Received: 22 March 2023 | Revised: 18 September 2023 | Accepted: 9 October 2023

DOI: 10.1002/pmic.202300151

Proteomics
Proteomics and Systems Biology

RESEARCH ARTICLE

Identification of the native *Torpedo californica* nicotinic acetylcholine receptor's glycan composition after a multi-step sequential purification method using MALDI-ToF MS

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Funding information
National Institutes of Health, Grant/Award Number: 1R01GM098343; National Institute of General Medical Sciences (NIGMS), Grant/Award Numbers: P20GM103642, U54-MD007600; National Institute on Minority Health and Health Disparities (NIMHD); UPR-RP RISE Program, Grant/Award Number: 5R25GM061151-18; NIGMS, Grant/Award Number: P20GM103475; BioXFEL STC of the National Science Foundation (NSF), Grant/Award Number: 1231306

Abstract

The Cys-loop pentameric ligand-gated ion channels comprise a dynamic group of proteins that have been extensively studied for decades, yielding a wealth of findings at both the structural and functional levels. The nicotinic acetylcholine receptor (nAChR) is no exception, as it is part of this large protein family involved in proper organismal function. Our efforts have successfully produced a highly pure nAChR in detergent complex (nAChR-DC), enabling more robust studies to be conducted on it, including beginning to experiment with high-throughput crystallization. Our homogeneous product has been identified and extensively characterized with 100% identity using Nano Lc MS/MS and MALDI ToF/ToF for each nAChR subunit. Additionally, the N-linked glycans in the Torpedo californica-nAChR (Tc-nAChR) subunits have been identified. To study this, the Tc-nAChR subunits were digested with PNGase F and the released glycans were analyzed by MALDI-ToF. The MS results showed the presence of high-mannose N-glycan in all native Tc-nAChR subunits. Specifically, the oligommanose population Man8-9GlcNac2 with peaks at m/z 1742 and 1904 ([M + Na]+ ions) were observed.

KEYWORDS
Lc MS/MS, MALDI ToF/ToF, nAChR-DC, N-linked glycans, *Torpedo californica*

Abbreviations: nAChR-DC, Nicotinic acetylcholine receptor in detergent complex; nano LC-MS/MS, Nanoscale liquid chromatography coupled to tandem mass spectrometry; MALDI-ToF, Matrix-assisted laser desorption/ionization-time of flight; PNGase F, Peptide-N-Glycosidase F; MS, Mass spectrometry; Man, Mannose; GlcNAc, N-Acetylglucosamine.

1 | INTRODUCTION

For more than five decades, scientists around the world have been working to purify and characterize the native muscle-type nAChRs [1–24]. These receptors are integral membrane proteins that belong to the Cys-loop pentameric ligand-gated ion channels superfamily and are involved in mediating neurotransmission in both the peripheral and central nervous systems. The muscle-type nAChR, found in organisms like *Torpedo californica* (*Tc*), *Torpedo marmorata* (*Tm*), *Torpedo nobiliana* (*Tn*), and the electric *eel Electrophorus electricus*, acts as a glycoprotein located in skeletal muscle that mediates neuromuscular transmission [16]. The muscle-type nAChR is a heteropentamer composed of two α subunits, one β subunit, one δ subunit, and one ε subunit [25]. Similarly, the neuronal type is also distributed throughout the central and peripheral nervous system, as well as certain non-neuronal tissues [26]. In neurons, these receptors are involved in the fast excitatory synaptic transmission mediated by the neurotransmitter acetylcholine. In addition, they are also present in non-neuronal cells, where they play a role in cell-to-cell communication and various physiological functions, including immune responses and inflammation [27, 28]. The journey of purifying nAChRs began in 1970 when Jean-Pierre Changeux pioneered their selective binding and purification from *Electrophorus electricus* using α-bungarotoxin [29]. However, obtaining sufficient protein quantities for complete receptor characterization remained challenging. In 1972, Jonathan B. Cohen, Michel Weber, Monique Huchet, and Jean Pierre Changeux succeeded in isolating the muscle-type nAChR protein from *Torpedo marmorata*, marking the beginning of a new era in nAChR purification [1]. Since then, numerous studies have been conducted to further characterize the structure and function of nAChRs. For example, efforts have been made to determine the three-dimensional structure of these receptors using x-ray crystallography and cryo-electron microscopy [24, 30–32]. These studies have revealed important insights into the mechanisms of ligand binding and channel gating and have provided a foundation for the development of drugs targeting nAChRs for the treatment of various diseases, such as Alzheimer's disease, schizophrenia, and addiction [24, 30–32]. The muscle-type nAChR is a vital protein involved in the proper functioning of an organism. The early efforts to purify and characterize these receptors have paved the way for a more comprehensive understanding of their structure and function, which has important implications for the development of therapeutics targeting nAChRs.

For many years, the proper purification of nAChR's has been a significant challenge in molecular biology, leading to a limited identification and molecular characterization of its subunits [16, 18–20, 33–39]. The complex structure and heterogeneity of the nAChR protein make it difficult to obtain a sample that is both stable and functionally active while maintaining high levels of purity. However, recent advancements in protein extraction and purification techniques have allowed us to produce a stable nAChR in detergent complex with high levels of purity [22, 23]. This breakthrough has allowed researchers to carry out reproducible molecular level analyses, including studies of peptide identification and the identification of glycan composition [22].

Significance Statement

This study constitutes a pivotal investigation into the *Torpedo californica* nicotinic acetylcholine receptor (*Tc*-nAChR), a crucial member of the Cys-loop pentameric ligand-gated ion channels, and its fundamental role in organismal function. The successful isolation of a highly pure and functional *Tc*-nAChR represents a significant milestone, enabling more robust high-throughput structural studies. This involves the careful application of detergent analogs to mimic physiological lipid interactions and the deliberate inclusion of cholesterol. These measures collectively contribute to maintaining the native conformation of the nAChR, ensuring that our findings provide unparalleled insight into its biophysical properties and physiological relevance. Our work strongly indicates that our sequential purification processes not only preserve the functionality and structural integrity of the protein but also yield the most representative glycans and peptides in the native state of the *Tc*-nAChR. The identification and characterization of N-linked glycans in *Tc*-muscle type nAChR subunits bear substantial implications as potential biomarkers to be identified for the diagnosis of neuromuscular diseases.

With this new method, we can isolate nAChR protein complexes that maintain their functionality, leading to a deeper understanding of the structural and functional properties of this vital protein. This breakthrough in purification techniques will open doors for new studies on the nAChR protein and its involvement in the neuromuscular system, potentially leading to improved therapeutic strategies for neurogenic health issues.

Glycans, also known as carbohydrates or sugars, play a crucial role in the proper folding, stability, and function of proteins, including nAChRs [40]. Glycosylation, a post-translational modification process, involves the covalent attachment of glycans to proteins [41]. This process takes place in the endoplasmic reticulum and Golgi apparatus, where a diverse set of enzymes catalyze the assembly and processing of glycans. The specific composition and location of glycans on a protein can significantly influence its structural conformation, stability, solubility, and biological activity. Mutations in genes responsible for glycan biosynthesis can lead to defects in glycan development, resulting in a wide range of neurological disorders [42]. For instance, mutations affecting the glycosylation of nAChRs have been found to cause developmental defects in the neuromuscular junction, leading to congenital myasthenic syndromes [43]. Additionally, alterations in glycosylation have been linked to various diseases, including cancer, inflammatory disorders, and immune system dysfunctions [44–47]. Understanding the intricate role of glycans in the structure and function of nAChRs is not only crucial for unraveling the molecular mechanisms

underlying these disorders but also for developing innovative treatments and therapies.

Our study focusing on nAChRs from *Tc* has broader implications for our understanding of homologous receptors in humans and opens up new avenues of research in the field. By investigating the glycosylation patterns and functional consequences of nAChRs in this model organism, we can not only gain insights into the glycosylation processes specific to this species but also extrapolate this knowledge to better comprehend the glycosylation patterns and functional implications of homologous nAChRs in human systems. Glycosylation, as a post-translational modification process, plays a critical role in protein folding, stability, and function. The specific composition and location of glycans on a protein can significantly influence its structural integrity, solubility, interaction with ligands, and biological activity. Mutations in genes involved in glycan biosynthesis can lead to defects in glycan development, resulting in a range of neurological disorders in organisms. Congenital myasthenic syndrome (CMS) is characteristic for altering the neuromuscular synapse in a patient, generally in a postsynaptic manner, where we find the nAChRs directly involved [48, 49]. CMS is part of the genetic disorders of the neuromuscular junction, which alters the kinetics of muscle-type nAChR [48, 50]. This condition is rare, affecting about 140 people per million [51, 52]. The clinical picture is weakening of the muscles caused by a failure in the synaptic transmission between the nerve and the muscle causing fatigue [53]. Studies have found that mutating one of the glycosylation sites in any of its subunits implied a reduction in the expression levels of the nAChRs, thus causing the myasthenic syndrome [48, 49]. However, recent studies have unveiled another dimension to CMS pathology, highlighting the involvement of glycosylation sites in nAChR subunits [48, 49]. Glycosylation, a post-translational modification process, adds complex carbohydrates to proteins and is indispensable for their stability and proper functioning. Perturbations in glycosylation can disrupt protein folding, stability, and interactions with other molecules, thereby contributing to the onset and progression of various diseases, including CMS. For this reason, the study of glycans using alternative tools such as mass spectrometry analysis is important [54–56]. This type of study allows us to have the tools that give us the foundations to identify and characterize glycosylations that may have some relevance to metabolic disorders. Over the past decade, there has been a significant increase in interest in glycomics studies, which has been fueled by the use of new, high-resolution mass spectrometry tools [57]. This has helped us gain a better understanding of the implications that post-translational modifications can have at the atomic level of a molecule. As a result, the study of glycan alterations has the potential to be used as possible biomarkers to identify and diagnose various diseases.

Advancements in glycoproteomic technologies, such as mass spectrometry-based glycan analysis and glycan engineering, are revolutionizing our ability to study and understand the complex biology of glycosylation. These cutting-edge techniques enable the identification, characterization, and quantification of glycan structures attached to proteins, including nAChRs. By applying these tools, we can unravel the intricate glycosylation profiles of different nAChR subunits across species and tissues, revealing their functional implications and paving the way for a deeper understanding of their roles in health and disease. Our research on nAChRs and glycosylation provides valuable insights into the critical role of glycans in protein structure and function, particularly in the context of homologous receptors in humans. By expanding our knowledge of glycosylation processes and their effects on nAChRs, we contribute to the broader field of neurobiology and lay the foundation for the development of innovative therapeutic approaches targeting nAChRs and related neurological disorders. Our work presents a significant opportunity for advancing our knowledge of glycosylation-related diseases, such as CMS, and has the potential to contribute to the development of personalized treatment strategies. The extensive abundance and diversity of glycosylation, the primary post-translational modification in mammals, including humans, offer immense possibilities for enhancing protein therapeutics [58]. The impact of glycosylation on crucial factors such as folding, trafficking, ligand interactions, solubility, stability, safety, activity, pharmacokinetics, and pharmacodynamics makes these modifications capable of enhancing the entire spectrum of this essential therapeutic class.

In this study, we aimed to identify and characterize the peptide and glycan composition per subunit of the Native Tc-nAChR for the first time using a multi-step sequential purification method and state-of-the-art analytical techniques such as Nano Liquid Chromatography Tandem Mass Spectrometry (Nano Lc MS/MS) and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI ToF/ToF). Our goal was to validate and characterize our purification process, and to gain a much more comprehensive understanding of the protein's molecular integrity than was previously possible.

2 | EXPERIMENTAL SECTION

2.1 | Materials

Tc electroplax tissue was obtained from Aquatic Research Consultants, (San Pedro, CA). The 4800 Plus MALDI ToF/ToF Analyzer (SCIEX, MA, USA) has been purchased from SCIEX. The 4-Chloro-α-cyanocinnnamic acid (Sigma 94141), Trypsin sequencing grade/Roche (Sigma 11047841001), 2′,4′,6′- Trihydroxyacetophenone monohydrate (Sigma 91928), Water MS grade (Sigma 900682), Acetonitrile (Sigma 34998), Methanol (Sigma 67-56-1), Acetic Acid (Sigma 64-19-7), Ammonium Bicarbonate (Sigma 11213) and PNGase F proteomics grade, Roche (Sigma P7367) were acquired from Sigma Aldrich (St. Louis, MO). We have purchased the Formic acid Lc / MS Grade (Fisher 64-18-6) from Fisher. The Easy-nLC1200 (Thermo Fisher LLC140), Pierce C18 Spin Columns (Thermo Fisher 89870), PicoChip H354, REPROSIL-Pur C18-AQ 3 µm 120 A, Q-Exactive Plus and Easy-nLC1200 (Thermo Fisher LLC140) were purchased from Thermo Fisher. Coomassie Brilliant Blue R-250 (Bio-Rad 1610400), 2X Laemmli Sample Buffer (Bio-Rad 1610737), 10X Tris/Glycine/SDS (Bio-Rad 1610732), Precision Plus Protein Unstained Protein Standard (Bio-Rad 1610363), 2-Mercaptoethanol (Bio-Rad 1610710) and 4%–20%

Criterion TGX Stain-Free Gel (Bio-Rad 5678093) was procured from Bio-Rad. All other reagents reported in this article were purchased from Sigma Aldrich (St. Louis, MO).

2.2 | Sample preparation of the native nAChR-DC

The native nAChR was extracted from the *Tc electroplax* tissue obtained from Aquatic Research Consultants, San Pedro, CA, USA, according to the protocols described by Maldonado-Hernández [22]. Briefly, the crude membranes from *Tc* were solubilized in 35 mM LysoFos Choline 16 (LFC-16), 0.2 mM cholesteryl hemisuccinate (CHS), 300 mM NaCl and 40 mM Tris pH 7.4 for 1 h at 4°C followed by ultracentrifugation at 162,600 *g* for 1 h at 4°C. ÄKTA Pure 25 chromatography system was used for all multi-steps of the purification system. The first step of purification consisted of Bromoacetylcholine affinity chromatography. The nAChR was eluted from the column with 2 column volumes of 13 mM Carbamylcholine Chloride, 150 mM NaCl in DBS-1X Buffer (100 mM NaCl, 10 mM MOPS, 0.1 mM EDTA, and 0.02% NaN3). The Capto Lentil Lectin affinity chromatography (LLC), which is usually used for glycoproteins purification, was used as a second chromatographic step. Then, size exclusion chromatography (SEC) was used as the last step of purification with a Superdex 200 10/300 increase Gel filtration GE column. The protein purity was analyzed by micro-capillary gel electrophoresis (MCGE) and SDS-PAGE.

2.3 | In-gel tryptic digestion for each of the native nAChR subunits

The in-gel tryptic digestion was performed by following the method of Shevchenko with modifications [59]. Briefly, 70 µg of protein was separated in a 4%–20% Criterion TGX Stain-Free Gel and stained for 1 h with Coomassie Brilliant Blue R-250 dye. Then, the gel was incubated overnight with the destaining solution (50% methanol, 40% nanopure water, and 10% acetic acid). Subsequently, the gel bands corresponding to molecular weights of the nAChR subunits ($\alpha\beta\gamma\delta$) were excised using a sharp scalpel and placed into the Eppendorf tube to destain with a solution containing 25 mM ammonium bicarbonate and 50% acetonitrile at 37°C for 30 min. Afterwards, protein disulfide bond reduction was performed by 20 min incubation at 37°C with 10 mM dithiothreitol in 50 mM ammonium bicarbonate. Then, the cysteine alkylation step was carried out in the dark for 45 min by adding 55 mM iodoacetamide in 50 mM ammonium bicarbonate at room temperature. The gel bands were then dried and digested overnight with 16 ng/µL trypsin (proteomics grade, Roche) in 50 mM ammonium bicarbonate at 37°C. Last, peptides were extracted by vortexing for 15 min in 100 µL of extraction buffer (extraction buffer (1:2 (vol/vol) 5% formic acid/acetonitrile). The extracted peptides were then dried in a Speed Vac concentrator and resuspended in 20 µL of 5% formic acid for MALDI ToF/ToF or 0.1% formic acid for Nano Lc MS/MS experiments (Figure 1).

2.4 | MALDI ToF/ToF system and analysis conditions for the native-nAChR peptide mass fingerprinting

MALDI ToF/ToF analysis was performed on a 4800 Plus MALDI ToF/ToF Analyzer (SCIEX, MA, USA) and mass spectra were acquired in a reflector positive mode. Peptides were analyzed using 12 mg of 4-Chloro-α-cyanocinnnamic acid dissolved in 70% acetonitrile (vol/vol). External calibration was performed with the Cal Mix 5 Opti-ToF High-Resolution TIS Calibration Inset (SCIEX, MA, USA). Protein identification was achieved by searching the NCBIprot protein database using the Mascot search engine (Version 2.3.01). For coverage of sequence analysis, we used mMass Open-Source Mass Spectrometry Tool (http://www.mmass.org). The search parameters included trypsin as the enzyme for proteolysis. Also, the modifications included were the cysteine carbamidomethylation (CAM) +57.021 Da (C) modification and methionine oxidation (MetO) +15.995 Da (Mo).

2.5 | Lc-MS/MS system and analysis conditions for the native-nAChR peptide mass fingerprinting

Sample reconstitution was performed using 15 µL of 0.1 % formic acid in nanopure water (according to the In-gel tryptic digestion methods described in this article). Peptide purification and concentration were performed by injection on a reversed phase chromatography (Pierce C18 Spin Columns). A total of 3 µL of sample were transferred to a sample vial for injection using Easy-nLC1200 system. For peptide separation, a PicoChip H354 REPROSIL-Pur C18-AQ 3 µm 120 A (75 µm x 105 mm) chromatographic column (New Objective) was used. The separation was obtained using a total gradient time of 69 min, as follows: of 5%–40% of 0.1% of formic acid in 80% acetonitrile (Buffer B) for 45 min, 40%–95% of Buffer B for 6 min, 5%–95% Buffer B for an additional 12 min, and 95%–5% for 6 min. Flow rate is set at 300 nL/min, and the injection volume is of 2 µL per sample. The Q-Exactive Plus is operated in positive polarity mode and a data-dependent mode. The full scan (MS1) was measured over the range of 400 to 1600 m/z at resolution of 70,000. The MS/MS (MS2) analysis was configured to select the 10 most intense ions for fragmentation over the range of 200 to 2000 m/z. A dynamic exclusion parameter was set for 15 s.

2.5.1 | Database search

The Lc-MS/MS raw data were analyzed with Proteome Discoverer software version 2.1. The samples were searched within two different databases: all samples with a general Torpedo californica database and each sample with its individual protein subunit. They were all downloaded from UniprotKB and saved in a Fasta format that is compatible with the program. The search parameters included trypsin as the enzyme for proteolysis, in which two missed cleavages were allowed with minimal peptide length of 6

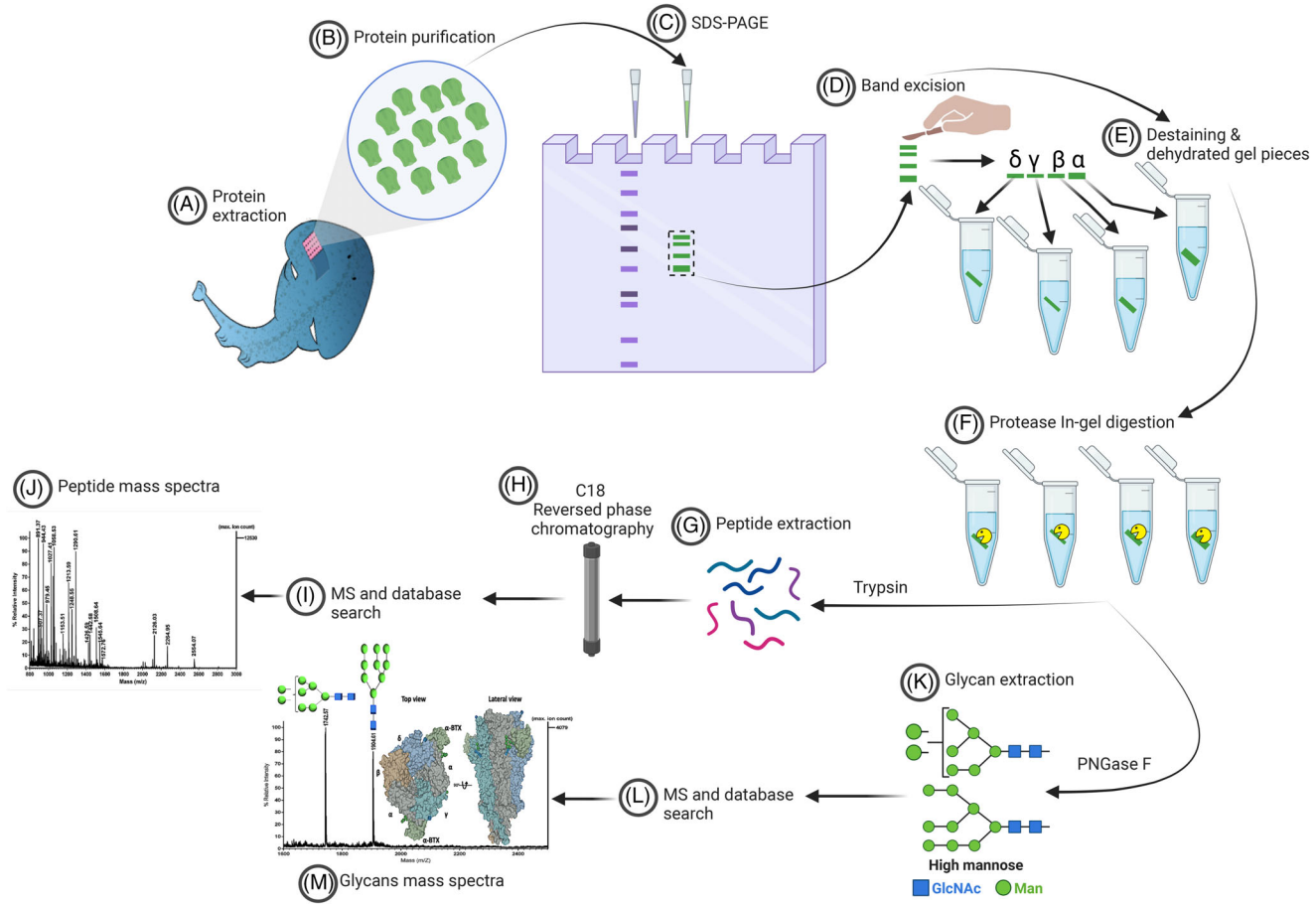

FIGURE 1 The native nicotinic acetylcholine receptor in detergent complex sample preparation. The illustration presents the (A) extraction and solubilization of native-nAChR from the *Tc electroplax* tissue, (B) was followed by the sequential purification chromatography to produce a highly pure, stable and functional nAChR in detergent complex. (C) The protein subunit was separated in an SDS-PAGE. (D) The gel bands corresponding to the nAChR subunits ($\alpha\beta\gamma\delta$) were excised using a sharp scalpel and placed into the Eppendorf tube to be (E) dried and destained. (F) After destaining the gel bands were digested overnight with (G) trypsin for peptide extraction or (K) PNGase F for N-glycans release. (H) The reversed phase chromatography was used to purified and concentrate peptide for each nAChR subunit. (I, L) Finally, the mass spectrometry analysis was performed for (j) peptide mass fingerprinting and (m) glycans profile identification. Created with BioRender.com (accessed on 14 March 2023).

and maximal length of 144. The modifications included were a static carbamidomethyl +57.021 Da I modification and a dynamic oxidation +15.995 Da (Mo) modification. The filters used in the sample analysis were of high confidence for positive and confident protein identification.

2.6 | The in-gel N-glycans release for each of the native-nAChR subunits

The in-gel N-glycans release was performed according to Harvey, D. J. 2009 with the following modification: nAChR-DC was separated in a 4%–20% Criterion TGX Stain-Free Gel and was then stained for 1 h with Coomassie Brilliant Blue R-250 dye [60]. Subsequently, the gel bands corresponding to the nAChR subunits ($\alpha\beta\gamma\delta$) were excised using a sharp scalpel and placed into the Eppendorf tube to destain with a solution containing 25 mM ammonium bicarbonate and 50% acetonitrile at 37°C for 30 min. Afterwards, protein disulfide bond reduction was performed by 20 min incubation at 37°C with 10 mM dithiothreitol in 50 mM ammonium bicarbonate. Then, the cysteine alkylation step was carried out in the dark for 45 min by adding 55 mM iodoacetamide in 50 mM ammonium bicarbonate at room temperature. Subsequently, N-glycans were released from 70 µg of the reduced and alkylated nAChR subunit by an overnight incubation at 37°C with 10 mU PNGase F. The glycans were extracted by vortexing for 15 min in 100 µL of extraction buffer (extraction buffer (1:2 (vol/vol) 5% formic acid/acetonitrile). The glycans extracted using a Speed Vac concentrator were resuspended in 20 µL of 1% formic acid. Mass spectrometry analysis was performed on a 4800 Plus MALDI ToF Analyzer (SCIEX, MA, USA) and mass spectra for glycopeptides were analyzed in the m/z range of 1600−2500 in the linear positive ion mode. Glycans were analyzed using 25 mg of 2,4,6 Trihydroxyacetophenone (THAP) matrix dissolved in 1 mL of 50% acetonitrile and 0.1% TFA. The 1.0 µL of glycans were mixed with 1.0 µL of matrix solution then spotted onto

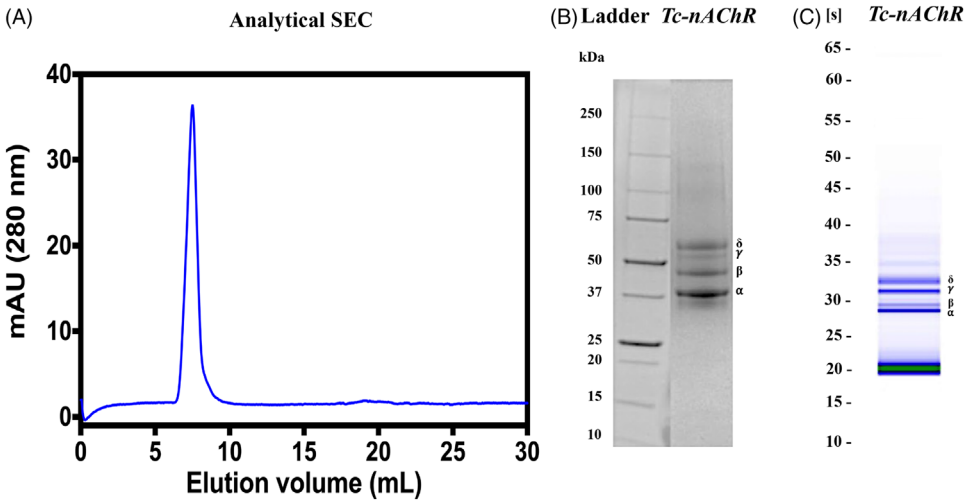

FIGURE 2 Sample preparation of the native nAChR-DC using a sequential purification process. The membrane solubilized is then subjected to the first step of affinity chromatography, eluted with 13 mM Carbamylcholine Chloride at 31.60 mL. Subsequently, the second step involves affinity chromatography with Capto Lentil Lectin, and the nAChR-DC is eluted using a continuous step of 0.2 M methyl α-D-mannopyranoside to 4.27 mL. (A) The last purification step is size exclusion chromatography (SEC), which is performed using a Superdex 200 Increase 10/300 GL gel filtration column, with an elution profile of 7.51 mL. The purity of the sample can be analyzed at each step of purification by (B) SDS-PAGE and by (C) micro-capillary gel electrophoresis, which measures the seconds [s] of protein migration.

an MALDI ToF target plate and allowed to dry at room temperature to co-crystallize through evaporation. Then the estimation of N-linked glycan composition was accomplished by input of the peak masses into the GlycoWorkbench software tool for rapid drawing of glycan structure.

3 | RESULTS

3.1 | Method optimization

In this study, we optimized our purification process for Tc-nAChR and identified a homogenous product with 100% identity for each subunit (Figure 2). To confirm the identity of our new preparation, we used both MALDI ToF/ToF and Nano Lc MS/MS methods. Peptide mass fingerprinting was performed for each subunit to analyze the individual peptides found in Tc-nAChR. For MALDI ToF/ToF analysis, mass spectra were acquired in a reflector positive mode using a 4800 Plus MALDI ToF/ToF Analyzer. Nano Lc MS/MS analysis was conducted on a Q-Exactive, and mass spectra were acquired in a positive polarity mode and a data-dependent mode. The full scan (MS1) was measured over the range of 400 to 1600 m/z and the resolution for higher energy collisional dissociation spectra was set to 70,000. The samples were searched within two different databases: all samples with a general Tc database and each sample with its individual protein subunit. These results demonstrate the successful optimization of our purification process, which allowed for the identification of a highly pure and homogeneous preparation of Tc-nAChR. The use of both MALDI ToF/ToF and Nano Lc MS/MS methods provided a comprehensive analysis of the peptide and glycan composition per subunit (Figures 3 and 4). These methods were effective in identifying each subunit individually and providing a complete analysis of the protein at the molecular level. The successful implementation of our multi-step sequential purification method combined with high-resolution mass spectrometry technologies is a significant contribution to the field of Tc-nAChR research.

3.2 | Ionization and fragmentation of different nAChR subunits for peptide composition

In our study, we aimed to validate and optimize a new purification process for nAChR-DC and characterize it using high-resolution mass spectrometry techniques. Our results demonstrated the successful identification of a homogenous product with 100% identity for each subunit. We utilized both MALDI ToF/ToF and Nano Lc MS/MS methods for peptide mass fingerprinting and confirmed the individual analysis of peptides found in Tc-nAChR for each subunit. To perform the peptide mass fingerprinting analysis, we selected peptides with higher intensity and compared the individual sequences of each subunit with the data provided by Uniprot. The proteolysis described in our methods used trypsin sequencing grade and experimental modifications for the addition of carbamidomethyl were included in the analysis. Our results suggest a 100% identity for the nAChR of Tc, with sequence coverages of 33%, 36%, 26%, and 35% for the subunits $\alpha\beta\gamma\delta$, respectively, using Nano Lc-MS/MS. When using MALDI ToF/ToF, the sequence coverages obtained were 35%, 38%, 26%, and 27%, for the subunits $\alpha\beta\gamma\delta$, respectively (Figure 3). These findings demonstrate the accuracy and reliability of our purification process and the high-resolution mass spectrometry methods used for characterization.

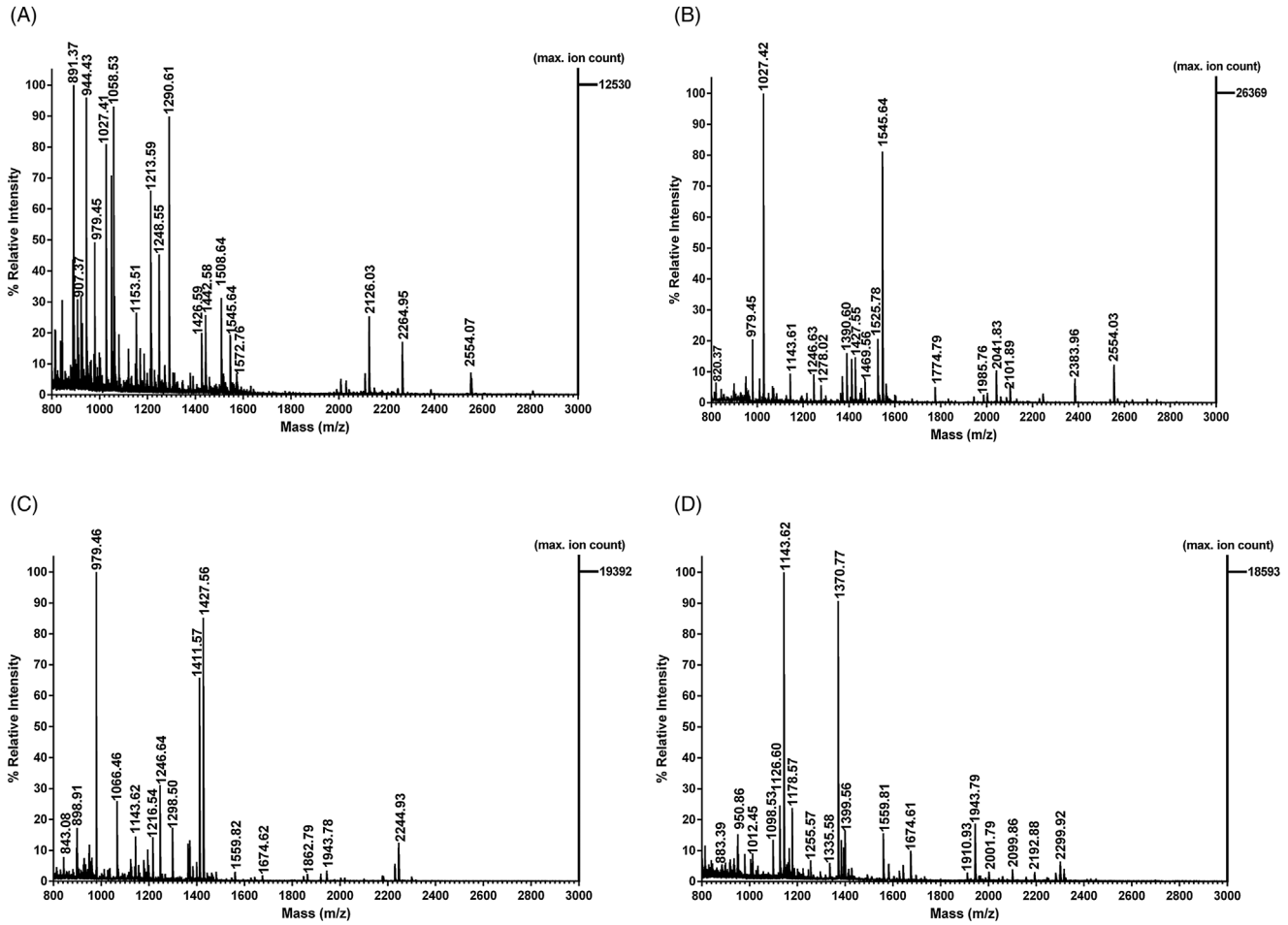

FIGURE 3 Peptide mass fingerprinting analysis of purified Tc-nAChR-DC by subunit. Peptide mass fingerprinting analysis of Tc-nAChR subunits was performed using matrix assisted laser desorption ionization time of flight (MALDI ToF/ToF) spectra. (A) alpha, (B) beta, (C) gamma, and (D) delta subunit spectra were acquired in a reflector positive mode and represent the digestion of nAChR using trypsin MS grade. The spectra were obtained over the full scan range of 800 to 3000 m/z for each subunit and represent high-resolution characterization of peptide fragments.

Overall, our study provides valuable insights into the development of a new purification process for nAChR-DC, which has enabled us to comprehensively analyze the peptide composition of each subunit. This information could contribute to further advancements in our understanding of the structure and function of nAChR and its potential role in metabolic disorders. Moreover, our use of high-resolution mass spectrometry techniques, such as Nano Lc MS/MS and MALDI ToF/ToF, has enabled us to accurately identify the individual peptides in each subunit. This could have significant implications in the identification and diagnosis of various diseases, as peptide alterations have been identified as possible biomarkers.

The following tables (Tables 1–4) provide a detailed breakdown of sequence data for each subunit of the protein obtained through Lc MS/MS analysis. These tables offer comprehensive information on the peptide sequences, including the total coverage of the protein sequence, the number of amino acids (#AAs) present in each peptide, and the molecular weight (MW) of each analyzed peptide.

3.3 | Ionization and fragmentation of different nAChR subunits for glycans composition

The identification of glycans in proteins such as nAChR is crucial to understanding their biological function and potential implications in diseases. In this study, we performed the glycan identification for each subunit of nAChR using the in-gel N-glycans release method, which allows for the analysis of glycans directly from the protein gel bands. This technique is advantageous as it eliminates the need for purification of glycans, which can be time-consuming and challenging due to their structural diversity and complexity. The in-gel N-glycans release was performed using the PNGase F enzyme, which cleaves N-glycans from the protein backbone. The released glycans were then analyzed using MALDI ToF/ToF equipment, which provides high sensitivity and resolution for mass spectrometry analysis. THAP matrix dissolved in acetonitrile and TFA was used for the analysis, which provides good peak resolution and sensitivity for oligomannose glycans. The estimation of N-linked glycan composition was

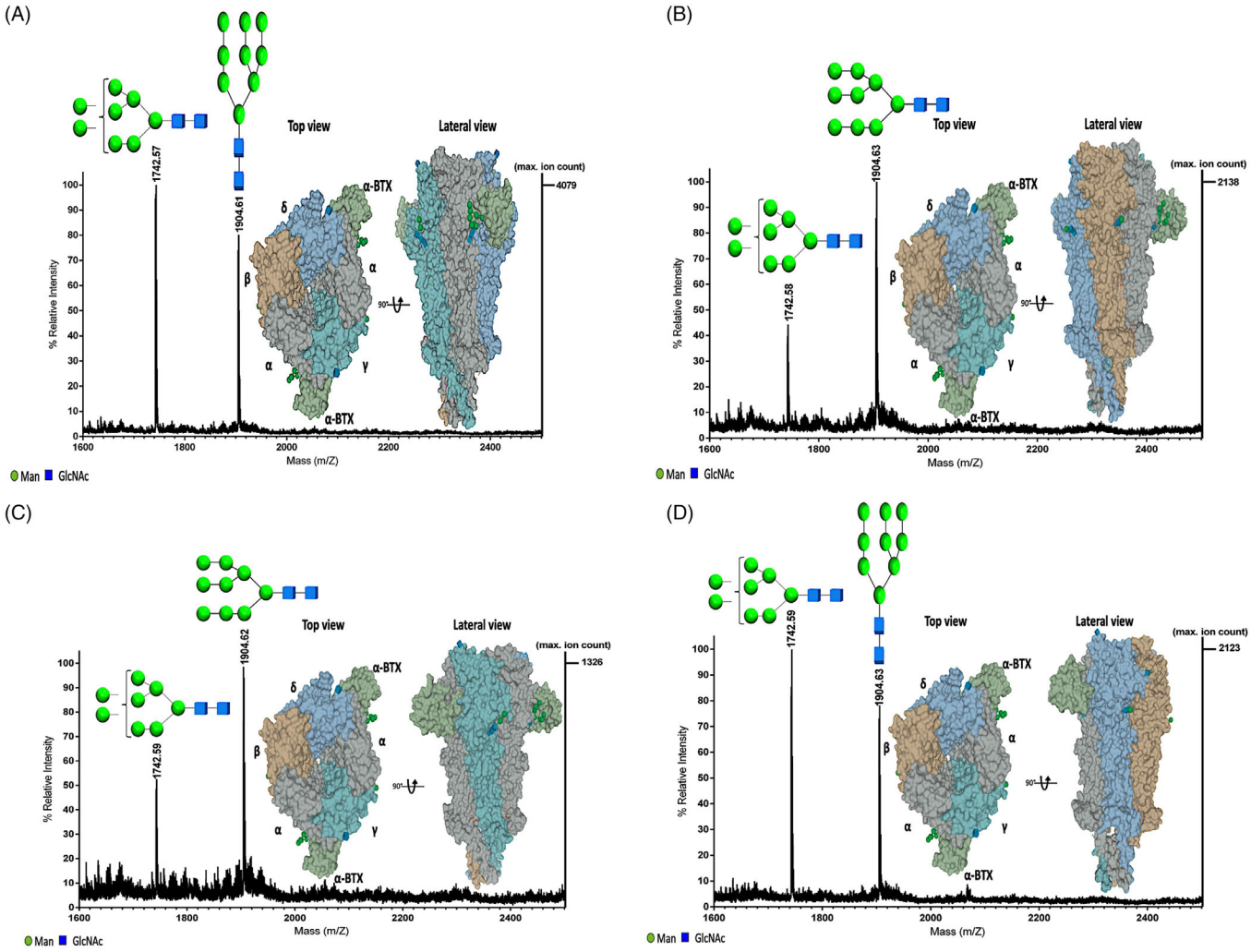

FIGURE 4 MALDI-ToF/ToF glycan profile of purified *Tc*-nAChR-DC by subunit. The spectra represent the in-gel N-glycans released by PNGase F treatment. The subunits (A) alpha, (B) beta, (C) gamma, and (D) delta spectra for glycan analysis. Symbols: •mannose & ■N-acetylglucosamine. N-glycans were released from 70 µg of the reduced and alkylated nAChR subunit by overnight incubation at 37°C with 10 mU PNGase F. The image of *Tc* nAChR structure at 2.69 Å resolution (PDB: 6UWZ) was created with library and tools for web molecular graphics MolVA / EuroVis Proceedings and RCSB PDB [62].

accomplished using the GlycoWorkbench software tool, which allows for the rapid drawing of glycan structures and the identification of the most abundant glycans. Our results show that the most abundant glycans in nAChR subunits are the oligomannose populations Man8-9GlcNac2, with peaks at m/z 1742 and 1904, respectively ([M + Na]+ ion for each nAChR subunit) (Figure 4). These results are consistent with previous studies on nAChR glycosylation, which suggest that oligomannose glycans are the predominant glycan structures in nAChR [30, 61]. Overall, our study provides valuable information on the glycan composition of nAChR subunits, which could lead to further advances in the understanding of glycosylation and its implications in disease. The use of high-resolution mass spectrometry techniques combined with in-gel N-glycans release and software tools such as GlycoWorkbench is a powerful approach for glycan identification and analysis.

4 | DISCUSSION AND CONCLUSION

The successful purification of *Tc*-nAChR-DC using the multi-step sequential purification protocol developed in our laboratory is a significant achievement as, historically, the preparation of nAChR-DC in its native state has been associated with numerous issues that affect the purity, functionality, and integrity of the protein [16, 18–20, 22, 23, 33–39, 63, 64]. Our purification method was validated through the use of two powerful analytical techniques, MALDI ToF/ToF and Nano Lc MS/MS, which identified 100% identity for each native subunit of *Tc*-nAChR-DC after purification (Figure 3). Peptide mass fingerprinting analysis was performed using MALDI ToF/ToF and Lc MS/MS, with each subunit digested separately and analyzed individually for peptide identification. This method allowed us to detect 105 target peaks for the nAChR peptide mass fingerprinting, which were confidently identified

TABLE 1 Subunit alpha: The peptide sequences, coverage, number of amino acids, and molecular weights of the identified peptides in the subunit alpha of the protein.

Description	Coverage	# AAs	MW [kDa]
Acetylcholine receptor subunit alpha OS=Tetronarce californica OX=7787 GN=CHRNA1 PE=1 SV=1	33	461	52.7
Sequence	**Modifications**	**# Missed Cleavages**	**Theo. MH+ [Da]**
QVTGEVIFQTPLIKNPDVK	N/A	1	2126.1
SPSTHTMPQWVR	N/A	0	1426.6
LRWNPADYGGIKK	N/A	2	1517.8
LRWNPADYGGIK	N/A	1	1389.7
LGIWTYDGTK	N/A	0	1153.5
SPSTHTMPQWVRK	N/A	1	1554.7
EKQENKIFADDIDISDISGK	N/A	2	2265.1
SAIEGVKYIAEHMK	N/A	1	1575.8
NPDVKSAIEGVK	N/A	1	1256.6
WNPADYGGIKK	N/A	1	1248.6
LVANLLENYNK	N/A	0	1290.7
QENKIFADDIDISDISGK	N/A	1	2007.9
LRQQWIDVR	N/A	1	1213.6
SDEESSNAAEEWK	N/A	0	1481.6
IFADDIDISDISGK	N/A	0	1508.7
QQWIDVR	N/A	0	944.4
LLLDYTGK	N/A	0	922.5
WNPADYGGIK	N/A	0	1120.5
SAIEGVK	N/A	0	703.3
SPSTHTMPQWVR	1xOxidation [M7]	0	1442.6
QQWIDVRLR	N/A	1	1213.6
IMWTPPAIFK	1xOxidation [M2]	0	1219.6
DYRGWK	N/A	1	824.4
LIELSQEG	N/A	0	888.4
YIAEHMK	N/A	0	891.4

using Proteome Discoverer software version 2.1 in the mass spectrometry analysis. The identification of these peaks included representative sequences to identify various important peptides with biological relevance in the *Tc*-nAChR. For example, we identified the peptide 824.35 of the alpha subunit as a necessary element for the ligand binding of α-neurotoxins, corresponding to the C-loop site [65, 66]. Additionally, peptide 1120.47 was identified as an essential peptide for the main immunogenic region of nAChRs [67]. We also identified a segment located between M3 and M4, which is part of the cytoplasmic domain, with the mass (m/z) of 1575.72 and 1256.57 (Table 1,2,3, and 4) [67].

The identification and characterization of these specific peptides hold paramount importance in advancing our understanding of the molecular and proteomic aspects of *Tc*-nAChR function and structure. The cytoplasmic domain, known for its pivotal role in receptor signaling and regulation, is particularly relevant in elucidating the intricate mechanisms underlying nAChR activity. In our study, we introduced a sequential purification process for *Tc*-nAChR, which yielded remarkable improvements in purity levels compared to conventional purification methods. With an average purity of 94%, the enhanced purification process showcases its effectiveness in isolating the target protein with enhanced integrity and reduced contamination [23, 68]. However, utilizing the highly sensitive Nano Lc MS/MS technology, we detected a minor fraction of impurities at 112 kDa (For more detail see Figure S1). Through detailed analysis, these impurities were identified as the Sodium/potassium-transporting ATPase subunit alpha, as evidenced by the data presented in the Supporting information material. This intriguing finding suggests a potential association or interaction between the ATPase and the nAChR complex in the *Tc* electroplax. The Na+/K+-ATPase enzyme complex, of which the ATPase subunit alpha is an integral part, comprises multiple subunits responsible for ATP hydrolysis and subsequent ion transport. These subunits are highly

TABLE 2 Subunit beta: The peptide sequences, coverage, number of amino acids, and molecular weights of the identified peptides in the subunit beta of the protein.

Description	Coverage	# AAs	MW [kDa]
Acetylcholine receptor subunit beta OS=Tetronarce californica OX=7787 GN=CHRNB1 PE=1 SV=1	**36**	**493**	**56.1**
Sequence	**Modifications**	**# Missed Cleavages**	**Theo. MH+ [Da]**
ANDEYFIRKPAGDFVCPVDNAR	1xCarbamidomethyl [C16]	1	2554.2
EVKEIVINKDAFTENGQWSIEHKPSR	N/A	2	3054.5
SYTYDTSEVTLQHALDAKGER	N/A	1	2384.1
KPAGDFVCPVDNAR	1xCarbamidomethyl [C8]	0	1545.7
EIVINKDAFTENGQWSIEHKPSR	N/A	1	2698.3
EIVINKDAFTENGQWSIEHKPSRK	N/A	2	2826.4
SYTYDTSEVTLQHALDAKGEREVK	N/A	2	2740.3
YIAEQLESASEFDDLKK	N/A	1	1985.9
DAFTENGQWSIEHKPSR	N/A	0	2001.9
SYTYDTSEVTLQHALDAK	N/A	0	2041.9
LQWDPAAYEGIK	N/A	0	1390.7
GEREVKEIVINK	N/A	2	1413.8
QIFIETLPPFLWIQRPVTTPSPDSKPTIISR	N/A	0	3577.9
ANDEYFIR	N/A	0	1027.4
DAFTENGQWSIEHKPSRK	N/A	1	2130.0
SPNTHTMPNWIR	N/A	0	1453.7
VRPAQTVGDKVTVR	N/A	1	1525.8
LQWDPAAYEGIKDLR	N/A	1	1774.9
LFSEMK	N/A	0	754.3
KPAGDFVCPVDNARVAVQPERLFSEMK	1xCarbamidomethyl [C8]	2	3060.5
VAVQPER	N/A	0	798.4
EIVINK	N/A	0	715.4
EAVEAIK	N/A	0	759.4

expressed in excitable tissues, including nerve and muscle cells, where their activities are crucial for proper cellular functioning and regulates synaptic efficacy [69, 70]. By uncovering these additional insights into the potential cross-talk and interplay between different protein complexes in *Tc*, our study contributes to a deeper understanding of the intricate molecular mechanisms underlying the neuromuscular system of this species.

Given the intriguing association between the ATPase subunit alpha and the nAChR complex in *Tc*, it is plausible to speculate on the potential implications of this interaction. One possible hypothesis is that the ATPase subunit alpha may play a regulatory role in the activity or assembly of the nAChR complex. The Na+/K+-ATPase enzyme complex, to which the ATPase subunit alpha belongs, is known to be involved in maintaining ion gradients across the cell membrane, particularly in excitable tissues such as nerve and muscle cells in rats [69, 71]. It is conceivable that the presence of the ATPase subunit alpha within the *nAChR* complex could contribute to the fine-tuning of ion flux and homeostasis in the muscle electroplax [72]. Furthermore, considering that the cytoplasmic domain of nAChR is crucial for receptor signaling and regulation, the interaction with the ATPase subunit alpha may influence downstream signaling pathways or modulate the receptor's response to neurotransmitter binding. This potential interplay between the ATPase subunit alpha and nAChR in *Tc* may thus contribute to the precise control and coordination of neuromuscular activity in this species. However, further investigation and experimentation are necessary to fully elucidate the exact nature and functional consequences of this interaction. Detailed studies involving mutagenesis, functional assays, and structural analysis could provide deeper insights into the mechanistic aspects of the ATPase subunit alpha and nAChR complex association in *Tc*.

Previous identification studies have been limited to low purity levels or expressed fragments of Tc-nAChR and have not been performed on highly purified nAChR samples per subunit using the new high-resolution mass spectrometry technologies [73–75]. The only documented oligosaccharide study on Tc-nAChR using mass spectrometry was conducted in 1989 and 2002, and at low resolution [75, 76].

TABLE 3 Subunit gamma: The peptide sequences, coverage, number of amino acids, and molecular weights of the identified peptides in the subunit gamma of the protein.

Description	Coverage	# AAs	MW [kDa]
Acetylcholine receptor subunit gamma OS=Tetronarce californica OX=7787 GN=CHRNG PE=1 SV=1	**26**	**506**	**58.1**
Sequence	**Modifications**	**# Missed Cleavages**	**Theo. MH+ [Da]**
YLGMQLEPSEETPEKPQPR	1xOxidation [M4]	0	2245.0
YLGMQLEPSEETPEKPQPR	N/A	0	2229.0
SELMFEEQKDR	N/A	1	1411.6
LIEKLLGDYDKR	N/A	2	1462.8
KPRSELMFEEQKDR	N/A	2	1792.9
SELMFEEQKDR	1xOxidation [M4]	1	1427.6
KNYNWQLTK	N/A	1	1194.6
KPRSELMFEEQKDR	1xOxidation [M7]	2	1808.8
SELMFEEQKDRHGLK	1xOxidation [M4]	2	1862.9
SELMFEEQKDRHGLK	N/A	2	1846.9
SSFGIMIKAEEYILKKPR	1xOxidation [M6]	2	2126.1
TLDHIIDVTLK	N/A	0	1267.7
LLGDYDKR	N/A	1	979.5
AEEYILKKPR	N/A	1	1246.7
EQNDSGSENENWVLIGK	N/A	0	1918.8
SCVEACNFIAKSTK	2xCarbamidomethyl [C2; C6]	1	1614.7
STKEQNDSGSENENWVLIGK	N/A	1	2235.0
SCVEACNFIAK	2xCarbamidomethyl [C2; C6]	0	1298.5
NYNWQLTK	N/A	0	1066.5
AEEYILK	N/A	0	865.4
SELMFEEQK	N/A	0	1140.5
HLFLGFLPK	N/A	0	1071.6
DLANFAPEIK	N/A	0	1117.5
LLGDYDK	N/A	0	823.4
SSFGIMIK	N/A	0	882.4
SELMFEEQK	1xOxidation [M4]	0	1156.5

In the 1989 study, as well as the 2002 study, the sample preparations followed classic extraction and purification protocols that are now known to affect protein function due to the use of certain detergents and chromatography column types. For instance, both studies employed Triton-X, and the 2002 study additionally used Chaps and n-octylglucoside to identify and analyze impurities in purified extracts of Tc-nAChR. However, it is known that these detergents have a potential impact on Tc-nAChR function [18]. This suggests that the results from those studies only provide a tentative characterization of a protein that is no longer in its native and functional state. In contrast, our current research has adopted a different approach by focusing on the use of the LFC-16 detergent in our preparations, aiming to mimic the most appropriate lipid environments. This specific detergent has been previously investigated by our team, and we have observed its effects on Tc-nAChR function [18, 23, 77]. Interestingly, LFC-16 possesses a carbon chain structure similar to the major lipids found in native unsolubilized membranes, providing a unique opportunity to extract the protein while minimizing detrimental effects on its function and preserving it in its native state [20]. By biomimicking the appropriate lipid environments, we aim to create a more physiologically relevant setting for Tc-nAChR purification, which enhances the preservation of its functional properties. This approach allows us to obtain a more accurate understanding of the protein's behavior and characteristics in its native context, opening doors for further studies exploring its structure-function relationship and interactions with other components of the cellular membrane.

Moreover, by using LFC-16, we aim to evaluate its compatibility with Tc-nAChR purification and determine if it provides advantages over other detergents in terms of preserving the native structure and functionality of the receptor. By comparing the findings from the

TABLE 4 Subunit delta: The peptide sequences, coverage, number of amino acids, and molecular weights of the identified peptides in the subunit delta of the protein.

Description	Coverage	# AAs	MW [kDa]
Acetylcholine receptor subunit delta OS=Tetronarce californica OX=7787 GN=chrnd PE=1 SV=1	**35**	**522**	**59.9**
Sequence	**Modifications**	**# Missed Cleavages**	**Theo. MH+ [Da]**
IGFGNNNENIAASDQLHDEIKSGIDSTNYIVK	N/A	1	3476.6
EKNAYDEEVGNWNLVGQTIDR	N/A	1	2450.1
ADESEQPDWQNDLKLRR	N/A	2	2100.0
NAYDEEVGNWNLVGQTIDR	N/A	0	2193.0
ADESEQPDWQNDLKLR	N/A	1	1943.9
SRSELMFEKQSER	N/A	2	1626.7
SRSELMFEKQSER	1xOxidation [M6]	2	1642.7
ADESEQPDWQNDLK	N/A	0	1674.7
IGFGNNNENIAASDQLHDEIK	N/A	0	2299.0
LPETALAVPLIGK	N/A	0	1321.8
VKQIFLEKLPR	N/A	2	1370.8
SELMFEKQSER	N/A	1	1383.6
FTALNYDANEITMDLMTDTIDGK	2xOxidation [M13; M16]	0	2624.1
LINDLLIVNKYNK	N/A	1	1559.9
SELMFEKQSER	1xOxidation [M4]	1	1399.6
QIFLEKLPR	N/A	1	1143.6
ILHMSRADESEQPDWQNDLK	1xOxidation [M4]	1	2428.1
SRSELMFEK	N/A	1	1126.5
AQEYFNIKSR	N/A	1	1255.6
TPSTHVLSTR	N/A	0	1098.5
SGIDSTNYIVK	N/A	0	1196.6
SELMFEKQSERHGLVPR	1xOxidation [M4]	2	2059.0
SGIDSTNYIVKQIK	N/A	1	1565.8
SGIDSTNYIVKQIKEK	N/A	2	1822.9
SRSELMFEK	1xOxidation [M6]	1	1142.5
ILHMSRADESEQPDWQNDLK	N/A	1	2412.1
LINDLLIVNK	N/A	0	1154.7
RSSSVGYISK	N/A	1	1083.5
SSSVGYISKAQEYFNIK	N/A	1	1920.9
FTALNYDANEITMDLMTDTIDGK	1xOxidation [M]	0	2608.1
VKQIFLEK	N/A	1	1004.6
SELMFEK	N/A	0	883.4
AQEYFNIK	N/A	0	1012.5
SELMFEK	1xOxidation [M4]	0	899.4
SSSVGYISK	N/A	0	927.4
HGLVPR	N/A	0	678.4

1989, 2002 studies and our current research, we can gain a comprehensive understanding of the impact of different detergents on Tc-nAChR purification and function. This knowledge is crucial for future studies, particularly those focusing on structural elucidation and functional characterization, where the choice of detergent can significantly influence the outcomes obtained through high-resolution mass spectrometry and other analytical techniques. Additionally, they only performed MALDI ToF without peptide identification by subunit or its impurities. However, the findings presented in this study showcase the attainment of exceptionally high levels of purity in native Tc-nAChR preparations using detergent analogs that mimic lipid environments, while simultaneously preserving the functional integrity of the receptor. This achievement holds significant implications for future endeavors in structural studies, as it enables a direct comparison between atomic structures and data acquired through high-resolution mass spectrometry. Moreover, our research capitalizes on the utilization of cutting-edge MS/MS techniques, allowing for a more accurate and precise identification of peptides and their modifications compared to previous studies [75, 76]. This enhanced identification capability not only contributes to a more comprehensive understanding of the structural aspects but also paves the way for in-depth investigations into the functional dynamics of Tc-nAChR. By leveraging these advancements, we are poised to unlock a wealth of information pertaining to the structural intricacies and functional mechanisms of Tc-nAChR. This comprehensive knowledge will prove invaluable for future studies, enabling researchers to delve deeper into the complex interplay between the Tc-nAChR structure and function.

On the other hand, we confirmed the presence of glycans in our sample through the N-glycans release experiment (Figure 4). Specifically, we identified the oligommanose population $Man_{8\text{-}9}GlcNac_2$ with peaks at m/z 1742 and 1904 ([M + Na]+ ion), which was in accordance with previous studies that showed similar receptors possess high-mannose glycans [30, 61]. Our results demonstrated that all *Tc*-nAChR subunits purified by our multi-step sequential purification protocol produce only high-mannose glycans $Man_{8\text{-}9}GlcNac_2$ [76]. The incorporation of glycoprotein purification in our multi-step sequential purification methods was facilitated by the fact that our glycoprotein is rich in mannose, which has a high binding affinity in the Capto Lentil Lectin affinity resin. This resin was implemented in our purification process, which allowed for the recognition of glycoproteins with mannoses in their structure [22, 23]. It is worth noting that the deglycosylation of the extracellular ligand-binding domain in *Tc*-nAChR has been shown to improve the diffraction quality of crystals, indicating that glycosylation may be an impediment to crystallization [78]. However, glycosylation plays an important role in the structural stability of nAChR, as highlighted by Corrie J.B. et al., [40] Therefore, understanding the glycosylation patterns of nAChR is crucial for the preparation of samples for structural studies. Overall, the successful purification of *Tc*-nAChR-DC using our laboratory's multi-step sequential purification protocol represents a significant achievement. The subsequent identification and characterization of the purified protein were conducted using advanced analytical instruments such as the 4800 Plus MALDI ToF/ToF Analyzer (SCIEX, MA, USA) and Q-exactive plus Nano Lc MS/MS System. These findings provide new insights into the structure and function of nAChR's and their role in organismal function. The characterization of the N-linked glycans in *Tc*-nAChR subunits may have important implications for drug development and therapeutic interventions targeting this important protein family.

Moreover, two studies utilizing proton nuclear magnetic resonance spectroscopy (NMR) were conducted to analyze glycans in Tc-nAChR, both of which involved digestion with endo-β-N-acetylglucosaminidase H (Endo H). The first study, conducted in 1986, revealed that Tc-nAChR exhibits a high-mannose type with the structures $Man_{8\text{-}9}GlcNac_2$, which are similar to our findings [61]. However, in 1992, the same group, performed a subsequent proton NMR analysis of Tc-nAChR [79]. This article provides a comprehensive analysis of the oligosaccharides present in the Tc-nAChR. Various types of oligosaccharides, including those with sialic acid residues, were identified through the utilization of chromatography and NMR techniques. The analysis revealed variations in the quantity of galactose residues and the presence or absence of fucose within the oligosaccharides. In contrast to these studies, our approach involved the implementation of MALDI-ToF techniques for rapid and high-throughput characterization of the composition and mass of the glycans present in Tc-nAChR, thereby validating the glycan subunit composition and confirming that they were not affected during our sequential purification process. However, glycan studies using proton NMR, as was the case in these articles, are required for a detailed understanding of the three-dimensional structural aspects and interactions of the glycans.

Is important to mention that the proton NMR studies employed traditional purification processes that involved the use of destructive detergents such as Triton-X, which have been shown to negatively impact Tc-nAChR stability and function [18]. Consequently, the glycan results obtained from these studies does not provide a native representation of a functional protein. Our approach utilizes lipid-analog detergent systems that effectively preserve the receptor's functionality, thus revealing the glycan profile of Tc-nAChR, as it would exist in a native membrane environment. By employing detergent analogs mimicking lipid-like properties, our results present a more realistic and reliable representation of the glycan composition associated with a fully functional Tc-nAChR. This crucial distinction enhances the validity of our data, as it aligns more closely with the glycan profile exhibited in its native membrane context.

Our findings, supported using lipid-analog detergents, represent a significant leap forward in achieving a more faithful depiction of a functional *Tc*-nAChR protein, enabling a clearer and more comprehensive identification of the peptides and glycan landscape associated with *C* in its native membrane state. The successful production of a highly pure *Tc*-nAChR-DC has paved the way for more robust investigations, including high-throughput crystallization experiments, which hold the potential to yield additional insights into the intricate structure and dynamic function of this protein. Moreover, the identification and characterization of N-linked glycans in *Tc*-nAChR subunits bear substantial implications for drug development and therapeutic interventions

targeting this protein family. The presence of high-mannose N-glycans, particularly the oligomannose population $Man_{8-9}GlcNac_2$, in all native *Tc*-nAChR subunits underscores the critical role of glycosylation in the proper functioning of these proteins (For more detail of sequencing see Figure S2). Collectively, our study underscores the need for continuous research endeavors aimed at comprehending the intricate biology of Cys-loop pentameric ligand-gated ion channels and their involvement in organismal function and disease.

AUTHOR CONTRIBUTIONS

Rafael Maldonado-Hernández (R.M.H.), Orestes Quesada (O.Q.), José A. González-Feliciano (J.A.G.F.), Abel Baerga-Ortiz (A.B.O.) and José A. Lasalde-Dominicci (J.A.L.D.) designed the experiments and optimized the protocol. R.M.H., J.A.G.F., O.Q., and A.B.O. performed the experiments and contributed to the data analysis. R.M.H. wrote the initial draft. R.M.H., O.Q., and J.A.L.D. edited and completed the final version of the manuscript.

ACKNOWLEDGEMENTS

This research was supported by The National Institutes of Health (NIH) with the grant number 1R01GM098343 and the National Institute of General Medical Sciences (NIGMS) under award number P20GM103642 (J.A.L.D. and R.M.H.); COBRE 1P20GM103642 (J.A.L.D. and R.M.H.). We are also grateful for the invaluable research infrastructure and services provided, in part, by the grant U54-MD007600 from the National Institute on Minority Health and Health Disparities (NIMHD), UPR-RP RISE Program (5R25GM061151-18) (O.Q. and R.M.H.), the PR-INBRE program supported by an Institutional Development Award (IDeA) from NIGMS under grant number P20GM103475, and BioXFEL STC of the National Science Foundation (NSF) Grant: 1231306 (R.M.H.). Furthermore, we acknowledge additional support from NIH grant 1S10OD025143-01A1, which was instrumental in acquiring the MALDI-ToF/ToF instrument. Also, the authors extend their sincere gratitude to Mallerie Carrasquillo-Rivera for her assistance during this research endeavor.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data associated with this article can be accessed at https://data.mendeley.com/datasets/zbfxf8kwvn/1, which is located in the Supporting information section at the end of the article.

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How to cite this article: Maldonado-Hernández, R., Quesada, O., González-Feliciano, J. A., Baerga-Ortiz, A., & Lasalde-Dominicci, J. A. (2024). Identification of the native *Torpedo californica* nicotinic acetylcholine receptor's glycan composition after a multi-step sequential purification method using MALDI-ToF MS. *Proteomics*, *24*, e2300151. https://doi.org/10.1002/pmic.202300151