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Development of a Sequence-Selective DNA Assay

Beata M. Kolakowski

A Thesis

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ABSTRACT

Development of a Sequence-Selective DNA Assay

Beata M. Kolakowski

The new assay employs single-stranded DNA bound to a microtiter plate, to which is added an unknown DNA sequence. Successful hybridization of a complementary sequence can then be demonstrated by the addition of a hybridization indicator. A number of indicators were tested, including ethidium bromide, 3,6-diaminoacridine (DAA), 9-aminoacridine (AAD), thionin, daunomycin, a histone H3-horseradish peroxidase conjugate, and a daunomycin-glucose oxidase conjugate. On the basis of its high molar absorptivity, strong association with DNA and the existence of an isosbestic point at a filter wavelength, DAA was chosen as the dye indicator. On the basis of its high enzyme activity, and strong signals, (daunomycin)$_4$-glucose oxidase was chosen as the enzyme-intercalant conjugate of choice.

Four methods of immobilization were attempted: a diazonium method, two carbodiimide methods and a biotin-avidin method. The biotin-avidin and carbodiimide (CovaLink^R) methods demonstrated comparable optimum DNA concentrations of 0.3 and 0.1 µg/ml, respectively, for immobilization with both intercalant (DAA) and intercalant-enzyme (daunomycin-glucose oxidase) detection. With optimized carbodiimide (CovaLink^R) immobilization, it was shown that enzyme detection (Abs = 0.303) generates a signal five times larger than that obtained with the dye (Abs = 0.056).

Methods of quantitating immobilized DNA were compared. On the basis of their detection limits, a chemical derivatization method using 3,5-diaminobenzoic acid and a voltammetric method were used to quantitate immobilized DNA.

ACKNOWLEDGEMENTS

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Abbreviations

A	Ampere
9-AA	9-aminoacridine
DAA	3,6 - diaminoacridine
DABA	3,5-diaminobenzoic acid
DM	daunomycin
DPA	diphenylamine
DPV	differential pulse voltammetry
dsDNA	double-stranded DNA
GOx	Glucose oxidase
ssDNA	single-stranded DNA

CHAPTER 1

INTRODUCTION

1.1 DNA Structure and Inherited Disease

Throughout history, the physical resemblances between siblings and between children and their parents have been observed. These observations were confirmed by the work of Mendel, who demonstrated that physical characteristics of offspring could be manipulated by selective breeding. For example, a line of tall plants would give rise to exclusively tall plants if two tall parents were crossed.[1]

In 1928, Griffith began experiments with the pneumococcus bacterium in mice. This bacterium can exist in an innocuous (nonencapsulated) form and in a lethal (encapsulated) form that can be deactivated by heating. Griffith found that a mixture of heat-treated encapsulated and nonencapsulated bacteria killed the mice, and postulated that the nonencapsulated bacteria were transforming themselves into encapsulated bacteria.[2] The "transformer" was fractionated and the activity of all constituents analyzed by Avery, MacLeod and McCarty.[3] Deoxyribonucleic acid (DNA) was determined and confirmed in further experiments to be the molecule of heredity.

The next major breakthrough was the elucidation of DNA structure by Watson and Crick in 1953. DNA was found to be a very long, threadlike polymeric macromolecule consisting of a great many nucleotide monomers. Each nucleotide possesses a nitrogenous base (guanine, cytosine, thymine or adenine), a sugar (deoxyribose) and a phosphate group. The sugar and phosphate groups play a structural

role, while the bases carry genetic information and permit the DNA to assume a helical structure. The DNA helix consists of two strands coiled around a common axis, as shown in Figure 1.1. The strands are held together by hydrogen bonds between bases - thymine and adenine form two hydrogen bonds while cytosine and guanine form three. The precise sequence of bases carries the genetic information.
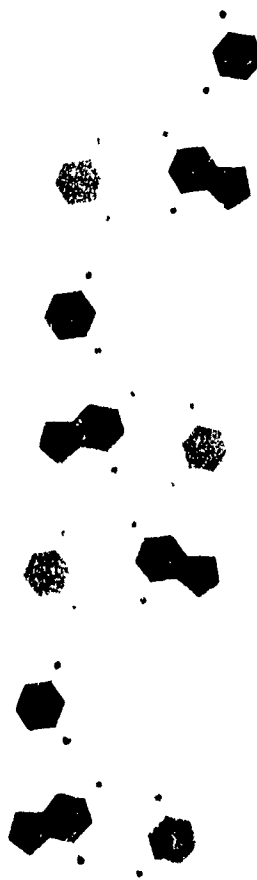
Figure 1.1. The DNA double helix with the hydrogen-bonding arrangement[4]

Changes in the base sequence of DNA in regions that code for proteins will alter the information transmitted. These changes or mutations can be harmless or even beneficial to the affected organism but many are lethal. In fact, a number of inherited diseases have been traced to specific changes in the base sequence. Examples of these diseases are shown in Table 1.1.

Table 1.1. Genetic Diseases in Humans.[5,6,7,8,9]

Disorder	Frequency/ 10^6 Births	Mode of Inheritance	Population Studied
Hypercholersterolemia	2000	Dominant	English Can. 4000 Fr.Can.
Sickle-Cell Anemia	1600	Recessive	Black
Cystic Fibrosis	500	Recessive	Caucasian
Thyroxine-Binding Globulin Deficiency	400	X-Linked	Male
Huntingdon's Disease	100	Dominant	European
Duchenne Muscular Dystrophy	77	X-Linked	Male
Osteodystrophy	72	Dominant	Caucasian
Phenylketonuria	64	Recessive	Caucasian
Hemophilia A	56	X-Linked	Male
Congenital Adrenal Hyperplasia	50	Recessive	Caucasian
Thalassemias	33	Recessive	Mediterr.

For example, cystic fibrosis is associated with a three base-pair deletion (ΔF508) on chromosome 7 while sickle-cell anemia results from the substitution of a thymine for an adenine residue in the β-globin gene. The high incidence of these and other inherited

conditions, and the rapid evolution of genetic therapies, makes safe, effective and rapid pre- and post-natal screening important. The aim of this work has been to develop an optical, sequence-selective DNA assay that exploits readily-available microtiter technology.

1.2 Sequence-Selective DNA Assays

A number of methods already exist for the diagnosis of disease based on DNA sequence detection. All make use of a DNA probe. This is a known sequence of at least five DNA bases (18 to several thousand bases are used in practice) to ensure that hybridization, when it occurs, is an exothermic process. The probe sequence is complementary to a target, or analyte DNA sequence, which may be the normal or the disease sequence. It is labelled with a chromophore, a fluorophore, a radioactive isotope or an enzyme. The labelling reaction can be performed by chemical modification, such as when DNA is labelled with fluorescein isothiocyanate, or by means of an enzymatic reaction, such as when ^{32}P-labelled nucleotides are added to DNA during incubation with DNase I and DNA polymerase I.[10]

1.2.1 Restriction Fragment Length Polymorphism

The first assay developed was the Restriction Fragment Length Polymorphism (RFLP), which employs the Southern blot. In this technique, DNA is incubated with restriction enzymes, which cleave native DNA into smaller, double-stranded fragments where they encounter a particular four- to eight-base sequence, known

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as a restriction site. Restriction fragment length polymorphisms result from sequence mutations or variations that occur at the restriction sites of the chosen enzymes, generating fragments that are either shorter or larger than those generated by the normal sequence. These polymorphisms are family-specific and can be used for genetic tracing, as well as disease detection. Once cleaved, the DNA fragments are separated electrophoretically on an agarose gel. The DNA is then transferred by a Southern blot to a nitrocellulose or nylon membrane.[11] A radioactively labelled DNA probe is then added and allowed to hybridize with its complementary sequence in the separated DNA fragments. In RFLP methods, a relatively nonselective DNA probe is employed, so that hybridization occurs with many fragments. Fragments that hybridize with the probe are detected as black bands on X-ray film by autoradiography while noncomplementary DNA shows no bands. Fragmentation patterns are thus used for diagnosis. Although this technique is very selective and useful, it has disadvantages of using radioactive isotopes, being very time-consuming and labour-intensive. Furthermore, RFLP is not universally applicable to diagnosis, because the disease-causing mutation must occur in a restriction enzyme's cleavage site.

1.2.2 DNA Amplification

The polymerase chain reaction (PCR) is a method for increasing or amplifying the amount of genetic material available for analysis. To the analyte DNA sequence are added the four deoxynucleotide triphosphate monomers, a thermostable DNA polymerase, and two "primer" oligodeoxynucleotides of about 20-base length. The primers

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are complementary to opposite DNA strands and bracket the region of interest in the analyte DNA. The first step in PCR is the denaturation of double-stranded DNA into single strands. This is done by heating the sample at 94°C. Primers are then annealed at 37-55°C, and in the final step (70°C), DNA catalyzes the extension of the primer sequences so that a copy of the analyte DNA is made. This sequence of three steps of denaturation, annealing and extension takes 2-4 minutes, and if repeated for twenty-five cycles, the amount of genetic material available for analysis is increased by one million fold.[12] PCR amplification can be made selective by a careful choice of primers, in the allele-selective amplification method, so that the presence or absence of a PCR product provides the assay result. PCR is also used in conjunction with most DNA sequence analysis methods.

1.2.3 Detection of Amplified DNA

The Dot Blot method was developed to overcome the need for electrophoresis and enzyme digestion. In this method, the DNA samples are amplified by PCR. The DNA is then immobilized by blotting onto a nylon or nitrocellulose membrane as a dot or slot. A labelled DNA probe is then added and allowed to hybridize. The presence of the label following rinse steps indicates the presence of the complementary sequence. Dot blots are commonly used in the detection of sickle-cell anemia.[13]

Amplified DNA can also be detected by the enzyme-linked oligonucleotide solution assay (ELOSA). In this method, the wells of a microtiter plate are first coated with streptavidin. Analyte DNA is then hybridized simultaneously to two DNA probes,

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one labelled with an enzyme and the other with biotin, which are complementary to different regions of the analyte DNA. Biotin interacts strongly with one of the four biotin-binding sites on streptavidin ($K = 10^{14}$ M^{-1})[14], thereby immobilizing both itself and the attached analyte DNA, which in turn is bound to the enzyme-labelled probe. Hybridization is then be detected following the addition of enzymatic substrate. The catalytic conversion of the substrate to the product results in a colour change that can be monitored spectrophotometrically with a microtiter plate reader.[14]

A recently developed method uses an anti-DNA monoclonal antibody which is specific for double-stranded DNA. Biotinylated probes were immobilized on streptavidin-coated polystyrene plates. The PCR-amplified analyte DNA was heat-denatured and incubated with the immobilized DNA. After rinsing, the anti-dsDNA was added and will react only with dsDNA. The bound antibody is detected by adding a HRP-labelled (horseradish peroxidase) rabbit anti-mouse IgG antibody. A substrate solution of tetramethylbenzidine/H_2O_2 was added and the reaction incubated in the dark for 30 minutes. After quenching the reaction with 1 N sulphuric acid, the absorbance was measured at 450 nm. This method has been successfully applied to the detection of the cystic fibrosis ΔF508 deletion.[29]

A recently introduced method, using the enzyme DNA ligase, can be applied to native or PCR-amplified DNA. In this method, two DNA probes are used, and these are complementary to immediately adjacent regions of one strand of the analye DNA, so that the 5' end of one is directly adjacent to the 3' end of the other. If the DNA sequence is exactly complementary across the junction, the two hybridized probes will be

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enzymatically ligated to form a single longer product strand. The product is detected by size following electrophoretic separation. The products of a successful ligation will appear as an intense band for the longer product and fainter bands due to unreacted probes. No ligated product will be present if the junction is noncomplementary, and intense bands for the unligated probes will be observed.[15]

These methods have been applied to the detection of viruses[16], of bacteria[17] and human DNA sequences. Table 1.2 shows some examples of DNA diagnostic methods.

Recent research in this laboratory[26-28] has demonstrated that DNA immobilized on carbon electrode surfaces can be detected using hybridization indicators; these are redox-active compounds such as tris(bipyridyl)cobalt(III), that associate selectively and reversibly with double-stranded DNA to preconcentrate in the DNA layer at the surface of the electrode. Hybridization of immobilized single-stranded DNA with its complementary (analyte) sequence can be detected voltammetrically, since peak currents for indicator reduction and oxidation are proportional to its local concentration at the surface of the electrode. An eight-fold increase in peak current for $Co(bpy)_3^{+3}$ reduction has been observed upon hybridization. This DNA biosensor with immobilized single-stranded DNA has been successfully applied to the detection of the cystic fibrosis $\Delta F508$ deletion.[28]

Table 1.2. Applications of DNA Detection Methods to the Diagnosis of Human Disease

Disease	Sequence	Detection Method	Ref.
AIDS	HIV-1 LAI Sequence	ELOSA	14
Fragile X	StB12.3 Sequence	Southern	18
Retinitis Pigmentosa	5'-CTGAGCTCAGGCCTTCGCAGCAT3' 5'-GGCAGTGGGGTCTGTGCTGAC-3' 5'-CAGCGCTCGGCAGCCACCTT-3' 5'-CTGGAGGAGCCATGGTCTGGA-3' 5'-CGTCCAGTTCCSAGCACTGTGG-3'	Southern + PCR	19
Hemoglobin E	5'-AGGGCCTTACCACCA-3'	Dot blot + fluores-cence	20
IDDM	5'-GGCGGCCTGTTGCCGSG-3'	Southern + PCR + ethidium	21
α_1-trypsin deficiency	5'GGCTGTGCTGACCATCGACG-3'	ELOSA	22
Non-Hodgkin's Lymphoma	5'-CCCTCCTGCCCTCCTTCCG-3' 5'-GGACCTTCCTTGGTGTGTTG-3'	Southern	23
Gaucher	5'-TACCCTAGAGCCTCCTGTA-3'	Dot Blot + fluor.	24
β-Thalassaemia	POSITION -101 C--> T POSITION -87 C--> G FRAMESHIFT CODON 6 (-A)	Southern	25

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1.2.4 Thesis Organization

The work described in this thesis is an optical counterpart to the voltammetric DNA biosensor. DNA is immobilized onto the well surfaces of microtiter plates, allowing up to 95 samples to be measured at a time. Optically-detectable hybridization indicators, such as intercalating dyes, allow the use of microtiter plate readers for detection by absorbance. This technology offers the advantages of ready automation of all steps such as reagent addition, washing and measurement steps, in addition to its adaptability to a clinical laboratory where enzyme-linked immunosorbent assays (ELISAs) are used extensively.

In our assay, single-stranded DNA is immobilized on a microtiter plate, and an analyte sequence is added and allowed to hybridize. Following rinse steps, intercalating dyes and intercalant-enzyme conjugates are used to distinguish between immobilized single- and double-stranded DNA.

Chapter 2 of this thesis concentrates on the development of a detection procedure. Two categories of indicators were investigated. The first category involves small DNA intercalants. The spectral properties of these species in the presence and absence of DNA, their association constants and the signal intensities observed with immobilized DNA were compared. The second category includes the products generated by coupling simple intercalants with enzymes. These species were characterized, and signal intensities observed for immobilized DNA with intercalant-enzyme conjugates were compared to those of the simple intercalating dyes.

Chapter 3 includes optimization and comparison of four DNA

immobilization procedures. These procedures were compared on the basis of the optimum concentrations of DNA during immobilization and hybridization and the signal increases observed on hybridization.

Chapter 4 is concerned with quantitating the amount of DNA immobilized. Methods were compared for their linear dynamic ranges, detection limits and reproducibility. Immobilized DNA was then quantitated.

Chapter 5 is a summary of the results presented in Chapters 2-4 and presents suggestions for future work.

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CHAPTER 2

DETECTION OF IMMOBILIZED DNA WITH DYES AND DYE-ENZYME CONJUGATES

2.1 Introduction

The sequence-selective DNA assay being developed in this work employs a DNA probe that is bound to the surface of a microtiter plate. Recognition of the analyte (target) DNA sequence results in hybridization, to form immobilized double-stranded DNA (dsDNA). The work described in this chapter is aimed at distinguishing between the immobilized single-stranded (ssDNA) probe, and the dsDNA product, using optically-detectable hybridization indicators.

An ideal hybridization indicator has the following characteristics: 1) it should bind selectively with dsDNA rather than ssDNA; 2) it should have a large association constant with dsDNA so that its binding is not significantly reversed in necessary wash steps; 3) as an absorbance signal is being monitored, the indicator should have a high molar absorptivity at the detection wavelength; 4) it should be commercially available or simple to prepare; 5) the indicator detection conditions should not be detrimental to DNA; and 6) it should be stable so that stock solutions can be prepared and used for many assays.

The first class of indicators studied were highly coloured organic dyes. These aromatic compounds interact with DNA through electrostatic interactions and by intercalation, that is, by insertion and stacking between the bases of DNA. This type of

interaction is generally accompanied by a red shift in the absorption spectrum of the bound dye in relation to the free dye, which results from the stacking interactions. The majority of these dyes can be detected either by absorbance or fluorescence measurements.[3] We have concentrated on five dyes: ethidium bromide, 3,6-diaminoacridine, 9-aminoacridine, daunomycin and thionin.[4] The structures of these DNA intercalants are shown in Figure 2.1.
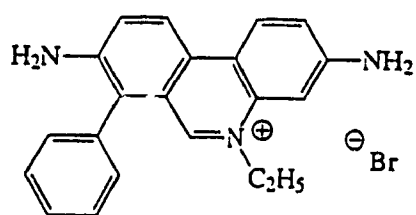
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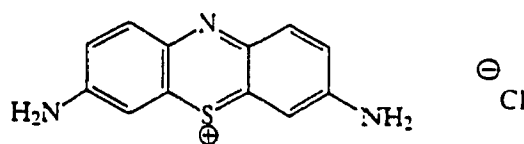
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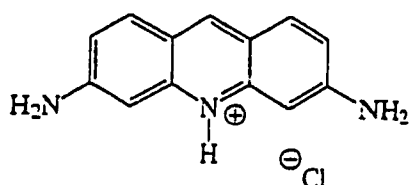
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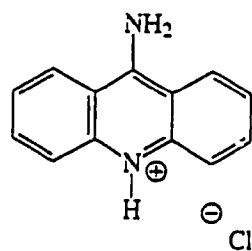
9-Aminoacridine Hydrochloride
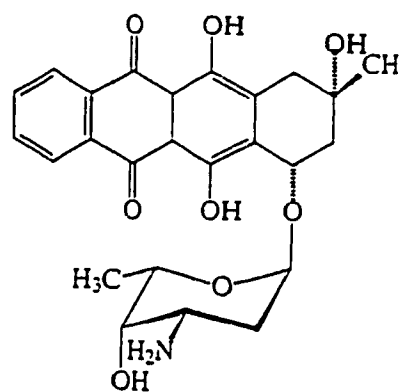
Daunomycin

Figure 2.1. The structures of the five dyes.

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As mentioned previously, an ideal hybridization indicator will generate strong signals at low concentrations, to allow easy and accurate detection of double-stranded DNA. With the knowledge that enzymatic systems tend to have lower detection limits than are usually possible with standard fluorometric or colorimetric techniques, due to their ability to generate multiple copies of an absorbing product, attention was focused on developing a detection system that could combine the selectivity of a DNA-binding species (like an intercalating dye or a histone protein) with the high specific activity and hence low detection limit of an enzyme. Ideally, the DNA-binding species and the enzyme would be combined in a complex whereby the intercalant has unlimited access to its normal binding sites in the DNA helix and the enzyme, with undiminished activity, remains outside the duplex. With the addition of substrate, catalytic conversion of substrate to product occurs and the appearance of a coloured product or the disappearance of a coloured reagent provides an amplified signal. As such a system is not available commercially, the synthesis of suitable enzyme-labelled species has been attempted.

Horseradish peroxidase (HRP) is a glycoprotein of 40 kD molecular weight, and is extensively used as a label in clinical and immunological assays.[6] It catalyzes the oxidation of dyes, such as o-dianisidine, by hydrogen peroxide, as shown below:

$$\text{H}_2\text{O}_2 + o\text{-dianisidine} \xrightarrow{\text{HRP}} \text{oxidized dye} + \text{H}_2\text{O} \qquad (1)$$
$$(\lambda\text{max} = 436 \text{ nm})$$
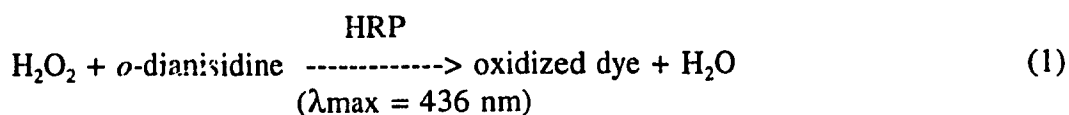
An attempt to prepare a histone (H3)- HRP conjugate involves modifying HRP with 1-fluoro-2,4-dinitrobenzene to block reactive amine groups, and oxidizing its polysaccharide

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with periodate to generate aldehyde groups which are reactive towards histone H3 amine groups. Sodium borohydride is used to reduce the Schiff base formed by reaction of the aldehyde with the amine groups.

Histones are DNA-binding proteins known to be rich in lysine and arginine residues. *In vivo*, they bind to double-stranded DNA as an octamer of 5 different proteins (H1, H2A, H2B, H3 and H4) through electrostatic interactions between the positively charged proteins and the negatively charged DNA backbone. H3 and H4 , being particularly enriched in lysine and arginine residues, are actively involved in binding to DNA while H2A and H2B stabilize this binding. This protein-associated DNA is the normal state of chromatin. Histone H3 is a 14.8 kD protein with ten lysine residues and fourteen arginine residues. Binding of H3 to DNA, which is mainly through arginine groups with the stabilizing influence of the lysine residues, can be reversed by a wash with a high ionic strength buffer[2] (in cortrast with, for example, antibodies to dsDNA). Histone H3 was bound to HRP and tested as a hybridization indicator.

Glucose oxidase (GOx) was also tested for use in a hybridization indicator. This enzyme is a 160 kD dimer isolated from *Aspergillus niger* with a very high selectivity for β-D-glucose. GOx possesses 66 free carboxylic acid groups on the surface of each monomer that can be used to covalently bind an intercalating dye, such as daunomycin, which possesses a primary aliphatic amine group. The preparation of these conjugates, and their characterization, used methods already in use in this laboratory.[11,12]

Daunomycin-glucose oxidase activity was measured in the presence of excess β-D-glucose, where hydrogen peroxide being formed as glucose is being oxidized.

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This peroxide, in turn, is reduced to water as the o-dianisidine dye is oxidized to an orange product that can be detected spectrophotometrically at 436 nm or with a 450 nm filter.

$$H_2O + O_2 + glucose \xrightarrow{\quad GOx \quad} H_2O_2 + gluconic\ acid \qquad (2)$$

$$H_2O_2 + o\text{-dianisidine} \xrightarrow{\quad GOx \quad} OXIDIZED\ DYE + 2H_2O \qquad (3)$$
$$\lambda_{max} = 436\ nm$$
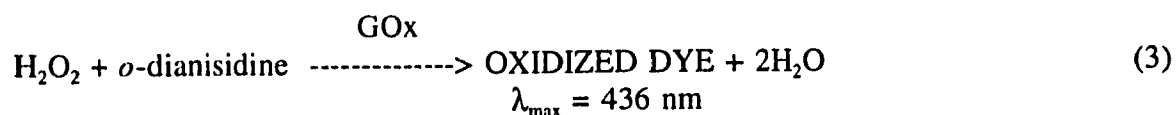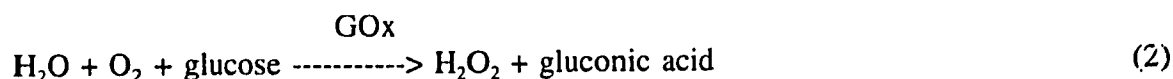
The enzyme conjugates were evaluated for their suitability for use as hybridization indicators in the sequence-selective DNA assay. The signals generated by the enzyme-intercalant complexes were compared to those generated with the intercalants alone.

2.2 Experimental

2.2.1 Materials

Histone H3 (Lot 262C8235), 3,6-diaminoacridine, 9-aminoacridine hydrochloric acid and thionin were purchased from Sigma. Two batches of ethidium bromide were used, one from Aldrich and one from Sigma. Horseradish peroxidase (Lot 53H9588) was purchased from Sigma. 1-fluoro-2,4-dinitrobenzene and sodium periodate, both A.C.S. grade, were purchased from Aldrich. Sephadex columns and packings were from Pharmacia. Sodium borohybride was obtained from Fisher. Glucose oxidase (Lot

12635525-33) from Boehringer Mannheim was used for preparing all daunomycin-GOx

conjugates and was used as received. The daunomycin was purchased from Sigma.

Anhydrous α-D-glucose (A.C.S. reagent) was bought from Sigma and was used for all

activity assays. o-Dianisidine dye was purchased from Sigma as were all buffer salts used

in buffer preparations including the acid and base forms of

tris(hydroxymethyl)aminomethane (Tris), and monobasic and dibasic potassium phosphate.

Sodium chloride was from Fisher. Ammonium sulphate (A.C.S. reagent grade) was

obtained from Anachemia. Coomassie blue was received from Bio-Rad and sodium

cyanoborohydride was from Aldrich. Calf thymus DNA (lot 79F9680 and 53H9536) was

obtained from Sigma. The dialysis membranes with a molecular weight cutoff of 5000 D

were obtained from Pharmacia.

The instrumentation used throughout was the Varian Cary 1 UV/visible

spectrometer, the DynaTech MR580 microplate reader and the BioTek EL307C microplate

reader. In addition, Amicon microconcentrators were used with Prod-Air ultrahigh purity

nitrogen as a gas pressure source. Nanopure water from the Sybron-Barnstead Nanopure

ion-exchange system was used throughout.

2.2.2 Methods

2.2.2.1 **Spectrophotometric Investigation of the Intercalants**

Solutions containing ethidium bromide, 3,6-diaminoacridine, 9-

aminoacridine, thionin and daunomycin, at concentrations of 5.148×10^{-4} M, 7.5×10^{-4}

M, 2.52 x 10^{-4}, 1.218 x 10^{-5} M and 1 mM, respectively, were prepared in 0.1 M potassium

phosphate, pH 7.0 buffer containing 50 mM NaCl. A solution containing 3.03 mM (in

base-pairs) of calf thymus DNA was prepared in the same buffer. The dyes, diluted 1:1

with either the DNA solution or with buffer, were scanned from 190 to 800 nm using

phosphate buffer as the blank.

2.2.2.2 UV-Visible Spectrophotometric Determination of the Association Constant of Thionin with DNA[5]

To determine the association constant, the quartz cuvette was filled with

2.500 ml of 14.8 μM thionin in 0.100 M phosphate buffer, pH 7.0. The initial absorbance

values at 600 and 616 nm were recorded. Aliquots of a 4.546 mM (base-pairs) calf

thymus DNA solution dissolved with the same concentration of dye in phosphate buffer

were added to the cuvette (25 μl at a time for 1.000 ml, then 100 μl aliquots for an

additional 1.500 ml) and the absorbance values recorded. With this method, the

concentration of dye remains constant and the concentration of DNA increases with each

addition. The association constant for the binding of thionin with DNA was determined

using the McGhee-von Hippel equation. This equation uses as a model for DNA a

homogeneous lattice consisting of a linear array of N identical repeating units. The ligand

(thionin) binding to the lattice covers n residues. Thionin is assumed to bind to all DNA

sequences with comparable affinities, and co-operativity in binding at adjacent sites is

assumed to be absent. The McGhee-von Hippel equation[5], in the form of a Scatchard plot,

is

$$r/c_{free} = K*(1-nr)*[(1-nr)/(1-(n-1)*r)]^{n-1}, \text{ where} \qquad (4)$$

$$r = c_{bound}/[BP]_T \qquad (5)$$

The free and DNA-bound dye concentrations, c_{free} and c_{bound}, are determined from absorbance measurements at the λmax values for the free and bound forms, using the molar absorptivities of the free and bound forms at each wavelength. A computer program (as presented in Appendix 1) calculated the concentration of free and bound dye after each addition of DNA. Having determined the values of c_{free}, c_{bound} and knowing the values of the total DNA base pair concentrations, $[BP]_T$, a Scatchard plot of c_{free} vs. r could then be prepared. Nonlinear regression was used to determine the association constant and the binding site size.

2.2.2.3 Preparation of the HRP-H3 Conjugate[6]

Horseradish peroxidase (5 mg) was dissolved in 1.00 ml of freshly prepared 0.3 M NaHCO$_3$, pH 8.1. To this was added 0.100 ml of a solution of 1 µl 1-fluoro-2,4-dinitrobenzene in 99 µl of absolute ethanol. In a sealed reaction vial, the reagents were stirred continuously at low speed for one hour at ambient temperature. Aqueous sodium periodate solution (1.00 ml, 0.08 M) was added and the reaction was incubated with stirring for 0.5 hour at room temperature. Then, 1.00 ml of 0.16 M solution of ethylene glycol was added. The sample was left for 2.5 hours. The reaction was dialysed against 3 x 1 L of 0.01 M Sodium bicarbonate buffer, pH 9.5, at 4°C, over 18 hours.

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The dialysed product was transferred to a vial, and 5 mg of histone H3 were added. The reaction proceeded, with stirring, for three hours at room temperature. NaBH$_4$ (5 mg) was added and the reaction continued overnight at 4°C. Following dialysis of the product for 3 hours, 6 hours and overnight against PBS buffer (150 mM NaCl + 150 mM sodium phosphate, pH 7.2), any precipitate formed was removed by centrifugation.

The product was pre-concentrated using an Amicon YM-30 filter. A 2.00 ml sample of the conjugate was purified using an 85 x 1.5 cm Sephadex G-100 column and eluted with PBS buffer. Fractions containing HRP were collected and characterized by the absorbance at 403 nm which corresponds to the wavelength maximum of the heme group of HRP. The aliquots were then stored at -20°C.

2.2.2.4 Activity Assay of the H3-HRP Conjugate[10]

The following solutions were prepared: a 0.800 µg/ml solution of enzyme based on heme content (modified and native), a 10 µg/ml solution of *o*-dianisidine, and a 0.015% solution of hydrogen peroxide in 0.1 M phosphate, pH 7.0 buffer.

Into disposable cuvettes were added 1.90 ml of buffer, 1.00 ml of peroxide solution, 10.0 µl of enzyme solution and 100 µl of dye solution. After 20 minutes, 10.0 µl of concentrated hydrochloric acid were added to stop the reaction and the absorbance was read at 460 nm against a blank containing everything but the dye solution. It is important to add the solutions in the order mentioned to prevent precipitation.

2.2.2.5 Preparation of Daunomycin-Glucose Oxidase Conjugates[11,12]

<u>Method A</u>

Daunomycin (8 mg) and glucose oxidase (94 mg) were dissolved in 40 ml of 0.1 M potassium phosphate buffer, pH 7.0. 5 ml of this solution was saved as a control. To the remaining 35 ml were added 36 mg of sulfo-NHS and 0.6 g of EDC. The reaction and control solutions were sealed with aluminum foil and incubated at 5°C overnight.

An Amicon microconcentrator (YM-30, 30kD cutoff filter) was used to concentrate the product mixture to 3.00 ml. This sample was applied to an 85 x 1.5 cm Sephadex G-15 column. When eluting with 0.1 M potassium phosphate, pH 7.0 buffer, two distinct bands were observed, the first red-orange band containing the species of interest.

<u>Method B</u>

Deglycosylation of the enzyme, to generate aldehyde groups to react with the aliphatic amine of daunomycin, was accomplished by incubating 50 mg of glucose oxidase in 5 ml of 0.1 M sodium periodate at 4°C for 5 h. The reaction was then quenched by 1.00 ml of ethylene glycol. After 30 minutes at room temperature, the sample was applied to a G-25 column and eluted with 50 mM sodium phosphate, pH 7.5. The first of the three bands was collected.

To this fraction was added 1.4 mg of daunomycin, 25 mg of sodium cyanoborohydride and 10 ml of 0.1 M potassium phosphate, pH 7.5. The reaction vial was

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covered with aluminum foil and incubated overnight at 4°C. The sample volume was then reduced to 3 ml by ultrafiltration (YM-30) and applied to a G-25 column and eluted with phosphate buffer. The first band was collected.

2.2.2.6 Characterization of the Daunomycin-Glucose Oxidase Conjugates

2.2.2.6.1 Stoichiometry

Quantitation of daunomycin and total protein in the reaction products was done by spectrophotometry to find the average number of daunomycin molecules bound to glucose oxidase.

Daunomycin was quantitated by measuring the absorbance at 476 and 550 nm, using 0.100 M phosphate buffer, pH 7.0 as a blank. The concentrations were obtained from Beer's law, using molar absorptivities of $\varepsilon_{476} = 9.62 \times 10^3 M^{-1}cm^{-1}$ and $\varepsilon_{550} = 5.56 \times 10^3 M^{-1}cm^{-1}$.[11]

To determine the enzyme concentration, a stock solution of 27.5 μM glucose oxidase was prepared. A range of concentrations, from 0.55 μM to 2.75 μM, was then prepared to 800 μl 0.1 M potassium phosphate buffer, pH 7.0. After the addition of 200 μl of Coomassie Blue reagent, vortexing and incubation for five minutes, the absorbance was read at 592 nm. A standard curve was prepared of absorbance as a function of enzyme concentration. The amount of protein in the conjugate was determined using a 50 μl aliquot diluted to 800 μl with buffer, submitting it to the same conditions

and comparing its absorbance to the standard curve.

2.2.2.6.2 Activity Assay[14,15]

Into a quartz cuvette was placed, 2.50 ml of 0.1 M potassium phosphate

buffer, pH 7.0 containing 70 µg/ml

o-dianisidine, 0.500 ml of 1 M glucose in phosphate buffer, 10 µl of peroxidase solution

(8.16 µg/ml in 3.2 M ammonium sulphate), and 50 µl of native or modified glucose

oxidase solution. Absorbance was monitored over three minutes at 436 nm against a blank

of all components, except the dye.

Activities were determined in the presence and absence of 1.52 mM (base-

pairs) of calf thymus DNA.

2.2.2.7 Immobilization and Hybridization of DNA in Microtiter Plates

2.2.2.7.1 Diazonium Method[7]

The wells of a polystyrene microtiter plate were first modified by a 47%

(v/v) concentrated nitric acid in concentrated sulfuric acid at 4°C for 20 minutes to

generate nitro groups, then reduced with a 6% (w/v) $Na_2S_2O_8$ at 70°C for 4 h to generate

primary, aromatic amines and reacted with a 1.25% sodium nitrite solution for 15 minutes

at 0°C to yield diazonium groups. These groups can react with brominated DNA bases.

A 3 µg/ml poly(dT) solution was incubated on ice for ten minutes with an

8 mM N-bromosuccinimide. 200 µl of this brominated DNA was added to the wells and

incubated overnight at 5°C. The excess was then rinsed away with water.

2.2.2.7.2 Carbodiimide Method[8]

This method immobilizes DNA to secondary amine groups present on CovaLink[R] plates by a phosphoramidate bond with the 5'-terminal phosphate group.

To an aqueous 3 or 10 μg/ml ssDNA or dsDNA solution was added 100 mM 1-methylimidazole buffer to a final concentration of 10 mM. To each well was added, 200 μl of the DNA solution and 67 μl of 0.2 M EDC in 10 mM 1-methylimidazole buffer. The plate was covered with parafilm and incubated at 50°C for 5 h. The plates were washed three times with 0.1 M potassium phosphate buffer for dsDNA or three times with 0.4 N NaOH + 0.25% SDS and three times with water for ssDNA.

2.2.2.7.3 Hybridization of DNA

The complementary ssDNA solutions were prepared in 0.1 M phosphate buffer containing 50 mM NaCl. 200 μl was added to each well and incubated overnight at 42°C for carbodiimide immobilization or at room temperature for 1 h for the diazonium method. The excess is rinsed away with buffer.

2.2.2.8 Detection Procedure in the Dye-Based Assay

A 1.5 mM 3,6-diaminoacridine (DAA) solution in 0.100 M potassium

phosphate buffer, pH 7.0 containing 1 mM NaCl was prepared. This was used as the pre-stain solution. 200 µl was added to the wells and incubated for 30 min at room temperature. This dye was replaced by 200 µl of 15 µM DAA in the same buffer. The absorbances of the wells were then measured at 450 nm against a blank of 200 µl of buffer. Control wells containing no DNA and DNA wells not exposed to the pre-stain solutions were included in the plate. This procedure was repeated 3 more times.

When determining the optimum concentration of dye to be used, this procedure was amended somewhat. The detection procedure was used before and after hybridization of immobilized ssDNA. Excess dye was removed (for hybridization) by rinsing the plate with deionized water at 100°C. This yielded data describing the interaction of the dye with both single- and double-stranded DNA and hence, the concentration that yielded the best discrimination between the two forms could be determined by comparison.

2.2.2.9 Detection with the Daunomycin-GOx Conjugate

GOx-catalyzed reactions were detected with o-dianisidine-HRP method. The wells of a microtiter plate were modified with either ssDNA that was subsequently hybridized or ds DNA. The wells were blocked with 12.5 µg/ml avidin overnight at 4°C, and the excess was rinsed away with buffer. The wells were blocked a second time the same way with 100 µg/ml bovine serum albumin. 300 µl of 2.75 µM solution of the daunomycin-GOx conjugate were added to each well. The plate was covered and incubated at 4°C for two hours to allow intercalation. Excess conjugate was removed in

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five rinse steps with 0.1 M potassium phosphate buffer, pH 7.0.

Detection was carried out in[14,15] 0.100 M potassium phosphate buffer, aerated for fifteen minutes prior to use. A 7 mg/ml solution of o-dianisidine was prepared, and a 0.500 ml aliquot is diluted to 50 ml with the aerated buffer. To this dilute dye solution were added, 3.58 ml of the aerated buffer and 4.60 ml of 1 M glucose in phosphate buffer. The indicator enzyme, peroxidase, was prepared as a 10 mg/ml stock solution in 3.2 M $(NH_4)_2SO_4$. 40 μl of this enzyme solution was mixed with 160 μl of 3.2 M ammonium sulphate, and 163 μl was added to the dye-glucose solution. After stirring, 250 μl of this solution was quickly added to each well and the plate was sealed to prevent atmospheric oxygen from interfering with the reaction. After 1, 2 and 24 hours, the absorbance of the wells at 450 nm was measured, using 250 μl of phosphate buffer as the blank and including controls which had not been incubated with modified glucose oxidase.

2.3 Results

2.3.1 Spectral Properties of Intercalants in the Presence and Absence of DNA

Figure 2.2 shows a typical UV/visible spectrum of an intercalating dye in the presence and absence of calf thymus DNA. In this case, the spectra of 0.75 mM DAA in the presence and absence of 1.52 mM DNA are presented. It can be seen that the free dye's λ_{max} is at 443.00 nm while that of the bound dye is 460 nm. Thus, the association of the dye with DNA causes a 17 nm shift in the spectra.

Similar data and the molar absorptivities of these dyes at the filter wavelengths of the microtiter plate reader are presented in Table 2.1.

Table 2.1. Spectral Properties of Intercalating Dyes[a]

Dye	λmax,nm Free	λmax,nm Bound	ε_{bound} 405[b]	ε_{bound} 450[b]	ε_{bound} 490[b]	ε_{bound} 570[b]	ε_{bound} 630[b]
Ethidium	484	520	206	578	766	334	0
DAA	443	460	427	2066	193	5	7
9-AA	422	429	1165	0	0	0	0
Daunomycin	479	510	88	142	251	29	7
Thionin	600	616	290	493	2027	18070	25630

[a] In 0.1 M phosphate buffer, pH 7.0, with 50 mM NaCl at 22°C.
[b] In the presence of calf thymus DNA, 1.52 mM base pairs

Another important consideration is the strength of interaction of the intercalants with DNA. Association constants, determined spectrophotometrically using the McGhee-von Hippel equation[5], are shown in Table 2.2. The values for thionin were determined in this work, and it is shown in Figure 2.3.

Table 2.2. DNA-Binding Properties of Intercalating Dyes[a]

Dye	K_a, M^{-1}	n, base pairs	Reference
Ethidium	1.07×10^5	2.9	4
DAA	1.00×10^5	2.8	4
9-AA	2.10×10^5	3.7	4
Daunomycin	2.6×10^6	3.0	16
Thionin	$(4.3 \pm 0.2) \times 10^4$	2.9 ± 0.1	this work

[a] 5 mM Tris buffer, pH 7.0 with 50 mM NaCl for ethidium, DAA, 9-AA
0.1 M sodium phosphate buffer, pH 7.3 + 0.001 M Na_2EDTA for daunomycin
0.1 M phosphate buffer, pH 7.0 for thionin
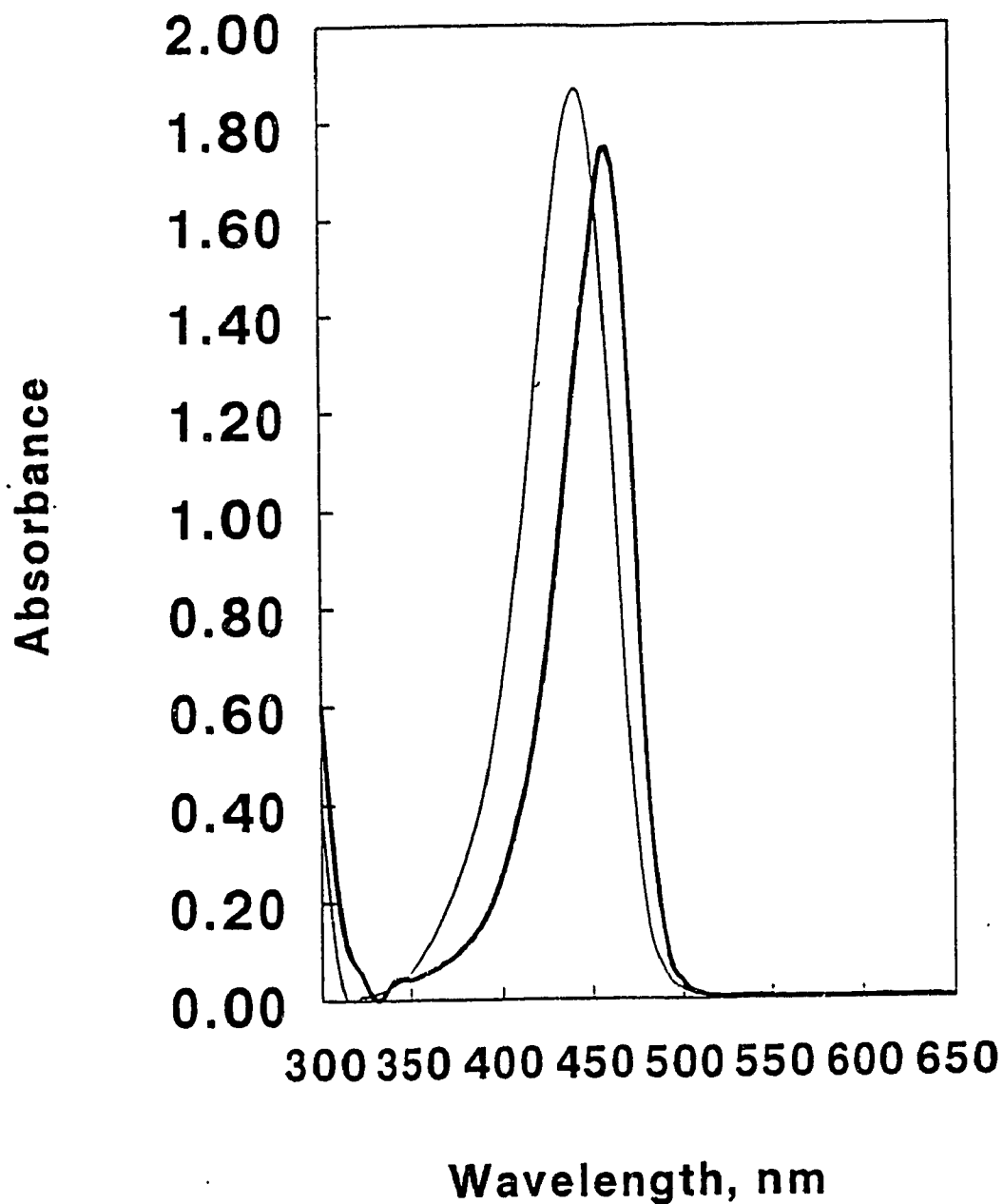
Figure 2.2. A typical spectrum of an intercalating dye in the presence and absence of DNA. The spectra of 0.7500 mM 3,6-diaminoacridine in 0.1 M potassium phosphate buffer, pH 7.0 + 50 mM NaCl in the presence and absence of 1.520 mM DNA in terms of base-pairs. The λmax (free) = 443.00 nm and λmax (bound) = 460.00 nm.
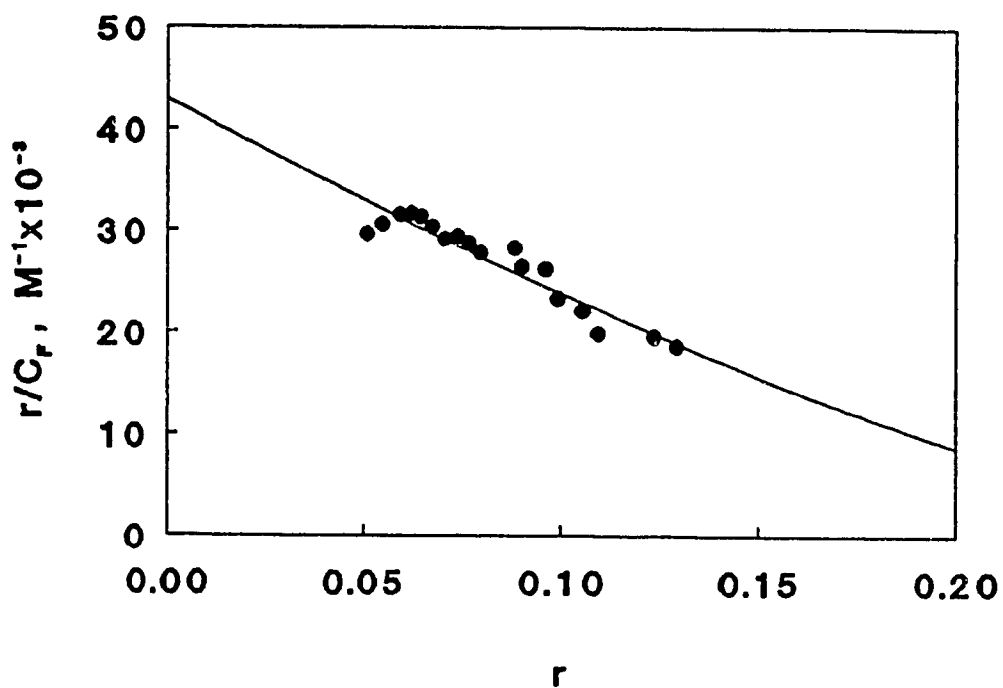
Figure 2.3. Scatchard plot for the binding of thionin to calf thymus DNA. Fitted line (according to equation 5) is for $K = (4.3 \pm 0.3) \times 10^4$ M^{-1} and $n = 2.9 \pm 0.1$ base pairs.

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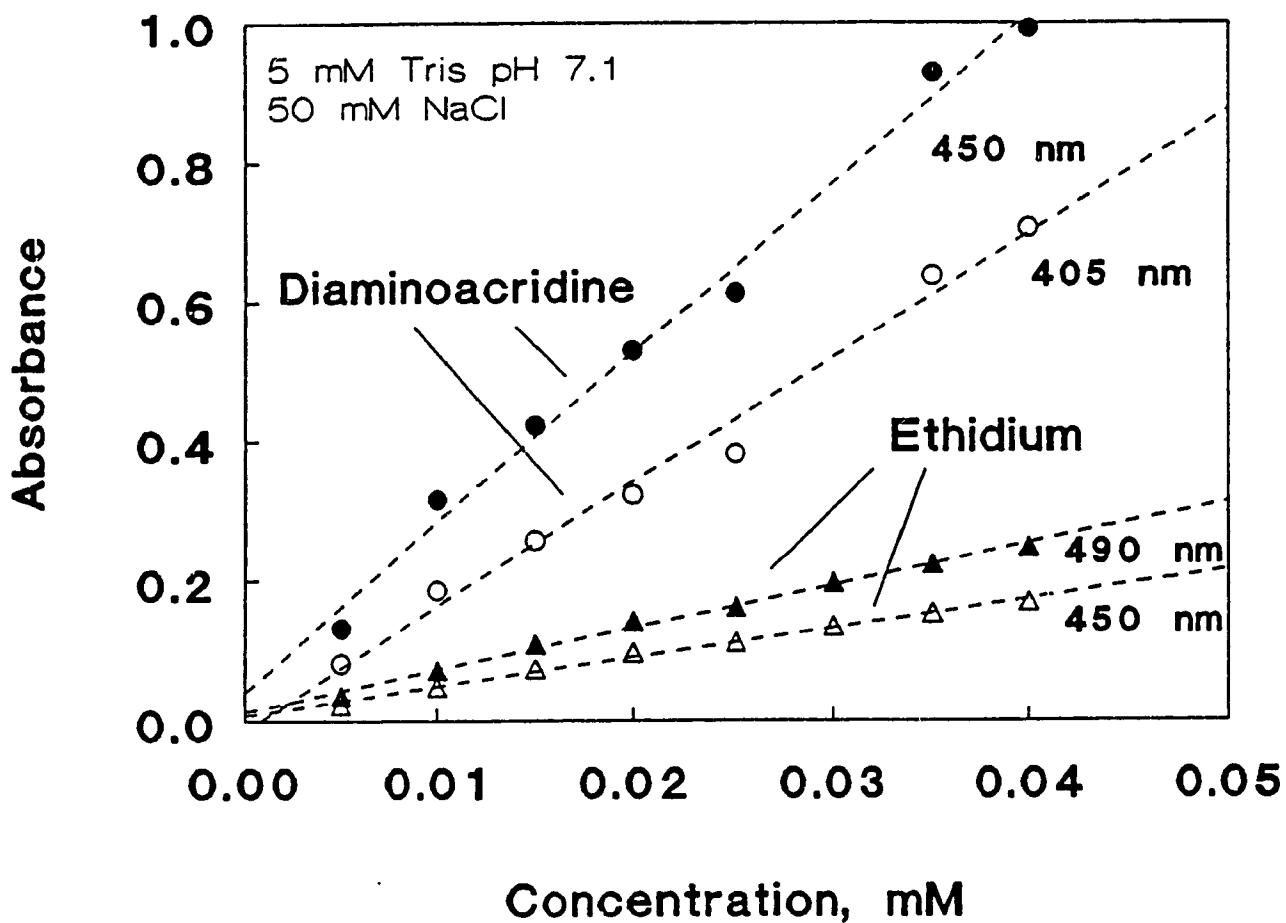
Figure 2.4. A Beer's law plot for 3,6-diaminoacridine and ethidium bromide in 5 mM Tris, pH 7.1 + 50 mM NaCl over the concentration range of 5-40 µM at the indicated filter wavelengths. 300 µl of solutions were used in polystyrene microtiter plates.

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Of these small intercalants, ethidium bromide and DAA offer the strongest signals at the filter wavelengths of 405 nm (DAA), 450 nm (DAA and ethidium), and 490 nm (ethidium) and associate strongly with DNA. These dyes were further tested to determine the concentration ranges over which they obey Beer's law at the wavelengths of available filters. Because of the positions of their maxima, ethidium was studied at 490 and at 450 nm and DAA at 405 and 450 nm. Figure 2.4 indicates linearity up to 40 μM for both species, but the 3,6-diaminoacridine yields a 5-fold larger signal at 450 nm as confirmed by the results in Table 2.1. This observation, in conjunction with the fact that 450 nm falls near an isosbestic point for the free and bound dye, was the reason this dye was selected as the hybridization indicator.

2.3.2 Detection of Immobilized DNA with 3,6-Diaminoacridine

The optimum concentration of DAA to use in the DNA assay is that which discriminates best between ssDNA and dsDNA. therefore, the dye detection method was used before and after hybridization of poly(dT), bound to microtiter plates by the diazonium method, with its complementary homopolymer, poly(dA). The pre-stain contained 1.5 mM DAA, while the concentration of DAA in the measurement solution was varied from 5 to 40 μM. Figure 2.5 shows a plot of the fractional increase in absorbance, (ADS-ASS)/ASS, as a function of DAA concentration. A clear optimum is observed at 15 μM dye. These results were obtained at 405 nm rather than the preferred 450 nm due to instrumental problems that made the latter wavelength unavailable. When these experiments were repeated with detection at 450 nm using the carbodiimide
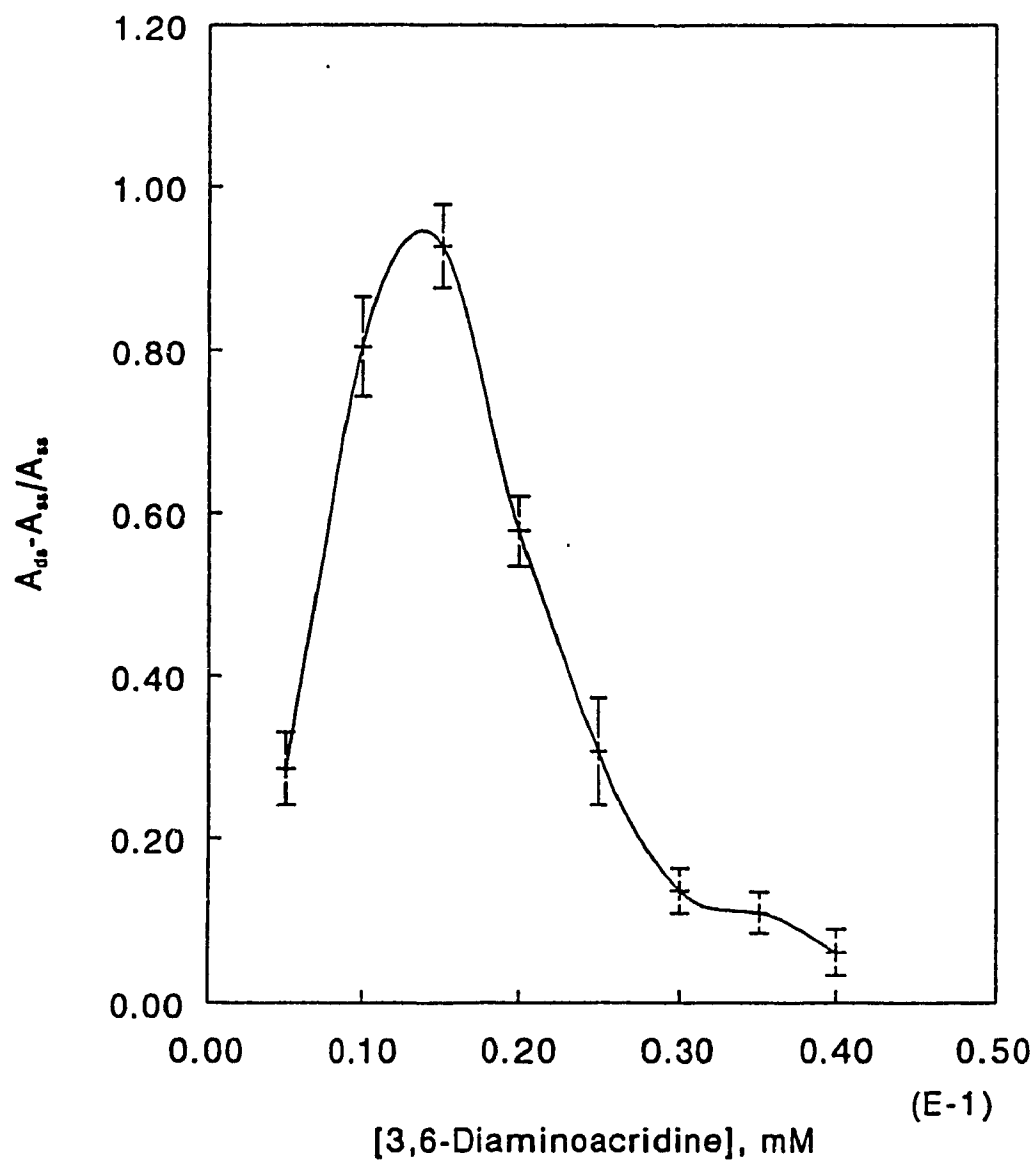
Figure 2.5. A plot of the fractional increase in absorbance upon hybridization as a function of dye concentration. 3 µg/ml of poly(dT) was immobilized by the diazonium method and A_{ss} was determined. It was then hybridized to 1 µg/ml of poly(dA) and the signal detected by the same procedure. Each point is the average of 8 measurements.

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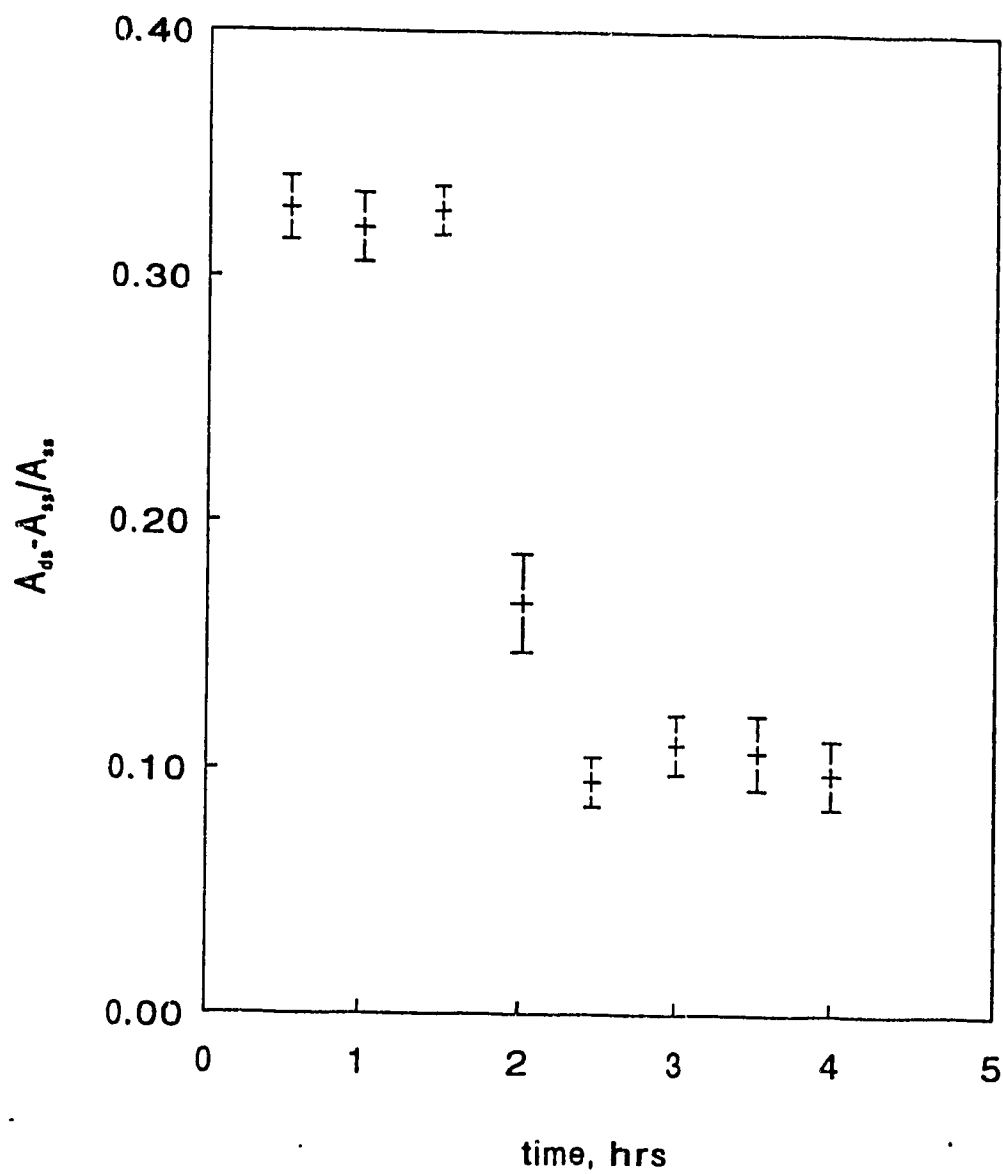
Figure 2.6. A plot of the fractional increase in absorbance upon hybridization as a function of dye exposure time. 3 µg/ml of poly(dT) was immobilized by the carbodiimide method and the background signal determined with the normal staining procedure. It was then hybridized to 1 µg/ml of poly(dA) and the signal detected by the same procedure. Replicates (n=8) were done at each exposure time. Error bars represent one standard deviation.

immobilization method, the optimum was also observed in the region of 10-20 µM over 10 plates, so that 15 µM was chosen as the best concentration.

Figure 2.6 shows the effect of exposure time to the pre-stain solution, using carbodiimide-immobilized DNA. No real difference was observed over the range of 30 - 90 minutes, but this signal is lost as the dye is exposed for longer periods of time. The drop may be due to nonspecific interactions of the dye with the microtiter plate, or may be due to DNA loss during rinse steps, which may be promoted by dye exposure.

Although the dye method was able to distinguish between ssDNA and dsDNA, the changes in absorbance values were actually quite small. In an effort to improve signal intensities on hybridization, intercalant-enzyme complexes were prepared and tested for their ability to detect duplex DNA.

2.3.3. Characterization of H3-HRP

To determine the activity of the enzyme, solutions containing equal concentrations of the native and modified HRP based on the heme absorbance were prepared. The activities of these solutions were determined in the presence of H_2O_2 and o-dianisidine by measuring absorbance at 460 nm as a function of time.[10] Table 2.3 presents the average (n = 3) absorbance changes observed over 3 minutes for these species and their relative activities. Due to the low (17%) activity of the HRP-H3 conjugate, it was not investigated further.

Table 2.3. The Activity of Modified and Native HRP

Sample	Absorbance Change	Relative Activity
Native HRP	0.92 ± 0.07	100 %
H3-HRP	0.16 ± 0.02	17 %

2.3.4 Characterization of Daunomycin-Glucose Oxidase Conjugates

Daunomycin in the DM-GOx conjugates was determined from its absorbance values measured at 476 and 550 nm. The amount of glucose oxidase was measured by the Coomassie Blue method. Table 3.4 lists the stoichiometries of the conjugates as well as the activities of the modified enzymes.

Table 2.4. Stoichiometries and Activities of Daunomycin-GOx Conjugates

Sample #	D per GOx	% Activity
Native	0	100
$(DM)_x$-GOx, Method A	4.0	56
$(DM)_x$-GOx, Method B	25	60

The two modified GOx species demonstrate similar activities, but have very different degrees of modification.

The spectra of $(DM)_4$-GOx in the presence and absence of DNA are shown

in Figure 2.7. (DM)$_4$-GOx in the free form has an optimum wavelength at 490 nm, while the DNA-bound form has an optimum at 500 nm. This 10 nm shift is a smaller shift compared with free daunomycin (Table 2.1), and suggests that intercalation is less extensive when daunomycin is attached to the bulky enzyme.

One important consideration was how the presence of DNA was affecting the activity of the enzyme in the (DM)$_4$-GOx conjugate. Activity assays were performed on solutions of 2.75 μM modified glucose oxidase in the presence and absence of 1.52 mM (base-pairs) calf thymus DNA. The results are presented in Table 2.5.

Table 2.5. The Activity of (Daunomycin)$_4$-Glucose Oxidase in the Presence and Absence of DNA

Sample	Activity (Units/mg)[a]
dGOx	7.21
dGOX + DNA	17.80

[a]performed in 0.100 m K-phosphate buffer, pH 7.0

The enzyme seems to be 2.5 times more active in the presence of DNA. The addition of nucleic acid to the solution of (DM)$_4$-GOx and its substrate caused it to become very viscous, and this would be expected to decrease enzyme activity. The reason for the increased activity in the presence of DNA is not known.

2.3.5 Detection of Immobilized DNA with Daunomycin-GOx Conjugates

Using the carbodiimide method, native (double-stranded) calf thymus DNA

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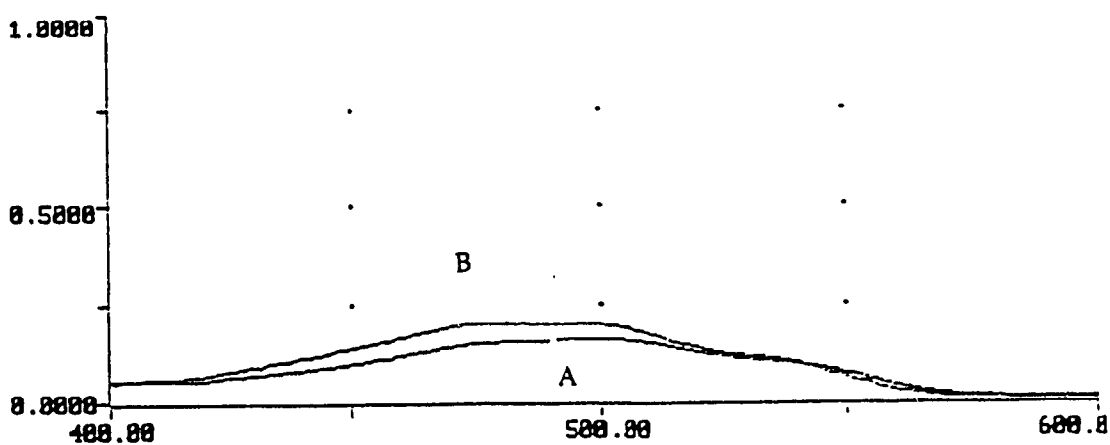
Figure 2.7. UV-visible spectra of 0.275 μM $(DM)_4$-GOx in the presence (A) and absence (B) of 1.52 mM calf thymus DNA.
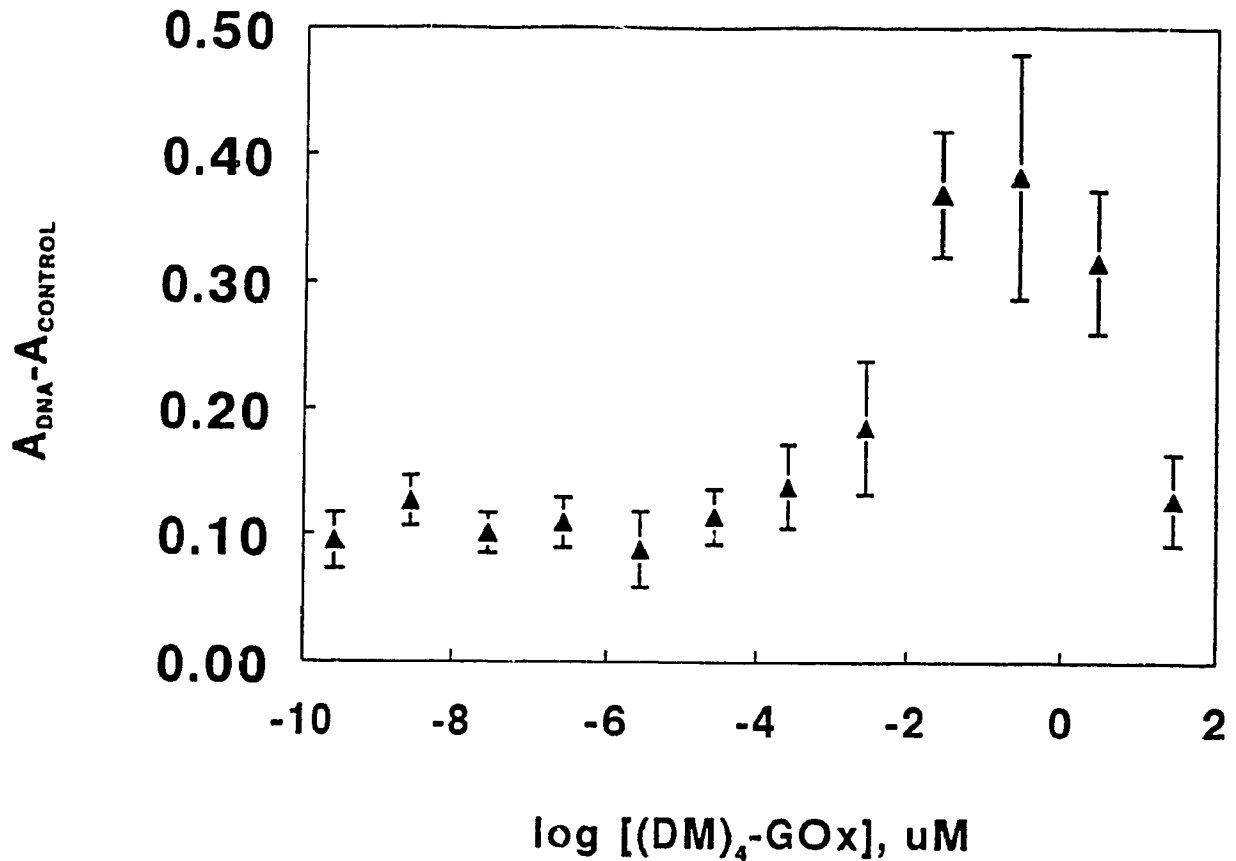
Figure 2.8. The effect of the conjugate concentration on the signal observed with conjugate detection. 10 µg/ml of native calf thymus DNA was immobilized by the carbodiimide method. The excess DNA was rinsed away. The wells were blocked with 100 µg/ml BSA protein. Various concentrations of $(DM)_4$-GOx were added. After allowing intercalation to proceed at 5°C, the excess was rinsed away and the substrate solution was added. After a two hour incubation at 22°C, the absorbance was read at 450 nm. The control included the substrate solution exposed to wells not modified with DNA, but exposed to the enzyme conjugate. All points represent the average of 8 measurements and the error bars represent a deviation of one standard deviation.

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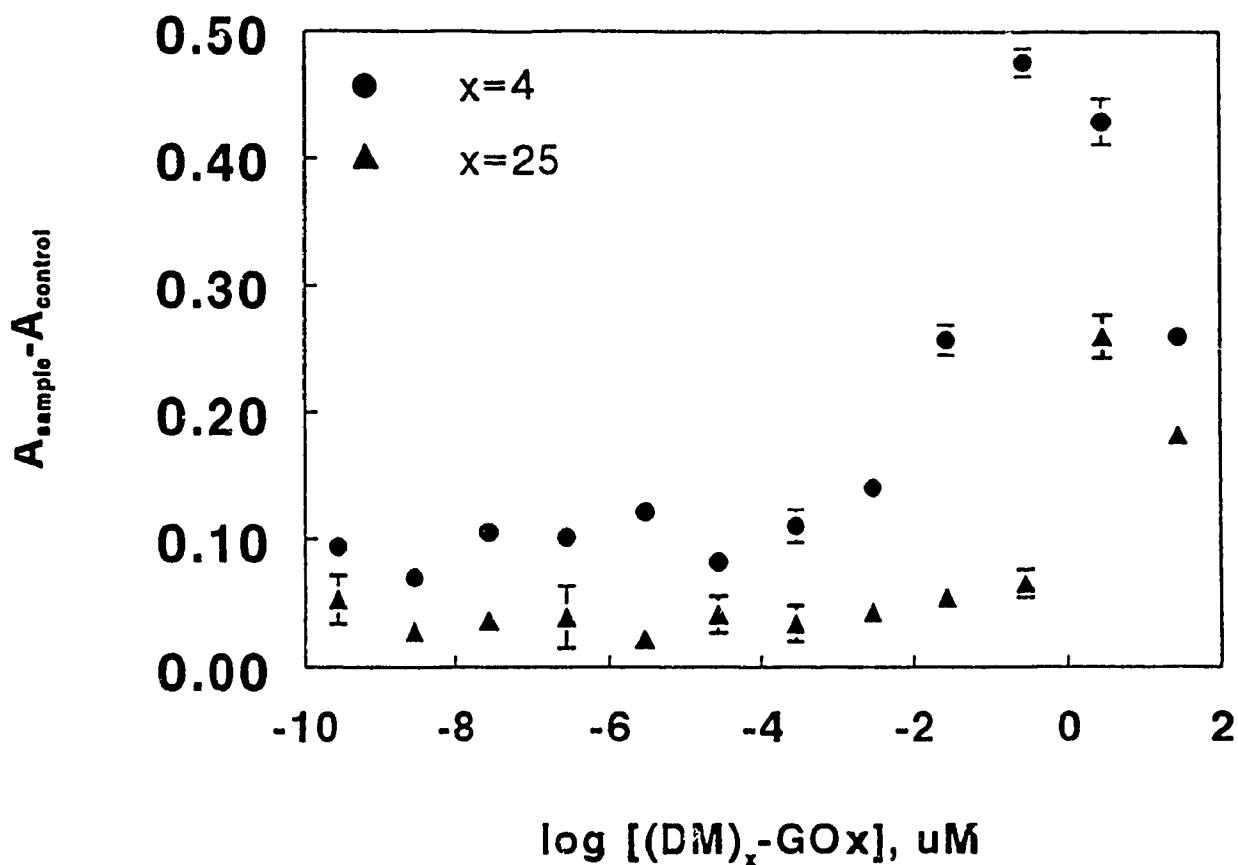
Figure 2.9. A comparison of the intercalation efficiency of two modified glucose oxidase products. 10 µg/ml calf thymus DNA was immobilized by the carbodiimide procedure. The unbound DNA was rinsed away. The modified enzyme was allowed to interact with the DNA for 2 h at 5°C. The excess enzyme was rinsed away. The substrate solution was added and the reaction was incubated at 22°C for 2 h and then the absorbance was measured at 450 nm. Each point represents the average of ± one standard deviation of 8 measurements.

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was immobilized onto CovaLink^R microtiter plates. Figure 2.8 shows the dependence of signal (absorbance of the o-dianisidine oxidation product) on the concentration of (DM)$_4$-GOx conjugate used during a 2 h, 5°C intercalation step. In these experiments, colour development proceeded during a 2 h, 22°C reaction with substrate. The control wells contained no immobilized DNA. A clear optimum can be seen at a (DM)$_4$-GOx concentration of 0.275 µM.

This experiment was repeated to compare the signals generated by (DM)$_4$-GOx and (DM)$_{25}$-GOx. The result, shown in Figure 2.9, again show the 0.275 µM optimum for (DM)$_4$-GOx, but show smaller signals and a higher optimum concentration of about 1 µM for the more extensively labelled (DM)$_{25}$-GOx. further experiments employed the (DM)$_4$-GOx.

Figure 2.10 shows the results obtained in a similar experiment, using immobilized poly(dA) and incorporating a hybridization step with poly(dT), and a blocking step with BSA to minimize nonspecific enzyme adsorption. The optimum (DM)$_4$-GOx concentration of 0.275 µM is similar to those obtained with calf thymus DNA, but slightly smaller signals were obtained. This may be caused by the length of the immobilized DNA, since the suppliers state that the two homopolymers are about 1 kilobase in length, while calf thymus DNA is highly polymerized.

Daunomycin has an experimentally determined tendency to associate more strongly with GC rather than AT pairs (K_a = 1.9 x 10^6 and K_a = 9.2 x 10^5)[17]. In fact, the preferred binding site is a triplet sequence with 5'-AGC-3' as the sequence on one strand.[10] It was important to know for the success of the assay whether this tendency would
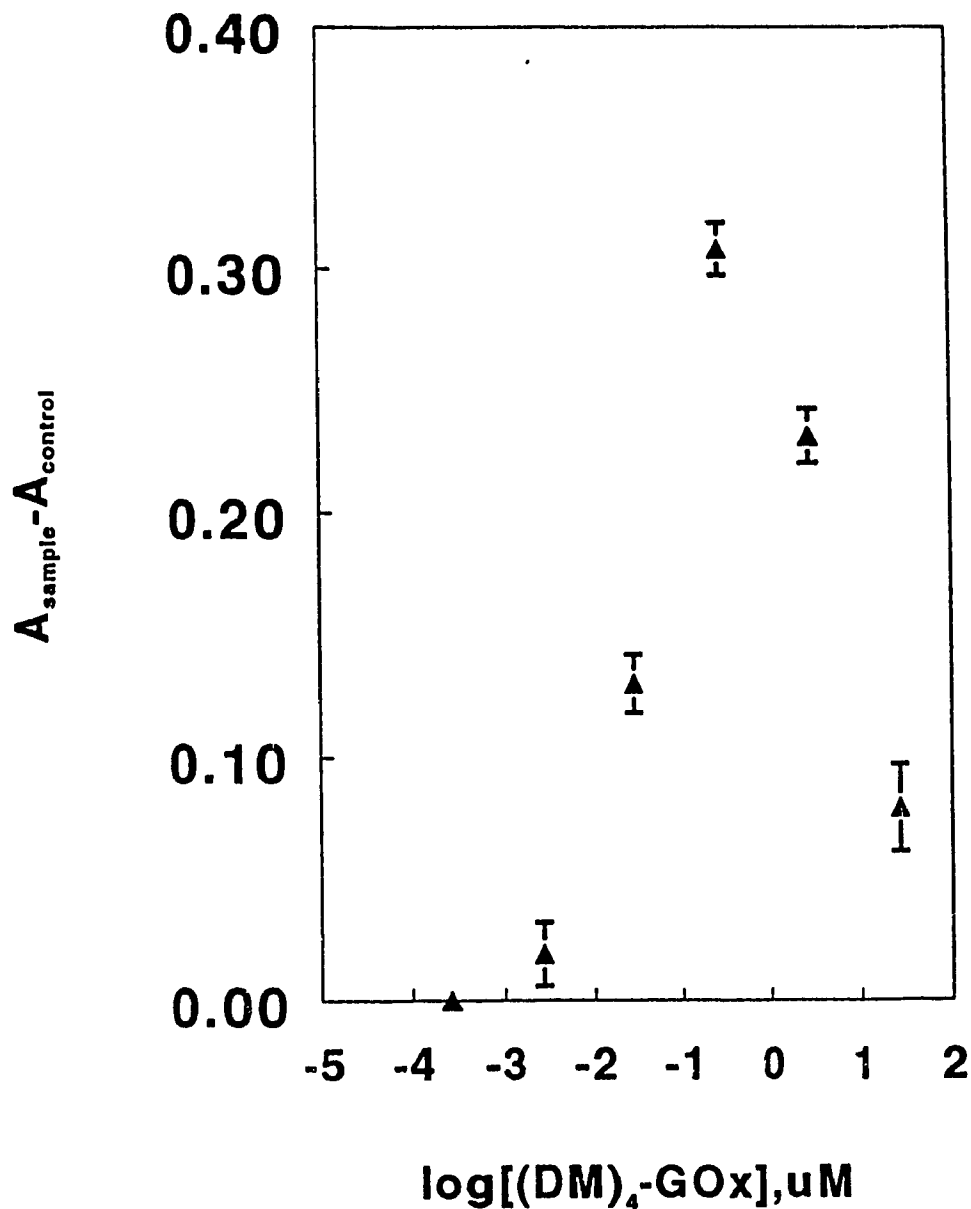
Figure 2.10. 3 µg/ml poly(dA) was immobilized by the carbodiimide method. The wells were blocked with 12.5 µg/ml avidin + 100 µg/ml BSA, and hybridized with 1 µg/ml poly(dT) (42°C, 18 h). (DM)$_4$-GOx was added and incubated at 5°C. After rinsing, the substrate solution was added. After 2 h at 22°C, the absorbance was read at 450 nm. The control included the substrate solution exposed to wells not modified with DNA, but exposed to the enzyme conjugate. Each point represents the average $\pm$ one standard deviation of 6 measurements.
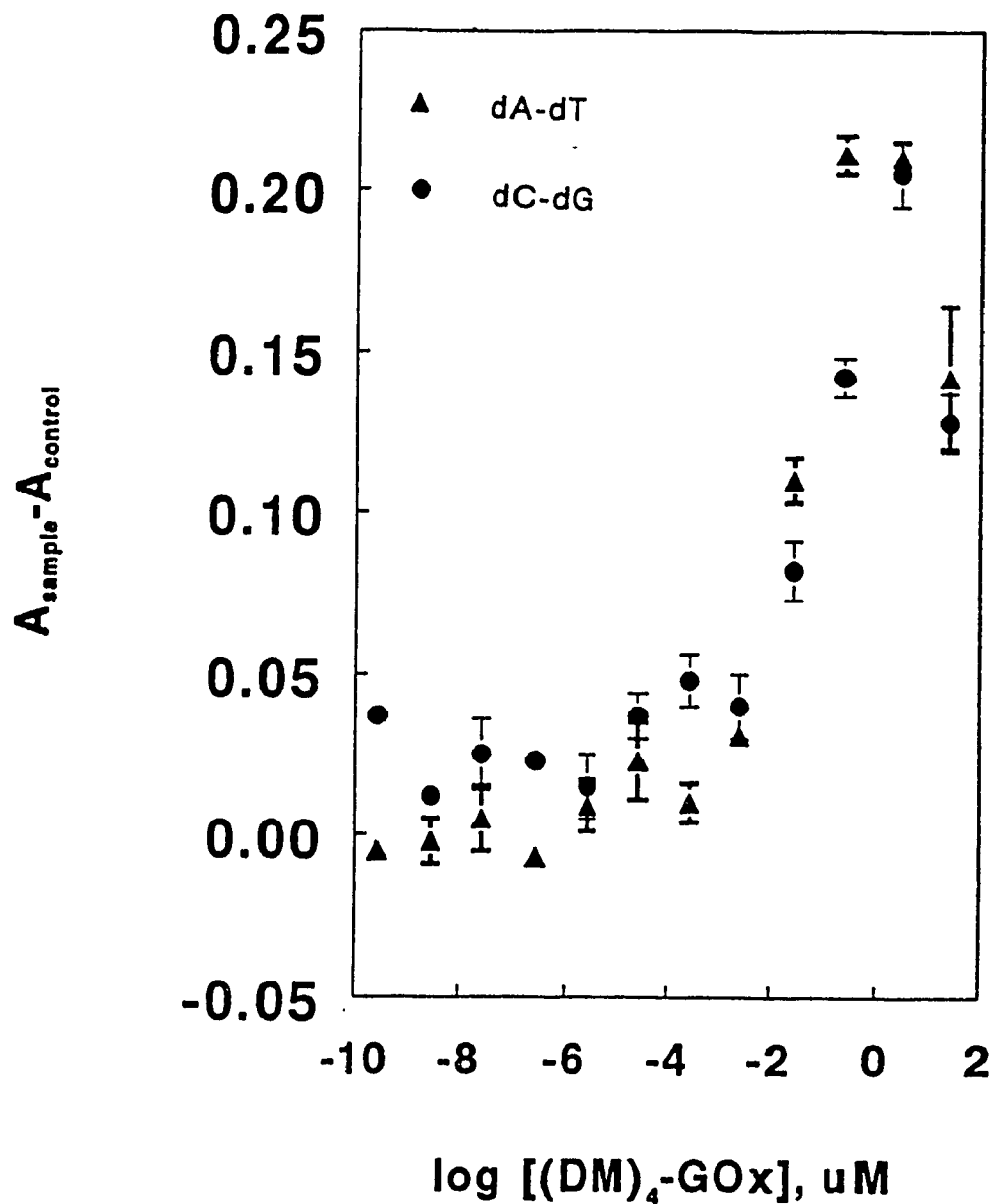
Figure 2.11. 3 µg/ml poly(dA) or poly(dC) was immobilized by the carbodiimide method. Blocked with 12.5 µg/ml avidin + 100 µg/ml BSA. Hybridized with 1 µg/ml poly(dT) or poly(dG) (42°C,18 h). (DM)$_4$-GOx was added and incubated at 5°C. After rinsing, the substrate solution was added. After 2 h at 22°C, the absorbance was read at 450 nm. The control included the substrate solution exposed to wells not modified with DNA, but exposed to the conjugate. Each point represents the average ± one standard deviation of 6 measurements.

continue if daunomycin were conjugated to glucose oxidase. To test this, identical concentrations of poly(dA) and poly(dC) were immobilized by carbodiimide method under identical conditions. After hybridization with identical concentrations of their complementary sequences, the two test plates were exposed to the same conditions of $(DM)_4$-GOx concentrations, and substrate solution and the results are presented in Figure 2.11. If $(DM)_4$-GOx truly interacts more strongly with poly(dC)-poly(dG) than with poly(dA)-poly(dT), it is expected that the signal intensity would be much higher or that the optimum concentration be much lower in the former case. This was not observed and it seems that the enzyme-intercalant complex interacts equally well with both types of DNA.

On the basis of these results, the enzyme detection was always carried out with 0.275 μM $(DM)_4$-GOx, regardless of the type of DNA used. The complex was exposed to the substrate solution for two hours at ambient temperature.

2.4 Discussion

The choice of a good indicator is crucial to the success of the DNA assay and to the optimization of the assay. It provides a means of assessing the impact of a parameter by indirectly measuring the quantity of immobilized DNA.

Five low molecular weight DNA intercalants were tested as indicators. These are molecules with an aromatic moiety in their structure which can interact with DNA through both electrostatic interactions and by intercalation. The electrostatic

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interactions are quite weak while the stacking interactions are quite strong. These stacking interactions are so strong that they lower the energy of $\pi \longrightarrow \pi^*$ transitions of these species and this can be observed by a shift of λ_{max} to longer wavelengths.[3] This trend certainly was observed with all the simple intercalants although the extent of the shift was a function of the intercalant species (see Table 2.1). The most profound shift was obtained with ethidium bromide (36 nm) and the smallest shift was associated with 9-aminoacridine (7 nm). The microplate reader operates on filters of defined wavelengths of 405, 450, 490, 570 and 630 nm which do not correspond to any of the λ_{max} of either the free or the bound forms of the dyes. Thus, these species were monitored at wavelengths closest to their maxima.

Thionin was found to have a binding site size of 3.0 base-pairs, but its association constant was found to be 4.5×10^4 which is about five times lower than for the other organic dyes (see Table 2.2). Because of its relatively low assoc. ation constant, this dye was rejected as a hybridization indicator. By way of contrast, daunomycin has an association constant of 2.6×10^6 with calf thymus DNA[16] which is the highest association constant of the five species used in this study.

3,6-Diaminoacridine was chosen as the best of the five intercalants for use as a hybridization indicator. Its molar absorptivity at the filter wavelength of 450 nm is 2.07 $mM^{-1}cm^{-1}$ when bound to DNA, and this wavelength is very close to an isosbestic point for the free and bound forms. The molar absorptivity of DAA at 450 nm is larger than all of the other intercalants, except thionin, which does not bind DNA as strongly.

Another important criterion for a hybridization indicator is the ability to distinguish double- and single-stranded DNA. Therefore, experiments using DAA to detect immobilized DNA plot the fractional increase in absorbance upon hybridization as a function of an experimental parameter. The optimum DAA concentration was found to be 15 µM, when DAA was immobilized by the diazonium method (Figure 2.5). When the same experiment was carried out with the carbodiimide immobilization procedure to CovaLink plates, the observed maximum varied from 10 - 20 µM (over 10 plates). It is encouraging that similar maxima are observed following two different immobilization methods. This makes the dye detection system flexible. The dye does not undergo nonspecific interactions or interact differently with the different surface-modified plates. However, a careful examination of the data reveals that only a 14% increase in signal was observed upon hybridization, due to the short path length through DNA-bound dye. Ideally, a much stronger signal is desirable.

In an effort to improve the signal, a DNA-binding species, H3 or daunomycin, was coupled to an enzyme like HRP or glucose oxidase. However, rather than detecting a bound DNA-binding species, the enzyme which is likely to be on the exterior of the double helix will be exposed to its substrate and undergo a catalytic conversion of substrate to product with a corresponding homogeneous change in colour that can be measured by absorbance. The first such species to be prepared was the histone H3-HRP complex.[6]

The enzymatic activity was determined in the presence of hydrogen peroxide and o-dianisidine dye. From Table 2.3, it can be observed that the modified

species retained only 17% of its activity relative to the same concentration of unmodified enzyme. Due to this low activity, studies of the H3-HRP conjugate were abandoned.

An alternative enzyme conjugate involves reacting daunomycin directly with glucose oxidase (Method A). This complex takes advantage of the high DNA binding constant of daunomycin and the high activity of the glucose oxidase enzyme. A further advantage of this species is that it is very simple to make, yielding an average stoichiometry of 4 daunomycins per glucose oxidase dimer.

A second synthetic procedure (Method B) was tested for preparing a daunomycin-glucose oxidase conjugate, involving deglycosylation of GOx followed by reductive coupling to daunomycin. The product of this reaction had 25 daunomycins per GOx dimer.

Given the two synthetic procedures, it was important to compare the relative activities and the signal intensities generated by the two products. From Table 2.4, it can be seen that the nature of the surface modification performed to bind daunomycin has no effect on the enzyme activity. This is consistent with the location of the enzyme's active site which is known to be deeply buried in the enzyme.[9] On the other hand, the deglycosylated enzyme can bind 25 daunomycin molecules while the unmodified enzyme can only bind 4. The presence of 21 extra daunomycin molecules should make for a stronger association of the enzyme-intercalant with the DNA double helix. However, it can be seen from Figure 2.9 that the glucose oxidase with only 4 daunomycin molecules attached actually interacts better with dsDNA. Had the $(DM)_{25}$glucose oxidase been a stronger DNA-binding species, it should have exhibited

stronger signals than $(DM)_4$glucose oxidase and its optimum concentration should have been much lower. Neither of these were observed so the less substituted species was used. In addition to providing better sensitivity, the smaller species is far easier and quicker to prepare because it does not require the deglycosylation of the enzyme.

With DAA, all plots featured the fractional increase in absorbance upon hybridization. This is due to the fact that this dye can be easily removed from a polystyrene plate by rinsing with a buffer solution or hot water. This offered the advantage that the same wells can be used for measuring the signal in the presence of single- and double-stranded DNA. The peroxidase reaction being studied has the disadvantage that it strongly and irreversibly stains the microtiter plate wells. Thus, wells cannot be re-used. To overcome this problem, the corrected absorbance of the species which takes into account all nonspecific interactions of the enzyme and background oxidation of the dye used in all plots.

From Figures 2.8 and 2.10, the optimum enzyme concentration is observed at about 0.3 µM, regardless of whether the species being immobilized is calf thymus DNA or the much smaller poly(dA)-poly(dT) system. The absorbance values were slightly larger when calf thymus DNA was used. Daunomycin is reported to show a preference for GC binding sites, particularly AGC binding sites.[16] A comparison of the signals generated by the modified daunomycin with poly(dA)-poly(dT) vs. poly(dC)-poly(dG) systems, shown in Figure 2.11, indicate no difference in signal magnitudes.

Thus, it appears that there are two effective detection systems available for detecting dsDNA. One of these uses the organic dye 3,6-diaminoacridine, the other the

(DM)$_4$-GOx. Both of these systems will be used in optimizing the immobilization and hybridization conditions employed in the DNA assay.

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CHAPTER 3

DNA IMMOBILIZATION ONTO MICROTITER PLATES

3.1 Introduction

The sequence-selective DNA assay begins with the immobilization of a single-stranded DNA (ssDNA) probe to the surface of a microtiter plate. Analyte ssDNA is then added. If the two sequences are complementary, hybridization can occur through Watson-Crick base-pairing. Hybridization is detected by one of the hybridization indicators described in the previous chapter.

For the assay to be useful, the immobilized DNA must be able to undergo hybridization and to retain its ability to distinguish complementary and noncomplementary sequences. The immobilization method must also be reproducible and quantitative. In an effort to achieve these goals, four immobilization methods were tested using two different types of microtiter plates.

Polystyrene plates, composed of polymeric units of styrene, can be chemically derivatized to primary amine or diazonium groups that can be used to bind DNA [1]. Alternatively, the physical adsorption of proteins, such as avidin and streptavidin onto polystyrene, can be used to immobilize biotinylated DNA probes.[2] The second type of microtiter plate used was the commercially available CovaLink[R] plate. The wells of this polystyrene plate have been derivatized with a secondary amine at the end of a 12 nm spacer.

3.1.1 DNA Immobilization by the Diazonium Method (Scheme 1)

Polystyrene microtiter plates can be derivatized by treating the wells with a fuming acid mixture (47% (v/v) concentrated HNO_3 in concentrated H_2SO_4) to nitrate the aromatic rings, as shown in Scheme 1.
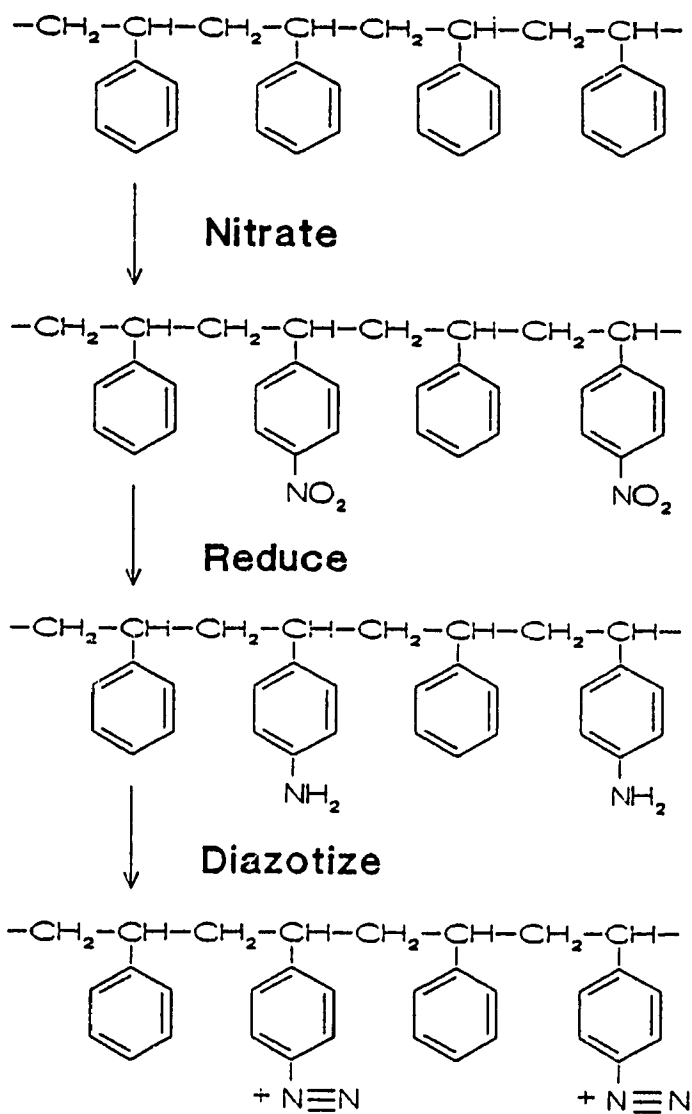
Scheme 1. Diazotization of Polystyrene

Addition of sodium nitrite and incubation on ice causes the primary amine groups to be converted to highly reactive diazonium groups. These groups have been shown to react with guanine, thymine and cytosine bases[4]. The adenine base is unreactive to this group. The reaction with the bases is a nucleophilic, S_N2 attack on the diazonium group by a brominated DNA base. The scheme for the four bases is similar to this reaction sequence for uridine, as shown in Scheme 2.[4]
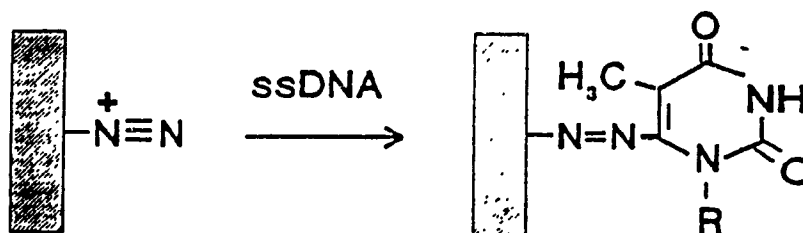
Scheme 2. Diazonium Immobilization of DNA

3.1.2 Carbodiimide Immobilization Methods

3.1.2.1 Plates with Aromatic Primary Amine Groups (Scheme 3)

In this method, polystyrene is derivatized by nitration and reduction, as shown in Scheme 1. DNA possessing a 5'-phosphate group is allowed to react with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) to produce an O-phosphonyl-isourea intermediate (Scheme 3). This species can react with amine groups on the microtiter wells to form a phosphoramidate bond.[3] Aromatic primary amine groups are known to be less nucleophilic than aliphatic amines, but may be reactive enough to allow DNA immobilization.

3.1.2.2 Plates with Aliphatic Secondary Amine Groups (CovaLink[R])

These commercially available plates can be used directly for DNA immobilization,

in the presence of EDC, as shown in Scheme 3. The secondary amine group is a good nucleophile, and successful ^{32}P-labelled DNA immobilization has been reported using these microtiter plates.[3]

$$-NHR \xrightarrow[\text{DNA}]{\text{EDC}} -NR-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O^-}{|}}{P}}-O-(5')-DNA$$

$$R = H, CH_3$$
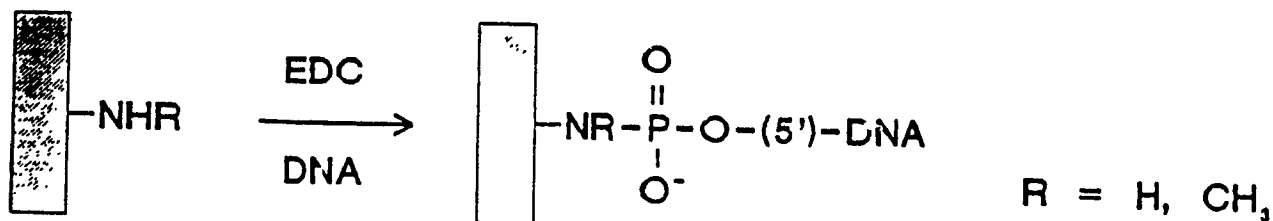
Scheme 3. Carbodiimide Immobilization Method

3.1.3 Biotin-Avidin Immobilization Method (Scheme 4)

Avidin is a glycoprotein obtained from egg white. It has a molecular weight of 45 kD and is tetrameric, with four biotin-binding sites. Its biotin association constant[5] is 10^{14}. Avidin adsorbs to polystyrene, and through its affinity for biotin, it can efficiently immobilize biotinylated species, such as biotinylated DNA.[5,6]

Biotinylated DNA is prepared using the enzyme terminal transferase. The DNA probe is incubated with a biotinylated nucleotide triphosphate, such as biotin-11-dUTP or biotin-16-ddUTP, this nucleotide will be added to the 3' end and hence introduce a biotin moiety .o DNA.[6] Biotinylated DNA is then immobilized according to Scheme 4.
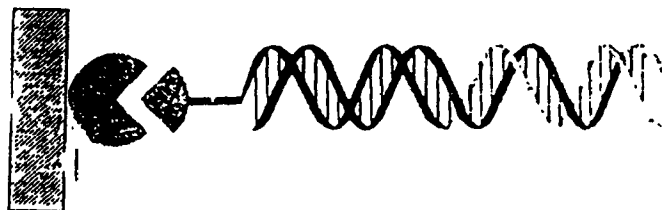
Scheme 4. Biotin-Avidin Immobilization.

3.1.4 Evaluation of Immobilization Methods

With each of the four DNA immobilization methods, an attempt was made to optimize conditions for the generation of surface functional groups, ssDNA probe binding, and analyte DNA hybridization. Detection of bound, double-stranded DNA proceeded using either the intercalating dye 3,6-diaminoacridine, or the (daunomycin)$_4$-glucose oxidase conjugate, as described in Chapter 2.

3.2 Experimental

3.2.1 Materials

The nitric, sulfuric and hydrochloric acids were ACS grade from Anachemia. The polystyrene plates were from Sigma and the CovaLink^R plates were from Nunc of Denmark. Sodium hydrosulfite was purchased from Sigma and sodium nitrite was obtained from Fisher. Tris HCl, Trizma base, sodium phosphate mono- and di-basic, and potassium phosphate mono- and di-basic were purchased from Sigma. 1-methylimidazole was obtained from Aldrich. 1-Ethyl-3-(3-dimethylaminopopyl)carbodiimide was purchased from Sigma. NaOH was obtained from Fisher. Sodium bicarbonate, ACS reagent grade, was purchased from Sigma. N-Bromosuccinimide was obtained from Sigma. Avidin, the terminal transferase enzyme kits, biotin-11-dUTP, biotin-16-ddUTP, bovine serum albumin and molecular biology grade solutions of phenol-chloroform-isoamyl alcohol were purchased form Boehringer

Mannheim. Tween 20 and Triton X-100 were obtained from Sigma. All other reagents used were of the highest quality available.

An anaerobic glove bag was generously provided by Elspeth Lindsay. Access to an Eppendorf microcentrifuge was generously provided by Drs. Turnbull and Joyce. Dynatech MR580 and BioTek EI307C microplate readers were used for measuring absorbance in the microtiter plates. A Thermoshaker incubator was used for all incubations at 37°C or 50°C. All 70°C or 94°C incubations were performed with Reacti-Therm heaters.

3.2.2 Methods

3.2.2.1 Diazonium Immobilization Method[1,4]

200 µl of 47% (v/v) concentrated nitric acid in concentrated sulfuric acid was added to each well of a polystyrene microtiter plate. The plate was covered and agitated for twenty minutes at 4°C. It was then carefully rinsed with distilled water at least three times. It was placed in an anaerobic glove bag with a nitrogen atmosphere and 6% (w/v) $Na_2S_2O_8$ in 2 M KOH was added over 14 minutes to each well. The plate was heated to 70°C and maintained at this temperature for four hours. The wells were then rinsed three times with 0.6 M HCl, three times with distilled water. This treatment generates the primary amine groups necessary for the EDC immobilization. The derivatized polystyrene plate was further modified by adding 200 µl of 1.25% sodium nitrite in 0.6 M HCl and incubating on ice for fifteen minutes. The plate was quickly

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washed three times with 1 mM HCl and three times with distilled water. This generated the diazonium groups. DNA was the immobilized as follows.

A 1 mg/ml stock solution of DNA, either calf thymus DNA or a homopolymer, was prepared in 1 M sodium bicarbonate buffer. The calf thymus DNA was heat-denatured to a single-stranded form while the homopolymer was used as received. 1 ml of the single-stranded DNA was then diluted to 5 ml with 1 M NaHCO$_3$ buffer. To this was added, 20 µl of a 8 mM solution of N-bromosuccinimide. The sample was vortexed and incubated on ice for ten minutes. This DNA solution was then diluted as required and added to the wells of the diazonium plate. After an incubation overnight at 5°C, the excess DNA was rinsed from the wells with distilled water. The signal in the presence of ssDNA was determined by the dye detection method described in Chapter 2. The ssDNA was then hybridized by adding the same concentration of DNA that was used in the immobilization and allowing the reaction to proceed at 22°C for one hour. The excess DNA was rinsed away with water and the signal in the presence of dsDNA determined by the same method used for ssDNA.

3.2.2.2 Carbodiimide Immobilization Methods[3]

3.2.2.2.1 Plates with Primary Aromatic Amine Groups[3]

Polystyrene plates derivatized to primary amines as described in Section 3.2.2.1 were used. A stock solution of 1 mg/ml DNA was prepared in 5 mM Tris buffer, pH 7.0 + 20 mM NaCl. If the DNA was double-stranded, it was denatured by heating at

95°C for twenty minutes. It was then plunged into an ice bath. Once the temperature of the DNA had dropped to about 5°C or with single-stranded DNA, 100 mM 1-methylimidazole buffer, pH 8.0 was added to a final concentration of 10 mM. Into each well was added 75 µl of DNA. To this was added 25 µl of EDC solution, 0.0363 g EDC/ml of 10 mM 1-methylimidazole, pH 8. The control wells contained 75 µl of the DNA in 1-methylimidazole buffer and 25 µl of water. The plate was then parafilmed and incubated overnight at 50°C. The plate was then removed, soaked for ten minutes with 0.4 N NaOH-0.25% SDS at 50°C, rinsed three times with the soaking solution and three times with water. The signal due to ssDNA was then determined by the 3,6-diaminoacridine dye method described in the previous chapter. The ssDNA was then hybridized with its complementary sequence (calf thymus DNA was heat-denatured before addition) for one hour at room temperature (22°C) and the signal in the presence of the duplex was then determined.

3.2.2.2.2 DNA Immobilization onto Aliphatic Secondary Amine Groups[3]

An appropriate concentration of either single- or double-stranded DNA, usually 3 or 10 µg/ml was prepared. Ice-cold 100 mM 1-methylimidazole buffer, pH 8.0 was added to a final concentration of 10 mM. 200 µl of this solution was added to each well including the control wells. Then, 66.7 µl of 0.2 M EDC in 10 mM 1-methylimidazole was added to each well, except to control wells to which 66.7 µl of water was added. The plate was covered with parafilm and incubated at 50°C for five hours. Only if the immobilized DNA was single-stranded, the wells were filled with

washing solution [0.4 N NaOH + 0.25% SDS], soaked for five minutes at 50°C, then

rinsed three times with washing solution and three times with 0.1 M potassium phosphate

buffer. If the immobilized DNA was double-stranded, the wells were rinsed three times

with phosphate buffer. The signal with ssDNA can thus be determined by either the dye

or the indicator-enzyme method. The ssDNA was hybridized with 1 μg/ml of the

complimentary sequence at 42°C overnight. After rinsing away excess DNA, double-

stranded DNA was detected by either the DAA or daunomycin-glucose oxidase methods.

3.2.2.3 Biotin-Avidin Immobilization of DNA[12,6]

A 12.5 μg/ml solution of avidin was prepared in 0.1 M potassium or

sodium phosphate buffer, pH 7.0. 250 μl of this solution was applied to each well of a

microtiter plate. The plate was parafilmed and incubated at 5°C overnight. The wells were

rinsed three times with buffer by filling the wells with 250 μl of buffer and gently

inverting the plate. The wells were blocked with a solution of 100 μg/ml bovine serum

albumin in the same buffer. After incubation at 5°C overnight, the wells were rinsed three

times with buffer. The plate was now ready for the biotinylated DNA.

In all cases, poly(dA) was used as the form of DNA to be modified with

a biotinylated nucleotide with the Boehringer Mannheim terminal transferase kit. 5 units

of poly(dA) were dissolved in 82.5 μl of water. 75 μl of this was transferred to an

Eppendorf tube. To this was added 10 μl of biotin-11-dUTP or biotin-16-ddUTP, 35 μl

of water, 60 μl of terminal transferase buffer, 10 μl of cobalt dichloride and 4 μl of

terminal transferase enzyme. The tube was gently inverted several times to mix the

contents. The reaction was incubated at 37°C. Two more additions of 4 µl of enzyme were made at four hour intervals. Finally, another addition of 4 µl of enzyme was made and the reaction incubated for twelve hours. The tube was then filled with an equal volume of phenol-chloroform-isoamyl alcohol solution. The tube was vortexed and centrifuged at 12000 x g for ten minutes. The aqueous layer and any DNA at the organic-aqueous interface were removed and placed in a separate Eppendorf. To this solution was added two equivalent volumes of cold ethanol and 1/10th the total volume of 2 M sodium acetate. This was incubated overnight at -20°C. The tube was then removed and centrifuged at 5°C at 12000 x g for ten minutes. The DNA was observed as a pellet and the supernatant was saved. The pellet was washed with 0.500 ml of 75% ethanol. The tube was centrifuged and the supernatant transferred to a separate tube. The pellet was then rinsed twice, once with 75% ethanol and once with 100% ethanol. The supernatants were saved in separate Eppendorfs. The pellet was allowed to air-dry for thirty minutes at room temperature. It was then dissolved in 165 µl of water. Its concentration and purity was determined spectrophotometrically from its absorbance at 260 nm (1 A.U. = 33 µg/ml of ssDNA) and the ratio of the absorbance at 260 nm to its absorbance at 280 nm should exceed 1.7 for a pure sample of DNA. This constitutes a stock solution of DNA.

Using the stock solution of biotinylated DNA, the appropriate concentration of DNA was prepared in 0.1 M potassium phosphate buffer. 250 µl of the biotinylated DNA was added to each well and the plate incubated overnight at 5°C. The excess DNA was then rinsed away with buffer. The DNA at this stage could be hybridized with its complementary sequence overnight at 42°C. The hybridized DNA could then be detected

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with either a 0.015 M solution of 3,6-diaminoacridine or with the (daunomycin)$_4$glucose oxidase detection.

3.2.2.4 Blocking Agents

A number of blocking agents were tested to decrease the non-specific interactions of (daunomycin)$_4$glucose oxidase with the CovaLink surface. The following table summarizes the blocking agent tested and the literature conditions that are recommended. In all cases, once the blocking agents had been incubated in the wells, the excess was rinsed away with 0.1 M potassium phosphate buffer. 200 μl of 2.75 μM of the enzyme-intercalant complex was added to each well and the plate was incubated at 5°C for two hours. Any complex that had not reacted with the plate was rinsed away. The enzymatic substrate solution was added and the absorbance after two hours was measured. In addition, controls which had been exposed to the blocking agent but not to the enzyme were used to study the colour development of the substrate solution in the absence of enzyme. The final absorbance signal was taken as the difference between the sample and control wells.

Table 3.1. Blocking agents and their recommended conditions.

Blocker	Time (hrs)	Temp (°C)	Reference
unmodified plate	2	22	
100 μg/ml BSA	18	5	13
10 μg/ml BSA	18	5	
100 μg/ml BSA + 10 μg/ml calf thymus DNA	18	5	
0.5% Tween 20	2	22	14
0.5% Triton X-100	2	22	15
5% skim milk	1	5	16
10% skim milk	1	5	
20% skim milk	1	5	
27.5 μM denatured GOx	1	5	
0.1 M PBS - 0.5% Tween 20	2	5	17
0.1 M PBS + 100 μg/ml BSA + 5% milk	18	5	18
0.1 M PBS + 0.5% Tween 20 + 100 μg/ml BSA	24	5	19
TTBS + 5% milk + 100 μg/ml BSA	0.5	22	20
0.1 M PBS + 100 μg/ml BSA	2	5	21
12.5 μg/ml avidin	2	5	12
12.5 μg/ml avidin + 100 μg/ml BSA	18	5	

TTBS = 50 mM TRIS-HCl, pH 7.5 + 150 mM NaCl + 0.1% Tween 20 + 5% milk + 0.1% BSA

BSA = bovine serum albumin

PBS = 0.1 M potassium phosphate + 150 mM NaCl

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3.3 Results

3.3.1 Diazonium Immobilization Method

Following a twenty-minute polystyrene nitration step, the times allotted for reduction to $-NH_2$ and diazotization were varied. For each of the three reduction times (2, 4 and 6 hours), diazotization times were varied from 15 minutes to 2 hours. The resulting diazotized microtiter plates were allowed to react with 3 μg/ml of brominated poly(dT), and incubated with poly(dA). Detection using 3,6-diaminoacridine (0.015 M) showed that the four hour reduction and 30-minute diazotization steps yielded the largest A_{450} values of 1.1 absorbance units, although the final A_{450} values only varied over a narrow range (0.98 - 1.10). Degassing the reducing agent prior to the 4-hour reduction step yielded an increase in the final A_{450} value to 1.4 a.u.

Using the optimized reduction and diazotization times, the temperature used during DNA immobilization was varied. The DAA dye-detection step was performed following ssDNA poly(dT) immobilization and again following hybridization with poly(dA). The results, shown in Table 3.2, indicate that low temperature immobilization (5°C) yield larger signals. The final column in Table 3.2 shows the fractional absorbance increase that occurs on hybridization $(A_{DS} - A_{SS})/A_{SS}$, and is intended to minimize signals due to nonspecific binding of the dye to immobilized DNA.

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Table 3.2. Effect of DNA Immobilization Temperature on Dye Absorbance (Diazonium Method)

Temp (°C)	A_{450} before Hybridization (A_{SS})	A_{450} after Hybridization (A_{DS})	$(A_{DS} - A_{SS})/A_{SS}$
5	0.384	0.468	0.132
22	0.706	0.740	0.048
37	0.605	0.549	0.000
50	0.883	0.916	0.037

Having established the optimum temperature, the effects of DNA concentration during diazonium immobilization and hybridization were tested. A range of poly(dT) concentrations was prepared and immobilized, then hybridized with 1 μg/ml poly(dA) and detected with 3,6-diaminoacridine. Figure 3.1 shows the fractional increase in absorbance as a function of log[poly(dT)] concentration. The plot reveals a clear optimum at 3 μg/ml.

Using 3 μg/ml poly(dT) during the immobilization step, experiments were carried out to assess the effects of hybridization time and the concentration of poly(dA) during hybridization. With dye detection at 450 nm, and hybridization of 1 μg/ml poly(dA) for times of 1 - 18 hours, the largest $(A_{DS} - A_{SS})/A_{SS}$ of 0.17 occurred with a 1 hour hybridization, and the value decreased to 0.08 with an 18 hour hybridization at room temperature. Thus, one hour at room temperature is optimal for these homopolymers, and was used throughout. Figure 3.2 shows the effect of poly(dA) concentration during hybridization. The data indicate that concentrations below 1 μg/ml result in inefficient

Figure 3.1.. A plot of the fractional increase in absorbance as a function of poly(dT) concentration during immobilization by the diazonium method. The single-strand was detected by the dye detection method. Poly(dT) was hybridized with 1 µg/ml of poly(dA) and then detected with 3,6-diaminoacridine.
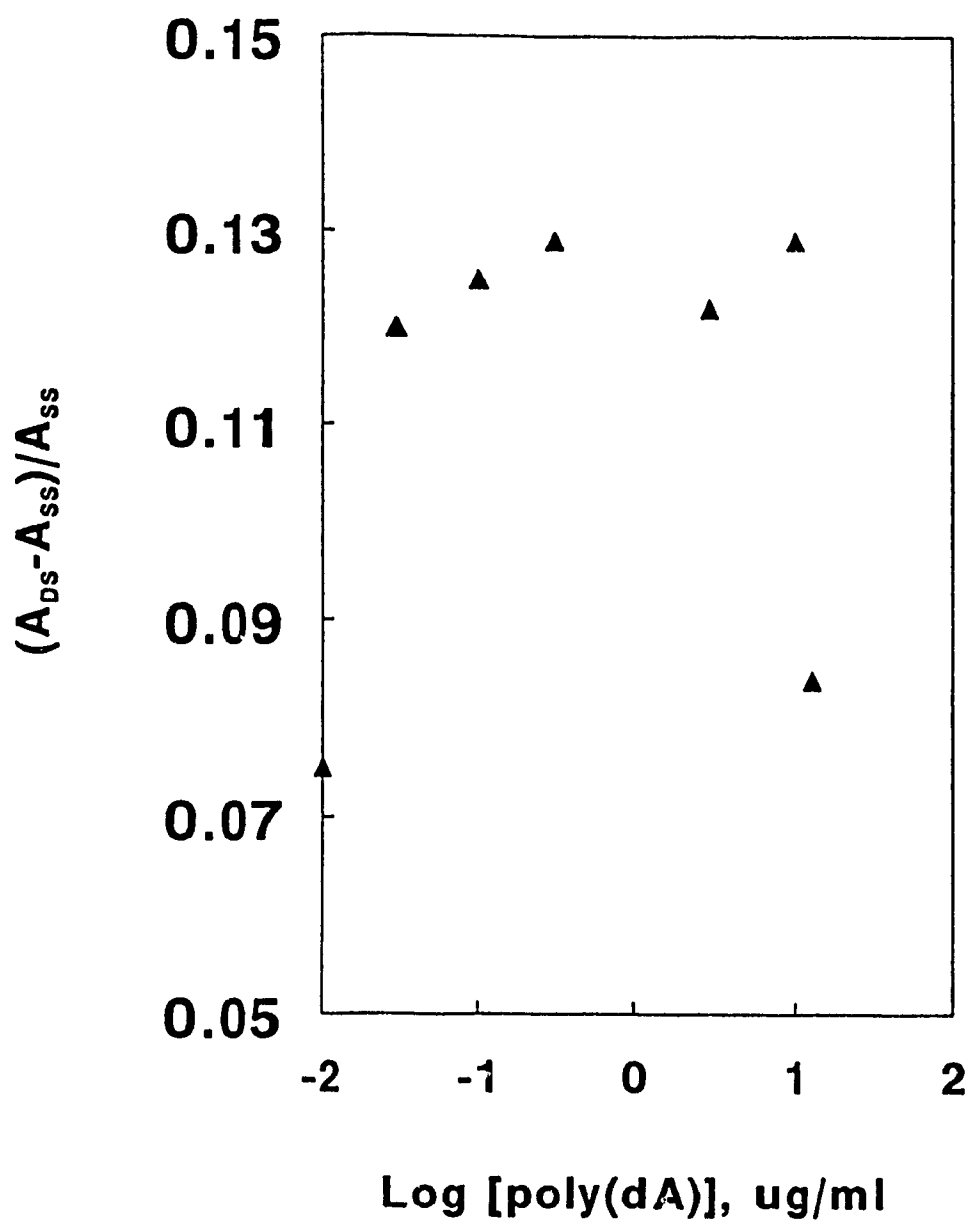
Figure 3.2.. Effect of poly(dA) concentration during hybridization on the fractional increase in absorbance at 450 nm. $(A_{DS} - A_{SS})/A_{SS}$ upon hybridization with diazonium-immobilized poly(dT). Hybridization occurred for 1 hour at 22°C.

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hybridization, that there is a strong increase in absorbance at 1 µg/ml poly(dA) and that the signal is essentially constant above 1 µg/ml. As 1 µg/ml produces essentially the same signal as 10 µg/ml, it was decided to use the lower concentration for hybridization.

3.3.2 Carbodiimide Immobilization Methods

3.3.2.1 Plates with Primary Amine Groups

Figure 3.3 shows the fractional increase in dye absorbance at 450 nm that occurs upon hybridization of 1 µg/ml poly(dA) (1 hour, 22°C) as a function of the poly(dT) concentration used during EDC-promoted immobilization onto nitrated and reduced polystyrene plates. It can be seen from these data that only a 5% increase in absorbance occurred at the optimum poly(dT) concentration of 0.3 µg/ml. Due to the small signal change, this DNA immobilization method was abandoned.

3.3.2.2 Plates with Aliphatic Secondary Amine Groups

This method employs the commercially available CovaLink[R] plates, derivatized to possess 10^{14} secondary amine groups per well.[3] Using the established procedure[3], the immobilization temperature was varied, and dye-detection of immobilized poly(dT), before and after hybridization with 1 µg/ml poly(dA) yielded the results shown in Table 3.3.
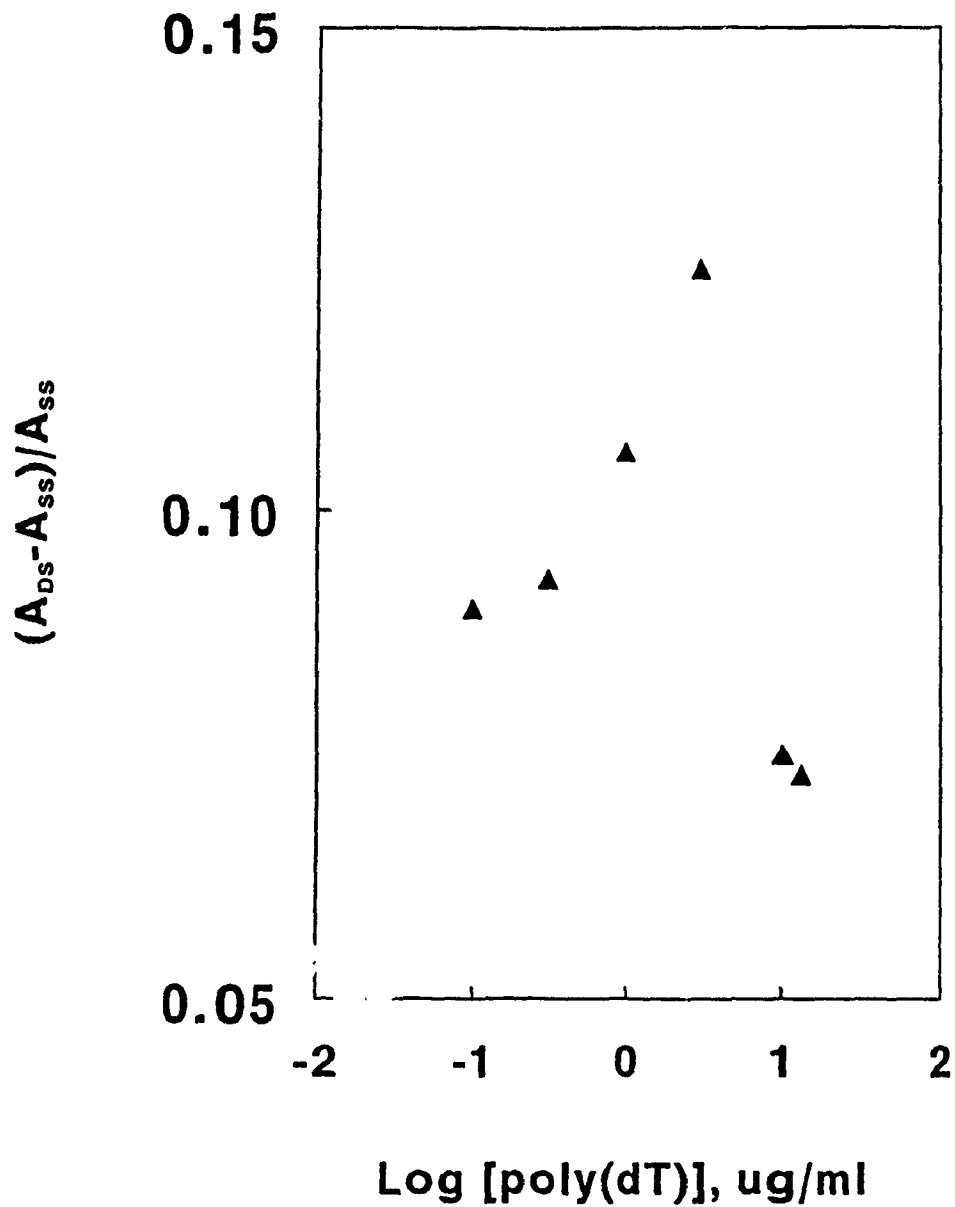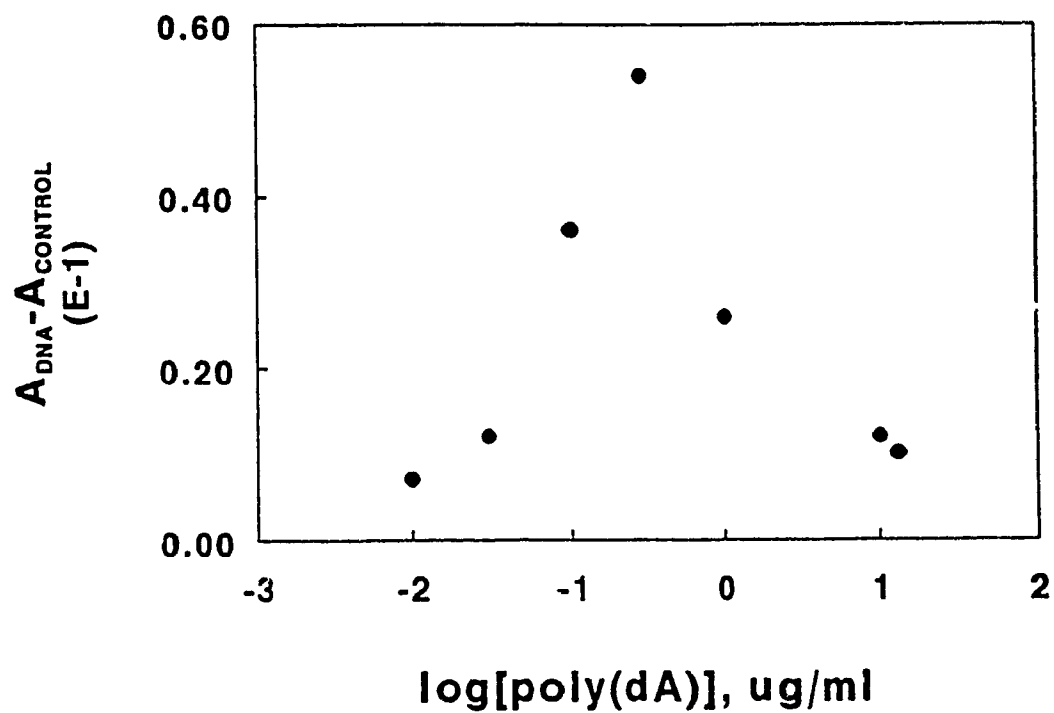
Figure 3.3. A plot of the fractional increase in absorbance as a function of poly(dT) concentration during immobilization by the carbodiimide method to an aromatic, primary amine. The single-strand was detected by 3,6-diaminoacridine. Poly(dT) was hybridized with 1 µg/ml of poly(dA) and then detected with DAA at 450 nm.

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Table 3.3. Dye Detection of EDC-Immobilized poly(dT) (CovaLink[R]) Before and After Hybridization

Temp (°C)	A_{450} before Hybridization (A_{SS})	A_{450} after Hybridization (A_{DS})	$(A_{DS} - A_{SS})/A_{SS}$
5	0.453	0.584	0.219
22	0.509	0.569	0.117
37	0.517	0.663	0.283
50	0.423	0.651	0.540

These results indicate that immobilization at 50°C provides optimal results, with a very large (64%) increase in signal upon hybridization.

Calf thymus DNA was used to investigate the effect of EDC concentration on the final 3,6-diaminoacridine absorbance at 450 nm. At the recommended[3] concentration of 0.2 M, an average absorbance value of 0.683 ± 0.061 was obtained following immobilization of 10 μg/ml DNA. Using 0.4 M EDC, A_{450} = 0.686 ± 0.030 was obtained. These results indicate that the both EDC concentrations yield the same amount of DNA binding to the CovaLink plates.

Earlier results (Chapter 2) showed that the (daunomycin)$_4$-glucose oxidase conjugate ((DM)$_4$-GOx) is a better hybridization indicator than the intercalating dye, 3,6-diaminoacridine. Thus, the next objective was to find conditions under which (DM)$_4$-GOx nonspecific binding to CovaLink plates could be minimized. The unmodified, secondary-amine-derivatized plates were then treated with blocking agents, rinsed and exposed to 2.8 μM native GOx for 2 hours at 5°C. The plates were then rinsed with 0.1 M phosphate buffer, pH 7.0 and substrate solution was added. After 1 hour at 22°C,

absorbance values were recorded at 450 nm. The results of this study are shown in Table 3.4. Absorbance values lower than 0.475, the value obtained with an unmodified plate, indicate lower nonspecific binding of the enzyme. The best blocking agents found were 10% skim milk (A = 0.236), 12.5 µg/ml avidin (A = 0.155) and 12.5 µg/ml avidin with 100 µg/ml BSA (A = 0.120). The avidin-BSA blocking agent was used in all subsequent experiments, and the blocking step occurred immediately prior to detection with the $(DM)_4$-GOx conjugate.

Figures 3.4 and 3.5 show the effect of DNA concentration used during the EDC-assisted immobilization of poly(dA) onto CovaLink[R] plates, on the absorbance signals obtained following hybridization with 1 µg/ml poly(dT). In both cases, the blocking step was included before detection with $(DM)_4$-GOx (Figure 3.4) and 3,6-diaminoacridine (Figure 3.5), and control wells containing no immobilized DNA.

It is clear from these results that the blocking agent minimizes nonspecific interactions of $(DM)_4$-GOx with the modified microtiter plates, and an optimum poly(dA) concentration of 3 µg/ml was obtained for the immobilization step. In Figure 3.5, however, nonspecific binding appears to occur over the entire range of poly(dA) concentrations studied, suggesting that the dye interacts with the blocking agent.

71
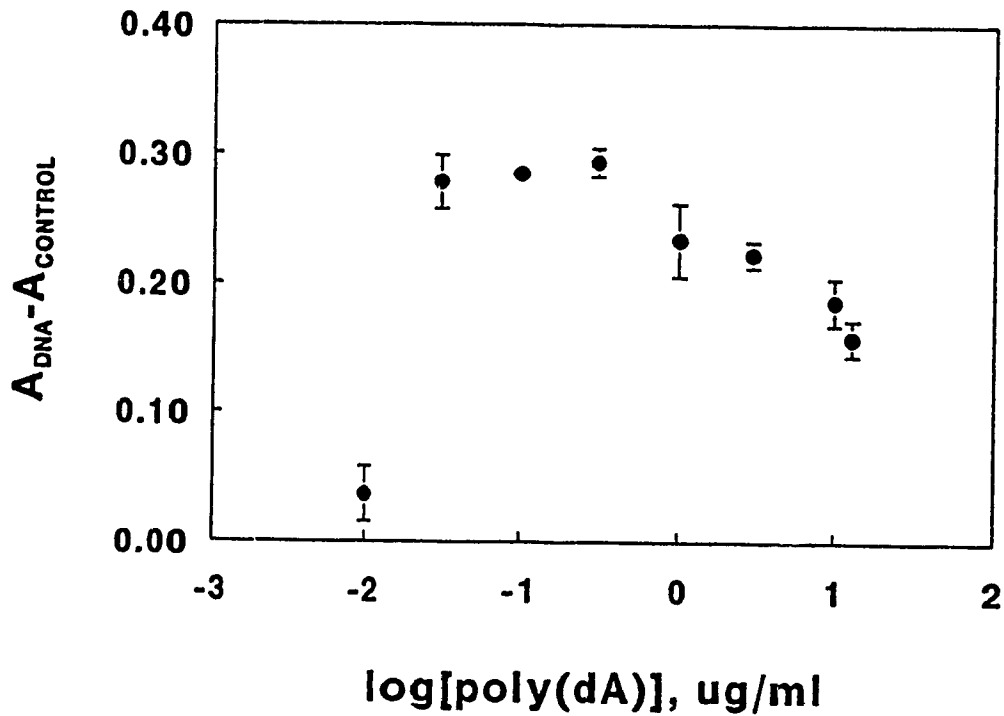
Figure 3.4. The optimization of the poly(dA) for immobilization by the carbodiimide (CovaLink[R]) method. Various concentrations of poly(dA) were immobilized, blocked with 12.5 µg/ml of avidin and 100 µg/ml of BSA, hybridized with 1 µg/ml poly(dT) and detected at 450 nm with $(DM)_4$-GOx. Each point represents the average ± one standard deviation of 8 measurements.
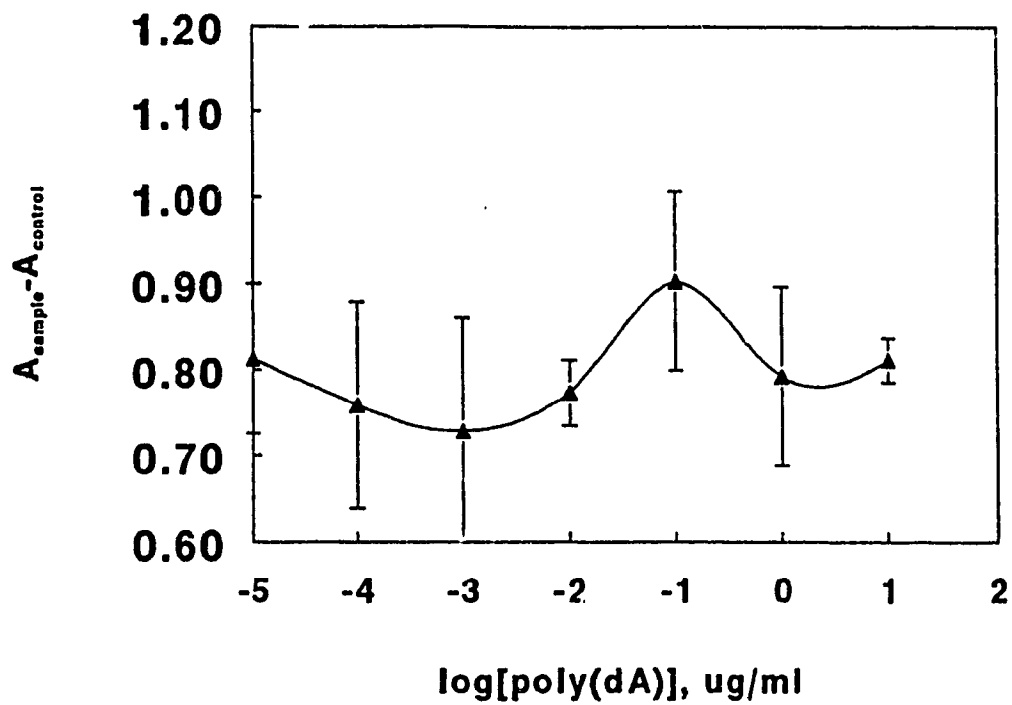
Figure 3.5. The optimization of the poly(dA) for immobilization by the carbodiimide (CovaLink[R]) method. Various concentrations of poly(dA) were immobilized, blocked with 12.5 μg/ml of avidin and 100 μg/ml of BSA, hybridized with 1 μg/ml poly(dT) and detected at 450 nm with DAA. Each point represents the average $\pm$ one standard deviation of 8 measurements.

Table 3.4. A comparison of blocking agents.[a]

Blocker	Time (hrs)	Temp (°C)	A_{SAMPLE}
unmodified plate	2	22	0.475
100 µg/ml BSA	18	5	0.348
10 µg/ml BSA	18	5	0.348
100 µg/ml BSA + 10 µg/ml calf thymus DNA	18	5	0.316
0.5% Tween 20	2	22	0.464
0.5% Triton X-100	2	22	0.460
5% skim milk	1	5	0.302
10% skim milk	1	5	0.236
20% skim milk	1	5	0.240
27.5 µM denatured GOx	1	5	0.396
0.1 M PBS - 0.5% Tween 20	2	5	0.613
0.1 M PBS + 100 µg/ml BSA + 5% milk	18	5	0.609
0.1 M PBS + 0.5% Tween 20 + 100 µg/ml BSA	24	5	0.615
TTBS + 5% milk + 100 µg/ml BSA	0.5	22	0.644
0.1 M PBS + 100 µg/ml BSA	2	5	0.529
12.5 µg/ml avidin	2	5	0.155
12.5 µg/ml avidin + 100 µg/ml BSA	18	5	0.120

[a] CovaLink plates were treated with blocking agents,then 2.75µM native GOx was added for 2 hours at 5°C. Substrate solution was added, and detection occurred at 450 nm after 1 hour

Figure 3.6 shows the variation in signal with poly(dT) concentration used during hybridization. There appears to be an optimum at about 10 ng/ml, and the increased values obtained around 10 µg/ml may result from triple-stranded DNA.[10]

Figure 3.7 shows the effect of $(DM)_4$-GOx concentration on signals generated following poly(dA) binding, poly(dT) hybridization and blocking of the CovaLink plates. It can be seen that identical results were obtained when the immobilization reaction was performed in imidazole or 1-methylimidazole buffers.

3.3.3 Biotin-Avidin Immobilization Method

Figures 3.8 and 3.9 show the detection of immobilized, biotinylated poly(dA), following hybridization with poly(dT), using $(DM)_4$-GOx (Figure 3.8) and 3,6-diaminoacridine (Figure 3.9). Optimum poly(dA) concentrations appear to be 0.1 µg/ml and 1 ng/ml, respectively, for immobilization. The reason for the 100-fold discrepancy in optimum poly(dA) concentration is not clear; however, an avidin 'blocking' step following biotin-avidin immobilization may result in the removal of some of the bound DNA. The large background signals seen with the dye-detection method again indicate significant nonspecific dye-binding to the modified plates.

The final step was to compare the detection methods. Table 3.4 presents the corrected absorbance obtained after immobilizing 10 µg/ml of poly(dA) by the CovaLink method, hybridizing with 3 µg/ml poly(dT) for one hour at 42°C and then detecting. There is a fivefold advantage in terms of signal intensity to using the $(daunomycin)_4$glucose oxidase conjugate as compared to the dye.

75
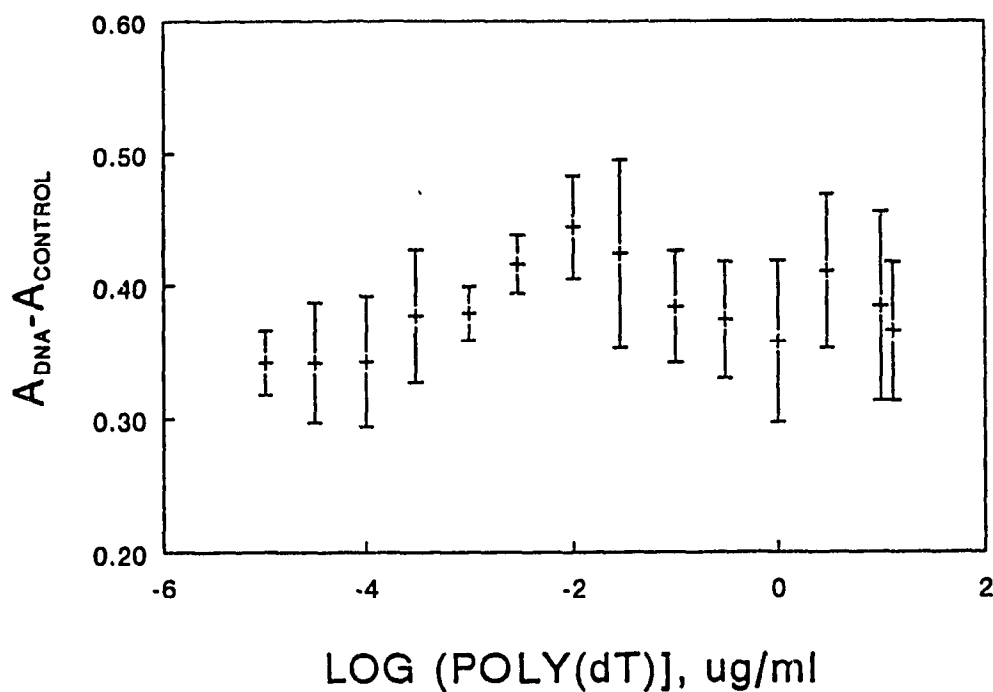
Figure 3.6.. The optimization of analyte DNA concentration. 3 µg/ml of poly(dA) were immobilized by the carbodiimide (CovaLink[R]) procedure, blocked with 12.5 µg/ml of avidin and 100 µg/ml of BSA, hybridized with 1 µg/ml poly(dT) overnight at 42°C and detected at 450 nm with (DM)$_4$-GOx. Each point represents the average ± one standard deviation of 6 measurements.

76
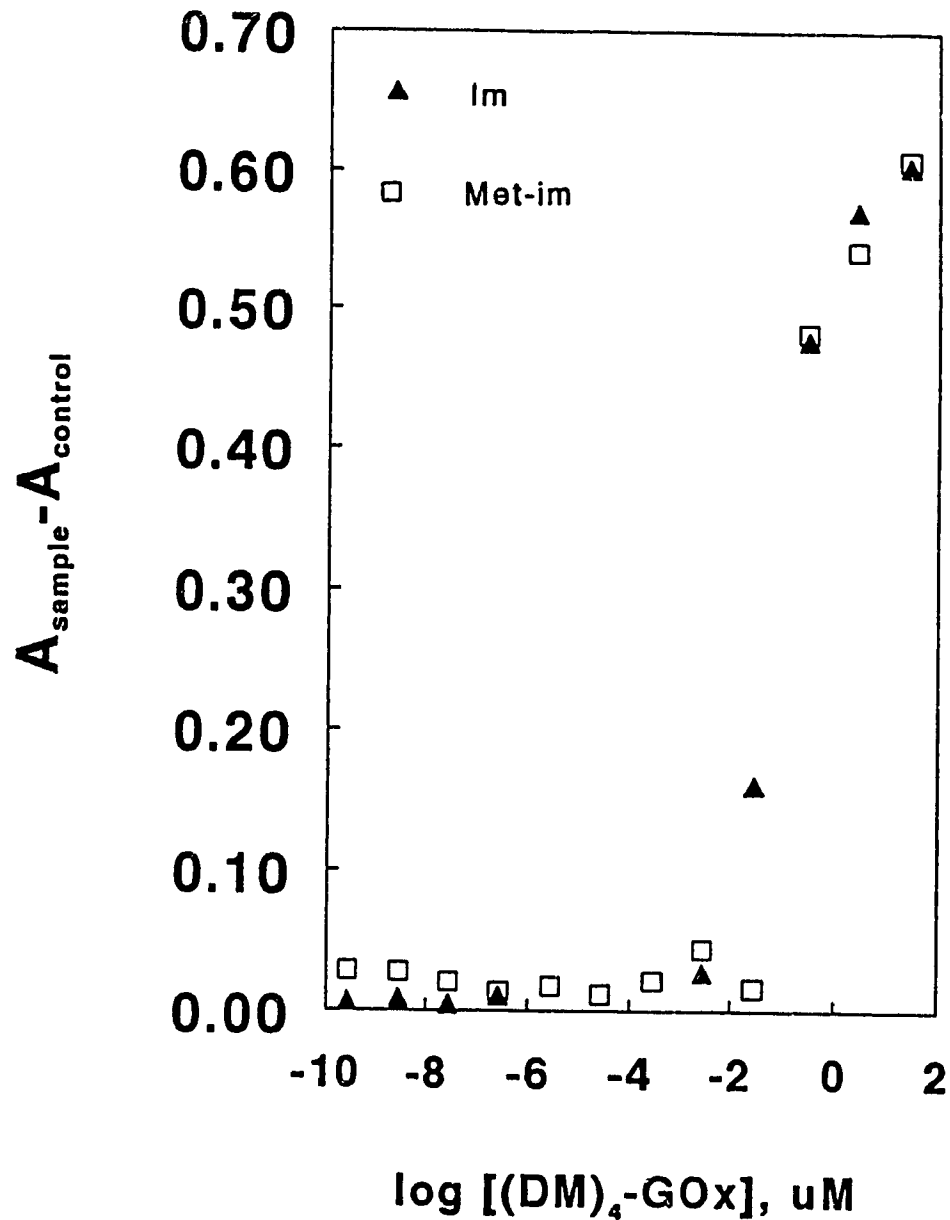
Figure 3.7. The effect of the immobilization buffer on the signal intensity with dsDNA. 3 µg/ml of poly(dA) was immobilized by the carbodiimide (CovaLink[R]) procedure using either 0.100 1-methylimidazole, pH 8.5 or imidazole, pH 8.5. The wells were blocked with 12.5 µg/ml avidin with 100 µg/ml BSA. The poly(dA) was hybridized with 1 µg/ml poly(dT) and the dsDNA detected at 450 nm with the (DM)$_4$-GOx complex.
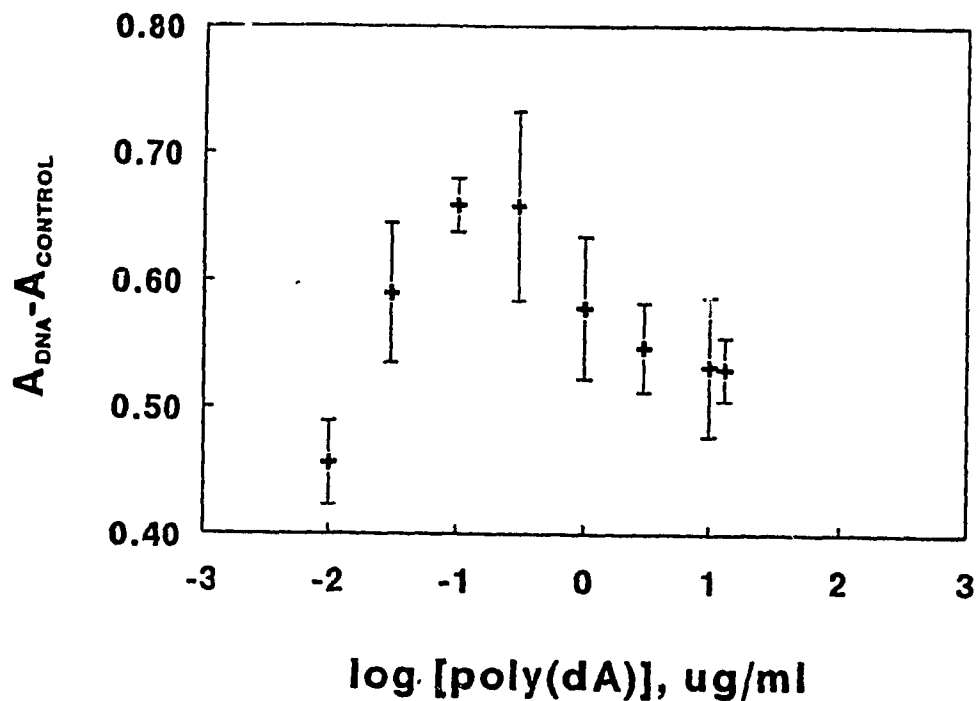
Figure 3.8. The optimization of the poly(dA) concentration with the biotin immobilization method. Various concentrations of poly(dA) were immobilized, blocked with 12.5 μg/ml of avidin and 100 μg/ml of BSA, hybridized with 1 μg/ml of poly(dT) and detected at 450 nm using $(DM)_4$-GOx. each point represents the average ± one standard deviation of 6 measurements.
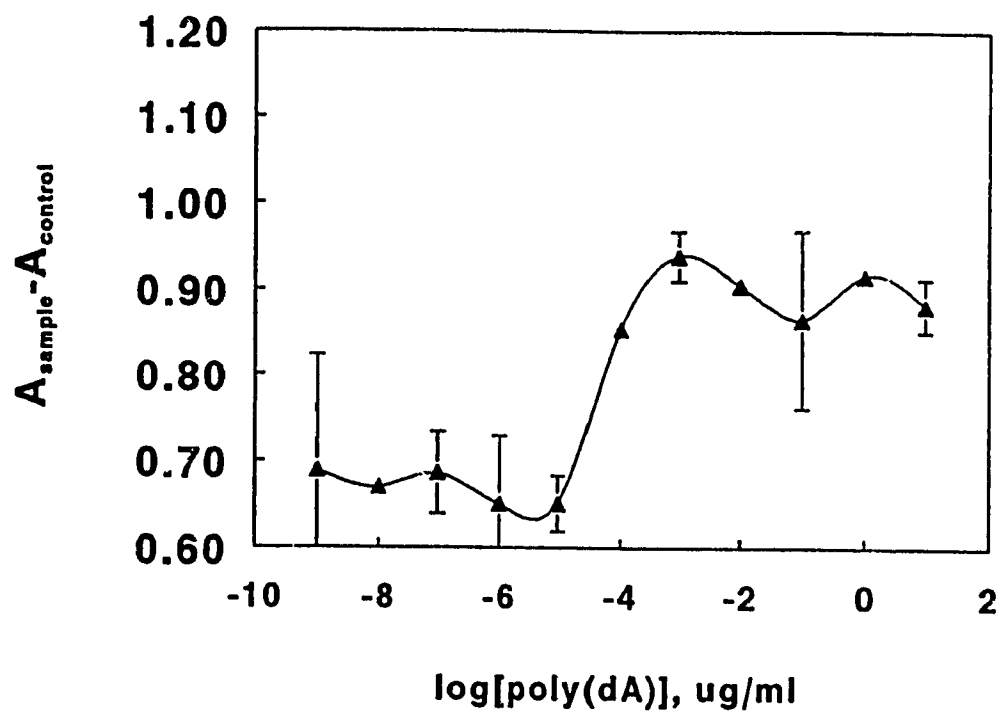
Figure 3.9. The optimization of the poly(dA) concentration with the biotin immohul. tir method. Various concentrations of poly(dA) were immobilized, blocked with ̇. ̇ ml of avidin and 100 μg/ml of BSA, hybridized with 1 μg/ml of poly(dT) and detected at 450 nm with 3,6-diaminoacridine. Each point represents the average ± one standard deviation of 6 measurements.

3.3.4 Summary of Optimized Methods

Table 3.5 summarizes the signals obtained using dye and dye-enzyme conjugate detection, for the four immobilization methods, with immobilized poly(dA) hybridized to poly(dT) for the latter three methods and vice versa for the diazonium method.

Table 3.5 The Absorbance Signals (450 nm) Obtained After Hybridization.[a]

Method	Dye Signal	Dye-Enzyme Signal
Diazonium	0.084	
EDC (1^c Aromatic)	0.064	
EDC (2° Aliphatic)	0.065	0.303
Biotin-Avidin	0.126	0.475

[a] Signals represent A_{DS} - $A_{CONTROL}$, where the control well had no immobilized DNA

3.4 Discussion

This assay is intended to detect the presence or absence of particular DNA sequences associated with genetic diseases such as cystic fibrosis and sickle cell anemia. To do so requires that one be able to immobilize a known DNA sequence that will be able to hybridize selectively with the disease sequence strand and not with a normal DNA sequence. To accomplish this, four immobilization procedures were tested.

The diazonium immobilization method requires the modification of polystyrene to yield diazonium groups. It is logical that the more such groups are

available, the more DNA can be immobilized. To improve the immobilization efficiency, two factors, time and the presence of dissolved oxygen, were tested for their ability to control the extent of modification. As mentioned in the results section, the optimum time for reduction is 4 hours and for diazotization, it is 30 minutes. These values are consistent with the literature procedure which recommends these reaction times.[1] This is also consistent with the reactivity of the groups involved. For instance, the nitro groups attached directly to the aromatic groups of styrene are very stable. An elevated temperature is required to catalyze the formation of primary amines. Too little time will cause only a part of the nitro groups to be converted. On the other hand, diazonium groups are generated quickly at low temperature, and are highly reactive. Once formed, all rinse steps and the addition of brominated DNA must be rapid to maintain as many diazonium groups as possible. The other factor to be considered is the effect of dissolved oxygen. The reduction of nitro groups to amine groups is an anaerobic reaction and is likely inhibited by oxygen.[1] Degassing the reducing agent minimizes the pathways by which oxygen can be introduced into the system and thus reduces to a minimum the dissolved oxygen. This was experimentally observed as much larger dye absorbance values. The diazonium method is most efficient at 5°C.

Using the optimized conditions, the optimum concentration of poly(dT) for immobilization was determined. A clear optimum was observed at 3 µg/ml. However, the signals observed are quite low (14% increase).

Then, the effect of hybridization time and the concentration of DNA used in the hybridization were assessed. The results indicate that the longer hybridization is

allowed to take place, the more the signals deteriorate. It is possible that the DNA could deteriorate over time at room temperature, but this effect should be investigated further. A hybridization time therefore of one hour was used throughout. A 1 µg/ml concentration of DNA was used during hybridization, and this concentration was associated with a broad maximum in dye absorbance (Figure 3.2).

One advantage of the diazonium method is that successful immobilization results in a colour change in the wells from yellow to brownish. However, there are problems with the diazonium method. Diazonium groups are reactive only with G, C and T bases. This places conditions on what DNA sequences can be immobilized.[4] In addition, immobilization through a base may interfere with the selectivity of strand association. The diazotization procedure also strongly discolours the plates and this raises the absorbance signal. This excludes the possibility of using an enzyme-intercalant detection method.

The problem of base selectivity was overcome by the use of phosphoramidate chemistry. The EDC-mediated immobilization reactions involve the 5'-terminal phosphate group of the DNA and are thus insensitive to the DNA sequence. The two methods differ in the nature of the amine group participating in the reaction, the one using a primary aromatic amine and the other a secondary aliphatic amine.

The former method required the derivatization of polystyrene to an aromatic, primary amine[1] and this was already optimized for the diazonium method. Using the optimized conditions[1,4], an optimum concentration of poly(dT) for immobilization of 0.3 µg/ml was observed. This is an order of magnitude lower than the optimum observed with the diazonium method. But the signal increase on hybridization was only 6%, which

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is too low to be useful, and so this method was abandoned.

The other method, the CovaLink[R] immobilization[3], has a number of advantages: 1) it immobilizes through a terminal group so that there is no preference for any base at the strand end nor is there anything that will interfere with hybridization selectivity or the association of strands into a double helix; 2) the plates are commercially available; 3) the immobilization procedure also does not require chemical or enzymatic modification of DNA; and 4) an immobilization protocol was available.

The first parameter to be assessed was the temperature. From Table 3.3, it can be observed that the most efficient immobilization was observed at 50°C, which is consistent with the manufacturer's protocol.[3]

Since the formation of the phosphoramidate bond is mediated by the presence of EDC, the effect of EDC concentration was assessed. Despite doubling the concentration of EDC used, the absorbance data indicated that no increase was observed with the higher concentration. Thus, the lower concentration of EDC, which corresponds to the manufacturer's protocol,[3] was used for all immobilization reactions.

Before optimizing this method for the DNA concentrations during immobilization and hybridization, the problem of the enzyme-intercalant conjugate having strong, nonspecific interactions with the microtiter plate was addressed. To reduce these effects, a number of blocking agents were investigated. The results, as presented in Table 3.4, indicate the best agents are 10% skim milk, 12.5 µg/ml avidin and 12.5 µg/ml avidin with 100 µg/ml BSA. The latter is the most efficient since the lowest signal was observed. Also, milk could be contaminated with bacterial DNA and was less efficient

than avidin. Avidin is a protein well-known to efficiently adhere to polystyrene[13] and hence can serve as a good blocking agent[13,18,19,20,21]. This was the agent chosen for all future studies, in conjunction with BSA which is also commonly used. However, it is interesting that the denatured GOx which should exhibit the same degree of interaction with polystyrene actually was less efficient than avidin at blocking the surface. The presence of the daunomycin groups must increase the extent of nonspecific interactions, probably because it changed the nature of the enzyme surface.

Figure 3.4 indicates that this blocking system does indeed inhibit the nonspecific interactions of the enzyme with the surface, but Figure 3.5 demonstrates that the dye is interacting with the blocking agent since there is little variation in the signal observed with various concentrations of poly(dA), which contradicts the manufacturer's protocol where a maximum of 7.5 µg/ml was observed with calf thymus DNA.[3] With the conjugate, an optimum concentration of 0.3 µg/ml was observed. This is similar to the optimum observed with the aromatic amine method and an order of magnitude lower than was achieved with the diazonium method.

The next step was to optimize the concentration of poly(dT) for hybridization. From Figure 3.6, an optimum was observed at 10 ng/ml. There appears to be a second peak at 10 µg/ml which may result from triple-stranded DNA,[9,10] via Hoogsteen base-pairing leads to the formation of DNA triplexes. For example, when an adenine homopolymer interacts with a thymine homopolymer, two hydrogen bonds are formed between each available adenine and thymine and this helix tends to wind very tightly. However, the major groove allows a third homopolymer to bind so that T x A x

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T or A x T x A helices can form.[9,10] Examples of triple-helix formation are given below.[10]
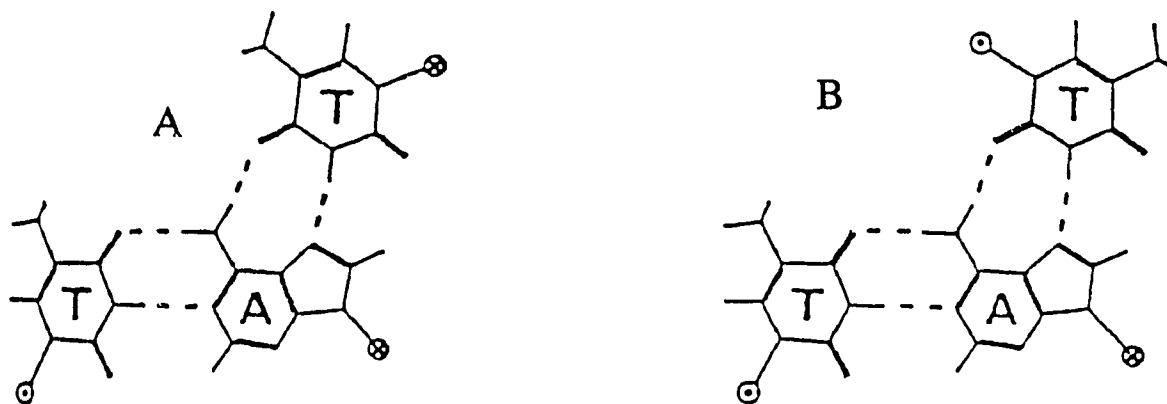
Scheme 5. Examples of (A) normal and (B) reverse Hoogsteen base-pairing.

There was concern that the 1-methylimidazole buffer interferes with the immobilization through polymerization of the DNA.[22] Figure 3.7 shows that the signals generated in the presence of 1-methylimidazole and imidazole buffers are virtually indistinguishable. This data cannot show directly whether a polymerization is taking place

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but they do indicate that this has no effect on the interaction of an intercalant-enzyme conjugate with DNA. To get a true idea of whether polymerization is taking place, the best method would be to electrophoretically analyze the DNA samples.

The last immobilization method tested used biotinylated poly(dA). The biotinylation reaction is performed by the terminal transferase enzyme which adds biotinylated nucleotides to any DNA strand and the immobilization is through biotin-avidin interactions.[6] This interaction does not interfere with any of the bases so that the ability of the DNA strand to find its complementary sequence and wind in the proper fashion is unaffected. The results indicate that the optimum concentration of poly(dA) for immobilization is 0.1 µg/ml with enzyme detection (Figure 3.8) and 1 ng/ml with dye detection (Figure 3.9). Thus, the biotin immobilization method also has an order of magnitude lower concentrations of DNA for immobilization and much stronger signals are observed, even with the dye detection method, than were observed with the diazonium method of immobilization but the optimum as determined by the enzyme is an order of magnitude higher than was observed with the CovaLink[R] procedure. This 100-fold discrepancy may result from the blocking step cutting off access of the dye to DNA by coating the nucleic acid or the blocking step may cause the removal of some of the bound DNA.

However, it is encouraging to see from Table 3.5 that the objective in preparing an enzyme-intercalant complex which was to increase the signal on hybridization was successful. The use of the enzyme-intercalant conjugate actually increased the signal observed by a factor of five with the EDC procedure using the

CovaLink^R plate and almost four-fold with the biotin system.

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CHAPTER 4

QUANTITATION OF IMMOBILIZED DNA

4.1 Introduction

In the previous chapters, various methods of immobilization and detection were presented. The observed signals are expected to depend on (a) the quantity of DNA immobilized, (b) the quantity of immobilized DNA that hybridizes, and (c) the quantity of the hybridization indicator that binds to the immobilized DNA. This chapter considers methods for independently quantitating the amount of DNA immobilized on the microtiter well surfaces. This will provide a useful criterion for comparing the immobilization methods.

With all the immobilization methods used in the DNA assay, the actual amount of DNA immobilized is likely to be quite low and hence a very sensitive quantitation method is required. In addition, many of the available methods for quantitating DNA require instrumentation or experimental conditions that are incompatible with a microtiter format. It was therefore decided to determine the amount of bound DNA by difference. Starting with a known quantity of DNA, aliquots of this sample were subjected to one of the immobilization procedures. After the immobilization, the contents of several wells were combined and the amount of DNA remaining was determined. Taking the difference between the initial and final concentrations of DNA, the amount of DNA immobilized could be calculated.

An important condition for the selection of a method is that good linearity

should be observed over the 0.1 - 10 µg/ml range, since initial concentrations of DNA are typically in the range of about 3-10 µg/ml.

A number of literature methods exist for the quantitation of DNA. Some of these methods require the chemical derivatization of DNA to produce chromophores or fluorophores while others leave the DNA intact. Initial tests of the methods were performed using calf thymus DNA.

The first assay attempted was the diphenylamine (DPA) assay. In this assay, diphenylamine is dissolved in glacial acetic acid and in the presence of a proton donor in the form of concentrated sulfuric acid undergoes a reaction with the bases of DNA. This reaction causes the DNA to be depurinated, the deoxyribose to be released and then converted to w-hydroxylevulinylaldehyde.

$$
\begin{array}{c}
\text{CHO} \\
|\\
\text{CH}_2 \\
|\\
\text{CHOH} \\
|\\
\text{CHOH} \\
|\\
\text{CH}_2\text{OH}
\end{array}
\quad \xrightarrow{\text{H}^{\oplus}} \quad
\begin{array}{c}
\text{CHO} \\
|\\
\text{CH} \\
\|\\
\text{CH} \\
|\\
\text{COH} \\
\|\\
\text{CH}_2
\end{array}
\quad \rightleftharpoons \quad
\begin{array}{c}
\text{CHO} \\
|\\
\text{CH} \\
\|\\
\text{CH} \\
|\\
\text{C}=\text{O} \\
|\\
\text{CH}_3
\end{array}
\qquad (1)
$$

This species reacts with diphenylamine (DPA) as follows:

$$
\begin{array}{c}
\text{CHO} \\
|\\
\text{CH} \\
|\\
\text{CH} \\
|\\
\text{C}=\text{O} \\
|\\
\text{CH}_3
\end{array}
\quad \xrightarrow[\text{Air}]{\text{2 DPA}} \quad
\qquad (2)
$$
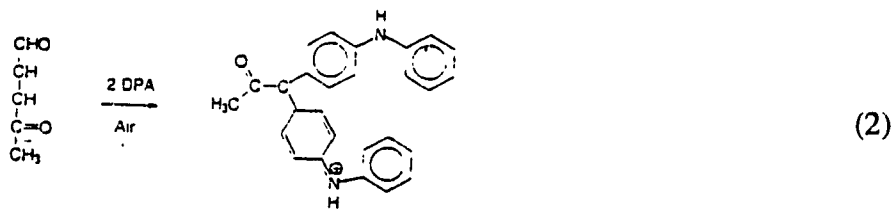
The blue product has an absorbance maximum at 600 nm. This method has a reported detection limit of 25 µg of DNA.[1,2,3]

The diphenylamine assay has recently been adapted to a microtiter format.

This opens up the possibility of doing multiple assays on small sample volumes. The same reactions are involved but a more dilute solution of perchloric acid is used. The absorbance due to the product can then be detected at 595 nm. However, our microplate reader is limited to filters at 570 and 630 nm, so measurements at both wavelengths were used. The literature reports a detection limit of 10 ng of DNA.[4]

DNA was also quantitated following chemical derivatization with 3,5-diaminobenzoic acid (DABA). In the presence of 1 M perchloric acid, DNA is depurinated, the deoxyribose is released and is then converted to w-hydroxylevulinylaldehyde, as in the DPA assay:

$$2 \ R{-}CH_2{-}CHO \longrightarrow R{-}CH_2{-}CH{=}\overset{\overset{\displaystyle R}{|}}{C}{-}CHO \qquad (3)$$
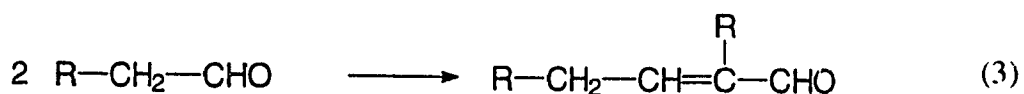
The DABA reagent is then added as an aqueous solution and reacts according to the following mechanism:

$$(4)$$
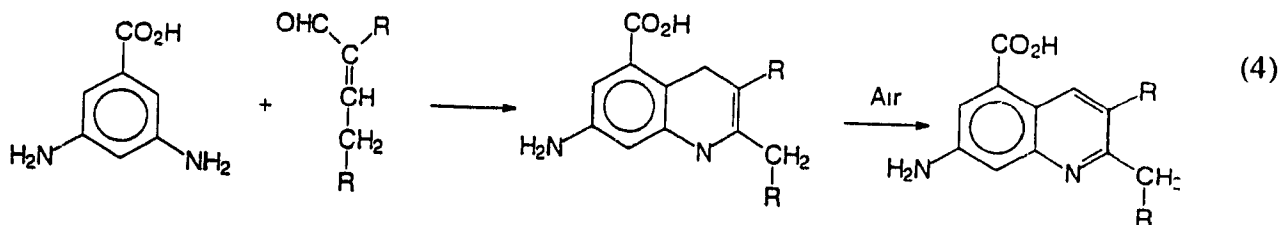
According to the literature, the optimum excitation and fluorescence wavelengths are 410 and 510 nm, and the detection limit is 30 ng.[1,6,7,8,9]

Two non-invasive techniques that do not require chemical derivatization were tested for quantitating DNA. One uses ethidium bromide with fluorescence detection

and the other is a voltammetric quantitation using a mercury-coated platinum electrodes.

Ethidium bromide (Figure 2.1) is a fluorophore which can intercalate into dsDNA. The intercalation of ethidium bromide leads to aromatic stacking. Hence, the free and bound dye has an excitation wavelength of 555 nm, but while the emission wavelengths are quite similar (595 vs. 606 nm for the free and bound forms), the fluorescence of ethidium increases about fifteen fold in the presence of DNA. The fluorescence intensity is linearly proportional to amount of dsDNA present.[10]

The voltammetric technique requires the adsorption of ssDNA onto a mercury electrode surface. Differential pulse voltammetry is then performed over the range of -0.4 to -1.6 volts. Differential pulse voltammetry is a process by which small amplitude pulses are applied and then superimposed on a staircase waveform. The current is measured immediately before and after the pulse, and this difference is plotted as a function of the applied potential. The resulting voltammograms shows a peak-shaped response. The difference in current is very small if the sample being analyzed is not undergoing a redox reaction. When the sample begins to undergo a redox process, near its $E^{o'}$, significantly changes its concentration at the electrode surface, this difference will increase to a maximum. At potentials close to the $E^{o'}$, the difference decreases to insignificant values.[12] This method is believed to have a detection limit of 100 pg/ml.[11]

In this chapter, the methods of quantitating DNA are tested, using calf thymus DNA as the analyte. Following this, two DNA quantitation methods are used to determine the actual amount of DNA immobilized by the carbodiimide (CovaLink[R]) method, using microtiter plates.

4.2 Experimental

4.2.1 Materials

Calf thymus DNA (Lot # 53H9536), diphenylamine, 3,5-diaminobenzoic acid, N-bromosuccinimide, Tris, sodium bicarbonate, acetaldehyde, fluorescein isothiocyanate, 1,6-diaminohexane and ethidium bromide were purchased form Sigma. Mercuric chloride and HEPES buffer were obtained from Aldrich. Glacial acetic acid, perchloric acid, sulfuric acid and ethanol were all ACS grade from Anachemia. Phenol-chloroform-isoamyl alcohol of molecular biology grade was purchased from Gibco-BRL. All other reagents were of the highest possible quality.

In the course of these experiments, the Cary 1 UV/visible spectrophotometer was used for all absorbance measurements, the BioTek E1307C microplate reader was used for all measurements in a microtiter plate, the Shimadzu model RF-5000 spectrofluorometer was used for all fluorescence measurements and the BAS-100 Electrochemical Analyzer was used for differential pulse voltammetry. Quartz absorbance and fluorescence cells from Fisher were used throughout. A platinum wire auxilliary electrode (Fisher), and a Pt disk working and a Ag/AgCl reference electrode (BAS) were used throughout.

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4.2.2 Methods

4.2.2.1 Large-Scale Diphenylamine (DPA) Assay[1,2,3]

Diphenylamine (0.75 g) was dissolved in 50 ml of glacial acetic acid. To this solution was added, 0.750 ml of concentrated sulfuric acid and 250 µl of 1.6% (v/v) acetaldehyde. This was the diphenylamine reagent solution, and was combined 1:1 with the DNA solution prepared in distilled water or immobilization buffer. The test tubes were sealed and heated in a boiling water bath for fifteen minutes, and cooled to room temperature. The absorbance was then measured at 600 nm using the diphenylamine reagent solution as a blank. This treatment tends to warp the microtiter plates so it is not amenable to a microtiter-based assay.

4.2.2.2 Microtiter DPA Assay[4]

The calf thymus DNA used in this method was sonicated for twenty minutes before use. 100 µl of a DNA sample was added to each well. To this was added 60 µl of 1:5 0.16% acetaldehyde in water : 20% (v/v) perchloric acid. Then, 100 µl of 4%(w/v) DPA in glacial acetic acid was added. The plate was sealed with parafilm and incubated at 37°C for 24 hours. The plates were then cooled to room temperature and the absorbance values were measured at 570 and 630 nm.

4.2.2.3 3,5-Diaminobenzoic Acid (DABA) Assay[1,6,7,8,9]

DNA standards were made up to a total volume of 1.000 ml in water or

immobilization buffer. To this was added 100 μl of concentrated perchloric acid to a final concentration of 1 M. The tubes were covered with parafilm and incubated for one hour at room temperature. DABA (32 mg) was dissolved in 80 μl of water, and equal volumes of DABA and DNA solutions were mixed and incubated at 55°C for one hour. The plate was then cooled to room temperature. Aliquots (250 μl) were diluted with 1.500 ml of 1 M HCl and the fluorescence was measured with a λ_{ex} = 408 nm and λ_{em} = 492.8 nm.

4.2.2.4 Ethidium Bromide Quantitation of DNA[10]

A range of DNA concentrations were prepared in 10 mM Tris, pH 8.0. A solution of 0.14 mM ethidium bromide was prepared in the same buffer. The fluorescence spectra of ethidium bromide were obtained in the presence and absence of DNA. The DNA standards were diluted 1:1 with the ethidium solution and the fluorescence intensities were measured using λ_{ex} = 540.8 nm and λ_{em} = 555.0 nm, the wavelength DNA-bound ethidium. Calibration curves were prepared of fluorescence intensity vs. DNA concentration.

4.2.2.5 Voltammetric Quantitation of DNA with Platinum Electrodes[13]

The first step was to electrochemically coat the platinum surface with mercury. A platinum electrode was placed in an electrochemical cell, thermostatted at 25°C, which contained 25.00 ml of a 0.1 M mercuric chloride solution, a platinum wire as an auxiliary electrode and a Ag/AgCl reference electrode. A voltage of -300 mV was

applied to the working electrode for 20 minutes. This caused the deposition of a layer of mercury on the electrode surface. The electrode was then rinsed with water and carefully blotted dry. The mercury-coated electrode was inverted, and 100 µl of a denatured DNA standard was applied to the surface and allowed to adsorb for five minutes. The excess DNA was rinsed off with distilled water and the electrode was carefully blotted dry. The DNA-coated electrode was placed into an electrochemical cell containing 25.00 ml of deaerated 0.2 M sodium phosphate buffer, pH 6.7, a platinum wire and a Ag/AgCl reference electrode. Deaeration with N_2 was continued for five minutes. With a nitrogen atmosphere above the solution, DPV was performed from -400 to -1600 mV. Other DPV parameters were: a 10 mV/sec scan rate, a 50 mV pulse amplitude and a quiet time of ten seconds at -400 mV prior to the scan. All other settings were left at default values.

To clean the electrode, DPV was performed over the +400 to +1600 mV range twice. The electrode was then rinsed, polished and sonicated in water for ten minutes.

4.3 Results

4.3.1 DPA Assays

The first method to be tested was the diphenylamine assay. The large-scale uses a very strong acid solution which was found to dissolve the polystyrene microtiter plates. This problem was overcome by performing the assay in stoppered, glass test tubes. Calf thymus DNA standars were prepared, subjected to the assay and then absorbance at 600 nm was recorded. Figure 4.1 indicates that the DPA assay is linear over the 5-1000 μg/ml concentration range. Linear regression of the results in Figure 4.1 yields a slope of $2.04 \times 10^{-3} \pm 8.58 \times 10^{-5}$ and a y-intercept of $0.24 \pm .06$, with $\sigma_y = 0.077$. The detection limit of this method, calculated as the DNA concentration needed to give a signal of $2\sigma_y$ above the blank, is 75 μg/ml. The immobilization procedures use low DNA concentrations (3-10 μg/ml), thus this assay is too insensitive to be useful for quantitation.

This assay, however, was recently adapted to a microtiter-based format with a lower detection limit. This was possible by making use of a more dilute acid medium which was much less corrosive and did not damage the wells. The assay was applied to our system, and the results are presented in Figure 4.2. In this format, the detection limit is 20 μg/ml. The lack of a filter at 600 nm may have compromised the sensitivity of the assay because of the need to work away from λ_{max}.

FIGURE 4.1. Calibration curve for the large-scale diphenylamine assay for the quantitation of DNA. A range of calf thymus DNA concentrations were used to prepare the calibration curve. The blue product was detected at 600 nm. Each point is the average ± one standard deviation of 5 measurements.
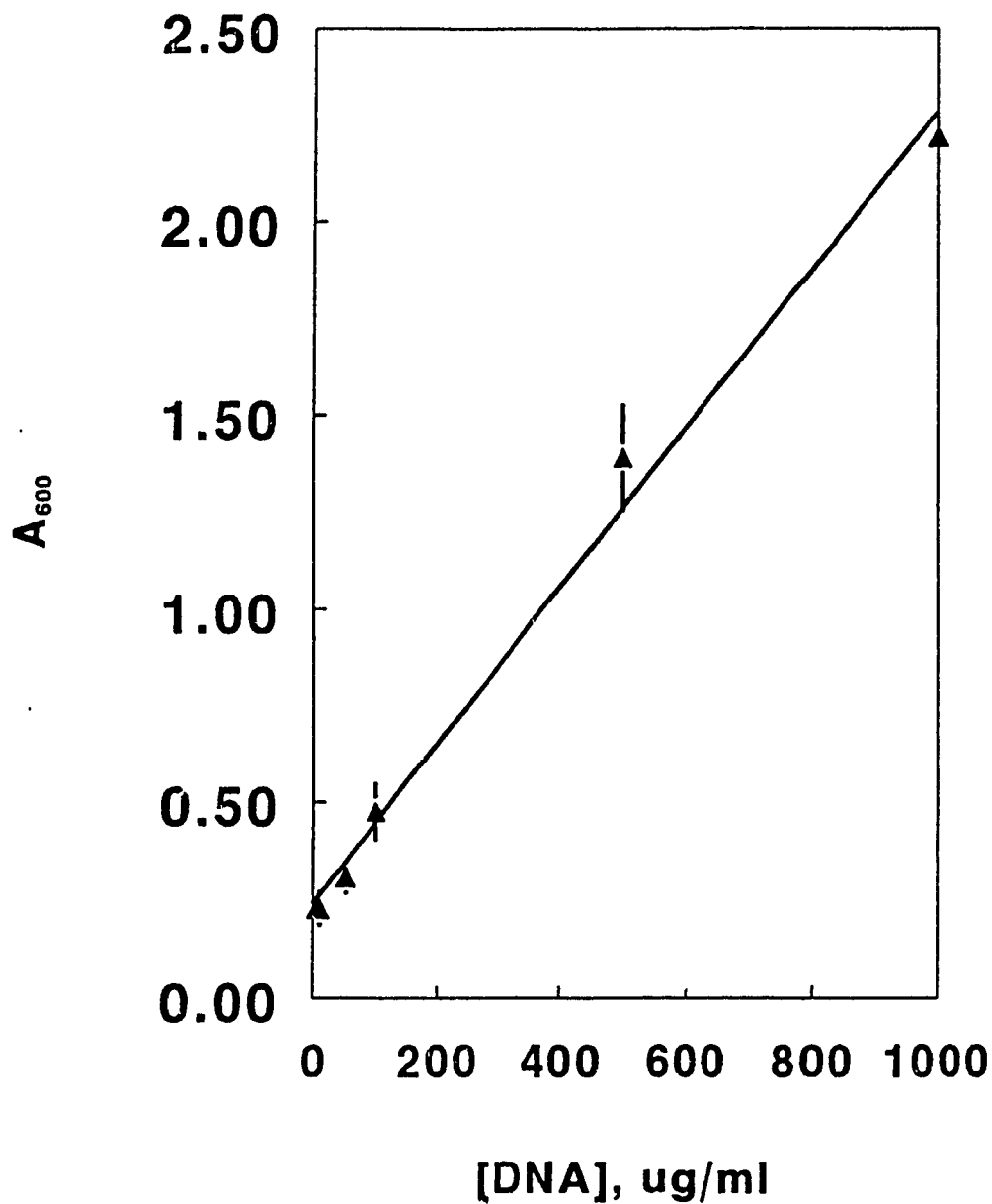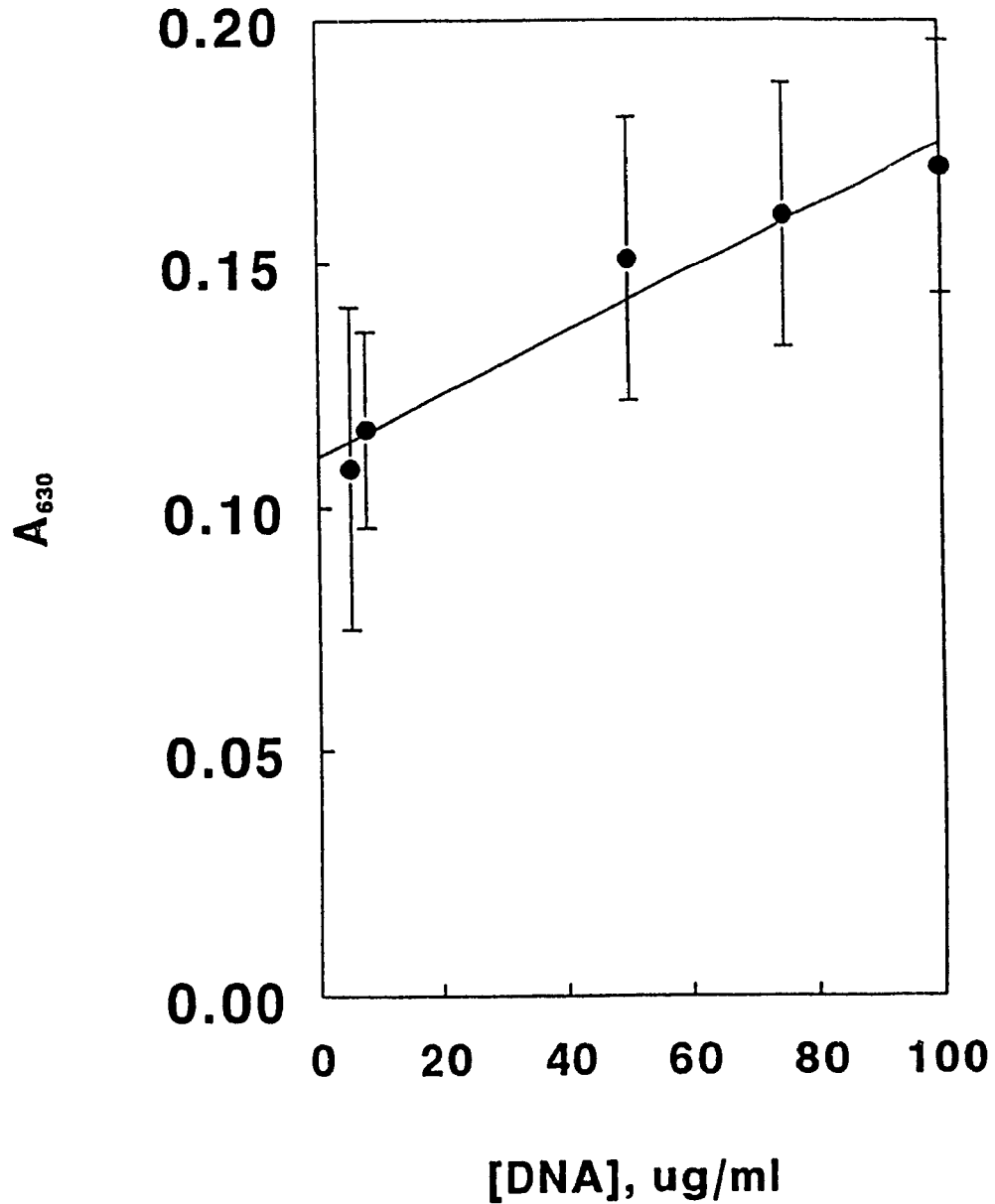
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FIGURE 4.2. Calibration curve for the microtiter-based diphenylamine assay for the quantitation of DNA. A range of calf thymus DNA concentrations were used to prepare the calibration curve. The blue product was detected at 630 nm. Each point represents the average ± one standard deviation of 4 measurements.

4.3.2 DABA Assay

The last chemical derivatization method is the 3,5-diaminobenzoic acid (DABA) assay. This assay requires modifying the DNA with DABA and then detecting the product, using an excitation wavelength of 408.00 nm and an emission wavelength of 492.8 nm. A plot of fluorescence intensity as a function of DNA concentration is presented in Figure 4.3. This plot is observed to be linear over the range of 1 to 5 μg/ml, and has a calculated detection limit of 130 ng/ml. This is a useful method for quantitation of immobilized DNA.

4.3.3 Ethidium Method

Table 2.1 (Chapter 2) shows that the absorbance maximum of ethidium in the presence of 1.52 mM DNA occurs at 520.0 nm. Using this excitation wavelength, an emission maximum was observed at 555.0 nm. Using these wavelengths, a calibration curve of flourescence intensity as a function of DNA concentration was prepared and is presented in Figure 4.4. This graph reveals that linear behaviour is being observed over the range of 0.5 to 1000 μg/ml. A detection limit ($2\sigma_y/m$) was calculated as 6.0 μg/ml. This method can be used for quantitating dsDNA but would not be useful for ssDNA. Any ssDNA would have to be hybridized prior to analysis.

4.3.4 DPV Method

The remaining method depends on the adsorption of DNA to an mercury-coated Pt electrode surface prior to differential pulse voltammetry. Plating can actually be observed as the adsorbed Hg appears as a visible white layer on the electrode surface.
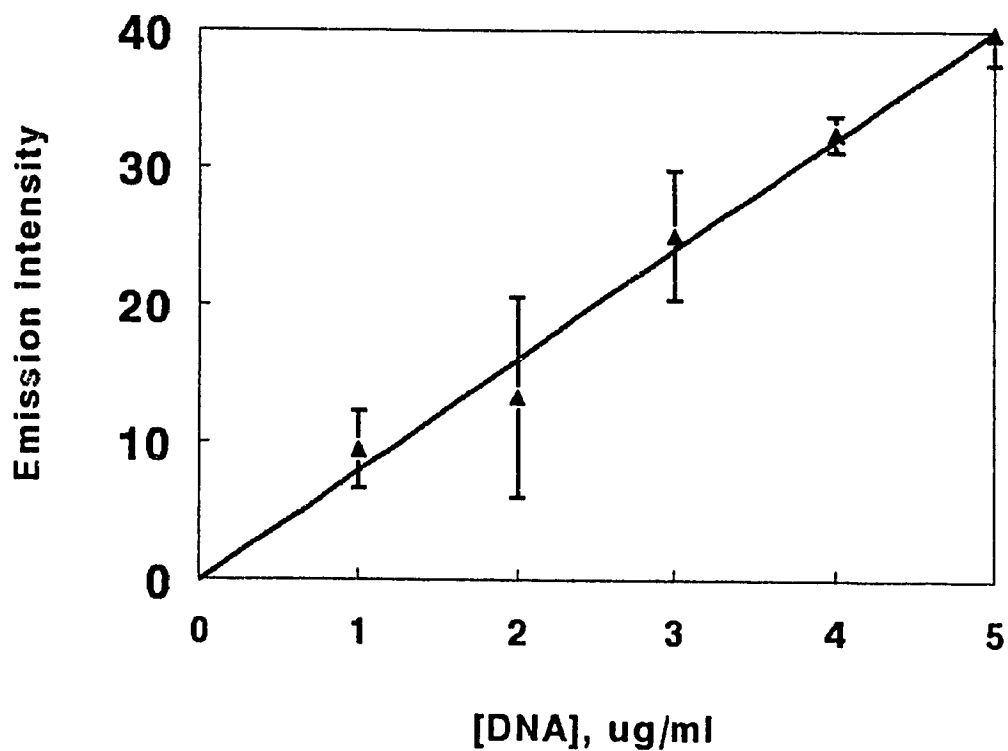
FIGURE 4.3. Calibration curve for the DABA method for the quantitation of DNA. A range of calf thymus DNA concentrations were used to prepare the calibration curve. The fluorescence intensity was measured with an excitation wavelength of 408.00 nm and an emission wavelength of 492.8 nm. Each point is the average ± one standard deviation of 3 measurements.

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After allowing denatured calf thymus DNA to adsorb to the mercury electrode surface, the amount of DNA can then be determined by DPV. Figure 4.5 shows a typical voltammogram in the presence of 0.4 μg/ml calf thymus DNA. One large peak is seen, at -1.344 V, vs. Ag/AgCl. This peak is used to quantitate adsorbed DNA. The resulting calibration curve is presented in Figure 4.6. There is a linear dependence of peak current with DNA concentration over the range of 0.4 - 6 μg/ml. The calculated detection limit $(2\sigma_y/m)$ for this method is 9.03 μg/ml. Therefore. the DPV method is useful for quantitating immobilized DNA.

Table 4.1. **Comparison of DNA Quantitation Methods**

Technique	Concentration Range (μg/ml)	Volume Required (ml)	R	Detection Limit (μg/ml)
Large-Scale DPA	5 - 1000	2.00	0.996	75
Microtiter DPA	5 - 100	0.100	0.978	20
DABA	1 - 5	0.125	0.992	0.13
Ethidium	0.5 - 1000	1.50	0.999	6.2
DPV	0.4 - 6	0.100	0.986	0.91

Table 4.1 compares the quantitation techniques. This table indicates that only the DABA and DPV methods provide low enough detection limits for the quantitation of immobilized DNA. The ethidium method cannot be used to quantitate ssDNA, so the DPV methods were used to quantitate the amount of DNA immobilized.

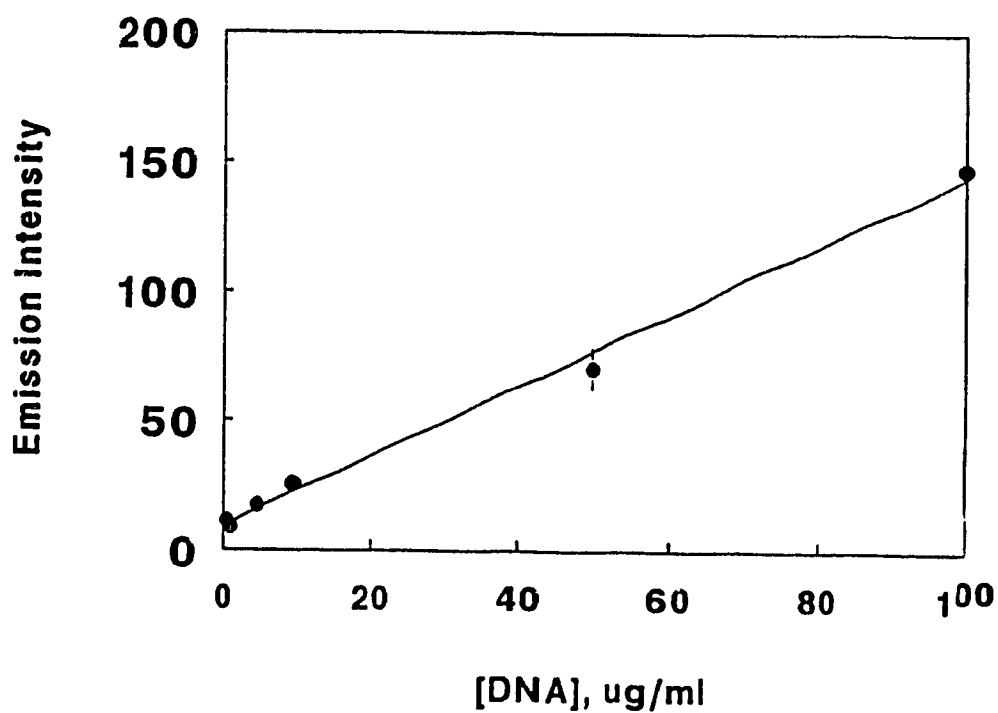**FIGURE 4.4.** Calibration curve for the fluorescence method for the quantitation of DNA using ethidium bromide as an intercalant. A range of calf thymus DNA concentrations were used to prepare the calibration curve. The fluorescence intensity was measured with an excitation wavelength of 540.8 nm and an emission wavelength of 555.0 nm. Each point represents the average ± one standard deviation of 3 measurements.

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EXP. CONDITIONS:

INIT E(mV)= -400
FINAL E(mV)= -1600
V (mV/SEC)= 10
PULSE AMPLITUDE(mV)= 50
SAMPLE WIDTH(mSEC)= 17
PULSE WIDTH(mSEC)= 50
PULSE PERIOD(mSEC)= 200

5uA   EXP. RESULTS:

PEAK POTENTIAL(mV)= -524
PEAK CURRENT(A)= 1.5059E -7

PEAK POTENTIAL(mV)= -914
PEAK CURRENT(A)= 3.0881E -7

PEAK POTENTIAL(mV)= -1344
PEAK CURRENT(A)= 1.6288E -5

0.400   -1.0   -1.600

E (VOLT)
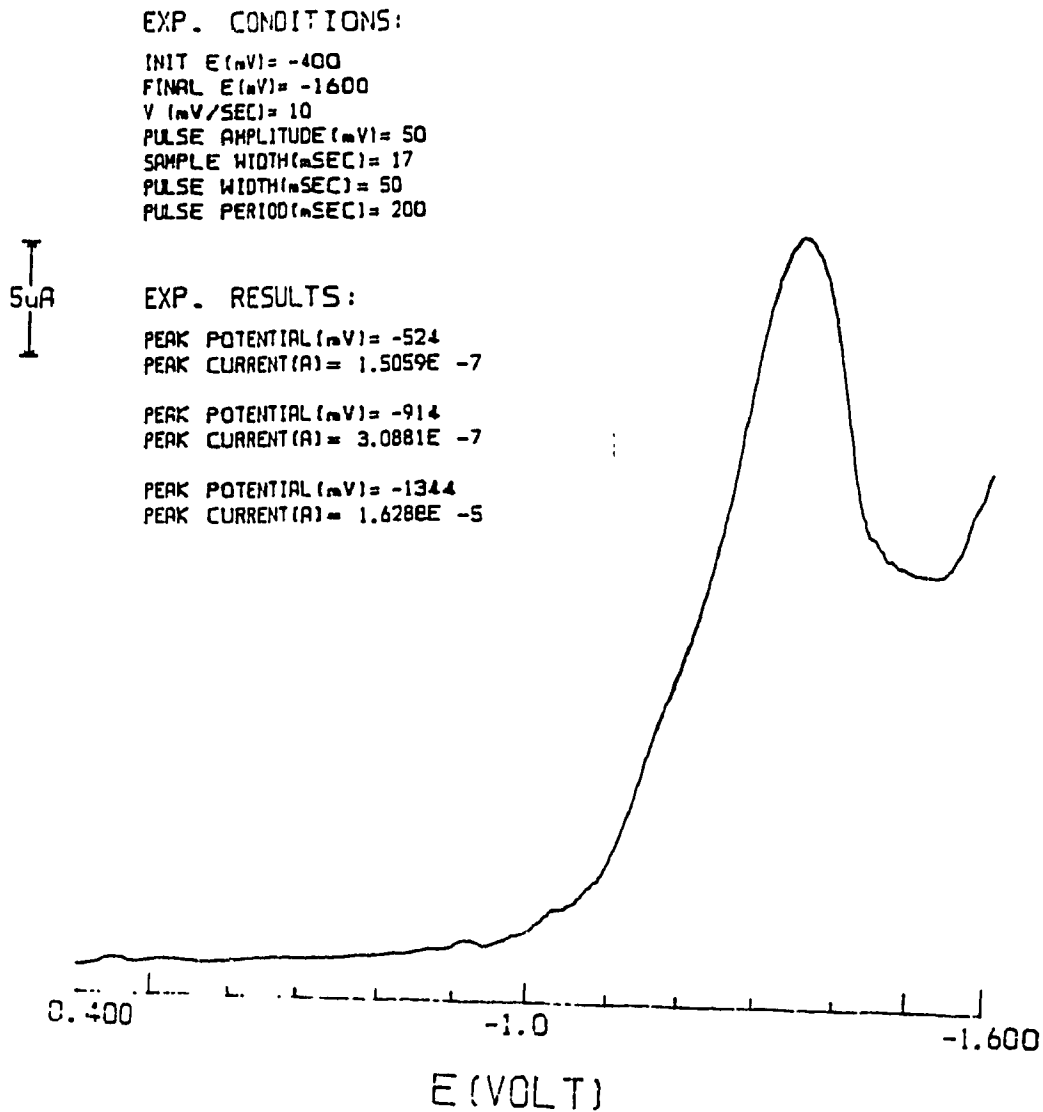
FIGURE 4.5. A typical voltammogram for the quantitation of DNA using a mercury-coated platinum electrode. A DNA standard of 0.4 µg/ml was adsorbed to the surface of the electrode. DPV was performed with a platinum wire as the auxiliary electrode and a Ag/AgCl reference electrode and the sample was scanned from -400 to -1600 mV.

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FIGURE 4.6. Calibration curve for the voltammetric quantitation of DNA using a mercury-coated platinum electrode. Denatured calf thymus DNA standars used to prepare the calibration curve. The DNA was analyzed by DPV over the range of -400 to -1600 mV. Each point represents the average ± one standard deviation of 3 measurements.

4.3.5 Quantitation of Immobilized DNA
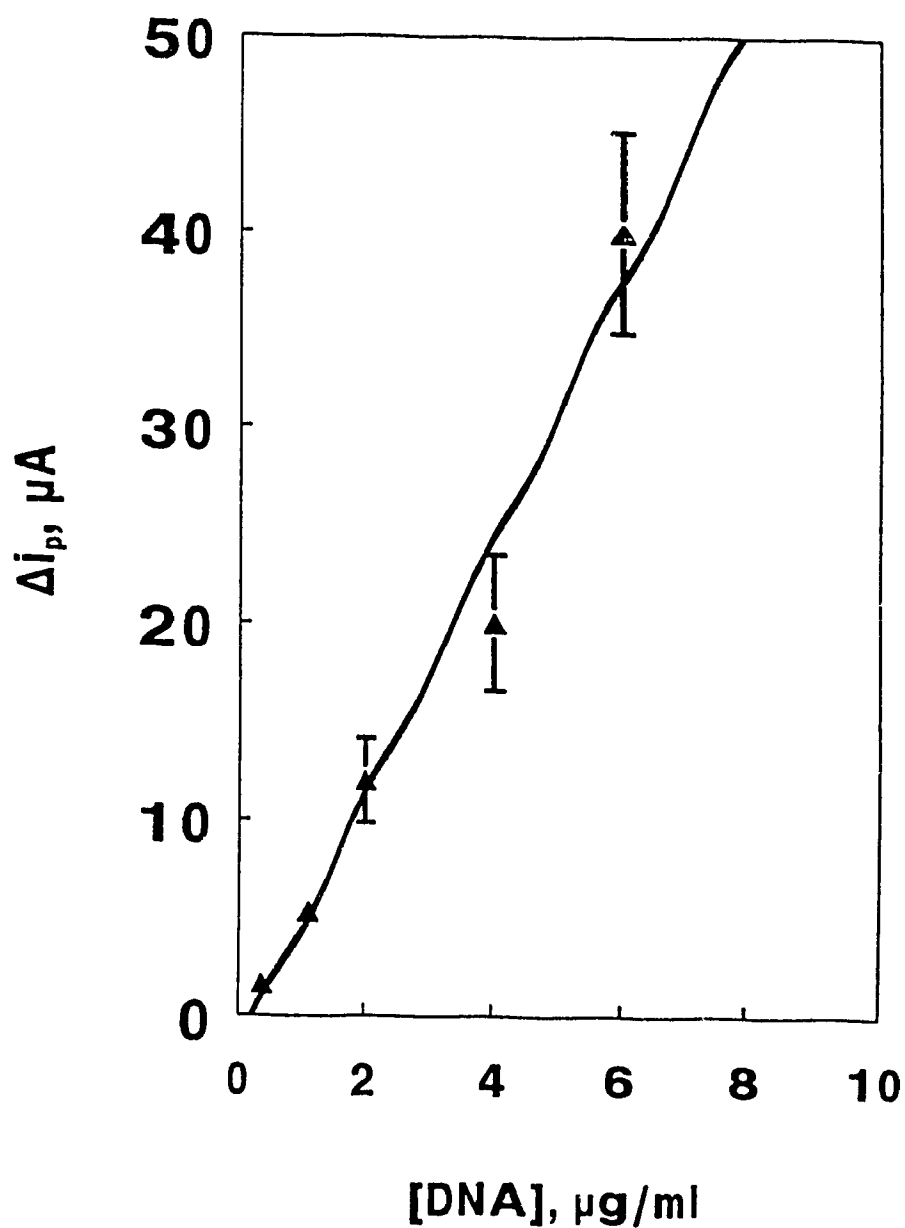
DNA (calf thymus) was immobilized onto the secondary amine-modified CovaLink^R microtiter plates, by the carbodiimide method (see Chapter 3, section 3.2.2.2.2). In one experiment, the initial DNA concentration was 3.0 µg/ml, and the DNA remaining in solution was quantitated by the DABA assay. In the second experiment, the initial DNA concentration was 10.0 µg/ml, and DPV was used to quantitate soluble DNA in the wells. The solutions remaining in the wells (200 µl) were combined to provide 1.00 ml for the DABA assay and 1.00 ml for the DPV assay. The results of these assays are presented in Table 4.2.

Table 4.2. DNA (µg/ml) Immobilized on Covalink^R Plates as determined by DABA and DPV methods

Method	[Initial]	[Final]	[Bound]	DNA Bound (ng/well)
DABA	3.0	2.0 ± 0.2	1.0 ± 0.1	200
DPV	10.0	2.48 ± 0.02	7.52 ± 0.04	1500

4.4 Discussion

The methods studied in this chapter provide an objective criterion to compare the amount of DNA actually immobilized using different methods. It has been observed in previous experiments that immobilization reactions did not always work,

leading to poor signal intensities and atypical behaviour upon hybridization and detection.

A method by which DNA could be quantitated following immobilization and/or hybridization would be useful in improving the well-to-well and plate-to-plate reproducibility of the DNA assay.

Given the facts that the amount of DNA actually immobilized is likely to be quite low and most of the methods described cannot be used directly in the microtiter plate, it was necessary to quantitate DNA by difference. The DNA remaining in solution after the immobilization reaction is compared to the initial concentration of DNA. The difference in concentration multiplied by the solution volume in the well is the amount of DNA that was bound.

In this chapter, five methods of DNA quantitation were used to prepare calibration curves for calf thymus DNA. Table 4.1 shows the results of these studies.

The microtiter plate-based DPA assay had the potential advantage that it uses absorbance detection, can be used to monitor immobilized DNA directly or by difference and it has a lower detection limit then the large-scale DPA assay. It was tested as a potential method for quantitating the DNA used in our assay. From Figure 4.2 and Table 4.1, it can be seen that the expected linear dependence of absorbance signal with DNA concentration was indeed observed over the range of 5 - 100 μg/ml, with a detection limit of 20 μg/ml. Considering that 100 μl of DNA were added per well, this indicates that the assay was able to detect 2.0 μg of DNA which is considerably higher than the literature value of 10 ng. This may be explained by the fact that the recommended wavelength was unavailable and measurements were performed 30 nm

away from the absorbance maximum of the product. The DPA method proved too insensitive to be used for our assay conditions and was thus abandoned.

The second method of quantitation by chemical derivatization was the DABA method. The product of the DABA reaction is detected fluorimetrically. This method showed a linear dependence of emission intensity as a function of DNA concentration over the 1 - 5 µg/ml range (Figure 4.3), with a detection limit of 13 ng in 100 µl. This value is lower than the literature detection limit of 30 ng[1,6,7,8,9], and could be due to the use of 10 mM 1-methylimidazole as the buffer. However, the use of fluorescence as the detection reduced the amount of DNA detected by almost an order of magnitude.

Although these methods were successful at quantitating DNA, they all suffer the disadvantage that they rely on a chemical reaction with DNA. Chemical reactions are time-consuming, do not always go to completion and are difficult to control. To overcome this disadvantage, the ethidium fluorescence and differential pulse voltammetric (DPV) methods were tested. Of these, only the DPV method is capable of quantitating ssDNA. The detection limit of 0.91 µg/ml achieved with the DPV method is low enough to allow quantitation of immobilized DNA on microtiter plates.

The DPV and the DABA assays were both found useful for quantitating immobilized DNA, and were tested in the quantitation of carbodiimide-immobilized DNA (Table 4.2). In both cases, about 30% of the concentration of DNA was immobilized, corresponding to 200 ng (original [DNA] = 3.0 µg/ml) and 1500 ng (original [DNA] = 10.0 µg/ml). These results indicate the importance of the initial DNA concentration on

the quantity immobilized, but it is not yet known what effect this would have on the final signals obtained with a hybridization indicator. Further work, involving DNA quantitation after immobilization and after hybridization, should be done to correlate these values with the final signals obtained. For this work, the DABA assay is preferred, because it tends to be more consistent and can be performed in an afternoon, whereas the electrochemical detection requires one hour per sample. Thus, the DABA assay is the method of choice to measure DNA concentration.

4.5 References

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CHAPTER 5

SUMMARY AND SUGGESTIONS FOR FUTURE RESEARCH

From the results presented in Chapters 2-4, the following general conclusions can be made:

1) While both a simple intercalating dye (3,6-diaminoacridine) and an intercalant-enzyme conjugate ((daunomycin)$_4$-glucose oxidase) can be used as hybridization indicators, the latter provides larger signals due to catalytic amplification.

2) Of the four DNA immobilization methods tested, the carbodiimide (CovaLink^R) and the biotin-avidin methods show the best reproducibility. They are also easier and less hazardous to perform. The biotin-avidin method is preferred because it does not result in polymerization of DNA.

3) The 3,5-diaminobenzoic acid (DABA) assay is the preferred method for DNA quantitation, and it was used in conjunction with the differential pulse voltammetry method to quantitate carbodiimide-immobilized DNA by difference. The amount of DNA immobilized was found to be 200 ng/well, and 1500 ng/well, with initial concentrations of 3.0 and 10.0 µg/ml, respectively.

On the basis of these experimental results, a number of recommendations

regarding future work can be made. The first step should be to extend the DNA quantitation techniques to homopolymers, such as poly(dA). The amount of DNA immobilized by the carbodiimide and biotin-avidin methods, using different DNA concentrations should then be compared with final signals obtained with the hybridization indicators.

Having related the final signal intensity to DNA concentration, the next step would be to determine the sequence-selectivity of the DNA assay.

This work must then be extended to shorter DNA sequences. Instead of immobilizing poly(dA), it would be useful to immobilize a 20-mer or a 30-mer synthesized with a DNA synthesizer, possibly with a biotin group added, to determine how the length of the immobilized DNA affects recognition of a complementary sequence and signal intensity. The assay should be optimized for short sequences.

Part of this optimization may involve improving the detection system. It may be useful to modify daunomycin or glucose oxidase with a bifunctional reagent that will bind to the other two species. In the process, it will become a spacer arm that may improve the ability of modified daunomycin to intercalate into double-stranded DNA. This should lead to higher signal intensities which will be helpful with short sequences, as the shorter the sequence, the fewer sites exist for binding an intercalant and the lower would be the observed signal. It would also be recommended that a search be launched for another enzymatic reaction that is equally or more sensitive but that will not irreversibly stain the plates.

Once these goals are met, the assay should be tried on a disease sequence

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to determine whether it can truly be used as a detection method. This would imply immobilizing a known sequence corresponding either to a disease state or a normal state. The wells would be divided into two groups. To one group would be added a complementary sequence, to the other a noncomplementary sequence. The signal intensities should be significantly different between the two groups if the method is successful.

APPENDIX 1

10 Input "dye conc in mM"; dyeconc

20 Input "Abs at 600 nm for free dye"; a600

30 efree 600 = 1000 * a600 / dyeconc

40 Input "Abs at 616 nm for free dye"; a616

50 efree 616 = 1000 * a616 / dyeconc

70 Input "Abs at 600 nm ofr bound dye"; b600

80 ebound 600 = 1000 * b600 / dyeconc

90 Input "Abs at 616 nm for bound dye"; b616

100 ebound616 = 1000 * b616 / dyeconc

110 Input " number of points"; n

120 for i = 1 to n

130 Input "DNA conc in bpy(M)"; bp

140 Input "Abs at 600 nm"; c600

150 Input "Abs at 616 nm"; c616

160 cfree = (c616 * ebound600 - c600 * ebound616) / efree 616 * ebound600 -efree600
*   ebound616

170 cbound = c616 * efree600 - c600 * efree616)/ebound616 * efree600 - ebound600 *
efree616)

180   LPrint "[bp]= "; bp; "cfree= " cfree; "cbound= "; cbound

185   Print "bp"; bp; "cfree="; cfree; "cbound="; cbound

190   r= cbound / (bp)

200   LPrint "r="; r; "r / cfree="; r / cfree

```
205        Print "r="; r; "r / cfree="; r / cfree

210 Next i

215 End
```