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Label-free detection and identification of protein ligands captured by receptors in a polymerized planar lipid bilayer using MALDI-TOF MS

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Abstract

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) coupled with affinity capture is a well-established method to extract biological analytes from complex samples followed by label-free detection and identification. Many bioanalytes of interest bind to membrane-associated receptors, however, the matrices and high vacuum conditions inherent to MALDI-TOF MS make it largely incompatible with the use of artificial lipid membranes with incorporated receptors as platforms for detection of captured proteins and peptides. Here we show that cross-linking polymerization of a planar supported lipid bilayer (PSLB) provides the stability needed for MALDI-TOF MS analysis of proteins captured by receptors embedded in the membrane. PSLBs composed of poly(bis-SorbPC) and doped with the ganglioside receptors GM1 and GD1a were used for affinity capture of the B-subunits of cholera toxin, heat-labile enterotoxin, and pertussis toxin. The three toxins were captured simultaneously, then detected and identified by MS based on differences in their molecular weights. Poly(bis-SorbPC) PSLBs are inherently resistant to nonspecific protein adsorption, which allowed selective toxin detection to be achieved in complex matrices (bovine serum and shrimp extract). Using GM1-cholera toxin B as a model receptor-ligand pair, the minimal detectable concentration of toxin was estimated to be 4 nM. On-plate trypsin digestion of bound cholera toxin B followed by MS/MS analysis of digested peptides was performed successfully, demonstrating the feasibility of using the PSLB-based affinity capture platform for identification of unknown, membrane-associated proteins. Overall, this work demonstrates that combining a poly(lipid) affinity capture platform with MALDI-TOF MS detection is a viable approach for capture and proteomic characterization of membrane-associated proteins in a label-free manner.

Keywords

polymerizable lipid; planar lipid bilayer; MALDI; ganglioside receptor; bacterial toxin

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

INTRODUCTION

Affinity capture coupled with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is a label-free method to detect and identify a peptide or protein ligand that binds to a receptor immobilized on a solid support [1-4]. In this approach, water-soluble receptors (typically antibodies) are used to extract the ligand from a complex mixture. The affinity capture surface is prepared by covalently immobilizing the receptor on a substrate surface that is compatible with MALDI-TOF MS analysis (the “direct” method [5]), or on a solid support from which the captured analytes are subsequently desorbed and deposited on a standard MALDI plate (the “indirect” method [2]). Multiple analytes can be captured from a complex mixture and identified if they have sufficiently different molecular masses [6]. The use of a gold substrate allows the direct method method to be combined with surface plasmon resonance (SPR) and SPR imaging [7-10]. SPR is used to detect ligand binding to the affinity capture surface followed by MS analysis of the captured ligands.

In principle, affinity capture coupled with MALDI-TOF MS could be a powerful approach for characterizing ligands that target receptors embedded in artificial phospholipid membranes, which are widely used as models of natural cell membranes and recognition processes that occur at these membranes [11,12]. A seminal paper by Marin et al. [13] demonstrated a strategy for MALDI analysis of a ligand bound to a receptor incorporated into an artificial membrane. Bovine rhodopsin (Rho) was reconstituted into His-tagged lipid nanodiscs that were captured onto a self-assembled monolayer (SAM) on Au. Transducin was incubated with the Rho/nanodisc/SAM assembly and then subsequently detected using MALDI-TOF MS. However, background peaks for Rho, lipids, and the nanodisc scaffold protein were also present in the mass spectrum, which illustrates a major drawback of the nanodisc strategy - matrix deposition and laser ionization causes dissociation of the entire assembly, producing a complex background spectrum. Ideally, the assembly would be less structurally complex, and matrix deposition/laser ionization would cause only the ligand to dissociate from the receptor, leaving the rest of the molecular assembly intact.

A structurally simpler alternative is a planar supported lipid bilayer (PSLB) that is deposited directly on a solid substrate [11,12]. However, conventional PSLBs are typically composed of fluid-phase glycerophospholipids. The relatively weak, noncovalent interactions in the bilayer and between it and the underlying planar substrate are insufficient to maintain the PSLB structure upon exposure to air, organic solvents, and high vacuum [14,15]; thus conventional PSLBs are not stable to the analysis conditions inherent to affinity capture coupled with direct MALDI-TOF MS.

PSLB stability can be greatly enhanced by polymerization of synthetic lipids bearing cross-linkable moieties [16]. PSLBs created using bis-Sorbyl phosphatidylcholine (bis-SorbPC) can withstand exposure to air and high vacuum; they also exhibit very low nonspecific protein adsorption and retain the functionality of embedded transmembrane proteins [15,17-23]. These attributes and a preliminary study [24] in which MS detection of affinity capture on poly(bis-SorbPC) was first demonstrated suggest that a polymerized PSLB could be a functional platform for MALDI-TOF MS detection of ligands captured by incorporated receptors.

In this proof of concept paper, we use bacterial toxins that target membrane-bound gangliosides to assess the suitability of polymerized PSLBs as an affinity capture surface for MALDI-TOF MS detection. Gangliosides are major membrane receptors for toxins such as cholera toxin [25,26], heat-labile enterotoxin [27,28] and pertussis toxin [29,30]. Poly(bis-SorbPC) PSLBs were doped with GM1, a monosialoganglioside that binds to the B-subunit of cholera toxin and heat-labile enterotoxin [25,27,31,32], and GD1a, a disialoganglioside that is a receptor for the B-subunit of pertussis toxin [29,33]. The three individual ganglioside-toxin B pairs were characterized first, followed by simultaneous detection and identification of all three toxins. Cholera toxin B (CTB) and GM1 were used to assess the minimal detectable toxin concentration, as well as detectability in a complex matrix. The results show that a poly(bis-SorbPC) PSLB is stable to MALDI-TOF MS analysis conditions; the mass spectrum of the dissociated toxin is largely free of background peaks due to other components in the molecular assembly. Finally, the feasibility of on-plate trypsin digestion of CTB bound to GM1 in a PSLB, followed by MS/MS analysis of digested peptides, also was demonstrated. Overall, these results show that combining a poly(lipid) affinity capture platform with MALDI-TOF MS detection is a viable approach for identification and proteomic characterization of membrane-associated proteins in a label-free manner.

MATERIALS AND METHODS

Materials

GM1 and 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) were purchased from Avanti Polar Lipids (Alabaster, AL). GD1a was purchased from Sigma-Aldrich (St. Louis, MO). 1,2-bis[10-(2',4'-hexadienoloxo)decanoyl]-*sn*-glycero-3-phosphocholine (bis-SorbPC) was synthesized as previously described [34] and purified by preparatory scale high performance liquid chromatography as described in Electronic Supplementary Material (ESM). For safety considerations, only the binding domains of toxins were used in this study. CTB and heat-labile enterotoxin B (LTB) were obtained from Sigma-Aldrich. Pertussis toxin B oligomer (PTB) was purchased from List Biological Laboratories, Inc (Campbell, CA). Water from a Barnstead Nanopure system with a minimum resistivity of 18 M Ω -cm was used. Stock toxin solutions were made by dissolving each toxin in nanopure water at 0.5 mg/mL. Phosphate buffered saline, pH 7.4 (PBS) contained the following components: 140 mM sodium chloride, 3 mM potassium chloride, 10 mM dibasic sodium phosphate, 2 mM monobasic potassium phosphate, 0.2 mM sodium azide. Silicon wafers were obtained from Wacker Chemie AG. Fetal bovine serum (FBS) was purchased from Invitrogen (Grand Island, NY). Sinapinic acid was purchased from Fluka Analytical (St. Louis, MO) and alpha-cyano-4-hydroxycinnamic acid (HCCA) was provided by Bruker Daltonics Inc. (Auburn, CA). Peptide calibration standard II, containing Angiotensin II, Angiotensin I, Substance P, Bombesin, ACTH clip 1-17, ACTH clip 18-39, Somatostatin 28, Bradykinin Fragment 1-7, and Renin Substrate Tetradecapeptide porcine, was supplied by Bruker Daltonics Inc. Cytochrome *c* and myoglobin were purchased from Sigma-Aldrich. Trypsin Gold, mass spectrometry grade, was purchased from Promega (Madison, WI).

Preparation of small unilamellar vesicles (SUVs) with incorporated GM1 and GD1a

Stock solutions of bis-SorbPC were prepared in pure chloroform. GM1 was dissolved in methanol and GD1a in 2/1 chloroform/methanol (v/v). GM1 and GD1a were mixed with bis-SorbPC at molar ratios of 1/99 and 1/4, respectively (expressed below as 1 mol% and 20 mol%, respectively). Organic solvents were evaporated from the lipid mixtures under a stream of argon, followed by vacuum drying for at least four hours. The lipids were then rehydrated with PBS to a concentration of 0.5 mg/mL, vortexed, and then sonicated in a Branson Sonicator with a cup horn at 35°C until the solution was visibly clear (usually 30 min).

Preparation of polymerized PSLBs

Silicon wafers (cut to 0.8 cm × 0.8 cm) were cleaned in piranha solution (7/3 concentrated H₂SO₄/H₂O₂) for 30 minutes and rinsed thoroughly in nanopure water. The silicon wafers were dried with a stream of nitrogen and incubated in 200 µL SUV solutions at 35°C on a hot plate for at least 15 minutes to form PSLBs. Unfused SUVs were rinsed away with copious PBS buffer (at least 10 mL) without exposing the PSLB to air. A low-pressure mercury pen lamp with a rated intensity of 4500 µW/cm² at 254 nm was directed through a bandpass filter (325 nm, 140 nm FWHM; U330, Edmund Optics) for 60 minutes to polymerize bis-SorbPC [15]. The distance between the lamp and the PSLB was 7.6 cm.

Mass spectrometric detection of toxins

The toxin solution (0.5 mL of 0.24 µM CTB, 0.24 µM LTB, and/or 1 µM PTB) was incubated with GM1- and/or GD1a- incorporated PSLBs on 0.8 cm×0.8 cm silicon wafers for 1 hour. PSLBs were then rinsed thoroughly with water and dried under a nitrogen stream. Nonspecific binding was assessed by incubating toxins with PSLBs that lacked gangliosides, followed by rinsing and drying. The MS mass calibration standard was prepared by mixing 0.5 µL of myoglobin solution (3.8 mg in 500 µL), 1.0 µL of cytochrome *c* solution (1.2 mg in 500 µL) and 8.5 µL of saturated sinapinic acid (SA) in 70/30/1 H₂O/ acetonitrile/trifluoroacetic acid. The dried silicon wafers were mounted onto a MALDI plate (a microtiter plate (MTP) adapter for Prespotted AnchorChip Targets (Bruker)) using double-sided tape. The calibration standard (1 µL) was spotted on each wafer for external calibration. Three or four different spots of 1 µL SA solution were added to the remaining surface of each wafer and the solvent was allowed to evaporate under ambient conditions, crystallizing the SA. The plate was mounted in a Bruker Ultraflex III MALDI TOF/TOF mass spectrometer (Bruker Daltonics) equipped with a Smartbeam laser (Nd: YAG laser, 355 nm; spot diameter at sample = 50 µm). After the laser ionization, ions were accelerated by a 20 kV electric field into the field-free flight tube and were detected in the positive ion linear detection mode. Spectra were exported as ASCII files and were processed using Origin 8 (OriginLab Corporation).

Preparation of shrimp extract

Shrimp extract was prepared using a modification of two published methods [35,36]. About 25 g of shrimp was weighed and an equal mass of PBS buffer was added to the sample. The

mixture was homogenized in a blender, then centrifuged at 3000 rpm for 1 h, and the supernatant was collected for use.

On-plate digestion and MS analysis of captured CTB

CTB was captured on a PSLB doped with GM1, as described above, and then enzymatically digested by spotting 10 μ L of 0.01 μ g/ μ L Trypsin Gold in 25 mM ammonium bicarbonate solution, pH 7.8, over the area of the PSLB that had been incubated with dissolved CTB. The digestion was carried out for 12 hours at 37°C in a humidified chamber to prevent solvent evaporation [37] after which the wafer was removed from the chamber and allowed to air-dry at room temperature. Different digestion times were tested and 12 hours was found to be optimal to maximize the intensities of the CTB peptide peaks for subsequent MS/MS analysis.

HCCA (20 μ g) was dissolved in 250 μ L of 50% acetonitrile, 2.5% trifluoroacetic acid and 47.5% nanopure water. One μ L of this matrix solution was spotted onto the dried spot where the enzymatic digestion had taken place. One μ L of peptide calibration standard II in HCCA solution was also spotted on the wafer. Wafers were mounted onto the MTP MALDI plate and analyzed using the MALDI TOF/TOF mass spectrometer as described above. The digested peptides were ionized, accelerated and detected in the reflectron mode for better resolution at lower mass-to-charge (m/z) ratios. After the full mass spectrum of the digested peptides was obtained, high energy (8 keV) collision induced dissociation was used to fragment the peptides to obtain sequence information (CID-LIFT mode with no added gas; background pressure is sufficient for CID as shown in the literature) [38]. Protein Prospector (University of California, San Francisco) was used to determine the theoretical m/z of the peptides generated from CTB digestion based on its amino acid sequence [39]. The experimental peaks were compared to the theoretical peak list to make the assignments. For MS/MS spectral interpretation, b ions and y ions were compared and assigned according to the theoretical m/z values generated by Protein Prospector.

RESULTS AND DISCUSSION

MALDI-TOF MS detection of CTB, LTB and PTB

Cholera toxin is composed of a dimeric A-subunit (M_r ~27,400) and five identical B-subunits [26], and the sequence is available [39]. Each B-subunit (M_r ~11,600) contains a binding site for its membrane receptor, GM1. Heat-labile enterotoxin and cholera toxin are very similar with respect to structure, function and immunology; they also share GM1 as the cell surface receptor [27,31,32,40]. Each B-subunit in LTB (M_r ~12,000) is slightly larger than a CTB subunit [28] (and the sequence is given in this reference). Pertussis toxin has an enzymatic component A protomer (S1, M_r ~28,000) noncovalently bound to the B oligomer which is the binding component. Four dissimilar subunits form PTB: S2 (M_r ~21,900), S3 (M_r ~21,900), S4 (M_r ~12,000) and S5 (M_r ~11,750) in a molar ratio of 1:1:2:1, respectively [30] (see this reference for the sequences of the subunits). Several studies have identified GD1a as a receptor for pertussis toxin [29,33].

To assess the feasibility of using MALDI-TOF MS to detect toxins captured on polymerized PSLBs doped with gangliosides, initial experiments were performed using poly(bis-SorbPC)

PSLBs doped with 1 mol% GM1 to capture CTB and LTB under conditions where the toxin concentration (0.24 μM) was sufficient to saturate the GM1 receptors in the PSLB (see Section 9 in ESM). Figure 1a shows a typical MALDI spectrum from a PSLB on which CTB was captured. The peak at 11,607 m/z corresponds to CTB monomer [26] while the doubly charged monomer peak appears at 5804 m/z. Detection of the CTB monomer rather than the pentamer is most likely caused by dissociation of the B-subunits during exposure to the matrix and organic solvent. It is also possible that laser ionization disrupts the non-covalent interactions between monomers. LTB was captured and analyzed under identical conditions. A typical MALDI spectrum from a PSLB on which LTB was captured is shown in Figure 1b; the pattern of singly and doubly charged peaks is similar to that observed for CTB. The singly charged LTB monomer peak was detected at 12,012 m/z [28] and the doubly charged LTB monomer peak appeared at 6006 m/z.

Pertussis toxin B was captured on poly(bis-SorbPC) PSLBs containing 20 mol% GD1a from a 1 μM solution, which was sufficient to saturate the GD1a receptors in the PSLB based on results reported in [33]. The ganglioside mol% was higher than that used for CTB and PTB based on preliminary experiments on poly(bis-SorbPC) PSLBs doped with GD1a at 10-40 mol% (see ESM Fig. S8). At 10 mol%, the intensities of the PTB peaks were too weak to be reproducibly resolved. The need for a higher mol% is consistent with the work of Janshoff et al. [33] who observed PTB binding only when a large mole fraction (40 mol%) of GD1a was incorporated into a POPC monolayer. Figure 1c shows a typical MALDI-TOF MS spectrum of a PSLB on which PTB was captured. Six peaks are assigned to the B-subunits described above [30]. S4 appears as a singly charged peak at 12,064 m/z ($[\text{S4}+\text{H}]^+$) and a doubly charged peak at 6033 m/z ($[\text{S4}+2\text{H}]^{2+}$), while S5 appears as a singly charged peak at 11,769 m/z ($[\text{S5}+\text{H}]^+$) and a doubly charged peak at 5886 m/z ($[\text{S5}+2\text{H}]^{2+}$). The broad, low intensity peak at 21,919 m/z is assigned to singly charged S2 and S3 ($[\text{S2}, \text{S3}+\text{H}]^+$). According to Tummala et al. [30], the masses of S2 (21,886 m/z) and S3 (21,864 m/z) differ by 22 m/z; however they are not resolvable under the experimental conditions employed here. Doubly charged S2 and S3 ions are indistinguishable as well, appearing as one peak at 10,968 m/z ($[\text{S2}, \text{S3}+2\text{H}]^{2+}$). The cause of the relatively low intensity of the S2 and S3 peaks is not known; however ionization efficiency differences and ion suppression effects may have played a role.

The spectra in Figures 1a-1c demonstrate successful capture of individual toxins on ganglioside-incorporated poly(bis-SorbPC) bilayers. Ganglioside recognition is clearly maintained after polymerization of the membrane, and the stability provided by cross-linking allows subsequent analysis by MALDI-TOF MS. The necessity for lipid polymerization was confirmed by attempting to capture CTB on a PSLB composed of 1 mol % GM1 and 99 mol% DPhPC followed by MALDI-TOF MS detection, following the same procedures used for PSLBs composed of 1 mol% GM1 and 99 mol% bis-SorbPC. No CTB peaks were detected in the mass spectrum (ESM Figure S1), demonstrating that a non-polymerized PSLB does not provide the stability required to conduct the MALDI MS analysis as described herein.

Background from nonspecific toxin adsorption and lipid membrane components

Control experiments were performed to assess the degree of nonspecific adsorption of toxins to PSLBs because in any non-competitive bioaffinity assay, nonspecific adsorption is usually the major source of background [41]. PSLBs composed of only poly(bis-SorbPC) (i.e., lacking gangliosides) were prepared and incubated with 0.24 μM CTB, 0.24 μM LTB, or 1 μM PTB, respectively. Nonspecific adsorption of CTB and PTB was not detectable (see ESM Fig. S2a and S2c). In the case of LTB, a small peak at ~ 12.0 k m/z was observed occasionally, indicating that nonspecific adsorption of LTB to poly(bis-SorbPC) is detectable (ESM Figure S2b for an example). However, this peak was only observed in some trials and in those cases, the S/N was less than three. These results show that poly(bis-SorbPC) PSLBs have a high resistance to nonspecific toxin adsorption which is consistent with previous studies demonstrating that these membranes are highly resistant to nonspecific adsorption of other proteins [21].

Another set of control experiments was performed to assess if lipid molecules, either gangliosides or bis-SorbPC monomers and/or oligomers, could be detected. PSLBs composed of 1 mol% GM1 and 20 mol% GD1a in poly(bis-SorbPC) were prepared and analyzed by MALDI-TOF MS, as described above, except that the toxin incubation step was eliminated. HCCA and SA were used as the matrices for low and high m/z ranges, respectively. In Figure 2, the HCCA matrix spectrum in the 500-1000 m/z range is compared with that of HCCA spotted directly on a MALDI plate. Matrix background peaks (e.g. m/z 568 and 757) were observed, as expected, but no lipid peaks appeared. The absence of a peak corresponding to the molecular weight (MW) of the bis-SorbPC monomer peak (MW = 786 Da) indicates that the amount of ionized monomers is too low to be detected, consistent with a high degree of lipid polymerization. The HCCA spectra in the 500-3000 m/z range are shown in ESM Figures S3a and S3b, respectively. No peaks corresponding to the molecular ions or fragment ions of GM1 (MW = 1564 Da) and GD1a (MW = 1836 Da) were detected, which suggests that ionization of these gangliosides is attenuated by their interactions with the polymerized bis-SorbPC membrane, and/or that the amount of ionized gangliosides and/or their fragment ions is too low to be detected. Mass spectra of SA spotted on a poly(bis-SorbPC) PSLB containing 1 mol% GM1 and 20 mol% GD1a and directly on a MALDI plate in the m/z range of 5-20 kD are shown in ESM Figures S4a and S4b, respectively. The background from the lipid bilayer is greater than that from the plate but is low relative to the intensity of the toxin peaks shown in Figure 1. Overall these data show that the lipid ionization background from ganglioside/poly(bis-SorbPC) PSLBs does not interfere with toxin detection under the conditions used herein.

Simultaneous detection of CTB, LTB and PTB

A number of label-free assays based on optical and electrochemical transduction principles have been developed for bacterial toxins, most notably for cholera toxin [42-48]. However, these detection methods lack specificity, i.e., the signals from the analytes and nonspecific binding cannot be distinguished. Additionally, if two bacterial toxins target the same membrane receptor, as in the case of cholera toxin and heat-labile enterotoxin, these label-free methods cannot discern which toxin is present. In contrast, toxins with different

molecular weights can be simultaneously captured on an affinity surface and identified in a label-free manner using MALDI mass spectrometry [6].

To assess the use of a ganglioside-doped lipid bilayer for simultaneous detection of CTB, LTB, and PTB, a poly(bis-SorbPC) PSLB containing 1 mol% GM1 and 20 mol% GD1a was incubated with a solution containing 0.24 μM CTB, 0.24 μM LTB, and 1 μM PTB. The mass spectrum is shown in Figure 1d. The doubly charged monomer peaks appeared in the 5000-6000 m/z range. Resolution of the singly charged monomer peaks in the 11000-13000 m/z range is shown in the inset in Figure 1d. The peaks at 11,606, 11,771, 12,007, and 12,056 m/z correspond to the CTB monomer ($[\text{CTB}+\text{H}]^+$), the PTB S5 monomer ($[\text{S5}+\text{H}]^+$), the LTB monomer ($[\text{LTB}+\text{H}]^+$), and the PTB S4 monomer ($[\text{S4}+\text{H}]^+$). Broad, low intensity peaks observed at ~ 21.9 k m/z and ~ 11.0 k m/z are assigned to S2 and S3 of PTB, as described above. The resolution at 12 k m/z is 1285 as calculated by FlexAnalysis (Bruker Daltonics); thus peaks separated by $12000/1285=9$ m/z can be resolved which is consistent with the Bruker specification of resolution >1100 in linear mode.

The correspondence of the peaks in Figure 1d with the individual toxin peaks present in Figures 1a-c demonstrate the capability to capture and detect three protein toxins simultaneously. This result suggests the possibility of using a poly(lipid)-based affinity capture platform, fabricated as a two-dimensional array (as shown in [24]), for high throughput screening of samples for ligands. The number of analytes presumably could be greater than three, assuming that the surface coverage of each analyte was sufficient for detection and they could be distinguished on the basis of differences in m/z .

Application of the affinity capture platform in complex samples

Typically, biological samples contain analyte(s) in a complex matrix of biomolecules and other components, such as tissue homogenate, fecal matter, blood serum, etc. To assess the utility of the poly(lipid)-based affinity capture platform in complex samples, analysis of CTB in both fetal bovine serum (FBS) and shrimp extract as the sample matrix was performed. CTB was spiked into either 10% (v/v) of FBS or 10% (v/v) of shrimp extract in PBS buffer, at a final CTB concentration of 0.24 μM , and the samples were incubated with poly(bis-SorbPC) PSLBs containing 1 mol% GM1, followed by rinsing, drying, and analysis as described above. Representative mass spectra are shown in Figure 3a and 3b. Peaks corresponding to singly and doubly charged CTB monomer peaks were clearly detectable despite the presence of a large excess of other proteins, although the peak intensities were generally lower than when the matrix was 100% buffer (compare to Fig. 1a). This decrease may be due to interaction of CTB with components in the complex sample matrices, which may have affected ionization efficiency. Two sets of control experiments were performed: a) 10% (v/v) of FBS and 10% (v/v) of shrimp extract were incubated with poly(bis-SorbPC) PSLBs containing 1 mol% GM1, rinsed, dried, and analyzed; the resulting spectra are shown in ESM Figures S5a and S5b. b) 10% (v/v) of FBS and 10% (v/v) of shrimp extract were spotted directly on a MALDI plate, and the resulting spectra are shown in ESM Figures S6a and S6b. These spectra show that some components in FBS and shrimp extract are detectable when spotted directly on a MALDI plate, as expected. However, they are not detectable when spotted on a poly(bis-SorbPC) PSLB, consistent with the low degree of

nonspecific adsorption described above. Overall these results demonstrate that CTB can be selectively captured on a PSLB when present in a complex biological matrix.

Minimal detectable concentration

The CTB-GM1 pair was used as a model system to estimate the minimal detectable concentration of protein captured on the PSLB-based affinity platform. Due to the semi-quantitative properties of MALDI-TOF MS, an approach based on the frequency with which CTB could be detected was used. After CTB was captured by a poly(bis-SorbPC) PSLB doped with GM1, SA matrix was spotted on different areas on the substrate. Multiple substrates were prepared and up to five matrix spots could be applied on each (the number of samples per substrate varied due to differences in substrate area). Due to the nature of dry-droplet matrix deposition, spatially heterogeneous crystal formation occurs across the matrix spot which causes significant variations in signal strength. To overcome this variable, the laser was scanned across entire area of each matrix spot at distance intervals of less than 50 μm (the diameter of the laser spot). Detection of a CTB monomer peak at 11.6 k m/z with a S/N ≥ 3 anywhere on the substrate was recorded as a detectable sample. In some published studies [49-51], internal standards have been used to generate a calibration curve and estimate detection limits for MALDI-TOF MS. In our platform, however, the amount of receptor is limited and the introduction of a competitive ligand will cause the minimal detectable concentration to be underestimated, therefore an internal standard was not used.

The minimal detectable concentration is defined here as the CTB solution concentration that produces a peak with S/N ≥ 3 for 100% of the samples analyzed. In these experiments, the mol% of GM1 was constant (1 mol% in poly(bis-SorbPC)) and the CTB concentration of the solution that was incubated with the PSLB was varied. A large CTB concentration range, from 0.5 nM to 1 μM , was screened, from which the minimal detectable concentration was determined to be in the range of 1-5 nM. A larger number of samples was prepared and analyzed in this concentration range, with each matrix spot counted as one sample. The number of samples that gave a CTB m/z peak with S/N ≥ 3 divided by the total number of samples is reported as a detection frequency in Table 1. At 4 nM CTB, the detection frequency is 100%, so this concentration is a conservative estimate of the minimal detectable CTB concentration. With respect to definitions used in clinical chemistry, 4 nM CTB is the concentration that produces a sensitivity of 100%, and since CTB was never detected in samples lacking the toxin, the specificity is 100%. The estimate of 4 nM is specific to the analysis conditions employed here, for example, the amount of GM1 is finite and the incubation time was selected to achieve an apparent steady state (but not equilibrium which, due to mass transport limitations, would have required much longer times). The minimal detectable concentration of CTB likely could be lowered by increasing the mol% of GM1 and the incubation time. The dissociation constant (K_D) for GM1-CTB also plays a role. Apparent K_D values reported for CTB-GM1 range from 4.55 pM to 370 nM [52], a very wide range attributed to significant differences in experimental parameters; thus it is also possible that at 4 nM, the amount of bound CTB is limited by its binding affinity to GM1.

Minimal detectable concentrations for cholera toxin using label-free SPR methods are in the nM range [42-44], comparable to the 4 nM reported here. Much lower limits of detection, down to the sub-attomolar [46] and attomolar levels [45] have been achieved using electrochemical methods. However, none of these methods provides the molecular specificity characteristic of the MS-based approach described herein.

On-plate tryptic digestion of captured CTB

Molecular weight information may be adequate to identify multiple analytes with resolvable molecular weights, as demonstrated above. However, when multiple analytes cannot be distinguished solely based on molecular weight differences, additional steps may be necessary. To further explore the applicability of the PSLB-based affinity capture platform, on-plate tryptic digestion of captured CTB was performed and the fingerprint spectra of CTB peptide fragments were obtained. A solution of 0.24 μ M CTB was incubated with a poly(bis-SorbPC) bilayer doped with 1 mol% GM1, rinsed with nanopure water, and dried. The trypsin concentration and on-plate digestion time were varied to obtain maximum amino acid coverage (data not shown). The optimal conditions were found to be 0.01 μ g/ μ L Trypsin Gold and 12 hours, respectively, and MALDI-TOF MS was performed on CTB peptides generated using these conditions.

A typical mass spectrum, in which the CTB peptides produced by digestion are labeled with asterisks, is shown in Figure 4. Other peaks are assigned mostly to trypsin autolysis products. The 12 CTB tryptic peptides that were detected are listed in Table 2; these peptides account for 57% coverage of the CTB amino acid sequence. In comparison to published on-plate digestion of proteins, 57% coverage is in the range that is considered acceptable [53,54]. Sequences 1-22 and 82-103 are connected through intra-chain disulfide bonds [55] and were not detected; it is possible that disulfide bridge reduction may be necessary to achieve full coverage. In comparison, van Baar et al. [39] used liquid chromatography-electrospray MS to characterize cholera toxin. They detected 13 peptides that covered the full sequence of CTB, and six of the peptides detected in their work matched peptides detected in our analysis (see Table 2).

In some cases, e.g. when analyzing multiple and/or unknown proteins, the m/z of the constituent peptides may not be sufficient to identify the proteins; in these cases, identification may be possible using MS/MS. Here the amino acid sequences of three peptides produced by on-plate tryptic digestion of captured CTB were obtained using MS/MS. These three peptides were selected for analysis because their relative yields were high in comparison to those of the other nine peptides (which were too low for this analysis). Spectra of sequences with MWs of 1216 Da, 1372 Da and 2042 Da are shown in Figure 5 and listed in Table 2. The mass difference between the measured m/z and the theoretical m/z of identified b ions and y ions is within 0.4 m/z , which is consistent with literature [56]. Overall, the results obtained from MALDI-TOF MS and MS/MS detection of peptides produced by on-PSLB tryptic digestion of CTB show that the PSLB-based affinity capture platform should be useful for identifying captured proteins with very similar molecular weights.

CONCLUSIONS

This work demonstrates that cross-linking lipid polymerization provides the stability necessary for implementation of a receptor-doped PSLB as an affinity capture platform for label-free protein detection using MALDI-TOF MS. Simultaneous capture and detection of CTB, LTB and PTB was performed, showing that differences in ligand molecular weight are sufficient to distinguish among multiple captured proteins. The high resistance of poly(bis-SorbPC) membranes to nonspecific protein adsorption is another feature that makes them useful for analysis of complex biological matrices. In some cases, differences in molecular weights among captured proteins may be inadequate for their identification. As demonstrated here, on-PSLB trypsin digestion can be employed to obtain the molecular weights of peptide fragments using MS/MS, which illustrates the potential use of the PSLB-based platform for proteomic identification of membrane-associated proteins. Finally, previous studies have shown that it is feasible to incorporate transmembrane proteins, such as bovine rhodopsin and ion channels, into poly(lipid) membranes with retention of activity [18,19,22,23]. This suggests the possibility of using the approaches described herein to capture and identify ligands that bind to transmembrane protein targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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a)

b)

FIG. 1.

MALDI-TOF MS spectra of: a) 0.24 μM CTB captured on a poly(bis-SorbPC) PSLB containing 1% GM1; b) 0.24 μM LTB captured on a poly(bis-SorbPC) PSLB containing 1% GM1; c) 1 μM PTB captured on a poly(bis-SorbPC) PSLB containing 20 mol% GD1a with subunits of PTB labeled as S1-S5.; and d) CTB, LTB and PTB captured simultaneously on a poly(bis-SorbPC) PSLB containing 1% GM1 and 20% GD1a. The inset in d) is an enlargement of the 11000-13000 m/z range. Masses are reported as means and standard deviations that were determined from at least three independent measurements on captured toxins.

FIG. 2. MALDI-TOF MS spectra in the 500-1000 m/z range of a) HCCA spotted on a poly(bis-SorbPC) bilayer containing 1% GM1 and 20% GD1a and b) HCCA spotted directly on a MALDI plate.

FIG. 3. MALDI-TOF MS spectra of 0.24 μ M CTB captured on a poly(bis-SorbPC) PSLB containing 1% GM1 in the presence of a) 10% (v/v) FBS and b) 10% (v/v) shrimp extract.

FIG. 4.

A representative MALDI-TOF mass spectrum of CTB captured on a poly(bis-SorbPC) PSLB containing 1% GM1 and subjected to on-plate tryptic digestion. The observed CTB peptide fragment peaks are marked with asterisks. The peaks marked with open circles are assigned to trypsin autolysis. The inset is an enlargement of the 1150-3000 m/z range, where CTB peptide peaks are most abundant.

a)

b)

FIG. 5. MALDI MS/MS spectra of peptides generated by on-plate tryptic digestion of CTB captured on a poly(bis-SorbPC) PSLB containing 1% GM1. The peptide MH^+ values are: (a) 1216 Da, (b) 1372 Da and (c) 2042 Da. Because these peptides were derived from a known protein (captured CTB), the m/z values of y ions and b ions were assigned by submitting the appropriate CTB sequence to Protein Prospector. In the case of an unknown protein, a search algorithm would be used. We tested this by applying the Mascot algorithm, although Mascot scores are known to vary with search parameters. Mascot scores for the spectra in (a)-(c), obtained with a typical set of search parameters, are 55, 38, and 96, respectively, with the score trend consistent with the S/N ratios apparent in the spectra. Mascot would have identified CTB based on the scores for the peptides in (a) and (c), but not (b).

Table 1

Estimation of the MALDI-TOF MS minimal detectable concentration for CTB bound to 1 mol% GM1 in poly(bis-SorbPC) PSLBs.^a

Concentration of CTB (nM)	Frequency of samples that gave a S/N $\geq 3^b$	Number of silicon wafers prepared
1	0/10	2
2	8/16	3
3	11/15	3
4	9/9	2
5	10/10	2

^aThis table focuses on a narrow CTB concentration range that was identified from screening samples over a larger concentration range.

^bFrequency is reported as the number of samples in which CTB was detected/total samples that were analyzed.

Table 2

Summary of peptide fragments observed in MALDI-TOF mass spectra of a CTB tryptic digest.

Fragment (amino acid start-end)	Sequence	Calculated MH ⁺ (Da)	occurrence frequency *	Confirmed by MS/MS?
64-67 [#]	(K)AIER(M)	488.28	3/4	
70-73	(K)DTLR(I)	504.28	1/4	
64-69 (1 Oxidation) or 68-73 [#]	(K)AIERMK(D) or (R)MKDTLR(I)	763.41	3/4	
68-73 (1 Oxidation)	(R)MKDTLR(I)	779.4	3/4	
74-81 [#]	(R)IAYLTEAK(V)	908.51	3/4	
36-43 [#]	(R)EMAIITFK(N)	952.52	4/4	
35-43	(K)REMAITFK(N)	1108.62	1/4	
35-43 (1 Oxidation)	(K)REMAITFK(N)	1124.61	4/4	
24-34 [#]	(K)IFSATESLAGK(R)	1215.62	4/4	Y
24-35	(K)IFSATESLAGK(R)	1371.72	4/4	Y
44-62 [#]	(K)NGATFQVEVPGSQHIDSQK(K)	2041.99	4/4	Y
44-63	(K)NGATFQVEVPGSQHIDSQKK(A)	2170.09	4/4	

* Occurrence frequency is the number of samples in which the fragment peak appeared/total samples. Four samples were analyzed.

[#] These peptides were also detected by van Baar et al. [39]