UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY TECH

Escuela de Ciencias Químicas e Ingeniería

TÍTULO: Protein aldehydes as precursors of a Biginelli/Hantzsch-assisted bioconjugation methodology design

Trabajo de integración curricular presentado como requisito para la obtención del título de Químico

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DEDICATORIA

Este trabajo se lo dedico a mis amados padres, hermanos, & mi abuelita Charito.

Alexis Andrés Eras Clavijo

AGRADECIMIENTO

"El agradecimiento es la memoria del corazón"

Lao Tsé

Esta tesis no había sido posible sin mis padres (Vinicio Eras y Virginia Clavijo) quienes con su gran amor, paciencia, esfuerzo y su gran apoyo incondicional brindado me han perimitido llegar a cumplir hoy un sueño más, una meta más. Les doy las gracias por inculcar valores como el esfuerzo, la valentía, perseverancia y la humildad para cumplir cada uno de mis objetivos en la vida y ser aplicados en cada peldaño de mi vida. Mis hermanos (Valeria Eras e Israel Eras) cuales me brindaron tod su cariño, apoyo incondicional, amor y confianza, durante todo mi vida y por estar conmigo en todos los momento, inspirándome y siendo un motor para cumplir todo lo que me proponga. También, agradezco especialmente a mi tutora Hortensia Rodriguez y co tutor Nelson Vispo, por su ayuda y apoyo. Además de todos mis profesores a lo largo de mi carrera universitaria por inculcarnos, guiarnos y enseñarnos valiosas lecciones de vida y de conocimiento para ser aplicadas profesionalmente con respectiva ética y moral que nos compete como profesionales y ciudadanos. Por otro lado, agradezco a mis amigos (Mayra Briones, Alexander Tipan, Melany López, Kevin Ruiz, Paula Cárdenas, Joselyn Delgado, Daniela Negrete, Xiomira Fiallos, Mayra Jiménez, Jazmín Gonzalez, Rebeca Rodríguez, entre otros), por ser las personas que se conviritieron en mi segunda familia en la universidad, quienes me apoyaron cuando más las necesito y brindarme grandiosos momentos, por permanecer conmigo a través de los buenos, y malos momentos, les agradezco infinitamente. Finalmente, le agradezco muy especialmente a una gran persona en mi vida y quien es mi corazón, Andrés Alomía. Les agradezco con todo mi corazón, siempre los llevare conmigo.

Alexis Andrés Eras Clavijo

ABSTRACT

Multicomponent reactions (MCR's) are very useful tools for modern organic chemistry, and generally, are one-pot reactions with three or more components, resulting in a product that incorporates atoms from all the starting materials and thus they are very suitable and convenient for a broad range of organic synthesis. Hantzsch and Biginelli are MCR's widely used to prepare interesting bioactive N-heterocycles. Hantzsch reaction involves an aldehyde, a beta-keto ester, and ammonia or ammonium acetate, which, through a multicomponent condensation plus oxidation of the dihydropyridine intermediate, results in a pyridine derivative, while Biginelli reaction uses urea as nitrogen source instead of ammonia to obtain 3,4-dihydropyrimidin-2-(1H)-ones. The MCR's like Biginelli and Hantzsch reactions showed the potential to be used for the development of novel bioconjugation methodologies. In this regard, the preparation of protein aldehydes is an essential part of this process and would be accomplished by oxidation methods (metal-catalyzed oxidation (MCO) and periodate oxidation). The aim of the present work is the generation of protein aldehydes as key precursors of Biginelli/Hantzsch-assisted bioconjugation methodologies design, boosting greener conditions and indeed low-cost for ligation/bioconjugation between biocarriers and drugs, and in future works, test its potential application for drug delivery systems (DDS´s). To tackle it, protein aldehydes were synthesized by metal-catalyzed oxidation (MCO) and periodate oxidation using BSA as proof of concept. The oxidized BSA fractions, which contain the aldehyde handles, were filtered, purified, and discriminated trough size exclusion chromatography (PD-10 column), and UV-nanodrop measurement. Moreover, they were characterized by spectroscopy techniques as HPLC (Photodiode Array Detector), and UPLC-MS to corroborate peptide changes and the generation of the aldehyde-containing modified biomolecules.

KEYWORDS: multicomponent reaction (MCR), Hantzsch reaction, Biginelli reaction, protein aldehydes, metal-catalyzed oxidation (MCO), periodate oxidation, bioconjugation.

RESUMEN:

Las reacciones multicomponente (MCR´s por sus siglas en inglés) son herramientas de la química orgánica moderna, que permiten la formación de un solo producto en el que se incorpora los atomos de los reactivos inicales, a partir de reacciones químicas donde intervienen tres o mas components. En este sentido, las reacciones de Biginielli y Hantzsch son MCR´s ampliamente usadas para preparar novedosos N-heterociclos con potencial y demostrada bioactividad. La reaccion de Hantzsch involucra un aldehído, un beta-cetoéster y amoníaco o acetato de amonio, los cuales a través de una condensación multicomponente, seguido de la oxidación de la dihidropiridina que se obtiene como intermediario, resulta en un derivado de piridina; mientras que la reacción de Biginelli usa urea en lugar de amoníaco como fuente de nitrógeno para obtener 3,4-dihidropirimidin-2-(1H)-onas . Las MCR´s como las reacciones de Biginelli y Hantzsch muestran un gran potencial para ser usadas en el desarrollo de nuevas metodologías de bioconjugación, donde la introducción de aldehídos en la estructura de las proteínas es clave en la estrategia sintética diseñada. Esta modificación de la estructura proteica puede lograrse a través de métodos de oxidación. El objetivo del presente trabajo es la generación de aldehídos proteicos como precursores claves de las metodologías de bioconjugación assistidas por las reacciones de Hantzsch o Biginelli, promoviendo condiciones amigables con el medio ambiente y además, el diseño de métodos de bioconjugación de bajo costo entre biomoléculas y agentes terapéuticos. Esto servirá de base para, en trabajos futuros, optimizar la bioconjugación y probar su potencial aplicación para sistemas de liberación de fármacos. Para abordar este objetivo, los aldehídos proteicos se sintetizaron mediante la oxidación catalizada por metal (MCO por sus siglas en inglés) y la oxidación asistida por periodato de la BSA (proteína usada como prueba de concepto). Las fracciones oxidadas de BSA, que contienen los grupos funcionales aldehídos, fueron filtradas, purificadas y discriminadas a través de cromatografía por exclusión de tamaño (columna PD-10) y midiendo su espectro de absorbancia UV usando el nanodrop. Además, las fracciones proteicas fueron caracterizadas por técnicas espectroscópicas como HPLC (con detector de arreglo de diodos) y UPLC-MS para corroborar los cambios en la estructura proteica, así como la generación de biomoléculas modificadas que contengan los grupos aldehído incorporados en su estructura.

PALABRAS CLAVE: reaccion multicomponente (RMC), Hantzsch, Biginelli, aldehídos proteicos, oxidación catalizada por metal (OCM), oxidation por periodato, bioconjugación.

INDEX

1 CHAPTER 1. INTRODUCTION – JUSTIFICATION

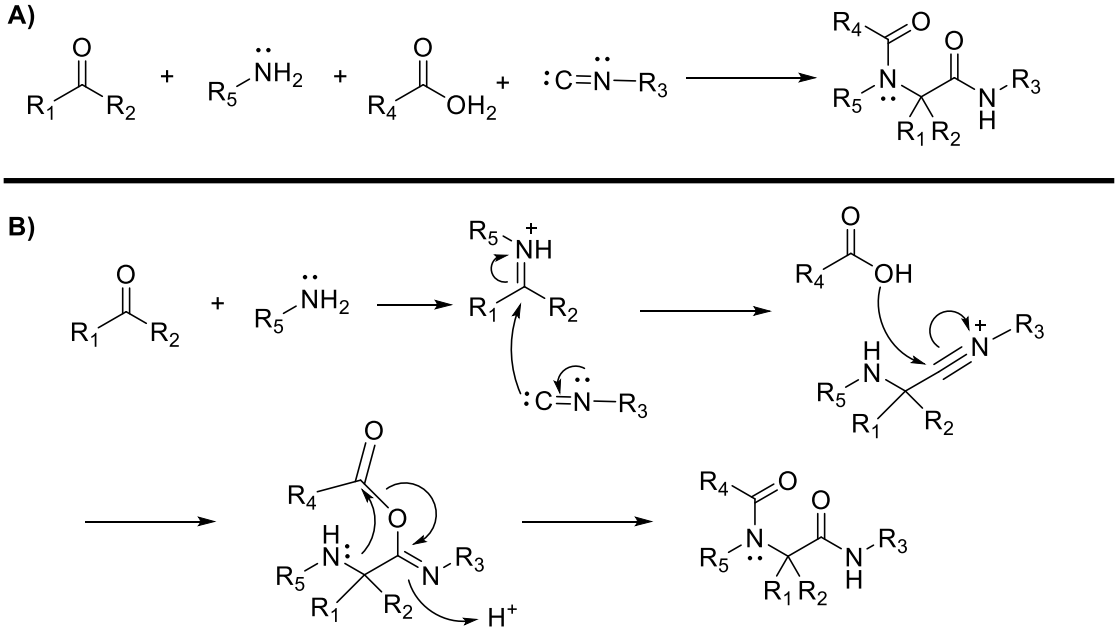
1.1 Multicomponent reaction-based synthetic strategy for drug delivery system

Multicomponent reactions (MCR's) are procedures that consist of at least three reactants, which combined in one pot results in a product. The MCRs are versatile that allow the incorporation of atoms of all reagents. These MCR's are well known recognized by their advantages like high atom economy, diversity, generator of molecules of high complexity, efficient, mild conditions, high convergence, and step economy.[1–3] They can be used in the drug delivery system (DDS), natural products, medicinal chemistry heterocycle, and carbohydrate chemistry, combinatorial chemistry, agrochemistry, organocatalysts development, peptide cyclization strategies, polymer synthesis, and biomolecular conjugations being the latter field in which this work will be focused on.[1,2] The synthesis of a variety of heterocyclic scaffolds trough MCR's since they are versatile tools to incorporate a broad range of functionalities and substituents in the desired product. MCR's serve as adducts that can be cyclized and re-functionalized, obtaining the synthesizer into the target heterocyclic structures. MCR's comprehends a great variety of reaction like Ugi,[4] Passerini,[5] vam Lausen,[6] Strecker,[7] Hantzsch,[8] Biginelli[9,10] or one of their variations to produce a great diversity of heterocyclic scaffold structures.[3,11,12] These heterocycles should be formed in such a way that products still contain in their structures function handles for further derivatization processes. The use of MCR's in conjunction with other strategies allows access to a wide number of functionalized heterocyclic scaffolds in an easy, cheap and fast way.[13]

Furthermore, MCR's can be used as advanced tools for sustainable organic synthesis because it fulfills the aim of green chemistry. Green chemistry, also known as sustainable chemistry, consists of designing chemical products and processes to reduce or eliminate the use or generation of hazardous substances, applying throughout the entire life cycle of a chemical, including its design, manufacture, and use. Besides, it emerges from the understanding that this chemical industry should cause the minimum impact in the environment or process that remediate chemical disaster that would happen to recover the natural balance of the world. Consequently, the objective of green chemistry is reformulating the existing methods, create novel methods, and most important, elaborate different synthetic pathways to include the environmental factor in the early stage of design and development experimental procedures. Thus, a MCR-based synthetic strategy plays a crucial role in novel organic synthesis due that they can manage the delicate chemical problem in an eco-friendly way.[2]

1.2 Ligation/bioconjugation techniques based on multicomponent reactions (MCR's)

The ligation /bioconjugation techniques based on multicomponent reaction where based principally on ugi reaction (**Scheme 1**). An Ugi four component reaction involves a ketone or aldehyde, a carboxylic acid, an isocyanide, and an amine. The mechanism of this reaction consit of formation of an imine, then a nucleophilic attack of the isocyanide is carried out which results in highly reactive nitrilium intermediate formation. After, this reactive intermedaite is attacked by the carboxylic acid, and a subsequente intramolecular Mumm rearrangement occur yielding a central bis-amide product[14].



Scheme 1. General reaction (A) and mechanism (B) of Ugi reaction. Adapted from Váradi, A.; Palmer, T. C.; Notis Dardashti, R.; Majumdar, S. Isocyanide-Based Multicomponent Reactions for the Synthesis of Heterocycles. Molecules . *2016. https://doi.org/10.3390/molecules21010019.*

- **Lipid-Lipid and lipid-Oligosaccharide conjugation.**

A procedure based on MCR allows to conjugate directly two lipid tails to an oligosaccharide moiety by Ugi reaction (I-MCR). It was achieved two variants in which a carbohydrate (oligosaccharides) is used as either the carboxylic acid or amino component. The oligosaccharides were functionalized with fatty acid and lipidic isocyanides, yielding glycolipids. Thus, this approach is capable of multi-functionalize biomolecules in one spot synthesis (**Figure 1**).[15]

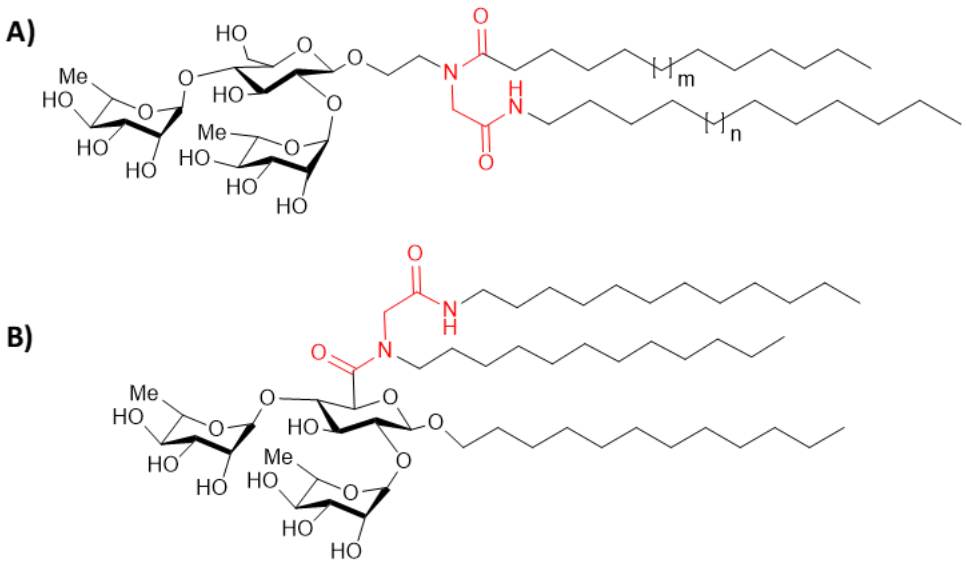


Figure 1. Lipid-Lipid and lipid-oligosaccharide conjugates. Final products using A) Oligosaccharides as amino component and B) as carboxylic component. Adapted from Pérez-Labrada, K.; Brouard, I.; Méndez, I.; Rivera, D. G. Multicomponent Synthesis of Ugi-Type Ceramide Analogues and Neoglycolipids from Lipidic Isocyanides. J. Org. Chem. ***2012***. *https://doi.org/10.1021/jo300462m*

- **Oligosaccharide-steroid conjugation.**

The synthesis of steroid-carbohydrate derivatives by I-MCR's was carried out with spirostanic steroids and β-chacotrioside functionalized as either amino or carboxylic acid component producing a library of cytotoxic analogs of diosgenyl-β-chacotrioside (tetrazole-based saponin mimic) for anticancer activity (**Figure 2**).[16] Even, to obtain more complex conjugation, it is ligated two oligosaccharide moieties to a bifunctional steroid, conjugation of four lactosyl fragments and a cholic acid-derived diisocynide by means a double Ugi reaction, rendering steroidal glycoconjugate (amphiphilic glycoconjugate), which the lactose units are located at the α face of concave cholanic skeleton (**Figure 2**). [17]

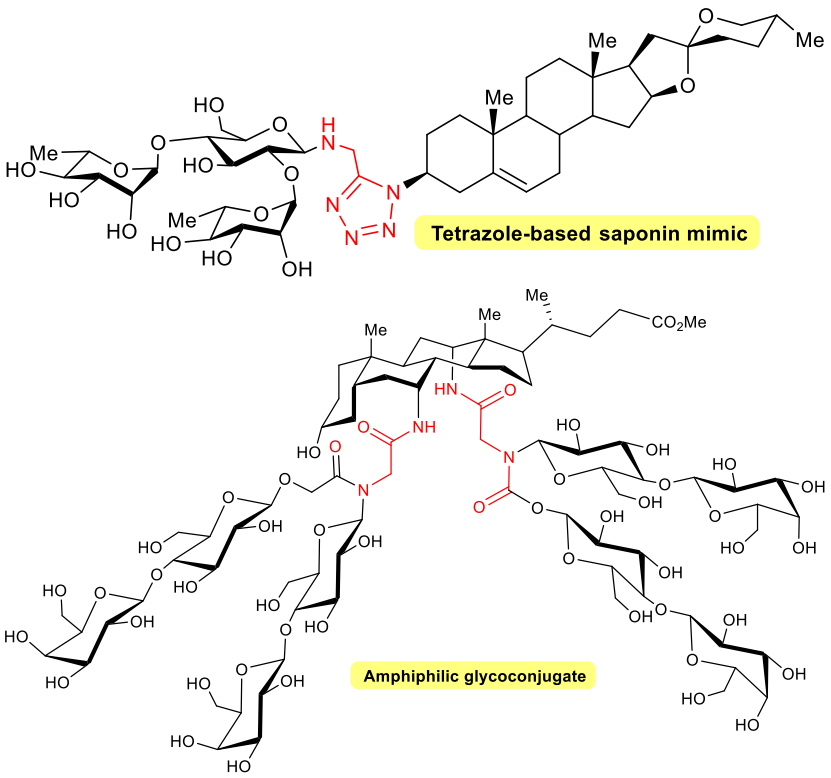


Figure 2. Oligosaccharide- steroid conjugates: Steroid-Carbohydrate conjugates by Multicomponent conjugation (MCC) of Oligosaccharide to steroidal isocyanides Adapted from Rivera, D. G.; Pérez-Labrada, K.; Lambert, L.; Dörner, S.; Westermann, B.; Wessjohann, L. A. Carbohydrate-Steroid Conjugation by Ugi Reaction: One-Pot Synthesis of Triple Sugar/Pseudo-Peptide/Spirostane Hybrids. Carbohydr. Res. 2012. https://doi.org/10.1016/j.carres.2012.05.003. Rivera, D. G.; León, F.; Concepción, O.; Morales, F. E.; Wessjohann, L. A. A Multiple Multicomponent Approach to Chimeric Peptide-Peptoid Podands. Chem. - A Eur. J. 2013. https://doi.org/10.1002/chem.201201591.

- **Peptide-Peptide and Carbohydrate-peptide ligation.**

The ligation of oligopeptides fragments by I-MCRs can be used as an approach for the Ugi multicomponent ligation (UML) of two "pseudo peptides fragments" to yield more stable and highly active peptides analogs by means of a highly diastereoselective UML procedure.[18–20] Moreover, the Ugi peptide ligation can be expanded by the development of "highly stereoselective organocatalytic" MCR method for peptide-peptide or sugar-peptide conjugation, which is based on use of enantiomerically enriched enol-hemiacetals as chiral inputs to conjugate isocyanopeptides to carbohydrates and aminopeptides to form peptidic hybrids (**Figure 3A**). [21] Furthermore, it is possible the multicomponent ligation (MCL) of urea-peptide, a lipidic aldehyde and a hybrid ribosyl-uridine isocyanide to produce a potent lipophilic antibacterial muraymicin analogue (**Figure 3B**).[22]

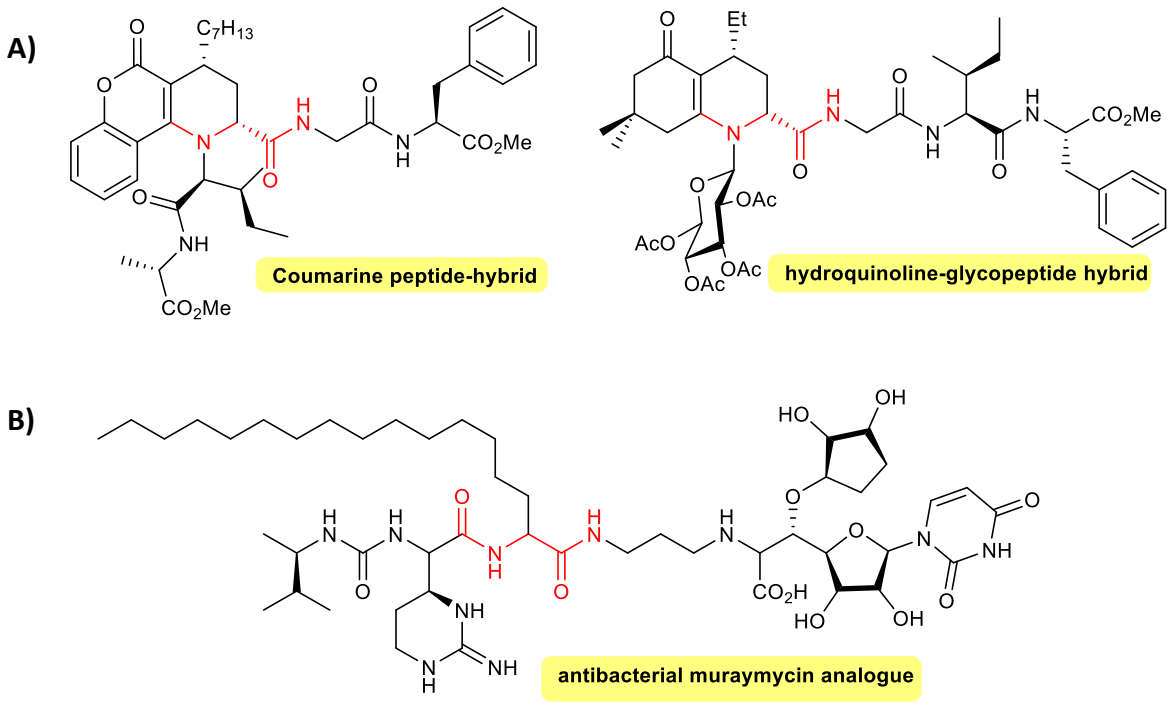


Figure 3. Peptide-Peptide and carbohydrate peptide conjugation. A) Final product fo diastereoselective multicomponent ligation (MCL) of peptide and sugar residues to chiral bifunctional building blocks adapted from Echemendía, R.; De La Torre, A. F.; Monteiro, J. L.; Pila, M.; Corrêa, A. G.; Westermann, B.; Rivera, D. G.; Paixão, M. W. Highly Stereoselective Synthesis of Natural-Product-like Hybrids by an Organocatalytic/Multicomponent Reaction Sequence. Angew. Chemie - Int. Ed. 2015. https://doi.org/10.1002/anie.201412074, B) Final product of MCL of urea-peptide, a lipid and aminoribosyl-5-C-glycyludrine adapted fromTanino, T.; Ichikawa, S.; Al-Dabbagh, B.; Bouhss, A.; Oyama, H.; Matsuda, A. Synthesis and Biological Evaluation of Muraymycin Analogues Active against Anti-Drug-Resistant Bacteria. ACS Med. Chem. Lett. 2010. https://doi.org/10.1021/ml100057z.

- **Peptide-Steroids and Peptide-Lipid conjugation.**

The synthesis of peptide-steroid conjugates can be achieved through multicomponent conjugation (MCC) of individual amino acids (AA's).[23] This concept can be expanded to the creation of a first-in-class family of peptide steroid conjugates (Unique class of *N*-steroidal peptide), highlighting the peptide backbone with the steroid skeleton as an N-substituent by means of a MCC of peptide carboxylic acid and isocyanopeptide or isocyanoacetate to a steroidal amine. These *N*-steroidal peptides can be further cyclized to yield "cyclopeptide-steroid conjugate" in which the cyclopeptide residue can be found in different positions of either steroidal or the side chain (**Figure 4**).[24]

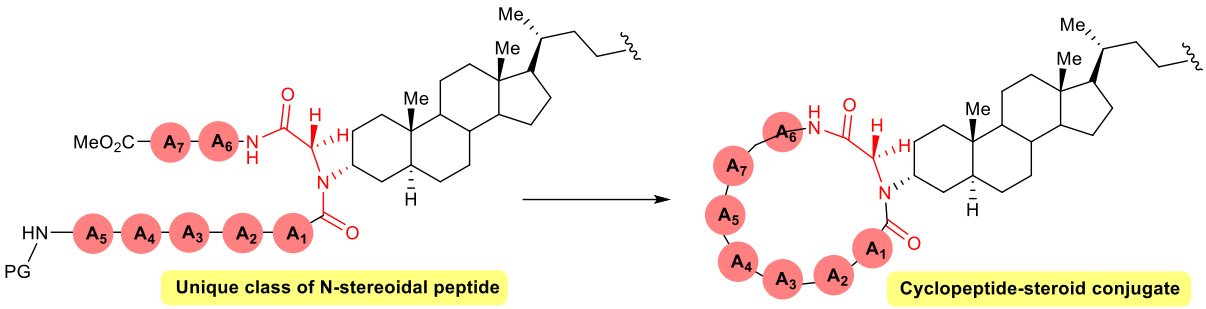


Figure 4. Peptide-Steroid and peptide-lipid conjugates. Multicomponent conjugation (MCC) of two peptide fragments previously linked to steroids for the construction of unique N-steroidal cyclopeptide adapted from Rivera, D. G.; Vasco, A. V.; Echemendía, R.; Concepciõn, O.; Pérez, C. S.; Gavín, J. A.; Wessjohann, L. A. A Multicomponent Conjugation Strategy to Unique N-Steroidal Peptides: First Evidence of the Steroidal Nucleus as a β-Turn Inducer in Acyclic Peptides. Chem. - A Eur. J. ***2014***. *https://doi.org/10.1002/chem.201403773*.

Moreover, the synthesis of antimicrobial lipopeptides is able to locate the lipidic tail either in the peptide termini, as an internal amide or side-chain substituent.[25,26] To obtain them, it was created a novel on-resin MCC of lipids and steroids to peptides based on a solid-phase methodology which enables the conjugation of either a lipid chain or a steroidal skeleton to resin-bound peptide (RBP)(**Figure 5A**). Whereas, subsequent AA's couplings allow the growth of the peptide obtaining the desired lipopeptides and peptide-steroid conjugate. Similarly, a double ligation of a RBP at the *N*-terminus was carried out allowing the *N*-terminal double lipidation and *N*-terminal lipidation and biotinylation (**Figure 5B**). Also, through a Ugi-azide reaction, it is obtained tetrazolo lipopeptides and tetrazolo peptidosteroid (**Figure 5C**).[25]

Furthermore, it can be produced efficiently microbial naturals products analogs (cyclic lipopeptides analogues) by Ugi and Passerini reactions in order to obtain a simultaneous cyclization and peptide lipidation. It let conjugation of either one or two exocyclic lipid tails along with the macrocyclic ring closure (**Figure 6**).[27] Likewise, it was proved the same procedure by an on-resin Ugi-Smiles reaction creating a multicomponent cyclo-ligation strategy that enables the simultaneous cyclization of peptide skeleton and its ligation to either lipidic moieties or fluorescent labels (**Figure 7**). [28,29]

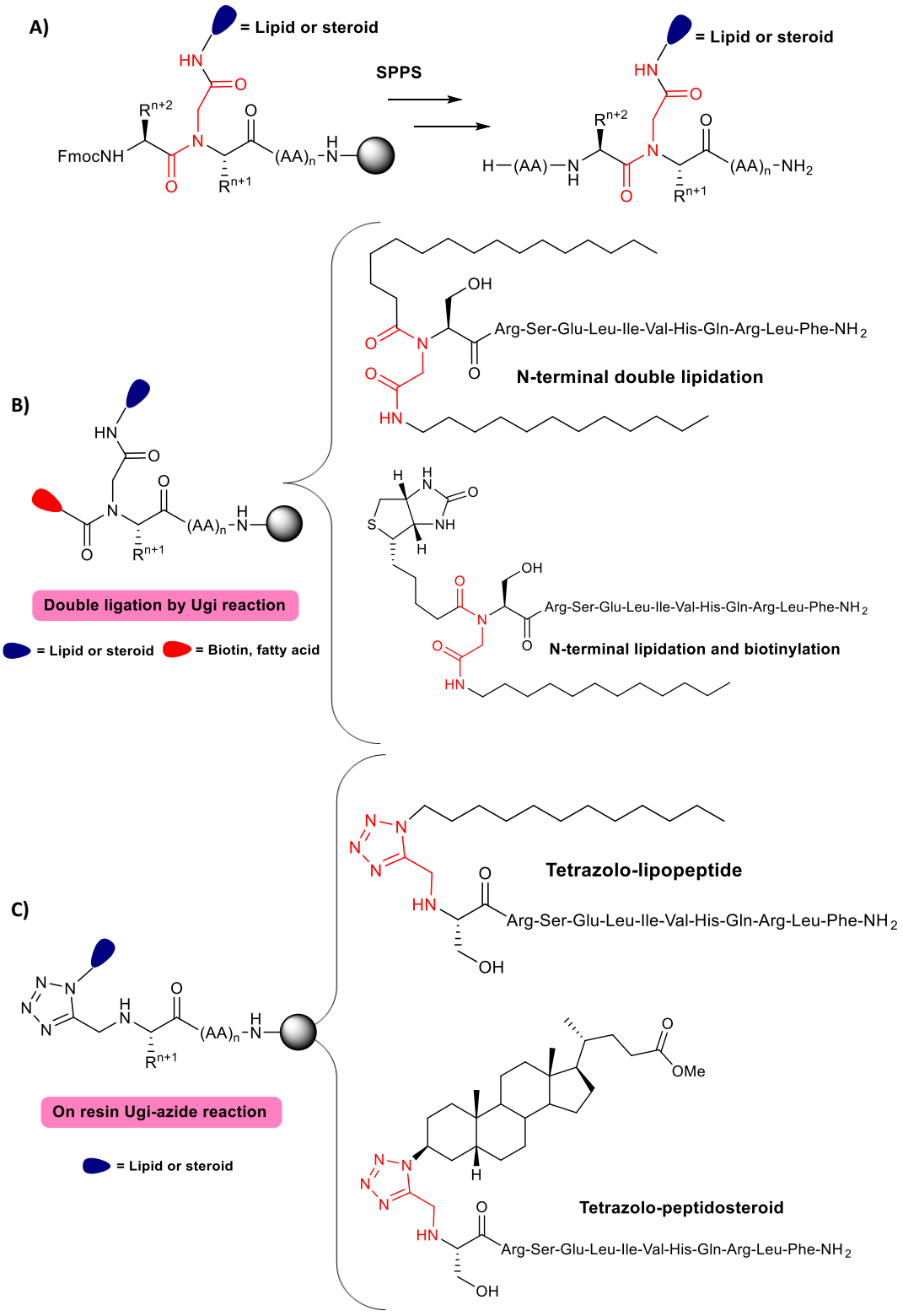


Figure 5. Peptide-Steroid and peptide-lipid conjugates. A) Solid Phase Ligation (SPL) of lipids and steroids by resin-bound peptide (RBP) using on-resin Ugi reaction. SPL of lipids, biotinylated steroids by RBP using on-resin Ugi and Ugi azide reaction: B) Double lipidation and lipidation and biotinylation by Ugi reaction, C) tetrazolo-lipopeptide and tetrazolo petidosteroid product obtained by Ugi-azide reactions. Adapted from Morales, F. E.; Garay, H. E.; Muñoz, D. F.; Augusto, Y. E.; Otero-González, A. J.; Reyes Acosta, O.; Rivera, D. G. Aminocatalysis-Mediated on-Resin Ugi Reactions: Application in the Solid-Phase Synthesis of n -Substituted and Tetrazolo Lipopeptides and Peptidosteroids. Org. Lett. 2015. https://doi.org/10.1021/acs.orglett.5b01147. Wessjohann, L. A.; Morejón, M. C.; Ojeda, G. M.; Rhoden, C. R. B.; Rivera, D. G. Applications of Convertible Isonitriles in the Ligation and Macrocyclization of Multicomponent Reaction-Derived Peptides and Depsipeptides. J. Org. Chem. 2016. https://doi.org/10.1021/acs.joc.6b01150.

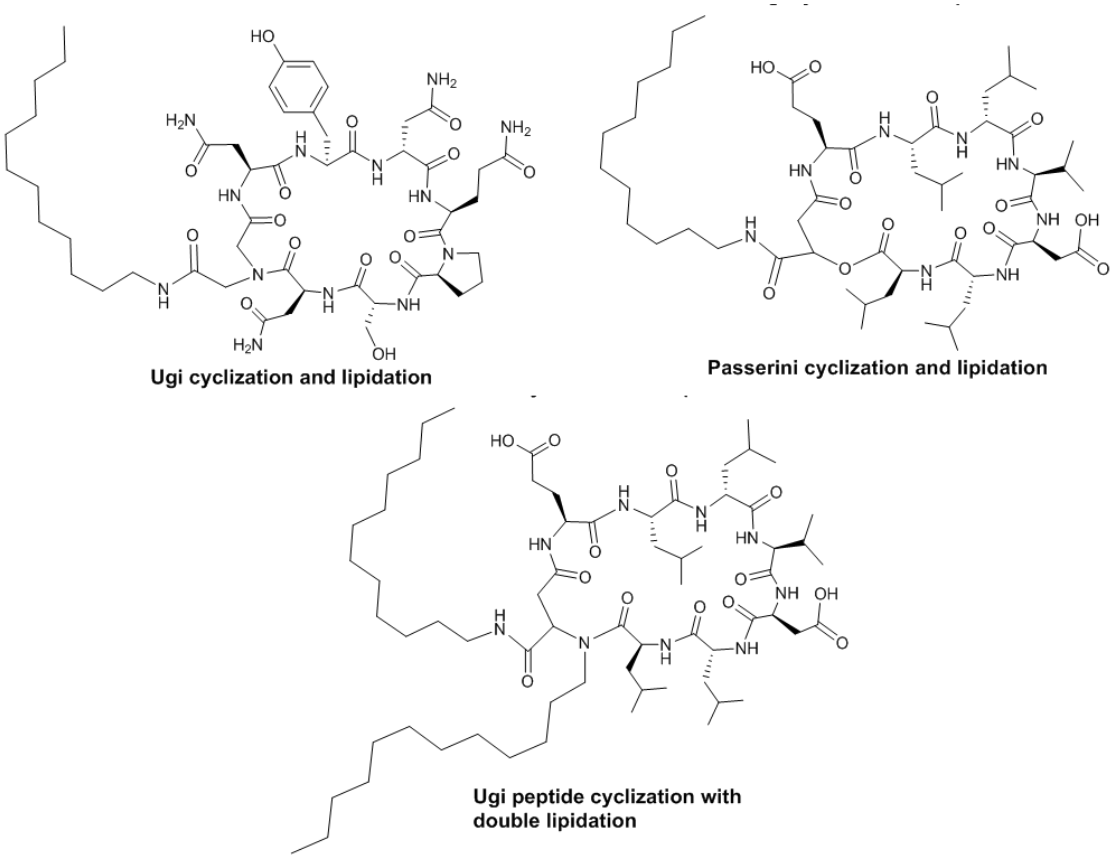


Figure 6. Peptide-Steroid and peptide-lipid conjugates: Simultaneous cyclization and lipidation of peptides by Ugi and Passerini reactions adapted from (27) Morejón, M. C.; Laub, A.; Kaluderović, G. N.; Puentes, A. R.; Hmedat, A. N.; Otero-González, A. J.; Rivera, D. G.; Wessjohann, L. A. A Multicomponent Macrocyclization Strategy to Natural Product-like Cyclic Lipopeptides: Synthesis and Anticancer Evaluation of Surfactin and Mycosubtilin Analogues. Org. Biomol. Chem. 2017. https://doi.org/10.1039/c7ob00459a.

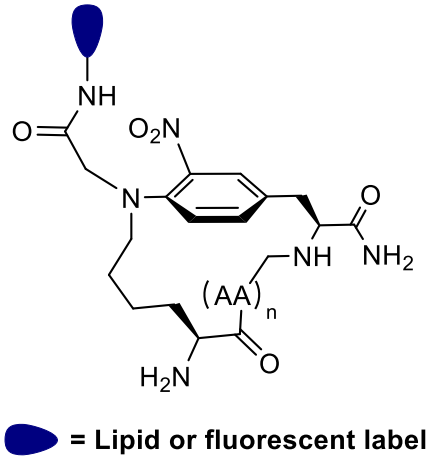


Figure 7. The pattern of the product obtained using on resin Ugi-smiles macrocyclization and ligation. Adapted from Morejón, M. C.; Laub, A.; Westermann, B.; Rivera, D. G.; Wessjohann, L. A. Solution- and Solid-Phase Macrocyclization of Peptides by the Ugi-Smiles Multicomponent Reaction: Synthesis of N-Aryl-Bridged Cyclic Lipopeptides. Org. Lett. 2016. https://doi.org/10.1021/acs.orglett.6b02001. El Kaïm, L.; Grimaud, L.; Oble, J. Phenol Ugi-Smiles Systems: Strategies for the Multicomponent N-Arylation of Primary Amines with Isocyanides, Aldehydes, and Phenols. Angew. Chemie - Int. Ed. 2005. https://doi.org/10.1002/anie.200502636.

- **Protein immobilization, labeling and glycoconjugate.**

Protein immobilization can be achieved through efficient Ugi reaction procedures immobilizing of the enzyme glucose oxidase on a polymeric carrier in which the glycoenzime undergoes periodate oxidation to obtain aldehyde group in glycosidic part. Then it is linked to amino-functionalized glycideyl methacrylate polymer using excess of acetic acid and cyclohexyl isocyanide to produce the "polymer-supported enzyme" (**Figure 8A**).[30] Also, it was prepared bovine serum albumin (BSA) and horseradish peroxidase (HRP) conjugates by Ugi reaction using either the carboxylic acid or amino groups at the biomolecules surface, monosaccharides and isocyano components to render several Ugi-derived glyconjugates (**Figure 8B**). Although, this procedure has a problem due to the long reaction times cause protein denaturation as a result of protein cross-linking, which can be solved by incorporating hydrazide-activated proteins in Ugi reaction to increase protein reactivity of amino group and decrease reaction time.[31,32] Moreover, it can be obtained polysaccharide conjugation by I-MCRs (Passerinin and Ugi reaction) to produce biocompatible synthetic hydrogels in order to be used for the immobilization of enzymes in polysaccharide networks.[33] Moreover, it was developed an amperometric enzyme biosensor for hydrogen peroxide by immobilization of HRP on a gold electrode which was coated with sodium alginate by Ugi reaction (**Figure 8C**). This method consists in the functionalization of polysaccharide with thiol groups by periodate oxidation and subsequent reductive amination. Once the polymer is attached to the gold electrode; it is achieved electrode-immobilized enzyme by means of Ugi bioconjugation procedure.[34] Similarly, using the Ugi bioconjugation strategy, it is suitable for preparation of thermostable neoglyconenzymes conjugating trypsin with sodium alginate and carboxymethyl cellulose to yield trypsin-polysaccharide glycoconjugates.[35] In addition, protein labeling can be obtained through a Mannich-type multicomponent process conjugation at Tyr residues, which consists of the mild reaction between Tyr phenol ring and "imines derived from aldehydes and electron-rich anilines carrying rhodamine tag to yield labeled proteins (**Figure 8D**).[36] Another procedure of site-selective protein labeling based on MCRs is the use of Cu-catalyzed A^3 couplings (**Figure 8E**). [37]

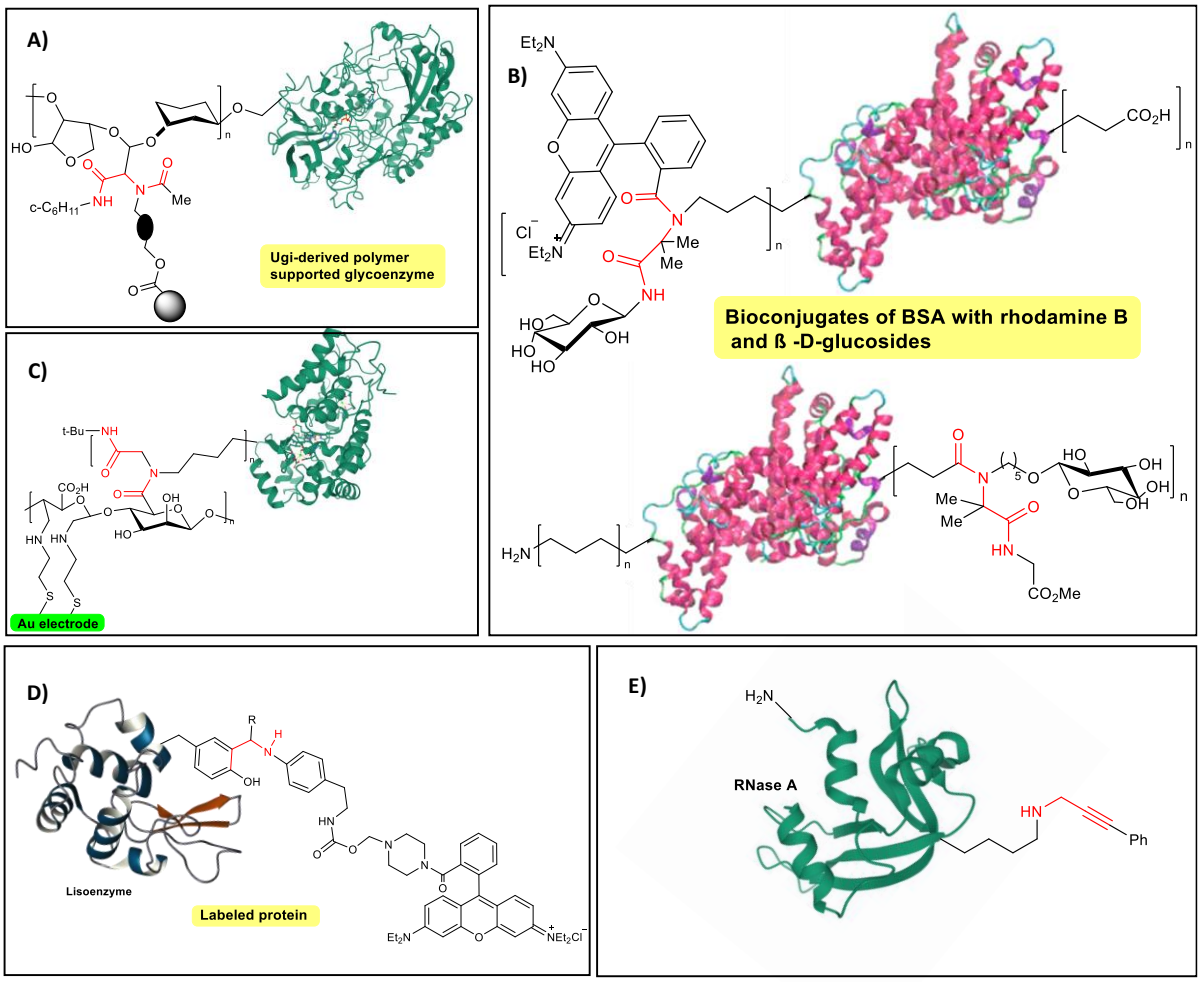


Figure 8. Protein immobilization, labeling, and glycoconjugates: A) Ugi-derived polymer-supported glycoenzime adapted from Marek, M.; Jarý, J.; Valentová, O.; Vodrážka, Z. Immobilization of Glycoenzymes by Means of Their Glycosidic Components. Biotechnol. Lett. 1983, 5 (10), 653–658. https://doi.org/10.1007/BF01386357.; B) Final products from multicomponent conjugation (MCC) to polymeric support (See A) and fluorescent label (Rhodamine B) and carbohydrate (ß-Dglucosides)employing Ugi reaction adapted from Ziegler, T.; Gerling, S.; Lang, M. Preparation of Bioconjugates through an Ugi Reaction. Angew. Chemie - Int. Ed. 2000. https://doi.org/10.1002/1521-3773(20000616)39:12<2109::AID-ANIE2109>3.0.CO;2-9. Méndez, Y.; Chang, J.; Humpierre, A. R.; Zanuy, A.; Garrido, R.; Vasco, A. V.; Pedroso, J.; Santana, D.; Rodríguez, L. M.; García-Rivera, D.; et al. Multicomponent Polysaccharide-Protein Bioconjugation in the Development of Antibacterial Glycoconjugate Vaccine Candidates. Chem. Sci. 2018. https://doi.org/10.1039/c7sc05467j; C) Multicomponent immobilization (MCI) of Horseradish peroxidase (HRP) on a polysaccharide-coated gold electrode adapted from Camacho, C.; Matías, J. C.; García, D.; Simpsom, B. K.; Villalonga, R. Amperometric Enzyme Biosensor for Hydrogen Peroxide via Ugi Multicomponent Reaction. Electrochem. commun. 2007, 9, 1655–1660; D) Site-selective protein labeling at Tyr residue by Mannich type MCC adapted from Joshi, N. S.; Whitaker, L. R.; Francis, M. B. A Three-Component Mannich-Type Reaction for Selective Tyrosine Bioconjugation. J. Am. Chem. Soc. 2004, 126 (49), 15942–15943. https://doi.org/10.1021/ja0439017; E). Site-selective protein labeling at only one Lys residue by Cu-catalyzed A3 coupling MCC adapted from Chilamari, M.; Purushottam, L.; Rai, V. Site-Selective Labeling of Native Proteins by a Multicomponent Approach. Chem. - A Eur. J. 2017. https://doi.org/10.1002/chem.201605938

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Finally, the development of multicomponent protein-polysaccharide conjugation method which consists of UML of functionalized capsular polysaccharide (CPs) of Streptococcus and Salmonella to carrier proteins like diphteria and tetanus toxoids ((DT & TT) activated by the reaction of glutamic and aspartic acid side chains with hydrazine (DT^a & TT^a) was reported.

The products of these procedures showed significant antigenicity and elicited goods titer of functional specific antibodies (**Figure 9A** and **B**). Similarly, it was conjugated two different polysaccharides to a protein using TEMPO-oxidized CPs14 (carboxylic component), periodate oxidized CPs7F (oxo component), and TT[a] (amino component) yielding a glycoconjugate that consists of two different polysaccharide antigens conjugated to a carrier protein which has dual polysaccharide antigenicity. This proved that this multicomponent bioconjugation method is efficient for the development of multivalent vaccine candidates (**Figure 9C**).[32]

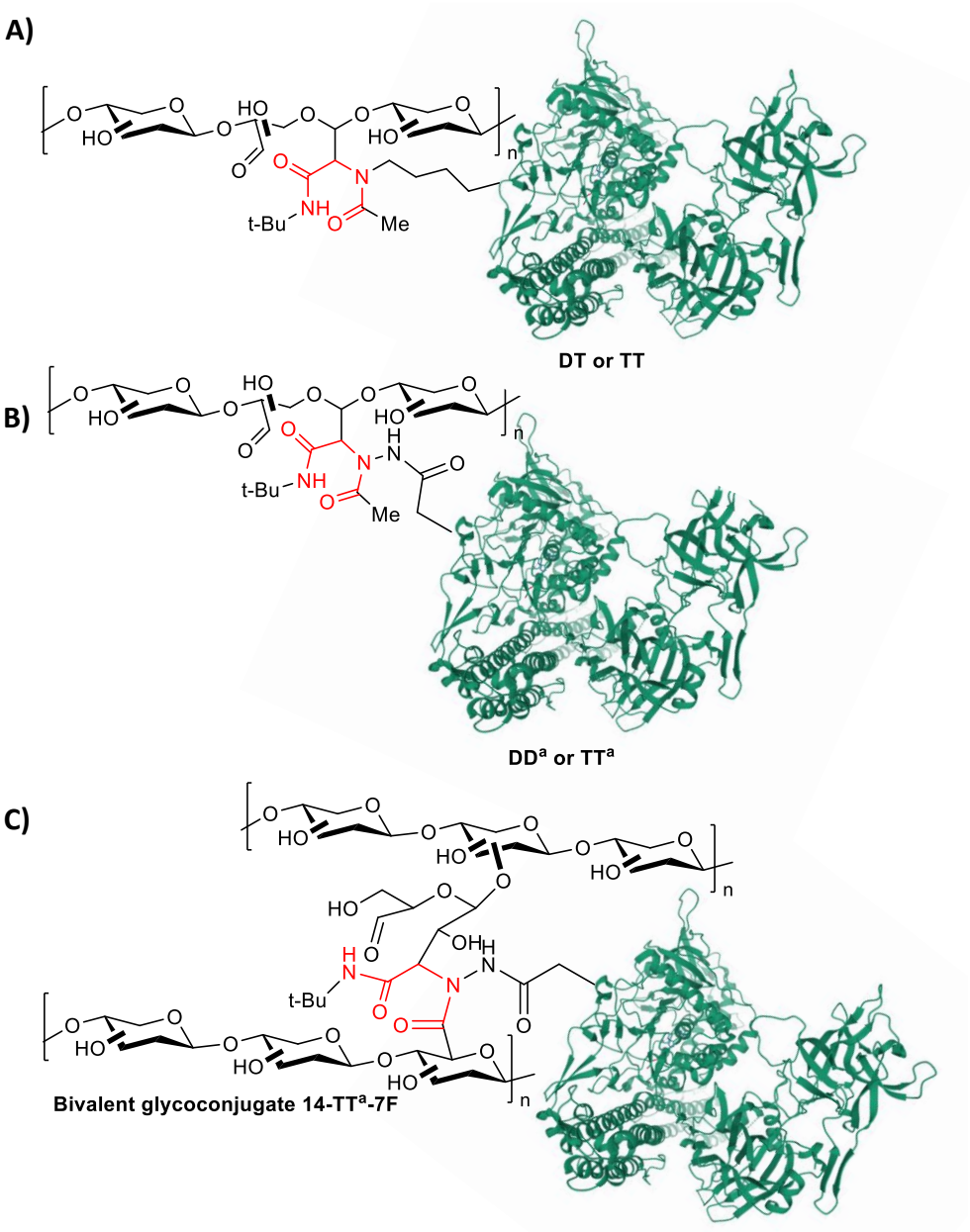


Figure 9. Protein-glycoconjugates obtained through A) Multicomponent Conjugation (MCC) of Oxo-functionalized Capsular Polysaccharide (CP) to nonactivated Diphtheria (DT) and tetanus toxoids (TT); B) hydrazide activated DT and TT (DDa or TTa); and C) MCC of two CPs to hydrazide-activated TT for the obtention of multivalent glycoconjugate Vaccines. Adapted from Méndez, Y.; Chang, J.; Humpierre, A. R.; Zanuy, A.; Garrido, R.; Vasco, A. V.; Pedroso, J.; Santana, D.;

1.3 Protein aldehydes

Protein aldehydes emerge as a result of novel strategies focused on unique unnatural functional handle incorporation into a protein due to the aldehyde moiety is a non-conventional functional group on an aminoacidic sequence of protein biomolecules. This aldehyde handle is a reactive carbonyl moiety with broad applications in synthetic chemistry.[38] Thus, the aldehyde functionality has been exploited as a novel method for site-selective protein modification where aldehyde allow us to perform chemistry like their use in multicomponent reaction-based synthetic strategies.

Aldehydes can be created trough three pathways to create reactive aldehydes at specific sites: (1) carbohydrates- or diols-containing molecules oxidation and (2) amino groups modification by aldehyde-containing/producing reagents and (3) protein oxidation. The first pathway includes the periodate oxidation of glycol and carbohydrates (glycoproteins),[39,40] and oxidase modification of sugar residues (e.g., galactose oxidase).[41] The second pathway embraces the modification of amines with NHS-aldehydes (i.e., SFB and SFAP),[42] glutaraldehyde[43] or pyridoxal 5-phosphate (PLP).[44] Also, genetic incorporation of a formylglycine (fGly) tag and non-native aldehyde containing amino acids by Formylglycine Generating Enzyme (FGE)(thiol-to-aldehyde generation), and tRNA/tRNA synthetase pairs (unnatural amino acid mutagenesis), respectively.[45–47] Additionally, enzymatic incorporation of farnesyl pyrophosphate analogs (e.g., farnesyl aldehyde pyrophosphate (FAPP)), aldehyde tags and 3-formyl-L-tyrosine by protein farnesyl-transferase (PFTase), lipoic acid ligase (LplA), and tubulin tyrosine ligase (TTL), respectively.[48–51] The third way is the oxidation of aminoacids residues by periodate or metal-catalyzed.[52,53]

Related to the protein oxidation methods, Metal catalyzed oxidation (MCO) can affect amino acid side chains by causing hydrogen abstraction, elimination, or addition reactions due to the oxidation process.[53] Metal catalyzed oxidation involves a series of reaction by which a Fenton reaction take place generating hydroxyl radicals, which indeed attack neighboring amino acids residues transforming some of them to carbonyl-containing derivates.[53,54] Where, the main carbonyl products of MCO of proteins are glutamic semialdehyde and aminoadipic semialdehyde.[53] On the other hand, periodate oxidation is useful to insert aldehydes residues

on unmodified N-terminal serine or threonine residues in proteins biomolecules by carbon-carbon bonds cleavage which contain in them either primary or secondary hydroxyls or amines resulting in glyoxyl species.[52] Even, it can be created the aldehyde residue by aminoacidic sequence oxidation trough common α-aminoacids oxidation resulting in aldehyde, carbon dioxide and ammonia formation.[55,56]

1.4 Problem statement

Multicomponent reactions (MCR's) emerge as a useful tool for modern organic chemistry One-pot MCR's of at least three components resulting in a product that incorporates atoms from all the starting materials. Thus, they become in a very suitable and convenient approach for a broad range of organic synthesis and bioconjugation procedures due to their advantages like high atom economy, diversity, generator of molecules of high complexity, efficient, mild conditions, high convergence, and step economy. The MCR's like Hantzsch and Biginelli, which are widely used to prepare interesting bioactive N-heterocycles, could be used for the development of new bioconjugation procedures, being the creation of protein aldehydes an essential part of this process.

In this regard, as **Hypothesis**, the search for the design of a multicomponent reaction-based synthetic strategy of novel linker 3,4-dihydropyrimidin-2-(1*H*)-ones (DHPM linker) and 1,4-dihydropyridine dicarboxylate (DHP linker) as linkers, would be carried out to develop bioconjugation techniques. Taking into account the importance of aldehyde proteins in the mentioned process knows oxidation procedures would be applied, and the results could be used like protein aldehydes generators.

1.5 General and specific objectives

- ***General objective***

  Study and develop synthetic strategies to obtain protein aldehydes for its use in Biginelli/Hantsch-assisted bioconjugation methodologies design.

- ***Specific objectives***
  - *To incorporate aldehyde residue on bovine serum albumin (BSA) as a biomolecule pattern by Metal catalyzed oxidation (MCO) and periodate assisted oxidation.*
  - *To filter, purify and discriminate protein and non-protein fraction trough size exclusion chromatography (PD 10 column), and UV-nanodrop measurement*

- *To analyze protein fraction trough UV spectra comparison of BSA oxidized fractions against a BSA standard.*
- *To analyze and compare retention times, purities, and UV absorbance of standard BSA and oxidized fraction BSA by HPLC (Photodiode array detector).*
- *To determine and compare the weight mass of standard BSA and oxidized fractions of BSA by UPLC-MS.*
- *To compare the Metal catalyzed and periodate oxidation methodologies applied to BSA as proof of concept.*

2 CHAPTER 2. METHODOLOGY

2.1 Chemistry

➢ Reagents

Commercial products and solvents were used as received without further purification. Bovine serum albumin (BSA) was purchased from LOBA Chemie (Mumbai, India), HEPES, magnesium chloride, L-ascorbic acid, EDTA, copper (II) chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diethyl malonate purchased from Merck (Darmstadt, Alemania). Ultrapure (Type I) water was obtained from the purification system (Direct-Q® 3UV), which was filtered through a Milli-Q system with 0.22 μm of the pore (resistivity over to 18 MΩ cm^{-1}). Dipotassium hydrogen phosphate, monobasic potassium phosphate, potassium chloride, urea, SDS, sodium sulphite, sodium periodate, ammonium hydroxide (30 %) were purchased from Fisher Scientific Company (Chicago, IL, USA). Pre-packed Shephadex™ G-25 (PD 10 column)containing columns were from GE Healthcare Europe GmbH (Freiburg, Germany).

➢ Equipment

MaxQ™ 4450 Benchtop Orbital Shakers (Digital Shaker) from Thermo Scientific™ Company (Waltham, Massachusetts, USA) was used for the incubation of Metal catalyzed oxidation reaction (MCO).

Nanodrop from Thermo Fisher Scientific™ Company (Waltham, Massachusetts, USA) was used to detected protein oxidized and protein conjugates fractions.

UPLC-MS spectrometry: reversed-phase column Acquity BEH C18 (2.1×100 mm, 3.5 μm) was from Waters (Ireland), and it was performed on a Waters instrument comprising a binary system

manager (ACQUITY UPLC® I-Class) with an automatic injector and Waters® SYNAPT® G2-S/Si as a mass spectrometer. Linear gradients of MeCN (0.01% acetic acid) into H_2O (0.01% formic acid) were run at flow rate of 0.4 mL/min. The solvents for UPLC were H_2O (Type I), and MeCN (MS quality).

HPLC spectrometry: An HPLC apparatus Ultimate 3000, C-18 column for HPLC Hypertensil GOLD^{TM} (150 x 4.6 mm) equipped with an autosampler, a quaternary pump, a column compartment, and a photodiode array detector (PAD) was used for the analysis of BSA and oxidized fractions at different wavelengths (230 nm, 254 nm, and 280 nm), but the absorbance at 254 nm was the wavelength which gives us better results for peaks analysis. The analysis was carried out using a reverse-phase C-18 column. Linear gradients of MeCN (0.01% acetic acid) into H_2O (0.01% acetic acid acid) were run at flow rate of 0.4 mL/min. The solvents for UPLC were H_2O (Type I) and MeCN (MS quality).

PD10-assisted purification: PD10 column Pre-packed Shephadex™ G-25 was used to purify protein-based samples. The purification of the oxidized samples is carried out after the column is equilibrated with buffer or water type I. Then, the oxidized sample is added and let it run with the constant addition of phosphate buffer or water type I until to ensure complete elution of the sample.

2.2 General procedure to obtain bovine serum albumin oxidized (BSA) (Protein aldehydes)

2.2.1 Metal catalyzed oxidation (MCO) of BSA

The MCO of BSA was carried out following the method previously reported[53] with slight modifications. BSA protein was dissolved at 1 mg/mL in a buffer (50 mM HEPES buffer, pH 7.4, 1000 mM KCl, and 10 mM $MgCl_2$ solutions) in Eppendorf tube. Then, to start oxidation, 400 μL neutral ascorbic acid (freshly prepared, 25 mM) and 400 μL of $FeNO_3.9H_2O$ (100 μM) are added into protein solution, and incubated on a digital shaker at 37°C, during 24 hours and at a shaking velocity of 150rpm overnight, Then, the reaction is quenched by addition 400 μL of EDTA (1 mM) during 30 minutes. After that time, the sample was undergone to filtration and purification with a PD 10 column at room temperature with the phosphate buffer, and then water type I. Finally, it is obtained the proteins fraction 1 and 2. Protein-containing samples were detected using nanodrop (**Figure 10**), and characterized through UV, HPLC, and UPLC-MS.

- A standard BSA sample was prepared in HEPES buffer solution and in water type I to compare with previously oxidized BSA samples.
- UV-nanodrop measurements [phosphate buffer or water type as blank]: each measurement is compared to BSA standard as the protein pattern
    - UV oxidized BSA Fraction A, phosphate buffer blank (λ, nm): 223 and 280
      UV BSA standard (λ, nm): 232 and 280
    - UV oxidized BSA Fraction 1 , water type 1 blank (λ, nm): 213 and 280
      UV BSA standard (λ, nm): 213 and 280
    - UV oxidized BSA Fraction 2, water type 1 blank (λ, nm): 228 and 280
      UV BSA standard (λ, nm): 215 and 280
- UPLC-MS [linear gradient H_2O/MeCN (5:95) over 10 min on Acquity BEH C18, 7μm RPC 2.1 × 50 mm column] :
    - Standard BSA: t_R, 3.86 min; BSA, Theor. Molecular Mass Calc.=66430.3 Da*; BSA (m/z) found = 66628.91 ± 115.23 Da.
      *The molecular weight of bovine serum albumin (BSA) was calculated based on its amino acid sequence.[57]
    - Oxidized BSA Fraction 1: t_R, 3.86 min, m/z found = 66465.98 ± 58.11 Da. Difference with standard BSA, 162.93 ± 0.01 Da.
    - Oxidized BSA Fraction 2: t_R, 4.11 min, m/z found = 66491.92 ± 63.78 Da. Difference with standard BSA, 136.99 ± 0.01 Da.
- HPLC spectrometry: [linear gradient H_2O/MeCN (95:5) over 10 min on Hypertensil GOLD™ C-18 column, 7μm particle size (4.6 × 150 mm column), λ=230nm]:
    - Standard BSA,t_R (min), Purity (%):2.95,1.44; 3.453, 29.15; 3.867, 69.41.
    - Oxidized BSA Fraction 1, t_R (min), Purity (%): 0.593, 29.27; 1.790, 7.77; 2.100, 53.33; 3.383, 9.63.
    - Oxidized BSA Fraction 2, t_R (min), Purity (%): 0.593,26.40; 1.780, 15.71; 2.097, 49.29; 3.383, 8.60.

2.2.2 Periodate oxidation on BSA

A protocol previously reported[43] was followed to carry out the BSA oxidation, with slight modifications depending on available reagents and equipment. BSA solution (2 mg/ml) was prepared using phosphate buffer (KH_2PO_4 / K_2HPO_4, pH ~7) in a sterile Eppendorf tube. Then, 500 μL of $NaIO_4$ solution in the buffer (2.5mM) is added to 1 mL of BSA solutions to start the

oxidation, and the reaction is stirring by three minutes at room temperature. After that, the reaction was quenched by the addition of 750 μL of Na_2SO_3 solution (12.5 mM), and protected from light exposure. After ten minutes, the mixture was purified using size exclusion chromatography (PD10 desalting column), and water as eluent. Finally, protein-containing fractions of 500 μL each one were collected and classified through UV nanodrop spectroscopy (**Figure 10**). It was obtained three protein fractions (fraction 3, 4, and 5) from this method.

- The same samples and spectral data (HPLC and UPLC) of standard BSA were used to compare with oxidized BSA samples with previously oxidized BSA samples.
- UV-nanodrop measurements [phosphate buffer or water type as blank]: each measurement is compared to BSA standard as the protein pattern
    - UV oxidized BSA Fraction B, phosphate buffer blank (λ, nm): 213.5 and 280
      UV BSA standard (λ, nm): 207.5 and 280
    - UV oxidized BSA Fraction C, phosphate buffer blank (λ, nm): 200.5 and 280
      UV BSA standard (λ, nm): 207.5 and 280
    - UV oxidized BSA Fraction 3, water type 1 blank (λ, nm): 201.5 and 280
      UV BSA standard (λ, nm): 213.5 and 280
    - UV oxidized BSA Fraction 4, water type 1 blank (λ, nm): 208 and 280
      UV BSA standard (λ, nm): 213.5 and 280
    - UV oxidized BSA Fraction 5¸ water type 1 blank (λ, nm): 205 and 280
      UV BSA standard (λ, nm): 213.5 and 280
- UPLC-MS: [linear gradient H_2O/MeCN (5:95) over 10 min on Acquity BEH C18, 7μm RPC 2.1 × 50 mm column]:
    - Oxidized BSA fraction 3: t_R, 3.97 min, m/z found = 66614.20 ± 50.82 Da. Difference with standard BSA, 14.71 ± 0.01 Da.
    - Oxidized BSA fraction 4: t_R, 3.83min, m/z found = 66614.34 ± 60.68 Da. Difference with standard BSA, 14.57 ± 0.01 Da.
    - Oxidized BSA fraction 5: t_R, 3.92 min, m/z found = 66601.45 ± 49.99 Da. Difference with standard BSA, 27.46 ± 0.01 Da..
- HPLC spectrometry: [linear gradient H_2O/MeCN (5:95) over 10 min on Hypertensil GOLD™ C-18 column, 7μm particle size (4.6 × 150 mm column), λ=230nm]:
    - Oxidized BSA Fraction 3, t_R (min), Purity (%): 0.593, 27.73; 1.777, 2.95; 2.100, 60.37; 3.383, 8.94.

- Oxidized BSA Fraction 4, t_R (min), Purity (%): 0.593, 34.22; 2.097, 55.18; 3.38, 10.60
- Oxidized BSA Fraction 5, t_R (min), Purity (%): 0.593, 34.71; 1.757, 15.14; 2.107, 39.75; 3.380, 10.39.

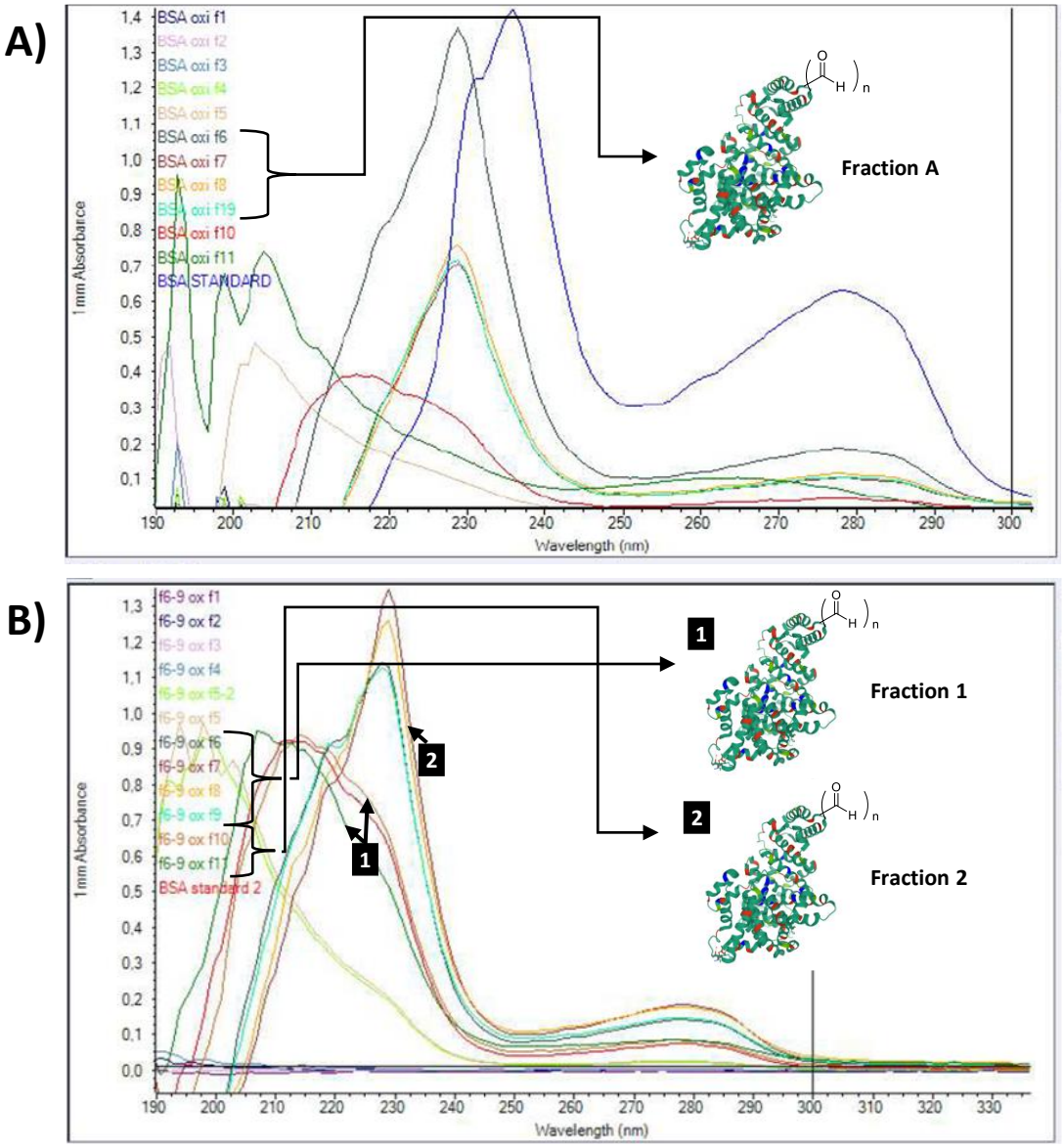


Figure 10. UV nanodrop analysis of fractions corresponding to size exclusion column chromatography of ***A) Final reaction mixture of MCO reaction B) Fraction A.***.

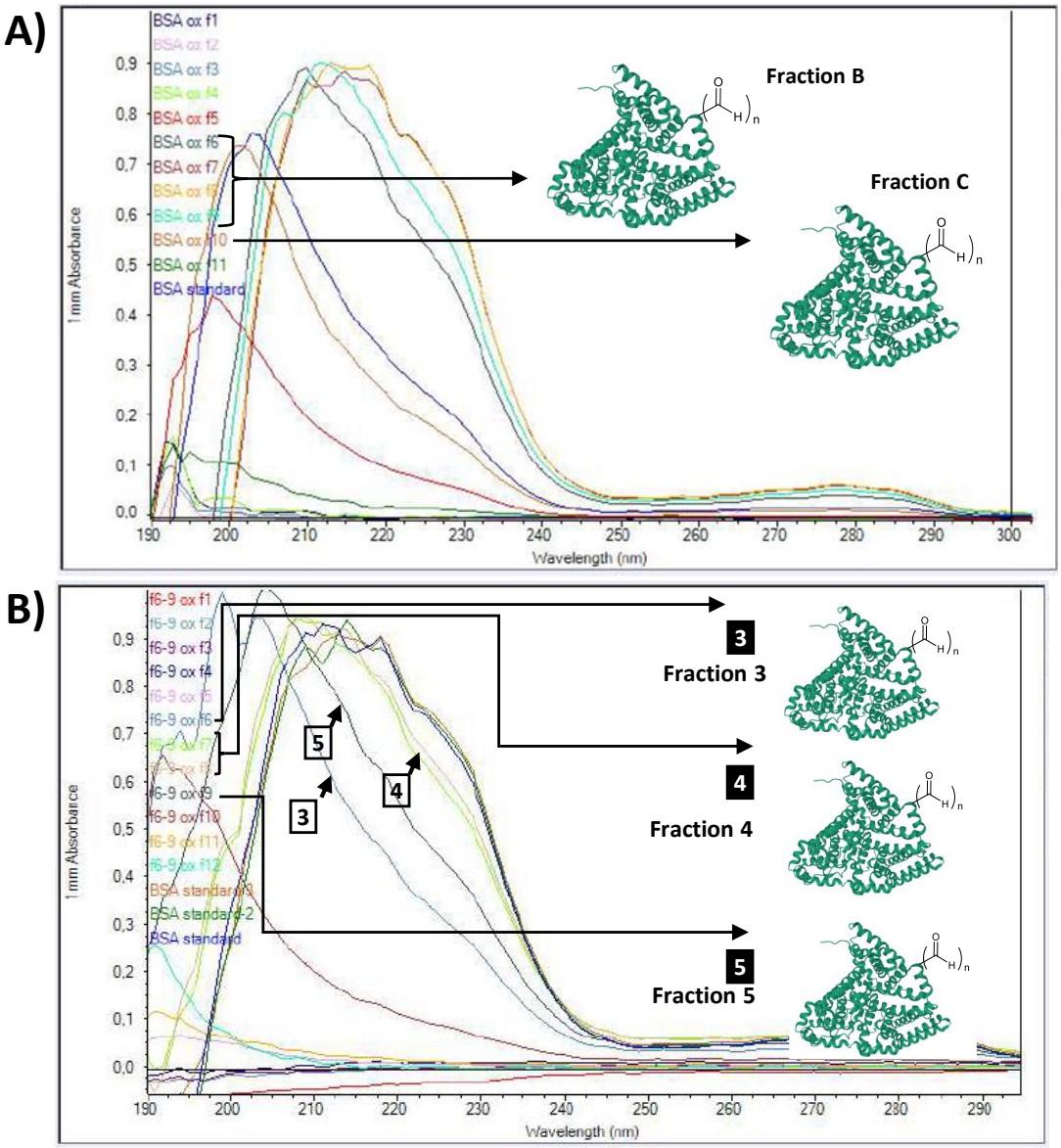


Figure 11. UV nanodrop analysis of fractions corresponding to size exclusion column chromatography of ***A) final reaction mixture of Periodate oxidation reaction*** *and* ***B) Fraction B.***

3 CHAPTER 3: RESULTS AND DISCUSSION

A first approach of the potential use of 3,4-dihydropyrimidin-2-(1*H*)-ones (DHPM linker) and 1,4-dihydropyridine dicarboxylate (DHP linker) as linkers for bioconjugation was designed, in order to find an efficient strategy for the chemical linkage of carriers and cargos. Both mentioned scaffolds (DHPM and DHP linkers) would be generated through slight modifications of the multicomponent reaction (MCR) Biginelli and Hantzsch, respectively. The MCR's used could allow us to carry out the one-pot reaction of the inserted aldehyde-functionalized biocarrier (oxidized protein, BSA as proof of concept), a nitrogen source (urea and ammonium hydroxide), and a β-ketoester from drug/cargo. The bioconjugation method would be enabled

us to generate novel Cargo/drug-DHPM/DHP-Biocarrier conjugates (**Figure 12** and **Figure 13**).

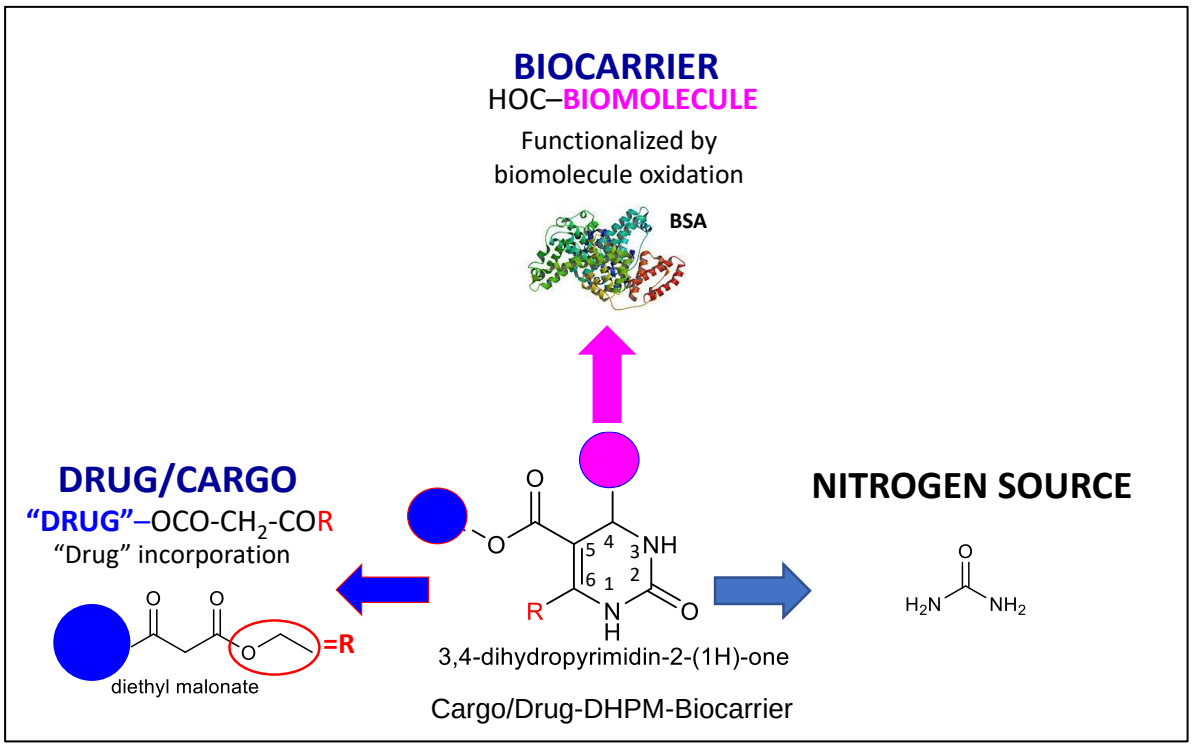


Figure 12. Bioconjugation technique using a DHPM linker resulted of Biginelli reaction

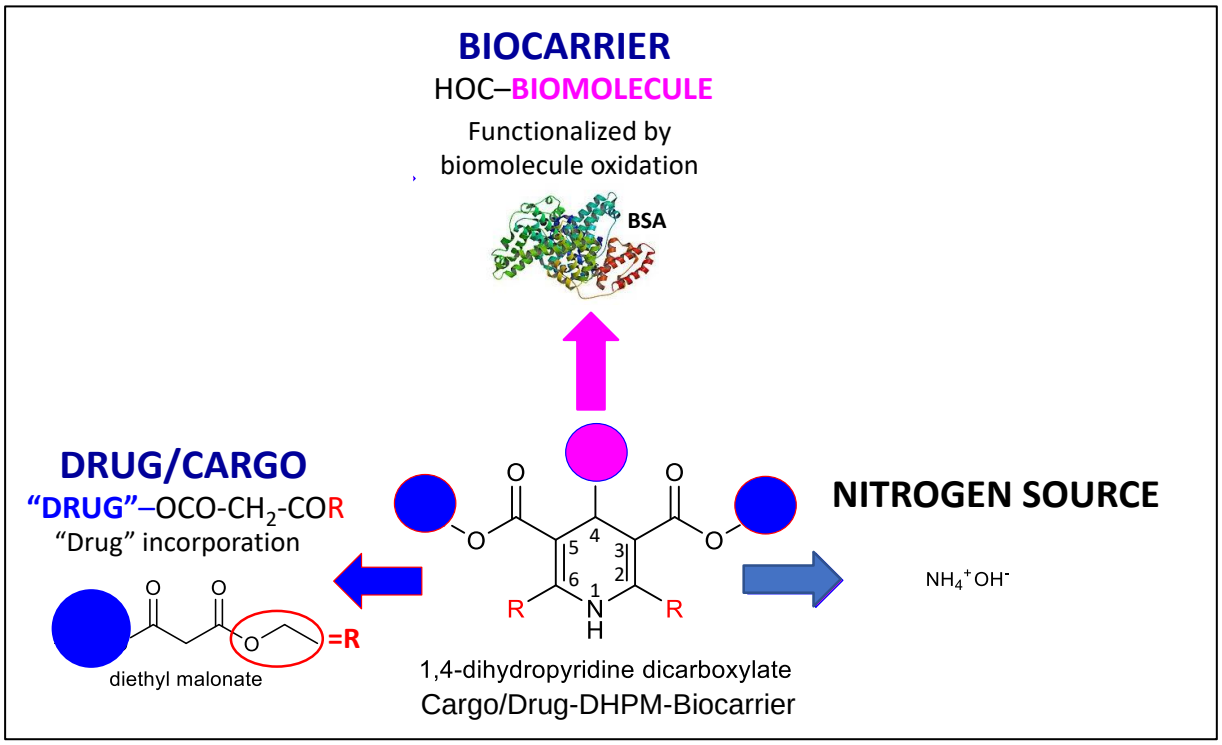


Figure 13. Bioconjugation technique using a DHP linker resulted of Hantzsch reaction

The chemicals functions needs in each MCR's would be introduced considering the reaction conditions and steric hindrances found in each bioconjugation procedure. The designed methodology of the present work consisted of previous oxidation of biomolecule in order to introduce aldehyde function, and carry out the bioconjugation reactions based on Biginelli and Hantzsch MCR's, where the cargo is incorporated through the β-ketoester moiety (**Figure 12** and **Figure 13**). This research would be focused on defining the advantages, and disadvantages of the use of different methodologies as the Metal catalyzed oxidation (MCO) and periodate oxidation to modified biomolecules introducing aldehyde function, using BSA as the pattern for the proof of concept. With protein aldehyde in hand, the design of MCR-assisted bioconjugation procedures would also be proposed for its application in future works.

3.1 Metal catalyzed oxidation and periodate oxidation of bovine serum albumin (BSA)

3.1.1 Oxidation of amino acids in proteins

Oxidative processes have a key role in disease and aging development since they cause tissue damage by free radical oxidation resulting in structure-activity alteration, normal protein interaction inhibition, side-chain modification of amino acids, backbone peptide cleavage, and protein-protein cross-linking .[54,58–60] The protein oxidation became important when it is used as peptides-based therapeutic agents taking into account that a slight change of amino acid residues results in modification on its activity *in vivo*. Protein oxidation can be carried out by exposure to oxidative species, through reactive oxygen intermediates from metabolic reactions, or as byproducts of oxidative stress reactions.[61] On the other hand, it can be achieved by the presence of strong oxidizing compounds that enable to selective damage of the structural protein. Chemical agents capable of oxidizing and modifying protein structure include reactive oxygen species (ROS), reactive iodinated species (RIS) such as periodate, metal salts (iron or copper salts) in the presence of oxygen species, but also reactive nitrogen species (RNS), hypobromous acid, performic acid, trichloromethyl peroxyl radical, metal-chelating compounds under right conditions, gamma radiation and UV light, among others.[58,62–64] Additionally, *in vitro* studies of protein oxidation indicate that all protein and peptides are prone to damage by the radical hydroxyl and superoxide anion, and the analysis of oxidation products shows the altered molecular weight (either fragmentation or oligomerization), net charge alteration, tryptophan destruction, and tyrosine dimerization. [65] Despite all the risks involved in the oxidation of proteins, it can be attained the oxidation proteins in a controlled condition to

obtain the desired biomolecule modifications with different biomedical and chemical applications.

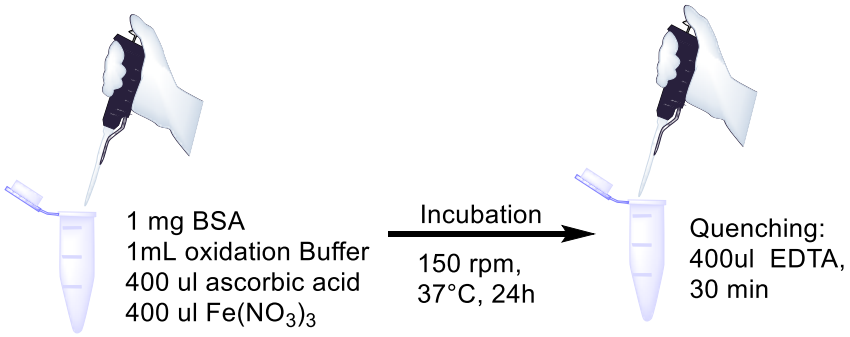
The aim of the present work is to developed a suitable and greener method to obtain a chemical linker between either biomolecules or drugs through Biginelli and Hantzsch reaction, which involve an aldehyde group to carry out the reaction. Thus, it is needed to incorporate the aldehyde groups by oxidation methods capable of modifying the side-chain of aminoacids on proteins and peptides to obtain the desired group to be used on a MCR-based bioconjugation strategy. Metal catalyzed oxidation (MCO) and periodate oxidation were the oxidation procedures chosen to introduce aldehyde function in the biomolecule pattern (BSA protein).

3.1.2 Metal catalyzed oxidation (MCO) procedure

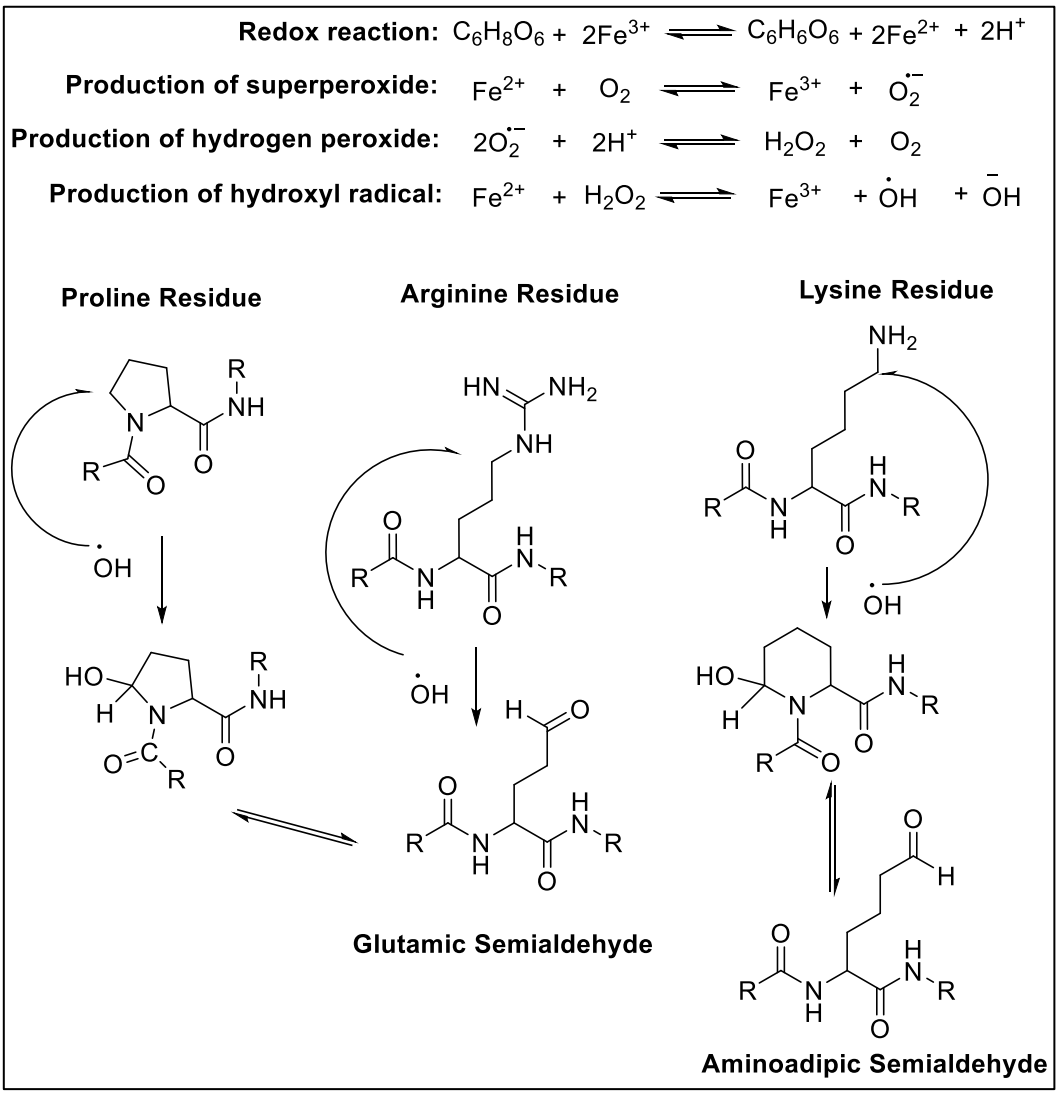
As was mentioned before, the BSA protein was used as a biomolecule pattern to carry out selected oxidations. MCO of BSA protein was accomplished by the mixture of the biomolecule with the freshly prepared oxidation buffer (10mM HEPES sol., 1000mM KCl sol., and 10mM $MgCl_2$), freshly prepared, L-ascorbic acid (25 mM), and ferric nitrate (100 µM) (**Scheme 2**). Taking into account a previous report,[53] oxidation of proline, arginine, and lysine residues would be expected through a radical mechanism (**Scheme 2**). The mixture was incubated on a digital shaker (T=37°C, t=24h, and v=150rpm) overnight.

Metal catalyzed oxidation (MCO) involves the reduction of iron (III) by a suitable electron donor (e.g., ascorbate), the oxidation of previously reduce Metal (Fe^{3+} to Fe^{2+}) to obtain superperoxide, continuing with the generation of hydrogen peroxide. The reduced metal Fe^{2+} and hydrogen peroxide allowed to produce the hydroxyl radical, which promoted the protein oxidation (**Scheme 3**). The reduced metal ions join specific metal-binding sites on proteins that react with peroxide to generate hydroxyl radicals (so-called Fenton reaction), which indeed attack neighboring amino acid residues, and some of them are transformed to carbonyl-containing derivates (i.e., aldehydes residues useful for our conjugation purposes).[53,54] Moreover, it was required to be careful with this procedure since oxidation potential sites within a peptide comprise the peptide backbone and side-chain amino acid groups. It can occur the hydrogen atom abstraction at the α-carbon of an amino acid chain upon reaction with hydroxyl radical to form a radical intermediate, and as a result of a subsequent reaction, peptide bond cleavage and protein fragmentation structure could happen. This potential secondary reaction often forms carboxylic acids or carbonyls (aldehydes or ketones).[43] During the entire time of the reaction, strict pH control was carried out, to avoid or reduce the possible damage to the

tertiary structure of the protein. The oxidation reaction was stopped with the addition of EDTA (1mM) solution (**Scheme 1**). The mixture was separated using a PD10 column.



Scheme 2. Metal catalyzed oxidation procedure

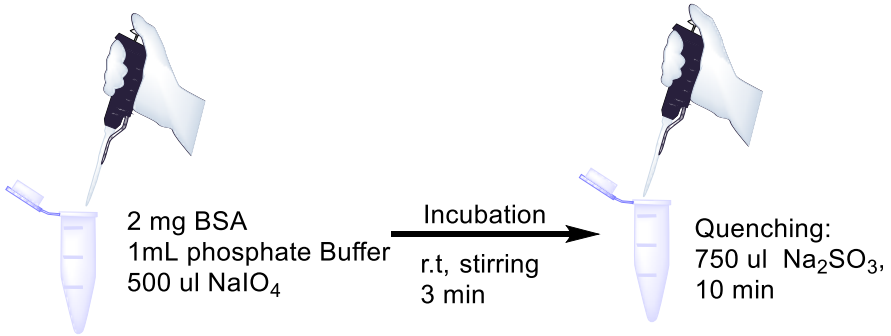


Scheme 3. Reaction scheme for the formation of glutamic and aminoadipic semialdehyde

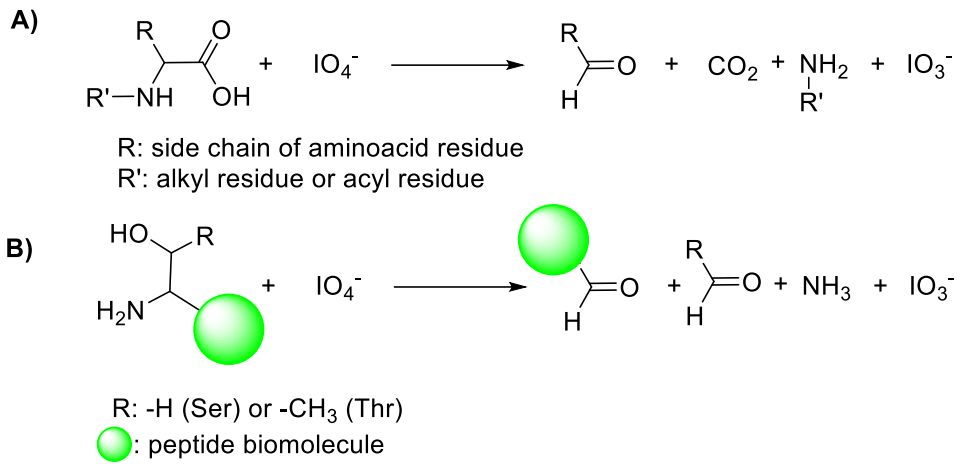
3.1.3 Periodate oxidation procedure

Another oxidation method was applied in which BSA protein was also used as a biomolecule pattern. Periodate oxidation of BSA protein was accomplished by the mixture of the biomolecule with the phosphate buffer (KH_2PO_4 / K_2HPO_4, pH~7), previously prepared, and sodium periodate ($NaIO_4$, 2.5 mM) (**Scheme 4**). Taking into account previously reports,[52,56] the oxidation by periodate of all amino acid residues at different rates would be expected through a radical mechanism (**Scheme 5**). Clamp and Hough[56] and Nicolet and Shinn,[55] established that the rates of oxidation of each residue vary with pH, being more faster at alkaline pH values, but also showed that the oxidation rates decrease when they are N-substituted like in protein peptides (**Scheme 5A**).The free amino acids more prone to be oxidized by periodate are serine, threonine, cysteine, methionine, proline, hydroxyproline, tryptophan, tyrosine, histidine, but also the cystine dimer.[56] Even, cysteine, cystine, methionine, tryptophan, tyrosine, and histidine could be oxidized either as free amino acids or as part of a polypeptide chain.[56] On the other side, if the peptide or protein contains N-terminal Ser or Thr residues, its lateral chain, which consists on $-CH_2(OH)$, is quickly oxidized at pH 7, due to periodate can form a cyclic intermediate which results in aldehyde handles generation (**Scheme 5B**).[52,55]

BSA periodate oxidation was carried out under pH and stoichiometry control and keeping minimum reaction times to avoid oxidative damage to other amino acid residues within the protein.[43,66] Thus, the mixture was incubated three minutes at room temperature and pH ~7. The pH was checked at the beginning and final of the oxidation reaction. The applied experimental conditions decrease the potential for side reactions by the very low periodate-to-protein molar ratios use.[52,67] The oxidation reaction (quenching) was stopped with the treatment of the oxidation mixture with sodium sulfite solution (Na_2SO_3, 1mM), in the dark and during ten minutes (**Scheme 3**). Finally, the mixture was separated using a PD10 column.



Scheme 4. Periodate oxidation procedure



Scheme 5. Periodate oxidation reaction for free amino acids(A) and N-terminal Serine or Threonine residues (B)

3.2 Separation, nanodrop measurements, and potential oxidized amino acids analysis of MCO and periodate BSA oxidized fractions

The mixture of MCO and periodate oxidation procedures were separated using a size exclusion chromatography (PD10 column) with phosphate buffer first to remove reagent remnant or low weight molecular compounds from oxidation reaction, but also to separate the protein and non-protein fractions. Those fractions with protein content were unified and separated again using water Type I to eliminate any interference or additional problems in the subsequent HPLC and UPLC-MS analysis (**Figure 14**). The fractions from PD10 separation of both MCO and periodate oxidation procedures were analyzed using nanodrop equipment. UV nanodrop spectroscopy allows as to discriminate between those non- protein or protein fractions. Protein fractions showed expected UV spectra with two well-defined maximums of absorbances around 240 and 280 nm (**Figure 15 - Figure 18**).

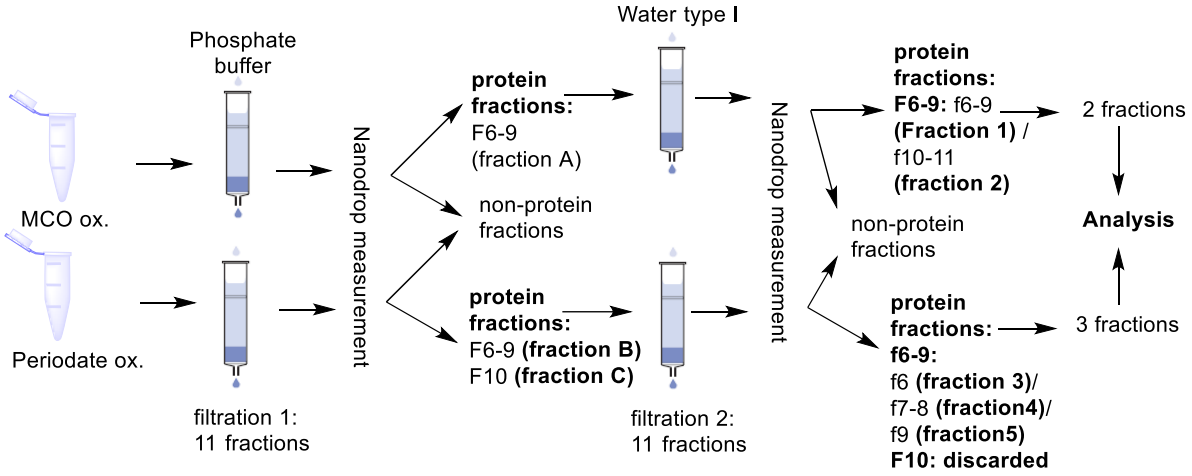


Figure 14. General scheme to obtain protein fractions

Related to the filtration and purification process of MCO procedure, the fractions 6,7,8 & 9 from filtration 1, which were obtained using phosphate buffer as mobile phase, had similar UV nanodrop profile compared to BSA standard (1mg/mL) (**Figure 15)**. Hence, mentioned fractions were joined and labeled as Fraction A. The UV nanodrop profile of Fraction A (**Figure 16)** showed a typical protein profile, but only a slight hypsochromically shifting on UV spectra was observed due to a change in BSA structure. Then, the fraction A was undergone to a second filtration using a PD10 column and water type 1 as mobile phase, to avoid problems related to the presence of phosphate buffer in UPLC-MS and HPLC-UV-vis measurements such us possible interferences with the analyte signals. After the analysis of UV nanodrop profiles of all fractions from the second purification, the fractions 6-9 and 10-11 were combined and labeled as Fraction 1 and 2, respectively, taking into account its quite similar spectra, which showed a slight bathochromic shift (**Figure 17**). Finally, the UV nanodrop profile for Fraction 1 and 2 were obtained and compared (**Figure 18**). Both fractions were analyzed through UPLC-MS and HPLC-UV-vis measurements.

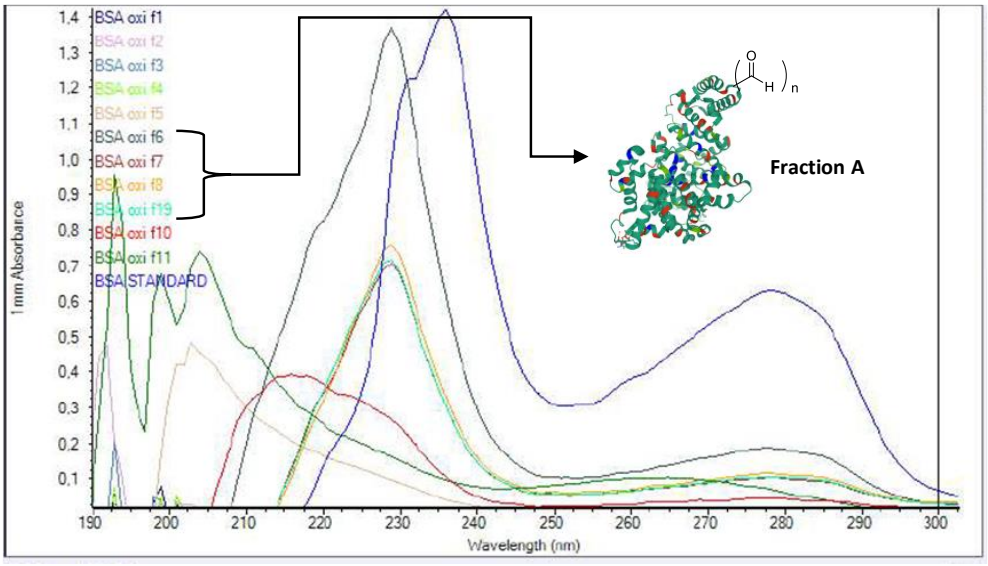


Figure 15. UV nanodrop analysis of fractions obtaining by size exclusion column chromatography of the MCO procedure (filtration 1, Phosphate buffer as mobile phase).

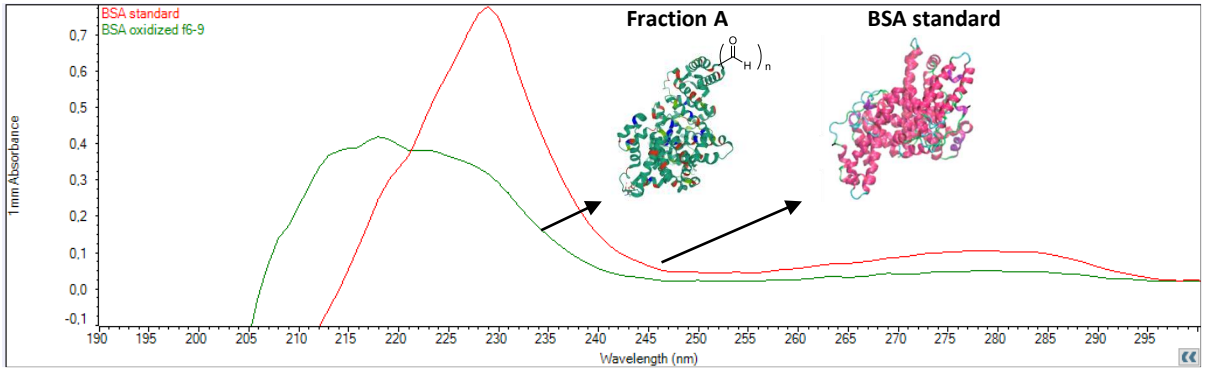


*Figure 16. UV nanodrop analysis of Fraction A compared with standard **BSA** (filtration 1, phosphate buffer as mobile phase).*

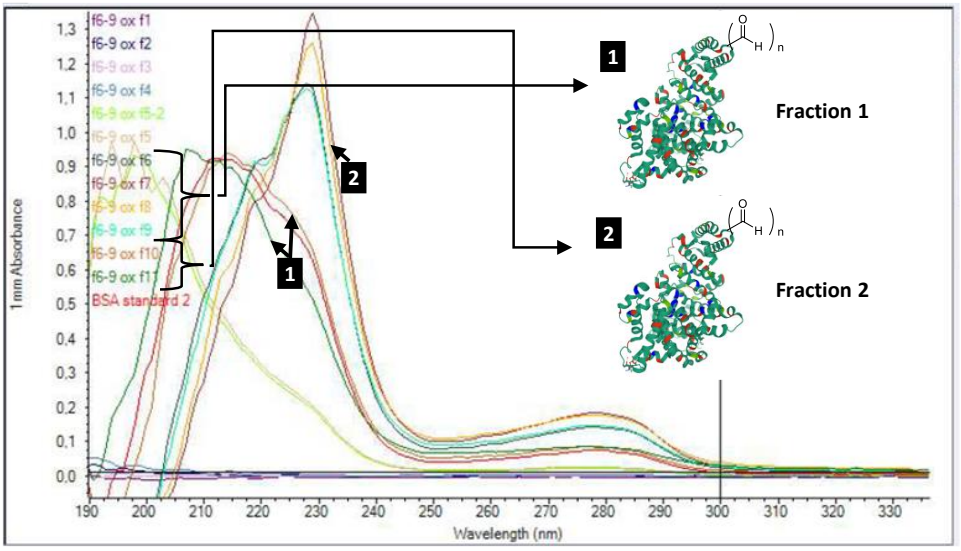


Figure 17. UV nanodrop analysis of fractions from Fraction A, obtaining by size exclusion column chromatography of the MCO procedure (filtration 2, water type 1 as tmobile phase).

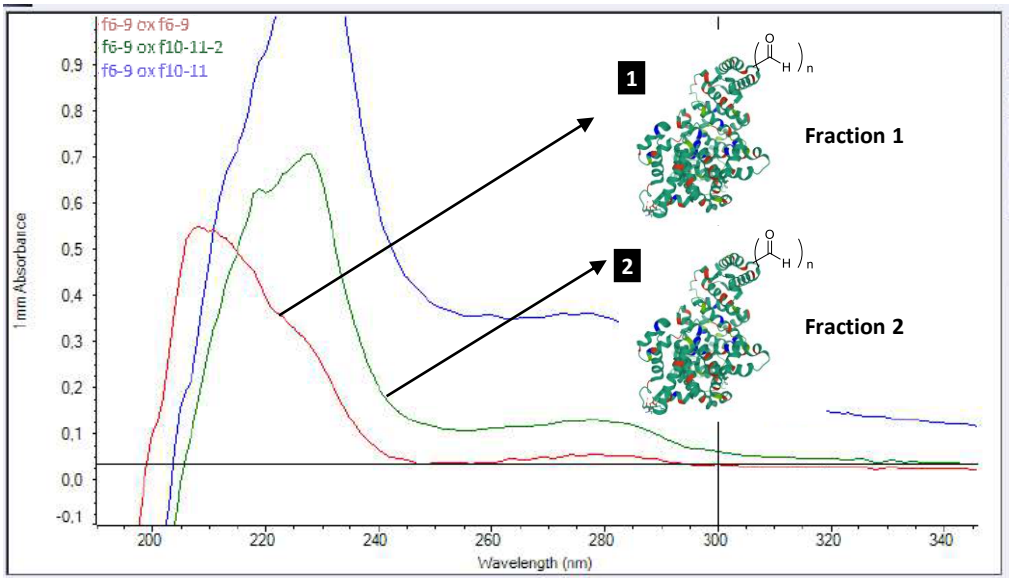


Figure 18. UV nanodrop profiles of Fractions 1 and 2 (filtration 2, water type 1 as mobile phase).

Concerning to the filtration and purification process of the reaction mixture obtained under periodate oxidation procedure, the fractions 6,7,8 & 9, were mixed and labeled as Fraction B, and fraction 10 was labeled as Fraction C from filtration 1 using phosphate buffer as mobile phase in the size exclusion chromatography (PD10 column), due to it showed similar UV nanodrop profile to standard BSA (1mg/mL) (**Figure 19**). Fractions B and C were also analyzed using nanodrop, and profiles like BSA protein were obtained with a slight shifting on UV spectra due to a change in BSA structure after the oxidation procedure (**Figure 20**). Fractions B and C were undergone to a second filtration and purification using size exclusion chromatography and water type 1 as the mobile phase, looking to eliminate problems related to the use of phosphate buffer in UPLC-MS and HPLC-UV-vis measurements, as previously described. After the second filtration, some fractions from Fraction B were mixed following the analysis of its UV nanodrop profiles, which showed similar and typical protein UV profile, but with a slight hypsochromic shift, allowing to obtain three diffrent Fractions 3, 4 and 5 (**Figure 21** and **Figure 22**). Fractions derivated from C were discarded due to the analysis of nanodrop UV profiles do not show like-protein UV profile (**Figure 23**). Fraction resulting from Fraction B was analyzed through UPLC-MS and HPLC-UVvis measurements.

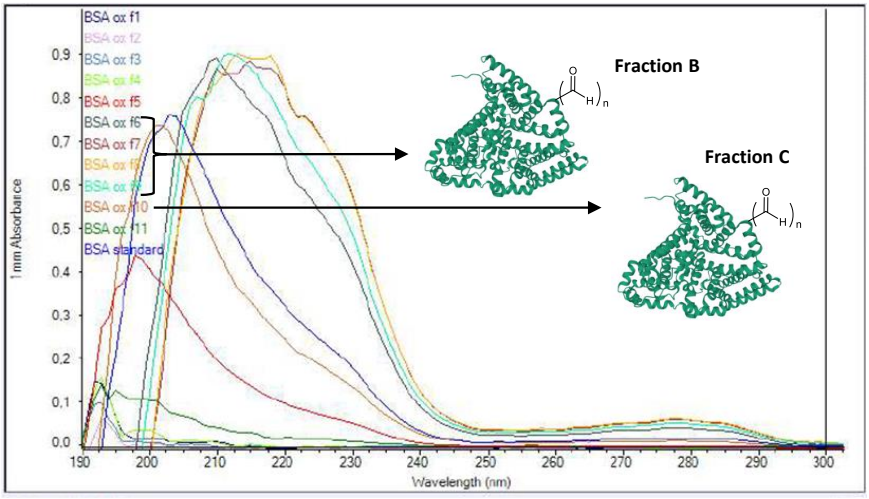


Figure 19. UV nanodrop analysis of fractions obtaining by size exclusion column chromatography of the Periodate procedure (filtration 1, Phosphate buffer as mobile phase).

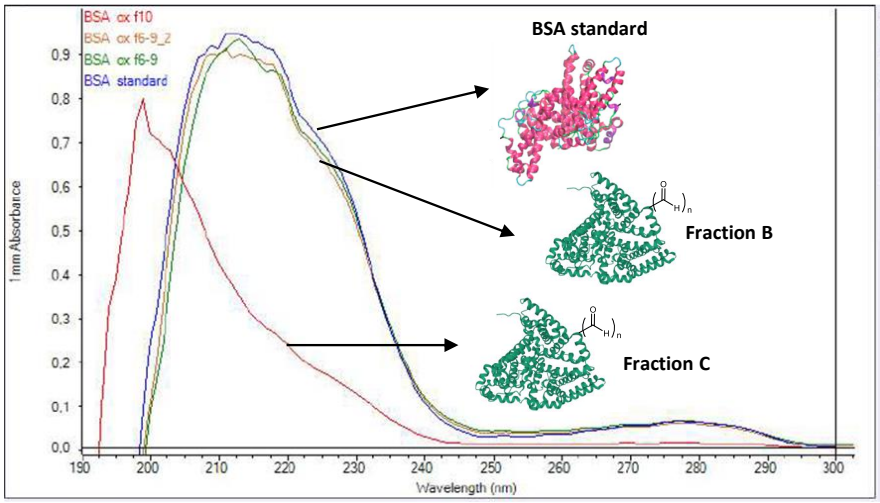


*Figure 20. UV nanodrop analysis of Fraction B and C compared with standard **BSA** (filtration 1, phosphate buffer as mobile phase).*

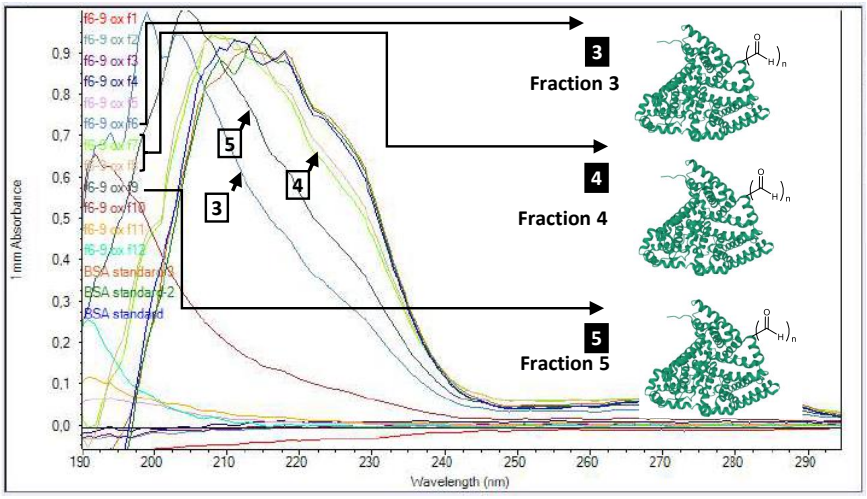


Figure 21. UV nanodrop analysis of fractions from Fraction B, obtaining by size exclusion column chromatography of the periodate oxidation procedure (filtration 2, water type 1 as tmobile phase).

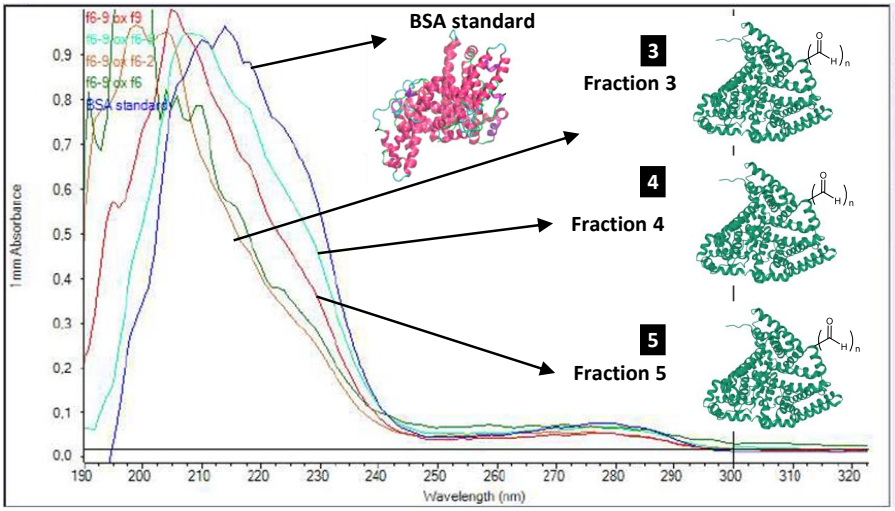


Figure 22. UV nanodrop profiles of Fractions 3, 4 and 5 compared with standard BSA (filtration 2, water type 1 as mobile phase).

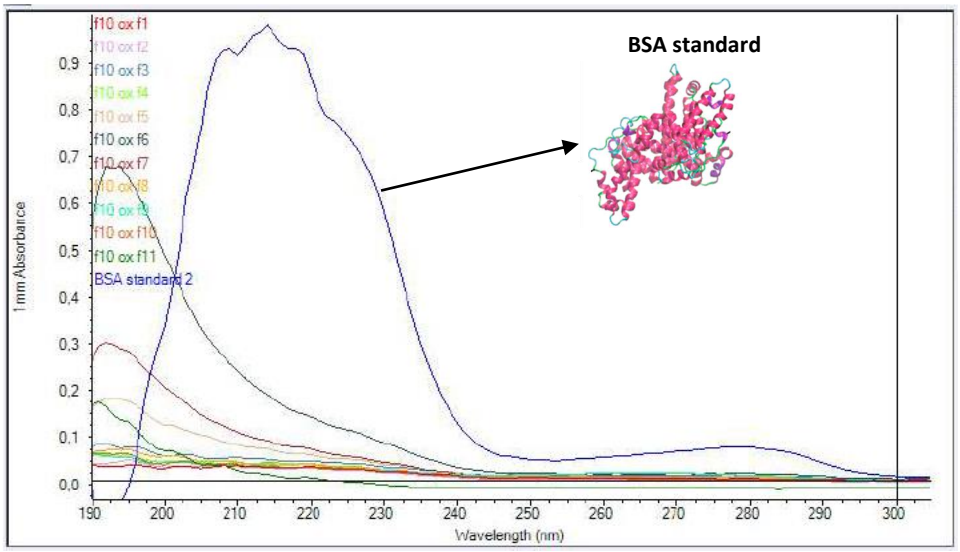


Figure 23. UV nanodrop analysis of fractions from Fraction C, obtaining by size exclusion column chromatography of the periodate oxidation procedure (filtration 2, water type 1 as tmobile phase).

The BSA sequence and structure are well known.[57,68–70] Related to oxidized amino acid analysis, **Table 1** showed the BSA amino acid composition highlighting the aminoacids that could suffer oxidation. BSA protein has 28 proline, 26 arginine and 60 lysine residues that could be selectively oxidized through the MCO procedure (highlighted in yellow), while all amino acids residues might be oxidized under periodate oxidation method. However, some reports considered Ser and Thr more prone to be oxidized faster than other amino acids under this procedure. Thus, BSA will undergo oxidation of the most exposed amino acid residues in each protocol performed in this work, allowed to obtain the oxidized BSA with aldehyde residues incorporated in the protein sequence (**Figure 24**).

Table 1. BSA Aminoacidic composition. MCO procedure selectively oxidize Lys, Pro, and Arg (Highly in yellow)

Ala48	Cys 35	Asp 41	Glu 58
Phe 30	Gly 17	His 16	Ile 15
Lys60	Leu 65	Met 5	Asn 14
Pro 28	Gln 21	Arg 26	Ser 32
Thr 34	Val 38	Trp 3	Tyr 21

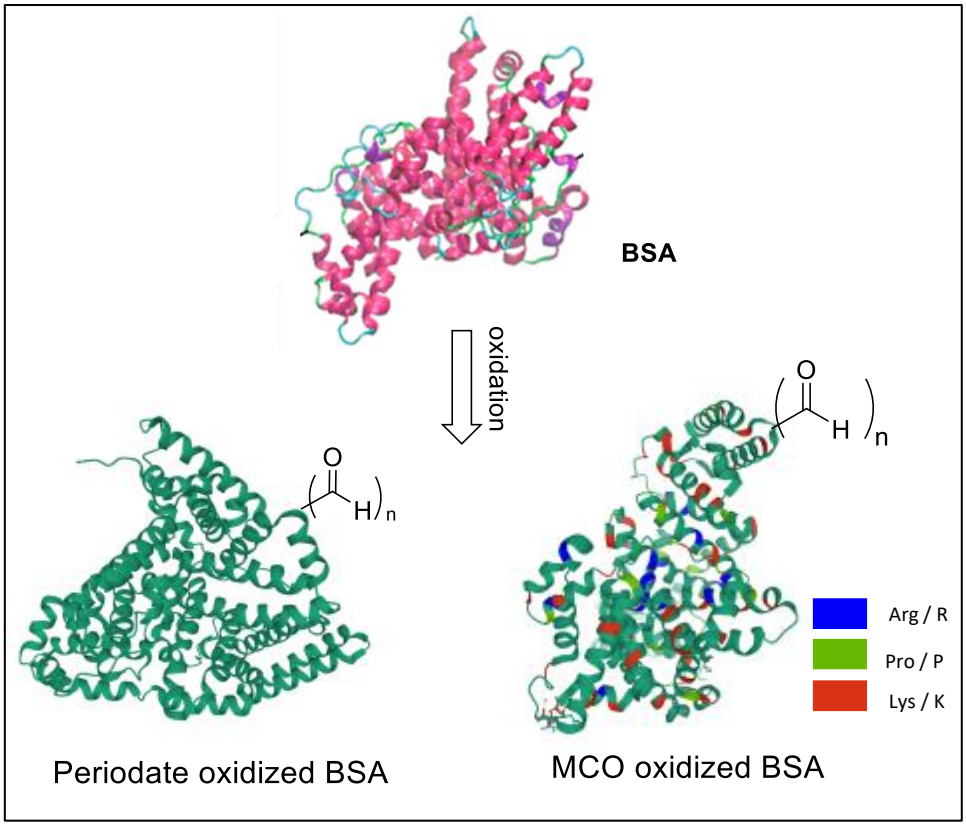


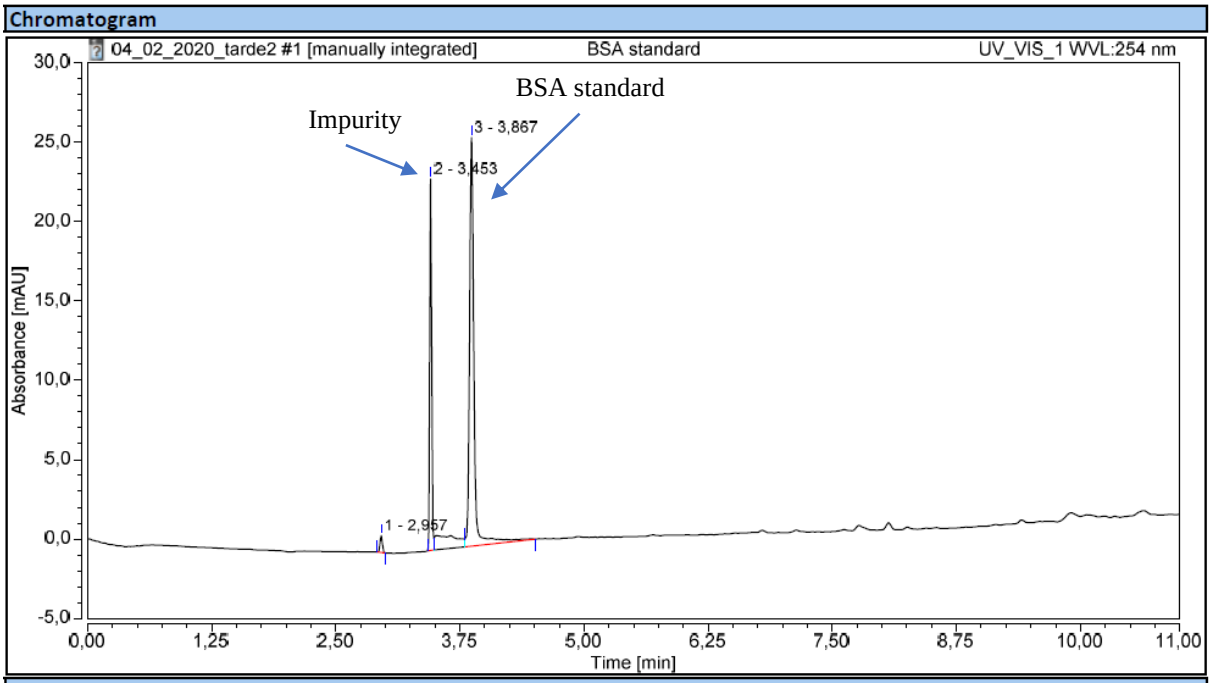
Figure 24.Representation of oxidized BSA under both, periodate and MCO procedure.

Finally, five final protein fractions, 1 and 2 from MCO procedure, and 3, 4, and 5 from periodate procedure were obtained. HPLC and UPLC-MS were carried out in order to analyze the amino acid modifications through each oxidation procedure.

3.3 HPLC (Photodiode array detector) analysis of MCO and periodate oxidized fractions

All oxidized protein fractions (Fractions 1 and 2 for MCO procedure; and Fractions 3-5 for periodate procedure) were characterized using HPLC spectrometry. The five oxidized BSA

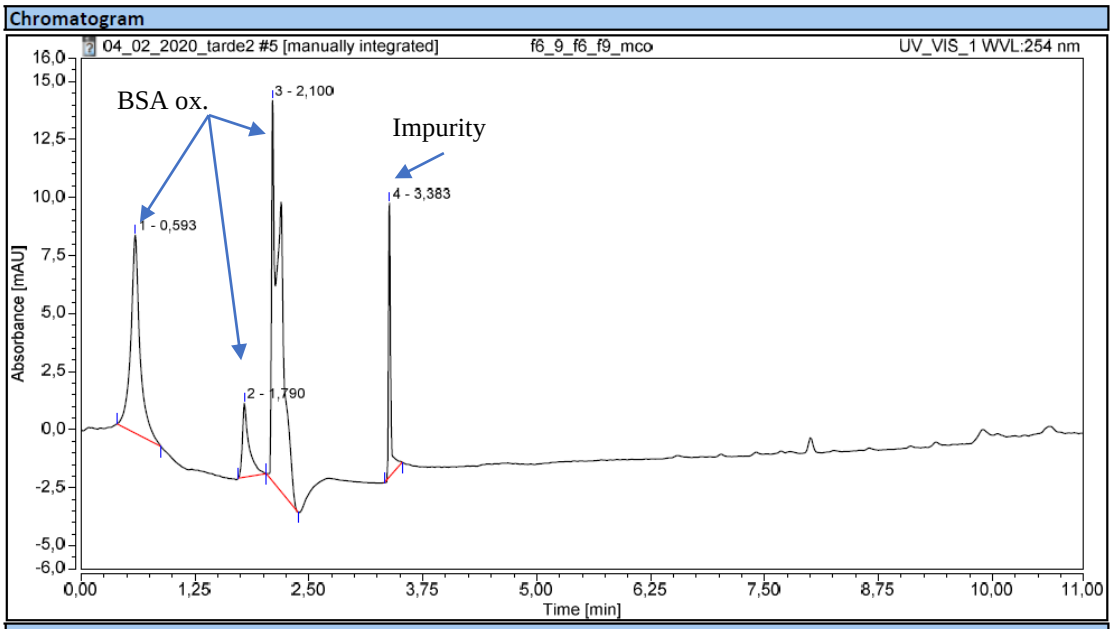
fractions showed similar results despite the use of the two different oxidation methods. A comparison of UPLC profiles at 254 nm of standard BSA and our oxidized BSA fractions showed expected differences. BSA standard spectrum shows two main peaks with retention times at 3.4 minutes (29.1 % of purity) and 3.8 minutes (69.4% of purity) (**Figure 25**). Unfortunately, it was not possible to confirm any of the hypotheses mentioned. Chromatogram for BSA oxidized fractions showed that the peak at 3.8 minutes disappear, and new peaks appear at shorter retention times, where the main broad peaks occur at 2.1 minutes, with similar purities of 54.7% as average, with the exception Fraction 5, with a lower purity of 39.7% (**Figure 26, Figure 27, Figure 28, Figure 29, & Figure 30**).



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		2,957	0,027	1,066	1,44	2,12	n.a.
2		3,453	0,554	23,394	29,15	46,62	n.a.
3		3,867	1,320	25,723	69,41	51,26	n.a.
Total:			**1,902**	**50,184**	**100,00**	**100,00**	

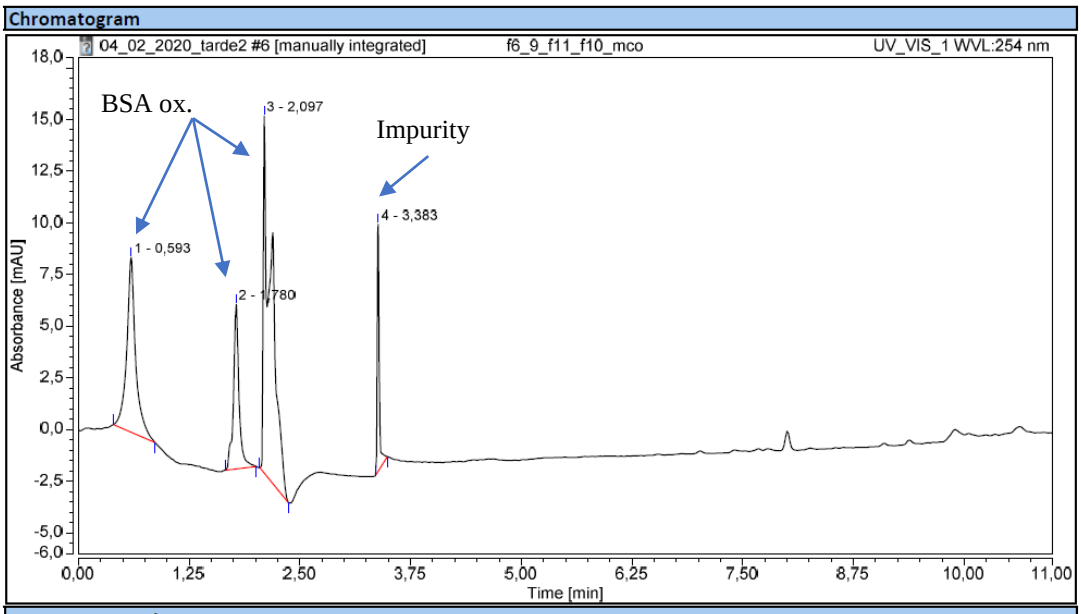
Figure 25. HPLC chromatogram of BSA standard.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		0,593	1,005	8,536	29,27	21,39	n.a.
2		1,790	0,267	3,196	7,77	8,01	n.a.
3		2,100	1,832	16,391	53,33	41,07	n.a.
4		3,383	0,331	11,789	9,63	29,54	n.a.
Total:			**3,435**	**39,912**	**100,00**	**100,00**	

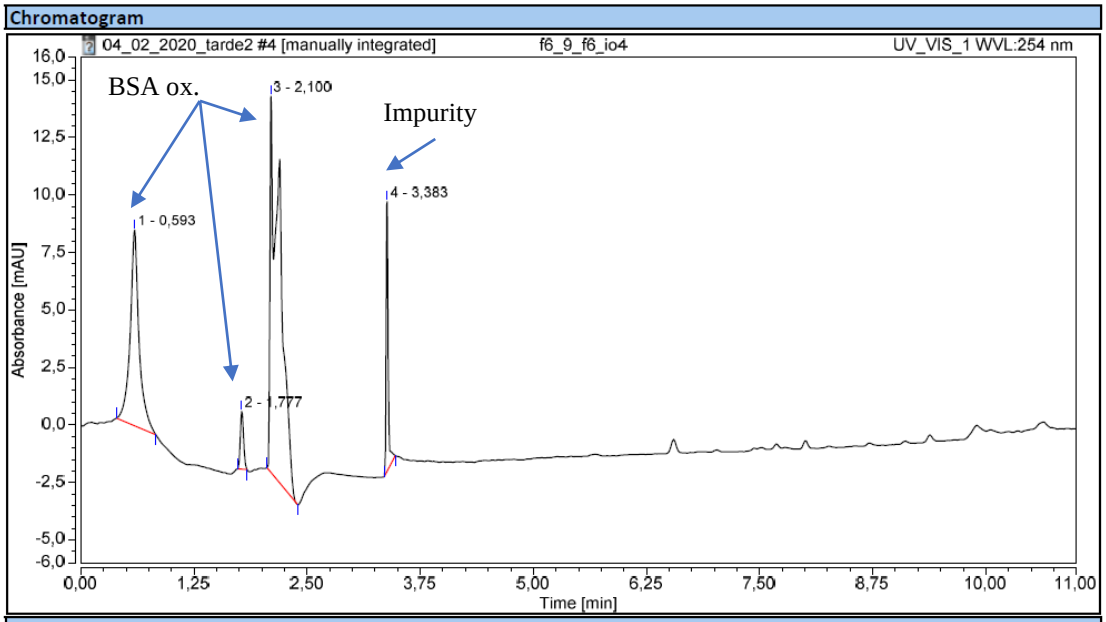
Figure 26. HPLC chromatogram of BSA oxidized Fraction 1 from MCO procedure.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		0,593	0,986	8,444	26,40	18,51	n.a.
2		1,780	0,587	7,956	15,71	17,44	n.a.
3		2,097	1,842	17,269	49,29	37,86	n.a.
4		3,383	0,321	11,948	8,60	26,19	n.a.
Total:			**3,736**	**45,618**	**100,00**	**100,00**	

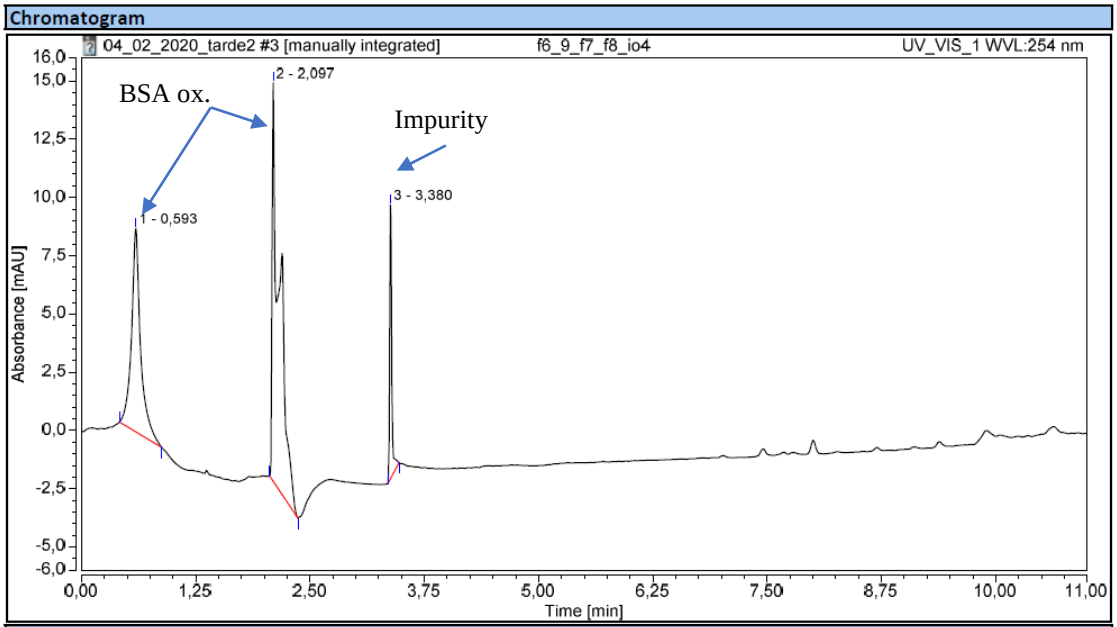
Figure 27. . HPLC chromatogram of BSA oxidized Fraction 2 from MCO procedure.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		0,593	0,971	8,515	27,73	21,77	n.a.
2		1,777	0,103	2,509	2,95	6,42	n.a.
3		2,100	2,113	16,362	60,37	41,84	n.a.
4		3,383	0,313	11,720	8,94	29,97	n.a.
Total:			**3,500**	**39,107**	**100,00**	**100,00**	

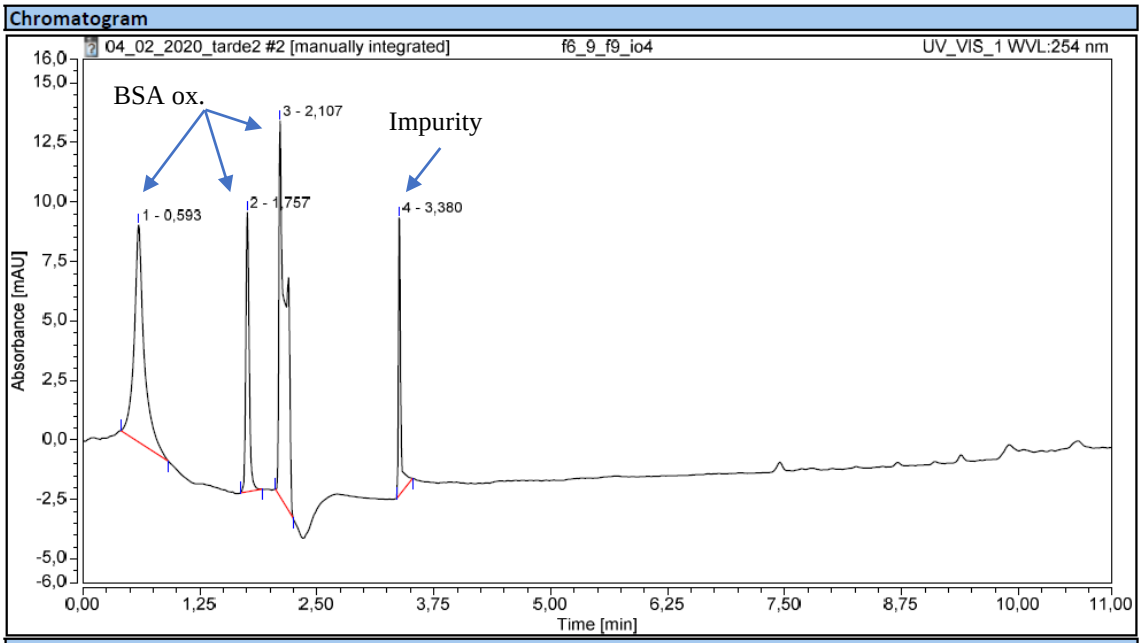
Figure 28. HPLC chromatogram of BSA oxidized Fraction 3 from periodate procedure.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		0,593	1,007	8,738	34,22	23,25	n.a.
2		2,097	1,624	17,113	55,18	45,53	n.a.
3		3,380	0,312	11,737	10,60	31,23	n.a.
Total:			**2,942**	**37,587**	**100,00**	**100,00**	

Figure 29. HPLC chromatogram of BSA oxidized Fraction 4 from periodate procedure.

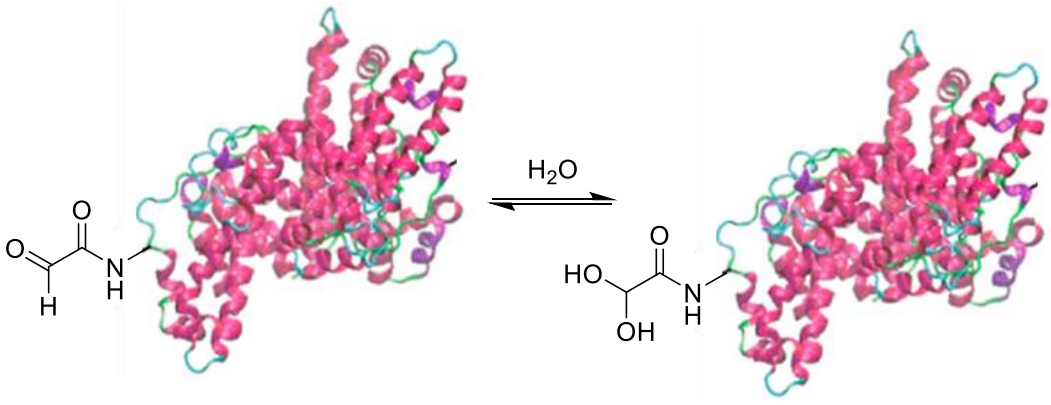


Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount n.a.
1		0,593	1,128	9,124	34,71	18,89	n.a.
2		1,757	0,492	11,733	15,14	24,29	n.a.
3		2,107	1,291	15,802	39,75	32,71	n.a.
4		3,380	0,338	11,647	10,39	24,11	n.a.
Total:			**3,248**	**48,307**	**100,00**	**100,00**	

Figure 30. HPLC chromatogram of BSA oxidized Fraction 3 from periodate procedure.

The HPLC profiles analysis allowed to conclude that the peak at 3.4 minutes present in all chromatograms could be due to some impurity of starting BSA, or it could be the signal corresponding to some compound previously retained in the column in previous elutions. On the other hand, the oxidized BSA elutes before non oxidized protein due to it exits as an equilibrium of the mixture of aldehyde and hydrate form (**Scheme 5**)[38].



Scheme 5. Equilibrium of the aldehyde and the hydrate form of the glyoxyl species

3.4 UPLC-MS analysis of MCO and periodate oxidized fractions

All purified oxidized protein fractions were characterized using UPLC-mass spectrometry, and all samples showed similar general results. First of all, a comparison of UPLC profiles of BSA and the oxidized BSA fractions 1-5 showed slight differences in its retention times. The BSA standard appears as a broad peak with two maximums at a retention time of 3.86, and 4.47 minutes (**Figure 31**). The oxidized BSA fractions also showed a wide peak with three maximums at 3.86, 4.91, and 3.97 minutes (**Figure 33**, **Figure 35**, **Figure 37**, **Figure 39**, & **Figure 41**). The differences UPLC profiles are expected, taking into account that the oxidation process could produce heterogeneous populations of modified proteins.

Regarding mass spectrometry analysis, the BSA and oxidized BSA were analyzed and compared. Under the positive mode ionization, ions with charge ranging from 38^+ to 63^+ are observed for the standard BSA (**Figure 32**). All these peaks correspond to the same molecule with different charges. A manual deconvoluted neutral molecular weight analysis from these peaks was carried out allowing to calculate a MW of 66628,9 ± 115.23 Da, which is very close to the reported value for BSA of 66430,3 Da.[57] On the other hand, mass spectra corresponding to the oxidized BSA fractions, showing multiple charge states from 38^+ to 63^+. The manual deconvolution of these peaks and the mass analysis indicate a loss of mass as a average of 150.0 Da resulted from MCO and 18.9 Da as a consequence of periodate oxidation, confirming that a modification has occurred. On the other hand, it can be noticed that BSA oxidized fraction 3 and fraction 4 from periodate oxidation procedure were combined since they had the same molecular weight and certainly belong to the same protein fraction. The spectra exemplify the concept of charge envelope, expected from the ionization of a larger molecule in mass spectroscopy (**Figure 34, Figure 36, Figure 38, Figure 38, Figure 40, & Figure 42**).

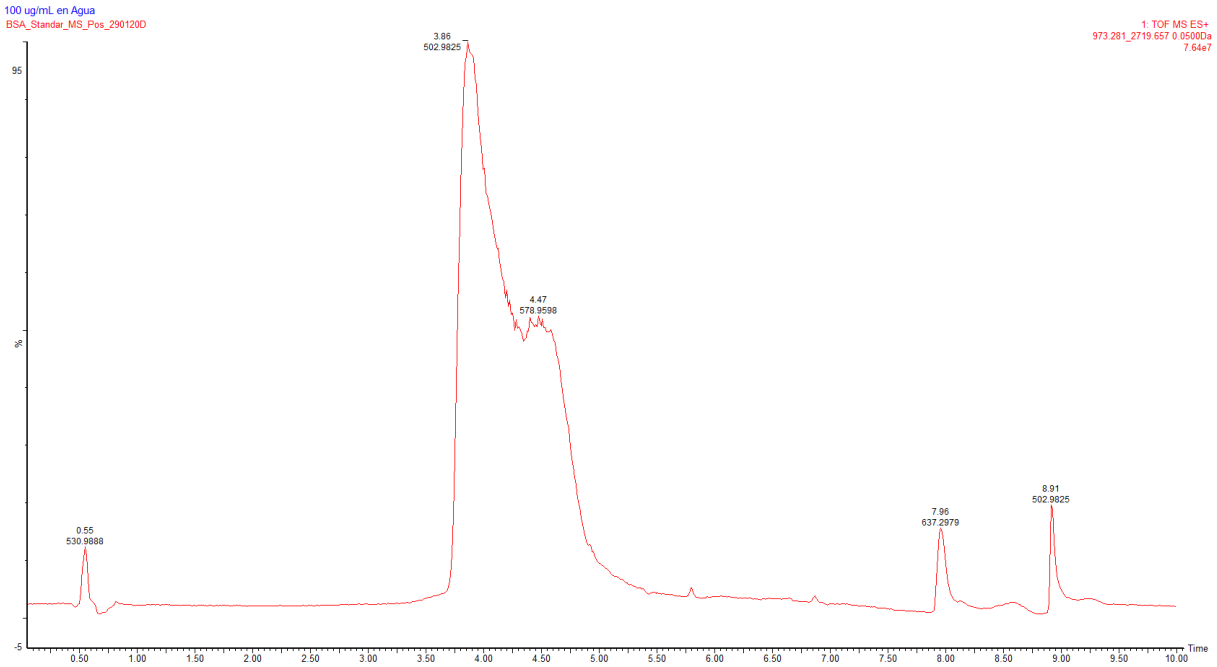


Figure 31. UPLC chromatogram of standard BSA.

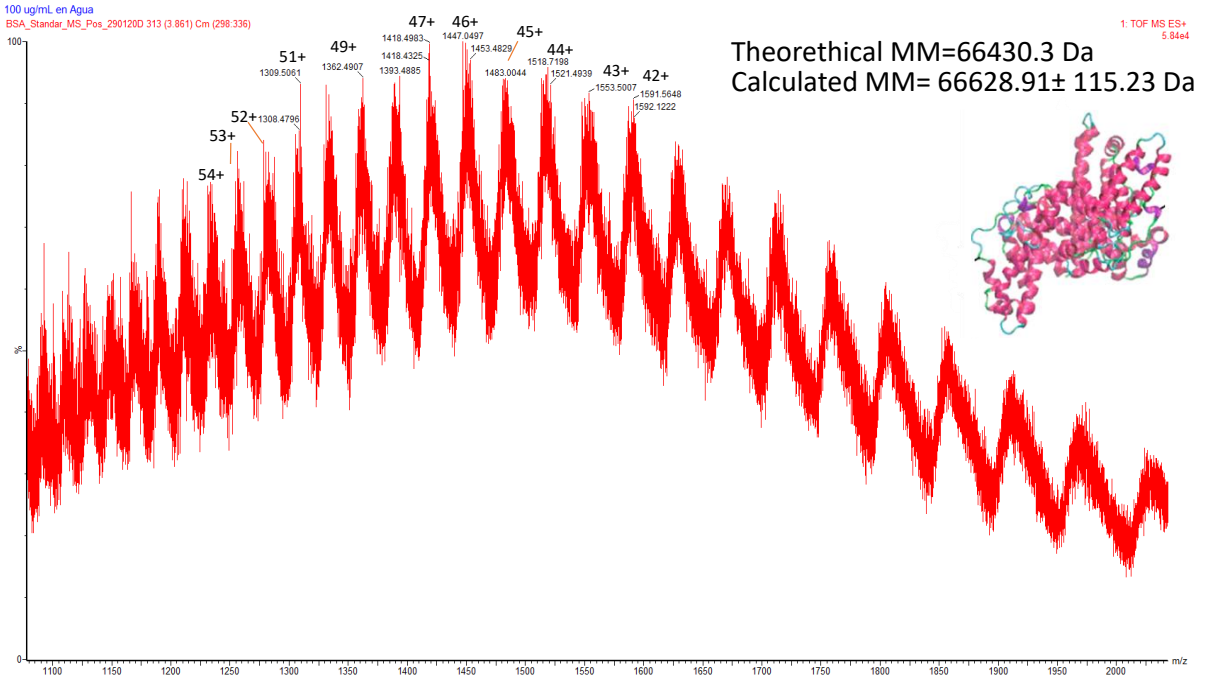


Figure 32. Mass spectrum of standard BSA.

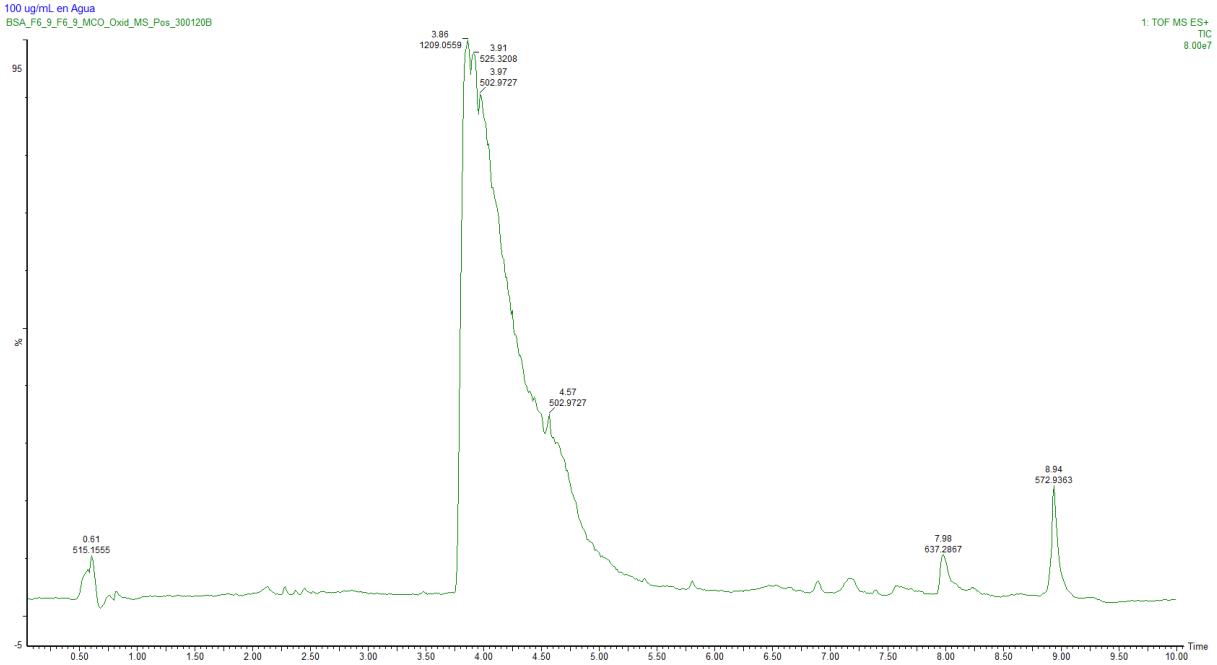


Figure 33. UPLC chromatogram of Fraction 1 corresponding to MCO procedure.

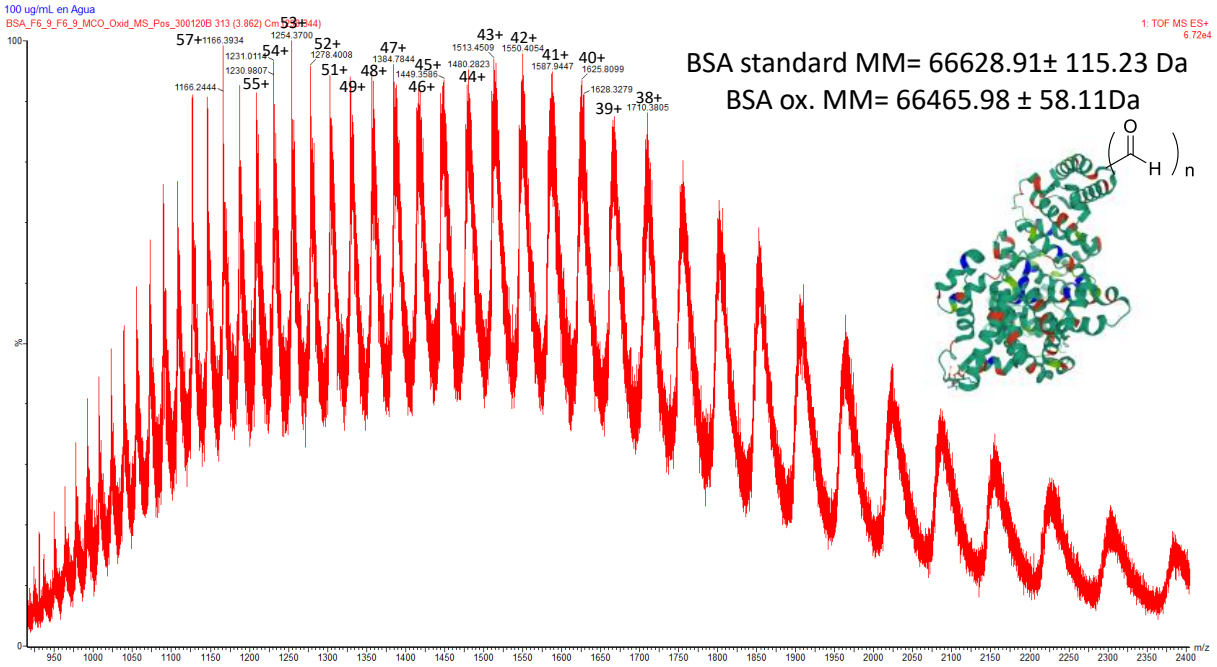


Figure 34. Mass spectrum of Fraction 1 obtained from MCO procedure.

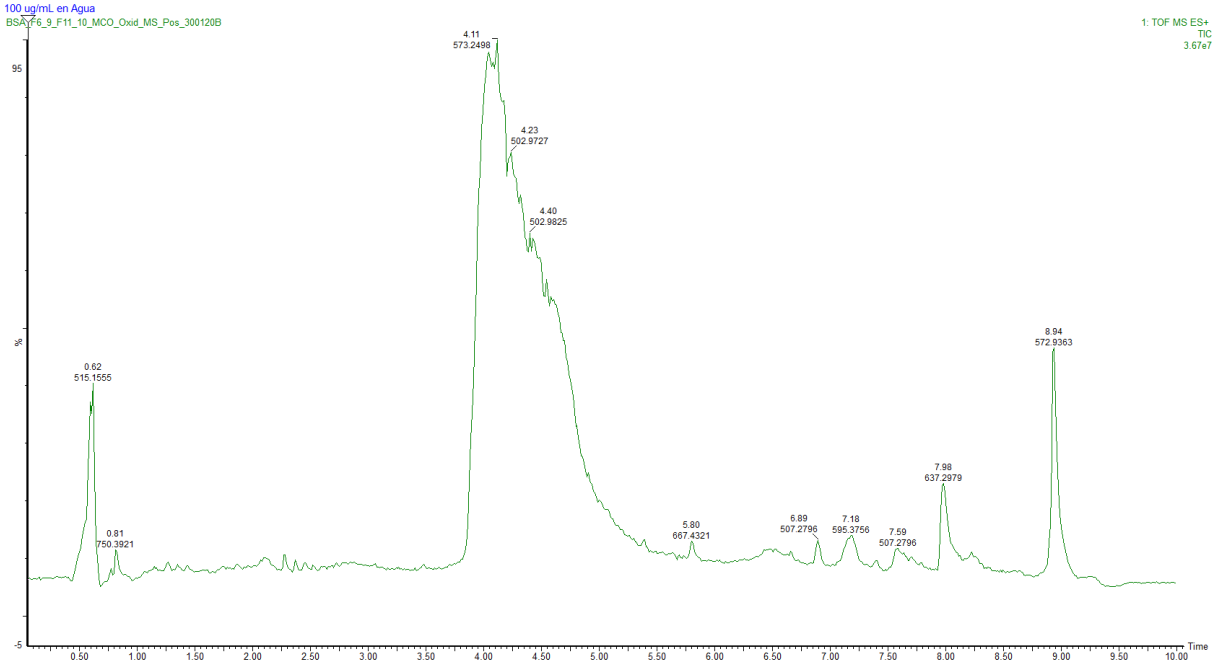


Figure 35. UPLC chromatogram of Fraction 2 corresponding to MCO procedure.

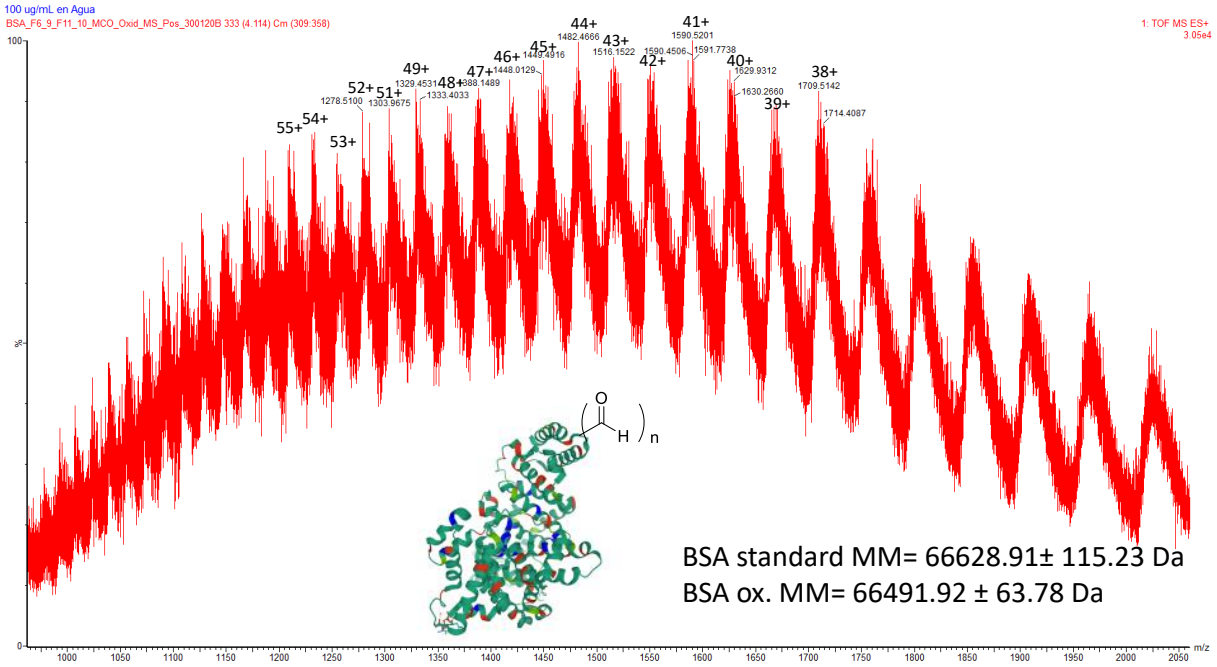


Figure 36. Mass spectrum of Fraction 2 obtained from MCO procedure.

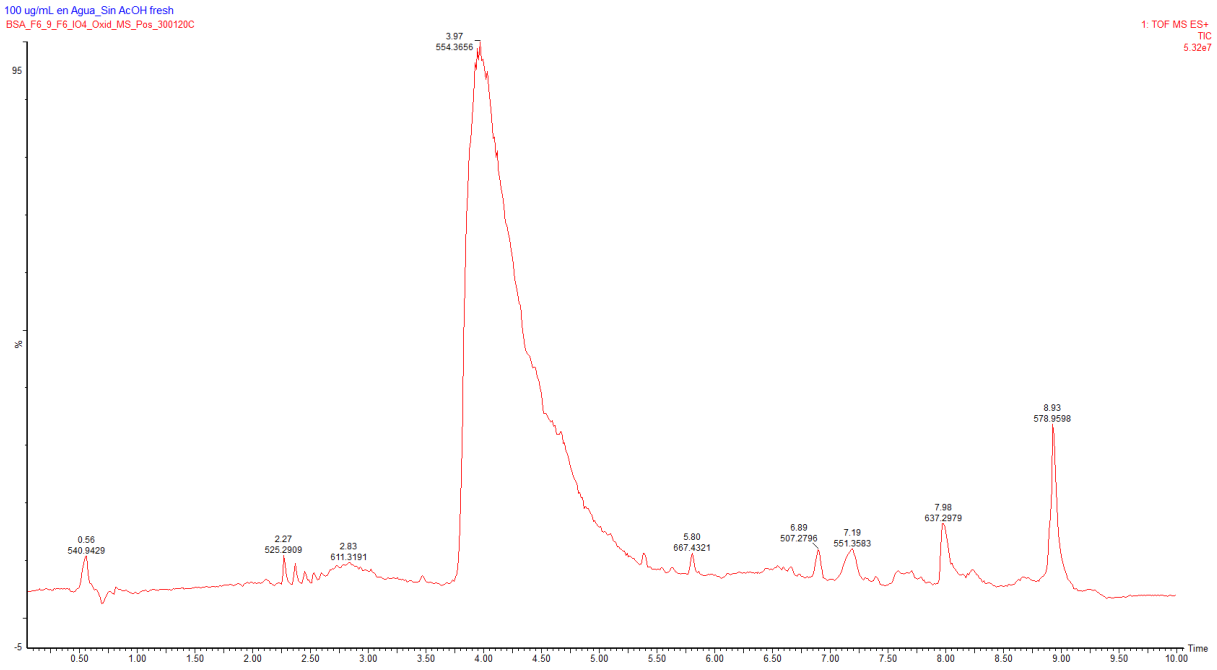


Figure 37. UPLC chromatogram of Fraction 3 corresponding to periodate oxidation procedure.

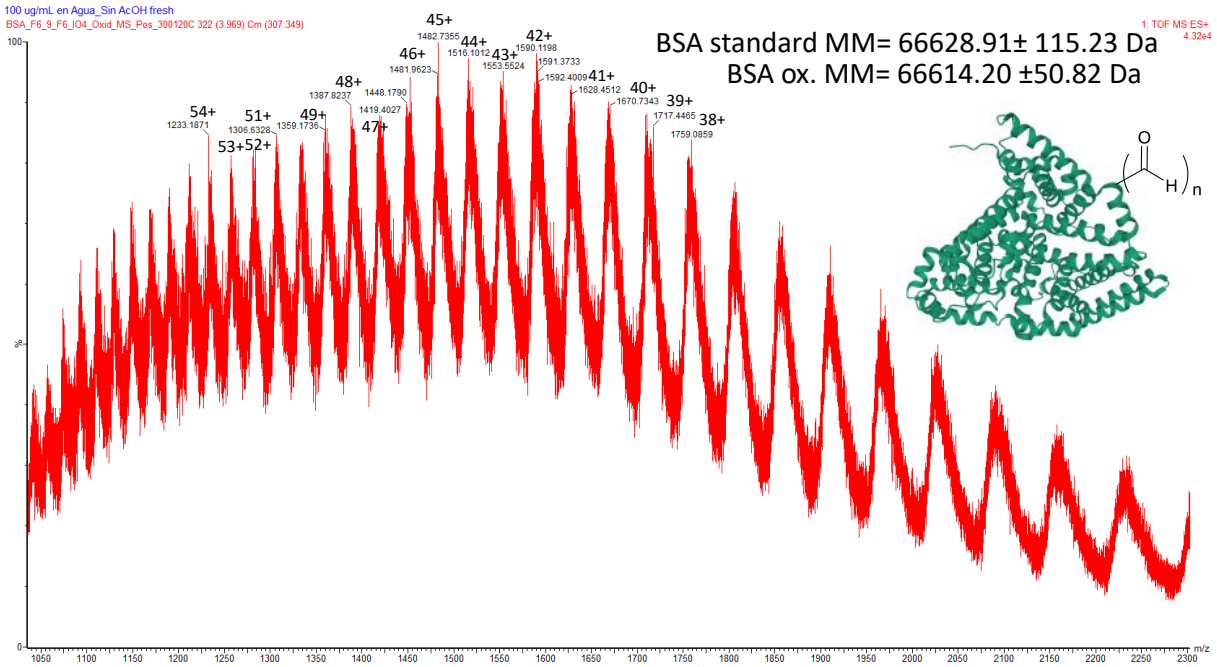


Figure 38. Mass spectrum of Fraction 3 obtained from periodate oxidation procedure.

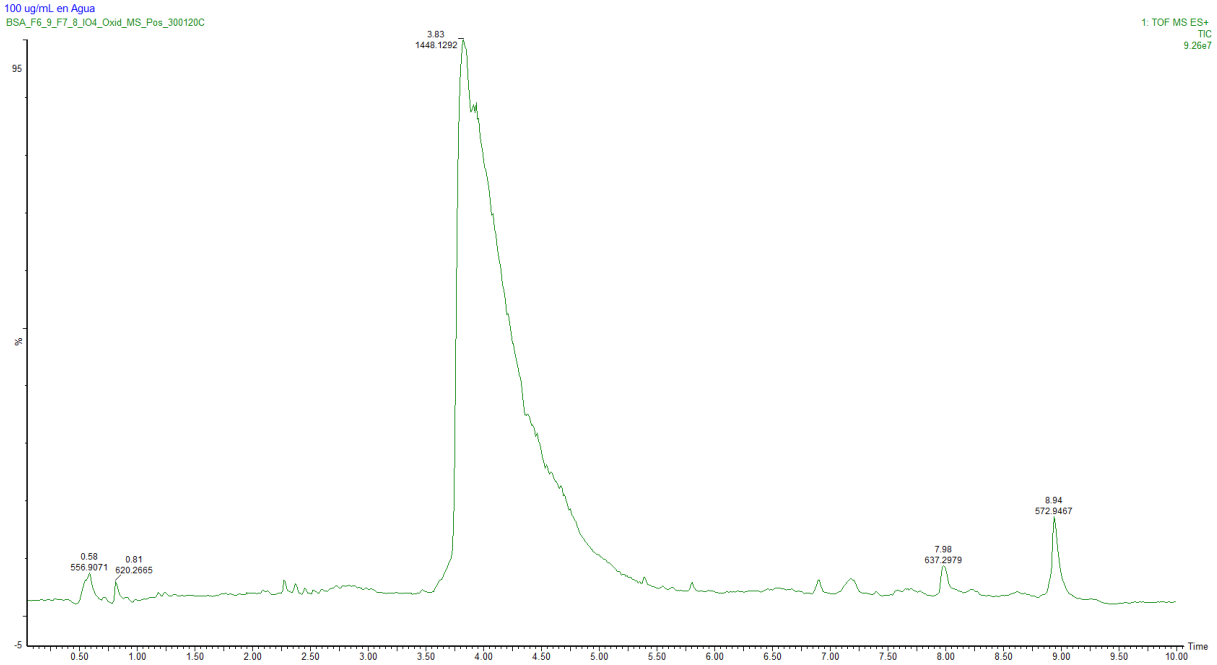


Figure 39. UPLC chromatogram of Fraction 4 corresponding to periodate oxidation procedure.

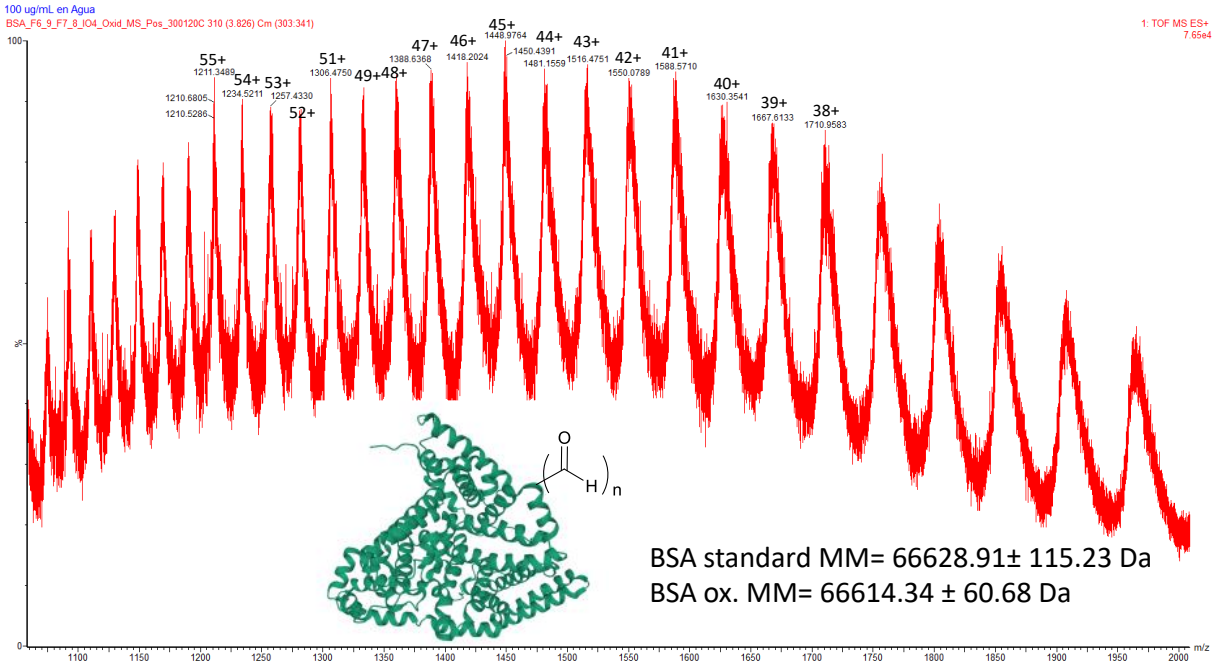


Figure 40. Mass spectrum of Fraction 4 obtained from periodate oxidation procedure.

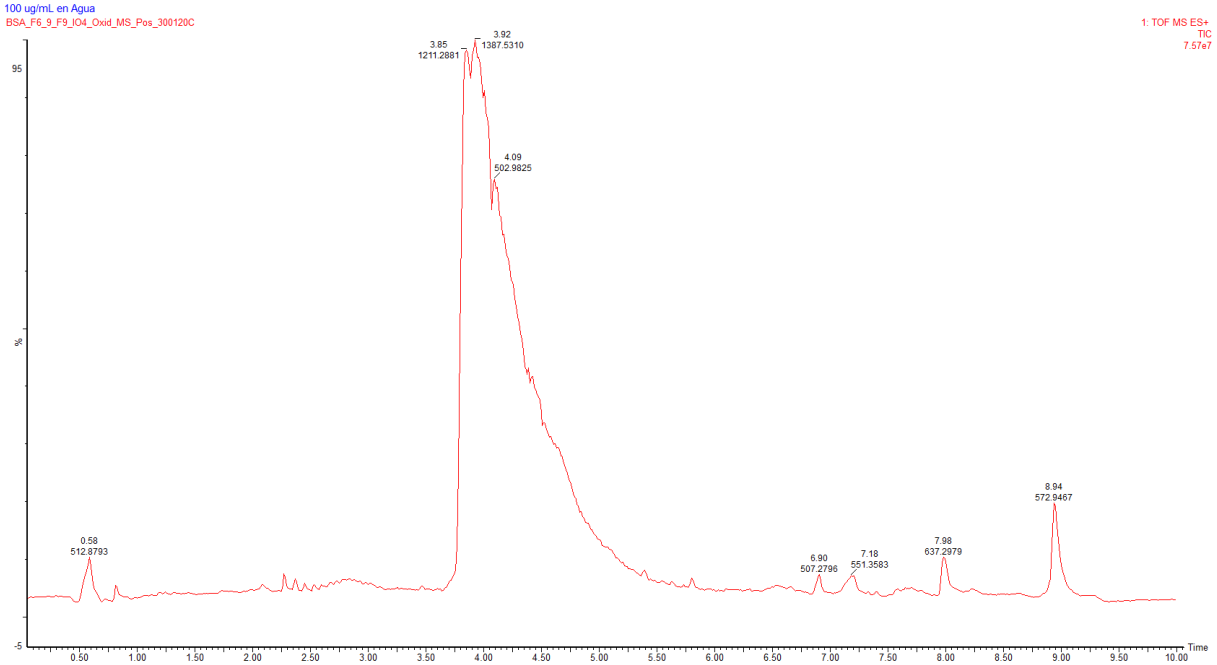


Figure 41. UPLC chromatogram of Fraction 5 corresponding to periodate oxidation procedure.

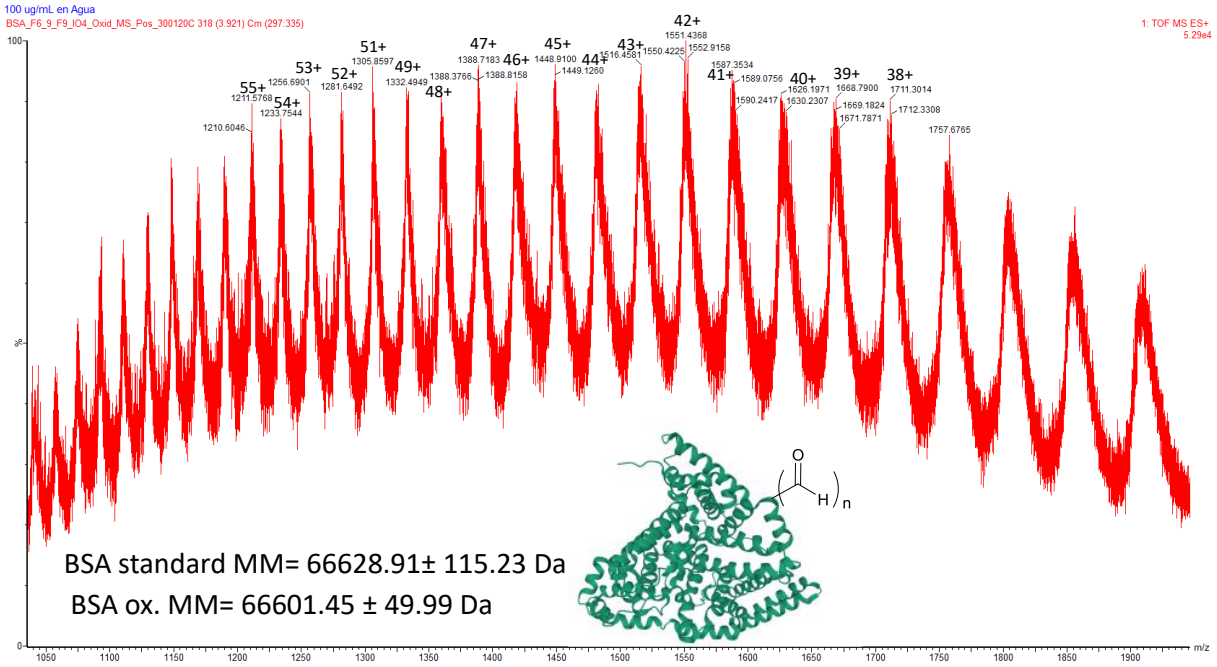


Figure 42. Mass spectrum of Fraction 3 obtained from periodate oxidation procedure.

The oxidized BSA spectra obtained through UPLC-MS allowed us to confirm modifications on BSA protein related to the oxidations of amino acids and the introduction of n aldehyde moieties on modified BSA.

The comparison between MW obtaining through deconvolution of mass spectra, allowing to conclude that both methodologies modified the protein structure, reducing MW of oxidized

BSA as it was expected. Taking into account that each oxidation method would be selected to different amino acid residues, with the tools in hand, it was not possible to determine how many and which amino acids generated the aldehyde residues due to the broad range of probabilities of oxidation. These different probabilities are evidenced in the wide peaks obtained for each fraction of UPLC profiles as a result of different populations of oxidized protein. Moreover, it can be suggested that it is more likely that the more exposed amino acids, the more prone to be oxidized and indeed generate aldehyde functional groups. All the mass spectral data are summarized (**Table 2.**

Table 2. Mass spectral data of BSA oxidized fractions

Sample	Standard BSA	Fraction 1 BSA ox.	Fraction 2 BSA ox.	Fraction 3 BSA ox.	Fraction 4 BSA ox.	Fraction 5 BSA ox.
method	-	MCO oxid.	MCO oxid.	IO4 oxid.	IO4 oxid.	IO4 oxid.
Mode	positivo	positivo	positivo	positivo	positivo	positivo
conc. [ug/mL]	200	100	100	200	200	200
elut. time [min]	3.86	3.86	4.11	3.97	3.83	3.92
MM (Da)	66628.91± 115.23	66465.98 ± 58.11	66491.92 ± 63.78	66614.20 ±50.82	66614.34 ± 60.68	66601.45 ± 49.99
MM Difference		162.93 ± 0.01	136.99 ± 0.01	14.71 ± 0.01	14.57 ± 0.01	27.46 ± 0.01

3.5 Comparison of Oxidation methodologies to obtain protein aldehydes

Both oxidation procedures could allow inserting aldehydes residues by modifying different BSA aminoacidic sequence sites. These modifications are evidenced in mass weight loss compared to native BSA and difference in spectrum data either in Nanodrop UV-measurements and HPLC-UVvis profiles. It is needed to highlight the broad range of oxidation probability that exists during these procedures, allowing differents modifications along the aminoacidic sequence of BSA, and as a consequence, more than one population of oxidized BSA could be obtained. On the other hand, it is required to be aware of the possible overoxidation and oxidative damage depending on the methods applied to biomolecules. Related to the mass weight loss, periodate-assisted oxidation was a softer method than MCO because of the less mass loss due to the difference of radicals generated and oxidation pathways in each procedure. Thus, in our opinion, is the use of the periodate the most suitable and more controlled oxidation procedure.

3.6 Design of bioconjugation reactions

Hantzsch and Biginelli are MCRs widely use to prepare interesting bioactive N-heterocycles. Hantzsch reaction involves an aldehyde, a beta-keto ester, and ammonia or ammonium acetate, which, through a multicomponent condensation plus oxidation of the dihydropyridine intermediate, results in a pyridine derivative. In contrast, Biginelli reaction uses urea as the nitrogen source instead of ammonia to obtain 3,4-dihydropyrimidin-2-(1H)-ones.[71] With oxidized protein in hand, we designed Biginelli and Hantzsch -assisted bioconjugation procedures. In this regard, we proposed that the cargo would be introduced using the β-ketoester moiety. In this way, from one MCR to another, we would only change the nitrogen source, using urea and ammonium for Biginelli or Hantzsch, respectively (Figure 43).

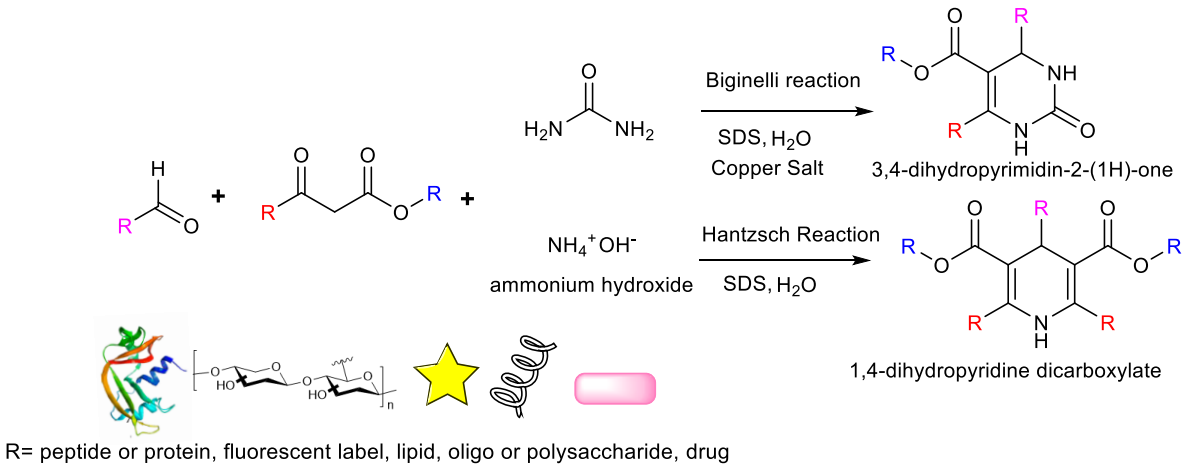


Figure 43. General Strategy proposed for bioconjugation based on Biginelli and Hantzsch reactions.

The next step would be to analyze the availability of synthetic strategies, which allow us to introduced potential cargos in bioconjugates, but also prove that mentioned reactions can work in biological media. This implies studying their degradation or the mechanisms through which the cargos could be released. All these aspects are currently being studied and will be the objective of future research work.

4 CONCLUSIONS AND RECOMMENDATIONS

Conclusions

- It was incorporated aldehyde residue on bovine serum albumin (BSA) as a biomolecule pattern by modifying different aminoacids along the aminoacidic sequence, using Metal catalyzed oxidation (MCO), and periodate assisted oxidation procedures.
- Protein, oxidized proteins, and non-protein fractions were separated by size exclusion chromatography and then discriminated by UV-nanodrop measurement.
- The analysis of protein fraction trough UV spectra comparison of BSA oxidized fractions against a standard BSA allows us to obtain five different protein fractions, two of them by MCO procedure, and three by periodate-assisted methodology. Protein fractions showed expected UV spectra with two well-defined maximums of absorbances at 240 and 280 nm, and were grouped by sharing similar UV-spectral profiles.
- The analysis and comparison of retention times, purities, and UV absorbance of standard BSA and oxidized BSA fractions by HPLC-PAD displays similar results between oxidized BSA fractions. Standard BSA spectrum shows two main peaks with retention times at 3.4 minutes (29.1 % of purity) and 3.8 minutes (69.4% of purity), being the first peak attributed to some impurity of starting BSA reagent. Whereas, oxidized fractions spectra showed that the peak at 3.8 minutes disappear, and new peaks appear at shorter retention times due to the existence of an aldehyde-hydrate form equilibrium, where the main broad peaks occur at 2.1 minutes, with similar purities of 54.7% as average, with the exception of Fraction 5, with a lower purity of 39.7%.
- UPLC-MS analysis of native BSA and oxidized fractions showed that the weight mass of standard BSA is of 66628,9 ± 115.23 Da, and for the oxidized fractions was 66557.58 Da as average. The oxidized BSA spectra obtained through UPLC-MS allowed us to confirm BSA modified protein resulting from aminoacids residues oxidations and the introduction of n aldehyde moieties on modified BSA by the reduction of MW of oxidized BSA. Mass spectral data indicate a loss of mass as an average of 150.0 Da resulted from MCO and 18.9 Da as a consequence of periodate oxidation procedure. Moreover, the broad peaks of UPLC profiles are generated as a result of different populations of oxidized protein.

- The comparison of the Metal catalyzed and periodate oxidation methodologies applied to BSA as proof of concept differ in oxidation reaction pathways, reactive radicals, and oxidation extensive. The periodate is the most suitable and more controlled oxidation procedure due to the less mass loss obtained experimentally.

Recommendations

- Include studies of the biomolecule integrity (protein unfolding and oxidative damage) after the oxidation procedures are applied.
- Deepen into the determination of amino acids involved in the generation of the aldehyde residues and its probability of oxidation in protein biomolecules with each oxidation protocol.

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6 ANNEXES

Annex I. Abbreviations and Acronyms

DDS	Drug delivery system
MCR	Multicomponent reaction
I-MCR	Isocyanide multicomponent reaction
MCC	Multicomponent conjugation
MCL	Multicomponent ligation
UML	Ugi multicomponent ligation
AA	Aminoacid
RBP	Resin bound peptide
HRP	Horseradish peroxidase
MCI	Multicomponent inmobilization
CP	Capsular polysacharide
DT & DT[a]	Diphteria toxoids and activated diphteria toxoids
TT & TT[a]	Tetanus toxoids and activated tetanus toxoids
SFP	Succinimidyl p-formylbenzoate
SFAP	succinimidyl p-formylphenoxyacetate
PLP	Pyridoxal 5-phosphate
fGly	formylglycine
FGE	Formylglycine generating enzyme
FAPP	Farnesyl aldehyde pyrophosphate
PFTase	Protein farnesyl transferase

LplA	Lipoic acid ligase
TTL	Tubuline tyrosine ligase
DHPM	3,4-dihydropyrimidin-2-(1H)-ones
DHP	1,4-dihydropyrimidine dicarboxylate
ROS	Reactive oxygen species
RIS	Reactive iodinated species
RNS	Reactive nitrogen species
MCO	Metal catalyzed oxidation
HPLC	High performance liquid chromatography
UV	Ultraviolet
UPLC	Ultra performance liquid chromatography
BSA	Bovine serum albumin
MeCN	Acetonitrile
AcOH	Acetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PAD	Photodiode array detector
r.t.	Room temperature
m/z	mass charge relation

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