Biophysical and electrochemical studies of protein-nucleic acid interactions

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Abstract

This review is devoted to biophysical and electrochemical methods used for studying protein-nucleic acid (NA) interactions. The importance of NA structure and protein-NA recognition for essential cellular processes, such as replication or transcription, is discussed to provide background for description of a range of biophysical chemistry methods that are applied to study a wide scope of protein-DNA and protein-RNA complexes. These techniques employ different detection principles with specific advantages and limitations and are often combined as mutually complementary approaches to provide a complete description of the interactions. Electrochemical methods have proven to be of great utility in such studies because they provide sensitive measurements and can be combined with other approaches that facilitate the protein-NA interactions. Recent applications of electrochemical methods in studies of protein-NA interactions are discussed in detail.

1. Background

1.1 Structures of nucleic acids

Nucleic acids play central roles in many cellular processes, particularly those involving the storage and expression of genetic information. There are two closely related types of nucleic acids (NAs): ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). As will become apparent in this review, these molecules have remarkably supple structures that can adopt bends, twists and many other more unusual shapes [1]. In terms of their chemistry, NAs such as DNA and RNA are polymers of nucleotides with a directional polarity, which occurs because the 3'-OH of one nucleotide is joined to the 5'-phosphate of the next by a phosphodiester linkage. Thus, one end of the molecule has a 5'-phosphate and the other end has a 3'-OH [1].

The most recognised activity of DNA is as a long-term storage molecule that contains the instructions necessary for the production of other cellular components, including proteins and RNA molecules. DNA is generally composed of two anti-parallel polymers of nucleotides that are joined together via a phosphodiester backbone. The nucleotides contain the nitrogenous bases adenine, thymine, guanine or cytosine, which coalesce into various combinations to produce precise genetic codes that are read in a 5' to 3' direction. The structural properties of DNA dictate its function and hence govern extensive cellular characteristics. The classical structure of DNA identified in the early 1950s is a right-handed helix known as B-form DNA. Other stable DNA variants include A-form DNA, which is also a right-handed helix, and Z-form DNA, which has a left-handed conformation [2]. The typical conformations of the sugars are distinct in each of these helical forms and the bases also interact differently. Factors such as DNA sequence, chemical modification, hydration and supercoiling can induce structural alterations so that DNA adopts non-B conformations. A wide range of unusual conformations of DNA exist, including quadruplexes, branched DNA, hairpin structures, Holliday junctions and single-stranded DNA (ssDNA) [1].

Although nucleotides are soluble in water, their bases are hydrophobic and prefer to avoid interactions with the aqueous environment. This promotes the formation of basepairs as the bases are held on the inside of the molecule and are kept relatively hidden from water, a phenomenon known as the hydrophobic effect. In addition to base pairing, DNA helices are stabilised by base-stacking interactions that occur between neighbouring bases [3]. The planar bases generally have unfavourable interactions with polar solvents, but, by 'stacking' on its neighbours, each base interacts mainly with another base, thus reducing its area that is exposed to solvent, which is usually water. Intuitively, one might expect that the structure adopted by a double-stranded molecule would be similar to that of a ladder, but such a structure leaves many gaps between the atoms of the molecule. Such gaps can be reduced if the ladder becomes 'skewed', which has the added advantage of optimising base–base stacking interactions. These hydrophobic interactions occur in both single- and double-stranded polynucleotides and can occur between all neighbouring bases of a sequence. Thus, base-stacking provides a large contribution towards the interactions that stabilise the overall three-dimensional structure of DNA. These stabilising effects have significant influences on the conformation of DNA molecules, and each helical form favours different types of stacking [2]. Together, these biophysical considerations highlight that the favoured conformation of DNA molecules are as spirals or helices. Theoretical calculations indicate that optimal conformations of the helix reduce the potential for interactions with water molecules and prevent unacceptably close contacts between neighbouring atoms [3]. The direction in which the phosphate and sugar backbone of each strand turns around the helix axis is also important as DNA can adopt helices that twist in either right- or left-handed directions.

In B-DNA, the two sugars linked to each base are located on the same side of the helix. A consequence of basepair stacking and the helical nature of the molecule is that the gap between these sugars forms continuous grooves in its surface, which are parallel to the sugar–phosphodiester backbone. The asymmetry present in basepairs leads to the formation of two types of grooves, referred to as 'major' and 'minor', which are 22 Å and 12 Å wide, respectively, in B-DNA. The widths

and depths of the grooves are related to the distances of basepairs from the axis of the helix and their orientation with respect to the axis. Thus, groove dimensions have specific characteristics dependent on the helical conformation. The B-form helix has wide major grooves and narrow minor grooves, which are established by the edge of the presented basepairs, and in the A-form helix the major groove is narrow and deep and the minor groove is wide and shallow. The Z-form helix has a major groove that is wide and shallow and a minor groove that is narrow and deep.

To allow large DNAs to be packaged in relatively small cells, it is clear that the molecules must undergo a high degree of bending, which is promoted by inherent flexibility in the double helix. It is also likely that some types of curvature promote the occurrence of biological processes on DNA, and localised bends in duplex DNA can be induced by external factors, such as protein binding. In addition to bending by proteins [4], some specific sequences adopt bent conformations preferentially – in other words, they have intrinsic curvature. There has been much speculation on the nature of such DNA bending in short regions of DNA [5], but it is clear that such conformations may promote binding of specific proteins, as is observed in the formation of packaging structures such as nucleosomes that are formed in eukaryotic cells.

RNA has a much higher rate of metabolic turnover than DNA, and is therefore more suited to its cellular roles of coordinated expression and regulation of genes. It has long been known that RNA is important for information transfer and protein synthesis due to its roles as mRNA, tRNA and rRNA [6,7]. Furthermore, recent studies have shown that small RNA molecules are involved in regulating the expression of genes [8,9]. Although RNAs are generally synthesized as single-stranded molecules, significant intra-strand base-pairing occurs within molecules and they have defined secondary and tertiary structures. Cellular RNA species vary in size from ~20 nucleotides upwards [10]. However, all double-stranded regions of RNA have a helical structure that is, typically, of a right handed sense and is closely related to the A conformation of DNA [11,12].

In summary, NAs have remarkable flexibility, allowing many different conformations to be adopted. Such a range of structures are likely to occur in cells, possibly for specific functions because they may allow recognition by distinct proteins or processes.

1.2 Protein-nucleic acid interactions

Many proteins have been identified that have the ability to directly interact with NAs and to modulate specific cellular processes, including DNA replication, transcription and DNA repair and maintenance. Protein-nucleic acid interactions show high variation in specificity and flexibility with certain proteins, such as structural proteins, able to bind non-specifically to any NA sequence, and other proteins, such as transcription factors, only able to bind to precise genomic regions.

As our current knowledge regarding protein-nucleic acid complex assembly continues to expand, the diversity and intricacy involved in these interactions becomes increasingly apparent. Prior to assembly into macromolecular protein-nucleic acid complexes, the individual formation of specific and homogenous three-dimensional structures of both protein and NA components are required. The thermodynamic tendency to bury nonpolar residues into the interior of a protein is a principal factor in stabilising protein folding. Similarly, restricting the surface exposure of planar surfaces of bases in NAs by base stacking is instrumental in stabilisation of duplex NAs [13]. The energies required for the formation of these folded entities must exceed the opposing force that is the decrease in configurational entropy. Crucially, the specificity of protein folding means individual types of amino acid residues are likely to be located at precise positions within the folded macromolecule [14]. Internal hydrogen-bonding alignments provide fundamental stability to the tertiary protein structure in conjunction with dipole-dipole and van der Waals interactions.

Proteins can further assemble into multi-subunit complexes consisting of other proteins and also with nucleic acid (NA) components through interactions with specific intermolecular interfaces. Electrostatic forces often significantly influence these interactions alongside hydrophobic bonding and, in some instances, ligand or ion binding [15]. The equilibrium stability of multi-subunit

complexes is also dependent on the concentration of the individual components and the solvent environment. As such, the addition of solvents including ethanol to the aqueous solution can strongly promote protein-protein and protein-nucleic acid interactions [16]. Crowding by other non-interacting molecular components, such as synthetic polymers or bovine serum albumin, can help to stabilise multi-subunit complexes by occupying elements of the solution that assemblies could potentially unfold or dissociate into [17]. Intracellular total salt (equivalent to monovalent cations such as K^+ or Na^+) concentrations vary between organisms but generally fluctuate between 100 – 200 mM. The highly negatively charged phosphate backbone of DNA destabilises its molecular structure and promotes denaturing of double-stranded DNA (dsDNA). Addition of salt neutralises this negative repulsive charge and so NA structures are stabilised at optimal ionic strengths [18]. The effect of salt on protein structure can either be stabilising or destabilising, depending on the specific charge distribution within the protein [19].

Protein-DNA interactions are generally flexible in nature and this is evident as, upon protein binding, the DNA conformation is often significantly perturbed yet the protein still remains bound [20]. Supercoiling, local unwinding and basepair breathing of DNA can occur following attachment of protein partners and such interactions must be accommodated by the protein in order to maintain the complex. Perhaps the best understood mode of protein-DNA interaction is the binding of a protein to a specific NA sequence, as is observed for restriction endonucleases and transcription factors. Proteins that bind in this sequence-specific manner are often able to overcome slight basepair alterations in the DNA [21], again demonstrating considerable flexibility.

Specific binding can be defined as a molecular association in which a particular molecule is bound tightly and exclusively in an energetically and kinetically stabilised complex [22]. This type of interaction is crucial for the functions of various regulatory proteins that act at precise locations on the genome. During sequence-specific DNA binding, protein interactions with NA sequences is primarily determined by recognition of hydrogen-bonding determinants situated in the major and

minor grooves of the DNA that interact with complementary recognition of the amino acids of the protein itself [23]. Hence, interactions are promoted when DNA bases are arranged in an optimal sequence. The hydrogen bond donator and acceptor patterns in the DNA grooves are recognised by complementary hydrogen bond donator and acceptors on the protein surface. The protein displaces water molecules and forms interfaces of complementary hydrogen-bonding patterns that are separated from the surrounding aqueous environment [16]. Electrostatic interactions between the hydrophilic and negatively charged DNA backbone and positively charged and dipolar amino acids stabilises the recognition surfaces. This type of interaction is heavily determined by structure and flexibility of both the DNA and protein. Although sequence-specific proteins associate tightly with recognition sites, the strength of binding does not prevent the protein from eventually dissociating from the substrate DNA once the downstream events of the interaction have been processed [24].

As described, certain proteins (e.g., transcription factors or restriction endonucleases) have strong binding affinity for target sequences, however most DNA binding proteins have the ability to bind non-specifically to DNA. This binding can be said to be a random molecular association as there are no exclusive over-arching determinants. The main component providing the necessary free energy to stabilise the interaction is the electrostatic attraction between positively charged amino acids and the negatively charged phosphodiester DNA backbone [25]. Usually, such binding does not require interactions to take place between the protein and bases, so the proteins tend to interact with features that can be determined from the backbone or the minor groove of DNA. These electrostatic, non-specific binding affinities are based on the displacement of counter-ions from the DNA and, thus, are not as tight as the previously described sequence-specific interactions. Consequently, proteins are able to move along DNA [26] by thermal motion in an exploratory fashion until the target binding site is located (Figure 1). Location of unique sites in the genome by proteins that are generally present in limited numbers would be considerably slower without the ability to translocate along DNA via these non-specific interactions. The process of protein diffusion in reduced dimensions includes short-range hopping along the same DNA strand and direct inter-strand transfer as well as

one-dimensional sliding (Figure 1). These processes reduce the volume through which the protein has to conduct its search for its target [27,28]. Upon contact with the target sequence, proteins can undergo reversible conformational changes and change from a non-specific complex dominated by electrostatic interactions to a conformation specific for tight association with target DNA basepairs [21].

It is recognised that the structure of DNA directly influences sequence-specific protein binding as molecular interfaces between interacting proteins and DNA are complementary in shape, allowing a close-fit association of the protein surface to the structure of the DNA [29]. This complementation in shape is determined by chemical contacts including hydrogen bonding, electrostatic interactions, van der Waals forces and hydrophobic interactions. The structure of the DNA has a significant impact on the strength of sequence-specific protein-DNA complexes, but some proteins recognise and bind with strong affinity to specific DNA structures in a sequence-independent fashion. Such DNA regions can be essential, specialised sites that require a non-B structure, some arise accidently during various cellular processes and others can be damage-induced and can be very detrimental. Denaturing of B-form DNA can occur following thermal fluctuations that induce basepair-breathing whereby dsDNA opens and closes spontaneously [30]. Specialised ssDNA binding proteins interact specifically to open single-stranded regions, independent of sequence, and act to stabilise the ssDNA and allow initiation of events such as replication [31]. Similarly to non-specific binding, the predominant component of this interaction is electrostatic.

2. Biophysical chemistry methods for studies of protein-nucleic acid interactions

A range of biophysical chemistry methods are available to study protein-nucleic acid interactions and some of these are outlined below. All techniques reviewed here have been used to study proteins interacting with DNA, but most of the methods have also been applied to studies of protein-RNA complexes [32]. Each technique can provide a wealth of knowledge of such interactions, but each has

limitations that usually restrict it from elucidating a full description of the mode of interaction between the protein and NA. Hence, alternative, complementary techniques are usually applied to the same system to provide a more complete description of these interactions.

A challenge can be that some conventional biophysical approaches are sensitive to variations in reaction conditions, making it difficult to recapitulate protein-nucleic acid interactions under conditions that are close to physiological. Mimicking intracellular ionic strength and pH values is of considerable priority for investigations of protein-DNA interactions under close to physiological conditions. The ionic strength of a solution has a substantial impact on the modes of many protein-DNA interactions due to the effective neutralisation of the electrostatic attraction between protein and DNA components at higher ionic concentrations. Therefore, sequence-independent interactions that generally rely heavily on charge [25] might be reduced under conditions that are suitable for high affinity, sequence-specific DNA binding.

2.1 Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assays (EMSAs) (sometimes referred to as “gel shift“ or “gel retardation“ assays) are among the most popular techniques for studying protein-nucleic acid interactions as they are relatively simple and straightforward to set up. These assays use electrophoretic separation of protein-nucleic acid mixtures and the speed at which different molecules move through the gel is determined by their size, charge and, importantly, their shape. When the protein binds to the NA, the less mobile complex of NA bound to protein will be 'shifted' up the gel compared to the NA alone. Thus, the ratio of bound to unbound NA on the gel reflects the fraction of free and bound NA molecules as the macromolecular complex enters the gel. If the starting concentrations of protein and NA are known, once the stoichiometry of the complex is determined the apparent affinity of the protein for the NA may be calculated [33]. Unless the complex is very long lived under the conditions of electrophoresis or dissociation during the

experiment is taken into account, the number derived is an apparent affinity of the protein for the NA.

Often, additional samples are analysed with a competitor oligonucleotide to determine the most favourable binding sequence for the binding protein. The use of different oligonucleotides of defined sequence allows the identification of the precise binding site by competition. Alternatively, an indicator oligonucleotide or longer DNA substrate can be utilized for indirect evaluation of protein binding to different competitor DNAs [34-36]. Variants of the competition assay are useful for measuring the specificity of binding and for measurement of association and dissociation kinetics.

In related experiments, an antibody that recognizes the protein can be added to this mixture to create an even larger complex with a greater shift. This method is referred to as a supershift assay, and is used to unambiguously identify a protein present in the protein-nucleic acid complex. Another way of confirming co-localisation of a specific DNA-binding protein with the retarded DNA in the gel represents combination of the gel shift assay with an immunoblotting technique [37,38].

These approaches tend to use polyacrylamide gel electrophoresis (PAGE), as this provides a relatively high level of resolution for identifying the regions of NA bound by the protein. However, the size of NAs used in PAGE is limited to a few hundred basepairs [39]. Agarose gel electrophoresis can also be used in EMSAs, allowing much larger NAs, to be analysed, such as plasmid DNAs. Such larger DNAs may differ in, for example, their topological states or the presence of alternative structures stabilized by DNA supercoiling, which may be hard to mimic using short oligonucleotides [40-42]. Thus, these approaches can be useful for studies of proteins that are influenced by DNA topology, such as topoisomerases [39,43]. However, the limitations of EMSA that are highlighted below tend to be exacerbated for such types of gel electrophoresis.

One disadvantage with traditional EMSA analysis is that experimental environments are restricted by the conditions required for electrophoresis. As mentioned above, the ionic strength of a solution has a substantial impact on the modes of protein-nucleic acid interactions meaning non-specific

interactions that generally rely heavily on charge are reduced in high salt environments [25], but stronger specific interactions that are dependent on other factors, such as base sequence, can remain. As modulating the salt levels during EMSA is problematic this can complicate analysis of the effects of ionic concentrations on macromolecular interactions.

One prevalent impediment of EMSAs is that protein-nucleic acid complexes must withstand changes in both chemical equilibrium and differences in stability between complexes in gel matrices compared to those in free solution [39]. The application of an electrical current during migration can also affect the stability of complexes, especially those influenced by electrostatic interactions. Indeed, it is possible that lower affinity interactions not governed by NA sequence, including those that are structure-specific, may remain undetected.

In summary, EMSA is a popular technique for studies of protein-nucleic acid interactions due to its ease of set-up and use, but it is most useful to produce reliable and reproducible data when the molecules have a high affinity for each other.

2.2 Footprinting

Another technique that makes use of gel electrophoresis is “footprinting“. However, important distinctions in the experimental set-up mean that this technique has different strengths and weaknesses compared to EMSA.

Typically, “footprinting“ experiments take advantage of enzymes or chemicals that attack NAs in a well-characterised manner, generating a highly predictable pattern for each specific NA [44]. The idealised arrangement for such experiments is to use agents to attack the NA independently of sequence. Enzymes such as DNase I and chemicals such as hydroxyl radicals are useful in this way, though even these agents are influenced to some extent by the altered arrangement of atoms and environment that occur at different sequences. Importantly, the base-pairing conformation tends to have a dramatic influence on such agents, especially in relation to whether the NA is in a single-

stranded or double-stranded form. Indeed, agents that attack NAs in a predictable way based on their structure (or sequence) can be useful to detect specific types of protein-nucleic acid interactions [44,45].

The standard “footprinting“ experiment is to initiate the interaction by adding the protein (or mixture of proteins or cell extract) to the relevant NA under defined reaction conditions, with the attacking agent then added at an appropriate time. Reactions are usually terminated by denaturation of the protein or NA (or cells), allowing samples to be analysed by gel electrophoresis at a later time. A recently developed electrochemical technique [46] utilizing DNA probes functionalized with click-transformable phenylazide reporters works on an analogous principle, although it does not provide single nucleotide resolution (for more details see Section 3.4).

In vitro “footprinting” studies have found widespread application since the use of defined reaction conditions is advantageous. It is more challenging to conduct such experiments inside cells because it is often difficult for the attacking agents to be added to the cellular environment under controlled conditions. Nevertheless, success has been achieved in using both modifying chemicals and enzymes in a range of cell types. Since such experiments tend to “kill“ the cells (or at least make them inviable), in some cases these experiments have been referred to as *in situ* rather than *in vivo* approaches. These types of experiments have been particularly successful for detecting the presence of unusual types of DNA structures [47-49], but have found some applications in studies of protein-nucleic acid interactions inside cells [50].

Footprinting methods are well suited to study relatively stable protein-nucleic acid complexes [45,51]. Interactions that have been particularly well studied include those between NAs and replication and transcription factors, including DNA and RNA polymerases, and proteins that are important for the formation of chromatin, as is found in the nucleosome [52].

2.3 Precipitation (”pull-down”) assays

A number of related biophysical approaches have been used to “pull-down” – or “precipitate” – macromolecular complexes. The term has been commonly applied as immunoprecipitation (IP) techniques, which use antibodies to “pull-down” their antigen out of solution. To allow recovery and analysis of the sample after the precipitation, the agent that recognises the complex is often immobilised to a substrate or surface. Magnetic beads (MB) represent a popular, versatile tool for various bioassays due to their convenient handling, easy repeated separation from (or resuspension in) the liquid phase and exchange of the medium. This is useful for efficient enrichment of the bioanalyte of interest at the large surface area of the MB, leading on to associated purification of the molecules under study from complex biological matrices. The MB can have attached to their surfaces various “biorecognition elements”, including oligonucleotides, streptavidin (to immobilize any biomolecule modified with biotin), antibodies or antibody-binding proteins (including protein A or protein G, as used in IP experiments), and so on. MB can be applied in a variety of bioaffinity assays and, as described below, they can be combined with diverse detection techniques, including electrochemistry [53].

A suite of related methods developed under the umbrella term of Chromatin Immunoprecipitation (ChIP) have found widespread applications to identify the site(s) of location at which proteins bind to genomic DNA within cells. Since such approaches have recently been widely reviewed [54,55], and because ChIP approaches tend not to assess the affinity of the interactions, they will not be discussed in detail within this review.

An important advantage of precipitation approaches is their significant degree of flexibility in terms of how the experiment can be set up, allowing for diverse reaction conditions to be studied. Major variations have been used in the way that the samples are pulled down and in relation to detection of the precipitated sample (protein or NA). Typical IP experiments use antibodies directed against proteins, but in protein-nucleic acid complexes the antigen can also target NAs via specific sequences or structures. For example, these approaches have been useful to study the interactions between

DNA and the tumour suppressor protein p53, which have been followed via pull-down of proteins [33] and by DNAs [35,36,56-58].

These recent studies also demonstrate how different approaches can be used to pull-down the same type of complex and to detect what molecules are present within it. For example, the connection between sample and precipitating factor, such as MB, can be via biotin and streptavidin, as used to detect p53 or MutS protein interacting with different DNA molecules [33,59,60]; one form of the arrangement can be set up with biotinylated NAs and streptavidin immobilised to the MB, as outlined in Figure 2A. Similar types of interactions have been studied using a range of antibodies against the p53 protein [35,36,56-58], as outlined in Figure 2B.

Recently, increased flexibility has been afforded to precipitation experiments by combining the approach with a wide range of detection techniques that can be made appropriate for the recovered analyte. These include labelling of samples with a reporter (radioactivity or fluorescence), analysis of the NA via enzymatic amplification and/or sequencing, immunodetection of the molecules (such as through combination with a western blot) and mass spectrometry. Such approaches have also been combined with electrochemistry techniques, as discussed further in Section 3.

Precipitation methods are well suited to studies of a wide range of protein-nucleic acid complexes. The flexibility of the assay in terms of its experimental set up mean that different types of information can be easily obtained. As outlined here, the system can be linked to a wide range of different biophysical methods, some of which generate quantitative information, allowing the affinity of the protein-nucleic acid interactions to be investigated.

2.4 High resolution techniques

Experimental methods that provide high-resolution, atomic information have been used to study a wide range of protein-nucleic acid interactions. Such approaches include x-ray crystallography and nuclear magnetic resonance (NMR), both of which have been reviewed for many types of proteins

and NAs [61-63]. NMR experiments are set up in solution and, thus, allow a range of reaction conditions to be studied, making them amenable to determine the affinity of interactions.

The major strength of these techniques is their ability to provide atomic details about the interactions and what influences them. However, the intricate nature of interactions required for formation of macromolecular complexes can prove troublesome. Thus, experimental conditions may not be easily identified to allow their use for all protein-nucleic acid complexes and they are most likely to be successful for interactions that have high affinity. Despite these potential problems, significant recent improvements in technology make them much more amenable to studies of such complexes and high-resolution structures have been determined for several large, multi-molecule complexes that are of fundamental biological significance, such as the nucleosome [52,64] and ribosome [65,66].

2.5 Activity assays

Among the most direct approaches to study the interactions between proteins and NAs are to use assays that detect some sort of enzymatic activity. If the protein acts on the NA to produce a measurable change in its structure or chemistry, then determination of the extent or rate the change can provide details about the level of interaction. Different types of biophysical approaches can be used in the measurements, but the most popular have traditionally used radioactivity or, more recently, chemically modified bases, which are often detected by fluorescent properties.

Examples of enzymes that have been widely studied using such approaches include enzymes that break or join the phosphodiester backbone, namely nucleases and DNA (or RNA) ligases, respectively; details that report on such types of analyses are provided in Section 3.5.

2.6 Calorimetric techniques

A variety of calorimetric technologies are available to study the energetics of chemical reactions and interactions [67,68]. The method most widely used for biophysical studies of protein-nucleic acid

interactions is isothermal titration calorimetry (ITC), and it is now established as an invaluable method for determining the thermodynamic constants, association constants and stoichiometry of such interactions [69-71]. The technique enables straightforward examination of the influence of reaction conditions on the interactions of the macromolecular complexes. Thus, it has found application within the drug discovery industry, which has utilized this approach to measure the interaction of protein-nucleic acid complexes with drug candidates.

2.7 Surface plasmon resonance

Surface plasmon resonance (SPR) biosensors are optical sensors that use electromagnetic waves to probe interactions between an analyte in solution and a biomolecular recognition element immobilized on the sensor surface [72-74]. Major application areas include the analysis of biomolecular interactions and these types of biosensors provide the important benefits of label-free, real-time analytical technology. Both qualitative and quantitative data can be obtained, and it is possible to obtain the kinetic parameters of the interactions between proteins and NAs. Like ITC, in addition to their use in basic biophysical chemistry research, SPR biosensors have found much interest in the drug discovery industry.

3. Electrochemical techniques

3.1 Electrochemistry of nucleic acids and proteins

NAs are electrochemically-active species that produce analytically useful polarographic, voltammetric and chronopotentiometric signals at different working electrodes (reviewed in [75]). Redox processes of NAs at mercury and amalgam electrodes in aqueous media involve reduction of cytosine and adenine moieties in natural DNAs or RNAs, manifested as a cathodic signal (peak CA, Table 1), and a chemically reversible reduction/oxidation of guanine giving rise to an anodic peak G. In addition, the NAs produce specific tensammetric (capacitive) signals at the mercury electrodes due to

adsorption/desorption (reorientation) processes at the electrically charged surface (Table 1, reviewed in [75,76]). Peak CA and the tensammetric responses are remarkably sensitive to changes in DNA structure, allowing differentiation not only between RNA and DNA, ssNAs and dsNAs, but, under specific conditions, also between dsDNAs containing or lacking free ends (see Sections 3.2 and 3.5 for applications). In summary, all nucleobases can be irreversibly oxidized at carbon electrodes and, in particular, signals due to oxidation of purine bases have found numerous applications in the electroanalysis of NAs (reviewed in [75]).

To improve sensitivity and selectivity of electrochemical analysis of NAs and to facilitate application of other electrode types (such as gold) that increase the flexibility and utility of the experiments, various external electroactive moieties have been applied. One group of such species include soluble redox indicators that interact with NAs non-covalently. Such compounds have widely been applied to differentiate between ss capture probes and hybrid duplex in sensors for DNA (typically, DNA intercalators or groove binders [75,77,78]). Another group includes covalently-bound redox labels that are used to encode a nucleotide sequence (used, for example, as a hybridization reporter probe or a specific DNA substrate for DNA-protein interaction studies) or a specific nucleotide (e.g., for analysis of sequence polymorphisms) [79]. Surface-confined terminally labelled capture probes have been designed as electrochemical “molecular beacons”, responding to DNA hybridization or interaction with a protein ligand by a structural transition accompanied by change of the distance of the redox moiety from the electrode surface, reflected in a change of electron transfer efficiency [75,80,81]. Covalently-labelled DNA can easily be prepared via chemical modification of natural DNA components (e.g., with osmium tetroxide complexes forming stable electroactive DNA adducts with a considerable selectivity for unpaired thymine residues [82], or analogous osmate complexes specifically modifying the 3’-terminal ribose in RNA [83]). Another, highly versatile approach involves incorporation of labelled nucleotides into DNA using DNA polymerases or terminal deoxynucleotidyl transferases and the corresponding modified deoxynucleotide triphosphates [79]. In this way, a number of novel redox DNA labels have been developed and applied, including those that are

reducible (such as nitrocompounds [46,84-86], benzofurazan [85,87], phenylazide [46]) or oxidizable (amino compounds [84,88], methoxyphenol [88]).

Similarly as for the NAs, intrinsic electroactivity of proteins is connected with the presence of electroactive or electrocatalytically-active moieties in their molecules, in this case represented by functional side groups of some amino acids (reviewed in [89]). Tryptophan and tyrosine residues are electrochemically oxidizable at carbon electrodes, yielding analytically useful signals that, under certain conditions, allow distinction between the two amino acids present in the same peptide molecule. Electrochemical behavior of proteins at the mercury and amalgam electrodes is closely related to the presence of cysteine. The thiol group of the latter amino acid exhibits a strong affinity to mercury, forming stable mercury thiolate. Detection of these species is possible via reduction of the sulfur-mercury bond. In the presence of cobalt ions, signals due to catalytic hydrogen evolution can be detected with cysteine-containing peptides and proteins (the so called “Brdicka reaction” [89,90]). Finally, practically all peptides or proteins (not only those that contain cysteine, depending on the conditions [91]) can produce signals due to electrocatalytic hydrogen evolution at the mercury-based electrodes in the absence of transition metal ions, giving rise to peak H. Recent studies by Palecek et al. using constant current chronopotentiometry have demonstrated that this technique is particularly useful for sensitive determination of peptides and proteins and for studies of protein (un)folding [92,93] or DNA-protein interactions [94] (also see Sections 3.2 and 3.3).

3.2 Techniques combining affinity separation at magnetic beads with electrochemical detection

Since the early 2000’s, when the first reports on the applications of MB technology in electrochemical DNA hybridization assays appeared [95-97], a number of techniques and analytical applications based on these principles have been proposed (reviewed in [53,98,99]). Basically, for studies of NA-protein interactions, the protein-NA complex can be anchored at the MB either via the NA substrate or via the protein, and can be built at the surface step-by-step as depicted in Figure 2, or pre-formed in solution and captured at the beads as a whole. After applying suitable washing

conditions to separate specific complexes from other species, the NA or protein is eluted (e.g., in increased salt concentration and/or at elevated temperature) and analysed electrochemically. For both NAs and proteins, which are firmly adsorbed at electrode surfaces, it is suitable to use an *ex-situ* (medium exchange, adsorptive transfer stripping) procedure that allows analysis of small-volume of the biomolecules [75,76]. Selection of the optimal electrochemical technique (such as cyclic voltammetry, square-wave voltammetry, differential pulse voltammetry, AC voltammetry or constant current chronopotentiometry) depends on the type of analyte to be analysed. Thus, a variety of electrochemical approaches have been applied to unlabelled DNA [35], RNA or protein [59,60,100] and DNA/RNA modified with a specific type of redox label [36].

To capture a nucleoprotein complex at the MB via the protein component (Figure 2B), IP is naturally convenient, provided that suitable antibodies against the protein are available. For example, various DNA binding modes of tumour suppressor protein p53 with different plasmid DNA substrates (supercoiled (sc), linear, containing or not containing a specific binding sequence) were studied using IP at protein G-coated MBs with two different anti-p53 monoclonal antibodies [35]. One of the antibodies (Bp53-10.1) has been known to influence the sequence-specific DNA binding by wild type p53 and hamper the protein's ability to bind scDNA with a high affinity [37,38]. For the detection of co-immunoprecipitated and subsequently eluted plasmid DNAs, label-free DNA structure-selective AC voltammetry at the mercury electrode was used, which provides a simple differentiation between scDNA (possessing no free ends and no tensammetric peak 3, see Section 3.1) and linear DNA (giving the peak 3) [35]. Another technique designed to assess p53-DNA binding via IP at the MB, followed by electrochemical detection, utilized double-stranded oligonucleotide probes armed with a single-stranded oligo(dT) tail, modified with an electroactive oxoosmium complex [82,101,102]. The osmium tags were measured by differential pulse voltammetry using a catalytic signal offering highly sensitive detection of the labelled DNA in the presence of unmodified DNA. The labelled probes were utilized to evaluate indirectly, in a competition mode, relative affinities of the p53 immuno complexes to different unlabelled plasmid DNA substrates.

To anchor the nucleoprotein complexes at the MB surface via the DNA component, oligonucleotides armed with biotin at one of its ends have typically been utilized (Figure 2A). Such dsDNA capture probes have been used, for example, for studies of DNA binding by MutS protein, which is the protein component of the mismatch repair pathway that recognizing basepair mismatches [33,59,60]. Biotinylated DNA duplexes, either fully complementary or involving a single base mismatch, were immobilized at streptavidin-coated MB and allowed to interact with the MutS protein in solution. After separation and washing, the DNA-bound protein was eluted and determined using the chronopotentiometric peak H at the mercury drop electrode [60] or using the peak Y due to tyrosine oxidation at a carbon paste electrode [59]. Besides a high sensitivity of protein detection (down to tens of attomoles in several microliters of the sample), this technique demonstrated a reliable discrimination among perfectly matched DNA homoduplexes and heteroduplexes, involving various single basepair mismatches (such as GT, AA or CA) and/or insertions.

Similar approaches have been applied to analyse interactions of protein targets with nucleic acid aptamers (synthetic NAs, *in vitro*-selected to bind target proteins with high selectivity and affinity [103], with potential applications in bioanalysis that are analogous to applications of antibodies). For example, streptavidin-coated MB were utilized for immobilization of a biotinylated lysozyme-specific DNA aptamer due to label-free electrochemical detection of the bound lysozyme (using oxidation signals of tyrosine and tryptophan at a pencil graphite electrode) [100]. Due to considerable specificity of the DNA aptamer-lysozyme interaction, the protein could be detected even in the presence of a large excess of other proteins or amino acids. In the field of bioanalytical applications of the aptamers, a number of concepts have been designed that utilise magnetic separation combined with electrochemical or electrochemiluminescence detection, involving various sandwich approaches, enzyme-linked detection systems and aptamer conjugates with nano-objects (such as gold nanoparticles, carbon nanotubes or graphene). The aptamer-based techniques have recently been reviewed [98,99,103].

3.3 Techniques and biosensors based on NA-modified electrodes

A typical biosensor consists of a signal transducer onto the surface of which is immobilized a biorecognition element, regardless of detection principle applied to generate the analytical signal (e.g., surface plasmon resonance, quartz crystal microbalance, electrochemistry). Attachment of an oligonucleotide probe onto a gold surface via a terminal sulfhydryl group is the most frequently used approach for construction of NA biosensors. In the area of detecting protein-nucleic acid interactions, reports devoted to various types of aptamer-based biosensors (aptasensors) represent the majority of the existing literature [103-112]. Similarly to the case of electrochemical biosensors for DNA hybridization, the electrochemical aptasensors for the detection of protein targets involve both label-free and redox label-based detection systems. Several examples are depicted in Figure 3. In general, label-free impedimetric biosensors are based on changes of the resistance of the biomolecular layer at the electrode surface upon binding of the analyte (here, protein) to the electrode surface-confined probe, towards electron transfer between the electrode and a soluble depolarizer, such as the frequently applied $[Fe(CN)_6]^{3-/4-}$ redox pair. When binding of a protein target (such as a bacterial outer membrane protein [107]) to the aptamer probe induces a structural change of the aptamer layer resulting in its increased compactness (Figure 3A), communication between the anionic ferro/ferricyanide redox pair and the electrode through the negatively charged aptamers layer becomes more difficult, which is reflected in an increase of the measured impedance. On the other hand, when the bound target protein bears considerable positive charge (such as lysozyme [108]), electrostatic attraction of the anionic depolarizer to the biomolecule layer facilitates electron transfer and the measured impedance decreases. Another strategy employs variants of the electrochemical molecular beacon concept based on a structural switch of the surface-attached aptamers upon binding of the target protein (Figure 3B). Some aptamers adopt, upon interaction with their target proteins such as thrombin [113,114], guanine quadruplex configurations that are relatively unstable in the absence of the target. A surface attached single stranded NA probe (aptamer), bearing a redox label (e.g. ferrocene) at its distal end (relative to the electrode surface),

allows the label to approach the electrode surface when in its unfolded form, resulting in efficient electron transfer and a high current signal. (Alternatively, the probe can be designed to adopt a hairpin structure in the absence of the target, bringing the label close to the electrode). The explanation proposed for this is that the target protein (thrombin) stabilises the aptamer strand in the quadruplex structure, thus reducing its motional freedom and increasing the average distance between the label and the electrode and leading to a diminished signal [113]. A “signal-on” alternative of this system involves a rigid duplex form of the aptamer that exists in the absence of the target protein, keeping the redox label away from the electrode (Figure 3C). When the target is present, the probe undergoes a structural transition resulting in folding of the distal part of one strand into the quadruplex conformation and release of a complementary stretch of the other strand, allowing the reporter to communicate with the electrode and the current signal to appear [114]. Similarly as for the MB-based techniques, a variety of aptasensors for different target proteins employing a variety of electrochemical detection schemes have been proposed and recently reviewed by other authors [103,112,115].

Electrochemical sensors working on the principle of DNA-mediated charge transfer have been designed for biologically relevant interactions of various proteins, including transcription factors, proteins involved in DNA repair or DNA methyltransferases. According to Barton and co-workers, dsDNA can conduct electrical current over long distances, provided that basepair stacking within the double helix is preserved [116-118] (Figure 4). When the basepair stacking is disrupted at a site between a donor and an acceptor of electrons tethered to the DNA (one of which may be an electrode to which the duplex DNA is attached and the other a redox-active moiety intercalated into the DNA duplex at its opposite end, Figure 4), the charge transfer is inhibited, resulting in diminution of the measured current signal. Typically, intercalators exhibiting reversible electrochemistry, such as methylene blue, Nile blue, daunomycin or metallointercalators, have been applied [116-120]. DNA-mediated redox cycling of these species have been measured by cyclic voltammetry or by amperometric systems involving a chemical oxidant $[Fe(CN)_6]^{3-}$ in the bulk of solution, which re-

oxidizes electrochemically-reduced intercalated methylene blue [118]. The stacking disruptions occur at sites of basepair mismatches, missing or flipped-out bases, bulges, bends or kinks. Such techniques have been applied to studies of TATA box-binding protein, which causes a considerable untwisting and bending of dsDNA when forming the specific complex with its recognition site [121]. In such structures the basepairs are unstacked considerably and the electron “wiring” through the DNA is inhibited. DNA methylases that catalyse methylation of cytosine at C5 act through formation of an intermediate, in which the enzyme is covalently bound to C6 of the cytosine moiety via a cysteine residue and the base is flipped out from the double helix, thus leaving a gap in the base stacks [120,122]. In both cases, binding of the protein is connected with diminution of the measured current responses. Similar sensors were applied to probe DNA interactions with MutY protein and uracil DNA glycosylase that are involved in DNA repair, restriction endonucleases and others [118,119,121].

Recently Paleček and co-workers applied constant current via the chronopotentiometric technique in the analysis of DNA complexes with DNA binding domain of the p53 protein (p53CD) [94]. It has been shown that p53CD sequence-specific binding to DNA strongly decreases the intensity of peak H when rapid potential changes at the thiol-modified mercury electrodes are used. This signal decrease is due to limited accessibility of the electroactive amino acid residues in the p53CD–DNA complex. Weaker non-specific binding can be eliminated or distinguished from the sequence-specific binding by adjusting temperature or optimal stripping current, which influences the rate of potential change. Notably, the technique is capable of distinguishing between different sequence-specific complexes differing in their thermodynamic stabilities. The technique is inherently easy to conduct as it does not involve specific immobilization of either of the interacting partners (protein or DNA) at the electrode; instead, the nucleoprotein complex is formed in solution followed by simple adsorption at the electrode surface.

3.4 Detecting DNA-protein interaction using a redox-labelled click-transformable DNA probe

A new electrochemical approach to the detection of DNA-protein interactions utilizes a DNA probe modified with electrochemically-reducible phenylazide that can be transformed into electrochemically-silent triazole via a copper-catalyzed click reaction with an acetylene derivative [46]. To obtain an independently measurable electrochemical signal that reveals the click reaction has taken place, 4-nitrophenylacetylene is applied in the click reaction. The nitro group in the resulting triazole derivative produces a reduction signal around -0.4 V (Figure 5). The click transformation of the phenylazide into the nitro-tagged triazole was feasible on naked DNA, whereas binding of p53CD to a specific DNA sequence modified with the phenylazide label protected its conversion. Simple electrochemical *ex-situ* voltammetric analysis was used to evaluate binding of the p53 protein to the specific reactive probe. Partial conversion of the azido into the nitro label (reflected in changes in the intensities of reduction peaks at -0.9 V and at -0.4 V, respectively) revealed binding of the p53CD to a target sequence spanning part of the DNA probe and protecting the azido tags within this region, while tags outside the region covered by the protein were click-transformed (Figure 5). Complete conversion of the azido groups was observed when a DNA non-binding protein, such as bovine serum albumin, was applied instead of p53CD. This assay is analogous to DNA footprinting techniques (Section 2.2), although it does not provide single-nucleotide resolution. The assay appears to be potentially suitable for fast preliminary screening purposes to select samples for more detailed studies of protein-nucleic acid interactions.

3.5 Electrochemical detection of enzymatic reactions acting on DNA

In addition to determining proteins simply binding to NAs, electrochemical assays have also been used to assess for changes in the chemical or conformational structure of the NA due to reactions catalysed by enzymes. Particularly good examples of such studies are provided by nucleases and ligases, which are of fundamental importance to cellular DNA metabolism and they have also been the cornerstone for widespread developments and applications in molecular biology.

Measurements of DNA responses at the mercury-based electrodes exhibit a high sensitivity to DNA structure, allowing discrimination between dsDNA molecules lacking any ends (such as covalently closed circular DNA, scDNA) from dsDNA containing strand ends (nicked circular DNA, linear DNA) and ssDNA or dsDNA containing extended ssDNA ends or internal "gaps" without any DNA labelling (Figure 6). Due to these properties, label-free electrochemistry at mercury or amalgam electrodes has been frequently used to detect DNA damage and products of enzymatic processing of the damaged DNA [123,124]. Some enzymes, namely those involved in DNA repair, such as T4 endonuclease V or *E. coli* exonuclease III, have been used to convert nucleobase lesions that do not significantly alter voltammetric responses of the DNA (at least at low DNA damage levels) into sensitively detectable strand breaks or ssDNA regions (Figure 6) [123]. On the other hand, using appropriate DNA substrates allows the same system to be used for detection and determination of corresponding enzymatic activities via measured changes in the DNA substrate structure. Thus, scDNA substrates (or scDNA-modified mercury-based electrodes) can be used for the detection of activities of endonucleases that introduce single- or double-stranded breaks in DNA [125] or repair endonucleases, which introduce nicks next to specific lesions they recognize [123]. Activities of exonucleases degrading one strand in DNA duplex (e.g. exonuclease III) can easily be probed using nicked circular DNA duplexes. The same detection system has been applied for the detection of activities of DNA ligases. Sealing of a single strand break in an open circular DNA substrate to form covalently closed circular DNA was detected via a decrease of the intensity of AC voltammetric peak 3 [126] (Table 1).

A range of other electrochemical assays have been developed for studies of the DNA ligases. These include application of electrode surface-confined nicked hairpins [127], molecular beacon-based approaches [128,129] a nanoparticle-based piezoelectric sensor [130] and a biometallization-based method [131]. A recently-described alternative approach used MB as a carrier for DNA substrates and alkaline phosphatase to generate a signal for the detection of ligation products [132]. As outlined in Figure 7A, this method presents a fast, sensitive and versatile assay of DNA ligase activity.

Electrochemical assays have also proven useful to detect ligation of DNA substrates immobilized on different surfaces through biotin-streptavidin interactions or direct linkage to the surface. For example, as shown in Figure 7B, a DNA hairpin attached to a gold surface was assessed for ligation due to voltammetric characterization of its ferrocene reporter [133]. By taking advantage of fluorescent labels, similar substrates have also been used to study the combined action of nucleases and ligases [127], which would allow extension of these types of assays to assess complete pathways of NA metabolism, e.g. DNA repair processes.

4. Summary

The interactions of proteins with nucleic acids are critical for many cellular processes, such as replication and transcription. Protein-nucleic acid interactions show high variation in specificity and flexibility, with some proteins recognising precise sequences whilst others bind to specific structures. A range of biophysical chemistry methods have enhanced our understanding of the wide scope of protein-DNA and protein-RNA complexes. Each technique can provide a wealth of knowledge of such interactions, but, importantly, each has limitations and alternative, complementary techniques are required to provide a complete description of such interactions. Electrochemical methods have proven to be of great utility in such studies because they provide sensitive measurements and can be combined with other approaches that facilitate the protein-nucleic acid interactions. The application of biophysical techniques to study protein-nucleic acid complexes continues to highlight the diversity and intricacy involved in these interactions.

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Table 1. Summary of electrochemical signals of natural nucleic acids and proteins, and selected DNA labels.

Signal	Potential[1]	Electrode	Medium	Moiety/ structure involved	Electrode process	Ref.
Peak CA	-1.5 V	mercury, amalgam	AFP[2]	cytosine, adenine	irreversible reduction	[75]
Peak G	-0.3 V	mercury, amalgam	AFP[2]	guanine	oxidation of G reduction product[3]	[75]
Peak 1	-1.2 V	mercury, amalgam	weak alkaline	DNA backbone	tensammetric	[75]
Peak 2	-1.3 V	mercury, amalgam	weak alkaline	distorted dsDNA	tensammetric	[75]
Peak 3	-1.45 V	mercury, amalgam	weak alkaline	ssDNA	tensammetric	[75]
Peak G^{ox}	+1.1 V	carbon	acetate pH 5.0	guanine	irreversible oxidation	[77]
Peak A^{ox}	+1.3 V	carbon	acetate pH 5.0	adenine	irreversible oxidation	[77]
Peak Y	+0.55V	carbon	phosphate pH 7.0	tyrosine	irreversible oxidation	[89,134]
Peak W	+0.7	carbon	phosphate pH 7.0	tryptophan	irreversible oxidation	[89,134]
Peak S	-0.6 V	mercury, amalgam	sodium tetraborate pH 9.5	cysteine (S-Hg bond)	reduction	[135]
Peak H	-1.75 V	mercury, amalgam	phosphate pH 7.0	cysteine, lysine, arginine, histidine[3]	catalytic hydrogen evolution	[89,134]
Brdička	-1.1 - -1.5	mercury,	ammoniacal buffer, pH >	cobalt complexes	reduction, catalytic	[89]

reaction	V^{5}	amalgam	9.5 , $[Co(NH_3)_6]^{3+}$	with cysteine-containing proteins	hydrogen evolution	
Peak NO_2^{red}	-0.4 - -0.5 V[6]	mercury, amalgam, carbon	AFP[2], sodium acetate pH 5.0	NO_2	irreversible reduction	[46,84-86]
Peak N_3^{red}	-0.9 V	mercury	AFP[2]	N_3	irreversible reduction	[46]
Peak BF^{red}	-0.8 V	mercury, amalgam, carbon	AFP[2], sodium acetate pH 5.0	benzofurazane	irreversible reduction	[86,87]
Peak α Peak β	-0.58 V -0.1 V	carbon	sodium acetate, pH 5.0	Os	reversible redox	[82,101,102]
Peak Os	-1.2 V	mercury, amalgam	Britton-Robison buffer, pH 4.0	Os	reduction, catalytic hydrogen evolution	[82]

[1] (V *vs.* Ag|AgCl|3 M KCl)
[2] 0.3 M ammonium formate, 50 mM sodium phosphate pH 6.9
[3] oxidation of 7,8-dihydroguanine generated at the electrode, see text for more details
[4] depending on pH and other conditions
[5] depending on the number and accessibility of cysteine residues and neighbouring amino acids
[6] depending on medium, conjugate group and nucleobase

Figure Legends

Figure 1 Mechanisms for diffusion of proteins along nucleic acids. DNA binding proteins can interact non-specifically with DNA, which facilitates location of specific target sites (in red) by reducing excursions of the protein away from the DNA. Three modes of protein translocation along nonspecific DNA have been proposed and are shown: A. One-dimensional sliding; B. Intra-strand hopping; C. Inter-strand transfer. If the protein detects its specific binding sequence it can undergo a conformational change resulting in a tighter association as represented in B.

Figure 2 *In vitro* magnetic beads DNA binding assays. A. Using immobilised nucleic acids. Biotinylated oligonucleotides are added to magnetic beads, followed by addition of free biotin molecules (to prevent unspecific protein binding to the beads). The protein of interest is then added and incubated with oligonucleotides and unbound protein is removed in the supernatant following collection of the beads/oligonucleotides/protein with magnets. Bound proteins are denatured with SDS, removed in supernatant and analysed alongside the unbound protein removed previously. B. Using immobilised protein. Protein and nucleic acids are incubated to allow interactions to occur, followed by addition of antibodies and then protein-G beads to pull down protein-nucleic acid complexes via attached antibodies. Magnetic force is applied to collect beads/antibody/protein/nucleic acid complexes and any unbound nucleic acid is removed in the supernatant. Following wash steps, bound nucleic acid is denatured, released from the protein and analysed e.g. by gel electrophoresis. Both procedures are compatible with electrochemical detection of the recovered proteins or nucleic acids.

Figure 3 Examples of general detection schemes used in electrochemical aptasensors for protein targets. (A) An impedimetric sensor using a soluble redox indicator ($[Fe(CN)_6]^{3-/4-}$, represented by the star) to probe changes in electron transfer resistance upon binding of the target protein to immobilized DNA aptamer [107]. (B) A “signal-off” electrochemical molecular beacon responding to

conformation change (quadruplex formation) of the aptamer upon interaction with the target protein by increasing the average distance of terminally attached redox label (L), reflected in a decrease of the redox current [113]. (C) A "signal-on" variant of the electrochemical molecular beacon. A rigid duplex form of the DNA aptamer prevents the terminally attached label from communicating with the electrode. In the presence of the target, part of one strand of the duplex DNA forms the quadruplex structure, liberating the complementary part of the other and allowing the label to approach the electrode surface [114].

Figure 4 Examples of sensors for DNA-protein interactions utilizing DNA-mediated charge transfer. Details are developed according to Barton et al. [118]. Double-stranded DNA with perfectly stacked basepairs can "wire" electrons between the electrode and a redox active moiety intercalated at the opposite end of the DNA duplex. When the basepair stacking is disrupted (e.g. due to untwisting and bending of the double helix upon interaction with TATA box binding protein [119] or due to base flipping-out as in the case of binding of a DNA methyltransferase [119,120]), the DNA-mediated electron transfer is inhibited and measured current signals diminish.

Figure 5 Assessment of DNA-protein interactions using a redox-labelled, click-transformable DNA probe. A phenylazide moiety introduced into a DNA sequence can be converted into a triazole derivative in a "click reaction" using an acetylene derivative, resulting in the switching-off of the azide reduction signal at around -0.9 V. When the acetylene bears another electrochemically-active group, a new signal appears after the click reaction (e.g. the signal of the nitro group reduction at around -0.4 V). Binding of a protein (such as the p53 core domain) to the phenylazide-functionalized DNA results in shielding of the reactive groups and the click reaction is prevented, which is reflected in the voltammetric responses of the probe after the click reaction in the presence of the protein [46].

Figure 6 Scheme describing label-free detection of enzymatic reactions on DNA catalysed by various nucleases or DNA ligases. Covalently closed circular (ccc) DNA (such as plasmid scDNA) does not give AC voltammetric peak 3 (specific for single-stranded DNA regions) at mercury-based electrodes. By contrast, circular DNA containing at least one single stranded break (ocDNA) or linear DNA yield the peak 3 due to its susceptibility to being unwound around the strand ends at the negatively charged electrode surface. Thus, formation of strand breaks upon interaction with endonucleases can be monitored by voltammetry at the mercury or amalgam electrodes [125]. The same principle is applicable for DNA repair enzymes recognizing specific base lesions and/or exonucleases generating single-stranded regions in double-stranded DNA [123] and, in reverse, for detection of DNA ligase activity converting the ocDNA into cccDNA, thus causing the peak 3 to diminish [126].

Figure 7 Use of reporter molecules for electrochemical detection of DNA nick-joining by DNA ligases. (A) Scheme of a DNA ligase assay employing magnetic beads and enzyme-linked electrochemical detection. A biotinylated nicked duplex oligonucleotide is captured on the streptavidin-coated magnetic beads and exposed to a DNA ligase. When the nick is ligated (but not in the absence of ligation), the 3'-half strand bearing digoxigenin (dig) label remains attached to the beads after removal of the template strand and can be detected using anti-dig antibody conjugate with alkaline phosphatase (ALP), which converts inactive 1-naphthyl phosphate into electrochemically-oxidisable 1-naphthol [132]. (B) Electrochemical ligase/nuclease assays use a hairpin probe that is attached to the electrode surface by its 3'-end and bears a redox label (ferrocene) at its 5'-end. In such configuration the label is positioned close to the electrode surface and produces an analytical signal. When a nick is present in the stem part of the hairpin, the labelled end of the probe is removed upon denaturation and the signal disappears. Ligation of the breaks prevents the labelled end of the probe from being lost and the signal obtained after ligase treatment followed by exposure to denaturing conditions indicates the ligase activity. Based on the same principle in reverse, action of a nuclease switches the signal off [127,133].

Figures

Figure 1

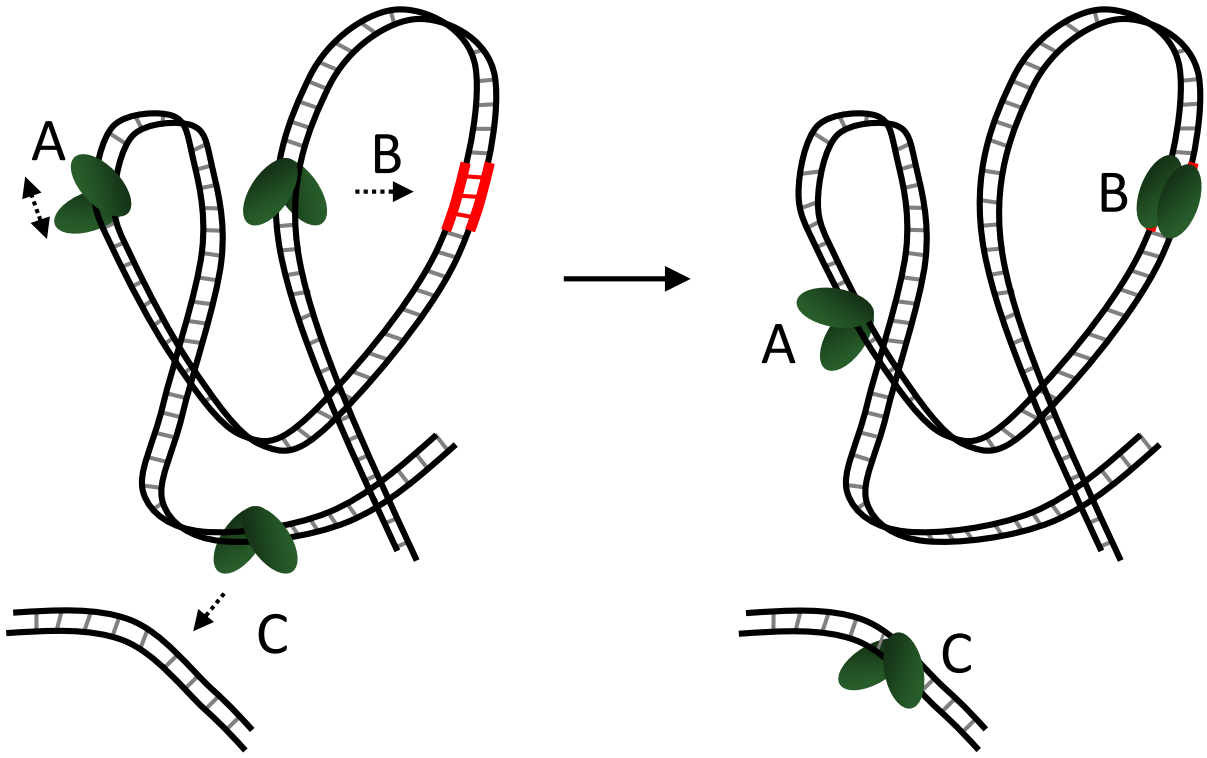
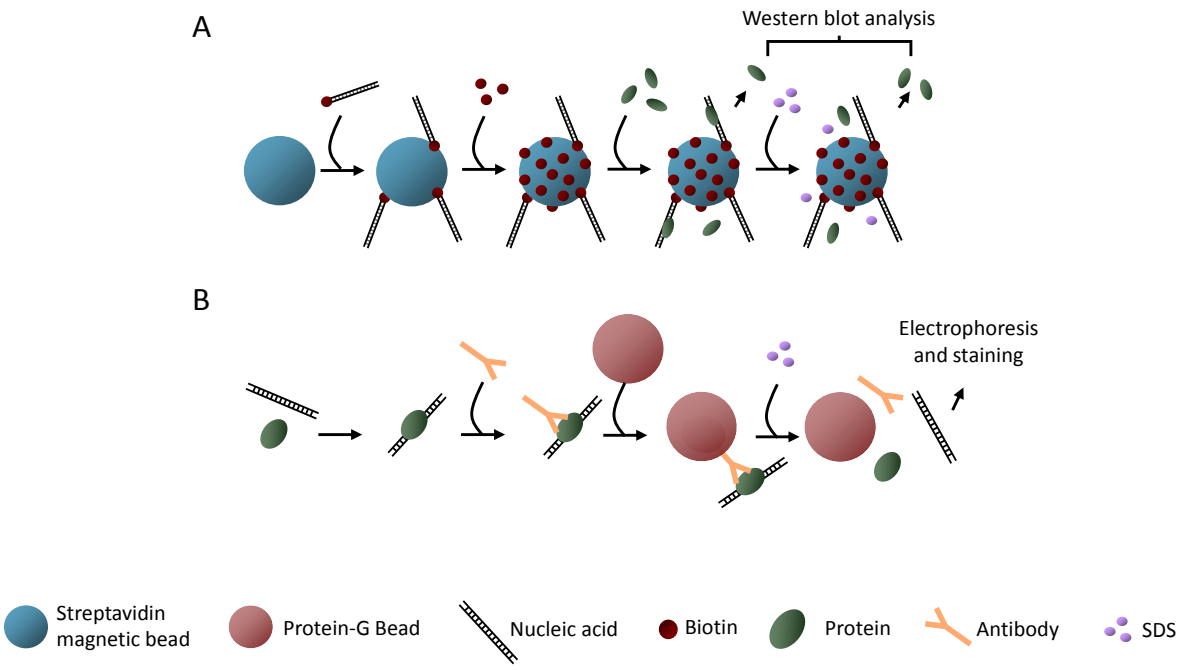


Figure 2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Protein
Antibody
SDS

Figure 3

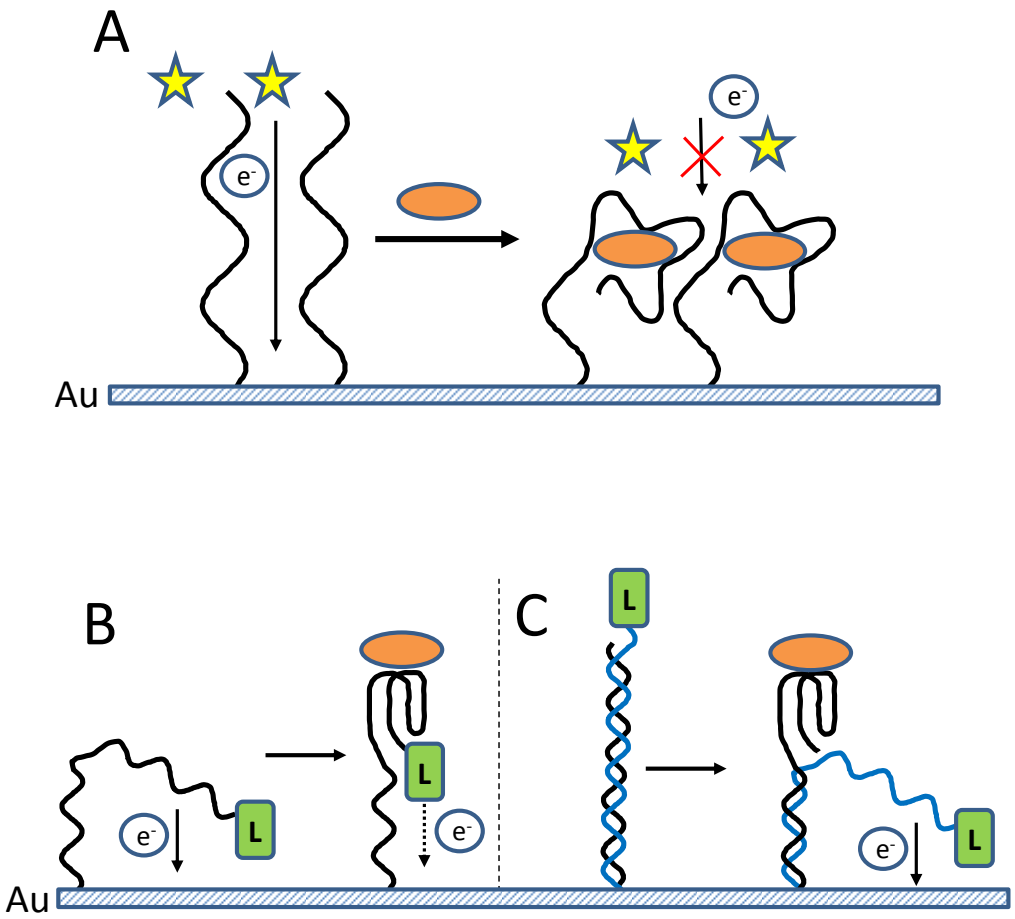


Figure 4

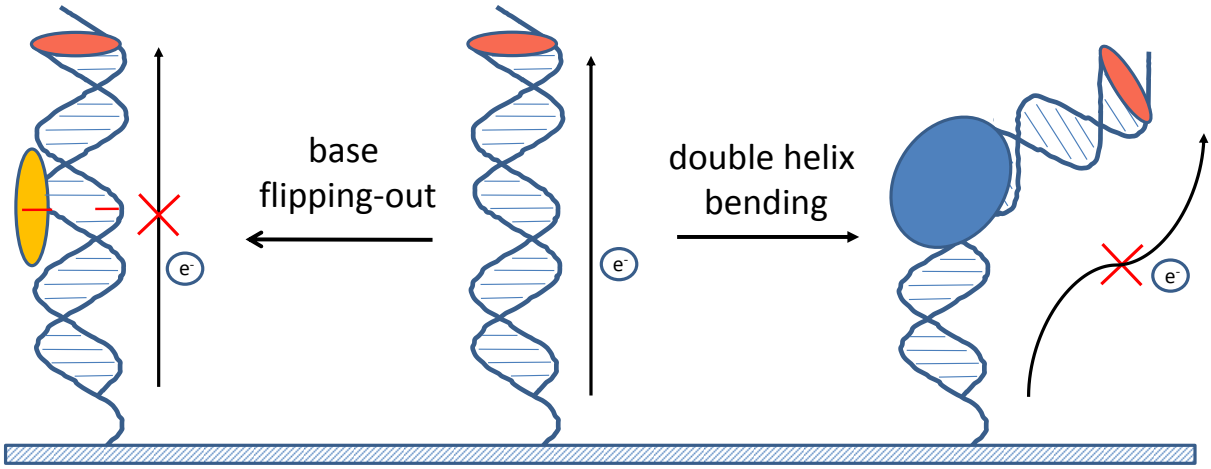
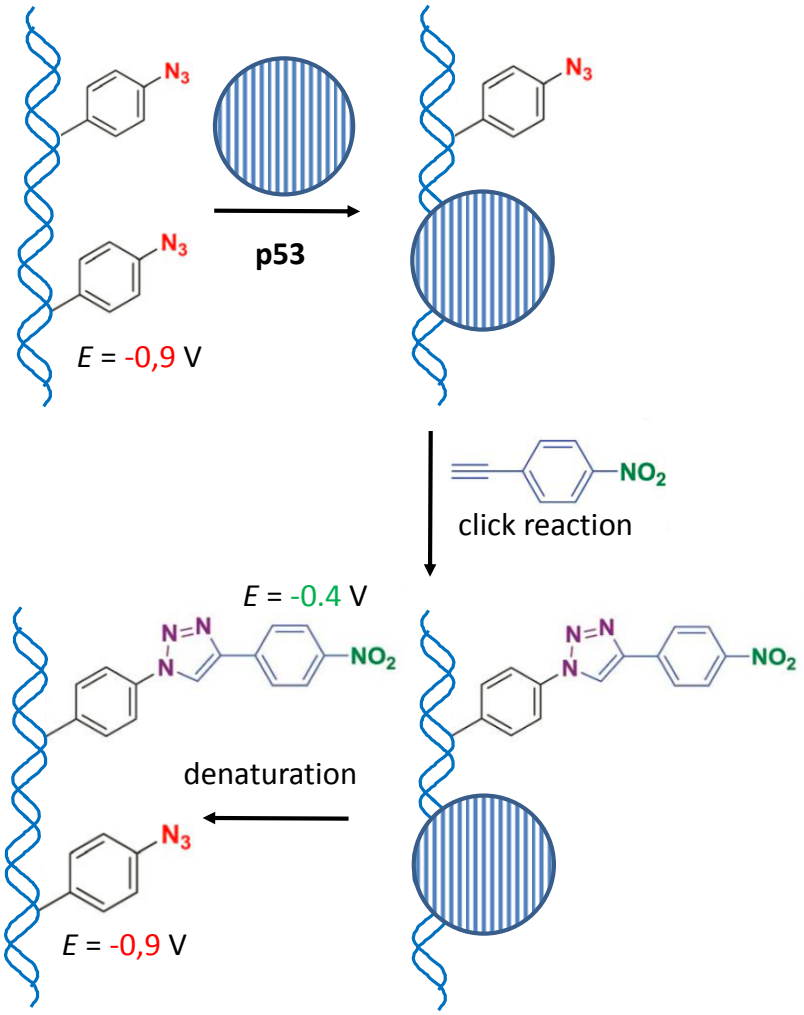


Figure 5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N3
p53
E = -0,9 V
N3
NO2
click reaction
E = -0.4 V
N=N
N
NO2
denaturation
N3
E = -0,9 V

Figure 6

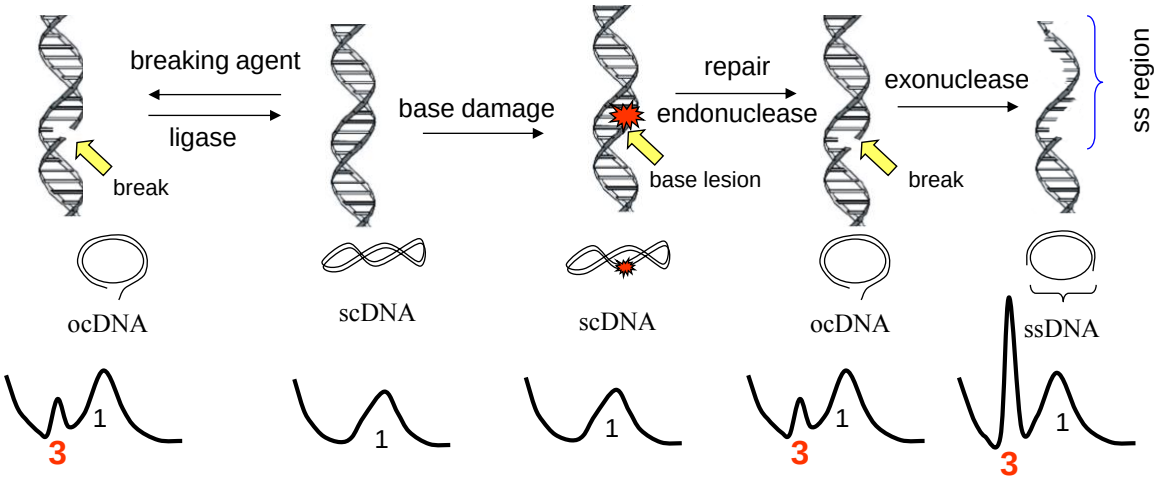
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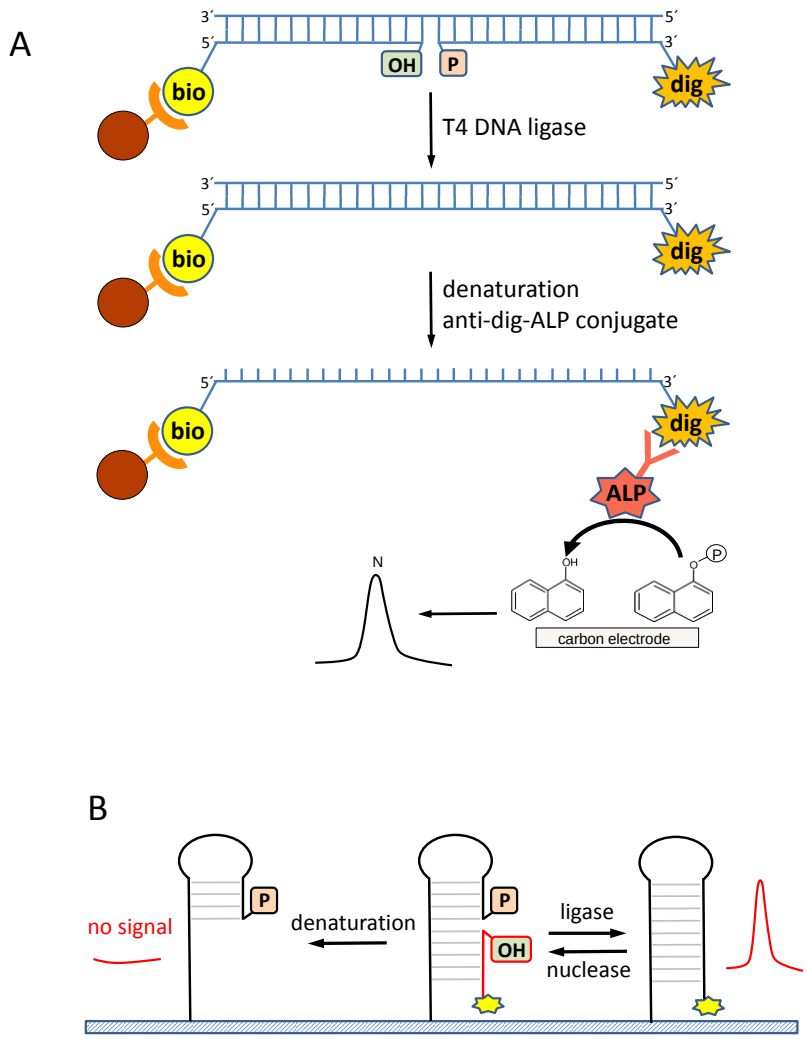
Figure 7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no signal
P
denaturation
ligase
nuclease
OH