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Fabrication of titanium based MALDI bacterial chips for rapid, sensitive and direct analysis of pathogenic bacteria

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ABSTRACT

For the first time, we report the fabrication of a titanium bacterial chip for MALDI-MS produced from a simple, cost effective and rapid heat treatment process. This bacterial chip can be reused many times and is highly versatile. These bacterial chips serve dual roles: (1) They can be applied as MALDI-MS target plates for direct and highly sensitive bacterial analysis. (2) They can be used as bacterial sensors for direct analysis of the captured bacteria using MALDI-MS. The sensitivity of these chips when used as bacterial sensors is $< 10^3$ cfu/mL. The lowest detectable concentration for direct MALDI-MS analysis was found to be 10^4 cfu/mL. The results were further justified by using standard plate counting method combined with Tukey–Kramer statistical analysis and fluorescence imaging followed by image processing for fluorescence quantification using ImageJ software to substantiate the MALDI-MS results.

1. Introduction

MALDI technique was initially introduced by Karas in 1987 (Karas et al., 1987) and then rapidly became an ideal (soft) ionization technique for the mass spectrometric analysis of biomolecules. The MALDI-MS technique uses a chemical matrix (Hillenkamp and Karas, 2007), that is typically a low molecular weight laser-absorbing organic molecule. Theory and applications of MALDI-MS have been described by Hillenkamp and Peter-Katalinic (Hillenkamp and Peter-Katalinic, 2007). MALDI-MS is widely used in many fields nowadays, especially in the realm of proteomics, where it is one of the foremost method for analysis of proteins and later become advantageous for high-throughput analysis of biological samples.

The Lab-on chip concept has been introduced into MALDI-MS analysis as early as 1994 by Zaluzec (Zaluzec et al., 1994), who used nylon-66 and positive charge-modified nylon (Zetabind) membranes for analysis of picomole amounts of adsorbed proteins and peptides. Pawel et al., very recently have published a review on the current lab-on chip domain associated with functionalized MALDI-MS targets (Urban et al., 2011).

Development of functionalized MALDI-MS targets for probing analytes such as phosphopeptides, peptides, biomolecules and nucleic acids has been reported by many authors. For example, Brockman and Orlando, used an antibody, lectin or receptor bound to the surfaces of the MALDI plates for probing weak cation-exchange focusing target solid-phase extraction/preconcentration (Brockman and Orlando, 1996). Navare et al., used polymer films for producing bioaffinity MALDI probes (Navare et al., 2009) and Wang et al., immobilized immunoglobulins to enhance capture efficiency (Wang et al., 1999). Neubert et al., in 2002 used an affinity capture polymer for selective capture of biotin-tagged proteins (Neubert et al., 2002). Koopman and Blackburn, used a DNA aptamer covalently attached to the surface of a glass slide for affinity capture of thrombin dick and ultrasensitive detection of hydrophobic proteins (Koopmann and Blackburn, 2003). McComb et al. (2007), used silylated DIOS chip molecules and Trauger et al. (2004) a polymer thin film, for selective isolation of avidin from a mixture of avidin, lysozyme and cytochrome c (McComb et al., 2007; Trauger et al., 2004). Li et al. (2005) used a poly(alkyl methacrylate-comethyl methacrylate) copolymer for protein analysis (Li et al., 2005). Shen et al. applied N-nitrilotriacetic acid Self asembled monolayer (NTA SAM) for gold on-target affinity capture, enrichment and purification of biomolecules (Shen et al., 2005). Muck et al. employed methacrylate-based copolymer enhanced protein adsorption (Muck et al., 2006), while, Xu et al. reported porous silicon iminodiacetic acid silyated DIOS chip for preconcentration of

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phosphopeptides (Xu et al., 2006). Blacken et al. demonstrated hydrophobic interaction of zirconium, titanium and hafnium oxide plates fabricated by reactive landing of gas-phase ions (Blacken et al., 2009). Johnson et al. applied gold with immobilized immunoglobulin G, and silica for capturing a biomarker protein out of a complex mixture (Johnson et al., 2009). Torta et al. developed a nanostructured TiO_2 film coated on stainless steel plates for phospopeptide enrichment (Torta et al., 2009). Wang and Bruening (Wang and Bruening, 2009) used silicon wafers with phosphopeptide-binding for phosphopeptide analysis. In all these cases, the strategy includes loading the samples onto a target plate functionalized with a material to which the analytes bind (with which they interact) in order to enhance signals and to improve detection sensitivity in MALDI-MS. All these methods work either as sensors or as MALDI-MS target plates for standard proteins samples. We report a bacterial chip that can capture bacteria from the surrounding solution and then be used for direct on-chip detection using MALDI-MS. Thus this chip can be used for sensing as well as detecting bacterial cells.

To date no report exists on the development of a MALDI-MS plate which can also function as a probe for bacterial analysis. The following work is unique in the following aspects: (i) This bacterial chip can be used as a bacterial sensor by directly immersing it into a bacterial solution to capture the bacteria from the solution. (ii) After sensoring the bacteria, it can also be directly used as a MALDI target plate for rapid, direct and sensitive bacterial analysis in MALDI-MS. This study optimizes the ideal conditions for obtaining the most effective Ti bacterial chip (TBC).

2. Materials and methods

2.1. Sample preparation

Commercially pure titanium grade-2 coupons (5 mm × 5 mm) were pickled in an acid bath containing nitric acid 400 g/L and hydrofluoric acid 40 g/L (Gopal et al., 2008a,b) and then ultrasonically cleaned using soap solution to remove all traces of acids from the surfaces. The chips were washed in running water and finally rinsed in distilled water and air dried. One set of these chips were retained as such prior to heat treatment and used as control in this study.

2.2. Heat treatment

The samples prepared as described above were subjected to heat treatment of 600, 700, 800 and 900 °C for 3 h. The calcined samples were removed from the furnace after the specified period and air-cooled. The control and heat treated TBCs were characterized using X-ray diffraction (XRD) studies (Bruker D8 advance, Germany) and scanning electron microscopy (SEM) (Field Emission Scanning Electron Microscope, Jeol JSM-6700F, Japan).

2.3. Evaluating the capture efficiency/bacterial sensor ability of TBCs

Staphylococcus aureus subsp. *aureus* (BCRC 10451) and *Pseudomonas aeruginosa* (BCRC 10303) standard pathogenic cultures were purchased from Bioresource Collection and Research Centre, Taiwan and used for these studies. One mL of *S. aureus* and *P. aeruginosa* containing approximately 2.35×10^9 and 1.05×10^9 cfu/mL of cells were inoculated into 20 mL of sterile 0.1% nutrient broth. This set up was incubated at 37 °C in an orbital shaker cum incubator (Firstek Inc., Taiwan) at 120 rpm for 6 h. The control, 600, 700, 800 and 900 °C heat treated TBC were exposed in dilutions containing 10^8 cfu/mL cells (in phosphate buffer) of *S. aureus* and *P. aeruginosa* respectively to identify the most efficient TBC surface showing enhanced capture. The optimized TBC was further tested for their sensitivity by exposing it in serial dilutions ranging 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 cfu/mL to detect the lowest detectable concentration. The initial concentration of the bacteria was determined by the standard plate counting method [22], by plating 10 μL of the serially diluted sample in nutrient agar. Colonies were counted after incubation and the total viable count was determined. The final concentration after exposure of TBCs was also obtained using the above method.

After exposure to the bacterial environment, the TBCs were removed from the medium and gently washed to remove loosely adhering cells and the bacterial cells on the coupons were dispersed into 1 mL sterile phosphate buffer (0.0425 g KH_2PO_4, 0.19 g $MgCl_2$ per litre) by ultrasonication for 10 min. The length of sonication for optimum recovery of cells was found to be 5 min. The ultrasonically cleaned surfaces were stained and observed to ensure complete recovery of cells. Serial dilutions of the bacterial cell suspension were prepared and 10 μL of each dilution was plated onto Nutrient agar. The plates were incubated for 24 h at 32 °C and then the colonies were counted. Mean TVC values were calculated for each TBC and the results are expressed as colony forming units (cfu) per cm^2 (Muraleedharan et al., 2003). Statistical analysis of the data was carried out using MYSTAT software. Three replicates were analyzed for each experimental condition (control, 600, 700, 800 and 900 °C). A Tukey–Kramer Multiple Comparison test was performed to assess the significance of the bacterial capture efficiency of all these test surfaces. A P-value >0.001 indicates that the difference between two data sets is extremely significant. Similarly, P-values of >0.01, >0.05 and <0.05 denote that the difference was very significant, significant and not significant respectively.

Another set of the TBCs that were exposed to bacterial culture were used for direct fluorescent microscopic observation. The TBCs were gently washed with sterile water and air dried in a sterile chamber and their surface was flooded using acridine orange (0.1% solution in distilled water). After 2 min, the excess stain was drained off and the TBCs were washed in sterile water, dried and observed. Acridine orange, a fluorescent dye, differentially stains single stranded RNA and double stranded DNA. Acridine orange, fluoresces orange when intercalated with the former and green while complexing with the latter when observed under an ESPA FI40 (NIB-100F, ESPA systems Co. Ltd., Taiwan) inverted epifluorescence microscope (excitation filter BP 490; barrier filter O 515). Thus, the number of orange fluorescing cells depicts the actively metabolizing cells on the TBCs (Gopal et al., 2004). The fluorescence emitted by the cells on the TBC surfaces were quantified using ImageJ software, which use Java for image processing. The images acquired from the various test surfaces using the fluorescence microscope were quantified using the ImageJ program. The higher the fluorescence the greater the bacterial cells present on that surface. The relative fluorescence of the various test surfaces is presented as a pie-diagram.

2.4. Evaluating the efficiency TBC to function as a MALDI target plate for direct bacterial analysis

After exposure, the TBCs were gently washed with sterile water and air dried in a sterile chamber. Then the surface was loaded with about 50 μL of 50 mM SA matrix (0.05 M sinapinic acid (SA) in 3:1 Acetonitrile: Water containing 0.1% TFA) and airdried before analyzing the TBCs using MALDI-MS. All mass spectra were obtained in positive ion mode using MALDI-TOF MS (Microflex, Bruker Daltonics, Bremen, Germany). The MALDI source was equipped with a nitrogen laser (337 nm) for and