Conjugation Ahead

Developments in peptide-nucleic acid conjugates are enabling novel means of drug delivery capable of passing the cell membrane, with exciting consequences for the future of RNA therapeutics.

Advances in peptides and nucleic acids synthesis are promoting the development of bioconjugates, where these molecules are either cross-linked to each other or to other compounds to yield products with distinct properties. There are many applications in biomedicine where linking two active molecules can lead to up- or down-regulation of gene expression. These bioconjugates are not limited to peptide-nucleic acid derivatives, but either of these two moieties can be substituted by another compound such as a polysaccharide, lipid, synthetic polymer or small molecules. Depending on the bioconjugates' nature they can have applications in qualitative/quantitative assays, targeting of specific cells for diagnostic and/or therapeutic purposes, transfer of compounds across cell membranes, modulation of certain biological activities and other uses.

Synthetically produced peptides and nucleic acids allow for the introduction of modifications, such as residues with certain functional groups (hydroxyl, thiol, amine and others) to select the chemical reaction needed to link both moieties of bioconjugates covalently. They also allow the insertion of a spacer with an optimal length to enable the cross-linking of both moieties without steric hindrance. Although bioconjugates are products with physical chemical properties different from their original free moieties – for example lipophilicity and charge – each cross-linked moiety largely retains its biological activity. This feature makes bioconjugates an attractive approach to allow particular compounds to circumvent certain barriers, enter the cell and exert their biological activities.

Although the first bioconjugates were largely proteins linked to peptides, enzymes, or small reporting molecules such as fluorescein, the most promising uses of bioconjugates are in the nucleic acid/oligonucleotide field, where these compounds are linked largely to peptides, and in some cases to lipids. While oligonucleotides are polar, hydrophilic and have a negative net charge, peptides can be polar and/or non-polar, have both hydrophilic and lipophilic regions and have a negative, positive or neutral net charge. Due to their properties, nucleic acids cannot pass across the cell membrane, which is lipophilic and negatively charged because of its sialic acid content. Conjugation of an oligonucleotide to some peptides that can move through the cell membrane would facilitate the passage of this conjugate across it for delivery into the cell's cytosol. Oligonucleotides can also be conjugated to lipids to yield derivatives with lipophilic properties that are capable of passing across the cell membrane.

Peptide-Oligonucleotide Conjugates

Peptide-oligonucleotide conjugates – a peptide that is covalently linked to an oligonucleotide – have two moieties: either an oligodeoxyribonucleotide (ODN) or an oligoribonucleotide (ORN). Alternatively, an oligonucleotide can be replaced with a peptidenucleic acid (PNA) that mimics the biological activity of that oligonucleotide. The oligonucleotide can interact with specific cell receptors to stimulate or inhibit biological responses, such as a gene expression. Peptide-oligonucleotide conjugates can deliver oligonucleotides intracellularly with a better efficiency and less cellular toxicity than other available methods, for example lipofectamine.

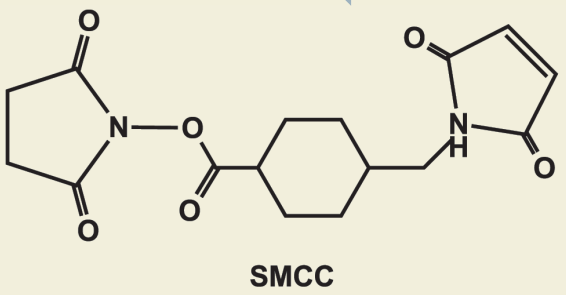
Miguel Castro at Bio-Synthesis Inc and Dante Marciani at Qantu Therapeutics

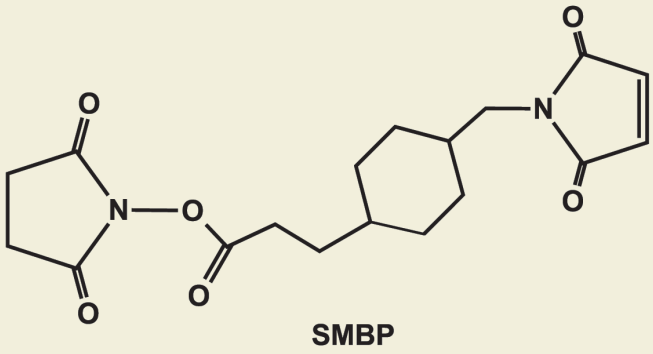
The Peptide Moiety

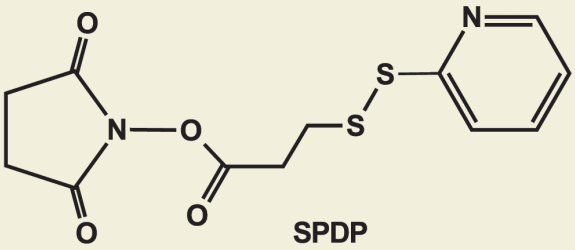
A major factor in the development of peptide-oligonucleotide conjugates was the discovery that peptides derived from certain proteins' transduction domains (PTD), called cell-penetrating peptides (CPP), can traverse the cell membrane, a process called protein transduction. The best studied PTDs are from HIV-1 Tat protein, HSV structural protein VP22 and the *Drosophila* homeoprotein antennapedia. In addition to the protein-derived CPPs, there are also artificial peptides that have been designed based on information derived from natural CPPs. These peptides are water-soluble and short – less than 30 amino acids – with the most effective being amphipathic and/or cationic. CPPs can translocate a broad variety of components covalently linked to them (cargo), such as low-medium molecular weight drugs, peptides, oligonucleotides, full size proteins, nanoparticles and liposomes. Several CPPs are arginine rich and tend to bind to nucleic acids, facilitating their transduction. The mechanism for this transduction is not known, but it seems that it does not require energy and is as efficient in living cells at 37°C as it is at 4°C. Though conformation of amphipathic peptides has been considered a factor to explain CPP's transducing properties

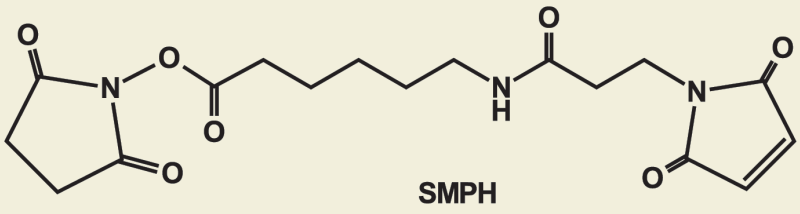
that small peptides with a minimum number of arginine and/or lysine are as efficient as large amphipathic peptides, research indicates that charge is the main characteristic of these peptides. The nature of the cargo apparently affects the mechanism of transduction, and in some cases it seems that this process can be partially mediated by endocytosis, an energy dependent process. In endocytosis an initial event appears to be the interaction between the CPP and the cell surface's glycosylaminoglycans. After the transduction across the cell membrane, which would be the first step, there is also interest in transduction across the membranes of cell organelles such as the nucleus and mitochondria: the nucleus because it contains genomic DNA, and the mitochondria due to their role in several diseases. Once the peptide-oligonucleotide conjugate is inside the cell, the nucleus can then be targeted by the use of nuclear localisation sequences (NLS): highly cationic peptides about 10 amino acids in length and with high levels of cell permeability. Although CPPs usually do not target mitochondria, targeting can be achieved by inserting certain unnatural amino acids, such as dimethyltyrosine, in the CPP or by using mitochondria leader peptides linked to low molecular weight polyethylenimine.

Figure 1: Examples of heterobifunctional crosslinkers. SMBP, SMCC and SMPH are non-cleavable linkers, while SPDP can be cleaved with thiol reagents







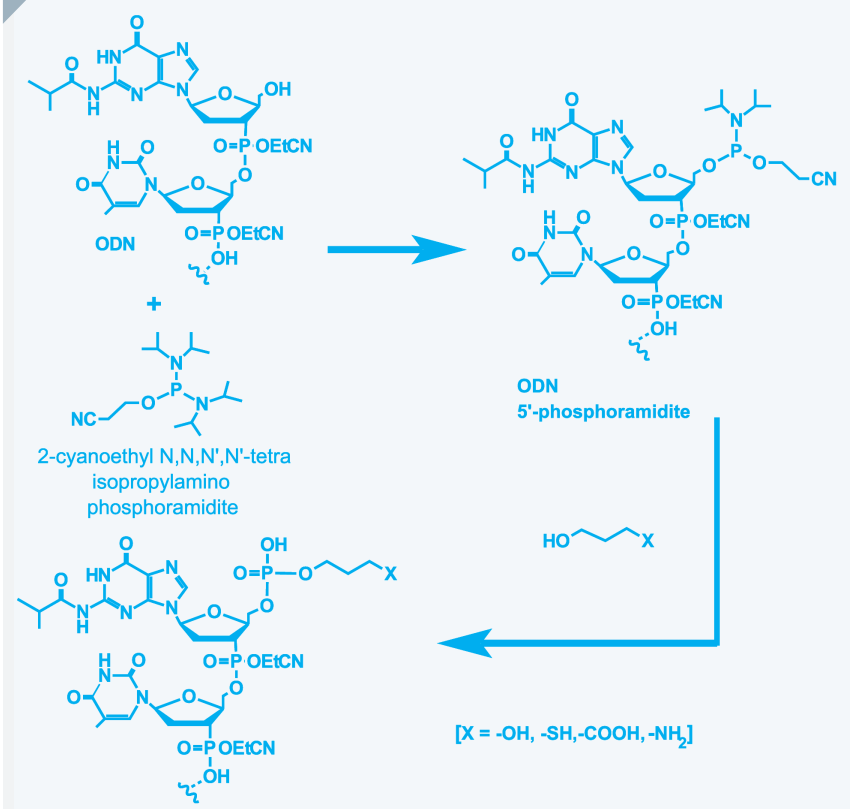


The Oligonucleotide Moiety

This moiety or cargo of the bioconjugate can be either a DNA/ODN or a RNA/ORN. Because of their anionic nature, they cannot pass across cell membranes unless they are conjugated to a CPP. Peptide-DNA conjugates are used in genetic therapy to replace a defective gene, or alternatively to inhibit the expression of a specific gene by antisense DNA. Peptide-DNA conjugates have shown higher resistance against nucleases as well as enhanced hybrid stability with ssDNA and dsDNA. Interestingly, antisense DNA-peptide conjugates have a significantly higher inhibitory activity than the unconjugated antisense DNA, chiefly against the telomerase activity. DNA can be replaced by a DNA mimic: a peptide nucleic acid (PNA) having an uncharged amide backbone to yield a PNA-peptide conjugate. These conjugates can enter live cells, target their chromosomal DNA and specifically down-regulate gene expression.

Peptide-RNA conjugates are largely used to inhibit the expression of certain genes by small interfering RNAs (siRNA) that are 20- to 25-nucleotide long double-stranded RNA. SiRNAs initiate a process that leads to the degradation of mRNA. Because of their relatively small size and capacity to block gene expression, siRNA are a potential source of new therapeutic agents. However, the capacity of siRNA to stimulate the production

Figure 2: Phosphitylated ODNs yield a 5′-phosphoramidite intermediate which is capable of reacting with a terminal hydroxyl or thiol group from either a homobifunctional or heterobifunctional linker



of pro-inflammatory cytokines may present some obstacles to its use as a therapeutic agent. Some progress in reducing its immune stimulatory properties have been achieved, firstly by avoiding the endosomes where siRNA is recognised by the innate immunity receptors TLR7 and TLR8 triggering the production of pro-inflammatory cytokines, and secondly by conjugating the siRNA to cholesterol. Another group of oligonucleotides that can be conjugated to CPPs are the locked nucleic acids (LNA), nucleic acid analogs with high affinity and specificity toward complementary DNA and RNA. An LNA is a modified ribonucleotide in which the ribose is modified by a bridge connecting the 2′ and 4′carbons. LNA nucleotides can be mixed with the nucleotides of DNA or RNA.

The Conjugation Process

Depending on the structure of the CPP and oligonucleotide moieties, cross-linking can be carried out using zero-length cross-linkers and homo- or heterobifunctional linkers. Conjugation of CPPs to oligonucleotides can be made in solution or in the solid phase. Functional groups in the CPPs suitable for conjugation are: amino groups from lysine; hydroxyl groups from serine and threonine; thiol groups from cysteine; and carboxyl groups from aspartate and glutamate. During the peptide synthesis it is also possible to add a terminal spacer carrying a functional group, such as aminohexanoate, cysteamine and others. To avoid reactions with other groups besides the terminal functional group of the peptide, the side groups are protected during the cross-linking reaction and deprotected at the end of the conjugation.

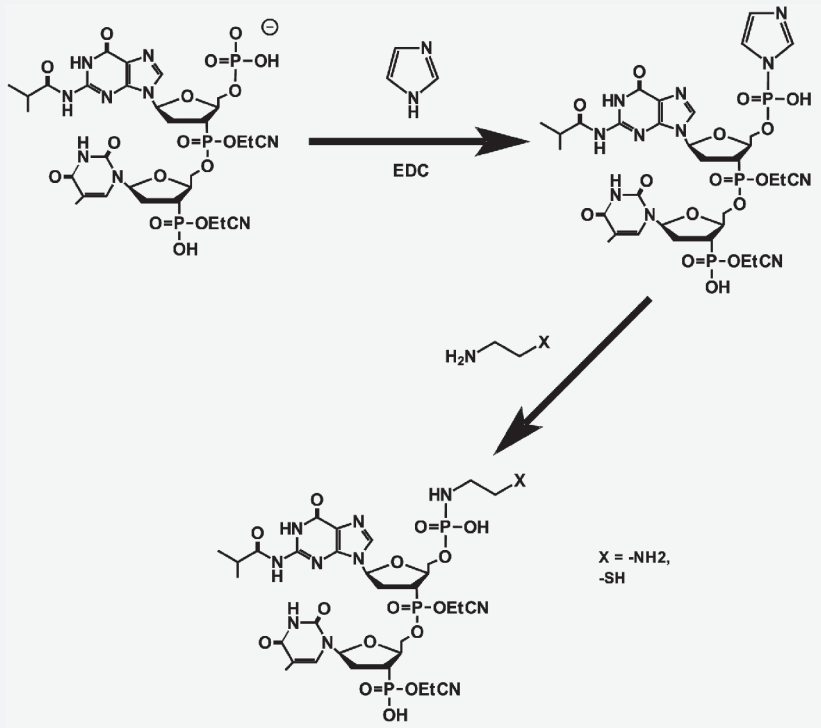
Unlike peptides, nucleic acids do not have readily reactive groups. However, it is possible to modify their bases to produce reactive derivatives capable of participating in cross-linking reactions. Reactive hydroxyl groups are found in the sugar portion of oligonucleotides: deoxyribose and ribose for ODNs and ORNs respectively. While in ODNs the available group would be the 5′ hydroxyl of the terminal deoxyribose, in ORN the terminal ribose would have a 5′ and 2′ hydroxyls while the rest of the riboses would have a free 2′ hydroxyl.

Another group available for conjugation in oligonucleotides is the single terminal 5′ phosphate group. To avoid steric hindrance during conjugation, a spacer, usually of six or more carbons in length carrying a functional group such as amino or thiol, is added to the peptide or the oligonucleotide. Covalent linking between both compounds is largely carried out using heterobifunctional cross-linkers in which some of the most common reactive groups are N-hydroxysuccinimide (NHS) ester, which reacts with primary amino groups to form an amide bond, and maleimide, which reacts specifically with thiols to yield stable thioethers, such as SMBP, SMCC and SMPH. Some cross-linkers carrying an NHS-ester group have a 2-pyridyl disulfide group at the other end that is capable of exchanging with thiols to form conjugates that have a cross-link with a disulfide bond that is cleavable by reduction with a thiol reagent such as 2-mercaptoethanol or DTT, such as SPDP.

The 5′ hydroxyl group of the ODNs' deoxyribose can be phosphitylated with 2-cyanoethyl-N,N,N′,N′ tetraisopropyl phosphoramidite to yield an ODN 5′-phosphoramidite intermediate. This intermediate can then react with a hydroxyl or thiol group of a linker that has a terminal hydroxyl, thiol, carboxyl or amino group capable of reacting with a functional group on the peptide or a heterobifunctional linker (see Figure 2).

The phosphoramidite procedure can also be applied to the 5′ hydroxyl of oligoribonucletides where the 2′ hydroxyl groups have been protected. The phosphoramidite approach has

Figure 3: Reaction of a terminal 5′ phosphate with EDC and imidazole to form a phosphorimidazolide intermediate that can react with the amine group of a homobifunctional or heterobifunctional linker, which subsequently can react with one of the groups available in a peptide



been used to introduce small molecules to ODNs such as fluorescein, biotin, anthraquinone, carbohydrates, lipids and others. However, in these cases the 5′ hydroxyl group of the ODN is reacted with the small molecule's phosphoramidite. The single terminal 5′ phosphate is another functional group suitable for conjugation. This group reacts with a water-soluble carbodiimide (EDC) and imidazole to form a phosphorimidazolide that reacts with the amine group of a homobifunctional or heterobifunctional linker, for example ethylenediamine or cysteamine, to yield a phosphoramidite bond and 5′ amino or thiol group (see Figure 3). This new 5′ group can react with a heterobifunctional linker and subsequently with one of the groups available in the peptide to yield a peptide-ODN conjugate.

Applications

Peptide-ODNs have immediate applications in gene therapy, by either introducing the correct copy of a gene, or alternatively by silencing that gene with anti-sense DNA-peptide conjugates. Targeting of the nucleus can be achieved by adding to the CPPs specific highly cationic NLS. Another cell's organelle that is important from the therapeutic point of view is the mitochondria that carry its own genetic information. Thus, abnormalities in mitochondrial function are usually the result of defective mitochondrial DNA. For instance, mitochondrial defects that have been implicated in several neuromuscular diseases (mitochondrial myopathies), diabetes mellitus, as well as several neuropathies, are all hereditary conditions. Because CPP-ODN conjugates cannot enter the mitochondria, targeting of this organelle demands some specific approaches such as the use of low molecular weight polyethylenimine linked to mitochondrial leader peptide sequences. Apparently a better penetration to mitochondria occurs with peptide-nucleic acids instead of ODNs, making PNAs an attractive alternative to develop peptide-oligonucleotides for use in these organelles. The siRNA and anti-sense DNA peptide conjugates are the focus of intense research due to their capacity to block gene expression potential therapeutic applications. Oligonucleotide conjugates with small molecules, such as reporter groups, have applications in research as well as in some diagnostic areas.

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