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Photonic crystals on copolymer film for bacteria detection

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ABSTRACT

The development of two-dimensional photonic crystals (PCs) on a copolymer film is described in connection with Fresnel reflection spectroscopy and fluorescence microscopy. Label-free detection of *Legionella pneumophila* was performed using a PC platform with a detection limit of 200 cells/mL. *L. pneumophila* is well known as the cause of Legionnaires' disease and a lesser form called Pontiac fever. Since death by *L. pneumophila* infection depends on the early anti-microbial treatment, rapid diagnosis of this disease is critical for efficient treatment and patient survival. Conventional assays have turn-around times measurable in several hours to days, and are limited in their detection of various serogroups. Due to the recent introduction of regulatory guidelines for routine testing of water cooling towers and treatment facilities, biosensors for the on-field detection of *Legionella spp.* are highly in demand. The versatile and economical immunochips described here can be easily adapted for the monitoring of *L. pneumophila* serogroups in clinical and environmental samples in a few minutes.
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1. Introduction

Nanoparticle-modified surfaces have impacted the growth of biosensors significantly in the past decade (Whitesides, 2003; Yurt et al., 2012). In particular, photonic crystals (PCs), which are periodic nanoarrays of dielectric scatters, have been extensively used for optical communication applications. In the past decade, PCs have been explored as detection platforms for surface-bound bimolecular interactions (Cunin et al., 2002; Li et al., 2003; Block et al., 2006; Chan et al., 2008; Endo et al., 2010a). PCs possess a photonic band gap, a strong light confinement with a band gap to prevent the propagation of light within a certain frequency range. Dielectric nanoparticles were used in the development of PC-based biological and chemical sensors for detecting creatinine and glucose (Moshe et al., 2006). Cunin et al. (2002) and Li et al. (2003) reported porous silicon-based PC for label-free biosensing. Block et al. (2006) and Chan et al. (2008) reported the development of metal deposited two-dimensional (2D)–PC for label-free detection of antigen–antibody reactions and DNA hybridization. Despite the promising potential in biosensing, the fabrication of these platforms involved sophisticated techniques, hazardous chemicals, and a high-cost apparatus. Therefore, a simplified and cost-effective fabrication technique was needed to accelerate the development of next-generation PC-based biosensors. To solve these problems, "printable photonics technology" based on nano-imprint lithography (NIL) was proposed for the fabrication of optical nanodevices (Guo, 2007). NIL is a low-cost and high-throughput technology applied to the fabrication of nanometer-scale patterns using polymers with high homogeneity (Endo et al., 2010b).

Since these films are also flexible and can be easily mass-produced at a low-cost, they show great promise for the development of miniaturized biosensor technologies. Some of the most powerful label-free detection techniques include surface plasmon resonance (SPR), ellipsometry, interferometry, grating couplers, gravimetry and reflectrometry. However, most of these techniques require expensive instrumentation, and the fabrication of these sensing surfaces is costly as well. SPR-imaging methods are highly sensitive, but only acquire information from a limited surface area due to the illumination source, light coupling optics, and uniformity of the sensors (Nikitin et al., 2000).

The goal of the present study is to investigate the potential of using NIL-based PC surfaces to develop immunochips for the detection of a Gram-negative bacterium, *L. pneumophila*. The *Legionella* genus comprises more than 50 species and 70 serogroups that inhabit natural and human-engineered aquatic

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environments (Benson and Fields, 1998). A review of drinking water-associated diseases in United States showed that *L. pneumophila* accounted for 29% outbreaks of Legionnaires' disease from 2001 to 2006 (Lau and Ashbolt, 2009). In several European countries there are strong regulations requiring regular controls of water systems for *L. pneumophila*, and there is a tendency for such regulation also in other countries. *L. pneumophila* infects humans through the inhalation of contaminated aerosolized droplets of water. This opportunistic pathogen targets respiratory tissues thereby, causing a severe pneumonia known as Legionnaires' disease and a lesser form called Pontiac fever (Fields et al., 2002; Duncan et al., 2011).

According to World Health Organization, mortality rate associated with Legionnaires' disease is up to 40% among average patients, and up to 80% among immuno-suppressed patients. Since death by *L. pneumophila* infection depends on the early anti-microbial treatment, rapid detection is critical for efficient treatment and patient survival.

The NIL-based PC surface is proposed to overcome the shortcomings of conventional methods, which are time-consuming, expensive, and can only detect limited serogroups of *L. pneumophila* (Svarrer et al., 2012). The conventional method for detection of *Legionella* from environmental sources is a complicated procedure and involves isolation on a selective medium, which takes several days and requires highly qualified personnel. In addition, it presents several problems including the presence of viable but non-culturable pathogens, loss of viability after collection and the long time required for confirmation. In addition, quantitative detection is difficult, since *L. pneumophila* can be inhibited and masked by the rapid or abundant growth of other microorganisms. Ontario Agency for Health Protection and Promotion (OAHPP) recently reported that up to 39% of culture confirmed cases of Legionnaires' disease were caused by other serogroups of *L. pneumophila* (Ng et al., 2009); therefore, new assays and antibodies are needed to capture different serogroups of *L. pneumophila*. Moreover, available screening test kits are not sufficient to provide the detections limits required by regulation. Our laboratory is collaborating with OAHPP for the development of new antibodies aiming to design a novel detection platform that would allow simple, quick, inexpensive, and sensitive on-chip assays for different serogroups of *L. pneumophila*. As shown in Fig. 1, throughout the preparation steps of immunochips, fluorescently-labeled biomolecules were utilized to allow optimization of surface immobilization events using fluorescence microscopy.

2. Experimental

2.1. Reagents

Alexa Fluor®555 donkey anti-rabbit secondary IgG antibody was purchased from Invitrogen (Burlington, ON, Canada). Rabbit anti- *L. pneumophila* primary antibody was kindly donated by the OAHPP. Paraformaldehyde (16%) was obtained from Canemco Inc. (Quebec, ON, Canada). Multi-functional copolymer was synthesized using poly (ethylene glycol) methyl ether methacrylate (PEGMA), 3-(trimethoxysilyl) propyl methacrylate (TMSMA), N-acryloxysuccinimide (NAS) and 2,2′- azobisisobutyronitrile (AIBN), which were purchased from Sigma-Aldrich (Milwaukee, WI). For the formation of multi-functional polymeric self-assembled monolayer, dimethyl sulfoxide (DMSO) was purchased from WAKO Pure Chemicals (Osaka, Japan). Ethanolamine and glycine were purchased from Sigma-Aldrich. Phosphate buffer saline (PBS, 50 mM, pH 7.4) was prepared with ultra-pure water (18.2 MΩ cm) from the Cascada Pall water system.

L. pneumophila serogroup 1 strain Lp02 was utilized as the target bacteria in this study. Green fluorescent protein (GFP) expressing *L. pneumophila* serogroup 1 was described previously (Duncan et al., 2011). Bacteria were cultured on buffered charcoal-yeast extract agar plates at 37 °C for 3 days before use. Bacterial colonies were re-suspended in PBS, and fixed in 4% paraformaldehyde for 1 h at RT. Bacterial concentration was determined using a Cell Density Meter Model 40 (Fisher Scientific) at 600 nm. Before treatment, bacterial suspension was washed twice and re-suspended using PBS. Serial dilutions were done using PBS to obtain different bacteria concentrations. For quenching experiments, 1.4×10^9 cells/mL were labeled with 19 μg/mL of primary antibody for 1 h, in a process called opsonization.

2.2. Apparatus

For the fabrication of PC, NIL was performed using a nanoimprint apparatus (X-300, SCIVAX Corp., Kanagawa, Japan). For the evaluation of surface characteristics, a miniaturized spectrophotometer (USB-4000-UV–vis, wavelength range: 200–1100 nm), a tungsten halogen light source (LS-1, wavelength range: 360–2000 nm), and an optical fiber probe bundle (R-200–7 UV/VIS, fiber core diameter: 200 μm, wavelength range: 250–800 nm) were purchased from Ocean Optics (Dunedin, USA). Atomic force microscopy (AFM) was performed using a commercial AFM unit (SPA-400, Seiko Instruments, Inc., Chiba, Japan) with a calibrated 20 μm xy-scan and 10 μm z-scan range PZT-scanner.

2.3. Design and fabrication of the PC copolymer film

In this report, PC copolymer films with 230 nm i.d. nanopillars were fabricated using NIL technology. In addition, the mold for heat transfer was fabricated using computer aided design (CAD) system, electron beam lithography, and nickel electro-casting. After fabrication of the mold, heat transfer was performed using a semi-automatic nano-imprinting apparatus.

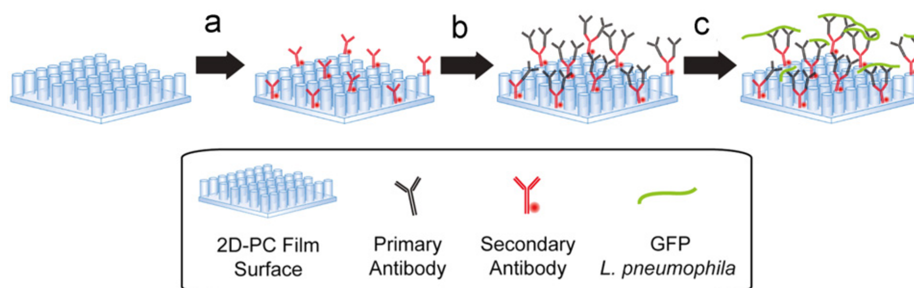

Fig. 1. Illustration for the experimental flow of the PC immunochips; (a) bioreactive groups on the PC surface covalently bound Alexa 555-labeled secondary antibody; (b) primary antibody was attached with secondary antibody, (c) green fluorescent protein expressing *L. pneumophila* was captured using the primary antibody layer on the surface.

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