1

Introduction

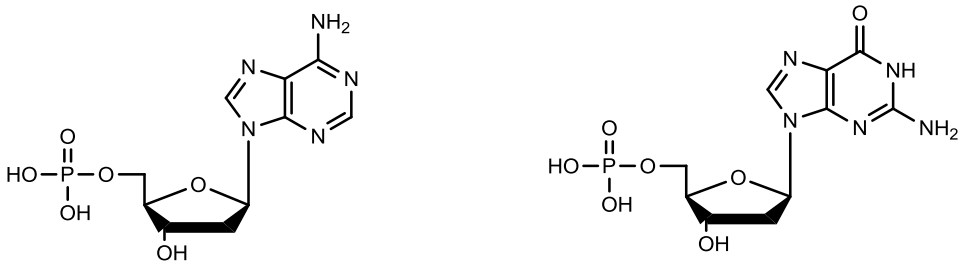
In a world where prevention is better than cure, there is an increasing need in medicine for biological probes which can detect disease states at increasingly lower detection limits, in more diverse applications. Completion of the human genome project in 2003 [1] has brought about a heightened knowledge of human genetic diseases and, as such, research into the science of deoxyribonucleic acid (DNA) for use in diagnostics has become more prevalent.

1.1 Deoxyribonucleic acid – DNA

There have been considerable advances in DNA science since research began less than 100 years ago.

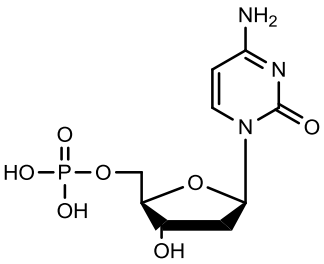
1.1.1 Primary structure of DNA

One of the first major advances in the determination of the primary structure DNA was reported by Klein and Thannhauser in 1935, [2] who discovered that DNA could be split into mononucleotides using an enzyme doped with arsenate. Their experiment produced four crystalline deoxyribonucleotides, whose specific structures were later confirmed by chemical synthesis undertaken by Todd *et al.* (**Fig. 1.1**). [3], [4], [5] The nucleotide structures are composed of a nitrogen-containing heterocyclic base, a pentose sugar and a phosphate residue.

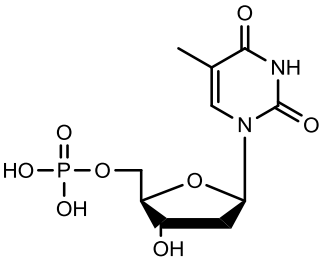


deoxyadenosine 5'- phosphate (dAMP)

deoxyguanosine 5'- phosphate (dGMP)



deoxycytosine 5'- phosphate (dCMP)



deoxythymidine 5'- phosphate (dTMP)

Figure 1.1 – *Structures of 5'- deoxyribonucleotides*

The nitrogenous bases of the deoxyadenosine and deoxyguanosine 5'- phosphates are purines, whilst the nitrogenous bases of the deoxcytosine and deoxythymidine 5'- phosphates are pyrimidines.

As well as the 5'- phosphates, Todd *et al.* were also able to synthesise the 3'- phosphates of the four deoxyribonucleotides. As a result of Klein and Thannhauser's original work it was thus possible to establish that the primary structure of DNA takes the form of a linear polynucleotide, in which each deoxyribonucleotide is attached to the next *via* a 3' – 5' phosphodiester linkage. [6] The variation in DNA that is important for the transfer of genetic information arises from the sequence order of the four deoxyribonucleotides.

1.1.2 Secondary structure of DNA

Enzymatic degradation of DNA undertaken by Edwin Chargaff in 1950, revealed that there existed a universal 1:1 ratio of adenosine to thymidine deoxyribonucleotides and, similarly, of guanosine to cytosine deoxyribonucleotides, regardless of the source from which the DNA was extracted. [7] This led to Chargaff's Rule of Base Pairing which states that, in any piece of DNA, the proportion of purines is always equal to the proportion of pyrimidines.

Probably the most famous discovery in the history of DNA followed in 1953. Using X-ray diffraction data (**Fig. 1.2**), reported by Wilkins [8] and Franklin, [9] Watson and Crick were able to deduce that DNA exists as a right-handed, double-stranded helix in which the two strands comprising the four deoxyribonucleotides run in opposing directions (antiparallel). [10] They proposed that the organic nitrogeneous bases lie on the inside of the helix, composing a hydrophobic core, while the charged phosphate groups of the 3'- to 5'- phosphodiester linkages comprise the water soluble outer 'backbone' of the helix (**Fig. 1.3**).

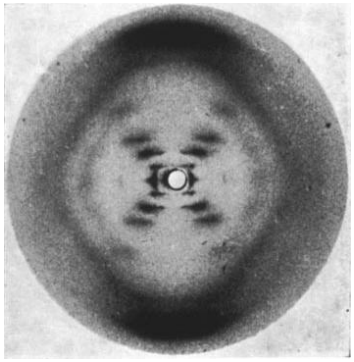


Figure 1.2 – *X-ray image of DNA as reported by Franklin. Reprinted by permission from Macmillan Publishers Ltd: Nature, Franklin, R.E., Gosling, R.G., 1953, 171, 740-74, copyright 1953*

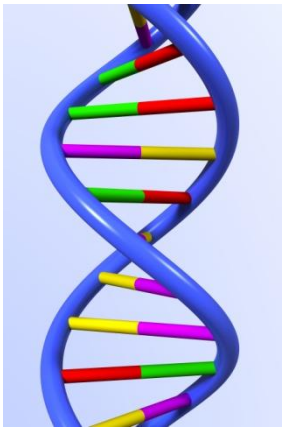


Figure 1.3 – *Schematic of DNA double helix, showing the hydrophilic phosphate backbone (blue) and the hydrophobic core of the four nitrogeneous bases (red, green, yellow, purple). Courtesy of the Royal Society of Chemistry.*

Watson and Crick anticipated that the two deoxyribonucleotide strands in the double helix were held together by way of hydrogen bonds formed between pairs of purine and pyrimidine bases in the plane perpendicular to the molecular axis. For bonding to occur one of the pair must be a purine and the other a pyrimidine. Assuming that the bases are structured in their tautomeric forms, it follows that adenine must bond to thymine and guanine must bond to cytosine (**Fig. 1.4**). Adenine pairs with thymine through two hydrogen bonds and guanine pairs with cytosine through three hydrogen bonds. The specific pairing of the bases in this manner is supported by Chargaff's Rule of Base Pairing. [7]

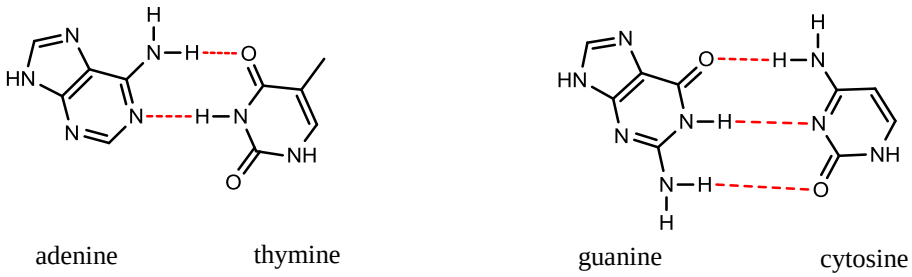


Figure 1.4 – *Hydrogen bonded base pairs adenine and thymine and guanine and cytosine, as proposed by Watson and Crick*

Watson and Crick's proposed structure supported the ability of DNA to self-replicate and, as such, pass on genetic information. From his discovery, Crick was able to postulate the 'central dogma of molecular biology' whereby the transfer of sequence information occurs from DNA to protein and not from protein to DNA as was originally thought. [11] The Watson and Crick model is now the universally accepted model for the secondary structure of DNA.

1.1.3 Synthetic DNA – a brief history

Elucidation of the structure of DNA naturally led to an interest in finding a method for the chemical synthesis of an oligodeoxyribonucleotide (hereafter simplified to oligonucleotide); a single strand of DNA containing two or more deoxyribonucleotides linked together.

The first chemical synthesis of an oligonucleotide with a natural 3' → 5' internucleotide linkage was reported by Michelson and Todd in 1955, who described the synthesis of a dithymidine nucleotide. [12] A few years later, a further development in the chemical synthesis of oligonucleotides was reported by Khorana *et al*. [13] A method of synthesis was described, which became known as the phosphodiester approach, and involved the direct coupling of deoxyribonucleotides, *via* their phosphate groups, using a coupling agent. Khorana's approach brought about an important innovation in oligonucleotide synthesis which allowed synthesis of longer nucleotide chains – the development of base protecting groups. [14] Whilst Michelson and Todd's synthesis of a dithymidine nucleotide required no base protecting groups, in the cases of adenine, cytosine and guanine the exocyclic amino groups on the nitrogeneous bases are vulnerable to reaction with activated phosphates, to form phosphoroamidates. As such, protecting groups for these functionalities had to be considered. Adenine and cytosine were protected as their 6-*N*-benzoyl and 4-*N*-(*p*-anisoyl) derivatives respectively, while guanine was protected as its 2-*N*-isobutyryl derivative (**Fig. 1.5**). These protecting groups are all base labile.

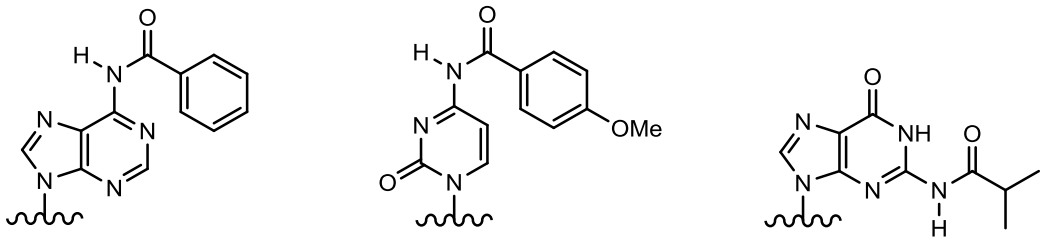


Figure 1.5 – *Protected adenine, cytosine and guanine bases for phosphodiester oligonucleotide synthesis*

A second important development in Khorana's phosphodiester approach was protection of the 5'- hydroxyl functionality of deoxyribonucleotides. [15] It was found that the classical triphenylmethyl (trityl) hydroxyl protecting group required strong acidic conditions for its removal, resulting in cleavage of the glycosyl bonds of the purine rings of adenosine and guanosine. As such, it was replaced with the more acid labile monomethoxytrityl (MMTr, *ca.* 10 x lability) and dimethoxytrityl (DMTr, *ca.* 100 x lability) moieties. This approach to 5'- hydroxyl nucleotide protection is central to automated oligonucleotide synthesis methods used today, where the acid-promoted cleavage of the 5'- hydroxyl protecting group is orthogonal to the base-promoted cleavage of the base protecting groups. This allows for a cyclic synthesis; whilst the 5'- hydroxyl protecting group requires removal in each cycle for linkage to the next deoxyribonucleotide in a sequence, for the base protecting groups to be effective, they should not be removed until the end of the total synthesis.

Khorana *et al.* also noted that cleavage of the methoxytrityl moieties was chromatographically detectable by a characteristic colour change and, in the case of DMTr protection, was quantifiable. [15] Indeed, quantification of DMTr cleavage by colorimetric detection is used in automated oligonucleotide synthesis today.

The phosphodiester approach dominated oligonucleotide synthesis for many years. Applying his methodology Khorana was able to report the first total synthesis of a gene in 1979. [16]

Some twenty years later, Letsinger and Lunsford reported a further innovation that is key to oligonucleotide synthesis methods used today. [17] They described the covalent linkage of deoxyribonucleotides in the absence of a coupling agent, using

nucleoside phosphordichloridites. These modified nucleotides were more reactive than those used previously since phosphorous exists in the P $^{(III)}$ state versus the traditional, more stable P $^{(V)}$ phosphoryl state. *In situ* oxidation of the oligodeoxyribonucleotides in iodine and water then generated the natural phosphoryl internucleotide linkage. The phosphordichloridites reported were, however, unstable to hydrolysis and oxidation in air. The instability of nucleoside phosphites was later overcome by Beaucage and Caruthers, with the development of stable nucleoside phosphoramidites, which could be stored as dried powders. [18] They also introduced a phosphoramidite activation step for formation of internucleotide phosphite linkages, using the mildly acidic 1 *H*-tetrazole.

2-cyanoethyl [19] -*N*, *N*-diisopropyl [20] phosphoramidites (**Fig. 1.6**) are now almost exclusively used in today's phosphoramidite-based solid phase oligonucleotide synthesis. The β-cyanoethyl phosphite protecting group is easily removed by treatment with concentrated aqueous ammonia, which is also used for cleavage of the nucleoside exoycyclic amino protecting groups, thereby simplifiying deprotection of chemically synthesised oligonucleotides to one step.

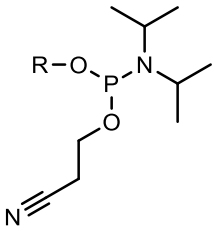


Figure 1.6 – *2-cyanoethyl-N, N-diisopropyl phosphoramidite, where R = deoxyribo-nucleotide*

1.1.4 Solid phase oligonucleotide synthesis

In the last two decades, almost all chemical synthesis of oligonucleotides has been achieved by solid phase synthesis [21] – the heterogeneous coupling of a protected deoxyribonucleotide in solution to a second deoxyribonucleotide, or oligonucleotide immobilised on a solid support. Solid phase synthesis allows the sequential addition of reagents in excess; all unreacted material being easily removed by a washing step such that only the oligonucleotide bound to the solid support is available for reaction

in the next step of the synthesis. A high yield of pure oligonucleotide is achieved by this technique – the yield of each coupling step is approximately 99%, such that:

$$\text{total oligonucleotide yield} = (0.99^{x}) \times 100$$

Where x = the number of coupling steps required for synthesis of a selected oligonucleotide sequence.

Solid phase synthesis is a fast, efficient process that can be automated, allowing for synthesis of both small quantities of oligonucleotides for biological research and multigram quantities of oligonucleotides for therapeutic applications.

1.1.4.1 Immobilisation onto the solid support

The solid support of choice for automated oligonucleotide synthesis is commonly controlled pore glass (CPG), functionalised with a long-chain alkylamine. [22] Solid phase oligonucleotide synthesis occurs in the 3' → 5' direction. As such, the first deoxyribonucleoside of a sequence, fully protected, is attached to the CPG solid support *via* its 3'- hydroxyl group. Immobilisation onto the support is achieved by formation of an ester linkage, *via* coupling of deoxyribonucleoside 3'-*O*-succinates to the amino substituted CPG beads. Oligonucleotide synthesis actually occurs within the regularly-sized pores of the glass beads, rather than on the surface. The solid support is contained in a column which can be placed on the automated synthesiser.

1.1.4.2 Oligonucleotide synthesis cycle

Solid phase oligonucleotide synthesis occurs in a four step cycle (**Fig. 1.7**):

1. Deblocking
2. Coupling
3. Capping
4. Oxidation

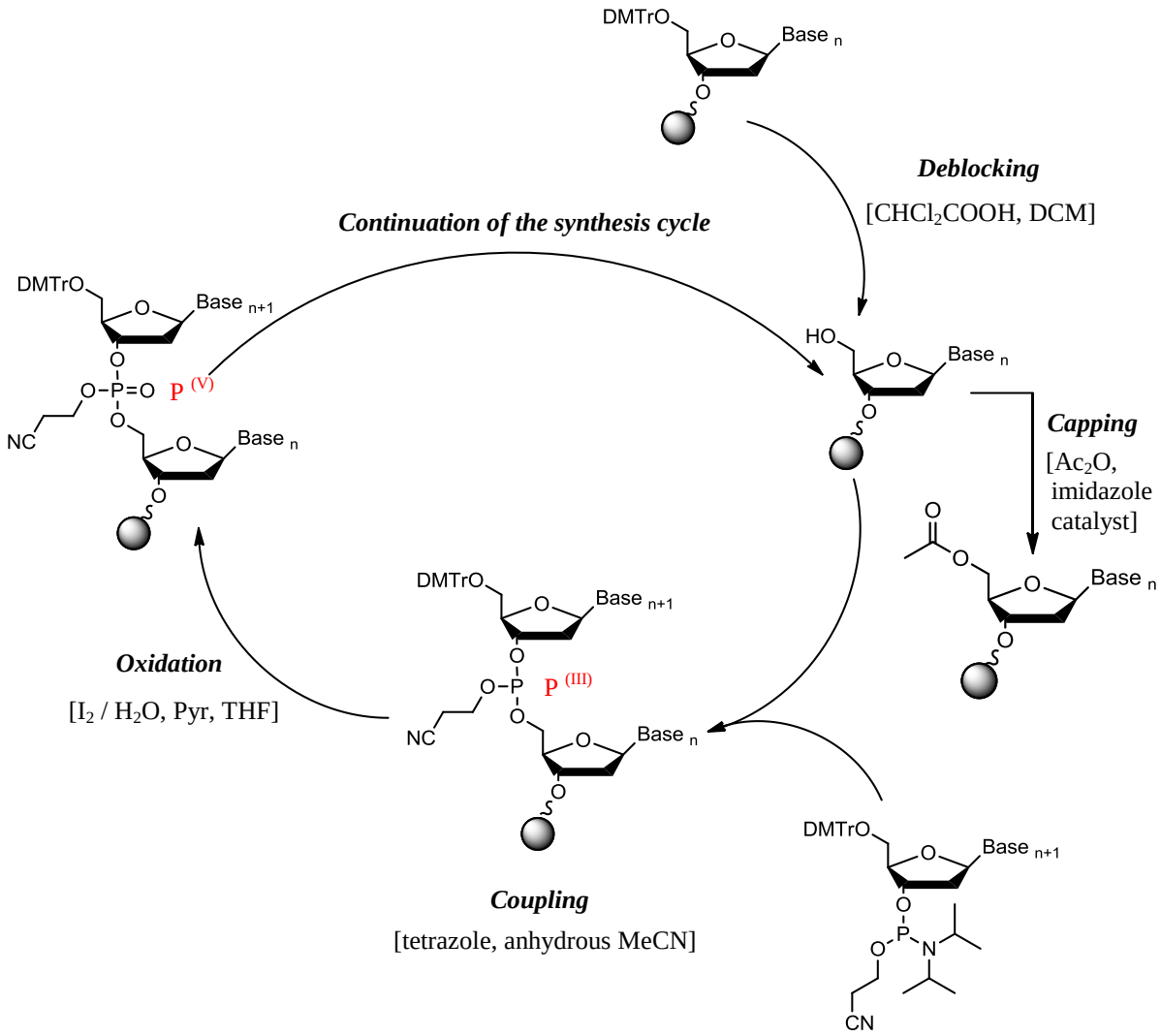


Figure 1.7 – *Solid phase oligonucleotide synthesis cycle by the phosphoramidite method*

1. Deblocking

The first step in the oligonucleotide synthesis cycle is removal of the 5'- hydroxyl DMTr protecting group. The ether linkage undergoes acid cleavage, by treatment with dilute dichloroacetic acid in dichloromethane. The free dimethoxytrityl cation formed in this process is a strong chromophore, with a vivid orange colour. By monitoring with a spectrophotometer at 498 nm (λ_{max}) it is possible to obtain an estimate of the efficiency of the previous coupling reaction in the cycle.

2. Coupling

The formation of the phosphite linkage between two deoxyribonucleosides is known as the coupling step. The free 5'- OH of the CPG bound deoxyribonucleoside is linked to the 3'- hydroxyl group of the next nucleoside in the sequence. This next nucleoside is added in the form of a phosphoramidite, previously generated *via* phosphitylation of the 3'- OH. The incoming phosphoramidite is activated for coupling using a tetrazole based catalyst, [18] which converts the *N*, *N*-diisopropyl group of the phosphoramidite into a good leaving group. Nucleophilic attack by the 5'- OH of the CPG bound nucleoside is directed towards $P^{(III)}$ of the incoming phosphoramidite monomer, resulting in formation of the dinucleoside phosphite.

3. Capping

As mentioned previously, the coupling step of solid phase oligonucleotide synthesis is only approximately 99 % efficient. Therefore, a small amount of the preceding deoxyribonucleoside, with unreacted 5'- OH functionality, remains bound to the solid support. Chain elongation from these unreacted nucleosides in the next coupling step of the synthesis leads to unwanted sequences and thereby increased difficulty in purification. Therefore, the free hydroxyl groups are "capped" by acylation with acetic anhydride, using an *N*-methylimidazole catalyst. [23] Excess reagents are removed by washing.

4. Oxidation

The coupling step of solid phase oligonucleotide synthesis results in formation of an unstable phosphite triester ($P^{(III)}$). Iodine, in a mixture of water and THF, is used to oxidise the phosphite triester to a stable phosphotriester ($P^{(V)}$). Pyridine, or 2, 6-lutidine is used to neutralise the HI formed in this process.

Following oxidation, the cycle is repeated, with addition of one deoxyribonucleotide to the sequence until the sequence of desired length has been synthesised.

1.1.4.3 Cleavage and deprotection

The final stage in solid phase oligonucleotide synthesis is cleavage from the solid support and removal of the protecting groups. Both are achieved simultaneously by treatment with concentrated aqueous ammonia. The synthesised oligonucleotide is released quickly from the solid support by ester hydrolysis; cleavage of the base protecting groups by hydrolysis and the β-cyanoethyl phosphate protecting groups by β-elimination takes a greater length of time – 40 ^{o}C, overnight or 2 days at room temperature.

1. 1.5 Chemical modification of DNA

The methodology for solid phase oligonucleotide synthesis allows for chemical modification of DNA. A variety of moieties (reporter groups such as fluorophores and biotin, intercalators, enzymes etc.), for the purposes of diagnostics or therapeutics, can either be attached to synthetic oligonucleotides *via* a specific functional group incorporated during chemical synthesis or directly incorporated into oligonucleotides *via* functionalised phosphoramidites. Suitable positions for oligonucleotide modification are the 5'- and 3'- termini, the C-5 position of a mid-sequence pyrimidine, or the exocyclic amino group of cytosine. For the purposes of this work, only 5'- and mid-sequence modification will be discussed.

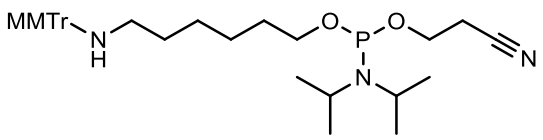


Figure 1.8 – *Commercially available amino linker phosphoramidite (Link Technologies)*

Modification at the 5'- terminus is the simplest method of oligonucleotide modification and requires a specially functionalised phosphoramidite that can be added in the final coupling step of automated oligonucleotide synthesis. Phosphoramidites for this purpose are commercially available, such as that shown in **Fig. 1.8**. Post oligonucleotide synthesis, treatment with acid removes the monomethoxytrityl (MMTr) protecting group, generating an aminohexyl phosphate at the 5'- terminus of an oligonucleotide which can then be coupled, to an acid derived moiety for oligonucleotide modification. It is also possible to directly modify an oligonucleotide sequence at the 5'- terminus, by synthesis of a phosphoramidite for 5'- modification *via* phosphitylation of a hydroxyl functionality contained in component to be incorporated into the sequence.

Mid-sequence modification of oligonucleotides is rather more complex. A modified deoxyribonucleoside phosphoramidite must be synthesised, involving functionalisation at the C-5 position of a pyrimidine base, protection of the 5'- OH of the sugar and phosphitylation at the 3'-OH for synthesis of the phosphoramidite. The modified nucleoside is incorporated into an oligonucleotide sequence by addition in an interim cycle in standard solid phase oligonucleotide sequence. A number of modified nucleosides are commercially available for this purpose. Among several applications, modified nucleosides can be used to increase the stability of a DNA duplex. [6]

1.1.6 Modified oligonucleotides for use as bioanalytical probes

The use of modified oligonucleotides for the detection of specific DNA sequences, based on the hybridisation of an oligonucleotide target with its complementary probe, is well documented.

DNA hybridization can be detected optically using a variety of techniques, including fluorescence. [24] for review However, most fluorescent DNA biosensors require surface immobilisation for specific sequence detection, to allow removal of any unhybridized probe sequences by washing for an accurate diagnosis. This thereby limits their use for sequence detection in cell samples.

One type of bioanalytical probe that employs fluorescence as a method for detection is the molecular beacon, first reported by Tyagi and Kramer. [25] A molecular beacon is a single-stranded oligonucleotide sequence that possesses a stem and loop structure (**Fig. 1.9**). The loop section contains a sequence complementary to the target; the stem comprises two complementary 'arm' sequences, hybridized to one another. Attached to one arm is a fluorescent moiety, while attached to the second arm is a non-fluorescent quenching moiety. The close proximity of these components, brought about by the hybridized stem, is such that the fluorescence of the fluorophore is quenched by fluorescence energy resonance transfer (FRET). [26] However, when the molecular beacon is hybridized to the target sequence, the hybrid formed is longer and more stable than the stem hybrid, thereby forcing the beacon to open and the arm sequences to move apart to reveal a fluorescent signal.

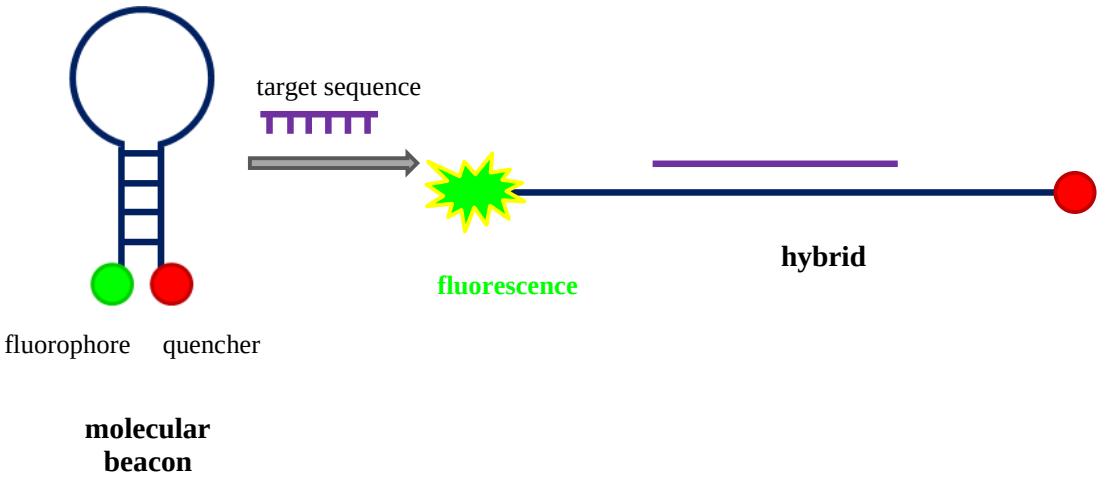


Figure 1.9 – *Schematic shown fluorescence detection by a molecular beacon of a target DNA sequence*

Using molecular beacons, Tyagi and Kramer were able to perform real-time monitoring, in solution, of the polymerase chain reaction (PCR) and proposed the use of molecular beacons for specific sequence detection in living cells. However, the cellular uptake of molecular beacons still remained an issue. Nitin and co-workers have since conjugated molecular beacons to a cell penetrating (Tat) peptide to enhance cellular uptake, for the detection of mRNA sequences in human fibroblasts. [27]

1.2 Cell penetrating peptides

Recent studies have uncovered the existence of certain proteins, including HIV-1 Tat transactivation protein, [28] *Drosophila* Antennapedia homoprotein [29] and the HSV-1 structural protein, VP22, [30] which have been shown to have the ability to traverse the cell membrane and reach the cell nucleus, whilst maintaining their biological activity. Their ability to do this has been attributed to short 'protein-transduction domains' (PTDs). [31], [32] It has since been reported that short peptide sequences derived from these PTDs have been internalised by cells and, when complexed or conjugated to bioactive molecules, have been able to deliver these molecules into the intracellular environment. [33] Such peptide sequences have been classified as cell penetrating peptides (CPPs). Further CPPs have been developed, whose design has not been based on PTDs. These include amphipathic peptides, [34] polycationic and guanidine-rich peptides such as polyarginine, [35], short arginine oligomers, [36] calcitonin-derived peptides [37] and peptide nucleic acid (PNA) internalisation peptides, otherwise known as Pip. [38] CPPs have shown delivery of a wide range of bioactive components into cells, including proteins and peptides, [33], [39] oligonucleotides, [40] PNA [41] and nanoparticles. [42]

1. 2.1 Tat peptide

The CPP discussed for the purposes of this research is Tat peptide, derived from the human immunodeficiency virus (HIV)-1 transcription-transactivating (Tat) protein, which is necessary for virus replication. [33] The Tat protein is 101 amino acids in length and comprises three functional domains: an acidic *N*-terminal region

required for its transactivation activity, a cysteine-rich DNA binding domain and a basic domain with nuclear localisation properties. [43] An important characteristic of the Tat protein is its ability, following secretion from virus-infected cells, to cross the plasma membrane of neighbouring cells. [44] Investigations of Tat proteins have shown their basic domains to be responsible for their cell penetrating properties, with the minimal sequence required for membrane translocation being confined to the 49-57 region: RKKRRQRRR. [31] This region incorporates eight basic amino acids. Tat peptide derivatives used for conjugation to biological cargo are based around this amino acid 9-mer, with other amino acid residues or functionalities attached to the *C*- and *N*- termini.

Tat peptides are used for transfer of a broad variety of biological macromolecules across cell membranes. Most often, they are used for cellular internalisation of oligonucleotides, *via* covalent oligonucleotide Tat peptide conjugates. [45]

1. 2.2 Cellular uptake mechanism of Tat peptide

There has been much debate around the mechanism(s) responsible for cellular uptake of CPPs and indeed the mechanism(s) remain controversial. A broad range of literature exists on this topic. The most appropriate conclusion to draw from these reports would appear to be that there is no single definitive mechanism and that cellular uptake varies with CPP type, its cargo and the cell type being studied.

Some studies into the translocation mechanism of CPPs, including Tat peptide, have shown that cellular uptake is not inhibited by low temperature, depletion of the cellular ATP pool or by endocytosis inhibitors, [31], [46] thereby indicating an energy-independent, non-endocytic mechanism of entry. Based on such observations, a proposed cellular uptake mechanism is adsorption of Tat peptide onto the plasma membrane, brought about by electrostatic interaction of the cationic peptide residues with the anionic charges of the phospholipid head groups, followed by formation of an inverted micelle, [46] in which the peptide and any cargo are internalised into the cell within a fully hydrophilic pocket before release into the intracellular environment (**Fig. 1.10**).

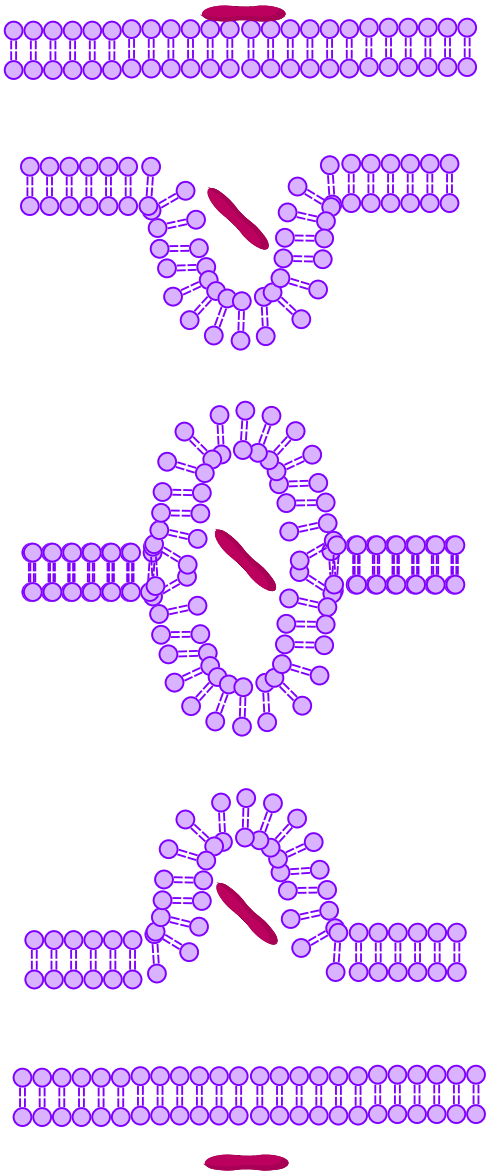


Figure 1.10 – *Schematic showing electrostatic absorption of a CPP onto the phospholipid cell membrane, followed by internalisation inverted micelle formation* [46]

A further proposed non-endocytic mechanism for cellular uptake of the Tat protein is direct penetration of the phospholipid bilayer, [47] initiated by interaction of the localised cationic charge of the peptide's PTD with the anionic charges of the phospholipid head groups. Following this, it is thought that the protein then passes through the cell membrane in an unfolded state and then refolds to its active form once inside the cell.

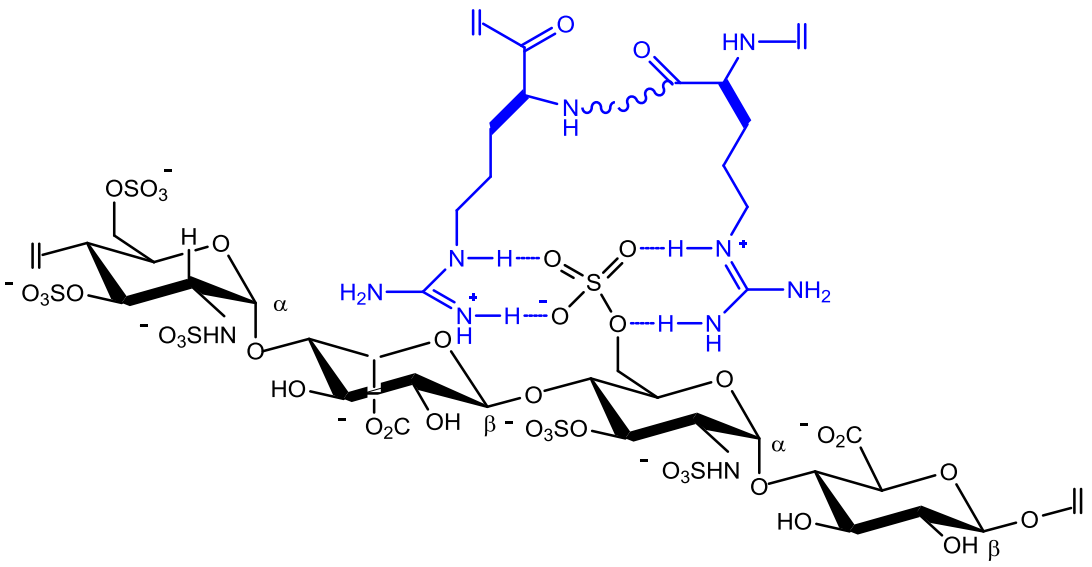


Figure 1.11 – *Schematic showing possible hydrogen bonds between two guanidinium groups of Tat peptide (**blue**) and a sulfuryl group of heparin sulfate (**black**)* [50]

Substitution of L-amino acids for D-amino acids has been shown to have no detrimental effect on internalization of CPPs, [46] indicating a non-receptor dependent mechanism for cellular uptake. However heparan sulfate proteoglycans, cell surface receptors expressed in most cell types, have been implicated in cellular uptake of Tat peptide. [48], [49] Internalisation of Tat peptide was shown to be inhibited on addition of heparin and on incubation with heparan sulfate-deficient cell lines. It has been proposed that the amino acid arginine, in which Tat peptide is rich, is a key component in binding of the peptide to these cell surface receptors, with the suggestion that the guanidinium functionality of arginine is involved in hydrogen bonding to the sulfuryl groups of the heparan sulfate cell receptors (**Fig. 1.11**). [50]

There *is* also evidence for membrane translocation of Tat peptide by endo-cytosis. [51] The majority of investigations into cellular uptake of CPPs are based on determination of cellular localisation of the peptides, or their cargo, by fluorescence analysis. Studies into artifacts arising during sample preparation and analysis bring into question non-endocytic cellular uptake mechanisms. Cell fixation has been shown to disrupt membrane barrier function, resulting in artifactual cellular uptake of fluorescein tagged Tat peptide. It has also been reported that fluorescence-activated cell sorter (FACS) analysis of cell studies with CPPs does not discriminate between membrane-bound and internalised fluorochrome. A trypsin digestion of cells,

following their incubation with fluorescein tagged Tat peptide, was performed as a method for removal of any surface-bound peptide. This showed that cellular uptake of the fluorescent peptide was significantly inhibited following incubation at a low temperature. A second fluorescence study showed, post trypsin digestion, that ATP depletion also reduced cellular uptake of the labelled Tat peptide, suggesting an energy-dependent, endocytotic pathway for cellular uptake.

There is further evidence in the literature for endocytosis as a mechanism for cellular uptake of Tat peptide. Studies have indicated that cellular uptake can occur by more than one endocytic pathway; macropinocytosis, [52), (53] clathrin-dependent endocytosis [54] and lipid raft-mediated caveolar endocytosis [55] have all been implicated in studies of the Tat peptide cellular internalisation mechanism.

1.3 Oligonucleotide peptide conjugates

As mentioned previously, use of oligonucleotides for diagnostic studies in living cells is limited by their poor cellular uptake. Translocation of oligonucleotides across cell membranes for delivery into common laboratory cell lines in culture (such as HeLa cells) has previously been achieved by complexation with cationic lipids; [56] however there are problems with cytotoxicity and stability associated with this method. [57), (58] An improved approach to enhancing cellular uptake efficiency of oligonucleotides can be achieved by their covalent linkage to certain peptide carriers. [59]

Chemical synthesis of both oligonucleotides and peptides is well established and includes solid-phase methodologies that can be automated. However, the respective chemistries of peptide and oligonucleotide synthesis are, for the most part, incompatible. This can be mostly attributed to the complexity of peptide synthesis, brought about by the diverse physical and chemical properties of the amino acid peptide building blocks. As such, the preparation of oligonucleotide peptide conjugates (OPCs) is a challenge.

There are numerous methods for the chemical synthesis of such oligonucleotide peptide conjugates (OPCs) reported in the literature. These can be separated into two key approaches – divergent methods and convergent methods.

1. 3.1 Chemical synthesis of OPCs by divergent methods: stepwise solid phase synthesis

This approach involves the sequential solid phase synthesis of the required peptide and oligonucleotide sequences on an automated synthesiser. Generation of OPCs can be achieved either by 'peptide first – oligonucleotide next' or 'oligonucleotide first – peptide next' methods. More frequently, the former methodology is adopted because this avoids exposure of the acid-labile oligonucleotides to the harsh conditions of peptide synthesis that can cause oligonucleotide cleavage and/or depurination. Peptide assembly is the first step, using Fmoc or Boc chemistry, followed by oligonuceotide synthesis as the next step, using phosphoramidite chemistry. [60] Stepwise solid phase synthesis of an OPC was first reported by Haralambidis *et al*. [61] $(Ala\text{-}Lys)_5$-Ala peptide was assembled first on CPG support then the immobilised peptide was functionalised with a hydroxyl group at the *N*-terminus using a protected α, ω-hydroxycarboxylic acid linker molecule (**Fig. 1.12**).

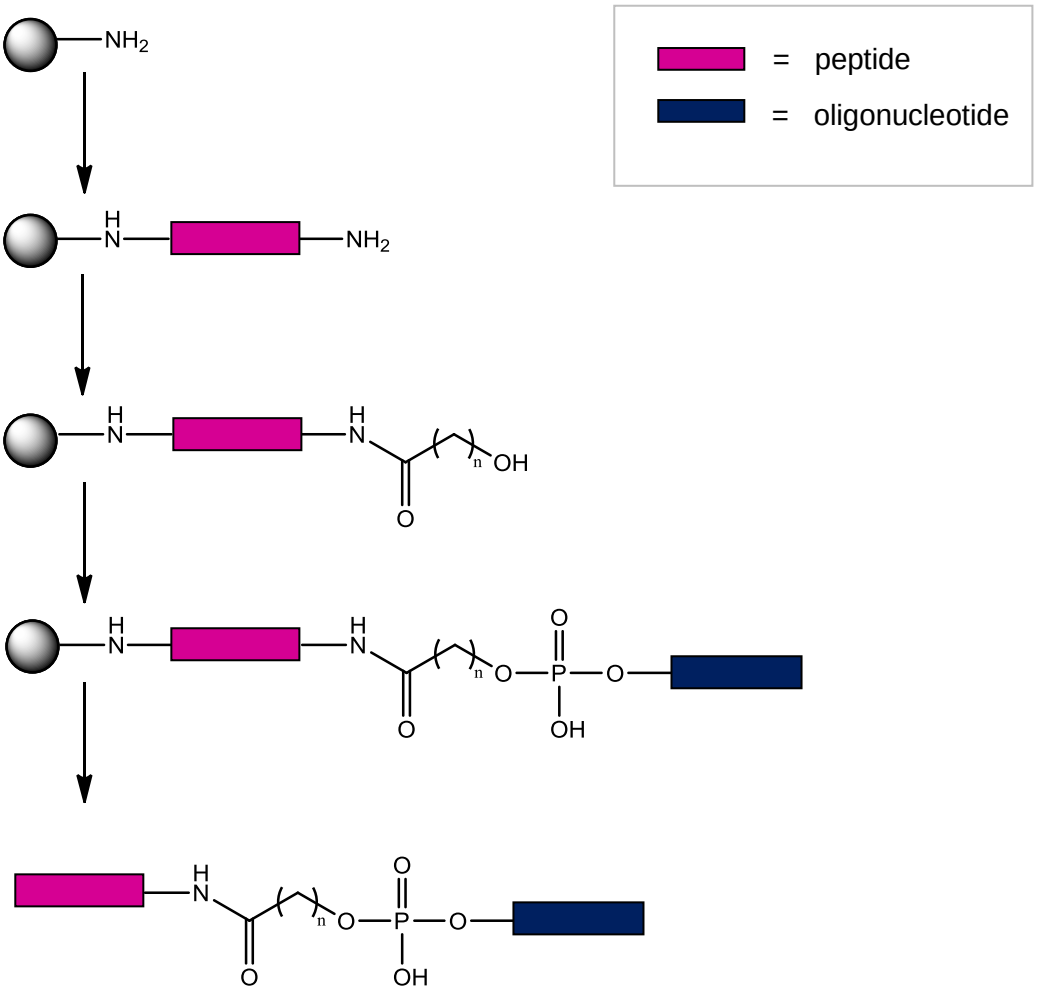


Figure 1.12 – *Schematic representation of first stepwise solid phase synthesis of an OPC, as reported by Haralambidis et al.* [61]

Following removal of the protecting group, the free hydroxyl group was reacted with the first nucleoside phosphoramidite of the oligonucleotide sequence then the remainder of the oligonucleotide sequence was assembled by phosphoramidite chemistry. Cleavage from the solid support by treatment with aqueous ammonia afforded the conjugate.

A second method for stepwise solid phase synthesis of OPCs uses an orthogonally protected, bifunctional branched linker molecule attached to a solid support. One of the most commonly used bifunctional linkers is lysine. De la Torre and co-workers report the use of a lysine functionalised PEG-PS support for OPC synthesis, [62] whereby the α-amino functionality of the amino acid is protected by a Boc group and the ε-amino functionality is protected by an Fmoc group (**Fig. 1.13**).

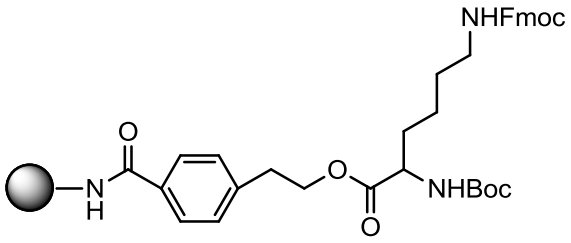


Figure 1.13 – *Lysine functionalised resin as a bifunctional linker for OPC synthesis, reported by de la Torre et al.* [62]

The peptide was assembled first, using standard Boc chemistry. Following deprotection of the ε-amino functionality, the *N*-terminus of lysine was modified with a spacer containing a DMTr-protected group, upon which standard phosphoramidite chemistry was performed for assembly of the oligonucleotide.

The main difficulty that must be overcome for stepwise solid phase synthesis of OPCs is in finding the correct combined protecting group strategy for both peptide and oligonucleotide synthesis. The harsh acidic conditions required in peptide synthesis for cleavage of the amino acid protecting groups by treatment with TFA are unsuitable to oligonucleotides while peptides may be unstable to ammonia deprotection used in oligonucleotide synthesis.

As a result, synthesis of OPCs must be either limited to incorporation of peptides composed of amino acids compatible with base-mediated deprotection and/or amino acids requiring no side chain protecting groups or new protecting groups and deprotection strategies must be developed. [63] For this reason, a more common approach to synthesis of OPCs is by convergent methods, whereby conjugation of the oligonucleotide and peptide sequences is performed post-synthetically.

1.3.2 Convergent methods: fragment conjugation

This approach avoids the difficulties posed by the incompatibility of oligonucleotide and peptide synthesis and deprotection chemistries. The peptide and oligonucleotide moieties are assembled separately on their respective solid supports

with functionalisation of each biomolecule in such a way that, following cleavage and deprotection, the two can be conjugated through their functionalities *via* a selective reaction. The point of conjugation in peptides can be at either the *C*- or the *N*-terminus, or on the amino acid side chains; the point of conjugation in oligonucleotides can be at either the 5'- or the 3'- terminus, on a mid-sequence base or on an internucleoside linkage. [60] Fragment conjugation can be performed either on a solid support, or in solution (**Fig. 1.14**).

A variety of chemical linkages have been used for fragment conjugation including disulfide, thioether, amide, urea, oxime, hydrazone and thiazolidine linkages. [60] for review Cycloaddition reactions have also been reported for linkage of oligonucleotides to peptides. For the purposes of this research, only linkages formed by solution phase fragment conjugation will be examined in detail on the proceeding pages.

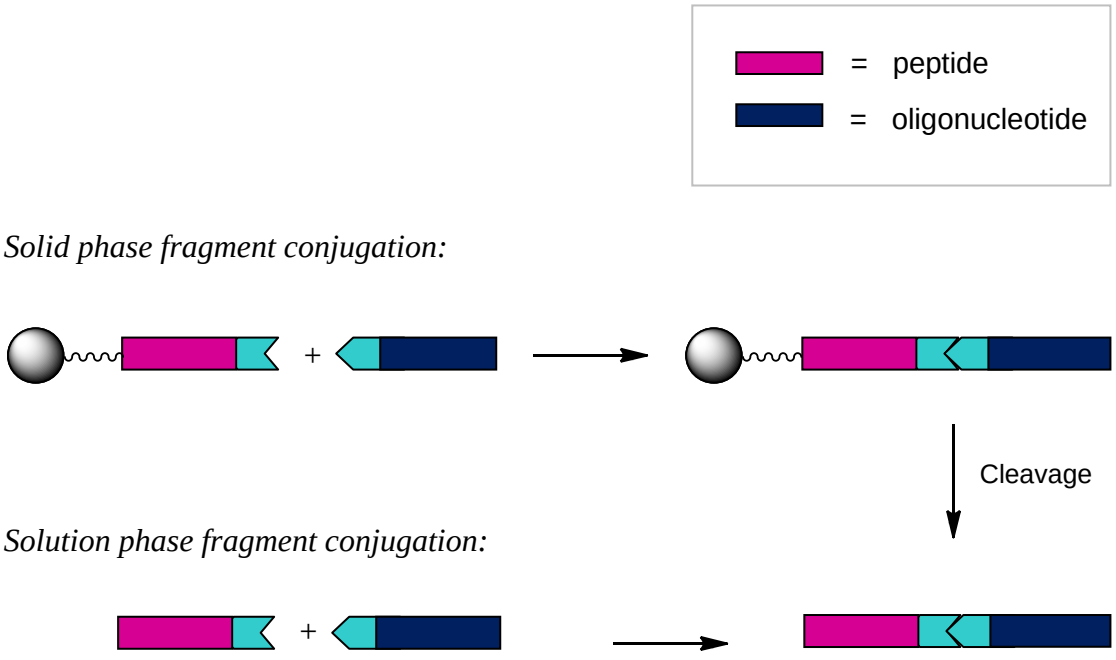


Figure 1.14 – *Schematic representation of solid and solution phase fragment conjugation*

1.3.2.1 Disulfide linkage

The disulfide linkage is frequently used for oligonucleotide conjugation to peptides. There are two main approaches to disulfide conjugation: [60] direct coupling of oligonucleotide and peptide thiols using a suitable reducing agent or thiol activation of either the oligonucleotide or the peptide fragment, followed by coupling to the second fragment (**Fig. 1.15**). Thiol activation is usually achieved by reaction with either bis-2, 2'-pyridylsulfide (Pys) [64] or bis-3-nitro-2, 2'-pyridylsulfide (Npys). In fact, the first reported OPC was synthesised by NPys activation of the *N*-terminus cysteine of the peptide fragment, followed by reaction with the 5'- thiolated oligonucleotide fragment, in the presence of TEAA. [65]

Disulfide linkage affords OPCs with simple peptides in high yields which can be purified easily by reverse-phase high performance liquid chromatography (RP HPLC). [65] However, oligonucleotide conjugation by disulfide linkage to highly cationic peptides results in precipitation of the reaction mixture, brought about by complexation of the two fragments through electrostatic interactions. [66] This can be counteracted by the introduction of denaturing agents [64] or high salt concentrations [66] to the reaction mixture; HPLC purification of such OPCs also requires denaturing conditions.

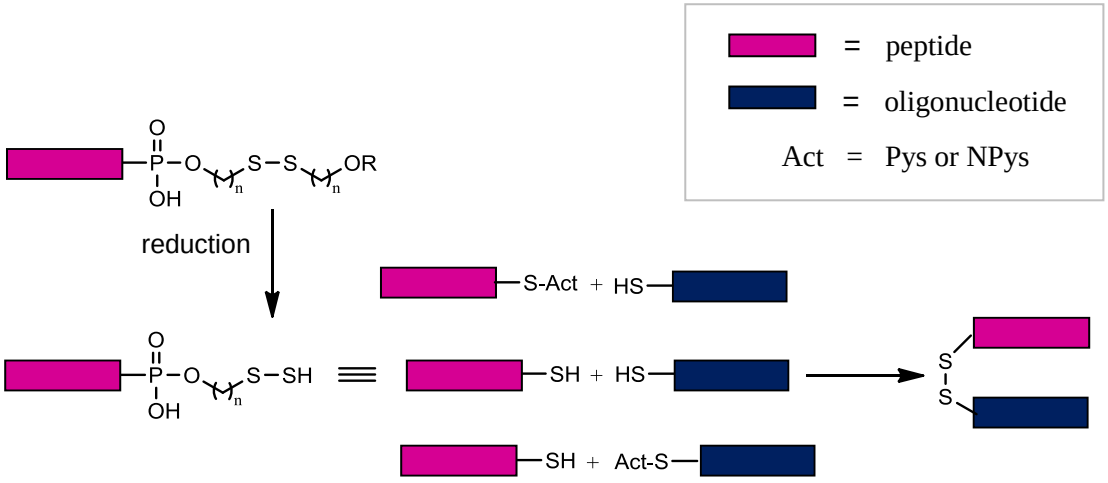


Figure 1.15 – *Schematic representation of methods for oligonucleotide conjugation to peptides by disulfide linkage*

1.3.2.2 Thioether linkage

Thioether linkages are also frequently used for oligonucleotide peptide conjugation; these are commonly synthesised by the maleimide-thiol protocol. In this strategy oligonucleotides or peptides functionalised with maleimide are reacted with peptides or oligonucleotides containing a thiol functionality, to form a maleimido-thioether linkage. Tung and co-workers have reported this methodology for OPC synthesis; [67] amino modified oligonucleotides were treated with a maleimido functionalised *N*-hydroxysuccinimidyl ester for derivitisation with maleimide then reacted with peptides containing a cysteine residue for formation of OPCs. Eritja and co-workers report a different approach to the maleimide-thiol protocol [65] whereby 5'- thiolated oligonucleotides were reacted with *N*-terminus maleimido functionalised peptides to form a thioether linkage for conjugation of antisense oligonucleotides to nuclear localisation sequence (NLS) peptides.

1.3.2.3 Amide linkage

There are a number of reports of the synthesis of OPCs through amide linkages, achieved by reaction of one fragment – functionalised with a carboxylic acid or a thioester – with the second fragment which is functionalised with a primary amine.

Bruick *et. al* report a template-directed chemical ligation method for amide linkage of oligonucleotides and peptides (**Fig. 1.16**). [68] This approach makes use of an oligonucleotide template and involves coupling of the peptide fragment, containing an activated thioester at the *C*- terminus, to a 5'- thiolated oligonucleotide (DNA-1), through a temporary thioester linkage. The DNA-1 of this intermediate is then hybridised to an oligonucleotide template. A second oligonucleotide (DNA-2, the sequence required for synthesis of the desired OPC), which is functionalised at the 3'- terminus with a primary amine, is also hybridised to the oligonucleotide template in close proximity to the DNA-1. This allows the peptide to migrate from the DNA-1 to the DNA-2, forming the desired OPC in good yield by means of a highly stable amide linkage.

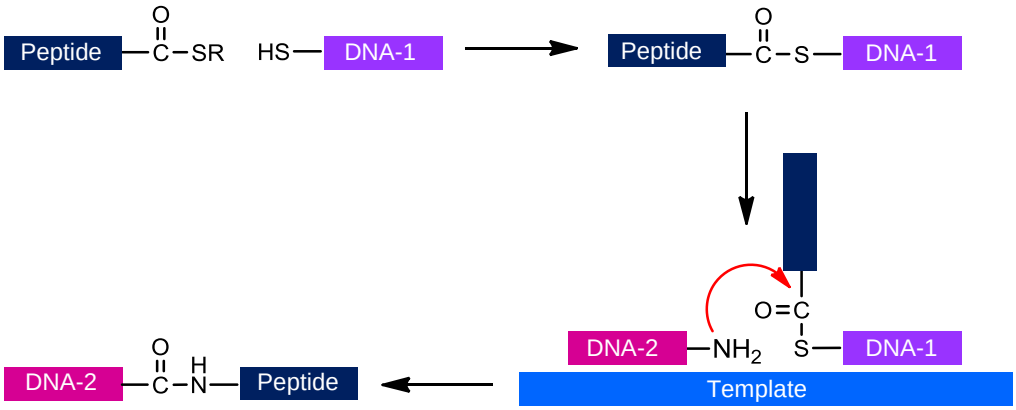
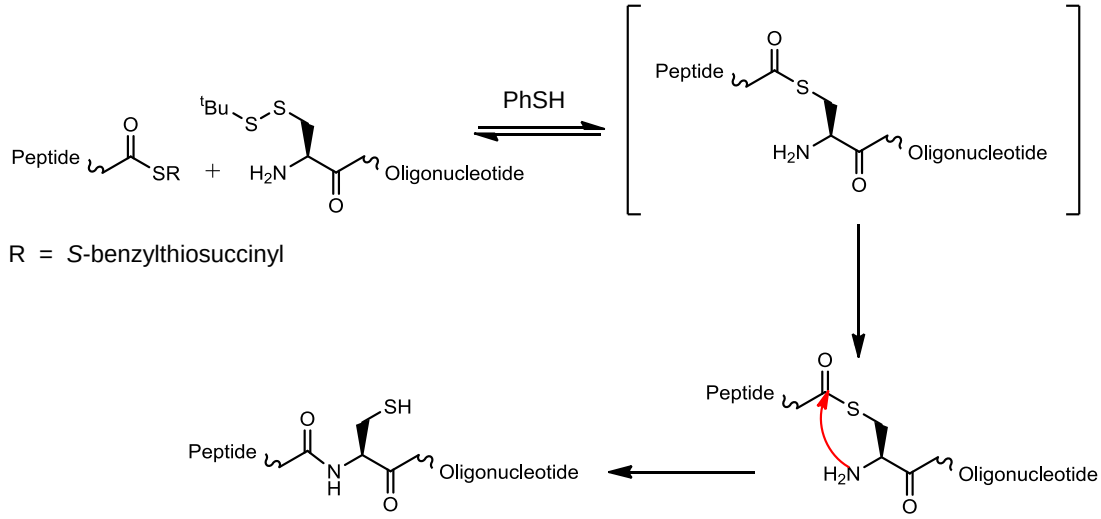


Figure 1.16 – *Schematic representation of template-directed ligation of an oligonucleotide to a peptide to form an amide linkage, as reported by Bruick et al.* [68]

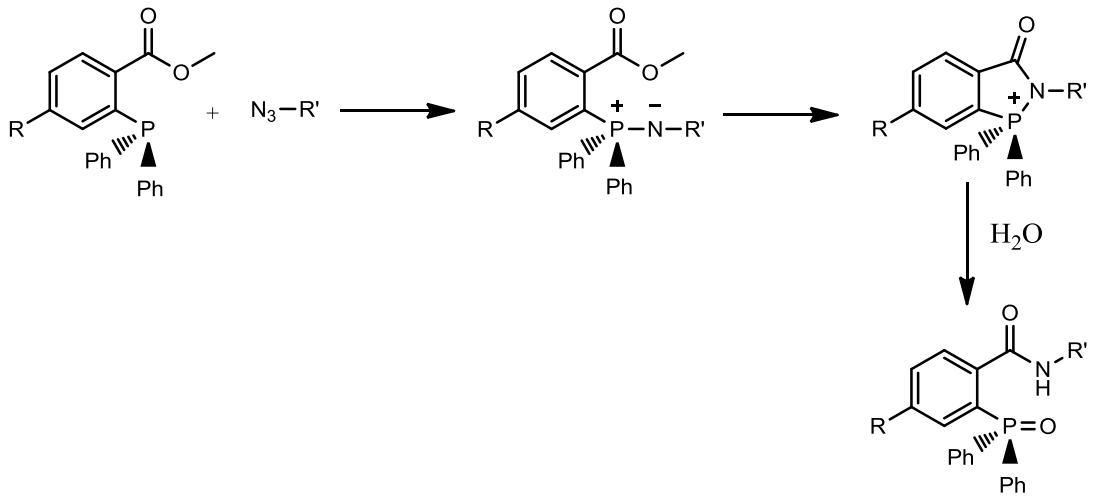
Stetsenko and Gait describe a powerful 'native ligation' method for OPC synthesis through amide linkage. [69] An *N*- terminal thioester functionalised peptide and a 5' -*S*-*tert*-butylsulfenyl-L-cysteinyl modified oligonucleotide were assembled using standard solid phase synthesis methodologies; the cysteine moiety was incorporated into the oligoinucleotide fragment using a specially synthesised L-cysteinyl modified phosphoramidite. The two fragments were then coupled in aqueous/organic solution to form a temporary thioester linkage, followed by ligation through reaction of the oligonucleotide's cysteinyl amine with the carboxyl of the peptide thioester, to form an amide linkage (**Scheme 1.1**). The *tert*-butylsulfenyl group was cleaved *in situ* and thiophenol was used to enhance conjugation.

Amide linkages between oligonucleotides and peptides have also been formed through coupling, in dimethylformamide (DMF), of oligonucleotides containing an amino modified thymine base with peptide *C*- terminal carboxylic acids using the 1-ethyl-3-(3- dimethylaminopropyl) carbodiimide (EDC) coupling agent.



Scheme 1.1 – *OPC synthesis by 'native ligation' to form an amide linkage as reported by Stetsenko and Gait* [69]

A further, well-known bioconjugation method by amide formation that is, as yet, unreported for oligonucleotide peptide conjugation, but could be a possible methodology for this, is the Staudinger ligation. The Staudinger reaction involves the reaction of an azide with a triarylphosphine to form an unstable aza-ylide, which is quickly hydrolysed in aqueous media to form an amine and the corresponding phosphine oxide. [70] Based on this, Saxon and Bertozzi designed an ester-modified aryl phosphine, [71] whereby the ester acts as an electrophilic trap which captures the aza-ylide before hydrolysis, resulting in conjugation of the azide and the phosphine by formation of a stable amide bond (**Scheme 1.2**). Raines and co-workers have since reported a 'traceless' Staudinger ligation, in which the phosphane oxide is cleaved during the hydrolysis step. [72] The Staudinger ligation is an attractive reaction for bioconjugations, being truly chemoselective; this is highly attractive when dealing with biomolecules which, by their nature, contain multiple reactive functional groups. The reaction has been used for the site-specific labelling of oligonucleotides, *via* conjugation of a 5'-azido modified oligonucleotide and a fluorescein modified phosphane. [73]



Scheme 1.2 – *The Staudinger ligation for bioconjugation by amide formation, as reported by Saxon and Bertozzi*

1.3.2.4 Oxime and thiazolidine linkage

Forget and co-workers have reported methods for OPC synthesis, using the chemoselective ligation of oxime and thiazolidine formation. [74] An aldehyde functionalised oligonucleotide was reacted with an oxyamine functionalised peptide to form an oxime linkage; a thiazolidine linkage was achieved by reaction of an aldehyde functionalised oligonucleotide with a peptide containing a cysteine residue. Both methodologies afforded OPCs in moderate yields (~ 50 %) in mild aqueous conditions, without the need for protecting groups on the biomolecules. Oligonucleotide conjugation to NLS peptides has been achieved by oxime formation; this linkage was found to be stable in phosphate buffers at pH 4 and pH 7 for up to 72 hours at 37 ^{o}C. However, the approach to synthesising an aldehyde functionalised oligonucleotide for this process was complicated, with deprotection and oxidation steps required post oligonucleotide synthesis for generation of the aldehyde.

1.3.2.5 Cycloaddition linkages

Synthesis of OPCs using the Diels-Alder 2, 4-cycloaddition has been reported by Grandas *et al*. [75] Dienyl modified oligonucleotides were conjugated to maleimide derived peptides in aqueous solution, using an acyclic diene for formation of the Diels-Alder adduct. Very recently OPC synthesis has also been achieved by peptide modification of a deoxyribonucleotide *via* a Diels-Alder cyclohexene linkage; [76] the

peptide-modified nucleotide was then incorporated into a DNA sequence during enzymatic polymerization.

The Diels-Alder reaction is fast, chemoselective and is enhanced in aqueous conditions, [77] thereby posing no solubility issues for OPC synthesis as both biomolecules are water soluble. The selective nature of the reaction is such that unwanted side reactions are minimal, although maleimides can be susceptible to Michael addition reactions with other nucleophilic centres on peptide side chains, such as cysteine. The oligonucleotide peptide linkage formed through the cycloaddition is also extremely stable, being comprised of carbon-carbon bonds.

The Huisgen 1, 3-dipolar cycloaddition has also been reported for OPC synthesis through formation of a triazole, in a so-called 'click' reaction between an azido functionalized peptide and 5'- alkynyl functionalized PNA. [78] Similar to the Diels-Alder cycloaddition, this is an attractive methodology for OPC synthesis being chemoselective, functional in both organic and aqueous media and forming a 1, 2, 3-triazole linkage that is highly stable.

1. 3.3 Oligonucleotide conjugation to Tat peptide

There are a number of reports in the literature of oligonucleotide conjugation to Tat peptide as a method for enhancing oligonucleotide cellular uptake. Many of these reports describe Tat peptide conjugation to antisense oligonucleotides for therapeutic effects and conjugation is mainly achieved by formation of a disulfide linkage. [45], [79], [80] However, conjugation of Tat peptide to molecular beacons (see 1.1.6 – *Modified oligonucleotides for use as bioanalytical probes*) has also been reported for bioanalytical purposes. [27], [81] Nitin *et al.* used three different linkages for covalent attachment of Tat peptide to a molecular beacon; biotin-streptavidin, maleimide-thiol and disulfide linkages were formed, [27] with the maleimide-thiol linkage above the others, being shown to have a negligible effect on binding of the molecular beacon to its target. An important feature of Nitin's work is the observation that conjugation of the molecular beacon to Tat peptide caused no impairment to its functionality.

Yeh and co-workers also report the use of a maleimide-thiol linkage for Tat peptide conjugation to a molecular beacon. [81]

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