentrations compared to the model. We used the fast Fourier transform (FFT) by Matlab to calculate the spectral intensity of the measured signals. The maximum frequency was calculated by $f_{\max} = 1/\Delta T$, where ΔT is the integration time. The dashed line represents $1/f$ curve while the dotted lines represent the FFT of the suggested model in the time domain. The PMT integration time was 1 s and the NA analyte concentrations were 8 and 13 ppm. We can see that the spectral density is independent on the NA analyte concentration, and matches the FFT of the kinetics model. All the plots have the $1/f$ behavior and can be expressed as:

$$\Rightarrow |F(f)| = \left(\frac{2\pi}{t_f w} + \pi \delta(w) \right) \quad (17)$$

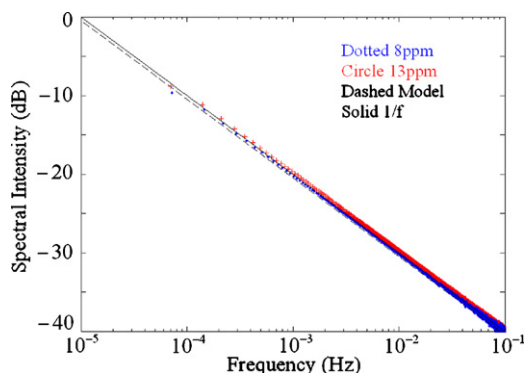


Fig. 4. Normalized spectral density of the bioluminescence signal for different NA analyte concentrations compared to the model. We used the PMT device with 1 sec integration time and NA analyte concentrations of 8 ppm and 13 ppm.

where $|F\{I\}|$ is the spectral density of the bioluminescence signal and w is the radial frequency. The effective bandwidth Δw of this signal, is bounded $2\pi/t_f \leq \Delta w \leq 2\pi/\Delta T$, and can be expressed as

$$\Delta w \equiv \int_{w_{\min}}^{w_{\max}} |F\{I\}| dw = \int_{w_{\min}}^{w_{\max}} \frac{2\pi}{t_f w} dw = \frac{2\pi}{t_f} \ln\left(\frac{t_f}{\Delta T}\right) \quad (18)$$

For example, for $t_f = 6 \times 10^3$ s (total measurement duration) and $\Delta T = 0.1$ s, we get $\Delta w/2\pi \approx 2$ mHz. Generally, the measurement time is very long ($t_f \gg \Delta T$), thus $\Delta w/2\pi \approx 1/t_f$. The only noise sources we have in our system are the shot noises: (1) the shot noise of the photon counting SPAD and (2) the biology shot noise (Hassibi et al., 2005). Thus, if we apply a digital low pass filter which width is equal to the effective bandwidth of the bioluminescence signal (cutoff frequency $f_c \approx 1/t_f$) on the measured signal, we will improve the SNR. The experimental measurement of the bioluminescence signal by the SPAD is shown in Fig. 5. The figure shows the bioluminescence signal vs. time for different NA analyte concentrations (20, 15, 5 ppm) with integration time of 100 msec and a digital low pass filter DSP (dotted black line) with cutoff frequency of 20 mHz. We subtract the average dark current ($\sim 300 \times 10^3$ counts/s) from the experimental results. The figure shows three significant issues: (1) the SPAD is sensitive enough to measure the bioluminescence signal, (2) the measured signals are proportional to the toxin concentration and (3) applying a digital low pass filter improves the

performance of the SPAD for weak signals for the first 50 min, similar to the performance of the PMT (Fig. 4). In order to characterize the influence of the digital low pass filter on the signal we can calculate the signal to noise ratio by

$$\text{SNR} = \frac{I_{\text{Avr}}}{|I_S - I_{\text{Avr}}|} \quad (19)$$

We are interested in the time interval where the signal is weak ($t < 100$ min), thus, we can assume that the signal has very weak dependency on the time (at least for short period), thus I_{Avr} is the average of the measured signal I_S for a specific interval. The SNR improvement by the digital low pass filter is illustrated in the insert of Fig. 5, for different cutoff frequency (10 mHz to 10 Hz). We normalized the SNR by the SNR at 10 Hz (which is equal to the SNR without digital low pass filter).

5. Conclusions

In this work we present new whole-cell bioluminescence biosensor based on a single photon avalanche diode and genetically engineered *E. coli* bacteria. We present a simple mathematical model which describes the kinetic process in bioluminescence based SOS response of *E. coli* bacteria. This model shows excellent agreement with the measured light signal. The model demonstrates the relation between the toxicant analyte and the signal. Furthermore, we found that the spectral density of the bioluminescence signal is inverse proportional to the frequency and the effective bandwidth is about 2 mHz. By applying a digital low pass filter we improve the performance detection of the SPAD and increase the signal to noise ratio. The SPAD is a photon counting device, so flicker noise (1/f noise) and thermal noise are absent. Biochemical enzymatic reactions can be described by Michaelis–Menten model, in the case that the disassociation rate is negligible (which mean that the signal peak is measured after very long time). We can describe the biochemical reaction kinetics by a second order polynom in the time domain and one over frequency in the frequency domain. Thus, photon counting devices have a significant advantage on other devices.

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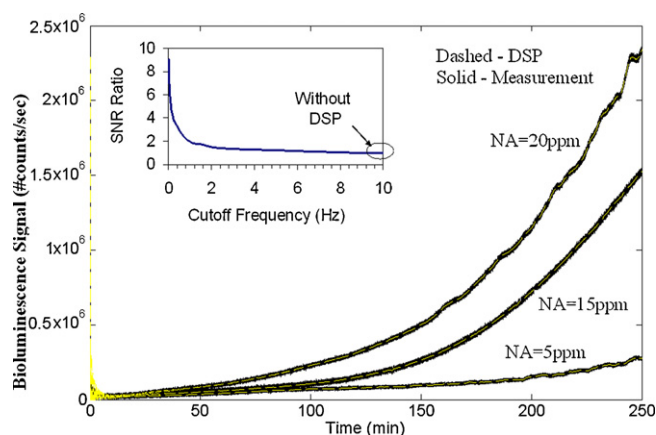


Fig. 5. Solid yellow line - experimental measurements of the bioluminescence activity for different NA analyte concentrations (20, 15, 5 ppm) with the SPAD, integration time 100 msec. The dashed lines are the filtered signal, after applying digital low pass filter (DSP) with cutoff frequency of 20 mHz. Insert of the figure: the normalized SNR calculation of the SPAD for different cutoff frequency. We normalized the SNR by its value at 10 Hz.

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