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Research Paper

A label-free optical biosensor based on nanoporous anodic alumina for tumour necrosis factor-alpha detection in chronic wounds

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ABSTRACT

Tumour necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine important to wound healing. In non-healing wounds, it has been suggested that the expression of TNF-α is prolonged and elevated which contributes to impaired healing responses. Hence it is of great interest to develop biosensors towards the detection of TNF-α in non-healing wounds. In this study, we have developed a label-free optical TNF-α biosensor based on interferometric reflectance spectroscopy (IRS) technique using a functionalized nanoporous anodic alumina (NAA) thin film transducer. The biosensor is fabricated by functionalizing NAA pore walls with anti-TNF-α antibodies using silanization chemistry. Binding of TNF-α to the bioreceptors within the pores causes a change in effective optical thickness (EOT) of the NAA thin film. Thus, analyte detection is achieved by monitoring EOT evolution with time. Label-free detection of TNF-α was demonstrated in buffer solution and in complex media such as simulated wound fluid. A limit of detection of 0.13 μg/mL was achieved. This study provides proof-of-concept evidence which sets foundations for further development of biosensors as point-of-care (POC) diagnostic tools for chronic wound care.

1. Introduction

Chronic wounds such as diabetic foot ulcers, pressure ulcers and venous leg ulcers are increasing health issues for the world population and present an immense economic burden to the health care system [1,2]. Annually, more than 400,000 Australians reportedly suffer from chronic wounds (Australian Wound Management Association) with a larger prevalence among older adults aged over 60 years. The cost of chronic wounds to the health care system in Australia is estimated as $3b annually. Moreover, it also causes immense mental and physical distress to the suffering patients [3]. Innovation in chronic wound management is imperative to provide better wound care by developing diagnostic tools that can serve as point-of-care devices to estimate wound healing trajectories. Diagnostic and theranostic sensors for detection of specific wound biomarkers have remarkable potential for improving wound management. Such diagnostic tools can tremendously reduce the frequent clinic visits by utilizing POC techniques or devices embedded within dressings allowing continual and rapid wound monitoring [4].

Due to the complexity of the wound healing process, there are an ample number of biomarkers that could be used to provide information about the wound [4]. TNF-α is one of the important biomarkers present in chronic wound environment which can be correlated to the status of wound healing. TNF-α is a pleiotropic pro-inflammatory cytokine that plays an important role in immunity and inflammation. Overproduction of TNF-α is associated with various diseased conditions such as persistent inflammation and tissue destruction [5]. It is found in significantly higher levels in wound fluid from non-healing wounds compared to healing wounds [6]. TNF-α is found three-fold higher in wound fluid from non-healing venous leg ulcers than in healing ulcers [7]. High levels of TNF-α also cause diabetic retinopathy and nephropathy [5]. It has been shown that neutralization of TNF-α in non-healing diabetic wounds aids in wound angiogenesis and wound closure [8]. Therefore, it is important to determine the levels of TNF-α in wound environment to assist in monitoring the wound healing status.

There is a strong impetus for developing new technologies that can detect the presence and concentration of TNF-α in chronic

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wound fluids. Assays for TNF-α detection such as ELISA [9], chemiluminescence assay [10], and immuno-PCR assay [11] already exist. However, these methods are time-consuming, expensive and cumbersome involving the use of sophisticated instruments as well as several steps making them difficult to use in a clinical setting. Other reported detection methods for TNF-α include techniques such as electrochemical voltammetric immunosensors [12], electrochemical voltammetric aptasensors [13] and electrochemical impedance immunosensors [14,15]. But none of these techniques have been demonstrated for wound applications and they have not been demonstrated to possess portability, cost effectiveness and ease-of-use required for application in wound monitoring.

Nanomaterial-based transduction platforms are highly desirable for developing high performing portable biosensing devices. Nanoporous anodic alumina (NAA) fabricated by self-ordering electrochemical anodization is a porous material that features highly ordered hexagonal arrays of cylindrical pores [16]. The unique optical properties of NAA such as photoluminescence (PL), transmittance, reflectivity and absorbance can be used as the detection principles of highly sensitive and selective biosensors [17–19]. Geometrical properties of NAA thin films such as pore diameter, film thickness, porosity and inter-pore distance can be finely tuned by adjusting electrochemical etch conditions and self-ordering of pore channels can be achieved in well-known regimes [20]. This allows to design suitable pore structure for ingress of biomolecules of interest. NAA also demonstrates high surface-to-volume ratio due to its porous structure which further enhances the analytical signals and allows the incorporation of larger amounts of biorecognition elements [21,22]. Moreover, the NAA surface features hydroxyl groups which allow chemical modifications to easily attach bio-receptors [23]. Other features of NAA that make it a suitable candidate for developing active sensing layers include scalability, chemical inertness and high stability in biological medium. These unique properties make it in some regards a better platform for biosensing in clinical applications compared to other popular porous sensor materials such as porous silicon (pSi).

Interferometric Reflectance Spectroscopy (IRS) is a highly sensitive optical detection method which is based on white light interference at thin film surfaces [24,25]. This is one of the most popularly used transducer techniques using porous materials like NAA and pSi [18,19,25–27]. In this technique, white light is illuminated on the NAA thin film surface which results in a Fabry-Perot interference fringe pattern. Fringe patterns arise from the Fabry-Perot interference of white light reflecting from the air-NAA and NAA-substrate interfaces. Physical characteristics of the porous thin film such as refractive index and thickness of the film can be extracted from the Fabry-Perot interference pattern. The wavelength of the peak maxima in the interference fringe pattern is determined by the Fabry-Perot relationship $m\lambda = 2nL$, where λ is the wavelength of the maximum constructive interference of order m, n is the mean refractive index of the porous film and its contents, and L is the geometric thickness of the porous thin film. The term "2nL" in the equation is called the effective optical thickness (EOT) and this can be calculated by applying a fast Fourier transformation to the interference spectra. Binding of analyte molecule to the biorecognition elements within the pores causes an increase in the effective refractive index of the porous layer. This causes a red shift in the spectral pattern and thus an increase in the EOT value. So the evolution of EOT over time (often called a sensogram) is monitored to detect and quantify analyte in the sample [28]. Interferometric reflectance spectra obtained from NAA in aqueous media exhibit low fidelity due to the low difference in refractive index between porous alumina and that of water. This limits the applicability of this material in aqueous media such as biological fluids. However, we have shown that this limitation can be significantly overcome by improving the reflectivity of the top interface of NAA by applying an ultrathin semi-reflective coating which improves the fidelity of interference fringe patterns in aqueous solutions and increases the signal-to-noise ratio [18].

In this work, we demonstrate an IRS optical biosensor based on NAA thin film material modified with monoclonal TNF-α antibodies as the transducer. A thin platinum (Pt) coating was applied to the top surface to improve the optical properties of NAA in aqueous media. Selective binding of TNF-α to the antibodies caused an increase in EOT which was monitored over time to determine the presence and quantity of TNF-α in the sample. We also demonstrate TNF-α detection in complex sample such as simulated wound fluid.

2. Materials and methods

2.1. Fabrication and characterization of NAA

NAA were fabricated by a two-step anodization process [16,29]. Aluminium (Al) discs of 15 mm diameter were first degreased by wiping with acetone using a lint-free tissue. Then the Al was electropolished in a 1:4 mixture of perchloric acid ($HClO_4$) and ethanol (EtOH) for 5 min at 20 V. After this, the samples were rinsed thoroughly using water and ethanol and dried under compressed air flow making sure all residual acid was removed. The first step anodization was carried out in 0.3 M oxalic acid at 40 V and 5 °C for 20 h. The disordered porous alumina layer was then dissolved by wet chemical etching in a mixture of 0.4 M phosphoric acid (H_3PO_4) and 0.2 M chromic acid for 3 h at 70 °C. Afterwards, the second step anodization was performed under same conditions as first anodization and it was done until a total charge of 20C was passed to obtain a layer of 5 μm thickness. Finally pore widening was done by wet chemical etching by immersing in 5% H_3PO_4 for different time periods.

Morphological features of the NAA samples were characterized using an environmental scanning electron microscopy (ESEM) FEI Quanta 600 (Hillsboro, OR, USA) operating at an accelerating voltage between 20 and 30 keV (for images in Fig. 1). Before observation with ESEM, NAA samples were sputter coated with a 15 nm layer of gold to increase the image quality. A Zeiss Gemini crossbeam 540 SEM at accelerating voltage 3–5 keV in "in-lens" mode was used to image the surface after surface modification (for images in Fig. 2b). Images were analyzed using ImageJ V. 1.46 software.

2.2. Surface modification of NAA

NAA samples were sputter-coated with a thin layer of Pt by magnetron sputtering for 1 min to provide high fidelity interferometric reflectance in both air and water. NAA samples were chemically functionalized to allow the attachment of antibodies on the pore surface. Samples were first boiled in hydrogen peroxide (H_2O_2) at 70 °C for 1 h to introduce hydroxyl groups on the surface. Samples were dried under nitrogen gas flow to remove any moisture on the surface. Samples must be completely dry as any residual moisture on the surface can react with the active groups on the organosilane. Hydroxylated samples were then immersed in 5% solution of 3-isocyanatopropyl triethoxy (ICN) silane in dry toluene for 30 min in a glass Petri dish. This step introduces isocyanate groups on the surface which can react with the amine groups in the antibody to covalently bind them to the surface. Silanized samples were then removed from the silane solution, washed thoroughly with dry toluene and dried under nitrogen stream. Silanized NAA samples were incubated with 500 μL of anti-human TNF-α antibody (Supplier: Australian biosearch, Western Australia; Manufacturer: Biolegend, San Diego, CA) in PBS buffer (50 μg/mL) for 2 h at room temperature. After incubation, the samples were thoroughly rinsed with PBS to remove any antibodies that were loosely bound on