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**Preparation of affinity surfaces for microbiological
diagnostics**

Příprava imunoafinitních povrchů pro mikrobiologickou diagnostiku

DOCTORAL THESIS

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Prague, 2025

DECLARATION

I declare that I have worked on this thesis under the guidance of my supervisor and that all sources of previous knowledge are appropriately cited. No part of this work was used or will ever be used to obtain any other academic degree than Ph.D. from Charles University.

Prague

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Mgr. Josef Dvořák

DECLARATION OF AUTHORSHIP

I declare that Mgr. Josef Dvořák contributed significantly to the experiments and all scientific publications in this Ph.D. thesis. He performed most of the experiments, substantially contributed to their planning, and took a significant part in the primary data interpretation and their preparation for publication.

Prague

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RNDr. Petr Pompach, Ph.D.

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ABSTRACT (IN ENGLISH)

Implementing matrix-assisted laser desorption/ionization (MALDI) mass spectrometry into clinical microbiology practice is rapidly advancing due to its ability to deliver fast, high-specificity results with minimal sample preparation. The functionalized MALDI-compatible chips prepared using ambient ion soft landing (AISL) technology represent a possible enhancement of the MALDI MS devices already present in the clinical microbiology laboratories. The AISL technology allows modification of the conductive surface with a biomolecule of interest, providing the modified surface with its biological function. Therefore, it is possible to prepare a variety of functionalized MALDI-compatible chips with a variety of applications, as demonstrated in this thesis by three distinct use cases:

- (1) Procalcitonin (PCT) – A sepsis biomarker detected directly from serum, allowing sepsis severity evaluation and intact and cleaved PCT forms differentiation. Therefore, it offers more profound insight into the patient condition.
- (2) *Clostridioides difficile* toxin B (TcdB) – A clinically significant bacterial toxin detected in complex stool samples, enabling specific confirmation of toxigenic *C. difficile* strains and potentially a species carrying toxin-coding genes obtained by horizontal transfer.
- (3) Botulinum neurotoxin A1 (BoNT/A1) – A highly potent neurotoxin identified through its enzymatic activity using substrate-cleavage monitoring up to 0.5 ng/mL. The developed assay may contribute to the effort to eliminate the need for animal-based assays.

These applications illustrate the flexibility and robustness of the MALDI-compatible chips platform and highlight its value as a potentially high-throughput, automatable, and clinically compatible tool for modern clinical microbiology laboratories.

keywords:

MALDI-TOF, mass spectrometry, ambient ion soft landing, MALDI chips, clinical microbiology, procalcitonin, botulinum neurotoxin A1, *Clostridioides difficile*, toxin B, biomarker detection, *in situ* enrichment, lab-on-a-chip diagnostics

ABSTRAKT (IN CZECH)

Zavádění hmotnostní spektrometrie s matricí asistovanou laserovou desorpčí/ionizací (MALDI) do praxe klinické mikrobiologie rychle postupuje, a to díky její schopnosti poskytovat rychlé a vysoce specifické výsledky s jednoduchou přípravou vzorku. Funkcionalizované čipy kompatibilní s MALDI MS, připravené pomocí metody měkkého přistávání iontů (AISL) představují možné rozšíření stávajících zařízení MALDI MS, která jsou již běžně využívána v laboratořích klinické mikrobiologie. Technologie AISL umožňuje úpravu vodivého povrchu biomolekulami, které povrchu propůjčují svoji biologickou funkci. Díky tomu je možné připravit celou řadu funkcionalizovaných MALDI čipů s různými aplikacemi. To je v této práci demonstrováno na třech konkrétních případech:

(1) Procalcitonin (PCT) – Biologický ukazatel sepse detekovaný MALDI hmotnostní spektrometrií přímo z lidského séra optimalizovanou metodou čipu s protilátkou proti procalcitoninu. Tento přístup umožňuje nejen vyhodnocení závažnosti sepse, ale také rozlišení intaktních a štěpných forem PCT. Optimalizovaná metoda tedy poskytuje hlubší vhled do patologického stavu pacienta.

(2) Toxin B *Clostridioides difficile* (TcdB) – Klinicky významný bakteriální toxin detekovaný v komplexních vzorcích stolice. Detekce přítomnosti aktivního toxinu umožňuje specifické potvrzení toxigenních kmenů *C. difficile* a potenciálně také druhů nesoucích geny kódující toxiny *C. difficile* získané horizontálním přenosem.

(3) Botulotoxin A1 (BoNT/A1) – Vysoce účinný neurotoxin byl identifikovaný na základě své enzymatické aktivity prostřednictvím sledování štěpení substrátu až do 0,5 ng/mL. Tento přístup potenciálně pomůže snížit nutnost použití standardní metody využívající živé myši.

Tyto aplikace ilustrují rozmanitost použití a robustnost platformy MALDI čipů a zdůrazňují její potenciál jako vysoce kapacitní, automatizovatelného a klinicky kompatibilního nástroje pro moderní laboratoře klinické mikrobiologie.

klíčová slova:

MALDI-TOF, hmotnostní spektrometrie, měkké přistávání iontů za atmosférického tlaku, MALDI čipy, klinická mikrobiologie, procalcitonin, botulotoxin A1, toxin B, *C. difficile*, detekce biomarkerů, *in situ* nabohacení, diagnostika na čipu

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ABBREVIATIONS

AISL	ambient ion soft landing
APD	autoprotease domain
BoNT	botulinum neurotoxin
CDI	<i>C. difficile</i> infection
CROPs	carries combined repetitive oligopeptides domain
CRP	C-reactive protein
CS	chemical sputtering
CT	cytotoxicity assay
CT -	C-terminal
CV	coefficient of variation
DDM	n-Dodecyl-B-D-Maltoside
DHAP	2,5-Dihydroxyacetophenon
DHB	2,5-Dihydroxybenzoic acid
DNA	deoxyribonucleic acid
DRBD	delivery and receptor-binding domain
E _{conv}	the extent of conversion
EIA	enzyme immunoassay
ELISA	enzyme-linked immunoassay
EMA	European Medicines Agency
EOC	ELISA on the chip
ESI	electrospray ionization
ESSI	electrospray sonic ionization
FDA	Food and Drug Administration
FT-ICR	Fourier-transform ion cyclotron resonance
GC	gas chromatography
GDH	glutamate dehydrogenase
GTD	glycosyltransferase domain
HA	hemagglutinin proteins
HCCA	α -Cyano-4-hydroxycinnamic acid
IA	immunoassay
ICP	inductively coupled plasma

ICU	intensive care units
IL	interleukin
ISL	ion soft landing
IVD	in vitro diagnostics
keV	kiloelectronvolt
LAMP	loop-mediated isothermal amplification
LC	liquid chromatography
LES	low-energy sputtering
LFA	Lateral flow immuno chromatography assay
LOD	limit of detection
m/z	mass-to-charge
MALDI	matrix-assisted laser desorption/ionization
MBA	mouse bioassay
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSn	multiple mass spectrometry
NAAT	nucleic acid amplification techniques
NAPs	neurotoxin-associated proteins
NGS	next-generation sequencing
NT-	N-terminal
NTNH	non-toxic non-hemagglutinin
PC	progenitor complex
PCR	polymerase chain reaction
PCT	procalcitonin
Q	quadrupole
qPCR	real-time polymerase chain reaction
RIS	reactive ion scattering
RL	reactive landing
RNA	ribonucleic acid
rRNA	ribosomal ribnucleic acid
S/N	signal-to-noise
SA	sinapic acid

SAM	self-assembled monolayers
SCP	single cell proteomics
SD	standard deviation
SELDI	surface-enhanced laser desorption
SID	surface-induced dissociation
SIM	single ion monitoring
SIMS	secondary ion mass spectrometry
SL	soft landing
SNAP	synaptosomal-associated protein
SRM	selective reaction monitoring
TC	toxigenic culture
TcdB	Toxin B
TIMS	traped ion mobility spectrometry
TNF- α	tumor necrosis factor α
TOF	time-of-flight
UDP	uridine diphosphate
UV	ultra-violet
VAMP	vesicle-associated membrane protein
WBC	white blood cells

1. Introduction

1.1 Clinical Microbiology

Clinical microbiology involves diverse pathogen isolation and identification procedures, strongly relying on culture-based and phenotypic methods. However, the fast-paced evolution of some microorganisms and the variety of pathogens represent an overall challenge for this field ^[1,2]. Therefore, a lot of effort is made towards developing new and optimizing current methods to obtain more efficient and robust tools. The most elementary routinely used methods are discussed in the following paragraphs ^[3].

1.1.1 Biochemical and phenotypical methods

Biochemical reactions and phenotypic characterization have been used since the early history of clinical microbiology for microorganism identification. The biochemical reactions were originally carried out and analyzed in separate reaction tubes. Therefore, the system was complicated and laborious ^[3]. However, commercial vendors simplified the testing by introducing instruments (Fig.1A) for automatic interpretation of panels (Fig.1B) that can also be interpreted manually.

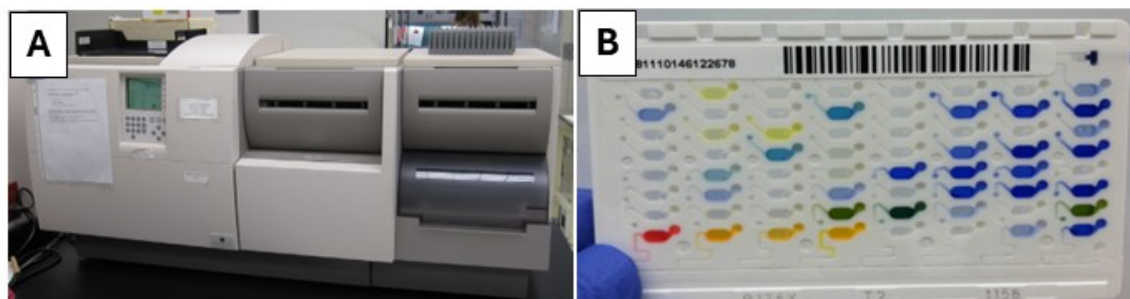


Figure 1 – A is a photograph of the Vitek 2 instrument used for automatized biochemical test reading of the biochemistry testing panel B, where each well represents a unique biochemical reaction.

Manual and automated systems compare the biochemistry tests to an organism database and provide identification with a percentage confidence score. Biochemical panels are cost-effective and simple to use. However, they are slow and vary in performance. Genus- and species-level identification rates of aerobic Gram-negative rods are in the range of 75 to 85 % ^[4]. The biochemistry tests are unreliable for inert, slowly growing, or infrequently isolated bacteria, including anaerobes, where the correct genus-level identification ranges from 70 to 90 % and 50 to 60 % for the species level ^[5,6].

Nowadays, biochemistry tests are still a good option for some microbiology labs because they are simple and cost-effective. However, higher accuracy, speed, and robustness are necessary for some applications. This fact led to the implementation of other methods, like enzyme-linked immunoassays (ELISA/EIA), molecular biology methods, and mass spectrometry [3].

1.1.2 Enzyme-linked immunoassays

Enzyme-linked immunoassay (ELISA/EIA) is a biochemical procedure utilizing a conjugate of the antibody with an enzyme for specific antigen detection. ELISA is a commonly used tool in multiple industries, including disease diagnostics and clinical microbiology [7,8]. There are three main types (Fig. 2): direct, indirect, and sandwich. In direct and indirect ELISA, the sample is added to a plate to bind antigens, followed by blocking with BSA or casein to prevent non-specific antibody binding. Direct ELISA uses a primary antibody with an enzyme, producing a detectable color change upon substrate addition. In contrast, indirect ELISA uses a secondary enzyme-conjugated antibody to bind the primary antibody's Fc region, enhancing versatility and cost-effectiveness [8,9]. Sandwich ELISA uses a capture antibody that binds the antigen first, followed by blocking and layering with primary and secondary antibodies, yielding higher sensitivity, making it the most widely used format [10].

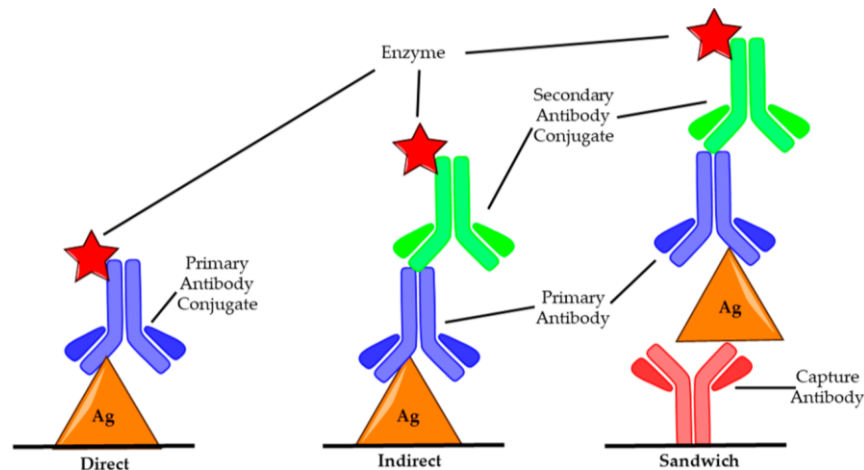


Figure 2 – The image of common ELISA setups. In Direct and Indirect ELISA, antigens (Ag – orange triangle) are first attached to the microtiter plate (black line), followed by an antigen-specific antibody binding. In Direct ELISA, this primary antibody (blue) is linked to an enzyme (red star), e.g., HRP, which produces a color change when exposed to a substrate. In Indirect ELISA, the enzyme is linked to a secondary antibody (green) that binds to the primary antibody, producing a detectable color change. In Sandwich ELISA, a capture antibody (red) first binds to a specific antigen on the plate, and the rest of the assay follows the same steps as indirect ELISA. As shown in [11].

Enzyme-linked immunoassay is one of the most extensively used *in-vitro* diagnostic testing methods. Routinely, ELISA can quantify diverse analytes such as proteins, hormones, antibodies, and drugs present in highly complex biological fluids. Therefore, it is suitable also for identifying pathogen infection. Although it is a valuable method that allows quick decisions, it suffers interferences from exogenous and endogenous sources. The interference might result in false positive and negative results ^[12]. Another alternative is molecular biology testing ^[13].

1.1.3 Molecular biology

Microscopy, microbial culture, and immunodiagnostics have served as reliable tools for over 100 years and represent a gold-standard approach for detecting many pathogens. There is little reason to expect the presence of these proven methods to fade with time. However, complementary diagnostic strategies based on molecular biology have dramatically changed in recent decades ^[13].

One of the first US Food and Drug Administration (FDA) approved molecular *in vitro* diagnostics (IVD) of infectious diseases were probe-based assays without nucleic acid amplification ^[14]. The molecular probes are single-stranded oligonucleotides (DNA, synthetic DNA mimetics, RNA) that hybridize with complementary microbial nucleic acids ^[15]. The probe is directly or indirectly conjugated to a chromogenic enzyme, radioisotope, or fluorophore. Probes can be applied directly or to the fixed/sectioned tissue, where hybridization occurs ^[16]. Non-amplified probes have been traditionally used as a substitute for biochemical identification ^[17].

The huge breakthrough in the field came in the early 1980s when K. Mullis developed the polymerase chain reaction (PCR) ^[18]. The method is based on the DNA-polymerase ability to synthesize double-stranded DNA *in vitro* using a single-stranded DNA template and 5'-oligonucleotide primer. The target sequence can be selectively amplified from a complex nucleic acid mixture by applying primers to both forward and reverse strands. After the first steps of denaturation, annealing, and elongation, specific double-strand DNA serves as a template, and the number of amplified target sequences doubles each cycle (n). Therefore, the resulting number of the amplified products can be characterized as 2^n ^[13]. The initial PCR steps are visualized in Figure 3 on the following page.

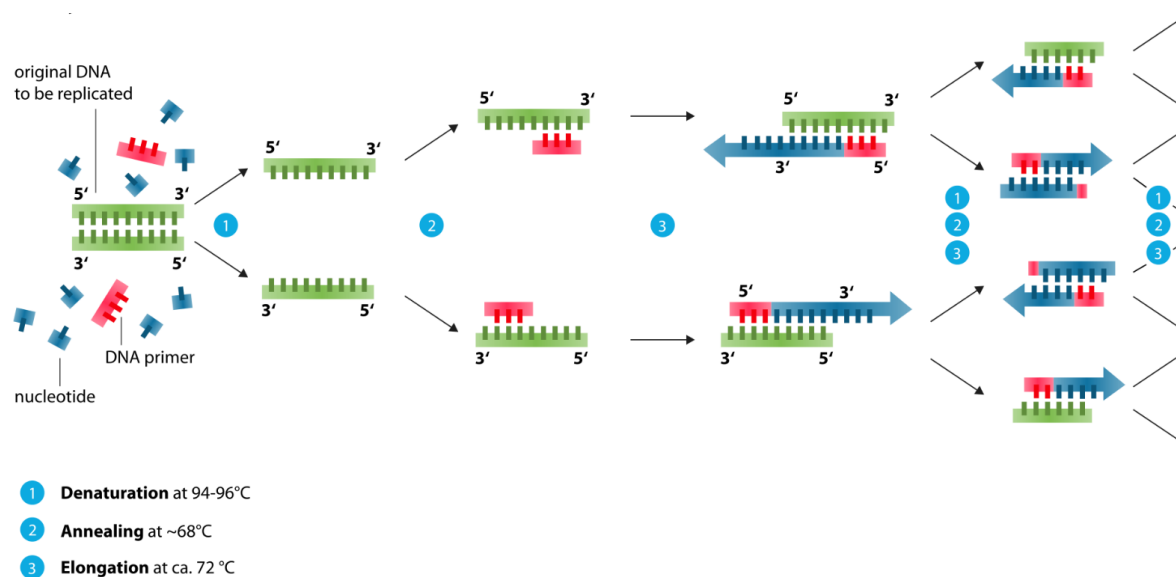


Figure 3 – Scheme of PCR steps. In the first initial step, double-stranded DNA is denatured by high temperature 1. In the following step, the primers are annealed on the 5' end of the single-strand DNA 2. In step 3, the DNA polymerase is used to elongate the complementary strand. In the following step, amplification of the specific DNA fragment takes place to produce 2^n products, where „n“ is the number of cycles. Used from [19].

A key PCR modification can be made using a reverse transcriptase enzyme. The aim is no longer double-stranded DNA, but it targets RNA to cDNA, which allows PCR to amplify the genomes of RNA viruses or mRNA and rRNA from any microbial target [20]. The diagnostic utility of PCR relies on paired methods for the amplicons. Therefore, PCR is combined with molecular probes that allow simultaneous amplification and detection – quantitative/real-time PCR (qPCR). The detection mechanism is exceptionally diverse. However, they share one basic mechanism of using one or more labeled oligonucleotides binding the amplicon and facilitating an empirical readout. One good milestone example was the use of fluorescence [21].

Nucleic acid amplification techniques (NAAT) became an integrated component of the microbiology routine practice. They have become very useful as a fast tool for substituting bacterial growth, especially for pathogens whose growth demands exceed routine capabilities [22]. However, the evolution of modern nucleic acid sequencing methods, such as next-generation sequencing (NGS), provides valuable information. Sequencing can be employed for microbial DNA/RNA characterization. The nucleic acid is purified directly from clinical samples or individual strains isolated in culture. Several sequencing assays have gained national IVD status [23].

The trends of clinical microbiology have changed several times throughout the years, from microscopy to cell culture, ELISA, PCR, and NGS. Technological progress provides various

methods, each with advantages and disadvantages ^[24]. Different methods are useful in different conditions and analysis requirements, such as time, cost, scale, and robustness. Although molecular methods have largely increased the time efficiency of the detection, there is a need for cost reduction using simplified operations when it comes to large-scale clinical applications. This fact also divides the focus towards developing mass spectrometry tools ^[24].

1.2 Mass spectrometry in clinical microbiology

The ideal clinical diagnostic approach emphasizes being cost-effective, highly specific, and sensitive, having a wide dynamic range, and allowing a rapid high-throughput analysis ^[25]. Besides all the mentioned properties, a key role has a low false positive and negative rate due to increased unnecessary expenses connected with the wrong treatment and prolonged patient stay in the hospital ^[26]. Today's peak methodology is the fruit of more than fifty years of development, providing a far-reaching impact on disease diagnosis and treatment. The most commonly used methods discussed in the previous section include traditional clinical microbiology, enzyme-linked immunosorbent assays, and nucleic acid amplification assays. However, their limitations have driven the search for alternative approaches. One of the most rapidly advancing fields is the application of mass spectrometry (MS) in clinical diagnostics. Beyond diagnostics, MS also plays a vital role in clinical research ^[27].

Mass spectrometry has gotten into the spotlight due to its advantages – high sensitivity, high specificity, and high-throughput analysis ^[28]. Mass spectrometry is crucial in research, helping to understand the specific mechanism of change in the relevant disease, which in turn provides a reliable hit for the early diagnosis of diseases and lays the practical foundation for developing clinical diagnostics methods ^[25]. Mass spectrometry has a wild card when connected with highly effective separation techniques, providing a powerful analytic tool commonly coupled with gas (GC) or liquid (LC) chromatography. Other setups used for clinical diagnostics are a combination of inductively coupled plasma (ICP) and matrix-assisted laser desorption/ionization (MALDI) with MS. These combinations were reported in many studies to be applied in different clinical application fields. Including treatment monitoring, discovery of metabolite biomarkers, drug detection/monitoring, metal poisoning, and protein identification ^[29–32].

Mass spectrometry delivers ideal analytical performance for clinical biological sample analysis, but its implementation requires sample pretreatment steps or a time-consuming

chromatographic separation process ^[25]. This fact fuels the exploration of novel MS and chromatographic instruments and approaches in clinical analysis. Ambient ionization mass spectrometry with little or no sample pretreatment dramatically simplifies the experimental complexity and operation time while improving sample analysis efficiency ^[33]. The exploration also led to the development of the MassSpec pen ^[34]. However, the removal of chromatographic separation can cause, in some cases, potential interference in the analysis since the samples are complex matrixes (blood, urine, tissue, hair); therefore, ambient ionization is used chiefly for clinical surgery (iKnife, MasSpec Pen) or breath testing ^[34–36]. The MS instruments have several scanning modes (Fig. 4): full scan, single ion monitoring (SIM), selective reaction monitoring (SRM), and multiple reaction monitoring (MRM). In the full scan mode, the instrument records all ions within a set range, which allows qualitative analysis of an unknown substance. Based on the full scan spectra, all fragment ions can be obtained by secondary (MS/MS) or multiple mass spectrometry (MSⁿ) analysis. A SIM can be performed if there is a need to exclude the interference of the other ions. SIM is often used as a pre-step for SRM (MS/MS) and following MSⁿ, where the parental ion is selected for further fragmentation, increasing the obtained information about the ion while decreasing other noise ^[25]. The MRM has evolved from SRM and has the same characteristics as SRM, meaning that multiple SRMs are performed simultaneously when multiple compounds are measured simultaneously. Full scan mode is always performed for qualitative analysis to obtain more information. SIM, SRM, and MRM are preferred modes for quantitative analysis to improve the signal intensity of known ions. SRM or MRM mode is preferred for complex samples with a high matrix background ^[25].

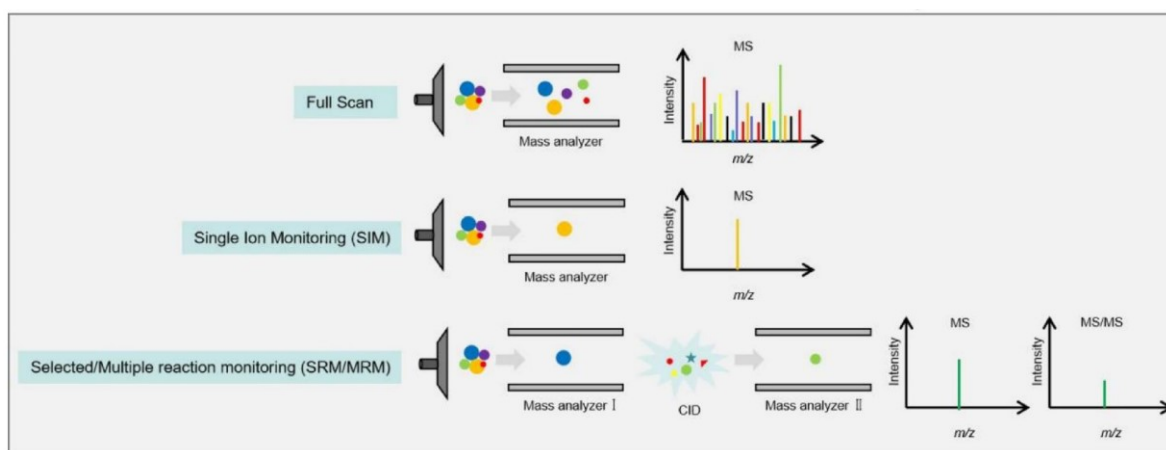


Figure 4 – Schematic diagram of mass spectrometry scanning modes. Provided by [25].

As mentioned in the previous paragraphs, mass spectrometry is also used in clinical research, where GC-MS, LC-MS, MALDI-MS, and ICP-MS play the main role [29,30,32]. Some techniques were certified by the US Food and Drug Administration. Numerous studies have shown the importance of MS in the field of clinical application, e.g., inborn errors of metabolism [37,38], vitamin testing [39], hormone detection [40], drug monitoring [41], metabolite detection [42], and, most importantly, for this thesis – microorganism [43] and protein identification [44].

MS protein identification – MS proteomics approaches were commonly used during the work, and the use of MS in clinical microbiology is the primary focus of this thesis.

1.2.1 MALDI-TOF MS in clinical microbiology

Karas and colleagues originally proposed matrix-assisted laser desorption/ionization mass spectrometry in 1985. Organic molecules were used as matrices to assist in the desorption and ionization of the analyte under UV laser [45]. The time-of-flight (TOF) analyzer was coupled with MALDI MS in 1988 for macromolecule analyses emphasizing proteins [46]. The scheme representing the instrument layout is in Fig. 5. The MALDI-TOF MS evolved throughout the years to be a powerful instrument for analyzing various molecules, particularly nonvolatile, large, and thermally labile compounds such as polymers, oligonucleotides, and proteins. The instrument's versatility across biological molecules laid the basis for using mass spectrometry in biomedical research, being awarded the Nobel Prize in Chemistry in 2002 [47].

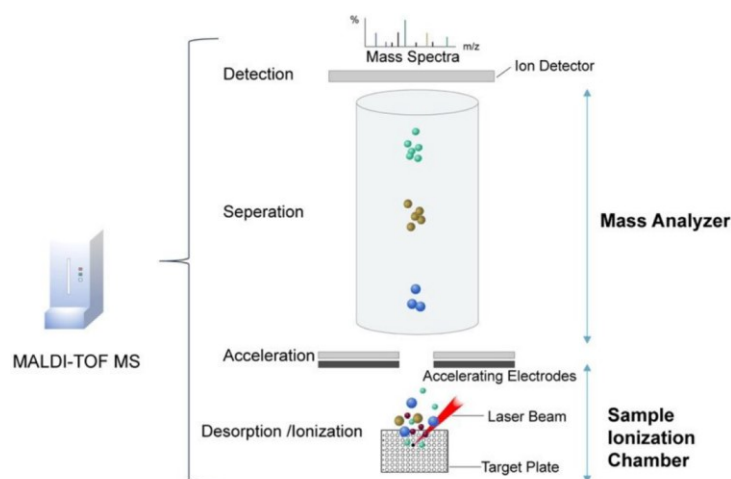


Figure 5 – MALDI-TOF mass spectrometer analysis scheme showing the overall setup of the instrument. An analyte on the conductive target plate overlaid with a matrix is ionized by a laser beam. The ions travel through the evacuated time-of-flight analyzer and hit the detector. Reprinted from [24].

The instrumentation and application of MALDI-TOF MS was developed preferably for proteomics and metabolomics studies through the qualitative and quantitative analysis of proteins and metabolites [24]. Due to its easy handling, high throughput, and high contamination tolerance (salts, buffers, detergents), MALDI-TOF is standardly used across clinical microbiology laboratories worldwide for various clinical applications. Several MALDI-TOF MS systems were registered as medical devices for clinical use, e.g., Bruker MALDI Biotyper, bioMérieux Clinical diagnostics VitekMS, and others [48,49]. In recent years, a large number of research review articles have been published, mostly focused on the application of microbiology and microorganism identification [50], antibiotic resistance testing [51], and bloodstream infection diagnostics [52].

Microorganism identification by MALDI-TOF MS became a standard and efficient approach since its introduction to clinical microbiology laboratories in 2009 [53]. Major improvements have been made during the last decade and a half since its introduction. The most important improvement was the bacterial sample preparation, demonstrating that fresh bacterial colonies can be streaked directly on MALDI plates with a simple preparation. The simplified protocol contributed to the practical application of the MALDI-TOF MS technology to be implemented worldwide [53]. The basic microbial identification workflow is shown in Figure 6.

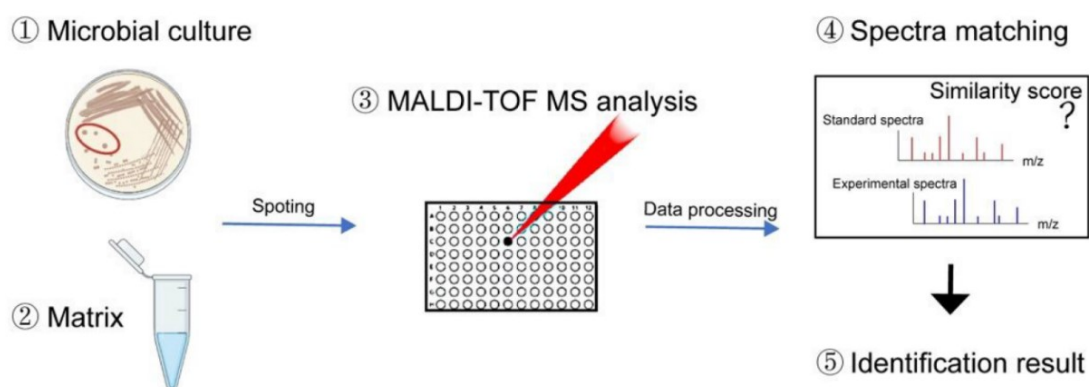


Figure 6 – Typical microbial identification workflow representation. In the first steps, a bacterial monoclonality is mixed with the matrix and applied to the MALDI target. The resulting spectra are matched with the database available with a similarity score. Reprinted from [24].

In 1975, Anhalt and colleagues attempted to identify bacteria by MS for the first time [54]. Since the 1990s, when the MALDI-TOF was introduced for identifying bacterial species, studies declared its ability [55]. In 1994, Cian et al. noted that the bacteria can be determined based on the mass spectra of the water-soluble proteins obtained from the bacteria. This method is called bacterial fingerprinting since the obtained spectra of the tested colony are compared with the database of clinically important bacteria on the species level [56]. Claydon and colleagues demonstrated in 1996 a possibility of MALDI-TOF analysis directly from single colonies of Gram-negative and Gram-positive bacteria in the intact state within minutes [55]. Several FDA-approved systems have been available for routine identification of bacterial, yeast, and fungi species throughout the years of database, software, instrumentation, and sample preparation development [57,58].

One of the limitation factors is the requirement of purified single colonies obtained by bacterial culture to reach high accuracy. A sample pretreatment, e.g., bacterial enrichment by magnetic beads [59], microfluidic techniques to shorten or avoid bacterial culture [60], development of new matrixes or surface enhancement to acquire more molecular information [61], or developing new algorithms or data analysis framework can be used to overcome this limitation [62].

In addition to microbial identification, MALDI-TOF MS has provided the possibility to assess the antimicrobial resistance of pathogens for the best possible treatment guidance [63]. There are several mechanisms of antimicrobial resistance, e.g., formation of enzymes against antibiotics, generation of novel pumps to exclude substances, generation of novel protective proteins, alterations of enzymes making bacteria less susceptible to antibiotics, or alterations in metabolic pathways related to the response against antibiotics [63]. MALDI MS showed an ability to target some of the mechanisms and uncover the resistance of some pathogens, making it complementary to the routinely used methods [63].

It was reported recently that MALDI-TOF MS could also be performed on liquid cultures and samples, such as urine, blood, or complex samples like stool. Despite the published research, no currently available instrument offers the possibility of identifying a mixture of pathogens. Due to its complexity, sample multistep pretreatment protocols enriching the analyzed microorganism are used [24,48]. This means that clinical microbiology diagnostics of complex human samples require specialized enriching protocols, where protein chips compatible with MALDI-TOF MS suitable for *in situ* enrichment can provide an alternative way for complex sample purification prior to analysis.

1.3 MALDI-compatible protein chips

1.3.1 Affinity carriers compatible with MALDI mass spectrometry

As mentioned in the previous paragraphs, MALDI MS is considered a powerful tool for biomolecule analysis. However, the analysis requires a certain sample purity ^[24]. Biological samples, e.g., serum, cell lysate, and stool, represent a challenge since they are a complex mixture of multiple biomolecules, organic compounds, and salts in addition to the targeted analyte ^[64]. The additional substances can disrupt the mass spectrometric analysis of the favored analyte. Therefore, prior to the analysis, a purification step is needed to discard unwanted molecules (mainly salts and lipids) and increase the abundance of the analyte in the sample. The analyte is purified based on its physical and chemical properties using several methods, including electrophoretic separation, liquid chromatography, and affinity enrichment ^[65].

Affinity enrichment methods use materials with a high affinity to the targeted molecules, such as gold for protein and titanium oxide for phosphopeptides enrichment. The low specificity is a significant disadvantage of these materials for some applications ^[66,67]. High specificity plays a crucial role in cases when enrichment of specific proteins and analytes is required. Surface-modified affinity carriers are commonly used for particular enrichment. The carriers, most widely beads or stationary phase, are surface-modified with an immobilized ligand or an antibody specifically binding the targeted molecule ^[68,69]. In 1993, a MALDI variation called surface-enhanced laser desorption ionization (SELDI) was published, where surface-modified chips are used for the specific analyte enrichment prior to the mass spectrometry analysis ^[70]. Multiple variations of SELDI chips surface modifications (Fig. 7, page 22) provide a highly useful and faster alternative for the enrichment of analytes in the context of MALDI MS. The biomolecules-modified chips provide high specificity for the analyte enrichment and can be manufactured with the ion soft landing technology ^[71].

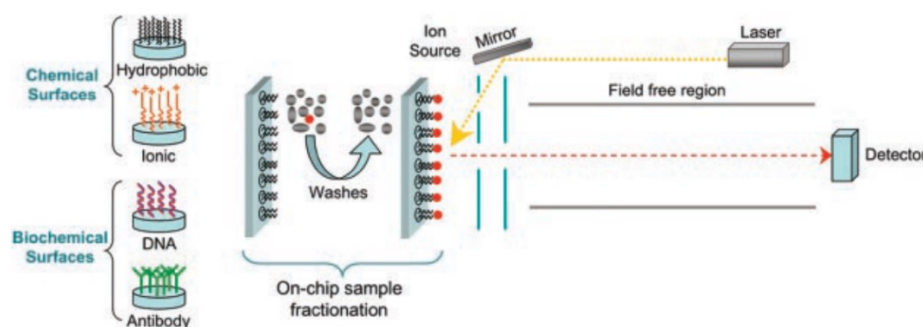


Figure 7 – SELDI-TOF MS principle showing different variations of SELDI surfaces. The enrichment is followed by washes to discard all non-specifically bound molecules. The enriched molecules are then analyzed using MALDI-TOF MS. Used from [72].

1.3.2 Ambient ion soft landing

One possibility for manufacturing biochemical affinity surfaces is using the ion soft landing, which is one of the processes occurring during collisions of ions with the surface (Fig. 8). In addition to the soft landing, several other competitive processes can occur depending on the ionization kinetic energy [73].

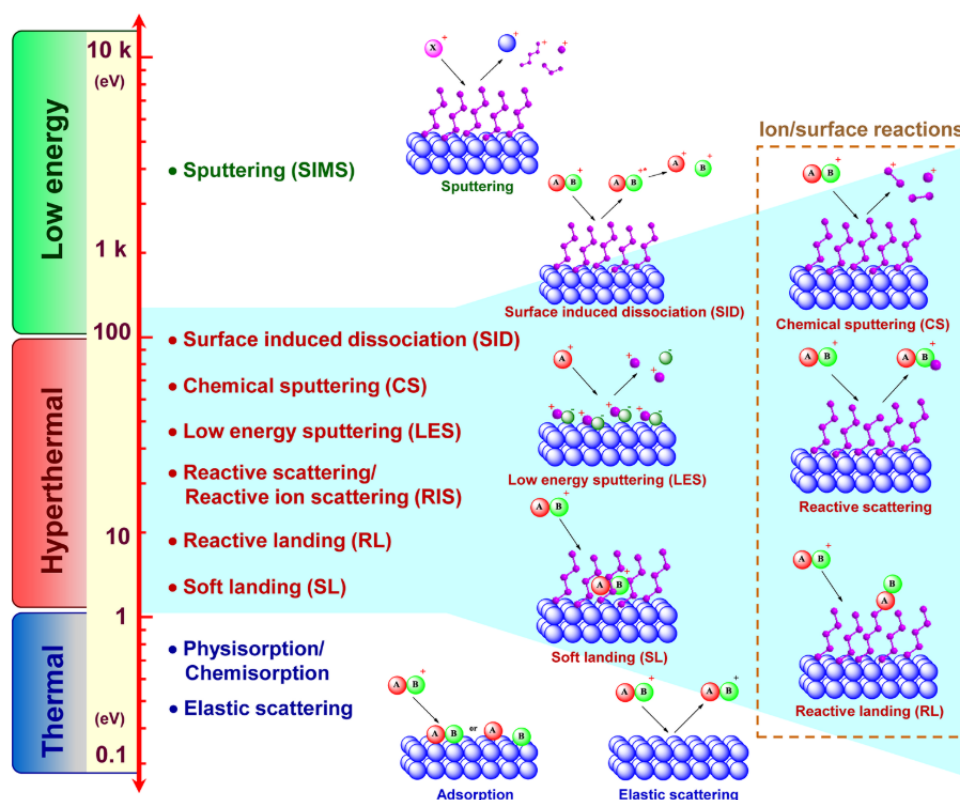


Figure 8 – The essential types of ion/surface collisions sorted by increasing energy regimes. It is important to note that soft landing is in the low-kinetic energy region. Therefore, it is suitable for surface modification in cases where there is an emphasis on retaining the structure of the molecule modifying the surface, e.g., biomolecule modification. Used from [73].

The first ion/surface collision observed by J. J. Thomson is the ion-induced ejection of secondary species from a surface ^[74]. Arnot and Milligan laid the origin of MS-based ion/surface experiments by investigating the secondary ion yield and energy distribution of negative ions induced by the collision of positive ions with the surface ^[75]. This type of mass spectrometry was later named secondary ion mass spectrometry (SIMS). In SIMS, a bombardment of kiloelectronvolt (keV) energy primary ion beams at a condensed-phase interface is used to release charged species that are mass analyzed. The SIMS setup didn't allow ionization without harming the molecular structure, so it was primarily used for elemental analysis ^[76]. In the 1970s, ionization technology evolved to low ion dose static SIMS for the nondestructive analysis of molecular surfaces, including organic materials ^[77]. Simultaneously with SIMS development, the surface-induced dissociation (SID) experiments took place. During the SID process, molecular ions collide with the surface (kinetic energy of 20-200 eV), resulting in fragmentation due to internal energy uptake competition with the neutralization process ^[78]. The SID is a powerful method complementary to collision-induced dissociation (CID), which is the most common activation technique in tandem mass spectrometry, activating target ions through collisions with collision gas ^[79]. The SIMS, SID, and CID are defined as non-elastic processes, the first historically observed, occurring during collisions of kinetic energy in the order of 10^2 - 10^4 eV. These non-elastic processes were predecessors to the elastic processes occurring during collisions of kinetic energy in the order of 1-100 eV, termed hyperthermal collisions ^[80,81]. The hyperthermal collisions include chemical sputtering (CS), low-energy sputtering (LES), reactive ion scattering (RIS), and essential for this work, reactive landing (RL) and ion soft landing (ISL). Competition among these processes depends mainly on the collision energy but also on the nature of the collision partners, e.g., elastic ISL competes with inelastic SID and additionally with several reactive collision processes ^[73].

During the CS and LES, ionization and sputtering of the atoms or molecules already present on the surface are caused by the colliding ions. The LES and CS are valuable tools for surface characterization ^[82]. The low-energy processes in which new chemical bonds are broken or formed during the ion/surface collisions are called reactive scattering or reactive collisions. During reactive collisions, an exchange of atoms or groups of atoms between the molecular surface and the projectile ion occurs. Gas-phase-generated molecular ions or clusters can be directed at a surface with only a small kinetic energy of a few electronvolts to achieve

2 processes ^[73]. The first is RL, where ions are covalently immobilized onto a molecular surface. The second process essential for this thesis is the nondestructive deposition of polyatomic ions on a surface called the ion soft landing – ISL ^[83].

The non-destructive nature of the RL and ISL makes them suitable for surface modification with molecular ions that retain their structure, providing desired properties to the surface ^[73]. For this thesis, we selected the surface modification conditions to reach the kinetic energy at lower limits of the hyperthermal processes; therefore, the ISL is the statistically most relevant process. The ISL can be used to modify conductive and semi-conductive surfaces, where the ion charge is not transferred to the modified surface. Instead of accumulating the charge from ions on the surface, the charge is neutralized by applying a voltage of opposite polarity on the modified surface, which increases the efficiency of the modification because it eliminates the repulsive force of the ions already immobilized on the surface ^[84]. The self-assembled monolayers (SAM), metal surfaces (stainless steel, gold, copper), and materials surface-modified with a thin layer of a metal oxide, e.g., glass slides modified with a thin layer of indium-tin-oxide for MALDI imaging, are routinely used for surface modification ^[84–87].

The ISL allows the deposition of untouched polyatomic ions on the modified surface ^[88]. The particle landing on the surface distributes its kinetic energy into the vibrational and electronic degrees of freedom of the projectile molecule and the surface without disturbing its own covalent bonds ^[73]. The first ISL experiments were conducted in the vacuum using the MS ionization and separation (Fig. 9), which allowed the selection of ions of a required chemical, structural, or isotope purity ^[89–91]. In 1977, Cooks and Franchetti demonstrated surface modification in the vacuum with polyatomic ions using the carbon and sulfur compounds example (CS^{2+} , CS_2^{2+} , CS^+ , S^+ , S_2^+), where a low and high energetic deposition of these ions was compared ^[90].

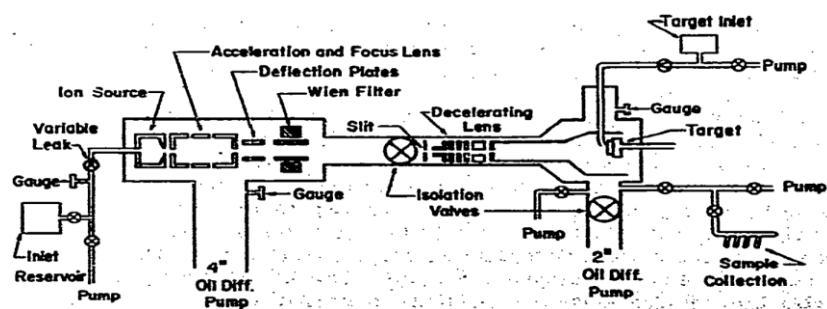


Figure 9 – A schematic diagram of the soft landing apparatus for the mass-selected ions deposition on the metal of frozen surfaces that Cooks and Franchetti published in 1977 ^[90].

The ion selection based on the m/z is a significant advantage of the soft landing in the vacuum ^[92]. However, the instrumentation requirements and small surface modification efficiency make this approach expensive and time-consuming. The ambient ion soft landing at atmospheric pressure (AISL) is an alternative without mass selection, simplifying the instrumentation requirements and increasing the yield of the final ions immobilized on the surface with a significantly denser ion beam compared to the vacuum instrumentation ^[83,93–95]. This method was used to prepare arrays of nanotubes, nanostructures, fullerenes, proteins, etc ^[96–99].

The ion soft landing apparatus (Fig. 10) is commonly composed of a soft ionization source, mostly electrospray sonic ionization (ESSI) or electrospray ionization (ESI), desolvation tube, and in case the collecting electrode (modified surface) is not in the horizontal line with the ion beam, a deflector electrode directs ions to the collecting electrode. The deflector electrode can be used to separate the ions from the particles without a charge, which helps to purify the ion beam landing on the surface at the cost of a smaller ion yield ^[83,99].

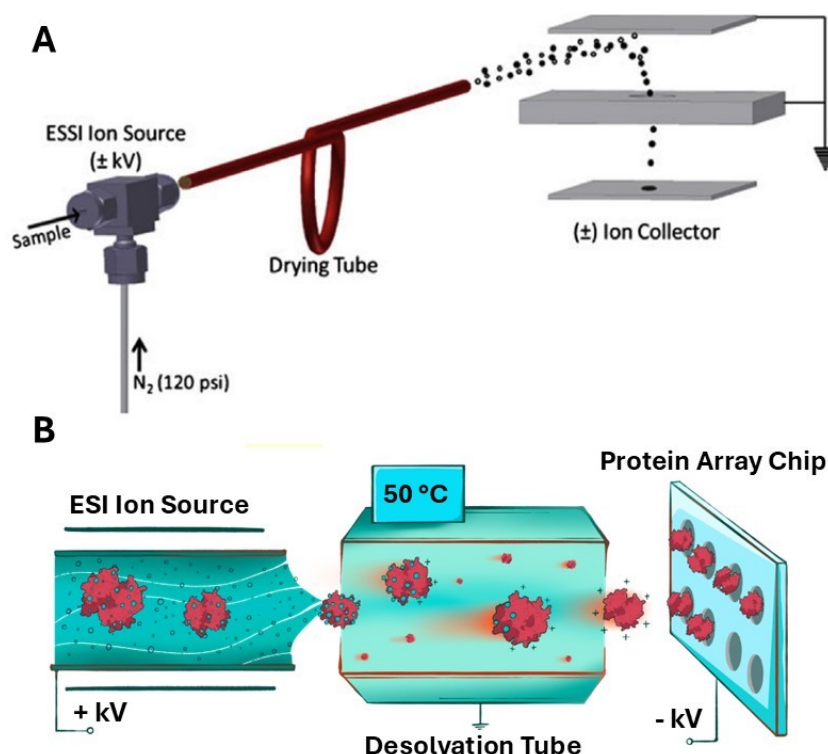


Figure 10 – (A) AISL apparatus with a collecting electrode perpendicularly to the ion beam requiring a deflector electrode. Reprinted from ^[95]. A soft landing apparatus with horizontal settings is shown in scheme (B). The sample solution pump connected to the electrospray ionization using inert carrier gas (N_2) and desolvation tube for dry ions deposition to the collector electrode (modified surface) is common for both set-ups. Scheme B was inspired by ^[100], which was discussed and produced by Kuba Scientific Draws.

1.3.3 MALDI protein chips – Application of the ion soft landing

Cooks and Gologan published a study in 2004 where a lysozyme was soft-landed on liquid glycerol surfaces using the classical vacuum set-up of the soft landing. It was noted that lysozyme retained its biological activity ^[101]. In the next study, Deng et al. used scanning tunneling microscopy to identify the influence of the electrospray deposition on the spatial arrangement of Cytochrome C as a model protein molecule. Cytochrome C was selectively deposited using charge selection (concentration of formic acid), showing that in high charge states ($z = +12$ to $+18$), the molecule is immobilized on the surface unfolded or refolded on the weakly interacting surfaces. The molecule is preferably folded in the lower ($< +10$) charge states ^[102]. Examples of each scanning tunneling microscopy measurement for each molecular state are shown in the following Figure 11.

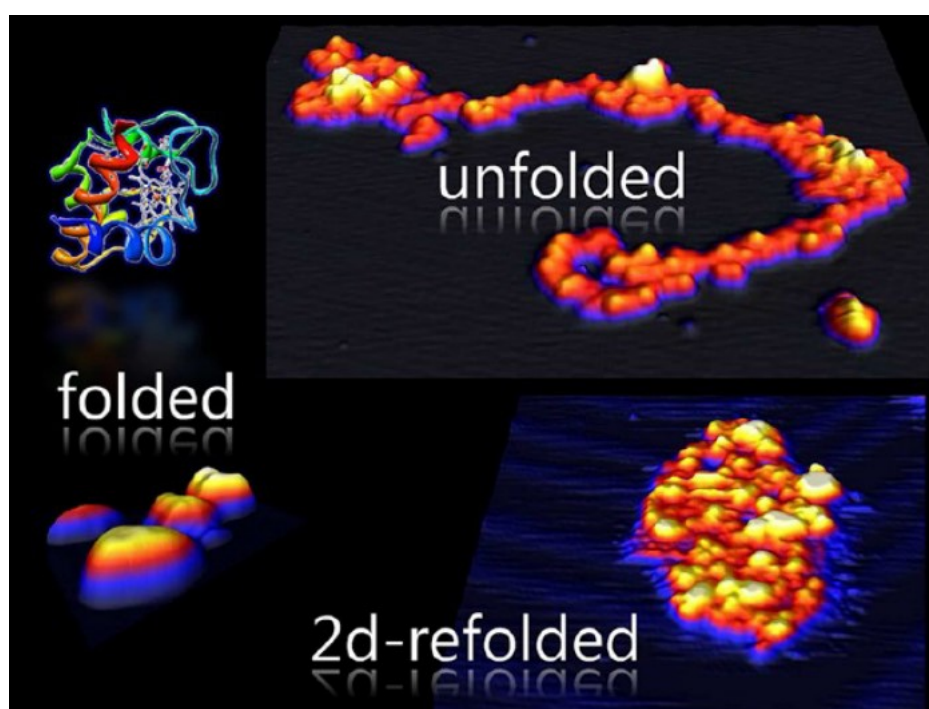


Figure 11 – Visualisation of the scanning tunneling data representing each of the possible states of Cytochrome C after soft landing deposition on the surface. Reprinted from ^[102].

Cooks, Deng, and their colleagues' research shows that the ion soft landing is suitable for surface modification with proteins that retain their spatial arrangement and biological activity. This opens a new possibility of functionalized protein chips for various applications ^[71,86,103].

Preparing functionalized MALDI-compatible protein chips is one of the possible applications [86,103–106]. In this approach, the immobilized protein of interest on the indium-tin-oxide or stainless steel surface provides biological activity to the modified surface. The use of proteases, glycans, and antibodies was published, showing the method's versatility in expanding the MALDI TOF MS applications. The overall protocol (Fig. 12) consists of multiple steps. The first step is preparing the MALDI chip using AISL, followed by sample application on the functionalized chip, e.g., enrichment of the analyte from a complex sample. The next steps are incubation, washing, and application of MALDI matrix. The enriched analyte is analyzed using MALDI TOF mass spectrometry in the last step.

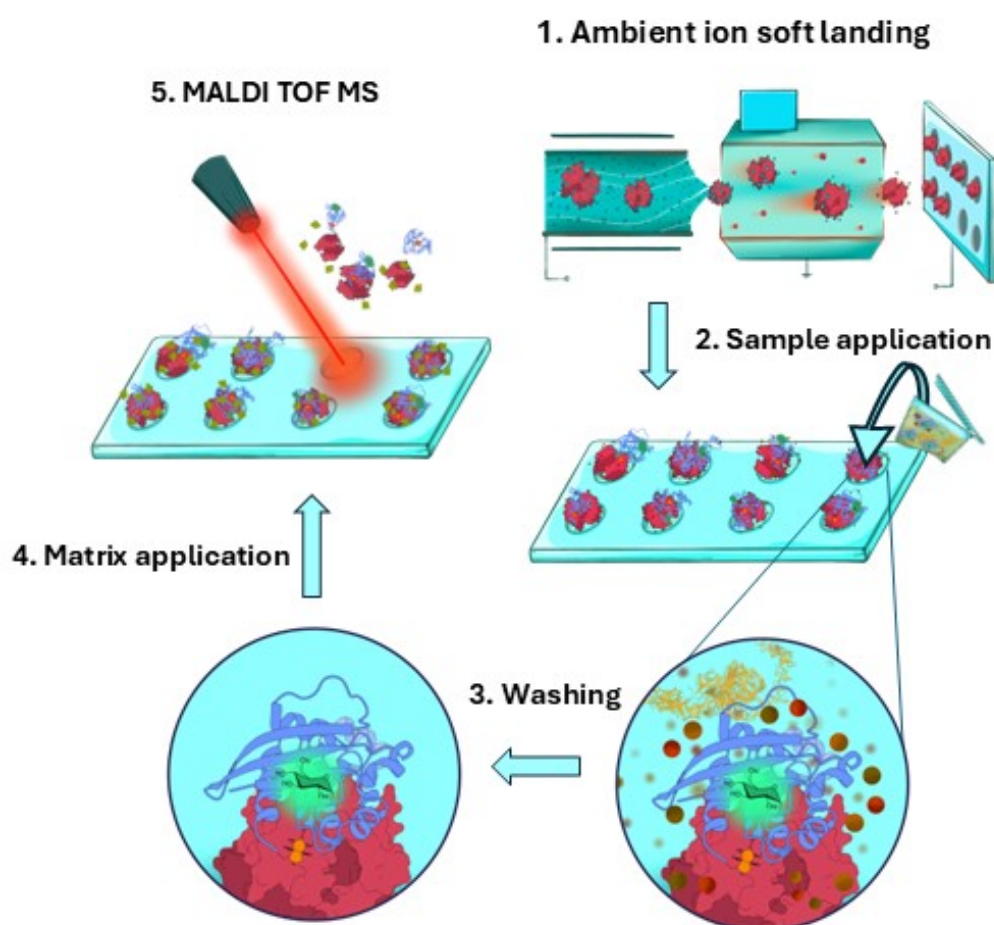


Figure 12 – A workflow (clockwise) of typical AISL functionalized chips experiment. The first steps include preparing the chip and enriching the desired analyte. Followed by washing protocol, removing the excess material, and applying MALDI matrix. The enriched analyte is analyzed using MALDI TOF mass spectrometry in the last step.

There are several applications of functionalized protein chips in combination with MALDI TOF mass spectrometry. These include limited *in situ* proteolysis on the chips with immobilized proteases, enrichment of glycopeptides by lectin chips [99], and haptoglobin phenotype detection or carbohydrate-deficient serotransferrin analysis as a pathological

marker of alcoholic patients by antibody chips ^[86,107]. Chips with immobilized antibodies represent a valuable alternative to the classical immunoprecipitation methods, with the advantage of smaller sample consumption and the possibility of direct analysis with MALDI MS ^[69]. Functionalized chips were used in this work for several applications. NeutrAvidin chips were used for *C. difficile* Toxin B detection in the stool samples and for *C. botulinum* neurotoxin detection in the animal food samples. Chips with immobilized antibodies were used to quantify septic biomarker procalcitonin from the human serum.

1.4 Diagnostics of sepsis

1.4.1 Sepsis

Sepsis is a common, expensive, and complex health condition associated with high mortality and, for many survivors, causes long-term morbidity ^[108]. The related clinical criteria to sepsis are systemic inflammatory response syndrome, severe sepsis, and septic shock. The sepsis and other criteria were internationally defined in 2016 on the Third Consensus Definition for Sepsis and Septic Shock (Sepsis-3) ^[109]. Systemic inflammatory syndrome is characterized by a certain threshold body temperature, heart rate, respiratory rate, and white blood cell count. Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. Severe sepsis is defined with evidence of acute organ dysfunction (hypotension, lactic acidosis, reduced urine output, reduced PaO₂/FIO₂ ratio, raised creatinine or bilirubin, thrombocytopenia). The septic shock is defined as sepsis with persistent hypotension after fluid resuscitation and the need for vasopressor therapy to increase mean arterial pressure ^[108–110].

Most data suggests that the incidence of sepsis is in developed countries, where 2.8 million deaths per year are attributed to sepsis ^[111]. It was reported that in the year 2001, the incidence of severe sepsis in the USA was more than 750 000 cases per annum, 300 cases per 100 000 population ^[112]. In the UK, it is reported that 27 % of all intensive care unit admissions are sepsis compared to 12 % in the USA ^[113]. This difference can be explained by the fact that in the USA, there are many more ICU beds available, which might misrepresent reality ^[108].

Several factors define the incidence and outcome of sepsis. Demographics should be considered. Women tend to have a lower incidence of severe sepsis ^[114]. In the USA, the incidence of sepsis is higher in non-whites, especially African Americans. The difference was described in subset states, showing data were not influenced by one region or more of

them ^[112]. Older adults are also susceptible. A study of discharge data from 500 hospitals in the United States reported that patients 65+ years old comprise 12 % of the population but nearly 65 % of sepsis cases ^[115]. Besides demographic influence, conditions that suppress innate and adaptive immunity are risk factors for sepsis. A study in multiple French intensive care unit centers estimated that immunosuppression is connected with a higher incidence of severe sepsis ^[116]. The immunosuppressing conditions include patients with HIV, solid organ transplantation, neutropenia, solid or hematologic cancer, inflammatory disorder, and primary immunodeficiency ^[117]. Last but not least, risk-increasing factors can be genetic predispositions and other modifiable factors, such as alcohol consumption and cigarette smoking ^[118–120].

Causes of sepsis can be divided into two main groups. The first is rare and described as the previously mentioned systemic inflammatory response syndrome, where patients develop a clinical picture of sepsis without an identifiable infection ^[121]. The second and most common case is the infectious cause. A large study gathered data from over 14 000 adult patients in 1,256 intensive care units from 75 countries. Of the 7 000 patients classified as infected, the infection was primarily localized in the lungs (64 %). The lungs were followed by the abdomen (20 %), bloodstream (15 %), and renal or genitourinary tract (14 %). Of the infected patients with positive microbiology, 47 % of isolates were Gram-positive, and 62 % were Gram-negative ^[122]. The abdominal infectious source of sepsis was mainly Gram-negative bacteria ^[123].

1.4.2 Pathophysiology of sepsis

Sepsis causes damage at an organ, cellular, and molecular level. At the organ and tissue level, sepsis progresses from local infection to systemic inflammation and septic shock. The cardiovascular system undergoes perturbations ^[124]. The perturbations can partially be responsible for tissue hypoxia, reflected as increased lactate in the patients ^[125]. The endothelium, which can be thought of as an organ covering nearly 1000 m² area, is also harmed. The endothelium has essential roles in regulating vasomotor tone, moving cells and nutrients into and out of tissue, and balancing anti-inflammatory and inflammatory signaling ^[126]. Alterations to the endothelium during sepsis include increased leukocyte adhesion, procoagulant state shift, vasodilatation, and loss of barrier function. The combination leads to widespread tissue edema ^[127]. The endothelial changes in severe sepsis are associated with altered organ barrier function. The increase in lung capillary permeability

results in the accumulation of edema fluid in the interstitial spaces. The changes result in perfusion-ventilation mismatch, arterial hypoxemia, and reduced lung compliance [128]. The other organ damage includes acute kidney injury and widespread tubular necrosis, encephalopathy, corruption of the blood-brain barrier, perivascular edema, oxidative stress, and widespread neurotransmitter alterations, summarized in Figure 13 [129–131].

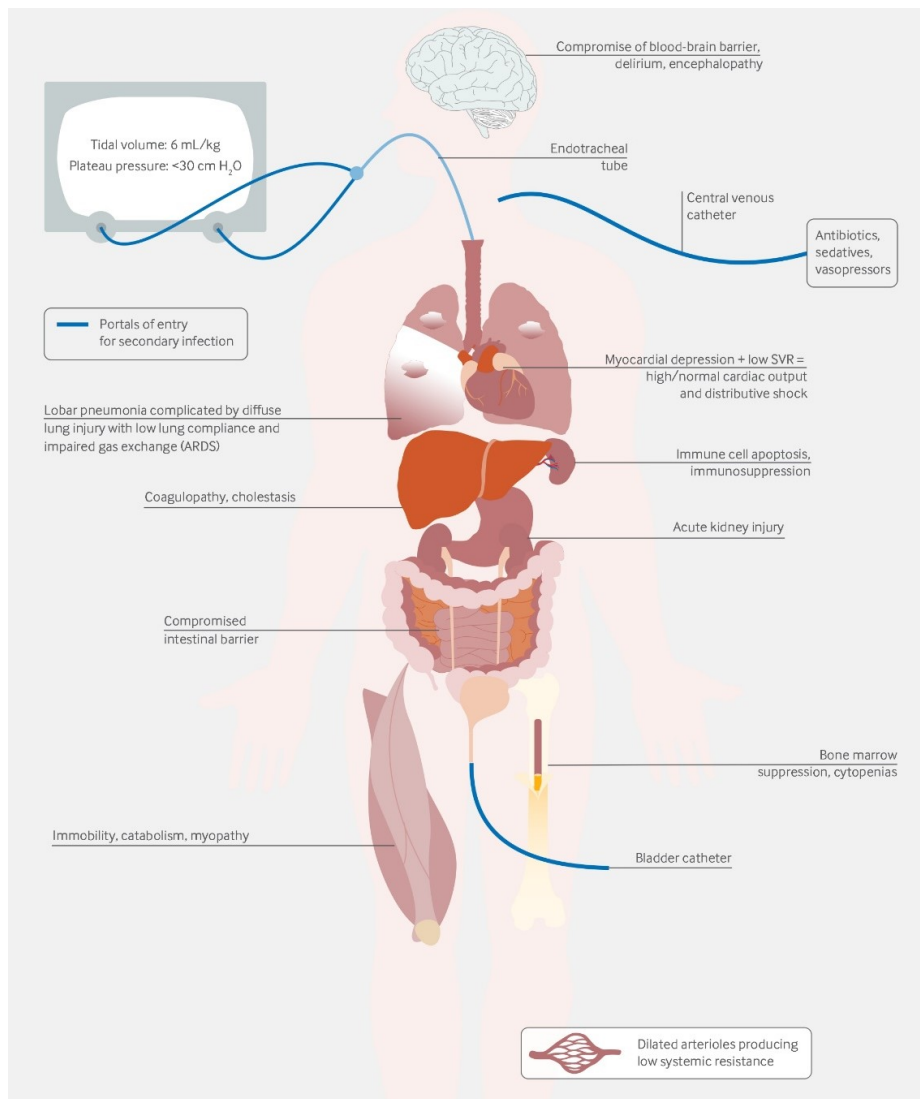


Figure 13 – Visualization of the critically ill patients with septic shock summarizing the organ failure due to acute respiratory distress syndrome. Reprinted from [110].

On the cell and molecular level, the molecular cascades released during severe infection are complex, temporally dynamic, and partially dependent on the particular virulence factors of the pathogen [132]. However, key pathways and themes were described. A recombinant tumor necrosis factor α (TNF- α) was introduced into rats during one study. Rats developed tachypnea, lactic acidosis, and lethal shock within three hours. They were found to have

areas of ischemia and hemorrhage of the lungs, gut, kidneys, pancreas, and adrenal glands ^[133].

Various proven, effective treatments have been implemented to achieve consistent improvements in sepsis survival. The early introduction of septic patients plays a key role in their survival. It was described that each hour of delay in antibiotic treatment after the onset of shock was associated with an almost 12 % decrease in patient survival. Another more recent study evaluated 18 000 patients from 165 intensive care units with severe sepsis or septic shock, where steadily increasing mortality was connected with a delay in antibiotic administration ^[134]. Timely diagnosis of sepsis is the most efficient tool to elevate a patient's survival expectancy. Therefore, several biomarkers and rapid tests were described as a result of the effort to fight sepsis.

1.4.3 Sepsis biomarkers

As was mentioned in the previous paragraphs, sepsis is a global health concern and remains a major contributor to mortality. Sepsis is most common in ICU patients, pregnant women, neonates, young children, older adults, immunocompromised, and individuals with underlying chronic conditions (HIV, renal disease, liver cirrhosis, malignancy). The study performed in 2020 estimated that nearly 50 million cases have been reported globally and 11 million sepsis-related deaths, which account for 20 % of all deaths reported globally ^[135]. Sepsis's nature and essence logically lead to the frequent use of broad-spectrum antimicrobials, contributing to the development of antimicrobial resistance, which increases the incidence of serious infections ^[135]. As mentioned in the previous paragraphs, suitable biomarkers can help to determine the early stages of sepsis, which helps to increase the chances of a patient's survival and can help in the correct antimicrobial therapy administration ^[136].

The National Institute of Health defines a biomarker as a characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention. The ideal biomarker must be highly specific and sensitive, related to the disease, provide information about prognosis, be reproducible and change following clinical evolution, and be easy and cost-effective ^[137].

A large number of sepsis biomarkers have been proposed with varied times of peak concentration in plasma or body fluids following infection or inflammation ^[138]. Over 200

different biomarkers mostly released from affected cells or tissues have been described for their role in sepsis's timely recognition, i.e., elevated counts of white blood cells due to cytokine activity, C-reactive protein (CRP), interleukin 6 (IL-6), presepsin, CD64, TNF- α , and the last but not least procalcitonin (PCT) ^[136]. However, no single biomarker is sufficient enough as an ideal biomarker due to the heterogeneous nature of sepsis ^[139,140]. Efficient diagnosis must combine biomarkers with clinical evaluation scores to correctly diagnose sepsis ^[136]. Moreover, biomarker-guided therapy is a valuable tool for optimizing antimicrobial treatment.

According to the Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock, biomarkers have a crucial role in antibiotic therapy management compared to clinical evaluation alone ^[141]. Procalcitonin is one of the biomarkers used for antibiotic therapy management. Overall, it was demonstrated that PCT-guided therapy positively influences treatment outcomes ^[142–144].

1.4.4 Procalcitonin

Procalcitonin is a pro-hormone of calcitonin and undergoes proteolytic cleavage ^[145]. It is produced in healthy individuals by thyroid C cells from a CALC-1 gene located on chromosome 11. The mRNA product is called preprocalcitonin, modified to 116 amino acid procalcitonin. It is cleaved into active calcitonin, which is involved in calcium homeostasis, katalcaltitonin, and N-terminal procalcitonin – aminoprocalcitonin (Fig. 14) ^[146].

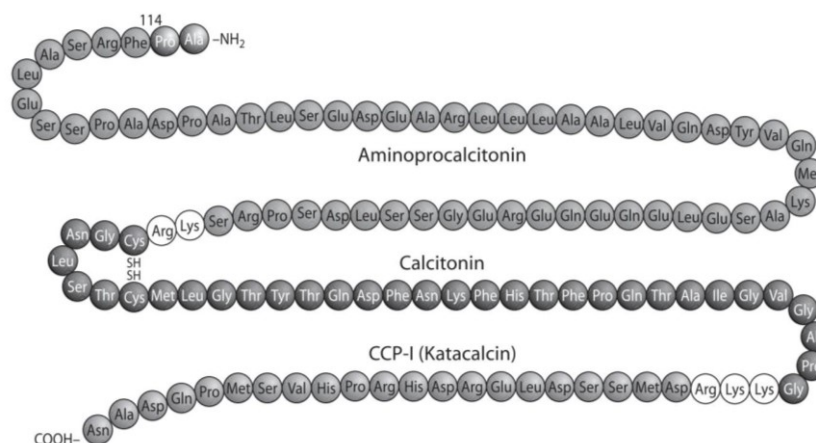


Figure 14 – Scheme of the preprocalcitonin showing a posttranslational modification cleavage site into the final products. Used from ^[147].

In healthy individuals, the PCT formed primarily in the thyroid C cells is converted to calcitonin. Therefore, the PCT levels in healthy individuals are very low (≤ 0.1 ng/mL). The

release of PCT during inflammation is independent of the thyroid cells. Its production is upregulated by two alternative mechanisms — the direct pathway induced by lipopolysaccharides or other toxic metabolites from microbes and the indirect pathway induced by inflammatory mediators, e.g., IL-6 or TNF- α [148–150]. A study performed on hamsters compared PCT production in several different tissues of healthy and septic hamsters. Compared to the healthy hamsters, where PCT was produced only in thyroid cells, the gram-negative-bacteria-infected hamsters produced PCT in various tissues. White blood cells, kidneys, spleen adipocytes, pancreas, colon, and brain showed elevated levels of PCT mRNA [148]. The upregulated PCT from patients with severe sepsis was characterized using MALDI-TOF mass spectrometry, suggesting additional cleavage of the N-end Ala and Pro amino acids by dipeptidase. The resulting truncated 114 amino acid long product was identified in the mass spectrum as m/z 12 628 for the singly and 6 314 for the doubly charged ion [151].

Procalcitonin has improved other traditional biomarkers, including CRP and WBC, due to its role during sepsis and rapid elevation in concentration (2-6 hours) ten or more times compared to healthy individuals [152]. It was described that PCT is combined with other biomarkers to determine the severity of sepsis [153]. An international expert consensus recommended PCT cut-off levels for assessing the probability of bacterial infection in critically ill patients and for antibiotic therapy guidance [154]. The comparison of PCT and other biomarkers and PCT cut-offs are visualized (Fig. 15). Rapid detection and diagnosis of bacterial infections are crucial for the right antimicrobial therapy to avoid unnecessary higher costs of treatment associated with prolonged hospitalization [155]. Therefore, methods to determine PCT presence in patients should be fast and sensitive, minimizing falsely negative results.

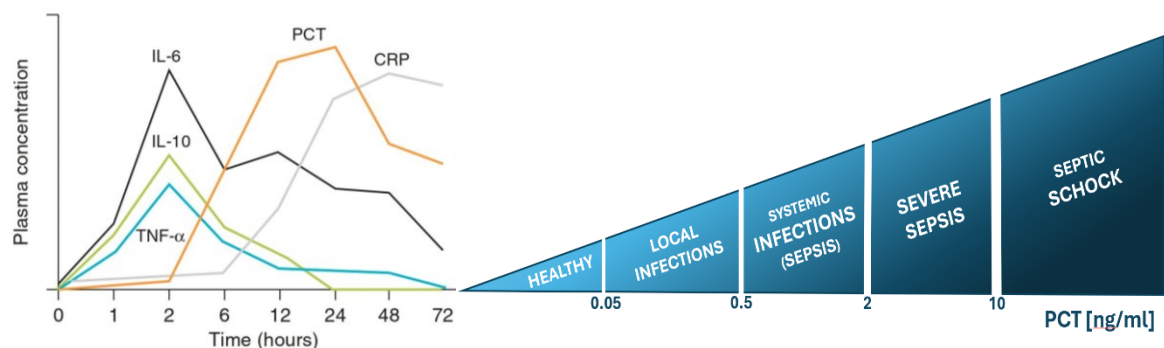


Figure 15 - Concentration curve in time showing different peaks of different biomarkers during sepsis (on the left). The severity of sepsis in the context of PCT concentration (on the right). Based on and used from [156,157].

1.4.5 Routinely used methods for procalcitonin detection

Every currently available assay for procalcitonin quantification uses immuno-based technology. The first commercial PCT assay was the BRAHMS PCT LIA[®] produced by Thermo Fisher. It was a manual luminometric immunoassay. It was followed up by a rapid, more sensitive automated assay named BRAHMS PCT Kryptor[®], the first assay cleared by the FDA in 2008 [158,159]. Several companies have partnered with BRAHMS to develop assays for PCT detection on their platforms, with minor changes in the analytical characteristics. There is a slight difference in functional sensitivity and measuring range. Different detection technologies are also used (enzyme-linked immunofluorescent, electrochemiluminescent, chemiluminescent, immunoluminometric). A study comparing different PCT assays regarding generally used cut-offs was performed in 2015 [160]. It compared four assays (DiaSorin Liaison, Vidas, Roche E601, Siemens Advia Centaur) with BRAHMS PCT Kryptor[®]. The study notes that all methods are aligned with BRAHMS Kryptor and can be used to predict the risk of progression to systemic inflammation in patients using conventional diagnostic thresholds [160].

1.5 *Clostridioides difficile* infection diagnosis

1.5.1 *C. difficile* infection

Clostridioides difficile were classified as one of the top five urgent threats to human health by the Centers for Disease Control and Prevention. *C. difficile* is an obligate anaerobic Gram-positive sporulating bacteria with a heterogeneous clinical picture [161]. The clinical picture can vary from an asymptomatic picture and mild symptoms to life-threatening conditions. It has emerged as the leading cause of nosocomial diarrhea, with increasing incidence in recent years [162]. It was also shown as a primary feces isolate of the antibiotics-treated patients, e.g., with clindamycin. Additionally, *C. difficile* was recognized as the primary cause of pseudomembranous colitis in 1978 [163].

Due to the highly resistant spores to the routinely used disinfection methods in hospitals, the *C. difficile* infection (CDI) exceeds 120 000 cases in Europe and 250,00 in the US annually, making it a significant economic burden on healthcare systems with a 3 % mortality rate. Additional costs of up to 1 billion US dollars annually are mainly due to the prolonged hospital stay of the patients [164–166]. In the early 2000s, the incidence of CDI increased in the US, Canada, and Europe [167]. The increase is due to several outbreaks of hypervirulent

strains such as RT027 in the US ^[168]. The leading causes of the *C. difficile* virulence are its toxins ^[169].

1.5.2 *C.difficile* virulence

Studies aiming at defining the virulence of *C. difficile* suggest that understanding the disease caused by the pathogen depends strongly on the detailed analysis of the activity of its two toxins – Toxin A (TcdA) and Toxin B (TcdB) ^[168]. Therefore, TcdA and TcdB are considered the main virulence factors ^[170].

Toxin A and Toxin B are encoded on the pathogenicity region within the chromosome of *C. difficile*. They are expressed during the late log and stationary growth phases as a response to environmental stimuli. Both toxins (TcdA and TcdB) have glucosyltransferase activity and belong to the group of large clostridial toxins ^[169]. TcdA (308 kDa) and TcdB (270 kDa) are single-chain proteins considered one of the largest bacterial toxins ^[168]. They consist of four conserved domains varying in function ^[171]. The toxin carries combined repetitive oligopeptides domain (CROPs), contributing to toxins binding to cell receptors and other domains that help it to interact with host cell apparatus, such as the delivery and receptor-binding domain (DRBD) and autoprotease domain (APD) to mature the toxin inside the host cell ^[172]. The host cell entry mechanism and toxin activation are visualized in Fig. 16. The N-terminal end carries a glucosyltransferase domain (GTD) responsible for Ras GTPase family inactivation due to the mechanism described in the following paragraph.

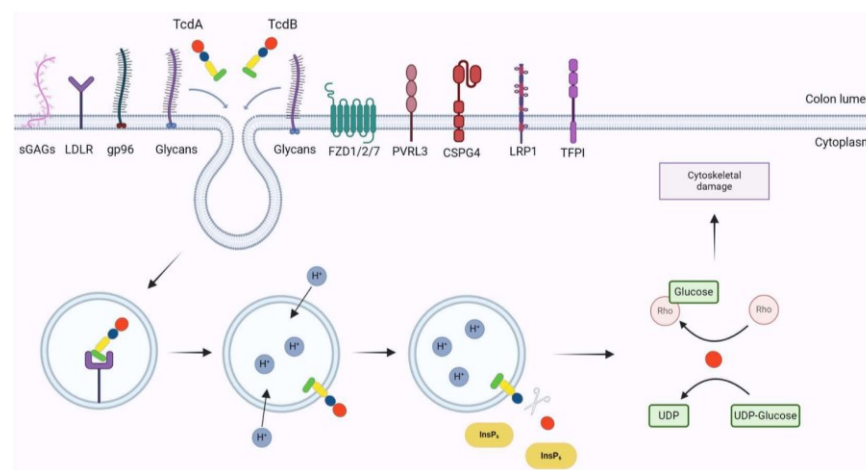


Figure 16 – The mechanism of TcdA and TcdB action. Toxins bind to the surface receptors of the colon epithelium and are endocytosed in acidic endosomes, where low pH initiates a conformational change in the toxins. The conformational change results in pore formation and release of the glucosyltransferase domain (red circle). The GTD inactivates the Rho and Ras GTPases by transferring the glucose from the UDP-glucose. The GTPase inactivation causes cytoskeleton rearrangement and further cytoskeletal damage. Interpreted from ^[173].

As mentioned in the previous paragraph, TcdA and TcdB are glucosyltransferases using the UDP-glucose as a co-substrate that irreversibly modifies the conserved threonine (involved in the Mg^{2+} coordination) of the Ras GTPases, preferably RhoA, where toxins modify the Thr37 [174,175]. The modification results in the inactivation of the Rho GTPases due to the conformation change caused by the Thr modification and the inability to bind GTP. Since these proteins serve in the signal pathways transduction as molecular switches of many signaling processes, most importantly, they serve as master regulators of cytoskeleton-building and govern various cellular mobile functions, e.g., phagocytosis, cellular trafficking, and migration [176,177]. Also, Rho proteins participate in the regulation of apoptosis and cell cycle progression [177].

The inactivation of the Ras/Rho GTPases is followed by the redistribution of the actin cytoskeleton and results in dramatic cell morphology changes [178]. The changes include the loss of stress fiber formation, rounding of cells (cytopathic effect), and cell body contraction, resulting in irregular structures [179]. Additionally, the deactivation of Rho GTPases leads to apoptosis and disrupts the cell cycle progression and the cell-to-cell contact, increasing the epithelial permeability [180–182]. In extreme cases, represented as an animal model, high levels of toxins combined with increased colon permeability can lead to systemic toxemia, where the toxin and bacteria can reach other organs via the bloodstream [183].

In addition to toxins' contribution to the disease, these two toxins are also used as primary markers for diagnosing CDI [164].

1.5.3 Diagnostics of *C. difficile* infection

There are several diagnostic methods, and the testing begins with a suspicion of the CDI. The first sign of the patient is unexplained diarrhea, defined as 3 or more unformed stools in 24 hours ^[184]. The diagnostic algorithm is shown in Fig. 17, where several techniques are combined and used in a defined order ^[185].

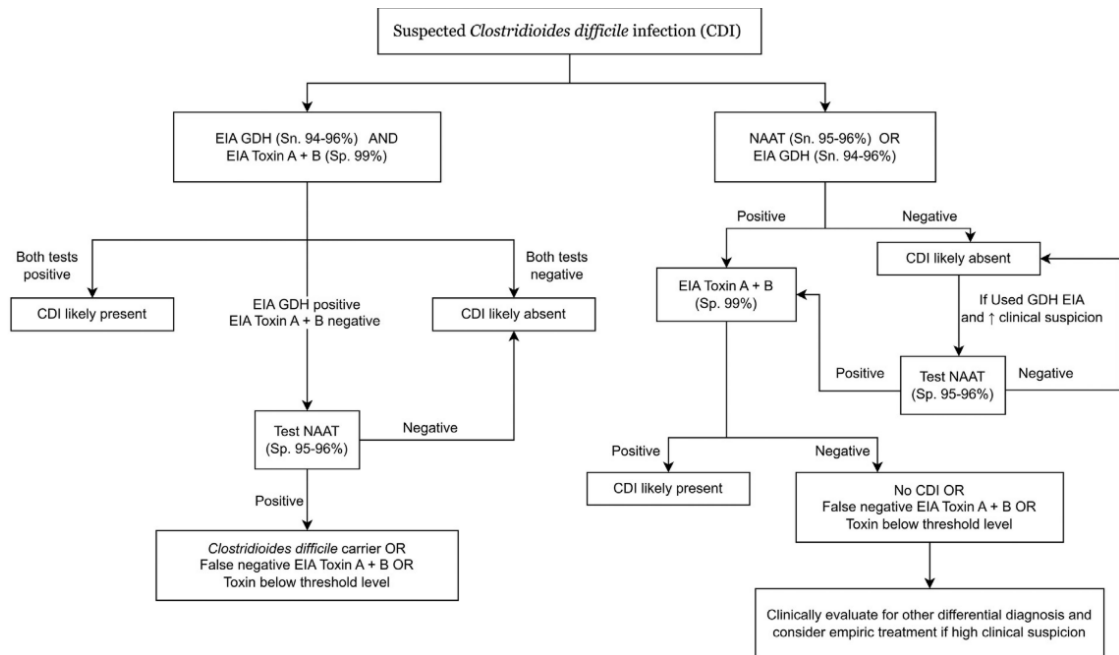


Figure 17 – The diagnostic algorithm using glutamate dehydrogenase (GDH), Toxin A, and Toxin B as biomarkers. The combination of enzyme immunoassays (EIA) and NAAT is used to determine false negative and false positive patients. The method sensitivity (Sn) and specificity (Sp) are defined. As displayed in ^[185].

As shown in Fig. 17, several methods can be used to diagnose CDI, including EIA, NAAT, and GDH. The GDH test uses glutamate dehydrogenase antigen, a very sensitive method with a high negative predictive value, to determine toxic and nontoxic strains of *C. difficile* since both produce this enzyme in reasonable quantities ^[186]. Regarding toxic and nontoxic *C. difficile* strain determination, EIA targeting the toxin presence and NAAT aiming for the presence of the gene encoding toxin are used ^[185]. The EIA and NAAT are complementary tests that are necessary to combine with GDH ^[186]. The NAAT methods are very sensitive but are susceptible to CDI overdiagnosis, resulting in patients undergoing the wrong treatment that can lead to prolonged stays in the hospital and additional costs ^[187].

A broad genetic variability of *C. difficile* must also be considered when diagnosing. There are strains producing both or only one of the two toxins. Additionally, there is considerable variability in the toxin activity of toxins produced by different strains across the species ^[1].

There is a question of causation between the CDI severity and toxin concentration ^[188]. Due to these facts, the presence and concentration of the toxins are essential information. However, they don't represent the complete picture. There are cases in which a CDI patient provided multiple negative tests using different testing methods in various facilities ^[189]. Methods like cytotoxicity neutralization assay and toxigenic culture are gold standards in giving information on toxin activity. However, these methods are time-consuming and require specialized equipment and professionals, which makes them unsuitable for clinical practice ^[185,186].

1.6 Detection of botulinum neurotoxin

1.6.1 *Clostridium botulinum*

Clostridium botulinum (Fig. 18) is a gram-positive anaerobic spore-forming bacillus. It

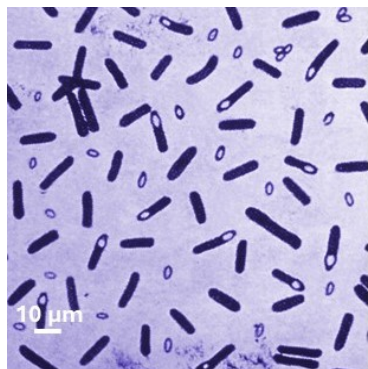


Figure 18 – *Clostridium botulinum* microscopic picture. Used from the Public Health image Library, #2107.

produces one of the most toxic substances in the world – botulinum neurotoxin (BoNT). *C. Botulinum* occurs wherever soil is and can be isolated from fruits, vegetables, marine sediments, and shellfish ^[190,191]. Under suitable conditions, the spores generate toxins, which enable the bacteria to proliferate ^[192]. Ingestion or inhalation of bacterial toxins, spores, or cells can result in paralysis that may require ventilation and administration of BoNT antitoxin ^[193]. The neuroparalytic medical condition caused by the *C. botulinum* bacteria and its neurotoxin is called botulism ^[192].

1.6.2 Botulism

Botulism (from Latin *botulus*, "sausage") is not a frequent medical condition, and it is mostly associated with outbreaks ^[194]. However, it can be potentially fatal. The study of *C. botulinum*'s neurotoxin-caused disease began in Europe in the 19th century when patients exhibited symptoms of food poisoning after consuming spoiled sausage. It was identified after the isolation of sporulated bacillus from ham ^[195].

It is a severe neuroparalytic human and animal disease caused by BoNT. Human botulism starts with blurred or double vision, problems with speaking, and dry mouth. It proceeds to muscle weakness followed by descending flaccid paralysis, including the neck and respiratory muscles, resulting in death. The patient is conscious but cannot control any muscle. ^[196]. Additional symptoms are abdominal pain, nausea, vomiting and dizziness. The progression and severity depend on the amount and type of BoNT and the toxin entry route ^[197]. Therefore, there are multiple types of botulism: foodborne, wound, infant, adult intestinal, inhalation, and iatrogenic ^[192]. The incidence of each botulism throughout the years 2001 to 2018 is visualized in the following graph (Fig. 19, page 40).

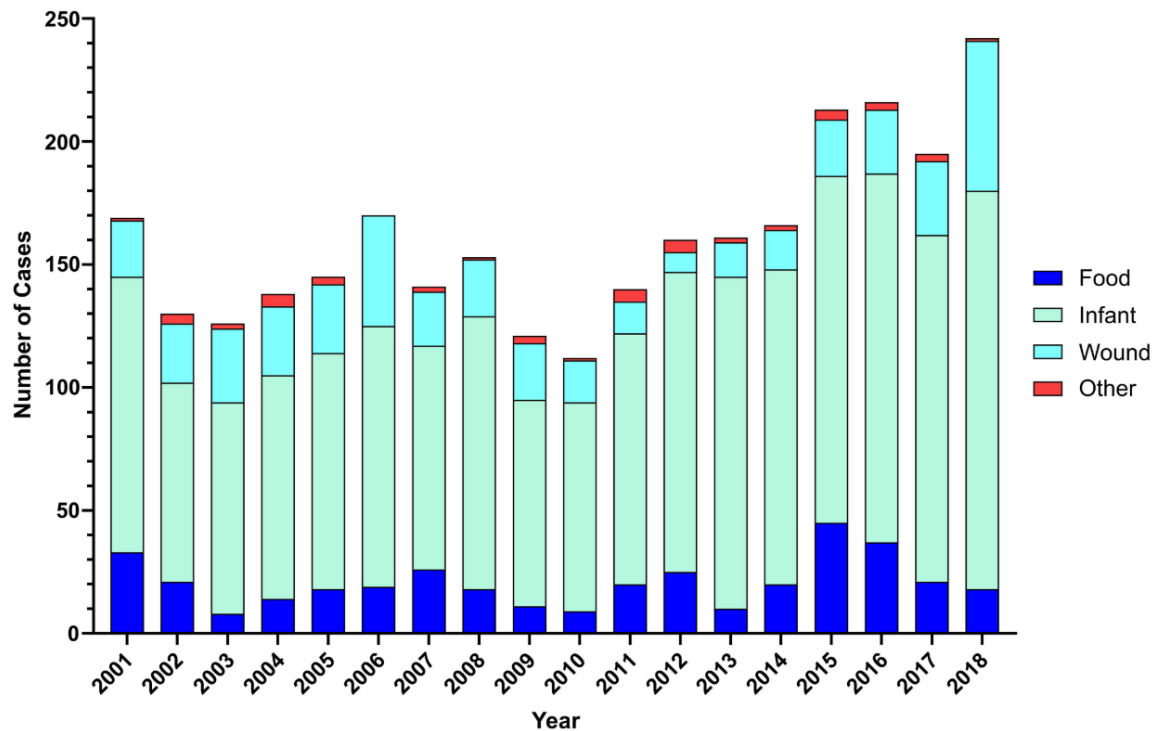


Figure 19 – Reported number of cases of each type of botulism in the US in 2001-2018. Data from National Botulism Surveillance ^[198]. The figure is used based on ^[199].

1.6.2.1 Foodborne botulism

Foodborne botulism is caused by the ingestion of contaminated food with BoNT. Only as little as a few milligrams of contaminated food can cause symptoms, followed by death if the proper treatment is not provided in time ^[200]. Symptoms appear 12-72 hours after consumption of the food containing BoNT. The exact time is influenced by the quantity of the contaminated toxin ingested ^[193]. It is historically the most common form of botulism, which is usually associated with food that is inadequately processed or conserved in other ways than cooking. Fermentation or pickling followed by bottling or canning without previous heating provides the ideal anaerobic environment for spore germination and bacterial growth ^[196]. The number of outbreaks decreased after introducing the food control measures “botulinum cook” (3 mins, 120 °C), refrigeration below 4 °C, and freezing ^[201].

The foodborne botulism outbreaks can have significant commercial implications for the food and drink companies involved. In one case in the US, inadequate refrigeration and/or heat treatment of cans of chili sauce caused product compromise, necessitating the recall of 111 million cans, which led to the closure of the canning company ^[202].

Food-associated botulism is a prevalent disease in domesticated and wild animals, where outbreaks in farm animals and wetland birds spread rapidly. The fast-spreading animal botulism can lead to potentially devastating epidemics because of the disease transmission cycle in the environment ^[203].

1.6.2.2 Infant botulism

In healthy, mature individuals, the digestive system is not susceptible to environmental *C. botulinum* spores, which pass through it routinely. The healthy individual's gut is not conducive to clostridial spore germination ^[196]. On the other hand, an infant's (up to 12 months of age) immature gastrointestinal tract is vulnerable to *C. botulinum* colonization after spore ingestion and germination ^[204]. The vulnerability is a result of immature gut physiology and underdeveloped gut microbiota. The colonization is followed by endogenous production of the neurotoxin, causing the phenomena of so-called “floppy babies” unable to suck or swallow. The symptoms typically start 18 to 36 hours after the ingestion ^[205].

It is the predominant form of human botulism cases in the U.S. (Fig. 19, page 40) ^[198,206]. Most cases are connected to the consumption of untreated natural food, e.g., corn syrup and honey ^[207]. Spores can also occur in contaminated powdered baby formula and household dust ^[208,209].

1.6.2.3 Other less common forms of botulism

Adult intestinal botulism is an infrequent condition caused by ingesting spores from contaminated food, followed by *in vivo* BoNT production due to *C. botulinum* colonization of the gastrointestinal tract. The colonization is possible due to the depletion of the natural gut microbiota. An article published in 2018 reported that only 33 patients suffered from this condition since 1980 ^[210].

Another extremely rare form is inhalation botulism. It does not occur naturally and is caused by accidental or intentional inhalation of toxins in aerosol form. It is connected to laboratory accidents or bioterrorism attacks. The clinical picture is close to the foodborne form with an incubation period of 1 to 3 days. Patients suspected of inhalation botulism should be decontaminated ^[211].

Botulinum toxin products are commonly used for both therapeutic and cosmetic use, with a low probability of side effects if the therapy is applied appropriately on suitable patients, the correct dosage, and the injection technique ^[212]. The wrong injection technique can cause the

dissemination of the BoNT into the surrounding muscles and glands, where effects differ depending on the application site. The number of cases has been growing with the increasing use in cosmetic and bariatric fields. In 2023, 87 cases of iatrogenic botulism were reported after surgery for weight loss in Türkiye. Iatrogenic botulism is mainly a result of a high dosage of toxin [213].

As mentioned in the previous paragraphs, the BoNT is the main toxigenic factor of the bacteria. In the following paragraphs, toxin types and toxicity mechanisms will be discussed.

1.6.3 Botulinum neurotoxins

C. botulinum species is historically defined as all bacteria producing a BoNT. This simplification made it a highly diverse species [214]. The taxonomy was accepted to avoid confusion when scientists and clinicians were handling or diagnosing the potentially hazardous bacterium. The species has four phylogenetically distinct lineages known as *C. botulinum* groups I-IV. The groups are divided based on genetics and physiological factors [215]. The difference between *C. botulinum* groups is more extensive than across other clostridial species; the only common denominator for the groups is the production of BoNT [214]. The diversity is visualized in Figure 20.

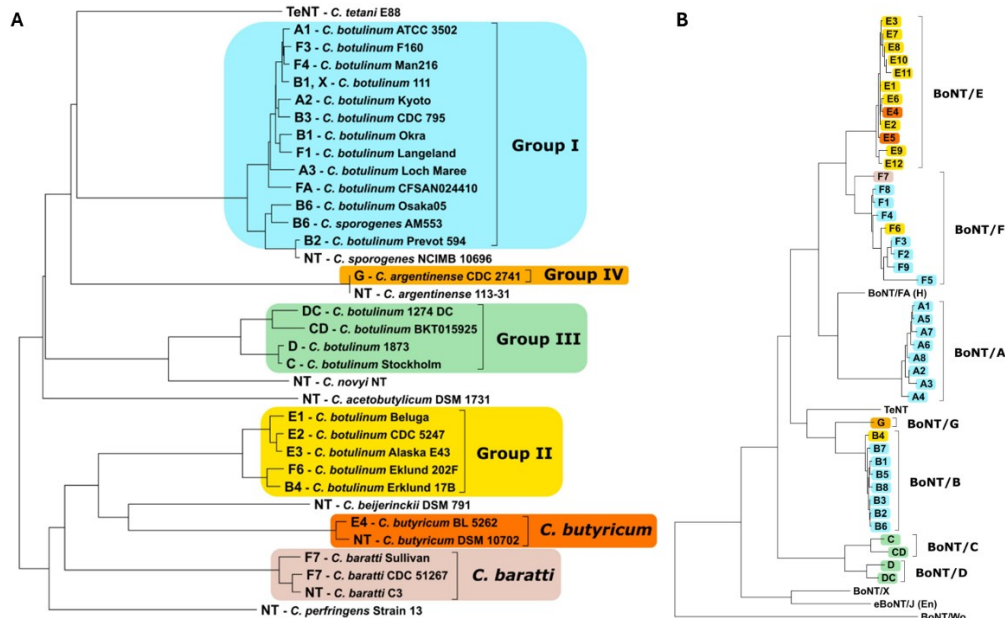


Figure 20 – (A) A phylogenetic dendrogram of 4 different *C. botulinum* groups and 2 other representative botulinum toxin-producing clostridioides groups. (B) – different strain examples differed into groups based on the toxin produced. Based on [199].

Most human botulism cases are associated with strains from Groups I and II. The Group III strains are connected with avian and mammalian cases. The *Clostridium argentinense* –

Group IV produces BoNT/G and rarely causes illness ^[216]. Other neurotoxicogenic strains that produce BoNT/B, BoNT/E, and BoNT/F neurotoxins are called *Clostridium sporogenes* (classified in Group I), *Clostridium baratii* (Group V) and *Clostridium butyricum* (often referred to as Group VI) ^[217]. The types of BoNT responsible for different types of botulism are visualized in Figure 21, from which it is evident that the most common types of BoNT are A and B.

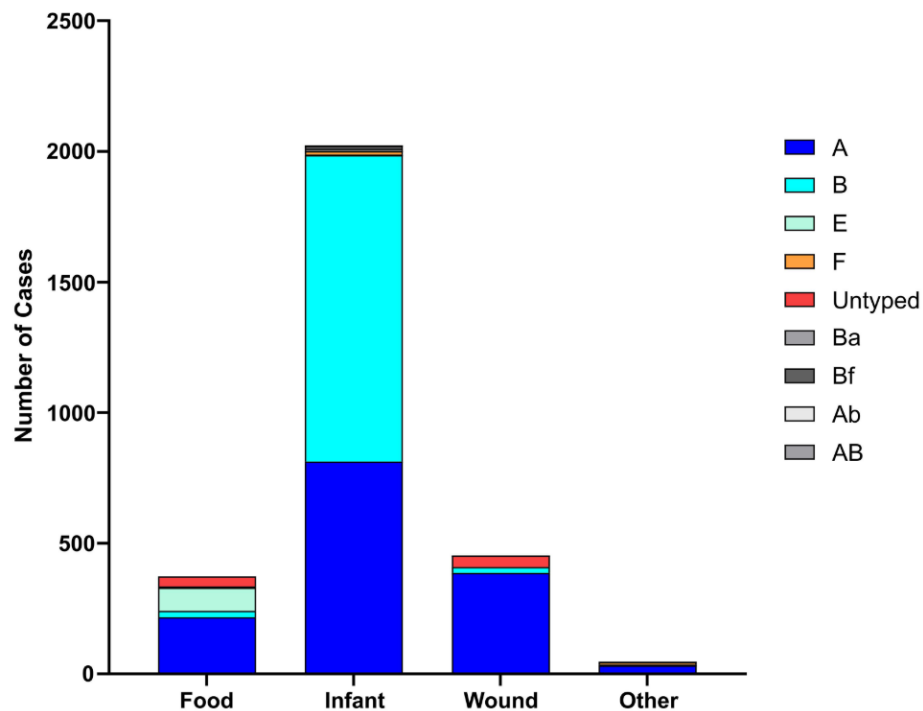


Figure 21 – Causative toxin type distribution across different forms of botulism. Picture used from ^[199]. Data is sourced from ^[198].

Botulinum neurotoxins have been traditionally classified into seven serotypes marked from A to G, where each group has subtype variants. The serotypes are classified based on the toxin's ability to be neutralized by monoclonal antibodies ^[215]. Several deviations and chimeric toxins have been reported throughout the years. For example, the chimeric toxin BoNT/FA was thought to be a new serotype BoNT/H until deeper analysis showed the chimeric nature of the toxin ^[218]. It is a well-known fact that strains from different groups have the ability to produce the same toxins serotype, and subtypes of one serotype vary in biological activity ^[219]. The presence of the toxin gene clusters on bacteriophages and plasmids and the presence of mobile genetic flanking elements in several species suggests a horizontal gene transfer has a role in the large diversity of BoNT-producing species and the production of chimeric toxins ^[220]. The use of whole-genome sequencing in 2015 uncovered

the first non-clostridial BoNT homolog in *Weissella oryzae* and, in the following years, BoNT-like toxins in other species ^[221,222]. These latest findings corrupt a dogma where BoNT is connected exclusively to *Clostridioides*.

The BoNT/A1-producing strains induce the most severe form of human botulism. The overall properties of BoNT/A1 make it a great choice for therapeutics and aesthetics ^[223]. Therefore, understanding the mechanism of action of this toxin is crucial for life sciences.

1.6.4 Botulinum neurotoxins structure and mechanism of action

All neurotoxin serotypes are encoded by *bont* genes, which, as previously mentioned, are located on either phage, plasmid, or chromosomal elements ^[224]. All serotypes are translated as an over 150 kDa polypeptide ^[225]. The active mature toxin produced after cleaving 11 amino acids from the premature toxin is a di-chain holoenzyme composed of two chains, a 100 kDa heavy chain, and a 50 kDa light chain, linked together by a single interchain disulfide bond and non-covalent interactions ^[226]. The structure of BoNT and composition scheme are in the Figure 22.

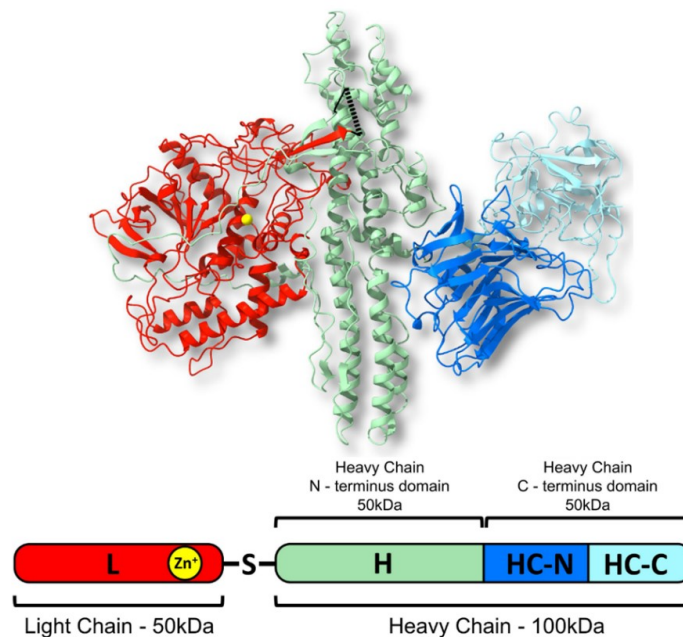


Figure 22 –A ribbon structure (up) and scheme describing each part of the single polypeptide chain translated from the *bontA1* representing a typical botulinum neurotoxins structure. The example is shown on a BoNT from *C. botulinum* strain ATCC 3502. The single 1295 amino acid chain is composed of a light chain (red – L), which is connected with the heavy chain (light green – H) by a disulfide bond between Cys430 and Cys454 residues (black dashed line – ribbon structure). The heavy chain comprises an N-terminal translocation domain (light green) and a receptor binding domain on the C-terminus, with two subunits: HC-N and HC-C. The light chain is a zinc metalloprotease (Zn²⁺ shown as Zn⁺ in yellow). Based on the figure from ^[199] that based the figure on ^[227], used structure source (PDB ID: 3BTA).

The light chain has catalytic metalloprotease activity with zinc cation as a cofactor. The zinc cation is bound to a HEXXH motive, sheltered in the structure's core ^[228]. The heavy chain can be divided into N-terminal translocation and C-terminal receptor-binding domains. The C-terminal receptor-binding domain consists of two subunits, with the interface between the subunits having enough space for the glycosylated receptors gSV2C of the synaptic terminal to bind ^[229]. The N-terminal translocation domain includes a peptide belt that wraps around the catalytic light chain, preventing any catalytic activity before separating from the heavy chain and facilitating translocation from the vesicles into the synaptic cytosol ^[230,231].

The mature BoNT with one or more neurotoxin-associated proteins (NAPs) form a progenitor complex (PC) ^[232]. The scheme summarizing the following step-by-step formation of the PC for the case of BoNT/A1 is in Figure 23.

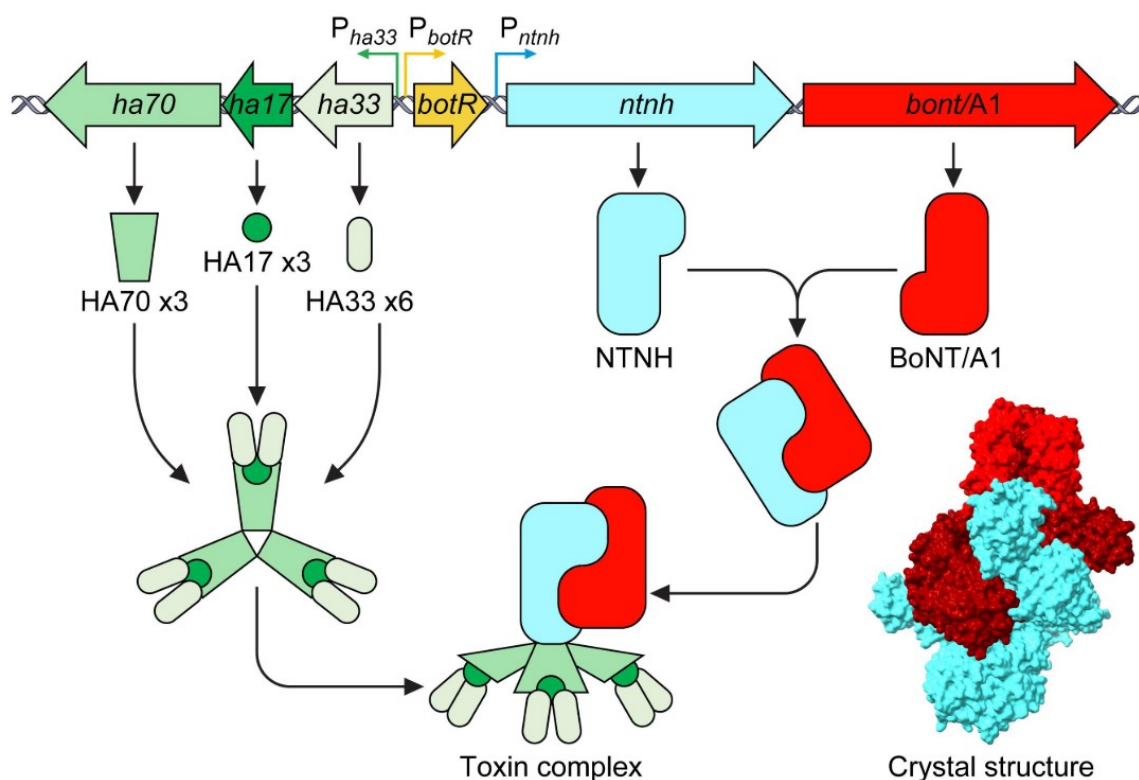


Figure 23 – A scheme of PC formation on the example of the BoNT/A1. The BoNT/A1 forms a heterodimer with NTNH, interacting with and forming a PC with the tripod-structured complex of three HA proteins. The crystal structure (PDB ID: 3VOA). The figure is taken from ^[199] and based on ^[233].

The mechanism is the same across all serotypes, where the mature BoNT forms a heterodimer with the non-toxic non-hemagglutinin (NTNH) protein, structurally similar to the toxin without the zinc-binding motive ^[233]. The BoNT-NTNH heterodimer complex associates with other NAPs depending on the strain. The hemagglutinin proteins (HA) and OrfX proteins are the main NAPs associated with forming a complex with the BoNT-NTNH

heterodimer ^[234]. In the case of BoNT/A1, the Has form a trigonal three-armed where the BoNT-A1/NTNH-A1 fits, and together, they make a “phage-like” structure. The NTNH in the resulting structure probably protects the toxin from enzymatic or chemical attacks, e.g., decaying biological matter, digestive tract proteases, and low pH ^[235]. The HA complex is believed to form a docking station on the intestinal lumen because it contains multiple carbohydrate binding sites, which allows the efficient transport of the BoNT through the intestine epithelium into the bloodstream. However, the mechanism still remains unclear ^[236,237]. The HA proteins increase the overall oral toxicity of BoNTs because they disrupt the intestinal epithelial barrier and compromise the integrity of the epithelium. The HA protein’s effect on the epithelium facilitates the absorption of the toxin ^[238]. The Orf-formed PC structures remain unknown, but they are thought to perform a similar function to the HA proteins ^[239].

The PC complex of BoNT enters the blood circulation by the lymphatic system in the case of toxin ingestion. It is then dispersed throughout the host tissue to localize at peripheral nerve endings ^[240]. The BoNT selectively targets the presynaptic membrane of these nerves using a dual receptor-binding approach and enters the presynaptic cell via receptor-mediated endocytosis ^[241,242]. The acidic pH caused by the neurotransmitter refilling causes a structural change of the heavy chain translocation domain to form a pore-like structure. The structural change allows the translocation of the unfolded light chain from the vesicles to the cytosol ^[243]. The disulfide bond connecting the heavy and light chains is reduced, causing the release of the catalytic light chain of the toxin. The light chain subsequently folds, allowing for the specific cleavage of the SNARE targets ^[244].

The SNARE proteins are part of the neurotransmitter exocytosis machinery. They allow the fusion of the neurotransmitter vesicle with the neural membrane ^[245]. The effect of BoNT on the SNARE proteins prevents postsynaptic excitation due to the neurotransmitter release blockage ^[246]. This results in the typical symptoms of botulism cases described in the previous chapters. The mechanism of action is summarised in Figure 24 (page 47). The BoNT targets the vesicle-associated membrane protein (VAMP), syntaxin, and the 25 kDa synaptosomal-associated protein (SNAP-25) ^[247]. Syntaxin and SNAP-25 are located on the inner leaflet of the neuron membrane. The light chain metalloprotease recognizes and cleaves specifically peptide bonds of one of the SNARE proteins amino acid chains based on the BoNT serotype. The cleavage causes the removal of a segment of one of the SNARE

proteins, preventing the formation of the SNARE complex crucial for neuronal vesicle docking to the inner presynaptic membrane [246].

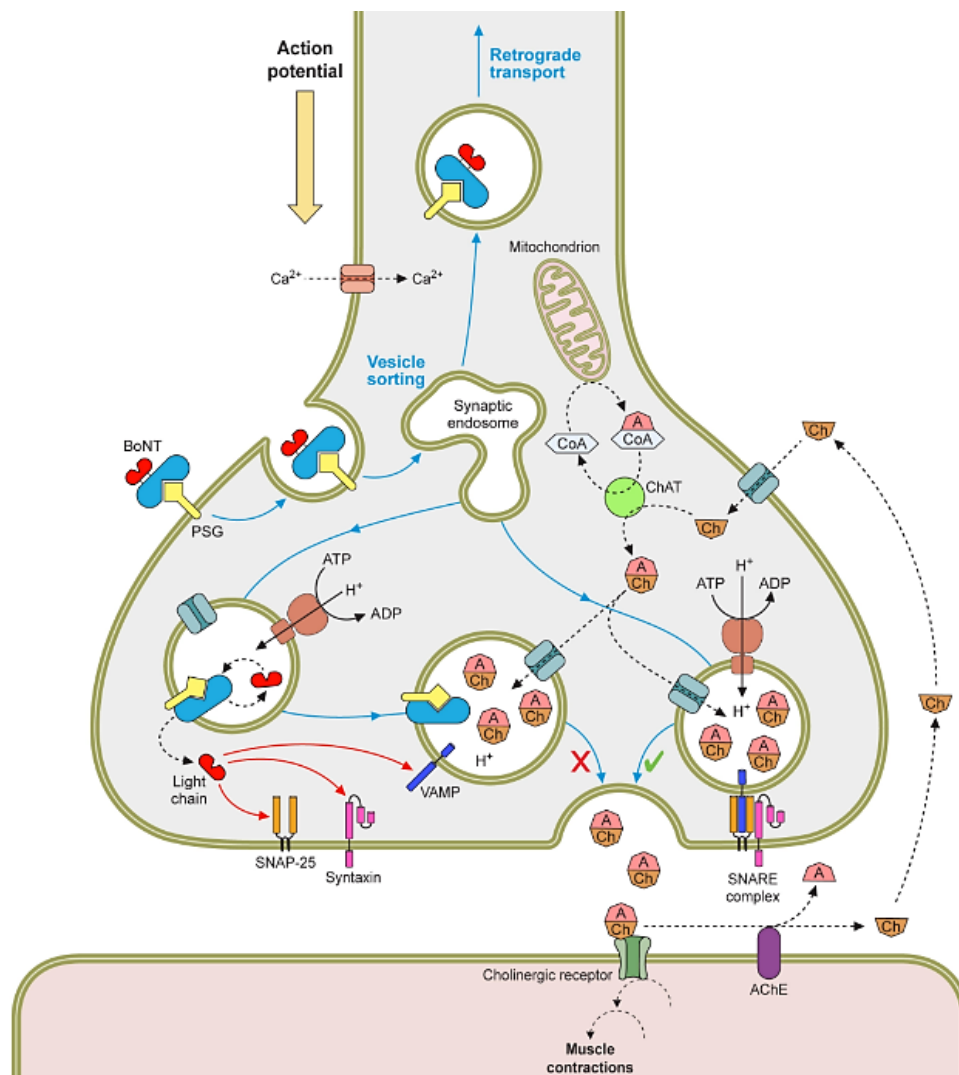


Figure 24 – The toxicity mechanism of BoNTs at the nerve ending. The neurotransmitters mediate the signaling between the pre- and postsynaptic cells. In the case of neuromuscular junction, the neurotransmitter is acetylcholine. Acetylcholine is stored in membrane-bound synaptic vesicles inside the neuronal cytosol. Endocytosis of the empty vesicles initiates the V-ATPase proton pump to generate a H^+ concentration gradient across the vesicular membrane. The pH gradient drives the newly synthesized neurotransmitter molecules to enter the vesicle. The vesicles with the neurotransmitter bind to the interleaflet of the presynaptic membrane using the VAMP that forms a SNARE complex with the SNAP-25 and syntaxin; the process is called docking. The SNARE complexes form around the vesicle during the docking, resulting in the vesicle fusing with the membrane, known as priming. The depolarization of the presynaptic nerve causes Ca^{2+} influx, resulting in the SNARE proteins conformational change and release of the neurotransmitter from the vesicle to the synaptic cleft. The neurotransmitter binds to the receptors on the postsynaptic cell membrane, causing excitation. The cycle is repetitive for future depolarisations. The BoNT inhibits the neurotransmitter release from the presynaptic nerve terminal. The toxin is endocytosed as described previously, and after the release of the catalytic light chain, causes truncation of one of the SNARE proteins dependent on the serotype and, therefore, inhibits the formation of the SNARE complex, which prevents the vesicle docking and fusion to the presynaptic membrane and the subsequent release of the neurotransmitter. Taken from the [199].

The long duration of BoNT/A intoxication is a combination of its retention in the nerve termini and the short nature of the SNAP-25 truncation by the toxin ^[248,249]. The length of the truncation effect can be observed in contrast with BoNT/E, which causes the shortest exocytosis blockade in humans to be no longer than 4 weeks. The BoNT/E removes 26 residues segment, preventing SNARE complex formation compared to the case of BoNT/A serotypes, cleaving only 9 residues segment of the SNAP-25. The 9-residual truncation does not prevent the SNARE complex formation, yet the complex does not have its function. The dominant negative effect due to the described truncation is also believed to enhance the persistence of the BoNT/A function in neurons ^[249,250].

As mentioned in the previous paragraphs, the risk of foodborne botulism is still an actual issue, as well as the popularity of using BoNTs for medicinal purposes is increasing the probability of iatrogenic botulism. Last but not least, there is a threat of using BoNTs as biological weapons. All of the above is a good argument to call for an efficient detection method to diagnose botulism as fast as possible to increase the survival rate and decrease the hospitalization time, saving treatment expenses.

1.6.5 Current detection methods

The method for BoNT detection should be quick to increase the efficiency of the treatment, and it should be specific and sensitive, too. It must also be compatible with complex food and environmental samples. BoNT testing has applications outside clinical diagnostics, such as food testing ^[251]. Detection of BoNT is a popular research topic for finding an effective way to replace the current gold standard method requiring animal testing. It was reported in 2013 that the worldwide consumption of mice is 600 000 worldwide. The ethical aspect, costs, and requirements for skilled human resources call for exploring replacement detection methods ^[251]. The following paragraphs discuss the current gold standard, two FDA-approved, and potential replacement methods.

1.6.5.1 FDA-approved methods

The mouse bioassay (MBA) is the first FDA-approved, most commonly used method and is considered a gold standard ^[252]. Samples suspected of containing the toxin are introduced into the living mice, which are then observed for symptoms within 48 h after injection (wasp waist, piloerection, hind limb paralysis, dyspnoea) and, last but not least – death by respiratory paralysis. The toxin is quantified with the maximum sample dilution that results in the death of mice and the minimum dilution that does not cause the death of mice. The test might be repeated if the minimal dilution is not found for the first time ^[253]. The toxin serotype is determined by administering serotype-specific antitoxins into mice before the toxin introduction. Mice are observed as described previously to confirm which specific antitoxin is protective. Therefore, it takes 4-6 days to carry out the assay. The assay is very sensitive, with a 10 pg/mL detection limit targeting the active toxin, which is another major advantage compared to the immunological methods ^[251]. The ethical issues of harming living animals, high costs, and the time-consuming process requiring specialized facilities are the main drawbacks ^[254].

Cell-based assays have become a promising *in-vitro* alternative to the gold-standard MBA, offering similar or greater sensitivity ^[255]. Over the past years, assays utilizing stem cells and neurogenic cell lines have been developed to quantitatively determine the potency of various BoNTs, providing a reliable method of measuring biological activity ^[252,256,257]. These assays involve incubating cells with the toxin, followed by detecting SNARE protein cleavage through western blotting, ELISA, and immunofluorescence, providing an accurate measure of toxin activity ^[255,258]. Although cell-based assays are approved by the FDA, EMA, and Health Canada, they still face challenges, such as sample-to-sample variability, limited sample capacity per assay, and the need for specialized facilities and workforce ^[259]. The cell-based assays help to decrease the number of animals used for testing, but the drawbacks described and the comparable run time to the MBA leave a market opportunity for faster and easier assays ^[252].

1.6.5.2 Other methods under development

Lateral flow immuno chromatography assay (LFA) for BoNT detection is a hand-held assay utilizing antibodies conjugated to colloidal gold to obtain a color response in the presence of a toxin ^[260]. The sample is applied on the sample pad, where the toxin is attached to the antibodies with the colloidal gold. The toxin-antibody complex migrates the nitrocellulose

strip and is captured on the other antibody spot, providing the first color response line. The excess material and gold-conjugated antibody travel further to another control antibody spot, providing the second control color response. The positive test has two lines ^[260]. This fast and cost-efficient assay provides results in less than 15 minutes. The main disadvantage is that it is impossible to determine between the active and denatured toxins ^[260,261].

Another approach is the ELISA. As described in the first section of this text, it is a biochemical method utilizing antibody-conjugated enzymes to detect the presence of specific antigens. Indirect ELISA is the most commonly used setup for BoNTs detection. Serotype-specific antibodies allow the toxin serotyping. The quick serotype determination allows the administration of the right antitoxin quickly, which is crucial for successful botulism treatment ^[262]. The typical assay length is around 6 hours, with the detection limit from 2 pg/mL to 2 ng/mL ^[251,262]. The main drawback of the immunological assays is the presence of NAPS, as described in the previous paragraphs. The neurotoxin-associated proteins shield the toxin-binding sites for antibodies. Therefore, many assays were developed using highly purified BoNT samples, which are unsuitable for diagnostics and food testing ^[263]. This fact has initiated several research projects to develop new antibodies to overcome this issue ^[264,265].

It is possible to perform ELISA on the chip (EOC), resulting in sequential cross-flow immuno-chromatographic analysis of the BoNT/A serotype ^[266]. The assay design consists of two molded plastic polycarbonate plates with two traversing channels recessed underneath and a horizontal flow adsorption pad. The immuno-strip contains two serotype A-specific antibodies between the top and bottom polycarbonate plates. Two antibodies are used for two specific roles in the EOC device. One is used as a capture antibody immobilized on the membrane. The second detection antibody is conjugated with HRP. The EOC setup provides a color signal correlative with the applied BoNT/A sample concentration. The EOC system can be used up to 2 ng/mL with an analysis time of up to 30 mins ^[267].

The biosensor technologies for detecting BoNT utilize surface plasmon resonance, fluorescence, refractometer, and chemical luminescence platforms. A good example is fluorescence-based biosensors that use the evanescent wave technology. Commonly, an immunological sandwich assay with two antibodies for immobilization and quantification on the sensor surface is used. BoNT serotype E and B sensitivity ranges from 150 to 200 ng/mL ^[268,269]. These results are optimized primarily in simplified buffer systems, which complicates the transfer of the method to real-life complex samples and limits broader

applications in diagnostics and food testing. However, the BoNT/A serotype can be detected up to 50 ng/mL LOD in complex food matrices ^[270]. Biosensor platforms typically take more than 20 minutes but allow the detection of multiple substances at once, which makes biosensor assays potentially valuable for diagnostics and food testing. The fast results come at a sensitivity price, as seen with the LFAs assays. However, recent improvements in biosensor sensitivity, especially in transducer parameters and biorecognition elements, have the potential to be transferred from research to commercial use ^[271].

Molecular biology techniques like PCR can be used for the detection of BoNTs as well. One example can be a PCR variation called Immuno-polymerase chain reaction (Immuno PCR), an ELISA test using PCR for signal amplification. This method relies on the formation of the antigen-antibody complex as with regular ELISA, where instead of an enzyme, there is a DNA fragment of a known sequence bound to the antibody. Once the binding occurs, the complex is formed, and the amplification of the DNA fragment bound to the anti-toxin antibody is performed using traditional or real-time PCR. The immuno-PCR enhances detection sensitivity 1000-fold ^[272]. It can provide the same LOD as a mouse assay and determine active toxin levels as well. The immuno-PCR variation reached the 1 pg/mL LOD for serotype A ^[273]. One main drawback is transferring the PCR methods for food and clinical diagnostics. Compared with the mouse assay, the PCR-based methods take around 9 hours ^[273].

It is also possible to use a colorimetric assay, which operates on the principle of a color change occurring in the presence of an analyte. Gold nanoparticles have been commonly used for the development of a diverse portfolio of biosensor platforms ^[274]. The color change of the gold colloid nanoparticle of 5-50 nm diameters is typically exhibited in the UV-vis spectra with absorption peak in the wavelengths of 515-535 nm ^[275]. Adding salt neutralizes the localized surface plasmon resonance of the gold colloidal particles and shifts the absorption peak wavelength to 700 nm ^[276]. For BoNT detection, an assay was developed using the gold colloidal particle's color change in the presence of sodium chloride as a monitoring principle. It is based on the BoNT biological ability to cleave SNARE proteins. When the BoNT cleaves SNAP-25, the protective layer of protein on the colloidal gold particles is reduced, making the nanoparticles more susceptible to aggregation, which results in a detectable color change ^[277]. This assay has demonstrated sensitivity in detecting BoNT/A down to 370 fg/mL with a price per sample of less than 1 dollar and a rapid time

of 7 minutes. The colorimetric assay is a promising method for BoNT detection but has not yet been approved by the FDA [277].

The list of newly developed methods for BoNT detection is very long [11]. In the previous chapters, the most important were discussed. Venn diagram summarizing and evaluating each method (Figure 25) based on the 3 criteria: speed, sensitivity, and cost.

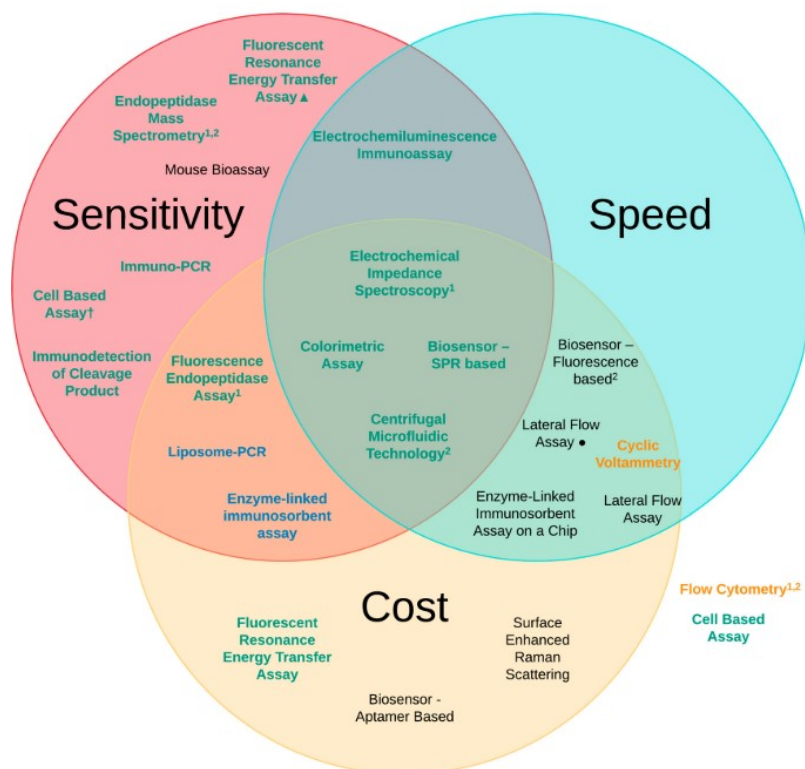


Figure 25 – Venn diagram summarizing available methods for BoNT detection. The evaluation was compared with the MBA, in which the LOD is 10 pg/mL. The green-highlighted text means the method can distinguish active toxins and is more sensitive than the MBA. The orange indicates that the assay determines active toxin but has poorer LOD than the MBA. The blue text represents methods that are not able to determine toxin activity. Additional annotation: dark circle marks methods combined with endopeptidase activity, dark triangle marks immunoseparation test, cross marks cell line coupled with ELISA, “1” marks multiple serotypes testing, and “2” complex samples matrices tested. Used from [11].

The mass spectrometry research teams didn’t fall far behind; they caught up with several mass spectrometry applications for BoNT detection and introduced methods, which are discussed in the following section.

1.6.5.3 MS-based methods

The mass spectrometry-based methods mainly use the toxin activity as well as several other available research methods, e.g., the fluorescence resonance energy transfer approach ^[254].

The main mass spectrometry method introduced in 2005 by J. Barr et al. is the endopeptidase mass spectrometry assay Endopep-MS. The Endopep-MS employs serotype-specific antibodies conjugated to magnetic beads, which are used to enrich the BoNTs. Magnetic beads with enriched toxin are separated from the tested sample and washed to remove excess material. In the initial publication, a synthetic peptide mimics the substrate protein sequence and is introduced to the beads. Toxin on the beads cleaves the substrate peptide specifically; therefore, it is easy to identify the substrate peptide with its cleavage products using MALDI-TOF mass spectrometry. ^[278]. The following Figure 26 shows each step of the Endopep-MS.

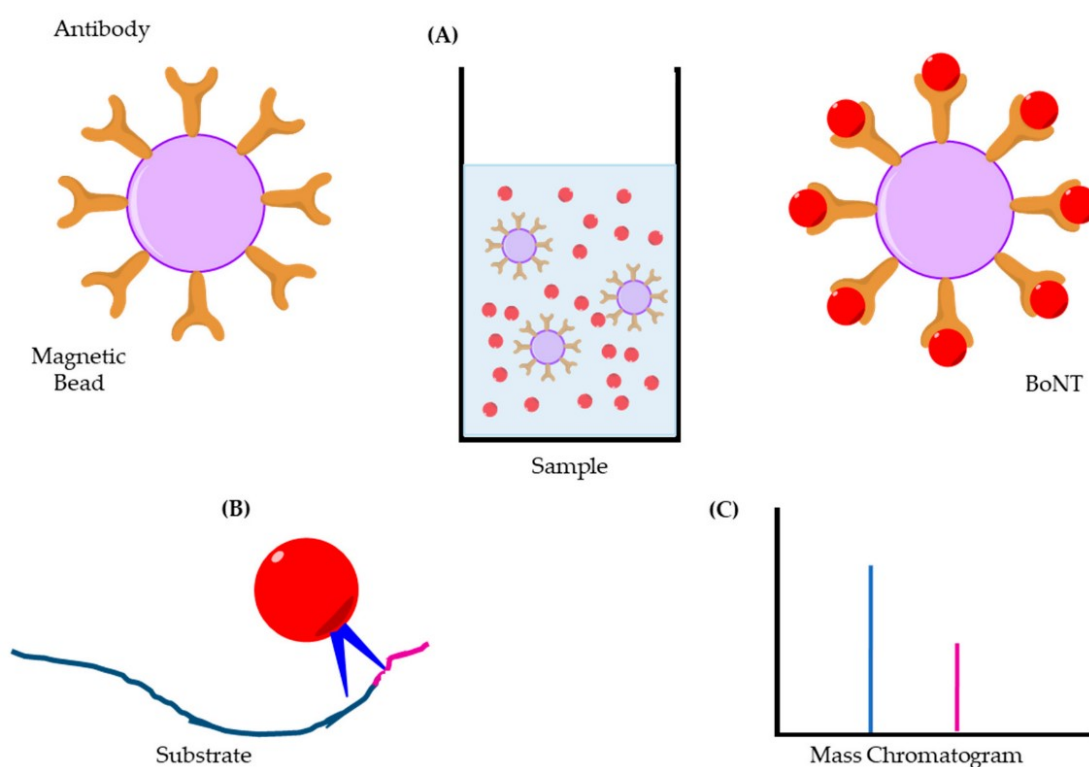


Figure 26 – Endopep-MS protocol steps overview, showing each step of the assay. The first step (A) shows the introduction of the toxin-containing sample to the magnetic beads with the conjugated antibody. The second step is the toxin-beads complex isolation and washing to remove excess impurities that could influence the following step (B), where a synthetic peptide in the reaction buffer is added to the enriched toxin. The N-terminal and C-terminal cleavage products present in the sample are then analyzed by the MALDI-TOF MS (C). As displayed in ^[11].

Assay determining toxin activity is particularly interesting for production testing in pharma companies ^[279]. The assay takes around 4-8 hours, but the procedure's potential automation allows processing hundreds of separate samples in this time range. It is also possible to target

multiple toxin serotypes simultaneously using the multiplex platform, allowing the detection of BoNT/A, B, and E. The multiplex assay minimizes the sample volume and reduces the testing time, improving assay usability, particularly in the human diagnostics and food testing field ^[280].

Depending on the sample matrix, the assay's sensitivity to detect toxin concentrations ranging from 100 fg/mL to 1 ng/mL is a good competition for the MBA. A wide range of different sample matrices, including serum, stool, diverse food samples, and culture supernatants, were tested, showing potentially extensive applications for this assay. Diverse matrix compatibility is a concern mainly for the food-testing application, compared to the pharmaceutical sector, where simple buffers are the matrix of choice ^[281]. The Endopep-MS was recently validated for clinical specimens, showing a sensitivity comparable to or better than MBA, with no cross-reactivity between different serotypes and no false positive results ^[282].

An interesting case study of a 7-month-old infant who was admitted to the pediatric intensive care unit suspected of botulism was reported in 2015. A mouse bioassay, bacterial culture, and PCR provided negative results. However, BoNT/A was detected in the infant's serum by Endopep-MS assay. The infant was treated with botulinum antitoxin, which returned the patient's ability to swallow three weeks after the admission. Conventional PCR from the stool showed positive results for BoNT two weeks after hospitalization ^[283].

In addition to Endopep-MS, mass spectrometry is valuable for epidemiologic and forensic investigations. A bottom-up proteomic technique was established to determine the serotype, subtype, and variant of BoNT. These techniques showed the ability to detect BoNTs and their NAPs, and differentiate the toxin from other toxins even in cases where a 99,9 % identity is observed. The techniques are compatible with complex matrix samples, such as stool, bacterial culture, or food; no DNA is required ^[284].

2. Aims of the study

The thesis aims to extend the number of potential clinical microbiology applications of the affinity chips prepared by ambient ion soft landing technology compatible with MALDI-TOF MS detection.

The specific goals were:

- Optimize the preparation of affinity chips surface-modified with anti-procalcitonin antibody and NeutrAvidin.
- Develop a suitable enrichment protocol, including sample treatment
- Optimize the reaction conditions for the indirect *in situ* enzymatic assays (Toxin B, BoNT/A1)
- Optimize the wash protocol to discard any impurities prior to MALDI-MS measurement.
- Test multiple MALDI matrices, including their preparation protocols, and optimize suitable MALDI-TOF measurement methods for the best possible signal intensity and resolution.
- Statistically evaluate the developed assays.

3. Methods

The manuscripts attached to this thesis include detailed descriptions of methods, used materials, and instruments. The description is sufficient enough for the experiments to be reproduced. Therefore, this chapter contains only the list of commonly used methods.

The list of used research methods

- ambient ion soft-landing for the preparation of functionalized chips compatible with MS
- *in situ* enrichment of analyte from a complex matrix
- SDS-PAGE western-blot
- *in situ* enzymatic reaction in a complex matrix
- MS analysis of enriched proteins and enzymatic products of the *in situ* reaction with toxin in the complex matrix
 - Autoflex speed MALDI-TOF in linear positive mode, FT-ICR, TIMS-TOF SCP Q-TOF LC-MS in LC-MS/MS mode with electrospray ionization

4. Results and Discussion

The main aim of the thesis was to tailor the use of MALDI-compatible chips for clinical microbiology. Therefore, all publications aim to use surface-modified chips with affinity proteins to enrich the analyte from a complex sample matrix or the substrate prior to the *in situ* enzymatic reaction.

4.1 Development of chips compatible with MALDI mass spectrometry for clinical microbiology

As mentioned previously, chips compatible with MALDI-TOF MS are prepared using AISL. The work with chips starts with developing the manufacturing protocols, which are generally followed by multiple steps to optimize each.

The general workflow using the MALDI-compatible chips can be divided into multiple steps:

- the chip manufacturing
- sample preparation
- complex sample incubation on the chip/*in situ* enzymatic reaction on the chip
- wash protocol
- matrix application
- MALDI-TOF MS analysis

The technology described in this thesis can potentially simplify current workflows used in clinical practice. It can even provide valuable additional information to improve clinical microbiology diagnostics when working with complex human sample matrices (stool, serum). This methodology can be compared with routinely used FDA-approved methods such as immunoassays (IA), nucleic acid amplification techniques (NAAT), e.g., PCR, cytotoxicity assay (CT), toxigenic culture (TC), and mouse bioassay.

In general, an assay using MALDI-compatible chips provides mass spectra in the range in which the analyte is measured. It is an easy-to-use technology with a potential for automatization. Therefore, the assay prevents false positive results due to the possibility of detecting the analyte's mass compared to the FDA-approved methods that use the antibody-antigen interaction, such as ELISA, in which non-specific impurities can interfere

with the measurement, or on the other hand, NAAT, which only aims at the presence of the encoding gene or the mouse bioassay that is non-ethical. Another advantage of the MALDI MS-based assay is the relatively easy implementation of the technology in clinical microbiology laboratories, considering that the MALDI-TOF MS is a routinely used instrument for microorganism biotyping in a high percentage of these facilities.

In this thesis, three applications for MALDI-compatible chips are described. The first publication aims to enrich the sepsis biomarker procalcitonin from the human serum. The second and third publications were based on indirectly monitoring the activity of two crucial bacterial toxins (Toxin B, Botulinum Neurotoxin A), which induce enzymatical change on the bound substrate on the NeutrAvidin chip. Each approach will be described in the following paragraphs.

4.1.1 Publication 1

Title: The Rapid Detection of Procalcitonin in Septic Serum Using Immunoaffinity MALDI Chips

The main goal was to study the potential of MALDI-compatible chips modified with anti-procalcitonin antibody for procalcitonin (PCT) enrichment as an alternative to routinely used immuno-based assays described in Chapter 1.1.2. In comparison, the discussed methods in the chapter are based on monitoring the antigen (PCT) bound on the antibody using various technologies (enzyme-linked immunofluorescent, electrochemiluminescent, chemiluminescent, immunoluminometric); the enriched PCT on the developed chip technology allows MALDI-TOF MS detection, which provides an opportunity to target the analyte directly from the measured mass spectrum and minimize false positivity caused by non-specifically bound molecules using the provided mass spectra.

The first step of this study was to optimize method measurement settings. Two commonly used MALDI matrices for protein detection were tested (SA, DHAP). The DHAP matrix provided significantly higher overall resolution and intensity in lower concentrations than SA. The MALDI-TOF MS settings were also optimized and are described in the method section of the attached publication.

The anti-PCT antibody chips assay limit of detection (LOD) was tested using the dilution series of the commercially available recombinant PCT in the serum pool of healthy individuals (Fig. 27, page 59). Two main PCT ions could be identified in the mass spectra:

the singly charged at the m/z 14 029, and at 7 014, the doubly charged. The doubly charged ions of the recombinant PCT provided better peak resolution and signal intensity. The lowest concentration detected using this approach was 0.5 $\mu\text{g/mL}$ with the signal-to-noise ratio 12.

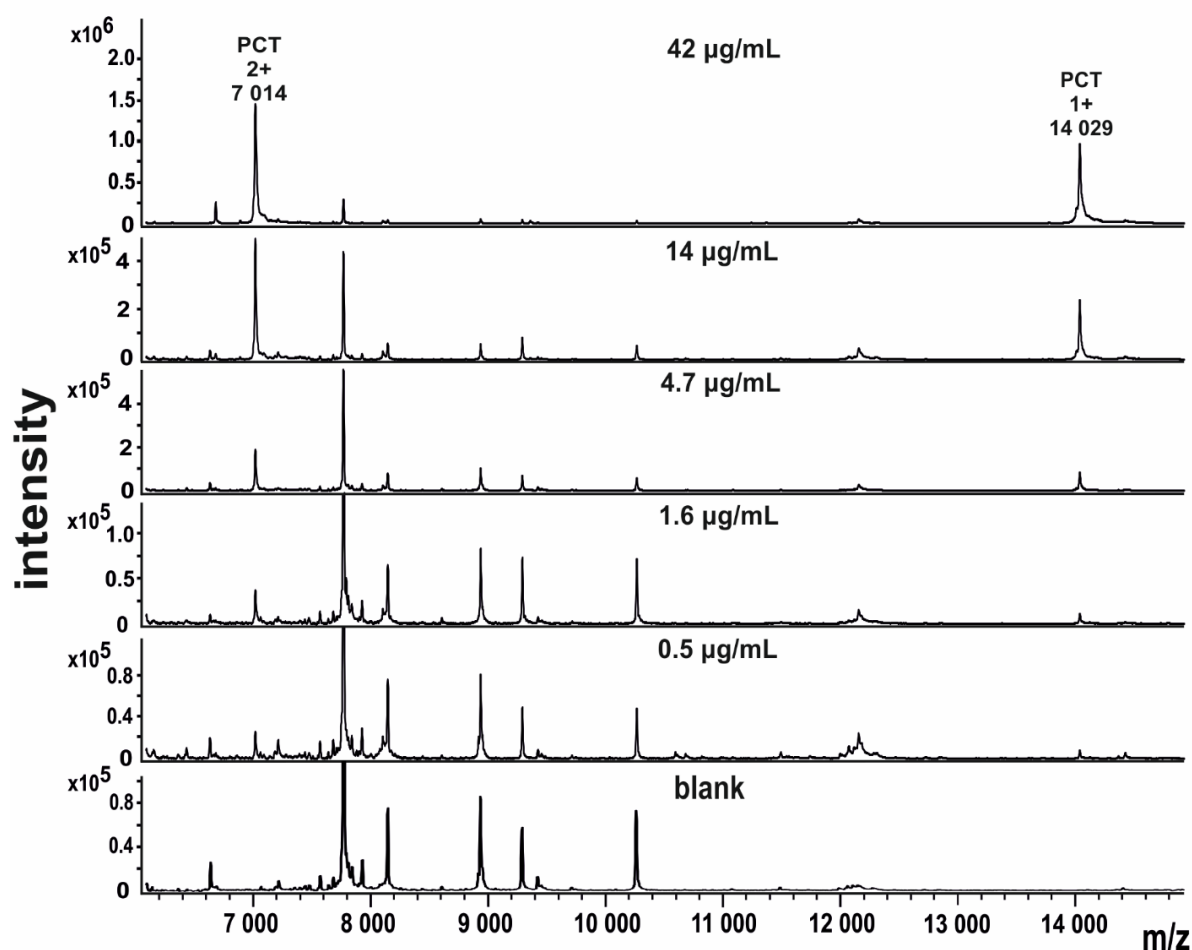


Figure 27 – Dilution series of the recombinant PCT spiked into healthy human serum enriched on the anti-PCT chips analyzed by MALDI-TOF MS. The bottom panel shows the spectrum of serum with no PCT added (blank).

The limit of detection needed improvement to meet the requirements for measuring PCT in septic patients. Therefore, a suitable precipitation protocol was necessary to remove excess material and enrich the sample in the PCT. Several protocols were tested, and the best results were achieved using the addition of acetonitrile in the ratio of 1:4.5 to the doubly diluted serum. The concentration of the PCT was increased with a factor of 15 based on the comparison of ion signal intensities (Fig. 28A, page 60). The LOD was improved to 10 ng/mL by using the acetonitrile protocol. The Figure 28B (page 60) shows the method's dynamic range, where it is clear that the higher concentrations (500 and 1 000 ng/mL) are out of the linear range. The graph visualizes intensities measured for each concentration of the dilution series, and numbers at each point represent the signal-to-noise ratio

A

Intensity $\times 10^5$

m/z

B

Intensity $\times 10^4$

m/z

○ PCT (2+)

● PCT (1+)

30

45

62

80

20

242

262

356

6

8

2

3

c_{PCT} [ng/ml]

60

The method was statistically evaluated using 16 samples containing 140 ng/mL of spiked recombinant PCT because it is in the linear detection range and simulates the PCT blood concentration in patients suffering severe sepsis. The coefficient of variation (CV) was estimated to be 25 % for the singly and 22 for the doubly charged PCT ion, respectively. The chip-to-chip CV was 14 % for singly and 10 % for doubly charged ions.

The optimized method was tested on 13 patients with confirmed sepsis. The elevated PCT concentration was quantified by ELISA prior to the testing by the anti-PCT chips MALDI assay. Three patient samples are shown as the representation of the whole cohort (Fig. 29).

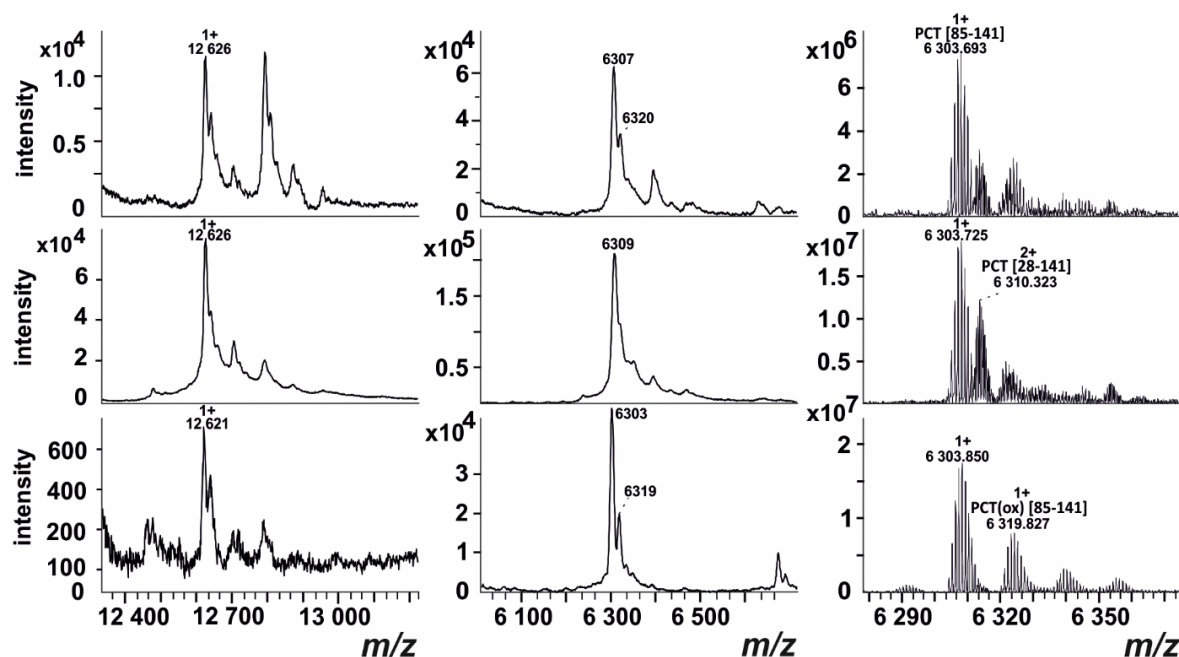
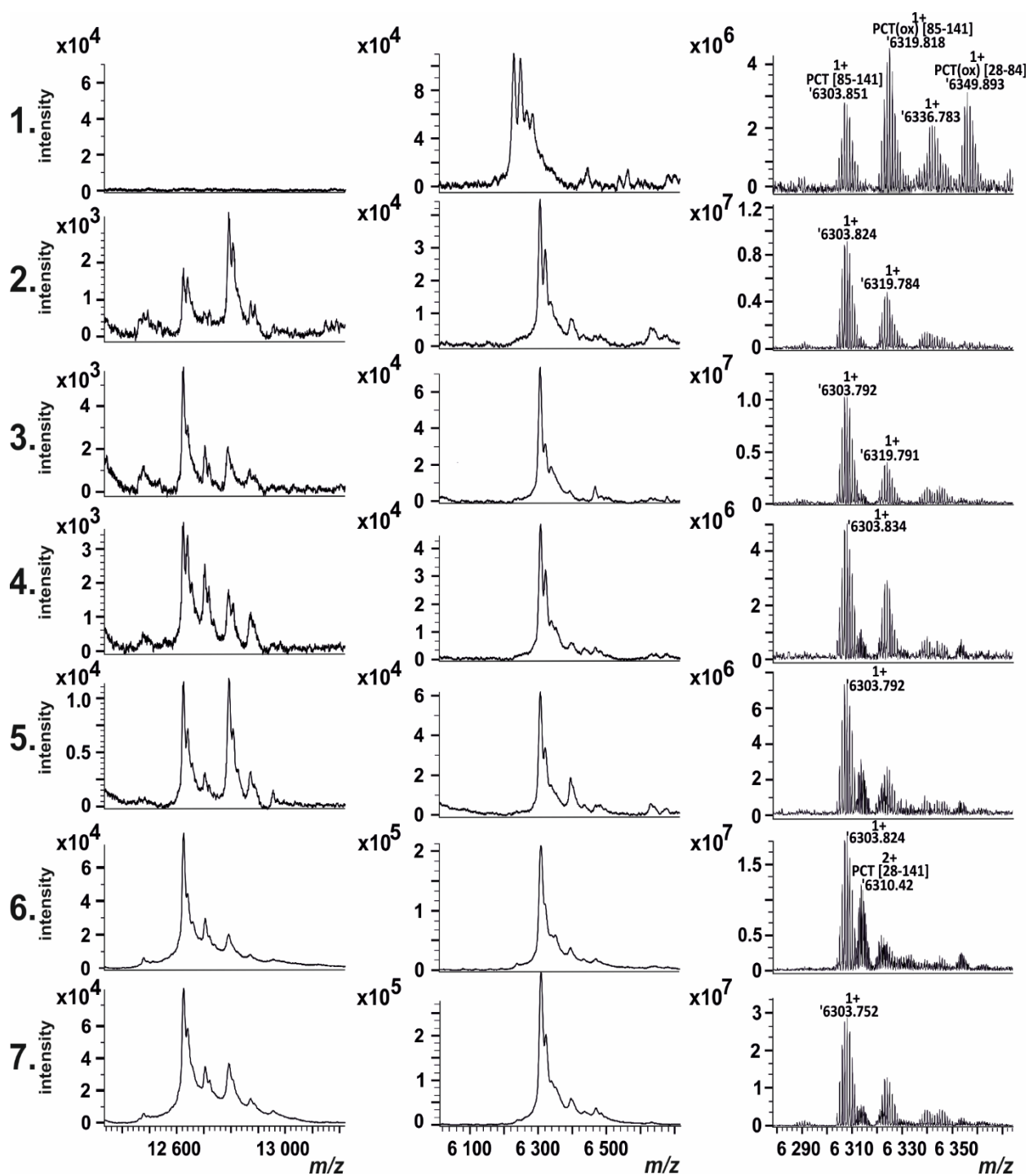


Figure 29 – PCT detection was carried out using MALDI chips for samples of the three septic patients from top to bottom. The figure represents detailed spectra using singly (left column) and doubly (middle column) charged PCT ions. The detailed FT-ICR measurement in the mass range where the doubly charged ion is expected (right column) also shows the presence of the singly charged ion at the m/z 6 303.850, which fits well to be a PCT fragment based on its exact mass that FT-ICR MS obtained. The PCT fragment is most noticeable in the third patient sample, suggesting the conversion from the original PCT to truncated form was the most significant.

Interestingly, patient three displayed the singly charged ion (m/z 6 303.85), which was believed to be a truncated PCT most dominantly. The conversion to the truncated PCT form was believed to be more significant due to the increased proteolytic activity during sepsis. This observation was done in most of the thirteen measured samples. The following spectra illustrate the presence of the truncated PCT forms, including signals of their oxidized forms in some cases (Fig. 30, page 62 & 63).



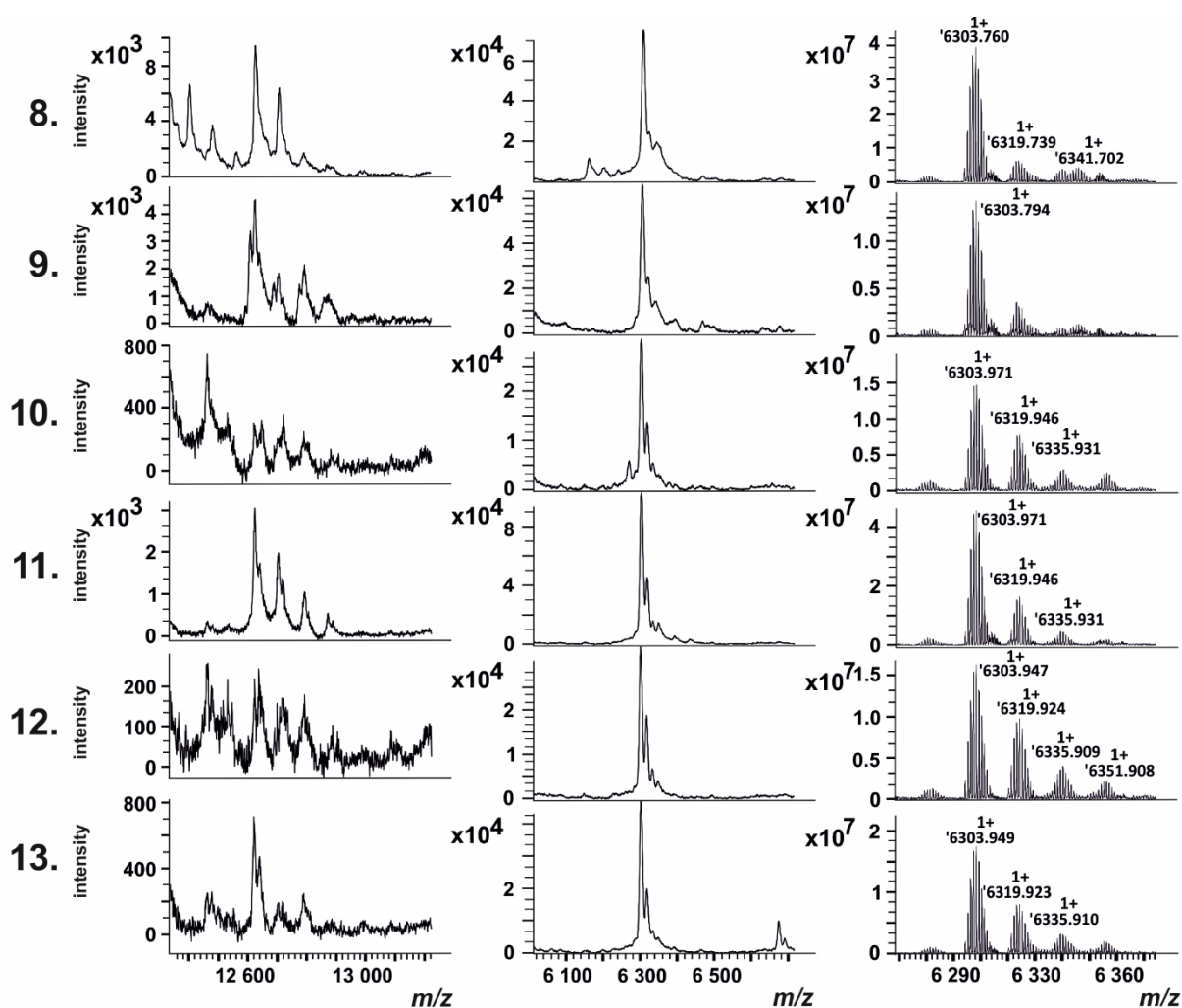


Figure 30 – In-situ detection of PCT in 13 patient samples. Starting from the left - detailed mass spectra from MALDI-TOF MS of the singly and doubly charged PCT ions and high-resolution data on the right show the presence of the truncated PCT species and their oxidized forms.

The cleavage site was targeted to be at the R84-C85 based on the exact mass of the truncated forms obtained from the high-resolution FT-ICR mass spectra. An investigation of the sample processing protocol was conducted to evaluate the origin of the PCT fragments. Pooled healthy human serum was spiked with the recombinant PCT, incubated at 37 °C for 3 hours, and subsequently processed using the acetonitrile concentration protocol. The detection was performed on the MALDI chips using MALDI FT-ICR (Fig. 31, page 64). A truncation on the N-terminus was detected. However, no signals suggested any analogical truncation to the one in the septic serum. Therefore, we can imply the presence of the truncated forms in the septic patients is not due to the protocol used. This indicates the observed truncation in the patient samples is due to the sepsis-related proteolytic activity rather than an artifact of sample processing.

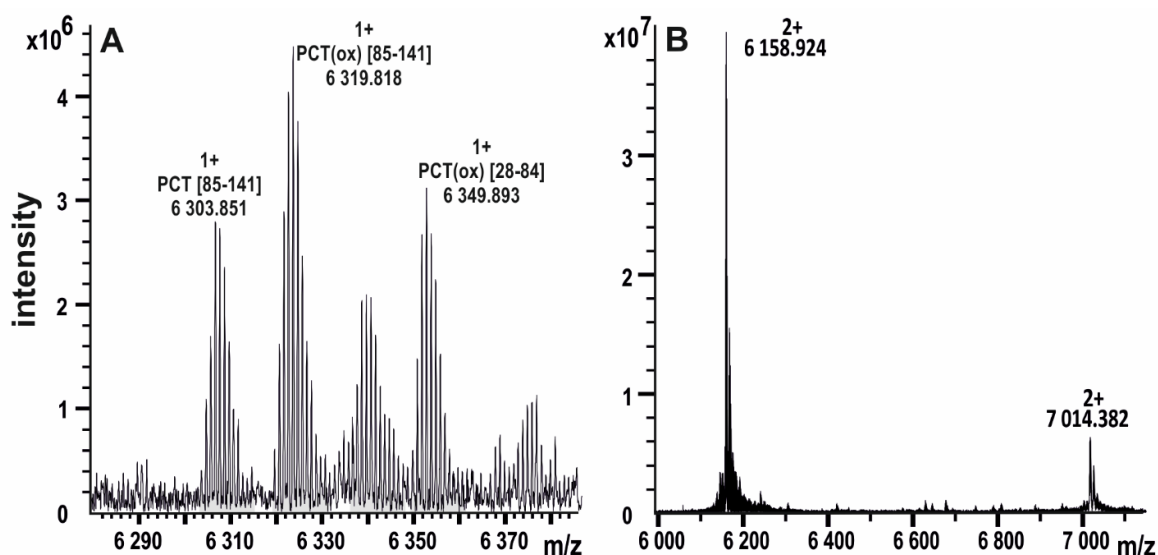


Figure 31 – (A) Detailed MALDI FT-ICR spectrum representing the septic patients' cohort with the signals of the truncated PCT forms. (B) MALDI FT-ICR spectrum of the recombinant PCT incubated in the healthy serum at 37 °C for 3 hours and processed using the standard sample processing protocol. The spectrum shows no equivalent fragments when compared to septic serum signals. Therefore, the presence of PCT fragments is not related to sample degradation.

As mentioned at the beginning of this section, this study aimed to develop a simple and rapid method for the detection of PCT enriched from the septic patients' sera based on the widely available MALDI-TOF MS [285], with the main advantage of the enrichment and discarding excessively bound material from the sample followed with the detection taking place on a single functionalized MALDI-compatible chip.

The anti-PCT chips used in this work have a strong base in the previous research, confirming the functionalized chips' overall universal utility. The chips were modified using the ambient ion soft landing technology with various biomolecules, e.g., antibodies, proteases, and glycopeptides [84,87,100,286,287]. The biomolecules' spatial structure and biological activity are retained because the AISL is gentle enough, providing a suitable chip for an analyte enrichment from the complex sample, as demonstrated by Darebná et al. [106].

The detection limit was enhanced by employing the acetonitrile serum precipitation protocol. This approach aims to precipitate highly abundant serum proteins while leaving PCT in the supernatant, as proved by Western blot (part of the supplemental material of the publication). It was possible to improve the method's detection limit to 10 ng/mL of the recombinant PCT spiked into healthy serum. While sufficient for detecting PCT in patients, the sensitivity is not high enough to detect PCT in healthy individuals (typically <0.1 ng/mL) or in the case of

mild infection (around 2 ng/mL), which is possible using conventional methods [288]. However, it is suitable for elevated PCT level detection in patients suffering severe sepsis. Our structural characterization was in agreement with the previous findings by Weglohner et al., who reported truncation on the PCT's N terminus and absent glycosylation despite the presence of a glycosylation motif in the PCT sequence [151].

The other experimental approaches employing mass spectrometry for PCT detection, such as bottom-up workflows, target PCT on the peptide level. While providing below ng/mL sensitivity, they don't offer valuable information about the presence of intact/truncated PCT [289]. In contrast, the MALDI affinity chip-based approach allows the direct detection of intact PCT captured *in situ* directly from the sample, including its truncated variants, which are also present in the spectrum. Matrix-assisted laser desorption/ionization connected to the TOF and FT-ICR analyzers confirmed the consistent presence of the previously described truncated PCT forms and their oxidized states across all the tested patient samples. We hypothesize that the truncated PCT forms are present due to the increased proteolytic activity during sepsis. Therefore, the increased proteolysis results in the mixture of intact PCT and the observed truncated forms. The possibility of the sample treatment influence was ruled out by the PCT incubation in the serum, resulting in the MS spectra shown in Fig. 31(B) on the page 64. The experiment suggests that the PCT fragmentation results from pathological processes rather than sample handling artifacts.

All the benefits described in this discussion provide additional valuable information to the conventionally used ELISA, where non-specific interactions can influence the measurement and no truncated PCT forms can be detected.

In addition, an interesting observation was made in one of the patient samples, in which LC-MS/MS identified RTX-1 toxin from *Actinobacillus pleuropneumoniae*. However, no pathogen was detected by conventional microbiological methods. In this sample, all PCT was turned into truncated forms, suggesting that the severity might be linked to the ratio of intact to cleaved PCT forms.

4.1.2 Publication 2

Title: A proof-of-concept MALDI-TOF-MS assay for detection of Toxin B enzymatic activity in *Clostridioides difficile* infection

The second publication aimed to develop the assay using MALDI-compatible chips for indirect toxin detection based on its enzymatic activity. In the last decade, MALDI-TOF MS became a gold standard method for microbial identification in clinical microbiology labs, as mentioned in the previous paragraphs ^[290]. However, the MALDI biotyping has certain limitations. A pure single-species colony is necessary to compare the mass fingerprint with the database. In the case of strictly anaerobic bacteria, cultivation can represent a costly, time-consuming, and laborious process ^[291]. In this project, a proof-of-concept assay using *C. Difficile* as a model organism was developed to overcome the limitations of MALDI biotyping of the anaerobic species. Additionally, our study provides valuable information about the toxin activity in the complex stool sample to the routinely used methods for *C. Difficile* detection, like immunoassays and nucleic acid amplification tests focused on the load of the pathogen and its virulence factors ^[189].

While NAATs aim for the presence of the encoding genes, the immuno-based assays target the presence of the bacterial glutamate dehydrogenase or its two toxins (Toxin A, Toxin B). The introduced MALDI-compatible method employs NeutrAvidin chips and recombinantly produced biotin-tagged substrate protein to assay *C. Difficile* toxin activity by monitoring the substrate conversion. The substrate protein design is based on the toxins' natural target protein, RhoA, while the biotinylation allows the interaction with NeutrAvidin chips. The biotin-RhoA protein bound on the NeutrAvidin MALDI chips is modified due to the toxins' activity by adding a glucose molecule from the UDP-glucose to its threonine 37.

Selecting the suitable MALDI matrix was a crucial step in the method optimization to achieve optimal ion intensity while avoiding matrix adduct formation that could disrupt the observation of the glycosylation product. The most suitable candidate was the HCCA matrix prepared using the protocol published in 1996 ^[292], which outperformed other candidates like SA, DHAP, and DHB while minimizing the interfering adduct formation. The comparison can be seen in the attached publication in the supplemental figure 3.

Surprisingly, the stability of the substrate biotin-RhoA was dependent on the enrichment protocol, where the prior enrichment of the substrate from the stock solution provided significantly better results compared to just mixing the substrate with the sample solution

followed by the *in situ* enrichment. However, multiple other factors needed to be optimized to reach the desired substrate stability, where the addition of the DDM and Mg^{2+} ions was necessary. Last but not least, the hostile environment of the stool sample is full of unknown proteases. This is why the right combination of protease inhibitors was necessary, which can be observed in the comparison of samples with and without protease inhibitors in the supplemental figure 4 of the attached publication.

For the enzymatic reaction, the time of incubation plays a significant role. In theory, the longer the substrate is incubated with the toxin, the better conversion is reached. However, the influence of the complex hostile environment and the time efficiency of the assay have to be considered. Therefore, the optimum time was estimated to be between 30 and 80 minutes.

Toxin modification of the substrate protein can be detected directly from the chip by MALDI-TOF MS as a mass shift of 162 for the singly and 81 units for the doubly charged biotin-RhoA ions (Fig. 32). This means that the original mass transforms from 22 063 (1+) and 12 534 (2+) for the non-modified substrate to 22 225 (1+) and 12 615 (2+) mass units for the modified. The transformation reflects the addition of glucose from the UDP-glucose.

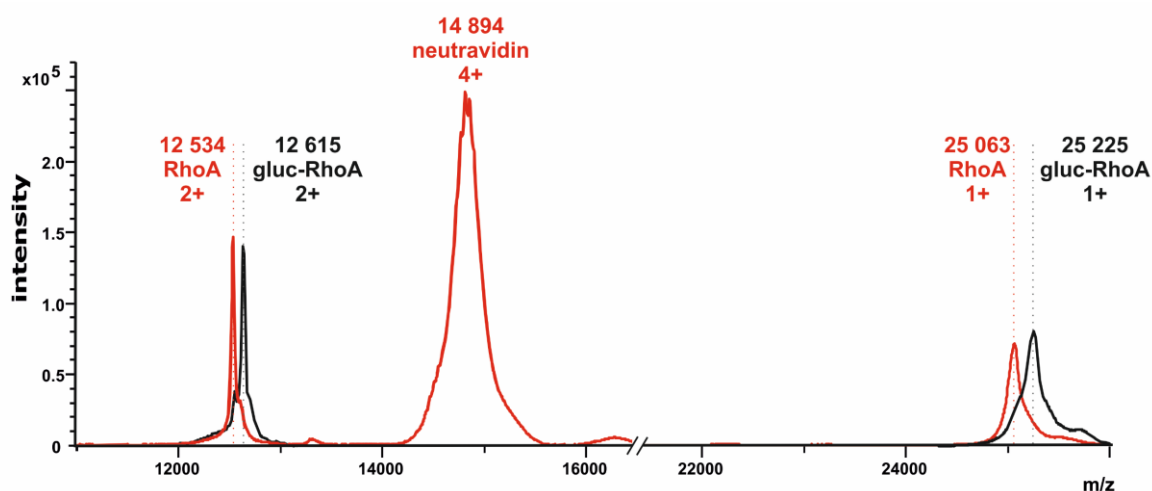


Figure 32 – MALDI-TOF MS spectra of intact RhoA (red) and glycosylated RhoA (black), acquired on a NeutrAvidin-coated chip. The spectra display a mass difference of 162 Da for singly charged ions and 81 Da for doubly charged ions between intact and glycosylated RhoA. This shift in m/z values reflects the glucosylation mediated by Toxin B glycosyltransferase activity. Additionally, a signal observed at m/z 14 894 corresponds to quadruply charged NeutrAvidin, which is also desorbed from the surface.

The doubly charged signal was selected for the testing because it showed the best overall intensity and resolution between the biotin-RhoA forms. The optimized assay demonstrated

the detection limit up to 32 ng/mL for recombinant Toxin B spiked into a healthy stool (Fig. 33). The signal-to-noise ratio at this concentration was 17 for the modified substrate.

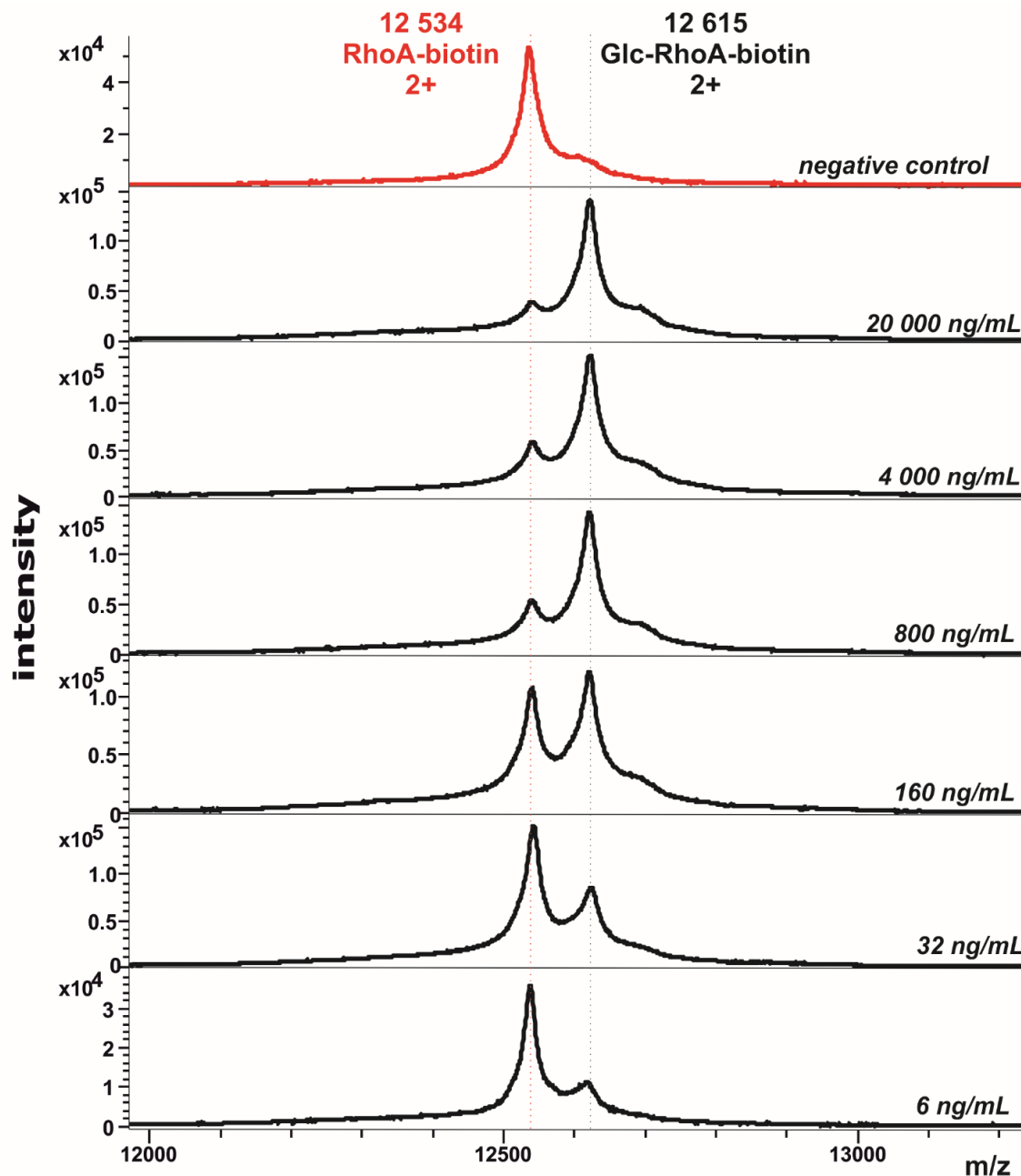


Figure 33 – MALDI-TOF MS spectra of the doubly charged substrate exposed to stool samples spiked with recombinant Toxin B in varying concentrations due to the dilution series compared to the negative control. The glucosylated form of the substrate was clearly detected at toxin B concentrations starting from 32 ng/mL. The dilution series was analyzed using a technical pentaplicate on a single MALDI chip.

The extent of conversion to glycosylated form was estimated using the formula (Formula 1) described in the attached publication. Additionally, to the extent of glycosylation, the standard deviation (S_D) and coefficient of variation (CV) were also estimated at 2 lowest

concentrations of the dilution series. The extent of conversion was 0.6 ($S_D = 0.08$; CV = 13 %) for 32 ng/mL and 0.3 ($S_D = 0.3$; CV = 3 %) for 6 ng/mL of spiked Toxin B.

The assay reproducibility was evaluated by spiking stool samples with recombinant Toxin B at 4 and 0.8 $\mu\text{g/mL}$, followed by analysis on two different NeutrAvidin MALDI chips in 20 technical replicates each. Resulting in 40 replicates in total. The resulting CV obtained with chip 1 was 6 % ($S_D = 0.05$) at a Toxin B concentration of 4 $\mu\text{g/mL}$ and 8 % ($S_D = 0.06$) at 0.8 $\mu\text{g/mL}$. For chip 2, the CV was 8 % ($S_D = 0.06$) at 4 $\mu\text{g/mL}$ and 12 % ($S_D = 0.08$) at 0.8 $\mu\text{g/mL}$. The inter-chip CV was 7 % ($S_D = 0.05$) at 4 $\mu\text{g/mL}$ and 10 % ($S_D = 0.07$) at 0.8 $\mu\text{g/mL}$ Toxin B concentration. The optimized assay was used to analyze 20 stool samples from CDI-suspected patients (Table 1).

*Tab. 1 – The table summarizes the results of patient sample testing using the optimized MALDI assay. It compares it with the routinely used approaches such as immunoassays (IA) and loop-mediated isothermal amplification (LAMP). A **plus** indicates a positive, and a **minus** indicates a negative result. If the GDH IA was negative, no further testing was performed. The MALDI assay was performed for every sample. Additionally, only a subset of samples where GDH IA was positive and toxins' IA was negative was further tested using the LAMP (patients 1 and 8). The used protocol in the hospital complied with the recommended testing workflow described in the theoretical introduction of the thesis.*

sample number	IA			LAMP			IA/LAMP assay	MALDI assay
	GDH	TcdA	TcdB	GDH	TcdA	TcdB		
1	+	-	-	+	+	+	+	-
2	-	N/A	N/A	N/A	N/A	N/A	-	+
3	+	+	+	N/A	N/A	N/A	+	+
4	-	N/A	N/A	N/A	N/A	N/A	-	-
5	+	+	+	N/A	N/A	N/A	+	-
6	+	+	+	N/A	N/A	N/A	+	+
7	-	N/A	N/A	N/A	N/A	N/A	-	-
8	+	-	-	+	-	-	-	-
9	+	+	+	N/A	N/A	N/A	+	-
10	-	N/A	N/A	N/A	N/A	N/A	-	-
11	-	N/A	N/A	N/A	N/A	N/A	-	-
12	-	N/A	N/A	N/A	N/A	N/A	-	-
13	-	N/A	N/A	N/A	N/A	N/A	-	-
14	-	N/A	N/A	N/A	N/A	N/A	-	-
15	-	N/A	N/A	N/A	N/A	N/A	-	-
16	-	N/A	N/A	N/A	N/A	N/A	-	-
17	-	N/A	N/A	N/A	N/A	N/A	-	-
18	-	N/A	N/A	N/A	N/A	N/A	-	+
19	-	N/A	N/A	N/A	N/A	N/A	-	-
20	-	N/A	N/A	N/A	N/A	N/A	-	-

Toxin B activity was identified in four samples using the MALDI assay (#2, #3, #6, #18). The MALDI-TOF MS spectra showing the double-charged and modified substrate of each positive patient are shown in Figure 34. The results were consistent with the hospital data in two cases (#3 and #6). Conversely, in three samples (#1, #5, and #9), Toxin B activity wasn't observed by MALDI assay despite the positive results obtained from the standard hospital protocol. The difference in the results can be caused by the different nature of the standard tests aiming at the presence and the MALDI assay targeting active toxins.

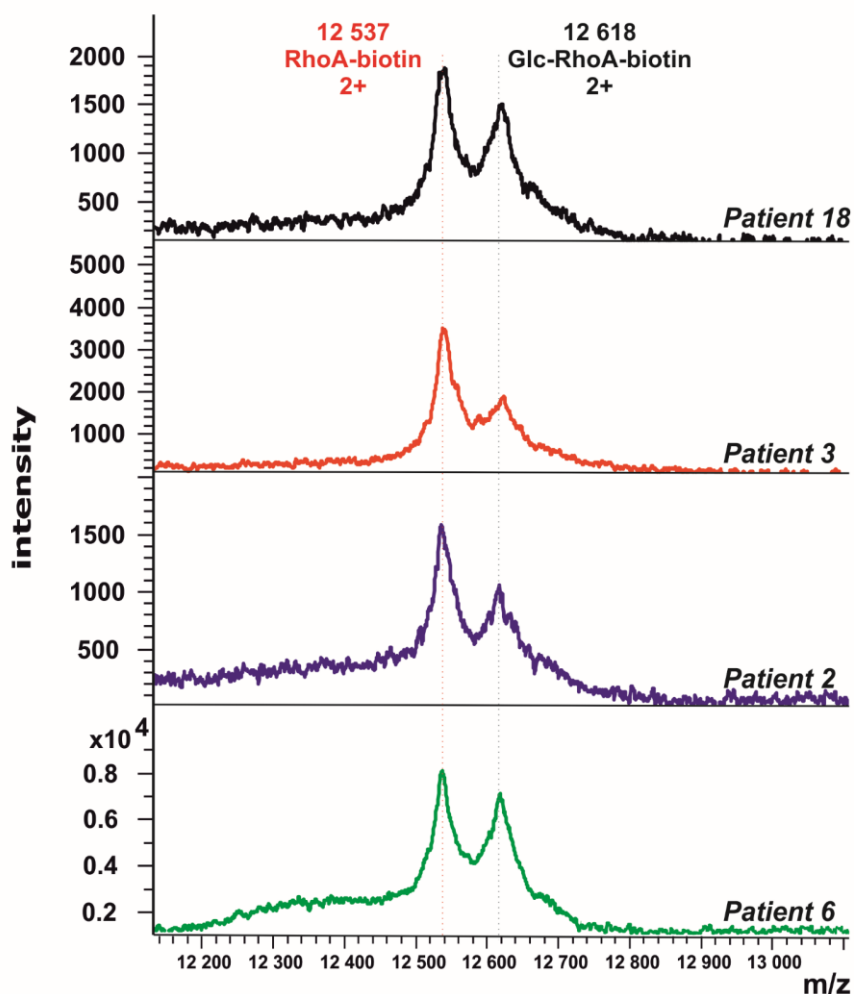


Figure 34 - Mass spectra of the RhoA substrate are shown for patient samples (#2, #3, #6, and #18) in which the MALDI-based assay indicated the presence of Toxin B. The observed levels of modification across these samples imply the likely presence of the active form of the toxin. Each sample was analyzed in technical duplicates using a single MALDI chip.

Table 2 (page 71) summarizes the extent of conversion into the glycosylated RhoA for patients (#2, #3, #6, #18) performed in duplicates. The ratio within the duplicates is reproducible and varies across the four patient samples. This observation indicates different Toxin B activity in each sample.

Tab. 2 – MALDI MS signal intensities of the modified and non-modified substrate RhoA in four positive patient samples. The extent of conversion was calculated using the formula described in the attached publication 2. The extent of conversion is expressed as a percentage. The samples were measured in the technical duplicates.

sample	replicate	intensity modified	intensity non-modified	the extent of conversion (E_{conv}) [%]
18	1	1815	2278	44
	2	8786	10949	45
3	1	2338	3937	37
	2	3737	7841	32
2	1	4309	6126	41
	2	1513	2015	43
6	1	1383	1513	48
	2	10988	12101	48

This study demonstrates the utilization of MALDI-TOF MS assay targeting the active Toxin B in the stool sample of CDI-suspected patients. The described assay offers additional information about the extent of the active toxin in the sample compared to the standard methods, which only target the toxins' presence without considering their biological ability. Targeting activity instead of concentration may provide a more valuable approximation of the potential threat of the CDI since the concentration seems to not correlate with the CDI potency ^[188].

The activity-based approach represents several advantages. Significant genetic variability of the *C. difficile* strains, in which the toxins are highly divergent, and the toxin production also varies ^[1]. The potential horizontal gene transfer to the other species must also be considered ^[293,294]. Thus, we believe that an assay capable of targeting the toxins' activity rather than simply their presence can be highly valuable in clinical microbiology. The simplified approach targeting just the presence can also indicate a high percentage of the non-active toxin, which can lead to wrong diagnosis or overdiagnosis. Overdiagnosing increases the patient's stay in the hospital and the treatment costs ^[187,189,295].

As mentioned in the previous paragraphs, employing the MALDI-TOF MS offers possibilities for a rapid and cost-efficient diagnostic approach. The assay also provides a shortcut to standard biotyping, where anaerobic cultivation or complex sample preparation steps would be required ^[296,297].

Nevertheless, several challenges remain. The stability of the substrate RhoA in the hostile stool matrix is a significant limiting factor for the assay's performance. A mixture of the protease inhibitors in combination with the suitable buffer and stabilizing agents was used to minimize the influence of the instability, which improved the assay's reproducibility and sensitivity. Additionally, discrepancies between activity-based and presence-based assays suggest that a combination of these divergent diagnostic strategies may offer the most reliable approach for diagnosing CDI ^[188].

In summary, this proof-of-concept study provides a potential addition to the diagnostics toolbox for the CDI by enabling the direct measurement of Toxin B activity. However, further validation in larger patient cohorts is necessary to establish its role in clinical decision-making.

4.1.3 Publication 3

Title: Novel activity assay for botulotoxin A1 detection using functionalized chips and matrix-assisted laser desorption/ionization mass spectrometry

This study indirectly assays the presence of toxin by monitoring its enzymatic activity products. As described in the introduction, *Clostridium botulinum* is a species that produces multiple variations of its neurotoxin (BoNTs), targeting the SNARE proteins and preventing postsynaptic excitation due to the neurotransmitter release blockage. The BoNTs are as considered one of the most lethal substances in nature. The most common cause of human botulism is the BoNT serotype A.

The study declares that it is possible to transfer an assay for the BoNT detection described by J. Barr ^[282,284] from magnetic beads to MALDI-compatible functionalized chips, which simplifies the workflow due to the possibility of performing both the reaction and the detection directly on the chip. This approach praises the work of J. Barr and shows its potentially more straightforward incorporation to the routinely used BoNT detection approaches due to possible automatization and high throughput by using the chip layout of the assay.

The moral controversy surrounding the routine use of the FDA-approved mouse bioassay was also an important motivation. The mouse bioassay requires the high consumption of living mice to test the suspected food sample for the BoNT presence ^[11]. The toxin presence is determined based on the effect on the mice, and the toxin serotyping requires additional testing with mice introduced to the different serotype antitoxins and followingly feeding with the intoxicated food sample.

Some newly accepted assays for the detection of BoNTs have been approved by the FDA ^[298]. However, they require the following validation with the mouse bioassay anyway. Our study contributes to the other studies trying to minimize animal casualties required by the traditional mouse assay.

The basic principle of the developed assay was the use of a peptide corresponding to a toxin-recognized sequence from one of its natural substrate SNARE proteins. Two types of chips were prepared and tested for the detection of the BoNT/A1 in this study (Fig. 35). The substrate peptide varied depending on the principle of the chip type. The first approach used NeutrAvidin chips combined with a substrate peptide containing biotinylation on both the

N- and the C- ends. NeutrAvidin was selected as the best candidate, providing consistently best results from the tested group of three biotin-binding proteins (Avidin, Streptavidin, and NeutrAvidin). In the second approach, anti-BoNT/A1 antibodies were immobilized on the chip, and the toxin was pulled from the sample solution prior to the reaction with the specially designed substrate peptide.

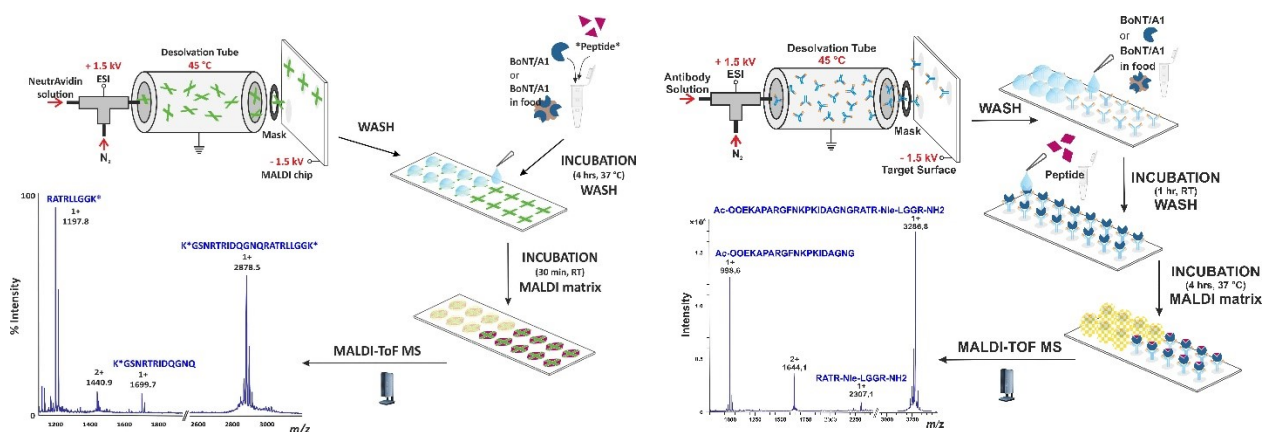


Figure 35 – workflow schemes for MALDI chips with immobilized NeutrAvidin (left) and immobilized anti-BoNT/A1 antibody (right). In the case of the NeutrAvidin (left) chip, the reaction with the toxin occurs in a separate tube. The biotinylated products are captured and enriched on the chip. This is in contrast to the antibody chip approach (right), where the toxin is enriched in situ before the reaction on the chip with the stock solution of the substrate peptide in the reaction buffer. In both cases, the final step of the workflow is the MALDI-TOF MS analysis.

In both cases, MALDI-TOF MS measurement was performed after the matrix application. The resulting spectra were compared with the positive and negative control. The negative control showed the mass of the intact substrate peptide, and the positive control showed the masses of two cleavage product peptides. Typical mass spectra contained both the cleaved products and the intact substrate based on the toxin quantity in the sample. Figure 36 (page 75, left) shows spectra obtained using the NeutrAvidin chips assay. The doubly charged substrate protein provides the signal at m/z 1440.9, and the singly charged CT-product at m/z 1197.9, and the NT-fragment at m/z 1699.7. The same BoNT/A1 enzymatic activity NT- and CT-products can be identified in the MALDI from food matrix are shown in Figure 36 (right).

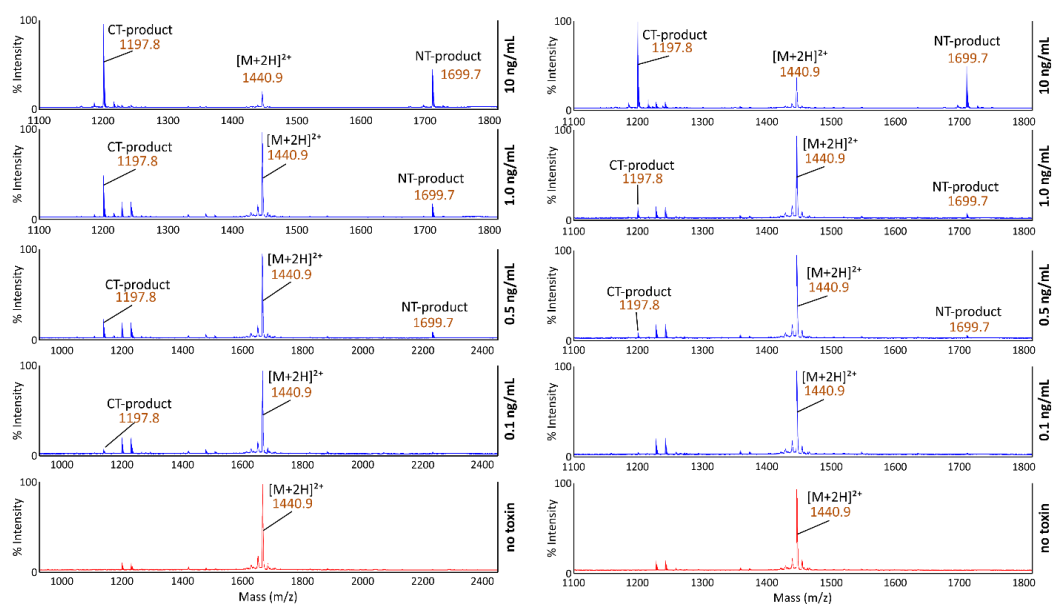


Figure 36 – MALDI-TOF MS spectra of C- and N- terminal peptide as a product of the BoNT/A1 activity in the reaction buffer (left) and food matrix (right) enriched in situ using NeutrAvidin chips. BoNT/A1 concentration is decreasing due to the dilution series. Compared to the negative control (red).

Figure 37 represents the same comparison as Figure 36, but it is for the anti-BoNT/A1 antibody-modified chips. The main difference comparing antibody to the NeutrAvidin modified-chips is the substrate peptide with a different amino-acid sequence. Therefore, with the different m/z 1 643.8 for the doubly charged ion. The singly charged NT fragment was observed at m/z 2 307.2, and the CT fragment was observed at m/z 998.7.

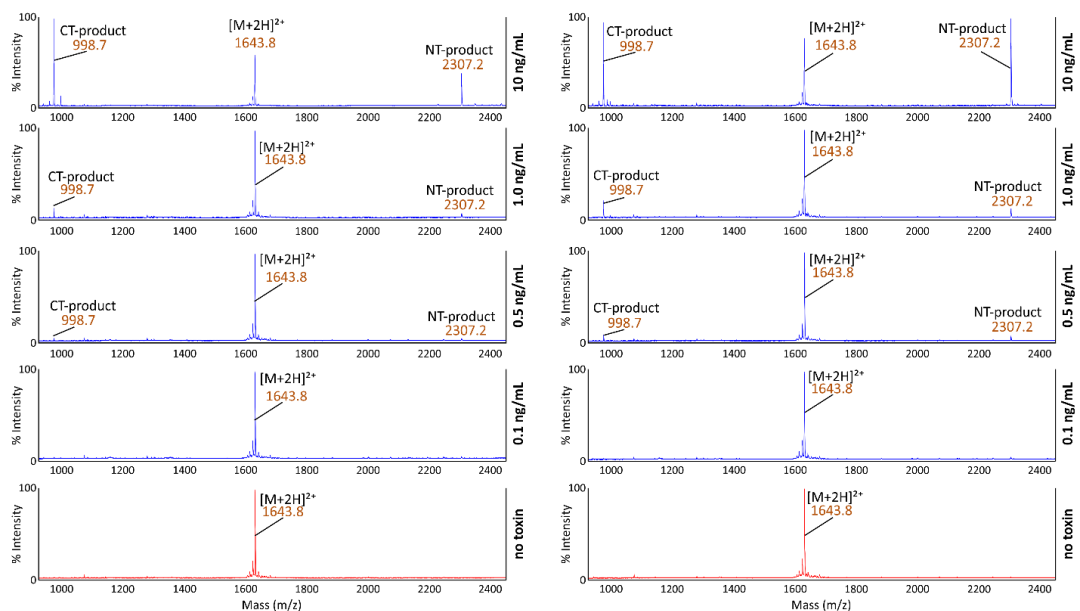


Figure 37 – MALDI-TOF MS NT- and CT- products resulting from the toxin activity in the reaction buffer (left) and food matrix (right) modified by the toxin in situ enriched on the HcA78 antibody chip. BoNT/A1 concentration is decreasing due to the dilution series. Compared to the negative control (red).

The assay successfully detected BoNT/A1 activity at a concentration of 0.1 ng/mL in both the reaction buffer and food matrix samples. The NeutrAvidin chips performed better in the reaction buffer, providing higher intensities for both fragment peptides. However, the antibody chips offered better results in the food matrix. Suggesting that the antibody chips are better suited to testing complex matrices.

The LOD was established to be 0.5 ng/mL for both types of chips and reaction conditions. The signal-to-noise ratio for fragment peptides was consistently above 8. At a toxin's concentration of 0.1 ng/mL the S/N dropped below 8, indicating reduced detection reliability.

The study introduces two MALDI-TOF-MS-based assays for toxin detection based on its activity, offering a rapid, cost-effective, and ethical alternative to the traditional mouse bioassay. Unlike PCR, ELISA, or lateral flow assays, which detect toxin present or genetic material without assessing functional activity, introduced MALDI assays provide the potential to evaluate toxicity by estimating the toxin activity ^[273].

The transfer of the assay from the magnetic beads to the microliter volume on the MALDI chip can provide a significant simplification while supporting multiplexing and high-throughput screening ^[86].

Both assays (NeutrAvidin, antibody) comply with international standards ^[299] with their 0.5 ng/mL LOD, while the antibody assay provides superior performance in complex matrices. However, limitations include potential matrix effects and non-specific substrate degradation, which may require additional substrate stability in complex sample optimization. Further validation in clinical and field applications is necessary.

However, as described in this publication, a promising alternative to the mouse bioassay combines speed, robustness, and ethical advantages with potential applications in clinical diagnostics, food safety, and biodefense.

5. Summary

This thesis was focused on developing and applying MALDI-compatible functionalized chips for various clinical microbiology applications. The research aimed to expand the utility capabilities of the matrix-assisted laser desorption/ionization mass spectrometry through the application of ambient ion soft landing (AISL) for chip fabrication. The results were summarized in three stand-alone scientific publications, each demonstrating the technology's versatility for different diagnostic and analytical targets. The specific outcomes are summarized in the following points:

- MALDI-compatible chips functionalized via ambient ion soft landing for various applications were developed, enabling immunoaffinity enrichment and *in situ* biochemical reactions followed by direct MALDI-TOF MS detection of target analytes.
- The first study demonstrated the detection of procalcitonin (PCT) in septic serum, utilizing immunoaffinity MALDI chips functionalized with anti-PCT antibodies. The assay enabled the detection of both intact and truncated PCT forms, providing clinically relevant information regarding sepsis severity.
- In the second publication, a functional assay for detecting the product of the enzymatic activity of Toxin B in *Clostridioides difficile* infection patient samples was established. Neutravidin-modified MALDI chips were used to immobilize biotinylated RhoA as a substrate for glycosylation by the *C. difficile* toxins in the sample. Therefore, offering an activity-based diagnostic tool that provides valuable diagnostic insights complementary to the routinely used techniques.
- The third study focused on the BoNT/A1 detection, utilizing functionalized NeutrAvidin and antibody MALDI chips. The assay allowed the detection of specific cleavage products from SNAP-25-derived substrate peptides up to the detection limit of 0.5 ng/mL in both buffer and complex matrices.
- All three approaches demonstrated the advantages of MALDI chip-based assays over traditional techniques, including faster analysis times, avoiding pathogen cultivation, and providing potential for high-throughput automation while reducing the non-specific interactions or the need for complex sample preparation.

- The developed assays provide functional and activity-based detection, distinguishing between active and inactive toxins, critical for clinical decision-making, infection severity assessment, and food safety applications.
- The combined work highlights MALDI-compatible chip technologies' versatility and clinical potential as robust, cost-effective, and in some cases ethically preferable alternatives to conventional diagnostic assays.

LIST OF PUBLICATIONS

Publications included in this thesis:

1. Dvorak J., Novakova J., Kraftova L., Studentova V., Matejovic M., Radej J., Karvunidis T., Horak J., Kralovcova M., Hrabak J., Kalaninova Z., Volny M., Novak P. & Pompach P. (2023)
The rapid detection of procalcitonin in septic serum using immunoaffinity MALDI chips. Clin Proteom 20, 20 (2023). <https://doi.org/10.1186/s12014-023-09410-3>
2. Dvorak J., Fojtík L., Adámková L., Vlkova K., Studentova V., Chudejova K., Geigerová L., Volny M., Novak P., Hrabak J. & Pompach P. (2025)
Proof-of-concept MALDI-TOF-MS assay for the detection of Toxin B enzymatic activity in *Clostridioides difficile* infection. Microbiol Spectr 0:e02453-24. <https://doi.org/10.1128/spectrum.02453-24>
3. Kalaninova Z., Dvorak J., Dresler J., Volny M., Novak P. & Pompach, P. (2025).
Novel activity assay for botulotoxin A1 detection using functionalized chips and matrix-assisted laser desorption/ionization mass spectrometry. Expert Review of Proteomics, 1–8. <https://doi.org/10.1080/14789450.2025.2482933>

Other publication as co-author:

1. Indra R., Jelínková S., Kollárová K., Zahumenská P., Dvořák J., Dušková Š. & Dračinská H. The tyrosine kinase inhibitor lenvatinib is oxidized by rat cytochromes P450 and affects their expression in rat liver. Acta Pharmaceutica, 2024, Sciendo, vol. 74 no. 3, pp. 441-459. <https://doi.org/10.2478/acph-2024-0027>

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ATTACHED PUBLICATIONS

ARTICLE I

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RESEARCH

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The rapid detection of procalcitonin in septic serum using immunoaffinity MALDI chips

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Abstract

Background Sepsis is a common worldwide health condition with high mortality. It is caused by a dysregulated immune response to the pathogen. Severe infections resulting in sepsis can be also determined by monitoring several bloodstream biomarkers, one of them being pro-hormone procalcitonin (PCT). PCT concentration in the bloodstream correlates well with sepsis and in severe cases increases up to a thousand times from the healthy physiological values in a short time. In this study, we developed a rapid technique for PCT detection by MALDI-TOF mass spectrometry, that uses in-situ enrichment directly on the specialized immuno MALDI chips that are utilized as MALDI plates. The method's ability to detect PCT was confirmed by comparing the results with LC-MS bottom-up workflow. The new method detects intact PCT by its m/z and uncovers its alternations in septic serum.

Methods The MALDI chips used for the detection of PCT were prepared by ambient ion soft landing of anti-PCT antibody on an ITO glass slide. The chips were used for the development of the rapid MALDI-TOF MS method. A parallel method based on affinity enrichment on magnetic beads followed by LC-MS/MS data-dependent peptide microsequencing was used to prove PCT presence in the sample. All samples were also tested by ELISA to determine PCT concentration prior to analyzing them by mass spectrometry methods.

Results The MALDI chip method was optimized using recombinant PCT spiked into the human serum. The PCT detection limit was 10 ng/mL. The optimized method was used to analyze 13 sera from patients suffering sepsis. The PCT results were confirmed by LC-MS/MS. The measurement of the intact PCT by the MALDI chip method revealed that sera of patients with severe sepsis have other forms of PCT present, which show post-processing of the primary sequence by cleavage of PCT, resulting in the formation of N and C termini fragments.

Conclusions Procalcitonin from human serum was successfully enriched and detected using immunoaffinity MALDI chips. The intact PCT was characterized in 13 septic patients. The method is more specific compared to non-MS-based immunoaffinity techniques and allows observation of different variants of PCT in septic patients.

Keywords Procalcitonin, MALDI-TOF, Sepsis, Immunoaffinity, Ion soft landing

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Background

Sepsis represents a severe clinical syndrome with very high mortality and long-term morbidity of its survivors [1, 2]. It is noted that there are over 31 million cases of sepsis annually worldwide, resulting in more than 5 million deaths per year [2]. In 2016 an updated definition of sepsis was introduced to address advances made in the pathobiology, management, and epidemiology of sepsis, suggesting the need for reexamination [3]. According to the document, known as Sepsis-3, sepsis should be defined as life-threatening organ dysfunction caused by a dysregulated host response to an infection. To prevent the fatal consequences of sepsis and to properly manage the therapy, fast and accurate diagnosis is crucial [4]. There are multiple clinical diagnostic criteria that include body temperature, heart rate, systolic blood pressure, respiratory rate or altered mentation as reviewed by Singer [3]. Besides these well-established diagnostic options, another avenue to diagnose sepsis if tools of laboratory medicine are available, is offered by analytical determination of various biomarkers, one of them being PCT [5].

Procalcitonin is 116 amino acids (AA) long precursor protein of the hormone calcitonin truncated at the N-end to form 114 AA bloodstream-present form at molecular mass around 12.6 kDa [6–8]. In healthy individuals, PCT is produced by thyroid C-cells. Its normal concentration in healthy individuals is less than 0.1 ng/mL. However, the concentration might increase up to a thousand times during sepsis (hundreds of ng/mL). PCT is sensitive and highly specific for bacterial infections, possessing rapid kinetics usually reflecting response to antibiotic therapy. These facts make PCT a useful biomarker for clinical diagnostics of sepsis [9, 10] with known advantages over other blood-based markers (e.g. C-reactive protein, or white blood cells count). Once the PCT level is at a maximum value, it doesn't change unless the patient's condition improves after successful treatment. Therefore PCT isn't only a valuable biomarker of sepsis and septic shock, but it can also be used as an indicator of treatment effect [11].

All currently FDA-approved detection methods are immunoassays that use anti-PCT antibodies to determine PCT concentration, e.g. ELISA [12]. Although these methods are sensitive, they may suffer from false positivity caused by antibody cross-reactivity. Immunoaffinity approaches coupled with mass spectrometry overcome the problem with false positivity by detecting precise molecular weight and identifying the exact forms of the molecular target, such as PCT. On the other hand, MS-based techniques are usually time-consuming, less robust, and can have problems to achieve ELISA limits of detection.

Here, we present a new method that combines in-situ enrichment of PCT on antibody-functionalized MALDI chips with MALDI-TOF MS detection.

The affinity surfaces used as MALDI chips were prepared by ambient ion soft landing of PCT antibody. This technology, previously well-described [13–15], allows modification of surfaces with a wide variety of ions. In ambient ion soft-landing ions are generated by soft electrospray ionization at atmospheric pressure, desolvated in a heated tube, and landed on a conductive surface such as MALDI plates. This approach allows effective and unique modification of surfaces without dramatical loss of ions while keeping their biological activity [16]. Several applications of MALDI surfaces prepared by ambient ion soft-landing for life science and clinical diagnostics have been recently presented [15, 17–21]. Detection of PCT represents a challenging task because of its low abundance in human serum and throughput requirements of clinical assays. The workflow described in the presented work uses detection from serum by in-situ enrichment on MALDI chips modified with commercially available anti-PCT antibody. The lab-on-plate sample preparation workflow is followed by standard MALDI-TOF MS detection. The assay was optimized by using recombinant PCT and successfully used to analyze samples of 13 septic patients with PCT

Methods

Chemicals and materials

Glass slides coated with a thin layer of indium-tin-oxide (ITO) were purchased from Bruker Daltonics (Massachusetts, USA). Recombinant PCT was purchased from BioVendor (Brno, Czech Republic) and an anti-PCT antibody from Fitzgerald (Massachusetts, USA). Magnetic beads Dynabeads™ Co-Immunoprecipitation Kit was purchased from Thermo Fisher Scientific (Massachusetts, USA). All other chemicals were obtained from Merck (Darmstadt, Germany).

Manufacturing of immunoaffinity MALDI ITO chips

Immunoaffinity chips were prepared using a previously described lab-built apparatus for an ambient ion-soft landing [22]. The ambient ion-soft landing technique was used to immobilize the anti-PCT antibody on standard ITO-coated glass slides (Bruker Daltonics, USA). The geometry of 16 spots array per chip was defined by an adhesive foil mask with a 2 mm spot diameter and 9.0 mm center-to-center distance. Anti-PCT antibody was sprayed from 0.6 mg/mL solution of 50 mM ammonium bicarbonate buffer (AMBIC), pH 7.8. The solution was delivered by the syringe pump at a flow rate of 60 μ L/h through the 20- μ m capillary connected to the microspray emitter. 1.5 kV of positive voltage was applied

to the syringe needle to create an electrospray. Nebulization was further supported by the stream of nitrogen gas preheated to 40–50 °C. The newly prepared chip was washed in water after soft landing to remove any excess material to only allow antibody bound to the surface to stay on the chip.

Optimizing the PCT detection method

In-situ enrichment and detection of recombinant PCT

Recombinant human PCT (BioVendor, Czech Republic) was used as a standard for optimization of in-situ enrichment and MS detection. Procalcitonin was diluted in human serum at concentrations of 42 µg/mL, 14 µg/mL, 4.7 µg/mL, 1.6 µg/mL and 0.5 µg/mL. One microliter of the sample was loaded onto the spot of landed antibody on the ITO chip and incubated for 1 h at room temperature inside the Petri dish to minimize evaporation of the sample. After incubation, the chip was washed three times for 5 min in PBS buffer and 1 min in deionized water and allowed to dry at room temperature. MALDI matrix (2,5-dihydroxyacetophenone, DHAP) was prepared according to a previously published protocol [21]. 0.6 µL of DHAP solution was pipetted on the chip and gently mixed several times by the pipette tip to obtain homogenous crystals.

Chips with enriched analyte and deposited MALDI matrix were placed into the MALDI slide adapter II holder (Bruker Daltonics) and aspirated into the MALDI-TOF mass spectrometer (Autoflex Speed, Bruker Daltonics). Procalcitonin was analyzed in a positive linear mode in the mass range 4 400–16 800 m/z. The laser frequency was set to 1 kHz, and 20 000 shots were accumulated for each sample using the partial random walk function. Each sample was in-situ enriched and measured at three different positions. In a different set of experiments, PCT was also analyzed by MALDI FT-ICR mass spectrometer (15 T solariX XR, Bruker Daltonics) in the mass range 800–16 000 m/z. Twenty-four spectra were accumulated with data acquisition of 2 M. MALDI-TOF MS data were processed by FlexAnalysis 3.0 and MALDI-FT-ICR data were processed by DataAnalysis 5.0 software (Bruker Daltonics).

Procalcitonin sample treatment

To improve the detection limit of the method, recombinant procalcitonin spiked in human serum (100 µL) was diluted with LC–MS water in the 1:2 ratio and vortexed for 10 s. The sample was further diluted by acetonitrile in the 1:4.5 ratio, vortexed, and sonicated for 10 min in an ultrasonic bath with ice. After centrifugation at 8000×g for 15 min at 4 °C, the supernatant containing PCT was lyophilized using a SpeedVac concentrator.

Procalcitonin detection in patient's serum

Patient samples

Samples of human venous blood (5 mL) were collected using the VACUETTE blood-collection system (Greiner Bio-One) containing 5 mM EDTA. Serum was separated by 10 min centrifugation at 1 700×g and immediately frozen to – 80 °C. The concentration of PCT in each sample was determined by ELISA. Each serum sample was then split into two aliquots and further analyzed either by magnetic beads or by functionalized MALDI chips.

In-situ enrichment of PCT using functionalized MALDI chips

The patient's sera were analyzed similarly to the recombinant PCT: 100 µL of serum was mixed with 200 µL of water and 450 µL of acetonitrile. Further processing was identical as described before for recombinant PCT samples. Samples were deposited in triplicates on the functionalized spots on the MALDI chip and analyzed by MALDI-TOF and MALDI FT-ICR mass spectrometers. Both mass spectrometers were externally calibrated using myoglobin and cytochrome C.

The limit of detection and the dynamic range were determined by measuring recombinant PCT spiked into a human serum at concentrations 1000 ng/mL, 500 ng/mL, 100 ng/mL, 50 ng/mL, 10 ng/mL and 5 ng/mL. A 6×100 µL of each sample was concentrated by acetonitrile enriched on the MALDI plate and analyzed using MALDI-TOF and MALDI FT-ICR. For determination of the coefficient of variation, the recombinant PCT was spiked into a human serum pool at a concentration of 140 ng/mL and split into 16×100 µL fractions, and treated and analyzed as described above.

Enrichment of PCT using magnetic beads

Magnetic beads (Dynabeads™ Co-Immunoprecipitation Kit, Thermo Fisher Scientific) were prepared according to the manufacturer's instructions, the brief summary of the protocol follows. The PCT antibody used for modification of magnetic beads was the same as the PCT antibody used for the functionalization of the MALDI chips. One milliliter of C1 solution (included in the Dynabeads kit) was used to rinse 7.5 mg of beads and the rinsing solution was separated in the magnetic rack. In the next step, 375 µL of 0.15 mg/mL anti-PCT antibody in C1 solution was added and mixed with the beads, followed by the addition of 375 µL of C2 solution. The beads were incubated overnight on a rotator at 4 °C and then rinsed as follows: 1×800 µL HB solution, 1×800 µL LB solution, 2×800 µL SB solution and again with 1×800 µL of SB solution for 15 min on rotator at laboratory condition with suspension transfer to a new microtube, 4×800 µL 1×IP solution containing 150 mM NaCl and Halt™ Protease and

Phosphatase inhibitor cocktail (Thermo Scientific). The magnetic beads with coupled antibody were stored in 700 μ L SB solution with 0.02% NaN_3 .

Before the use, magnetic beads were rinsed 3 $\times$ in 800 μ L of PBS with 0.1% BSA. The patient serum was incubated with the beads for 45–60 min on a rotator at 4 $^{\circ}\text{C}$. After immunoprecipitation, the liquid phase was removed using a magnetic rack and a pipette. The beads were rinsed 3 $\times$ in 800 μ L of PBS with 0.1% BSA and then washed 1 $\times$ in 800 μ L of LWB solution for 5 min using a rotator at laboratory conditions. Beads transferred to a new microtube were washed 3 $\times$ with 800 μ L of LWB solution.

Proteins were eluted from the beads by the addition of 2 $\times$ 300 μ L of elution buffer (0.5 mM EDTA, 0.3 mM NH_4OH in LC–MS water). Samples were dried by Speed-Vac concentrator and resuspended in 20 μ L of 5% acetic acid in LC–MS water with 10 mM dithiothreitol (DTT). For intact mass spectrometry, two microliters of sample were spotted onto the MALDI steel plate and covered with 0.6 μ L of DHAP matrix.

Bottom-up detection of PCT enriched by magnetic beads

For LC–MS/MS analysis, five microliters of sample were mixed with 20 μ L of 50 mM ammonium bicarbonate. The sample pH was adjusted with NH_4OH solution to pH 7–8. The sample was incubated at 60 $^{\circ}\text{C}$ for 40 min. After cooling down to room temperature, iodoacetamide was added to the final concentration of 30 mM, and the sample was incubated for 30 min in the dark. Additional DTT was added to the final concentration of 50 mM to stop the alkylation reaction. Trypsin (0.1 $\mu\text{g}/\text{mL}$) was added, and the reaction mixture was incubated overnight at 37 $^{\circ}\text{C}$.

Samples were analyzed using a liquid chromatography system Agilent 1200 (Agilent Technologies) connected to the timsTOF Pro PASEF mass spectrometer equipped with Captive spray (Bruker Daltonics). The mass spectrometer was operated in a positive data-dependent mode. Five microliters of peptide mixture were injected by autosampler on the C18 trap column (UHPLC Fully Porous Polar C18 2.1 mm ID, Phenomenex). After 5 min of trapping at a flow rate of 20 $\mu\text{L}/\text{min}$, peptides were eluted from the trap column and separated on a C18 column (Luna Omega 3 μm Polar C18 100 $\text{\AA}$, 150 $\times$ 0.3 mm, Phenomenex) by a linear 35 min water–acetonitrile gradient from 5% (v/v) to 35% (v/v) acetonitrile at a flow rate of 4 $\mu\text{L}/\text{min}$. The trap and analytical columns were both heated to 50 $^{\circ}\text{C}$. Parameters from the standard proteomics PASEF method were used to set timsTOF Pro. The target intensity per individual PASEF precursor was set to 6000, and the intensity threshold was set to 1500. The scan range was set between 0.6 and 1.6 V s/cm² with

a ramp time of 100 ms. The number of PASEF MS/MS scans was 10. Precursor ions in the m/z range between 100 and 1700 with charge states $\geq 2+$ and $\leq 6+$ were selected for fragmentation. The active exclusion was enabled for 0.4 min.

The raw data were processed by PeaksStudio 10.0 software (Bioinformatics Solutions, Canada). The search parameters were set as follows: enzyme–trypsin (specific), carbamidomethylation as a fixed modification, oxidation of methionine, and acetylation of protein N-terminus as variable modifications. Database: UniProt (human, virus and bacteria, 01/2021). The mass spectrometry data have been deposited to the ProteomeX-change Consortium via the PRIDE partner repository with the dataset identifier PXD035456 [23].

Results

The MALDI chips modified by the ambient landing of the human PCT antibody were first evaluated by analyzing samples of human recombinant PCT spiked into the human pooled serum of healthy individuals at different concentrations. The ions corresponding to singly and doubly charged recombinant PCT were observed at m/z 14 029 and 7 014 (Fig. 1). The doubly charged ions show better peak resolution and signal intensity. That is why DHAP was a preferred MALDI matrix over sinapic acid, because it supported their formation [21]. Figure 1 shows analysis of serum samples with spiked PCT at different concentration using the MALDI chips prepared by soft landing of the anti-PCT antibody. The signal-to-noise ratio of doubly charged ion of PCT at the lowest detected concentration (0.5 $\mu\text{g}/\text{mL}$) was 12.

To improve the limit of detection, serum samples with recombinant PCT were concentrated by acetonitrile precipitation of a major amount of serum proteins to leave the PCT in a supernatant. Two different approaches for concentration using acetonitrile were tested. The efficiency of the concentration method was confirmed by immunostaining using a PCT antibody. The best results were achieved when acetonitrile was added to the diluted serum at a 1:4.5 (v/v) ratio without addition of acid (Additional file 1: Fig. S1).

The concentration procedure increased the sensitivity of the method by the factor of 15 calculated based on the comparison of ion signal intensities (Fig. 2A). The limit of detection was improved to 10 ng/mL (Additional file 2: Fig. S2). Highly concentrated samples (500 ng/mL and 1000 ng/mL) were out of the linear dynamic range of the method (Fig. 2B).

The coefficient of variation (CV) of the PCT in serum was determined using 16 samples with spiked recombinant PCT at a concentration of 140 ng/mL that were concentrated using the acetonitrile treatment. This

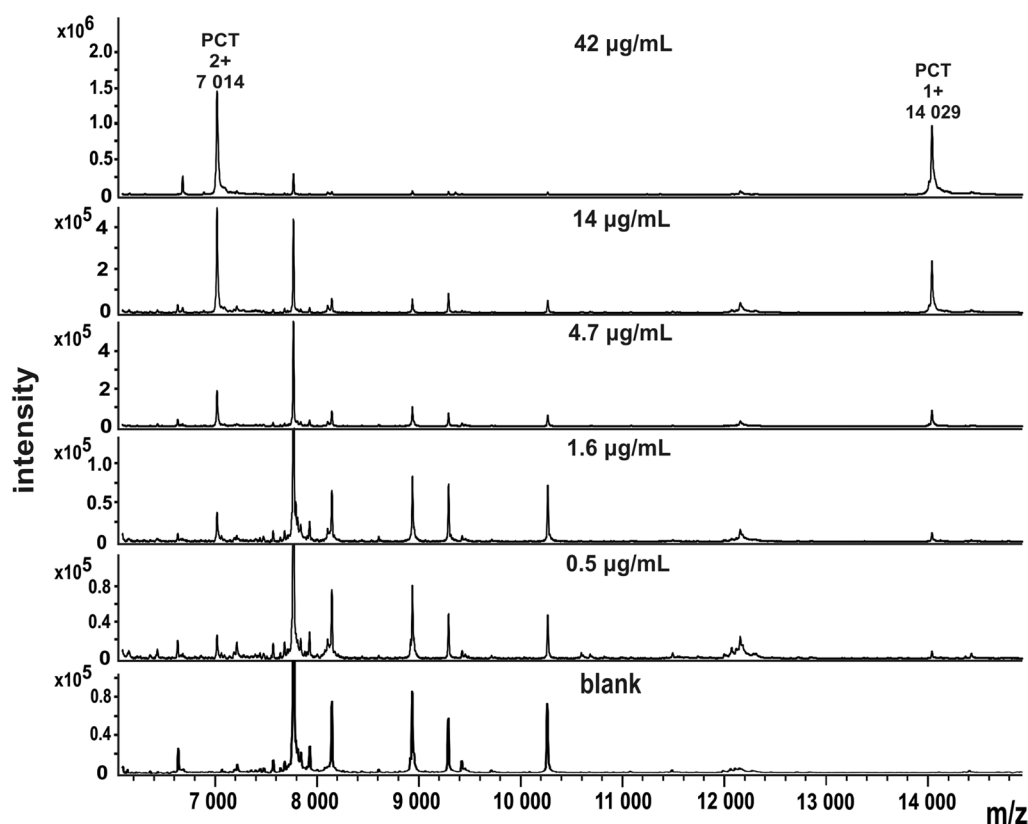


Fig. 1 MALDI-TOF MS spectra of recombinant PCT spiked in human serum at different concentrations enriched and analyzed by functionalized MALDI chips. The bottom panel shows spectrum of serum without spiked PCT (blank)

concentration level is within the linear detection range and simulates PCT blood concentration during severe sepsis. The CV value was 25% for singly and 22% for doubly charged PCT ions. The chip-to-chip CV was 14% for singly charge ion and 10% for doubly charge ions.

Detection of PCT in patient's samples

The developed technique was tested for the detection of PCT in patient samples. The presence of PCT was first determined by using the ELISA method. Thirteen samples of septic patients with PCT concentrations above 100 ng/mL were selected for evaluation of MALDI chips. Each patient's serum was divided into two aliquots. One was used for enrichment on magnetic beads with LC-MS detection and the other for enrichment on functionalized MALDI chips with MALDI-TOF MS detection. Samples for MALDI chip enrichment were processed by the acetonitrile concentration procedure. The physiological human PCT is different from the recombinant protein used for optimization: the primary sequence does not include the Histidine tag anchor and the 2 N-terminus amino acids are cleaved. That is why the physiologically occurring PCT has a

different molecular mass and it is detected by MALDI-MS at different m/z values (12626 for singly charged and 6308 for doubly charged ions) than the recombinant version. To confirm that the accurate mass of PCT corresponds to the expected sequence, each sample was also analyzed by a high-resolution accurate mass MALDI FT-ICR mass spectrometer (Additional file 3: Fig. S3). It confirmed the presence of PCT by detection of the doubly charged ion at m/z 6310.32, but a singly charged ion was not observed.

Interestingly, analysis of PCT in patient samples also revealed the presence of other forms of PCT as shown in Fig. 3. A dominant singly charged ion at m/z 6303.85 and its oxidized form at m/z 6319.83 were detected. This ion was interpreted as a PCT fragment $C^{85}GNLSTCM LGTYTQDFNKFHTFPQTAIGVGAPGKKRDMSSD LERDHRPHVSM PQNAN^{141}$ with cysteines forming a disulfide bond. A doubly charged ion at m/z 6310.32, which corresponds to noncleaved PCT, was still present in most samples, but the modified forms were dominant. Figure 3 shows an example of three selected patient samples. All PCT MALDI-TOF MS spectra from septic

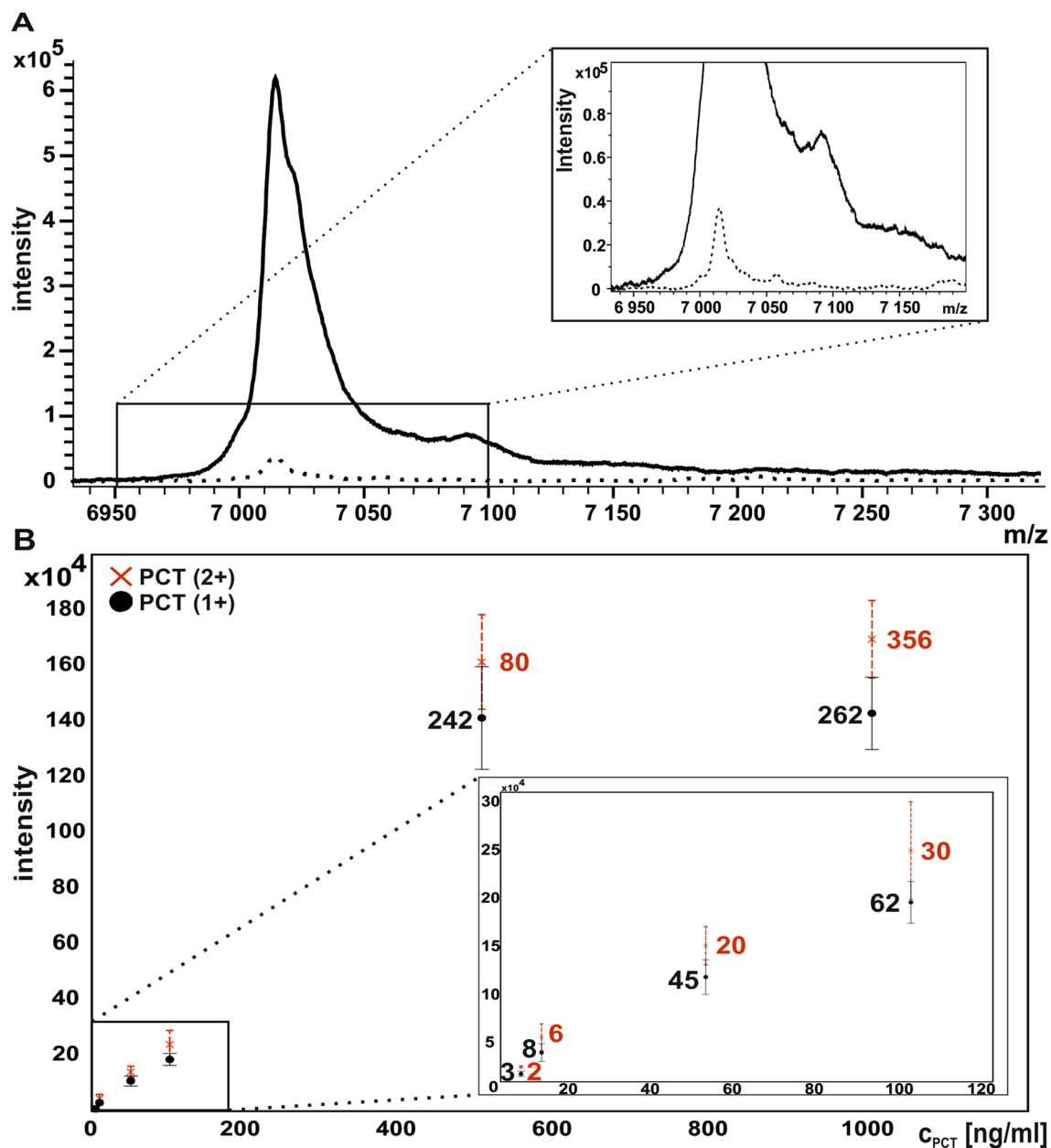


Fig. 2 **A** MALDI-TOF MS spectra of in-situ enriched recombinant PCT (140 ng/mL) concentrated by acetonitrile (solid line) and without concentration treatment (dashed line). **B** Relative intensities of singly and doubly charged ions at different concentrations of recombinant PCT. The PCT concentrations are 5; 10; 50; 100; 500; 1000 ng/mL. The numbers represent S/N ratios for singly (black) and doubly (red) charged ions

patients' sera samples are shown in the Additional file 3: Figure S3.

Another fragment ion at m/z 6349, (1+) that was observed in the patient's serum was assigned as a PCT fragment:

F²⁸RSALESSPADPATLSEDEARLLLAALVQDY-VQMKASELEQEQUEREGSSLDSPRSKR⁸⁴ (Fig. 4A).

To investigate if the fragments originate simply from sample degradation, human pooled serum spiked with

recombinant PCT was incubated for 3 h at 37 °C. The serum was processed by acetonitrile concentration procedure and PCT was detected using MALDI chips. The recombinant PCT was truncated at its N-terminus, but PCT cleavage fragments that are present in the patient samples (Fig. 4A) were not observed in the incubated sample (Fig. 4B).

The second aliquot was enriched on anti-PCT antibody-modified beads. Interestingly, samples that were

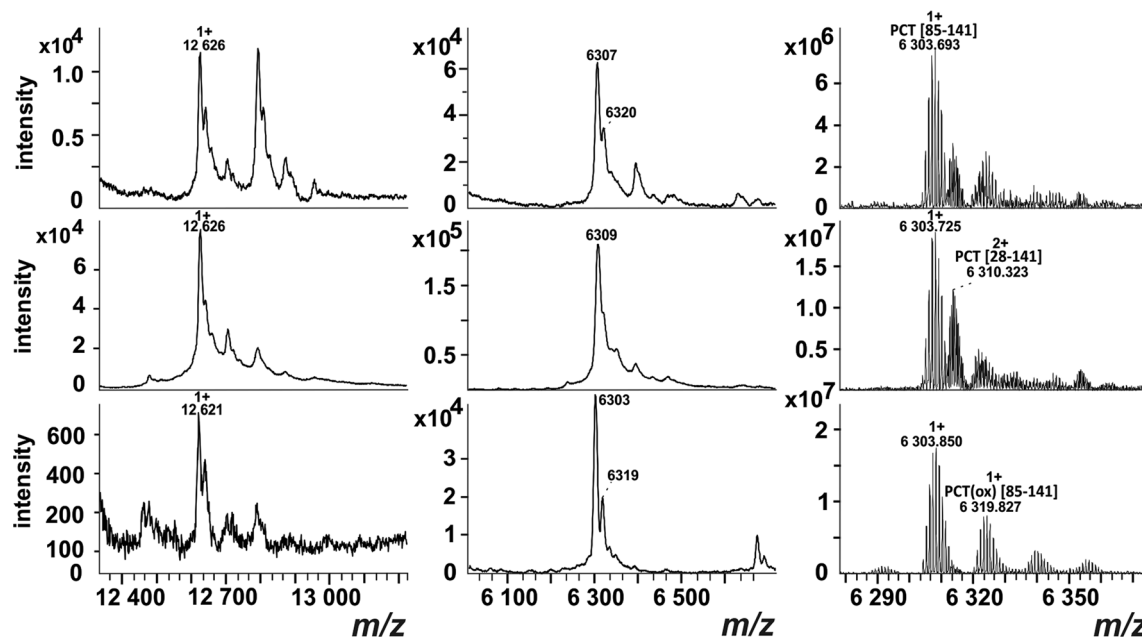


Fig. 3 Detection of PCT using the MALDI chips in three selected septic patients (from top to bottom). The figure shows detailed spectra of singly and doubly charged ions of PCT and its cleaved forms measured by MALDI-TOF MS (left and center) and by MALDI FT-ICR (right). In the third patient's sample, the intensity of the doubly and singly charged ions of PCT is very low compared to the intensity of singly charged fragment ion at m/z 6 303.850, which indicates that most of the PCT was converted into the fragment form

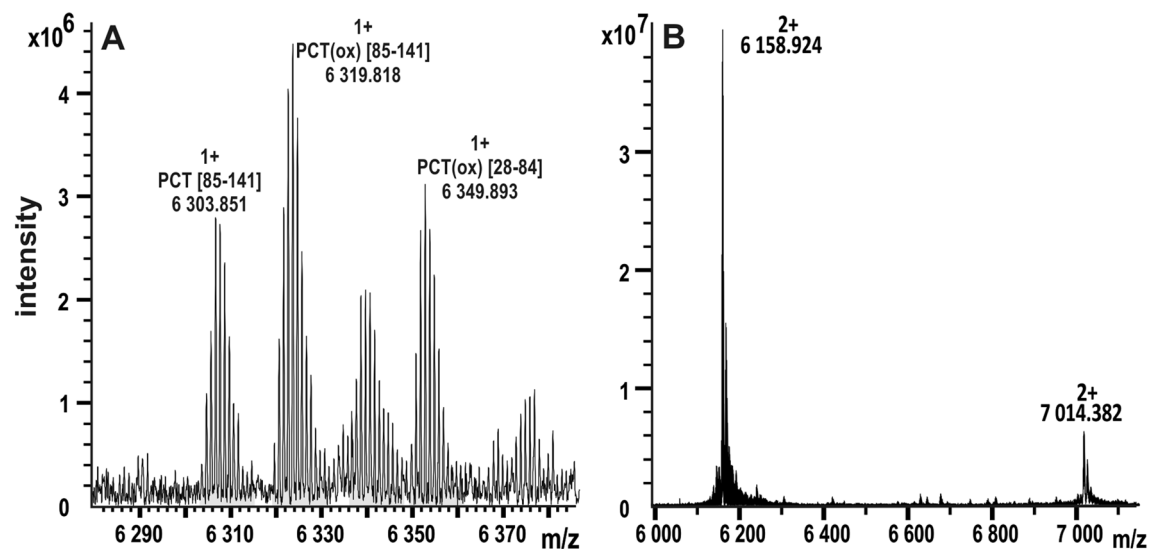


Fig. 4 **A** Detailed MALDI FT-ICR spectrum of PCT from septic patient enriched by MALDI chip. Fragments of PCT and their oxidized forms corresponding to cleavage at the R84-C85 site were detected. **B** MALDI FT-ICR spectrum of human recombinant PCT spiked in serum and incubated at 37 °C shows no equivalent fragments. This indicates that the origin of the fragments is not related to sample degradation

enriched using magnetic beads, eluted, and analyzed by MALDI-TOF MS showed more complex spectra in comparison with samples enriched and analyzed directly on MALDI chips (Additional file 4: Fig. S4). The aliquot eluted from the beads was also analyzed by a bottom-up

LC-MS/MS workflow. The qualitative results confirmed PCT in all 13 samples in accordance with the analysis on MALDI chips. (Because the bottom-up results were searched against a microbiology proteome database as well, it was identified that one of the patient samples

contained the RTX-I toxin from *Actinobacillus pleuropneumoniae*. The RTX-I toxin was matched by 41 peptides with an FDR value <1% covering 38% of the protein sequence (Additional file 5: Fig. S5). The PCT enriched from the serum with RTX-I toxin was fully converted into its cleaved forms. The full list of proteins identified in the patient's samples analyzed by LC-MS/MS is available in (Additional file 6: Table S1).

Discussion

The purpose of this work was to develop a mass spectrometry technique for the detection and characterization of human PCT that would be compatible with the requirement for clinical management of the sepsis. MALDI-TOF MS was chosen as a technique that is relatively simple, provides rapid results, and is common in clinical microbiology laboratories and has been used in clinical chemistry as well [24]. The specific advantage of our approach is that the entire workflow can be performed in-situ on the MALDI chip and detected by any standard MALDI-TOF mass spectrometer, including standard microbiology biotypers.

MALDI chips used in the current work were prepared by ambient ion soft-landing of antibodies. This technique was previously reported as a platform for detecting proteins in complex matrices. Several applications of MALDI chips and lab on plate concept have already been presented, including protein digestion by different proteases, glycopeptide enrichment by immobilized lectins, haptoglobin phenotyping, or quantification of human carbohydrate-deficient transferrin using immobilized antibodies [15, 19, 21, 25, 26]. The current study demonstrates a possibility to immobilize antibodies by ambient ion soft landing with retention of their immunoaffinity and use them effectively for the immunoMALDI workflow. This workflow allows effective detection of analytes in relatively complex clinical assays, as described before [27].

We utilized the acetonitrile concentration procedure, which precipitated major amount of serum proteins, while leaving PCT in the acetonitrile phase, to reduce the complexity of the sample matrix and to improve the limit of detection. The acetonitrile procedure in combination with MALDI chips allowed the detection the recombinant PCT by MALDI-TOF MS at a concentration level of 10 ng/mL. This is not a sufficient limit of detection for monitoring PCT in healthy individuals (0.1 ng/mL or less), nor in patients with mild infections (2 ng/mL) [28], but can be used for the detection of high levels of PCT that are present in the serum during severe sepsis.

Procalcitonin in a similarly small cohort of septic patients (PCT concentration >100 ng/mL) was analyzed in a 2001 study by Weglohner et al. [8]. The results

showed that two amino acids from the N-terminus are missing. This is in agreement with our results that also show the N-terminal truncated form of PCT. No N-glycosylation of PCT was found in our results, although the primary amino acid sequence of PCT contains N-glycosylation motive NLS. This is again consistent with results obtained by Weglohner [8].

More recently, a complex bottom-up LC-MS/MS workflow that includes two solid phase extraction steps was published [29]. The bottom-up LC-MS/MS approach allowed to achieve sub ng/mL limits of detection, but it is elaborate and provides quantification on the peptide level, without information about different PCT variants in the sample. The presented method based on MALDI chips and intact PCT detection is less sensitive, but the measurement of intact protein provides information about the different forms of primary structure in septic patients. The mass spectrum of human PCT analyzed by MALDI chips with MALDI-TOF MS detection contained singly and doubly charged ions of PCT, corresponding to its primary amino acid sequence. Oxidation of PCT, in the form of the addition of 16 Da was also present. The PCT fragments were identified by high-resolution accurate mass MALDI FT-ICR and appeared in the spectra of all septic patients. It seems that for severe septic states, the PCT exists as a mixture of intact and cleaved forms. The cleavage of PCT in sera from septic patients might be caused by the increased presence of proteases during sepsis. To exclude possible PCT degradation during sample processing, recombinant PCT was incubated in healthy individual serum at 37 °C for 3 h, processed as described before, and detected by MALDI-TOF MS and MALDI FT-ICR MS. It was shown that incubation under physiological conditions and follow-up sample processing, including acetonitrile concentration, did not cause cleavage of PCT into two observed forms. Only a partial truncation of the PCT N-terminus was observed. As mentioned above, detailed information about PCT fragments can not be obtained by ELISA methods that are not specific enough or by LC-MS/MS bottom-up approaches that only detect PCT on the level of tryptic peptides. The MALDI chip technique that detects all intact forms of PCT represents not only specific and rapid approach, but also offers rich information about the biochemical transformation of PCT in severe sepsis. Interestingly, it was identified by LC-MS/MS that one patient sample contained RTX-I, a toxin from *Actinobacillus pleuropneumoniae*. Using classical bacteriological examination (i.e., cultivation of blood and bronchoalveolar lavage), however, we detected no significant pathogen including *A. pleuropneumoniae* (data not shown). The identified RTX-I toxin may indicate the presence of a bacterium in the bloodstream. Furthermore, the serum of

this patient contained no intact PCT, but only its cleaved forms. This could indicate that sepsis severity is reflected in the ratio of intact and cleaved PCT forms. To monitor this parameter, only intact mass spectrometry methods can be utilized, because ELISA is not specific enough and bottom-up approaches detect the PCT on the level of digested peptides.

Conclusions

The in-situ enrichment and detection of PCT from human serum using functionalized immunoMALDI chips have been reported in this study. The MALDI-compatible chips were prepared by ambient ion soft landing of PCT antibody on ITO glass slides. The method was optimized using human serum spiked with recombinant PCT protein and then used on patient serum samples. The limit of detection was estimated to be 10 ng/mL. The MALDI chip technique is capable of detecting and distinguishing different forms of PCT. It was observed that PCT of patients suffering severe sepsis undergoes cleavage and exists in several forms. The advantage of MALDI chip technology is in rapid detection and the ability to describe PCT variants, including truncated and modified forms.

Abbreviations

AA	Amino acids
AMBIC	Ammonium bicarbonate
DHAP	2,5-Dihydroxyacetophenone
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FDR	False discovery rate
FT-ICR	Fourier-transform ion cyclotron resonance
ITO	Indium-tin-oxide
LC-MS	Liquid chromatography mass spectrometry
MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
PASEF	Parallel accumulation and serial fragmentation
PBS	Phosphate-buffered saline
PCT	Procalcitonin
PRIDE	Proteomics identifications database
TOF	Time of flight

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12014-023-09410-3>.

Additional file 1: Figure S1. Monitoring of concentration efficiency by immunostaining. **M**—protein standard; **1**—supernatant after acidic concentration; **2**—pellet after acidic concentration; **3**—serum with recombinant PCT; **4**—supernatant after acetonitrile concentration; **5**—pellet obtained after acetonitrile concentration.

Additional file 2: Figure S2. MALDI-TOF spectrum of in-situ enriched intact recombinant PCT spiked in human serum at LOD concentration 10 ng/mL.

Additional file 3: Figure S3. In-situ detection of PCT in 13 septic patients. Detailed spectra of singly and doubly charged ions of PCT measured by MALDI-TOF (left and center) and detailed spectra of PCT measured by MALDI FT-ICR (right).

Additional file 4: Figure S4. MALDI-TOF of intact PCT from septic patient's serum using MALDI chips (**A**) and using immunoaffinity magnetic beads (**B**).

Additional file 5: Figure S5. Sequence coverage of Calcitonin and RTX-I toxin. The blue lines represent identified peptides by LC-MS/MS

Additional file 6: Table S1. The list of proteins identified in the patient's samples analyzed by LC-MS/MS.

Author contributions

JD performed the experiments, wrote the main manuscript. JN, LK, VS, MM, JR, TK, JH, MK, JH collected the samples and reviewed the manuscript. ZK, MV reviewed the manuscript. PN, PP reviewed the manuscript and designed the study. All authors read and approved the final manuscript.

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Availability of data and materials

The LC-MS/MS data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD035456.

Declarations

Ethical approval and consent to participate

All individuals who provided venous blood samples were enrolled into the study in full accordance with the guidelines approved and monitored by the Ethics Committee of the University Hospital in Pilsen.

Competing interests

All authors declare no competing interests.

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ARTICLE II

Dvorak J., Fojtík L., Adámková L., Vlkova K., Studentova V., Chudejova K., Geigerová L., Volny M., Novak P., Hrabak J. & Pompach P.

Proof-of-concept MALDI-TOF-MS assay for the detection of Toxin B enzymatic activity in *Clostridioides difficile* infection

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Proof-of-concept MALDI-TOF-MS assay for the detection of Toxin B enzymatic activity in *Clostridioides difficile* infection

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ABSTRACT Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometers have become an integral part of all modern clinical microbiology laboratories. They serve as the key tool for pathogen identification and antibiotic resistance determination. However, certain limiting conditions must be fulfilled. The pathogen cannot be tested directly from the sample and requires the cultivation of a pure colony, which means that the standard protocol takes additional time, workforce, and consumables. The testing protocol is also more complicated when it comes to anaerobes. In our work, we focused on the functional detection of *Clostridioides difficile*, an important nosocomial human pathogen that is responsible for diarrhea and can lead to life-threatening colitis, as a model diagnostic problem. The virulence of *C. difficile* is mainly caused by two toxins, Toxin A and Toxin B. Established diagnostic methods, including nucleic acid amplification testing methods and immunoassays, detect the presence of the microorganism or the presence and concentration of the toxins, with limited ability to gauge infection severity based on the actual biochemical activity of the toxins and thus their potency to cause harm. This work presents proof-of-concept assays that indirectly determine the toxin activity in the human stool, a very complex matrix sample, using the natural RhoA protein as substrate. The RhoA protein substrate was recombinantly prepared with biotin tag modification, which allows its attachment to the NeutrAvidin MALDI chips. In the assay, the RhoA substrate anchored on the MALDI chip undergoes enzymatic glycosylation when exposed to the Toxin B in the stool sample, and the reaction product is then detected by MALDI-TOF mass spectrometry directly from the MALDI chip. The entire assay, from sampling to final mass spectrometry detection, was performed *in situ*, on the NeutrAvidin MALDI chip, which was prepared by unique surface modification technology also described in this work. The assay was successfully tested for the detection of Toxin B in a cohort of patient samples as well as in cell culture of *C. difficile*.

IMPORTANCE The diagnostics of *Clostridioides difficile* infection is usually based on the identification of the bacterial pathogen and/or on the detection of the Toxins A and B. Due to the variance in Toxins A and B activity across species, the toxin concentration determined by standard methods does not necessarily correlate with the severity of the disease. Assays that would target toxins' enzymatic activity are not routinely used because the requirements are unsuitable for clinical laboratories. In this study, we demonstrate a new approach that determines the presence and potency of Toxin B indirectly by determining its enzymatic activity rather than its concentration. This is performed by detecting mass difference due to glycosylation of RhoA substrate by Toxin B, which is then determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The presented proof-of-concept assay thus offers the possibility to quickly determine the activity of *C. difficile* toxins directly in the stool samples without pathogen cultivation.

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KEYWORDS *C. difficile*, Toxin B, MALDI-TOF-MS, diagnostics

During the last decade, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has become a standard instrument in clinical microbiology laboratories. MALDI biotyping is a common tool for quick pathogen identification and strain classification based on measuring fingerprint mass spectra of lysed bacterial cells, including strain classification of *Clostridioides difficile* (1, 2). It is a cost-effective, reproducible, and reliable method for the rapid identification of bacteria and fungi (3). Due to its accuracy and the small number of bacteria required, the MALDI-TOF-MS has become the primary tool for identifying bacterial infections. However, in most cases, biotyping still requires cultivation, which is a drawback for some bacteria (4). In some cases, anaerobic cultivation needs additional steps and resources, such as specialized consumables, equipment, or enrichment prior to cultivation (5). MALDI-TOF-MS has also become one of the key inventions that allow the concept of syndrome-based pathogen detection that shapes the current clinical microbiology and personalized medicine. This approach is based on multiplex testing of specific panels in the context of the patient's symptoms (6). However, gastrointestinal tract pathogens, which are mostly anaerobic, require a more complicated cultivation protocol that limits the use of MALDI-TOF-MS. Thus, personalized medicine assays that target anaerobic species are mostly based on molecular-genetic techniques (e.g., multiplex PCR) (7).

We focused our proof-of-concept study on a clinically important severe nosocomial gut pathogen, *C. difficile*, as a model for the method optimization and demonstration of its potential clinical utility. *C. difficile* is a Gram-positive, strictly anaerobic, spore-forming gut bacterium that requires time-consuming and laborious handling (8, 9). Therefore, their anaerobic cultivation does not align with the modern clinical laboratory demands for low-cost tests and time efficiency (5, 8, 10). The *C. difficile* infection (CDI) provides a heterogeneous clinical picture from asymptomatic carriage and mild or moderate diarrhea to colitis that can result in colon perforation, leading to sepsis and possible death (10–12). The spores persist even in extreme antibacterial healthcare conditions, e.g., heat or common disinfectants, which make CDI a leading cause of healthcare-associated infection, with almost half a million cases per year, leading to nearly 30,000 deaths. In Europe, the number of CDI cases is around 120,000 per year, with 3% attributable mortality (10, 13). A fast method for detecting the CDI in patients while evaluating the potential severity of the infection is thus crucial to provide optimal care, which would increase the patient's survival rate (11, 13, 14).

The nucleic acid amplification testing (NAAT) methods provide rapid, quantitative data on anaerobic species and are commonly used in determining a CDI presence as a part of the recommended workflows (10, 15). The first step in one of the recommended workflows of CDI identification is the immunoassay (IA) test for glutamate dehydrogenase (GDH), which identifies a possible non-specific infection in the colon. This is followed by NAAT or IA aiming directly at the presence of *C. difficile*. In some cases, the GDH test is simultaneously performed with the IA test for Toxin A and Toxin B. If these tests have inconclusive results, additional testing is performed using a different type of test than previously used. However, the actual algorithms for diagnosing *C. difficile* vary between laboratories (16). More laborious tests to confirm CDI in case of inconclusive results are toxigenic culture (TC) or stool cytotoxicity assay (CTA), which are usually considered to be optional only and are not frequently requested. Another possibility to decide cases with inconclusive results from the laboratory testing is colonoscopy (10, 17, 18).

The NAAT and IA tests are used to detect the presence and concentration of the main virulence factors responsible for the *C. difficile* potency—Toxin A and Toxin B (10, 12, 18). Both these toxins enzymatically modify small Rho GTPases by glycosylation. RhoA protein is the most common target of this modification—its Thr37 is modified by adding glucose from the UDP-glucose substrate. This modification results in RhoA inactivation. Since RhoA participates in several cell processes, including cytoskeleton regulation and

apoptosis, its inactivation during colitis may lead to cytoskeleton disorganization and cell death, which, on the macroscale, can result in the perforation of the colon (19, 20).

Importantly, *C. difficile* strains differ in toxin activity. For instance, the strain RT017, which was responsible for outbreaks of CDI in several countries around the world, is missing a gene for Toxin A and only exhibits Toxin B activity (21). Because of the difference among strains, the detection by standard laboratory assays (IA and NAAT) lacks the ability to estimate infection severity (18, 21, 22), which calls for assays based on the activity of the toxins. Another diagnostic complication arises from the fact that the toxins can be produced by other bacteria, e.g., *Paenibacillus sordellii*, due to the virulence transfer. This is a result of the fact that the virulence genes, including those encoding for toxins, can also be spread in microbial populations by horizontal gene transfer (23–25). That is why assaying the actual toxin activity can improve the accuracy of the diagnosis by adding complementary information orthogonal to standard methods. As mentioned, there are two diagnostic methods, TC and CTA, that can evaluate pathogen potency based on the cytotoxic activity (7, 11, 14). However, they are not routinely used because they are time-consuming, laborious, and require experienced workers and specialized equipment (10).

This work demonstrates a proof-of-concept assay that can screen Toxin B enzymatic activity using the same MALDI-TOF mass spectrometers that are already available in clinical microbiological laboratories. The assay can be done in complex human samples without a need for cultivation. The detection assay presented here was based on monitoring the enzymatic activity of Toxin B on the affinity-tagged (by biotinylation) recombinant RhoA, which was prepared in-house using BirA-producing *Escherichia coli* (26). The substrate underwent an enzymatic modification *in situ* on the surface of the NeutrAvidin MALDI chips by Toxin B in the stool sample. The NeutrAvidin chips were manufactured using ambient ion soft landing technology. This is a unique surface modification technique that allows the attachment of protein molecules to different inert surfaces with the retention of the protein's original biological activity (27–30). The immobilized RhoA substrate was modified by glucose addition, and the mass difference was then detected by MALDI-TOF-MS. The described MALDI assay was optimized and tested using the stool samples of a cohort of CDI-suspected patients. The importance of this proof-of-concept assay needs to be seen in the context of a recent study that shows that the potency of CDI does not directly correlate with the concentration of the toxin in the stool (31). That is why a fast instrumentation-based assay that can measure the actual activity of the toxins instead of concentration provides important clinical information complementary to standard techniques while avoiding the laborious cultivation and CTA or TC approaches.

RESULTS

Fast protein liquid chromatography (FPLC) purification and mass spectra of RhoA substrate

After GST-tag removal, the RhoA was purified by the FPLC with a gel filtration column. The peripheral membrane protein nature of the RhoA GTPase suggests that the first fraction (8–10 minutes) in the chromatogram (Fig. 1) could be a RhoA aggregate. The purity of the collected RhoA fraction (11–13 minutes) was evaluated with direct electrospray ionization Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometry, and the deconvoluted spectra confirmed the presence of the desired RhoA-biotin of desired purity, which could be used for further experiments (Fig. 1).

The measured monoisotopic mass was 24,959.625 Da, corresponding to the theoretical monoisotopic mass with an error of 0.6 ppm. The purified biotin-tagged RhoA protein was further used as a substrate in enzymatic reactions with Toxin B. The biotin tag on expressed RhoA allows the substrate to be bound on the in-house-prepared affinity chips with immobilized NeutrAvidin molecules (20).

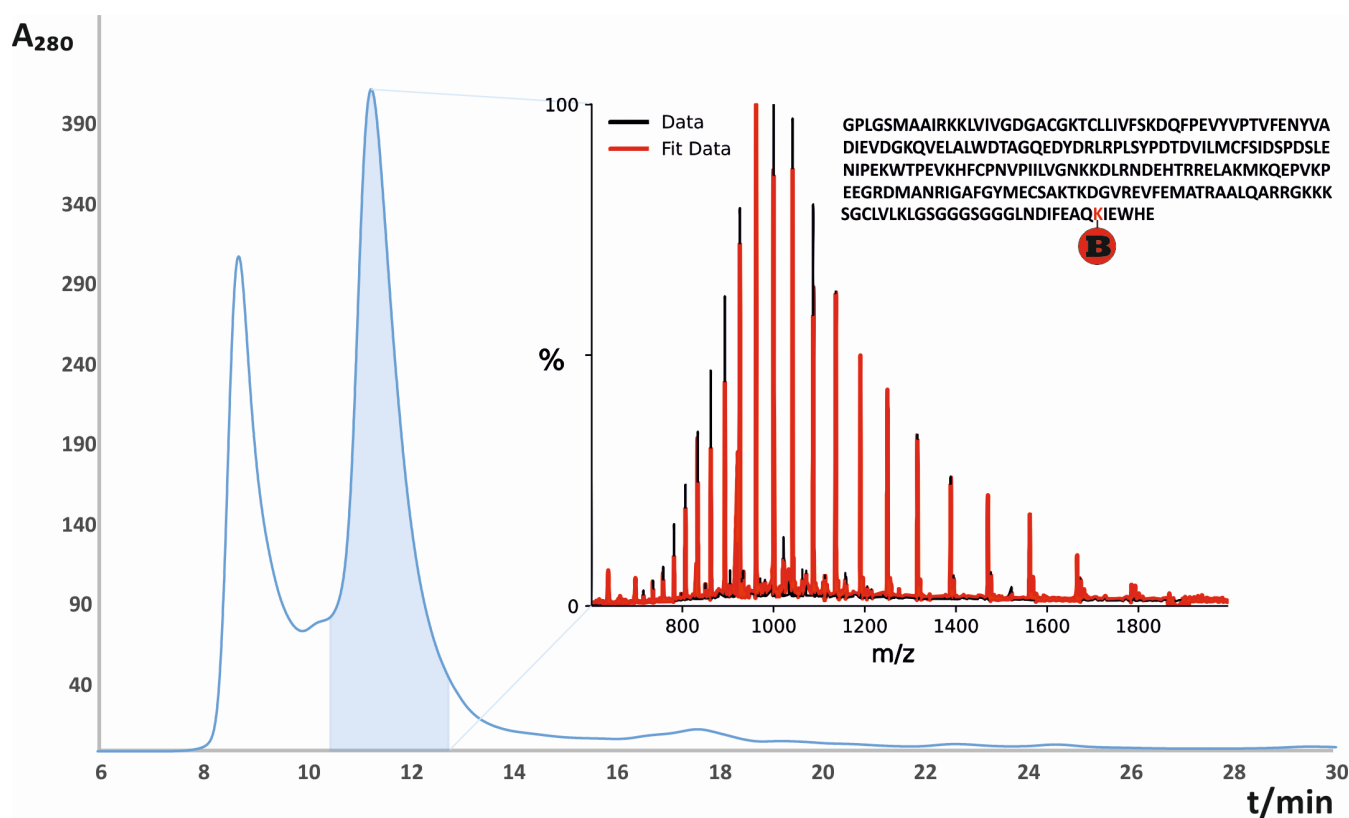


FIG 1 The results of RhoA gel filtration purification. The FPLC chromatogram shows a fraction at the retention time of 11–13 minutes, corresponding to the RhoA protein, as confirmed by Electrospray ionization (ESI) FT-ICR mass spectrometry (inset mass spectra). Fractions that elute between Retention time (RT) 8 and 10 minutes consist of impurities.

Optimization of the *in situ* enzymatic assay

Several MALDI matrices were tested to achieve the highest ion intensities while minimizing the formation of matrix adducts. The α -Cyano-4-hydroxycinnamic acid (HCCA) matrix, prepared by the published protocol as described in the methods section, was demonstrated to perform better than Sinapinic acid (SA), 2,5-Dihydroxyacetophenone (DHAP), HCCA, ferulic acid (FA), and 2,5-Dihydroxybenzoic acid (DHB) matrices prepared by the standard protocols provided by the manufacturer. Some of the tested matrices formed adducts that interfered with the modified protein signal on the same m/z , and some suffered from low ionization yield of the substrate RhoA protein (Fig. S3). Interestingly, when mixed with stool extract, the immobilized substrate RhoA on the NeutrAvidin chip was more stable than the free RhoA directly added. Additional stability was achieved by adding n -dodecyl- β -D-maltoside (DDM) and Mg^{2+} into the washing buffer and utilizing the protease inhibitor mix (Fig. S4).

The experiments showed that reaction time between 30 and 80 minutes allowed the detection of the toxin activity in most stool samples (Fig. S5). The optimized workflow is shown in the following Fig. 2.

Mass spectrometry detection of Toxin B activity using indirect assay

The Toxin B presence in the sample based on its activity was monitored by detecting the ion at m/z 25,063 corresponding to intact RhoA protein and the ion at m/z 25,225 corresponding to glucosylated form of RhoA using MALDI-TOF mass spectrometer operated in linear positive mode (Fig. 3).

To determine the detection limit of our method indirectly assaying the Toxin B activity in the complex matrix (stool), the dilution series of spiked recombinant Toxin B was

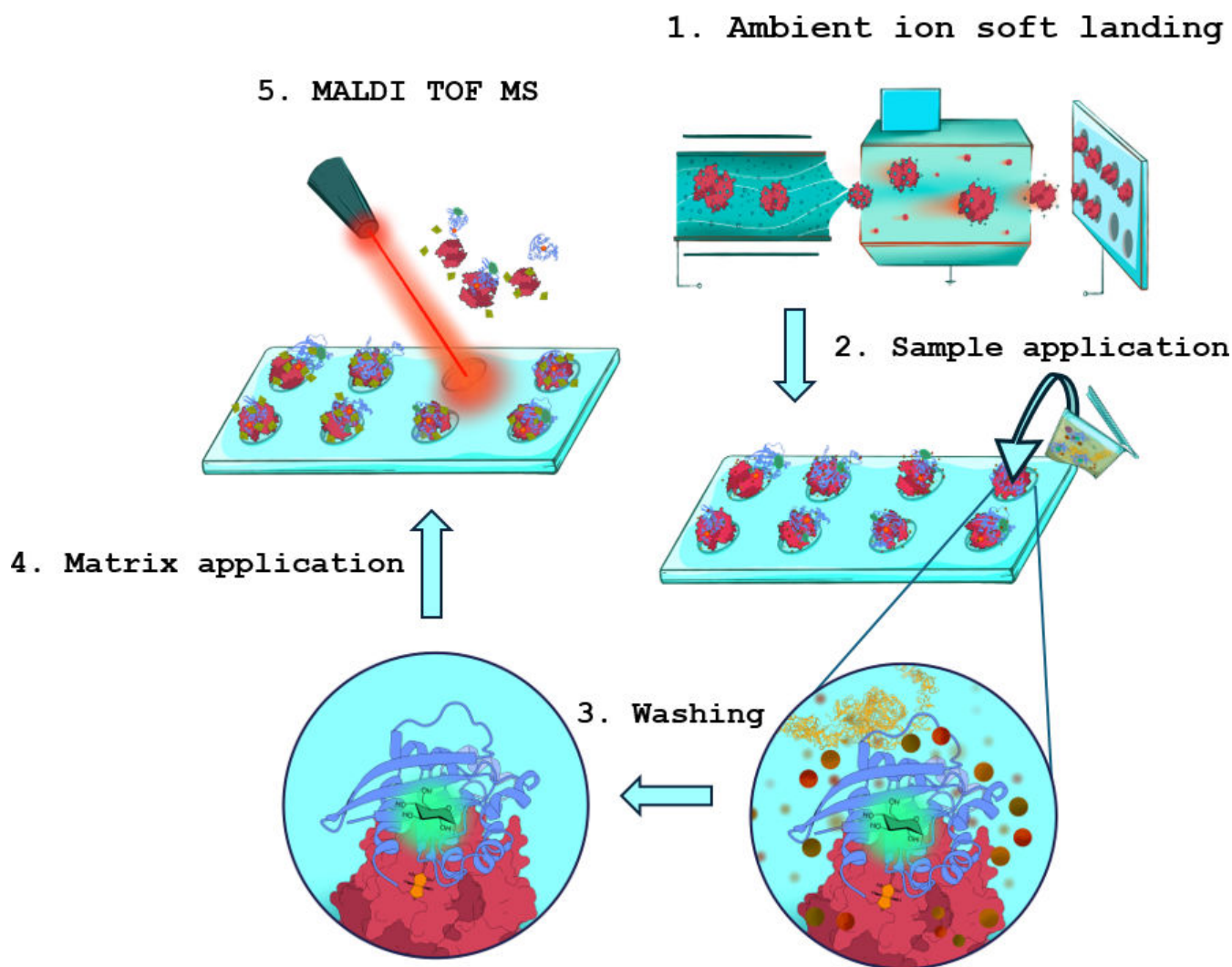


FIG 2 The optimized workflow consists of multiple steps. In the first step, the soft landing is used to prepare the NeutrAvidin chips, followed by the immobilization of the RhoA-biotin substrate on the chip. In the second step, a stool sample in the reaction buffer is applied on the spots with substrate and incubated for 30–80 minutes at 37°C for the substrate to undergo the enzymatic reaction due to the present toxin. The spots with modified/non-modified substrate are washed and overlaid with the HCCA matrix to be measured using the MALDI-TOF-MS.

prepared and used as described in the methods section. The data for each Toxin B concentration represented by signals of glucosylated/non-glucosylated RhoA substrate were obtained using MALDI-TOF mass spectrometry (Fig. 4). The signal intensity of the glucosylated substrate decreases as Toxin B is progressively diluted. For the Toxin B concentration of 32 ng/mL, the signal of the ion corresponding to the glucosylated form of RhoA had an average signal-to-noise ratio of 17. We monitored the signals of the doubly charged ions of both forms of RhoA at m/z 12,534 and 12,615, as they exhibited better resolution and higher intensity compared to the singly charged ions (Fig. 4).

The MALDI spectrum of doubly charged RhoA protein, modified by glucose after treatment with the *C. difficile* strain, is shown in Fig. S6.

The extent of conversion to glycosylated form as defined by the formula in the method section (Formula 1) and corresponding SD and coefficient of variation (CV) was 0.6 (SD = 0.08; CV = 13%) for 32 ng/mL and 0.3 (SD = 0.3; CV = 3%) for 6 ng/mL of spiked Toxin B.

For a variation test, recombinant Toxin B was spiked into the stool extract at final concentrations of 4 μ g/mL and 0.8 μ g/mL. The RhoA glucosylation level was calculated as a ratio of glucosylated and RhoA protein signal intensities using the formula described

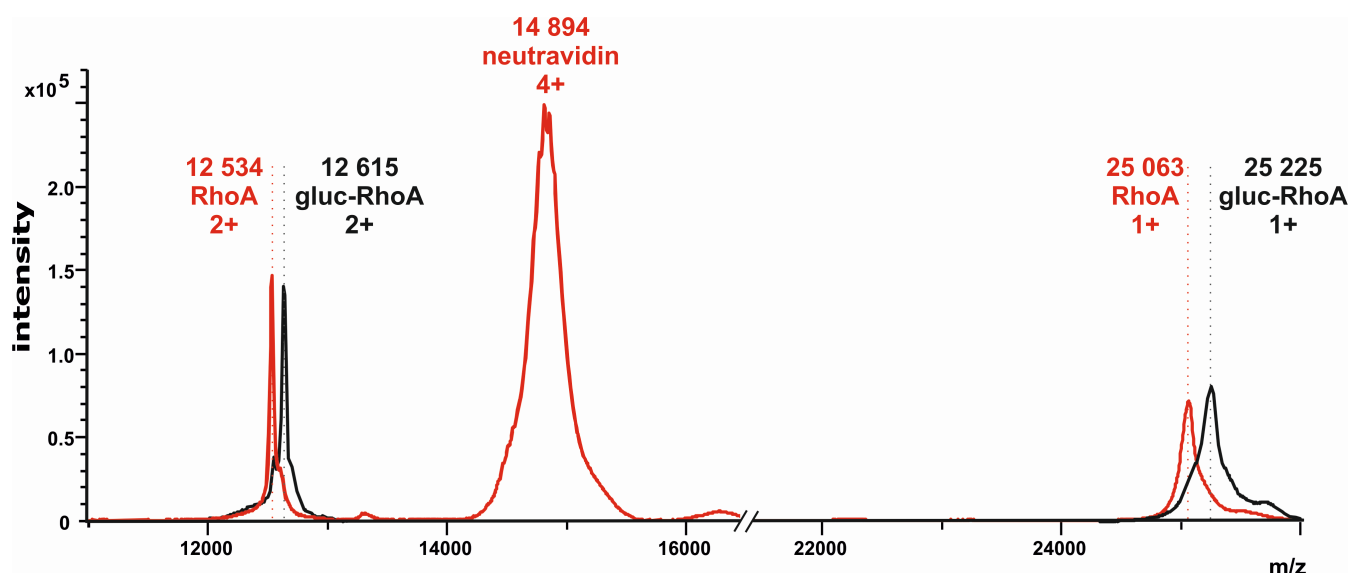


FIG 3 MALDI-MS spectra of intact RhoA (red) and glucosylated RhoA (black) on a NeutrAvidin MALDI chip. Signals obtained for intact RhoA and glucosylated RhoA differ by a mass of 162 (singly charged ions) and 81 (doubly charged ions). The glycosylation of intact RhoA that produces the difference in m/z is a product of Toxin B glucosyltransferase activity. The signal at m/z 14,894 corresponds to quadruply charged NeutrAvidin also released from the surface of the MALDI chip.

in the method section (Formula 1). Twenty technical replicates were prepared on each of the two chips, resulting in a total of 40 replicates for the statistical evaluation of the method (Fig. 5).

The CV obtained using chip 1 at a Toxin B concentration of 4 $\mu\text{g/mL}$ was 6% (SD 0.05), and at a Toxin B concentration of 0.8 $\mu\text{g/mL}$, it was 8% (SD 0.06). The CV obtained using chip 2 at a Toxin B concentration of 4 $\mu\text{g/mL}$ was 8% (SD 0.06), and at a Toxin B concentration of 0.8 $\mu\text{g/mL}$, it was 12% (SD 0.08). The CV between both chips was 7% (SD 0.05) for a Toxin B concentration of 4 $\mu\text{g/mL}$ and 10% (SD 0.07) for a Toxin B concentration of 0.8 $\mu\text{g/mL}$.

The use of the optimized assay on CDI-suspected patient samples

The optimized assay was used to detect Toxin B activity in 20 stool samples (Table 1) of patients suspected of CDI (Fig. S5). All samples were tested in the Pilsen Faculty Hospital by IA test that simultaneously detects GDH, Toxin A, and Toxin B or by the loop-mediated isothermal amplification (LAMP) assays for both toxins. The subsequent LAMP assay was performed only if the GDH test was positive in IA but negative for Toxin A and B. Only two patient samples showed these results; thus, there were only two LAMP tests performed (samples #1 and #8). The samples were then shipped to be tested by the presented toxin activity assay based on the MALDI-TOF-MS detection. The MALDI assay was performed in technical duplicates. Details about the assay's protocols are included in the methods section.

Toxin B activity was detected by MALDI assay in four samples (#2, #3, #6, and #18); two of them agree with the hospital data obtained (#3 and #6). The MALDI-TOF-MS spectra for positively detected samples are in Fig. 6. In the other three samples (#1, #5, and #9), no toxin activity was detected by MALDI, even though the hospital methods provided positive detection.

Table 2 shows the summary of the modification ratios between intact and glucosylated RhoA (extent of RhoA conversion) for four patient samples (#2, #3, #6, and #18) performed in duplicates. The ratio is reproducible within the duplicates, but each of the four samples has a different extent of conversion, which indicates different Toxin B activity in the samples.

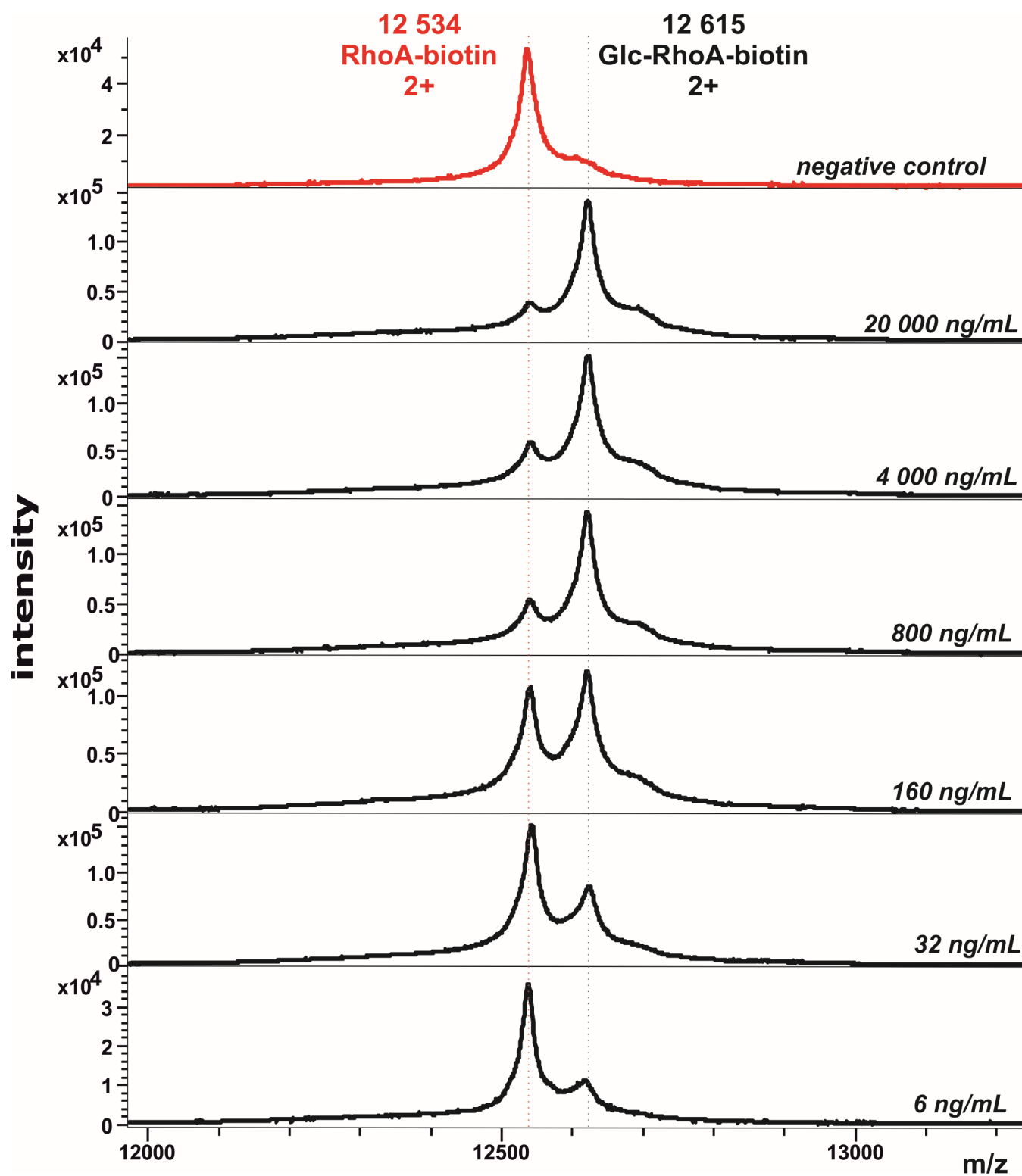


FIG 4 Mass spectra of doubly charged RhoA protein, exposed to stool spiked with different concentrations of recombinant Toxin B in the reaction buffer. The peak of the glucosylated form of RhoA was clearly observed, starting with the Toxin B concentration of 32 ng/mL. The dilution series was measured as a technical pentaplicate on one chip.

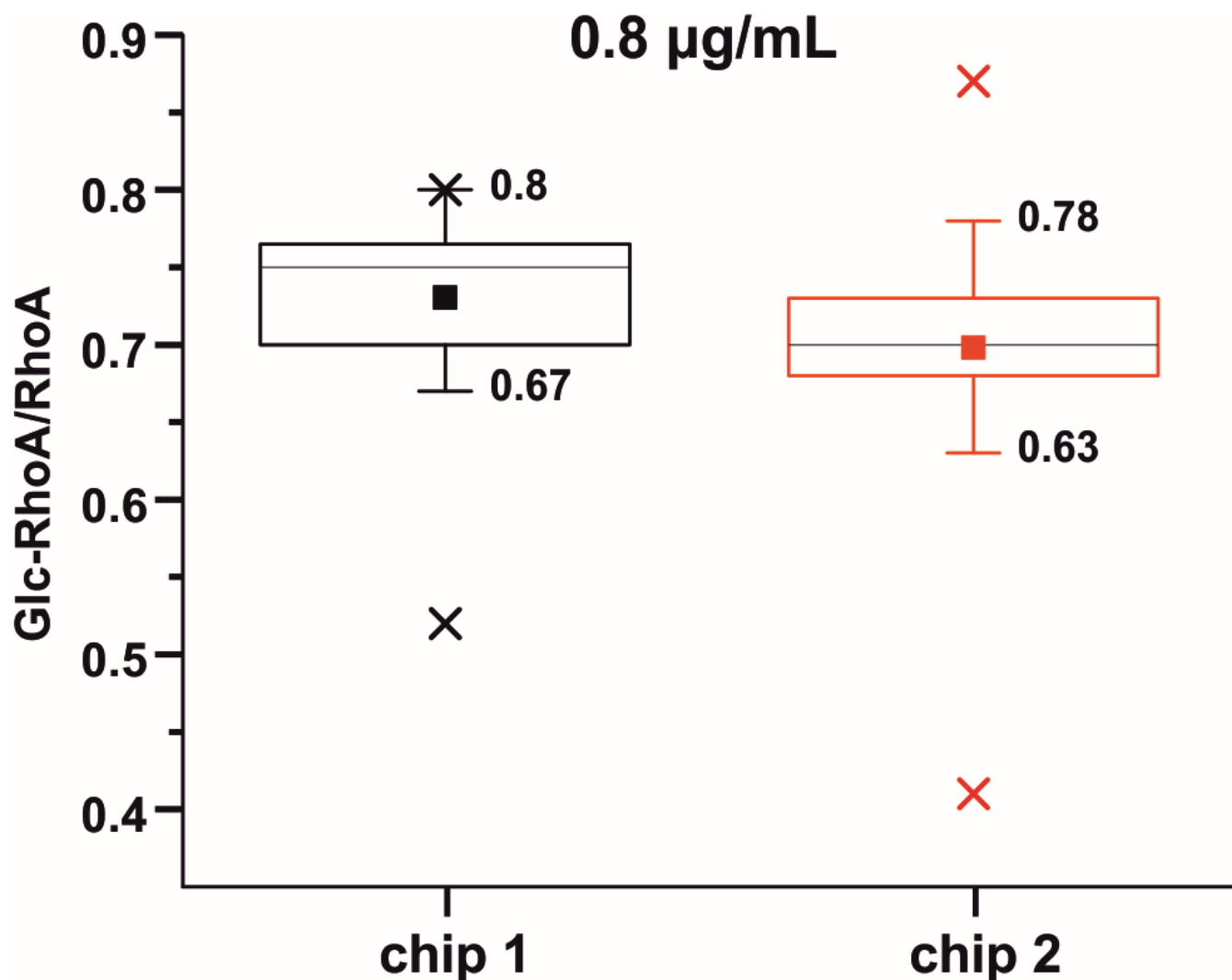


FIG 5 A box plot visualization of the variation test for two NeutrAvidin-modified chips tested using a Toxin B concentration of 0.8 µg/mL. The black box represents the range of values for chip 1 and the red for chip 2. The full square inside the range box represents an average level of modification across the replicate, and the gray line inside the range box represents the median value. The numbers represent minimum and maximum statistically valid extremes, and the cross signs outside the box represent the outlier values. The experiment was performed in 20 technical replicates on each of two different chips, resulting in a total of 40 repetitions.

DISCUSSION

Colonization of the gastrointestinal tract by *C. difficile* can happen without the manifestation of typical clinical symptoms of infection. Obtaining positive or negative results only by the highly sensitive genetic NAAT techniques can lead to misidentification of the pathogenesis of the gastrointestinal tract infection and lead to inappropriate therapy. Unfortunately, toxins with similar activity can also be produced by other bacteria, e.g., *P. sordellii*, due to horizontal gene transfer. This is an important mechanism of bacterial evolution (23), especially, but not only, in the spread of antibiotic resistance. This mechanism of bacterial genome plasticity has been described for different virulence genes, and in *Clostridium* spp., horizontal transfer of toxins encoding genes has been documented (32). That is why the determination of free toxins A and B in feces is often performed, especially in CDI-suspected children patients (33), but the results from IA assays do not describe the actual activity of the present toxins. Functional assays oriented on detecting the toxins' activity, irrespective of the source of microbial

TABLE 1 The table represents the results of patient sample tests obtained by standard hospital techniques (GDH and IA against the toxins) and by MALDI assay^{a,b,c}

Sample number	IA			LAMP			IA/LAMP assay	MALDI assay
	GDH	ToxA	ToxB	GDH	ToxA	ToxB		
1	+	–	–	+	+	+	+	–
2	–	N/A	N/A	N/A	N/A	N/A	–	+
3	+	+	+	N/A	N/A	N/A	+	+
4	–	N/A	N/A	N/A	N/A	N/A	–	–
5	+	+	+	N/A	N/A	N/A	+	–
6	+	+	+	N/A	N/A	N/A	+	+
7	–	N/A	N/A	N/A	N/A	N/A	–	–
8	+	–	–	+	–	–	–	–
9	+	+	+	N/A	N/A	N/A	+	–
10	–	N/A	N/A	N/A	N/A	N/A	–	–
11	–	N/A	N/A	N/A	N/A	N/A	–	–
12	–	N/A	N/A	N/A	N/A	N/A	–	–
13	–	N/A	N/A	N/A	N/A	N/A	–	–
14	–	N/A	N/A	N/A	N/A	N/A	–	–
15	–	N/A	N/A	N/A	N/A	N/A	–	–
16	–	N/A	N/A	N/A	N/A	N/A	–	–
17	–	N/A	N/A	N/A	N/A	N/A	–	–
18	–	N/A	N/A	N/A	N/A	N/A	–	+
19	–	N/A	N/A	N/A	N/A	N/A	–	–
20	–	N/A	N/A	N/A	N/A	N/A	–	–

^aThe check mark indicates positive results. If the GDH test was negative, follow-up testing by IA was not performed. The MALDI assay was performed for every sample, irrespective of the prior GDH results. Only some samples tested positive (patients 1 and 8) by IA were also tested by LAMP.

^b(+) - positive; (-) - negative; N/A - not applicable.

^cLAMP - Loop-mediated isothermal amplification; IA - immuno-assay; GDH - glutamate dehydrogenase; ToxA - toxin A; ToxB - toxin B.

infection determined by genomic techniques, could thus significantly improve microbial diagnostics and subsequent therapeutic decisions.

In the present proof-of-concept assay, we demonstrated the ability of MALDI-TOF MS to detect glucosylation of substrate RhoA protein by *C. difficile* Toxin B. The major difficulty of the assay is that it is influenced by the complex matrix of the feces samples. The reaction buffer composition was optimized based on observations made in a previous literature report, where 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and Mn^{2+} , Mg^{2+} , and K^{+} ions were proposed for enhancing the toxin activity (34). The stool samples contain many other enzymatically active proteins; thus, adding an optimized mix of protease inhibitors was a necessity. Metalloprotease inhibitors could

TABLE 2 Signal intensities for intact and modified RhoA as detected by MALDI-MS in four positive patient samples^{a,b}

Sample	Replicate	Intensity modified	Intensity non-modified	The extent of conversion (E_{conv}) (%)
18	1	1,815	2,278	44
	2	8,786	10,949	45
3	1	2,338	3,937	37
	2	3,737	7,841	32
2	1	4,309	6,126	41
	2	1,513	2,015	43
6	1	1,383	1,513	48
	2	10,988	12,101	48

^aThe extent of conversion was calculated according to the formula in the method section and expressed as a percentage. The samples were measured in the technical duplicate.

^bIntensity modified - intensity of modified substrate; Intensity non-modified - intensity non-modified substrate

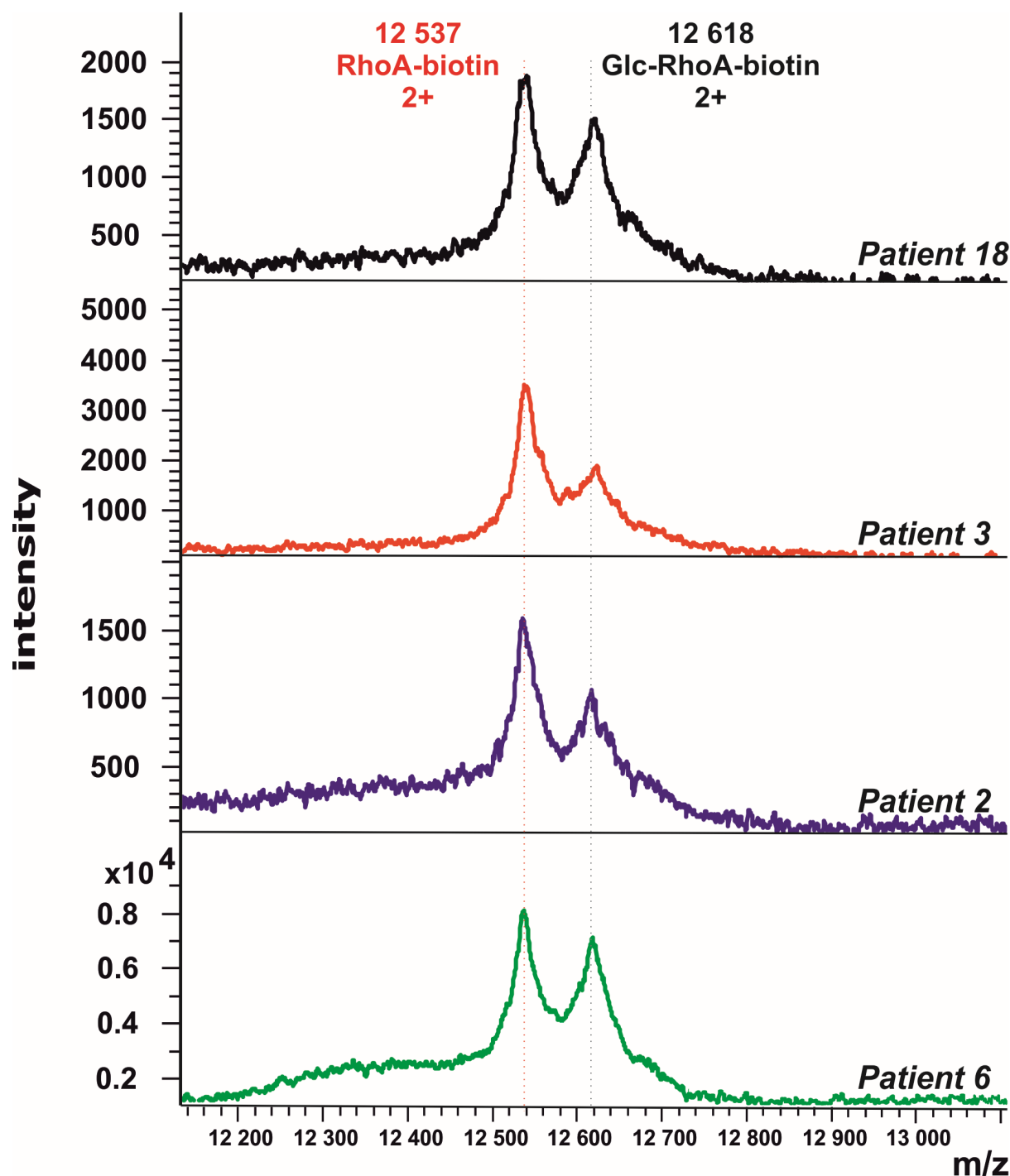


FIG 6 Mass spectra of RhoA substrate for the samples where MALDI assay detected Toxin B (patient samples #2, #3, #6, and #18). The amount of modification in all the samples suggests a possible presence of the active toxin in the sample. The samples were measured in technical duplicates on one chip.

not be used due to the need to keep the defined ion strength and composition of the buffer. Strictly from the enzymatic kinetics point of view, the ideal reaction time would be the longest practically possible because, in theory, the longest reaction time leads to the maximum RhoA conversion to the modified Glc-RhoA form. However, the presence of the proteases in the sample matrix limits the lifespan of RhoA in the sample, even if the inhibitors are added to the sample (Fig. S4). Thus, the optimal reaction time is a compromise of these two different factors.

The assay was developed and optimized so that it can be performed *in situ* on the MALDI-compatible chip by applying a stool isolate, which contains the enzymatically active Toxin B. The assay is indirect because it aims to determine the level of glycosylation of RhoA—Toxin B's substrate—to Glc-RhoA. The RhoA substrate is anchored on the MALDI protein chip by integration between NeutrAvidin immobilized on the chip surface and the biotin tag on the RhoA substrate. This approach overcomes the need for time-consuming cultivation necessary for the routinely used MALDI biotyping and provides information about the actual activity of Toxin B. The optimized assay was tested on the cohort of patient samples and showed results that were for the most part in agreement with the results obtained by the reference methods used in the hospital that provided the samples. Two samples in the cohort (#5 and #9 in Table 1) provided negative results from the new activity assay, although the samples were positive from the IA assay performed in the hospital. Sample #1 was also a false negative, although the hospital detected the toxin by LAMP and not by IA assay. We hypothesize that the different results were caused by the impacted activity of Toxin B in the samples. Thus, assays oriented on measuring the activity and assays oriented on measuring the concentration could not provide the same result. This then manifested as a false negative result, even though the information might mean that the Toxin B in the samples was inactive. Another two samples (#2 and #18) provided positive results by activity assay but were negative in the hospital. It is possible that a toxin with the same activity was present in these samples but originated from other bacterial species and was not detected by IA. The results thus showed two potentially false positive and three false negative results (out of 20 total samples). We speculate that in the case of false negative results, the new activity MALDI-TOF-MS assay was correct, and the Toxin B in the samples was not active at the time of the analysis. It is important to note here that the MALDI-TOF-MS assay is not proposed as a direct replacement for the current methods. It is merely intended as an expansion of the armory of detection tools, which would provide complementary information about the Toxin B activity and thus help to complete the clinical image of the infection severity and of the patient's condition.

In conclusion, the results show that the presence of virulent *C. difficile* strains can be established based on monitoring products of the Toxin B enzymatic activity in the stool extracts by detecting the level of RhoA protein glycosylation using MALDI-TOF-MS. The assay can be used to detect Toxin B activity in the stool samples of real patients with suspected and confirmed CDI. However, this report is only a proof-of-concept, and further optimization and large-scale evaluation are planned for the follow-up study.

The assay is complementary to other approaches because it provides information about the active Toxin B but not about its total chemical concentration. Another interesting feature of the assays is that by reporting toxin activity, it can identify other toxin-producing bacterial species that obtained the *C. difficile* gene for toxin production through horizontal plasmid transfer (24, 25, 35–37). The main limitation of the assay is the reliance on the stability of RhoA during the assay incubation. Unfortunately, the stability of the RhoA protein in human stool can be impacted by the sample matrix, which is extremely complex. This adversely impacts the assay's sensitivity, which depends on the incubation time and requires RhoA to be stable enough for the reaction to produce sufficient yields of the glycosylated RhoA variant. Finally, we argue that the implementation of the assay would be relatively easy and economical because the majority of clinical microbiology labs are already equipped with the MALDI-TOF Biotypers that can be used for this assay without any necessary modifications. It does not require cultivation, which makes it very time efficient. The workflow and data interpretation can be easily automated and adjusted for high throughput, thanks to the existing MALDI-TOF and laboratory automation.

MATERIALS AND METHODS

Chemicals and materials

Indium tin oxide (ITO)-coated glass slides were purchased from Bruker Daltonics (Massachusetts, USA). Recombinant Toxin B and phosphoramidon were purchased from R&D Systems, Inc. (Minnesota, USA) and NeutrAvidin protein from Thermo Fisher Scientific (Massachusetts, USA). The 10× concentrated cOmplete mini EDTA-free inhibitor cocktail, pepstatin, and bestatin were purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany).

Stool samples of 20 patients were provided by the Department of Microbiology, Faculty of Medicine, and University Hospital in Pilsen. The samples were recovered from patients who suffered from diarrhea suspected to be caused by CDI during August and September 2023. All specimens were diagnosed in a routine diagnostic process by GDH, Toxin A, and Toxin B production using fluorescence IA and interpreted using Standard F2400 reader as recommended by the manufacturer (Lab Mark, Prague, Czech Republic). The inconclusive IA resulting samples (GDH positive, toxins negative) were simultaneously alarmed using LAMP for *gdh*, *tcdA* (ToxA), and *tcdB* (ToxB) genes (eazyplex *C. difficile* Kit, AmplexDiagnostics GmbH, Gars-Bahnhof, Germany). Based on this combination of tests, five samples were identified as positive for *C. difficile*.

Manufacturing of affinity MALDI-compatible chips

A previously described apparatus for ambient ion soft landing (38) was used to prepare NeutrAvidin affinity chips on ITO glass slides. The geometry of the deposited NeutrAvidin array on the ITO glass was defined using sticker masks. The 0.6 mg/mL NeutrAvidin solution in 20 mM ammonium bicarbonate buffer, pH 7.8, was delivered by the syringe pump at a flow rate of 60 μ L/h using 100 μ m fused silica capillary connected to the micro-spray emitter with an applied voltage of positive 1.5 kV to create an electro-spray. A stream of preheated nitrogen gas (50°C–60°C) supported the nebulization and evaporation of droplets. After the soft landing, a washing step with deionized water was performed to remove any excess material, allowing only NeutrAvidin bound to the surface to remain.

Cultivation of *C. difficile*

Isolates of *C. difficile* were cultivated on *C. difficile* Selective agar (Oxoid, Hants, UK) in an anaerobic atmosphere developed by the Anaerocult system (Merck, Prague, Czech Republic) at 37°C for 48 hours. For toxin detection, colonies identified using the MALDI Biotyper Sirius System (Bruker Daltonics GmbH, Bremen, Germany) were used for inoculation of Oxoid Fastidious Anaerobe Broth (Oxoid, Hants, UK) and incubated in an anaerobic atmosphere for another 24 hours. Bacteria were collected by centrifugation (12,000 g, 2 minutes) and used for further experiments.

Biotin-tagged RhoA recombinant protein expression

The substrate RhoA-biotin was prepared as described by (39, 39) with several modifications leading to the production of recombinant RhoA protein with a biotin tag. A human RhoA sequence was modified with the avi-tag linker necessary for biotinylation by BirA biotin ligase. The pGex-6p-1 expression plasmid was used as the final construct (Fig. S1), and the insert (Fig. S2) was used to produce the biotin-tagged GST-RhoA fusion protein. The expression was made in the Luria-Bertani (LB) media using the BL-21 Bir. A producing *E. coli* with a biotinylation ability under Isopropyl β -D-1-thiogalactopyranoside (IPTG) induction control. When the OD₆₀₀ reached 0.9, the IPTG and the biotin were added and left to incubate overnight at 18°C. The protein was purified using GST-Sepharose. The GST-hRhoA-Avi-tag product was cleaved overnight on the column at 6°C using a PreScission protease. Purified protein was aliquoted into a 1 mg/mL stock solution and stored at –80°C.

***In situ* enrichment of biotin-tagged RhoA**

One microliter of RhoA with a biotin Tag at a concentration of 0.5 mg/mL was applied on each spot of the affinity MALDI chip modified with NeutrAvidin and incubated in a humidity chamber for 60 minutes. The incubation was followed by a fast wash with a buffer solution containing 20 mM HEPES, 50 mM NaCl, 10 mM MgCl₂, 1 mM tris(2-carboxyethyl) phosphine (TCEP), 0.17 mM DDM, pH 7.5, and consecutive wash with deionized water. The chip was dried at room temperature. The total amount of substrate protein deposited was 20 pmol.

***In situ* enzymatic reaction with recombinant Toxin B**

The following protocol prepared samples for the assay optimization and evaluation. The feces specimen was diluted to the final concentration of 1% (wt/wt) in 20 mM HEPES, 100 mM KCl, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM TCEP, and 0.17 mM DDM, with protease inhibitors cocktail (cOmplete Mini EDTA free, pepstatin, phosphoramidon, and bestatin) reaction buffer, pH 7.5. Recombinant Toxin B was added into the feces at different concentrations as a 5× dilution series from 20–0.006 µg/mL. The dilution series was measured on one chip in a technical pentaplicate.

One microliter of each sample with a different concentration of Toxin B was applied on the MALDI surface with immobilized RhoA protein serving as a substrate. After 1 hour of incubation at 37°C, the affinity chip was washed three times with a washing buffer and twice with deionized water and let dry at room temperature. Each spot was overlaid by an HCCA prepared as a saturated solution in formic acid, acetonitrile, and water mixed in the 1:2:3 ratio as described previously by Cohen et al. (40).

The optimized assay was tested on the CDI-suspected patient samples obtained from Pilsen Faculty Hospital. The samples were diluted with the same HEPES reaction buffer as previously to provide a 1% solution, but no recombinant Toxin B was added. Twenty patient samples were analogically applied on the NeutrAvidin chip with immobilized substrate RhoA in technical duplicates of 1 µL of stock solution per spot.

MALDI mass spectrometry and data processing

The samples from MALDI chips were detected using an Autoflex speed MALDI-TOF mass spectrometer (Bruker Daltonics) equipped with a Smart beam-II laser and operated in the positive linear mode. A partial sample random walk and accumulation of 7,000 laser shots for each spot were set up for data acquisition. The MS data were acquired in the *m/z* range of 6,000–300,000 Th. The laser frequency was set to 500 Hz, and the detector gain was set to 2,860 V.

The method was evaluated using two different Toxin B concentrations spiked into the stool isolate using two different affinity chips. Samples at 4 and 0.8 µg/mL concentrations were applied on 20 spots in the technical replicate of 1 µL per spot on two different chips, resulting in 40 repetitions of both concentrations. The extent of conversion (E_{conv}) of the RhoA substrate to its glycosylated form was calculated as the following ratio:

$$E_{conv} = \frac{I_{glyco}}{I_{intact} + I_{glyco}} \quad \text{Formula 1}$$

Where I_{glyco} is the signal intensity of modified RhoA protein in the MALDI MS spectrum, and I_{intact} is the signal intensity of intact RhoA in the same spectrum.

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DATA AVAILABILITY

The raw mass spectrometry data were deposited under ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier [PXD055622](#) (41).

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Fig. S1 (Spectrum02453-24-s0001.tif). Scheme of plasmid and insert.

Fig. S2 (Spectrum02453-24-s0002.tif). Plasmid insert sequence.

Fig. S3 (Spectrum02453-24-s0003.tif). Comparison of the resulting spectra during the MALDI matrix optimization experiments.

Fig. S4 (Spectrum02453-24-s0004.tif). RhoA MALDI mass spectra without and with protease inhibitors.

Fig. S5 (Spectrum02453-24-s0005.tif). MALDI mass spectra of RhoA protein from all 20 patient samples.

Fig. S6 (Spectrum02453-24-s0006.tif). MALDI spectrum of doubly charged RhoA protein modified by glucose.

Legends (Spectrum02453-24-s0007.docx). Legends for supplemental figures.

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ARTICLE III

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Novel activity assay for botulotoxin A1 detection using functionalized chips and matrix-assisted laser desorption/ionization mass spectrometry.

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ORIGINAL RESEARCH



Novel activity assay for botulotoxin A1 detection using functionalized chips and matrix-assisted laser desorption/ionization mass spectrometry

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ABSTRACT

Background: Botulinum neurotoxins (BoNTs) are a group of neurotoxins produced by *Clostridium* bacteria. Their effect on neuro-muscular connections through cleaving proteins of the SNARE complex results in blocking acetylcholine signal transduction. The FDA-approved mouse bioassay, which involves exposing live mice to potentially contaminated food, is the most widely used method. However, this assay is costly, time-consuming, and raises ethical concerns. Therefore, there is a need for alternative assays that can enzymatically measure the activity of BoNTs.

Research design and methods: We present an approach that combines the EndoPep-MS assay with protein affinity chips fabricated using ion soft-landing technology. Toxic activity is indirectly assessed by monitoring the N- and C-terminal fragments of the substrate peptide. This new method employs a protein array with affinity molecules targeting either the BoNT/A1 or the substrate peptide. Both variants enable *in-situ* reaction and detection of substrate peptides via MALDI-ToF MS on the protein chip.

Results: This method demonstrated successful detection of active BoNT/A1 in both buffer and complex matrices, achieving a detection limit of 0.5 ng/mL.

Conclusions: This study reports the *in-situ* detection of botulotoxin A1 using functionalized MALDI chips. The advantages of the MALDI chip technology include speed, robustness, cost-effectiveness, and possible automatization.

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1. Introduction

Botulotoxins or botulinum neurotoxins (BoNTs) are infamously known as the most potent biological substances [1]. Produced under suitable conditions by germinated spores of the anaerobic bacterium *Clostridium botulinum*, these zinc-dependent metalloproteases target Soluble N-ethylmaleimide-Sensitive Factor Attachment Protein Receptor (SNARE) proteins in peripheral postsynaptic nerve terminals. Proteolytic cleavage of one of the SNARE proteins prevents the initiation of the protein multicomplex that guides vesicles with acetylcholine at the neuromuscular junction, preventing neural transmission to effector muscles [2].

The seven main BoNT serotypes, A-G, are defined by their response to the antisera [3,4]. Serotype A (BoNT/A) is the most commonly known cause of human botulism and is also the main available form of BoNTs for clinical medicine [5,6]. Furthermore, BoNT is considered a threat as a biological weapon because its lethal dose (LD₅₀) (1–5 ng/kg) is much lower than that of Sarin (420 mg/kg), Ricin (1.5 mg/kg) and others [7,8].

Human botulism can occur in multiple forms, namely foodborne, wound, infant, and inhalation botulism. In all cases, infection with the toxin causes blurred vision, difficulty

speaking, and oral dehydration, quickly followed by flaccid paralysis [9]. The highest incidence of botulism derives from the consumption of food contaminated with BoNTs (foodborne botulism) [10]. Flaccid paralysis can occur within 12–48 hours from exposure [11]. Botulism can be treated using polyclonal antibodies and other antitoxins [12]. Antitoxin treatment neutralizes free toxins in circulation but has no effect on toxin already delivered to nerve terminals. Therefore, treatment effectiveness depends on timely diagnostics [13].

The gold standard for BoNT detection is a mouse bioassay (MBA). This MBA is the most widely used test to assess the levels of active BoNTs [14]. To this end, mice are introduced to a potentially infected sample, followed by symptom observation. Symptoms typically manifest 48 hours after the injection. Both sensitive (10 pg/mL) and robust, this approach can be used for quantification and toxin serotyping [14]. However, the results can take 1–4 days, and the method requires specialized facilities and skilled personnel. The ethical problem of this method must also be addressed since many animals are harmed annually. In 2019, 400,000 animals were used for industrial BoNT batch testing in Europe alone [15]. To overcome the limitations of

Article highlights

- Botulinum neurotoxins are highly potent toxins disrupting neuromuscular signaling. The gold standard for detecting is the mouse bioassay, but it is slow, costly, and ethically problematic.
- This study introduces a new BoNT detection method that combines the EndoPep-MS assay with protein affinity chips using ion soft-landing technology. This allows *in-situ* enzymatic reaction and analysis via MALDI-ToF MS.
- Two types of MALDI chips were developed – one with neutravidin for capturing biotinylated substrate peptides and another with anti-BoNT/A1 antibodies for toxin enrichment.
- The method successfully detected active BoNT/A1 in both buffer and complex matrices, achieving a detection limit of 0.5 ng/mL.
- The MALDI chip-based approach offers speed, robustness, cost-effectiveness, and potential for automation, making it a promising alternative to the mouse bioassay and other BoNT detection techniques.

the MBA method, other BoNTs detection approaches have been developed [7,16], most of which based on enzyme-linked immunoassay (ELISA), polymerase chain reaction (PCR), lateral flow assay (LFA), fluorescent resonance energy transfer (FRET) or colorimetric assay (CA) [7,11,17–21]. Although these methods are good alternatives, they fail to provide information about toxin activity. For this reason, the MBA assay remains the gold standard for BoNT detection.

The presented method, based on *in-situ* detection of substrate peptides, employs a similar principle and the same substrate peptides as the method developed in J. Barr's lab [22,23] termed endopeptidase mass spectrometry (EndoPep-MS). The EndoPep-MS method involves three main steps, namely enriching BoNTs on magnetic beads with immobilized antibodies, incubating these beads with a specifically designed substrate peptide, and detecting cleavage products by MALDI-ToF mass spectrometry [7,22–24].

Building on EndoPep-MS, we present two approaches for detecting BoNT/A1 activity differing in the type of protein immobilized on the MALDI chip. Neutravidin-modified MALDI chips utilize a substrate peptide with biotin tags at both its N- and C-termini, while the enzymatic reaction takes place in the tube. The cleaved peptides, resulting from BoNT/A1 activity, are enriched *in-situ* and detected by MALDI-ToF MS. In contrast, antibody-modified chips rely on the enrichment of BoNT/A1 on the chip and the *in-situ* cleavage of the substrate peptide prior to MALDI-ToF MS analysis. Because these chips are prepared using ambient ion soft-landing (AISL) at atmospheric pressure, biomolecules used for surface modification retain their biological activity after landing on the conductive surface. Thanks to AISL, these chips can be prepared with a biomolecule-modified array [25–29]. As such, this study provides two novel approaches for BoNT/A1 detection, supporting MALDI-ToF MS analysis in combination with high-throughput and easy-to-use affinity chips.

2. Materials and methods

2.1. Preparation of functionalized surfaces

Modified MALDI surfaces were prepared by depositing proteins onto indium tin oxide (ITO) glass slides using an ion soft

landing apparatus. The solution of biotin-binding proteins (neutravidin, avidin, or streptavidin) or anti-BoNT/A1 antibodies (A1688 and HcA78) in 50 mM ammonium bicarbonate, pH 7.9, at a concentration of 10 and 4 μ M, respectively, was infused into the electrospray using a syringe pump at a 1 μ L/min flow rate and preheated (45°C) nitrogen as nebulizing gas. Charged ions were dried with a ground, 10-cm tube (4 mm bore) preheated to 45°C and deposited onto an ITO glass slide (75 $\times$ 25 $\times$ 1.1 mm). High voltage (+1.5 kV and –1.5 kV) was applied to a liquid junction of the spray tip and on the ITO glass slide, respectively. Spot geometry (2 $\times$ 8 or 4 $\times$ 12) was defined by taping a mask to the conductive side of the ITO glass. The diameter of the spot was 2 mm, and each spot was sprayed for 5 minutes. Before use, the mask was removed, and the glass was washed with deionized water for 5 minutes.

2.2. Selection of biotin-binding proteins

Three biotin-binding proteins, namely neutravidin, streptavidin, and avidin, were used to modify ITO glass to test the binding efficiency of the biotinylated peptides. The peptide targets were synthetic BoNT/A1 activity products, RATRLLGGK-biotin, and biotin-KGSNRTRIDQGNQ (Apigenex). At different concentrations (10.00; 3.33; 1.11; 0.37; 0.12; 0.04 and 0.01 μ M), one microliter of the biotinylated peptides was applied in triplicate to functionalized MALDI chips. After 30 min of incubation at room temperature in a humidity chamber, the chips were washed 5 $\times$ with 20 mM ammonium bicarbonate buffer and 3 $\times$ with water (LC-MS grade).

2.3. Monitoring BoNT/A1 activity using neutravidin-modified surfaces

BoNT/A1 (Robert Koch Institute) at various concentrations (200, 20, 2, 0.2, and 0.02 ng/mL) was mixed at a 1:1 (v/v) ratio with 20 μ M biotinylated substrate peptide (biotin-KGSNRTRIDQGNQRATRLLGGK-biotin). The reaction buffer (5 mM HEPES, 10 μ M potassium chloride, 10 mM dithiothreitol, 1 mg/mL bovine serum albumin, pH 7.3) was used to dilute the toxin and the substrate peptide. To monitor BoNT/A1 activity in the complex matrix, dry dog food was resuspended in the toxin solution. After 15 minutes, the food extract was mixed with 20 μ M substrate peptide at a 1:1 (v/v) ratio. After 4 h of incubation at 37°C, one microliter of the reaction mixture was applied in quadruplicate on neutravidin-functionalized spots, and the chips were incubated for 30 minutes at room temperature in a humidity chamber. MALDI chips were washed 2 $\times$ with 50 mM ammonium bicarbonate, pH 7.9, and 2 $\times$ with deionized water. The spots were covered with one microliter of α -Cyano-4-hydroxycinnamic acid (HCCA) matrix prepared by mixing HCCA-saturated methanol solution with 50% acetonitrile at 1:2 ratio (v/v).

Botulinum neurotoxin is highly toxic and must be handled with the utmost care, in compliance with strict safety measures required by the local regulatory authorities. To ensure proper containment and protection, all neurotoxins were handled in a level 2 biosafety cabinet. All standard safety and decontamination procedures for handling botulinum neurotoxins were strictly followed in this research.

2.4. Monitoring BoNT/A1 activity using anti-BoNT/A1-modified surfaces

Two mouse monoclonal antibodies, A1688 and HcA78 (Robert Koch Institute), targeting BoNT/A were immobilized onto MALDI chips by ambient ion landing to evaluate their efficiency in enriching botulotoxin. BoNT/A1 toxin diluted in either the reaction buffer (5 mM HEPES, 10 μ M potassium chloride, 10 mM dithiothreitol, 1 mg/mL bovine serum albumin, pH 7.3) or the complex matrix (dry dog food extract prepared as described above) at concentrations of 100, 10, 1, 0.1 and 0.01 ng/mL was applied in quadruplicate on anti-BoNT/A1-antibody modified spots. After 1 h of incubation at room temperature in a humidity chamber, the spots were washed 3 $\times$ with PBS and 2 $\times$ with deionized water. One microliter of substrate peptide (Ac-OOEKAPARGFNPKPIDAGNGRATR-Nle-LGGR-NH₂) at a concentration of 10 μ M was loaded on the spots and incubated for 4 h at 37°C in a humidity chamber. One microliter of the HCCA matrix solution was mixed *in-situ* with the sample and left to dry. Both mouse monoclonal antibodies, that is, A1688, which targets the translocation domain of BoNT/A1, and HcA78, which targets the heavy chain of the toxin, were kindly provided by the EuroBioTox consortium [30].

2.5. MALDI-ToF MS analysis

Peptide products of BoNT/A1 activity were analyzed on an AutoflexSpeed MALDI-ToF mass spectrometer (Bruker Daltonics) in a positive reflectron mode using a random walk function, totaling 10,000 laser shots at 500 Hz laser frequency. The detection range was 800–3500 m/z. The mass spectrometer was calibrated using Peptide calibration standard II (Bruker Daltonics). Data were processed in flexAnalysis software version 3.3 (Bruker Daltonics). The raw mass spectrometry data were deposited under ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD058240 [31].

3. Results

Three different MALDI chips modified with neutravidin, streptavidin, and avidin were prepared using ion soft landing technology. Each MALDI chip was tested for binding efficiency using BoNTA-specific biotin-conjugated peptides at various concentrations. All three biotin-binding proteins efficiently enriched the standard biotinylated peptides *in-situ* (Supplemental Table S1 and Supplemental Figure S1). The neutravidin surface was more sensitive to the N-terminal peptide than the streptavidin and avidin surfaces. Only neutravidin MALDI chips were used in all further experiments.

As shown in Figure 1, we validated two techniques for monitoring BoNT/A1 enzyme activity. For the MALDI chip with anti-BoNT/A1 antibody, the immobilized antibody first captures the toxin from the surrounding matrix, which then reacts with the substrate peptide on the chip. For the MALDI chip with neutravidin, the reaction with the toxin occurs in the tube before loading the biotinylated products on the chip for enrichment and mass spectrometric analysis.

Figure 2(a) shows a MALDI spectrum of peptide products resulting from the activity of the toxin enriched on neutravidin-modified chips. The ions corresponding to the substrate peptide were observed at m/z 2878.9 (1+) and 1440.1 (2+); the N-terminal peptide (biotin-KGSNRTRIDQGNQ), at m/z 1699.7 (1+); and the C-terminal peptide (RATRLLGGK-biotin), at m/z 1197.9 (1+). Figure 2(b) shows a MALDI spectrum of peptide products measured using anti-BoNT/A1 antibody chips. The ions corresponding to the substrate peptide were detected at m/z 3287.1 (1+) and 1643.8 (2+); the N-terminal fragment (Ac-OOEKAPARGFNPKPIDAGNG), at m/z 2307.2 (1+); and the C-terminal fragment (RATR-Nle-LGGR-NH₂), at m/z 998.7 (1+). The concentration of toxin was 10 ng/mL.

The MALDI spectra of NT- and CT-products from the reaction buffer and food matrix enriched on neutravidin chips, which were spiked with the toxin at various concentrations, are shown in Figure 3.

For the neutravidin-modified surfaces, the intensities of CT- and NT-peptides at toxin concentrations of 0.5 and 1.0 ng/mL were higher in the reaction buffer than in the food matrix

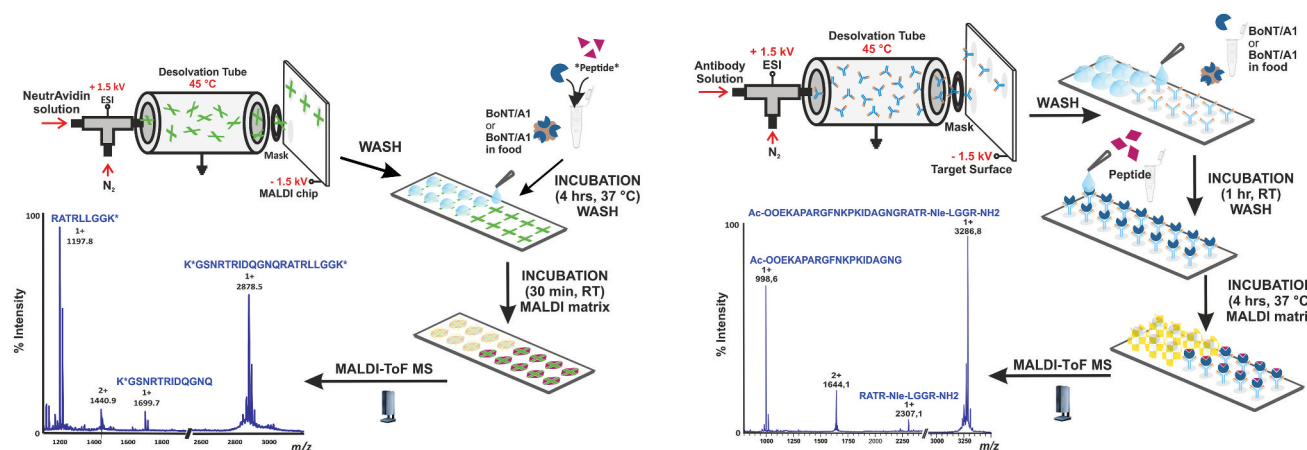


Figure 1. Workflow schemes for MALDI chips with immobilized neutravidin and immobilized anti-BoNT/A1 antibody. Left – MALDI chip with neutravidin: the reaction with the toxin takes place in the tube, and the biotinylated products are enriched using neutravidin. Right – MALDI chip with anti-BoNT/A1 antibody: the immobilized antibody first captures the toxin from the matrix and then the toxin reacts with the substrate peptide on the chip. Nle stands for Norleucine.

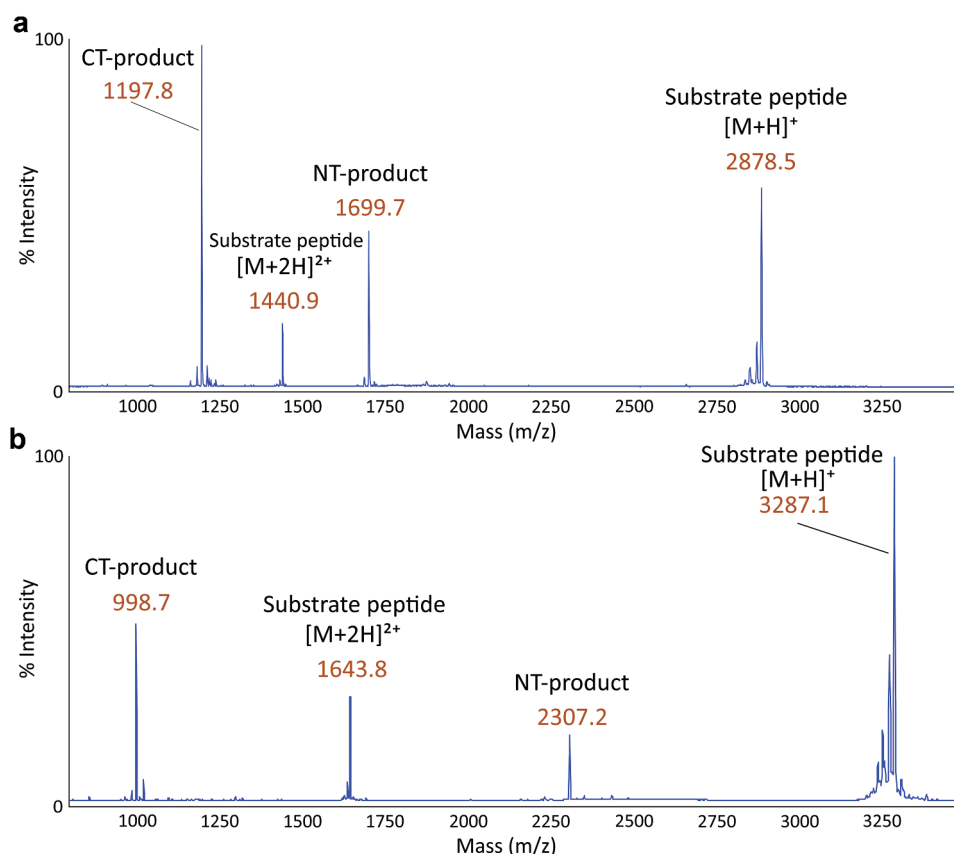


Figure 2. Representative MALDI-ToF spectrum of BoNT/A1 enzyme products enriched *in-situ* using MALDI chips modified with neutravidin (a) and HcA78 anti-BoNT/A1 antibody (b). The concentration of BoNT/A1 was 10 ng/mL. CT-product stands for the C-terminal peptide product and NT-product for the N-terminal peptide product. Both substrate peptides are observed as singly and doubly charged ions.

(Figure 3). Conversely, for both antibodies, that is, HcA78 (Figure 4) and A1688 (Supplemental Figure S2), the CT- and NT-peptides intensities were higher in the food matrix than in the reaction buffer.

The LoD of the method in the reaction buffer and in the food matrix was determined by measuring the ion intensities of CT- (Figure 5(a)) and NT- (Figure 5(b)) peptides at different BoNT/A1 concentrations. The LoD of neutravidin and anti-BoNT/A1 antibodies is 0.5 ng/mL. The S/N ratio of NT- and CT- peptides for 0.1 ng/mL toxin was lower than 8.

4. Discussion

Botulotoxins have long been identified as some of the most toxic substances. These lethal substances are primarily associated with food poisoning, but other forms of intoxication are also possible. Despite the decreasing incidence of food poisoning thanks to improvements in the food industry, the botulism threat remains a high risk. The number of botulism cases has also increased due to the popularity of the use of this toxin for medical purposes. The threat of botulism also fuels efforts to develop alternatives to the widely accepted mouse bioassay, with multiple ethical misgivings.

Developed by J. Barr's group, the EndoPep-MS assay provides a fast, efficient, and easy method for detecting and differentiating all BoNT serotypes. This method utilizes toxin

activity to cleave a substrate peptide based on its natural target, SNARE proteins in neuromuscular junctions. Unique to each serotype, peptide fragments resulting from toxin activity are then detected by MALDI-ToF mass spectrometry. This method was optimized using magnetic beads with two possible enrichment mechanisms to enhance its performance in complex biological matrices. In the first approach, a substrate peptide biotinylated on both C- and N- termini and streptavidin beads are used to enrich biotin-tagged peptide fragments. In the second approach, antibody magnetic beads are used to enrich toxins from samples before the enzymatic reaction.

This study aimed to transfer the assay from magnetic beads to affinity chips prepared by ambient ion soft landing technology. The modified chips are commercially available glass slides with a thin indium tin oxide layer. These slides are conductive and compatible with soft landing technology and MALDI mass spectrometry. This approach simplifies the assay by removing the beads enrichment protocol and may be used for high-throughput screening and multiplex assays.

Here, we present two optimized assays for detecting the most common serotype of botulotoxin, BoNT/A1, based on its proteolytic activity. The two assays differ in the modification of the affinity chips, but in both cases, the substrate peptides derive from the natural target of BoNT/A, SNAP-25, one of the proteins involved in the formation of the SNARE complex.

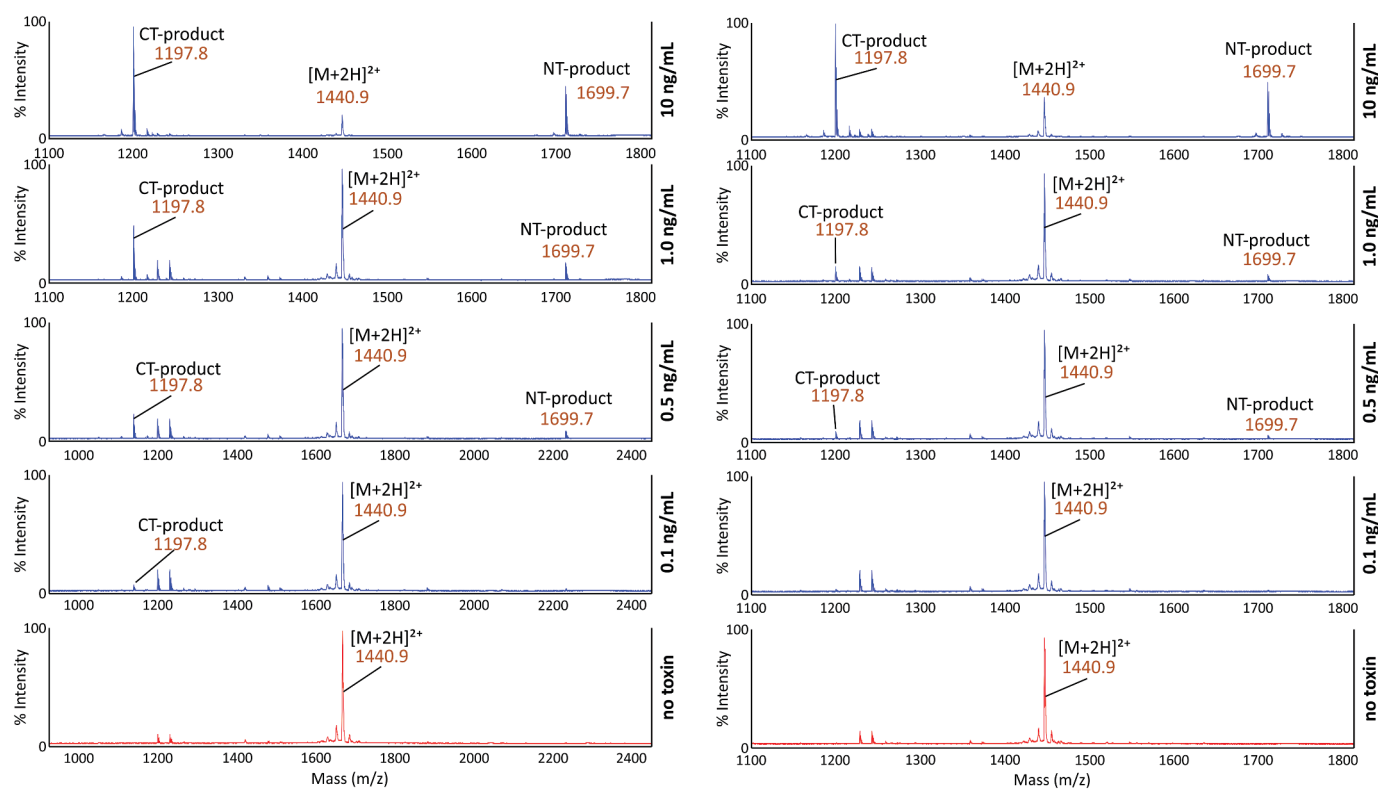


Figure 3. MALDI-ToF spectra of BoNT/A1 enzymatic reaction products from the reaction buffer (left) and food matrix (right) measured after *in-situ* enrichment on a neutravidin chip at different concentrations of spiked botulotoxin.

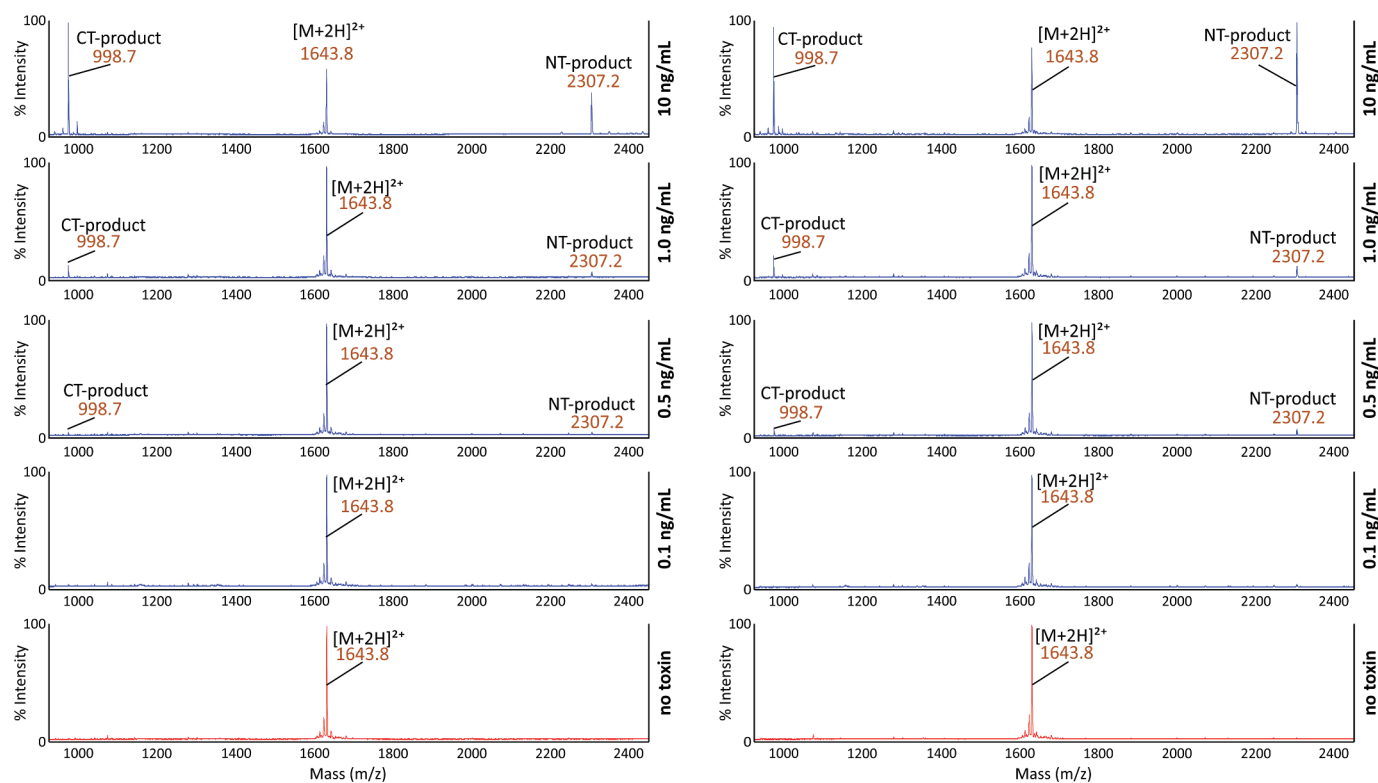


Figure 4. MALDI-ToF spectra of CT- and NT- peptides resulting from BoNT/A1 activity of the reaction buffer (left) and food matrix (right) and enriched *in-situ* using MALDI chips modified with the HcA78 antibody.

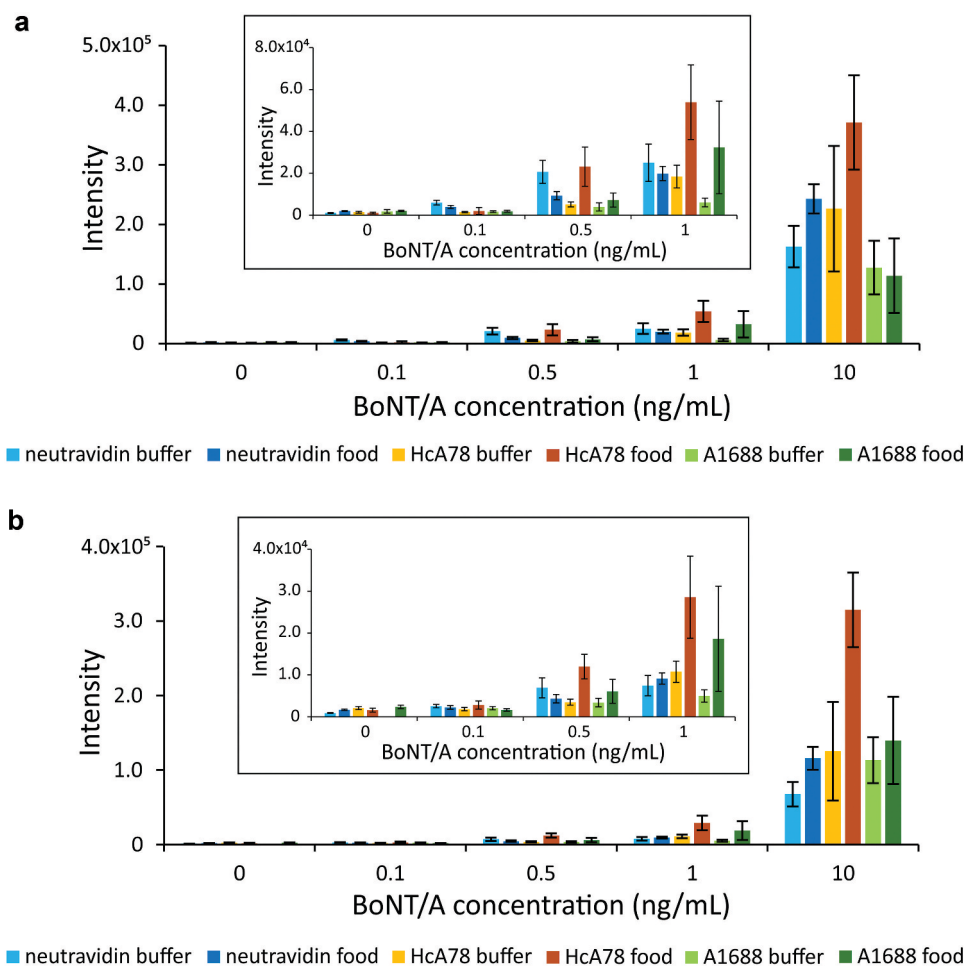


Figure 5. Intensities of (a) CT- and (b) NT-peptides at different toxin concentrations for MALDI chips with neutravidin and A1688 and HcA78 anti-BoNT/A1 antibodies.

The first approach was developed by testing chips modified with biotin-binding proteins. Biotin-binding proteins play key role in many affinity protocols, primarily due to their cost-effectiveness and to the strength of biotin-avidin interactions. Neutravidin was chosen for its slightly stronger binding to the substrate peptide compared to avidin and streptavidin. The substrate peptide biotin-KGSNRTRIDQGNQRATRLGKG-biotin was designed based on a previous study [32]. In the sample, BoNT/A1 cleaved the substrate peptide, producing C- and N- terminal products. Together with the remaining substrate peptide, these products were then enriched on neutravidin-modified affinity chips before applying the MALDI matrix, and peptides were detected directly on the chip by MALDI-ToF MS. Once the reaction conditions were optimized, the assay was transferred to a complex matrix and optimized. The dry dog food was ground to fine powder and spiked with either reaction buffer or toxin. The suspension was incubated with the substrate peptide.

Despite their cost-efficiency and strong affinity for substrate peptides, neutravidin chips have a limited use with complex sample matrices. Since the targeted toxin is typically found in complex matrices, ranging from food to human biological samples, matrix effects and nonspecific cleavage of

the substrate peptide by naturally occurring proteases can negatively affect the analysis, leading to false positive or false negative results. Therefore, the toxin must be purified prior to interaction with the substrate peptide.

In this study, the previously published immunoaffinity assay based on magnetic beads was adapted to MALDI chips modified with anti-BoNT/A1 antibodies by applying ambient ion soft landing technology. These immunoaffinity chips were used to enrich the toxin from either the reaction buffer or complex samples before the enzymatic reaction. Complex samples were prepared as for the neutravidin-modified chips assay. Two mouse monoclonal antibodies were employed, namely A1688, which targets the translocation domain of BoNT/A1, and HcA78, which targets the heavy chain of the toxin, thus ensuring that the toxin activity remained intact. In this assay, the substrate peptide (Ac-OOEKAPARGFNKPKIDAGNGRATR-Nle-LGGR-NH₂), previously designed based on its natural target, was added to the enriched toxin, and the enzymatic reaction was carried out directly on the chip [33].

The resulting LoD of BoNT/A1 (0.5 ng/mL) for both MALDI chip methods is consistent with the LoD specified in the international proficiency test for detecting and quantifying botulinum neurotoxins [34].

5. Conclusion

This study reports two approaches to *in-situ* detection of botulotoxin A1 using functionalized MALDI chips. MALDI-compatible chips were prepared by depositing neutravidin and anti-BoNT /A1 antibodies onto ITO glass slides through ambient ion soft landing. The feasibility of both approaches was evaluated by detecting toxin activity products in samples spiked with various concentrations of botulotoxin. Using this method, active toxin can be detected in both buffer and complex matrices, with a LoD of 0.5 ng/mL. MALDI chip technology provides several advantages over other methods, including speed, robustness, cost-effectiveness, and potential automation.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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All authors agree to be accountable for all aspects of the work.

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DECLARATION OF AUTHORSHIP

- I. Dvorak, J., Novakova, J., Kraftova, L. et al. The rapid detection of procalcitonin in septic serum using immunoaffinity MALDI chips. Clin Proteom 20, 20 (2023). <https://doi.org/10.1186/s12014-023-09410-3>

I declare that Josef Dvořák contributed ~ 70 % to **publication I**. He was responsible for MALDI-compatible chips preparation and method optimization, including the suitable sample preparation protocol. He was also responsible for testing patients' samples using the optimized method. Josef also wrote the first draft of the article.

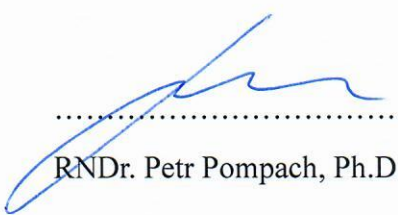
- II. Dvorak J, Fojtík L, Adámková L, Vlkova K, Studentova V, Chudejova K, Geigerová L, Volny M, Novak P, Hrabak J, Pompach P. Proof-of-concept MALDI-TOF-MS assay for the detection of Toxin B enzymatic activity in Clostridioides difficile infection. Microbiol Spectr 0:e02453-24. <https://doi.org/10.1128/spectrum.02453-24>

I declare that Josef Dvořák contributed ~ 80 % to **publication II**. He was responsible for MALDI-compatible chips preparation and method optimization, including the *in situ* enzymatic reaction conditions and wash protocol. He was responsible for optimizing the measurement condition – especially the matrix selection. He was also responsible for testing patients' samples using the optimized method. Josef also wrote the first draft of the article.

- III. Kalaninova, Z., Dvorak, J., Dresler, J., Volny, M., Novak, P., & Pompach, P. (2025). Novel activity assay for botulotoxin A1 detection using functionalized chips and matrix-assisted laser desorption/ionization mass spectrometry. Expert Review of Proteomics, 1–8. <https://doi.org/10.1080/14789450.2025.2482933>

I declare that Josef Dvořák contributed ~ 20 % to **publication III**. As a co-author, he helped to prepare samples and optimize the incubation device for the *in situ* reaction. He contributed to the manuscript by writing the Introduction section, participating in the Results and Discussion section, and editing the final article.

In Vestec 16.4.2025



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