UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Químicas e Ingeniería

TÍTULO: NOVEL LINKER TO BIOCONJUGATION BASED ON TETRAHYDROTHIADIAZINE-2-THIONE (THTT) SCAFFOLD

Trabajo de titulación presentado como requisito para la obtención del título de Químico(a)

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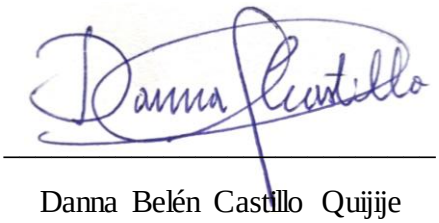

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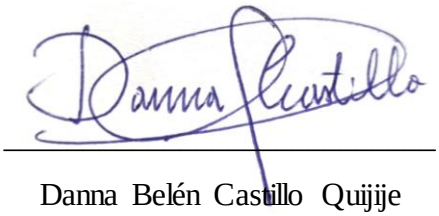

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Dedicatoria

Para mis amados padres, por su lucha y constancia diaria en dar lo mejor de sí mismos por la educación tanto académica como ética de sus hijas. Haber nacido de ustedes fue la más grande fortuna.

Danna Belén Castillo Quijije

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Danna Belén Castillo Quijije

Resumen

El cáncer es una de las enfermedades con la tasa de mortalidad más alta y cuyos tratamientos para mitigarlo generalmente son tan fuertes e invasivos que terminan debilitando al paciente por lo que la cura puede ser tan peligrosa como la enfermedad. Desde hace algún tiempo hasta hoy, para reducir la tolerancia generada por la administración constante del medicamento y mejorar su farmacocinética, los científicos han estado desarrollando tecnología de sistemas de administración de medicamentos (DDS, por sus siglas en inglés). Para maximizar la efectividad de los medicamentos administrados al querer que lleguen específicamente al área afectada por la enfermedad, pero también para reducir los posibles efectos secundarios. Varias biomoléculas como los eritrocitos, los liposomas y los anticuerpos, pero también las nanopartículas han sido usados como portadores, y los ensayos más exitosos están relacionadas con el uso de la conjugación química covalente para promover la reticulación entre la biomolécula y el fármaco. Centrados en los conjugados de medicamentos con anticuerpos (ADC, por sus siglas en inglés), actualmente, cuatro de ellos se han comercializado durante los últimos años como un prometedor tratamiento del cáncer.

Los ADC se definen como una entidad híbrida generada mediante bioconjugación química entre un agente citotóxico y un anticuerpo monoclonal (mAb) a través de un conector. El enlazador es responsable de proporcionar los grupos funcionales capaces de enlazar tanto el medicamento como el mAb. Estos pueden ser clasificados como no escindible, lo que indica que es estable y mantiene el mAb y el fármaco unidos hasta llegar a la célula objetivo, y también se puede clasificar como un conector escindible, lo que indica la descomposición del conjugado en el entorno de la célula objetivo liberando el medicamento.

Teniendo en cuenta la importancia del enlazador dentro del conjugado, así como la necesidad de generar una reticulación eficiente en un ADC novedoso, el objetivo de este trabajo es desarrollar un enlazador eficiente basado en tetrahidrotiadiazin-2-tiona (THTT) para su uso en bioconjugación Para abordarlo, se sintetizó un mono-THTT usando un residuo de glicina en la posición N3, N5, además de otras cinco bis-THTT que fueron usadas para realizar el estudio de acidólisis UV para establecer si el THTT podría usarse como un conector escindible o no escindible. El conector THTT fue usado para llevar a cabo la bioconjugación con la proteína BSA y el anticuerpo anti-alfa-tubulina. Ambos conjugados se caracterizaron por técnicas de espectroscopía como UV y ULPC-MS para corroborar si la conjugación tuvo lugar.

PALABRAS CLAVE: enlazador, conjugado anticuerpo-fármaco, tetrahidrotiadiazin-2-tiona, acidólisis, bioconjugación, albúmina de suero bovino, anti-alfa-tubulina.

Abstract

Cancer is one of the diseases with the highest mortality rate and whose treatments to mitigate it usually are so strong and invasive that they end up weakening the patient so that that cure can be as dangerous as the disease. From some time ago until today, to reduce the tolerance generated by the constant administration of the drug and improving its pharmacokinetics, scientists have been developing drug delivery systems (DDS´s) technology. To maximize the effectiveness of the drugs administered by wanting them to reach specifically to the affected area by the disease, but also to reduce the potential side effects. Several biomolecules like erythrocytes, liposomes, and antibodies, but also nanoparticles have been used as carriers, and the most successful experiences are related to the use of covalent chemical conjugation to promote the crosslinking between the biomolecule and the drug. Recently, four of Antibody Drug Conjugates (ADC´s), have been commercialized during the last years as the promising treatment of cancer.

ADC´s are defined as a hybrid entity generated through chemical bioconjugation between a cytotoxic agent and a monoclonal antibody (mAb) through a linker. The linker is responsible for providing the functional groups capable of binding both the drug and the mAb. It can be classified as non-cleavable and cleavable linker. In the first case indicates that it is stable and maintain both the mAb and the drug linked then whole conjugate reach the target cell and the drug is release inside it. In the second case, in the cleavable linker, breakdown the bonds from conjugate is given at the surrounding of target cell and hence releasing the drug.

Taking into account the importance of the linker within the conjugate, as well as the need to generate efficient conjugation in novel ADC, the goal of this work is develop an efficient linker based on tetrahydrothiadiazin-2-thione (THTT) for its use in bioconjugation. To tackle it, a mono-THTT was synthesized using glycine residue at N3, N5 position, and other five bis-THTT was used to perform the acidolysis study to establish if the THTT could be used as a cleavable or non-cleavable linker. THTT linker was used to carry out the bioconjugation with BSA protein and anti-alpha-tubulin antibody. Both conjugates were characterized by spectroscopy techniques as UV and ULPC-MS to corroborate whether the conjugation took place.

KEYWORDS: Linker, antibody-drug conjugate, tetrahydrothiadiazin-2-thione, acidolysis, bioconjugation, bovine serum albumin, anti-alpha-tubulin.

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CHAPTER 1. INTRODUCTION – JUSTIFICATION

1.1 General Introduction

Drug delivery system (DDS) is a technology used to improve the targeted delivery or controlled release of pharmaceutical products in patients,[1,2] which is a field with major developments in the pharmaceutical industry. During the design and development of DDS´s is needed to take into account two main features: drug targeting, and controlled release. The first is related to the area where the expected drug arrives, and the second feature is related with two variables: rate and time of drug releasing. In other words, a DDS is defined by the rate and the time which will take to be released the drug in determined (specific) area.[3]

Regarding DDS development, the oral or intravenous route has been the most traditional ways to drug delivery administration.[3] In the beginning, preferred way of drugs formulations were made like a pill or capsule, which allowed the drug release when they have contact with water. However, pills or capsules do not allowed the drug release kinetic control.[4]

The first DDS modifications were carried out by oral administrated systems. In this regards, enteric coatings (i.e. keratin and shellac), which have a delayed effect in the drug release, were prepared at the end of the nineteenth century. The mentioned systems only break up at pH around 8 such intestinal regions, which it is not associated with stomach pH. This feature allowed both, stomach protection of gastric irritation, and the drug protection of being destroyed because of acidic gastric environment.[5,6]

Keratin and shellac were the first developed coating; however there were not useful because a high basic pH (9-10) is needed to dissolve them in the intestine region. In 1952, Spansule, the first technology related to the control of the drug release kinetics was reported.[5]Spansule is defined as capsules, which can be coated by either hydrophilic or hydrophobic polymers, or a combination of both. The coated tends to dissolve slowly, allowing medication release at a specific time. Spansule is considered a system with a zero-rate pharmacokinetic, because of its continuous release.[7]

Concerning drugs administration, it is possible to classify them in three different generations related to the chronological order in which they were developed. In the first generation (1G) (1950 to 1980 period), only oral and transdermal controlled release formulations were designed and developed through dissolution-controlled and diffusion-controlled systems. For the first generation of drugs, the oral administration was the most suitable. The second-generation (2G)

of drugs (1980 to 2010 period) have a release kinetic of zero-order, then, the drug absorption and kinetic release are biological barrier dependent, which sometimes is difficult to overcome it. Currently, scientific work on the third generation of drugs (3G) (2010- 2040), which is still in progress. The last generation should overcome both physicochemical and biological barriers, for example, be functional over the lifetime of drug delivery or being highly selective and specifics.[4]

The main goal of DDS is to reduce the side effects that usually cause by treatments to mitigate some diseases, especially cancer. Current cancer treatments are usually not very specific affecting not only sick organs and tissues but also the healthy others which end up weakening the patient so that cure can be sometimes as dangerous as the disease. DDS try to maximize the effectiveness of the drugs administered by aiming them to specific area affected by the disease.

In this context, novel techniques base on chemical conjugations has been carried out to find selective and effective drug delivery systems. Recently, a broad range of hybrid covalent entities based on erythrocytes, liposomes, nanoparticles, and antibodies linked to the drug have been developed as efficient drug carriers (**Figure 1**), and some of them, specifically four ADC`s have been commercializing, and about this topic later it will deepen. The most important approach to DDS obtained through chemical conjugation in the last years are summarized, and the main features, but also its efficiency as therapeutic agents are also discussed focus on ADC´s entities.

1.2 Drugs delivery systems based on Chemical Conjugation

To find effective and selective entities to counteract several human diseases, but to avoid or to minimize side-effects, differents kind of DDS have been reported. The present review focuses on those systems designed and prepared through the chemical conjugation of drugs with specific carriers. Bioentities such as erythrocytes, [8-21] liposomes,[22-37] or antibodies[53-75] have been used as bio carriers. Additionally, a combination of biomolecules have also been used as potential DDS.[76-80] On the other hand, nanoparticles as a conveyor in DDS [38-52] are also summarized (**Figure 1**).

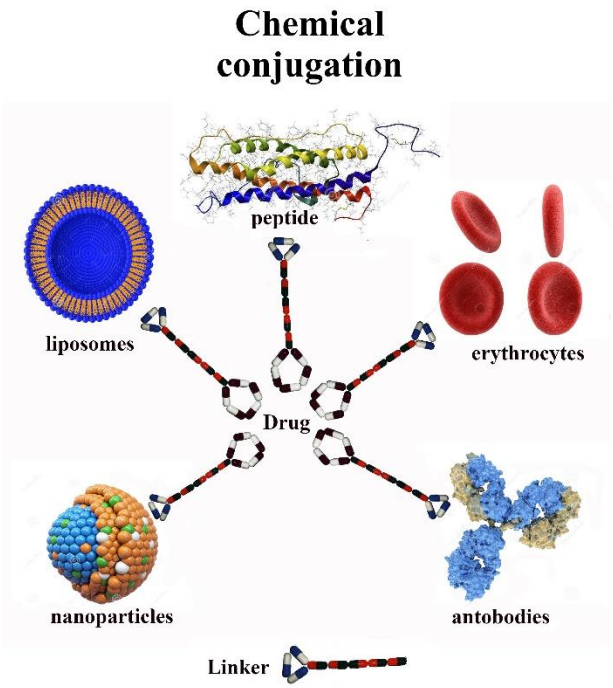


Figure 1. Chemical conjugation in drug delivery systems based on erythrocytes, liposomes, nanoparticles, peptides, and antibodies covalently linked to the drug.

1.2.1 Erythrocytes (Red Blood Cell, RBC) as a carrier in DDS

Blood is one of the most important components of the circulatory system. It is responsible for irrigating the entire human body fulfilling, and erythrocytes, also called Red Blood Cell (RBC), are the most numerous cells present in the blood. RBC transport and exchange oxygen and carbon dioxide between the lungs and tissues of other organs.[8,9] Erythrocytes are biocompatible, biodegradables, and non-immunogenics, which make them interesting to be used in DDS´s development. Erythrocytes are being successfully used in some pharmacological therapies due to its potential to transport molecular cargos in two different ways, inside it (embedded) or chemically linked to drugs.[10] In this regards, we highlighted the second way in which chemical conjugation is present.

- *Erythrocytes (RBC) conjugated through a streptavidin-biotin technique*

Kumkum Ganguly et al. reported erythrocytes and tissue plasminogen activator (tPA or rPA) conjugation through of streptavidin-biotin technique with the purpose to using it in thromboprophylaxis. Previously to conjugation between erythrocytes and tPA/rPA, biotinylation of both biomolecules were carried out.[11,12] Biotinylation, also known as biotin

labeling, is most commonly performed through chemical means, although enzymatic methods are also used. Nowadays, a wide variety of biotinylation reagents are available, so chemical methods provide greater flexibility in the type of biotinylation needed. All biotinylation reagents have similar features, where Biotin group is present, but also a reactive moiety, which gives biotinylation reagents distinct characteristics that are ideal for different types of experiments (**Figure 2**).[13] Because biotin has extremely high affinity to the protein streptavidin, biotinylated erythrocytes (b-RBC) join streptavidin (SA) non-covalently, and the hybrid obtained entity is then conjugated to biotinylated alteplase (b-tPA) or arteplase (b-rPA) (**Figure 3**). The synthesized final conjugate made possible improve the resistance of tPA to PAI-1 inhibitors and hence these have a longer intravascular life[12]. Using the same streptavidin-biotin technique, the conjugation of urokinase plasminogen activator (scuPA) to RBC as a carrier has been also reported. [14]

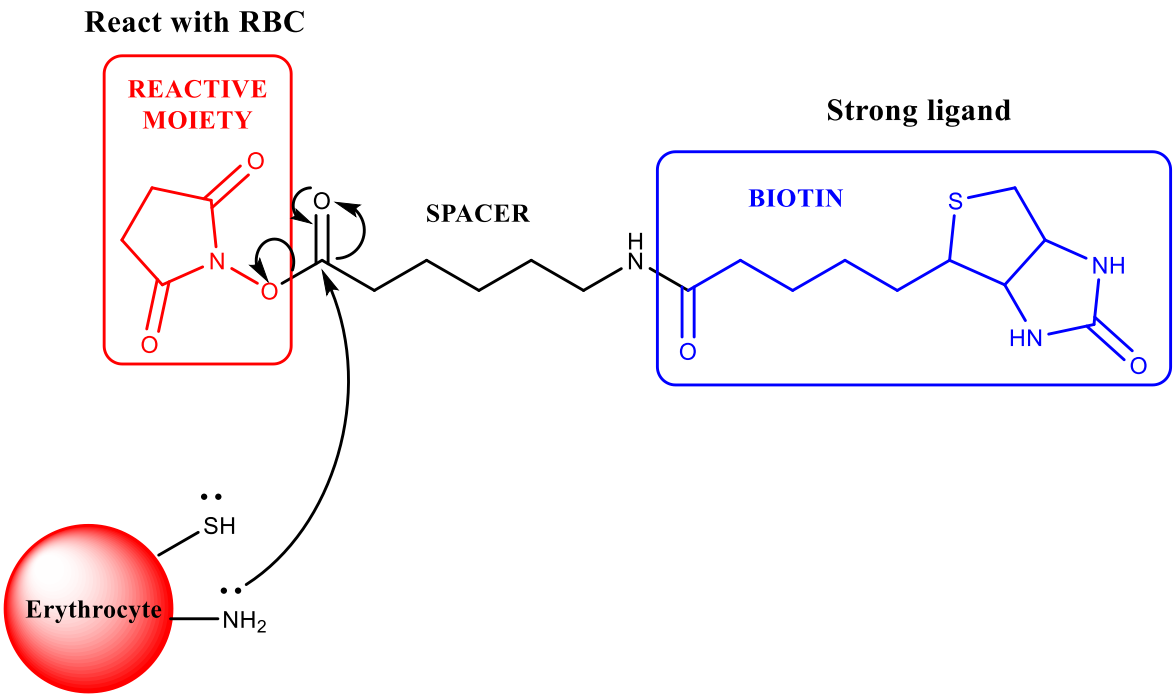


Figure 2. Biotinylation reagents general structure. The reagent shown is NHS-LC-Biotin

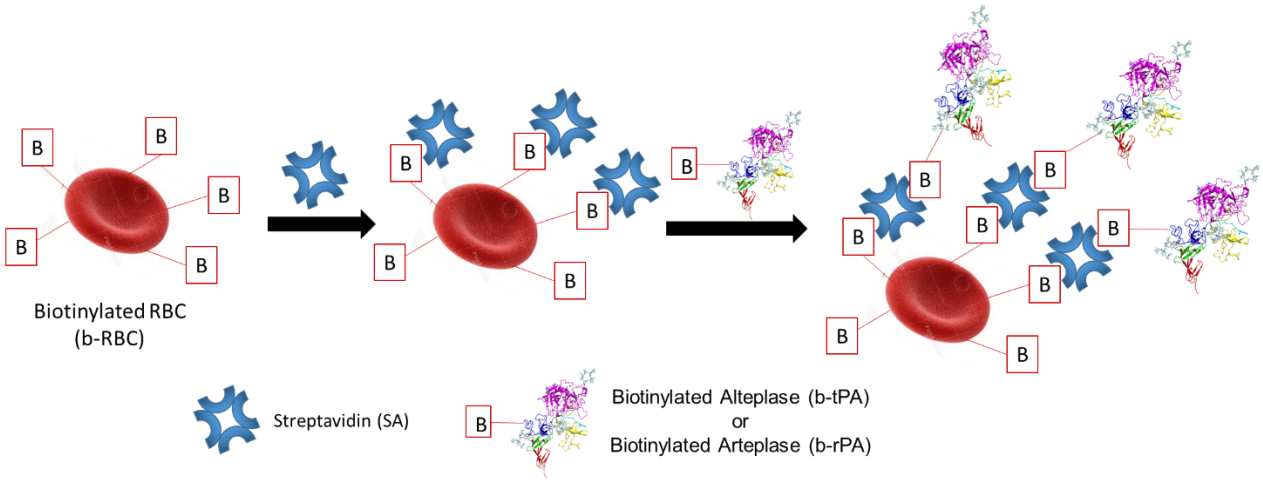


Figure 3. A streptavidin-biotin technique using RBC as a carrier.

The purpose of the conjugation of both receptors (b-RBC and b-tPA/b-rPA) is to prevent blood coagulation called thromboprophylaxis. Venous thromboembolism (VTE) is a condition in which blood clots form in the veins of the leg, groin or arm, called deep vein thrombosis. These clots can travel through the circulation until the lungs, appearing a pulmonary embolism and can be a direct cause of death.[15,16]. The plasminogen activator (PA) like tPA or rPA are useful due to their ability to lyse clots that are beginning to develop, but during of thromboprophylaxis is eliminated very quickly from the blood. The authors prepared previously mentioned conjugate with PA and RBC which has a greater anticoagulant effect than when PA is administrated alone. [11,12,14]

- *Engineered binding to erythrocytes (RBC)*

Another example of erythrocytes use in DDS was reported by Lorentz et al.,[17] which described the conjugation of Escherichia coli L-asparaginase-II (ASNase) with some copies of glycophorin A-binding peptide (ERY1). The procedure started with the chemical conjugation between lysine residues of ASNase and peptide ERY1 using succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) as linker (**Figure 4A**). SMCC is one of the most usefull bifunctional linkers using in bioconjugation, which allowed the first reaction via amide formation (free amines from biomolecule or cargo, and succimidyl esther from SMCC), and then, the maleimide moiety would react with sulfhydryl groups of another entity (biomolecule or payload).[18] After that, high-affinity binding of ASNase to erythrocytes was achieved through chemical conjugation of several copies of glycophorin A–binding peptide (ERY1). This peptide (ERY1) was conjugated to ASNase through its cysteine residue [17] (**Figure 4B**).

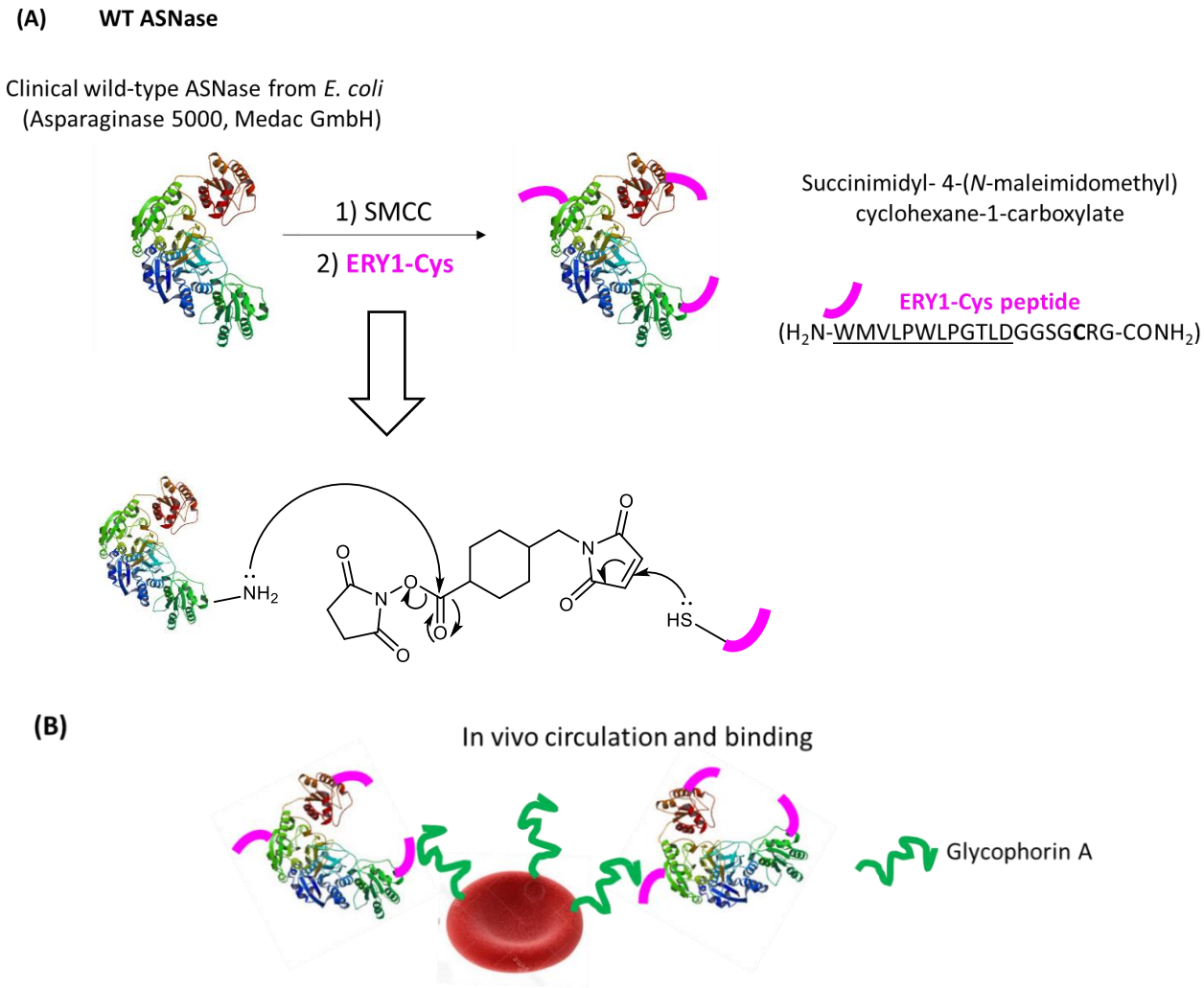


Figure 4. A streptavidin-biotin technique using RBC as carrier.

The conjugation described above allows reducing the development of antibodies due it induce antigen-specific tolerance. Erythrocytes conjugated with ASNase have a drug pharmacodynamics effect greater than ASNase administrated alone. Alizée et.al[19] used the same ERY1 peptide chemically conjugated the erythrocyte to ovalbumin through of sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) as a linker. This conjugation aim improving the response of CD4+ and CD8+ T cells to an antigen because these were induced an antigen tolerance previously.[19]

- *DDS based on Erythrocytes as Commercial Drugs*

Nowadays, there are no FDA approved conjugate erythrocytes, but there are studies about non-conjugated erythrocytes in phase I, II and III.[13,20] Erythrocytes as carriers of DDS is complicated to bring to market because of implicating be careful with the parameters that a clinical product must-have in both, conjugated and non-conjugated biomolecules. Among parameters that make

difficult their market development are: i) cell material source, which refers if the erythrocytes come from analogous or homologous donated blood; ii) manufacturing process, which refers to the sterility of the cell suspension and the reduction of leukocytes; iii) product storage, regarding the solution where the erythrocytes are reserved to theirs prompt administrated because they tend to degrade.[21]

1.2.2 Liposomes as a carrier in DDS

Liposomes are spherical vesicles which can be make from a suspension of phospholipids in a hydrophobic medium; this solvent is slowly removed or replaced by another which is hydrophilic, usually an aqueous solution, using dilution, evaporation or dialysis processes. This change of solvent causes the phospholipids to group together spontaneously, and in this way form spherical bilayers and trap a large amount of the aqueous medium in which they are found. When working with drugs or hydrophilic entities, they are trapped inside the bilayer; and the hydrophobic remain in the phospholipid layer (**Figure 5**). [22,23]

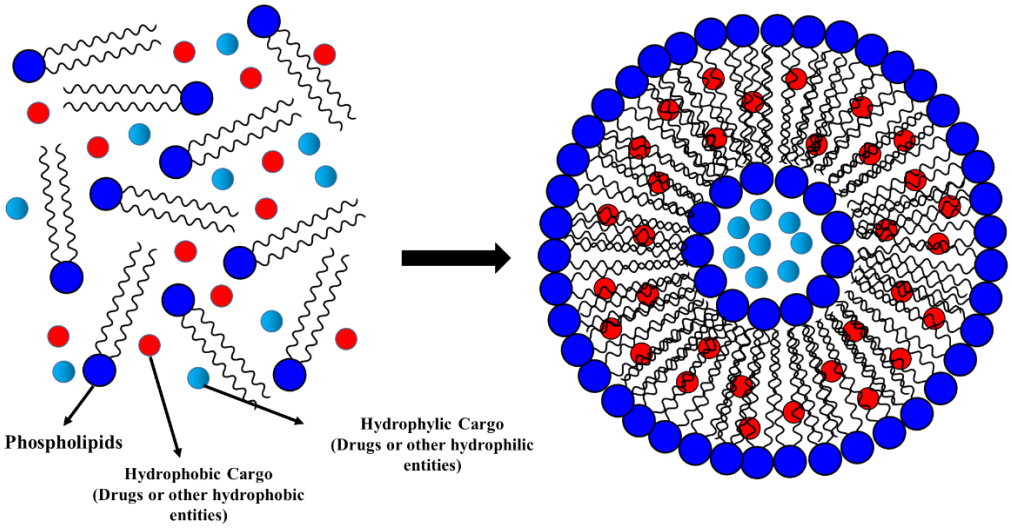


Figure 5. General liposome formation.

This carrier is one of the most studied to be used in DDS due to some positive features that liposomes have, for example, they have a high level of loading and also a long period time in circulation, changing drug pharmacokinetics in comparison of the drug administered alone.[23,24] Liposomes as carriers are safe, biodegradable and biocompatible, which made of them very useful to theranostics [25] and even with imaging purpose when these are conjugated with quantum dots or gold nanocluster.[25,26]

In general, liposomes are able of transporting drugs in different ways: trapped inside the bilayer (hydrophilic drug); remain in the phospholipid layer for hydrophobic drugs, and covalently linkage to liposome surface (**Figure 6**). Regarding chemical conjugation, ester and amide bonds have been extensively used in two ways: i) to join phospholipid to the drug, and then carry out the liposome formation; and ii) to decorate the liposome surface.

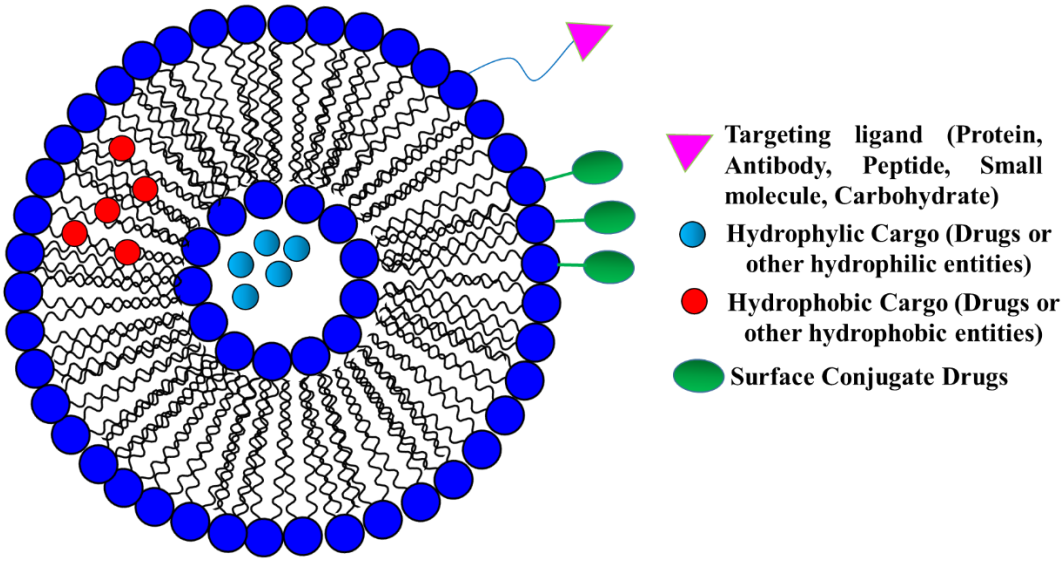


Figure 6. General structure of DDS based on functionalized liposomes.

As was mention before, DDS is developed as a therapeutic alternative, which avoiding or minimizing the harmful effects caused by other chemical treatments (especially for cancer). In this regards, liposomes have been using as bio carrier, and its conjugation with microbubble,[27] some protein[25] or antibodies,[28] allowed to obtain even DDS with anticancerigen activity. Different chemical approaches with different functional groups are used to carried out the conjugation, but the more used are:

- Ester bonds: It is formed between a carboxylic acid group and a hydroxyl group that could have the drug and the lipid, respectively. To facilitate this conjugation is used linker or spacer such as succinic acid.[29] Another linker useful to lead the conjugation is succinic anhydride functionalized.[25,26,28,30.]

  Amide bonds: It is generated between a terminal carboxylic acid of the lipid and an amine group present in the drug.[31,32]

➢ *DDS based on Liposomes as Commercial Drugs.*

DDS based on liposomes approved by FDA are scarce, and most of them are PEG (Polyethylene Glycol) conjugated liposome. PEGylation is a successful technique in the development of a

drug delivery system using liposomes as a carrier. For instance, the antibiotic doxorubicin used in chemotherapy was combined with liposome conjugated with PEG (Doxil®) to improve circulation time of the drug and reduce the toxicity.[33] It was the first DDS based on liposome approved by FDA in 1995.[34] Twelve years later Liposomal doxorubicin in combination with another anticancer drug bortezomib (Lipodox®) for relapsed or refractory multiple myeloma was developed by Ning et al.[34,35] Lipodox® has not been approved yet by FDA, but given the shortage of Doxil was accepted the importation of Lipodox® because of it has the same active ingredient, dosage, strength, and route of administration as the previously FDA-approved drug Doxil. [36] Additionally, some others DDS based on PEGylated liposomes are still in clinical trials (phases I, II, or III), and as good examples are Thermosensitive doxorubicin in phase III which is a promising treatment of liver cancer [37] or Irinotecan in phase I which could be used in advanced refractory solid tumors.[38]

1.2.3 Nanoparticles (NPs) as a carrier in DDS

Nanoparticles (NPs) are defined as entities with an ultra-small size; generally with dimension less than 100 nanometers, these dimensions confer different physical, chemical or/and biologycal properties in comparison with a macro or micro-sample for the same material.[39,40] Some useful features of NPs interns of DDS are: i) suitable to be used in intravenous delivery, ii) a good behavior as site-specific drug targeting for treating of several diseases, iii) a higher surface to volume ratio as compared with bulk material would provide a diminution in the dose of the drug and therefore a reduction in the toxicity; and iv) a prolonged circulation in the blood, among others, make them suitable for its use as DDS and promised results have been reported.[41]

In general, drugs can be aggregated on the external or internal surface of a nanocarrier or can be linked through chemical bioconjugation. Focused on DDS based on nanoparticles with an emphasis in chemical conjugation, different kind of NPs as polymeric (i.e. PLGA, PLA, PEG, hydrogel, chitosan analog), magnetic NPs and structures base on carbon have been extensively described[42]. Chemical conjugation normally take place on the surface of NPs and different strategies are used for the conjugation of NPs with drug like: amine-carboxyl, thiol-meleimide, thiol-thiol, hydrazide-aldehyde and gold-thiol are the most useful reactions used in the conjugation with NPs, allowed to generated amide, thioether, disulfide, hydrazine, gold-thiol, and triazole ring as covalent linkage, respectively. [42] In this regards, the structure of drug or ligand determining the type of reaction (**Figure 7**).

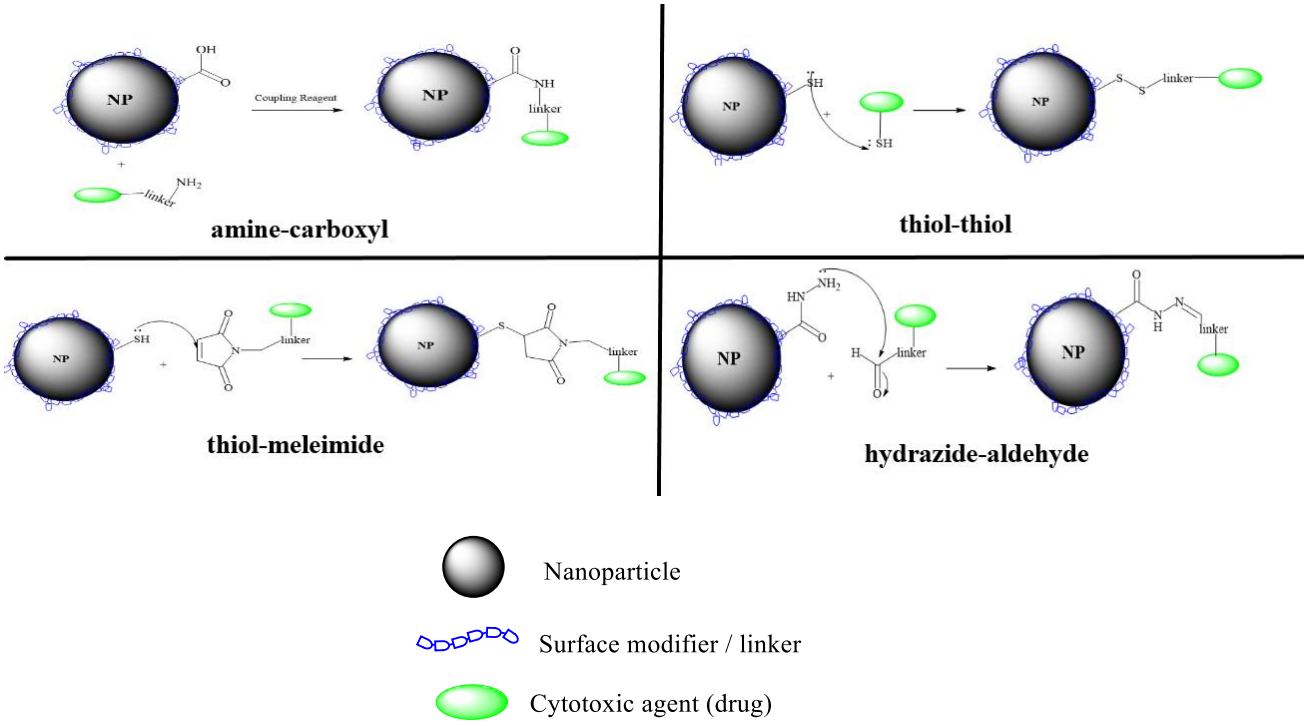


Figure 7. Strategies of conjugation for DDS based on the modified nanoparticle surface.

The potential use of nanoparticles with therapeutic purposes generated an emerging field called nanomedicine, whose goal is developing new systems or nanocarriers for drugs or imaging agents with high selectivity, safety, and less toxicity. NPs have been conjugated with the aim of improving the pharmacokinetic and the pharmacodynamic properties of this cargos, allowing overcome inconveniences like multidrug resistance.

- *DDS based on NPs as Commercial Drugs*

Nowadays, in nanomedicine PEGylated polymers nanoparticles are the most used and the more available in the market. For instance, Oncaspar® which was one of the first nanotherapeutic approved by FDA in 1994[43]. NPs of Oncaspar® are formed by L-asparaginase aminohydrolase covalently conjugated to monomethoxypolyethylene glycol (mPEG) through non-specific random PEGylation of ε-amino groups on the lysine residues of the enzyme (**Figure 8A**), which is used in chemotherapy for the treatment of acute lymphoblastic leukemia (ALL).[44] Also using PEGylation procedures, Krystexxa® was developed in 2010, [45] which the NPs consists of a pegloticase and mPEG covalent conjugate, and was designed for the treatment of severe debilitating chronic tophaceous gout.[46] Four years later Plegridy® is introduced in the pharmaceutical market,[47] to the treatment of relapsing-remitting multiple sclerosis.[48]

PEGylation of interferon beta-1a using mPEG and O-2-methylpropionaldehyde linker was reported as its formation reaction of NPs. [47,48]

Other successfully example is the Abraxane®, [49] an albumin-bound nanoparticle formulation of paclitaxel without phospholipids (**Figure 8B**), which improved the potential activity of paclitaxel for the treatment of recurrent breast cancer and avoiding the hemolysis reaction of the liposome.[50]

The last polymer-drug conjugated approved was ADYNOVATE[51] in 2015, where is covalently conjugated ADVATE molecule with PEG. This is used to treat and control of hemophilia A[52].

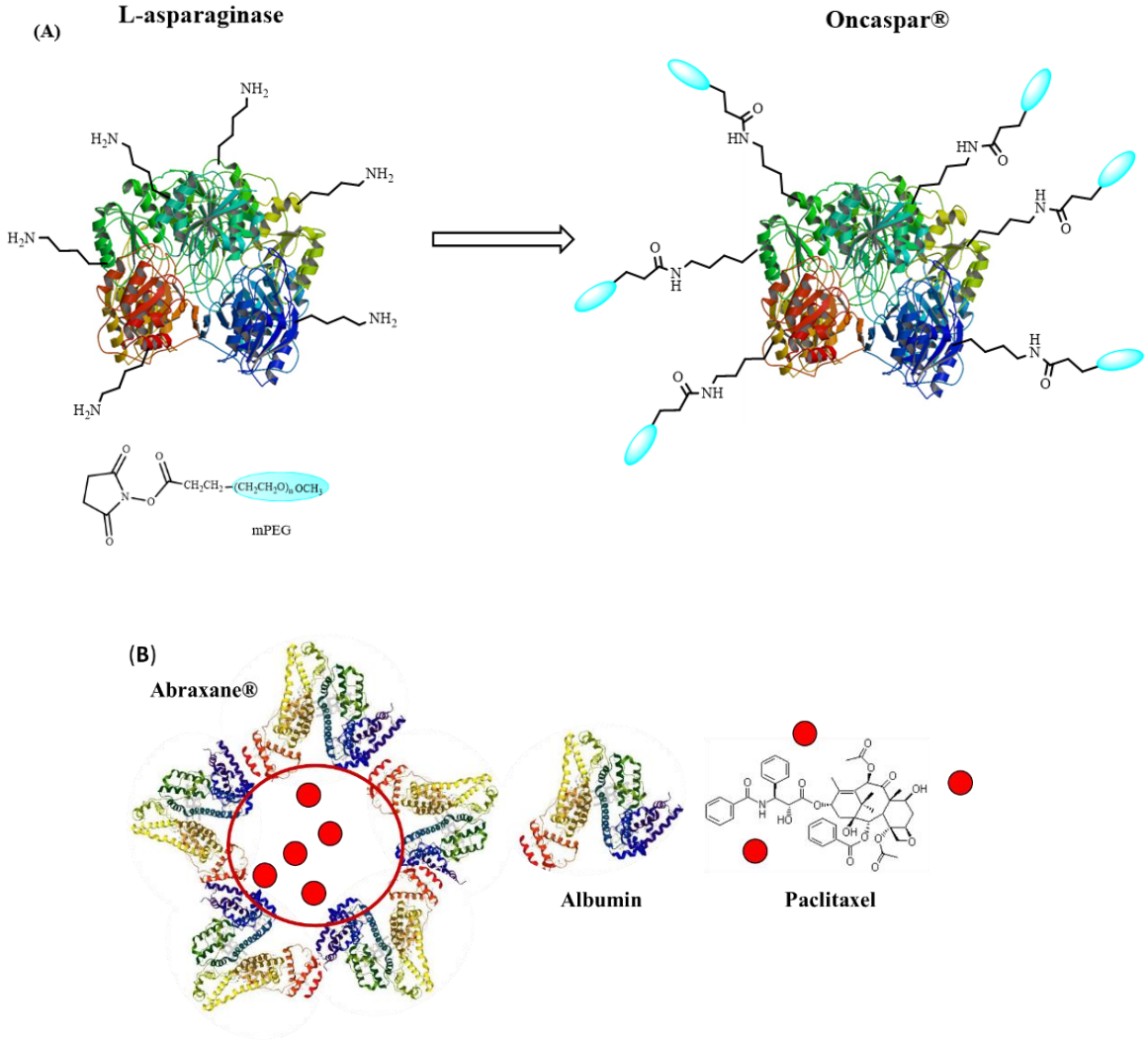


Figure 8. General structures of Nanotherapeutic products approved by the FDA. **A** Pegylation of L-aspariginase to obtain Oncaspar®; **B**, General structure of Abraxane®.

Other several of these pharmaceuticals products with novel technologies attract research community attention. Invega Trinza®[53] with nanocrystal technology based on Paliperidone

Palmitate structure, releases paliperidone slowly over a long period for the treatment of schizophrenia and schizoaffective disorder. Sublocade® (buprenorphine extended-release),[53] with the Atrigel technology in situ gel-forming system polymer (biodegradable 50:50 poly (DL–lactide–co–glycolide) and a biocompatible solvent, N–methyl–2–pyrrolidone (NMP) is thought to be a revolutionary product administrated for the treatment of moderate to severe opioid use disorder. The most important Sublocade® disanvantage is that it can only be administered via subcutaneous, in fact serious harm or even death would result if this drug is administered intravenously.

1.2.4 Antibody-Drug Conjugates as a carrier in DDS

An antibody is a Y-shaped protein generated by the immune system in response to antigens such as infectious agents or foreign substances. The main characteristic of an antibody is its ability to bind to a specific region or epitope of the corresponding antigen, which allows the antibody to be specific and very precise and therefore has demonstrated applications in the treatment of some disease, i.e. cancer.[54] In general, monoclonal antibodies (mAb) are generated through hybridoma technique,[55] which use murine B cells and murine myeloma cells to produce murine mAbs with a unique specificity. The mAbs have been used to developed bioconjugation techniques, using chimeric and humanized antibodies with therapeutic purposes and hence in bioconjugation. [56,57]

Undoubtedly, one of the most successful DDS therapeutic alternatives are the Antibody-Drug conjugates (ADCs), due to the high specificity of these biomolecules, but also the structural facility (several functional groups available) to load the drug (**Figure 9**). For this reason, the amount and concentration of the cytotoxic agent used for the treatment are lower in comparison with the treatment of naked antibody or drug only.[58] Since the objective is to focus cytotoxicity towards tumor cells, patients tend to tolerate ADC treatment for a bit more time. Another advantage of ADCs is that it takes time to develop resistance than their constituent mAb,[59], which allows an increasing in the therapeutic window and a more aggressive fight against cancer. In general, an ADC is formed by the mAb and the drug, and both entities are joined through crosslinking achieved with a variety of reactive groups such as amines, thiols, alcohols or even carboxylic acids present in either, mAb or drug. Another successful way to achieve the bioconjugation is the use of a linker. The linker can be a homo- or hetero-biofunctionalized molecule, regarding if the reaction take places through the same functional group or two different kind. In typical homobifunctional crosslinkers conjugation occurs through the same

functional group (e.g. conjugation between two amines or two sulfhydryl groups), On the other hand, on the denominated heterobifunctional crosslinkers, the conjugation takes place through two different functional groups (e.g. conjugation between an amine and a thiol groups).(**Figure 10**)

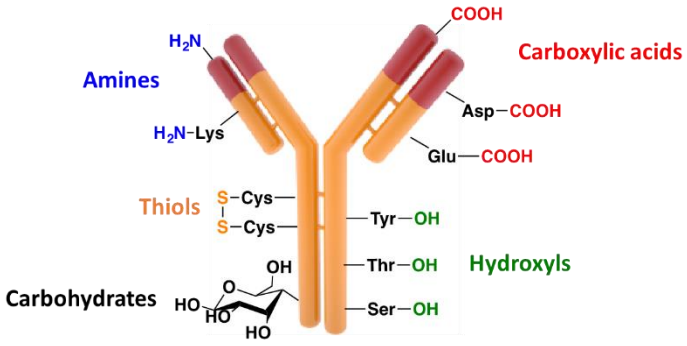


Figure 9. Representation of all potential available antibody functional groups to suffer bioconjugation.

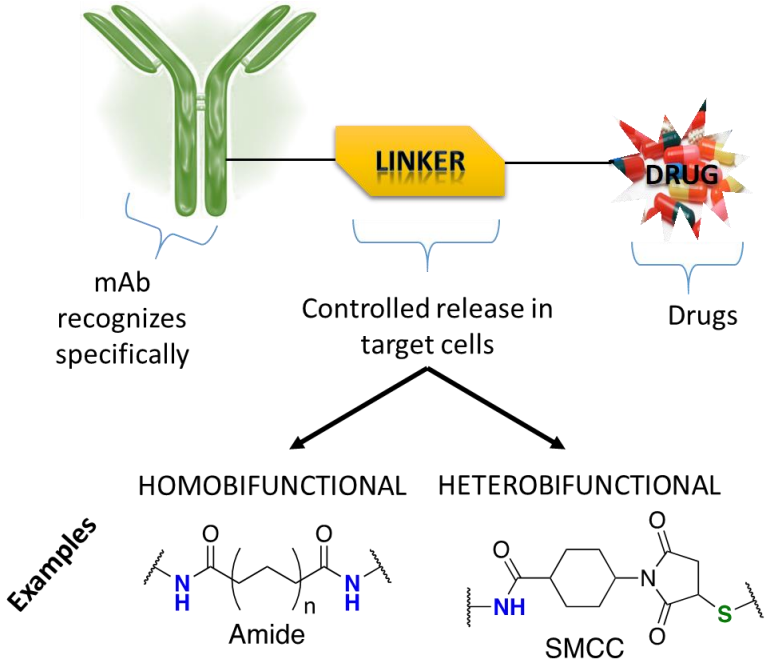


Figure 10. General ADC structure

- *Linker properties*

As was mentioned before, the drugs could be conjugated directly to the antibody, but to facilitate bioconjugation, the linkers have been very useful in the preparation of ADCs. Among the advantages of using a linker are prevention premature drug release, improving the liberation

of the active drug at the target, and facilitating the stability in the production process. The most important aspect of the linker is its physicochemical properties, which allows defining two main different kinds of linker:

- Cleavable linker: It has the characteristic of breaking one of its chemical bond that binds the two molecular entities. This kind of liker has been successful in the elaboration of ADCs due to its stability in the synthesis process as well as in the blood circulation of the conjugate for a long time. It allows the releasing of the drug under some conditions at the surrounding of the target cell or tissue-like overexpression of enzyme or acidic environment.[60,61]
- Non-cleavable linker: Its main function is maintain linked both biomolecule and drug. This feature represents a greater advantage over cleavable linkers because it has greater stability in the plasma, thus increasing the specificity. It means that the drug will not be released outside the target cells because the mAb degradation is carried out within the lysosome.[61,62]

➢ *Chemical conjugation*

Chemical conjugation in the ADC uses covalent bond formation. This delivery system uses a linker between the cytotoxic agent and antibody.[63] Preferred sites to carry out on antibody is the amine group of a lateral chain of exposed lysines or the sulfhydryl group of the exposed cysteines. In this regards, the covalent coupling can be made mainly through two methods: lysine amide or cysteine coupling.

- *Lysine amide coupling:* this method binds payload and lysine residues on the antibody using linkers containing activated carboxylic acid esters. However, this conjugation can give multiple ADC species with a different drug to antibody ratio (DAR). With a DAR around of 3-4 because a typical antibody has an average of 10 residues of lysine chemically available of around 80 lysines that it would have. Therefore, this way of chemical conjugation generates heterogeneous mixture with some species difficult to characterize and to purify, which could provoke effects on the binding to the antigen and even in the safety profile of the ADC.[63,64]
- *Cysteine coupling:* this method binds payload previously modify with the thiol-reactive group and cysteine residues of the antibody, generally using maleimide-type linkers.[63,64] Cysteine is in less amount in Abs in comparison with lysines, for example in the antibody IgG type (commonly used in ADC) 16 cysteines are present: twelve intra-chain

and four inter-chain disulfide bonds. These four inter-chain disulfide bonds are the target for conjugation because these are not important for structural stability,[65] allowing limited conjugation sites (2-8 free thiols) due to reactivity of thiol group. There is still possibility to end up with a heterogeneous mixture of ADC, although less extent than lysine conjugation. Cysteine coupling has been more used in clinical trials because it has a higher DAR (approximately between 0-8) than lysine amide coupling and beside the conjugates obtained with this technique are easier to characterize.[62,64]

Regarding cancer disease, the major advantage of these systems is the delivery of the drug, which could have high toxicity, directly in the tumor.[66] The possibility to combine the favorable binding properties of mAbs with the activity of potent cytotoxic agents promises to increase the therapeutic index of therapeutic payloads.

Borek et al.[67] reported a chemical conjugation of scFvF7-Fc antibody fragment through its cysteine residues to cytotoxic agent monomethyl auristatin E (MMAE) using valine-citruline linker and maleimidocaproyl spacer.[68] In this case, enzymatic and selective cleave occurs in the bond between the peptide with the termini valine-citrulline and the corresponding drug (**Figure 11**). This ADC can be internalized into cancer cells due to its composition, i.e., the antibody and linker. This study was carried out in search of an alternative in the treatment of gastrointestinal cancer, which has a high incidence of mortality with relatively short survival rates.

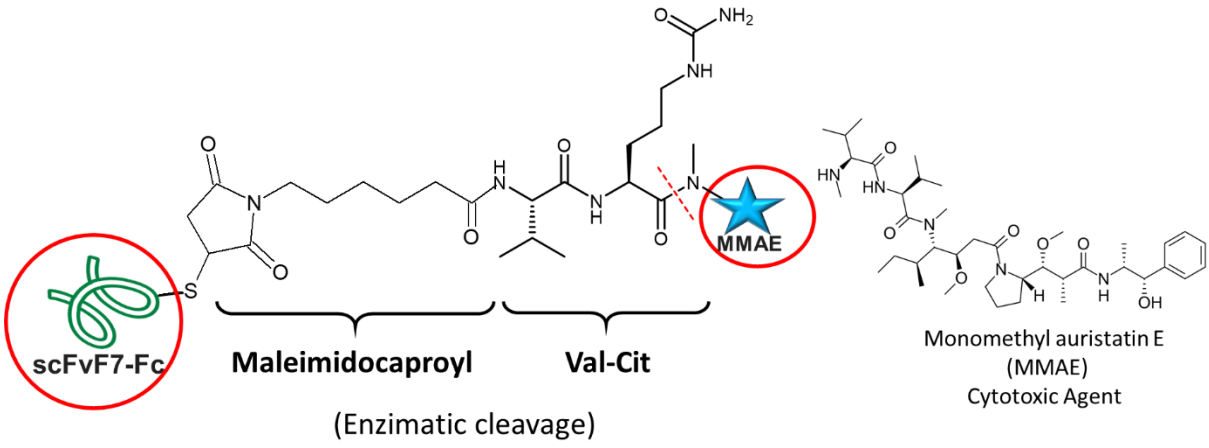


Figure 11. Structures of drug (MMAE) and mAb-drug conjugate.

On the other hand, numerous studies using lysine residues of ADCs have been carried out. In general, lysine mediated chemical bioconjugation allows to obtain heterogeneous conjugates mixtures with 0-8 DAR, and consequently, each of them has different toxicity,

pharmacokinetics, and efficacy. Although both cysteine and lysine assisted conjugation is still used, some alternative strategies have also been implemented in order to generate more homogeneous ADC´s. For example, Nanna et al.[69] reported the conjugation of the humanized anti-hapten monoclonal antibody (mAb) h38C2 through its most exceptionally nucleophilic lysine present at 11-Å deep hydrophobic pocket in your structure. The mentioned lysine is capable of catalyzing aldol and retro-aldol reactions, thus it can be selectively conjugated with β-lactam derivatives (i.e., β-lactam functionalized monomethyl auristatin F (MMAF)), generating a non-cleavable and stable amide bond, until its enzymatic degradation inside the target cells take place (**Figure 12**). The described conjugate could avoid premature releasing of the cytotoxic agent. The additional achievement of this study were generate homogeneous ADC's. [69]

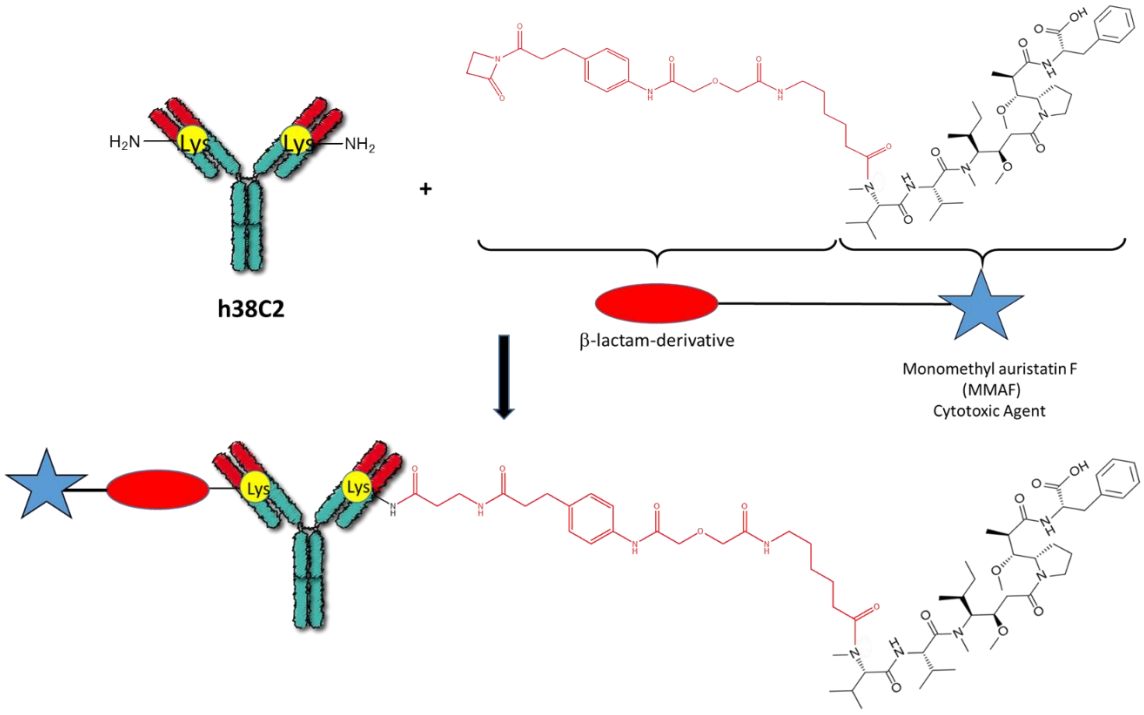


Figure 12. Conjugation of the humanized anti-hapten monoclonal antibody (mAb) h38C2 through nucleophilic lysine with β-lactam derivatives (i.e. β-lactam functionalized monomethyl auristatin F (MMAF).

- *DDS based on ADCs as Commercial Drugs*

At present, four ADC products hold market authorization for the therapy of certain types of cancers: Adcetris™,[70] Kadcyla™,[71,] Besponsa™,[72] Mylotarg™[73,74] (**Figure 13**). Adcetris™ (Brentuximab Vedotin, BV) is an anti-neoplastic agent used in the treatment of Hodgkin

lymphoma and systemic anaplastic large cell lymphoma. BV is obtained through the covalent conjugation of the mouse-human chimeric monoclonal antibody IgG1 antiCD30 (cAC 10), with monomethyl auristatin E (MMAE), a toxin which is a synthetic anti-tubulin analog, using an enzymatically cleavable dipeptide linker (**Figure 13A**). On average, each antibody molecule was conjugated to four groups of MMAE. [75]

On the other hand, Kadcyla™ (Trastuzumab emtansine) is an innovative, unique, and selective antineoplastic drug used in patients with advanced breast cancer (HER2+). This ADC is composed of the antiHER2 antibody trastuzumab (Herceptin®) and the cytotoxic microtubules agent, DM1, bound through a bifunctional succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) as a non-cleavable linker. This drug acts selectively on HER2 + tumors cells, exercising, on one hand, the mechanisms of action of the trastuzumab, and on the other hand, the powerful cytotoxic effect of DM1 **(Figure 13B).** The main advantage of this ADC is that it has a selective release, allowed to minimize its side effects in comparison to other agents for the same pathology. [71, 76]

Besponsa™ (Inotuzumab ozogamicina) is used in adults with acute lymphoblastic leukemia (ALL) whose disease has stopped responding to conventional chemotherapy (recurrence), or never responded to it (refractory). The carrier of Inotuzumab ozogamicina is a monoclonal antibody that attacks the CD22 protein, which is produced in excess on the surface of lymphoblastic leukemia cells. The antibody binds to a compound called calicheamicin that kills cancer cells using hydrazone linker **(Figure 13C).** Once inotuzumab antibody binds to CD22 in the cancer cells, the calicheamicin is released into the cell where it damages cellular DNA and causes its death. [74, 72]

Finally, Mylotarg™ (Gemtuzumab ozogamicin) is used for the treatment of acute myeloid leukemia (AML), a bone marrow cancer. Conjugation is similar to Besponsa, however different antibody is used. In this case, Inotuzumab ozogamicin consists of a recombinant humanized IgG4 kappa CD22-targeting monoclonal antibody covalently attached to calicheamicin derivative, which is a potent DNA-binding cytotoxic agent **(Figure 13C)**. [73,74] Mylotarg was removed from the market in 2010 due to the high incidence of death in the patients but it was restituted in 2017 combined with induction chemotherapy.

1.2.5 Hybrid conjugates as carriers in DDS

In general, DDS uses only one biomolecule or nanoparticle as a carrier, and sometimes, it is not effective. Scientists are conducting various experiments where a combination of biomolecules

or biomolecules with nanoparticles has been made to find novel hybrid systems to therapeutic use in front of a certain disease, especially cancer. Taking into account that this disease has a variety of types, it is not possible to have "general" DDS to treat it.

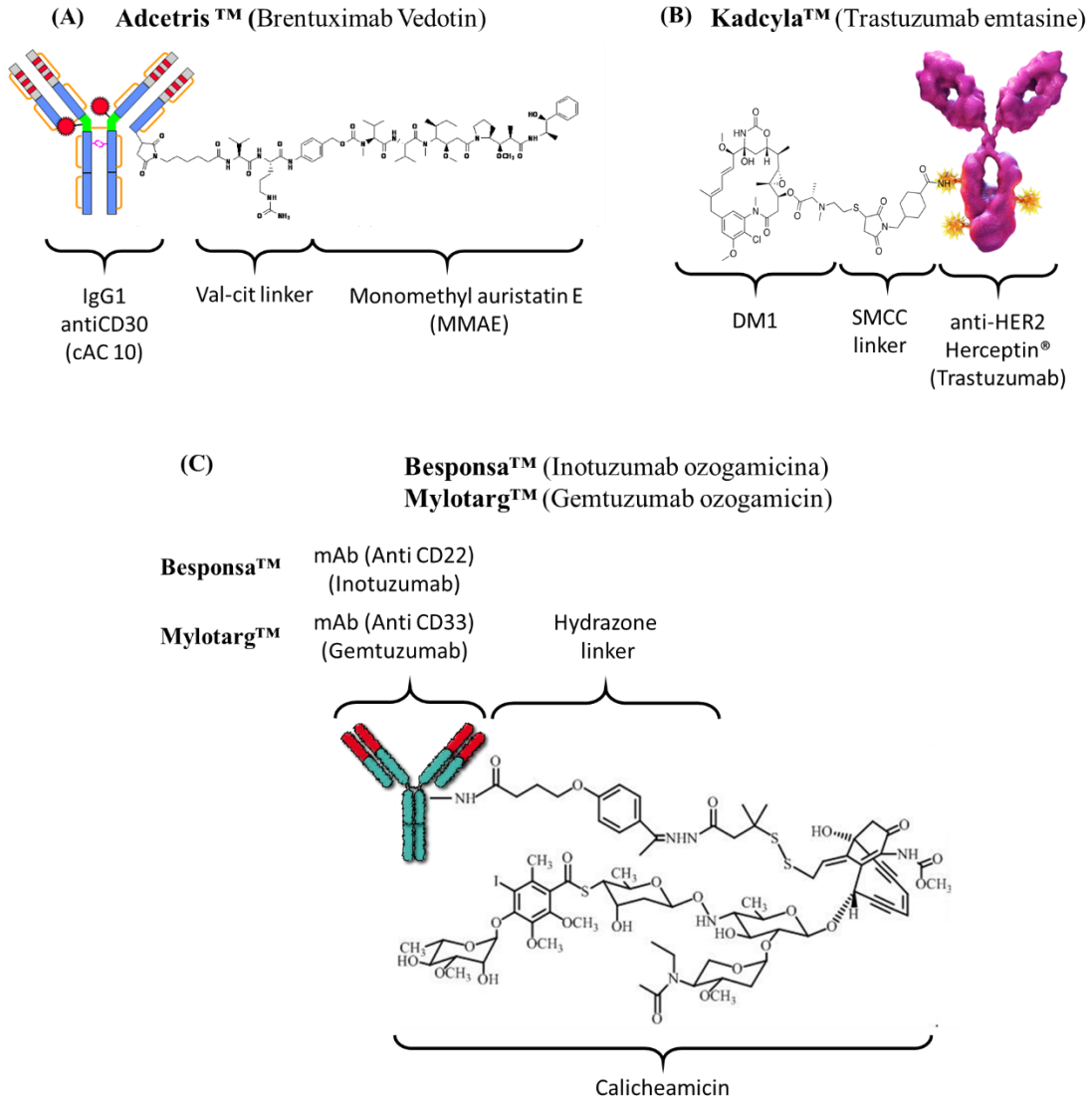


Figure 13. General structures of ADC´s approved by FDA. **A,** Adcetris™ (Brentuximab Vedotin, BV); B: Kadcyla™ (Trastuzumab emtansine); C: Besponsa™ (Inotuzumab ozogamicina) and Mylotarg™ (Gemtuzumab ozogamicin).

For example, in the search of a conjugation a brain delivery systems able to crossing the blood brain barrier (BBB), Huang et al [77] studied lactoferrin (Lf) protein like active principle conjugated to PEGylated liposomes (PL). The hybrid conjugate was prepared through the thiolization of Lf using Traut's reagent, allowing to have a sulfhydryl group at the N-terminus. At the same time, PL was functionalized with maleimide in order to promote a subsequent

thioether formation between the previously thiolated Lf and maleimide PL derivatives. Pharmacokinetics and pharmacodynamics properties of the Conjugate Lf-PL were compared with PEGylated liposomes (PL), taking into account that PEGylation is useful to improve the properties mentioned above.[78] The results showed a greater uptake of the conjugate system, mediated by Lf receptor present in the surface of the glioblastomas.[79]

The research of Lin et al.[80] present interesting DDS base on liposome and antibody. It was achieved by generating a half-antibody with free thiol groups available for the subsequent formation of thioether. Selected maleimide-pegylated liposomes loaded with Triptolide (TPL), which has a cytotoxic effect of reducing tumor growth, were conjugated with the mAb and tested in lung tumor cells. Taking into account that carbonic anhydrase enzyme IX is present on the lung tumor cells surface, and to achieve the selectivity and precision in this type of DDS, carbonic anti-anhydrase antibody IX conjugated to the surface of a liposome was used to target tumor in the lungs. Lung cancer is one of the most afflicted to the world population and it has lower rates of long-term survival.[81]

All the consulted bibliography allows concluding that DDS is an emergent field in Medicinal Chemistry. DDS based on Erythrocytes, Liposomes, Nanoparticles, and mainly mAb has been successfully developed, yielding entities able to avoid or minimize drug side effects or circumventing the resistance of the disease to certain drugs. Many of these conjugates are already in the market, and many others are already in clinical trials. Therefore, the search for more efficient DDS is continuously under development.

1.3 Problem statement

ADCs are an emerging field in the development of novel drug administration systems. Their main characteristic is very selective and specific in the treatment of several diseases, especially cancer, which represent one of the most common causes of death. However, from the concept of targeted drug administration in 1913 by Paul Ehrlich to the present days, there are only four ADCs approved by the FDA. Despite countless efforts in the development of controlled drug delivery systems, the search for new conjugates continues to be a challenge and a necessary. The development of new linkers, in addition to the selection of the antibody and the drug, is an essential part of this process.

In this regards, as **Hypothesis**, the search for a new type of linker based on Tetrahydro-thiadiazine-2-thione (THTT) scaffold for bioconjugation, would be carried out to develop study of the new and efficient kind of linker to be applied conjugation procedures. In turn, the stability under different pH could indicate the potential use of THTT as a cleavable or non-cleavable linker.

1.4 General and specific objectives

- ***General objective***

Design and develop a new linker based on tetrahydrothiadiazine-2-thione (THTT) scaffold for its use in bioconjugation.

- ***Specific objectives***
  - Synthesize the dithiocarbamate as linker precursor, using glycine as starting reagent and cargo.
  - Perform the acidolysis study of THTT derivatives to determine if the potential linker is cleavable or non-cleavable, using UV nanodrop analysis.
  - Carry out the bioconjugation with a protein (Bovine serum albumin, BSA) and an antibody (anti-α Tubulin).
  - Characterize the bioconjugates using UV nanodrop spectroscopy and ULPC mass spectrometry.

CHAPTER 2. METHODOLOGY

2.1 Chemistry

Reagents

Commercial products and solvents were used as received without further purification. Glycine, bovine serum albumin (BSA), anti-alpha-tubulin antibody, potassium hydroxide, formaldehyde solution 37 wt.%, diethylether, and dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). 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}). Phosphate buffer solution (pH 7.2), hydrochloric acid, and ethyl ether were purchased from Fisher Scientific Company (Chicago, IL, USA). Pre-packed Shephadex™ G-25 containing columns were from GE Healthcare Europe GmbH (Freiburg, Germany).

Equipment

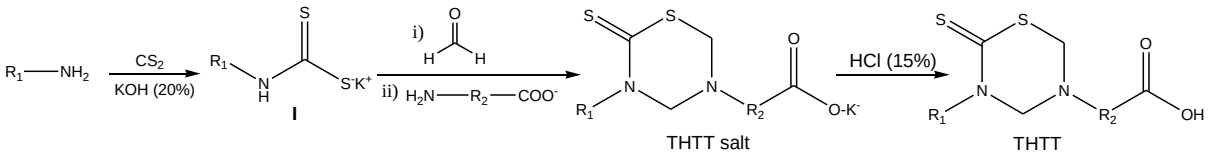
Nanodrop from Thermo Fisher Scientific Company (Waltham, Massachusetts, USA) was used to detected protein and antibodies conjugates fractions but also was used to carry out the acidolysis study through UV absorbance's detection.

UPLC-MS spectrometry: reversed-phase column SunFire™ C18 3.5 µm (2.1×100 mm) 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% formic acid) into H_2O (0.01% formic acid) were run at flow rate of 0.3 mL/min. The solvents for UPLC were H_2O (Type I), and MeCN (HPLC quality).

2.2 General procedure to obtain 3,5-disubstituted tetrahydro-2*H*-1,3,5-thiadiazine-2-thione derivative.

The synthesis of THTT has been widely reported in the literature.[82] The most used procedure to obtain 3,5-disubstituted tetrahydro-(2*H)*-1,3,5-thiadiazine-2-thione is carried out in two steps (Scheme 1). In the first step, equimolecular amounts of an amine (R_1-NH_2), carbon disulfide (CS_2) and potassium hydroxide (KOH at 20% aqueous solution) react at room temperature by 4 hours, to give the dithiocarbamate potassium salt (I). Generally, this salt is not isolated. To this first step is followed by cyclocondensation with a double equimolecular amount (regarding the first reagents) of formaldehyde (HCHO) for a further 1 hour, and subsequent addition of equimolecular amount of a second compound containing free amine, generally a salt of an

amino acid (NH_2-R_2-COO^-K^+), which, is stirring by 2 hour allowing to obtain the THTT salt. The solution with a pH 7 is put in an ice bath. To the neutral cold solution was added HCl (15%) until pH 2, let to precipitate THTT product. The precipitate is filtered and washed with cool diethylether allowing to obtain the final THTT generally with moderate to high yield.

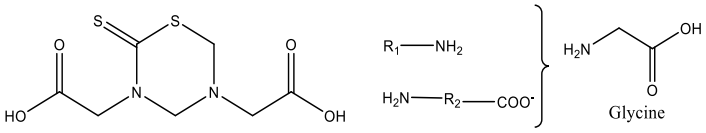


Scheme 1. General procedure for the synthesis of THTT derivatives.

3,5-(1´-carboxymethyl)- tetrahydro-2*H*-1,3,5-thiadiazine-2-thione

Using glycine (Gly) residue (0.005 mol, 0.375 g) as a substituent at position 3 and 5, and according to the reported procedure, the THTT should have precipitated. In our case, at pH=1 there was no presence of precipitate. Then, two extractions with diethylether (around 20 mL in each extraction) were carried out. The organic extracted solution was rotoevaporated to dryness. To eliminate water residues of our mixture, addition of little amounts of diethyl ether and subsequent rotovaporate were carried out. Having removed the water, the round bottom flask was put in a desiccator under vacuum for 7 days. Final compound obtained was a light yellow powder (1.19×10^{-4}mol, 29.7 mg).

Yield	2.4%
Melting point (decomposition)	124 °C
UV λ_{max}	200 nm, 220nm



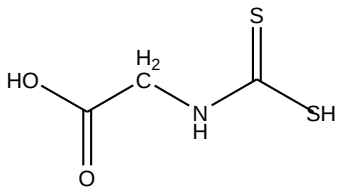
Preparation of the linker precursor: Dithiocarbamic acid (DTC) synthesis, glycine-N-dithiocarboxylic acid (II)

To a stirred solution of glycine (0.005 mol, 0.375 g) in water (25 mL) in a 250 mL flask 10 mL of 20% aqueous potassium hydroxide (0.005 mol, 0.28 g) was added and carbon disulfide (0.005 mol, 0.3 mL). This mixture was stirred during 4 hours at room temperature. Then, the result solution was washed with 20 mL of ether. After, the aqueous solution is cooled and acidulate using HCl (15%) drop by drop until acidic pH (pH=1).

Following the reported procedure for others aminoacid-DTC; the dithiocarbamic acid should have precipitated. In our case, at pH=1, there was no presence of precipitate. Then, two

extractions with diethylether (20 mL by each extraction) were carried out. The organic extracted solution was rotoevaporated to dryness. To eliminate water residues of our mixture, the addition of little amounts of diethyl ether and subsequent rotovaporate was carried out. Having removed the water, the round bottom flask was put in a desiccator under vacuum for 5 days. The final product was a light brown powder (50.1 mg).

Yield	6.6%
Melting point (decomposition)	130 °C



2.3 Acidolysis study

Acidolysis study was carried out in five bis-thiadizine-2-thione derivatives*, named JH (A-E).

* The five compounds were synthesized by the Suárez research group at Organic Synthesis Laboratory of the University of Havana.

2.4.1 Preparation of samples

In general, 1 mg of each sample was diluted in 200 µL of DMSO and 800 µL of water (Type-I) in an Eppendorf of 1.5 mL. All samples were diluted in the 200 uL of DMSO, but when the water type 1 was added, the solutions showed turbidity except JH-A. For this reason, all samples (except for JH-A) were placed for 10 minutes in the sonicator at 40 ° C of temperature to improve the solubility. As they still showed some turbidity, samples B- E were centrifuged for 10 minutes at 14,000 rpm. After centrifugation, the mother liquors were decanted to another Eppendorf.

Before to perform acidolysis study, all initial sample concentrations (1mg/mL) were adjusted through subsequent water dilutions in order to carry out correct monitoring that will be done with UV nanodrop spectroscopy. The dilutions were different for each sample, to obtain the concentrations which allowed us to obtain a suitable absorbance for monitoring in UV nanodrop spectroscopy, i.e. absorbance around or under 1.

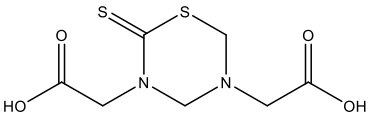
2.4.2 Acidolysis

The acidolysis study was carried out treating the previously diluted samples with HCl solution at pH 6. Degradation process was monitored by UV nanodrop at 0, 10, 20, 30 minutes and at 1,

24 and 48 hours. In general, 50 µL of each samples at concentrations between 0.2 µg/ µL to 0.002 µg/µL was mixed with 50 µL of an HCl solution in a Eppendorf of 2 mL.

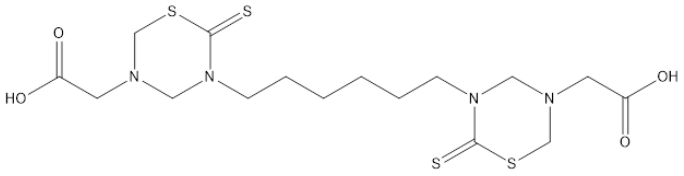
- ***Mono-THTT***

  Final concentration: 0.05 µg/ µL



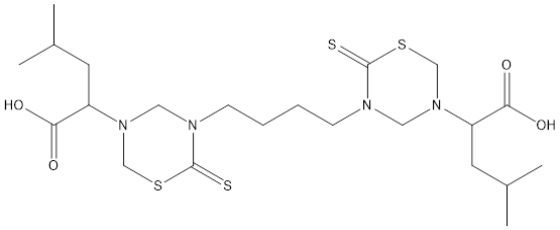
- ***JH-A***

  Final concentration: 0.01 µg/ µL



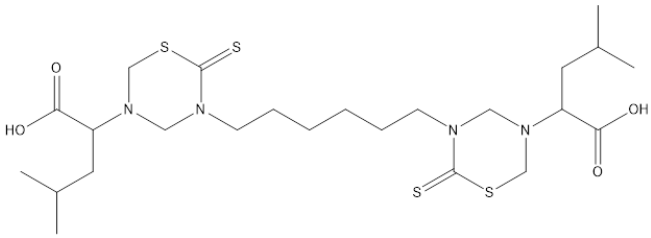
- ***JH-B***

  Final concentration: 0.1 µg/ µL



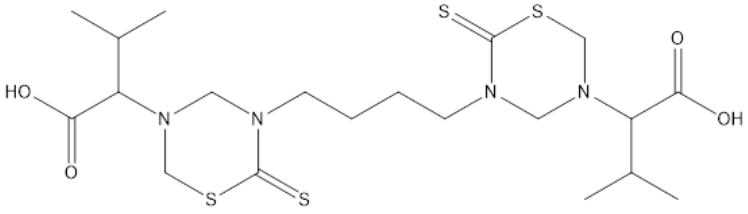
- ***JH-C***

  Final concentration: 0.1 µg/ µL



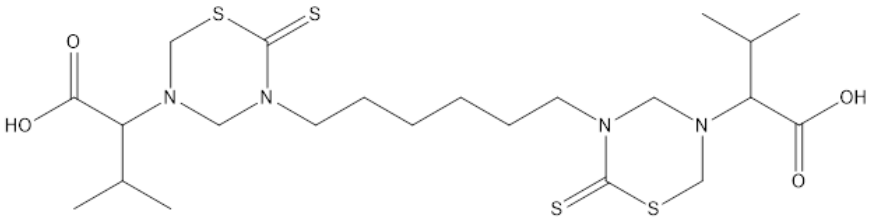
- ***JH-D***

Final concentration: 0.05 µg/ µL



- ***JH-E***

Final concentration: 0.05 µg/ µL



2.4 Bioconjugation

2.5.1 BSA-THTT conjugation (Conj-BSA-(1-3))

To carry out the BSA conjugation, first of all, to a corresponding solution of dithiocarbamic acid (DTC, **II**) in PBS, the right amount of formaldehyde was added (**Table 1**), and the mixture was left to react in a shaker during 1 hour at room temperature. The obtained mixture was added dropwise upon a corresponding solution of Bovine Serum Albumin (BSA) (**Table 1**) previously dissolved in distilled and deionized water (Class-I) and left to react in a shaker under established conditions (**Table 1**). The mixture was purified using size exclusion chromatography (PD10 desalting column), and water as eluent. Finally, protein-containing fractions of 500 µL each one was collected and classified through UV nanodrop spectroscopy (**Figure 14**). Three conjugates were obtained using different amounts of the reagents, but also different conjugation conditions (**Table 1**).

	BSA (mg-µmol)	DTC, II (mg-µmol)	PBS (µL)	H_2O (µL)	CH_2O (µL- µmol)	T(°C)/t_{reac}(h)
Conj BSA-1	21.8 – 0.33	7.2 – 47.6	1400	600	2.6-95.2	RT/24h
Conj BSA-2	11 – 0.165	7.3 – 48.28	700	300	2.6-95.2	RT/24h
Conj BSA-3	11 – 0.165	7.3 – 48.28	700	300	2.6-95.2	37° C/5h

Table 1. Reaction conditions of BSA conjugation.

UPLC-MS: [linear gradient H_2O/MeCN (5:95) over 8 min on BioSuite pC18, 7µm RPC 2.1 × 50 mm column] t_R (BSA)=3.36 min t_R (Conj BSA)= 3.46 min. BSA, Theor. Molecular Mass Calc.=66430.3 Da*; BSA m/z found = 66646.7522 ± 37.258 Da. Conjugate BSA-3 , Molecular Mass theor. Calc.= 69496.7522 Da; m/z found = 69415.8548±264.412 Da.

*The molecular weight of bovine serum albumin (BSA) was calculated based on its amino acid sequence.[83]

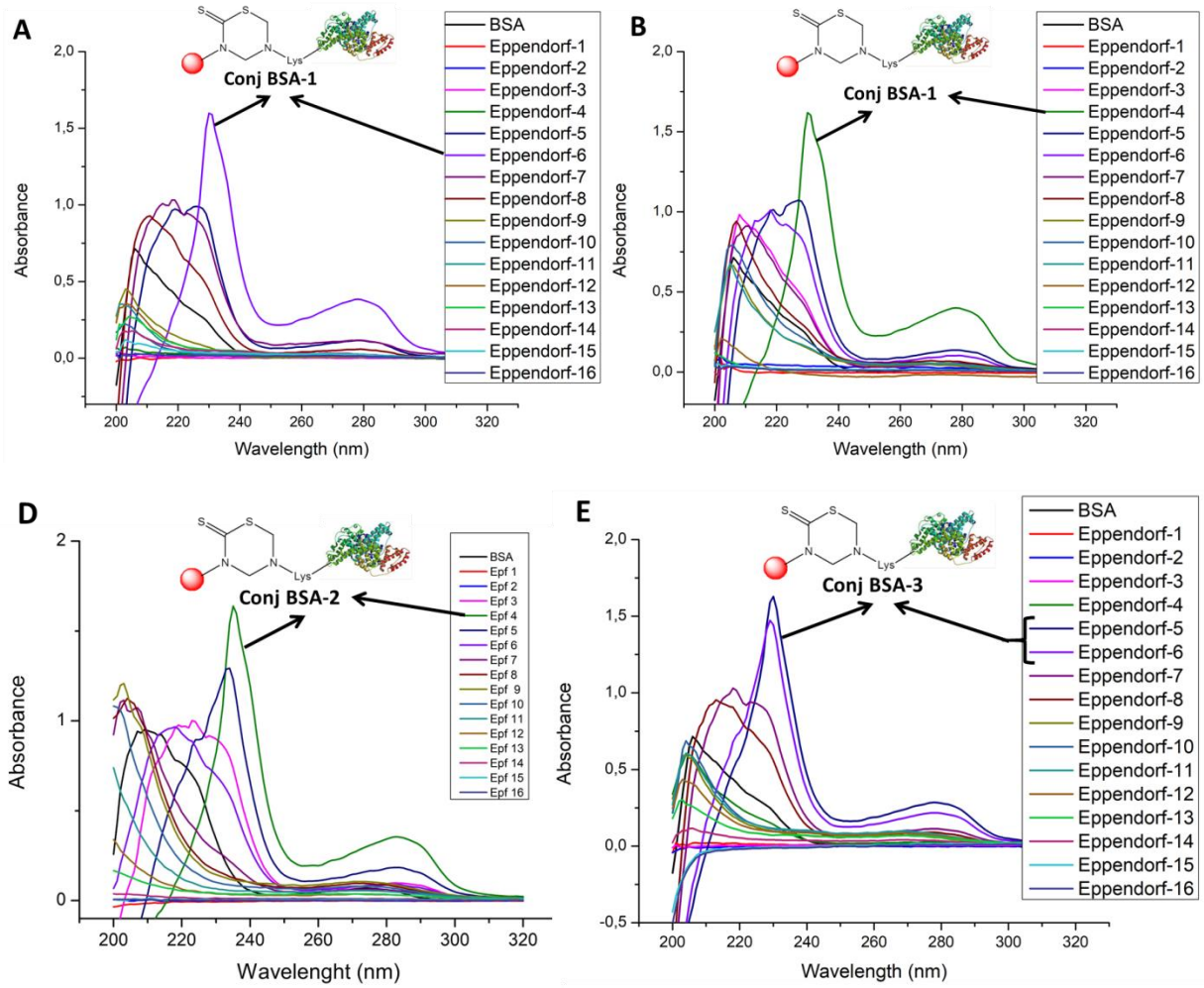


Figure 14. UV nanodrop analysis of fractions corresponding to size exclusion column chromatography of **A, B) Conj BSA-1, C) Conj BSA-2** and **D) Conj BSA-3**.

2.5.2 Anti-alpha tubulin (anti-α-T)-THTT conjugation

In order to carry out the anti-tubulin-THTT conjugation(**anti-α-T-Conj 1,2**), first of all, to a corresponding solution of dithiocarbamic acid (DTC, **II**), in PBS for **anti-α-T-Conj 1**, and in water for **anti-α-T-Conj 2**, excess of formaldehyde was added (**Table 2**), and the mixture was left to react in a shaker for 1 hour at room temperature. The obtained mixture was added dropwise upon a corresponding solution of anti-α-tubulin, a mAb (**Table 2**) previously dissolved in distilled and deionized water (Type I), and left to react in a shaker at room temperature during 5 hours. Finally, the mixture was purified using size exclusion chromatography (PD10 desalting column), and water as eluent. Finally, protein-containing fractions of 500 µL each one was collected and classified through UV nanodrop spectroscopy (**Figure 15**).

	mAb* (µL)	DTC, II (mg-µmol)	PBS (µL)	H_2O (µL)	CH_2O (µL)	T(°C)/t_{reac}(h)
anti-α-T -Conj-1	10	10 – 66.1	500	-	3.6-0.13	RT/5h
anti-α-T -Conj-2	10	10 – 66.1	-	500	3.6-0.13	RT/5h

Table 2. Reaction conditions of anti-α tubulin-THTT conjugation(**anti-α-T-Conj 1,2**).

*[anti-α-T] = 1 mg/mL

UPLC-MS: [linear gradient H_2O/MeCN (5:95) over 8 min on BioSuite pC18, 7µm RPC 2.1 × 50 mm column] t_R (anti-α-tubulin)=6.33 min t_R (anti-α-T-Conj)=6.27 min. anti-α-tubulin, Theor. Molecular Mass Calc.= 50000 Da*; anti-α-tubulin m/z found = 50592.39 ± 1700.27 Da. anti-α-T-Conj-2 m/z found = 53838.92±1515.245 Da.

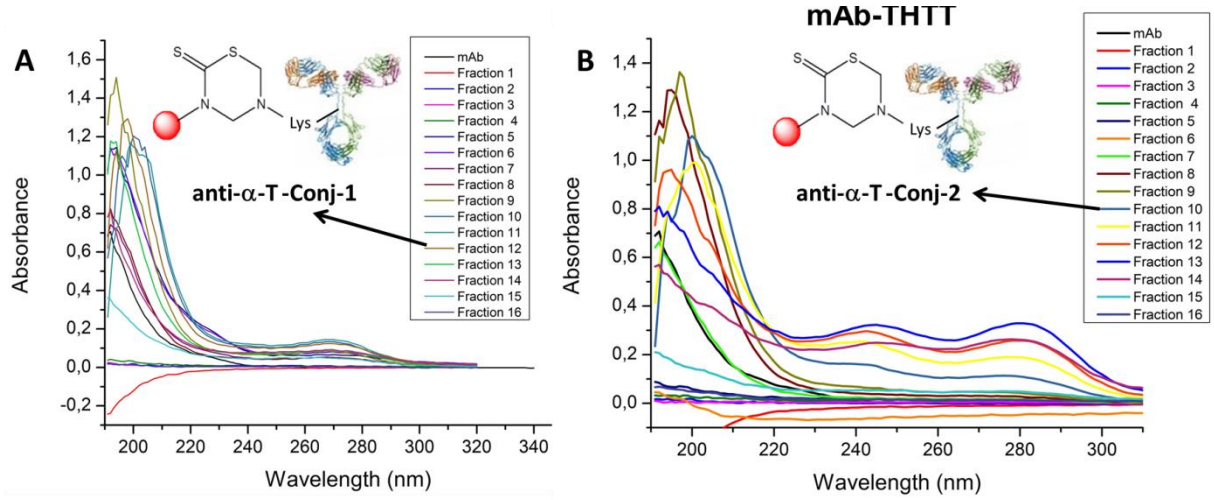


Figure 15. UV nanodrop analysis of fractions corresponding to size exclusion column chromatography of **A**) **anti-α-T -Conj-1** and **B**) **anti-α-T -Conj-2**

CHAPTER 3. RESULTS AND DISCUSSION

To find an efficient strategy for bioconjugation, the potential use of tetrahydro-2*H*-1,3,5-thiadiazine-2-thione (THTT) scaffold was studied. In this sense, the reported procedure to obtain this heterocycle allows us to use the amine-functionalized cargos (in our case Glycine, as proof of concept), but also the amine residues of lysines present in different bio carriers, i.e. protein (BSA) or antibodies (anti-α-tubulin), as substituents of the positions 3 and 5, respectively. The bioconjugation allowed generating novel Cargo-THTT-Biocarrier conjugates (**Figure 16**).

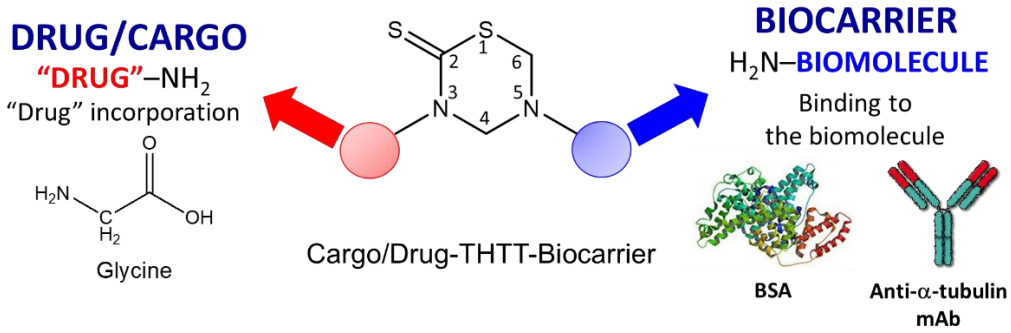
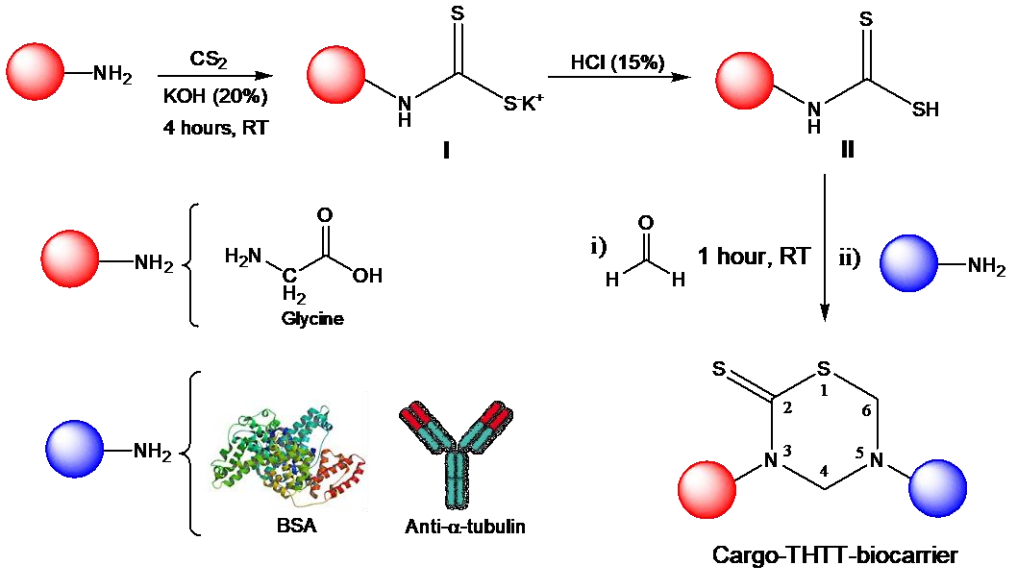


Figure 16. General bioconjugation using THTT scaffold.

The position for each entity was established, taking into account the reaction conditions associated with each step for the corresponding amines during the bioconjugation procedure. In that way, first of all, one of the free amines is left to react with carbon disulfide in the presence of a strong basic solution (KOH 20%) to generate the dithiocarbamate (DTC), while the other amine involved is added at the end of the procedure at neutral pH. In this context, the most robust amine from the cargos or drugs must be introduced in the first step, at N3 position, while the bio carriers must be introduced at the end, under milder conditions (**Scheme 2**).

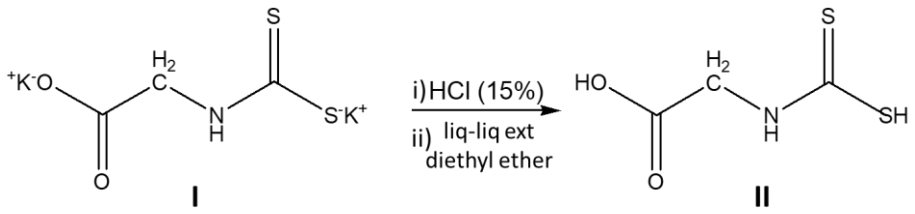
Despite the fact that of the previously described synthetic methodology to obtain THTT derivatives could be carry out without isolated the DTC intermediary, our bioconjugation strategy goes through the previous isolation of the intermediate dithiocarbamic acid (II), by the liberation of the corresponding dithiocarbamic salt (I) through the acidulation of the reaction medium (**Scheme 2**). With II in hand, a fresh solution is prepared and left to react with formaldehyde (37%) by 1 hour, after that, the obtained mixture is dropwise over the selected bio carrier solution, and they are allowed to react during the corresponding time. Finally, the conjugates are purified using PD10 desalting columns. The specificities of each step, as well as the UV and MS characterization of the conjugates, are analyzed below.



Scheme 2. General strategy of THTT-based bioconjugation.

3.1 Synthesis of dithiocarbamic acid, glycine-N-dithiocarboxylic acid (II).

As was mentioned before, firstly the free amine from glycine react with carbon disulfide (CS_2) in KOH (20%) solution, and at room temperature to give place to the dithiocarbamate (DTC) salt (I). The basic media promoted the formation of ionic species in solution, but also decrease the decomposition speed of the DTC potassium salt. To isolate this intermediate, which will be used in the subsequent bioconjugations, the reaction mixture was cooled and then acidulated at pH=1 by addition of hydrochloric acid (HCl, 1 M). Dithiocarbamic acid (**II**) did not precipitate from the mixture, as expected according to bibliography reported for similar compounds.[84] Liquid-liquid extractions using cold diethyl ether, following by the evaporation of the organic phase, allowed obtain II as a light brown amorphous solid with a slight sulfur smell with low yield (~7%) (**Scheme 3**).



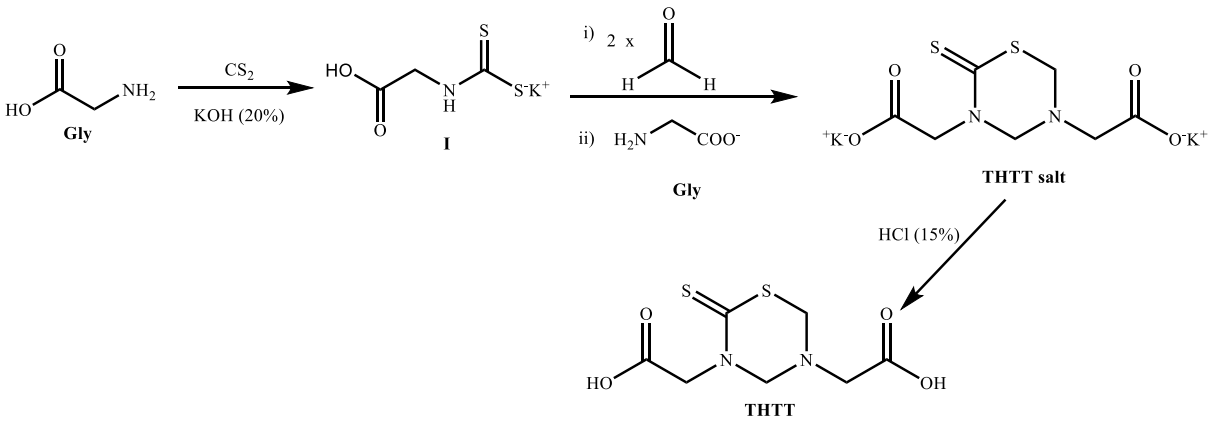
Scheme 3. Synthesis of glycine-*N*-dithiocarboxylic acid (II).

The low yield is due to the loss of part of the product due to the high expected solubility of this compound in an aqueous medium, hindering the extraction process. Glycine-N-

dithiocarboxylic acid (II) was then used as linker precursor in the bioconjugation procedures with selected bio carriers, Bovine Serum Albumin (BSA) and the mAb anti-α-tubulin.

3.2 Synthesis of 3,5-carboxymethyltetrahydro-(2*H*)-1,3,5-thiadiazine-2-thione.

In the synthesis of this kind of heterocycle, having a mono THTT as a pattern in which to carry out preliminary acid degradation studies, the synthesis of 3,5-carboximetiltetrahydro-(2*H*)-1,3,5-tiadiazine-2-tiona was carried out using the procedure previously and extensively reported.[82,84,84,85] Briefly, obtaining the intermediary dithiocarbamic salt I, without previous isolation, it was condensed with the double of moles of formaldehyde during 1 hour, and then equimolar amount of the amino acid (glycine) was added in a buffer suspension (pH=7-8), after two hours of reaction at room temperature, the mixture was cooled and acidulated to pH= 1, expecting the precipitation of the THTT in the form of an amorphous solid to get the corresponding mono-thiadiazine salt (**Scheme 4**).



Scheme 4. Synthesis of 3,5-carboxymethyltetrahydro-(2*H*)-1,3,5-thiadiazine-2-thione (**mono-THTT**).

According to the literature,[85] most products of THTT obtained from this synthetic methodology tend to precipitate like amorphous white solids after the acidulation of the aqueous media. In our case, the mono-THTT did not precipitate from the mixture. Liquid-liquid extractions using cold diethyl ether, following by the evaporation to dryness of the organic phase, allowed obtaining THTT as a light yellow amorphous solid with a very low yield (~2,4 %).

The yield obtained was lower than reported in the literature. In our experimental trial, it was possible to confirm the total conversion of starting reagent (glycine) to mono-THTT as the final product through performing a thin layer chromatography (TLC). Thus, it is a fact that the mono-

THTT was synthesized, but a large amount of the product was lost during extraction. Therefore, it would possible to improve the extraction method and increase the yield either by changing the solvent for a more polar than diethylether or turn performing successive extractions, in other words increasing the number extractions but using a smaller volume of solvent.

3.2.1 UV Spectroscopic

UV spectroscopic was used to carry out qualitative monitoring of the degradation of the analyzed THTT. The main goal of the degradation study is to determine if the THTTs would be used as a cleavable or non-cleavable linker for bioconjugation.

First, the UV spectra of the mono-THTT and the bis-THTT´s were recorded using water type 1 as a solvent, and cover from 180 to 240 nm of wavelength. In general, all samples showed similar UV profiles, is possible to observe two main absorption bands at 200 and 220 nm. The lower energy band (at 200 nm) corresponding to the transition n-π* from the carboxylic chromophore group (COOH, **Figure 17**, highlighted purple), present as substituents in the N1 and N3 positions for the mono-THTT, or as substituents at N5 for both heterocycles present in the bis-THTTs. The other band observed in the 220 nm region corresponds, for all THTT´s compounds, with the n-π * transitions of the thione chromophore group bound to nitrogen and sulfur (C=S, **Figure 17**, highlighted blue). As an example, the UV spectrum of the mono-THTT is shown (**Figure 17**).

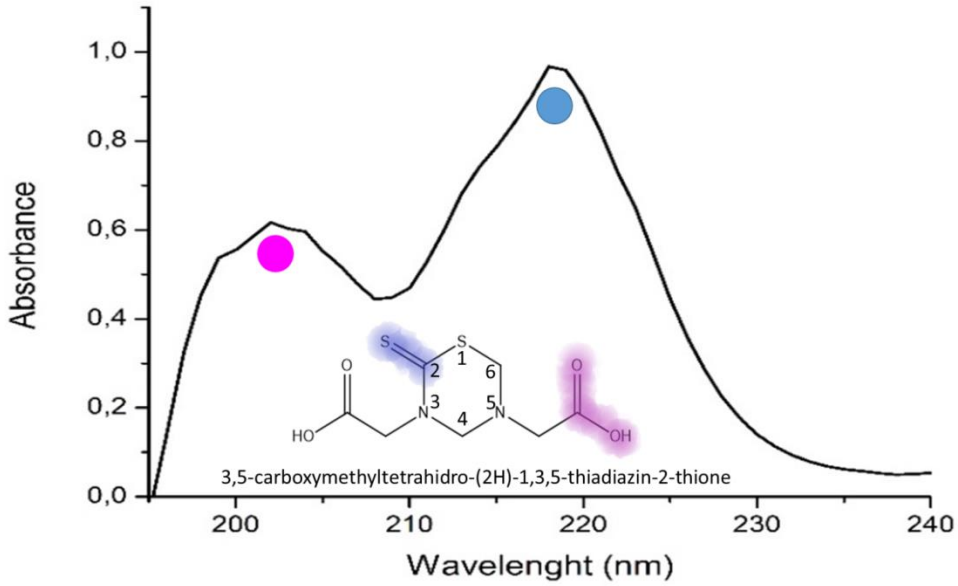


Figure 17. UV spectrum of 3,5-carboxymethyltetrahydro-(2*H*)-1,3,5-thiadiazine-2-thione (mono-THTT).

With all UV profiles in hand, we carried out the degradation study following the changes that occurred in these profiles over the time, mainly the increase or decrease of the intensity of the two main bands mentioned above.

3.3 Degradation study of THTT´s.

The degradation study was carried out with the mono-THTT and five bis-THTT previously synthesized by Suárez research group at the Laboratory of Organic Synthesis of the University of Havana (**Figure 18**). Inside of the bis-THTTs family, we have two main groups, which differ in the alkyl chain that joins both rings, for three of them (JH-A, JH-C, and JH-E) the alkyl chain has six carbon atoms, and for the other two (JH-B, and JH-D), the heterocycles are linked by an alkyl chain of four carbon atoms (**Figure 18**).

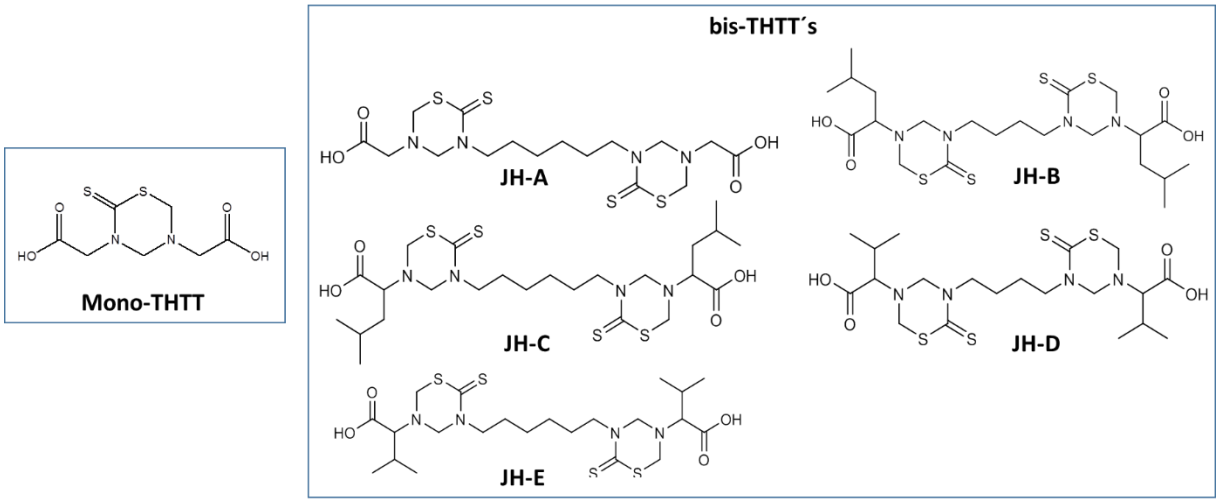


Figure 18. Structures of mono-THTT and bis-THTT´s (JH-A-E) using in the degradation study.

To assess the degradation behavior of the THTT´s in a slightly acidic medium, the compounds were treated with a solution of HCl at pH=6. The mentioned pH was selected, taking into account the hypothesis related to the presence of a slightly acid media in the micro-environment of cancer cells. If some evidence of degradation is detected, the THTT´s would be used as a cleavable linker to bioconjugation, on the contrary, if the THTT are stable under the mentioned conditions, means that would be used as a non-cleavable linker. For the last case, the carrier should ensure the correct internalization of the conjugate inside the cell.

The UV spectra of all acids solutions of our THTT´s were recorded at 10, 20, and 30 minutes, but also at 1, 24, and 48 hours. The profiles comparison (**Figure 19 A-B, See Annex 2)** allowed to define a general behavior, in which the band at 200 nm decrease its intensity. These results could indicate the degradation of THTT´s involving the hydrolysis of the ring in the degradation mechanism, although they are not definitive and further studies need to be carry out.

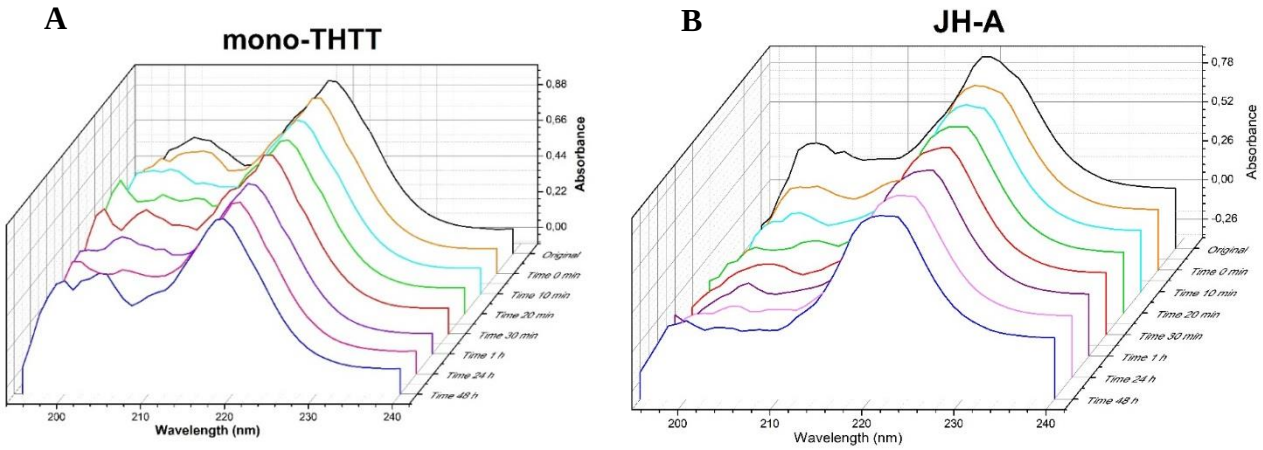


Figure 19. Degradation study using UV nanodrop profiles for A) JH-A and B) mono-THTT

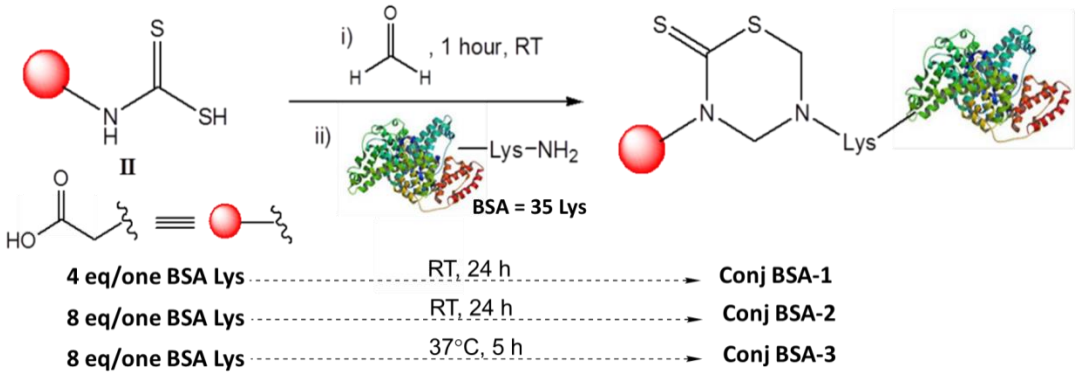
Degradation study results using UV spectroscopy, allowed to partially concluding that THTT hydrolyzed in slightly acidic medium, and could be used as a cleavable linker in bioconjugation procedures. As was mention before, further studies need to be carry out to deepen in the degradation mechanism.

3.4 Bioconjugation

To finally prove if the THTT moiety would be feasible in bioconjugation procedures 'through lysine amino group of proteins and antibodies, the DTC **II** previously obtained was activated through its reaction with formaldehyde during 1 hour at room temperature. With activated **II** in hand, bioconjugation of a protein and a monoclonal antibody was tackled (**Scheme 5** and **6**).

3.4.1 BSA conjugates

The bioconjugation to bovine serum albumin (BSA) was performed by adding the activated dithiocarbamate **II** dissolved in PBS to a solution of BSA (11 mg/mL) in water (Type-I) using three different bioconjugation conditions (**Scheme 5**).



Scheme 5. BSA Lys-mediated conjugation with **II**, previously activated with HCHO.

Three bioconjugation conditions allowed to obtain the expected conjugates, which were purified using size exclusion column (PD10) and water as eluent, to eliminate the excess reagents (HCHO, activated DTC), but also to separate the conjugates from free BSA. UV nanodrop analysis was used to identify and select the correct fractions after purification procedures. As an example, the UV nanodrop profiles of all fractions of the conjugate mixture are shown (**Figure 20**).

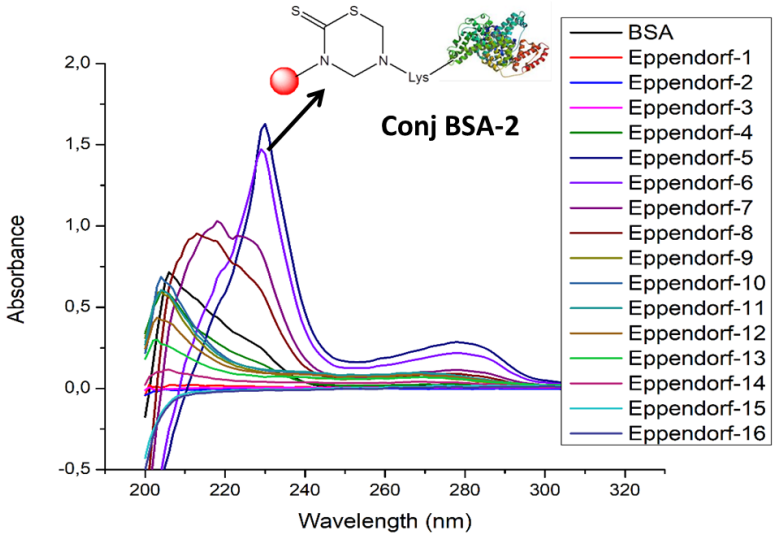


Figure 20. UV nanodrop analysis of fractions corresponding to size exclusion column chromatography of **Conj BSA-2**.

UV nanodrop analysis allowed us a correct separation of the fractions corresponding to the bioconjugates from an excess of reagents or non-conjugated protein. However, it was not possible differentiates between the conjugate profile from BSA pure profile (**Figure 21**). In this regards, the UPLC-MS analysis let us corroborate the bioconjugation unequivocally.

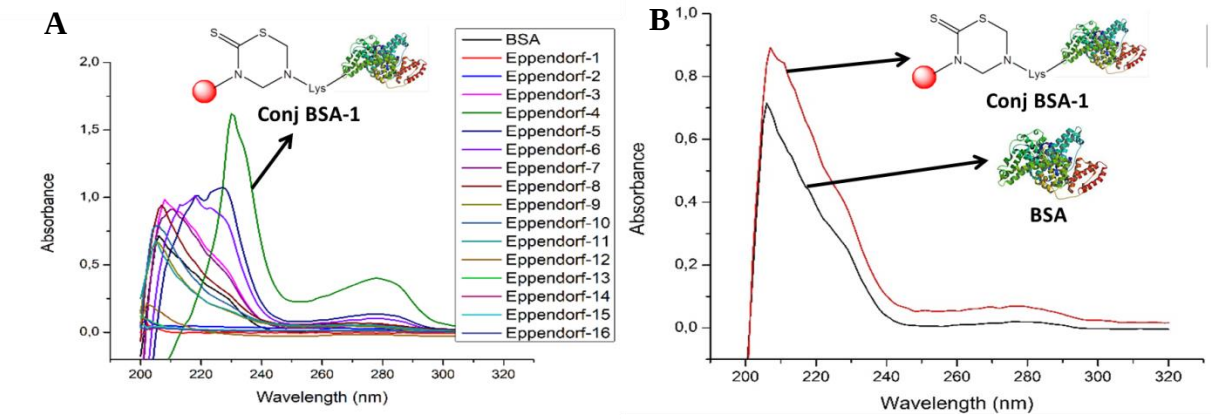


Figure 21. A) UV nanodrop analysis of fractions corresponding to size exclusion column chromatography of **Conj BSA-1**,and **B)** UV nanodrop analysis of diluted of fraction 4 corresponding to **Conj BSA-1** compared with **BSA**.

All purified conjugates were characterized using UPLC mass spectrometry, and the three BSA conjugates showed similar results. First of all, a comparison of UPLC profiles of BSA and our conjugates showed a difference in its retention time, while the BSA pure has a retention time of 3,36 minutes, the peak corresponding to BSA conjugates appears to 3,46 minutes (**Figure 22 A,B**).

To carry out the mass spectrometry analysis, the mass spectra corresponding to BSA pure and its conjugates obtained at 3,36 and 3,46 minutes, respectively, were analyzed and compared (**Figure 23** and **Figure 24**).

The BSA spectrum at 3,36 minutes showing multiple charge states of the protein from 43 to 60 charges (**Figure 23**), which exemplify the concept of a charge envelope to show the ionization of a larger molecule in mass spectroscopy. All these peaks correspond to the same molecule, just with different charges. A deconvoluted neutral molecular weight analysis from these peaks was carried out manually and allowed to found a MW of 66436,7522 ± 37,528 Da, very close to the calculated and reported value for BSA of 66430,3 Da[83].

Related to the BSA conjugates, the mass spectrum at 3,46 minutes also showing multiple charge states for a conjugate from 43 to 56, and the deconvolution of these peaks and the mass analysis define that each BSA acquires 16-17 payload (**Figure 24**).

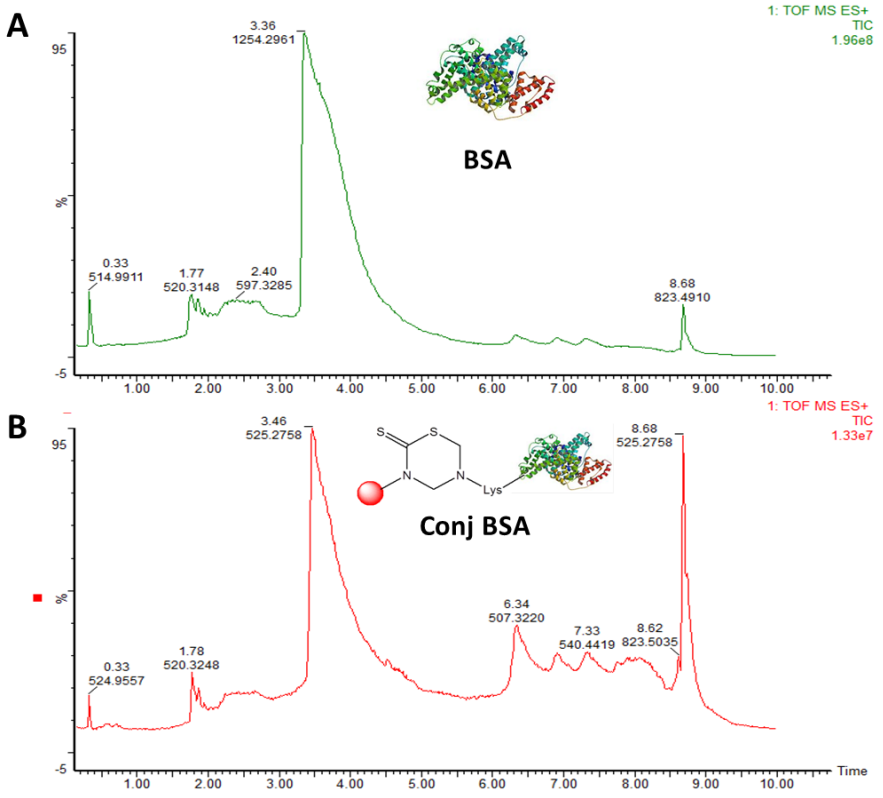


Figure 22. UPLC analysis of **A) BSA** and **B)** BSA conjugate.

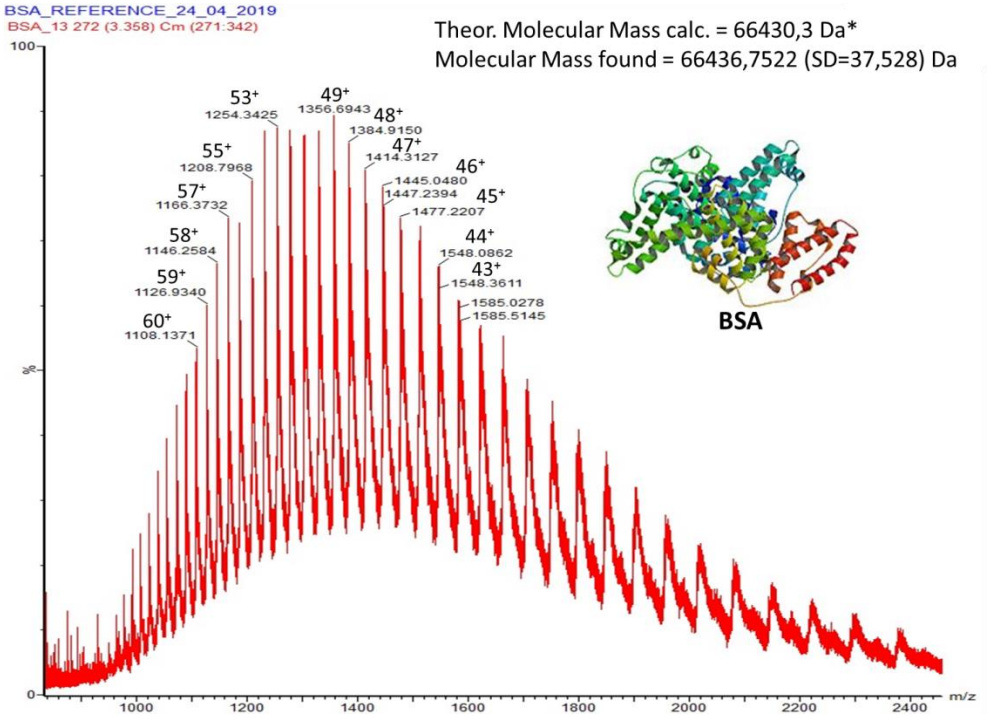


Figure 23. Mass spectrum at 3,36 minutes showing multiple charge states of BSA. *The theor. molecular weight of bovine serum albumin (BSA) was calculated based on its amino acid sequence.

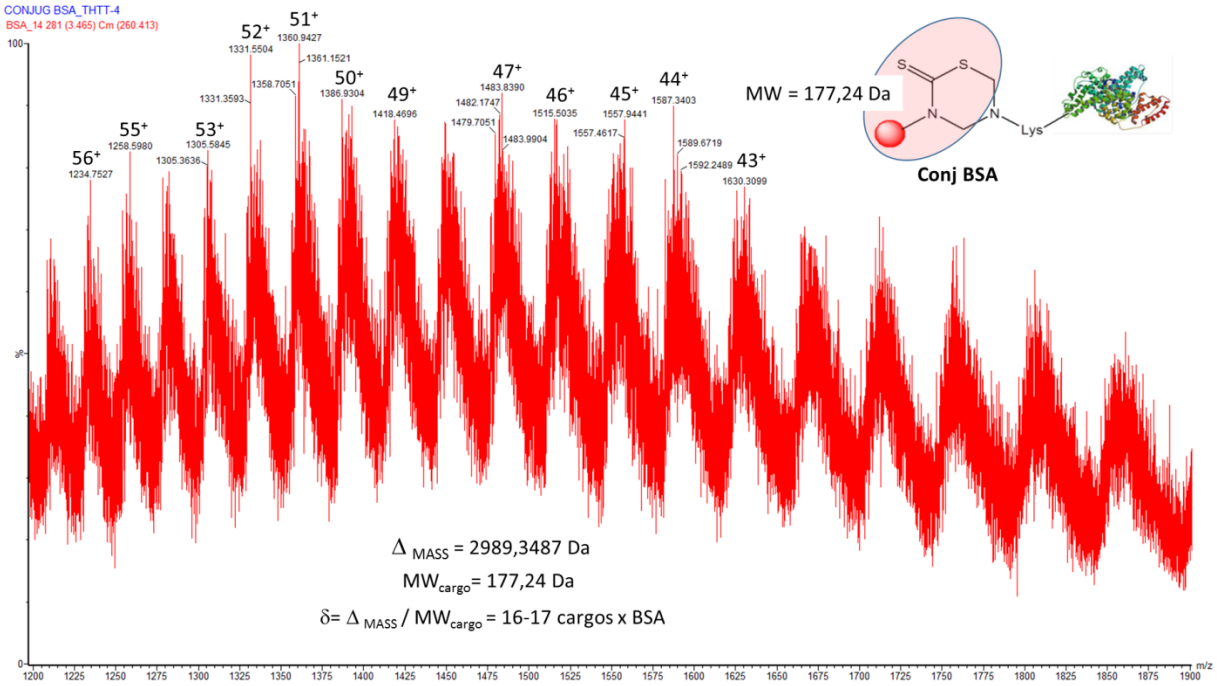
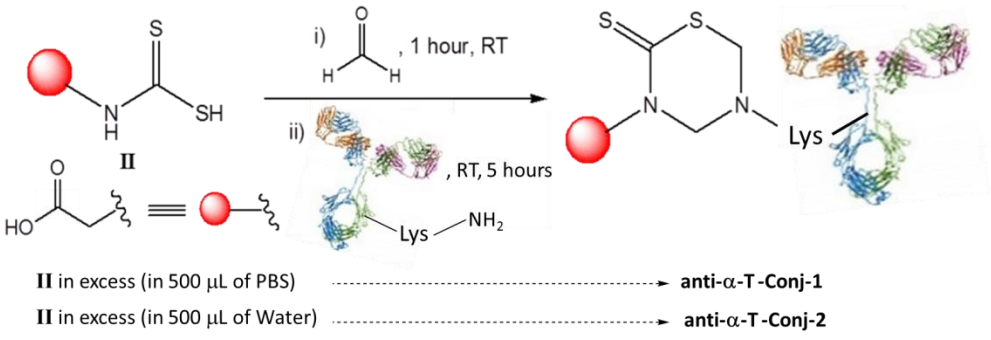


Figure 24. Mass spectrum at 3,46 minutes showing multiple charge states of BSA conjugate.

The UPLC-MS analysis allowed concluding that bioconjugation was efficient enough because the mass analysis determined that 16-17 molecules of our cargo (THTT moiety) were attached to the BSA surface using either 4 or 8 eq. of activated **II** by each BSA Lys. The UPLC-MS technique unequivocally confirms that conjugation occurs.

3.4.2 Anti-alpha tubulin (anti-α-T)-THTT conjugates

After observed that lysine bioconjugation was possible into a protein sequence (BSA), we ventured to the bioconjugation with the same general methodology into a more complex system. For this purpose, bioconjugation to a monoclonal antibody (anti-α tubulin) was performed (**Scheme 6**).



Scheme 6. Anti-α-Tubulin Lys-mediated conjugation with **II**, previously activated with HCHO.

The two conjugates **anti-α-T Conj-1** and **anti-α-T Conj-2** were generated under similar reaction conditions (excess of **II**, previous activation of **II** by its reaction with formaldehyde 1 hour at RT, and finally the bioconjugation with 10 µL of anti-α-tubulin (1 mg/mL)), except by the solvent use to dissolve the DTC (**II**) before the addition of formaldehyde, for which PBS and water were used for **anti-α-T Conj-1** and **anti-α-T Conj-2**, respectively. Regardless of the solvent used to dissolve **II**, the results for both synthesized conjugates were very similar.

UV nanodrop analysis of synthesized conjugates, after its purification by PD-10 columns (size exclusion chromatography), reveals some changes into the conjugates spectra, in comparison to the naked antibody, allowing us the correct separation of fractions corresponding to anti-α-T conjugates(**Figure 25**).

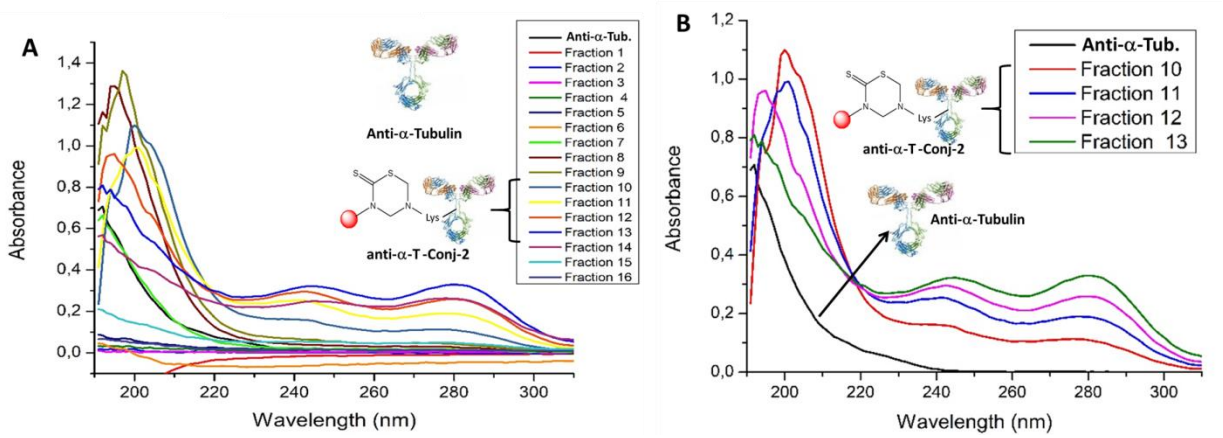


Figure 25. A) UV nanodrop analysis of fractions corresponding to size exclusion column chromatography of **anti-α-T-Conj-2**, and **B)** UV nanodrop analysis of fractions 10-13 corresponding to **anti-α-T-Conj-2**, in both cases compared with **anti-α tubulin**.

To corroborate the **anti-α tubulin** conjugation through its exposed lysine's, UPLC mass spectrometry was carried out (**Figure 26** and **Figure 27**). From the UPLC chromatogram profiles for both, naked antibody and the conjugate **anti-α-T-Conj-2**, it was not possible to obtain much useful information. The chromatographic profiles showed some coincident peaks, and the mass analysis for each peak allowed to concluded that those peak at 6,33 and 6,27 minutes corresponding to the naked antibody and to the conjugate, respectively (**Figure 26**). On the other hand, it was not possible to identify the other entities that could be present in the mixtures, which are not a protein nature.

As the mass analysis depicted, mostly all the conjugates maintain its integrity after the conjugation step. The analysis of the molecules attached to the antibodies was determined by

comparing the naked antibody mass spectrum with the respective conjugate mass spectrum (**Figure 27**).

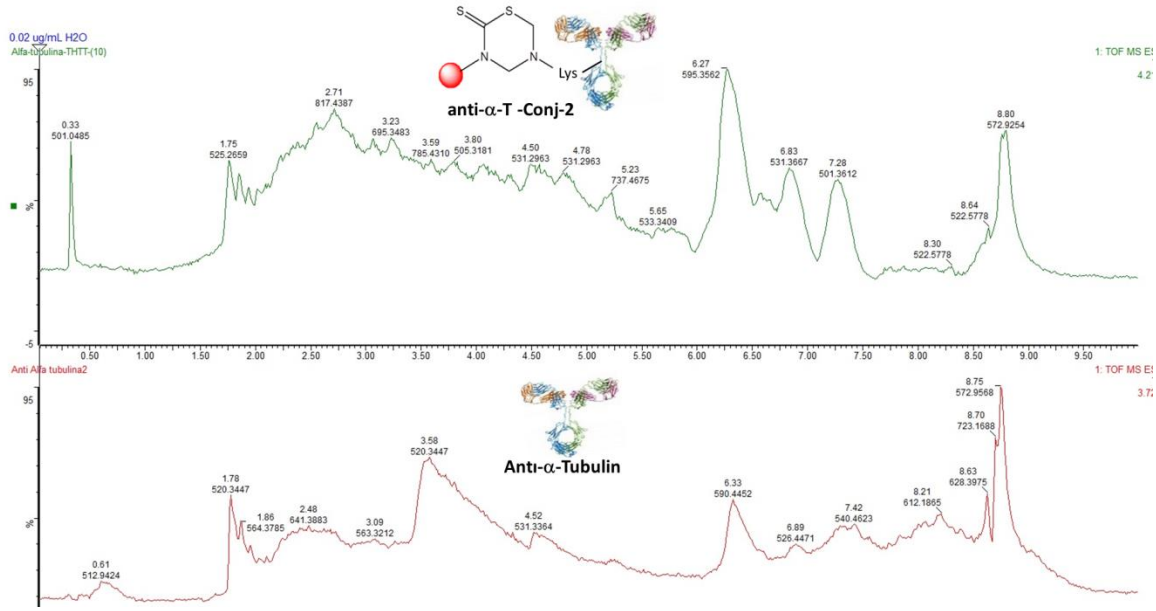


Figure 26. UPLC analysis of **A) anti-α-T-Conj-2** and **B) anti-α-tubulin**.

The **anti-α-T-Conj-2** and the **anti-α-Tubulin** (naked antibody) mass spectra showed multiple charge states from both entities from 41 to 49 charges (**Figure 27A, B**), with the expected charge envelope shape. A deconvoluted neutral molecular weight analysis from these peaks was carried out manually, and allowed to found an MW of 50592,39 ± 1700,27 Da for the **anti-α-Tubulin** (naked antibody), very close to its reported value of 50000 Da; and also allowed to define that each mAb acquires 18 payloads.

Additionally, more than one envelope charge pattern was observed in both mass spectra, which would be indicating a heterogeneous population of the **anti-α-tubulin**, and as a consequence, the same behavior for the corresponding conjugates (**Figure 27A, B**).

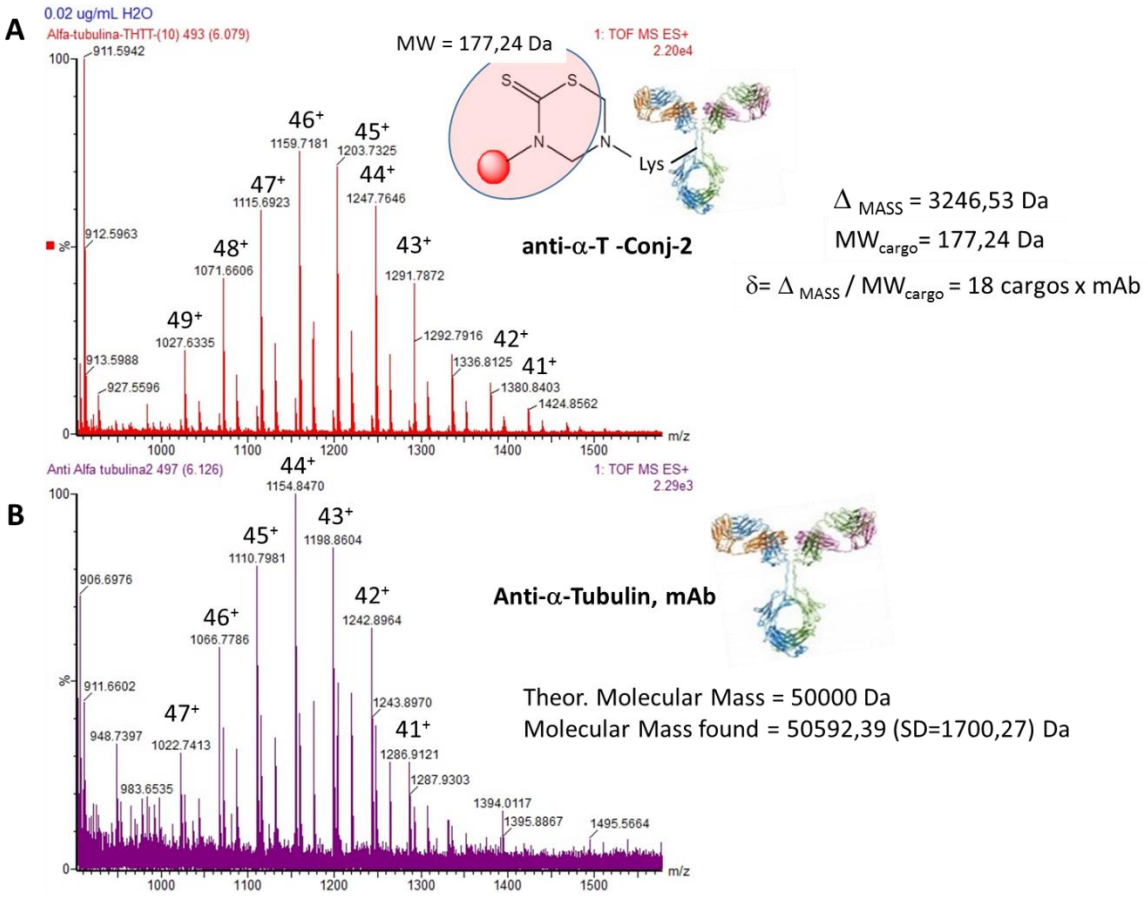


Figure 27. Mass spectra **A. anti-α-T-Conj-2** and **B. anti-α-Tubulin** (naked antibody), both at 6,1 minutes, showing multiple charge states.

The UPLC-MS analysis allowed us to conclude that bioconjugation to **anti-α tubulin** occurs. The mass analysis determined a Drug-to-Antibody ratio (DAR) of 18 molecules of our cargo (THTT moiety), which were attached to the mAb surface using an excess of activated **II** by each mAb, and using either PBS or water as a solvent. Although the UPLC-MS technique unequivocally confirms that conjugation occurs, for most complex systems like mAb´s, a preliminary analysis of all samples using diode array would be desirable. Generally, the accurate quantification of the molecules conjugated to the antibodies is limited due to the poor sensitivity of its compounds but also related to the heterogeneous mixture of mAb. Thus, completely antibody reduction or enzymatic digestion should also improve the analysis sensitivity.

Our results showed that THTT moiety could be used as a cleavable linker for bioconjugation. Firstly, a preliminary UV degradation studies under pH=6 give us clues related to the ring

degradation under slightly acidolysis conditions, replicating the cancer cells environment, which is responsible for the drug release close to the tumor. Besides, reported bioconjugation procedures were viable for proteins (BSA), and also for antibodies (anti-α tubulin); nanodrop UV analysis was a useful tool to discriminate and separate synthesized conjugates from the mixture. UPLC-MS analyses corroborated the bioconjugation while allowing us to determine that with the reported methodology, the BSA and the anti-a tubulin are loaded with 16-17 and 18 cargos, respectively. These results, although preliminary, encourage us to deepen this study, to optimize the conjugation procedure to reduce the load, to analyze the integrity of the biomolecule after the conjugation, or to check the activity, but also the toxicity of the conjugates in comparison with the drugs only.

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

- New linker for bioconjugation based on tetrahydrothiadiazin-2-thione (THTT) scaffold was designed and develop.
- The acidolysis study using UV nanodrop analysis of the prepared mono-THTT, and the five bis-THTT given by the Laboratory of Organic Synthesis of the University of Havana, allowed us to suggest that the novel THTT linker could be cleavable because seems its decompose over the time in slightly acidic media.
- The bioconjugation was carried out through the dithiocarbamic acid **II**, previously obtained from amine-functionalized cargo (Glycine), and the lysine amines exposed in proteins (i.e., **BSA**) or antibodies (i.e., **anti-α tubulin**).
- UPLC-MS analyses corroborated the bioconjugation, while allowing us to determine that with the reported methodology, the **BSA** and the **anti-α tubulin** are loaded with 16-17 and 18 cargos, respectively.

Recommendations

- Deepen the acidolysis study of THTT using HPLC-MS to detect and characterize the degradations products.
- Test the bioconjugation procedure using more bulky cargos or drugs, or modified the methodology to reduce the number of cargos / bio carriers.
- Include studies of the biomolecule integrity after the conjugation, checking the activity, but also the toxicity of the conjugates in comparison with the drugs only

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ANNEXES

Annex I. Abbreviations and Acronyms

DDS	Drug delivery system
ADC	Antibody drug conjugate
FDA	Food and drug administration
THTT	Tetrahydro-thiadiazin-2-thione
UV	Ultraviolet
UPLC	Ultra performance liquid chromatography
BSA	Bovine serum albumin
MeCN	Acetonitrile
DTC	Dithiocarbamate
DMSO	Dimethyl sulfoxide
RT	Room temperature
m/z	mass charge relation
mAb	Monoclonal antibody
DAR	Drug-to-Antibody ratio

Annex II. Profile from degradation study of THTT´s

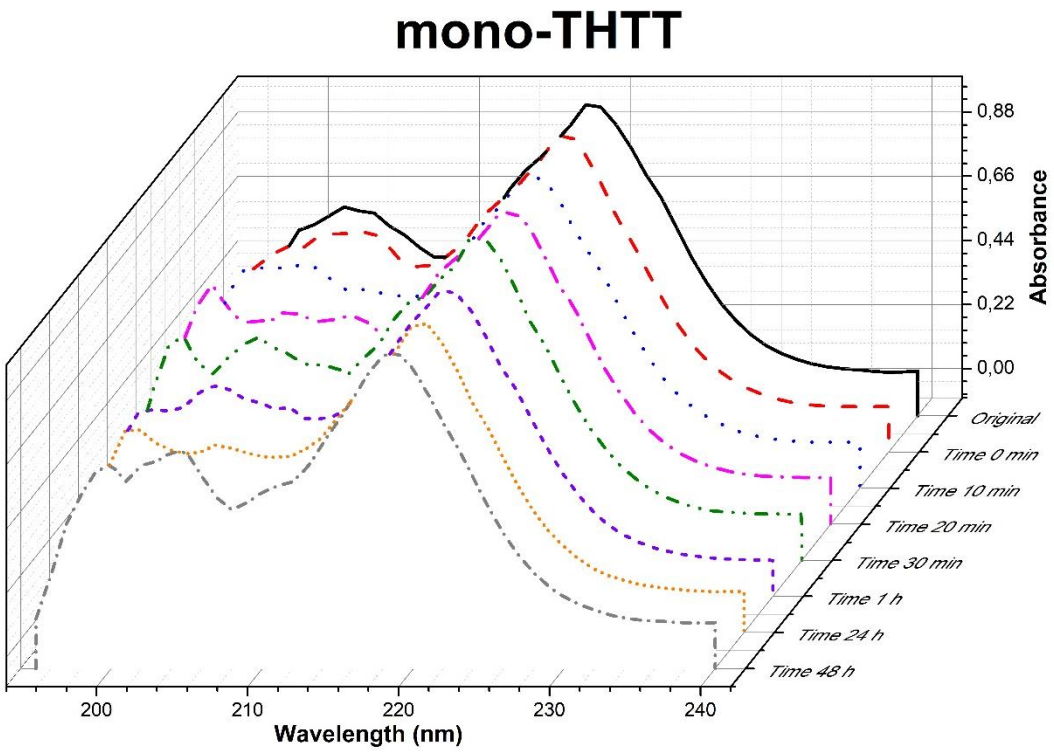

Figure 28. Degradation study using UV profiles for mono-THTT

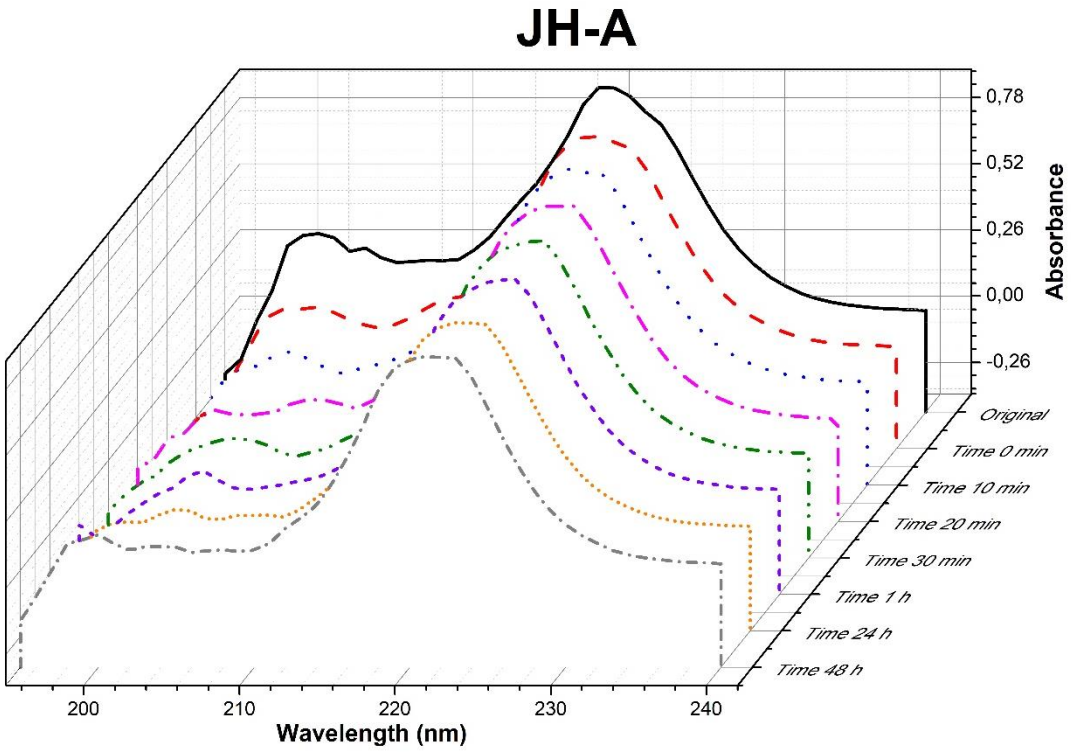

Figure 29. Degradation study using UV profiles for JH-A

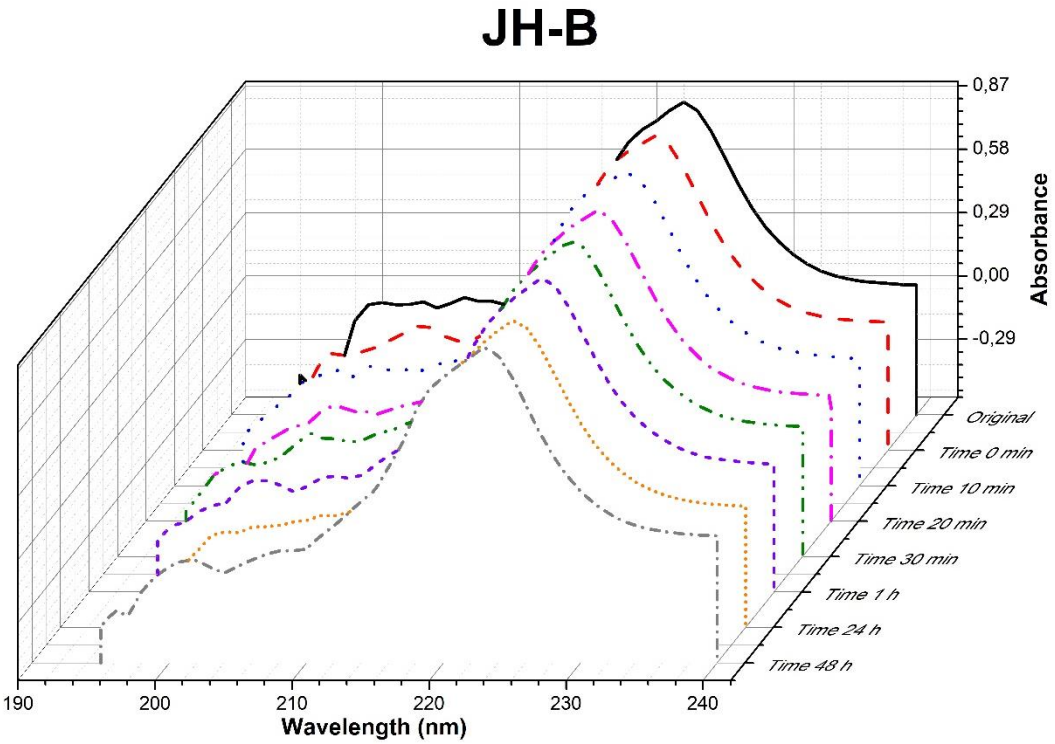

Figure 30. Degradation study using UV profiles for JH-B

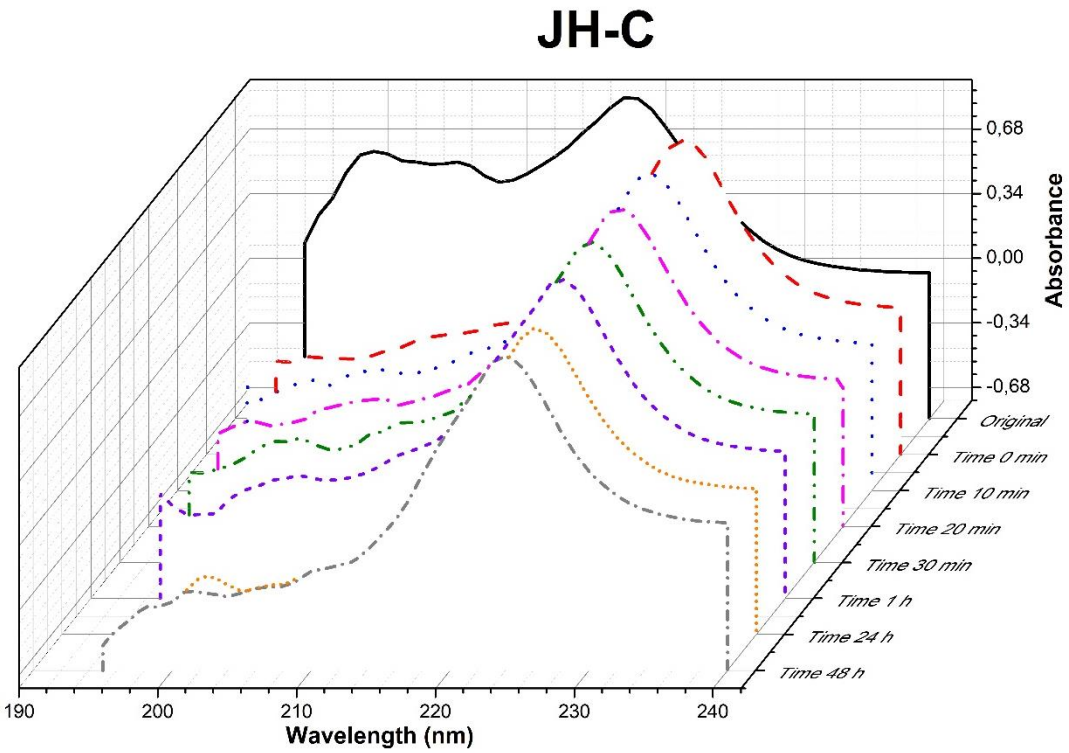

Figure 31. Degradation study using UV profiles for JH-C

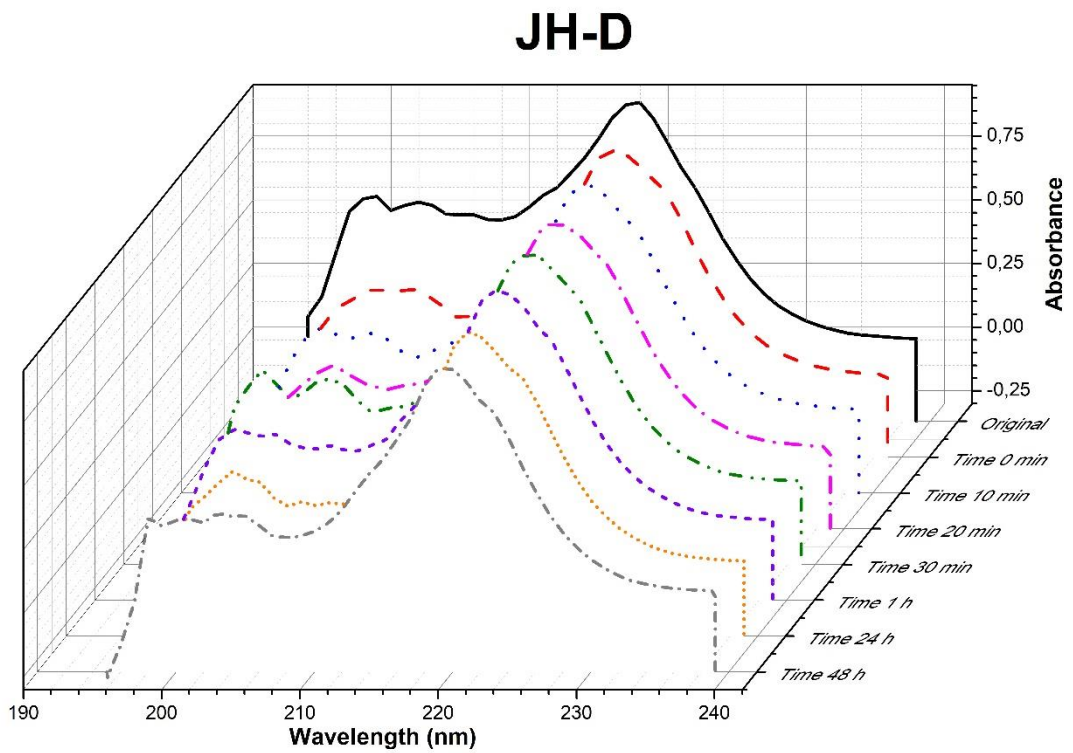

Figure 32. Degradation study using UV profiles for JH-D

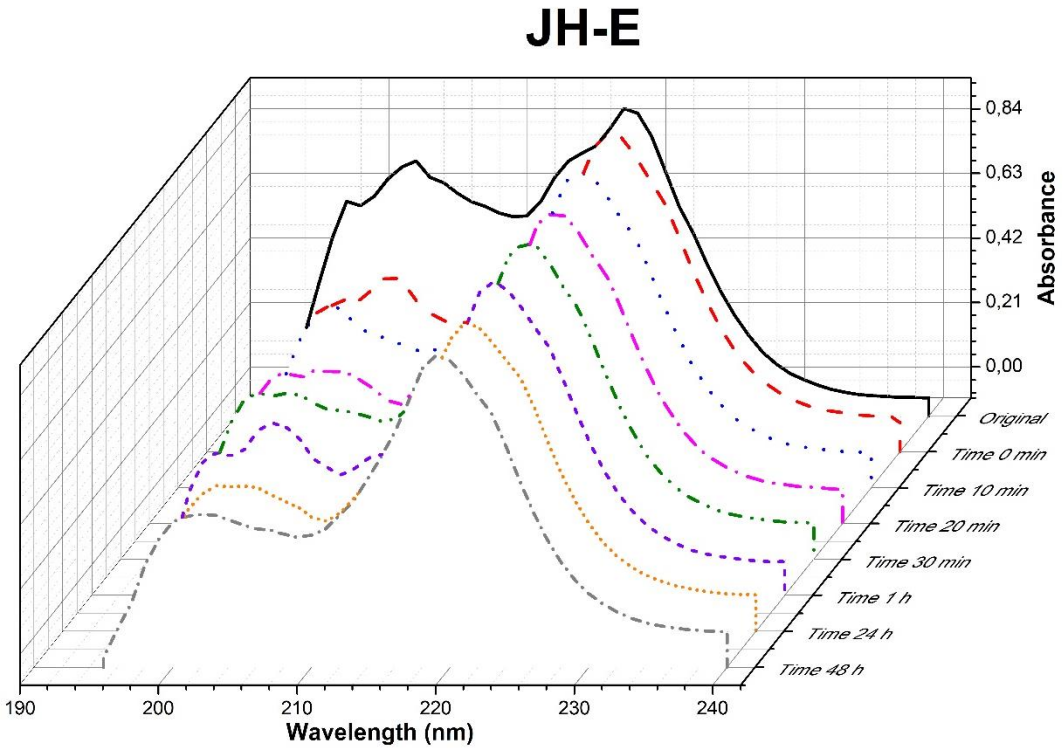

Figure 33. Degradation study using UV profiles for A) JH-E