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Studies of non-covalent small molecule DNA interactions and protein-DNA interactions

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STUDIES OF NON-COVALENT SMALL MOLECULE DNA INTERACTIONS AND PROTEIN-DNA INTERACTIONS

A thesis submitted in (partial) fulfilment of the requirements for the award of the degree

DOCTOR OF PHILOSOPHY

from

UNIVERSITY OF WOLLONGONG

by

Amit Kapur, BSc (Hons)

Department of Chemistry
November, 2002
CERTIFICATION

I, Amit Kapur, declare that this thesis, submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy, in the Department of Chemistry, University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Amit Kapur
November 2002
To those people who made it possible for me to get here. I would like to thank:

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Sections of the work described in this thesis have been reported in the following publications:

Kapur A. Beck JL. Brown SE. Dixon NE. Sheil MM.
Use of electrospray ionization mass spectrometry to study binding interactions between a replication terminator protein and DNA.

Gupta R. Kapur A. Beck JL. Sheil MM.
Positive ion electrospray ionization mass spectrometry of double-stranded DNA/drug complexes.

Beck JL. Colgrave ML. Kapur A. Ianetti-Tito P. Ralph SF. Sheil MM. Weimann A. Wickham G.

Kapur A. Beck JL. Sheil MM.
Observation of daunomycin and nogalamycin complexes with duplex DNA using electrospray ionisation mass spectrometry.
Electrospray ionization mass spectrometry has been used to study the non-covalent interactions between Tus and TerB. The Tus-Ter complex was very stable using a spray solvent of 10 mM ammonium acetate at pH 8. Initial attempts to distinguish binding affinities of Tus and mutant Tus proteins for Ter DNA were unsuccessful. Increasing the ammonium acetate concentration in the electrospray solvent (800 mM) increased the dissociation constants sufficiently such that relative orders of binding affinities for Tus and various mutant Tus proteins for various DNA sequences could be determined. A dissociation constant of $700 \times 10^{-9}$ M for the binding of the mutant Tus protein A173T (where residue 173 is changed from alanine to threonine) or Ter DNA was estimated, compared with a value of $\leq 2 \times 10^{-9}$ M for Tus where A173T was unchanged. These results were in agreement with solution studies and are the first example in which ESI-MS has been used to compare binding affinities for a DNA-binding protein with mutant proteins for specific DNA recognition sequences. Overall, this work demonstrates that ESI is a powerful technique for the observation of non-covalent DNA-drug and DNA-protein interactions.

In the second part of this work, the use of ESI-MS for studying the non-covalent interactions between the anti-tumor drugs daunomycin and nogalamycin to duplex DNA. Ions corresponding to the complex were most abundant relative to free DNA when prepared in the pH range 8-9 and acquired using gentle ESI-MS conditions on a triple quadrupole mass spectrometer. Titration experiments gave ESI-mass spectra in which the most intense ions correspond to three molecules of nogalamycin or 4 molecules of daunomycin bound to the duplex 8 mers.
(d(CGCGCGCG)\textsubscript{2} and d(GGCTAGCC)\textsubscript{2}) and 4 molecules of nogalamycin or 6 molecules of daunomycin bound to the duplex 12 mer (d(TGAGCTAGCTCA)\textsubscript{2}). These data are consistent with the neighbour exclusion principle. Competition experiments involving a single drug in an equimolar mixture of two oligonucleotides (d(TGAGCTAGCTCA)\textsubscript{2} with either d(CGCGCGCG)\textsubscript{2} or d(GGCTAGCC)\textsubscript{2}) showed that the intercalators bound preferentially to d(CGCGCGCG)\textsubscript{2} compared to d(GGCTAGCC)\textsubscript{2} relative to the 12 mer. This shows that ESI-MS has the potential to detect differences in sequence selectivity. ESI-mass spectra from experiments in which both drugs were reacted with the same oligonucleotide were more complicated and as such, a clear preference for one drug could not be established.

Similar studies were done using longer 16 mer self-complementary oligonucleotides (d(ATATATATATATATAT)\textsubscript{2}, d(ATATACGTATATAT)\textsubscript{2} and d(CCATACGTATATGG)\textsubscript{2}). These data showed only small amounts of dsDNA-drug complex. Most ions observed were from single stranded oligonucleotides complexed to drug. Non-self-complementary oligonucleotides were subsequently used to avoid ambiguities in assigning dsDNA-drug peaks. Extensions of these studies using a quadrupole-time-of-flight instrument, showed no evidence of these non-specific single stranded DNA-drug complexes. Different CID experiments (cone voltage, capillary voltage, desolvation temperature and collision energy) all showed that the order of binding of the intercalators was ethidium->nogalamycin->daunomycin. Preliminary results on the observation of the retamycin-dsDNA complex were also presented.
ABBREVIATIONS

A  Adenine
T_b  bulged sequence
C  cytosine
Dn  daunomycin
DNA  deoxyribonucleic acid
dsDNA  double-stranded DNA
Eb  ethidium bromide
EDTA  ethylenediaminetetraacetic acid
ESI  electrospray ionization
ESI-MS  electrospray ionization mass spectrometry
G  guanine
Hd  hedamycin
me5C  5-methyl-deoxyCytidine
Ng  nogalamycin
pS  phosphorothioate
Rt  retamycin
RNA  ribonucleic acid
ssDNA  single-stranded DNA
T  thymine
TE  Tris-EDTA buffer
Wt  wild-type
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1 General Introduction

Nucleic acids play a key role in all cellular function. Genetic information from deoxyribonucleic acid (DNA) is transcribed into ribonucleic acid (RNA) which is in turn translated into a protein. Of particular interest for the work described here is the central role DNA plays in cell reproduction, which in turn makes it a potential target for chemotherapeutic agents aimed at preventing the reproduction of diseased cells (as in tumour growth and/or cancer) (Garrett and Workman, 1999; Sikora et al., 1999). Some of these DNA-interactive drugs include bleomycin, daunorubicin (daunomycin), cisplatin, actinomycin D, mitomycin and cyclophosphamide (Hecht, 1996). Most of these agents have a number of damaging side effects resulting predominantly from the poor selectivity for these agents for diseased cell states so that damage to normal cells that reproduce rapidly also occurs. Additionally, there are problems associated with the tumour cells developing resistance to some of these drugs. Hence there is an on-going need for the development of new anti-tumour drugs that display a greater selectivity for diseased cells.

Nature is an attractive source of new therapeutic candidate compounds that possess interesting reactive functionalities and therefore potentially novel modes of DNA interaction (Cragg et al., 1997). Indeed, over 60% of the chemotherapeutic agents approved by the Food and Drug Administration (U. S. Department of Health and Human Services) as of 1999 were derived from natural products (Cragg and Newman, 2000). Unfortunately, problems associated with accessing the source of these natural products and obtaining appropriate amounts often limits clinical
applications. There is an on-going need for synthetic analogs of these potent natural compounds either to ensure that adequate supply is achieved and/or to produce analogs that may be more effective than their natural counterparts.

In order to design more effective anticancer drugs that target DNA, a better understanding of the precise nature of the binding of these ligands (or drugs) to DNA at the molecular level must first be determined (Workman, 2001; Workman and Clarke, 2001). Improvements in high resolution molecular characterization methods namely X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy have enhanced our understanding of the structures of DNA-ligand complexes (Neidle, 1994). Data obtained on the nature of the interaction of these agents with DNA have to a degree indicated that many of these drugs interact with DNA in a sequence-specific manner. The publication of the working draft of the human genome sequence (Lander et al., 2001; Venter et al., 2001) has raised the possibility of developing drugs that can be used to target particular regions of the genome e.g. certain guanine rich regions of oncogenes. Examples of these novel nucleic acid-recognising drugs include modified oligopeptides and synthetic oligonucleotides of high sequence specificity (Jayaram and Beveridge, 1996; Rizzo et al., 1989; Schuerman et al., 1996; Sun et al., 1995).

1.1 DNA-ligand interactions

DNA is comprised of nucleotides linked together via phosphodiester bonds. Under physiological conditions (pH 7), DNA exists as a polyanionic species due to the negative charges on the phosphate backbone. In DNA, base-pairing occurs between
two complementary strands that line up antiparallel to each other resulting in the formation of a double helical structure or double-stranded DNA (dsDNA). The secondary and tertiary structures of DNA are to a large extent sequence dependent (Dickerson et al., 1983a; Dickerson et al., 1983b). It follows therefore that sequence-specific interactions between DNA and ligands (both small molecules and proteins) are related to the various conformations of the DNA (Dickerson and Drew, 1981). DNA has a number of potential sites for binding of ligands either covalently or via non-covalent interactions. DNA interactive agents are numerous and varied and can include mutagens, carcinogens, antibiotics, dyes and metal complexes (Blackburn, 1990).

1.1.1 Covalent DNA-ligand interactions

Covalent reactions can occur between small molecules and a variety of sites on DNA. In general, nucleophiles can attack the pyrimidine residues of nucleic acids at C-6 or C-4 whilst reactions at C-6 of adenine or C-2 of guanine are more difficult (Blackburn and Gait, 1990). Electrophilic attack occurs most frequently at O-6, N-6 and N-7 in the major groove and N-1 and N-2 in the minor groove. Examples of such compounds include metal complexes such as cisplatin and most DNA alkylating compounds including cyclophosphamide (Walker and Gait, 1990), the nitrogen mustards (Wilman and Connors, 1983) and halogenated ethylnitrosureas (Kohn and Gibson, 1986) (which encompass many chemotherapeutic drugs, carcinogens and mutagens) (Blackburn and Gait, 1990). For instance, known carcinogens such as vinyl chloride, dimethyl sulphate and methyl methanesulphonate are known alkylation agents that interact directly with DNA and form covalent bonds principally
to the nitrogen and oxygen atoms of purine bases. Other compounds are converted into alkylating agents by metabolic processes in the cell. An example of this is the conversion of amines by biological oxidation to their hazardous aryl-hydroxylamine derivatives (Guengerich et al., 1999). These converted compounds bind preferentially to the C-8 of deoxyguanosines. A further example is the metabolic conversion of polycyclic aromatic hydrocarbons (PAHs) to diol epoxides via oxidation by cytochrome P-450 enzymes (Yang, 1988).

Several anticancer agents bind covalently to DNA via a combination of alkylation and intercalation (described in detail below). Examples of such drugs include hedamycin, DC92-B and CC-1065 (Hecht, 1996). This ‘dual pronged’ attack ensures greater sequence selectivity of alkylation of DNA by these compounds. The binding of the antibiotic hedamycin which was examined in part in this thesis is discussed in detail in Chapter 4.

1.1.2 Non-covalent DNA-ligand interactions

There are three main modes of non-covalent DNA interactions involving DNA. First, interactions can occur between cations (Na⁺, Ca²⁺ and NH₄⁺) and the polyanionic backbone of DNA. Such interactions are important because most nucleic acid conformations are possible only because of reversible interactions with metal ions (as well as water). Changes in amounts of water present, changes in salt concentration (ionic strength) and interaction with organic molecules can change the conformation of DNA.
Second, groove binding interactions involve direct interactions of the ligand with the edges of base pairs in either the major or minor groove. Minor groove binders typically contain several aromatic rings linked such that there is torsional freedom to allow movement complementary to that of the DNA minor groove (Blackburn and Gait, 1990). In addition, electrostatic interactions and favourable van der Waals contacts with the walls of the minor groove provide additional components to the free energy of binding. Minor groove binding-molecules typically are crescent shaped and have N-H groups on the interior of the crescent that are favourable for H-bonding with adenine:thymine (A:T) base pairs. Binding to guanine:cytosine (G:C) bases is restricted by N-3 in guanine. Examples of these include Hoechst 33258, distamycin and netropsin (Fox et al., 1999; Wang et al., 2000).
The third mode of non-covalent DNA-ligand binding involves intercalation. Intercalation involves insertion of a planar or approximately planar aromatic molecule between the stacked base pairs of the hydrophobic interior of helical dsDNA. Intercalation forms the major focus of the latter part of the work described in this thesis.

A number of molecules are capable of binding to DNA via intercalation (Brana et al., 2001). Some of these compounds include simple intercalators such as ethidium bromide and daunomycin (Blackburn and Gait, 1990), threading intercalators such as nogalamycin (Caceres-Cortes and Wang, 1996; Das et al., 1974; Egli et al., 1991; Fox et al., 1985) and bisintercalators such as echinomycin (Park and Choi, 1995).

Intercalation between DNA base pairs has several characteristics. First, a change of the physical properties of the double helix occurs. The DNA double helix must undergo structural changes in order to accommodate an intercalator. The double helix is unwound; and the amount of unwinding is dependent on the nature of the intercalator. In B-DNA, base pairs are rotated approximately 36° with respect to the next. Once the drug is intercalated, however, the double helix will rewind and decrease this degree of rotation (Moore et al., 1989; Waring, 1970; Zhang and Patel, 1990). For instance, once intercalated, ethidium unwinds DNA by 26° (Wilson et al., 1985) whilst daunomycin unwinds DNA by 11° per bound molecule (Wang et al., 1987). As a result of this rotation about torsional bonds, the creation of the intercalator site results in separation of the base pairs and lengthening of the double helix resulting in a distorted phosphodiester backbone. This increase is typically about 3.4Å which is the thickness of typical aromatic ring systems.
Generally, when an intercalator binds to any one available site in the double helix, the sites adjacent to it become unavailable for binding. This is known as the neighbour exclusion principle (Rao, 1987) and is particularly relevant to the discussion of the results of binding studies involving DNA intercalators and is discussed in more detail in Chapter 3.

Finally, the binding of small molecules to DNA is covered in more detail in many books and reviews (Blackburn, 1990; Blackburn and Gait, 1990; Chaires, 1998; Hecht, 1996; Hemminki et al., 2000; Nieto and Jones, 2001).

1.2 Non-covalent DNA-protein interactions

Interactions between proteins and DNA are the basis for many important processes in the cell. First, some proteins play an architectural role for the chromosome in the cell. These include the histone class proteins, integration host factor (IHF), high mobility group (HMG) 1 proteins and HMG-box containing proteins, the sex determining factor, SRY and the lymphoid enhancing factor, LEF-1 (Bewley et al., 1998).

Second, DNA binding proteins have roles in DNA replication. DNA replication has been most extensively studied in bacterial systems such as *E. coli* (Kelman and O’donnell, 1995). These proteins include DnaA (an initiator protein) (Messer et al., 1999; Speck et al., 1999), a helicase for relaxing supercoiled DNA (Weigelt et al., 1999), a primase to synthesise primer RNA (Mustaev and Godson, 1995), ten
subunits of polymerase III, ATPases, exonucleases as well as a protein (Tus protein) to terminate the process (Kelman and O'donnell, 1995). Similar proteins can also be found on viral and eukaryotic systems.

Another class of DNA binding proteins includes the transcription factors. These proteins include the E. coli trp repressor (Lane and Jardetzky, 1987; Lane et al., 1987; Lefevre et al., 1987), Fos and Jun (Mechta-Grigoriou et al., 2001), the vitamin D receptor (Hsieh et al., 1999; Issa et al., 1998), estrogen receptor (Yudt et al., 1999), and the homeobox transcription factors (Gehring et al., 1994).

A full description of the many varied DNA-protein interactions is beyond the scope of this thesis. Several recent reviews cover these interactions in greater detail. (Alberts et al., 1994; Dean and O'donnell, 1996; Suck, 1994).

1.3 Structural characterisation of non-covalent DNA-ligand and DNA-protein complexes.

Probing the nature of non-covalent interactions between DNA and either proteins or small molecules is important in gaining a better understanding of the mechanism of action of many carcinogens, mutagens and anti-tumour drugs. Information on nucleic acid binding specificity and ligand-induced conformational transitions obtained from studying interactions of small molecules with DNA has also been used as a basis to predict the nature of interactions of DNA with large molecules. For instance, detailed studies into the binding of aromatic amino acids to DNA sequences has been used to predict DNA-protein interactions (Rachez et al., 1996).
A variety of structural techniques have been employed in order to study these interactions. Two of the most common techniques used to study these non-covalent interactions, however, include X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) (Hensley, 1996). X-ray crystallography is a technique that has been employed extensively for the structural analysis of nucleic acids. Crystallographic methods have provided most of the highly detailed information available not only on DNA structure but also on the DNA complexes formed with drugs and proteins (Neidle, 1994). Whilst this technique is capable of providing detailed 3-D structural information, it has a major limitation in that it requires that the samples must be capable of being crystallized. This is often difficult to achieve and can be quite time consuming. Furthermore, as the samples are in solid form for analysis, it has been argued that these data are not always directly applicable to the behaviour of biomolecules in physiological environments.

Nuclear magnetic resonance spectroscopy does enable structures to be determined in solution, largely by means of measurements of proton-proton coupling constants and through-space nuclear Overhauser effect (NOE) derived distances (Neidle, 1994). It has an apparent advantage over X-ray crystallography as the structures determined by NMR in solution are more relevant to physiological processes. NMR has a major disadvantage in that it is a technique of low sensitivity as large concentrations of sample (μM-mM) are often required. Samples at these concentrations are more prone to solubility and/or precipitation problems. Even though X-ray crystallography and NMR have some limitations, these two techniques
have complemented each other to provide invaluable structural information for nucleic acids and DNA-ligand complexes.

Spectroscopic techniques including circular dichroism (CD) and light scattering also provide valuable information on the structures of non-covalent DNA-ligand complexes (Dykstra and Lisy, 2000; Hensley, 1996). Circular dichroism (CD) is a spectroscopic method which measures the difference in absorbance of left- and right-handed circularly polarized light by a material, as a function of the wavelength. Nucleic acids are chiral and show circular dichroism in their ultraviolet absorption bands. This property can thus be used as an indication of secondary structure of both free and drug-bound DNA (Eriksson et al., 2001). In light scattering studies, when a sample containing the DNA-ligand complex is illuminated with light, a portion of the incident light can be "deflected" by fluctuating electrons. The intensity of "light scattering" depends on the mass of the scattering object (Robinson and Drobny, 1995; Robinson et al., 1997). Whilst both these techniques have advantages in speed and sensitivity, they typically work better with oligonucleotides longer than 100 base pairs in length (Eriksson et al., 2001; Robinson et al., 1997) and also only provide information on gross structural changes.

The use of a relatively recent spectroscopic technique, Surface Plasmon Resonance (SPR) has become increasingly applied in the structural analysis of non-covalent DNA-ligand complexes (Buckle and Figures, Carrasco et al., 2001; Pieper et al., 2002). This technique exploits the differing refractive indices of two transparent media and their refractive and reflective properties. If the interface between these media is coated with a thin layer of metal (typically gold or silver), the reflected light
is monochromatic, polarized, and has a reduced intensity which results in a shadow (termed surface plasmon resonance). This SPR signal (expressed in resonance units) has a direct linear relationship with the mass concentration of biomolecules and can therefore be used to observe ligand association and dissociation and ultimately rate and equilibrium constants (Buckle and Figures).

Mass spectrometry (MS) has emerged relatively recently as a powerful method which can provide data that are complementary to other high resolution techniques used for the structural characterization of DNA-ligand complexes. It has major advantages pertaining particularly to its sensitivity and speed. The following sections give a brief overview of the previous applications of mass spectrometry in the general area of the structural analysis of DNA and DNA-ligand complexes.

1.4 Mass Spectrometry

Mass spectrometry involves the mass measurement of individual molecules by transforming them into ions \textit{in vacuo} and then measuring the response of their trajectories in either an electric or magnetic field or a combination of both (Fenn \textit{et al.}, 1989). The mass of the molecule of interest is determined by measurement of the mass-to-charge ratio ($m/z$) of its gaseous ions. The technique was applied first to organic molecules in the petrochemical industry for the analysis of volatile components of oil. During the 1960s and 1970s, mass spectrometry and later gas chromatography (GC-MS) was developed and applied to an increasing array of organic molecules and mixtures. Extensions of these studies to the analysis of intact thermally labile biopolymers including peptides and nucleic acids however, proved
problematic owing to the difficulty of transforming these large involatile polar species to gaseous ions. Earlier ionisation techniques, electron ionization (EI) and chemical ionization (CI) involved heating the sample for volatilization that had the disadvantage that thermal decomposition of the biopolymer occurred. Between the late 1960s and early 1980s, a variety of softer ionization techniques such as field desorption (FD) (Beckey, 1969), plasma desorption (PD) (MacFarlane and Torgerson, 1976) and fast atom bombardment (FAB) (Horning et al., 1974) were introduced. These new techniques opened up the door for the direct analysis of thermally labile and involatile species (Loo, 1995). The role of mass spectrometry in biological research however, was profoundly expanded by the introduction of two ‘soft’ ionization techniques, matrix assisted laser desorption ionization (MALDI) (Karas and Hillenkamp, 1988) and electrospray ionization (ESI) (Fenn et al., 1989).

In comparison to the earlier ionization techniques, both ESI and MALDI have the ability to effectively ionize very large involatile molecules and are thus useful for the determination of structural and molecular weight properties of proteins, oligonucleotides, and their complexes. As only ESI-MS was used for the work described in this thesis, a detailed discussion of this technique is given below.

1.5 Electrospray Ionization Mass Spectrometry

Electrospray ionization involves the production of gas-phase ions directly from solution by spraying a solution of analytes in the presence of a strong electric field to form charged droplets which, through evaporation and desolvation in a bath gas at atmospheric pressure, generate a dispersion of gas-phase ions (Fenn et al., 1989). The technique was pioneered in the 1960s with the work of Dole (Dole et al.,
The application of electrospray as an ionization method for mass spectrometry was first demonstrated with the nearly simultaneous reports by Yamashita and Fenn (Yamashita and Fenn, 1984), and Aleksandrov (Aleksandrov et al., 1984a; Aleksandrov et al., 1984b). These studies clearly demonstrated the use of electrospray for the analysis of large macromolecules that had previously been too involatile or too complex or fragile to be analysed using other ionization techniques. These results fuelled the use of ESI as an ionization technique in the field of biological research.

The enormous potential of ESI became apparent with the successful analysis of an intact protein of mass 76000 Da (Fenn et al., 1995). This study also highlighted a remarkable feature of this technique in that their analysis was made possible by multiple-charging of the protein ions. This phenomenon has a number of implications. First, the $m/z$ range of the mass analyzer need not be large because as a result of multiple-charging, ions generally fall within the range of $m/z$ 1000-4000 (Jardine, 1990). Second, as the mass of the species being analysed is determined from more than one peak, the mass accuracy is increased (Deroussent et al., 1995).

During the electrospray process, a solution of analyte is introduced through a metal (usually stainless steel) capillary maintained at an elevated voltage relative to the counter electrode as shown in Figure 1.2. The electrode also has an orifice to allow for a flow of nitrogen bath gas to entrain the movement of ions and/or charged droplets into the high vacuum region of the mass spectrometer. The potential difference between the capillary tip and the counter electrode results in charge accumulation on the surface of the liquid emerging from the capillary terminus.
which causes dispersion of the liquid into a fine spray of charged droplets. A flow of warm bath gas (usually nitrogen) is typically employed in order to remove the solvent vapour and further to exclude large droplets and other uncharged particles. Additionally, evaporation of the solvent increases the charge density on the droplet surface. Once the electrostatic repulsion is greater than surface tension, the droplets explode (Coulombic repulsion) and the resulting ions pass through a small orifice where the analyte ions are separated from the drying gas and solvent vapour. These analyte ions and then accelerated into the higher vacuum region of the instrument for detection and analysis.

Figure 1.2: A schematic showing a configuration of a typical electrospray ionisation source. Note that in the Z-spray™ configuration, the capillary is perpendicular to that in conventional systems.
Typical ESI mass spectra are dominated by ions from intact molecules owing to the gentle nature of the ionization process. Although this data can help confirm the identity (by mass) of the biomolecule in the sample mixture, little information regarding its molecular structure can be discerned. Tandem mass spectrometry or MS/MS is very useful technique for the characterisation of molecular structure (Loo, 1995; Loo et al., 1994; Siuzdak, 1994). Tandem MS involves two stages of mass analysis. In the first, a precursor ion of interest is selected from the other precursor ions produced in the ionization source. Following this stage, the selected ion is introduced into a collision cell where it is fragmented by a transfer of energy. This is by caused by inducing ion collisions with an inert gas (usually argon or helium). This process is known as collision-induced dissociation (CID). The resulting charged fragments (product ions) are analysed by a second mass analyzer, resulting in a product ion spectrum.

Electrospray ionization has been coupled to a number of different mass analysers. Quadrupoles have the advantage of relatively low cost and tolerance to high pressures (Loo et al., 1994) and have been used most commonly with ESI. Magnetic sector analysers has also been combined with ESI. The latter analysers have the advantage for higher m/z resolution and high-energy CID for MS/MS studies. However, it is the combination of ESI with time-of-flight (TOF) instruments that has been shown to be one of the most powerful tools for the analysis of biomolecules (Morris et al., 1996). The advantages of time-of-flight instruments include the extended m/z range, sensitivity, speed and resolution. The advent of orthogonal acceleration has greatly improved the sensitivity and resolution of these instruments as it provides a means of accumulating and focusing the ions before they are
accelerated into the TOF analyzer (Verentchikov et al., 1994). Other mass analysers that have been coupled to ESI include the quadrupole ion trap (McLuckey et al., 1994) and Fourier Transform (FT) mass analysers (Buchanan and Hettich, 1993). Recently, hybrid mass analysers such as quadrupole-time-of-flight have been produced with ESI sources (Morris et al., 1996). In this study, the mass analysers used included the quadrupole mass analyser and the quadrupole-time-of-flight mass analyser. Both are described in Chapter 2.

1.6 ESI-MS of non-covalent oligonucleotide complexes.

In addition to the inherent advantages of ESI for the characterisation of primary structure, ESI-MS is emerging as an exciting method by which to study biomolecular non-covalent interactions. ESI-MS has been used for example, to detect non-covalent complexes of DNA with metal ions, drug molecules and proteins (Beck et al., 2001). ESI has an advantage over other ionization methods in that it is gentle and therefore does not result in molecular fragmentation. This “gentleness” also allows for the detection of weakly bound non-covalent complexes.

ESI-MS has a number of possible advantages over other techniques commonly used for studying these interactions. These advantages are often referred to as the ‘S’ advantages of mass spectrometry (Mclafferty, 1981). The first advantage of the technique is specificity. ESI-MS is capable of providing information regarding not only the identity (by mass) of a binding partner but its relative binding affinity (Loo, 1997). Coupled to this idea of specificity is another obvious advantage of ESI-MS – stoichiometry. The stoichiometry of a complex can be easily be obtained from mass
spectra where the molecular weight of the complex can be measured with sufficient accuracy.

The greatest advantages ESI-MS has over other techniques, however, are speed and sensitivity. NMR and X-ray crystallography often require relatively large quantities (mg) of material. ESI-MS on the other hand, has a great advantage over these techniques in that picomolar to femtomolar quantities of sample are required (Fitzgerald et al., 1996; Hofmann et al., 1999). Furthermore, NMR and X-ray crystallography are very slow compared to a typical ESI-MS experiment which takes a fraction of the time. It is recognized that mass spectrometry does not provide the same detailed structural information. Mass spectrometry can be a power adjunct to these techniques. For instance, MS might be used to establish relative binding affinities prior to more detailed structural studies by NMR or X-ray crystallography.

Unfortunately, typical solvent conditions normally used in ESI-MS to achieve maximum sensitivity are often not optimal for maintaining non-covalent interactions. These interactions are highly dependent on conditions such as the pH and ionic strength (including type of ionic species) of the solution. ESI-MS, on the other hand, is intolerant to the presence of high salt concentrations and non-volatile buffer components. Solution pHs used for ESI-MS are typically in the range 2-4 (for positive ions) and 8-10 (for negative ions). Furthermore, organic solvents such as methanol and acetonitrile are often added to solutions to obtain maximum sensitivity and signal stability. These solvent conditions are not, however, optimal for the formation of non-covalent complexes that require aqueous environments at near or around neutral pHs. It has thus been a challenge to obtain ESI-MS data
under more physiological conditions. Indeed, numerous ESI-MS studies have now been done using aqueous solutions without the addition of organic solvents and typical salts (Gale et al., 1994; Gale and Smith, 1995; Veenstra, 1999; Veenstra et al., 1998a; Veenstra et al., 1995; Veenstra et al., 1998b; Veenstra et al., 1997; Veenstra et al., 1998c).

1.6.1 ESI-MS of non-covalent drug-DNA complexes

There are relatively few reports on the use of ESI-MS to detect non-covalent drug-DNA complexes. The first report of an ESI-MS study on drug-DNA complexes involved observing the non-covalent complex between actinomycin D and seven different single-stranded DNA sequences (Hsieh et al., 1994). This study used MS/MS as a tool to determine the differences in the non-covalent binding affinities between the different sequences used and actinomycin D. Pocsfalvi and coworkers also examined the interaction between a single stranded DNA (14 and 6 mers) with beauvericin (Pocsfalvi et al., 1997). They determined the relative binding affinities of the drug for different oligonucleotides and the drug using competition experiments and therefore demonstrated how ESI-MS can be used to determine relative binding affinities of non-covalent complexes. In both of these studies, the use of single-stranded DNA (ssDNA) and organic solvents (acetonitrile and methanol, respectively), makes the physiological relevance of this study unclear.

The first study reporting the detection of a non-covalent complex between a drug and duplex DNA involved the minor groove binder distamycin (Gale et al., 1994). In this study, the 1:1 and 2:1 complexes between distamycin and a self
complementary 12-mer, \(d(CGCAAATTTGCG)_2\) were detected. These researchers used various buffer solutions and determined that the most effective buffer for the observation of the drug-duplex DNA complex was an aqueous 10 mM ammonium acetate/10 mM ammonium citrate, pH 8.3 solution. These experiments were elaborated upon in future work with other drugs such as Hoechst 33258 and netropsin binding to self-complementary 14-mers (Gale and Smith, 1995). In these studies, the effect of various instrumental parameters (capillary temperature, capillary voltage and cone voltage) on the resulting ESI mass spectra were investigated. They determined that gentle interface conditions in the mass spectrometer increased the relative abundance of the non-covalent complex species in the gas phase. A greater abundance of non-covalent species were also observed when aqueous volatile buffers (10 mM ammonium acetate or a 10 mM ammonium acetate/10 mM ammonium citrate mixture) were used and when the DNA in the sample had been annealed prior to analysis by ESI-MS.

The first intercalated complex observed by ESI-MS was the daunomycin-\(d(CGATCG)_2\) complex (Triolo et al., 1997). These researchers, however, reported that the observed triply charged dsDNA-daunomycin peak was in extremely low abundance compared to either the ssDNA or the dsDNA. Conversely, similar experiments by these researchers on minor groove binders showed abundant ions from the complexed DNA which suggested that complexes containing groove binders are more stable than intercalated drug-DNA complexes under conditions used for ESI-MS.
A detailed study on the gas phase stability of double-stranded DNA as well as non-covalent DNA-drug complexes was carried out by Wan and coworkers (Wan et al., 2000b). These workers examined the binding stoichiometries and affinities for distamycin, Hoechst 33258, Hoechst 33342, berenil, actinomycin D, porphyrin H$_2$TmpyP-4, metalloporphyrins (CuTmpyP-4, FeTmpyP-4 and MnTmpyP-4) and Ru(II) 12S$_4$dppzCl$_2$ to 6 and 12 mer self-complementary DNA strands. These studies were carried out using an ion trap instrument. These researchers were able to distinguish between the binding of the various ligands of different types but did not present dissociation profiles of structurally similar compounds. For example, dissociation profiles distinguishing the unbound d(ATGCAT)$_2$, distamycin-d(ATGCAT)$_2$ (minor groove binder), actinomycin D-d(ATGCAT)$_2$ (intercalator) and MnTmpyP-4-d(ATGCAT)$_2$ (minor groove/intercalating) were presented. On the other hand, data presenting differences between the binding groups were not presented. Furthermore, these researchers also highlighted problems with ambiguity as self-complementary (double-stranded) oligonucleotides with even numbers of drug molecules bound and even numbers of charges have the same m/z value as corresponding single-stranded oligonucleotides with half the number of drug molecules bound (e.g. [2M+4I-4H]$^{4-}$ has the same m/z as [M+2I-2H]$^{2-}$ where I represents a bound drug). Following this study, the same researchers (Wan et al., 2000a) used the same drugs and extended their ion-trap MS studies by looking at these complexes with either AT rich or CG rich 6 mers. They determined that minor-groove binders show a marked preference for AT rich regions whilst intercalators prefer binding to CG rich sequences. This is consistent with data obtained using other techniques (Brana et al., 2001; Fox et al., 1985; Fritzsche et al., 1987; Haq and Ladbury, 2000).
Similar studies were also carried out by Gabelica and coworkers using an ion trap mass spectrometer (Gabelica et al., 1999). In these studies a non-self-complementary set of oligonucleotides (5'-GGGGATATGGGG-3' and 5'-CCCCTATACCCC-3') were used to enable unambiguous interpretation of spectra. The drugs used in that study included the minor groove binders netropsin, berenil, Hoechst 33258, DAPI, distamycin, and the intercalators ethidium bromide, amsacrine and ascididemin. These researchers used thermal denaturation curves and CID experiments to distinguish between the binding of the various drugs to DNA. It is important to note that whilst binding data differentiated between the various minor-groove binders, there were no comparisons distinguishing binding of the various intercalators to DNA. The intercalated drugs were displaced by a minor-groove binding compounds in these studies indicating that intercalation is generally a weaker mode of binding compared to minor-groove binding under these conditions. Furthermore, in a subsequent study presented by the same researchers (Gabelica et al., 2000), no data were presented to distinguish between the binding of various intercalators. Interestingly, the researchers used a 80:20 ratio of 100 mM aqueous ammonium acetate:methanol solution in the latter study (Gabelica et al., 2000). This is unique as previous ESI-MS studies on non-covalent drug-dsDNA complexes had used aqueous buffers containing volatile salts at near or around neutral pH.

It is important to note that most of the studies described above were primarily done using 6 mer DNA strands which may not fully reflect binding to DNA since one turn of a DNA helix requires 10 base pairs (Blackburn and Gait, 1990). Non-covalent
complexes formed with longer pieces of DNA are therefore expected to be more representative of a structure(s) which might occur in cells.

All the above studies involved measurement of dsDNA complexes as negative ions. Indeed, the first reported ESI mass spectra of dsDNA were obtained in the negative ion mode (Ganem, 1993). The use of negative ion spectra seems logical considering that at physiological pHs, phosphate groups are fully ionized (negatively) and a statistical contribution of counter ions ensures electroneutrality (Jayaram and Beveridge, 1996; Maher, 1998). Recent related work from our laboratory presented ESI mass spectra acquired as positive ions of complexes of DNA with cisplatin, daunomycin and distamycin (Gupta et al., 2001). The use of positive ion mass spectrometry on these DNA complexes is unusual as most earlier studies had utilised negative ion acquisition. Surprisingly, these positive ion spectra showed ions of only negligible intensity arising from single strands. It is important to point out that this study used longer more biologically-relevant DNA sequences (16 mer) and post-dated the work on intercalators conducted as part of the work described in this thesis.

1.6.2 ESI-MS of non-covalent protein-DNA complexes

ESI-MS has been used to observe a number of different types of non-covalent interactions with proteins. These include protein-metal, protein-protein, protein-peptide and protein-oligonucleotide interactions. The first non-covalent ligand complexes successfully observed by ESI-MS were myoglobin complexed to its heme group (Katta and Chait, 1991) and the FKBP-FK506 receptor-ligand complex
(Ganem et al., 1991). Since then, many other ESI-MS studies of non-covalent interactions have been reported (Loo, 1997; Veenstra, 1999).

ESI-MS has also been applied to protein-oligonucleotide complexes. Of the many studies on the ESI-MS of non-covalent protein-ligand interactions with proteins, only a relatively small number have been devoted to studies on the ESI-MS of DNA-protein complexes.

The first reported ESI-MS study of a DNA-protein complex involved the interaction between BSA and ss 20-mer phosphothioate oligonucleotides (Greig et al., 1995). These researchers observed a 1:1 ssDNA:BSA complex. Titration experiments of the BSA with DNA resulted in the observation of a 2:1 ssDNA-BSA complex when the concentration of DNA in solution was sufficiently high (3 \mu M). Dissociation constants for this complex varied depending on solvent conditions. For instance, when the solvent was changed from water to 10 mM NH₄OAc/imidazole, pH 7.5, there was a greater than 100-fold increase in Kᵦ. Increasing the concentrations of NH₄OAc whilst keeping the imidazole concentration constant in the sample mixture resulted in an increase in the dissociation of the complex. The dependance of Kᵦ on the ionic strength and pH of the buffer clearly suggest that electrostatic forces contribute significantly to the binding energy of the DNA-BSA complex.

The gene V protein from bacteriophage f1 stabilizes ssDNA during phage replication and also prevents the synthesis of the complementary DNA strand during the later stages of phage infection (Fulford and Model, 1988). The stoichiometry of binding of the gene V protein to several 13 – 16 mer ssDNA strands was investigated using a
Fourier transform ion cyclotron resonance mass spectrometer (FTICR) (Cheng et al., 1996a). Relatively gentle ESI conditions were used in these studies with a lower than normal capillary temperature used. The complexes were prepared in 10-50 mM NH$_4$OAc, pH 7.0. It was noted that oligonucleotides shorter than 15 bases formed a 2:1 ssDNA-gene V protein complex, whereas complexes containing the 16 mer ssDNA had a 4:1 ssDNA:gene V protein stoichiometry. Changes in the concentration of NH$_4$OAc in the reaction mixture did not affect the binding of the protein to the oligonucleotide.

The first ESI-MS study of a protein complexed to dsDNA involved the DNA-binding domain of the transcription factor PU.1 (PU.1-DBP) (Cheng et al., 1996b). This protein has been implicated in regulating gene expression during a variety of biological processes (Nye et al., 1992; Wasylyk et al., 1993). The same instrument and instrumental parameters were the same as described in the previous study (above) by the same group (Cheng et al., 1996a). A 1:1 complex was observed in 10 mM NH$_4$OAc, pH 7. Heating of the capillary resulted in dissociation of the non-covalent complex. In competition binding experiments it was determined that a consensus sequence (i.e. 5'-GGAA-3') was necessary for the formation of the complex. Thus a 17 mer oligonucleotide incorporating the 5'-GGAA-3' sequence formed a complex, whereas on the other hand, a 19 mer oligonucleotide without the consensus sequence did not bind to the PU.1-DBP as effectively. This result was confirmed by gel-electrophoresis and showed that ESI-MS can be used as a rapid and efficient method for the determination of DNA-binding oligonucleotides to their respective proteins.
Nuclear hormone receptors bind to regulatory DNA sequences and control the expression of target genes in the cell. In order for these receptors to bind to their target DNA sequences, Zn$^{2+}$ ions need to be present in high affinity zinc-finger binding sites (Freedman and Luisi, 1993). ESI-MS has been used to study the effect of Zn$^{2+}$ and Cd$^{2+}$ ions on the DNA binding properties of a nuclear hormone receptor-the vitamin D receptor DNA binding protein (VDR-DBP) to its specific DNA sequences termed vitamin D response elements (VDREs) (Veenstra et al., 1998a). The complexes were prepared in water and analysed in negative ion mode using gentle ESI conditions and a relatively low desolvation temperature of 80°C. The VDR-DBP was shown to bind to the VDRE only when 2 moles of Zn$^{2+}$ or Cd$^{2+}$ were bound per mole of protein. Further additions of the metal ions resulted in dissociation of the complex.

The xeroderma pigmentosum group A complementing protein (XPA) is believed to be involved in the process of nucleotide excision repair. XPA has been implicated in the recognition and binding of damaged DNA (Wood, 1997). The interaction between XPA and cisplatin-adducted oligonucleotides was studied using negative ion ESI-FTICR-MS (Xu et al., 1999). ESI conditions were again termed gentle with low capillary temperatures used as per previous studies by these researchers (Cheng et al., 1996a; Cheng et al., 1996b). The electrospray solvent used was 10 mM NH$_4$OAc, pH 6.7. Both the undamaged and cisplatin adducted 20 mer dsDNA were observed binding to XPA in a 1:1 ratio. A 2:1 complex between the XPA and the damaged cisplatin adducted oligonucleotide was also observed in these studies. As the mass difference between the 1:1 and 2:1 adducts only differed by 229 Da, they would be
difficult to detect using common biochemical techniques thus highlighting the advantages of ESI-MS in these experiments.

All of the ESI-MS studies of non-covalent DNA-protein interactions described above were carried out in negative ion mode. There have also been a number that employed positive ion ESI-MS. For example, *E. coli* trp apo-repressor protein (TrpR) is a DNA binding protein that is involved in the regulation of tryptophan biosynthesis. TrpR binds to its specific operator DNA sequence only when its co-repressor tryptophan is also bound. Duckworth and coworkers reported a comprehensive ESI-MS study on non-covalent interactions between TrpR, tryptophan and its specific operator DNA (Potier *et al.*, 1998b). A time-of-flight (TOF) mass spectrometer with gentle ESI conditions (low capillary, cone and desolvation temperatures) were used in these studies. This study highlighted the importance of sample preparation. For instance, when the complex was prepared in 5 mM NH₄OAc, pH 6, two distinct charge envelopes were observed which indicated that the TrpR existed in two different conformations and further that the protein existed as dimers or trimers. In addition, there was no evidence of the DNA-TrpR-*trp* complex when this buffer was used. In contrast, when the complex was prepared in 20 mM Na₃PO₄, pH 6, 90 mM NaCl, followed by dialysis against 10 mM NH₄OAc, pH 6, a 1:1 protein-dsDNA complex was observed. The application of ESI-MS for assessing sequence specificity of protein-DNA complexes was also highlighted using competition experiments. In these studies, the only complex observed had the specific DNA bound suggesting that the ESI-MS results reflected solution-phase data. The effect of the two isomeric forms of tryptophan (D and L) on the stability of these complexes was also investigated. D- tryptophan appeared
to bind to the complex with poor specificity and affinity whilst the L-isomer has a high affinity for the complex.

The vitamin D receptor (VDR) and retinoid X receptor-α (RXRα) belong to a family of ligand-dependent transcription factors termed steroid hormone receptors (SHRs). These factors bind to a specific DNA sequence (osteopontin vitamin D response element – OP VDRE) to enhance or suppress gene transcription. ESI-MS has been used to analyse the binding of VDR and RXRα to the OP VDRE and to assess the influence of ligands such as 1α, 25-dihydroxyvitamin D₃ (1,25-[OH]₂D₃) and 9-cis-retinoic acid (9-c-RA) (Craig et al., 1999). These researchers observed the 4:1:1:1 complex of Zn²⁺:VDR:RXRα:Op VDRE which is to date the largest DNA-protein complex studied by ESI-MS (Mr 118.2 kDa). The study also reported that addition of excess 1,25-[OH]₂D₃ resulted in increases of complex containing the VDR protein whilst increases in 9-c-RA resulted in a greater abundance of complex containing RXRα. ESI interface conditions used in this study were harsher than the conditions typically used for the analysis of non-covalent complexes (higher source temperatures and capillary voltages).

GCN4 is a sequence specific DNA-binding protein responsible for gene transcription and for the general control of amino acid synthesis in yeasts (Hinnebusch, 1984). ESI-MS has been used to detect the tetramolecular non-covalent complex between the GCN4 dimer and its specific DNA binding partner (5’-ATGA(C/G)TCAT-3’) (Deterding et al., 2000). In addition, combining limited proteolysis of GCN4-DNA complexes with ESI-MS analysis compared to identical experiments with non-
complexed peptide allowed for the identification of specific amino acids believed to be involved in DNA binding.

These studies clearly indicate that ESI-MS has considerable promise as a tool for the study of non-covalent DNA-protein interactions. The bulk of these studies have emphasized the use of gentle electrospray ionization conditions namely low source temperatures and lower than normal capillary and cone voltages. Furthermore, most studies use aqueous volatile salts such as \( \text{NH}_4\text{OAc} \) and \( \text{NH}_4\text{HCO}_3 \) with no organic solvents present. These conditions prevent premature dissociation of the complex whilst still in solution.

1.7 Relationship between gas phase interactions and solution behaviour?

It is clear from the above that the mass accuracy of modern MS combined with the gentle nature of ESI can provide important stoichiometric information concerning the non-covalent binding between biomolecules in the gas phase. An important question that needs to be considered before the technique becomes widely accepted is the extent to which non-covalent interactions in the gas-phase are representative of solution behaviour. A second issue is whether gas phase studies can provide new, fundamental information concerning the nature of biomolecular interactions in the absence of solvent.

It has been quite a challenge to obtain ESI-MS data under conditions that more closely parallel physiological environments. Typical solvent conditions for
maintaining non-covalent interactions are highly dependent on the pH and ionic strength (including type of ionic species) of the solvent. On the other hand, ESI-MS is generally intolerant to the presence of high salt concentrations and non-volatile buffer components. The solution pH used for positive ion ESI-MS is typically in the range 2-4 whilst for negative ions it is typically in the range, 8-10. Furthermore, organic solvents including methanol and acetonitrile are often added to solutions to improve stability of the spray and therefore sensitivity. Unfortunately, typical ESI-MS solvent conditions are not, however, optimal for the formation of non-covalent complexes that usually require aqueous environments at near or around neutral pHs. Despite this, a number of ESI-MS studies have now been done using aqueous solutions without the addition of organic solvents and typical salts (Gale et al., 1994; Gale and Smith, 1995; Veenstra, 1999; Veenstra et al., 1998a; Veenstra et al., 1995; Veenstra et al., 1998b; Veenstra et al., 1997; Veenstra et al., 1998c).

In some cases higher ligand stoichiometries have been observed in binding studies with biomacromolecules than those measured in solution. (Smith and Light-Wahl, 1993; Smith et al., 1992). It has been suggested that these result from non-specific gas-phase aggregation. Additional studies have been presented illustrating examples where ESI-MS data do not concur with solution-phase characteristics. For example, work done on acyl coenzyme-A binding protein and acyl coenzyme-A analogues (Robinson et al., 1996) could not differentiate the various solution dissociation constants of the protein to its ligand using ESI-MS. Furthermore, changes in the length of the hydrocarbon acyl chain which greatly affected solution binding did not appear to have any effect on the stability of the gas-phase complex. Similarly, the gas-phase stabilities of the non-covalent complex between bovine
carbonic anhydrase and para-substituted benzenesulfonamide inhibitors were found to have no correlation to solution studies (Wu et al., 1997). These observations reinforce the major role the solvent (water) plays in many non-covalent interactions (Robinson et al., 1996; Schwabe, 1997). This is especially the case where differences in binding may be as a result of hydrophobic interactions. Care must therefore be taken when interpreting ESI-MS data acquired on non-covalent complexes in relating these data to solution behaviour.

Another difference between solution and gas phase interactions is that electrostatic interactions are thought to be strengthened *in vacuo* because of enhanced Coulombic stabilization of binding partners with opposite charges caused by the molecule being in a solvent-free environment (Loo, 1997). For example, the spermine-SBP (acidic spermine-binding-peptide) complex was readily detected using ESI-MS even though it has a weak binding constant (10^4 M) in solution (Feng et al., 1995). This complex also proved to be extremely stable in the gas-phase with increases in collision energy resulting in covalent bond dissociation prior to the dissociation of the non-covalent complex.

Although the above results indicated some ambiguities between gas phase and solution phase data, a number of studies using ESI-MS have supported solution data. For example, in competition studies of the binding of various phosphopeptide inhibitors to Src SH2 domain protein similar conclusions were drawn to other solution-phase studies (Loo et al., 1997). In that study the relative abundances of the Src SH2 protein-phosphopeptide complexes observed in the ESI-mass spectrum were consistent with their measured solution-phase binding constants. ESI-MS
experiments have also been used to generate Scatchard plots for the measurement of the binding constants of vancomycin antibiotics with tripeptide ligands (Lim et al., 1995). The gas-phase generated plots were in reasonable agreement with solution-phase data reported previously.

All the results described above clearly indicate that whilst ESI-MS has shown promise in the study of non-covalent complexes, the general applicability of ESI-MS has yet to be established conclusively and therefore there is a need for further, more detailed studies in this area.

1.8 Outline of the project

The major aim of this work was to use ESI-MS to examine a range of non-covalent interactions between dsDNA and either a replication terminating protein – Tus or a range of intercalators. The specific aims of the project include

To optimize instrument and experimental parameters for the analysis of DNA-intercalator and Tus-Ter complexes using ESI-MS.

To determine binding stoichiometries of each of the complexes, and;

To explore the utility of ESI-MS for the determination of binding affinities and the structural characterization of these complexes in general.

ESI-MS analysis of a non-covalent DNA-protein complex – the Tus-Ter complex has been described in Chapter 3. The Micromass QToF2™ time-of-flight mass spectrometer (described in Chapter 2) was used for these studies. Optimum conditions for the detection of the complex are described in detail. Differences in
the binding of the various DNA variants to the Tus mutants were investigated using a combination of MS techniques including cone voltage, desolvation temperature as well as collision energy. Furthermore, the dissociation of these complexes due to increasing concentrations of NH₄OAc are also described in the latter section of Chapter 3.

The VG Quattro™ triple quadrupole mass spectrometer (described in Chapter 2) was used for the analysis of the non-covalent complexes between dsDNA and the anthracycline antibiotics, nogalamycin and daunomycin. This work is described in Chapter 4. Optimisation of sample conditions including DNA annealing as well as optimization of buffers (determination of the concentration of NH₄OAc and solution pH) are described. A range of studies involving optimization of instrument parameters for the observation of the complexes including cone voltage, capillary voltage and desolvation temperature are also described. Details of drug titrations and competition experiments using 8 and 12 mer self-complementary DNA strands are also described in this chapter. The final section of this chapter describes difficulties encountered in the characterization of non-covalent complexes of self and non-self-complementary DNA duplexes with the anthracyclines.

Extensions of the DNA-intercalator studies are described in the latter sections of Chapter 4. These studies involved a non-self-complementary 16 mer and a range of intercalators including nogalamycin, daunomycin, ethidium bromide, hedamycin and retamycin. The Micromass Qtof2™ mass spectrometer (described in Chapter 2) was used in these studies. Instrument parameters including cone voltage, desolvation temperature and collision energy were used to differentiate between the binding of
the various drugs. Additionally, the use of positive ion ESI-MS for the observation and analysis of these complexes was also explored.
2 Materials and Methods

2.1 Reagents

All reagents used were of analytical grade. Milli-Q™ water was used in all experiments. Daunomycin, nogalamycin and ethidium bromide were obtained from Sigma Chemicals (St. Louis, MO, USA). Retamycin samples were kindly donated by Dr Gabriel Padilla (University of Sao Paolo, Brazil). Hedamycin was donated by Bristol-Myers Co. (Wallingford, CT, USA). Other commonly used reagents and solvents were ammonia, acetic acid, acetonitrile (HPLC grade), methanol (HPLC grade) (Asia Pacific Specialty Chemicals, Seven Hills, NSW, Australia). Ammonium acetate, sodium chloride, 1,4-dithiothreitol, ethylenediamine tetraacetic acid and polyethylene glycol were purchased from Sigma (St. Louis, MO, USA). Tris-HCl was purchased from ICN Pharmaceuticals (Aurora, OH, USA) and caesium iodide from Merck (NJ, USA).

2.2 Oligodeoxyribonucleotides

The experiments involved in these studies used a number of different oligonucleotides. Their sequences and theoretical masses are listed in Table 2.1. Oligonucleotide masses were calculated using an oligonucleotide mass calculator available as freeware online (Leuven, 1998).
Table 2.1: Sequences and masses of DNA used in these studies.

<table>
<thead>
<tr>
<th>Oligodeoxyribonucleotide</th>
<th>Mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Studies with intercalators</td>
<td></td>
</tr>
<tr>
<td>5'-CGGCAGCGC-3'</td>
<td>2411.6</td>
</tr>
<tr>
<td>5'-GGCCAGCC-3'</td>
<td>2410.6</td>
</tr>
<tr>
<td>5'-TGAGCTAGCTCA-3'</td>
<td>3645.4</td>
</tr>
<tr>
<td>5'-ATATATATATATATATAT-3'</td>
<td>4877.3</td>
</tr>
<tr>
<td>5'-ATATATACGTATATAT-3'</td>
<td>4878.3</td>
</tr>
<tr>
<td>5'-GGATATACGTATATCCC-3'</td>
<td>4880.3</td>
</tr>
<tr>
<td>5'-CCCCCCCCGGGGGGGGG-3'</td>
<td>4885.2</td>
</tr>
<tr>
<td>5'-CTCGTGGACGATCGAC-3'</td>
<td>4808.2</td>
</tr>
<tr>
<td>5'-GATCGAATCGAGACGAG-3'</td>
<td>4955.3</td>
</tr>
<tr>
<td>Studies with Tus protein</td>
<td></td>
</tr>
<tr>
<td>5'-ATGATAGGTTGTAAGAAAGG-3' (TerB)</td>
<td>6492.4</td>
</tr>
<tr>
<td>5'-CTTATGTTCAACACATCT-3' (TerB)</td>
<td>6354.2</td>
</tr>
<tr>
<td>5'-GATCGTATGTTGAACTAGC-3' (TerH)</td>
<td>6426.3</td>
</tr>
<tr>
<td>5'-AGATGCTATACACGAC-3' (TerH)</td>
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<tr>
<td>5'-ATGATAGGTTGTAAGAAAGG-3' (posn5 TerB)</td>
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<td>6330.2</td>
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<tr>
<td>5'-CTTATGAGCTTAAAGCTACAAG-3' (non-specific 24 mer)</td>
<td>7350.9</td>
</tr>
</tbody>
</table>

2.2.1 Preparation and purification of oligonucleotides

All oligonucleotides were purchased from Geneworks (formerly Bresatec) Adelaide, South Australia with a dimethoxytrityl protecting group attached to the 5' terminus.

Reversed phase HPLC using a C-18 octadecysilyl column (Waters 8 x 100 mm Delta Pak (Radial Pak)) was used to purify the oligonucleotides. Oligonucleotides were supplied in 33% ammonia and were dried prior to HPLC purification. This was typically done by gently bubbling gaseous nitrogen over the sample. Once dry, the samples were then redissolved in 10 mM NH₄OAc, pH 6.8. The first stage of HPLC
purification involved separation of the tritylated oligonucleotide from unwanted products of the synthesis including failure sequences. As the detritylated group is more hydrophobic, separation is enhanced on the non-polar C-18 column. This first step uses a linear gradient ranging from 0-60% acetonitrile in 10 mM NH₄OAc, pH 6.8 over 30 minutes at a flow rate of 1 mL/min. The fractions containing the trityl-on oligonucleotide were then collected, freeze dried and detritylated by treatment with 80% acetic acid at room temperature for 20 minutes, followed by addition of an equal volume of ethanol. Each detritylated sample was then freeze dried, resuspended in 10 mM NH₄OAc, pH 6.8 and further purified on the HPLC using the same conditions as those prior to detritylation. The detritylated, purified fractions were then collected, pooled and freeze dried. These samples were redissolved in water and stored at -20 °C. Aliquots of purified oligonucleotides (50-100 μM for the Quattro™; 1-20 μM for the Qtof2™) were dissolved in 5 mM NH₄OAc, 50% CH₃CN) and the purity of each was assessed by ESI-MS.

2.2.2 Determination of oligonucleotide concentration

Concentrations of single-stranded oligonucleotides were estimated by measuring the absorbance at 260 nm (A₂₆₀) on a model 265 Shimadzu UV-Visible spectrophotometer. The ε₂₆₀ value for each oligonucleotide was determined by summation of individual ε values of each base pair – 15400 M⁻¹cm⁻¹ (A), 11700 M⁻¹cm⁻¹ (G), 7300 M⁻¹cm⁻¹ (C) and 8300 M⁻¹cm⁻¹ (T) (Sambrook et al., 1989). These values were then used by application of Beer's Law.
2.3 Preparation of Drug-DNA complexes

2.3.1 Drug-DNA complexes analysed using the VG Quattro™ triple quadrupole mass spectrometer

Daunomycin and nogalamycin were dissolved (with the aid of sonication) in water or acetonitrile, respectively, giving 1 mM stock solutions. Solutions were stored at -20°C. All reactions were carried out in the dark.

In a typical experiment, adducts of daunomycin or nogalamycin with oligonucleotides were prepared by addition of 250 μL of a stock anthracycline solution to 50 μL of 1 mM (duplex) oligonucleotide in water. This example gives a drug/duplex DNA ratio of 5:1. In other experiments where this ratio was varied, or in competition experiments, volumes of stock drug or oligonucleotide solutions were changed accordingly. These mixtures were freeze dried and re-dissolved in 25 μL of 0.1 M aqueous NH₄OAc, pH 8, giving final concentrations of duplex DNA in all cases of 1-2 mM. Each mixture was heated in a water bath to ≥20°C above its calculated melting temperature. The melting temperatures of the oligonucleotides were calculated using a freeware online Oligonucleotide Properties Calculator (Leuven, 1998). Nogalamycin and daunomycin were shown by ESI-MS to be stable to thermal degradation under these experimental conditions. After 15 minutes, the heater was turned off and the reaction mixture was allowed to cool overnight to ambient temperature. The reaction mixtures were diluted 10-fold with water just prior to analysis by ESI-MS giving a final dsDNA concentration of 50-100 μM and a final NH₄OAc concentration of 10 mM.
Experiments carried out to examine the effect of pH (range 4-11) on complex formation, the pH of 0.1 M NH₄OAc solutions used for redissolving freeze-dried mixtures was adjusted using acetic acid or ammonia solution as required. To investigate conditions for annealing oligonucleotides, an experiment was conducted in which 5'-GGCTAGCC-3' was treated with a 5-fold molar excess of nogalamycin under the following conditions:

(a) annealing was carried out as outlined above,
(b) after heating to ≥20°C above Tm, reaction mixtures were transferred to ice and left overnight, and
(c) after heating to ≥20°C above Tm, reaction mixtures were transferred to ice for 10 minutes, then left at ambient temperature overnight.

All other variations on sample preparation have been outlined in the relevant text (Chapter 4).

2.3.2 Drug-DNA complexes analysed using the Micromass Qtof2™ mass spectrometer

In later experiments (chapter 4) using the Qtof2™ mass spectrometer, sample preparation methods were similar to those outlined above. Differences arose, however, in that after annealing the sample (duplex DNA concentration of 1 mM) in 0.1 M NH₄OAc, pH 8.5, these samples were diluted 100-fold with 0.1M NH₄OAc, pH 8.5 prior to analysis by mass spectrometry giving a final dsDNA concentration of 10 μM and maintaining the NH₄OAc concentration at 0.1 M. The drugs used in the studies in Chapter 5 were daunomycin, nogalamycin, ethidium bromide, hedamycin.
and retamycin. Ethidium bromide and hedamycin were dissolved (with the aid of sonication) in water or acetonitrile, respectively, giving 1 mM stock solutions. These solutions were stored in the dark at -20°C. All reactions were carried out in the dark. Retamycin was dissolved in 50% methanol/water giving 1 mM stock solutions. These solutions were also stored at -20°C in the dark.

2.4 Protein (Tus)-DNA complexes

2.4.1 Proteins

All Tus proteins were generously donated by Dr Nick Dixon (Research School of Chemistry, Australian National University, Canberra). Native Tus, his6Tus, A173T (his6Tus where Ala 173 was changed to Thr), R198A (his6Tus where Arg 198 was changed to alanine), and K89A (his6Tus where Lys 89 was changed to alanine) were expressed in E. coli, purified, and stored as previously described (Neylon et al., 2000). These protein samples had been characterized previously by mass spectrometry giving masses in agreement with calculated values (Neylon et al., 2000). Protein concentrations were determined by measurement of UV absorbance at 280 nm, using molar absorption coefficients of 39,700 M⁻¹cm⁻¹ (Coskun-Ari et al., 1994).

2.4.2 Ter and non-specific DNA sequences

Double-stranded DNA was prepared by heating complementary single-stranded oligonucleotides (2.5 mM in 0.1 M NH₄OAc, pH 8.0) to greater than or equal to 20°C
above melting temperature and allowing the solution to cool slowly overnight. Annealed DNA was stored at 4°C before use.

2.4.3 Preparation of Protein (Tus)-DNA complexes

Tus protein was supplied in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 20 % w/v glycerol. Tus-DNA complexes were prepared by dialyzing Tus (1-15 μM) against 10 mM NH₄OAc, pH 8.0, at 4°C, followed by mixing it with an equimolar amount of dsDNA (typically 500 μL of protein to 1 μL of DNA in 0.1 M NH₄OAc). The mixture was left on ice for 1.5 hr before injection into the mass spectrometer. All these experiments were carried out using a Micromass Qtof2™ mass spectrometer. In experiments in which the NH₄OAc concentration was varied, a small volume of 10 M NH₄OAc, pH 8.0, was added to the mixture 1 hr before mass spectrometry. In competition experiments, the two Tus protein samples were mixed in 800 mM NH₄OAc at pH 8.0 and allowed to equilibrate for 30 min, followed by addition of an equimolar amount of dsDNA, giving a final concentration of each component of the mixture of 10 μM. The mixtures were left on ice for 1.5 h before direct injection into the mass spectrometer. ESI mass spectra of samples of pure proteins used in these mixtures were acquired just before and after ESI mass spectra of mixtures to ensure that there had been no drift in calibration.

For the experiment aimed at comparing the relative ESI-MS response factors of free Tus and a Tus-TerB complex, the his₅tus-TerB complex was prepared and titrated with A173T in 800 mM NH₄OAc at pH 8.0. ESI mass spectra were acquired of mixtures in which TuS_total (the total concentration of all Tus protein, bound and free
in the reaction mixture) was maintained at 10 μM. The following relative amounts of A173T and his6Tus-TerB, respectively, were used in this experiment: 0 μM and 10 μM, 2 μM and 8 μM, 4 μM and 6 μM, 6 μM and 4 μM, 8 μM and 2 μM.

2.5 Electrospray mass spectrometry

Two mass spectrometers were used in these studies. Studies involving anthracycline-DNA interactions were carried out using a VG Biotech Quattro™ (now Micromass, Wyntheshawe, UK) (Chapter 3). Subsequent studies on drug-DNA interactions as well as all studies carried out on protein-DNA interactions were performed using a Micromass Qtof2™ (Micromass, Wyntheshawe, UK) mass spectrometer equipped with a Z-spray source (Chapters 3 and 4).

2.5.1 Quattro™ triple quadrupole mass spectrometer

The Quattro™ mass spectrometer shown schematically in Figure 2.1 has a quadrupole/hexapole/quadrupole mass analyser configuration. The instrument was originally a Quattro 1 which was subsequently upgraded in 1998 to meet Quattro 2 specifications. It has a range up to m/z 4000. Solvent was introduced through PEEK tubing by a Harvard Model 22 syringe pump (Natick, MA, USA) at a flow rate of 5-20 μL/min. In the case of experiments involving drug-DNA interactions, the solvent was water. The formation of a stable spray was aided by using a flow of nitrogen gas concurrent with the stainless steel capillary in the probe at a flow rate of ~10 L/hr (nebuliser gas). To enhance droplet evaporation, a flow of dry N2 gas at 60°C except where otherwise described counter-current to the solvent flow was used at a
rate of \( \sim 500 \ \text{L/hr} \). This flow is higher than commonly used for routine analysis \( (\sim 300 \ \text{L/hour}) \).

![Schematic representation of the VG Quattro™ triple quadrupole mass spectrometer. Adapted from Micromass Quattro™ Operators Manual.](image)

All ESI mass spectra were acquired in the negative ion mode using multichannel analyses (MCA) at a scan rate of 100 \( m/z \) per second over the range 400–2000. All data were acquired and manipulated using the Micromass MassLynx™ software system (versions 3.2-3.5). Typically, 15-25 scans were summed to obtain representative spectra. These summed spectra were smoothed using a 2 \times 2.5-m/z window with a Savitzky Golay algorithm and were presented subtracted with a polynomial of 11, 40% below the curve. The typical source parameters in the majority of these experiments are represented in Table 2.2. Instrument calibration was achieved using a standard CsI solution (0.75 \( \mu \text{M} \)) over the same \( m/z \) range of the ESI mass spectrum of the sample to be analysed.
Table 2.2: Typical operating parameters used on the Quattro™.

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<th>Source Parameters</th>
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<th>HV Lens</th>
<th>Cone</th>
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<th>Lens 3</th>
<th>Lens 4</th>
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<td>100 V</td>
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<td>350 V</td>
<td>210 V</td>
<td>50 V</td>
<td>650 V</td>
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</tbody>
</table>

2.5.2 Qtof2™ mass spectrometer

The Qtof2™ mass spectrometer has a quadrupole/hexapole/orthogonal accelerator time-of-flight mass analyser configuration as shown in Figure 2.2. It has a range of up to \( m/z \) 10 000 by virtue of the time-of-flight section. It is equipped with a Z-spray ion source. Samples were injected directly into the source using a Harvard Model 22 syringe pump at flow rates between 5 and 20 \( \mu \text{L/min} \). The formation of a stable spray was assisted by a flow of nitrogen concurrent with the stainless steel probe at \(~10\ \text{L/hr}\) (nebuliser gas) with a counter current flow of \( \text{N}_2 \) gas (desolvation
gas) at approximately 250 L/hr at 240°C for the DNA protein interaction studies (Chapter 3) or 40°C for later DNA drug interaction studies (Chapter 4). All data were acquired and manipulated using the Micromass MassLynx™ software system (Version 3.5).

Figure 2.2: Schematic representation of the Micromass Qtof2™ mass spectrometer. Adapted from Micromass Qtof2™ Operators Manual.

All ESI mass spectra acquired in studies of the DNA-protein interactions were acquired in the positive ion mode as continuum spectra over the \( m/z \) range of 1000-7000. Typically, 25-30 acquisitions at 500 \( m/z \) per second were summed to give representative spectra. These summed spectra were smoothed using a 2 x 30 \( m/z \) window with a Savitzky-Golay algorithm and are presented without background subtraction. The typical instrument parameters used in the acquisition of these
complexes are shown in Table 2.3 below. Note that in all experiments on protein-DNA interactions, the collision gas (argon) was left on at a pressure of ~15 psi.

Spectra used in studies of intercalator-DNA complexes were acquired in both positive and negative modes as continuum spectra. Positive ESI mass spectra were acquired using a probe tip potential of 2600 V and a cone voltage of 50 V. The source block temperature was always 40 °C whilst the desolvation temperature varied from 40 – 90 °C. The spectra were acquired over the range $m/z$ 1400 – 3000 with typically 50 to 70 acquisitions at 100 $m/z$ per 1 second summed to obtain representative spectra. The instrument was calibrated against a standard CsI solution (750 µM) over the same $m/z$ range.

Negative ion spectra were acquired using a probe tip potential of 2500 V and a cone voltage of 50 V. Source temperatures (source block and desolvation) were kept consistent with those in positive ion studies. Furthermore, the spectra were acquired and calibrated over the same range and at the same rate as those in the positive ion studies. Typical instrument conditions (both positive and negative ion modes) are shown in Table 2.4.
Table 2.3: Typical operating conditions used for ESI-MS experiments involving DNA-protein interactions studied on the Qtof2™ mass spectrometer.

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Table 2.4: Typical operating conditions (both negative and positive ion) for ESI-MS experiments of DNA-drug interactions studied on the Qtof2™ mass spectrometer.

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<td>Mass 2</td>
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</table>
3 ESI-MS of the Tus-Ter complex

3.1 INTRODUCTION

Much that is known today about DNA replication is largely a result of studies involving the bacterium *Escherichia coli*. Procaryotic DNA replication, a three-step process consisting of initiation, elongation and termination is known to occur in a bidirectional manner along the bacterial chromosome at a rate of about 1000 base pairs/second (Hyrien, 2000).

Replication begins at a point on the chromosome, the origin or oriC, and terminates in a region opposite the origin as shown in Figure 3.1. Two main components dictate replication arrest (Baker, 1995; Bierne *et al.*, 1994; Bussiere and Bastia, 1999; Sharma and Hill, 1995). One of these is a set of 6 termination DNA sequences, termed Ter. Each contains a consensus element of about 20 base pairs (Table 3.1).

*Table 3.1: Sequences of the various Ter sites of *E. coli*. The dashes indicate that the base is the same as in the TerA sequence. Only one strand of the complementary pair is shown.*

|        | T | T | T | A | G | T | T | A | C | A | A | C | A | T | A | C | T | A | A | T | T |
| TerA   | T | T | T | A | G | T | T | A | C | A | A | C | A | T | A | C | T | A | A | T | T |
| TerB   | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | - | A | T | A | T |
| TerC   | A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | C | - | A | T | A | T |
| TerD   | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | A | A | T | G |
| TerE   | C | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | T | T | A | A |
| TerF   | C | G | C | - | - | - | - | - | - | - | - | - | - | - | - | - | G | A | A | G | G |

These DNA termination sequences are sometimes also referred to as replication fork barriers (Hyrien, 2000). The other element is a *trans*-acting protein encoded by the

![Diagram of E. coli chromosome showing terminus sites](image)

**Figure 3.1:** A schematic of the circular *E. coli* chromosome showing the location of the six terminus sites. Two replication forks initiate at the origin, approach each other and encounter two groups of terminators (Ter sites). Those in the clockwise fork trap can arrest anticlockwise replication (Coskun-Ari and Hill, 1997).

### 3.1.1 The Tus gene and Protein

The **tus** gene lies ten base pairs downstream of the **TerB** site (Hill *et al.*, 1989). This gene codes for a protein of 309 residues and a mass of 35,652 Da (Hidaka *et al.*, 1989), (Hill *et al.*, 1989). The protein sequence has no homology with any other DNA binding motif. Tus has more basic residues compared to acidic ones with a predicted isoelectric point of 10.1 (Hill *et al.*, 1989).

The physical and biochemical characteristics of Tus have been determined using sucrose density gradient centrifugation as well as gel filtration column chromatography (Coskun-Ari *et al.*, 1994). These authors determined that the sedimentation coefficient ($S_{20,w}$) of Tus was 3.2 and it had an experimental
mass of 36,190 Da. This value was in good agreement with the calculated mass of 35,652 based on the amino acid composition. These data suggest that Tus exists as a monomer in solution (Coskun-Ari et al., 1994; Sista et al., 1989). Gel filtration data also indicated that the protein would cover 13 base pairs of DNA on binding which was in agreement with earlier footprinting studies (Sista et al., 1989).

The actual isoelectric point of native Tus (pH 7.5) is significantly different from that estimated from amino acid content (pH 10.1). The difference indicates that the tertiary structure of Tus perturbs the ionisation of positively charged residues (presumably in the central cleft of the protein). Circular dichroism spectroscopy indicated that the protein comprises 40% α-helix, 0% in β-sheet, 15% in turns and 45% in aperiodic structures (Coskun-Ari et al., 1994). Subsequent X-ray crystallographic data, however, indicated that the binding cleft of Tus contains some β-sheet structure (Kamada et al., 1996).

Several mutants of Tus have been isolated. The first naturally occurring mutants of Tus involved point mutations which changed the alanine residue observed in the wild type protein to threonine (A173T) or valine (A173V) (Skokotas et al., 1994). The A173T mutant had a 4100-fold lower affinity for Ter sites as compared with the wild type protein based on studies which measured DnaB-catalyzed strand displacement in an *in vitro* helicase assay. The A173V mutant on the other hand bound 130-fold less tightly to Ter.

The following year, the same researchers (Skokotas et al., 1995) discovered three new Tus mutants based on cell culture colony numbers. They named these Tus
P42S, Tus E49K and Tus H50Y. The relative replication arrest activities of these mutants compared to the wild type (1.04) were 0.37, 0.38 and 0.81, respectively (Skokotas et al., 1995).

The X-ray crystal structure of the complex was determined by Kamada and coworkers (Kamada et al., 1996). It is important to note that the crystal structure was derived from a Tus-\textit{TerA} complex. The structure is divided into three distinct regions – 2 $\alpha$-helical regions and a central $\beta$-structure. These together form a large positively charged cleft. The \textit{Ter} DNA is accommodated into this cleft with the two $\alpha$-helical regions and the central $\beta$-structure embracing 13 base pairs of the duplex DNA.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{ribbon.png}
\caption{Ribbon drawing of the complex viewed perpendicularly to the helical axis of the DNA (left) and above the helical axis of the DNA (right). Figure adapted from Kamada et al. (Kamada et al., 1996).}
\end{figure}

These two domains are connected via well defined, twisted anti-parallel $\beta$-strands (bF-bG and bH-bI) and the extended L4 loop (D283-L296). These strands contribute to the contact with the bases of the major groove and the sugar phosphate
backbones of *Ter* DNA and cross each other owing to bulges and two proline residues.

Reference to this structure enabled descriptions of the interactions that had been weakened in complexes of *TerB* with the mutants described above. For example, it is believed that the replacement of A173 by bulkier residues inhibits DNA binding. In addition, Kamada *et al.*, postulated that the E49K and H50Y mutations resulted in conformational changes of the flexible L1 loop of the protein, hence affecting DNA binding (Kamada *et al.*, 1996). Several other amino acids make contact with the DNA backbone and bases. The points of contact in each of these is summarized in Table 3.2 below.

*Table 3.2: The amino acids that make contact with the double-stranded DNA either via the backbone or via direct contact with the bases.*

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<tr>
<th>Amino acid contacts with DNA backbone</th>
<th>Amino acid contacts with DNA bases</th>
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</tr>
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<td>T136</td>
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<td>A91</td>
<td>A173</td>
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52
Based on the X-ray structure of the Tus-*TerB* complex, Kamada and coworkers (Kamada et al., 1996) also screened a variety of mutants using molecular modeling techniques. They determined that four mutants could disrupt the hydrogen bond network within the complex (K175E, R232S, Q237R and Q252R). It is believed that the hydrogen-bond and water network is critical for the sequence recognition in the major groove of DNA. Other mutations screened were found to be responsible for destabilizing the hydrophobic interactions and thus preventing binding to *Ter* DNA. These mutants include P95H, P95L and P238L. Two other mutants were discovered that would lead to perturbation of the protein domains. For instance, the L150Q mutation destabilizes the hydrophobic region between the \( \alpha IV \) and \( \alpha V \) helices in the amino domain. In addition, the Y156C mutation disrupts the hydrogen bond to the phosphate on A12 and disturbs the hydrophobic region of Tus lending support to the theory that the orientation of the \( \alpha IV \) and \( \alpha V \) region is important in replication arrest.

Recently, surface plasmon resonance (SPR) studies have been used to study binding between Tus and *Ter* DNA. In addition, the binding between three new mutants – K89A, R198A and Q250A and *TerB* DNA was examined (Neylon et al., 2000). The mutants K89A and R198A are both at the end of the protein that blocks the replisome - the non-permissive face. Most of the sequence-specific contacts are made at this end and are therefore of interest in investigating fork arrest. Residue Q250 is not in the central cleft of the Tus protein but can also make sequence specific contacts with the *Ter* DNA at the permissive face.
Mutational analysis of Tus and X-ray crystal structure information (Kamada et al., 1996) indicated that the amino acids in the L1 loop were likely to play an important role in replication arrest. This led to site directed mutagenesis being used to produce a new mutant, E47Q (Henderson et al., 2001). This mutant had a greater affinity for the Ter site but a reduced ability for replication arrest (Henderson et al., 2001). These results support the idea that these residues in the L1 region have a role in the protein-protein interactions responsible for Tus function and indeed that replication arrest has more variables than simple DNA binding.

These mutants and/or mutations identify a number of residues important in replication arrest. Many confirm the importance of specific amino acid - DNA binding. Others have more of an effect aligning the protein to properly accommodate the DNA.

### 3.1.2 The Ter Sequences

The first nucleotide sequence identified that corresponded to a terminator region (Ter) was found in the plasmid R6K (Bastia et al., 1981a; Bastia et al., 1981b; Germino and Bastia, 1981). These studies identified a region that spanned 216 base pairs. This region was then narrowed down in subsequent experiments to a 20 base pair region (Horiuchi and Hidaka, 1988).

Later, it was found that this region contained two loci-termed T1 and T2, and that they required the trans-acting factor Tus to inhibit the progress of replication forks (Hill et al., 1988a; Hill et al., 1988b; Pelletier et al., 1989). These workers also
reported that the terminator signal sequence was dependent on the orientation in the chromosome, both in the R6K plasmid and on the *E. coli* chromosome. Further studies on the Col E1 plasmid (Pelletier *et al.*, 1989) showed that these T1 – T2 regions could block replication forks *in vivo*. *In vitro* studies in replication systems prepared from cell extracts proved that terminator sites could result in replication arrest (Khatri *et al.*, 1989).

The known *Ter* sequences were originally identified as 22 –23 base pairs in length based on sequence identity between *TerA*, *TerB* and *TerC* (Hidaka *et al.*, 1988; Hill *et al.*, 1988b). As chromosome mapping identified additional sites (*Ter D-J*), it became clear that the essential conserved elements of the *Ter* site was on an 11 base pair core sequence (positions 9-19) (Coskun-Ari and Hill, 1997) as shown in Figure 3.3. Experiments involving nucleoside analogs used to map the determinants of Tus binding (Duggan *et al.*, 1996; Duggan *et al.*, 1995) show that G residues at positions 10, 13 and 17 within the core sequence contribute to both major and minor groove interactions (Figure 3.3). The G residue at position 6 has little or no effect on the stability of the Tus-*Ter* complex. In addition, these researchers showed that hydrophobic interactions occur with thymidine methyl groups at positions 8, 9, 12, 14, 16 and 19 (Figure 3.3).

![Figure 3.3](Image)
The question has been raised as to whether variations in the DNA sequence can change the binding properties in the Tus-Ter complex. Studies on the effect of base pair substitution on the arrest of DNA replication were carried out using competition essays (Coskun-Ari and Hill, 1997). In this study, a series of DNA sequences in and around the core 11 base pair region were varied. The numbering terminology used in the following discussion is that used in that study (Coskun-Ari and Hill, 1997).

The first set of substitutions was done at positions 6 and 7 (Figure 3.3). Position 6 is a well-conserved G-C base pair. Substitution studies on that position showed little change suggesting that the nucleotide in that position contributes little to the stability of the Tus-Ter complex. This is consistent with earlier competition assay experiments (Duggan et al., 1995).

In contrast, position 7 is not well conserved in Ter sites and the X-ray structure reveals that it does not contact any amino acid residues (Kamada et al., 1996). It is therefore not surprising that changes to this base yielded little or no change in replication arrest activity.

The A-T base pair at position 8 is well conserved in all known chromosomal Ter sites. However, several plasmid (including R6K) Ter sites have a G-C base pair instead. Tus makes two contacts with the thymidine residue at position 8 via K89 (oxygen atom) and T139 (major groove contact). When A-T was substituted, G-C made the smallest difference whilst C-G and T-A had a substantial impact on replication arrest. This has been rationalised by the suggestion that the G-C substitution retains contact with K89 and loses contact with T139. The other two
base pair changes eliminated both contacts and had a reduced replication arrest activity. These data suggest that the base pair at position 8 plays a significant role in both Tus binding and replication arrest.

Substitutions were also performed on positions 9 – 19, which constitute the 11 base pair core sequence. Substitutions in this region imposed severe energetic penalties on DNA binding and loss of replication activity. In particular, substitutions at positions 10, 12 – 16 and 19 precluded replication arrest whilst single substitutions at positions 17, 18 and multiple substitutions at positions 9 and 11 were tolerated. Substitutions in position 11 were tolerated better than any other in the core region. The amino acid V234 makes contact with the substitution at position 6 where G.C was substituted for C.G.

### 3.1.3 The **Tus-Ter** Complex

Data accumulated via various experiments involving mutations of both the Tus and the DNA have proven invaluable in generating a better understanding of the Tus-Ter complex. In addition, experiments including DNA footprinting and dissociation kinetics on these protein mutants and gene sequences from *E. coli*, *B. subtilis* and plasmids like R6K have generated vast amounts of valuable information. Recently, the crystal structure of the complex (Kamada *et al.*, 1996) has also been determined. The question of the exact binding mechanism in Tus-Ter complexes has still to be resolved.
Several mechanisms have been proposed for this Tus-Ter replication arrest. The first proposed mechanism is a clamp system (Kamada et al., 1996). In this model, there is complete dissociation of Tus from the DNA prior to the passage of a helicase into the Ter site. The major drawback of this mechanism, however, lies in its inability to explain polar arrest. If helicases are hindered using a thermodynamic rationale, then it would appear likely that similar energetics would be required for the traveling fork in either direction and the fork would be blocked in either direction. To explain polarity therefore, mechanistic and kinetic arguments must be added to the model. For example, a more complex mechanism has the clamp working in two stages. In the first step, the DNA arrives in the correct orientation causing a conformational change in the DNA binding residues leading to non-specific binding. The second step then includes either a tight specific binding of the DNA or its removal from the system.

A more sophisticated clamp model was proposed to be a zipper-like interaction (Neylon et al., 2000). In a similar fashion to the simple clamp model, the first step involves non-specific interactions. The non-specifically bound protein then ‘scans’ the DNA searching for a Ter site (Berg et al., 1981; Shimamoto, 1999). Once found, specific recognition of the DNA requires rearrangement of the β-strands in the core region such that the DNA aligns to allow for strong sequence specific contacts. Once bound, the C-domain tightens about the DNA rendering it bent. This is consistent with the crystal structure (Kamada et al., 1996).

Another model proposed involves protein-protein interactions. These interactions are proposed to exist within Tus as well as between Tus and other proteins of the
replisome. This model seems rather complex, however, and has significant limitations. For example, in this model the complex can be formed without the need for other replication proteins to be present. As other replication proteins have been discovered, the ideas behind this model make it less attractive.

### 3.2 Outline of this work

The Tus-Ter replication system is very well characterized with data obtained from a variety of sources. It was therefore considered to be an excellent system for an examination of the potential of ESI-MS to study DNA-protein interactions for a number of reasons. These include both the availability of solution-phase data on Tus-Ter itself and a variety of available protein and DNA mutants. Furthermore, there are unresolved questions concerning the nature of interaction to which ESI-MS might make some contributions.

The first challenge was to prove that the instrument configuration was sufficiently sensitive to detect the complex. Once detected, the instrument and sample conditions needed to be optimized to obtain the best possible signal-to-noise ratio and resolution. These results are described in the early part of this chapter. Once optimized, we explored the differences in the binding between various DNA variants/Tus mutants in an attempt to compare these data to solution phase data and therefore gain an improved understanding of differences between the solution and gas phase behaviour of this type(s) of complexes.
3.3 ESI-MS of Tus protein

One of the first experiments performed involved acquiring an ESI mass spectrum of Tus alone in 10 mM NH₄OAc, pH 5, and changing the cone voltage whilst keeping other instrument parameters constant.

In these preliminary experiments, ESI mass spectra of Tus protein were acquired using conditions that might be used for non-covalent complexes. Desolvation temperature was kept low (40°C) as previous studies had shown that low temperatures were essential to maintain the integrity of non-covalent complexes (Kapur et al., 1999a; Loo, 1997). Tus at 20 V shows a typical spectrum of a protein with a fairly symmetrical charge envelope distribution. On increasing the cone voltage to 60 or 100 V, there is an increase in the abundance of species of higher mass to charge ([M+15H]¹⁵⁺, [M+16H]¹⁶⁺, and [M+17H]¹⁷⁺). It is possible that this may be the result of a decrease in the abundance of lower m/z ions from fragmentation resulting in an increase in the abundance of these species of higher mass to charge. The expected values of m/z for Tus and Tus-TerB are shown in Appendix 1. The Tus spectrum acquired using a cone ramp of 20-50 V shows a broader charge envelope similar to that seen in the spectrum at 20 V but with slightly better signal-to-noise ratios for the ions at higher m/z ratios.

A second series of experiments shown in Figure 3.5 was carried out in which the spectra of Tus (10 mM NH₄OAc, pH 5) alone were recorded at different capillary voltages with cone voltage and desolvation temperature kept constant (50 V and 40°C, respectively).
Figure 3.4: ESI mass spectra of Tus (10 μM) in 10 mM NH₄OAc, pH 5 at different cone voltages of: (a) 20 V, (b) 60 V, (c) 100 V and at (d) a ramp of 20-50 V. Capillary voltage (2.0 kV) and desolvation temperature (40° C) were kept constant.

These spectra again show charge envelopes typical for proteins. The spectrum at 1.6 kV (Figure 3.5a) has more intense ions at lower m/z with the most abundant ion being [M+31H]^{31+} (m/z 1151.1). An increase in capillary voltage to 2.0 (Figure 3.5b) and 2.5 kV (Figure 3.5c) results in a change whereby the most abundant ions
correspond to the \([\text{M}+30\text{H}]^{30+} \ (m/z \ 1189.4)\) and \([\text{M}+27\text{H}]^{27+} \ (m/z \ 1321.6)\) ions, respectively.

![Graphs showing mass spectra](image)

**Figure 3.5:** ESI mass spectra of Tus (10 \(\mu\)M) in 10 mM NH4OAc, pH 5, at different capillary voltages of (a) 1.6, (b) 2.0, and (c) 2.5 kV. Cone voltage (50 V) and desolvation temperature (40°C) are kept constant.

When determining conditions to study the non-covalent binding of Tus with its DNA binding partner, it was important to achieve sample conditions that did not compromise the biological integrity of the protein or the oligonucleotide. For example, the conditions should maintain the native conformation of the protein. Most studies of non-covalent biochemical complexes use traditional buffers, which contain non-volatile salts such as sodium and potassium. These alkali metal ions are however not appropriate for ESI. ESI sample preparation usually involves the use of volatile salts and therefore compromises on sample preparation conditions need to
Figure 3.6: ESI mass spectra of Tus (10 μM) in 10 mM NH₄OAc, at pH values of (a) 5.0, (b) 6.0, (c) 7.0, and (d) 8.0. Cone voltage (50 V), capillary voltage (2.0 kV) desolvation temperature (60°C) are kept constant.

be made. Figure 3.6 shows the ESI mass spectra of Tus that had been dialysed in 10 mM NH₄OAc in the pH range of 5 to 8. At pH 5 (Figure 3.6a), the spectrum showed multiple ions with a typical charge envelope with the most abundant peak being [M+25H]²⁺ (m/z 1427.1). Upon increasing pH, the charge distribution becomes markedly different with the most abundant ions having higher m/z values.
([M+13H]^{13+} \text{ at } m/z 2743.5) \text{ in Figure 3.6b-d. The change in the charge distribution on acidification of the protein solution can be explained in terms of unfolding of the protein leading to exposure of a greater number of basic residues resulting in sharper peaks at lower pH (Chowdhury and Chait, 1990; Jarrold, 1999; Konermann and Douglas, 1998a; Konermann and Douglas, 1998b). At pHs from 6 to 8, the peaks are very broad (peak width at half height \sim 115 m/z units). NMR studies carried out by Dixon and Otting (unpublished data, private communication) suggest that Tus is fully folded at pH 8 and unfolds at pH less than 6. In addition, broader peaks at higher pH levels indicate that the protein has water and salt entrapped in its structure when folded. It was thus determined that ammonium acetate at pH 8 represented the optimum solution for maintenance of a folded protein conformation.}

### 3.4 ESI-MS of the Tus-\textit{TerB} complex-preliminary experiments.

#### 3.4.1 Initial optimisation for the Tus-\textit{TerB} complex.

In preliminary experiments, a Tus-\textit{TerB} complex was prepared by dialysing the binding partners together against various concentrations of NH$_4$OAc at pH 8. Figure 3.7 shows the ESI mass spectra of the complex when prepared by dialysis against 5 mM NH$_4$OAc (Figure 3.7b) and 50 mM NH$_4$OAc (Figure 3.7a), pH 8. In Figure 3.7b, the most abundant ions observed are from Tus-\textit{TerB} complex (m/z 3032.2, 3234.6 and 3464.8). There are also ions of low abundance from free Tus (m/z 3241.9, 2972.2 and 2743.6). Furthermore, a small amount of complex containing 2 Tus with 1 \textit{TerB} is also evident (m/z 4008.7, 4209.4, 4431.1). This is presumably as a result of non-specific associations during the ionisation process as the crystal structure of Tus only allows for a single TerB molecule to fit into the Tus cleft (Kamada \textit{et al.},
A complex with the Tus dimer binding to Ter has however, been noted previously (Sista et al., 1989) where it was thought to result from non-specific interactions. There has been no corroborating data supporting a biological role for a dimer. The dimer was not observed consistently and following optimisation of the remaining variables involved in preparation of the complex, this non-specific complex was not observed (more below). When the Tus-TerB complex was prepared in this way but dialysed against 50 mM NH₄OAc, pH 8, ions from free Tus were predominant (Figure 3.7a).

These observations are consistent with a proposal put forward by Dixon and his coworkers (Neylon et al., 2000) for the mechanism of Tus-TerB binding. In that proposed mechanism, Tus and TerB form a relatively weak complex held together by electrostatic interactions. Presumably when Tus and TerB are mixed together in 50 mM NH₄OAc, pH 8.0, then this weak complex is not readily formed. In the proposed mechanism, however, once the weak complex is formed, a conformational change takes place that allows for more specific and tighter interactions e.g. A173 with thymine (Kamada et al., 1996). This is also consistent with later experiments that showed that the complex formed by mixing Tus and TerB that had been dialysed separately in 10 mM NH₄OAc, pH 8 and then allowed to equilibrate 1-2 hours for complex formation was very stable to increasing salt concentrations (see for example Figure 3.17; Figure 3.19).
Figure 3.7: ESI mass spectra of the Tus-TerB complex prepared by dialysis of the mixture under different salt conditions; (a) 50 mM NH₄OAc, pH 8 and (b) 5 mM NH₄OAc, pH 8. (●) Ions from free Tus protein; (◆) Ions from the Tus-TerB complex; (★) Ions from the 2Tus-TerB complex.

Figure 3.8 shows the effect of changing cone voltage on the Tus-TerB complex prepared by dialysis of Tus and TerB together in 10 mM NH₄OAc, pH 8. The most notable differences are in relation to peak widths and non-specific binding. At 25 V, the [M+15H]¹⁵⁺ ion (observed m/z 3234.6) has a peak width-at-half height of m/z 39 whilst at 50 and 100 V, the same peak is 21 and 41 m/z units wide, respectively. Ions from the non-specific interaction resulting in two Tus molecules binding to one TerB molecule (observed m/z 4015.3, 4210.4 and 4431.3) are also more abundant at 100 V. Since this interaction is not thought to be biologically relevant, conditions to minimise its observation are preferred. In both respects, therefore, it is apparent that a cone voltage of 50 V results in the best spectra of the Tus-Ter complex.
The use of a small voltage applied to the collision cell and a relatively low pressure of collision gas has been shown to improve the sensitivity of the Qtof2™ for the detection of large proteins. It is thought that an elevated pressure of gas (usually argon) in the collision cell reduces the energy spread of the ions entering the time-of-flight analyzer. In addition, these collisions can help remove water and salt clusters bound to the molecule resulting in narrower peaks.
The Tus-\textit{TerB} complex was subjected to limited increases in collision energy as a means of improving signal sensitivity and these spectra are shown in Figure 3.9. For, the ion $[\text{M}+15\text{H}]^{15+}$ (observed $m/z$ 3237.0) shows a peak width at half height of 85 $m/z$ units when in the cell with collision gas on but no collision energy. Furthermore, there is a large amount of dimeric complex observed in this spectrum. Increasing the applied voltage to 5 (Figure 3.9a), 15 (Figure 3.9b) and 20 V (Figure 3.9c) results in sharpening of the peak from 35 to 25 to 20 $m/z$ units respectively for the given peak (in this case $m/z$ 3237.0) and also results in a marked reduction in the amount of dimeric complex observed. This is most likely from dissociation of the weak interactions holding the non-specific dimer together. It is thus clear that limited increases in voltage applied to the cell result in sharper, more intense peaks as well as diminishing the non-specific 2Tus-\textit{TerB} complex.

Figure 3.10 shows the effect of increasing desolvation temperature on the Tus-\textit{TerB} complex. The most significant differences between these spectra are in the peak widths. At 40°C, for example, the peak width at half height of the $[\text{M}+15\text{H}]^{15+}$ ion (observed $m/z$ 3237.2) is 29 $m/z$ units. At 100°C, the same peak has a peak-width-at half height of 24. Increased temperatures of 160 and 200°C result in further narrowing of the peaks to 22 and 15 $m/z$, respectively, in the case of the $[\text{M}+15\text{H}]^{15+}$ ion. It thus follows that the best ESI mass spectra of Tus-\textit{TerB} complex are obtained at a desolvation temperature of 240°C. These observations do not, however, correlate well with earlier studies that emphasise the need for lower temperatures to maintain the structural integrity of non-covalent dsDNA-protein complexes (Cheng \textit{et al.}, 1996a; Deterding \textit{et al.}, 2000; Potier \textit{et al.}, 1998a; Veenstra \textit{et al.}, 1998a; Xu \textit{et al.}, 1999). For example, in studies involved in looking
at the binding between the gene V protein to DNA (Cheng et al., 1996a), the researchers stressed the use of extremely mild interface conditions in order for the

![ESI mass spectra of the Tus-TerB complex under different collision conditions](a) 0 V, (b) 5 V, (c) 15 V and (d) 20 V. Other instrument parameters (capillary 2.5 kV, cone 50 V and desolvation temperature 120°C) were kept constant. (●) Ions from free Tus protein; (◆) Ions from the Tus-TerB complex; (★) Ions from the 2Tus-TerB complex.

Figure 3.9: ESI mass spectra of the Tus-TerB complex under different collision conditions; (a) 0 V, (b) 5 V, (c) 15 V and (d) 20 V. Other instrument parameters (capillary 2.5 kV, cone 50 V and desolvation temperature 120°C) were kept constant. (●) Ions from free Tus protein; (◆) Ions from the Tus-TerB complex; (★) Ions from the 2Tus-TerB complex.
experiments to work. They used a heated capillary (60°C) which was cooler than typically used. Deterding and coworkers (Deterding et al., 2000) also used a very low temperature desolation gas at 35 °C in their studies on the complex between the DNA-binding leucine zipper peptide dimer and double stranded DNA. Similarly in our own work investigating complexes of DNA with drugs, a temperature of 60°C was used (discussed in detail in Chapter 4) (Kapur et al., 1999a). Earlier ESI-MS studies on DNA-protein interactions have involved low source temperatures as a

![Figure 3.10: ESI mass spectra of the Tus-TerB complex (10 μM) in 10 mM NH₄OAc pH 8, at different desolation temperatures; (a) 40°C, (b) 100°C, (c) 160°C and (d) 240°C. Other instrument parameters remained constant (cone 50 V, capillary 2.5 kV). (●) Ions from free Tus protein; (♦) Ions from the Tus-TerB complex; (*) Ions from the 2Tus-TerB complex.](image-url)


means to avoid dissociation of these non-covalent complexes in the ESI source region. The rationale behind this is that increases in temperature result in an increase in the energy of the molecules with concomitant dissociation of the molecule. The differences noted here may be explained by the tight binding of Tus to TerB that ensures that the complex is not dissociated at higher temperatures. Under these conditions, it is expected that there may be some removal of water molecules from the complex. It is therefore important to point out that this may be a vital difference in solution versus gas phase studies of this interaction.

The results pertaining to changing instrument conditions and the effect these changes have on the peak widths of Tus-TerB complex are summarised in Table 3.3.

Table 3.3: Peak-width-at-half-height values for the [M+15H]\(^{+}\) ion using the different instrument parameters- desolvation temperature, cone voltage and collision energy. The best conditions are highlighted in the grey boxes.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Peak width at half height (m/z)</th>
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<td>26</td>
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<td>20</td>
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The optimal instrument conditions for analysis of the complex therefore are capillary 2.5 kV, cone voltage 50 V, desolvation temperature 240°C and a collision energy of 20 eV.

3.4.2 Optimisation of sample preparation for detection of Tus-TerB

For all the experiments outlined above (Figure 3.4 - Figure 3.10), the complex had been prepared by mixing together Tus and TerB in Tris-HCl buffer supplied by our collaborators (Dixon and Brown, ANU) (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 20% w/v glycerol) incubating on ice at 4°C for 30 minutes, and then dialysing overnight at 4°C against 3 changes of 10 mM NH₄OAc, pH 8. Even though the best instrument conditions were determined, it was observed that there was some degree of variation in the complex sample from day to day (data not shown). The differences included ratio of free protein versus complexed protein, the presence of small amounts of 2Tus-TerB, and the presence of a precipitate seen in the dialysis tubing.

Hence, a series of experiments was conducted in an attempt to develop better complex preparation conditions, which might give more reproducible ESI mass spectra of the Tus-TerB complex. For these experiments Tus protein was dialysed alone in 10 mM NH₄OAc, pH 8. After dialysis of the protein, an equimolar amount of TerB stock (2.5 mM in 100 mM NH₄OAc, pH 8) was added to the protein. Typically, only small volumes of the TerB stock was added to the protein (1-2 μL in 1 mL of protein solution) thereby ensuring that the final NH₄OAc concentration was approximately 10 mM NH₄OAc at all times. The mixture was then left to stand on ice for one and a half hours. Care had been taken to ensure that the protein
concentration was as close to 10 μM as possible and further that the total volume was no less than 500 μL. An ESI mass spectrum of the Tus-TerB complex prepared in this way was obtained using optimal instrument conditions described in Table 3.3. The spectrum shown Figure 3.11a shows that almost all the protein exists in a complex with TerB. Further, little or no precipitation was observed and there were no ions from the non-specific complex. Also shown in the figure (Figure 3.11b) is the spectrum of the complex prepared in the previous manner which shows close to 40% free protein. Subsequent experiments were thus performed using stock TerB added to the already dialysed protein giving reproducible ESI mass spectra of the Tus-TerB complex in which there were negligible amounts of free protein and no ions from 2Tus-TerB.

Figure 3.11: Differences observed in complex preparation techniques. In (a), the complex was prepared by addition of a stock solution of TerB (2.5 mM in 100 mM NH₄OAc, pH 8) to the already dialysed Tus (10 μM in 10 mM NH₄OAc, pH 8). In (b), the complex was prepared by addition of TerB in TE buffer to Tus in the same buffer and dialysing them together. (●) Ions from free Tus protein; (●) Ions from the Tus-TerB complex; (★) Ions from the 2Tus-TerB complex.
3.5 ESI-MS of \( Ter \) variants- preliminary experiments

3.5.1 Influence of DNA sequence

Complexes of Tus and a range of \( TerB \) variants were prepared in an attempt to explore the capacity of ESI-MS to distinguish between the binding affinities of Tus and different DNA sequences.

\[ \text{Table 3.4: Sequences of dsDNA used in this work; only one strand of each is shown. The dots indicate that the base in that position was the same as in the TerB sequence.} \]

<table>
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<tr>
<th>Sequence</th>
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</tr>
<tr>
<td>posn7TerB</td>
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<tr>
<td>posn10TerB</td>
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<tr>
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</tbody>
</table>

The DNA sequences used during the course of this study were derived from 33bp oligonucleotides used by Coskun-Ari and Hill and were termed position 6, 8 and 11 substitutions in their studies (Coskun-Ari and Hill, 1997). In our work, we used 21 bp oligonucleotides which contained the core sequence necessary for binding to Tus. We termed these substituted oligonucleotides pos5\( TerB \), pos7\( TerB \), and pos10\( TerB \) (since our DNA sequence had a different start point to that used by Coskun-Ari and Hill (Coskun-Ari and Hill, 1997)). \( TerH \) was a sequence that had been found in a database search of the \( E. coli \) chromosome and was proposed to be a 'moderately strong (binding) site' (Coskun-Ari and Hill, 1997). Finally, a non-specific 24 mer DNA sequence was also used to assess the influence of non-specific binding. These DNA sequences are shown in Table 3.4.
The solution behaviour of these complexes had been well characterised by Coskun-Ari and Hill (Coskun-Ari and Hill, 1997). They observed that the $K_D$ were in the order $\text{TerB} < \text{Posn5} < \text{Posn7} < \text{Posn10}$, respectively.

Figure 3.12 shows ESI mass spectra acquired under optimal conditions (as summarised in section 3.4.1) of complexes of Tus with the different $\text{Ter}$ sequences. In all spectra of complexes of Tus with the $\text{Ter}$ variants, the predominant ions are from the complex (denoted by •) with little or no free protein (denoted by ●). Thus the expected differences expected in binding affinity (Coskun-Ari and Hill, 1997) cannot be distinguished from these spectra under these conditions.

Importantly, however, in the spectrum of the complex of Tus with non-specific DNA (Figure 3.12f), prepared and acquired under the same conditions the predominant ions observed were those of free Tus. There was also some Tus-non-specific DNA complex observed in the spectrum. A possible explanation for this association is that the first step in the proposed binding mechanism of Tus to $\text{TerB}$ involves electrostatic interactions of positively charged residues of Tus with the anionic phosphodiester back bone of DNA. It is important to note that these electrostatic interactions are non-specific (Neylon et al., 2000). In addition, non-specific interactions are often observed in studies of complexes of DNA and DNA-binding proteins by a whole range of different methods (Ha et al., 1992).
Figure 3.12: Positive ion ESI mass spectra of complexes of Tus with different Ter sequences; (a) TerB, (b) TerH, (c) pos5 TerB, (d) pos7 TerB, (e) pos10 TerB and (f) non-specific 24 mer self-complementary DNA. Instrument conditions are outlined in the methods section. (●) Ions from free Tus protein; (◆) Ions from the Tus-TerB complex.
3.5.2 Influence of collision energy and cone voltage

Since no differences were observed in the extent of binding of Tus to *TerB* compared to the *Ter* variants, a variety of techniques were used to see if differences could be detected in the extent to which different complexes dissociate under different conditions whereby the energy transferred to the complex is increased.

In many of the subsequent studies carried out in this work, His₆Tus, rather than wild-type Tus was used. Dixon and coworkers (Neylon *et al.*, 2000) using SPR studies showed that the dissociation constants for Tus and His₆Tus were indistinguishable and hence easily substituted. Indeed, no significant differences were noted between the ESI-mass spectra of Tus and His₆Tus complexes with *TerB* at any salt concentration in the range 10 to 2200 mM. Furthermore, no significant differences were noted between ESI-mass spectra of Tus-*Ter* and His₆Tus-*Ter* complexes.

The first technique used an increase in the voltage applied to the collision cell to attempt to dissociate the complexes. Collision induced dissociation (CID) experiments either in the source or collision cell have been proposed previously as a measure of the stability of the binding interaction of non-covalent complexes in ESI-MS studies (Lightwahl *et al.*, 1994; Schwartz *et al.*, 1995). As described earlier, the use of limited collision cell voltage and low gas pressure helps reduce the energy spread of ions entering the TOF analyzer. Increasing the voltage applied to the collision cell increases the energy of the ions undergoing collisions with the
argon gas such that at higher voltages more energy is available to dissociate a given complex. Figure 3.13 shows the spectra of His₆Tus:TerB complex as well as the His₆Tus-pos10TerB complex with increasing collision cell voltage. It is important to note that the Tus-TerB complex has been shown to bind 1204 times more tightly than the corresponding complex with pos10TerB (Coskun-Ari and Hill, 1997). In ESI mass spectra of the His₆Tus-TerB complex, increasing the collision cell voltage from 5 V (Figure 3.13a) to 60 V (Figure 3.13d) resulted in a decrease in the quality of the spectra but the complex remained intact. The complex was still present at 60 V but spectral quality was poor. The spectra of the His₆Tus-pos10TerB complex (Figure 3.13e-f) once again show a decrease in quality when subjected to higher collision cell voltages but the complex remains intact even at higher energy. There is thus no difference in the binding of the two TerB variants detected using variations in collision cell voltages.

Increasing the sampling cone voltage results in fragmentation in the ion transport region of the electrospray source. This fragmentation, which is caused by the ions gaining extra kinetic energy and breaking apart with collisions with other gaseous molecules (bath gas). It is therefore important to keep the cone voltage low in studies of non-covalent complexes by mass spectrometry. Indeed, earlier studies (Chowdhury and Chait, 1990; Kapur et al., 1999a; Loo, 1997; Potier et al., 1998a) have shown that increasing cone voltage results in the disruption of non-covalent complexes. The use of cone voltage as a tool to attempt dissociation of protein-DNA complexes has been employed previously (Potier et al., 1998a).
Figure 3.13: The effect of increasing collision cell voltage on ESI mass spectra of His₆Tus-TerB ((a) at 5 V, (b) at 10 V, (c) at 45 V and at (d) 60 V) and His₆Tus-pos10-TerB ((e) at 5 V, (f) at 10 V, (g) at 45 V and at (h) 60 V) each in 10 mM NH₄OAc, pH 8. (●) Ions from the Tus-TerB complex; (●) Ions from free protein.
The effect of increasing cone voltage on the ESI mass spectrum of the His$_6$Tus-\textit{TerB} complex in 10 mM NH$_4$OAc was judged by acquiring spectra at 25, 50, 75, 100 and 150 V. At 25-75 V, the spectra show sharp, intense peaks corresponding to the Tus-\textit{TerB} complex ions as previously observed with no ions from free His$_6$Tus (data not shown). When we increased the cone voltage to 100 V, the Tus-\textit{TerB} complex remained intact. There was however a skewing to lower \textit{m/z} suggesting that there may have been some fragmentation of DNA in the complex. This phenomenon was also reported in earlier experiments involving depurination of DNA in a Trp repressor complex (Potier \textit{et al.}, 1998a). The resolution of this spectrum was, however, inadequate to discern species dissociated from the complex. In a separate experiment resolution was sufficient to enable observation of an ion corresponding to a mass loss of $\sim$134 Da (data not shown) consistent with the loss of adenine. At 150 V, evidence of both free and complexed protein is seen indicating that the complex is breaking up. The spectral quality at 150 V is however very poor and as such detailed quantitative data cannot be determined. The two dissociation techniques did not reveal differences in binding affinities between either Tus mutants or \textit{Ter} variants owing to the extremely strong nature of the binding.

3.6 Titrations of Tus with \textit{TerB}

Scatchard plots have been generated for a number of non-covalent systems using ESI-MS. These include vancomycin antibiotic complexes with tripeptides (Lim \textit{et al.}, 1995) and also aminoglycoside-RNA complexes (Sannes-Lowery \textit{et al.}, 2000). Titrations of Tus with \textit{TerB} were carried out under experimental conditions which gave the best ESI mass spectra of the Tus-\textit{TerB} complex in an attempt to measure
an equilibrium dissociation constant via a Scatchard-type analysis. Figure 3.14 shows the ESI mass spectra of Tus treated with increasing amounts of TerB under optimal conditions for the preparation of the complex. The spectrum of Tus alone shows the typical protein charge envelope expected for an uncomplexed protein. On addition of TerB giving a ratio of TerB to Tus of 0.25, no ions corresponding to the complex were observed. An increase in TerB : Tus to 0.5 resulted in the appearance of ions in the ESI mass spectrum corresponding to the Tus-TerB complex with the [M+16H]^{16+} (m/z 3032.2), [M+15H]^{15+} (m/z 3234.3) and the [M+14H]^{14+} (m/z 3465.3) ions observed. In addition, ions from the free Tus were also present. The spectrum of 1:1 TerB:Tus solution exhibits mainly complex with little free Tus and free TerB. These observations were encouraging since it was expected that the amount of complex observed would increase as the amount of TerB added was increased. Subsequently, however, the problem of protein precipitation observed on addition of TerB was explored in more detail. Precipitation of component(s) of the mixture was monitored using UV-vis spectroscopy at 360 nm. The results of the experiment are shown in Figure 3.15. The maximum amount of precipitation observed after 1 hour of incubation is that when the Tus:TerB ratio is 1 : 0.1. The 1:0.25 ratio shows a similar amount of precipitation although the precipitation occurs at a more rapid pace at the start of the reaction. At the 1:0.5 Tus:TerB ratio, precipitation is less again than when smaller amounts of Tus were added. The least precipitation was observed in the 1:1 sample mixture. This precipitation is a problem for determining dissociation constants in titration experiments under these conditions the protein concentration in the sample cannot be known. In addition, problems encountered with probe capillary blocking can be avoided if sufficient TerB was added to the protein so as to avoid precipitation.
Figure 3.14: ESI mass spectra of Tus with increasing amounts of TerB in 10 mM NH₄OAc, pH 8. (a) Tus with no TerB, (b) 1:0.25 Tus:TerB, (c) 1:0.5 Tus:TerB and (d) 1:1 Tus:TerB. (●) Ions from the Tus-TerB complex; (●) Ions from free protein.

Similar experiments were also performed using the Tus mutant, A173T Tus and His₆Tus. A173T Tus and His₆Tus have been shown using SPR to have extremely different K_D values for binding to TerB, of 2000 and 0.53 nM, respectively (Neylon et al., 2000). That is, A173T Tus binds to TerB about 4000-fold less tightly than the
wild type protein. ESI mass spectra of A173T Tus and His₆Tus complex with TerB (data not shown) indicated only of ions arising from the complex and not from the free protein under the optimal conditions. This suggests at first glance that ESI-MS is not sensitive enough to distinguish between the different Tus mutants binding affinities under the conditions of these experiments. Previously, the binding between His₆Tus and various Ter sequences could not be distinguished using ESI-MS (Section 3.5.1).

Figure 3.15: Precipitation profile of Tus with increasing amounts of TerB added to the reaction mixture.

3.7 Influence of salt concentration.

Initially, the failure to distinguish between these binding affinities was a cause for concern especially as they had been previously differentiated in solution studies. Previous ESI-MS studies had stressed the use of gentle ESI conditions in order to maintain non-covalent interactions. Thus we consider that these interactions were
maintained. Under solution conditions and using $K_{obs}$ measured for the weakest complex, $\text{Tus-pos10}^{\text{TerB}}$ $(1204 \times 10^{-13} \text{ M;}$ (Coskun-Ari and Hill, 1997)), we calculated the expected percentage of free Tus if the binding in the gas phase was at least as tight as in solution (calculation presented in appendix 2).

Using the $K_D$ for binding of Tus to $\text{TerB}$ measured in solution studies, we expect that $[\text{Tus}_{\text{free}}] = 0.03 \mu\text{M}$ whilst $[\text{Tus}_{\text{complex}}] = 9.97 \mu\text{M}$. The percentage of total Tus that is free would thus be 0.23%. Any ions from the uncomplexed protein would not be readily observable in the spectrum. The argument assumes that the response factors which gas phase ions can be formed in the source region of the mass spectrometer of the free Tus as well as the complexed Tus are similar (discussed in detail later).

3.7.1 Influence of increasing NH$_4$OAc on the Tus-$\text{Ter}$ complex

Therefore, in order to be able to measure relative binding affinities of Tus-$\text{TerB}$ complexes, experimental conditions clearly had to be changed. In many biochemical binding studies where dissociation constants are very low, interactions between the binding partners need to be weakened to enable measurement of bound and free partners in a single reaction mixture (Neylon et al., 2000). Hence, NH$_4$OAc was added to the system to weaken electrostatic interactions in the Tus-$\text{TerB}$ complex and lower the binding affinity.

Furthermore, we could not carry out titration experiments owing to the precipitation observed when Tus:$\text{TerB}$ was not 1:1. It therefore became necessary to use the 1:1
Tus: TerB ratio to avoid precipitation problems and use other techniques to weaken the binding between the two species such that semi-quantitative analyses could be performed on the system using ESI-MS.

In SPR experiments with His\textsubscript{6}Tus and Tus, Dixon and coworkers (Neylon \textit{et al.}, 2000) were only able to determine dissociation constants when the binding interaction was weakened by KCl. Similarly, we reasoned that increasing the concentration of NH\textsubscript{4}OAc in the spray solvent would weaken the binding such that distinction between complexes of Tus and mutants with DNA using ESI-MS may be observed. In most ESI-MS studies of non-covalent complexes, typically 10-50 mM NH\textsubscript{4}OAc is used. One study used 150 mM NH\textsubscript{4}OAc, pH 7, to acquire a dsDNA spectrum (Hofstadler and Griffey, 2001).

Figure 3.16 shows the effect of adding a small volume of 10 M NH\textsubscript{4}OAc, pH 8, to a solution containing His\textsubscript{6}Tus-TerB complex. At 800 mM NH\textsubscript{4}OAc, the spectrum shows complex (●) predominantly with small amounts of free Tus (■) and free DNA. On addition of more salt to 1400 mM, there is an increased amount of free protein and DNA. At 2600 mM NH\textsubscript{4}OAc, the spectrum shows evidence of a predominantly free TerB and protein with only a small amount of complex present.

Typical ESI-MS experiments do not normally involve such high amounts of salt. As a volatile salt, NH\textsubscript{4}OAc was used and the desolvation temperature used was high (240°C) it is expected that adducted salt would be removed from the complex. In addition, the Z-spray source used in the Qtof2™ instrument ensures that any excess salt not nebulised would end up on the backing plate and not end up on the cone.
where undesirable blocking and/or charging effects could occur. The highest concentration of ammonium acetate used was 3.4 M in experiments involving the wild-type Tus (data not shown).

Figure 3.16: ESI mass spectra of the His$_6$Tus-TerB complex at different NH$_4$OAc concentrations showing free DNA; (a) 800 mM NH$_4$OAc, (b) 1400 mM NH$_4$OAc and (c) 2600 mM NH$_4$OAc. (●) Ions from free protein; (◆) Ions from the Tus-TerB complex.

3.7.2 Analysis of Tus mutants with addition of NH$_4$OAc

In order to enable more direct comparisons with mutant Tus proteins (A173T and R198A), which carry the hexahistidine tag, His$_6$Tus rather than the wild type species was used in the analysis. Indeed, no significant differences were noted between the
ESI mass spectra of Tus and His\textsubscript{6}Tus complexes with TerB at any NH\textsubscript{4}OAc concentration in the range 10 to 2200 mM.

Figure 3.17 below shows ESI mass spectra of His\textsubscript{6}Tus-TerB and the A173T Tus-TerB complexes acquired using a range of NH\textsubscript{4}OAc concentrations, all at pH 8. It is important to note that the spectra represented show a shortened range of \textit{m/z} 2500-4000 and thus the free DNA is not shown here. At 10 and 800 mM NH\textsubscript{4}OAc, the spectrum of the complex containing His\textsubscript{6}Tus shows predominantly complex (\textbullet) with very little protein (\textbullet\textbullet). On addition of stock NH\textsubscript{4}OAc to 1400 mM, there is an increased amount of free protein although the amount of complex is still in excess. This trend continues to 2000 mM NH\textsubscript{4}OAc where the predominant species in the spectrum is free protein and DNA (not shown) with a significant amount of the His\textsubscript{6}Tus-TerB complex remaining. This complex does not dissociate until the concentration of ammonium acetate is greater than 2400 mM. The ability to weaken the binding between His\textsubscript{6}Tus and TerB suggested that it might be possible to choose an ammonium acetate concentration where it would be possible to distinguish the relative binding affinities of TerB for Tus and Tus mutant proteins. In order to test this proposal, the effect of varying the concentration of NH\textsubscript{4}OAc on the ESI mass spectra of the complex of A173T Tus-TerB was examined. The results are also shown in Figure 3.17. As for the His\textsubscript{6}Tus-TerB complex, increasing the concentration of ammonium acetate results in dissociation of the complex as evidenced by the increasing intensity of ions from the free A173T Tus protein. Ions from free DNA also increased in intensity (data not shown).
Figure 3.17: ESI mass spectra of the Tus-dsDNA complexes- His$_6$Tus-TerB at: (a) 10 mM NH$_4$OAc, (b) 800 mM NH$_4$OAc, (c) 1400 mM NH$_4$OAc and (d) 2000 mM NH$_4$OAc and A173T Tus-TerB at: (e) 10 mM NH$_4$OAc, (f) 800 mM NH$_4$OAc, (g) 1400 mM NH$_4$OAc and (h) 2000 mM NH$_4$OAc. (●) Ions from free protein; (♦) Ions from the Tus-TerB complex.
When comparing the His\textsubscript{6}Tus-\textit{TerB} complex with the different A173T Tus-\textit{TerB} complex, the trend of complex dissociation continues. The difference noted between the two systems however involves the different ammonium acetate concentration which results in dissociation of the complex. For instance, at 1400 mM and 2000 mM NH\textsubscript{4}OAc concentrations (Figure 3.17e-f), there is little or no visible complex noted in the A173T Tus-\textit{TerB} complex system. This technique therefore shows that one can distinguish the relative binding affinities of Tus and Tus mutants for \textit{TerB} under these conditions.

A similar experiment was carried out for the R198A Tus-\textit{TerB} complex. The ESI mass spectra are not shown. This information is represented graphically. This is achieved by determining the intensities of each peak corresponding to the free Tus as well as the complex. Thus by plotting Tus(complex)/Tus(total) \times 100\% versus [NH\textsubscript{4}OAc], a dissociation profile is observed. A comparison of complex with free DNA was not attempted as it seemed unlikely that the response factor for DNA would the same as for free Tus or the complexed Tus. The plot of Tus(complex)/Tus(total) \times 100\% versus [NH\textsubscript{4}OAc] is shown in Figure 3.18. This plot shows the differences in the stabilities of complexes of \textit{TerB} with His\textsubscript{6}Tus, R198ATus and A173T Tus. The Tus-\textit{TerB} and His\textsubscript{6}Tus-\textit{TerB} complexes show dissociation profiles that are essentially the same with close to 20\% intact complex noted even at [NH\textsubscript{4}OAc]>2200 mM (data not shown). Approximately 50\% dissociation occurs at 1530 mM NH\textsubscript{4}OAc. The R198A Tus-\textit{TerB} complex forms the next most stable of the Tus mutant complexes. The complex is still observed at 1800 mM NH\textsubscript{4}OAc and it is 50\% dissociated at 1210 mM NH\textsubscript{4}OAc. The mutant that binds the least tightly to \textit{TerB} is the A173T Tus mutant. This is evident as the complex is almost 90\% dissociated by 1000 mM.
NH₄OAc and that 50% dissociation is observed at 775 mM NH₄OAc. It can therefore be concluded from the above data that the order of binding affinity of the proteins investigated was His₆Tus > R198A Tus > A173T Tus.

![Graph showing stability of complexes of His₆Tus and Tus mutants with TerB.](image)

**Figure 3.18:** Stability of complexes of His₆Tus and Tus mutants with TerB. The data show the decreasing amounts of Tus (or mutants) in the complex with dsDNA (Tus\textsubscript{complex}) as a percentage of the total amount of Tus (Tus\textsubscript{total}), as a function of [NH₄OAc]. These values were determined by summing the intensities of all ions from Tus\textsubscript{free} and all ions from Tus\textsubscript{complex}, Tus\textsubscript{free} + Tus\textsubscript{complex} = Tus\textsubscript{total}. (●) His₆Tus-TerB, (▲) R198A Tus-TerB, (■) A173T Tus-TerB.

In SPR studies, the K\textsubscript{D} values measured in 250 mM KCl for His₆Tus, R198A Tus and A173T Tus-TerB complexes were 0.5, 130 and 2000 nM respectively (Neylon et al., 2000). The relative order of binding affinities measured using ESI-MS are hence in very reasonable agreement with solution studies.
3.7.3 Analysis of *Ter* variants with addition of NH$_4$OAc

In a similar way to the experiments described above for Tus mutants, an experiment was carried out over a range of salt concentrations in order to compare differences in binding between the *Ter* variants: *TerB*, *TerH*, pos5*TerB*, pos7*TerB* and pos10*TerB* and His$_6$Tus. Previously, when ESI mass spectra of these complexes were obtained using 10 mM NH$_4$OAc, pH 8, as solvent, the relative binding affinities of these complexes could not be distinguished (Section 3.5.1).

Differences in the binding affinities of different *Ter* sequences for Tus can also be determined by measuring the relative amounts of free and bound Tus as a function of [NH$_4$OAc] from ESI mass spectra. The sequences used in the study are listed in Table 3.4. Figure 3.19 compares ESI mass spectra showing the dissociation of His$_6$Tus-*TerB* and His$_6$Tus-pos10*TerB* complexes on addition of NH$_4$OAc. In the case of the His$_6$Tus-*TerB* complex, no dissociation is observed until the complex was treated with 1400 mM NH$_4$OAc, pH 8 solution. Even at this concentration, there is predominantly complex observed. On the other hand, the His$_6$Tus-pos10*TerB* complex shows a greater degree of dissociation with increasing NH$_4$OAc concentration. Almost all the complex has dissociated at 1400 mM NH$_4$OAc. This stands in contrast to earlier experiments where the spray solvent was 10 mM NH$_4$OAc (data not shown).
Figure 3.19: ESI mass spectra of the Tus-dsDNA complexes- His₆Tus-TerB at: (a) 10 mM NH₄OAc, (b) 800 mM NH₄OAc, (c) 1000 mM NH₄OAc and (d) 1400 mM NH₄OAc and His₆Tus-pos10Tus-TerB at: (e) 10 mM NH₄OAc, (f) 800 mM NH₄OAc, (g) 1000 mM NH₄OAc and (h) 1400 mM NH₄OAc. (○) Ions from free protein; (●) Ions from the Tus-TerB complex.
Similar experiments were also performed on the different Ter variants, TerB, TerH, pos5TerB, pos7TerB and pos10TerB. When depicted graphically as shown in Figure 3.20 differences between the binding of these different Ter variants can be noted. For example, the NH₄OAc concentrations at which 50% dissociation occurs for TerB, TerH, pos5TerB, pos7TerB and pos10TerB are 1960, 1780, 1490, 1180 and 1150 mM respectively. Table 3.5 shows the ammonium acetate concentrations at which ESI mass spectra that ~50% of the complex had dissociated. The relative binding affinities of His₆Tus for the Ter sequences can be ranked according to these data: TerB > pos5TerB > TerH > pos7TerB = pos10TerB.

Table 3.5: The concentration of ammonium acetate necessary to cause 50% dissociation in the different His₆Tus-TerB variant complexes.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>[NH₄OAc] (mM) needed for 50% dissociation</th>
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<tbody>
<tr>
<td>TerB</td>
<td>1960</td>
</tr>
<tr>
<td>Pos5TerB</td>
<td>1780</td>
</tr>
<tr>
<td>TerH</td>
<td>1490</td>
</tr>
<tr>
<td>Pos7TerB</td>
<td>1180</td>
</tr>
<tr>
<td>Pos10TerB</td>
<td>1150</td>
</tr>
</tbody>
</table>

These results are consistent with values of $K_{obs}$ measured in solution studies of $9 \times 10^{-13}$, $16 \times 10^{-13}$, $16 \times 10^{-13}$, $139 \times 10^{-13}$ and $1204 \times 10^{-13}$ M for TerB, posn5TerB, pos7TerB and pos10TerB, although it is difficult to ascertain from the data the relative binding order of pos10TerB relative to pos7TerB. The His₆Tus-pos10TerB complex dissociates over a shorter [NH₄OAc] range compared to the His₆Tus-pos7TerB complex. Database searches identified TerH as being a potential termination site on the E. coli chromosome (Coskun-Ari and Hill, 1997). On the basis of base pair substitution studies it was proposed to be a moderately strong site and indeed our present results support that. The pos5TerB variant has a G .C substitution, which results in the removal of an interaction between R198 of Tus.
and the N-3 atom of guanine of the DNA. The pos7 TerB variant has an A-T base pair changed to A-C resulting in loss of interaction of the oxygen atom of the thymine with K89 and losing a major groove interaction of thymine methyl group with Thr139 (Coskun-Ari and Hill, 1997; Kamada et al., 1996). These two variants therefore bind less tightly to His6Tus as compared to the modified TerB.

![Graph showing stability of complexes of His6Tus with variant Ter DNAs](image)

*Figure 3.20: Stability of complexes of His6Tus with variant Ter DNAs. The data show the decreasing amounts of Tus in the complex with dsDNA (Tus_{complex}) as a percentage of the total amount of Tus (Tus_{total}), as a function of [NH₄OAc]. These values were determined by summing the intensities of all ions from Tus_{free} and all ions from Tus_{complex}. Tus_{free} + Tus_{complex}=Tus_{total}. (●)His6Tus-TerB, (▲)His6Tus-pos5TerB, (■)His6Tus-TerH, (×)His6Tus-pos7TerB, (+)His6Tus-pos10TerB.*

Similarly, the relative binding affinities of the His6Tus-TerB, His6Tus-pos10TerB, A173T Tus-TerB, and A173T Tus-pos10TerB complexes were compared and are represented graphically with Tus_{complex}/Tus_{total} x 100 (%) vs [NH₄OAc] in Figure 3.21. Clearly both complexes containing His6Tus form stronger complexes with each of TerB sequences (50% dissociation for His6Tus-TerB and His6Tus-pos10TerB occurs at 1550 and 945 mM NH₄OAc, respectively, whilst for the A173T
Tus-TerB and A173T Tus-pos10TerB occurs at 790 and 475 mM NH₄OAc, respectively). In addition, complexes containing TerB are more stable at higher salt concentrations compared to the pos10TerB variant. When comparing data for the complexes of TerB and pos10TerB (Figure 3.21) with each of His₆Tus and A173T Tus, the order of stability of these complexes in increasing [NH₄OAc] is His₆Tus-TerB > His₆Tus-pos10TerB > A173T Tus-TerB > A173T Tus-pos10TerB. Quite clearly, complexes containing His₆Tus from stronger bonds than any complex containing A173T Tus. Furthermore, complexes containing TerB form stronger bonds with the protein than the pos10TerB DNA. These results are seen to be consistent with previously measured Kₒ values of 9 and 1204 × 10⁻¹³ M (Coskun-Ari and Hill, 1997).

Figure 3.21: Stability of complexes of His₆Tus and A173T Tus with TerB and pos10TerB. The data show the decreasing amounts of His₆Tus or A173T Tus in the complex with dsDNA (Tus_complex) as a percentage of the total amount of Tus (Tus_total), as a function of [NH₄OAc]. These values were determined by summing the intensities of all ions from Tus_free and all ions from Tus_complex + Tus_free + Tus_complex = Tus_total. (◊)His₆Tus-TerB, (▲)His₆Tus-pos10TerB, (■) A173T Tus-TerB, (×) A173T Tus-TerB.
As the A173T Tus-TerB complex has not as yet been compared with the A173T Tus-pos10TerB complex in solution, no specific $K_D$ data is available for comparison. However, changing two residues involved in the binding; one an important sequence-specific contact in the protein (alanine substituted for threonine) as well as changing a base pair in the DNA results in a much weaker interaction than changing a single residue in either one of the binding partners.

3.7.4 Influence of cone voltage on the Tus-Ter complex in 800 mM NH$_4$OAc, pH 8.

In preliminary experiments, CID experiments carried out by varying cone voltage could not differentiate between the binding affinities of the various Tus mutants and/or the TerB sequences. In those experiments, the ammonium acetate concentration was 10-20 mM. However, as shown above, increasing the solution concentration of NH$_4$OAc, caused interactions between the DNA and the protein to be sufficiently weakened such that differences in relative binding affinities could be measured.

Figure 3.22 shows ESI mass spectra of cone voltage experiments involving the His$_6$Tus-TerB and A173T Tus-pos10TerB complexes in solutions containing either 10 mM and 400 mM NH$_4$OAc, pH 8. First, for the His$_6$Tus-TerB complex, it is clear that there is very little dissociation of the complex at 10 mM NH$_4$OAc under all cone voltages tested as no free protein was observed even at 100 V. At this cone voltage, however, the peaks are broad and the distribution becomes skewed to lower $m/z$. When these experiments were repeated using 800 mM NH$_4$OAc, pH 8 as the
solvent, a small change is observed whereby there is a small amount of His_{6}Tus protein (•) as compared to the complex (♦) at 100 V.

This change is very small and the most intense ions in the spectrum are still the ions from the complex. It is important to note that any free DNA in solution is observed at lower cone voltages but higher cone voltages are not conducive to the observation of intact DNA (Gupta et al., 2001). At 10 mM NH₄OAc, the A173T Tus-pos10TerB complex does not appear to dissociate. On the other hand, at 400 mM NH₄OAc, the A173T Tus-pos10TerB complex shows some dissociation at 50 V as judged by the peaks from the free protein (•). These peaks are observed at high intensity when the cone voltage was increased. At 100 V, the peaks from free A173T Tus (•) are slightly more intense than the peaks from the complex(♦).

Similar experiments were also performed in order to test differences in binding between the different Tus mutants with TerB (data not shown). The spectra of R198A Tus-TerB predominantly showed complex with increases in cone voltage. At 100 V, there is an increased amount of free protein but this still represented a very small amount of dissociation. On the other hand, the spectra of the A173T Tus-TerB complex show a greater amount of free protein even at 25 V. The abundance of free protein increases dramatically with increases in cone voltage. At 75 and 100 V, the free protein was in excess of the complex observed. The use of cone voltage to measure stabilities of complexes was thus made possible in this system owing to some refining of sample conditions such that non-covalent interactions were sufficiently weak enough to dissociate in the mass spectrometer.
Figure 3.22: ESI mass spectra of the Tus-dsDNA complexes- His$_2$Tus-TerB shown in Panel I ([NH$_4$OAc] = 10 mM with cone voltages of (a) 25 V, (b) 50 V, (c) 75 V and (d) 100 V) and Panel II ([NH$_4$OAc] = 800 mM with cone voltages of (e) 25 V, (f) 50 V, (g) 75 V and (h) 100 V). Spectra of A173T Tus-pos10Tus-TerB at different cone voltages are shown in Panel III ([NH$_4$OAc] = 10 mM with cone voltages of (i) 25 V, (j) 50 V, (k) 75 V and (l) 100 V) and Panel IV ([NH$_4$OAc] = 800 mM with cone voltages of (m) 25 V, (n) 50 V, (o) 75 V and (p) 100 V). (●) Ions from free protein; (◆) Ions from the Tus-TerB complex.
3.7.5 Dilution as a tool for estimating dissociation constants.

ESI-MS has been used to generate Scatchard plots (for measuring $K_D$) for non-covalent complexes (Lim et al., 1995). As titration of Tus with TerB proved to be problematic (Section 3.6) we circumvented this by accumulating data from serial dilution experiments. In these experiments, complexes were prepared as outlined in Chapter 2, but were subjected to 2-fold serial dilutions effectively changing the initial concentrations of the binding partners. Under these conditions, precipitation of a component of the mixture was not observed. Figure 3.23 shows plots of Tus (complex) / Tus (total) x 100 (%) vs [Tus total] in 800 mM NH$_4$OAc, pH 8, for His$_6$Tus, R198A and A173T Tus complexes with TerB. The His$_6$Tus-TerB and R198ATus-TerB complexes showed little change in the relative amount of complex observed over the concentration range (0.23-15 μM). The subtle changes observed at the low end of the [Tus] scale were a consequence of the lower signal-to-noise ratio associated with samples of lower concentration. The A173T Tus-TerB complex dilution series gave rise to changes in the relative amount of complex compared to free protein. By plotting this change, and comparing it to an theoretical plot, estimated by using a dissociation constant of $700 \times 10^{-9}$ M, one can estimate that under the experimental conditions the $K_D$ for the experiment involving A173T Tus-TerB is approximately $700 \times 10^{-9}$ M. By comparison, the fact that no changes were observed for the His$_6$Tus-TerB and R198A Tus-TerB complexes upon dilution suggests that the $K_D$ of these complexes is $\leq 2 \times 10^{-9}$ M (assuming that the ions arising from free Tus can be observed in the ESI spectrum once their total intensity (Tus$_{\text{free}}$) is about 6% of the total amount of Tus protein in solution (Tus$_{\text{total}}$)). It was
also interesting to note that we were able to study the system at extremely low sample concentrations (50 µL of a 0.23 × 10^{-6} M solution).

![Graph showing data on Tus complex formation](image)

**Figure 3.23:** The data show the amounts of His{sub}6 Tus (or mutants) in the complex with TerB in 800 mM NH{sub}4OAc, pH 8.0, (Tus{sub}complex), as a percentage of the total amount of Tus (Tus{sub}total), plotted against Tus{sub}initial. These values were determined by summing the intensities of all ions from Tus{sub}free and all ions from Tus{sub}complex. Tus{sub}complex = Tus{sub}total. (+)His{sub}6 Tus-TerB, (▲) R198A Tus-TerB, (■) A173T Tus-TerB.

### 3.8 Competition experiments used to determine relative binding affinities of Tus mutants

The relative binding affinities of the various Tus mutants could also be determined using competition experiments where various protein and DNA samples were mixed together in a 1:1:1 (10 μM of each). For instance in order to determine the binding affinity differences between R198A Tus and A173T Tus for TerB, mixtures were set up such that 1:1:1 ratios of R198A Tus : A173T Tus: TerB and R198A Tus: A173T Tus: pos10TerB were mixed and the resulting spectra compared. Results of these types of experiments are summarized in Table 3.6. The results clearly indicate that
each protein binds to both TerB variants in the order His6Tus > R198A Tus > A173T Tus. These results are consistent with those observed in Figure 3.17; Figure 3.18 as well as with SPR experiments (Neylon et al., 2000). The relative binding affinities of the different TerB variants were not however easily determined using this technique because the difference between A•T and G•C of 1 Da could not be resolved.

Table 3.6: Mixture components were present in 1:1:1 molar ratios in 800 mM NH4OAc, pH 8.0. In the ESI mass spectra of the mixtures only one complex was observed. (x) ions not observed; (✓) ions observed; (-) not a mixture component in the experiment.

<table>
<thead>
<tr>
<th>Mixture components</th>
<th>Ions observed in the ESI-mass spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>His6Tus</td>
</tr>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>His6Tus: A173T:</td>
<td>✓</td>
</tr>
<tr>
<td>TerB</td>
<td>✓</td>
</tr>
<tr>
<td>His6Tus: R198A:</td>
<td>✓</td>
</tr>
<tr>
<td>TerB</td>
<td>✓</td>
</tr>
<tr>
<td>R198A: A173T:</td>
<td>-</td>
</tr>
<tr>
<td>TerB</td>
<td>✓</td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>His6Tus: A173T:</td>
<td>✓</td>
</tr>
<tr>
<td>posn10TerB</td>
<td>✓</td>
</tr>
<tr>
<td>His6Tus: R198A:</td>
<td>✓</td>
</tr>
<tr>
<td>posn10TerB</td>
<td>✓</td>
</tr>
<tr>
<td>R198A: A173T:</td>
<td>-</td>
</tr>
<tr>
<td>posn10TerB</td>
<td>✓</td>
</tr>
</tbody>
</table>

3.9 Conclusion

The binding of the replication terminating protein (Tus) to its specific DNA recognition sequences (Ter) on the E. coli chromosome was investigated by ESI-MS.
Conditions were devised whereby the 1:1 Tus-\textit{TerB} complex was observed using ESI-MS. In order to be able to observe this complex, both instrumental those pertaining to solution control needed to be determined firstly for the free protein and subsequently the complex thereafter.

Initial attempts to distinguish binding affinities of the different Tus mutants to \textit{TerB} as well as the different Ter variants to Tus proved unsuccessful due to the stability of the Tus-Ter complex. Therefore, in order to sufficiently increase the dissociation constants of these complexes, the concentration of ammonium acetate in the electrospray solvent was raised (from 10 \textmu M to 3.2 M \textit{NH}_4\textit{OAc}). The relative order of binding affinity for Tus and its mutants to \textit{TerB} and variants was thus determined in this way.

As initial titration experiments used to determine the \textit{K}_D of the Tus-\textit{TerB} complex proved to be unsuccessful, a method of serial dilution was employed. The \textit{K}_D of A173T mutant to \textit{TerB} was estimated using this method to be $700 \times 10^{-9}\text{M}$ whilst those of His_{6}Tus and R198A were less than or equal to $2 \times 10^{-9}\text{M}$. However, the question arises as to how valid these dissociation constants are when measured in the gas phase. For instance, the ionization process involving drying of the ions may perturb the structure of the complex and thus also the equilibrium position. The presence of water on the DNA-protein binding interface is thought to be useful in acting as a form of glue whereby a form of structural adaptability is maintained (Schwabe, 1997).
These water molecules are thought to be involved in the specificity of DNA recognition by the proteins and furthermore, more water molecules remain at the binding interface in non-specific complexes (Schwabe, 1997).

In the Tus-TerB complex, water molecules are involved in H-binding between the DNA and protein (Kamada et al., 1996). On the other hand, studies have shown that the hydrophobic effect (along with the release of cations) is generally thought to be a major driving force for the formation of specific protein-DNA complexes (Ha et al., 1989). Electrostatic interactions are also thought to be strengthened in vacuo. Thus, contributions including hydrogen bonding, electrostatic interactions, hydrogen bonding and van der vaals interactions will influence the stabilities and hence dissociation constants of the non-covalent complexes (Loo, 1997).

One can therefore not predict how the formation of ions of the complex will affect its binding properties. It is therefore clear that a number of experiments using numerous wall characterized non-covalent complexes must be carried out using both ESI-MS and solution studies such that detailed similarities and/or differences can be noted. Indeed, our experiments as well as those on antibiotic binding by other researchers (Lim et al., 1995; Sannes-Lowery et al., 2000) shows that the relative order of binding affinity in the gas phase is in agreement to solution studies.
4 ESI-MS of non-covalent DNA-drug complexes

4.1 INTRODUCTION

As noted in Chapter 1, the utility of ESI-MS for the analysis of non-covalent drug-DNA complexes is now well established (Gabelica and De Pauw, 2001; Gabelica et al., 1999; Gabelica et al., 2000; Gale et al., 1994; Gale and Smith, 1995; Gupta et al., 2001; Hsieh et al., 1994; Kapur et al., 1999b; Pocsfalvi et al., 1997; Wan et al., 2000a; Wan et al., 2000b). Prior to the commencement of this study (in early 1999), only three reports on the observation of intact non-covalent DNA-drug complexes had appeared in literature (Gale et al., 1994; Gale and Smith, 1995; Triolo et al., 1997). These studies mainly focused on the non-covalent complexes formed between short dsDNA strands (6 mers) and the minor groove binders distamycin and netropsin. There was only one reported study of an intercalator-dsDNA complex observed by ESI-MS (Triolo et al., 1997). In that study, a dsDNA-daunomycin peak was observed in extremely low abundance compared either the ssDNA or the dsDNA.

Studies on the ESI-MS of non-covalent interactions between the anthracycline antibiotics, daunomycin and nogalamycin complexes on the Quattro™ triple quadrupole mass spectrometer are described in detail in the first part of this chapter. However, extensions of these studies using 16 mer self-complementary oligonucleotides exposed problems associated with possible non-specific interactions between the single-stranded DNA (ssDNA) and the anthracycline antiobiotics and possibly with intramolecular base pairing of self-complementary strands to form duplex structures. These studies were revisited using a more sensitive instrument –
the Qtof2™ mass spectrometer (Micromass, Wyntheshawe, UK) and non-self-complementary (the two strands were complementary to each other) dsDNA and are described in the latter part of the chapter. In addition, comparisons of the binding of the anthracycline antibiotics to double-stranded DNA (dsDNA) compared with other intercalators such as ethidium bromide and the alkylating intercalator hedamycin were also examined. Preliminary studies on a novel intercalator retamycin are also reported here.

4.2 Ligands used in this study

4.2.1 Anthracycline antibiotics

The DNA-binding properties of two anthracycline antibiotics, daunomycin and nogalamycin formed the bulk of the studies described in this chapter. The anthracycline antibiotics are DNA intercalators. Some of these compounds have exhibited effective anticancer properties and have been used in the treatment of tumours since the 1960s (Smith et al., 1996). The precise mechanism for these antitumour effects is not completely understood. One fact however remains strikingly clear – these antibiotics have an affinity for DNA (Capranico et al., 1986a; Capranico et al., 1986b).

Current anthracycline therapies target chromosomal DNA with a view of binding to DNA thus inhibiting DNA replication as well as DNA – directed RNA synthesis (Fisher and Aristoff, 1988). In general, these effects can be explained by intercalation into DNA (Arcamone et al., 1975). This disturbs the action of DNA and RNA polymerases
and also interferes with the DNA unwinding and supercoiling enzyme topoisomerase II (Zunino and Capranico, 1990).

Unfortunately biochemical and biophysical studies suggest that DNA binding is relatively non-specific and as a result all fast growing cells \textit{in vivo} are also affected by anthracyclines. This results in several side effects such as cardiotoxicity (Fisher and Aristoff, 1988). Recent evidence has shown that anthracyclines can cross-link DNA (Cutts and Phillips, 1995; Skladanowski and Konopa, 1994b). However, in order to cross-link DNA, they have to be enzymatically activated and it is assumed that this process can occur \textit{in vivo} (Skladanowski and Konopa, 1994a).

4.2.1.1 Daunomycin

In 1962, an antibiotic was isolated from strains of \textit{Streptomyces caeroleorubidus}. At about the same time, it was isolated in \textit{Streptomyces peucetius} (DiMarco \textit{et al.}, 1963; Gause \textit{et al.} 1964). The structure of this compound which was subsequently named either daunomycin or daunorubicin is shown in Figure 4.1. Daunomycin is now used more commonly and this name is used throughout this thesis.

![Figure 4.1: The structure of daunomycin (Mr 527.51).](image)

(Rizzo \textit{et al.}, 1989; Zunino \textit{et al.}, 1975)
Daunomycin has found clinical use in the treatment of acute leukemias and subsequently in the treatment of non-small cell lung carcinoma and Kaposi sarcoma (Bepler and O'Briant, 1998; Tulpule et al., 1998).

It was realized early in studies on daunomycin, that intercalation was the basic mode of DNA binding (Calendi et al., 1965). A recent model by Chaires (Chaires et al., 1996) proposes that daunomycin is more than an intercalator and possesses three functional domains. First, there is the anthraquinone ring system, which intercalates but in a more complex manner than the simple intercalators like ethidium bromide and proflavine. The long axis of the inserted daunomycin chromophore is perpendicular to the long axis of the DNA base pairs (Williams and Gao, 1992). The second domain is the daunosamine sugar, which acts as a minor groove binder. The third domain proposed involves the substituents of the A-ring (Figure 4.1). These substituents form hydrogen bonds with DNA serving as an anchor and thus stabilizing the complex.

Several methods have been used to determine the sequence selectivity as well as the structure of the bound dsDNA-daunomycin complex. For example, X-ray crystallography has been carried out on various complexes. These include d(CGTACG)\textsubscript{2} – daunomycin (Quigley et al., 1980; Wang et al., 1987), d(CGATCG)\textsubscript{2} – daunomycin (Frederick et al., 1990; Moore et al., 1989), d(TGATCA)\textsubscript{2} – daunomycin (Nunn et al., 1991) and d(TGTACA)\textsubscript{2} – daunomycin (Nunn et al., 1991).

Furthermore, crystal structures of dsDNA-daunomycin analogs have been solved (Gao and Wang, 1991; Williams et al., 1990a). No crystal structure has provided
concrete evidence for the basis of the sequence selectivity of daunomycin. It has been proposed, however, that the mobility of the amino sugar in solution is critical to sequence selectivity and further that the thermal mobility is greater than the rest of the complex resulting in varying sequence selectivity (Lipscomb et al., 1994; Williams et al., 1990b). An example of a crystal structure is presented below.

![Crystal Structure](image.png)

**Figure 4.2:** The crystal structure of d(CGTACG)$_2$ complexed with two daunomycin molecules. Note the DNA molecule is represented in grey whilst the daunomycin residue is represented in blue. The anthraquinone ring system intercalates between the base pairs (Wang et al., 1987).

Similarly, short DNA sequences have been used to identify the sequence selectivity of daunomycin on DNA using NMR spectroscopy. Recently, Veselkov et al., (Veselkov et al., 2001a; Veselkov et al., 2001b) studied the interaction between daunomycin and d(TACGTA)$_2$, d(CGCGCG)$_2$ or d(CGTACG)$_2$. These studies showed that the drug preferred binding to a CpG step flanked by an AT pair and further that this binding required at least 3 base pairs.
In 1994, spectroscopic techniques like fluorimetry, UV-Vis absorption, calorimetry and stopped-flow techniques were used to ascertain the site selectivity of daunomycin (Roche et al., 1994). They determined using these techniques that the CpG step was the preferred sequence compared to the TpA or CpT steps.

The d(ACG)₂ and d(TCG)₂ sites have been suggested to be preferential binding sites of daunomycin by theoretical studies (Chen et al., 1985). These results were complemented by footprinting studies which suggested the preferred binding sequence to be d((A/T) CG) or d((A/T)GC) (Chaires, 1990). In addition, fluorescence quenching experiments indicate that daunomycin prefers binding to duplexes containing GC base pairs as part of alternating purine pyrimidine sequences (Remeta et al., 1993).

Recent NMR experiments have again shown the sequence selectivity of daunomycin to be the CpG site (Mazzini et al., 1998). Thus this study in conjunction with other spectroscopic studies propose that the binding sequence of daunomycin is the CpG or GpC steps and that this step is better when flanked by ApT or TpA steps. The results of these studies have been summarized in Table 4.1.

4.2.1.2 Nogalamycin

Nogalamycin was first extracted from *Streptomyces nogalator* in 1965 (Wiley et al., 1968). It was soon realized that nogalamycin showed promise as an anti-tumour agent. For instance, selective inhibition of DNA-directed RNA synthesis was noted *in vivo* (Ennis, 1981; Fok and Waring, 1972). Furthermore, this drug was found to be
active against gram-positive bacteria and experimental tumours (Bhuyan and Dietz, 1967; Bhuyan and Reusser, 1970). Problems with drug administration, in particular solubility have, however, resulted in nogalamycin having less importance clinically than daunomycin.

Table 4.1: Summary of the recent spectrometric techniques used to study the interaction between daunomycin and dsDNA.

<table>
<thead>
<tr>
<th>Sequences used</th>
<th>Technique</th>
<th>Preferred binding step</th>
<th>Researchers</th>
</tr>
</thead>
<tbody>
<tr>
<td>d(CGTACG)$_2$</td>
<td>X-ray crystallography</td>
<td>CpG</td>
<td>(Quigley et al., 1980; Wang et al., 1987)</td>
</tr>
<tr>
<td>d(CGATCG)$_2$</td>
<td>X-ray crystallography</td>
<td>CpG</td>
<td>(Frederick et al., 1990; Moore et al., 1989)</td>
</tr>
<tr>
<td>d(TGTACA)$_2$</td>
<td>X-ray crystallography</td>
<td>TpG and CpA</td>
<td>(Nunn et al., 1991)</td>
</tr>
<tr>
<td>d(TGATCA)$_2$</td>
<td>X-ray crystallography</td>
<td>TpG and CpA</td>
<td>(Nunn et al., 1991)</td>
</tr>
<tr>
<td>d(TAGTGA)$_2$</td>
<td>NMR</td>
<td>CpG</td>
<td>(Veselkov et al., 2001a; Veselkov et al., 2001b)</td>
</tr>
<tr>
<td>d(CGCGCG)$_2$</td>
<td>Fluorimetry, Absorption calorimetry</td>
<td>CpG</td>
<td>(Roche et al., 1994)</td>
</tr>
<tr>
<td>d(TACGTA)$_2$, d(CGCGCG)$_2$, d(CGTACG)$_2$</td>
<td>Theoretical studies</td>
<td>CpG</td>
<td>(Chen et al., 1985)</td>
</tr>
<tr>
<td>d(ATCG)$_2$, d(ATGC)$_2$</td>
<td>Footprinting</td>
<td>CpG</td>
<td>(Chaires, 1990; Chaires et al., 1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluorescence quenching</td>
<td>GpC</td>
</tr>
<tr>
<td>d(CGATCG)$_2$, d(CGTA CG)$_2$</td>
<td>NMR</td>
<td>CpG</td>
<td>(Mazzini et al., 1998)</td>
</tr>
</tbody>
</table>

The structure of nogalamycin shown in Figure 4.3 shares common features with the other antracyclines, namely that it possesses a planar anthraquinone ring system. It however differs from daunomycin in that it possesses two bulky side residues—a hydrophobic sugar nogalose and a bicyclic aminoglycose moiety. It therefore
assumes the approximate shape of a dumbbell. Even though the dumbbell structure might be energetically unfavourable in forming a complex with DNA owing to steric effects, nonetheless it actually forms a stable complex (Kersten et al., 1966; Plumbridge and Brown, 1979; Searle et al., 1988; Sinha et al., 1977; Waring, 1970; Williams et al., 1990a; Zhang and Patel, 1990).

Figure 4.3: The structure of nogalamycin ($M_r 787.83$).

Whilst the precise mechanism for the nogalamycin binding is unclear, several models have been proposed. Collier et al., (Collier et al., 1984) proposed that transient DNA melting is required in order to allow entry of the bulky nogalamycin into the DNA. This model is consistent with evidence of slow association and dissociation rates (Fok and Waring, 1972; Fox et al., 1985). A more recent model proposes that the DNA base pairs would be unstacked and buckled in order to accommodate the nogalamycin molecule (Egli et al., 1991). This model, however, lacks the inherent simplicity of the earlier model and is not easily explained by recent spectroscopic data (Fok and Waring, 1972; Fox et al., 1985).

Several methods have been employed in order to determine the sequence selectivity of nogalamycin to DNA. Several X-ray crystal structures of complexes of dsDNA with
nogalamycin and analogs have been determined. These include: d(CGT(pS)ACG)$_2$-nogalamycin; d($^{m6}$CGT(pS)$^{m6}$CG)$_2$-nogalamycin (Liaw et al., 1989); d($^{m6}$CGT(pS)$^{m6}$CG)$_2$-nogalamycin (Egli et al., 1991); d(CGT(pS)$^{m6}$CG)$_2$-nogalamycin; d($^{m6}$CGT(pS)$^{m6}$CG)$_2$-nogalamycin (Gao et al., 1990; Smith et al., 1996; Williams et al., 1990b); d(TGATCA)$_2$-nogalamycin (Schuerman et al., 1996; Smith et al., 1995) and d(CCCGGG)$_2$-nogalamycin (Cruse et al., 1996). In most X-ray crystal structures, the CpG step was the preferred binding site for nogalamycin, except in the case of the d(TGATCA)$_2$ complex where TpG and CpA were the preferred binding sequences.

NMR spectroscopy has also been used extensively to determine the sequence selectivity of nogalamycin. The complexes studied by this method include: d(AGCATGCT)$_2$-nogalamycin (Zhang and Patel, 1990); d(GCATGC)$_2$-nogalamycin (Searle et al., 1988); d(GCGT)$_2$-nogalamycin (Van Houte et al., 1993) (NB: $T_b$ is bulged) and d(CT$_b$GTACG)$_2$-nogalamycin (Caceres-Cortes and Wang, 1996). All these NMR experiments showed that the CpG step was the preferred site for nogalamycin binding.

Other techniques have also been used to determine the sequence selectivity of nogalamycin to DNA. Fluorescence studies on poly (dA-dT) and poly (dC-dG) showed that nogalamycin had a preference for the AT site (Richardson et al., 1981). This preference can be explained in terms of the bulky nogalose moiety which would prefer the easily unwound AT sites. As fluorescence is better able to detect the initial conformational changes in molecules, this result is reasonable. The same result was again noted in stopped-flow experiments using similar sequences (Fox
and Waring, 1984) in which case ApT was the preferred binding site although the researchers maintain that over a period of time, the nogalamycin may indeed move to a different site. These researchers also studied the association-dissociation rates of the nogalamycin-DNA complex (Fox et al., 1985; Fox and Waring, 1981). They determined that nogalamycin dissociates from poly (dA-dT) 160 times faster than from poly (dC-dG). However, the rate of association to poly (dA-dT) was also higher. The results have been summarized in Table 4.2.

Thus there is still not a clear consensus for the major binding site(s) for nogalamycin to dsDNA. X-ray crystallography and NMR indicate that the preferred site is CpG. Fluorescence studies indicate that the rate of association of ApT is higher than CpG owing to the ease of unwinding of DNA sequences with greater numbers of ApT steps. Thus we can assume that the preferred binding sequence for nogalamycin is similar to that of daunomycin whereby a CpG step is flanked by AT sequences. It is again important to point out that the NMR and X-ray studies were performed on DNA sequences shorter than 10 base pairs. These sequences thus lacked the full helical shape attributed to the DNA double helix. Therefore, it would be important to direct future work in this field using larger DNA sequences in order to preserve the attributes of the DNA found in vivo.

A connection between the biological effectiveness of the drug and the lifetime of the DNA-anthracycline complex has been proposed (Chaires, 1985; Chaires et al., 1985; Fox et al., 1985; Fritzsche et al., 1987). Evidence shows that the rate of complex formation is similar in all anthracyclines, although nogalamycin is slightly lower than the rest of the other antibiotics. This evidence lends support to the model that
proposes that the DNA exists in a relaxed and denatured state in order to allow for intercalation. Once anchored, however, the rate of dissociation determines how cytotoxic the compound is. Thus nogalamycin having the lowest rate of dissociation is the most cytotoxic of the anthracycline antibiotics.

Table 4.2: Summary of the recent spectrometric techniques used to study the interaction between nogalamycin and dsDNA.

<table>
<thead>
<tr>
<th>Sequences used</th>
<th>Technique</th>
<th>Preferred binding step</th>
<th>Researchers</th>
</tr>
</thead>
<tbody>
<tr>
<td>d(CGT(pS)ACG)$_2$</td>
<td>X-ray crystallography</td>
<td>CpG</td>
<td>(Liaw et al., 1989)</td>
</tr>
<tr>
<td>d(me$^5$CGT(pS)Am$^5$CG)$_2$</td>
<td>X-ray crystallography</td>
<td>CpG</td>
<td>(Liaw et al., 1989)</td>
</tr>
<tr>
<td>d(me$^5$CGT(pS)Am$^5$CG)$_2$</td>
<td>X-ray crystallography</td>
<td>CpG</td>
<td>(Egli et al., 1991; Gao et al., 1990; Smith et al., 1996; Williams et al., 1990a)</td>
</tr>
<tr>
<td>d(CGT(pS)Am$^5$CG)$_2$</td>
<td>X-ray crystallography</td>
<td>CpG</td>
<td>(Smith et al., 1996)</td>
</tr>
<tr>
<td>d(TGATCA)$_2$</td>
<td>X-ray crystallography</td>
<td>TpC and CpA</td>
<td>(Egli et al., 1991; Gao et al., 1990; Schuerman et al., 1996; Smith et al., 1996; Williams et al., 1990a)</td>
</tr>
<tr>
<td>d(CCCGGG)$_2$</td>
<td>X-ray crystallography</td>
<td>CpG</td>
<td>(Cruse et al., 1996)</td>
</tr>
<tr>
<td>d(AGCATGCT)$_2$</td>
<td>NMR</td>
<td>CpG</td>
<td>(Zhang and Patel, 1990)</td>
</tr>
<tr>
<td>d(GCATGC)$_2$</td>
<td>NMR</td>
<td>GpC</td>
<td>(Searle et al., 1988)</td>
</tr>
<tr>
<td>d(GCGT)$_2$</td>
<td>NMR</td>
<td>CpG</td>
<td>(Van Houte et al., 1993)</td>
</tr>
<tr>
<td>d (CT$_6$GTACG)$_2$</td>
<td>NMR</td>
<td>CpG</td>
<td>(Caceres-Cortes and Wang, 1996)</td>
</tr>
<tr>
<td>poly (dA dT)</td>
<td>Fluorescence</td>
<td>ApT</td>
<td>(Richardson et al., 1981)</td>
</tr>
<tr>
<td>poly (dC dG)</td>
<td>Stopped-flow experiments</td>
<td>ApT</td>
<td>(Fox and Waring, 1984)</td>
</tr>
</tbody>
</table>
4.2.2 Ethidium bromide

Ethidium bromide shown in Figure 4.4 is a dye used to stain DNA and RNA. The ethidium molecule intercalates between nucleotides, and the complex fluoresces when exposed to ultraviolet light. The DNA/ethidium bromide complex strongly absorbs UV light at 300 nm and fluoresces at 590 nm. Whilst it is the most common intercalator compound in routine laboratory use, there are very few studies on the ethidium-DNA complex in the literature. Indeed ethidium bromide is used more as a probe to determine secondary DNA structure (Boger et al., 2001; Bugs and Cornelio, 2001). As ethidium bromide is considered to be a simple intercalator, without bulky substituents on the aromatic ring, comparisons between it and other intercalators in terms of binding affinity as well as sequence specificity could yield data of interest (Eaton et al., 2000; Haq and Ladbury, 2000).

![Figure 4.4: The structure of ethidium (M, 314.4 Da).](image)

4.2.3 Retamycin

The antibiotic retamycin shown in Figure 4.5, has been isolated from *Streptomyces olindensis* (Cotias, 1971). It has been shown to have antitumour activity (Cotias,
1971; Delle Monache, 1970). Relatively little is known about the drug in terms of the structure of its complexes with DNA. The anthracycline ring system in retamycin is similar to that in the well-characterized intercalators, daunomycin and nogalamycin and it is therefore expected to be an intercalator. Complexes of retamycin with DNA were studied here in analogous experiments to those carried out using daunomycin and nogalamycin. The structure of the complexes with retamycin must be ascertained so that its possible future use as a chemotherapeutic agent can be determined.

$$\text{OH} \quad \text{O} \quad \text{OH}$$

Figure 4.5: The structure of retamycin ($M, 772.1$ Da).

4.2.4 Hedamycin

Hedamycin is a naturally occurring antitumour antibiotic belonging to the pluramycin family. It was isolated from *Streptomyces griseoruber* (strain C-1150) and was subsequently shown to have the ability to directly inhibit RNA and DNA polymerases (Joel and Goldberg, 1970) and to inhibit the growth of HeLa cells in tissue culture (Schmitz et al., 1966), and certain transplanted rodent tumours (Bradner et al.,
Hedamycin binds non-covalently with double-stranded DNA, forming tight complexes that result in an increase in the melting temperature of the DNA double helix (White, 1969). Hedamycin is also involved in covalent reactions with DNA. Various studies of the sequence selectivity indicate that hedamycin alkylates DNA at guanine bases (Pavlopoulos et al., 1996; Prakash, 1995).

Hedamycin consists of a highly substituted 4-H-anthra-[1,2,6]-pyran-4,7,12-trione chromophore with two amino sugars, and an anglosamine sugar attached to the C10 position as shown in Figure 4.6. Hedamycin binds to DNA in a two stages. First, it binds to DNA via rapid intercalation followed by a slower covalent binding to the bases (Jernigan, 1978) with preference for 5'-PyGT-3’ and 5'-PyGG-3’ steps, in particular favouring 5'-CGT-3’ sites (Murray et al., 1995; Prakash, 1995; Sun et al., 1995). The anthrapyrantrione chromophore of hedamycin has been shown to intercalate between the 5’-PyG-3’ bases with two amine sugars placed in the minor groove and the bisepoxide chain located in the major groove (Hansen et al., 1995; Pavlopoulos et al., 1996; Sun et al., 1995). The anglosamine sugar is located to the 3’ side of the intercalation site whilst the N, N-dimethylvancosamine sugar is located to the 5’ side of the intercalation site.

Figure 4.6: The structure of hedamycin (M, 748.7 Da).
4.3 Aims of this study

Given that the non-covalent complexes of dsDNA with nogalamycin or daunomycin have been well characterized with data from a variety of techniques, it was thought that complexes of these drugs with DNA would provide a useful starting point for exploring the viability of ESI-MS to characterise drug-DNA complexes and to allow comparisons of solution and gas phase data. The first aim was to optimize sample conditions for the preparation of drug-dsDNA (8 mer) complexes for their detection using the VG Quattro™ instrument. In the early part of this work, the Quattro™ was the only available ESI mass spectrometer in our laboratory. Thus, the effect of pH, different salts in the sample buffer as well as different annealing conditions were explored. The effect of various instrumental parameters including cone voltage, capillary voltage and desolvation temperature on the quality of the spectra of these complexes were also examined.

Following optimization of parameters for the detection of these complexes, a range of experiments including titration experiments and competition experiments with 8 and 12 mer dsDNA were examined. It was hoped that ESI-MS would show the capacity to detect differences in sequence selectivity and further that more light may have been shed on the controversy of the preferred binding sequences of daunomycin and nogalamycin. These studies were also extended to more biologically (structurally) relevant, 16 mer sequences.
Extensions to these studies using a more sensitive mass spectrometer allowed for approximately 10-fold less sample to be used, and the use of non-self-complementary DNA meant that all ions could be assigned unambiguously. The range of intercalators was extended to nogalamycin, daunomycin, ethidium bromide, hedamycin and retamycin. In-source CID experiments and ESI-MS/MS experiments were carried out in an attempt to discern differences in stabilities of the various complexes. Finally, ESI mass spectra of some complexes were acquired in positive ion mode for comparison with the results of negative ion experiments.

4.4 Determination of optimal conditions for the observation of the DNA-anthracycline antibiotic complexes using a triple quadrupole mass spectrometer.

Since no successful studies had been reported on DNA-intercalator complexes using ESI-MS prior to this work, it was necessary to first optimize both sample and instrumental conditions for the observation of these complexes by ESI-MS. Experiments were carried out using a VG Biotech Quattro™ mass spectrometer (now Micromass) with a triple quadrupole mass analyzer as described in Chapter 2. These studies have been divided into two main sections. The first deals with the determination of optimal experimental parameters – both sample and instrumental to observe the best ESI-mass spectra of these intercalated drug-DNA complexes. The second section deals with studies involving titrations of DNA with the anthracycline drugs, nogalamycin and daunomycin as well as competition
experiments. Some of these studies are revisited using a quadrupole time-of-flight analyzer (Qtof2™, Micromass) later in the chapter.

### 4.4.1 Sample Conditions

In an initial experiment, the DNA annealing conditions were tested and success was judged by measuring the intensities of ions from dsDNA relative to those from ssDNA. Annealing involves heating of the oligonucleotide to greater than or equal to 20°C above its melting temperature and cooling slowly to an ambient temperature (Blackburn and Gait, 1990). This process ensures that the strands undergo a bimolecular pairing process and therefore form a duplex structure. Figure 4.7 shows the ESI-mass spectra of a 5:1 complex of nogalamycin-d(GGCTAGCC)₂ prepared using a variety of annealing conditions. Detailed assignments of peaks observed are given in Table 4.3. Note that in these assignments we have assumed that the nogalamycin molecule is a neutral species. Since nogalamycin has a pK'a of 7.45 (Mankhetkorn and Garniersuillerot, 1998) it follows that at pH 8 up to 50% of the drug molecules could be positively charged. Hence an assignment [2M+3Ng-5H]⁵⁻ could also be represented as [2M+3(NgH)⁺-8H]⁵⁻. As both assignments have identical m/z values, only the former is used for simplicity. In addition, it is important to realize that only ions carrying an odd number of charges or an odd number of drug molecules can be unequivocally assigned as either the dsDNA molecule or dsdrug-DNA complex. For example, [2M+4Ng-5H]⁶⁻ (J) and [2M+3Ng-4H]⁴⁺ (M) correspond to dsDNA-drug complexes. On the other hand, an assignment such as [2M+4Ng-6H]⁶⁻ (H) has the same m/z as the single-stranded [M+2Ng-3H]³⁺ (H). As individual isotopes could not be resolved in the quadrupole mass analyzer, these ions could not be assigned unambiguously. However, as we could observe the
[2M+xNg-5H]^{5-} (G, I, J, L) ions in each series and each series had a charge envelope distribution expected in ESI-mass spectra, these ions can be reasonably assigned to dsDNA-drug complex. Other ions observed in these spectra include the nogalamycin ion [Ng-H]^{5-} at 786.5 (C), as well as those from the uncomplexed ssDNA (m/z 480.4 (A), 601.4 (B) and 802.5 (D)).

When the sample was annealed by cooling slowly to room temperature (Figure 4.7a) the most intense ion observed was that from [2M+3Ng-5H]^{5-} at m/z 1435.8 (I). The intensity of this peak relative to those for free ssDNA (B and D) was only slightly greater in (a) compared to other conditions where the complex was left on ice overnight (b) or plunged into ice for 10 minutes and left at room temperature overnight (c). The spectra (Figure 4.7b and c) were very similar. Rapid cooling is expected to decrease annealing of DNA strands. The spectra in Figure 4.7 show that there was little difference in relative amounts of ssDNA and dsDNA under the annealing conditions described above. However, as nogalamycin was present in this reaction mixture, this result can be explained as intercalators have been reported to stabilize DNA in the duplex form (Kersten, 1966; Kersten et al., 1966).
Solution studies on the DNA-nogalamycin interaction have involved the use of Tris buffers and alkali salts such as NaCl, KCl, Tris and MgCl₂ (Blackburn and Gait, 1990). These buffers have, however, been reported as being unsuitable for use in ESI-MS (Gale et al., 1994; Gale and Smith, 1995). That is, volatile salts such as ammonium acetate and ammonium citrate are required for optimum sensitivity. Figure 4.8 shows a selection ESI mass spectra acquired using various salt and solvent conditions. Assignments are given in Table 4.3. In the ESI-mass spectrum of the sample that had been annealed in 1 M NH₄OAc, pH 8, diluted 10-fold and then acquired using 100 mM NH₄OAc, pH 8.5 as delivery solvent (Figure 4.8a), the most
abundant drug-DNA peak is that from $[2\text{M}+3\text{Ng}-5\text{H}]^{5-}$ (I) at $m/z$ 1435.7. This peak is, however significantly less intense than either peaks from the ssDNA (B and D). Conversely, the ESI-mass spectrum of the sample annealed in 100 mM NH$_4$OAc, pH 8, diluted 10-fold and then acquired using 10 mM NH$_4$OAc, pH 8, as delivery solvent has the most intense drug DNA peak ($[2\text{M}+3\text{Ng}-5\text{H}]^{5-}$ (I)). This ion is more intense than any ssDNA peak observed (B and D) although not significantly so. When the same sample used in (b) was injected using water as a delivery solvent (Figure 4.8 (c)) the $[2\text{M}+3\text{Ng}-5\text{H}]^{5-}$ (I) peak is significantly more intense than either of the ssDNA peaks (B and D). It is therefore clear that using water as delivery solvent

![Figure 4.8](image)

Figure 4.8: ESI mass spectra of 5:1 mixtures of nogalamycin and d(GGCTAGCC)$_2$ acquired using different delivery solvents. The dsDNA-nogalamycin complex was prepared as described in Chapter 2; (a) 100 mM NH$_4$OAc, pH 8.5; (b) 10 mM NH$_4$OAc, pH 8.5; (c) water. Assignments of the various ions are given in Table 4.3.
rather than 10 or 100 mM NH₄OAc, pH 8, significantly improves the signal intensity of ions corresponding to drug-dsDNA species relative to ions from ssDNA. Furthermore, in experiments where increasing amounts of salt were used, the source region became increasingly dirty and hence signal sensitivity decreased. In addition, having increased concentrations of NH₄OAc at lower temperatures results in more species being present in the gas phase. It is known that analyte ion sensitivities decrease with increasing species concentration (Ikonomou et al., 1990; Sterner et al., 2000). This result was also noted by Triolo and co-workers (Triolo et al., 1997) where they obtained the highest sensitivity at 10 mM NH₄OAc compared to 100 mM NH₄OAc. Similarly, previous ESI-MS studies of distamycin-DNA complexes had used either a combination of 10 mM ammonium acetate/10 mM ammonium citrate (Gale et al., 1994) or 10-20 mM ammonium acetate or ammonium citrate (Gale and Smith, 1995).

Table 4.3: Assignments (expected average m/z) of major ions in ESI mass spectra shown in Figures 4.7-4.11.

<table>
<thead>
<tr>
<th>Ion</th>
<th>( M = d(GGCTAGCC)_2 )</th>
<th>( I = \text{Nogalamycin} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( [M-5H]^{5-} )</td>
<td>786.5</td>
<td>C</td>
</tr>
<tr>
<td>( [M-4H]^{4-} )</td>
<td>480.4</td>
<td>A</td>
</tr>
<tr>
<td>( [M-3H]^{3-} )</td>
<td>601.4</td>
<td>B</td>
</tr>
<tr>
<td>( [M+I-3H]^{5-} )</td>
<td>802.5</td>
<td>D</td>
</tr>
<tr>
<td>( [M+2I-3H]^{5-} )</td>
<td>1064.9</td>
<td>E</td>
</tr>
<tr>
<td>( [M+2M+2I-6H]^{5-} )</td>
<td>1278.1</td>
<td>G</td>
</tr>
<tr>
<td>( [M+I-2H]^{2-} )</td>
<td>1598.2</td>
<td>K</td>
</tr>
<tr>
<td>( [2M+3I-6H]^{6-} )</td>
<td>1196.0</td>
<td>F</td>
</tr>
<tr>
<td>( [2M+3I-5H]^{5-} )</td>
<td>1435.8</td>
<td>I</td>
</tr>
<tr>
<td>( [2M+3I-4H]^{4-} )</td>
<td>1795.7</td>
<td>M</td>
</tr>
<tr>
<td>( [M+2I-3H]^{3-} )</td>
<td>1327.4</td>
<td>H</td>
</tr>
<tr>
<td>( [2M+4I-6H]^{5-} )</td>
<td>1593.6</td>
<td>J</td>
</tr>
<tr>
<td>( [M+2I-2H]^{2-} )</td>
<td>1992.0</td>
<td>N</td>
</tr>
<tr>
<td>( [2M+5I-5H]^{5-} )</td>
<td>1751.6</td>
<td>L</td>
</tr>
</tbody>
</table>
The use of acetonitrile as a delivery/annealing solvent was also tested (data not shown). Acetonitrile is expected to give a significantly higher response for ssDNA in ESI-MS (Bleicher and Bayer, 1994). In our experiments aimed at detecting dsDNA, acetonitrile resulted in a decrease in the abundance of ions from dsDNA. This was not unexpected as organic solvents such as acetonitrile are expected to disrupt most electrostatic interactions, dissociating dsDNA. A similar result has also been noted by other researchers where on addition of acetonitrile to an annealed DNA sample, dsDNA peaks of low relative abundance were observed (Ding and Anderegg, 1995).

Figure 4.9 shows the effect of varying solution pH during the annealing step on the ESI-mass spectra of the d(GGCTAGCC)₂-nogalamycin complex. At pH 4 (Figure 4.9d), peaks from the ssDNA (B, D and E) are significantly more intense than any peak corresponding to drug-dsDNA complex. The most abundant drug-dsDNA ion is \([2M+3Ng-5H]^{5^-}\) at \(m/z\ 1435.5\). On increasing the pH to 7 (Figure 4.9c), the intensity of this peak (I) increases relative to the ssDNA peaks. Furthermore, the peaks in the ESI-mass spectrum show less tailing. Increasing the solution pH to 8 (Figure 4.9b) results in further improvements in spectral quality as well as better signal intensity of the drug-dsDNA peaks relative to the ssDNA peaks. However, this trend did not continue when solution pH was increased to 11. At pH 11 (Figure 4.9a), the most intense peaks in the spectrum are those from ssDNA (A, B, D and E) with the drug-dsDNA ions being of low relative abundance. Hence, increasing the pH above 8-9 apparently results in the denaturing of the complex. Annealing the sample in NH₄OAc, pH 8, therefore results in the best ESI mass spectrum. This
result is consistent with conditions used by Smith and co-workers to study distamycin-dsDNA complexes in which case the buffers were either at pH 8.3 (Gale et al., 1994) or at pH 8 (Gale and Smith, 1995).

Figure 4.9: ESI mass spectra of 5:1 mixtures of nogalamycin and d(GGCTAGCC)_2 acquired using different solution pH. Assignments of the major ions are given in Table 4.3. (a) pH 11, (b) pH 8, (c) pH 7 and (d) pH 4.
4.4.2 Instrumental parameters

Instrumental parameters including cone voltage, capillary voltage and source/desolvation temperature each can influence the rate and extent of desolvation, in-source CID and focusing/transport of non-covalent complexes. The influence of each was examined in this study.

Figure 4.10 shows the effect of varying cone voltage on the ESI-mass spectra of a 5:1 mixture of nogalamycin-d(GGCTAGCC)_2. At 20 V (Figure 4.10d), the most intense peaks are from ssDNA (B, D) with smaller peaks from the drug bound complex (E-K). These peaks are however, still relatively small compared with the ssDNA. Increasing the cone voltage to 60 V (Figure 4.10c) results in the nogalamycin ion (C) at $m/z$ 786.6 being the most intense with other dominant ions being $[M-3H]^3^-$ (D) and $[M-2H]^2^-$ (F). Ions from drug-dsDNA complexes are of only low abundance: i.e. $[2M+4Ng-5H]^5^-$ (J) and $[M+Ng-2H]^2^+[2M+2Ng-4H]^4^+$ (K). When using a cone voltage ramped from 15 V to 60 V (Figure 4.10b) from $m/z$ 400-2000, ions corresponding to drug-dsDNA complexes are apparent. For example, the dominant ions in this spectrum again include the ssDNA ions (A, B and D) with evidence for the complex noted with peaks E, F, M, I, J and K observed (Table 4.3). The best conditions for the acquisition of the ESI-mass spectrum of d(GGCTAGCC)_2-nogalamycin complexes were obtained using a cone ramp of 15-30 V (Figure 4.10a). In this spectrum, the major ions include those of the ssDNA (A, B and D). The ions corresponding to the complexes are, however, relatively more intense than under any of the other cone voltage conditions. The most intense drug-dsDNA peak in this spectrum is the $[2M+3Ng-5H]^5^-$ ion (I) with other drug-dsDNA complex ions also observed. Clearly, the use of a cone ramp from 15-30 V gives the best
distribution of the ions from drug-dsDNA complexes and improves spectral quality compared with acquisitions at 20 V or 60 V alone.

Figure 4.10: ESI mass spectra of 5:1 mixtures of nogalamycin and d(GGCTAGCC)_2 acquired using different cone voltages; (a) 15-30 V, (b) 15-60 V, (c) 60 V and (d) 20 V. Assignments of the major ions are given in Table 4.3.

Increasing the cone voltage increases the energy at which the ions are accelerated through the intermediate pressure region in the source (Katta and Chait, 1991). Hence, when these ions undergo collisions with bath gas molecules, then

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dissociation occurs. Since these drug-DNA complexes are held together via relatively weak non-covalent forces, it follows therefore in order to optimize conditions necessary for their observation in the gas phase, cone voltage must be kept low. The first study of the daunomycin-dsDNA complex studied by ESI-MS used a cone voltage of 100 V (Triolo et al., 1997), which may explain why these researchers were unable to observe these complexes.

Electrospray as an ionization technique uses voltages on the stainless steel capillary to nebulise and ionize the sample. Figure 4.11 shows the ESI-mass spectra of 5:1 mixtures of nogalamycin-d(GGCTAGCC)_2 acquired using different capillary voltages. The ESI-mass spectra acquired using a capillary voltage of 2.0 kV is of a higher quality compared with that acquired with a capillary voltage of 3.5 kV. At 2.0 kV, species from both the ssDNA and drug-dsDNA can be clearly observed and assigned. At 3.5 kV, the peaks are broader and are quite difficult to assign owing to the significantly lower signal-to-noise ratio. Interestingly, the same result has also been noted in earlier ESI-MS studies. For instance, Gale and Smith (Gale and Smith, 1995) used a capillary voltage of 2.2 kV. Conversely, Triolo and his co-workers utilized a higher voltage of 4 kV resulting in spectra of poorer quality. Thus it follows that the lower capillary voltages give rise to ESI-mass spectra with improved signal-to-noise ratios.
Figure 4.11: ESI mass spectra of 5:1 mixtures of nogalamycin and d(GGCTAGCC)₂ acquired using different capillary voltages of (a) 3.5 kV and (b) 2.0 kV. Assignments of the major ions are given in Table 4.3.

In keeping with the idea of maintaining gentle ionization conditions in order to minimize the dissociation of complexes, it follows that the desolvation temperature should also have to be kept low. Figure 4.12 shows ESI-mass spectra of a 5:1 mixture of nogalamycin-d(GGCTAGCC)₂ at desolvation temperatures of 60°C (Figure 4.12b) and 90°C (Figure 4.12a). Both spectra show the same ions except for the absence of the [M+2Ng-2H]²⁺/[2M+4Ng-4H]⁴⁻ (N) ion at 90°C. Overall, the intensities of all the ions from the drug-dsDNA complexes were lower at 90°C than 60°C. It seems likely that increasing the desolvation temperature increases the internal kinetic energy of the droplets/ions resulting in a greater proportion of complex dissociation. The use of a desolvation temperature of 60°C was surprising given that with water as a delivery solvent, higher desolvation temperatures (above 100°C) are usually recommended to ensure complete vaporization for aqueous
solutions. To compensate, a counter current flow of nitrogen at 500 L/h was used as a desolvation gas. This flow rate is somewhat higher than that normally used but was found to be necessary to reduce both salt and water adducts.

![Graph showing mass spectra](image)

**Figure 4.12:** ESI mass spectra of 5:1 mixtures of nogalamycin and d(GGCTAGCC)2 acquired using different desolvation temperatures; (a) 90°C and (b) 60°C. Assignments of the major ions are given in Table 4.3.

Thus, optimal sample and instrumental conditions on the triple quadrupole mass spectrometer for the observation of intercalator-dsDNA complexes involved first annealing the sample in 100 mM NH₄OAc, pH 8, diluting this sample 10-fold using water and then using water as a delivery solvent to introduce the sample into the mass spectrometer. The critical instrument parameters were: a ramped cone voltage of 15-30 V, a capillary voltage of 2.0 kV and a desolvation temperature of 60 °C with a countercurrent nitrogen gas flow at 500 L/hr.
4.5 Stoichiometric analysis of the anthracycline drugs binding to DNA.

4.5.1 Titration of d(CGCGCCG)_2 with nogalamycin or daunomycin

A series of experiments was carried out in which either nogalamycin or daunomycin was added to dsDNA over the range from no drug to a ten-fold excess over dsDNA under optimal sample preparation and instrument conditions as outlined above. Figure 4.13 shows the ESI-mass spectra of 0:1, 2:1 and 5:1 mixtures of nogalamycin and daunomycin with d(CGCGCCG)_2. The expected m/z values for these ions are presented in Appendix 5. In the absence of drug (Figure 4.13a), ssDNA predominates with ions of low abundance from dsDNA observed (*). The only ion that can be unambiguously assigned as dsDNA is [2M-3H]^{3-}. On addition of drug (2:1 drug: dsDNA, ions from drug-dsDNA are observed for both nogalamycin (Figure 4.13b) and daunomycin (Figure 4.13e). The ssDNA peaks however, remain dominant. Addition of a greater excess of drug drug (5:1) (Figure 4.13c & f) results in more abundant drug-dsDNA ions. These increases can also be expressed graphically. Figure 4.14a compares the relative intensities of the [2M+xNg-5H]^{5-} ions (x = 1–5) of complexes of nogalamycin bound to d(CGCGCCG)_2 versus ratio of drug over the range of 1:1 to 10:1 nogalamycin to dsDNA. Data are shown for [2M+xNg-5H]^{5-} ions (Figure 4.14a) and for [2M+3Ng-xH]^{x-} ions (Figure 4.14b) since they could be unambiguously assigned as arising from dsDNA. The data are consistent with reaction stoichiometry. For example, once the ratio of drug to dsDNA exceeds 2:1, then the complex containing 2 nogalamycin molecules bound to dsDNA becomes significant. Similarly the complex containing 3 molecules of daunomycin is more significant once the ratio of drug to duplex DNA exceeds 3:1. It
is apparent when looking at the data (Figure 4.14a), that the complexes containing either one or five molecules of nogalamycin are less abundant regardless of the ratio of excess drug present in the reaction mixture. This therefore suggests that the 1:1 and 5:1 nogalamycin stoichiometries are less favoured. The complexes containing two and three bound nogalamycin molecules are more favourable with 3:1 nogalamycin:dsDNA molecule being the most abundant. Simple intercalators such as ethidium bromide obey the “neighbour exclusion” principle (Rao, 1987). That is, the intercalator can bind between every other base pair. If this principle is obeyed, up to four nogalamycin molecules can bind to d(CGGCGCCG)₂. Thus these results are consistent with this binding principle. The fact that very little [2M+5Ng] complex is observed suggests that even at high concentrations of nogalamycin in solution there is only a very small contribution from non-specific interactions. Furthermore, this graph shows that at ratios above 5:1 nogalamycin:dsDNA, there is a plateau suggesting that the binding sites on the dsDNA have become saturated. In the experiments in subsequent sections, the amount of drug added to dsDNA never exceeded a 5-fold excess.

These spectra were also analysed by comparing the relative intensities of the [2M+3Ng-γH]⁻ ions (Figure 4.14b). This was done so as to further reduce the contributive effect of non-specific interactions between nogalamycin and the DNA i.e. ensure that there was no contribution to the abundance of ions from complexes with ssDNA. In all nogalamycin-dsDNA ratios from 1:1 to 10:1 the intensity of the 5⁻ ion > the intensity of the 4⁻ ion > intensity of the 6⁻ ion. If non-specific binding were to occur at high concentrations of drug e.g. 10:1 drug:dsDNA (between the negatively

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Figure 4.13: ESI mass spectra of d(CGCGCCCG)₂ with either nogalamycin ((a) no drug, (b) 2:1 and (c) 5:1 drug-dsDNA mixtures) or daunomycin ((d) no drug, (e) 2:1 and (f) 5:1 drug-dsDNA mixtures). (●) ssDNA; (✱) uncomplexed dsDNA; (◆) drug-dsDNA complex.
Figure 4.14: Relative intensity of selected ions vs ratio (0:1 to 10:1) of nogalamycin to \(5'-d(CGGCGCCG)\_2-3'\). (a) Ions with the same charge and different numbers of bound drug, i.e. \([2M+xNg-5H]^\pm\) ions of complexes where \(x = 1-5\) molecules of nogalamycin. (b) Ions with three molecules of bound nogalamycin and different charge, i.e. \([2M+3Ng-\gamma H^\pm]\), where \(\gamma = 4-6\). Intensities have been scaled relative to the base peak in each spectrum.

4.5.2 Titrations of \(d(CGGCGCCG)_2\), \(d(GGCTAGCC)_2\) and \(d(TGAGCTAGCTCA)_2\) with nogalamycin and daunomycin.

Similar titration experiments to those described in the section above were carried out using different oligonucleotide sequences. Figure 4.15 compares the binding of charged phosphate groups and the positively charged drug) differences might be expected in the charge distribution.
both nogalamycin (a) and daunomycin (b) (in a five-fold molar excess) with
d(CGGCGCCG)$_2$, d(GGCTAGCC)$_2$ and d(TGAGCTAGCTCA)$_2$. These data were
compiled using the sum of the intensities of $[2M+x\text{Ng-5H}]^{5-}$ ions in the case of the 8
mers, and the $[2M+x\text{Ng-7H}]^{7-}$ ions for the 12 mer. These ions, rather than the sum
of ion currents for all charge states of a given species were used to ensure that
there were no contributions to the data from peaks that comprised both ssDNA and
dsDNA.

In the case of the drug complexes with the 8 mer DNA strands, the most abundant
ions in the case of the nogalamycin were those in which 3 nogalamycin molecules
were bound to dsDNA. The results for daunomycin differ in that whilst the complex
with 3 daunomycin molecules bound was still the most intense drug-dsDNA ion,
evidence of 4, 5 and, to a much lesser extent, even 6 drug molecules were bound to
dsDNA. In ESI mass spectra of complexes of d(TGAGCTAGCTCA)$_2$ with nogalamycin,
the most abundant ion observed was that corresponding to 4 bound nogalamycin
molecules with ions of low abundance corresponding to 3 bound with no ions from 5
or 6 nogalamycin molecules bound to dsDNA. Similarly, the most abundant ion in
the case of daunomycin complexed with d(TGAGCTAGCTCA)$_2$ was the complex with
4 daunomycin molecules. However, there were abundant ions from dsDNA with 5
and 6 bound daunomycin molecules. These ions were relatively more intense than
those observed for the 8 mer. This is expected since longer oligonucleotides have a
greater number of potential binding sites (up to 6 for 12 mers based on the
neighbour exclusion principle). It is not possible to ascertain whether the $[2M+5\text{Dn}]$
and $[2M+6\text{Dn}]$ species observed for the 8 mer dsDNA are bound intercalatively and
therefore in violation of the neighbour exclusion principle.
A number of non-classical intercalators are known to stack on opposite sides of the same base pair in violation of this principle (Robledo-Luiggi, 1991; Veal, 1990). These intercalators include tetrapeptide amines containing lysine moieties (Robledo-Luiggi, 1991) as well as bis-9-aminoacridines (Veal, 1990). Alternatively, these \([2M+5Dn]\) and \([2M+6Dn]\) complexes may result from the ligands being bound by a combination of intercalator and/or electrostatic interactions between the positively charged amine of daunomycin and the negatively charged phosphate backbone of

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*Figure 4.15: Comparison of the relative intensities of the major ion/drug dsDNA ion series for (a) nogalamycin and (b) daunomycin complexes observed in 5:1 reaction mixtures of drug (I) with different oligonucleotides. For \(d(TGAGCTAGCTCA)\) the data show the intensity of \([2M+xI-7H]^+\) normalised to the sum of ion intensities for \(x = 1-6\). For \(d(GGCTAGCC)\) and \(d(CGCGCGCG)\), the data show normalised intensity of \([2M+5H]^+\).*
DNA. It is interesting nogalamycin was not observed to bind with a stoichiometry of greater than 4 bound molecules to any great extent. This might be explained simply by the greater size and therefore steric hindrance for the binding of nogalamycin to DNA and/or the need for the dsDNA to ‘open up’ or ‘breathe’ to accommodate the intercalator (Gao et al., 1990; Liaw et al., 1989).

4.5.3 Competition experiments

Two types of competition experiments were carried out. In the first, competition between nogalamycin and daunomycin for the same dsDNA was studied. In the second, differences between the affinities of the different dsDNA sequences for the respective anthracyclines were examined.

Figure 4.16 shows the spectra of a solution in which daunomycin was mixed in a five-fold excess with a 12 mer, d(TGAGCTAGCTCA)_2 and either of two 8 mers, d(CGGCGCCG)_2 or d(GGCTAGCC)_2. The major ions in these spectra are assigned in Table 4.4. Differences were noted between these spectra and those in which only one sequence was present with daunomycin (data presented in Figure 4.13). For instance, only 1 or 2 daunomycin molecules were bound to the dsDNA 8 mer sequences compared to 3, 4 and to a lesser extent 5 bound daunomycin molecules in the spectra of mixtures of the drug and either of the sequences in the absence of d(TGAGCTAGCTCA)_2 (Figure 4.16b). This is not unexpected as the same number of drug molecules are now competing for binding sites on two different duplex DNA molecules rather than one. Similarly for the dsDNA 12 mer in the presence of either d(CGGCGCCG)_2 or d(GGCTAGCC)_2, only complexes containing 2 to 4 bound
daunomycin molecules were observed compared to up to 6 when d(TGAGCTAGCTCA)₂ was present alone. The key finding noted when comparing the differences in binding between either the dsDNA 8 mer sequences and the dsDNA 12 mer sequence was the observation that complexes of daunomycin with d(CGGCGCCG)₂ are more abundant compared to those containing d(GGCTAGCC)₂. Comparison of the intensities of the ions from the 8 mer dsDNA complexed with daunomycin, [2N+2I-5H]₅⁻ (n) with the corresponding ions for the 12 mer dsDNA, [2M+3I-6H]₆⁻ (Q) in Figure 4.16 show that the intensity of the ion (n) containing d(CGGCGCCG)₂ is approximately twice as abundant as that for d(GGCTAGCC)₂.

Figure 4.16: ESI mass spectra from competition experiments in which daunomycin was reacted in 5-fold excess with an equimolar mixture of two oligonucleotides. (a) d(CGGCGCCG)₂ and d(TGAGCTAGCTCA)₂ and (b) d(GGCTAGCC)₂ and d(TGAGCTAGCTCA)₂. Assignments are given in Table 4.4.
Table 4.4: Assignments of major ions in ESI mass spectra shown in Figure 4.16.

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<th>Species</th>
<th>m/z</th>
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<th>(a) m/z</th>
<th>Label</th>
<th>(b) m/z</th>
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<td>I (Daunomycin)</td>
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<td>I</td>
<td>525.9</td>
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<tr>
<td>M</td>
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</tr>
<tr>
<td>[M-4H]⁻</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td>1213.4</td>
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<td>832.8</td>
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</tr>
<tr>
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<td>1190.0</td>
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</tr>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>T</td>
<td>1173.7</td>
<td>T</td>
<td></td>
</tr>
<tr>
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<td>U</td>
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<td>U</td>
<td></td>
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<tr>
<td>[2M+4I-6H]⁺</td>
<td></td>
<td></td>
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<td>V</td>
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<tr>
<td>[2M+4I-5H]⁺</td>
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<td></td>
<td>1879.6</td>
<td>W</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>N=d(CGCGCGCC)₂</td>
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<td></td>
<td></td>
<td></td>
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<td>d</td>
<td>480.6</td>
<td>d</td>
<td></td>
</tr>
<tr>
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<td>e</td>
<td>601.1</td>
<td>e</td>
<td></td>
</tr>
<tr>
<td>[N-3H]⁻</td>
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<td></td>
</tr>
<tr>
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<td>g</td>
<td>1203.6</td>
<td>g</td>
<td></td>
</tr>
<tr>
<td>[2N+I-3H]⁺</td>
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<td>h</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[N+I-4H]⁺/[2N+2I-8H]⁺</td>
<td>733.1</td>
<td>k</td>
<td>732.8</td>
<td>k</td>
<td></td>
</tr>
<tr>
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<td>978.2</td>
<td>m</td>
<td>978.0</td>
<td>m</td>
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</tr>
<tr>
<td>[2N+2I-6H]⁺</td>
<td>1173.8</td>
<td>n</td>
<td>1173.7</td>
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<td></td>
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<tr>
<td>[2N+2I-5H]⁺</td>
<td>1467.7</td>
<td>o</td>
<td>1467.3</td>
<td>o</td>
<td></td>
</tr>
</tbody>
</table>

The data in Figure 4.16 can be represented by summing intensities of the ions of the drug-d(TGAGCTAGCTCA)₂, drug-d(GGCTAGCC)₂ and drug-d(CGCGCGCC)₂ complexes and plotting them as a percentage of the total intensities of all ions as shown in Figure 4.17. There is also data represented on this graph for a similar experiment where nogalamycin was used instead of daunomycin. For example, ESI
mass spectra of the reaction mixture containing \(d(TGAGCTAGCTCA)_2\), \(d(CGGCGCCG)_2\) and nogalamycin (Figure 4.17a) shows that almost half of the ions are from nogalamycin-\(d(CGGCGCCG)_2\) complex. When comparing this to the reaction when \(d(GGCTAGCC)_2\) was substituted for \(d(CGGCGCCG)_2\) (Figure 4.17c), the nogalamycin-\(d(GGCTAGCC)_2\) complexes account for only 18% of the drug bound DNA (i.e. 82% of the nogalamycin complexes contain \(d(TGAGCTAGCTCA)_2\)). This suggests therefore that nogalamycin binds to \(d(CGGCGCCG)_2\) in preference to \(d(GGCTAGCC)_2\). Similarly, for the reaction containing \(d(TGAGCTAGCTCA)_2\), \(d(CGGCGCCG)_2\) and daunomycin (Figure 4.17b) just over 31% of the complexes involve the dsDNA 8 mer. However, when substituting \(d(GGCTAGCC)_2\) for \(d(CGGCGCCG)_2\), (Figure 4.17d) the former only accounts for 10% of the complex. These competition experiments indicate first, that the anthracycline antibiotics appear to display some sequence selectivity for the GC rich oligonucleotide.

![Figure 4.17: Normalised intensity (% of the drug bound to each respective dsDNA (either 8 mer and 12 mer) in competition experiments in which drug was reacted in a 5-fold excess with an equimolar mixture of two oligonucleotides- a 12 mer and an 8 mer. (a) \(d(TGAGCTAGCTCA)_2\)/\(d(CGGCGCCG)_2\)/ nogalamycin; (b) \(d(TGAGCTAGCTCA)_2\)/\(d(CGGCGCCG)_2\)/ daunomycin; (c) \(d(TGAGCTAGCTCA)_2\)/\(d(CGGCGCCG)_2\)/ daunomycin; (d) \(d(TGAGCTAGCTCA)_2\)/\(d(CGGCGCCG)_2\)/ daunomycin.](image-url)
A second interesting aspect of these experiments is that nogalamycin apparently binds more efficiently to either of the dsDNA 8 mer sequences compared with daunomycin. At first examination, this seems surprising since nogalamycin is a bulkier molecule and therefore is expected to require a greater degree of unwinding of the double helix to intercalate. Once bound, however, the molecule remains bound more tightly compared to daunomycin. There is some evidence that nogalamycin binds preferentially to AT rich regions owing to the ease of unwinding of AT rich regions (i.e. they “breathe” better) (Fox and Waring, 1984; Richardson et al., 1981). Once bound however, nogalamycin has been shown to dissociate 160 times more slowly from CG rich regions compared to AT rich regions (Fox et al., 1985). Indeed the general consensus for the preferred binding sequence of nogalamycin to dsDNA involves having the preferred CG step embedded into AT rich regions (Caceres-Cortes and Wang, 1996; Fox et al., 1985; Gao et al., 1990; Gao and Wang, 1991; Liaw et al., 1989; Smith et al., 1996; Smith et al., 1995; Zhang and Patel, 1990). It is also important to point out that the competition experiments compared a dsDNA 12 mer with dsDNA 8 mers. It should be noted that in all mixtures, the intensities of ions from drug-12 mer DNA complexes were more intense than drug-8 mer ions. This is predictable based on the greater number of binding sites in the absence of any clear sequence preferences. However, to fully explore this observation, a more detailed series of experiments taking into account possible differences in ionization efficiency of each complex needs to be conducted. In order to carefully compare sequence preferences, it will be important to use DNA sequences of similar length as they would be expected to have relatively similar ionization efficiencies. If there is no sequence preference, the DNA of the same
length would be expected to bind the same number of intercalator molecules. A complicating factor is that DNA sequences of the same length have similar molecular weights. For example, 5’-GGCTAGCC-3’ has a mass of 2410.6 Da whilst 5’-CGGCGCCG-3’ has a mass of 2411.6. However, the resolution of the mass spectrometer would not be able to differentiate between these complexes. In future experiments it might be useful to compare complementary pairs of say 12 mers with 13 mers.

A second type of competition experiment compared equimolar amounts of each of daunomycin and nogalamycin with d(CGGCGCCG)₂ (Figure 4.18). Similar experiments were also carried out with d(GGCTAGCC)₂ and d(TGAGCTAGCTCA)₂ (data not shown). The resulting spectra were significantly more complicated than others seen previously owing to the overlap of peaks at higher m/z values. For instance [2M+4Ng-5H]⁵⁻ occurs at m/z 1593.5 whilst [2M+2Ng+3Dn-5H]⁵⁻ occurs at m/z 1594.9. These data were therefore presented graphically. Figure 4.18 shows the sum of the intensities of [2M+M-5H]⁵⁻ ions of complexes formed on reaction of a 2.5:2.5:1 mixture of daunomycin and nogalamycin with d(CGGCGCCG)₂ (Figure 4.18a) compared with 5:1 nogalamycin-d(CGGCGCCG)₂ (Figure 4.18b) and 5:1 daunomycin-d(CGGCGCCG)₂ (Figure 4.18c). The drugs bound to the oligonucleotide in a number of different stoichiometries. When either of the drugs was present on its own, the most abundant complex was that in which 3 drug molecules were bound to dsDNA. The most abundant complexes when nogalamycin and daunomycin were present together were those with two drugs bound (either 2 nogalamycin or 1 each of nogalamycin and daunomycin) suggesting that the binding of both types of drugs decreased the number of intercalator sites. There were also
relatively abundant species with 3 bound drug molecules (3 nogalamycin, 2 nogalamycin and 1 daunomycin or 1 nogalamycin and 2 daunomycin). Small quantities of complexes containing 4 or 5 bound drug molecules were also observed. Furthermore, when both drugs were present together, there were no complexes observed that had daunomycin bound exclusively whereas complexes containing nogalamycin alone were observed. The significance of these results remains unclear and thus warrants further investigation. A possible explanation for this phenomenon may be that once nogalamycin intercalates into the DNA and its nogalose and bicyclic aminoglucose moieties wrap around the dsDNA, the bulkier sugars in the drug molecule present mechanistic problems for intercalation requiring conformational changes in the DNA. Once bound, the nogalamycin molecule remains more tightly bound compared to daunomycin (Fox and Waring, 1984; Richardson et al., 1981) thereby sterically restricting daunomycin intercalation into the dsDNA.

A full turn of the DNA double helix spans 10 base pairs. The structure of our 8 mer is therefore only an approximation of a double helix. To date, most studies aimed at probing daunomycin and nogalamycin interactions with DNA have used self-complementary DNA less than 8 base pairs in length. A summary of these techniques and experiments has been presented in Table 4.1 and Table 4.2 respectively. An advantage of mass spectrometry is that the technique has developed so that longer pieces of DNA can be studied. In the experiments discussed below, the binding of nogalamycin and daunomycin to two dsDNA 16 mer sequences was examined.
Figure 4.18: Normalised intensity of $[2M+xI-5H]^-$ ions in ESI mass spectra of $d(CGGCGCCG)_2$ and (a) an equimolar mixture (2.5-fold excess) of nogalamycin and daunomycin (b) 5-fold excess of nogalamycin and (c) 5-fold excess of daunomycin.
Experiments using 16 mer DNA sequences

Self-complementary 16 mer DNA

The experiments described earlier in this chapter used self-complementary 8 mer and 12 mer dsDNA sequences. To explore further possibilities of using sequences which may have higher order structure, self-complementary oligonucleotides containing 16 bases were used in experiments described in this section. Figure 4.19 shows the ESI-mass spectra of a 5:1 mixture of daunomycin or nogalamycin with d(ATATATATATATATAT)₂. Assignments are given in Table 4.5. At first glance, these spectra appear similar to other drug-DNA ESI-mass spectra observed. However, on closer inspection it is apparent that most of the ions observed are in the form [2M+\(\pm\) nh\(\pm\)H]\(\pm\) where both \(x\) and \(y\) are even integers. For example, in the complexes containing daunomycin (Figure 4.19a) the only ions that can be assigned specifically to dsDNA complexes include the [2M+3Dn-7H]\(\pm\) (P), [2M+4Dn-7H]\(\pm\) (Q) and [2M+3Dn-6H]\(\pm\) (S) ions. The relative intensities of these ions account for only 8\%, 2\% and 3\% relative to the base peak respectively. Similarly the only ion in the spectrum of the mixture containing nogalamycin that can be unequivocally assigned as arising from a complex with dsDNA is the [2M+5Ng-8H]\(\pm\) ion (n) which accounts for less than 1.5\% of the base peak (Figure 4.19b).
Figure 4.19: ESI mass spectra of d(ATATATATATATATAT)₂ with either (a) daunomycin or (b) nogalamycin. Assignments of the major ions are given in Table 4.5.

The data in Figure 4.19 can also be plotted by summing the intensities of [2M+2I-H]⁺ and [2M+4I-H]⁺ ions (all charge states) containing a given number of intercalator molecules and assuming they are from dsDNA. This information is presented in Figure 4.20. A striking pattern is observed. Complexes containing even numbers of drug molecules predominate. Clearly the only ion that can be attributed to arising from a dsDNA complex is the [2M+4Dn-7H]⁻ peak which only accounts for 1.3% of all related species. This indicates that the complexes observed are clearly not from [2M+2I-H]⁺ and [2M+4I-H]⁺ but from the ssDNA ions; [M+I-\(\sqrt{2}\)H]⁺ and [M+2I-\(\sqrt{2}\)H]⁺ (where \(z = \sqrt{2}\)) respectively.
Table 4.5: Assignments of all the major ions in Figure 4.19.

<table>
<thead>
<tr>
<th>Ion</th>
<th>M=d(ATAATATATATATAT)_2 I=Daunomycin</th>
<th>M=d(ATAATATATATATAT)_2 I=Nogalamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m/z</td>
<td>Label</td>
</tr>
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</tr>
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</tr>
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<td>811.4</td>
<td>G</td>
</tr>
<tr>
<td>[M-5H]^5^-</td>
<td>973.7</td>
<td>h</td>
</tr>
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<td>[2M+3I-6H]^6-</td>
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Figure 4.20: Normalised intensity of [2M+2I-yH]^+ and [2M+4I-yH]^+ ions in ESI mass spectra of d(ATATATATATATAT)₂ and either daunomycin or nogalmycin.
Similar results were obtained using other self-complementary 16 mer sequences including d(ATATATACGTATAT)\textsubscript{2} and d(GGATATACGTATATCC)\textsubscript{2} (spectra not shown). These results have been plotted using the total ion current of each of the species in Figure 4.21. The data show the results for all charge states added together assuming that all complexes occur with dsDNA. These results are similar to those in Figure 4.20. There is some evidence for the dsDNA complex with 3 and 5 drug bound species observed binding to d(GGATATACGTATATCC)\textsubscript{2}. There is also some binding of 5 nogalamin molecules to d(ATATATACGTATAT)\textsubscript{2} (ATATATATATATAT)\textsubscript{2} observed (Figure 4.21a). These complexes can be assigned unequivocally as arising from dsDNA. These complexes are, however, in relatively low abundance. This phenomenon is even more pronounced when considering the complexes of daunomycin with the 16 mer sequences (Figure 4.21b). The major species observed here include complexes with 2, 4 and 6 bound drug molecules bound to dsDNA (or 1, 2, and 3 drug molecules bound to ssDNA). There is still evidence of some genuine dsDNA-drug complexes with 3 daunomycin molecules bound to d(ATATATATATATAT)\textsubscript{2} and (ATATATACGTATAT)\textsubscript{2}. These complexes are again in relatively low abundance to complexes with ssDNA.

A possible explanation for the lack of complexes containing odd numbers of intercalator molecules is that all the complexes are with ssDNA. That is the complexes with 2, 4 or 6 molecules of intercalator assumed to be bound to dsDNA are actually complexes with 1, 2 or 3 intercalator molecules bound to ssDNA.
Figure 4.21: Comparison of the relative intensities of the major ion/drug dsDNA ion series for (a) nogalamycin and (b) daunomycin complexes observed in 5:1 reaction mixtures of drug (I) with different 16 mer oligonucleotides. Note that the data compiled assumed that all the complexes were arising from dsDNA.

Longer DNA is more stable in duplex form than shorter sequences owing to increased numbers of hydrogen bonds between longer strands (Blackburn and Gait, 1990). Therefore, it is difficult to understand how complexes involving dsDNA would not be observed for 16 mer DNA, but are observed for the 8 mer and 12 mer DNA. Furthermore, the longer DNA lengths used were expected to be more representative of the true structure of B-DNA than shorter sequences. As daunomycin and nogalamycin are known intercalators, another possibility is that all the complexes
with self-complementary 16 mer DNA are double-stranded, but consist of one self-complementary strand folded back on itself as outlined schematically in Figure 4.22. This is only possible with self-complementary DNA. Various DNA sequences are known to form hairpin structures. Examples of these include sequences that contain a central GAA loop (Colgrave et al., 2002; Colgrave et al., 2001; Williams et al., 2002; Zhu et al., 1996; Zhu et al., 1995), ATC in the central loop (Amiraslani et al., 1996) as well as a number of different 16 mers that contain differing combinations of the central sequence (reviewed in Vallone et al., 1999).

![DNA structure](image)

Figure 4.22: A schematic describing the most likely scenario possible for the binding of the intercalator to ssDNA.

### 4.6.2 Binding to non-self complementary DNA

To preclude the problem outlined above with the self-complementary 16 mers, a variety of non-self complementary sequences of DNA capable of associating to form dsDNA was used. By using these sequences, problems arising from ambiguous peaks could be avoided and further, that any non-specific binding to ssDNA could be accounted for. Furthermore, each of the single strands used was not expected to fold over and form hairpin structures.
Figure 4.23 shows the ESI-mass spectra of one single strand of a complementary pair 5'-CTCGTCCGATTGACGATC-3' with nogalamycin, the other strand of the complementary pair 5'-GATCGAATCGGACGAG-3' with nogalamycin and the dsDNA 5'-CTCGTCCGATTGACGATC-3'/ 5'-GATCGAATCGGACGAG-3' with nogalamycin. The spectrum of 5'-CTCGTCCGATTGACGATC-3' with nogalamycin (Figure 4.23a) shows ions from ssDNA with no complexed nogalamycin (A, B, C, D, F, H, K and P) (accounting for 55.9% of the total ions observed) as well as ions from association of the ssDNA with nogalamycin. These ssDNA complexes have either 1 nogalamycin bound (G, J, M and R), 2 bound (Z, L, O) and 3 bound drugs (N, Q). These ions account for 18.2, 17.8 and 3.7% of the total ion current respectively. Similarly the spectrum of 5'-GATCGAATCGGACGAG-3' with nogalamycin (Figure 4.23b) also shows ions from ssDNA (a, b, c, d, f, h, k and q) (58.0% of the total ions observed) as well as the complex containing 1 drug bound to the single strand (g, j, m) (23.3%), with 2 drugs bound (i, l, o) (8.9%) as well as that with 3 drugs bound (n, q) (3.0%). Note that percentage values given in parenthesis are the relative amounts of each complex compared to the total ion current. When 5'-CTCGTCCGATTGACGATC-3' and 5'-GATCGAATCGGACGAG-3' were annealed together with nogalamycin (Figure 4.23c), the resulting spectrum is significantly different. ssDNA is still evident with ions from each strand present (B, C, D, F, K, c, d, f, k). There are also a smaller proportion of ions from the non-intercalative (single-stranded) 5'-CTCGTCCGATTGACGATC-3'-nogalamycin complex present (F, J, M). There are also a number of ions from complexes involving dsDNA observed in the spectrum. These ions contain either 3 bound drugs ($\Delta_1$, $\Delta_4$), 4 bound drugs ($\Delta_2$, $\Delta_5$) and 5 bound drugs ($\Delta_3$, $\Delta_6$). The drug-dsDNA complexes, however, only account for 9.0 % of the total ions observed in the spectrum.
While the use of the non-self-complementary DNA enabled distinction of complexes involving dsDNA from ssDNA non-specific complexes, the abundance of complexes from drugs bound to dsDNA was still relatively low.

![Figure 4.23: ESI mass spectra of nogalamycin with (a) dsDNA 5'-CTCGTCCGATTGATTCGATC-3'/5'-GATCGAATCGGACGAG-3'; (b) 5'-GATCGAATCGGACGAG-3' and (c) the other strand of the complementary pair 5'-CTCGTCCGATTGATTCGATC-3'. Assignments are given in Figure 4.23.](image-url)
### Table 4.6: Assignments of major ions in Figure 4.23.

<table>
<thead>
<tr>
<th>Ion</th>
<th>M₁=5’-GATCGAATCGGACGAG-3’</th>
<th>M₂=5’-CTCGTCGCGATTGATC-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M₁</td>
<td>m/z</td>
</tr>
<tr>
<td>[M-10H]⁺⁻⁻</td>
<td>A</td>
<td>480.5</td>
</tr>
<tr>
<td>[M-9H]⁺⁻⁻</td>
<td>B</td>
<td>533.6</td>
</tr>
<tr>
<td>[M-8H]⁺⁻⁻</td>
<td>C</td>
<td>600.8</td>
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<tr>
<td>[M-7H]⁺⁻⁻</td>
<td>D</td>
<td>685.7</td>
</tr>
<tr>
<td>Nogalamycin</td>
<td>E</td>
<td>786.3</td>
</tr>
<tr>
<td>[M-6H]⁺⁻⁻</td>
<td>F</td>
<td>800.2</td>
</tr>
<tr>
<td>[M+Ng-6H]⁺⁻⁻</td>
<td>G</td>
<td>931.5</td>
</tr>
<tr>
<td>[M-5H]⁺⁻⁻</td>
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<td>960.5</td>
</tr>
<tr>
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<td>1061.9</td>
</tr>
<tr>
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<td>1118.2</td>
</tr>
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<td>[M-4H]⁺⁻⁻</td>
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<td>[M+3Ng-4H]⁺⁻⁻</td>
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<td>[M+Ng-3H]⁺⁻⁻</td>
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<td>[Δ+3Ng-8H]⁺⁻⁻</td>
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<td>Δ₂</td>
<td>1614.4</td>
</tr>
<tr>
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<tr>
<td>[Δ+4Ng-7H]⁺⁻⁻</td>
<td>Δ₅</td>
<td>1844.9</td>
</tr>
<tr>
<td>[Δ+5Ng-7H]⁺⁻⁻</td>
<td>Δ₆</td>
<td>1657.6</td>
</tr>
</tbody>
</table>

### 4.7 Studies using the quadrupole-time-of-flight instrument

The experiments outlined above (interactions of self-complementary dsDNA with nogalamycin or daunomycin) were performed using the VG Quattro™ triple-quadrupole mass spectrometer and injecting samples along with water as a carrier solvent. The later studies the interactions of non-self-complementary dsDNA with a range of intercalators were revisited and/or extended using a quadrupole-time-of-flight mass spectrometer (Micromass Q-Tof2™) that is more sensitive and possesses a greater m/z range than the older instrument. Furthermore, the instrument was
fitted with a Z-spray source which allowed for higher concentrations of salt in the sample solutions.

In our laboratory, Gupta et al. determined optimal sample and instrument conditions necessary for the observation of non-covalent complexes of daunomycin with dsDNA using the Q-Tof2™ mass spectrometer (Gupta et al., 2001). These conditions are described in Chapter 2. Briefly, DNA strands (1 mM) were annealed in 100 mM NH₄OAc, pH 8.5. Samples were diluted 100-fold with the same solvent (100 mM NH₄OAc, pH 8.5) just prior to analysis by ESI-MS.

4.7.1 Titration of complementary DNA strands

In order to prepare drug-dsDNA complexes, equal amounts of each complementary strand of DNA must be annealed. The concentrations of solutions of each strand were determined by UV-vis spectroscopy and application of Beer’s Law. Since errors can arise especially when there is any secondary structure present, we routinely titrated the strands together to account for any inaccuracies. Figure 4.24 shows a series of negative ion ESI-mass spectra following titration of one strand of DNA, 5’-GATCGAATCGGACGAG-3’ (M₁) with its complementary strand 5’-CTCGTCCGATTCGATC-3’ (M₂). It is important to note that the final total concentration of DNA in solution as judged by UV spectroscopy, was 10 μM. In the ESI mass spectra, the peaks denoted with lower case letters correspond to the [M-4H]⁺ (a) and [M-3H]⁻ (b) ions of the ssDNA M₁. The letters A and B denote the corresponding ions of M₂. In this experiment the abundances of ions from either ssDNA strands were lowest when the two strands were annealed in the ratio 0.5
dsDNA is denoted by $\Delta_1$ and $\Delta_2$ for $[\text{dsDNA-6H}]^6$ and $[\text{dsDNA-5H}]^5$, respectively. In numerical terms, the relative amounts of dsDNA compared to ssDNA based on the intensities of the peaks in the $0.4 \text{ M}_1 : 0.6 \text{ M}_2$, $0.5 \text{ M}_1 : 0.5 \text{ M}_2$ and $0.6 \text{ M}_1 : 0.4 \text{ M}_2$ were 48%, 74% and 36%, respectively. This gave us confidence that the concentrations of each of the strands determined by UV-vis spectroscopy for this mixture were reasonably accurate.

Figure 4.24: Negative ion ESI mass spectra of DNA strand titration ($5'-\text{GATCGAATCGGAG-3'} (\text{M}_1)$ and $5'-\text{CTCGTCCGATTCGATC-3'} (\text{M}_2)$) to determine correct ratios for optimal duplex formation; (a) $0.3 \text{ M}_1 : 0.7 \text{ M}_2$, (b) $0.4 \text{ M}_1 : 0.6 \text{ M}_2$, (c) $0.5 \text{ M}_1 : 0.5 \text{ M}_2$, (d) $0.4 \text{ M}_1 : 0.6 \text{ M}_2$ and (e) $0.7 \text{ M}_1 : 0.3 \text{ M}_2$. Ions corresponding to $5'-\text{GATCGAATCGGAG-3'} (\text{M}_1)$ are denoted in lowercase whilst those corresponding to $5'-\text{CTCGTCCGATTCGATC-3'} (\text{M}_2)$ are denoted in uppercase. $\Delta$ denotes dsDNA ions.
4.7.2 Complexes of dsDNA with nogalamycin and daunomycin

Figure 4.25 shows the negative ion ESI-mass spectra of the nogalamycin-dsDNA and daunomycin-dsDNA complexes prepared by addition of drugs to dsDNA in the ratio 5:1. Assignments for the labelled peaks are given in Table 4.7. The ESI-mass spectrum of the daunomycin-dsDNA mixture contained ions corresponding to 1 daunomycin molecule bound (D, E), 2 bound (F, G, H), 3 bound (I, J, K) and 4 bound (L, M, N). Of these, the complexes where 2 and 3 daunomycin molecules were bound to dsDNA exhibited the most intense peaks (33% and 27%, respectively) when the intensities of all ions from each species were summed and expressed as a percentage of total ion current. The spectrum of the nogalamycin-dsDNA mixture contained ions corresponding to 1 nogalamycin bound (c, d, e), 2 bound (f, g, h) and 3 bound (i, j). Of these, the complexes where 1 and 2 nogalamycin molecules were bound to dsDNA were most abundant with their ions accounting for 44% and 39% of the total ion current, respectively.

Figure 4.25: Negative ion ESI mass spectra of 5:1 mixtures of nogalamycin or daunomycin with dsDNA with using optimal conditions developed for the Qtof2™ mass spectrometer. Peaks are assigned in Table 4.7.
These spectra are quite different to those described in the preceding sections (see for example Figure 4.23. First, there is no evidence of non-specific ssdrug-DNA complexes. It is important to point out that the conditions employed here were subtly different from those in which the Micromass Quattro™ instrument was utilised. For instance, the sample was injected with a final ammonium acetate concentration of 100 mM compared to 10 mM in the earlier studies using the triple quadrupole instrument. Additionally, the ESI conditions used on the Quattro™ (capillary = 2.0 kV, cone = 15-30 V ramp and temperature = 60°C) were different to those used on the Qtof2™ (capillary = 2.5 kV, cone = 50 V ramp and temperature = 40°C). Furthermore, owing to the increased sensitivity of the Qtof2™, a lower final concentration of dsDNA prior to injection was used (i.e. 10 μM) compared with 100 μM in experiments performed on the triple quadrupole mass spectrometer. The use of a lower analyte concentration is thought to discourage non-specific interactions (Gale et al., 1994; Gale and Smith, 1995).

Table 4.7: Assignments of major ions from negative ion spectra in Figure 4.25. Δ = dsDNA (M₁ + M₂).

<table>
<thead>
<tr>
<th>Ion</th>
<th>Δ+I-7H</th>
<th>[M₁-4H]⁻</th>
<th>[M₁-4H]⁻</th>
<th>Δ+I-6H</th>
<th>[Δ+I-5H]⁻</th>
<th>Δ+2I-7H</th>
<th>Δ+2I-6H</th>
<th>Δ+2I-5H</th>
<th>Δ+3I-7H</th>
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<th>Δ+4I-5H</th>
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<td></td>
<td></td>
<td>1201.0</td>
<td>1237.9</td>
<td>1544.7</td>
<td>1714.4</td>
<td>2057.4</td>
<td>1506.5</td>
<td>1802.3</td>
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<td>2109.6</td>
<td>1620.1</td>
<td>2268.4</td>
<td>2377.7</td>
</tr>
<tr>
<td>I=Daunomycin</td>
<td>a</td>
<td>1201.2</td>
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<td>1506.5</td>
<td>1757.8</td>
<td>2109.6</td>
<td>1619.1</td>
<td>1889.0</td>
<td>2266.9</td>
<td>1731.6</td>
<td>2020.4</td>
<td>1731.6</td>
<td>2020.4</td>
<td>1731.6</td>
</tr>
<tr>
<td>I=Nogalamycin</td>
<td>b</td>
<td></td>
<td></td>
<td>c</td>
<td></td>
<td>d</td>
<td>e</td>
<td>f</td>
<td>h</td>
<td>g</td>
<td>i</td>
<td>j</td>
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</table>

M₁=5'-GATCGAATCGGACGAG-3'
M₂=5'-CTCGTCCGATTCGATC-3'

Table 4.7: Assignments of major ions from negative ion spectra in Figure 4.25. Δ = dsDNA (M₁ + M₂).
4.7.3 Titration of dsDNA with intercalators

A series of experiments was carried out whereby either nogalamycin, daunomycin, or ethidium bromide were added to dsDNA \((M_1 + M_2)\) over the range from no drug to a 9-fold excess using the optimal experimental conditions described above (and in Section 2.6.2). Figure 4.26 shows the negative ion ESI-mass spectra of 1:1, 3:1, 7:1 and 9:1 mixtures of nogalamycin with the duplex non-self-complementary 16 mer DNA \((M_1 + M_2 = \Delta)\). The assignments are given in Table 4.8. All peaks in these spectra can be assigned unambiguously. In the spectrum of the 1:1 nogalamycin:dsDNA mixture (Figure 4.26a), the major peaks observed are those arising from the complex where either one (D) or 2 (G) nogalamycin molecules are bound to dsDNA. There is also some evidence of ssDNA (A, B) although these are of low abundance compared to the \([\Delta+2Ng-6H]^{6-}\) peak (G) (24% (A) and 17% (B), respectively). On increasing the drug concentration to 3:1 nogalamycin:dsDNA (Figure 4.26b), the \(\Delta+3Ng\) species becomes evident (I, J, K). However, the ions corresponding to \(\Delta+1Ng\) and \(\Delta+2Ng\) remain predominant (C, D, E and F, G, K, respectively). Increasing nogalamycin to 7:1 drug:dsDNA (Figure 4.26c) results in ions arising from the \(\Delta+4Ng\), \(\Delta+5Ng\) and \(\Delta+6Ng\) species (L, M; N, O; and Q respectively). These ions are more abundant than those from \(\Delta+3Ng\). Ions from \(\Delta+Ng\) and \(\Delta+2Ng\) are of low abundance in this spectrum. Increasing the drug again to 9:1 nogalamycin:dsDNA (Figure 4.26d) results in spectra of poorer quality with ions from \(\Delta+5Ng\) and \(\Delta+6Ng\) becoming more intense. There is also some evidence in this spectrum of the nogalamycin trimer (3Ng).
These data are summarized in Figure 4.27. In this graph, the relative intensity of each species (y-axis) is plotted against the number of drug molecules bound to dsDNA (x-axis) for each drug-dsDNA mixture from 1:1 to 9:1. Each titration curve is plotted on the same graph in order to compare these data. First, it is important to...
note that these data are consistent with the reaction stoichiometry. For instance, in
the curve for the 3:1 nogalamycin:dsDNA mixture (Figure 4.27a) (■), the Δ+3Ng
complex becomes significant. Likewise, in the 5:1 nogalamycin:dsDNA titration
curve (▲), the Δ+5Ng complex arises although not as intense as the Δ+3Ng species
(12% vs 40% respectively). As the relative amount of nogalamycin increases
however, the binding sites on the dsDNA become saturated as shown by the overlap
of the curves for the 7:1 (X) and 9:1 mixtures (•) mixtures.

Table 4.8: Assignments of the major ions in the negative ion ESI mass spectra in
Figure 4.26.

<table>
<thead>
<tr>
<th>Ion</th>
<th>( \text{M}_1=5'\text{-GATCGAATCGGACGAG-3'} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{M}_2=5'\text{-CTCGTCCGATTGATC-3'} )</td>
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<tr>
<td>( [\text{M}_2-3\text{H}]^{3-} )</td>
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</tr>
<tr>
<td>( [\Delta+1-7\text{H}]^{7-} )</td>
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</tr>
<tr>
<td>( [\Delta+1-6\text{H}]^{6-} )</td>
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<td>( [\Delta+1-5\text{H}]^{5-} )</td>
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\( \text{I = Nogalamycin} \)

\( \text{m/z} \) \( \text{Label} \)
A 1651.0
B 1601.7
C 1506.5
D 1757.7
E 2109.7
F 1619.0
G 1889.1
H 2266.8
I 1731.7
J 2020.3
K 2425.0
L 1844.2
M 2151.5
N 1956.6
O 2283.1
P 2069.2
Q 2414.5
Figure 4.27: Curves showing relative amounts of drug-dsDNA \((M_1 + M_2)\) complexes of a particular stoichiometry in mixtures of drug:dsDNA over the range 1:1 to 9:1, as a percentage of total intensity of all ions in the mixture. (a) nogalamycin, (b) daunomycin and (c) ethidium bromide.
Similar results were obtained for the mixtures of daunomycin with the dsDNA (Figure 4.27b). These data are also consistent with reaction stoichiometry. The curves for the various complexes with daunomycin also indicate that more drug molecules are bound to the dsDNA than in the corresponding mixture containing nogalamycin. For example, in the 3:1 daunomycin:dsDNA mixture (■), evidence of [Δ+4Dn] complex is observed. The [Δ+4Ng] complex is not, however, observed in the 3:1 nogalamycin:dsDNA mixture. Similarly in the 5:1 nogalamycin:dsDNA mixture (▲), the maximum number of drugs bound to the dsDNA is 5, whilst the [Δ+6Dn] complex is observed in the 5:1 daunomycin:dsDNA mixture.

Data for ethidium bromide:dsDNA mixtures are shown in Figure 4.27. In negative ion ESI mass spectra of 3:1 ethidium bromide (Eb):dsDNA mixtures (Figure 4.27c), ions from the [Δ+5Et] complex were present. They were, however, of low intensity (1.3% of all ions). In the case of ethidium bromide, the curves for the 7:1 and 9:1 mixtures do not exhibit the same degree of overlap as those for nogalamycin or daunomycin mixtures suggesting that the DNA binding sites for ethidium are not yet saturated. If ethidium can bind between every other base pair (neighbour exclusion principle (Rao, 1987)) and there is no non-specific binding, then up to 8 molecules of ethidium could be bound to 16 mer dsDNA. The intercalators discussed to this point approach saturation of the dsDNA binding sites for that particular drug in the order nogalamycin>daunomycin>ethidium. The result might be explained simply in terms of steric effects. For example, as the ethidium molecule is smaller than either of the anthracyclines, lack of steric hindrance might enable ethidium to intercalate between base pairs that are close together. As daunomycin lacks the bulky sugars
possessed by nogalamycin, it may be capable of intercalation with fewer steric effects.

4.8 In-source C.I.D experiments used to estimate binding strength

4.8.1 Influence of cone voltage on relative complex stabilities

The effect of varying cone voltage on the ESI mass spectra of non-covalent complexes has been investigated in a number of studies (Loo, 1997; Potier et al., 1998). Increasing the cone voltage increases the acceleration of the ions in the intermediate pressure region in the source; thereby increasing the energy of collisions with bath gas ($N_2$), in many cases increasing the dissociation of weakly bound complexes. Altering the cone voltage, however, may change the proportion of ions focused into the mass spectrometer from the source (Hunt et al., 1998) such that interpretation of results from these experiments must consider both collision and focusing effects.

Figure 4.28 shows the negative ion ESI mass spectra of a 3:1 daunomycin:dsDNA mixture acquired using different cone voltages. The spectrum acquired at 25 V is one of poor quality (low signal-to-noise ratio), presumably because the focusing is not optimised. Nevertheless, ions from drug-dsDNA complexes, though very broad (peak width at half height ~40 $m/\chi$) are present. Increasing the cone voltage to 50 V (optimal cone voltage) results in significant sharpening of peaks (peak width at half height < 2.5 $m/\chi$) suggesting that water-buffer salts are lost from the complex.
Figure 4.28: Effect of cone voltage on negative ion ESI mass spectra of a 3:1 mixture of daunomycin with dsDNA (M₁ + M₂): (a) at 25 V, (b) at 50 V, (c) at 75 V and (d) at 100 V. (♦) Daunomycin-dsDNA complexes; (●) ssDNA (either M₁ or M₂).

under these conditions. Once again, the peaks observed predominantly arise from daunomycin-dsDNA complexes (♦). There are, however some ions from ssDNA (●), although these only account for ~11% of the total ion current. Increasing the cone voltage to 75 V results in an increase in the amount of ssDNA present (42% of total
ion current). Ions corresponding to the drug-dsDNA complexes are still observed. Increasing the cone voltage to 100 V results in further reduction in the intensity of the daunomycin-dsDNA ions and an increase of the ssDNA ions (50% of total). Interestingly, there were no ions of significant intensity in any of the spectra that were from dsDNA with no drug molecules bound suggesting that the drug-dsDNA complex is relatively stable. In the spectrum recorded at 100 V, there is also some evidence of oligonucleotide fragmentation in the spectrum ($m/z$ 1000-1500).

Similar experiments were also carried out for mixtures of nogalamycin and ethidium with dsDNA (3:1) and also for uncomplexed dsDNA. The relative amount of drug-dsDNA complex as a percentage of the total ion current (ssDNA and drug-dsDNA) has been plotted against cone voltage in Figure 4.29. Ions from dsDNA with no drug molecules bound were insignificant in all of the spectra of drug-dsDNA mixtures. That is, dissociation of complexes involved separation of DNA strands rather than dissociation of drug molecules resulting in free dsDNA. Complexes with ethidium bromide appear to be the most stable with respect to increases in cone voltage in these experiments. On going from 50 V to 100 V, there appeared to be a small increase in the amount of complex present. This can be explained in terms of enhanced signal for the ions at high $m/z$ at higher cone voltages (Hunt et al., 1998). Complexes containing nogalamycin and daunomycin are less stable than those containing ethidium bromide.
Figure 4.29: The effect of cone voltage on negative ion ESI mass spectra of uncomplexed dsDNA (M₁ + M₂) and the complexes of dsDNA with nogalamycin, daunomycin and ethidium.
The relative stabilities of these complexes in solution have not been measured. There are at least three factors that must be considered in discussing the relative stabilities of the complexes with respect to cone voltage. First, it has been widely reported that intercalators stabilize DNA and thus increase the melting temperature of the DNA (Ali et al., 1998; Bhattacharya and Mandal, 1997; Escude et al., 1995). It has also been reported that the denaturation caused by raising the cone voltage can be described as thermal and thus closely related to DNA melting curves (Gabelica et al., 1999). Accordingly, the results show that negatively charged complexes of dsDNA with these drugs are more stable to strand separation compared with uncomplexed dsDNA over the range of cone voltages from 50 to 65 V. At higher energies, however, there is no stabilization of dsDNA by these drugs. At cone voltages higher than 65 V, however, [Δ+Eb] is clearly more stable than [Δ]> [Δ+Ng] ~ [Δ+Dn]. A possible reason for the clear result with [Δ+Et], is that in this mixture, the [Δ+3Eb] complex was most abundant. The more intercalator molecules bound, the more the stabilization. This proposal can be examined more closely by plotting the amounts of each species [Δ+3I], [Δ+2I], [Δ+3I] (I = intercalating drug) as a percentage of all ions as a function of cone voltage as shown in Figure 4.30. For all drugs (nogalamycin, daunomycin and ethidium bromide), the [Δ+1I] complex is the least stable to increases in cone voltage. The [Δ+2I] and [Δ+3I] complexes have very similar responses to cone voltage for complexes containing daunomycin and ethidium bromide. A more substantial difference is noted between the [Δ+2I] and [Δ+3I] complexes of nogalamycin. A second factor is the collisional cross-section of the ions. In the 3:1 drug:dsDNA mixtures, there are 2-3 drugs bound in the complexes with dsDNA as judged by negative ion ESI-MS. The complexes containing nogalamycin or daunomycin are bulkier than those with
ethidium. The larger complexes have a higher probability of undergoing collisions with bath gas molecules. Similar observations have also been made in other ESI-MS studies (Barton et al., 1997; Jasieczek et al., 1996). A third factor is the charge on these molecules. In these experiments, we studied negative ions. The effect of cone voltage on positive ion spectra also needs to be examined (Section 4.8.4). It is also important to consider that the relative response factors for the various complexes and uncomplexed dsDNA (molecular mass 9763.5) could be different.

![Figure 4.30: The effect of cone voltage on the \([\Delta+1I], [\Delta+2I] \text{ and } [\Delta+3I] \) (I=drug) complexes of nogalaminic, daunomycin, and ethidium bromide from negative ion ESI mass spectra. The 5-, 6- and 7- charge states were used to sum these data (Ions with higher or lower numbers of charges were insignificant in these spectra).]
4.8.2 Influence of desolvation temperature on DNA-drug complexes

Increases in the distribution of internal energy of ions by increasing the desolvation temperature can result in dissociation of non-covalent complexes (Gabelica et al., 2000; Goodlett et al., 1994). Figure 4.31 shows the negative ion ESI mass spectra of complexes of ethidium bromide:dsDNA mixtures (3:1) acquired with increasing desolvation temperatures ranging from 40°C to 280°C. The spectrum of the ethidium:dsDNA mixture at 40°C (Figure 4.31a) shows that the majority of the ions correspond to complexes. There is also some ssDNA present (~11% of total ion current). Increasing the temperature to 120°C results in a slight increase in ssDNA peaks (13% of total ions). This trend continues such that at 200°C (Figure 4.31c) where the ssDNA peaks account for 43% of the total ion current. The most significant changes in the spectrum occur between 200°C and 280°C. In the ESI mass spectrum acquired using a desolvation temperature of 280°C, ions from ssDNA account for 80% of the total ion current. The predicted solution melting temperature of uncomplexed DNA used these experiments is 46°C (Cao et al., 2002). The Tm for this DNA complexed with ethidium has not been measured, however, intercalators are thought to stabilize dsDNA (Beck et al., 2001; Blackburn and Gait, 1990; Brana et al., 2001; Chaires et al., 1985; Fox et al., 1985; Haq and Ladbury, 2000; Rizzo et al., 1989). It is also important to point out that all complexes appear to be relatively stable to 200°C. The gas phase temperature required to dissociate dsDNA to ssDNA is therefore quite high and it would be interesting in future work to compare values of Tm measured in solution with the gas phase. Furthermore, the temperature experienced by ionized macromolecules in droplets formed during the electrospray process is difficult to judge since evaporation of droplets causes cooling (Lee et al., 1998). Relating solution Tm to
applied desolvation temperatures, therefore, would require a more sophisticated experimental arrangement whereby the applied temperatures could be calibrated in a meaningful way.

These spectra can also be analysed graphically. For example, comparing the intensities of ions from complexed dsDNA (DNA\textsubscript{complex}) as a percentage of total DNA (dsDNA\textsubscript{complex} + dsDNA\textsubscript{free} + ssDNA\textsubscript{free}) and plotting against desolvation temperature yield interesting dissociation curves. Figure 4.32 compares the stability of free dsDNA and the various complexes containing either nogalamycin, daunomycin or ethidium bromide. In all of the spectra of mixtures containing the intercalators, ions from uncomplexed dsDNA were of low abundance. The uncomplexed dsDNA at no time exceeded 9.8, 4.7 and 2.9% of the total ion current for mixtures containing nogalamycin, daunomycin and ethidium, respectively. The drug-dsDNA complexes dissociated yielding free ssDNA and free drug. All the complexes are quite stable to 200°C. Complexes containing ethidium bromide appear to be the most stable to temperature increases. Nogalamycin appears to form the next most stable complexes whilst daunomycin appears to form drug-dsDNA complexes that are least stable. The uncomplexed dsDNA shows the least stability to increases in desolvation temperature above 160°C. The order of stability for complexes with respect to desolvation temperature are ethidium>nogalamycin~daunomycin. The overall differences in stability determined in these experiments are not great, and this method may not be the most useful for studying stabilities of these complexes. This result is interesting as most previous non-covalent DNA ligand studies (Gale et al., 1994; Gale and Smith, 1995; Gupta et al., 2001; Kapur et al., 1999b; Wan et al., 2000a; Wan et al., 2000b) all used low desolvation temperatures (≤60°C).
Figure 4.31: Effect of desolvation temperature on negative ion ESI mass spectra of a 3:1 mixture of ethidium bromide with dsDNA (M₁ + M₂) at: (a) 40°C, (b) 120°C, (c) 200°C and (d) 280°C. (▲) Ethidium bromide-dsDNA complexes; (●) ssDNA (either M₁ or M₂).
Figure 4.32: The effect of desolvation temperature on negative ion ESI mass spectra of uncomplexed dsDNA (M1 + M2) and the complexes of dsDNA with nogalamycin, daunomycin and ethidium bromide.
4.8.2.1 ESI-MS/MS experiments

The stabilities of the intercalator-dsDNA complexes were also probed using ESI-MS/MS. This involves selecting an ion of interest in MS1 (the quadrupole), passing then through the collision cell where dissociation is induced upon increasing the energy or by increasing gas pressures. In the experiments described below, dissociation was monitored upon increasing the collision energy.

Figure 4.33 shows a series of ESI-MS/MS spectra of the precursor ions \([\Delta+\text{Ng}-6\text{H}]^6^-\) and \([\Delta+2\text{Ng}-6\text{H}]^6^-\) (where \(\Delta\) is the dsDNA). Assignments of the major ions are given in Table 4.9. At 5 eV, the \([\Delta+\text{Ng}-6\text{H}]^6^-\) ion at \(m/z\) 1757.3 (E) remains intact. Increasing the collision energy to 10 eV results in loss of nogalamycin from dsDNA (C, D) with some separation of the dsDNA to ssDNA (A, B). The \([\Delta+\text{Ng}-6\text{H}]^6^-\) ion is still, however, the dominant species (82% of the total ion current). At 15 eV, the product ions (ssDNA) increase in intensity. At 25 eV unbound DNA (free dsDNA and ssDNA) predominate. There is still, however, some complex observed although it is greatly reduced (33%). Similarly, in the spectrum of \([\Delta+2\text{Ng}-6\text{H}]^6^-\) at 5 eV, the major species present is the \([\Delta+2\text{Ng}-6\text{H}]^6^-\) ion at \(m/z\) 1888.7 (e). This ion still remains very much in excess at 10 eV even though small amounts of \([\Delta+\text{Ng}-5\text{H}]^5^-\) (f) are noted (less than 10% of the total ions). Increasing the collision energy to 15 eV results in the observation of free DNA (both ssDNA (a, b) and dsDNA (d)). The \([\Delta+2\text{Ng}-6\text{H}]^6^-\) ion remains the most abundant ion (82%). At 25 eV there is evidence of a greater degree of complex dissociation as evidenced by the increased abundance of ions from free DNA (a, b, d). The drug-bound dsDNA is still present with evidence of both \([\Delta+2\text{Ng}-6\text{H}]^6^-\) (e) and \([\Delta+\text{Ng}-5\text{H}]^5^-\) (f). The percentage of ssDNA in this instance is \(~26\%\) compared with \(~44\%\) for \([\Delta+\text{Ng}-6\text{H}]^6^-\). These
results show that \([\Delta+2Ng]\) is more stable than \([\Delta+Ng]\) in these experiments. This is consistent with the proposal that complexation of dsDNA with greater numbers of intercalator molecules stabilize the dsDNA structure.

Similar experiments were carried out using the intercalators daunomycin and ethidium bromide. The results can be expressed graphically by comparing the total abundance of ions from drug-dsDNA complexes and normalizing this data to the total ions present (Note that the calculated values for the \(E_{acc}\) have been calculated and presented in Appendix 6). These values can be then plotted against collision energy. Figure 4.34 presents such a plot comparing the dissociation of \([\Delta+I-6H]^{6-}\) and \([\Delta+2I-6H]^{6-}\) where I is either nogalamycin, daunomycin or ethidium bromide. One point is clear when examining these data. That is, the \([\Delta+2I-6H]^{6-}\) ions are more stable than their \([\Delta+I-6H]^{6-}\) counterparts for each intercalator over the range 15-25 eV. A possible explanation for this is that removal of two intercalated drug molecules requires more energy than the removal of a single drug molecule. For \([\Delta+I-6H]^{6-}\) and \([\Delta+2I-6H]^{6-}\) complexes, the intercalators stabilize the dsDNA (between 15 and 25 V) in the order nogalamycin>ethidium bromide>daunomycin. This overall order of stability differs from the studies on the effect of cone voltage and desolvation temperature where complexes containing ethidium were the most stable.
Figure 4.33: Product ion spectra of $[\Delta + \text{Ng-6H}]^{\pm}$ at: (a) 5 eV, (b) 10 eV, (c) 15 eV and (d) 25 eV, and of $[\Delta + 2\text{Ng-6H}]^{\pm}$ complexes at: (e) 5 eV, (f) 10 eV, (g) 15 eV and (h) 25 eV. Assignments are given in Table 4.9. $\Delta$ = dsDNA.
Figure 4.34: The effect of collision energy on [Δ+1I-6H]^− and [Δ+2I-6H]^− complexes (I = nogalamycin, daunomycin or ethidium bromide). Δ = dsDNA
Table 4.9: Assignments of major ions from negative ion ESI mass spectra in Figure 4.33.

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4.8.3 Comparison between positive and negative ion ESI-MS.

Figure 4.35 shows the spectra of 3:1 mixtures of daunomycin or ethidium bromide with dsDNA acquired using either positive or negative ion modes. Assignments are given in Table 4.10. The spectrum of the daunomycin:dsDNA mixture acquired in negative ion mode has been presented previously (Figure 4.25). A major difference between the positive and negative ion spectra is that ions from uncomplexed dsDNA are significant in positive ion spectra (E, F, G, H). In negative ion spectra (Figure 4.35b) complexes containing 1 daunomycin (I, J, K), 2 daunomycin (L, M, N) and 3 daunomycin molecules (P, Q) are present with the most intense ion being the [Δ+2Dn-6H]⁺ ion (m/z 1802.3) (M). In negative ion ESI mass spectra, the [Δ+Dn] and [Δ+2Dn] species account for 21.1% and 42.8% of the total ion current, respectively. When the ESI mass...
spectrum of the same sample was acquired in positive ion mode (Figure 4.35a), the resulting spectrum is clearly different. First, there are no ions from ssDNA. Secondly, ions from dsDNA are present in the spectrum (E, F, G, H). The most intense ion in this spectrum is \([\Delta + \text{Dn} + 6\text{H}]^-\) (J) at \(m/z\ 1716.2\). These \([\Delta + \text{Dn}]\) and \([\Delta + 2\text{Dn}]\) species account for 37% and 14.3% of the total ion current, respectively. There are also ions of low abundance from ssDNA (A, B) \((m/z < 1300)\).

The spectrum of the complex containing ethidium bromide acquired in negative ion mode (Figure 4.35b) shows several different species. These include the complexes \([\Delta + \text{Eb}]\) (i, j, k), \([\Delta + 2\text{Eb}]\) (l, m, n), \([\Delta + 3\text{Eb}]\) (o, p, q), \([\Delta + 4\text{Eb}]\) (r, s, t) and \([\Delta + 5\text{Eb}]\) (u, v). There is also evidence of single-stranded oligonucleotide (a, b, c) as well as uncomplexed dsDNA (e, f). The most intense ions in this spectrum are \([\Delta + 2\text{Eb} - 6\text{H}]^-\) at \(m/z\ 1730.3\) (m) and \([\Delta + 3\text{Eb} - 6\text{H}]^-\) at \(m/z\ 1782.8\) (p). When the ESI spectrum of the same sample was acquired in positive ion mode, the resulting spectrum was very different. There is no evidence of any drug-dsDNA complex. While it is possible complexes containing ethidium are unable to form positive ions, this seems unlikely as the ethidium molecule is expected to be positively charged at pH 8 (Figure 4.4). Alternatively, the positively charged ethidium does not readily remain bound to positively charged DNA. Some work is currently being done in our laboratory on the preparation of these complexes under different pH conditions and analysis of the ESI mass spectra. Perhaps these experiments will give us some insight as to how positive or negative ions of the complexes are formed in the gas phase.
Figure 4.35: ESI mass spectra of mixtures of 3:1 daunomycin:dsDNA and ethidium:dsDNA showing differences when acquired using positive and negative ion modes. (a) daunomycin/dsDNA, positive ions; (b) daunomycin/dsDNA, negative ions; (c) ethidium/dsDNA, positive ion and (d) ethidium/dsDNA, negative ions. Assignments are given in Table 4.10.
Table 4.10: Assignments of the major ions of spectra showing the differences in positive and negative ion modes in spectra of solutions of 3:1 daunomycin:dsDNA and ethidium:dsDNA shown in Figure 4.35. • indicates an species that is not observed.

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4.8.4 Effect of cone voltage and desolvation temperature on positive ion ESI mass spectra of complexes of nogalamycin or daunomycin with dsDNA.

The effects of increasing cone voltage on positive ion ESI mass spectra of 3:1 complexes of nogalamycin or daunomycin with the 5’-GATCGAATCGGACGAG-3’/5’-CTGTCGATCGATC-3’ duplex are shown in Figure 4.36. The data are less complicated than the corresponding results from negative ion spectra (Figure 4.29). A difference in all the positive ion spectra (dsDNA alone or drug-dsDNA mixtures) compared with negative ion spectra was that there were significant amounts of uncomplexed dsDNA over the range of cone voltages. This is shown in Figure 4.37. In these experiments, nogalamycin stabilized the dsDNA, relative to daunomycin. A similar order of stability was observed in experiments where desolvation temperature was varied (Figure 4.38).

4.8.4.1 Effect of collision energy on positive ion ESI mass spectra of complexes of nogalamycin and daunomycin with dsDNA.

Figure 4.39 shows the results of ESI-MS/MS experiments acquired in positive ion mode for the [Δ+I+6H]^{6+} and [Δ+2I+6H]^{6+} ions (I = nogalamycin or daunomycin). The differences in dissociation between the [Δ+I+6H]^{6+} and [Δ+2I+6H]^{6+} complex ions as a function of collision energy are not as great as those noted in negative ion mode (Figure 4.33; Figure 4.34). For each intercalator the complexes containing two drug molecules are clearly more stable with increasing collision energy compared with those containing one drug molecule. When comparing the data for the [Δ+I+6H]^{6+} complexes, those containing nogalamycin are more stable than
Figure 4.36: The effect of cone voltage in positive ion mode on uncomplexed DNA and the complexes of dsDNA with nogalamycin and daunomycin.
Figure 4.37: ESI mass spectra of complexes of dsDNA with nogalamycin (Panel I) and daunomycin (Panel II) acquired using both negative ion mode at 50 V (b, f) or 75 V (d, h) and positive ion mode at either 50 V (a, e) or 75 V (c, g). (●) ssDNA; (⋆) uncomplexed dsDNA; (◆) drug-dsDNA complex.
Figure 4.38: The effect of desolvation temperature on ESI mass spectra acquired in positive ion mode on uncomplexed dsDNA and the 3:1 mixtures of nogalamycin and daunomycin with dsDNA.
Figure 4.39: The effect of collision energy on positive ion ESI-MS/MS of $[\Delta+1I+6H]^+$ and $[\Delta+2I+6H]^+$ complexes ($I =$ nogalamycin and daunomycin).
those with daunomycin. The data are less distinctive for the $[\Delta+2I+6H]^6^+$ ions. Overall, ESI-MS/MS studies of these complexes with dsDNA acquired using both and negative ion modes are consistent with an order of stability of nogalamycin>daunomycin.

### 4.9 ESI-MS studies of retamycin and dsDNA

Figure 4.40 shows the ESI mass spectrum of a 5:1 retamycin:dsDNA mixture acquired in negative ion mode. Assignments are given Table 4.11. The spectrum shown is of poorer quality than those obtained previously for other drugs. A number of different complexes are evident in this spectrum. The most predominant complexes contain retamycin with 1 sugar lost ($R_{ta}$) (Figure 4.41). These species include $[\Delta+R_{ta}]$ (C, D), $[\Delta+2R_{ta}]$ (E, F), $[\Delta+3R_{ta}]$ (G, H) and $[\Delta+4R_{ta}]$ (I, J). There are also complexes of dsDNA with the intact retamycin ($R_t$) molecule. These include $[\Delta+R_t]$ (K, L) and $[\Delta+2R_t]$ (M, N). These complexes are, however, of lower intensity compared with those containing $R_{ta}$. Furthermore, only two intact retamycin molecules were bound to the dsDNA compared to four of the $R_{ta}$ molecule. This observation probably has little to do with the fact that the larger retamycin molecule would be sterically hindered from intercalation but more to do with the fact that the $R_t$ molecule is very much in excess of the intact retamycin and thus more drug molecules are available for binding. Further work including purification of the stock drug solution and further observations of the drug-dsDNA complex must be done in order to fully understand the mode of binding of retamycin to dsDNA.
Figure 4.40: Negative ion ESI mass spectra of a 5:1 mixture of retamycin with dsDNA. Assignments of the major ions are given in Table 4.11.

Table 4.11: Assignments of the major ions in spectra from Figure 4.40.

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4.10 ESI-MS studies of hedamycin and dsDNA.

The data for the intercalating, alkylating agent hedamycin, were considered separately as experiments with this drug are complicated in that it can undergo covalent reactions with DNA (Pavlopoulos et al., 1996). Figure 4.42 shows the negative ion ESI-mass spectra of 1:1, 3:1 and 5:1 mixtures of hedamycin with the non-self-complementary 16 mer dsDNA ($M_1 + M_2 = \Delta$) used in the experiments described in sections 4.6-4.7. The assignments are given in Table 4.12. In the spectrum of the 1:1 hedamycin:dsDNA mixture, the major peaks observed are those arising from the uncomplexed duplex, $[\Delta]$ (C, D) and the $[\Delta+Hd]$ complex (E, F, G). These peaks account for 31% and 40% of the total ion current, respectively. There is also evidence of ssDNA (A, B) and the $[\Delta+2Hd]$ (J) and $[\Delta+3Hd]$ (K) complexes observed in the spectrum. These species are, however, of low abundance (11%, 6% and 9% respectively). An increase in the ratio of drug to 3:1 (Figure 4.42b) results...
in an increase in the \([\Delta+3\text{Hd}]\) complex (K, L, M). This species accounts for 26% of all the species observed in the spectrum. The \([\Delta+2\text{Hd}]\) complex is also evident although this is of low abundance (11%). This spectrum also shows evidence of intrastrand fragmentation not noted in any spectra of the other drug-dsDNA mixtures. Fragmentation is not unexpected since alkylation weakens the glycosidic bond in DNA resulting in enhanced fragmentation (Iannitti et al., 1997). These peaks include the \([a_4(M_1)+\text{Hd}-6\text{H}]\)\(^{6-}\) ion (a) and the \([\text{G}+\text{Hd}-1\text{H}]\)\(^{1+}\) ion (b) (the nomenclature for the fragmentation of oligonucleotides is derived from McLuckey and coworkers (McLuckey and Habibigoudarzi, 1993)). These ions account for 19% and 24% of all the major ions observed in the spectrum. In the spectrum of the 5:1 hedamycin:dsDNA mixture (Figure 4.42c), the major peaks observed are the \([w_{11}+\text{Hd}-6\text{H}]\)\(^{6-}\) (M_1) (a) and the \([\text{G}+\text{Hd}-\text{H}]\)\(^{1+}\) ions (b). Fragmentation of the complex yields the \([\text{G}+\text{Hd}-\text{H}]\)\(^{1+}\) ion which can arise from either strand. The \(m/z\) region ranging from 500 to 1000 shows evidence of intrastrand fragmentation although because of the extensive overlap of those ions, the majority have not been assigned. There is also evidence of the \([\Delta+3\text{Hd}]\) (K, L) and \([\Delta+4\text{Hd}]\) (N, O) complexes. These ions are, however, of low abundance. The observation of the \([\Delta+4\text{Hd}]\) complex was surprising because hedamycin shows preference for intercalation at CG steps and then alkylation preferentially to guanine sites (Murray et al., 1995; Prakash, 1995; Sun et al., 1995). In shorter segments of DNA, binding to the terminal guanines has been observed (Iannitti, 1999). This may account for the binding of the fourth hedamycin to the dsDNA. In the DNA used in the study (5’-GATCGAATCGGACGAG-3’/5’-CTGTCGGATTCGATC-3’), there are 3 such CG pairs available that are separated sufficiently to allow binding without steric hinderance.
Figure 4.42: Negative ion ESI mass spectra of a titration of dsDNA with hedamycin: (a) 1:1, (b) 3:1, and (c) 5:1 hedamycin:dsDNA. Assignments are given in Table 4.12.

MS/MS spectra of several ions from the hedamycin-dsDNA complexes were examined to determine the sites of binding of hedamycin to the dsDNA oligonucleotide. Figure 4.43 shows the product ion spectrum of the \([\Delta+2\text{Hd-6H}]^6\) ion. Assignments are given in Table 4.13. Fragmentation of this ion yields product ions from both strands of the dsDNA sequence \(M_1\) and \(M_2\) \((M_1 + M_2 = \Delta)\). Ions observed arising from 5'-GATCGAATCGGACGAG-3' \((M_1)\) include the \([\text{w}_{11}+\text{Hd-6H}]^6\) ion \((b)\), the \([a_{13}-5H]^5\) ion \((c)\), the \([a_{10}-3H]^3\) \((f)\), \([w_6 -2H]^2\) ion \((f)\) and the \([M_1-(\text{Hd+G})-3H]^4\) \((g)\). Ions observed arising from 5'-CTCGTCCGATTGATC-3' \((M_2)\)
include the \([M_2-a_4^{-} a_4+Hd-4H]^4\) ion (a), and the \([w_3-1H]\) ion (e). These data are also represented schematically in Figure 4.44 below.

**Figure 4.43**: Negative ion ESI-MS/MS of the \([\Delta+2Hd-6H]^4\) ion. Assignments are given in Table 4.13.

**Figure 4.44**: Schematic showing the major sites of binding/cleavage observed using fragment ion data shown in Figure 4.43. The residues circled are the sites of binding; cleavage of the strands (indicated by dashed lines) occurs at the bonds directly proceeding the nucleotide. The assignment of each fragment is given in Table 4.13.
There is evidence of alkylation at a number of guanine residues in these sequences. Binding was observed at the guanines at positions 5, 10, 11 and 13 on the M₁ sequence. Interestingly, cleavage of the strand between the two guanines at positions 10 and 11 is not expected as the predicted fragmentation pattern should see fragmentation at either side of the guanine residues. This may suggest that there is perhaps an equal opportunity for the binding of hedamycin to either of these two guanine residues. Binding on the complementary strand (M₂) was observed at all the expected guanine binding sites on the DNA sequence. In the spectrum shown (Figure 4.43), the peak corresponding to the fragmentation at the 5'-CGT-3' site (a, e) is more intense (11% and 55%, respectively relative abundance to base peak) compared to peaks corresponding to fragmentation at 5'-CGA-3' (b and c) (6% and 9%, respectively), 5'-CGG-3' (f) (10%) and 5'-CGG-3' (a) (11%). Earlier work in our laboratory (Wickham et al., 1995a; Wickham et al., 1995b) which was consistent with other sequencing studies using other techniques (Prakash, 1995) had indicated that hedamycin has a much higher affinity for 5'-CGT-3' compared with 5'-CGA-3' sites.

Hedamycin was chosen to see if the initial intercalation step was sufficiently rapid relative to the alkylation step such that the behaviour of hedamycin may have been compared to that of the other intercalators examined here. The extent of fragmentation observed in the mass spectrometer alone (induced by alkylation weakening the glycosidic bond) suggests that alkylation occurs too rapidly to enable intercalation to be studied in isolation.
Table 4.12: Assignments of major ions from negative ion ESI-MS spectra of the different hedamycin:dsDNA mixtures as shown in Figure 4.42.

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<tr>
<th>Ion</th>
<th>$M_1$=5'-GATCGAATCGGACGAG-3'</th>
<th>$M_2$=5'-CTCGTCCGATTGATC-3'</th>
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<td>$[\Delta+1-7H]^{7-}$</td>
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<td>E</td>
</tr>
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<td>F</td>
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<td>2101.3</td>
<td>G</td>
</tr>
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<td>H</td>
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<td>M</td>
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<td>$[w_{11}+Hd-6H]^{6-}$ (M$_1$)</td>
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<tr>
<td>$[G+Hd-1H]^{1-}$</td>
<td>896.5</td>
<td>b</td>
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Table 4.13: Assignments of major ions from a negative ion ESI-MS/MS spectrum of $[\Delta+2I-6H]^{6-}$ as shown in Figure 4.43.

<table>
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<th>$[\Delta+2I-6H]^{6-}$; I = Hedamycin</th>
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<td>b</td>
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<td>770.0</td>
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<td>$[G+Hd-1H]^{1-}$</td>
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<td>$[a_{10}^-3H]^{3-}/[w_{6}^-2H]^{2-}$</td>
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<td>923.1</td>
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<td>f</td>
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<td>979.1</td>
<td>g</td>
<td>$[\Delta+2I-6H]^{6-}$</td>
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5 Conclusions

ESI-MS was used to study the binding of DNA to both intercalators and the replication terminator protein (Tus). As these interactions are non-covalent in nature, conditions were developed whereby two main criteria had to be met. First, formation of these non-covalent complexes require aqueous solutions with salts such as NaCl present so as to stabilize their structures. These conditions are however not ideal as ESI-MS experiments typically require organic solvents with no salt present. Thus conditions had to be determined whereby the observation of these non-covalent complexes by ESI-MS was successful.

In our earlier studies of the dsDNA-drug complexes using daunomycin and nogalamycin, conditions – both instrumental (using the VG Quattro™) and sample were developed. The ESI mass spectra of these complexes of intercalators with dsDNA (8 and 12 mer) became the first published in which abundant duplex ions were observed.

Drug titration studies were carried out on d(CGGCGCCG)$_2$, d(GGCTAGCC)$_2$ and d(TGAGCTAGCTCA)$_2$ under optimal conditions. The complexes observed appear to be as a result of specific intercalative interactions as even in the presence of a 10-fold excess of drug. The most abundant complexes for the 8 mers were those containing three drugs for the 8 mer and four drugs for the 12 mer. There was however some evidence of non-specific interactions with evidence of small amounts of [2M+5Dn], [2M+6Dn] and [2M+5Ng] complexes observed (M = 8 mer).
Two different types of competition experiments were carried out. In the first, one of each of the 8 mers was added to the 12 mer oligonucleotide and were annealed together in the presence of either nogalamycin or daunomycin. The data obtained from this experiment indicated a preference of binding of the intercalators to CG rich regions. Furthermore, more nogalamycin-dsDNA (8 mer) complexes were observed in these ESI mass spectra. In the other competition experiment, both drugs were added to d(CGGCGCCG)₂. Complexes containing nogalamycin alone were observed. Complexes containing daunomycin exclusively on the other hand were not observed. Both competition experiments therefore suggest that once bound, nogalamycin is difficult to remove thus forming stable complexes. These studies require further investigation using different sequences that are at least 10 mer in length and have distinct CG or AT rich regions.

The extension of the above work using self-complementary 16 mers resulted in predominantly negative results. The majority of the complexes noted arose from single stranded DNA folded over (hairpins) to accommodate 1-3 bound drug molecules. In order to combat this problem, non-self-complementary strands (capable of forming a duplex) were used to avoid problems arising from ambiguity in assigning drug-dsDNA peaks. Unfortunately, these experiments done on the VG Quattro™ also showed evidence of non-specific interactions where the single stranded oligonucleotides were forming complexes with the intercalators.

Following these problems, these studies using the non-self-complementary strands were then carried out on the Micromass Qtof2™ using daunomycin, nogalamycin, ethidium bromide, retamycin and hedamycin. Interestingly, no evidence of
non-specific interactions between the non-self-complementary duplex and drug were noted. Different experiments were then carried out using this dsDNA. Titration experiments indicated that the complexes formed were as a result of specific intercalative interactions as the neighbour-exclusion principle was obeyed in all reactions. More ethidium was able to bind to the duplex at lower titration values compared to daunomycin or nogalamycin.

Data from the different CID experiments (cone voltage, desolvation temperature and collision energy) indicated in that the order of stability of the complexes were ethidium bromide > nogalamycin > daunomycin. Data for hedamycin showed that alkylation was occurring and thus comparisons were not made between this drug and the others studied.

The use of positive ion ESI-MS was also explored. These data yielded interesting results. For example, when the dsDNA-ethidium bromide mixture was acquired, the resulting spectrum showed no evidence of dsDNA-drug complex. A study using different pHs in the mixture solution during acquisition is currently underway.

These studies need to be extended to include a wider range of intercalators with a greater variety of oligonucleotides (non-self-complementary) that have differing ratios of CG-rich and AT-rich regions. Competition experiments using shorter sequences (10-12mer) and the different intercalators would be required in order to extend earlier studies done on the VG Biotech Quattro™.
The binding of the replication terminating protein (Tus) to its specific DNA recognition sequences (Ter) on the *E. coli* chromosome was investigated by ESI-MS. Conditions were devised whereby the 1:1 Tus-TerB complex was observed using ESI-MS. In order to get to this stage, several parameters, both instrumental those pertaining to solution control needed to be determined firstly for the free protein and subsequently the complex thereafter.

Initial attempts to distinguish binding affinities of the different Tus mutants to TerB as well as the different TerB variants to Tus proved unsuccessful due to the stability of the Tus-Ter complex. Therefore, in order to sufficiently increase the dissociation constants of these complexes, the concentration of NH₄OAc in the electrospray solvent was raised (from 10 μM to 3.2 M NH₄OAc). The relative order of binding affinity for Tus and its mutants to TerB and variants was thus determined in this way.

As initial titration experiments used to determine the Kᵥ of the Tus-TerB complex proved to be unsuccessful, a method of serial dilution was employed. The Kᵥ of A173T mutant to TerB was estimated using this method to be 700 x 10⁻⁹M whilst those of his₆tus and R198A were less than or equal to 2 x 10⁻⁹M. However, the question arises as to how valid these dissociation constants are when measured in the gas phase. For instance, the ionization process involving drying of the ions may perturb the structure of the complex and thus also the equilibrium position. The presence of water on the DNA-protein binding interface is thought to be useful in acting as a form of glue whereby a form of structural adaptability is maintained (Schwabe, 1997). These water molecules are thought to be involved in the
specificity of DNA recognition by the proteins and furthermore, more water molecules remain at the binding interface in non-specific complexes (Schwabe, 1997). In the Tus-\textit{TerB} complex, water molecules are involved in H-binding between the DNA and protein (Kamada \textit{et al.}, 1996). On the other hand, studies have shown that the hydrophobic effect (along with the release of cations) is generally thought to be a major driving force for the formation of specific protein-DNA complexes (Ha \textit{et al.}, 1989). Electrostatic interactions are also thought to be strengthened in vacuo. Thus, contributions including hydrogen bonding, electrostatic interactions, hydrogen bonding and van der vaals interactions will influence the stabilities and hence dissociation constants of the non-covalent complexes (Loo, 1997).

One can therefore not predict how the formation of ions of the complex will affect its binding properties. It is therefore clear that a number of experiments using numerous wall characterized non-covalent complexes must be carried out using both ESI-MS and solution studies such that detailed similarities and/or differences can be noted. Indeed, our experiments as well as those on antibiotic binding by other researchers (Lim \textit{et al.}, 1995; Sannes-Lowery \textit{et al.}, 2000) shows that the relative order of binding affinity in the gas phase is in agreement to solution studies.

Additional work needs to be carried out in order to add information that may add some insight into the most correct binding model. For example, substituting the different anions in the salt titration experiments and applying the Hofmeister series could determine if it were hydrophobic or electrostatic effects that had the most influence on the binding of Tus to \textit{TerB}. If nitrate, acetate and sulphate anions were
compared in these experiments for example, the predominant non-covalent influence could be deciphered in the binding model.

All in all, ESI-MS has shown promise as a technique used in the observation of DNA-drug and DNA-protein interactions. In general, our results concurred with findings from similar solution studies especially in studies on the Tus-TerB complex. Data obtained from studies on the DNA-drug complexes did not however fully complement similar solution-phase studies and as such further conditions need to be determined so as to ensure that the systems studied yield ‘real’ results.
6 References


Appendix 1. Expected $m/z$ value for all binding partners and complexes from Chapter 3.

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<th>2 Tus + Ter</th>
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Appendix 2. Using $K_D$ to determine the relative concentrations of $\text{Tus}_{\text{free}}$ and $\text{Tus}_{\text{complex}}$ in the gas phase

$\text{Tus} \rightleftharpoons \text{Tus}_{\text{free}}[\text{TerB}_{\text{free}}]$

\[ \cdot K_D = \frac{[\text{Tus}_{\text{free}}][\text{TerB}_{\text{free}}]}{\text{TusTerB}} \]

Now, let $\text{Tus}_{\text{free}} = x$

\[ \cdot \text{Since } \text{Tus}_{\text{free}} = \text{TerB}_{\text{free}}, \text{ then } \text{TerB}_{\text{free}} = x \]

\[ \cdot K_D = \frac{x[x]}{10 \times 10^{-6} - x} \]

\[ \cdot \text{ in } \mu \text{M}, \]

\[ 1.204 \times 10^{-7} = \frac{x^2}{10 - x} \]

\[ x^2 + (1.204 \times 10^{-7})x - (1.204 \times 10^{-6}) = 0 \]

\[ \cdot x = 0.03 \ \mu \text{M}, \]

$[\text{Tus}_{\text{free}}] = 0.03 \ \mu \text{M}$ and $[\text{Tus}_{\text{complex}}] = 10 \ \mu \text{M} - 0.03 \ \mu \text{M} = 0.97 \ \mu \text{M}$
Appendix 3. Calculations used to generate data on the
dissociation of the Tus-Ter complex by sample dilution.

\[
\text{Tus-Ter} = [\text{Tus}_{\text{free}}][\text{Ter}_{\text{free}}]
\]

\[
K_D = \frac{[\text{Tus}_{\text{free}}][\text{Ter}_{\text{free}}]}{\text{TusTer}}
\]

Now, let \([\text{Tus-Ter}] = x\) and \(K_D = 700 \times 10^{-9} \text{M}\)

\[
K_D = \frac{[\text{Tus} - x][\text{Ter} - x]}{x}
\]

Now, for \([\text{Tus}] = 15 \times 10^{-6} \text{M}\),

\[
700 \times 10^{-9} x = (15 \times 10^{-6} - x)(15 \times 10^{-6} - x)
\]

\[
700 \times 10^{-9} x = 225 \times 10^{-12} - 30 \times 10^{-6} x + x^2
\]

\[
0 = 225 \times 10^{-12} - 3.07 \times 10^{-5} x + x^2
\]

\[
x = \frac{3.07 \times 10^{-5} - \sqrt{9.4249 \times 10^{-10} - 9 \times 10^{-10}}}{2}
\]

\[
x = 1.21 \times 10^{-5} \text{M} = [\text{Tus-Ter}]
\]

\[
\% \text{Tus in complex} = \frac{12.1}{15} = 80.7\%
\]

\[
\% \text{Tus free} = \frac{15 - 12.1}{15} = 19.3\%
\]

Similarly, solving for \(x\) for different dilutions yields the following data:

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Appendix 4. Comparison of the relative ESI-MS response factors of Tus\textsubscript{free} and Tus\textsubscript{complex}

In the work described (Chapter 3), the intensities of the ions from free and complexed Tus were compared to measure the percentage dissociation of the complex. For this to be valid, ESI-MS response factors for complex and free protein must be similar. For adequate determination of response factors, the final concentration of each sample must be equal as there is often a limit in the number of ions detected by ESI-MS. For example, it has been shown that analyte ion sensitivities decrease with increasing concentration above $10^{-5}$ M (Ikonomou \textit{et al.}, 1990). Another problem encountered in electrospray is ion suppression whereby larger molecules can suppress the ionisation of smaller molecules (Ikonomou \textit{et al.}, 1990; Sterner \textit{et al.}, 2000). This is presumed to be as a result of ion-ion interactions where larger, more highly charged ions can collisionally transfer transitional energy to smaller ions with less charge (Sterner \textit{et al.}, 2000). These problems have serious ramifications when attempting to quantify multicomponent samples.

Even though the ESI response will include a contribution from the relative ionization efficiencies of solution components, pKa values of each component alone do not adequately predict the extent of ion formation (Cech and Enke, 2000; Constantopoulos \textit{et al.}, 1999; Wang and Cole, 1994).

To explore this further in relation to the work in Chapter 3, an experiment was thus performed in which free A173T Tus was added to a His6Tus-\textit{TerB} complex such that at all times the final total concentration of all Tus species in the solution was 10 $\mu$M. That is, Tus\textsubscript{free} + Tus\textsubscript{complex} = 10 $\mu$M. The solution contained 800 mM NH\textsubscript{4}OAc, pH 8 such that the complex was maintained at a salt concentration where there was no dissociation. Similarly, free A173T Tus protein was at a salt concentration where formation of a complex was not favourable. The figure below shows a plot of Tus\textsubscript{free}/Tus\textsubscript{total} and Tus\textsubscript{complex}/Tus\textsubscript{total} determined by addition of free A173T Tus to a His\textsubscript{6}Tus-\textit{TerB} complex in varying ratios as described in Chapter 2. The plot shows that on varying the ratios whereby protein became in excess of the complex and
amounts of each have a mirror image effect. The two straight line curves firstly have a good correlation coefficient (0.99). The intersection of the curves occurs at 50% on the y-axis and at 47% on the x-axis indicating that within experimental error, the response factors of the free protein (which will be almost identical to the other Tus mutants) is very similar to that of Tus in the complex.

Figure A1: Comparison of the relative ESI-MS response factors of free (A173T Tus; Tus\textsubscript{free}) and complexed Tus (His\textsubscript{6}Tus-TerB; Tus\textsubscript{complex}) Tus\textsubscript{total} = Tus\textsubscript{free} + Tus\textsubscript{complex}. A plot of Tus\textsubscript{free}/Tus\textsubscript{total} (sum of intensities of all ions from A173T/sum of intensities of all ions from His\textsubscript{6}Tus-TerB + sum of intensities of all ions from A173T Tus) against Tus\textsubscript{complex}/Tus\textsubscript{total} added to the solution. In this experiment, the His\textsubscript{6}Tus-TerB complex was prepared and titrated with A173T Tus in 800 mM NH\textsubscript{4}OAc, pH 8.0. ESI mass spectra were acquired of mixtures where Tus\textsubscript{total} was maintained at 10 \mu M. (●) Tus\textsubscript{free}/Tus\textsubscript{total} in the ESI mass spectrum (gas phase); (■) Tus\textsubscript{complex}/Tus\textsubscript{total} in the ESI mass spectrum. The slope of the lines are similar suggesting that the response factors of protein and complex are the same.

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Appendix 5. Expected $m/z$ value for all binding partners and complexes from Chapter 4.

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Appendix 6. $E_{\text{acc}}$ calculations for $[\Delta+I-6H]^6-$ and $[\Delta+2I-6H]^6-$ ions.

$E_{\text{available}} = \frac{m_{\text{gas}} \times E_{\text{ion}}}{m_{\text{ion}} + m_{\text{gas}}}$

$E_{\text{available}}$ = energy available for conversion into internal energy (centre of mass)

$E_{\text{ion}}$ = kinetic energy of the ion

$m_{\text{ion}}$ = mass of ion of interest

$m_{\text{gas}}$ = mass of collision gas (Ar)

Now for the $[\Delta+Ng-6H]^6-$ ion at an applied voltage of 5 eV:

$(E_{\text{ion}} = zV = 6 \times 5 = 30 \text{ eV})$

$E_{\text{available}} = \frac{39.95 \times 30}{10551.3 + 39.95}$

$E_{\text{available}} = 0.113$ eV

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