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A Bioinformatics Method for the Production of Antibody-Drug Conjugates Through Site-Specific Cysteine Conjugation

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Additional information is available at the end of the chapter

Abstract

Antibody-drug conjugates (ADCs) have emerged as a promising class of targeted anticancer therapy, and it is distinguished from traditional chemotherapeutic approaches by its potential to kill cancer cells with limited side effects. Site-specific conjugation is one of the current challenges in ADC development because it allows for controlled conjugation and production of homogeneous ADCs. This chapter describes a computational method for the generation of antibody-drug conjugates as PDB files through site-specific cysteine conjugation, given the PDB files of a drug, a linker, and an antibody. The drug and linker are reconfigured using the rotation and translation functions of an affine transformation, which is brought in appropriate positions for the bonds to occur between the three molecules. The hydrogen and disulfide bonds are employed to connect the linker and drug as well as the linker with the antibody, respectively. Examples of conjugates produced with the presented method have been demonstrated.

Keywords: bioinformatics, cancer, targeted therapy, antibody-drug conjugates, cysteines

1. Introduction

Antibodies are large proteins produced by the immune system against an invader substance called antigen. As proteins, they consist of one or more chains of amino acid residues, two types of which are cysteines and lysines. Antibodies can also be manufactured and used as stand-

alone therapeutic agents for a number of diseases including cancer. The largest part of the amino acid sequence of an antibody is conserved in all antibodies, with the exception of a small part that is called a hypervariable (HV) region. Due to the specialized and unique amino acid sequence of the HV region, an antibody has the ability to recognize and bind a specific antigen with higher affinity than different antigens—a property called specificity. The specificity and long half-lives of antibodies are valuable features to their therapeutic effect. However, the performance of manufactured antibodies as standalone therapeutic agents is impeded by their limited potency. On the contrary, traditional chemotherapeutic drugs are more potent but have short half-lives and no specificity, targeting all cells indiscriminately. A class of targeted anticancer therapy (TAT), that utilizes the best qualities of antibodies and cytotoxins by combining them in one molecule, is antibody-drug conjugates (ADCs). ADCs are therapeutic agents formed by three elements: an antibody whose target is a cancer-specific molecule, a cytotoxic agent, also called the drug, and a linker molecule that connects them. An ADC ideally delivers the conjugated cytotoxic molecule directly into cancer cells, limiting damage to healthy tissues [1–8].

The typical route of an ADC molecule upon its insertion to the bloodstream can be described briefly with the following steps. At first, the ADC circulates in the plasma remaining stable, while the drug remains inactive. When the target antigen is encountered, the antibody binds to it. Typically, the ADC-antigen complex undergoes antigen-mediated or antigen-independent internalization by the cancer cell. Subsequently, the ADC is degraded in its parts through a process that depends on the type of linker used. ADCs containing a non-cleavable linker usually undergo lysosomal digestion. The antibody is degraded to its amino acids and the final active drug complex is the drug with the linker and a single amino acid. On the other hand, the ADCs, containing a cleavable linker, are usually degraded through hydrolysis or enzymatic cleavage. At that stage, the drug is activated and kills the cell, in a way that depends on the type of the cytotoxic agent used. For example, auristatins and maytansinoids disrupt the microtubule assembly of the cell, while calicheamicins and duocarmycins target its DNA structure. An alternative ADC strategy is targeting the endothelial cells of the tumor vessels in order to deprive the cancer cells from blood [6, 9–12].

Conjugation usually occurs at solvent-accessible reactive amino acids of the preserved regions of the antibody, leaving the hypervariable regions of the antibody available for antigen binding. The reactive thiol groups of cysteine residues, being made available for conjugation after the reduction of the inter-chain disulfide bonds, as well as the amino groups of lysine residues, are typical conjugation sites. However, these methods by default produce ADCs with variable drug to antibody ratio (DAR) and conjugation sites, and therefore unstable pharmacokinetic properties. For example, in cysteine conjugation, ADCs with a DAR of eight have a significantly shorter circulation time compared to the unconjugated antibodies. On the other hand, ADCs with a DAR of four have a longer circulation time than ADCs with the double DAR, but the same therapeutic effect as them. More homogeneous ADCs can be produced by site-specific conjugation methods, one of which is cysteine engineering. THIOMABs are engineered antibodies in which cysteines have been introduced to the amino acid sequence by substituting original residues. The ideal sites for residue substitution can be identified with phage display techniques. Eight residues on the light chain (LC-V205C, LC-S168C, LC-A153C,

LC-S127C, LC-S121C, LC-S114C, LC-V110C, LC-V15C) and five residues on the heavy chain (HC-T116C, HC-S115C, HC-A114C, HC-S113C, HC-S112C) have been investigated as potential cysteine insertion sites (Kabat numbering). The above amino acids were substituted with cysteines, generating THIOMABs, which were subsequently conjugated with biotin-malei‐mide. Considering the conjugation of two molecules per antibody as 100% conjugation efficiency, most THIOMABs demonstrated more than 90% conjugation efficiency, proving to be suitable for site-specific conjugation of thiol reactive probes. THIOMAB-drug conjugates (TDCs) are the product of the conjugation of a drug to a THIOMAB [11, 13–28].

The process of drug discovery and development is rather strenuous, taking up to 15 years to produce a new drug from the early *in vitro* discovery stages to the time it is available as a treatment option. However, drug discovery can be facilitated by bioinformatic techniques. The three-dimensional structure of a molecule can be described computationally in various formats, a prevalent one being the Protein Data Bank (PDB) format [29–31]. Computational drug design and molecular mechanics methods provide a means to model molecules and assess their features. Even though there is a variety of general computer-aided drug design tools, the more specific field of ADC computational design is less evolved. JSDraw–Antibody-Drug Conjugates is an editor tool that can be used for ADCs, although it is drawing-oriented and uses 2D coordinates. In addition, a mathematical model was recently developed to describe mechanistically the pharmacokinetic behavior and preclinical efficacy of THIOMAB-drug conjugates [32–41].

An attempt to contribute to the study of ADC computational modeling was made in [42] describing the computational construction of ADCs through lysine conjugation. The three input PDB files of the antibody, the linker, and the drug were processed and merged to a PDB file that represented an ADC molecule. The linker and drug molecules were reconfigured and aligned with the selected lysine amino acid, while hydrogen bonds linked the three molecules [30, 31].
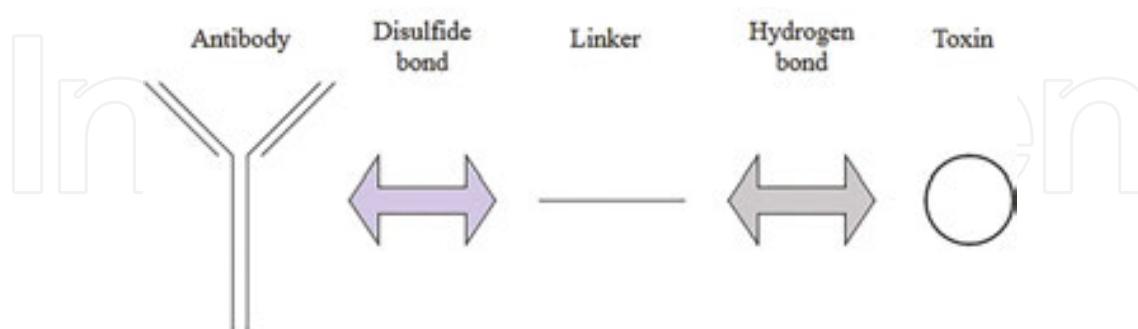

Figure 1. Scheme of the bonds formed between the drug, the linker, and the antibody molecules, according to the method described in this paper.

The present chapter aims to extend the above-mentioned technique to a more site-specific conjugation method, connecting the linker-drug complex to engineered cysteines instead of lysines. The molecule $C_{12}S$ has been used as a non-cleavable linker. The linker and drug form

a hydrogen bond (H bond), while the linker-drug complex and the engineered cysteine form a disulfide bond (SS bond) (**Figure 1**). The configurational change of the linker and drug is accomplished using the rotation and translation functions of an affine transformation. Molecular modeling software was used for functions such as the visualization of molecules, the addition of hydrogen atoms to the antibody PDB files, the insertion of cysteine amino acids to the antibody sequence, as well as the generation of the linker PDB file.

2. Methods

The main aspects of the cysteine conjugation method have been analyzed here. Firstly, the steps of the method are presented in detail. The Affine Transformation subsection contains the definition of the affine transformation, as well as details concerning its application to this project. The Rotation and Translation subsections focus on the application of the rotation and translation functions to the reconfiguration of the molecules. The Axes and Distances subsections contain the specifications made in relevance to the axes of the molecules involved and the distances between them. Finally, the implementation of the method is described.

2.1. Method steps

First, the antibody, the cytotoxic agent, and the linker that will form the conjugate are determined (step S1). The antibody PDB files we have used were obtained from the RCSB database, while the drug PDB files from the NCI database. The linker PDB file was created with the molecular modeling software UCSF Chimera [36]. Given an antibody PDB file, it is possible to produce the PDB file of an engineered antibody (THIOMAB) by replacing selected antibody residues with cysteines using molecular modeling software (step S2). The antibody was selected to have solvent-accessible residues not in the hypervariable region of the antibody, that are suitable to be replaced with cysteines and to be conjugated. The drug was selected so as to have at least one hydrogen (H) atom bonded covalently to an electronegative (EN) atom, in order to be able to form a hydrogen bond with the linker (**Figure 1**). The linker molecule was designed to be able to form a disulfide bond with a cysteine. For that purpose, a sulfur (S) atom was included. Also, the linker was designed to be long enough to allow for the drug to be linked to the cysteine without colliding with the nearby residues. For that purpose, 12 carbon (C) atoms were incorporated following a linear, instead of circular, layout. Finally, the linker should theoretically be able to release the drug from the antibody inside the cancer cell. For the molecule $C_{12}S$ we used, there are three possible ways for that to happen. Breakage of the hydrogen bond between the drug and linker could occur. Also, the disulfide bond between the linker and drug could break due to disulfide exchange, a phenomenon associated with cleavable linkers. In addition, lysosomal digestion of the antibody could occur, in case the disulfide bond between the cysteine and the linker does not break, a phenomenon associated with non-cleavable linkers. In that case, the final active drug complex would contain a single residue (the cysteine connected to the linker), the linker, as well as the drug.

Even though the PDB format has more than 40 types of PDB records, the three types of records that are crucial for the representation of three-dimensional molecular structure include the PDB ATOM records, which contain information about the atoms of standard amino acids and nucleotides. The PDB HETATM records contain information about additional "non-standard", non-polymer chemical structures of the molecule, and the PDB CONECT records specify the connectivity between the atoms of the molecule [29].
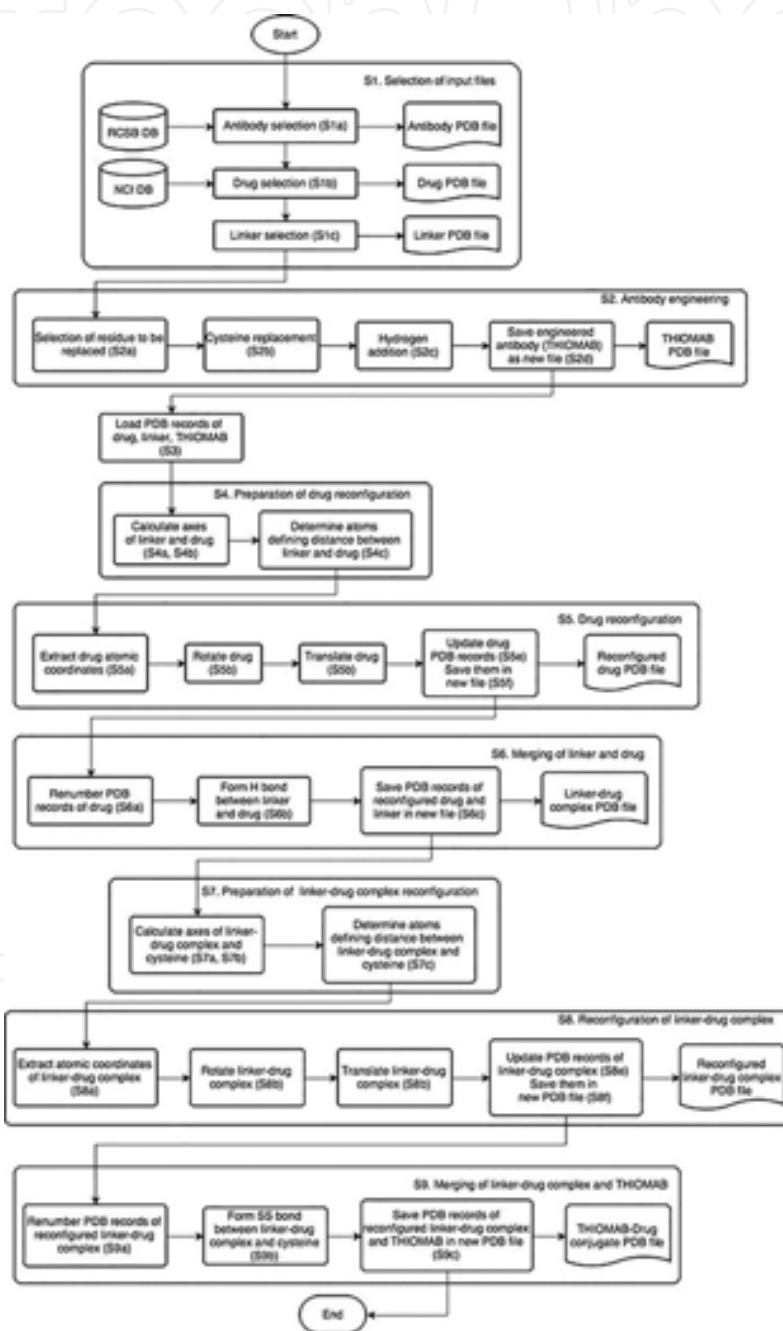

Figure 2. Flowchart of the cysteine conjugation method described in this paper. S1. Determine the three molecules (antibody, drug, linker) that will build the antibody-drug conjugate. S1a. Select the antibody PDB file from the RCSB database (e.g., ab.pdb) according to the criteria described in Section 2.1. S1b. Select the anticancer drug PDB file from the

NCI database (e.g., drug.pdb). S1c. Determine the linker PDB file (e.g., linker.pdb). S2. Engineer the antibody (ab.pdb) to create a THIOMAB (e.g., thiomab.pdb), using molecular modeling software. S2a. Select one (or more) native residue(s) of the antibody (ab.pdb) to be replaced by a cysteine residue. The residue should be solvent-accessible, on the surface of the antibody, in order to be accessible to the linker-drug complex, as well as suitable for cysteine replacement and site-specific conjugation. S2b. Replace the selected residue(s) with a cysteine residue. S2c. Add hydrogen atoms to the antibody, since the antibody PDB files from the RCSB database are provided without them. S2d. Save the engineered antibody (THIOMAB) to a new file (thiomab.pdb). S3. Load the necessary PDB records from the PDB files of the engineered antibody (thiomab.pdb), the linker (linker.pdb) and the drug (drug.pdb). Those include (i) the PDB ATOM, HETATM, and CONECT records of the engineered antibody; (ii) the PDB HETATM and CONECT records of the linker; (iii) the PDB HETATM and CONECT records of the drug (the drug and linker files do not contain PDB ATOM records). S4. Prepare the reconfiguration of the drug. S4a. Calculate the axis of the drug as specified in Eqs. (16–18). Briefly, the drug axis has been specified as the line connecting the drug atoms participating in the hydrogen bond with the linker, which are a hydrogen (H) atom and the electronegative (EN) atom bonded covalently to it. S4b. Calculate the axis of the linker as specified in Eqs. (19–21). Briefly, the linker axis has been specified as the line connecting the two most distant non-hydrogen (NH) atoms of the linker, since that line is quite representative of the shape of the linker. Given the chemical formula of the linker used ($C_{12}S$), those were always the sulfur (S) atom and its most distant carbon (C) atom. S4c. Find the atoms that will define the distance between the drug and linker according to Eqs. (30–32). Briefly, the distance between the drug and linker can be described as the distance between the atoms participating in the hydrogen bond between the two molecules, which are a hydrogen drug atom and an electronegative linker atom. S5. Reconfigure the drug. S5a. Obtain the coordinates of the drug atoms from the drug PDB HETATM records (drug.pdb). S5b. Rotate the drug using the affine transformation (Eqs. (1–15)). Apply the affine transformation to the drug for $a = 1$, $T_x = T_y = T_z = 0$ and the values of the variables r_x, r_y, r_z in [−180°, +180°] that satisfy a specific condition, as defined in Eq. (28). Briefly, the condition can be specified as the minimization of the absolute value of the angle between the axes of the drug and linker. S5c. Update (optional) the PDB drug records with the modified coordinates from step S5b. S5d. Translate the drug using the affine transformation (Eqs. (1–15)). Apply the affine transformation to the drug for $a = 1$, $r_x = r_y = r_z = 0$ and the values of the variables T_x, T_y, T_z in [−D, +D] that satisfy a specific condition, defined in Eq. (36). Briefly, the condition can be specified as the minimization of the absolute value of the difference between the distance of the two molecules (defined in step S4c) and the official length of the hydrogen bond. S5e. Update the PDB records of the drug with the modified coordinates. S5f. Save updated PDB drug records in a new PDB file (e.g., drug_rt.pdb). S6. Merge the PDB files of the linker and the reconfigured drug to produce the PDB file of the linker-drug complex. S6a. Renumber the sequence numbers of the PDB records of the reconfigured drug, since they will be placed after the PDB records of the linker in step S6c. S6b. Form computationally a hydrogen bond between the drug and linker by updating the PDB CONECT records of the drug and linker. S6c. Save the necessary data to a new file that will represent the linker-drug complex (e.g., linkerdrug.pdb). Those data include the (i) PDB HETATM records of the linker (linker.pdb; from step S1c); (ii) renumbered PDB HETATM records of the reconfigured drug (from step S6a); (iii) updated PDB CONECT records of the linker and drug, including the one that represents the new hydrogen bond (from step S6b). S6d. Load the records of the linker-drug complex (from step S6c) into appropriate structures, to use in the steps S7–S9. S7. Prepare the reconfiguration of the linker-drug complex. S7a. Calculate the axis of the linker-drug complex as specified in Eqs. (22–24). Briefly, the axis of the linker-drug complex has been specified as the line connecting the two most distant non-hydrogen (NH) atoms of the linker-drug complex. S7b. Calculate the axis of the selected engineered cysteine as specified in Eqs. (25–27). Briefly, the axis of the cysteine residue has been specified as the line connecting the alpha carbon (Cα) atom and the sulfur (S) atom of the cysteine side chain. S7c. Find the atoms that will determine the distance between the linker-drug complex and the cysteine residue, as specified in Eqs. (33–35). Briefly, the distance between the linker-drug complex and the cysteine has been specified as the distance between the atoms participating in the disulfide bond between the two molecules. S8. Reconfigure the linker-drug complex. S8a. Get the atomic coordinates of the linker-drug complex from its PDB HETATM records (linkerdrug.pdb). S8b. Rotate the linker-drug complex using the affine transformation (Eqs. (1–15)). Apply the affine transformation to the linker-drug complex for $a = 1$, $T_x = T_y = T_z$ and the values of the variables r_x, r_y, r_z in [−180°, +180°] that satisfy a specific condition, as defined in Eq. (29). Briefly, the condition is the minimization of the absolute value of the angle between the axes of the linker-drug complex and the cysteine residue. S8c. Update (optional) the PDB records of the linker-drug complex with the modified coordinates (from step S8b). S8d. Translate the linker-drug complex using the affine transformation (Eqs. (1–15)). Apply the affine transformation to the linker-drug complex for $a = 1$, $r_x = r_y = r_z = 0$, and the values of the variables T_x, T_y, T_z that satisfy a specific condition, as defined in Eq. (37). Briefly, the condition is the minimization of the absolute value of the difference between the distance of the two molecules and the official length of the disulfide bond. S8e. Update the PDB records of the linker-drug complex with the modified coordinates. S8f. Save the updated PDB records of the linker-drug complex in a new PDB file (e.g., linkerdrug_rt.pdb). S9. Merge the PDB files of the THIOMAB and the reconfigured linker-drug complex to produce the PDB file of the THIOMAB-drug conjugate. S9a. Renumber the PDB records of the linker-drug complex (from step S8), since they will be placed after the antibody records in step S9c.

S9b. Form computationally a disulfide bond between the cysteine and the linker-drug complex, by updating the PDB CONECT records of the antibody and linker. S9c. Save the necessary data to a new file that will represent the final THIOMAB-drug conjugate (e.g., tdc.pdb). Those data include (i) the PDB ATOM and HETATM records of the antibody (thiomab.pdb) (from step S2); (ii) the renumbered PDB HETATM records of the reconfigured linker-drug complex (from step S9a); (iii) the updated PDB CONECT records of the antibody and the linker-drug complex, including the one that contains the disulfide bond (from steps S9a, S9b).

Once the input files of the engineered antibody, linker, and drug are available, the necessary PDB records are loaded into instances of suitable data structures (step S3). The remaining process can be divided in two basic stages, which are the synthesis of a linker-drug complex molecule from the linker and drug (steps S4–S6), and the synthesis of an antibody-drug conjugate from the linker-drug complex and engineered antibody (steps S7–S9). More specifically, while the linker remains fixed, the drug undergoes rotation and translation, brought in a specific position near the linker (step S5). Following that, the two molecules are linked through a hydrogen bond, and their PDB files are combined to form a linker-drug complex (step S6). While the antibody remains fixed, the linker-drug complex undergoes rotation and translation (step S8). The two molecules are linked through a disulfide bond and their PDB files are combined to produce the final THIOMAB-drug conjugate (step S9). Since a drug molecule can contain more than one pair of atoms that can form a hydrogen bond with the linker, it is possible to either select the drug conjugation site, or produce more than one final TDCs. The computational method proposed in this chapter is described more analytically with the following steps (S1–S9), depicted in **Figure 2**.

2.2. Affine transformation

The modification of atomic coordinates serves the purpose of altering the relative positions between the different molecules. However, it should not disrupt the initial lines and distances between the atoms of a single molecule, since that would interfere with the validity of its chemical structure. To that end, an affine transformation was employed for the reconfiguration of a molecule [42–45]. According to [45], the transformation of a rigid body in three-dimensional space can be described with the following equations:

$$x' = R_{00} * x + R_{01} * y + R_{02} * z + R_{03} \tag{1}$$

$$y' = R_{10} * x + R_{11} * y + R_{12} * z + R_{13} \tag{2}$$

$$z' = R_{20} * x + R_{21} * y + R_{22} * z + R_{23} \tag{3}$$

$$R_{00} = a * \cos r_z * \cos r_y \tag{4}$$

$$R_{01} = a * \left(\cos r_z * \sin r_y * \sin r_x - \sin r_z * \cos r_x \right) \tag{5}$$

$$R_{02} = a * \left(\cos r_z * \sin r_y * \cos r_x + \sin r_z * \sin r_x \right) \qquad (6)$$

$$R_{03} = T_x \qquad (7)$$

$$R_{10} = a * \sin r_z * \cos r_y \qquad (8)$$

$$R_{11} = a * \left(\sin r_z * \sin r_y * \sin r_x + \cos r_z * \cos r_x \right) \qquad (9)$$

$$R_{12} = a * \left(\sin r_z * \sin r_y * \cos r_x - \cos r_z * \cos r_x \right) \qquad (10)$$

$$R_{13} = T_y \qquad (11)$$

$$R_{20} = -a * \sin r_y \qquad (12)$$

$$R_{21} = a * \cos r_y * \sin r_x \qquad (13)$$

$$R_{22} = a * \cos r_y * \cos r_x \qquad (14)$$

$$R_{23} = T_z \qquad (15)$$

where x, y, z are the initial coordinates of a point of the rigid body, x', y', z' are the coordinates of the same point after the application of the transformation, α is the scaling factor, r_x, r_y, r_z are the rotation angles of the body around the x, y, z axis, respectively, and T_x, T_y, T_z are the distances according to which each point will be translated in the x, y, z direction, respectively. From Eqs. (1–15), we can deduce that if we apply the values $a = 1$, $r_x = r_y = r_z = T_x = T_y = T_z = 0$, it will be $x' = x$, $y' = y$, $z' = z$.

The term point used above refers not to a set of constant coordinates but to an element of the body with variable coordinates. In the context of molecular reconfiguration, each molecule was considered as a rigid body. Each atom of a molecule was seen as a single point, since an atom is assigned one set of three-dimensional Cartesian coordinates according to the PDB format. The Cartesian coordinates of an atom of a molecule correspond to the variables x,y,z, x',y',z' of Eqs. (1–15). The scaling factor α was always set to the value of 1, because scaling the molecules would result in the disruption of their structure.

2.3. Rotation

According to the method presented in this chapter, in order to perform the rotation of a molecule A in reference to a fixed molecule B, the affine transformation is applied to A without using the translation and scaling functions, for the parameter values $a = 1$, $T_x = T_y = T_z = 0$. The values of r_x, r_y, r_z that satisfy a certain condition (defined in Eqs. (28, 29)) are searched for in the interval [−180°, +180°]. Finally, the affine transformation is applied to molecule A for the found r_x, r_y, r_z values. According to **Figure 2**, rotation occurs in two occasions in our method. First, the drug adopts the role of molecule A and the linker adopts the role of molecule B (step S5b). Later, the linker-drug complex adopts the role of molecule A and the cysteine takes the role of B (step S8b).

2.4. Translation

According to the method presented in this chapter, in order to perform the translation of a molecule A in reference to a fixed molecule B, the affine transformation is applied to A without using the rotation and scaling functions, for the parameter values $a = 1$, $r_x = r_y = r_z = 0$. The values of T_x, T_y, T_z that satisfy a certain condition (Eqs. (36, 37)), are searched in the interval [−D, +D]. Usually, the value $D = 200$ was sufficient to complete the search successfully; otherwise, a larger D value was applied. Finally, the affine transformation is applied to molecule A for the found T_x, T_y, T_z values. According to **Figure 2**, translation occurs in two occasions in our method. First, the drug adopts the role of the molecule A undergoing translation, and the linker adopts the role of the fixed molecule B (step S5d). Later, the linker-drug complex adopts the role of the molecule A undergoing translation, and the cysteine adopts the role of the fixed molecule B (step S8d).

2.5. Axes

As mentioned in Section 2.3, the rotation of a molecule A (i.e., the drug or linker-drug complex) in relation to its corresponding fixed molecule B (i.e., the linker or antibody, respectively) is performed for $a = 1$, $T_x = T_y = T_z = 0$ and the values of the variables r_x, r_y, r_z that satisfy a certain condition. That condition is the minimization of the absolute value of the angle between the axes of the two molecules (Eqs. (28, 29)).

In the context of this project, the axis of each molecule has been defined by taking into account the desired final layout of the three molecules. In the lysine [34] and cysteine conjugation methods, the same kind of bond connects the linker with the drug. Therefore, in both cases, the drug axis is defined by the drug atoms participating in the hydrogen bond with the linker, which are a hydrogen (H) atom and the electronegative (EN) atom covalently bonded to it [Eqs. (16–18); **Figure 2**, step S4a]. The linker axis has been specified so as to connect the two most distant non-hydrogen linker atoms because that line is quite representative of the linker shape [Eqs. (19–21); **Figure 2**, step S4b]. Given the chemical structure $C_{12}S$ of the linker used in cysteine conjugation, those two atoms are always the sulfur (S) atom and its most distant carbon (C) atom. On a similar note, the axis of the linker-drug complex connects the two most distant non-hydrogen atoms of the linker-drug complex [Eqs. (22–24); **Figure 2**, step S7a]. The

cysteine axis has been defined as the line going through the alpha carbon (C_α) and sulfur atoms (S) of the cysteine side chain [Eqs. (25–27); **Figure 2**, step S7b]. This line was chosen for its direction towards the exterior of the antibody, which is the desired direction for the linker-drug complex. In the lysine conjugation of [34], the linker $C_{15}N$ was used, therefore the linker axis was defined by the nitrogen (N) atom with its most distant carbon (C) atom. Also, the lysine axis went through the alpha carbon (C_α) and nitrogen (N) atoms of the lysine side chain.

$$p_{d1} = (x, y, z)_{H \, atom \, of \, drug} = (x_{Hd}, y_{Hd}, z_{Hd}) \tag{16}$$

$$p_{d2} = (x, y, z)_{EN \, atom \, of \, drug} = (x_{ENd}, y_{ENd}, z_{ENd}) \tag{17}$$

$$L_d : (x, y, z) = p_{d1} + t_d * (p_{d1} - p_{d2}) = (x_{Hd}, y_{Hd}, z_{Hd}) + \\ t_d * (x_{Hd} - x_{ENd}, y_{Hd} - y_{ENd}, z_{Hd} - z_{ENd}), -\infty < t_d < +\infty \tag{18}$$

$$p_{l1} = (x, y, z)_{NH \, atom \, of \, linker} = (x_{NHl}, y_{NHl}, z_{NHl}) \tag{19}$$

$$p_{l2} = (x, y, z)_{S \, atom \, of \, linker} = (x_{Sl}, y_{Sl}, z_{Sl}) \tag{20}$$

$$L_l : (x, y, z) = p_{l1} + t_l * (p_{l1} - p_{l2}) = (x_{NHl}, y_{NHl}, z_{NHl}) + \\ t_l * (x_{NHl} - x_{Sl}, y_{NHl} - y_{Sl}, z_{NHl} - z_{Sl}), -\infty < t_l < +\infty \tag{21}$$

$$p_{ld1} = (x, y, z)_{NH \, atom \, of \, linker-drug} = (x_{NHld}, y_{NHld}, z_{NHld}) \tag{22}$$

$$p_{ld2} = (x, y, z)_{S \, atom \, of \, linker-drug} = (x_{Sld}, y_{Sld}, z_{Sld}) \tag{23}$$

$$L_{ld} : (x, y, z) = p_{ld1} + t_{ld} * (p_{ld1} - p_{ld2}) = (x_{NHld}, y_{NHld}, z_{NHld}) + \\ t_l * (x_{NHld} - x_{Sld}, y_{NHld} - y_{Sld}, z_{NHld} - z_{Sld}), -\infty < t_{ld} < +\infty \tag{24}$$

$$p_{cys1} = (x, y, z)_{S \, atom \, of \, Cysteine} = (x_{Sc}, y_{Sc}, z_{Sc}) \tag{25}$$

$$p_{cys2} = (x, y, z)_{Ca\ atom\ of\ Cysteine} = (x_{Cc}, y_{Cc}, z_{Cc}) \tag{26}$$

$$L_{cys} : (x, y, z) = p_{cys1} + t_{cys} * (p_{cys1} - p_{cys2}) = (x_{Sc}, y_{Sc}, z_{Sc}) +$$
$$t_{cys} * (x_{Sc} - x_{Cc}, y_{Sc} - y_{Cc}, z_{Sc} - z_{Cc}), -\infty < t_{cys} < +\infty \tag{27}$$

$$r_x, r_y, r_z : \angle(L_d, L_l) = 0° \tag{28}$$

$$r_x, r_y, r_z : \angle(L_{ld}, L_{cys}) = 0° \tag{29}$$

2.6. Distances

As mentioned in Section 2.4, the translation of a molecule A (i.e., the drug or the linker-drug complex) in relation to its corresponding fixed molecule B (i.e., the linker or the antibody, respectively) is performed for $a = 1$, $r_x = r_y$ $r_z = 0$, and the values of the variables T_x, T_y, T_z that satisfy a certain condition. That condition is the minimization of the absolute value of the difference between the distance of the two molecules and a fixed value l (Eqs. (36, 37)). The definitions of the distance between two molecules and the fixed value are given below.

Taking into consideration the desired connectivity between the three molecules, in the context of this project, the distance between two molecules was defined as the distance between the atoms that will participate in the intended intermolecular bond (hydrogen or disulfide bond, **Figure 1**). As a consequence, the fixed value l was defined as the official length of that intermolecular bond ($l_{Hb} = 1.5$–2.5 Å for the Hydrogen bond, $l_{SSb} = 2.05$ Å for the disulfide bond).

In more detail, in both lysine and cysteine conjugation, the distance between the drug and linker was defined by the drug hydrogen (H) atom and the linker electronegative (EN) atom participating in the H bond between the linker and drug [Eqs. (30–32); **Figure 2**, step S4c]. In regard to the distance between the linker-drug complex and the conjugation residue, in lysine conjugation, it was defined by the linker nitrogen (N) atom and the lysine hydrogen (H) atom bonded to the side chain nitrogen atom. In cysteine conjugation, however, it has been defined as the distance between the sulfur (S) linker atom and the sulfur atom of the cysteine side chain [Eqs. (33–35); **Figure 2**, step S7c]. The translation of the drug in relation to the linker is performed for the values of the variables T_x, T_y, T_z for which the distance between the two molecules takes the official length $l_{Hb} = 1.5$–2.5 Å of the hydrogen bond (Eq. (36)). The translation of the linker-drug complex in relation to the cysteine is performed for the values of the variables T_x, T_y, T_z for which the distance between the two molecules takes the official length $l_{SSb} = 2.05$ Å of the disulfide bond (Eq. (37)):

$$p_d = (x, y, z)_{H\ atom\ of\ drug} = (x_{Hd}, y_{Hd}, z_{Hd}) = p_{d1} \tag{30}$$

$$p_{l1} = (x, y, z)_{EN\ atom\ of\ linker} = (x_{ENl}, y_{ENl}, z_{ENl}) = (x_{Cl}, y_{Cl}, z_{Cl}) \tag{31}$$

$$D_{d-l} = p_d - p_{l1} \tag{32}$$

$$p_{l2} = (x, y, z)_{S\ atom\ of\ linker} = (x_{Sl}, y_{Sl}, z_{Sl}) \tag{33}$$

$$p_{cys} = (x, y, z)_{S\ atom\ of\ Cysteine} = (x_{Scys}, y_{Scys}, z_{Scys}) \tag{34}$$

$$D_{cys-l} = p_{cys} - p_{l2} \tag{35}$$

$$T_x, T_y, T_z : |D_{d-l} - l_{Hb}| = 0 \tag{36}$$

$$T_x', T_y', T_z' : |D_{cys-l} - l_{SSb}| = 0 \tag{37}$$

where the variables T_x, T_y, T_z of Eq. (36) refer to the translation of the drug (**Figure 2**, step S5d), while the variables of T_x', T_y', T_z' Eq. (37) refer to the translation of the linker-drug complex (**Figure 2**, step S8d).

2.7. Implementation

The steps S1 and S2 (see the **Figure 2**) that involve the selection and preparation of the input files of the engineered antibody, the linker, the drug, as well as the selection of the conjugation cysteine residue, are carried out manually. The steps S3–S9 have been developed as a C++ program, and therefore can be executed automatically. In order to make the necessary data management, a number of classes and functions were created in regard to the PDB records used, the three-dimensional points and lines, as well as the affine transformation.

3. Results

The method described in the previous section has been applied to various antibody and drug combinations, taking about 10–13 seconds for each TDC to be generated. In **Figure 3**, the basic stages of the production of a THIOMAB-drug conjugate are depicted. First, the drug and linker are formed in two independent PDB files. The axis of each molecule is depicted as a white line, and the initial angle between them is 98.2° (**Figure 3a**). The drug is rotated, and the angle between the two axes becomes 0.08° (**Figure 3b**). The initial distance between the atoms that will form the hydrogen bond (the C atom of the linker and the H atom of the drug, bonded

covalently to an O drug atom) is 28.02 Å. The drug is translated and the distance between the two atoms becomes 1.51 Å (**Figure 3c**). The linker has remained fixed. The two molecules are merged into a linker-drug complex, whose new axis is depicted as a white line (**Figure 3d**). The residue valine of the antibody with PDB id 4GAG, with sequence number 206 on the light chain (LC-VAL206), is replaced by a cysteine, whose axis is calculated (**Figure 3e**). The initial angle between the axis of the linker-drug complex and the axis of the cysteine is 105.6°, and the distance between the sulfur atoms of the cysteine and the linker is 54.75 Å. The linker-drug complex is rotated and translated, so that the angle becomes 0.083° and the distance becomes 2.07 Å. Finally, the linker-drug complex is connected with the cysteine (**Figure 3f**).
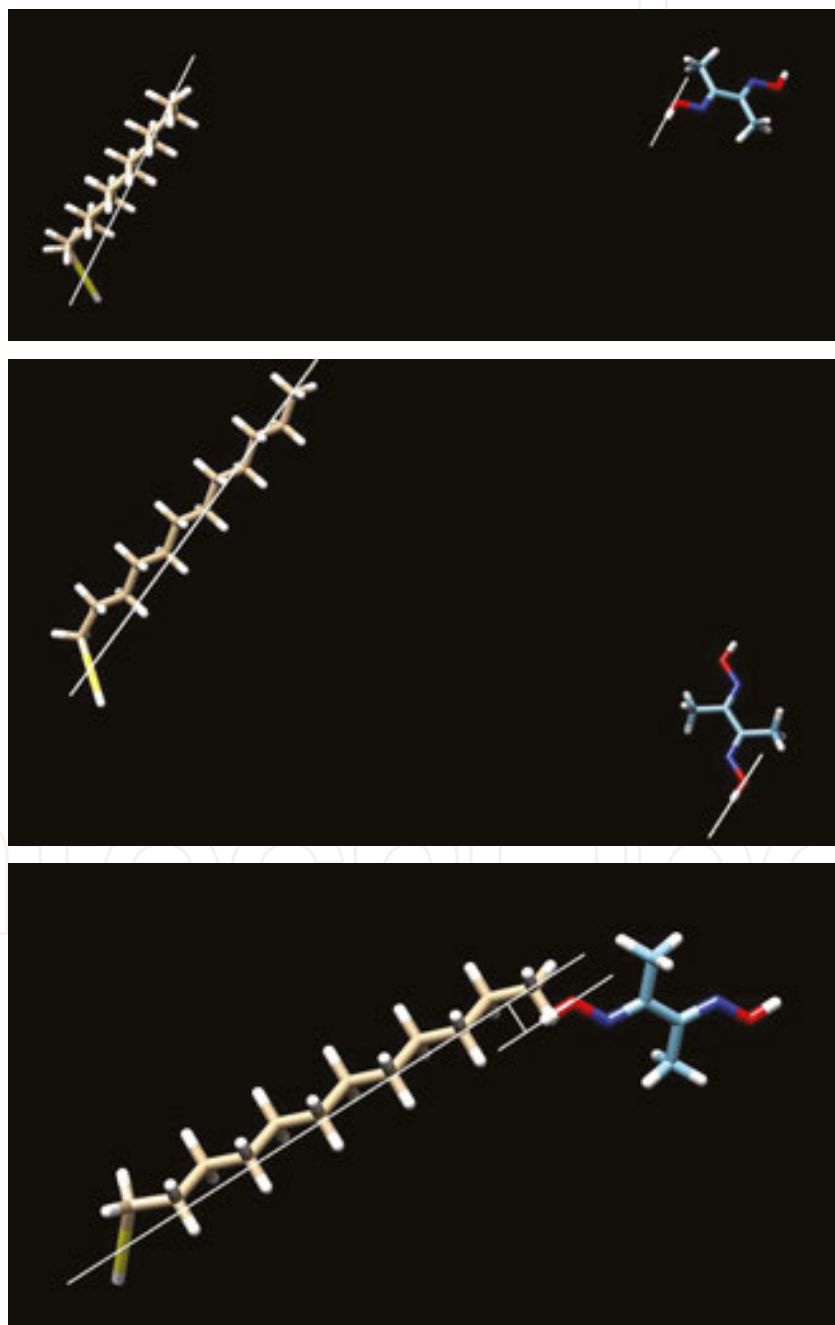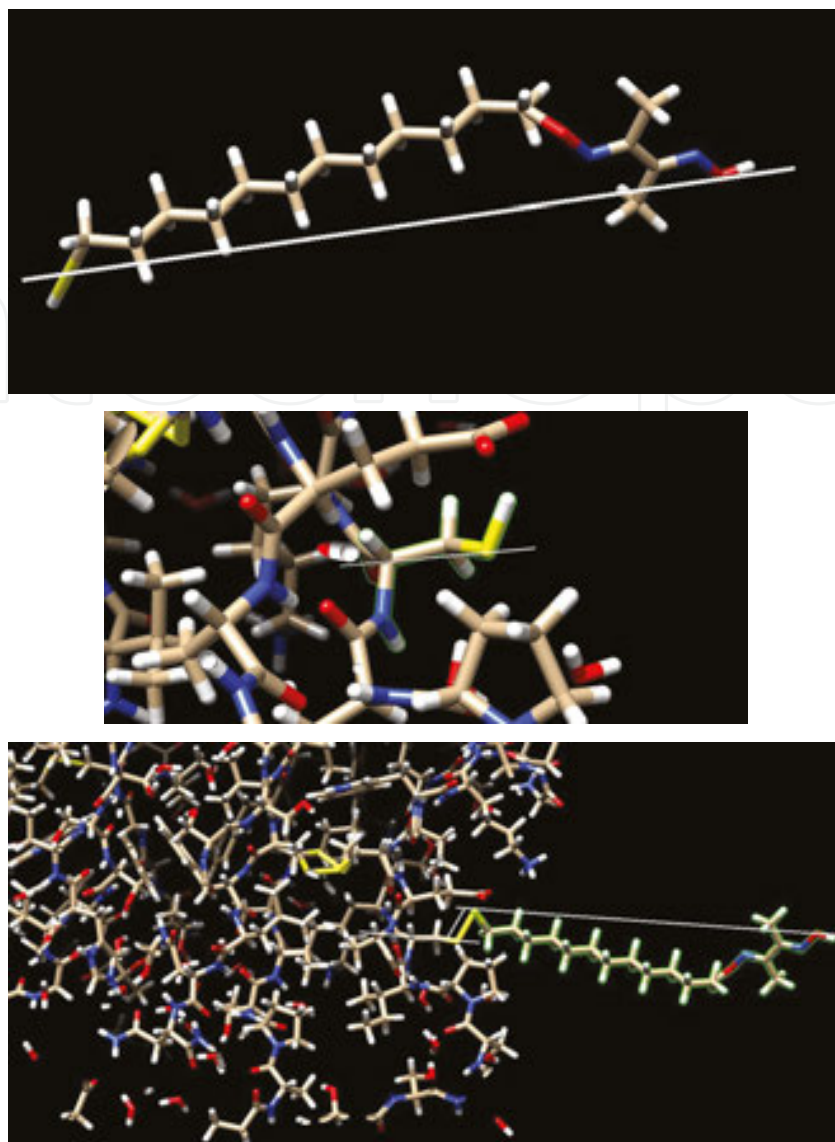
Figure 3. The basic stages of the computational production of a TDC. (a) Left: The linker molecule $C_{12}S$. Right: The drug with NCI sequence number 9, before its reconfiguration. Angle between the two axes: 98.2°. (b) The linker and the rotated drug. Angle between the two axes: 0.08°. Distance between the H atom of the drug and the C atom of the linker: 28.02 Å. (c) The linker and the translated drug. Distance between the H atom of the drug and the C atom of the linker: 1.51 Å. (d) The linker-drug complex. (e) The engineered cysteine that has replaced the residue LC-VAL206 on the antibody with PDB id 4GAG. Initial angle between the axes of the cysteine and the linker-drug complex: 105.6°. Initial distance between the two S atoms: 54.75 Å. (f) The linker-drug complex conjugated to the engineered cysteine. Angle between the axes of the cysteine and the linker-drug complex: 0.084°. Distance between the two S atoms: 2.07 Å.

More examples of produced TDCs are demonstrated in **Figure 4**. The TDC composed by the antibody with PDB id 4GAJ, the linker molecule $C_{12}S$ and the drug with sequence number 5 (nci_5.pdb) is depicted in **Figure 4(a)**. A hydrogen bond connects the drug with the linker, and a disulfide bond connects the engineered cysteine on the antibody with the linker-drug complex. The cysteine has replaced the solvent-accessible amino acid alanine with residue sequence number 114, at the heavy chain of the antibody (HC-ALA114). The same TDC is depicted from a closer distance at the right of the figure. In **Figure 4b**, a similar TDC composed

by the antibody with PDB id 4GAJ, the linker molecule C12S and the drug with sequence number 7 (nci_7.pdb) is depicted. The above technique can be used for the conjugation of TDCs with more than one drug molecules per antibody. **Figure 4c** depicts a TDC with a DAR of 2, composed by the antibody with PDB id 4GAG, to which two drug molecules with sequence number 1 (nci_1.pdb) have been conjugated. The engineered cysteines that serve as conjugation sites have replaced the solvent-accessible residues alanine with sequence number 114 at the heavy chain and valine with sequence number 206 at the light chain (LC-VAL206). The conjugation areas of the same TDC are demonstrated from a closer distance at the right of the figure. The linker and the drugs used in the examples are depicted in **Figure 5**.
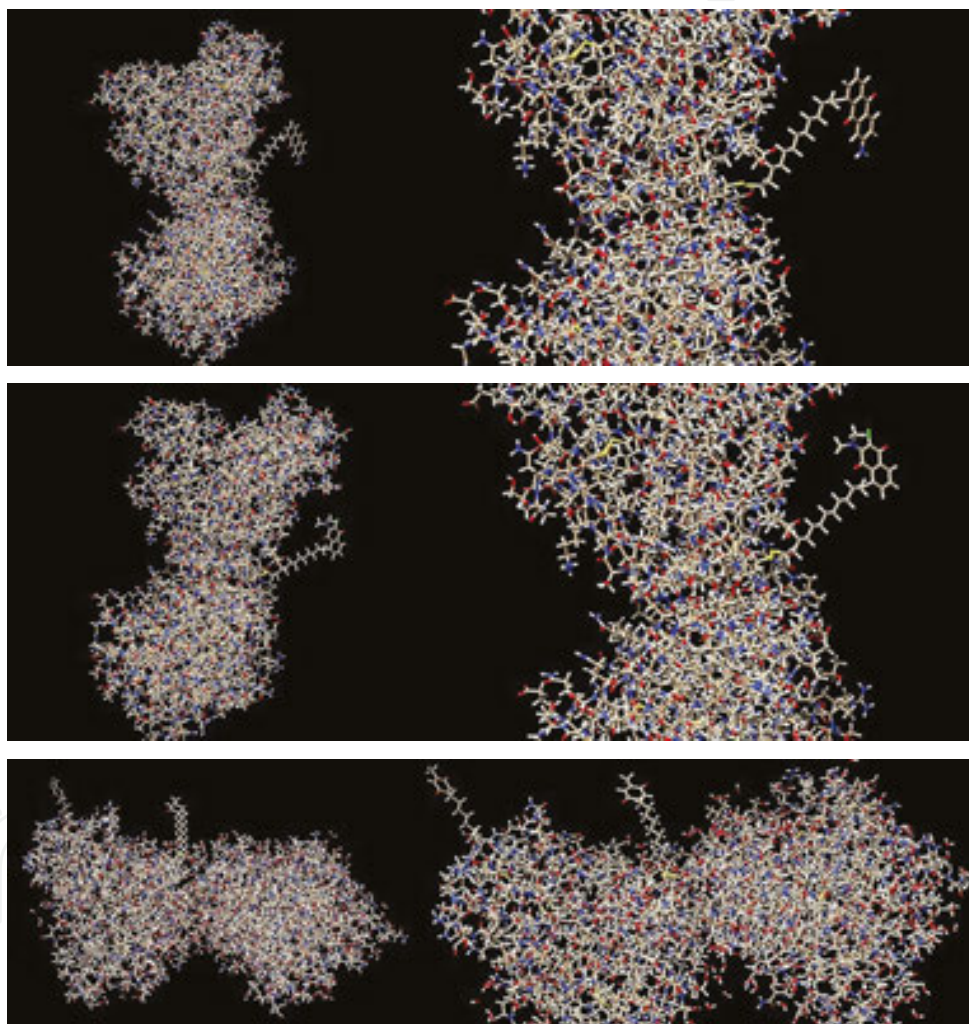

Figure 4. (a) Left: TDC composed by the antibody with PDB id 4GAJ, the linker molecule $C_{12}S$, and the drug with sequence number 5. The drug has been conjugated through the linker to the engineered cysteine that has replaced the residue HC-ALA114. Right: The same TDC from a closer distance. (b) Left: TDC composed by the antibody with PDB id 4GAJ, the linker molecule $C_{12}S$, and the drug with sequence number 7. The drug has been conjugated through the linker to the engineered cysteine that has replaced the residue HC-ALA114. Right: The same TDC from a closer distance. (c) Left: TDC composed by the antibody with PDB id 4GAG, two linker molecules $C_{12}S$, and two drug molecules with sequence number 1. The antibody conjugation sites are engineered cysteines that have replaced the residues HC-ALA114, and LC-VAL206. Right: The same TDC from a closer distance.
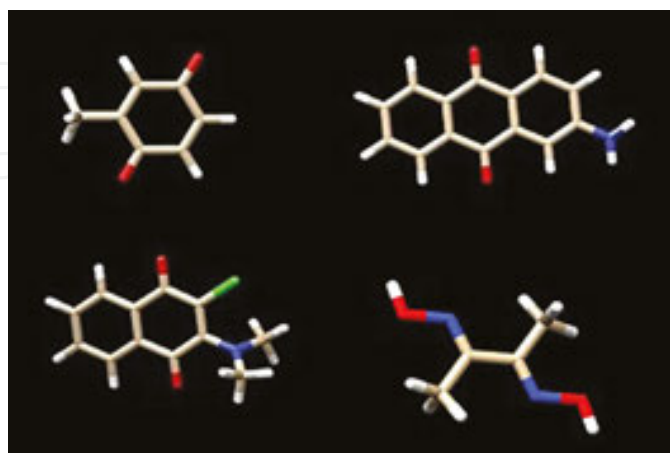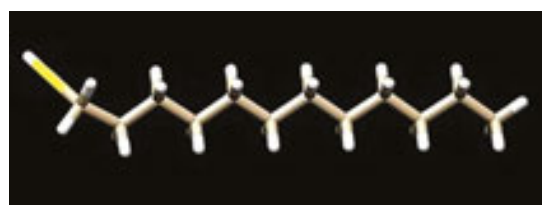
Figure 5. (a) The linker molecule C$_{12}$S. (b) The cytotoxic drugs used in **Figures 3** and **4**. Top left: the drug with sequence number 1 (nci_1.pdb). Top right: the drug with sequence number 5 (nci_5.pdb). Bottom left: the drug with sequence number 7 (nci_7.pdb). Bottom right: the drug with sequence number 9 (nci_9.pdb).

4. Discussion

This chapter has presented a computational method for site-specific formulation of ADCs, by conjugating the cytotoxin and the linker to engineered cysteines on the surface of the antibody. A hydrogen bond is employed to connect the linker with the drug, forming a linker-drug conjugate, which is then attached through a disulfide bond to the selected engineered cysteine. The change of the atomic coordinates of a molecule was implemented with an affine transformation. The generation of the linker molecule C$_{12}$S, as well as the cysteine replacement, was achieved through molecular modeling software [36, 42].

The present chapter proposes a general way to implement computationally ADC molecules using data from established databases. The common antibodies used do not target cancer-specific molecules, as opposed to antibodies that are parts of real ADCs. The employment of antibodies with cancer-specific antigens was not feasible, since their three-dimensional structures are not available. However, considering that the conjugation occurs in the preserved regions of the antibody, the same techniques could be applied using the PDB file of any full antibody. In addition, conventional anticancer drugs were used, since the three-dimensional structures of the more potent drugs used in ADCs currently are not available. It is important to note that the results of the presented method are not proposed as real anticancer drugs. The presented method is an attempt to simulate ADCs using programming techniques and available data.

Site-specific conjugation is a substantial goal in ADC development, since it gives the ability to control the pharmacokinetic and therapeutic properties of the produced ADCs. Aside from the replacement of residues with cysteines, mentioned in Section 1, a similar concept is the substitution of cysteines with serines in order to reduce the number of inter-chain cysteines available for conjugation. Another technique is the introduction of non-native amino acids in the antibody sequence, such as selenocysteine, acetylphenylalanine, para-acetylphenylalanine, and para-azidophenylalanine, utilizing their ability to provide orthogonal conjugation chemistries that are not available from original residues. Enzymes have been used to help form bonds between the antibody, the linker and drug, a method called enzymatic conjugation. An additional strategy is the conjugation at the glycosylation sites of the antibody, leaving the amino acid sequence of the antibody and the cell culture condition intact. For example, sialic acid moieties can be introduced enzymatically to the antibody, which can then be oxidized to introduce aldehyde groups. The resulting antibodies can then form stable oxime bonds with drugs containing aldehyde-reactive aminooxy groups [22, 46–59].

A number of actions could be taken to improve and expand the work described in this chapter. Some of our future goals is the implementation of more site-specific conjugation ways, the use of antibody forms alternative to the full monoclonal antibody, such as scFvs, diabodies, and minibodies, as well as other non-cleavable and cleavable linker molecules. In addition, the automatic recognition of all amino acids available for conjugation given a specific antibody would be interesting, as well as the estimation of the best drugs and linkers given a conjugation method. Finally, the conditions and equations used could be modified in order to take into consideration other factors.

Acknowledgements

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