

**BIO CONJUGATION IN THE PHARMACEUTICALS OCCURRENCE, BEHAVIOUR AND APPLICATIONS.**

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Date of Acceptance: 21<sup>st</sup> September 2017)**ABSTRACT**

Conjugation is the process of chemically joining two or more molecules by a covalent bond. The availability of several chemical groups in proteins and peptides make them targets for conjugation and for study using crosslinking methods. The same reaction is applied to amino acid and nucleic acid surface modification and labeling. This area of chemistry is known as bioconjugation and includes crosslinking, immobilization, surface modification and labeling of biomolecules. Recent advances in the understanding of biomolecules and bioconjugation enabled their application to numerous fields like medicine and materials. In the present review, the authors are very much interested in making the review on bioconjugation, cross linkers and modifiers, types of reactive groups for conjugation, background of going for conjugation, research work carried out in conjugation, applications and in the development of Active pharmaceutical ingredients (API) and its application.

**Keywords:** bioconjugation, cross linkers, modifiers, drug delivery, biomedical research

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## INTRODUCTION

**Bioconjugation** is a chemical strategy to form a stable **covalent link** between two molecules, at least one of which is a biomolecule. Bioconjugation is a burgeoning field of research. Novel methods for the mild and site-specific derivatization of proteins, DNA, RNA, and carbohydrates have been developed for applications such as ligand discovery, disease diagnosis, and high-throughput screening. These powerful methods owe their existence to the discovery of chemoselective reactions that enable bioconjugation under physiological conditions—a tremendous achievement of modern organic chemistry and in Pharmaceuticals. (Jeet kalia and Ronald T. Raines *et al* 2010, Stephanopoulos and N. Francis, M. B 2011)

### II. Cross linkers

Cross linkers contain at least two functional groups which target common functional groups found in bio molecules such as protein and nucleic acids. The most important property of a cross linker is its reactive chemical group. The reactive group establishes the method and mechanism for chemical modification.

Crosslinking reagents contain reactive ends to specific functional groups (primary amines, sulfhydryls, etc.) on proteins or other molecules. Common types of bioconjugation reactions are coupling of lysine amino acid residues, coupling of cysteine residues, coupling of tyrosine residues, modification of

tryptophan residues, and modification of the N- and C- terminus.

#### a. Types of Cross linkers

There are two types of cross linkers.

1. Homobifunctional cross linkers.
2. Heterobifunctional cross linkers.

**1. Homobifunctional crosslinkers** have identical reactive groups at either ends of a spacer arm. Generally, they must be used in one-step reaction procedures to randomly "fix" or polymerize molecules containing like functional group. This is useful for capturing a snapshot of all protein interactions, but it cannot provide the precision needed for other types of crosslinking applications.

**2. Heterobifunctional cross linkers** possess different reactive groups at either end. These reagents not only allow for single-step conjugation of molecules that have the respective target functional group, but they also allow for sequential (two-steps) conjugations that minimize undesirable polymerization or self-conjugation. In sequential procedures, heterobifunctional reagents are reacted with one protein using the most labile group of the crosslinker first. After removing excess unreacted cross linker, the modified first protein is added to a solution containing the second protein where reaction through the second reactive group of the crosslinker occurs. Molecular properties of cross linkers and modifiers is shown in the table 1.

### III. Different types of reactive groups for conjugation.

- **Amine - reactive chemical groups**
  - *N*-hydroxysuccinimide esters (NHS esters)
  - Imidoesters
- **Carboxylic acid-reactive chemical groups**
  - Carbodiimides (EDC and DCC)
- **Sulphydryl-reactive chemical groups**
  - Maleimides
  - Haloacetyls
  - Pyridyl disulfides
- **Carbonyl-reactive chemical groups**
  - Carbonyls (aldehydes) as crosslinking targets
  - Hydrazides
  - Alkoxyamines
  - Reductive amination
- **Nonspecific-reactive chemical groups**
  - Aryl azides
  - Diazirines
- **Chemoselective ligation**
  - Chemoselectivity of azide-phosphine reactions

Popular crosslinker reactive groups for protein conjugation is provided in the table 2.

### Background of going for bio conjugation

In the past decade we have seen major advances in the development of suitable, effective and tolerable drug compounds, which aim to deliver drugs more specifically to target tumor cells while sparing healthy tissue. Following the identification of candidate drugs and suitable carrier bonds, the concept of developing drug conjugates to optimize drug effects and patients' tolerance has progressed from *in vitro* and *in vivo* models to the achievement of promising results in early clinical trials.

#### a) Ease of developing Active pharmaceutical ingredient (API) or Pharmaceutical formulations

The development of drug conjugates suffered an early setback with the anti-CD33 compound gemtuzumab ozogamicin (Mylotarg®). This drug gained accelerated FDA-approval for acute myeloid leukemia (AML) in 2000, but in 2010 (FDA Safety Information, posted June 2010) a confirmatory post-approval trial indicated new safety concerns and failed to demonstrate a benefit, leading to the withdrawal of the product by the manufacturer. (Wolf-Dieter Janthur *et al* 2012)

Mylotarg (gemtuzumab, Wyeth) is the only immunoconjugate that has been approved by the FDA for the treatment of cancer. This immunoconjugate consists of humanized anti-CD33 mAb linked to the cytotoxic antibiotic ozogamicin (N-acetyl-γ calicheamicin). The linker consists of two cleavable bonds. Mylotarg has been approved for the treatment of

CD33+ acute myeloid leukemia (AML) in elderly patients who are not eligible for other chemotherapies and who are suffering from their first relapse. Mylotarg demonstrated clinical efficiency in pediatric patients with advanced CD33+ AML. (Yashveer Singhet *al* 2008)

#### b) Combining Biological compound with synthetic components.

The advantages of combining biological structure and function with synthetic components is provided by protein-polymer conjugates. Modern polymer chemistry allows the bulk physical properties of synthetic macromolecules to be fine tuned with a high degree of control. When appended to proteins, such polymers can alter solubility, provide responsiveness to various environmental conditions including temperature and pH, and facilitate the integration of proteins into manufactured devices. However, it is difficult to obtain conjugates without affecting native biochemical properties. As a result, the ability to manipulate both attachment site and the nature of the polymer holds promise for both biotechnology and medicine. (Matthew B Francis and Isaac S Carrico 2010)

#### c) Herbal drug with Synthetic compound increases the permeability.

Curcumin-diclofenac conjugate as been synthesized by esterification of phenolic group of curcumin with the acid moiety of diclofenac. Diclofenac was selected as the drug to synthesize curcumin-diclofenac conjugate (CDC) because of its high permeability, which could potentially increase the permeability of curcumin. Additionally,

conjugation with diclofenac could provide stability to the curcumin molecule in the gastrointestinal tract because of steric hindrance in CDC, and may thereby prevent its intestinal metabolism. Synthesis of curcumin-diclofenac via a chemical bond to form CDC appears to be a novel approach to create new drug-drug conjugates with complementary therapeutic activities to obtain substantially enhanced activities of either one or both e.g. in this case CDC would be highly beneficial for the treatment of RA, adopted to increase the bioavailability of curcumin hence its enhanced anti-inflammatory activity. (S.K Jain *et al* 2014, Ireson CR *et al* 2002, Vareed SK *et al* 2008, Mishra S *et al*)

#### **V. Research Work carried Out in bio conjugation**

The different types of research work carried out in various fields. They are a) Bioconjugation in pharmaceutical chemistry, b) Bacterial conjugation : a potential tool for genomic engineering, c) Bacterial conjugation based antimicrobial agents, d) New frontiers in protein conjugation, e) Structure and role of coupling proteins in conjugal DNA, f) Drug delivery strategy utilizing conjugation, g) Growing applications of click chemistry for bioconjugation in contemporary biomedical research, and h) Role of polymer-drug conjugates in organ specific delivery systems.

#### **a. Bioconjugation in pharmaceutical chemistry**

Polymer conjugation is of increasing interest in pharmaceutical chemistry for delivering drugs of simple structure or complex compounds such as peptides,

enzymes and oligonucleotides. For long time drugs, mainly with antitumoral activity, have been coupled to natural or synthetic polymers with the purpose of increasing their blood permanence time, taking advantage of the increased mass that reduces kidney ultrafiltration. Labile oligonucleotides, including antisense drugs, were also successfully coupled to polymers in view of an increased cell penetration and stabilization towards nucleases.

#### **b. Bacterial conjugation: a potential tool for genomic engineering**

Bacterial conjugation is a mechanism for horizontal DNA transfer with potential for universal DNA delivery. The conjugal machinery can be separated into three functional modules, the relaxosome, the coupling protein and a type IV protein secretion system. Module interchangeability among different conjugative systems opens up the possibility of engineering of DNA delivery into virtually any cell type. (Matxalen Lloa and Fernando de la Cruz 2005)

#### **c. Bacterial conjugation-based antimicrobial agents**

Bacterial conjugation based antimicrobial agents generating radically different antibacterial technologies will give a solution for the conventional chemical antibiotics usage. Bacterial conjugation based technologies (BCBT), is a new frontier of drug discovery. One of the objectives of BCBT is to exploit plasmid biology for combating the rising tide of antibiotic-resistant bacteria. This concept utilizes conjugationally

developed plasmids as antimicrobial agents. (Marcin Filutowicz *et al* 2008)

#### **d. Protein conjugation**

Protein modifications involve the coupling of synthetic molecules to native or artificial amino acid side chains, typically yielding bio conjugates with chemical linkages that differ significantly from those that are produced biosynthetically. However, there are many instances in which it is useful to replace a particular side chain group with synthetic functionality that more closely mimics a particular post-translational modification. In some cases it is also advantageous to introduce a particular side chain after protein expression and folding have taken place. The recent progress in the use of synthetic transformations to convert specific amino acid residues on an expressed protein into other native or modified native groups. These "Chemical Mutations" allow mimics of post-translationally modified proteins to be prepared, and can also be used as components of "traceless" protein purification strategies. A particular emphasis has been placed on dehydroalanine, as this group can be introduced directly as an artificial amino acid, or it can be generated through the oxidation and subsequent elimination of a cysteine side chain. Once present in the protein sequence, these electrophilic sites can be coupled to thiols and potentially even organometallic reagents to generate a range of modified protein targets. (Matthew B Francis *et al* 2010)

#### **e. Structure and role of coupling proteins in conjugal DNA**

Type IV secretory systems are transmembrane bacterial multiprotein

complexes. They are pivotal for conjugation, bacterial-induced plant tumour formation, toxin secretion and mammalian pathogen intracellular activity. These systems are involved in the spread of antibiotic resistance genes among bacteria by enabling conjugative DNA transfer. When such translocons transport DNA, they require the assistance of multimeric integral inner membrane proteins, the type IV coupling proteins. Its structural prototype is plasmid R388 TrwB protein, responsible for coupling the relaxosome with the DNA transport apparatus during bacterial conjugation. Its monomeric molecular structure is reminiscent of ring helicases and AAA ATPases. The quaternary structure is made up by six equivalent protomers featuring a flattened sphere resembling F<sub>1</sub>-ATPase, with a central channel traversing the particle, thus connecting cytoplasm and periplasm. (Xavier Gomis-Ruth. *Fet al* 2002)

#### **f. Drug delivery strategy utilizing conjugation via reversible disulfide linkages: role and site of cellular reducing activities.**

The first disulfide linkage-employing drug conjugate that exploits the reversible nature of this unique covalent bond was recently approved for human use. Increasing numbers of drug formulations that incorporate disulfide bonds have been reported, particularly in the next generation macromolecular pharmaceuticals. These are designed to exploit differences in the reduction potential at different locations within and upon cells. The recent characterization of a novel redox enzyme in endosomes and

lysosomes adds more excitement to this approach. This review focuses on understanding where and how the disulfide bond in the bioconjugate is reduced upon contact with biological milieu, which affects delivery design and the interpretation of the delivery strategies. (Saito G *et al* 2003)

### **g. Growing Applications of Click Chemistry for Bioconjugation in Contemporary Biomedical Research**

Click chemistry has become a burgeoning strategy of bioconjugation in the development of bifunctional molecules. Bioconjugation involves the attachment of synthetic labels to biomolecular building blocks, such as fusing two or more proteins together or linking a carbohydrate with a peptide, and covers a wide range of science between molecular biology and chemistry. Although bioconjugation is applicable to the *in vivo* labeling of biomolecules, only a handful of reactions are actually useful. The possibility of applying click chemistry in bioconjugation was first applied for the preparation of peptidotriazoles via solid state synthesis. Their goal was to develop new, more efficient synthetic methods to prepare various [1, 2, 3]-triazole pharmacophores for potential biologic targets. This initial report makes possible the introduction of various novel functional and reporter groups into biomolecules, such as peptides and proteins, for DNA labeling and modification, and for cell-surface labeling. (Kido New and Martin W. Brechbiel *et al* 2009)

### **h. Role of polymer-drug conjugates in organ-specific delivery systems.**

Polymers have been utilized to deliver the drug to targeted site in controlled manner, achieving the high-therapeutic efficacy. Polymeric drug conjugates having variable ligands as attachments have been proved to be biodegradable, stimuli sensitive and targeted systems. Numerous polymeric drug conjugates having linkers degraded by acidity or intracellular enzymes or sensitive to over expressed groups of diseased organ/tissue have been synthesized during last decade to develop targeted delivery systems. Most of these organs have number of receptors attached with different cells such as Kupffer cells of liver have mannose-binding receptors while hepatocytes have asialoglycoprotein receptors on their surface which mainly bind with the galactose derivatives. Such ligands can be used for achieving high targeting and intracellular delivery of the drug. This review presents detailed aspects of receptors found in different cells of specific organ and ligands with binding efficiency to these specific receptors. This review highlights the need of further studies on organ-specific polymer-drug conjugates by providing detailed account of polymeric conjugates synthesized till date having organ-specific targeting. (Paramjot *et al* 2015)

#### **Applications of bio conjugation:**

- Attachment of Fluorescent Labels to Biological Molecules
- Biological Assays: Detection and monitoring of a protein or nucleic acid finding the function of genes/proteins, monitoring the progress of a disease, etc.

- Cell Biology: mechanistic studies of biological transformations in cells
- Genomics, Molecular Biology, Proteomics, Medical Diagnostics.
- Metabolic labelling
- Cell surface crosslinking
- Cell membrane structural studies
- Subunit crosslinking and protein structural studies(Keld Fosgerau and Torsten hoffmann2015)
- The biolabeling "market" is lucrative; "kits" available for most jobs
- New fluorescent labels: metal centers, quantum dots, conjugated polymers, etc.
- Targeted drug delivery( C.W.Pouton 1985)
- Antibody-drug conjugation technology used in monoclonal antibodies.(Hans peter Gerber et al 2009)
- *In vivo* labelling of bio molecules (Tornoe CW et al 2002)**Figure: 1**
- Biotinylation

Possesses one of the strongest binding interactions with avidin / streptavidin ( $K_d \sim 10^{15} M$ )

Contains four identical binding sites for interaction with biotin. It is shown in the **figure 2**.

**Conclusion**

Application of bioconjugation range from fundamental science to industrial and clinical research.Choosing the optimal bioconjugation linkage for a particular application is crucial. It is imperative to ensure that the linkage is stable during the course of its use and generating the desired bioconjugate quickly. These imperatives present interesting challenges of substantial importance. As a consequence, new conjugation modalities are being pursued with vigor. In conclusion, the bioconjugation methods, types and application revealed as a potential field in developing various Pharmaceutical active ingredient to reduce toxic effects and improve the quality of drugs.

Biotin-Streptavidin interaction  
 Biotin (Vitamin-H):Can be conjugated to different bio molecules

**Table 1** :Molecular Properties of Crosslinkers and Modifiers.

Chemical specificity	The reactive target(s) of the crosslinker’s reactive ends. A general consideration is whether the reagent has the same or different reactive groups at either end (termed homobifunctional and heterobifunctional, respectively).
General reaction conditions	The buffer system required to perform bio conjugation. Variables include pH, buffer concentration and protein concentration.
Spacer arm length	The molecular span of a crosslinker (i.e., the distance between conjugated molecules). A related consideration is whether the linkage is cleavable or

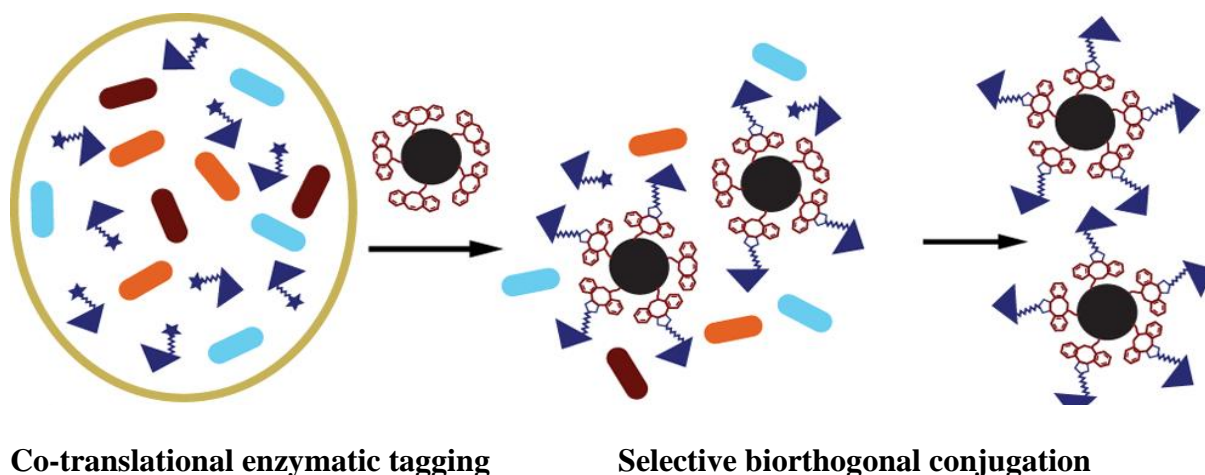


	reversible.
Space arm composition	The chemical groups found within the spacer arm.
Space arm cleavability	The availability of a cleavage site within the spacer arm between the chemical reactive groups.
Chain structure	The presence of a straight or branched chain.
Water solubility and cell membrane permeability	Affect whether a crosslinker or modifier can permeate into cells and/or crosslink hydrophobic Proteins within membranes. These properties are determined by the composition of the spacer arm and/or reactive group.

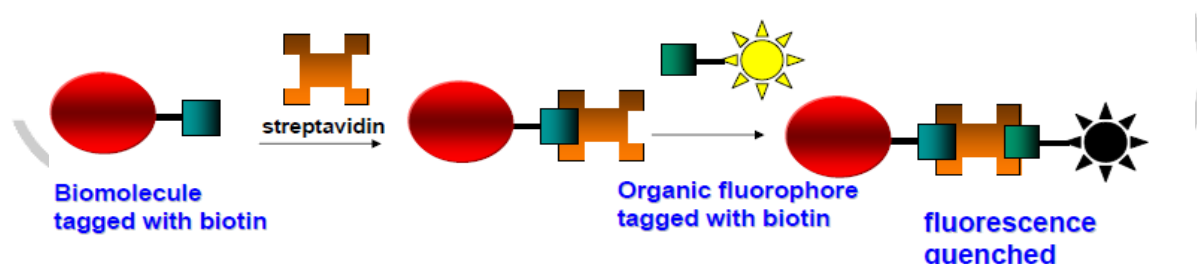
**Table 2:** Popular crosslinker reactive groups for protein conjugation.

Reactivity Class	Target Functional group	Reactive chemical group
Amine-reactive	-NH <sub>2</sub>	NHS ester Imidoester Pentafluorophenyl ester Hydroxymethyl Phosphine
Carboxyl-to-amine reactive	-COOH	Carbodiimide (e.g., EDC)
Sulfhydryl-reactive	-SH	Maleimide Haloacetyl (bromo-or iodo-) Pyridylsulfide Thiosulfonate Vinylsulfone
Aldehyde-reactive i.e., oxidized sugars	-CHO	Hydrazide Alkoxyamine
Photo-reactive i.e., non selective, random insertion	Random	Diazirine Aryl azide
Hydroxyl ( non aqueous)-reactive	-OH	isocyanate
Azide-reactive	-N <sub>3</sub>	Phospine

**Figure 1:** *New trends: in vivo labeling with bio-orthogonal chemistry*



**Figure 2:** *Bioconjugation of biotin with streptavidin*



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