



Short communication

TiO₂ nanoparticle assisted mass spectrometry as biosensor of *Staphylococcus aureus*, key pathogen in nosocomial infections from air, skin surface and human nasal passage

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ABSTRACT

For the first time, we have demonstrated the use of mass spectrometry as a biosensor for detecting a clinically important bacterium: *Staphylococcus aureus* in air, nasal passage and skin samples using culture-free, rapid, direct analysis via TiO₂ nanoparticles (NPs) assisted MALDI-MS. When this bacterium is predominating, the nasal passage of an individual is observed to lead to wound infections especially when the individual has a surgery or some wounds. This study indicates that even at very low concentrations of an individual bacterium can be directly detected from a mixture of bacteria using the MALDI-MS analysis without the requirement of any culturing steps or any other sample pretreatment techniques. The current approach is extremely simple, rapid, straightforward and sensitive which could be widely applied for the detection of this deadly pathogen in clinical as well as environmental samples.

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1. Introduction

Staphylococcus aureus (*S. aureus* or “*Staph*”) has long been recognized as one of the most important bacteria that can cause severe morbidity and often rapidly fatal infections in humans. It is the main cause of skin and soft tissue infections such as abscesses (boils), furuncles, and cellulitis. Although most *Staph* infections are not serious, *S. aureus* can cause serious infections such as bloodstream infections, pneumonia, or bone and joint infections (Mulligen et al., 1993; Waldvogel, 1995). This organism is often resistant to multiple antimicrobial agents, including β -lactams, clindamycin, erythromycin, sulfonamides, tetracycline, chloramphenicol, macrolides, aminoglycosides and quinolones. The incidence of *Staphylococcus aureus* related infections has increased dramatically since the emergence of methicillin resistant strains and high rates of mortality and morbidity are occurring world-wide (Oliveira et al., 2002). Rapid differentiation of MRSA from methicillin-sensitive *S. aureus* (MSSA) is essential for appropriate therapy and timely intervention for cross infection control. Isolates sent to diagnostic laboratories have long been identified by traditional biochemical tests and then subtyped by a variety of

methods (Ip et al., 2003; Fang and Hedin, 2003; Jury et al., 2006). However, criteria for the identification of species are still equivocal. Some strains have been misidentified with closely related species. Given the clinical importance of nosocomial infections, failure to unequivocally identify between species can have far reaching consequences clinically and epidemiologically.

Most current techniques for the epidemiological identification of microorganisms are laborious and time consuming. Currently, the most popular methods for bacterial identification are based on microbiological procedures, antibody recognition, and PCR amplification. Traditionally, microbiological methods are culture-based assays that examine the presence of bacterial species. These methods provide high sensitivity and specificity, but their efficiency is limited by the complexity of the procedures, including culture, selection, isolation, and morphologic and biochemical characterization, which usually take 48 h or longer. Serologic methods are presumptive and confined to the availability of antibodies and to bacteria that are included ahead in the assays. Molecular biology techniques, particularly PCR, have been regarded as non-culture based methods with high efficiency and specificity (Dworzanski and Snyder, 2005). However, they are completely dependent on the known genetic sequences of the target bacteria. Molecular subtyping methods, e.g., pulsed field gel electrophoresis (PFGE), provide better discrimination than the traditional phage-typing technique, but PFGE takes several days to be performed. Mass spectrometry (MS) with its capability of de novo protein/peptide

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sequencing (such as electrospray ionization or matrix assisted laser desorption/ionization (MALDI)MS for Tandem MS) or its high efficiency for proteome profiling (particularly MALDI-TOF MS) has been suggested as an alternative for microbial identification (Pershing et al., 2003; Holland et al., 1996; Krishnamurthy and Ross, 1996). Therefore, rapid, identification of this bacterium using MALDI MS can be an important application in the diagnosis of infections caused by this bacterium, which would help in early detection and control. Early reports involved solvent extraction and chemical modification before analysis, but some methods use simple specimen preparation and direct analysis via appropriate ionization methods. Recent studies have indicated the possibility for direct analysis of bacteria using MALDI-MS, by picking up bacteria grown in agar plates and placing them on MALDI target plates for analysis (Lakshani et al., 2009; Katussevani et al., 2002).

Usually as described above, samples collected from various sources are grown on agar plates and after 48 h the colonies are spotted on target plates and analyzed; so far this was what is being called as 'direct analysis' of bacteria using MALDI-MS. The present work takes the realm of MALDI-MS based direct analysis and identification of bacteria to a higher dimension, in that we report the direct analysis of *S. aureus* from samples collected from nasal passage without preliminary culturing. This technique would give real meaning to rapid analysis using MALDI; since samples are taken as such and after a simple two-step preparation procedure, are directly used for MALDI-MS analysis assisted by TiO₂ nanoparticles (NPs). Additional experiments were also conducted to confirm the presence of *S. aureus* using direct analysis technique as reported in earlier papers. This protocol involved isolating this bacterium from air, human skin surface and nasal passage and growing the bacteria in agar plates and then analyzing the individual colonies using MALDI-MS. Moreover, this paper also reports the sensitivity of TiO₂ assisted MALDI-MS to selectively detect/distinguish a particular bacterial species (in this case *S. aureus*) from a mixture of unknown bacteria collected from air, skin and the nasal passage.

2. Experimental

2.1. Requisites

All the glassware and media used for the studies were autoclaved at 15 lb pressure for 15 min. All aqueous solutions were prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA). All the chemicals used were of analytical grade.

2.2. Procedure for direct analysis

The bacterial swabs were collected from forehead surface and nares of a female human volunteer. The air sample was collected by exposing a swab wetted in sterile water which was exposed to air for 2 h. Using sterile cotton, swabs were collected from nares and forearm skin surface of the volunteer. The respective swabs were washed into 200 μ L of sterile water. These samples were vortexed (VM 2000, Digi System Laboratory, Taipei, Taiwan) for 5 min and spotted for direct analysis on the MALDI target plates. Each spot was overlaid with 5 μ L of matrix (3, 5-dimethoxy-4-hydroxycinnamic acid 0.05 M in acetonitrile: water (3:1, v/v) containing 0.1% TFA) and air dried; all experiments were performed in triplicates.

2.3. TiO₂ synthesis and characterization methods

Indigenously prepared TiO₂ NPs following standard protocols reported in literature (Qourzal et al., 2006), were used to enhance the bacterial signals in the real samples. The freshly synthesized TiO₂ NPs were calcined in a Thermolyne furnace (model FB 1315 M, Thermo Scientific, Dubuque, IA, USA) for 2 h at 400 °C to obtain

anatase form of TiO₂. The TiO₂ nanoparticles were characterized using HR-TEM (TEM-3010, JEOL, Tokyo, Japan) to confirm the particle size and shape. The UV–vis spectrum was acquired from the UV–vis spectrophotometer (U3501, Hitachi, Tokyo, Japan). X-ray diffraction (XRD) was used to confirm the presence of anatase form of TiO₂ NPs.

We had recently evaluated the threshold concentration of these indigenously prepared TiO₂ nanoparticles (NPs) for bacterial analysis with respect to *S. aureus* (unpublished data). 1 mg/mL concentration of TiO₂ was observed to work well for enhancement of *S. aureus* signals. All the experiments conducted in this paper used 1 mg/mL of concentration for assisting the real sample analysis. 5 μ L TiO₂ (1 mg/mL) was mixed with 5 μ L of the real sample and incubated at room temperature for 1 h. 10 μ L of SA matrix was added to this and hand incubated (by shaking back and forth) and 5 μ L of this sample solution was spotted on the target plates for MALDI analysis.

2.4. Procedure for bacteria analysis after culture

100 μ L of the swab samples from air, skin and nose were suspended in 200 μ L sterile water and plated on sterile Luria Bertani agar plates. After an incubation period of 48 h at 37 °C (Firstek, Orbital Shaking incubator, Firstek Scientific Co., Ltd.), the colonies developed were counted to obtain the total viable count (TVC) which is the standard method to quantify bacteria in an unknown sample. The bacterial count is expressed as cfu/mL. These colonies were then scraped up into 500 μ L sterile water, vortexed and then spotted (5 μ L) on the MALDI plates. The MALDI-MS mass spectra of a standard *S. aureus* subsp. aureus BCRC 1045, purchased from the culture collection of the Bioresource collection and research center (BCRC), Hsin-Chu, Taiwan was used as the standards to identify this bacterium in the unknown samples. Fig. S1(a) of supporting information displays the schematic representation of the methodology applied in this study.

2.5. MALDI-MS analysis

All mass spectra were obtained in the positive ion mode using a MALDI-TOF MS (Microflex, Bruker Daltonics, Bremen, Germany). The MALDI source was equipped with a nitrogen laser (337 nm) for irradiation of analyte and the accelerating voltage was obtained at +20 kV. All experiments were performed in the linear mode with 200 laser shots at the laser energy of 63.2 μ J.

3. Results and discussion

3.1. Isolation and direct analysis of bacteria

Fig. S1(b) shows the yellow colonies predominating the plate grown from nasal swab. Although similar yellow colonies were also obtained from air and skin swabs, maximum colonies were observed from nasal samples compared to those from the surfaces of the skin and air. This is understandable because compared to the air and skin surfaces which are dry environments, the nares are moist and serve as better environments for bacterial colonization. Supporting Fig. S2(a–c) gives the mass spectra obtained from direct analysis of the air, skin and nasal passage. As can be observed from these spectra, it is evident that the bacterial concentration was below the MALDI-MS detection limit and so very less peaks in the case of the nasal samples (FigS2(b)) were observed; in the case of the air and skin samples, no signals were detected (FigS2(a and b)). The total bacteria in these samples were enumerated by the plate counting method. The bacterial count in the air sample was 9×10^2 cfu/mL, in skin swab; 7×10^2 cfu/mL and nasal

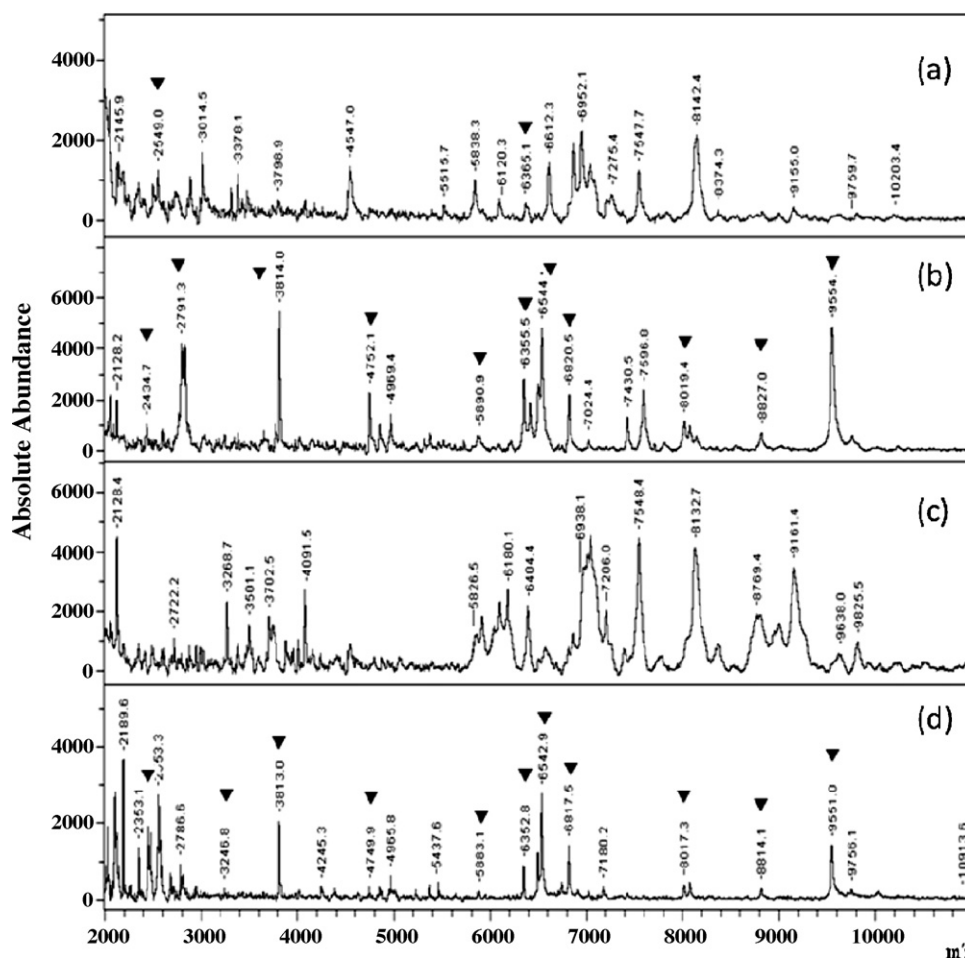


Fig. 1. MALDI-MS spectra showing TiO_2 assisted analysis with SA (50 mM) matrix of (a) air, (b) nasal, and (c) skin samples compared with the (d) standard *S. aureus* (BCRC 10451). TiO_2 NPs resulted in selective enrichment of the bacterial peaks and lead to identification of *S. aureus* peaks in the air and nasal samples. The peaks matching the standard are shown with the symbol (▼).

passage was 4.6×10^2 cfu/mL. Since, the lowest detectable concentration of MALDI-MS for bacteria analysis is above 10^7 cfu/mL (Gant et al., 1999), it is reasonable that direct analysis did not yield bacterial signals.

3.2. Characterization of indigenously prepared TiO_2 NPs

The particle sizes of the synthesized TiO_2 NPs were obtained from the HR-TEM studies (Fig. S3(a)). The particle sizes were in the range of 10–20 nm and the particles were ovoid in shapes. The UV-vis spectrum showed that the NPs exhibited the maximum absorption wavelength at 290 nm, which is the typical wavelength of TiO_2 NPs (Fig. S3(b)). Fig. S3(c) gives the XRD results of the TiO_2 NPs showing strongly crystalline peak at 2θ values of 25.2 (101) corresponding to anatase TiO_2 phase and also at three other planes such as (004), (200), and (105) which confirmed the presence of anatase TiO_2 NPs according to the database available at JCPDS-ICDD card no. 78-2486.

3.3. TiO_2 assisted MALDI-MS for selective sensing of *S. aureus*

In order to obtain significant bacterial signals, TiO_2 NPs were used to enhance the bacterial detection from direct MALDI-MS analysis. Fig. 1 gives the comparative mass spectra where addition of TiO_2 NPs resulted in significant enhancement of bacterial peaks. Fig. 1(a) shows the mass spectra obtained from direct analysis of the air samples after incubation with TiO_2 NPs for 1 h. Addition

of TiO_2 NPs in addition to the organic matrix resulted in significant enhancement of the bacterial peaks in the air (Fig. 1(a)), nasal (Fig. 1(b)) and skin (Fig. 1(c)) samples. Fig. 1(d) belongs to the mass spectra obtained from the standard *Staphylococcus aureus* purchased from BCRC. As we can clearly observe, many peaks from the nasal samples and a couple of peaks from the air samples could be matched with the standard *S. aureus* peaks. This confirms the presence of *S. aureus* in these samples and the efficacy of TiO_2 NPs to selectively enrich the bacterial peaks in real samples. In fact, the samples from the nasal passage would contain a lot of other proteins, but TiO_2 NPs were able to selectively enhance the bacterial proteins. TiO_2 exists in three polymorphic forms anatase, rutile and brookite. But among these three forms of TiO_2 , anatase shows more affinity and ability to interact with bacterial cells. The specificity of anatase TiO_2 for bacteria has been reported and the interactions of these NPs resulting in inhibition or killing of bacterial cells has been also reported. (Saito et al., 1992; Sakai et al., 1994; Muraleedharan et al., 2003; Gopal et al., 2004). However, the role of TiO_2 NPs functioning as affinity probes for MALDI-MS studies of bacteria from real samples is never reported and that is the uniqueness of this study. In this study, we have demonstrated the beneficiary role played by the anatase TiO_2 NPs when used at optimum concentrations. We have previously studied the use of one such NPs possessing bactericidal activity, namely ZnO NPs and have reported the existence of a critical concentration at which they would function as affinity probes for bacterial studies. Above this critical concentration, these bactericidal NPs would show inhibitory properties

leading to quenching of bacterial signals rather than the expected enhancement. Hence, we use the ideal critical concentration (1 mg/mL) of TiO_2 NPs (unpublished data) for the purpose of sensing the *S. aureus* in the real world samples. Use of higher concentrations than these lead to quenching of bacterial cells due to the pronounced bactericidal property exhibited by the TiO_2 NPs in those regimes.

Fig. 2 gives the mass spectra comparing the standard Staph spectra (Fig. 2(a)) with the spectra acquired from the nasal samples with addition of TiO_2 NPs (Fig. 2(b)), showing appearance of peaks matching that of Staph. In the case of the direct analysis without TiO_2 NPs (Fig. 2(c)) to the same sample, no Staph peaks were obtained. This signifies the unequivocal role of TiO_2 NPs in bringing out the bacterial peaks by selective enrichment.

3.4. Confirmation of the new methodology using the existing direct analysis method

A further confirmation to verify whether the peaks obtained were indeed from bacteria was made by scraping the bacterial colonies grown from the samples obtained from air, nare and skin and analyzing them using MALDI-MS. To make sure that the peaks in the direct sample analysis using TiO_2 NPs are not skin based or from nasal proteins or from organic molecules, this experiment was conducted. Fig. 3(a)–(d) reconfirms that similar to the direct sample using the culture free technique, air (Fig. 3(a)) and nasal samples (Fig. 3(b)) alone showed peaks matching the standard Staph (Fig. 3(d)). The skin samples (Fig. 3(c)) showed just a couple of peaks matching that of the standard Staph.

Nanoparticle based affinity probes for enhanced detection of biomolecules have been reported. Nanoparticles (NPs) provide a high surface to volume ratio which can increase the binding/capture efficiency of bacteria. The affinity separation techniques are an attempt to selectively concentrate trace levels of bacteria from biological and food samples and have been reported earlier (Li et al., 2010). We previously have reported the use of various unmodified and modified NPs as affinity probes to increase the detection sensitivity of MALDI-MS (Gopal et al., 2010; Shrivastava et al., 2009; Shrivastava and Wu, 2008) for biomolecule analysis. TiO_2 is also reported (Nazim et al., 2010) to enhance detection of proteins using MALDI-MS. Therefore, it is no doubt that the role of TiO_2 in this study is straightforward and has led to the enhancement of the bacterial signals. It even could be applied on culture free, direct and rapid analysis of bacteria in real samples in MALDI-MS.

S. aureus is reported to be present in all these three sources analyzed in this paper, namely, air, skin and nares. They can be found in the air, dust, water and human faces, and can be present on clothing and utensils handled by man. *Staphylococci* are normal part of the microflora of the nose, throat and skin. It can be found on other parts of the body but the nasal passage is the most significant site. The carrier rate varies with different populations and studies have found a carriage rate of 10–40% in adults outside the hospital environment. Carriage may be intermittent or continuous over a long period of time (Noble et al., 1967; Hocking, 2003). *S. aureus* can exist in this form without harming its host or causing symptoms. However, if there is a break in the patient's skin from a wound or surgery, or if there is a depression in the person's immune system, then the colonizing *S. aureus* can cause an infection. Staph may also

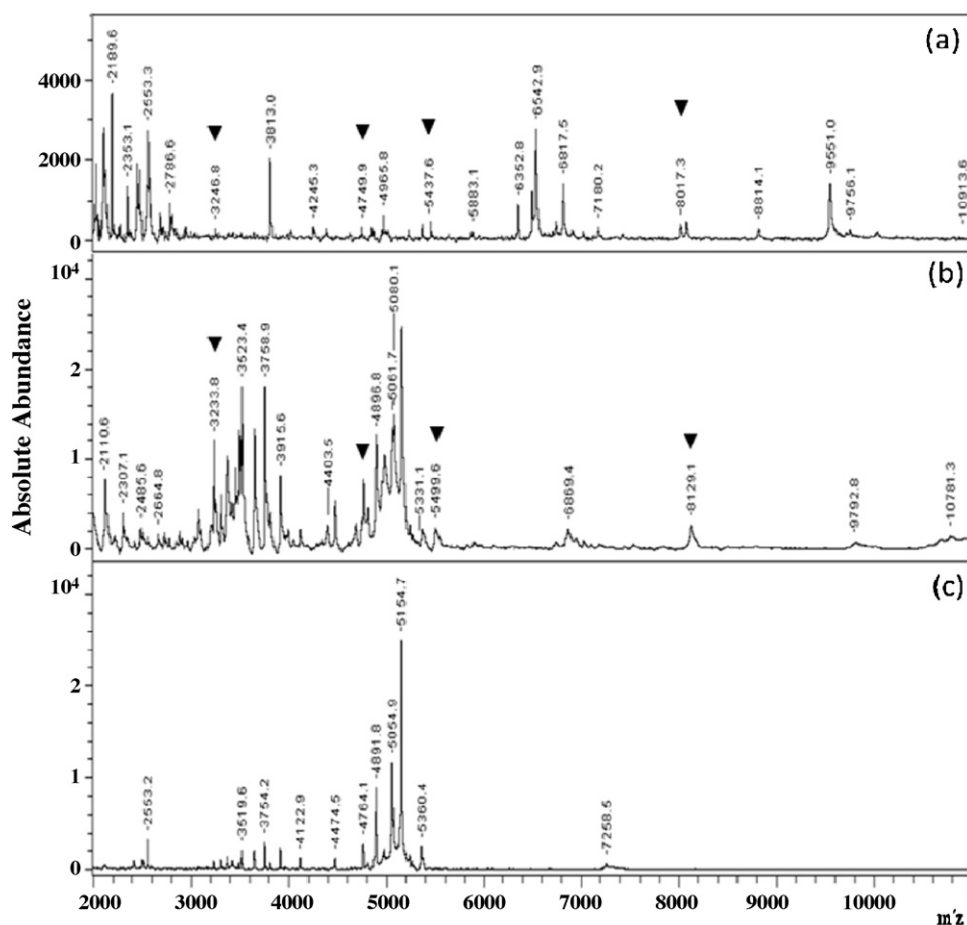


Fig. 2. Figure showing efficient role of the indigenously prepared TiO_2 NPs leading to enrichment of bacterial peaks and culture free identification of *S. aureus* in the nasal sample. MALDI-MS spectra of (a) standard Staph, (b) TiO_2 assisted direct analysis of nasal sample and (c) direct analysis of nasal sample.

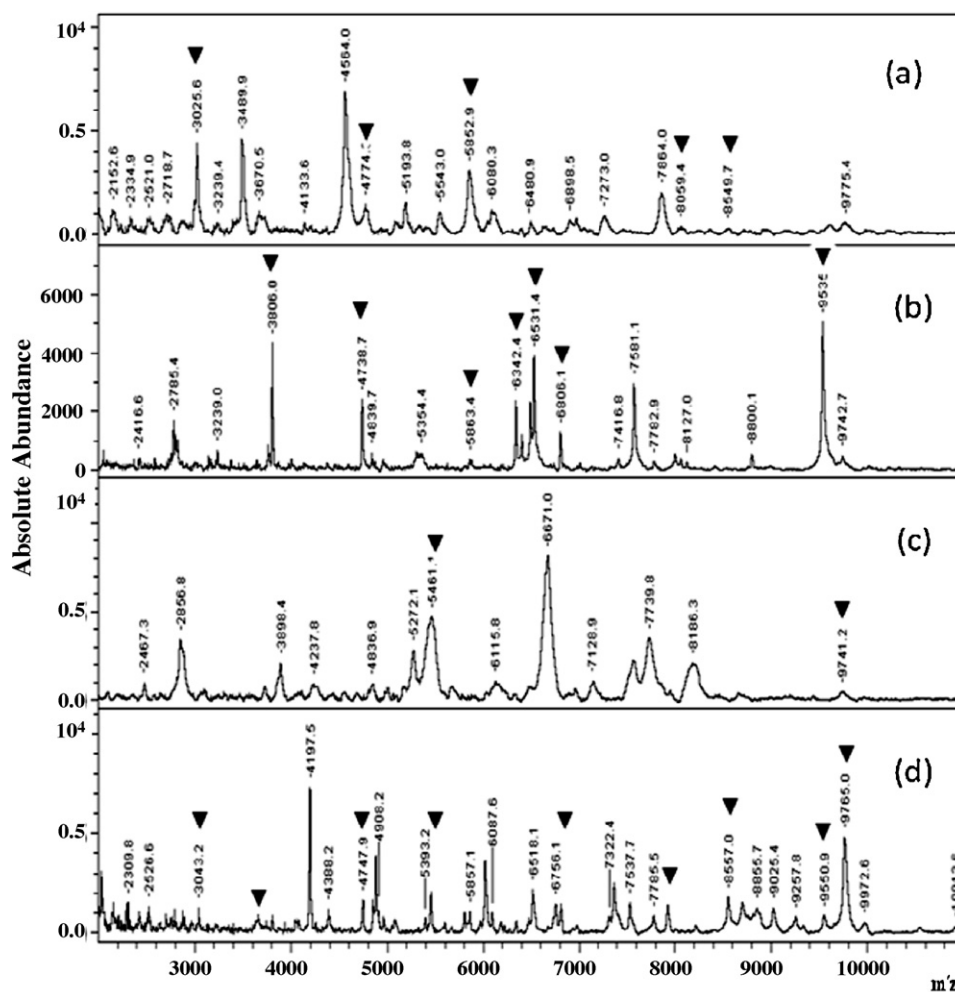


Fig. 3. MALDI-MS spectra of bacteria from (a) air, (b) nasal passage, and (c) skin which were grown on agar plates and then colonies scraped up and suspended in 500 μ L sterile water. Spectra were acquired using TiO_2 combined with SA (50 mM) matrix. The peaks matching the (d) *S. aureus* standard are shown with the symbol (▼).

infect others as it can be passed from both infected and colonized people to other people through skin contact or through sharing contaminated objects, such as towels or razors. A serious implication has been reported that nasal carriage of *S. aureus* is a major risk factor for wound infections after cardiac surgery by Kluytmans et al. (1995).

Katussevani et al. (2002) reported the occurrence of various strains including the clinically important *S. aureus* resistant to methicillin (MRSA) which have been responsible for an increasing number of outbreaks in hospitals throughout the world and the methicillin-sensitive *S. aureus* (MSSA). MALDI-MS with its high speed and reliability can be extended to rapid differentiation between pathogenic and non-pathogenic bacteria in clinical samples, which could help in administering appropriate therapy and timely intervention for cross infection control.

4. Conclusion

We have demonstrated that the *S. aureus* isolated from human nasal passage of a healthy person can be directly detected using MALDI-MS as the biosensor assisted by TiO_2 NPs without the requirement of any culturing steps or other sample pretreatment techniques. We believe that the current approach would be very important for bacterial proteomics and it will prove to be an effective tool for the medical society.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.06.035.

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