Published in final edited form as:

IEEE Sens J. 2012 June ; 12(6): 1668–1672. doi:10.1109/ISEN.2011.2176930.

High Throughput Micropatterning of Optical Oxygen Sensor for Single Cell Analysis

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Abstract

In this paper, we present our results from process development and characterization of optical oxygen sensors that are patterned by traditional UV lithography. An oxygen sensitive luminescent probe, platinum octaethylporphyrin (PtOEP), was encapsulated in commercially purchased photoresist (AZ5214) to form uniform thin sensor films on fused silica substrates. Plasticizer ethoxylated trimethylolpropane triacrylate (SR454) was added to the dye-photoresist sensor mixtures to improve the oxygen sensitivity. The optimum sensor mixture composition that can be patterned with maximum sensitivity was identified. The microfabrication process conditions, cell adherence and oxygen sensitivity results from patterned structures were characterized in detail. Down to 3 μm features have been fabricated on fused silica substrates using the developed techniques. The result implies the developed methods can provide a feasible way to miniaturize the optical sensor system for single cell analysis with precise control of sensor volume and response

I. INTRODUCTION

The traditional population-averaged physiological measurements on a large number of cells measure only the average cellular response to a stimulus [1]. It soon becomes apparent that measuring physiological parameter at the single cell level is highly desired to understand cell metabolism, heterogeneity and disease formation at the early stage. Among the many physiological parameters, oxygen consumption rate (OCR) is one of the most important indicators of cellular viability. The recent research effort on single cell OCR measurement [2] are mainly based on fluorescence sensor system because they are non-invasive, disposable, and can facilitate remote measurement of biochemical parameters. To detect OCR at the single cell level, corresponding sensor system is required to be miniaturized to facilitate easy integration of the sensor with the analysis platform. Till now, there has been very little progress made toward patterning optical sensor at the micro-meter range [3–4]. Most of them have drawbacks of low throughput, limited repeatability and reproducibility, and poor control of the pattern size and sensor volume, which are critical to the success of single cell OCR measurement. The goal of this paper is to report our results from development of a high throughput, reliable, and reproducible patterning technique to outcome the problem associated with current patterning technique.

II. LID-ON-TOP CONFIGURATION

It has been shown that oxygen consumption rates of single cells can be measured by placing individual cells in microfabricated microwells and subsequently producing hermetically sealed microchambers by means of placing a cover on top of the microwells [2]. Due to the proximity of the sensor to the cells, this configuration poses potential risks associated with

the chemical toxicity and/or phototoxicity of the sensor that may interfere with normal cell function. Similar issues become even more important in multiparameter cell phenotype analysis, where several sensors are utilized for the detection of multiple physiological parameters. In order to minimize the close proximity effects of platinum-porphyrin-based dissolved oxygen sensors, and to alleviate the stringent biocompatibility requirements of potential upcoming sensors, we conceived and implemented a “lid-on-top” architecture for the microchamber. (Fig.1)

In this configuration, the sensors are selectively deposited (patterned) in the top part of the microchamber (“lid”), while the cell resides in a vertically aligned microwell on the bottom substrate. Since the diameter of each microwell is in the range of 50–100 μm , the size and volume of the sensor pattern need to be precisely controlled to provide sufficient sensitivity with high signal-to-noise ratio. Though this project is focused on only oxygen sensor patterning, it is desired to further miniaturize the sensor system to allow deposition of other type of sensor in the same lid.

The lid is attached to the piston by polydimethylsiloxane (PDMS) or a commercially available double-sided adhesive tape. This layer confers compliance on the lid and uniformly distributes the force exerted by the piston on the lid surface. Once the piston is lowered onto the microwells on the bottom substrate, mechanical force is exerted on the lid through the piston to produce a hermetic seal between the lid containing the sensor and the base chip with the cell located within a microwell. The resulting sealed microchamber containing both cell and sensor is excited by a narrowband light source and the sensor emission intensity is measured as a function of time. By sensor molecular design, emission intensity is directly correlated with analyte concentration providing an accurate measure of analyte concentrations within the sealed microenvironment.

III. EXPERIMENTS

A. Material selection

In this study, platinum octaethylporphyrin (PtOEP: Frontier Scientific, UT) was chosen as the oxygen probe for the following reasons. PtOEP is one of most studied optical sensors, exhibiting a large Stokes shift (difference in the wavelength of the band maxima of the absorption and emission spectra $> 100 \text{ nm}$) for reducing re-absorption problems. Also, the relatively long life time of the phosphorescent excited state (10 – 100 μs) and the efficient triplet-triplet energy transfer from PtOEP probes to oxygen molecules result in its higher oxygen sensitivity than that of ruthenium complexes [5] Photoresist AZ 5214 (MicroChemicals, NY) were used as the polymer matrices to encapsulate PtOEP dye for the following reasons: (i) to make use of the readily available protocols of conventional UV lithography technique, which have been developed for the semiconductor industry for decades (ii) to achieve wafer level fabrication of miniaturized oxygen sensors, which is critical to realize high throughput sensor fabrication at a biological relevant scale. AZ5214 consist of a matrix (cresol novolak resin), solvent (1-methoxy-2-propanol acetate) and a photoactive compound (PAC: diazonia-phthoquinone-sulfonic ester) [6–7] All the solvent present in the photoresist will be completely driven off during the photoresist bake process leaving behind just the solid phase, which consists of the PtOEP dye, photoresist (PR) matrix and PAC.

B. Sensor mixture preparation

The sensor mixture preparation started with mixing selected amount of PtOEP powders with 1g of chloroform, and 30 min sonication was used to completely dissolve the sensor powder in chloroform. The resulted mixture was then mixed with 1g of AZ5214 at room temperature

using glass rod. The amount of the AZ5214 is fixed at 1g during study and the amount of the PtOEP was increased from 5mg to 20mg. The mixing ratio resulting complete dissolving, good patternability, and acceptable sensitivity during the subsequent process and measurement were used for this study.

C. Sensor patterning

After the sensor mixture is prepared, 4-inch fused silica wafer was RCA cleaned to get rid of organic impurities and other contaminants. A dehydration bake (120°C, 10 min) was performed to prepare the surface of the wafer. This operation promotes adhesion between the sensor mixture and wafer by evaporating any moisture present on the surface of the wafer. Before dispensing the sensor mixture, an adhesion promoter hexamethyldisilazane (HMDS) was spin coated (4000rpm, 30s) on the wafer. Immediately thereafter, PtOEP/AZ5214 sensor mixture was dispensed on the wafer using the same spin parameter. It was then soft baked (95°C, 3 min) using a hot plate to drive away the solvent from the resist. This is a critical step and failure to sufficiently remove the solvent will affect the resist profile, as will excessive baking, which destroys the photoactive compound and reduces sensitivity. Soft bake was followed by broadband UV exposure (75mJ/cm²) using a mask, which defines which areas of the resist will be exposed to light and those that will be covered. The exposure was followed by a post exposure bake (PEB: 105°C, 2.5 min), which thermally activates the chemical process of image reversal and the areas exposed to light were selectively crosslinked. Thereafter, a flood exposure of 200mJ/cm² (without mask) was performed that converted the regions which were not exposed in the first step, and soluble in the developer. Hence upon development in AZ developer for 1 min, the areas that were exposed in the first step remained. After development, the resulted features were inspected using an optical microscopy to ensure the features are fully developed. Hard baking of AZ 5214 was not performed since the PEB has already been performed at elevated temperatures. Figure 2 summarizes in detail the optimized process recipe steps required for oxygen sensor fabrication using AZ5214 and PtOEP sensor mixtures.

D. Sensor characterization

The selected Oxygen sensor mixtures were micropatterned using above mentioned process on 13.1 mm × 13.1 mm fused silica chips. The sample chip was mounted at 45° angle in the fused silica cuvette containing Britton-Robinson (B-R) buffer at pH 7.0, which composes of acetic acid, boric acid, phosphoric acid, and sodium hydroxide. The cuvette was then inserted into the optical path of the spectrofluorophotometer. The cuvette was equipped with an air tight stopper and a gas manifold was employed to flush the cuvette with oxygen and nitrogen mixtures of different ratios to achieve different dissolved oxygen concentrations within the sample cell. After each change, the sample was allowed to equilibrate for 10 minutes before the next luminescence measurement was carried out. Fluorescence spectra of the sensing films were excited at 380 nm.

E. Cell adherence test

For single cell analysis, patterned sensors must pass the cell adherence test to maintain intact growing environment for normal living cell activities. During this test, human esophageous precancer CP-A cells were used. Selected sensor mixture (PtOEP/AZ5214) was micropatterned on 13.1 mm × 13.1 mm RCA cleaned fused silica chips and baked on a hot plate at 100 °C for 5 minutes. The chips were then glued onto the bottom of the culture dishes using polydimethylsiloxane (PDMS) as the biocompatible glue and left overnight for curing. The CP-A cells were then cultured on the surface of the sensor pattern in an incubator at 37 °C with 5% CO₂ for different incubation times – 1 hour, 3 hours and 15 hours.

IV. RESULTS AND DISCUSSION

A. Micropatterned Sensor Features

The sensor mixture composition for confocal fluorescence microscopy measurement is 1g of AZ 5214, 11 mg of PtOEP, and 1 g of chloroform. Down to 3μm sensor dots has been successfully fabricated using this developed process and composition with over 90% repeatability. Dektak 150 contact profilometer was used to measure the thickness of the patterned oxygen sensing dot, which was found to be 2.1 μm. Figure 3 shows the confocal fluorescence image of the micropatterned sensor dots. 402 nm laser was used to excite the oxygen probe PtOEP under confocal fluorescence microscopy and the red emission was collected using a 605/75 nm filter set.

B. Sensor response and Sensitivity Improvement

Figure 4 shows the excitation and emission spectra of the patterned sensor. The red emission was observed with a peak at 650 nm. This indicated that PtOEP emission wavelength is not affected by the photoresists. Also the excitation spectra shows three peaks at 381 nm, 501 nm and 535 nm corresponding to the Soret band and Q bands of platinum porphyrin PtOEP [8]. This clearly indicated that there was no electronic interaction between PtOEP and AZ5214 at the ground state and hence AZ5214 is acting as chemically inert matrices for PtOEP.

Oxygen response of the patterned sensors was investigated. It was found the oxygen sensing of the thin films followed a linear Stern-Volmer equation

$$\frac{I_0}{I} = 1 + K_{SV} [O_2] \quad (1)$$

where K_{SV} is the Stern-Volmer quenching constant and $[O_2]$ is the dissolved oxygen concentration. I_0 and I are the steady-state fluorescence signals measured at various dissolved oxygen concentration, respectively. The dissolved oxygen concentration $[O_2]$ is proportional to the partial pressure of oxygen, pO_2 , in the gas used to saturate the liquid. At 23 °C under air condition with the oxygen partial pressure of 21.3 kPa, the $[O_2]$ in the B-R buffer is 8.6 mg L⁻¹.

The dynamic range (or sensitivity) of the optical probes in the polymer matrix was calculated using I_0/I_{100} , where I_0 and I_{100} represent detected fluorescence intensity from PtOEP under deoxygenated condition and oxygenated condition. As shown in Table 1, the sensitivity of PtOEP in AZ5214 matrix acquired is 1.41. To further improve the sensitivity, a plasticizer which has higher oxygen permeability was added into the matrix. Mills et al. carried out a comprehensive investigation of various polymers on the oxygen sensitivity for both ruthenium and porphyrin complex doped thin films [9–10]. He demonstrated that by adding plasticizer to a dye-polymer combination, it was possible to produce a range of oxygen sensors in which the oxygen sensitivity improved with increasing level of plasticizer present. Herein, we use SR454 (Sartomer Company, PA), ethoxylated trimethylolpropane triacrylate, as the plasticizer. SR454 is a monomer. It can be polymerized during our processing at 95°C to form an oxygen permeable gel. Different amounts of SR454 were added to the PtOEP/AZ 5214 dye-polymer combinations to tune the sensitivity. Table 1 summarizes the composition and resulted sensitivity of the non-patterned sensing films with and without the addition of plasticizer SR454.

It is observed that higher SR454 content will lead to higher sensitivity, however, in case of higher SR454 content films (eg. 1.1g SR454), the films simply peeled off during the development process leading to poor patternability. This seemed to be primarily an adhesion

issue. Substrate pre-treatment using HMDS spin coat followed by oven bake at 150 °C for 30 minutes was tried to improve the adhesion of the sensor film. It didn't help much and the higher content SR454 oxygen sensor films continued to peel off during the pattern develop process. It was therefore concluded that adding SR454 beyond a particular threshold changes the chemistry of the mixture so it adversely affects the adhesion of the sensor film with the substrate.

C. Cell Adherence Test

To test the biocompatibility of the microfabricated sensor dots, the cell adherence test is performed. At the end of each incubation period, the cell morphology was observed using an inverted microscope (figure 6). Upon visual inspection after 1 hour of incubation, the cells showed good adhesion to the film which was a critical pre-requisite for subsequent cell growth. And after 3 hours and 15 hours of incubation, the cells demonstrated spreading and proliferation across the entire micropatterned chip, similar to cells cultured on blank fused silica chip. From these experimental results, it can be concluded that micropatterned sensor features do not cause any noticeable changes in cell morphology and furthermore do not significantly alter growth and proliferation of cells even after 15 hours of incubation. It was therefore concluded that micropatterned sensor features should be biocompatible to CP-A cells. Moreover, this was the worst case test scenario since in the proposed "sensor on top" configuration for our analysis system, the cells would never be in direct contact with the sensor and hence it further eliminates the possibility of toxicity.

V. CONCLUSION

In conclusion, a high throughput micropatterning process for oxygen optical sensing at microscale has been developed for integration with the single cell OCR measurement system. The PtOEP sensor probe was mixed with AZ5214 photoresist and SR454 plasticizer with various ratios to tune the sensitivity to extracellular oxygen concentration changes. The sensor mixture was spin-coated onto the fused silica substrate, and standard lithography process was performed to pattern the sensor film to desired feature size and shape. Down to 3 μm sensor dots were successfully produced using the developed process. The excitation and emission spectra of the patterned sensor are very close to that obtained with pure PtOEP which indicates no spectra change due to the photolithography process. The patterned sensor feature has no negative effect on the cell adherence, can quickly respond to the dissolved oxygen concentration changes, and have a maximum sensitivity of 2.2 for the oxygen partial pressure range of 0–101kPa. The acquired results imply that the micropatterned sensor structure fabricated by this developed method can be implemented in the single cell study with precise control of sensor volume and response.

Acknowledgments

This work was supported by NIH National Human Genome Research Institute, Centers of Excellence in Genomic Science, Grant Number 5 P50 HG002360. The authors also would like to thank the staff of CSSER (Center for Solid State Electronic Research, Arizona State University) for their technical help on the microfabrication process.

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Fig.1.

Lid-on-Top configuration. Sensors are selectively deposited and patterned on the top lid which is separate from the bottom cell-containing microwell

Fig.2.
Sensor micropatterning process flow.

Fig.3.
Confocal fluorescence image of the micropatterned Sensor dots.

Fig.4.
Measured Excitation and Emission spectra of PtOEP/AZ 5214 sensor after the patterning.

Fig.6.
Cell adhesion study using micropatterned sensor dots

TABLE 1COMPARISON OF SENSITIVITY (I_0/I_{100}) OF PtOEP SENSOR FILM

AZ5214/PtOEP	SR 454	I_0/I_{100}
1g/11mg	0	1.41
1g/11mg	0.275g	1.73
1g/11mg	0.55g	2.75
1g/11mg	1.1g	13.54