*Review*

# An Overview of the Optical and Electrochemical Methods for Detection of DNA – Drug Interactions

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## Abstract

A large number of inorganic and organic compounds is able to bind to DNA and form complexes. Among them, drugs are very important, especially chemotherapeutics. This paper presents the overview of DNA structural characteristics and types of interactions (covalent and non-covalent) between DNA molecule and drugs. Covalent binding of the drug is irreversible and leads to complete inhibition of DNA function, what conclusively, causes the cell death. On the other hand, non-covalent binding is reversible and based on the principle of molecular recognition. Special attention is given to elucidation of the specific sites in DNA molecule for drug binding. According to their structural characteristics, drugs that react non-covalently with DNA are mainly intercalators, but also minor and major groove binders.

When the complex between drug and DNA is formed, both the drug molecule, as well as DNA, experienced some modifications. This paper presents the overview of the methods used for the study of the interactions between DNA and drugs with the aim of detection and explanation of the resulting changes. For this purpose many spectroscopic methods like UV/VIS, fluorescence, infrared and NMR, polarized light spectroscopies like circular and linear dichroism, and fluorescence anisotropy or resonance is used. The development of the electrochemical DNA biosensors has opened a wide perspective using particularly sensitive and selective electrochemical methods for the detection of specific DNA interactions. The presented results summarize literature data obtained by the mentioned methods. The results are used to confirm the DNA damage, to determine drug binding sites and sequence preference, as well as conformational changes due to drug–DNA interaction.

**Keywords:** DNA, drug, interaction, spectroscopic methods, electrochemical DNA-biosensors.

## 1. Introduction

Deoxyribonucleic acid, DNA,[1–3] is a natural product of an enormous importance for understanding the mechanism of genetic processes such as growth, differentiation and aging of the cell. Binding of small organic and inorganic molecules to DNA can influence numerous biological processes in which DNA participate, like transcription and replication.[2,3] These processes begin when DNA receives the signal from regulatory protein which binds to its particular part. If, instead of the regulatory protein, some other small molecule binds to DNA, its function is artificially changed – inhibited or activated. Such interference can retard or even prevent the cell growth, or, on the other hand, it can lead to excessive production of some protein and uncontrolled cell growth. In the case when the activation or inhibition of the DNA function act in the way to cure or control the disease, small molecule is denoted as a drug, but otherwise it is cytotoxic agents. Accordingly, the growing interest in studying the process of interaction between DNA and chemotherapeutic drugs is understandable. Extensive chemical and biochemical studies have characterised a variety of molecules that react with DNA, which are classified as antibiotic, antitumor, antiviral or antiprotozoal agents. Some of them are used in clinical practice, while the others are still under clinical trial.

When the complex between small molecule and DNA is formed, both molecule, as well as DNA, experienced some modifications. Detection and explanation of these changes makes a great challenge for new instrumental methods. The development of crystallographic and NMR methods[4–6] in the last twenty years made it possible to ascertain more about the conformational flexibility of DNA molecule, the influence of base sequence, and the role of water molecules and counterions. The challenge to obtain the similar information about DNA–drug complexes arise. The aim of the contemporary research is to distinguish the rules governing sequence specific binding, to understand the correlation between DNA structure, sequence and activity, to determine drug binding sites, as well as conformational changes due to the DNA–drug interaction. These facts are useful for DNA–drug interaction mechanism elucidation, what leads to rational drug design, and development of new anticancer agents.

The intention of this review is to give an overview of the present state of the DNA–drug interaction cognition. The presented results summarize literature data obtained by the application of selected optical and electrochemical methods, their operation and detection principles. In order to facilitate the discussion about the stability of obtained complexes and the modes of the binding interactions, a brief introduction to the DNA chemical structure should be considered.

## 2. Structural Characteristics of DNA Molecule

Deoxyribonucleic acid, DNA,[1–3] consists of two long polynucleotide chains composed of four types of nucleotide subunits. The nucleotides themselves are composed of a five-carbon sugar (deoxyribose) to which one or more phosphate groups and a nitrogen-containing base are attached. Base may be either adenine (A), cytosine (C), guanine (G), or thymine (T). The nucleotides are covalently linked together in a chain through the sugars and phosphates, which thus form a "backbone" of alternating sugar and phosphate (Figure 1). The way in which the

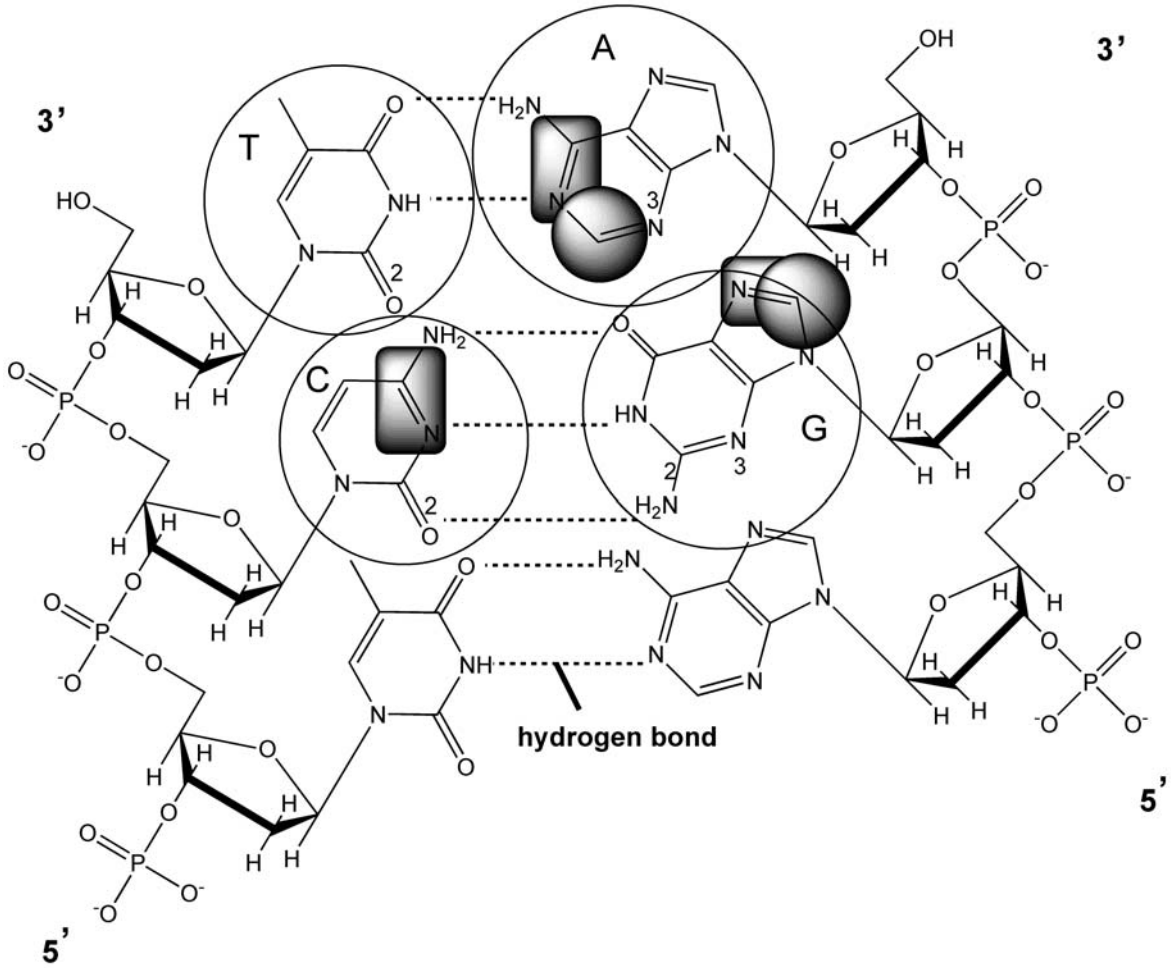



**Figure 1.** Structure of double stranded DNA. Squares denote electroactive groups that can be reduced at mercury electrodes, and circles show sites oxidized at carbon electrodes.

nucleotide subunits are lined together gives a polarity to a DNA strand. If each sugar is presented as a block with a protruding top (the 5' phosphate) on one side and a hole (the 3' hydroxyl) on the other, each completed chain, formed by interlocking tops with holes, will have all of its subunits lined up in the same orientation. Moreover, the two ends of the chain will be easily distinguishable, as one has a hole (the 3' hydroxyl) and the other a top (the 5' phosphate) at its terminus. This polarity in a DNA chain is indicated by referring to one end as the 3' end and the other as the 5' end. The DNA secondary structure is represented by the double helix which consists of two antiparallel polynucleotide chains that are held together by hydrogen bonding between the bases of the different strands, with all the bases on the inside of the double helix, and the sugar-phosphate backbones are on the outside. This complementary base-pairing enables the base pairs to be packed in the energetically most favourable arrangement in the interior of the double helix and each strand of a DNA molecule contains a sequence of nucleotides that is exactly complementary to the nucleotide sequence of its partner strand. Parts of a DNA molecule that can interact with small molecules are: negatively charged phosphate backbone (electrostatic interaction), the hydrogen accepting and donating sites in the minor and major grooves (H-bonds), the phosphate oxygen atoms and aromatic hydrophobic components (van der Waals interactions). One should always keep in mind that DNA is polymorphic, and the geometry of double helix, including the depth and width of the minor and major grooves, is different in different conformations (A, B and Z form). Besides, hydration plays very important role in the stability of DNA molecule, as well as DNA–drug complex, since this process is believed to be governing by the base sequence, also.

## 3. Modes of DNA–drug Interactions

Large number of drug substances, inorganic and organic, both natural and synthetic, are able to form complexes with DNA molecules, mostly when DNA is in double stranded form. Drugs interact with DNA molecule both covalently and non-covalently.

**Covalent binding** in DNA is irreversible and undoubtedly leads to complete inhibition of DNA functions and subsequent cell death. A major advantage of covalent binders is the high binding strength. Moreover, covalent bulky adducts can cause DNA backbone distortion, which in turn can affect both transcription and replication, by disrupting protein complex recruitment.[7] Three modes of covalent binding to DNA are possible: replacement of nitrogenous bases, inter and intra-strand cross linking and alkylation of nitrogenous bases.

The most famous covalent binder is cisplatin (Table 1), which is used as an anticancer drug. When used for clinical practice, cisplatin is administered intravenously. In the extracellular environment, where the chloride concentration is high, it does not undergo appreciable hydrolysis. When cisplatin passes the cell membrane the reduced intracellular chloride concentration allows the chlorido ligands to be replaced by water molecules to form *cis*-$[Pt(H_2O)(NH_3)_2Cl]^+$ and *cis*-$[Pt(H_2O)_2(NH_3)_2]^{2+}$. It is generally accepted that these two cations bind covalently to the electron-rich sites on DNA such as N-donor ligands.[8] The preferred target in DNA is guanine (G) since it has the highest electron density of all four nucleobases.[9] It is believed that this reaction is responsible for the anticancer effect of cisplatin which is able to induce apoptsis/necrosis of the cancer cell.[10]

Besides cisplatin, another two antitumor antibiotics found the application in clinical practise: mitomycin C and anthramycin (Table 1). Mitomycin C interacts covalently with guanine base only in reduced state, i.e., after the reductive activation. The activated antibiotic forms a cross-linking structure between guanine bases on adjacent strands of DNA thereby inhibiting single strand formation. Anthramycin covalently binds to N2 nitrogen of guanine, placed in the minor groove of DNA molecule.

Another type of covalent binders are called alkylating agents. These agents are capable of adduct formation by attaching an alkyl group to DNA.[11] Alkylating drugs, like temozolomide, chlorambucil and nimustine are the oldest class of anticancer drugs but still commonly used in the treatment of several types of cancers.

**Non-covalent binding** of drugs to DNA is reversible, and considering drug metabolism and potential toxic effects, it is more desirable comparing to covalent. However, non-covalent DNA interacting agents can change DNA conformation, DNA torsional tension, interrupt protein–DNA interaction, and potentially lead to DNA strand breaks. One of the main principles of DNA chemistry is molecular recognition, the process when molecules (small or large) selectively recognize each other. This is manifested through a couple of interaction modes: electrostatic, hydrogen bonds, and van der Waals (dipole–dipole) interaction. The stability of the formed complex DNA–drug depends on the intensity of the mentioned interactions.

Drugs that react non-covalently with DNA are classified in following categories:[12]

1. Intercalating agents
2. Minor groove binders
3. Major groove binders
4. External binders

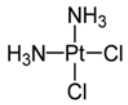
### 3. 1. Intercalators

Intercalators are molecules that stack perpendicular to the DNA backbone without forming covalent bonds and without breaking up the hydrogen bonds between the DNA bases. The only known forces that sustain the stabi-

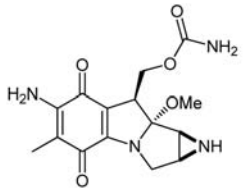
lity of the DNA–intercalator complex, even more than DNA alone, are van der Waals, hydrogen bonding, hydrophobic, and/or charge transfer forces.[13–17] Intercalation stabilizes, lengthens, stiffens, and unwinds the DNA double helix.[18] Intercalators contain planar heterocyclic groups which stack between adjacent DNA base pairs, forming complex which is stabilized by π–π stacking interactions between the drug and DNA. Intercalators can be mono- or bifunctional, depending on the number of aromatic moieties. There are simple mono-intercalators like acridine derivatives: proflavine, acriflavine, acriflavine neutral (euflavine), aminacrine, ethacridine, and more

**Table 1.** Drugs interacting with DNA, covalently and non-covalently

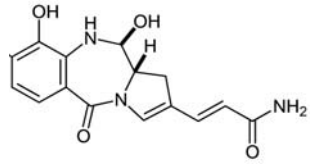
**Drugs that covalently bind to DNA**



Cisplatin



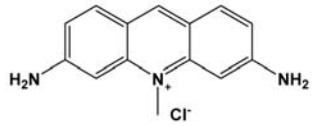
Mitomycin C



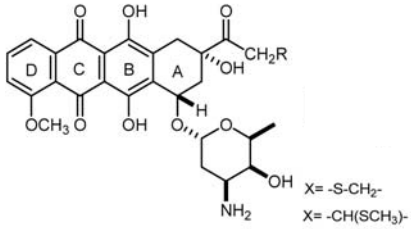
Anthramycin

**Drugs that non-covalently bind to DNA**

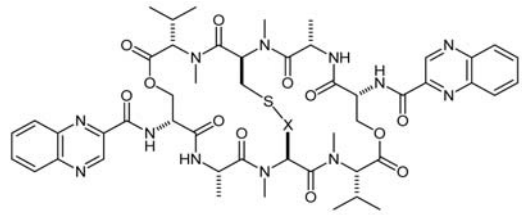
Mono-intercalators



Acriflavine



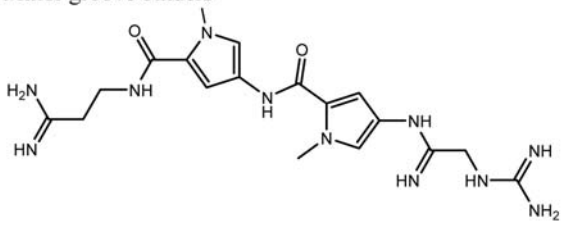
Bifunctional intercalators



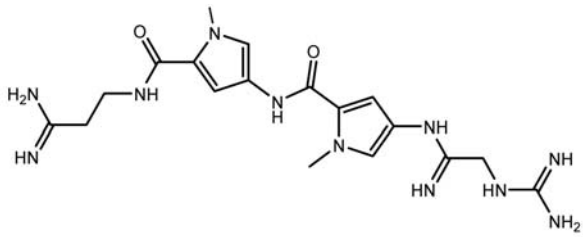
Triostin A
Echinomycin

R = H Daunomycin
R = OH Adriamycin

Minor groove binders



Netropsin



Berenil

complex like daunomycin and adriamycin, with four fused six membered rings with substituents in positions 7 and 9 (Table 1). These intercalators are often called the threading intercalators since they thread one of the substituents on opposite sides of the intercalating aromatic ring between the base pairs at the intercalation site.

In bifunctional intercalators, two planar aromatic moieties are present and separated by some voluminous system like fairly rigid cyclic peptide system stabilized through disulphide bridge as in the case of triostin A and echinomycin (Table 1.)

The phenomenon of intercalating involves the aromatic part of a drug molecule positioning itself between base pairs (Figure 2). Intercalating agents inure in hydrophobic interactions with DNA because the hydrophobic, aromatic side chains interact favourably with the aromatic environment of the base pairs. These agents introduce strong structural perturbations in DNA molecule by increasing the distance between the adjacent base pairs. In order for an intercalator to fit between base pairs, the DNA must dynamically open a space between its base pairs. Fortunately, the resultant helix distortion is compensated by adjustments in the sugar-phosphate backbone unwinding of the duplex. Stacking interactions between the bases and the intercalating molecule are the major stabilising factors for the complex formed. For example, there are direct hydrogen bonds between the functional groups of the drug that are essential for the drug action (like hydroxyl group of the ring A or $NH_3^+$ group in the 3' position in daunomycin) and functional groups N2 and N3 of guanine, or O2 of cytosine at the intercalation site. Very often, water-mediated contacts occur rather than a direct hydrogen bonded interaction. Anyway, the total amount of surface bound water is reduced after the complex formation. Besides this general scheme of complex formation, the interaction between the DNA and drug also depends on the sequence of the bases adjacent to the intercalation site, and on the chemical modifications of the intercalating drug.

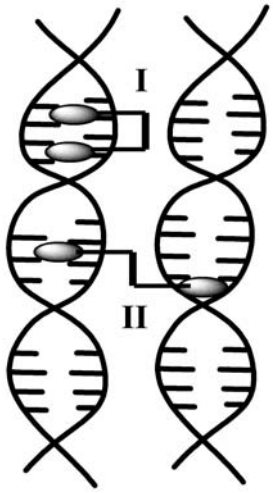



**Figure 2.** Types of intercalation of bifunctional intercalators in DNA molecule: I - intramolecular cross-link and II - intermolecular cross-link.

Bifunctional intercalators are able to intercalate in DNA molecule in two different ways: **I** – intramolecular cross-linking, when both aromatic moieties intercalate with the same DNA molecule, and **II** – intremolecular cross-linking, when intercalating moieties interact with two separate DNA molecules (Figure 2).

There are also multi-intercalators containing three or more intercalation rings, which were synthesized because, as potential drugs, their high DNA binding constants are expected to enhance their therapeutic activity.

## 3. 2. Minor Groove Binders

The most famous minor groove binding drugs are netropsin, berenil, distamycin and mithramycin (Table 1). They usually have crescent shape, which complements the shape of the groove[19,20] and facilitates binding by promoting van der Waals interactions. These drugs typically have several aromatic rings, such as pyrrole, furan or benzene connected by bonds possessing torsional freedom. Additionally, these drugs can form hydrogen bonds to bases, typically to adenine/thymine rich sequences. In all complexes the drug fits snugly into the minor groove and displaces the hydration layer. Thus, it might be expected that there is relatively little change in DNA itself due to the complexation, but the results indicates that this is not the case. In complex structure alternation of the helical twist is reversed from the uncomplexed alternating DNA. One of the bases rotates to allow hydrogen bonding to the drug. Sometimes hydrogen bonding is mediated by a water molecule between the drug molecules and the DNA acting as a major complex stabilising force. Van der Waals forces also significantly contribute to the stabilisation of the complexes and may lead to serious perturbations of the DNA helix from the normal Watson/Crick geometry.

Beyond the changes in DNA, these interactions also induce changes in the conformation of the groove binding drugs themselves.

## 3. 3. Major Groove Binders

Most of the DNA groove binding drugs chose minor groove as a target. These drugs are generally arc shaped, planar and unfused aromatic with a positive electrostatic potential that attracts them to the electronegative potential of the minor groove, and the arc matching the curvature of the DNA double helix. Considerably small number of substances is reported to bind to major groove. The reason for this probably lies in the fact that nitrogen and oxygen atoms in base pairs of wide and deep major groove are oriented towards the axis of the helix, what makes them accessible for proteins.[21] Proteins recognize these base sequences, and specific binding interaction takes place. In

order to inhibit these protein–DNA interactions it was necessary to find natural products or to design ligands that will act as a major groove binding agents.

First, solid evidence about the small molecules (methyl green) binding to major groove dates from 1993.[22] Later on some antitumor agents with acridine carboxamide skeleton,[21] aminoglycoside antibiotics tobramycin, and other agents as pluramyicins, aflatoxins, azinomycins, and neocarzinostatin were synthesized.[23,24] Unfortunately, number of these drugs is still very modest. A better understanding of DNA–ligand chemical interactions is necessary for the rational design of more efficient analogues as potential DNA major groove binding drugs.

### 3. 4. External Binders

This type of binding is electrostatic in nature. Some ligands are capable of forming non-specific, outside edge stacking interactions with the DNA phosphate backbone. This mode usually occurs when the ligand self-associates to form higher-order aggregates, which may stack on the anionic DNA backbone in order to reduce charge–charge repulsion between ligand molecules. Some metal complexes interact with DNA through external binding. This association mode was proposed for $[Ru(bpy)_3]^{2+}$ as the luminescence enhancement of this complex upon binding to DNA is strongly dependent on the ionic strength. Cations like $Mg^{2+}$, usually also interacts in this way.[25]

### 3. 5. Metal-Drug Complexes

DNA represents a fruitful target for metal complexes as well. Transition metal complexes are known to bind to DNA via both covalent and/or non-covalent interactions. In covalent binding the labile ligand of the complex is replaced by a nitrogen base of DNA such as guanine N7. On the other hand, the non-covalent DNA interactions include intercalative, electrostatic and groove (surface) binding of metal complexes along outside of DNA helix, along major or minor groove. Very important class of complexes that react with DNA are metal-drug complexes. Among numerous drugs, particularly quinolone antibiotics are very often reported to form such complexes.[26] DNA can provide three distinctive binding sites for quinolone metal complexes; namely, groove binding, electrostatic binding to phosphate group and intercalation.[27,28] Related to the great biological role of the quinolone antibiotics in humans, this behaviour deserves our attention. In synthesized ciprofloxacine complexes with $Mn^{2+}$, $Fe^{3+}$, $Co^{2+}$, $Ni^{2+}$ and $MoO_2^{2+}$,[29] ciprofloxacin is deprotonated and behave as bidentate ligand bound to the metal ion through the pyridine oxygen and one carboxylate oxygen. Each metal ion is six-coordinated with geometry around octahedronally distorted. Results showed that these complexes can bind to DNA by both intercalative and electrostatic binding mode. Other DNA–ciprofloxacine-metal complexes with $Mg^{2+}$,[30,31] $Cu^{2+}/Cu^{+}$,[32] and $UO_2^{2+}$,[33] as well as enrofloxacine-$Cu^{2+}$ complex are also reported.[34] Magnesium-ciprofloxacine in aqueous medium form colloidal particles that are believed to be involved in specific binding to GC containing sequences, most probably in the major groove of DNA. Mixed valence $Cu^{2+}/Cu^{+}$-ciprofloxacine complexes showed an interesting DNA cleavage activity which exact mode of action is not determined yet.[32]

### 3. 6. DNA–drug Interaction in Aqueous Environment

In aqueous solution DNA is polyanion attracting positively charged counter ions ($Na^{+}$, $Ca^{2+}$ or $Mg^{2+}$). Drug molecules are also often charged and thus associated with counter ions. The associated counter ions lie near the charged groups and are also partially solvated. When the binding occurs, it results in a displacement of solvent from the binding site on both the DNA and drug. Also, since there would be partial compensation of charges as the DNA and the drug are oppositely charged, some counter ions would be released into the bulk solvent fully solvated. Besides the counter ion effect, there are other interactions that must be considered, *e.g.* hydratation/dehydratation process which occurs through drug–solvent (hydration shell) and DNA–solvent interaction. Hydration plays very important role in the stability of DNA molecule, as well as DNA complex, since this process is believed to be governing by the base sequence, also.

The binding process is also associated with some structural deformation and adaptation of the DNA as well as the drug molecule, in order to accommodate each other. All these processes are associated with some enthalpic and entropic changes that lead to the binding free energy changes. It is confirmed that DNA double helix structure is more stable when complexated with intercalating agents, and the obtained complex shows a reduced heat of denaturation compared to bare DNA. Since no covalent bond formation is involved, the binding can be considered as an equilibrium process, and the corresponding equilibrium constants can be determined by measuring the free and bound fraction of drug.

## 4. Analytical Techniques for Detection DNA–drug Interaction

The aim of the contemporary research is to link measurable biophysical parameters with cytotoxicity, and to correlate the antitumor activity of the drug with its capability to intercalate into DNA double helix structure. Detection and explanation of the arise changes is a growing challenge and a basis for application of the renewed analytical techniques and methodologies for these causes. Large number of techniques is used to investigate

DNA–drug interactions from classical UV-VIS spectroscopy, along with 2D and 3D NMR spectroscopy, all the way to most recent optical and electrochemical biosensors.

## 4. 1. Optical Methods

The DNA–drug interaction can be detected by **UV-Vis absorption spectroscopy** by measuring the changes in the absorption properties of the drug or the DNA molecules. The UV–Vis absorption spectrum of DNA exhibits a broad band (200–350 nm) in the UV region with a maximum at 260 nm. This maximum is a consequence of the chromophoric groups in purine and pyrimidine moieties responsible for the electronic transitions. Slight changes in the absorption maximum and the molar absorptivity can occur with the variations in pH or ionic strength of the media. DNA–drug interactions can be studied by comparison of UV–Vis absorption spectra of the free drug and DNA–drug complexes, which are usually different. The binding with DNA through intercalation usually results in hypochromism and hypsochromism (blue shift) or bathochromism (red shift). As a consequence of intercalative mode of binding that includes a stacking interaction between an aromatic chromophore and the base pair of DNA, the extent of the hypochromism consistent with the strength of intercalative interaction can be observed.[35–37]

In the case of electrostatic attraction between the compound and DNA, hyperchromic effect is observed that reflects the changes of DNA conformation and structure after the DNA–drug interaction has occurred. The hyperchromic effect results in increase of absorbance of DNA upon denaturation. When the DNA double helix is treated with denaturing agents, the interaction force holding the double helical structure is disrupted. The double helix then separates into two single strands which are in the random coiled conformation. At this time, the base–base interaction will be reduced, increasing the UV absorbance of DNA solution because many bases are in free form and do not form hydrogen bonds with complementary bases. As a result, the absorbance for single-stranded DNA (ss-DNA) will be 40% higher than that for double stranded DNA (ds-DNA) at the same concentration. Furthermore, the hyperchromic effect arises mainly due to the presence of charged cations which bind to DNA via electrostatic attraction to the phosphate group of DNA backbone and thereby causing a contraction and overall damage to the secondary structure of DNA.[38]

Based upon the variation in absorbance, the intrinsic binding constant/association constant ($K$) of the drug with DNA can be determined according to Benesi–Hildebrand equation:[39]

$$\frac{A_0}{A - A_0} = \frac{\varepsilon_0}{\varepsilon - \varepsilon_0} + \frac{\varepsilon_0}{\varepsilon - \varepsilon_0} \times \frac{1}{K[\mathrm{DNA}]} \quad (1)$$

where $K$ is the binding/association constant, $A_0$ and $A$ are the absorbancies of the drug and its complex with DNA, respectively, and $\varepsilon_0$ and $\varepsilon$ are the absorption coefficients of the drug and the drug–DNA complex, respectively. The binding constant can be obtained from the intercept-to-slope ratios of $A_0/(A - A_0)$ vs. 1/[DNA] plots.

Many drug–DNA interactions are investigated by analysing the above mentioned changes in absorption spectra. The binding of three potential antibacterial agents: $Bu_3SnL$, $Cy_3SnL$ and $Ph_3SnL$ to DNA caused a progressive blue shift of 10, 8 and 4 nm, respectively. The peculiar hypochromism observed here is attributed to the intercalation of these drugs into the DNA base pairs. The reported binding constant values for these compounds are ranged from $2.30 \times 10^3$ to $6.05 \times 10^3$ $M^{-1}$. The highest value of $K$ was observed for the $Bu_3SnL$ due to additional hydrophobic nature of butyl group interacting with the bases of DNA.[40] The interaction of daunorubicin with calf thymus DNA (ct-DNA) has been investigated with the use of methylene blue dye as a spectral probe by the application of UV–Vis spectrophotometry, spectrofluorometry and voltammetry. The results showed that both daunorubicin and methylene blue molecules could intercalate into the double helix of DNA.[41] The thermodynamic parameters were also calculated and suggested that hydrophobic force might play a major role in the binding of daunorubicin to ct-DNA.[42]

Based on electrochemical and spectroscopic results reported by Kalanur at al.,[43] the interaction between the anticancer drug gemcitabine hydrochloride (GMB) and DNA is suggested to be the groove binding. With the addition of increasing amounts of DNA the absorbance of GMB increased at 207, 232, 248 and 283 nm (with red shift at 207 and 248 nm), while those at 331 nm decreased slightly. The high value of the binding constant, $K = 1.97 \times 10^6$ $M^{-1}$ suggests the strong interaction between drug and DNA.

11-Phenyl-substituted indoloquinolines have been found to exhibit significant antiproliferative potency in cancer cells but to show only moderate affinity toward genomic double-helical DNA. In the study of Riechert-Krause at all.,[44] parallel and antiparallel triple-helical DNA targets are employed to evaluate the triplex binding of indoloquinoline ligands. Compared to parallel triplexes, an antiparallel triplex with a GT-containing third strand constitutes a preferred target for the indoloquinoline drug. On the basis of pH-dependent titration experiments and the results obtained with Job's method of continuous variation, the binding of the drug and the stoichiometry of the complex was found to be pH strongly dependent.

The interaction of doxorubicin with polynucleotides and calf thymus DNA has been studied by several spectroscopic techniques in phosphate buffer aqueous solutions.[45] Obtained data showed that intercalation is the prevailing mode of interaction, and also reveals that the inte-

raction with AT-rich regions leads to the transfer of excitation energy to doxorubicin.

Multi-spectroscopic methods including resonance light scattering (RLS), ultraviolet spectra (UV), fluorescence spectra, $^1$H NMR spectroscopy, coupled with thermo-denaturation experiments were used to study the interaction of antitumor drug tamoxifen with calf thymus DNA.[46] The interaction caused a significant enhancement of RLS intensity, the hyperchromic effect, red shift of absorption spectra and the fluorescence quenching of tamoxifen, indicating the inserting interaction between tamoxifen and ct-DNA.

Recently, molecular docking and QSAR studies are carried out for the investigation of interactions between drugs and DNA. Eleven antitumor drugs (Doxorubicin, Epirubicin, Cisplatin, Fluorouracil, Daunorubicin, Carboplatin, Etopside, Cyclophosphamide, Dactinomycine, Dactinomycine and Mitoxantrone) are analyzed in the work of Perveen and coauthors.[47] Variation in spectral profile of these drugs on the addition of DNA was used to determine the values of formation constant ($K_f$) which is an indicator of the binding strength of the drug with DNA. Spectrophotometric studies are also used for systematic study of DNA binding properties of irinotecan (CPT-11) including binding constant, thermodynamic parameters, and thermal denaturation. The binding of CPT-11 to ds-DNA is quite strong as indicated by its remarkable hypochromicity, equilibrium binding constant and large positive enthalpy and entropy changes.[48]

The study of the metal ($Mn^{2+}$, $Fe^{3+}$, $Co^{2+}$, $Ni^{2+}$ and $MoO_2^{2+}$) complexes of the antibacterial agent ciprofloxacin (CF) interaction with ct-DNA performed with UV spectroscopy revealed that all investigated complexes could bind to DNA.[29] In combination with cyclic voltammetry the authors showed that Fe-CF complex exhibits much higher binding constant then other complexes. Complexes Mn-CF, Fe-CF and Mo-CF bind to DNA by both intercalation and electrostatic interaction, Ni-CF only by the intercalative mode and Co-CF can bind to DNA by electrostatic interaction.

**Fluorescence spectroscopy** is probably one of the most commonly used techniques to study interactions between small ligand molecules and DNA. The advantages of molecular fluorescence over other techniques are its high sensitivity, large linear concentration range and selectivity. The most intense and the most useful fluorescence is found in compounds containing aromatic functional groups with low-energy $\pi\rightarrow\pi$ transition levels. Compounds containing aliphatic and alicyclic carbonyl structures or highly conjugated double-bond structures may also show fluorescence, but the number of these transitions is small compared with those in aromatic systems.[49]

The orientation of fluorophoric ligands and their closeness to the DNA pairs of bases can be studied by fluorescence anisotropy or fluorescence resonance energy transfer.[50–52] Fluorescence quenching experiments give additional information concerning the localization of the drugs and their mode of interaction with DNA.[53] Fluorescence quenching refers to any process that decreases the fluorescence intensity of a sample. A variety of molecular interactions, such as excited-state reactions, molecular rearrangements, energy transfer and complex formation, can result in quenching. Quenching of the fluorescence is described by the Stern–Volmer equation[49]

$$\frac{I_0}{I} = 1 + K_{SV}[Q] \quad (2)$$

where $I_0$ is the fluorescence intensity in the absence of quencher while $I$ is the fluorescence intensity in the presence of quencher, and [Q] is the concentration of the quencher.

Fluorescence emission is very sensitive to the environment, and hence the fluorophore transfer from high to low polarity environments usually causes spectral shifts (10–20 nm) in the excitation and emission spectra of drugs.[50] Interaction with DNA usually causes a significant enhancement of the fluorescence intensity as a consequence of different factors.[54]

Thus, in the case of intercalating drugs, the molecules are inserted into the base stack of the helix. The rotation of the free molecules favors the radiationless deactivation of the excited states, but if the drugs are bound to DNA the deactivation through fluorescence emission is favored, and a significant increase in the fluorescence emission is normally observed. In the case when groove binding agents,[55,56] electrostatic,[57] hydrogen bonding[58] or hydrophobic interactions are involved, and the molecules are close to the sugar-phosphate backbone, it is possible to observe a decrease in the fluorescence intensity in the presence of DNA.[59] In the presence of quencher, in the case of intercalating agents the reduction in the Stern–Volmer quenching constant ($K_{SV}$) values is observed. $K_{SV}$ values were obtained from the slope of the plot of $I_0/I$ vs. [DNA] for several drugs.[53,60]

Different drugs were analyzed by spectrofluorimetric method with the aim to determine the binding mode, quenching constant and thermodynamic constants. Pazufloxacin, a new fluoroquinolone antibiotic, interacts with ct-DNA in the mode of groove binding,[61] and alkaloid Codeine, analgesic similar to morphine with uses similar to morphine, combines with the groove of nucleic acids through hydrogen bond or van der Waals force.[62] Other alkaloids were also successfully studied using fluorescent spectra.[63]

Ethidium bromide (EB) is a common fluorophore that bind to DNA. The fluorescence of EB increases in the presence of DNA, due to its strong intercalation between the adjacent ct-DNA base pairs. It was previously reported that the enhanced fluorescence can be quenched by the addition of a second molecule. Thus if the second molecu-

le intercalates into DNA, it leads to a decrease in the fluorescence intensity of the EB–DNA because it will compete with EB in binding with DNA. The extent of fluorescence quenching of EB bound to ct-DNA can be used to determine the extent of binding between the second molecule and ct-DNA.[64–66] The binding mode of diacetylcurcumin (DAC), a synthetic derivative with promising activity in threatening diseases like AIDS and cancer, was analyzed by competitive binding between ethidium bromide (EB) and DAC for ct-DNA. DAC was found to be a minor groove binder with a preference for the A-T region compared to the G-C region.[67]

Competitive binding studies of metal ($Mn^{2+}$, $Fe^{3+}$, $Co^{2+}$, $Ni^{2+}$ and $MoO_2^{2+}$) complexes of the antibacterial agent ciprofloxacin with EB showed that the complexes exhibit the ability to displace EB from the EB–DNA complex.[29] EB shows a single monomeric peak at 480nm. On adding ct-DNA its spectrum was changed showing the red-shifted absorption peak from 480 to 488 nm and significant decrease of the absorbance due to the formation of EB–DNA complex by intercalation. Addition of a second molecule, i.e. metal-ciprofloxacin complex, gradually displaces EB from the EB–DNA complex resulting in an increase of the absorbance of the free EB. These results explained the ability of each complex to gradually displace the DNA-bound EB, suggesting its strong competition with EB for the intercalative binding site.

**Infrared spectroscopy** (IR) has been widely used for the structural analysis of DNA because it can distinguish among A-, B-, and Z-forms of DNA, triple stranded helices, and other structural modifications. It has also been a useful tool to study interactions of nucleic acids with drugs and the effects of such interactions in the structure of DNA, providing some insights about the mechanism of drug action. A major advantage is that samples can be analyzed in different aggregation states, *i.e.*, as solids or crystals, and also in solution, making it possible to establish a comparison with results from other techniques. In addition, small quantities of sample are needed and collection of spectra is not time consuming. The characteristic IR bands of nucleic acids have been compiled and discussed.[68] Four regions are considered, each one containing marker bands reflecting either nucleic acid interactions and/or conformations. Approximate position of IR band regions are as follows: 1800–1500 $cm^{-1}$ region, sensitive to effects of base pairing and base stacking; 1500–1250 $cm^{-1}$ region, sensitive to glycosidic bond rotation, backbone conformation and sugar pucker; 1250–1000 $cm^{-1}$ region, sensitive to backbone conformation and 1000–800 $cm^{-1}$ region, sensitive to sugar conformation.

The infrared spectra of three different parallel-stranded DNAs (ps-DNA) have been studied by Fritzsche and co-authors.[69] They have used ps-DNAs and compared them with their antiparallel-stranded (aps) reference duplexes in a conventional B-DNA conformation. Significant differences have been found in the region of the thymine C=O stretching vibrations. They also showed that the interaction with three drugs known to bind in the minor groove of aps-DNA (netropsin, distamycin A and Hoechst 33258) induces shifts of the C=O stretching vibrations of ps-DNA even at low ratio of drug per DNA base pair. Those results suggest a conformational change of the ps-DNA and can be used to optimize the DNA–drug interaction.

Fourier transform infrared (FTIR) spectroscopy has been used alone or supporting other techniques to determine drug binding sites and sequence preference, as well as conformational changes due to drug-DNA interaction.[70,71] FTIR with self-deconvolution and second-derivative resolution enhancement were used to determine the drug binding mode, binding constant and the protein secondary structure in the presence of taxol in aqueous solution.[72] The observed spectral changes indicate a partial unfolding of the protein structure, in the presence of taxol in aqueous solution. In another research[73] FTIR difference spectroscopy method was used to characterize the nature of taxol–DNA interaction and to determine the taxol binding site, the binding constant, sequence selectivity, helix stability and biopolymer secondary structure in the taxol-DNA complexes *in vitro*.

5-Fluorouracil (5FU) is an anticancer chemotherapeutic drug which exerts cytotoxic effect by inhibiting cellular DNA replication. The binding of 5FU with DNA results in structural and conformational changes on DNA duplex. FTIR spectroscopic results[74] revealed that intercalation is the primary mode of interaction between 5FU and nitrogenous bases of the nucleic acid. The binding constant was found to be $9.7 \times 10^4$ $M^{-1}$; which is indicative of moderate type of interaction between 5FU and DNA duplex. Furthermore, the authors showed that 5FU disturbs native B-conformation of DNA though, DNA remains in its B conformation even at higher concentrations of 5FU. Similar results were obtain when FTIR spectroscopy and molecular docking studies were employed to investigate chlorambucil anticancer drug interaction with DNA.[75]

Multispectroscopic studies of paeoniflor [76] and mitoxantrone [77] interactions with calf-thymus DNA determined the modes of their binding. The results suggested that paeoniflorin molecules could bind to DNA *via* groove binding, while overall spectroscopic analysis revealed the intercalation of mitoxantrone between the DNA base pairs along with its external binding with phosphate–sugar backbone.

**Nuclear Magnetic Resonance** (NMR) is based on the fact that an atomic nucleus with odd number of protons or neutrons has a property called nuclear spin that will align with an applied magnetic field. The degree of this alignment depends not only on the strength of the magnetic

field, but also on the type of nucleus and its chemical environment. Each magnetically active nucleus is characterized by chemical shift, multiplicity, *J*-couplings, relaxation data and Nuclear Overhauser Effect (NOE), parameters that can be used to obtain detailed structural information about the molecule under study. NMR experiments can be performed at different temperatures, solvents, pH values, ionic strengths and dielectric constants.

Most common atomic nuclei available for the study of DNA are $^{1}$H, $^{13}$C, $^{15}$N and $^{31}$P. Among them $^{31}$P NMR is especially useful for studying the effects of ligand binding on the phosphate groups of DNA. Chemical shifts of $^{31}$P are sensitive to conformational changes in DNA, intercalating drugs cause downfield shift in the $^{31}$P signal, whereas divalent cations cause upfield shifts. The characteristic chemical shift for $^{1}$H NMR spectra of nucleic acids at room temperature has been reported about twenty years ago,[78] and any significant change in the chemical shift is attributed to the binding between ligand and DNA molecule.[78,79] The broadening of DNA $^{1}$H NMR resonances upon addition of a suitable minor-groove binding compound is usually taken as primary evidence of complex formation.

The most useful 2D-NMR experiments are: *Homonuclear Shift Correlated Spectroscopy* (COSY), which provides scalar coupling information which is transmitted over covalent bonds, and *Nuclear Overhauser Enhancement Spectroscopy* (NOESY), which provides information related to the spatial arrangements of atoms relative to one another and can show whether the double-helical is left or right handed.

Various DNA–drug complexes were studied using NMR spectroscopy. Netropsin antibiotic was reported to bind specifically on 5'-AATT sequence of DNA octamer duplex (DNA-5) in the minor groove.[80] The formation of netropsin–DNA complex caused $^{13}$C-NMR chemical shift. Mazzini et al.[81] described berberine binding to AT-rich sequences of DNA oligomer, and Park et al.[82] have also demonstrated the DNA-binding-induced $^{1}$H-NMR chemical shifts of berberine. In another study the same authors[83] have used UV and $^{1}$H-NMR methods to show that protoberberine interacts with the DNA duplex. However, the structural evaluation of the drug–DNA complex was not furnished.

In 2009, Mondelli group[84] has discussed the intercalation complex of topopyrone between GC base pairs of DNA, and Wu et al.[85] have shown the intra-strand GG cross-linked oxaliplatin complex with DNA. The interaction of the antibiotic drug norfloxacin with double-stranded DNA was studied by $^{1}$H NMR spectroscopy.[86] A highly selective broadening of the imino proton resonances assigned to central CpG steps was observed after addition of drug, indicating an intercalation-like interaction. In the same paper, using two-dimensional-NOESY spectra, authors reported that the planar two-ring system of norfloxacin partially intercalates into CpG steps and that the drug also exhibits non-specific groove binding. Another research group used $^{1}$H NMR spectroscopy (500 MHz) to quantify the complexation of the norfloxacin with DNA in the presence of caffeine.[87] They succeeded to determine the equilibrium parameters (induced chemical shifts, association constants, enthalpy and entropy) of the two and three-component mixtures. Investigations of the competitive binding of norfloxacin and caffeine with DNA show that the dominant mechanism influencing the affinity of norfloxacin with DNA is the displacement of bound norfloxacin molecules from DNA due to caffeine–DNA complexation (i.e. the protector action of caffeine). Similar results were obtained upon the complexation of three different anthracycline antitumour drugs with DNA in the presence of caffeine.[88]

The binding of mitoxantrone, a promising antitumor drug with reduced cardiotoxicity, to DNA has been studied by proton and phosphorous-31 nuclear magnetic resonance spectroscopy.[89] The stoichiometry reveals that 1:1 and 2:1 mitoxantrone–d(ATCGAT)$_2$ complexes are formed in solution. Authors suggest that mitoxantrone binds as a monomer at either or both ends of hexamer externally with side chains interacting specifically with DNA. This finding seems to be relevant to the understanding of pharmacological action of drug. Solid-state $^{31}$P–$^{19}$F REDOR nuclear magnetic resonance (NMR) experiments were performed to monitor changes in minor groove width of the selected oligonucleotide upon binding of the drug distamycin. In the hydrated solid-state sample, the minor groove width for the unbound DNA was 9.4 Å. Binding of a single drug molecule caused a 2.4 Å decrease in groove width. Subsequent addition of a second drug molecule results in a larger conformational change, expanding this minor groove width to 13.6 Å. These results demonstrate the ability of solid-state NMR to provide the direct spectroscopic measurement of minor groove width in nucleic acids.[90] The methodological approach of $^{23}$Na NMR spectroscopy to the study of the structure and dynamics of the DNA molecule was reviewed by Mariccola at. al.[91] In particular, the application of the $^{23}$Na NMR quadrupolar relaxation to investigate the perturbations on the polyion surface due to exogenous agents was discussed.

**Circular and linear dichroism** spectroscopies are useful techniques for studying non-covalent drug-DNA interactions, which affect the electronic structure of the molecules. Linear dichroism (LD) use polarized light and provides structural information in terms of the relative orientation between the bound drug molecule and the DNA molecular long axis.[92] LD spectroscopy involves measuring the difference in absorption of two linear polarizations of light, which usually are parallel and perpendicular to a sample orientation direction. In contrast to LD which depends only on the electric field vector, circular dichroism (CD) depends on both electric and magnetic interactions and provides additional structural details of the formed

complex.[92,93] CD signals of nucleic acids are observed in the UV region between 200 and 300 nm. In general B form of DNA has a positive signal, and A form tends to exhibit a positive and more intense signal. In contrast, CD of the left handed double helix of Z form DNA exhibits almost the reverse pattern to that of B form. These characteristic features of the CD spectra are the most useful for studding DNA conformational changes arising from drug binding. When an achiral and chromophoric drug binds to DNA, an induced circular dichroism (ICD) may appear within the adsorption band of the asymmetrically perturbed chromophore. On the other hand, DNA binding of a chiral chromophoric drug may result in a CD change within the absorption band of the drug as well as a CD change in the absorption region of the DNA due to the perturbations of structures with already optically active transition moments. Third possibility is DNA binding of a chiral drug which is silent because it contains weak UV-Vis chromophores which may give rise to CD changes within the absorption bands of the DNA only. The observation of an ICD immediately reveals that the drug actually binds to DNA, while its sign provides qualitative information about the binding mode: whether it is an intercalator or a groove binder, or binds on the outside of the polyphosphate backbone. In general, a strong, positive ICD is typical of a minor groove binder, while intercalators usually exhibit negative and weaker ICDs.

Circular dichroism was used to study the interaction of diacetylcurcumin (DAC) with ct-DNA,[67] and did not reveal any unwinding of the DNA helix on interaction with DAC, implying no conformational changes. Also, circular dichroism binding isotherms give stoichiometry of 0.25, and 0.5 for anticancer drug mitoxantrone molecules binding per duplex.[94] The CD spectra show blue shift and change in intensity of bands accompanied by appearance of induced bands. The results suggest stacking of aromatic chromophore of mitoxantrone with terminal base pair of DNA strand forming a sandwiched structure of mitoxantrone between four and two duplex molecules.

Neomycin is reported to be the most effective aminoglycoside in stabilizing a DNA triple helix without any effect on the DNA duplex. Triplex stabilization by neomycin is salt and pH dependent. CD binding studies indicate ~5-7 base triplets per drug apparent binding site, depending upon the structure of the triplex. The novel selectivity of neomycin is suggested to be a function of its charge and shape complementarity to the triplex W-H groove.[95]

Studies on the binding affinity of anticancer drug mitoxantrone to chromatin, DNA and histone proteins were performed employing UV/Vis, fluorescence, CD spectroscopy, gel electrophoresis and equilibrium dialysis techniques.[96] CD and fluorescence analysis showed that mitoxantrone interacts strongly with histone proteins in solution making structural changes in the molecule. The higher binding affinity of mitoxantrone to chromatin compared to DNA has been suggested, implying that the histone proteins may play an important role in the chromatin–mitoxantrone interaction process.

**Viscosity measurements**

Optical methods provide many crucial information about DNA–drug interaction, but still sometimes they do not offer sufficient clues to support a binding model. In the absence of the crystallographic structural data, viscosity measurements represents critical test of a DNA binding model in the solution. A viscosity measurement is hydrodynamic experiment that is sensitive to the change in molecule length. During the process of intercalation the lengthening of the DNA helix occurred since base pairs are separated to accommodate the bound ligand, leading to the increase of DNA viscosity. On the other hand, in the case of electrostatic interaction (out-binding mode) the compactness and aggregation of DNA may occur. The aggregation reduces the number of independently moving DNA molecules which results in lowering the solution viscosity. Groove binding drugs also induce a decrease in DNA solution viscosity. Such event is described by a bend or loop within the DNA helix, shortening the overall helical length.[97]

Viscosity measurements are performed using Oswald or Ubbelohde type viscometer measuring the flow time of the sample solutions after reaching the thermal equilibrium. The relative viscosity is calculated according to equation $\eta \sim (t - t_0)/t$, where $t$, and $t_0$ represents flow times of the sample and buffer solution respectively, and data are usually presented as $(\eta/\eta_0)^{1/3}$ *versus* DNA/drug binding ratio, where $\eta$ is the viscosity of the DNA in the presence and $\eta_0$ in the absence of the drug.

Viscosity measurements are often performed along with other optical methods. A class of novel carboline anti-tumor agents was analyzed.[98] Relative viscosity of the drug/DNA solutions in the ratios of 0–0.36 was measured and results indicate its increase, what is considered as a result of the intercalation. The same result was observed for polypyridyl ruthenium(II) complexes,[99] and ciprofloxacin drug based metal complexes.[100]

Neomycin is shown to be the first molecule that can selectively stabilize DNA triplex structures (polynucleotides, small homopolymer, as well as mixed base triplexes). Based on viscosimetric studies, this stabilization is shown to be based on neomycin's ability to bind triplexes in the groove with high affinity.[95] The interaction of potential anticancer drug, 4-nitrophenylferrocene (NFC) with DNA was also studied by viscosity measurements.[101] The plot $\eta/\eta_0$ with increasing concentration of NFC reveals negative change. Authors suggested that such a behavior is due to the electrostatic interaction that may cause the compactness and aggregation of DNA. Very interesting behavior was observed with acridin derivative, ACMA, potent antitumor agent.[102] From the viscosity studies it was shown that its apparent binding site depends upon the different structural or compositional domains (e.g., AT-rich

*vs*. GC-rich), which may display different affinity with the drug. ACMA interacts with the A–T and G–C bases quite differently; interaction with AT-rich domains is intercalation, while the interaction with GC-rich domains lead to the formation of two distinct complexes (partially intercalated and groove binding), depending on the ACMA content.

## 4. 2. Electrochemical Biosensors

During the last decade of the 20th century, DNA oligomers have been utilized as selective bio-recognition elements in new group of affinity biosensors. These so called DNA-based biosensors possess specificity of the response taking advantage of the bioaffinity properties of DNA. However, in contrast to conventional enzyme- and imuno-sensors, the DNA biosensors are mostly used for the investigation of interactions of DNA itself rather than for typical determination of the concentration of an analyte.[103–105]

According to International Union of Pure and Applied Chemistry (IUPAC) an electrochemical biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element.[106] Simply, it can be characterized as a devise that integrates DNA as the biological recognition element and an electrode as the physicochemical transducer. It is often presented as an electrode chemically modified by nucleic acid. Advantages and successful use of the electrochemical DNA biosensors have been reported and reviewed in numerous papers.[107–113]

**Operation Principles**

The signal obtained at the modified electrode surface is transduced into the electrical signal. The measurement can be carried out both amperometrically and potentiometrically. In amperometric measurement, an external potential is applied to oxidize or reduce an electrochemically active compound at its intrinsic redox potential, and the current produced during the process is measured. In potentiometric measurements, equilibrium is reached at the electrode surface without the need of the external potential, and as a consequence, the equilibrium potential between the electrode and the measured solution is generated and recorded.[106,114] The possibility of setting the working potential to the specific redox value of the analyte of interest provides the greater selectivity for the amperometric detection. The procedure usually includes the following three steps: 1) DNA immobilization on the electrode surface, 2) dipping the electrode into analyte solution, and 3) measuring of the current response. The measured current intensity depends on the concentration of the analyte following the Cottrel's equation:

$$I = nFA\frac{D^{1/2}}{\pi^{1/2}t^{1/2}}C \qquad (3)$$

where $A$ represents the area of the electrode, $D$ is diffusion coefficient, $C$ is concentration of the analyte, $t$ is time during which the measurement is performed, and $n$ is the number of exchanged electrons in the electrode process.

The electrode used as transducer element can be made up from different materials such as platinum, gold, mercury, pyrolytic graphite, glassy carbon or carbon paste. The use of solid conductors dispersed into polymeric nonconducting matrices – composites and nanostructured materials is growing over the last years. The last class of materials such as metal nanoparticles, magnetic nanoparticles or carbon nanotubes possesses very attractive features. The large surface and characteristic conducting properties allow them to achieve better response times, higher sensitivity and improved specificity.[115–117]

Depending on the electrode material, DNA can be immobilized on the electrode surface using different techniques: physical adsorption, electrochemical adsorption, covalent binding, the use of avidid/biotin complex, or the electropolymerized monomers like pyrrole, with the role of trapping the DNA molecules and binding them to the electrode.

**Detection Principles**

Regardless to the DNA immobilization technique the fundamental principle of the detection is based on the fact that the electrode detects the change at the DNA molecule. The resulting change may be due to the change in DNA concentration, orientation (conformation) or structure, caused by damage or denaturation. On the other hand, modification of the signal can also be caused by the noncovalent interactions of DNA with different ligands that lead to hybridization, association or complex formation. The most widely used substances that reversible bind to DNA are electroactive intercalators, and by measuring the change in redox signals derived from an electroactive intercalator, the interaction can be followed (Figure 3). The

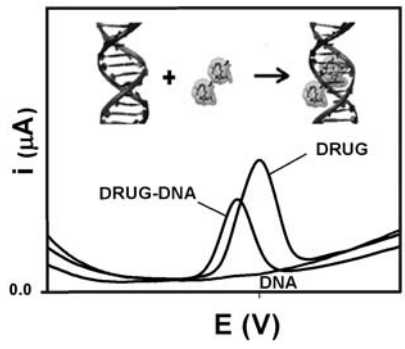



**Figure 3.** Schematic presentation of the change in the intercalator redox signal derived upon the interaction with DNA

third type of interactions that can be registered by electrochemical biosensor are the irreversible interactions among electroactive substances covalently bound to ds-DNA (double-stranded, native DNA), used mostly for increasing selectivity of analytical determination of DNA.

### 4. 2. 1. Electrochemical Activity of DNA

When nucleic acids interact with electrodes, they are usually strongly adsorbed. The adsorbed nucleic acid undergoes charge-transfer reactions, producing signals that can provide information about their concentration, changes in structure and orientation. Electrochemical activity of DNA is a consequence of the redox properties of nucleobases. Guanine and adenine residues are oxidized at carbon electrodes, while cytosine and adenine residues are reduced at mercury electrodes, in aqueous solutions at neutral pH. Thymine (T) is reduced only in non-aqueous media at highly negative potentials.[118–123] Electroactive groups that undergoes oxidation or reduction processes are presented in Figure 1.

Oxidation of guanine at glassy carbon electrodes occurs in two consecutive irreversible steps, yielding peaks at + 0.8 V and + 0.95 V *vs*. Ag/AgCl.[122,124] Adenine oxidation at glassy carbon electrodes is also irreversible, and occurs in three steps.[123] In cyclic voltammogram of adenine, two peaks are observable, at + 1.05 V and + 1.12 V *vs*. Ag/AgCl, and the third one, is detected after several scans, and corresponds to oxidation of adenine electroactive products formed at the electrode surface.[124] DNA oxidation can be followed at other carbon electrodes, such as pyrolytic graphite, carbon paste, modified carbon electrodes, as well as with the tin-oxide electrode.

At neutral and weakly acidic pH, adenine and cytosine residues in DNA produce reduction signals at mercury-based electrodes close to –1.4 V *vs*. Ag/AgCl, and in cyclic modes guanine yields an anodic signal, at about –0.3 V *vs*. Ag/AgCl, due to the oxidation of its reduction product back to guanine.[119] The pH of the solution has an important role in the detection process, since protonation of bases is involved in the electrode process. Nucleic acids strongly adsorb on electrodes, especially on mercury and carbon. Ideally smooth and highly reproducible surface of liquid mercury is very well suited for alternating current (ac) impedance measurements, which can provide information about DNA adsorption/desorption properties. This adsorption/desorption behaviour of DNA depends on the structure of the DNA molecules, and its orientation on the electrode surface. Reduction signals of adenine and cytosine are strongly influenced by DNA structure. For example, in differential pulse polarography, the reduction peak of native ds-DNA is almost two orders of magnitude smaller than the peak of denatured, ss-DNA. Also the non-Faradaic capacity signals are highly sensitive to changes in DNA structure, and can provide information about the bases interactions with the electrode.[125] Since the electrochemical signals are strongly influenced by the DNA structure, mercury electrodes are more suitable for studies of DNA structural transitions and local conformational changes. Experiments with mercury electrodes have identified single-strand interruptions in linear and circular DNA molecules, differences in the superhelix density of supercoiled DNAs, and superhelix density dependent structural transitions in DNA.[126,127] According to all this, electrochemical analysis of DNA can, in principle, be performed without introducing any labels and additional reagents into DNA. This kind of detection principle is often called **Label-free technique** and it is based on electrochemical and surface activity of DNA.[128,129] Electrochemical reduction and oxidation of nucleobases are irreversible and do not allow reusability of these kind of biosensors.

### 4. 2. 2. Non-covalent Drug–DNA Interaction

The use of DNA redox indicators is an alternative technique used to detect the presence and interaction events of immobilized DNA such as hybridization, damage, and association with another substance. The redox indicators usually show their electrochemical response at potentials different enough from the nucleobases, which is often reversible. Some of the indicators interact with DNA through electrostatic forces,[130] while others are present "free" in the solution phase. Special kind of redox indicators are the intercalators. Due to the accumulation within the immobilized ds-DNA layer, the bound indicator exhibits an increased voltammetric response.

The most commonly used redox indicators are drugs, especially anticancer agents. For example daunomycin can be oxidized at low potentials and high current density on a basal plane pyrolytic graphite electrode with an adsorbed DNA probe.[131] This kind of modified electrode detects electrochemical signal derived from an intercalator non-specifically bind to DNA. Voltammetric characteristics such as anodic and cathodic voltammetric peak potential and current of different drugs obtained at modified graphite electrode are presented in Table 2.[131]

Glassy carbon electrode was used for electrochemical study of *in situ* anticancer drug adriamycin oxidative damage to DNA.[132] In the study of Tiware et al.,[133] DNA modified glassy carbon fiber electrodes were used to study the interaction between the adriamycin and DNA. They reported a formation of an adriamycin radical which is able to oxidize the guanine in the ds-DNA, thereby causing the decrease in the guanine oxidation peak. Voltammetric measurements on glassy carbon electrode showed that antileukemia drug glivec binds to ds-DNA and leads to modifications in the ds-DNA structure, recognized through changes of the anodic oxidation peaks of guanine and adenine bases.[134] Using the same electrode, the decrease of the ds-DNA oxidation peaks are detected upon the interaction of microcystin-LR and nodularin with DNA due to the aggregation of DNA strands which may cause the mutations in

**Table 2.** Anodic and cathodic voltammetric peak potentials and currents of some intercalators in phosphate buffer, pH 7.0 (working electrode pyrolytic graphite, reference electrode Ag/AgCl, $v$ = 25 mVs$^{-1}$).[131]

| Intercalator | $E_{pa}$ (mV) | $i_{pa}$ (μA) | $E_{pc}$ (mV) | $i_{pc}$ (μA) |
|---|---|---|---|---|
| *Anthracycline antibiotics* | | | | |
| Daunomycin | 446 | 2.06 | 394 | 0.34 |
| Doxorubicin | 440 | 3.81 | 391 | 0.41 |
| Pirarubicin | 446 | 1.47 | 389 | 0.26 |
| *Tetraccycline antibiotics* | | | | |
| Tetracycline | 674 | 1.88 | – | – |
| Doxycycline | 663 | 2.79 | – | – |
| Minocycline | 385 | 3.58 | 155 | 0.42 |
| *Others* | | | | |
| 7-Aminoactinomycin D | 651 | 1.34 | 376 | 0.51 |
| Propidium iodide | 631 | 4.84 | 494 | 1.03 |
| Quinacrine mustard | 688 | 2.54 | –73 | 0.32 |
| Rifampicin | 718 | 3.49 | 44 | 0.57 |

the ds-DNA during the replication process.[135] Similar research involving another anticancer drugs, epirubicin[136] and mitoxantrone,[137] reported a decrease in peak current obtained at modified carbon paste electrode upon the interaction of these drugs with DNA. Recently, an anodically activated pencil graphite electrode (PGE) was employed for investigating the mechanism of the interaction between the anticancer drug leuprolide and fish sperm ds-DNA, immobilized into the electrode surface.[138]

Mercury based electrodes are also used to investigate intercalation reactions mostly based on reduction processes.[130,139,140] It is known that some intercalators could distinguish between ds-DNA and ss-DNA. Data obtained by voltammetric experiments using both the ss-DNA and ds-DNA modified electrode showed the type of binding of the intercalators. Bard and co-authors[141–143] reported that positive shifts of the peak potential were observed in the binding form via hydrophobic interactions (intercalation), while electrostatic interactions led to negative shift. Electrochemistry of anti-tumor drugs echinomycin,[144] and acridine,[145] as well as nicotinic partial agonist varenicline,[146] and their interaction with DNA were studied on hanging mercury drop electrode. In all cases a formation of drug–DNA complex was reported and the binding constant values determined.

Several other electrodes have been exploited in these studies. Among them modified gold surfaces are very often used. The self-assembled monolayers (SAMs) of an azidohexane thiol derivative were prepared on the Au electrode, and then used for the immobilization of ds-DNA and investigation of interactions of taxol, a very important anticancer drug, and DNA.[147] This was done by monitoring the guanine oxidation peak current. Sun at al.[148] used daunomycin intercalated into a ds-DNA as a biosensor to investigate the relation between peak current and the concentration of the DNA at modified gold electrode.

Due to the recent progress in the analysis of nucleic acids, peptides and proteins, the electrochemical methods appear ready for application to sequence-specific and nonspecific interactions of proteins with DNA. Peptides, polyaminoacids and proteins produce a structure-sensitive chronopotentiometric peak at mercury electrodes, which is due to the catalytic hydrogen evolution reaction. This characteristic reaction was used in the investigation of basic protein like histones, binding to DNA,[149] as well as in the research on the aggregation of α-synnuclein in Parkinson's disease,[150] and mutation of tumor suppressor proteins p53.[151]

### 4. 2. 3. Covalent drug–DNA Interactions

Electrochemically active DNA labels (tracers) are compounds covalently bound to DNA, which are used for the DNA detection with improved analytical selectivity and specifity.[113,130,152] Most commonly used labels are modified ferrocene,[153] nitrophenyl and aminophenyl groups, as well as osmium tetraoxide complexes with nitrogen ligands.[154–156]

There are number of drugs that interact covalently with DNA molecule.[9] Covalent binding in DNA is irreversible and undoubtedly leads to complete inhibition of DNA functions and subsequent cell death. The most famous covalent binder is cisplatin (Table 1), which is used as an anticancer drug. The use of electrochemical techniques for studying platinum–DNA interactions were reported by Brabec[157] and by Erdem et al.,[158] who studied the binding of cisplatin and a cisplatin-like chemotherapeutic agent to DNA which was coated on a wax- impregnated graphite electrode and a pencil graphite electrode, respectively, by using differential pulse voltammetry. Besides, Oliveira-Brett et al. reported the electrochemical determination of carboplatin in serum using a DNA-modified glassy carbon electrode.[159]

Mascini et al. have developed biosensors involving binding of cisplatin, carboplatin, platinum bipy and oxaliplatin[160–162] to double-stranded DNA immobilized on the

surface of screen-printed electrodes (SPEs). These biosensors use chronopotentiometry or square wave voltammetry (SWV), to rapidly and quantitatively measure the decrease of the oxidation peak of guanine. By calculating the ratio between the area (or height) of the guanine oxidation peak after interaction with the drug, and the area obtained by DNA alone, the percent of guanine sites in DNA that have not been modified by the interaction can be estimated.

The attempt to use non-platinum metal complexes as anticancer agents was initiated with an idea to find less toxic and more specific drugs. Some ruthenium and titanium complexes showed promising pharmacological properties as antitumor and antimetastatic agents. The interaction of ruthenium(III) complex, NAMI-A, with ds-DNA immobilized on screen-printed electrodes was studied as a screening tool for *in vitro* DNA–drug interaction.[163] Titanium(IV) complex titanocene dichloride showed a lower degree of interaction with DNA than cisplatin since the ionic interaction between Ti cation and external phosphate backbone produces a minor effect on the oxidation of G with respect to the direct coordination.[164]

This approach that has been successfully employed for the study of the interaction between a series of antitumor metallo-drugs and DNA offers information concerning the reactivity of the metal complex, the effect of anions acting as leaving ligands, the affinity of the generated electrophilic agent to DNA, and the strength of perturbation caused in the DNA chain by these metallo-drugs.

In the last decade, the use of nanostructured materials is spreading in the field of nanosensors. This class of materials such as carbon nanotubes, magnetic nanoparticles or metal nanoparticles possesses very attractive features. Carbon nanotubes have recently attracted considerable attention due to their unique structural, electronic, mechanical and chemical properties.[165] The large specific surface and characteristic conducting properties allow them to achieve better response times, higher sensitivity and improved specificity.[115] Aligned carbon nanotubes were used to detect a DNA sequence characteristic for genetically modified organisms with sensitivity in the nanomolar range.[116] A combination of magnetic beads for immunomagnetic separation and a later detection step using magnetic graphite-epoxy composite electrode has been recently employed for the detection of *Salmonella* in milk with the very low limit of detection.[117] Nanoparticles or nanocrystals of gold, indium, zinc, cadmium or lead chalcogenides have been used as well.[166]

## 5. Conclusions

Having in mind that DNA is one of the most important biomacromolecule, the possibility of detection and explanation of the changes resulting upon its interaction with chemotherapeutic drugs deserves a great attention. It is very important to use the most advanced methods in the analysis of drugs and toxic substances that may produce changes in DNA structure. The choice of the appropriate instrumental method allows the complete knowledge of the nature and the type of the mentioned interaction.

Compounds binding with DNA through intercalation usually results in hypochromism and hypsochromism (blue shift) or bathochromism (red shift) of the UV–Vis absorption spectra. In case of electrostatic attraction between the compound and DNA, hyperchromic effect is observed that reflects the changes of DNA conformation and structure. The advantage of molecular fluorescence over other techniques is its high sensitivity, large linear concentration range and selectivity. Interaction with DNA usually causes a significant enhancement of the fluorescence intensity. Fluorescence quenching experiments give information concerning the localization of the drugs and their mode of interaction with DNA. A major advantage that the application of IR spectroscopy offers is that samples can be analyzed in different aggregation states. Fourier transform infrared (FTIR) spectroscopy has been used to determine drug binding sites and sequence preference, as well as conformational changes due to drug–DNA interaction. Chemical shifts in NMR spectra are attributed to the binding between ligand and DNA molecule and are sensitive to conformational changes. Intercalating drugs cause downfield shift, and the broadening of DNA $^{1}$H NMR resonances upon addition of a suitable minor-groove binding compound is usually taken as primary evidence of complex formation. CD spectra may distinguish between chiral and achiral drug via an induced circular dichroism. The observation of an ICD immediately reveals that the drug actually binds to DNA, while its sign provides qualitative information about the binding mode: a strong, positive ICD is typical of a minor groove binder, while intercalators usually exhibit negative and weaker ICDs. The fundamental principle of the electrochemical biosensor detection is based on the fact that the electrode detects the change at the DNA molecule. Upon the interaction with the drug, changes in intensity and position of the voltammetric signals of both drug and DNA may occur. Positive shifts of the peak potential were observed in the binding form via hydrophobic interactions (intercalation), while electrostatic interactions led to negative shift.

The application of existing and development of new techniques and methods aims to expand research limits regarding to DNA selectivity and bioaffinity towards the drug, as well as for fundamental evaluation of effects of their interaction.

Although all mentioned techniques show their own specificity, their common aim is to correlate the measurable biophysical parameters with cytotoxicity i.e. anticancer activity of the drug, and to enforce the obtained knowledge for design new DNA ligands for *in vitro* and *in vivo* genetic diseases monitoring.

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## Povzetek

Številne anorganske in organske spojine se lahko vežejo na DNA in tvorijo komplekse. Mednje sodijo tudi zdravilne učinkovine, še posebno kemoterapevtiki. V tem prispevku je zbran pregled strukturnih značilnosti DNA in vrst interakcij (kovalentne in nekovalentne) med DNA in zdravilnimi učinkovinami. Kovalentna vezava učinkovine je ireverzibilna in vodi do popolne inhibicije delovanja DNA, kar posledično vodi do celične smrti. Nekovalentna vezava je reverzibilna in temelji na osnovah molekulske prepoznave. Posebna pozornost je namenjena ugotavljanju specifičnih vezavnih mest na DNA za vezavo učinkovin. Glede na strukturne značilnosti so učinkovine, ki se vežejo nekovalentno na DNA, večinoma interkalatorji, lahko pa se vežejo tudi na veliko ali malo brazdo DNA.

Ko se tvori kompleks med učinkovino in DNA, pride do delne modifikacije tako učinkovine kot tudi same DNA. Zbran je pregled metod za proučevanje interakcij med DNA in učinkovinami z namenom kako detektirati nastale spremembe in interpretirati rezultate. S tem namenom se uporablja številne spektroskopske metode kot so UV–Vis, fluorescenca, IR in NMR, spektroskopije z uporabo polarizirane svetlobe, kot sta cirkularni in linearni dikroizem ter fluorescenčna anizotropija ali resonanca. Razvoj elektrokemijskih DNA biosenzorjev odpira nova obzorja za uporabo zelo občutljivih in selektivnih elektrokemijskih metod za detekcijo specifičnih DNA interakcij. Predstavljeni rezultati podajajo pregled literature s področja omenjenih metod. S pomočjo obravnavanih metod lahko ugotavljamo poškodbe DNA, določamo vezavna mesta učinkovin in preferenčne sekvence, kot tudi konformacijske spremembe zaradi nastanka interakcij učinkovina–DNA.