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Dissecando a patogênese da doença de Chagas através de abordagens glicômicas e glicoproteômicas

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Dissecting the pathogenesis of Chagas disease by deep glycomics and glycoproteomics approaches

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RESUMO

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Trypanosoma cruzi é um protozoário unicelular responsável pela doença tropical negligenciada (DTN) denominada doença de Chagas (tripanossomíase americana), uma doença endêmica em 21 países da América do Sul e Central. A doença de Chagas também se apresenta como uma preocupação emergente de saúde global com casos reportados na América do Norte, Europa, Japão e Austrália. A doença pode apresentar diferentes formas clínicas e as opções de tratamentos disponíveis, Benznidazol e Nifurtimox, são limitadas devido à alta toxicidade, efeitos colaterais e reduzida eficácia do tratamento devido à resistência apresentada pelos parasitas. As cepas de *T. cruzi* são geneticamente diversas com implicações na patogenicidade e virulência, progressão e desfecho da doença, além da susceptibilidade/resistência a drogas. Nesta tese, será apresentada a aplicação da espectrometria de massas para elucidar os principais aspectos moleculares de *T. cruzi*, incluindo: **1)** modulação sistemática do proteoma entre estágios e entre diferentes cepas e espécies de parasitas e **2)** modulação de modificações pós-traducionais (PTMs) de *T. cruzi*. Relativo à parte proteômica, será apresentado **A)** A modulação de proteínas de membrana da cepa CL14 de *T. cruzi* durante a progressão da fase exponencial para a fase estacionária a fim de elucidar as mudanças moleculares durante os estágios iniciais de metaciclogênese e **B)** análise global de perfis de expressão de proteínas entre cepas de *T. cruzi* e espécies de tripanossomas intimamente relacionadas. A análise de PTMs por espectrometria de massas dará enfoque **A)** na modulação da S-nitrosilação de proteínas em tripomastigotas seguida pela incubação com a matriz extracelular do hospedeiro, **B)** na análise global das mudanças conformacionais de glicoproteínas e **C)** no mapeamento do glicoproteoma (N- e O-ligados), glicoma (N- e O-glicanos) e suas respectivas expressões diferenciais entre as cepas de *T. cruzi* e espécies de tripanossomas intimamente relacionadas. Considerando todos os achados, esta tese mostra a importância da proteômica no estudo das mudanças moleculares na expressão de proteínas e modificações pós-traducionais durante a interação patógeno-hospedeiro. Especificamente, os métodos desenvolvidos e implementados neste estudo serão úteis para a comunidade científica para estudar, além de infecções pelo *T. cruzi*, outros sistemas biológicos. Finalmente, este trabalho elucida o papel de proteínas específicas e modificações pós-traducionais, alvos para o diagnóstico e terapia da doença de Chagas.

Palavras-chave:

Trypanosoma cruzi, Proteômica, Modificações Pós-Traducionais, Glicoproteômica, glicômica, S-nitrosilação

ABSTRACT

Mule S. N. Dissecting the pathogenesis of Chagas disease by deep glycomics and glycoproteomics approaches, Thesis (PhD) – Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, 2021.

Trypanosoma cruzi is a unicellular protozoan parasite responsible for the neglected tropical disease (NTD) termed Chagas disease (American Trypanosomiasis), a disease endemic in 21 South and Central American countries. Chagas disease is also an emerging global health concern with cases reported in Northern America, Europe, Japan, and Australia. The disease presents in variable clinical forms, and the treatment options available, Benznidazole and Nifurtimox are limited by high toxicities, side effects and decreasing treatment efficiency due to resistance by *T. cruzi* parasites. *T. cruzi* strains are genetically diverse, with implications on pathogenicity and virulence, disease progression and outcome, and drug susceptibility/resistance. In this thesis, the application of mass spectrometry to elucidate key *T. cruzi* molecular aspects will be presented, including: 1) system-wide modulation of the proteome between growth stages and between different parasite strains and species, and 2) modulation of *T. cruzi* posttranslational modifications (PTMs). For the proteomics part, **A**) the modulation of membrane proteins of *T. cruzi* CL14 strain during progression from exponential to stationary growth phases to elucidate the molecular changes during the early stages of metacyclogenesis, and **B**) the systems-wide protein expression profiles between *T. cruzi* strains and closely related trypanosome species will be presented. Analysis of PTMs by mass spectrometry will focus on: **A**) the modulation of *T. cruzi* trypomastigote protein S-nitrosylation following incubation with host extracellular matrix, **B**) the systems-wide analysis of glycoprotein conformational changes, and **C**) the mapping of intact N- and O-linked glycoproteomes and N- and O-glycomes, and their differential expression between *T. cruzi* strains and closely related trypanosome species. Taken together, this thesis shows the importance of proteomics in studying the molecular changes in protein expression and PTMs during host-pathogen interaction. Specifically, the methods developed and implemented in this thesis will be useful for the scientific community to study not only *T. cruzi* infections but also other biological systems. Finally, this thesis sheds new lights on the role of specific protein and PTMs targets for Chagas disease diagnostic and therapy.

Key words:

Trypanosoma cruzi, Proteomics, Post translational modifications, Glycoproteomics, Glycomics, S-nitrosylation.

1. OVERVIEW

Trypanosomes regulate their gene expression post-transcriptionally through trans-splicing and polyadenylation of polycystronic mRNAs generated by of RNA polymerase II (1-3). The low correlation between trypanosome mRNA and protein levels validates proteomics for system-wide gene expression studies at protein level in trypanosomes. Coined by Marc Wilkins, the term 'Proteome' describes the entire protein complement of a cell, tissue and organism expressed at a specific time and space (4), and the study of the proteome is termed proteomics. The first *T. cruzi* proteomics study by Paba et al. in 2004 (5) combined two-dimensional gel electrophoresis (2-DE) followed by MALDI-TOF MS peptide mass fingerprinting to map and identify differential protein expression between the epimastigote, amastigote and trypomastigote developmental stages of *T. cruzi* parasites. In this study, Paba and colleagues identified 19 proteins from 26 2-DE spots including heatshock proteins, enzymes in the glycolytic pathway, tubulin, paraflagellar rod proteins and elongation factors (5). Another study applied isotopic labelling-based quantitative proteomic approach to evaluate the remodeling of the proteome during amastigogenesis of *T. cruzi* Berenice, where a total of 41 proteins were identified and quantified, with 9 and 3 proteins upregulated in the trypomastigote and amastigote stages, respectively (6). Etwood and colleagues employed large scale proteomics of the four developmental stages of *T. cruzi* CL Brener; epimastigotes, metacyclic trypomastigotes, amastigotes and bloodstream trypomastigotes, were they identified and quantified a total of 2784 proteins, with trans-sialidases, mucins and MASPs preferentially detected in the trypomastigote stage, and several HSPs, kinases and proteins involved in the trafficking from ER to Golgi were identified exclusively in the amastigote life stage (7).

Proteomics analysis have also been employed to decipher mechanisms of drug resistance in *T. cruzi* by exposing the parasites to different chemotherapeutic drugs and identifying and quantifying differentially modulated proteins and pathways. Andrade and colleagues analyzed the proteomes of benznidazole-resistant and susceptible *T. cruzi* strains using 2-DE and mass spectrometry (8), identifying differentially expressed protein spots between the resistant and susceptible trains. Among the proteins identified in the resistant strains included peroxiredoxins and iron superoxide dismutase, indicating a mechanism of removal of oxidative by-products generated by drug metabolism (8). Another study used 2-DE and mass spectrometry to analyze the proteomes of *T. cruzi* Y and Bolivia strains incubated with piplartine, a secondary metabolite from Piper longum Linn fruit, where,

in both strains, the overexpression of tryparedoxin peroxidase and methionine sulfoxide reductase enzymes was observed. These proteins are involved in protection against oxidative stress, and were proposed as candidates for future therapeutic targets (9).

Proteomics studies have been applied in the site-specific mapping of novel post translational modifications. A large-scale mass spectrometry-based proteomics was applied to *T. cruzi* Sylvio X10/1 strain, and a previously uncharacterized PTM termed protein arginylation was mapped for the first time in a trypanosome using unrestrictive PTM search algorithm (10). Another application of proteomics in *T. cruzi* studies was the correlation between levels of protein expression in different *T. cruzi* strains and their genetic heterogeneity. Telleira and colleagues used 2-DE and mass spectrometry to map the proteomes of several *T. cruzi* and the closely related *T. c. marinkellei* strains, and performed a hierarchical clustering which was compared to a MLEE (multilocus enzyme electrophoresis) based clustering, demonstrating a high correlation between *T. cruzi* phylogenetics and levels of protein expression (11). Proteomics studies have also been used to study host-parasite interaction, pathogenicity, parasite growth and differentiation, and subcellular protein expression profiling have been described (12-19).

In the first part of this thesis, mass spectrometry-based analysis of the molecular changes during transition of *T. cruzi* CL14 epimastigote cells from the exponential to the stationary growth phase was performed (20). This study identified and quantified approximately 3000 proteins between the two growth stages, and showed that ribosomal proteins were upregulated in the exponential stage, while autophagy-related proteins were upregulated in the stationary stage, indicating the early onset of metacyclogenesis. The expression profiles between the two growth stages revealed a subcellular rewiring during transition from the exponential to the stationary growth phase of *T. cruzi* epimastigote life stage. Furthermore, we focused on membrane proteins due to their importance as vaccine and therapeutic targets.

Besides studying the proteome modulation during growth phase transitioning, we looked at the differential proteome expression in the seven Discrete Typing Units (DTUs) and closely related trypanosomes. *T. cruzi* parasites are genetically highly diverse, and this genetic variability has been attributed as a contributing factor to the variable clinical course of the disease, virulence, pathogenicity, drug resistance, transmission cycles and ecological distribution. Different molecular markers are used to study the heterogeneity

in *T. cruzi* parasites, including spliced leader intergenic region (21), kinetoplast DNA (22), RAPDS (23) and multilocus sequence typing (24), which has been proposed as the gold standard for *T. cruzi* genotyping (25). *T. cruzi* is currently divided into seven genetic subdivisions termed discrete typing units (DTUs) (25); six human infective DTUs (TcI-TcVI), and the bat restricted TcVII.

Using large-scale mass spectrometry-based protein expression profiles, we have developed a novel technique termed "*PhyloQuant*" to infer evolutionary relationships between *T. cruzi* DTUs and closely related trypanosome species (26). Using MS1, iBAQ and LFQ quantitative features, we show close correlation between *T. cruzi* genetic subdivisions and levels of protein expression. The identification of synapomorphies, herein the proteins and their respective expression profiles that differentiate the *T. cruzi* DTUs and trypanosome species, will offer additional knowledge to understand the genetic variations and their impact on important aspects including virulence, drug resistance and the variable clinical progression of the disease.

In the second part of this thesis, we focused on the modulation of two post-translational modifications, S-nitrosylation and glycosylation, in *T. cruzi* in different biological conditions. In order to achieve these objectives, we show the development and application of novel analytical and computational methods to characterize these PTMs in complex biological systems.

For the first biological system, we focused on the interaction between *T. cruzi* trypomastigotes and host extracellular matrix (ECM). During a blood meal, infected triatomine insect vectors transmit *T. cruzi* metacyclic trypomastigotes to the mammalian host. During the early stages of the infection process, the parasites interact with the host's ECM prior to invading the host cells. This early step of interaction between *T. cruzi* and the mammalian hosts offers opportunities to decipher key molecular pathway modulations which prepare the parasites for the invasion and entry into the host cells. In this thesis, a novel approach to identify and quantify S-nitrosylated proteins in *T. cruzi* trypomastigote cells incubated or not in the presence of host ECM will be presented (27). A resin-assisted enrichment of thiols combined with mass spectrometry was applied to map site-specific *T. cruzi* proteins modified by S-nitrosylation. The nitrosylation-dependent rewiring of such processes as carbon and lipid metabolism and translation was identified, in addition to the identification of S-nitrosylated histone proteins (H2B and

H3), kinases, phosphatases and oxidoreductases. Moreover, the cross-talk between protein phosphorylation and S-nitrosylation was mapped, and revealed proteins involved in metabolic and signaling pathways targeted by these PTMs. The identification of S-nitrosylation as a modification of histones was first shown by this study, adding to the increasing PTMs of histones that have reported with roles in transcriptional regulation, including acetylation, glycation, lipidation, monoaminylation, serotonylation, dopaminylation, S-glutathionylation, and homocysteinylation (28). Lastly, the first time, previously unidentified putative nitric oxide synthase enzymes were detected in the genome of *T. cruzi* through bioinformatics-based domain mapping approaches.

The second PTM addressed in this thesis is protein glycosylation, a ubiquitous PTM which plays key roles including intrinsic and extrinsic recognition, protein stability, protein folding and solubility, cellular localization of proteins, and host-pathogen interaction, where glycans on the pathogen cell surface help to recognize, adhere and invade host cells, in addition to evading immune responses during infections. At the same time, host cell surface glycans function as recognition motifs for pathogen lectin-like proteins. Currently, the methods employed for structural analysis of proteins suffer from limitations in the elucidation of glycoprotein structures. X-ray crystallography is hindered by the need for highly purified protein crystals, NMR is limited by the requirement to have high concentrations of pure non-aggregating proteins (29), while sample structural homogeneity is a requirement for cryoEM. In addition, these methods cannot be applied to biological samples with complex protein mixtures. Here, a novel approach termed *Limited Deglycosylation Assay* (LDA), is demonstrated (30). This approach was used to study the conformational modulation of glycoproteins in a systems-wide approach of LLC-MK2 cells treated or not with DTT, a compound which induces ER stress leading to protein unfolding in the ER. The principle behind this approach is the differential accessibility of PNGase F enzyme to N-linked glycans, where correctly folded glycoproteins are less susceptible to N-linked glycan hydrolysis to PNGase F enzyme compared to unfolded/misfolded glycoproteins. Using mass spectrometry, we were able to map the differential accessibility by PNGase F enzyme, and show that glycoprotein misfolding is more pronounced in loops/turns compared to beta sheets and alpha helices. This approach can be applied to compare the structural conformations of glycoproteins between disease and health conditions, or upon administration of drugs in

pharmacokinetics to map the conformational modulation of glycoproteins in complex biological systems during treatment.

Protein glycosylation in *T. cruzi* is essential for parasite survival in the different developmental stages, mediating key roles such as cellular recognition, adhesion and invasion of host cells, and evasion of immune responses mounted by the host during infection. As such, glycoproteins are promising chemotherapeutic candidates, since the current options available for *T. cruzi*, benznidazole and nifurtimox, are limited by high toxicities, drug resistance, and long treatment regimens. In addition, since there are currently no vaccines available for CD, and the fact that glycans are able to stimulate immune response during infection, novel biomolecule targets such as glycans offer promising glycan-based vaccine options. Glycans expressed by *T. cruzi* parasites have been characterized using lectin blots, immune-agglutination assays, NMR spectroscopy and mass spectrometry-based techniques. These studies have shown that the glycans expressed on the surface of *T. cruzi* are diverse, and that protein glycosylation is strain and developmental-stage specific (31, 32). Glycoproteome and glycome characterization of *T. cruzi* parasites with divergent genetic variability is important to elucidate the role of the glycoproteins and glycans in host-parasite interaction, and can elucidate the importance of these molecules to the biology and pathogenicity of *T. cruzi* parasites during infections. Current knowledge of the intact glycoproteome of *T. cruzi* is confined to the Y strain (32). Here, we have investigated the intact glycoproteome of 17 strains using an in-depth mass-spectrometry-based characterization of *T. cruzi* and closely related trypanosome species' glycoproteome and glycome repertoires. Mass spectrometry-based analysis of HILIC enriched glycopeptides enabled the detection of intact N- and O-linked glycopeptides, while PCG-LC-ESI-MS/MS analysis enabled the characterization of N- and O-glycans. The evolutionary relationships between trypanosome species based on abundance levels were also mapped, revealing a revolutionary conservation of *T. cruzi* DTUs from closely related trypanosome species. Moreover, chemical inhibition of the oligosaccharyltransferase machinery was investigated as a potential therapeutic option.

4. PERSPECTIVES

This thesis focuses on the modulation of the proteome and PTMs of *T. cruzi* in different biological conditions. In order to achieve a deep knowledge on the molecular diversity of *T. cruzi*, analytical and computational methodologies were developed during this thesis including novel enrichment methods and application of algorithms to infer evolutionary relationships.

In the first part of the thesis, mass spectrometry analysis of *T. cruzi* proteome in the transition from exponential to stationary growth stage (page 24), and between *T. cruzi* representative of the seven DTUs, and closely related trypanosome species at the epimastigote life stages (page 53) was reported highlighting the role of proteomics in enhancing information on *T. cruzi* biology and their evolutionary relatedness. Future studies focusing on the elucidation of protein-based discrimination of *T. cruzi* trypomastigote cells will uncover differentially expressed DTU-specific proteins with potential applications to understand the variable clinical presentations and drug resistance described in different *T. cruzi* genetic subdivisions.

In the second part of the thesis, *T. cruzi* protein nitrosylation (page 65) and glycosylation (page 91) were studied. Besides the detailed quantitative and site-specific characterization of these PTMs, the works presented here have looked into their biosynthesis. Identification of possible *T. cruzi* NOS enzymes offer opportunities to manually characterize these proteins, and their identification can have chemotherapeutic potential in *T. cruzi* treatment using chemical inhibitors of NOS. Moreover, targeting the glycosylation machinery was shown to be a potential and still unexplored target for Chagas disease therapy.

From a methodological point of view, the limited deglycosylation assay presented in this thesis can be applied to study the global effect of *T. cruzi* chemotherapeutic agents, information that is currently missing (page 78). Moreover, this method is filling a current gap in determining the conformational changes in glycoproteins which are currently difficult to achieve with the technologies present today. Finally, the identification of differentially abundant *T. cruzi* strain and DTU-specific glycopeptides and glycans has expanded our knowledge on this PTM in *T. cruzi* offering novel diagnostic and chemotherapeutic targets against Chagas disease.

Although this thesis introduces several new potential targets to understand *T. cruzi*-host interaction, there are still several points that need more investigation.

For example, 1) the proteome repertoire of different *T. cruzi* strains in the trypomastigote stage will help in elucidating the cell surface proteins involved in invasion of the mammalian host and help in correlating it with the clinical phenotype. 2) chemical and genetic inhibition studies of the *T. cruzi* and host glycosylation machinery are needed to elucidate its role during parasite adhesion, invasion and replication. 3) The identification of host factors regulated upon *T. cruzi* infection is also needed to pinpoint potential hotspots for therapy. This should be done using different cell line models, phagocytic and non-phagocytic, using 2D and 3D in vitro models and also in vivo. 4) The identification of PTMs from a molecular and structural point of view needs to be improved in order to produce antigens to elicit a PTMs-based immune response against *T. cruzi*. 5) There is a bug need in applying proteomics technology in *T. cruzi*-invertebrate host interaction since the majority of the studies are still focused on the vertebrate one for possible vector control strategies. 6) The proteomics tools developed so far needs to be applied more to clinical samples collected from Chagas disease patients. Although, important contributions are in the literature, there is still a big gap in the application of these technologies in the clinic. Regarding to it, there is a big lack in diagnostic methods that are in line with the REASSURED criteria of the WHO for *T. cruzi* genotyping.

Recognizing these issues is an important aspect to continue the research in the field of Chagas disease and this thesis gives a contribution to it.

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