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Chip level agitation effects on the electrochemical sensing of alkaline-phosphatase expressed from integrated liver tissue

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ABSTRACT

Integrated "tissue on a chip" system with chip-level agitation apparatus has been tested for alkaline phosphatase (ALP) sensing from integrated live tissues on chip. The system was tested and the results are compared to the solutions of a simple 1D models. The models assume electrochemical monitoring of ALP activity with 1-naphtyl phosphate (1-NP) substrate in freshly recovered small tissue samples placed in a micro-electrochemical cell with and without agitation in the 0–12 Hz frequency range. The results indicated on more than an order of magnitude increase in the alpha-naphthol (α-NAP) product oxidation current for samples with agitation compared to non-agitated measurements. The models assume that ALP expressed in the cells (mainly localized in the microvilli of the hepatocytes canalicular membrane) and that part of it is secreted outside of the tissue. The product percolates through the extracellular matrix or leaks out via truncated blood vessels. Once out of the tissue, the product is rapidly distributed throughout the electrolyte and can be oxidized at the working electrode. The measured current and effective charge at long time periods increased as $t^{1/2}$ and $t^{3/2}$ respectively. This pointed toward diffusion-limited condition; however, convection should not be excluded. The agitation probably affects the rate of excretion of the product from the tissue and the transfer from the tissue to the electrolyte. Finally, we discuss the applicability of such method for point-of-care application. We present a short discussion and an approach to make such "tissue on a chip" useful for an automated point of care application. To improve accuracy, stability and reproducibility we discuss our approach for a post measurement digital signal processing of the output variable (e.g. current, charge, etc.), providing the best signal to noise ratio.

1. Introduction

The field of enzyme biosensors includes many detection methods [1–3], some of which are electrochemical, e.g., immunoassays [4–10] or amperometric detection [11–13]. During the last decade, responding to the growing needs for modern diagnostic medicine, micro and nano-fabrication methods were combined with life science technologies creating whole cell biosensors, where cells or tissues were integrated on the chip level. In the current paper we continue and expand our previous studies of whole cell biochip where enzymes expressed in bacterial cells or mammalian tissues were detected using integrated microelectrode [14–16]. Applying this approach, engineered microbes were used where toxic materials in the aqueous media around the microbes triggered their expression of an electroactive enzyme. Similar chips were applied for tissues measuring electrochemically active enzymes as biomarkers. There are reports for on-chip electrochemical detection of enzymes such as alkaline phosphatase, β-galactosidase and tyrosinase [16–24]. The current manuscript focuses on alkaline phosphatase (ALP) since it is a clinically significant enzyme, which can be found in most body tissues, including liver, bone, small intestine, kidney, placenta, and leukocytes [25,26]. Determination of ALP serum level has primary importance in detection of bone and

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liver diseases. Elevated serum values are attributed, among other clinical occurrences, to hepatitis, liver malignancy, Paget's bone disease, and osteomalacia (softening of the bone). Lower than normal ALP serum levels can be observed in various nutrition deficiencies including a rare form of rickets known as hypophosphatasia [27–30]. Lately, an apparatus similar to the one used in this paper was used to characterize the effect of formaldehyde on ALP activity [31]. The work presented here is concerned with two effects: (a) the effect of chip level agitation and (b) the mechanisms governing the enzymatic reaction product transport from the tissue and the electrolyte to the electrodes. The solutions of the transport models are compared to the experimental results explaining the effect of agitation and the character of the boundary condition formed by the tissue.

Unlike the case of time-dependent bacterial expressed ALP, when we deal with tissue samples the ALP concentration is rather fixed and depends on the pathology of the tissue. Therefore, we can assume a constant generation rate of the products in the tissue and a constant (after some time) leak of the products onto the microchip electrolyte. Since ALP can also leak onto the electrolyte it also affects the total product generation.

In this work we examine the direct measurement of ALP activity in small tissue samples of liver using 1-naphtyl phosphate (1-NP) as a substrate. We work with relatively high substrate concentration, therefore, assuming time-independent substrate concentration, which is uniformly distributed. The collected tissue samples were defined as healthy, non-pathological tissue samples. Tissue samples were collected from several individuals and representative samples will be discussed. The samples were placed on the biochips and the substrate was added shortly. The electrochemical current, which had been produced via chronoamperometry, was evaluated, and compared to a simple 1D transport model. Following is a brief discussion regarding the role of the agitation and the transport of the product as the rate-limiting factor in our system. Finally we present our conclusions regarding using such "tissue on a chip" bio electrochemical methods for point of care applications.

2. Experimental

Tissue samples were obtained as surgical material. Liver tissue samples were obtained from patients with chronic acalculous cholecystitis undergoing surgical intervention at Perm Regional Clinical Hospital. Prior to measurements, these normal healthy liver fragments have been stored at 4 °C for a few hours in phosphate buffered saline (PBS) solution. Autolytic changes in tissue samples at the time of the experiment were not observed.

Chronoamperometry measurements of ALP activity in liver samples were conducted using PalmSens® potentiostat (PalmSens BV, The Netherlands) connected to an in-house apparatus (Fig. 1A–D) providing electrical contacts for screen-print electrodes (custom made by Gwent, UK) composed of a planar carbon working electrode, carbon counter, and Ag/AgCl quasi reference electrode. The electrochemical micro-chambers (~300 μl volume) were produced by gluing a polystyrene cylinder, made of a bottom removed commercially available ELISA well strips (Corning Costar® Stripwell™ Plates, Cat. No. CLS2580). The attached cylinder confined the area of the three-electrode cell. The ~300 μl electrochemical chambers were micro-agitated by a suction-expulsion-based efficient stirring.

Prior to measurements, tissue samples were dissected to a ~2 mm × 2 mm × 2 mm pieces and incubated in PBS for 2 h at 37 °C. All electrodes were connected via an eight-channel multiplexer, continuously operating under mixing. Each biopsy slice was suspended in 225 μl PBS in the electrochemical chambers containing 1-naphthyl phosphate (Sigma, UK) at a final concentration of 0.1 mg/ml. Tissue samples were also tested without agitation. A potential of 300 mV vs. Ag/AgCl quasi reference electrode was applied. The added substrate 1-NP undergoes dephosphorylation by ALP, yielding the alpha-naphthol (α-NAP) product. Subsequently, the product is oxidized on the electrode at a potential of 300 mV. Each measurement was repeated for 3 samples taken from the same tissue. The chronoamperometry experiments were conducted in parallel, and the results presented in this paper are the average of the measurements of the three samples.

3. Results

Initially, we demonstrate the system response to agitation and to the addition of substrate. Fig. 2A and B presents typical response when substrate is added while Fig. 2C shows the output current when the substrate is missing. Without substrate the signal is negligible; within the system's noise level. Fig. 2A and B describes the oxidation current of α-NAP, resulting from ALP activity, with and without agitation for liver tissue samples. Both currents show a sharp initial decrease followed by an increase for time periods longer than ~1 min with agitation and ~10 min without agitation. The signal with agitation, in the range where the signal increases with time, was more than an order of magnitude higher than that without agitation. At longer time the signal follows a general trend proportion to the square root of time. Note that for shorter time the current is negative indicating on the presence of other substances in the solution that could be reduced. We will show later that those substances are probably also extracted from the tissue.

To assess the role of the agitation a similar experiment was conducted using tissue fragments from the same sample under different agitation levels (Fig. 2B). It was found that agitation improves the signal by over an order of magnitude when compared to similar system and tissue without agitation. The results yielded the following observations (related to the current measurements):

a. An initial decrease of the current which is proportional to $1/\sqrt{(\text{time})}$. That decrease is independent of the agitation frequency (within the experimental error).
b. A rapid increase of the measured current after some threshold time following a trend of $\sqrt{(\text{time})}$. The threshold time decreases as the agitation frequency increases from ~250 s without agitation to ~75 s with agitation at 10 and 12 Hz. It shows that agitation increases current signal, thus reducing the time required for detection.
c. In many samples there was a significant noise, especially some spurious ("pop corn") noise. We believe that this noise is due to the fact that the experiments were conducted in the point-of-care at the hospital and not in a research laboratory. Therefore, it was impossible to avoid electrical interference, both low frequency interference (LFI) and radio frequency interference (RFI), from other tools and power supply infrastructure, which are present in real-life (i.e. hospitals, point-of-care clinics, field use, etc.) environments. To overcome this we propose to use digital signal post processing for data smoothing and noise filtering, optimizing signal to noise ratio.
d. The agitation effect was significant and reproducible. It reproducibly increases the signal by more than an order of magnitude and the increase was consistent.
e. The measurements included some small negative background current, I_{BG}, which its value increased also with increasing the agitation frequency.

It was interesting to look at the effect of the agitation frequency on the signal. For example, see Fig. 2B and C for the current signal and Fig. 2D for the charge signal. Note also that in that experiment