UNIVERSITY OF ALBERTA

Development of a Binding-Induced DNA Assembly Assay for Carbohydrate Antigen 19-9

by

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ABSTRACT

Binding-induced DNA assembly (BINDA) was initially developed for protein detection. To demonstrate the homogeneous detection of carbohydrates using this principle, a BINDA assay for the pancreatic cancer marker carbohydrate antigen 19-9 (CA 19-9) was developed. The assay uses two DNA-antibody conjugates as probes, with the DNA oligonucleotides designed to have a complementary base-pairing sequence. Binding to CA 19-9 brings the probes into close proximity, allowing the complementary regions to hybridize. Enzymatic ligation joins the probe oligonucleotides and amplification and detection of the ligation product by real-time PCR is used to quantify CA 19-9. The principle was first demonstrated using streptavidin as a model target before developing and optimizing the BINDA assay for CA 19-9. CA 19-9 could be detected in both buffer and human serum. This study is the first description of the use of BINDA for carbohydrate analysis.

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LIST OF SYMBOLS, NOMENCLATURE, OR ABBREVIATIONS

α	alpha
β	beta
Δ	delta
ϕ	phi
ºC	degree Celsius
%	percentage
′	prime
A	adenine
ATP	adenosine-5′-triphosphate
AuNP	gold nanoparticle
BINDA	binding-induced DNA assembly
BSA	bovine serum albumin
C	cytosine
CA 19-9	carbohydrate antigen 19-9
CEA	carcinoembryonic antigen
Ct	threshold cycle
Da	dalton (1 g/mol)
DNA	deoxyribonucleic acid
ECPA	electrochemical proximity assay
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay

FBS	fetal bovine serum
FDA	Food and Drug Administration
FPLC	fast protein liquid chromatography
FRET	fluorescence resonance energy transfer
g	acceleration due to gravity
G	guanine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	human epidermal growth factor receptor 2
HPLC	high performance liquid chromatography
IgG	immunoglobulin G
K	equilibrium constant
K_a	association constant
K_d	dissociation constant
KCl	potassium chloride
Le	Lewis
M	molar concentration (mol/L)
MB	methylene blue
$MgCl_2$	magnesium chloride
MUC	mucin
NaCl	sodium chloride
NaOH	sodium hydroxide
OD_{280nm}	optical density, absorbance at 280 nm
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEA	proximity extension assay
PEG	polyethylene glycol
pH	potential of hydrogen
PLA	proximity ligation assay
PSA	prostate specific antigen
PVDF	polyvinylidene fluoride
RCA	rolling-circle amplification
rcf	relative centrifugal force
RNA	ribonucleic acid
ROX	5-carboxy-X-rhodamine
rpm	revolutions per minute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SELEX	systematic evolution of ligands by exponential enrichment
T	thymine
TEG	tetra-ethyleneglycol
T_m	melting temperature
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
U	units
VEGF	vascular endothelial growth factor
XX	spacer composed of two aminohexanoic chains

CHAPTER 1

Literature and Objectives

1.1 General Introduction

Proteins and carbohydrates serve a multitude of biological functions. In disease states, the expression and distribution of these biomolecules is often altered. Therefore, the detection of these biological markers, or biomarkers, in fluid or tissue samples can indicate the health status of patients and aid in the management of disease. Carbohydrate antigen 19-9 (CA 19-9) is a biomarker that is often present at elevated concentrations in cases of pancreatic cancer.

A great deal of research has been generated in regards to assays to qualitatively or quantitatively determine the presence, concentration, or activity of biomarkers. In the 1940s, the first affinity based assay used a fluorescein conjugated antibody to visualize the antigen in tissue [1]. Detection methods for proteins and carbohydrates in serum emerged in the 1960s with the advent of the radioimmunoassay [2]. Further developments included the sandwich immunoassay format, enzyme-linked immunosorbent assay (ELISA) [3], and immunosensors. While these technologies have been useful, there is still a drive to improve upon current techniques and develop new methods in order to detect lower concentrations, improve the sensitivity and specificity, simplify procedures and shorten analysis times, and/or measure several biomarkers in one assay. Beginning in the early 1990s, DNA-assisted immunoassays have been developed for protein or carbohydrate detection in an attempt to meet the challenges of

biomolecule analysis. As Fredriksson et al. [4] noted, "the technical ability to analyze nucleic acids is far more advanced than that for proteins." Ingenious methods have been developed including immuno-PCR, the proximity ligation assay (PLA), and molecular pincers that marry protein detection with the advantages of nucleic acid analysis. The binding-induced DNA assembly assay described in this thesis is another such DNA-assisted analytical technique.

This chapter provides a brief overview of CA 19-9 and its use as a serological marker of pancreatic cancer. The polymerase chain reaction (PCR) will be described, as real-time PCR is the technique used for detection of DNA in the assay developed for this thesis research. Developments in DNA-assisted assays for proteins and carbohydrates involving amplification of DNA, or assembly of DNA with subsequent generation of a fluorescent or electrochemical signal, will also be described. The objectives of this thesis research are then outlined.

1.2 Cancer Biomarkers

A biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic response to a therapeutic intervention" [5]. In carcinogenesis, a number of changes can occur to normal biological processes, resulting in the aberrant expression or alteration of biomolecules that can then serve as tumor markers. The changes can be exploited to aid in the detection of cancer, help determine the diagnosis or prognosis, predict the optimal therapeutic intervention,

monitor how the patient is responding to treatment, or detect the recurrence of disease [5, 6]. A survey of assays approved by the United States Food and Drug Administration (FDA) prior to 2009 revealed that there were 109 unique protein targets in serum/plasma. Of the other approved assays, 18 targeted post-translational modifications, including 6 that assessed glycosylation [7]. Glycans consist of covalently linked saccharides, and may form covalent complexes with proteins or lipids, or exist in free form [8].

1.2.1 Carbohydrate cancer biomarkers

In the progression of cancer, genetic alterations can cause an overexpression of enzymes involved in glycan biosynthesis, producing carbohydrate structures that are different than those carried by normal cells. The glycosylated molecules are released into the circulation by secretion or proteolytic cleavage [9], making blood-based tests possible. The glycans may also contribute to the progression of cancer, as they can play a role in cell proliferation, invasion, adhesion, metastasis, and angiogenesis, and can alter host immunity [8]. Many of the commonly used tumor markers are glycoproteins, including carcinoembryonic antigen (CEA) for colorectal cancer and cancer antigen 125 (CA 125) for ovarian cancer [9, 10]. The biomarker carbohydrate antigen 19-9 (CA 19-9), also known as sialyl Lewis a (sialyl Le^a), is a terminal glycan carried on mucins in blood. CA 19-9 is a FDA approved biomarker for monitoring pancreatic cancer [10]. An advantage of carbohydrate biomarkers over protein markers is that mucins and

glycoproteins often carry multiple carbohydrate chains, so the signals produced from detection are amplified [9].

1.3 Carbohydrate Antigen 19-9

1.3.1 History of discovery

In the late 1970s, Koprowski and co-workers immunized a BALB/c mouse with cells of the SW1116 human colorectal carcinoma cell line, and created hybridomas that generated a number of monoclonal antibodies including antibody 1116NS-19 of the IgG_1 isotype [11]. The idea that identification of the target antigens could help elucidate changes that occur during the development of cancer and open new avenues for diagnosis and treatment spurred further research. In a binding inhibition assay, binding of the newly discovered antibody to membrane extracts of SW1116 cells was significantly inhibited by the serum of patients with advanced colorectal carcinoma, pancreatic carcinoma, and gastric carcinoma, but was not inhibited by the serum of healthy volunteers [12]. The observation that treatment of SW1116 cells with neuraminidase prevented antibody binding while treatment with the protease ficin did not have an effect, and chromatography results of total lipid extracts of SW1116 cells, led to the conclusion that the cancer-associated antigen was a monosialoganglioside [13]. Gas chromatography-mass spectrometry analysis provided the first description of the carbohydrate structure, and the molecule was identified as sialyl Le^a [14].

1.3.2 Structure and synthesis

The CA 19-9 antigen is a sialylated derivative of the Le^a antigen with the sequence Neu5Acα2,3Galβ1,3(Fucα1,4)GlcNAc (Figure 1.1), and is therefore related to the Lewis (Le) blood group system.

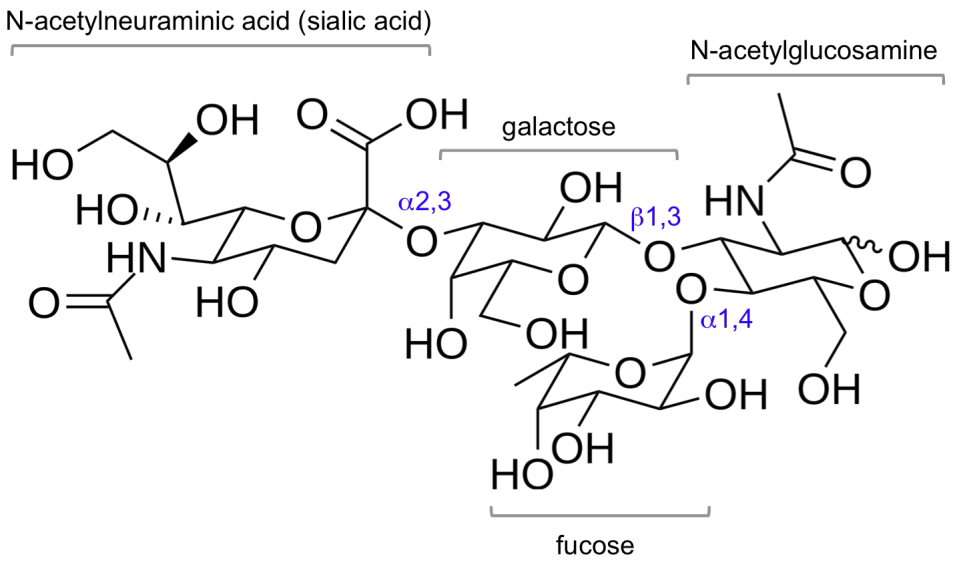

Figure 1.1 Structure of sialyl Le^a. Carbohydrate residues and glycosidic bond linkages are indicated. Adapted from a public domain image from reference [15].

The synthesis of Le^a, Le^b, and sialyl Le^a is illustrated in Figure 1.2. The Lewis antigens are soluble in plasma and will adsorb to red blood cells [16]. The Lewis gene (*FUT3*) and the secretor gene (*FUT2*) on chromosome 19 dictate the Lewis phenotype of an individual. An active secretor gene encodes for α1,2-fucosyltransferase that catalyzes the addition of fucose to the terminal galactose of type 1 carbohydrate chains (chain with a β1,3 linkage between galactose and N-acetylglucosamine) [17]. An active Lewis gene encodes for α1,4-fucosyltransferase that adds fucose to the subterminal N-acetylglucosamine [16].

This results in the production of Le^a if the precursor chain was unmodified, as would be the case with non-secretors (homozygous for an inactive secretor allele), and the individual will have Le^{a+b-} red cells. Le^b is produced if the precursor chain was first modified to produce H-type 1, as would occur in individuals with active Lewis and secretor genes, and will result in Le^{a-b+} red cells. If the Lewis gene is inactive, then the Le^{a-b-} phenotype results [16]. The enzyme α2,3-sialyltransferase catalyzes the addition of a sialic acid residue to the terminal galactose residue [18]. The addition of fucose by the action of α1,4-fucosyltransferase forms sialyl Le^a. It is thought that the increased serum level of sialyl Le^a associated with carcinogenesis could be due to increased expression of α2,3-sialyltransferase [18] or the loss of α2,6-sialyltransferase that would otherwise add an additional sialic acid to sialyl type 1 to form disialyl Le^a (not shown in Figure 1.2) [19]. The synthesis of sialyl Le^a is also influenced by the Lewis blood type; strong secretors produce Le^b and less of sialyl Le^a due to competition between α1,2-fucosyltransferase and α2,3-sialyltransferase, while Lewis negative individuals cannot synthesize sialyl Le^a [20].

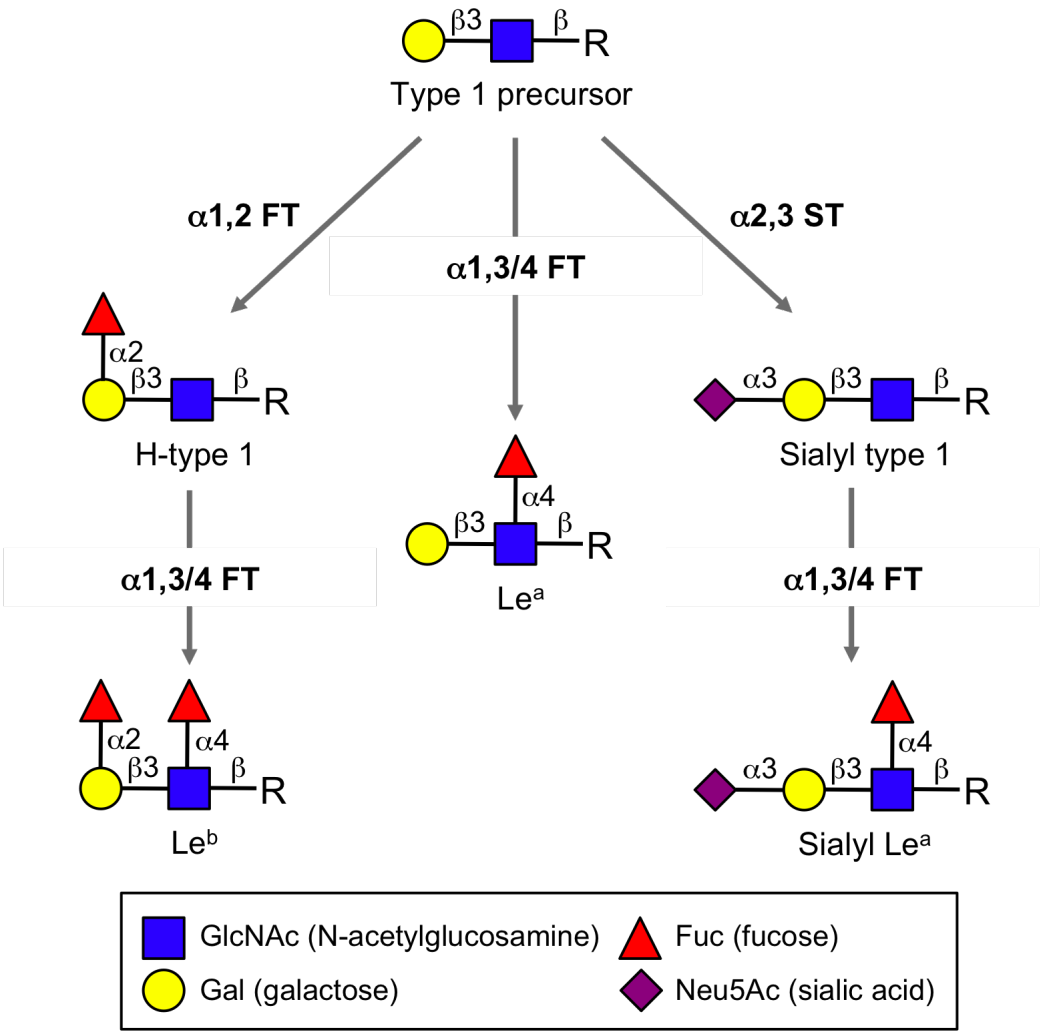

Figure 1.2 Schematic of the biosynthesis of Le^b, Le^a, and sialyl Le^a. R represents the precursor carbohydrate chain; FT, fucosyltransferase; ST, sialyltransferase. Adapted from references [17, 19-21].

In serum, CA 19-9 is found on mucins, such as MUC1, MUC5AC, and MUC16, and carcinoembryonic antigen (CEA) [22-25]. MUC1 from the serum of cancer patients was found to have increased levels of CA 19-9 relative to sera from control patients [22, 25]. More recent research involving immunoprecipitation of CA 19-9 from pooled sera and identification of the

proteins using mass spectrometry indicated that CA 19-9 is also carried on the proteins apolipoprotein E, ARVCF (armadillo repeat gene deleted in the Velo-Cardio-Facial syndrome), kininogen, and apolipoprotein B-100 [26]. However, there was no association between CA 19-9 levels on these proteins and pancreatic disease.

1.3.3 CA 19-9 as a marker of pancreatic cancer

Pancreatic cancer is not a common cancer, but it is one of the most lethal. In Canada, it was estimated there were 4600 new cases of pancreatic cancer and 4300 deaths in 2012 [27]. That same year, in the United States there were approximately 43920 new cases and 37390 deaths attributable to pancreatic cancer [28]. The unfavourable outcome is largely due to the fact that the majority of patients do not present with symptoms until they have advanced disease (Stage III or IV) where resection of the tumor is not possible [29, 30]. Pancreatic cancer is usually diagnosed based on the findings of imaging techniques such as ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI) [29, 31, 32], with confirmation by histology [29]. In an attempt to improve upon patient outcomes, many biomarkers have been investigated for their use in screening and early diagnosis of disease. However, their low specificity, selectivity, and lack of validation have limited their use. Thus far the only marker implemented for clinical use has been CA 19-9 [33].

The FDA has approved the use of serum CA 19-9 to monitor patients with pancreatic cancer [10]. Serial measurements can be used to monitor how patients

are responding to therapy; generally, a decrease in the serum level of CA 19-9 is associated with prolonged survival and an increase may be indicative of cancer progression [34, 35]. CA 19-9 has also been evaluated for other uses in patient management. Typically, 37 U/mL is designated as the cut-off value to distinguish normal from elevated CA 19-9 levels [29]. A review of 22 studies found that elevated serum CA 19-9 had a median sensitivity of 79% and median specificity of 82% for the diagnosis of pancreatic cancer [36]. A preoperative CA 19-9 level less than 1000 U/mL and a postoperative level of less than 200 U/mL were predictors of longer survival times [37]. After patients undergo surgery to remove the tumor, serum CA 19-9 can be monitored to aid in the detection of recurrent or metastatic disease [35].

However, caution should be exercised regarding the use of CA 19-9 as a pancreatic cancer marker. CA 19-9 can be elevated in several other adenocarcinomas, especially those of gastrointestinal origin, and may also be elevated in benign conditions such as pancreatitis, cholangitis, obstructive jaundice, and cirrhosis [30, 35]. CA 19-9 is not a suitable marker for all patients as it cannot be expressed by individuals with the Le^{a-b-} phenotype; this corresponds to approximately 5% to 10% of the Caucasian population [32, 35, 38]. Therefore, it is important that the patient's Lewis blood type has been determined prior to making clinical decisions based on the measurement of CA 19-9. One study suggested that measurement of CA 19-9 on the specific protein carriers MUC5AC or MUC16 could be useful for the subset of patients who show no elevation with standard CA 19-9 assays [39].

1.3.4 Analysis methods

In the early 1980s, shortly after the development of the antibody 1116-NS-19-9 and discovery of CA 19-9, a radioimmunometric sandwich assay was developed to measure the antigen in serum [40, 41]. Currently, automated immunoassay systems are used for the analysis of CA 19-9 in clinical centres, while ELISA and chemiluminescence immunoassay (CLIA) kits are available for research use. Table 1.1 provides a brief description of the assay format, detection limit, and measurement range without dilution for some of the common automated systems that support CA 19-9 assays. Though the recommended cut-off value to distinguish normal and elevated levels may vary depending on the assay manufacturer, many methods recommend 37 U/mL, similar to the reference value of 40 U/mL established using the radioimmunoassay in a clinical study conducted in the 1980s [40-42]. The ADVIA Centaur was the first automated assay to be approved by the FDA [43]. Many of the assays are non-competitive, two-step sandwich immunoassays that use the antibody 1116-NS-19-9 [43]. Due to methodological differences between the assays, the same method should be used when monitoring a patient's CA 19-9 level [43-46].

Table 1.1 Summary of automated systems for CA 19-9 analysis

Instrument	Assay format	Detection limit (U/mL)	Range (U/mL)	Refs
ADVIA Centaur (Siemens Healthcare Diagnostics)	2-step sandwich assay using antibody 1116-NS-19-9; magnetic particle separation, chemiluminescence detection	0.43 (1.2)	700	[43, 47]
ARCHITECT i2000 (Abbott Laboratories)	2-step sandwich assay using antibody 1116-NS-19-9; magnetic particle separation, chemiluminescence detection	0.23 (2.0)	(2 – 500)	[43, 45, 46]
AxSYM (Abbott Laboratories)	sandwich enzyme immunoassay; fluorescence detection	(2)	(2 – 500)	[46]
Dimension Vista (Siemens Healthcare Diagnostics)	homogeneous sandwich chemiluminescent immunoassay; LOCI technology to generate signal	2.0	2.0 – 1000	[44]
Elecsys E170/1010/2010 (Roche Diagnostics)	sandwich assay using antibody 1116-NS-19-9; magnetic particle separation, chemiluminescence detection	0.07 (0.6)	(0.6 – 1000)	[42, 43, 46, 48]
KRYPTOR (Thermo Scientific BRAHMS) not cleared by FDA	TRACE technology (non-radiative energy transfer) when immunocomplex forms; fluorescence detection	(1.2)	(1.2 – 700)	[46, 49]
UniCel Dxl 800 Access GI Monitor assay (Beckman Coulter)	2-step immunoenzymatic sandwich assay using antibody C192 and paramagnetic particles; chemiluminescence detection	0.07, <0.8 <1.05 at one centre	(0.8-2000)	[43, 48]

Manufacturers' claimed values in brackets
LOCI®: Luminescent oxygen channeling immunoassay
TRACE: Time-resolved amplified cryptate emission

1.4 Polymerase Chain Reaction

The polymerase chain reaction (PCR) has been a revolutionary scientific technique that has enabled the exponential amplification of small amounts of a specific nucleic acid sequence *in vitro*. As real-time PCR is the technique used for this thesis research as the means to amplify and detect the DNA representing CA 19-9, the following section is devoted to describing the history of its development and a general procedure for PCR.

1.4.1 Development of PCR

In 1971, Kjell Kleppe and H. Gobind Khorana first conceived the possibility of replicating a specific DNA sequence when studying what they termed "repair replication" of DNA oligonucleotides representing segments of the yeast gene for alanine transfer RNA [50]. It was not until 1983 when Kary Mullis, then an employee of Cetus Corporation, successfully combined previously known laboratory procedures to develop a means to exponentially amplify a nucleic acid sequence *in vitro*. As Mullis would tell it, he thought of the idea while driving to his cabin [51]. As his thoughts drifted back to the lab, "lurid blue and pink images of electric molecules injected themselves somewhere between the mountain road and [his] eyes" [52] and he realized that two primers on opposite strands would allow him to target a specific DNA sequence. In his opinion, the breakthrough was influenced by psychedelic drug use, as his mind had learned how to "get down" to the molecular level, allowing him to "sit on a DNA molecule and watch

the polymerase go by" [53]. Quite the interesting character, Mullis was awarded the Nobel Prize in Chemistry in 1993 for his invention.

Mullis and his technician Fred Faloona initially demonstrated the technique by amplifying a 25 base pair fragment from the pBR322 plasmid [54]. PCR involved denaturation of the source DNA by increasing the temperature in a solution containing deoxyribonucleoside triphosphates (dNTPs) and an excess of two oligonucleotides that are complementary to different strands of the target sequence [55]. When the temperature is lowered, the oligonucleotides anneal to each strand, and the Klenow fragment of DNA polymerase I from *Escherichia coli* catalyzes the addition of nucleotides to extend the annealed oligonucleotides over the template DNA. Fresh DNA polymerase had to be added after each cycle, and temperature cycling of the reaction was performed by hand [55]. After temperature cycling, PCR products were separated and detected using gel electrophoresis. Early applications of PCR included analysis of genomic mutations for sickle cell anemia [56] and the detection of viral nucleic acid sequences [57].

Randall Saiki and others at Cetus improved PCR by using the DNA polymerase from the thermophile bacterium *Thermus aquaticus* (*Taq*) instead of the Klenow fragment [58]. *Taq* DNA polymerase is stable at high temperatures, allowing the denaturation step of PCR to be performed at temperatures up to 95°C. The use of *Taq* instead of the Klenow fragment conferred several advantages: the heat stability of *Taq* meant that the enzyme did not need to be replenished after each cycle, thereby simplifying the procedure and eliminating a

source of error; the annealing step could be performed at a higher temperature, which reduced nonspecific binding of primers; the increased specificity resulted in fewer nonspecific products competing for the polymerase, thereby increasing the efficiency and target yield; and longer DNA sequences could be amplified [58].

1.4.2 Development of real-time PCR

Despite the potential uses of PCR, Russell Higuchi and his co-workers at Roche Molecular Systems noted that factors such as high cost, labour intensive detection methods, and the potential for sample contamination, were hindering the widespread use of PCR in clinical settings [59]. Their solution was a "homogeneous" PCR assay where amplification and detection of the DNA was possible without opening the reaction tube, thereby eliminating downstream processes such as gel electrophoresis or DNA hybridization. To accomplish this, ethidium bromide dye was included in the reaction mixture. When ethidium bromide intercalates between the bases of double-stranded DNA, its fluorescence is enhanced relative to the fluorescence of ethidium bromide bound to single-stranded DNA, or to the dye alone. By measuring the fluorescence at the annealing/extension temperature when DNA will be double-stranded, amplification can be followed over the course of PCR. Their prototype system used a bifurcated optical fibre to both deliver excitation light from a spectrofluorometer to the PCR tube in a thermocycler, and to return the emitted fluorescence from ethidium bromide to the spectrofluorometer [59]. The next

system they developed used a CCD camera, which allowed them to simultaneously monitor the amplification of an array of samples [60]. As opposed to traditional PCR with end-point detection of the amplification product, real-time PCR produces a signal at each cycle and the amount of DNA in the exponential amplification phase can be used to quantitate the initial amount of template DNA [61, 62].

1.4.3 General procedure for PCR

The procedure for PCR, shown in Figure 1.3, involves the repetition of denaturation, annealing, and extension steps; temperature cycling via the use of a thermocycler is imperative to the process. The nucleic acid template must be single-stranded in order for the *Taq* polymerase to synthesize a complementary strand. Therefore, the first step of the cycle is to denature the target DNA by increasing the temperature to 94 or 95°C for usually 1 minute [63]. The temperature is then lowered to 55 to 60°C (dependent on the melting temperature of the primers, and the concentration of primers and $MgCl_2$) to allow the primers to anneal to the complementary sequence on the DNA template strand [63]. In the extension step, the primers are extended from the 3′ end at a temperature that is optimal for polymerase activity (usually 72°C for *Taq* polymerase). Some assays employ a two-step temperature cycling profile; for example, 12 to 15 seconds at 94 or 95°C followed by 30 to 60 seconds at 60°C [63]. During the exponential amplification phase, assuming 100% efficiency, the amount of DNA product is doubled at each cycle.

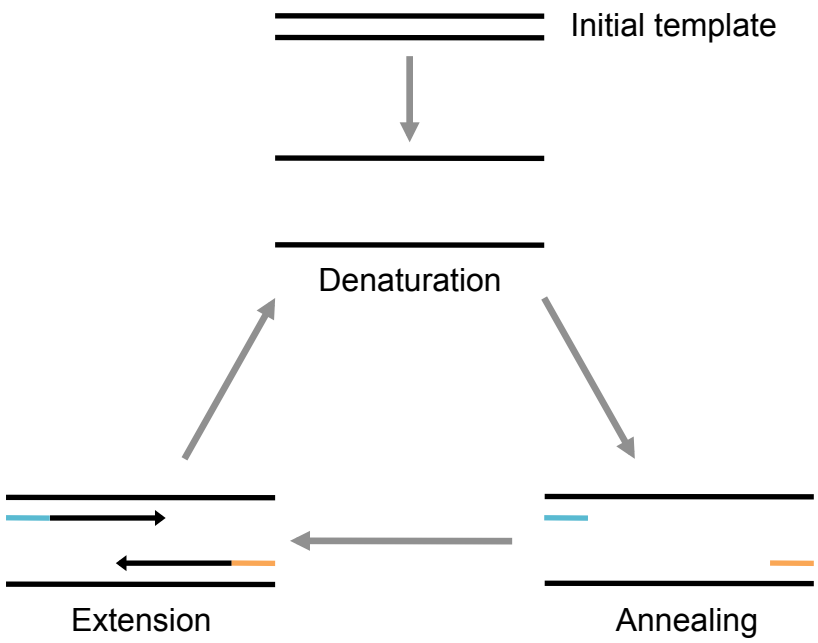

Figure 1.3 Schematic of the steps involved in the polymerase chain reaction. Adapted from reference [64].

In real-time PCR, the amount of DNA is monitored by changes in fluorescence as the reaction progresses. One detection method is to include an intercalating fluorescent dye, such as SYBR Green, in the PCR reaction mixture [65]. SYBR Green fluoresces only when bound to double-stranded DNA, and as the reaction proceeds, more DNA will accumulate and the fluorescent signal will increase. The method is highly sensitive, but specificity may suffer as SYBR Green will indiscriminately bind to any double-stranded DNA. TaqMan probes, molecular beacons, and scorpion primers are oligonucleotide probes with an attached fluorophore and quencher, and can enhance the specificity of detection because a fluorescent signal is produced only upon complementary binding of the probe to the DNA template [62, 65].

1.5 Immuno-PCR

Immunoassays rely on the binding specificity of antibodies for detection of antigens. ELISA, first described in 1971, is a commonly used technique that detects the target using an enzyme labelled antibody that catalyzes a substrate to produce a detectable signal [3, 66]. Sano et al. [67] sought to improve upon the sensitivity of antigen detection by coupling antibody recognition to PCR amplification. In a 1992 edition of *Science*, they described their system termed immuno-PCR, which is similar in format to ELISA, except the detection antibody is conjugated to a strand of DNA instead of an enzyme. The use of PCR amplification has allowed a 100 to 10000-fold improvement in the limit of detection compared to the common ELISA [68].

In the first description of immuno-PCR, the DNA-antibody conjugate was created by linking a monoclonal antibody against bovine serum albumin (BSA) to biotinylated linear plasmid DNA (pUC19) via a streptavidin-protein A chimera [67]. After the antigen BSA was immobilized to the wells of a microtiter plate, the DNA-antibody conjugate was added, and unbound conjugates were removed by a series of washing steps. PCR was then performed to amplify the DNA, and agarose gel electrophoresis with ethidium bromide staining was used to quantify the antigen. Use of immuno-PCR for BSA analysis enhanced the detection sensitivity by five orders of magnitude compared to ELISA performed with a chimera-alkaline phosphatase conjugate [67].

Subsequent studies used different means of preparing DNA-antibody conjugates, due to the limited availability of chimeric proteins and nonspecific

absorption of protein A. Avidin and streptavidin, with their strong binding affinities for biotin, were used to link biotin labelled oligonucleotides and biotinylated antibodies [69-71]. Covalent linkage of the DNA to the antibody allowed conjugates to be prepared in advance, and by linkage of unique DNA oligonucleotides to each type of antibody, multianalyte detection was possible [72]. A concern is that conjugation of oligonucleotides could alter antigen binding and cause antibody aggregation. Recent research has involved site-specific DNA conjugation of antibodies using genetically encoded unnatural amino acids, which enhanced the sensitivity and selectivity of HER2 detection relative to antibodies nonspecifically conjugated [73].

Early methods of immuno-PCR used gel electrophoresis or microplate-based methods, such as PCR-ELISA, to detect the products of PCR [67, 74, 75]. The advantages of detection of DNA in the exponential phase of amplification, as opposed to end-point detection, were applied to immuno-PCR by Sims et al. [76], by the use of a TaqMan probe. The assay was capable of detecting as low as 0.2 pg/mL (0.005 pM) of vascular endothelial growth factor (VEGF), a glycoprotein that may be elevated in cancer. A number of quantitative immuno-PCR assays have since followed [77-79].

Immuno-PCR requires the reporter antibody to be labelled with the oligonucleotide to be amplified (Figure 1.4). A modified method, termed immuno-aptamer PCR (iaPCR), eliminates the need to attach DNA to the antibody by using a RNA aptamer in place of a secondary antibody (Figure 1.4c) [80]. Quantitative reverse transcription-PCR is then used to determine the target

concentration. The limit of detection for VEGF was 100 fg, a 100-fold improvement over an ELISA kit using the same antibody pair, with a dynamic range of about four orders of log magnitude.

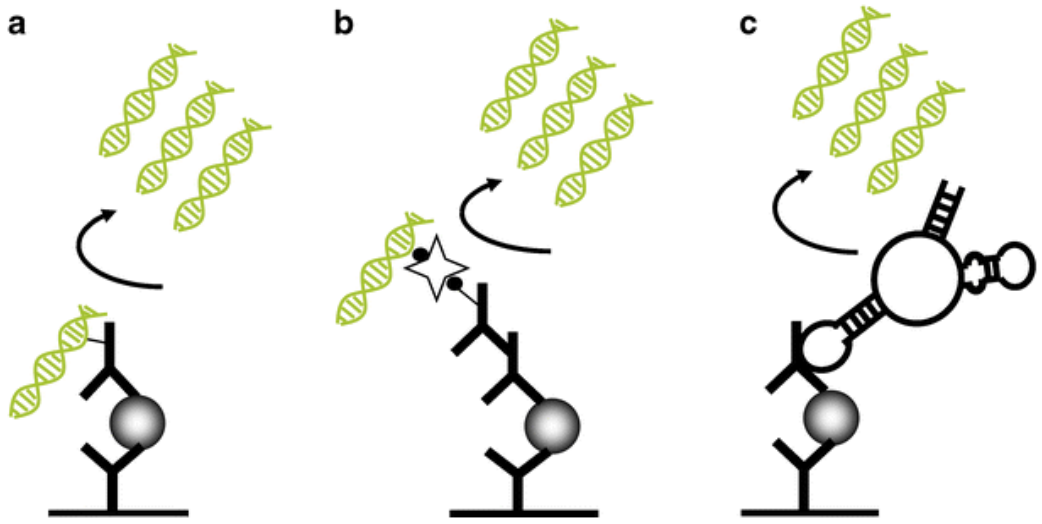

Figure 1.4 Examples of different reporter antibody assemblages for immuno-PCR. (a) Direct immuno-PCR uses an antibody preconjugated to DNA. (b) Biotinylated DNA can be linked to the antibody via a streptavidin-biotin bridge. A generalized method uses a secondary antibody to detect the bound antibody. (c) In immuno-aptamer PCR, an aptamer recognizes the bound antibody and is amplified to determine the target concentration. Reprinted from reference [80] with kind permission from Springer Science and Business Media, copyright 2009.

Immuno-PCR, in various formats, has been applied to the detection of cancer biomarkers, including the aforementioned VEGF [76, 80], prostate specific antigen (PSA) [81, 82], tumor necrosis factor-α (TNF-α) [82], CEA [84], HER2 [73], and MG7-Ag, a gastric carcinoma-associated antigen [85]. A cellular

immuno-PCR assay was developed to detect the ganglioside GM3 on the surface of tumor cells [86].

1.6 Assays Based on Target Induced DNA Assembly

The previous section described assays that use amplification of DNA oligonucleotides as reporters for the detection of the analyte. Methods have also been developed that require the assembly of DNA induced by target binding to produce a signal representing the target concentration.

1.6.1 Proximity ligation assay

The proximity ligation assay (PLA), initially referred to as proximity-dependent DNA ligation assay, was developed in 2002 by researchers at Uppsala University under the guidance of Ulf Landegren [87]. They noted that PCR allowed for sensitive and specific detection of nucleic acid sequences due to dual and proximal primer recognition of the target sequence, and applied those characteristics to develop a homogeneous assay for proteins that did not require washing or separation steps. The basic format of the assay is shown in Figure 1.5. The original PLA was developed for the analysis of the homodimeric cytokine platelet-derived growth factor-BB (PDGF-BB). To create the pair of probes, different oligonucleotide extensions of approximately 40 nucleotides were added to DNA aptamers with affinity for PDGF-BB. A connector oligonucleotide was designed to hybridize to 10 oligonucleotides at the end of each probe. The two probe types are incubated with the sample, and when a pair of corresponding

probes binds to PDGF-BB they are brought into close proximity. The components required for ligation, including the connector oligonucleotide in molar excess, and amplification, are then added. Hybridization of the probes to the connector oligonucleotide facilitates bringing the free probe ends together, and T4 DNA ligase joins the ends to form a new DNA sequence. This new DNA template is detected using real-time PCR and serves as a surrogate for the amount of PDGF-BB. Compared to an ELISA for PDGF-BB, 1000-fold fewer molecules could be detected by PLA [87].

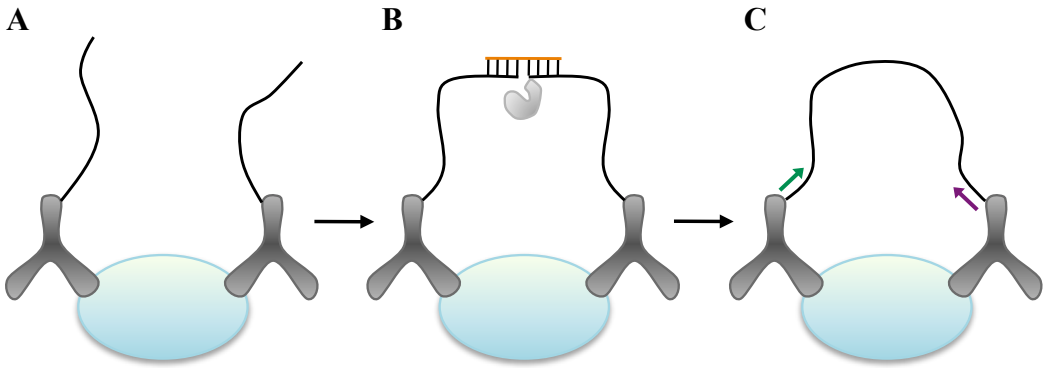

Figure 1.5 Schematic of the homogeneous proximity ligation assay. (A) The proximity probe pair binds to the target. (B) A connector oligonucleotide hybridizes to a portion of each probe, and ligation of the free probe ends creates a new DNA strand (C) that can be amplified and detected by real-time PCR. Adapted from references [88, 89].

Since its conception, the PLA has undergone several changes. In order to extend the PLA to the analysis of other biomolecules, the method was adapted to use polyclonal antibodies or a matched pair of monoclonal antibodies as the affinity ligands of the probe, as was first demonstrated by the detection of several

cytokines [90]. The probes could be prepared by direct conjugation of the 3′ or 5′ end of an oligonucleotide to the antibody via a bifunctional crosslinker, or by conjugating the oligonucleotides to streptavidin and then combining the conjugates with biotin labelled antibodies [90]. Another modification was the use of an asymmetric connector with one side having fewer complementary bases to hybridize to one of the probes. This reduced target-independent ligation (background), allowing for higher probe concentrations to be used to increase the dynamic range [91].

The solid-phase format of PLA involves the capture of the target via an antibody attached to a solid support, such as a magnetic microparticle, and after wash steps the proximity probes are added [92]. Solid-phase PLA is useful for complex biological samples, as the washing steps remove potential interferences and unbound probes [92]. However, since the homogeneous PLA format does not require washing steps the procedure is simpler, and it only requires 1 μL of sample compared to 5 μL for solid-phase PLA [92]. PLA in homogeneous or solid-phase formats has been applied to the analysis of proteins [87, 90, 92-94], protein complexes [95, 96], inhibitors of protein-protein interactions [97], protein aggregates [98], the interaction between DNA-binding proteins and regulatory DNA sequences [99], and bacteria and virus particles [100].

Variants of the original dual probe PLA assay have been developed. In a triple-binder PLA referred to as 3PLA, three target recognition events are required for assembly of the DNA oligonucleotides, resulting in increased specificity and reduced background [101]. Two probes consist of antibody-linked

oligonucleotides with block oligonucleotides hybridized to their ligatable ends to prevent DNA assembly in the absence of target, while the third probe consists of an antibody linked to a connector oligonucleotide. When all three probes bind to the target, the probe containing the connector oligonucleotide will outcompete the blocks and hybridize to the two other probes. A cassette oligonucleotide hybridizes to the middle of the connector and is ligated to the two probes to create an amplifiable DNA strand. The 3PLA method was applied to the analysis of the biomarkers troponin I, VEGF, and PSA. Compared to 2PLA, 3PLA increased the sensitivity of detection of VEGF by 100-fold and PSA by 4×10^4-fold [101]. The 4PLA method was demonstrated for the analysis of prostasomes, microvesicles that are secreted by prostate acinar cells [102]. The assay involves recognition of five different epitopes on the multiprotein prostasomes. One antibody captures the prostasome on a solid support while the binding of four PLA probes allows the DNA oligonucleotides to hybridize and guide hybridization of the connector oligonucleotide. Enzymatic ligation of the connector to two probes creates the amplifiable DNA template. When the assay was used to analyze plasma samples, elevated prostasome levels were found for patients with aggressive prostate cancer [102].

Multiplex assays are of value, as they allow for simultaneous analysis of many different analytes. A homogeneous PLA was created for the simultaneous detection of six cancer biomarkers, with individual real-time PCR detection using primers specific for the unique ligation product of each marker [4]. The method was then applied to the analysis of 21 plasma biomarkers of pancreatic and

ovarian cancer, of which CA 19-9 was included, using panels of 6 or 7 analytes per assay [103]. CA 19-9 was also among 74 biomarkers detected in only 1 μL of human plasma sample using four 24-plex assays [88]. This assay introduced the use of green fluorescent protein detection by PLA to serve as a non-human internal reference to account for enzymatic inhibition of DNA ligase by serum components, thereby improving assay precision. A multiplex PLA was used to investigate 21 biomarkers in the plasma of patients with pancreatic ductal adenocarcinoma and healthy controls in an attempt to identify potential biomarkers to use in combination with CA 19-9 [104]. A solid-phase assay for 35 proteins using next-generation sequencing to quantify the ligation product was used to identify markers of cardiovascular disease [94]. Solid-phase PLA has also been combined with dual-tag microarray readout in order to investigate levels of proteins and protein-protein interactions [96].

Landegren and co-workers also developed *in situ* PLA (Figure 1.6) to visualize the subcellular localization of individual pairs of interacting biomolecules in fixed cultured cells and tissue sections [105]. Instead of PCR, this method uses the linear amplification process called rolling circle amplification (RCA) that proceeds under isothermal conditions [105, 106]. Upon coincident binding of the antibodies of the probe pair to adjacent target molecules, the oligonucleotides are brought into close proximity and function as templates for the circularization of two connector oligonucleotides that are joined by enzymatic ligation [105]. One of the probe oligonucleotides primes the RCA reaction after the addition of ϕ29 DNA polymerase. The product of RCA is a long single-

stranded sequence that collapses into a bundle and contains tandem repeats that are complementary to the DNA circle. The product is detected using fluorophore-labelled hybridization oligonucleotides and the use of fluorescence microscopy [105, 107] or flow cytometry [108]. A generalized method was created that used secondary proximity probes that would recognize the primary antibodies that had bound the target [109].

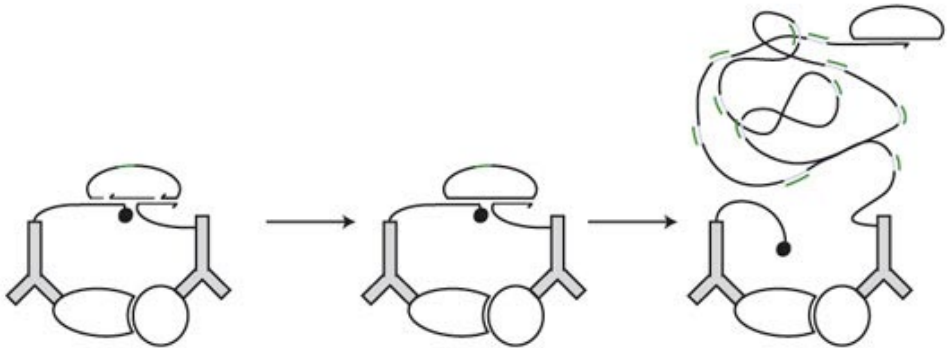

Figure 1.6 Example of the *in situ* proximity ligation assay. Protein-protein interactions are detected by a pair of proximity probes that bind to the targets and guide formation of a DNA circle. The DNA is amplified by RCA and is visualized by fluorescent hybridization probes. Reprinted by permission from Macmillan Publishers Ltd: Nature Methods [105], copyright 2006.

In situ PLA has been used to investigate protein-protein interactions [105, 108, 110], protein phosphorylation [109], inhibitors of phosphorylation [107], and interactions of proteins with DNA [111] or RNA [112]. The method has also been applied to study mucins and the associated glycans. MUC2 was found to be a major carrier of the carbohydrate antigen sialyl-Tn in cases of intestinal metaplasia and gastric carcinoma [113]. To identify the glycan modifications on

mucins, Pinto et al. [114] screened mucinous adenocarcinomas from different organs using an *in situ* PLA with an antibody directed against one of several mucins and the other antibody directed at a carbohydrate antigen. The major carriers of sialyl Le^a (CA 19-9) were MUC1 and MUC2; MUC5AC and MUC6 were also identified as carriers of CA 19-9.

1.6.2 Proximity extension assay

Inspired by the concept of proximity ligation, an assay was developed based on a proximity extension reaction that occurred upon dual aptamer binding to the target, and subsequent rolling-circle amplification (RCA), as shown in Figure 1.7 [115]. The feasibility of the method was demonstrated by analyzing human thrombin using aptamers that recognize distinct epitopes. One aptamer was linked to a template loop to create the circular DNA necessary for RCA. The linear aptamer was extended with a poly(T) tail that at the 3′ end contains six complementary bases to the template loop. Binding of the aptamers to thrombin increases the local concentration of the constructs and favours hybridization of the complementary region. The linear aptamer construct serves as a primer for polymerase mediated extension and isothermal RCA of the circular template that is monitored in real-time by the measurement of SYBR Green fluorescence. The detection limit was 30 pM, almost 3 orders of magnitude lower than the K_d value of each aptamer, and the linear range was reported as 10 pM to 5 nM [115].

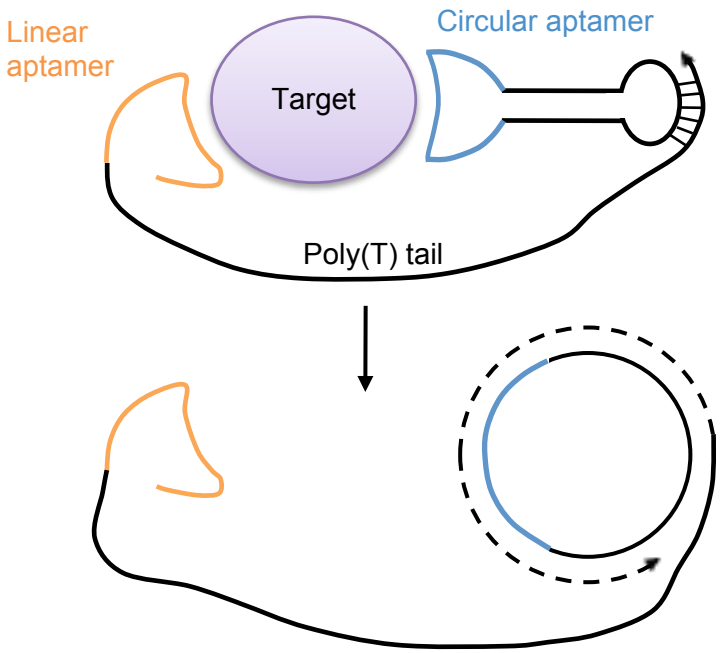

Figure 1.7 Proximity extension assay using a circular DNA aptamer and rolling-circle amplification. Adapted from reference [115].

DNA hybridization, extension, and amplification by PCR was applied by Liu and co-workers to monitor covalent bond formation and cleavage [116], and to identify ligand – target binding pairs from libraries of proteins or small molecules [117]. In the presence of the target molecule, the two DNA probes bind the target and the short complementary regions form a stable intramolecular duplex. The hairpin structure that forms as a result of target binding is extended from the 3′ end, producing a strand that contains the primer binding sequences required for PCR amplification. Since double-stranded nucleic acids exhibit a higher melting temperature with intramolecular hybridization compared to intermolecular hybridization [118], hairpin templates are amplified more efficiently than linear templates [116]. The format could potentially be applied to

the analysis of multivalent analytes via noncovalent binding interactions, as was demonstrated by the detection of as low as 2×10^{-19} mol (200 zmol) of streptavidin using two DNA strands conjugated to biotin [117].

After the Fredriksson research group found that the DNA ligase used in the PLA was prone to enzymatic inhibition when analyzing plasma samples, they exchanged the use of ligase for DNA polymerase and created another variation of the proximity extension assay (PEA) [119]. PEA involves two proximity probes each with a different 40 base oligonucleotide strand conjugated to an antibody. One of the probes also has a 56 base hybridization oligonucleotide with a complementary region to the oligonucleotide of the other proximity probe. If the target of interest is present in the sample, binding of the antibodies to the target will bring the probes into close proximity and the hybridization oligonucleotide of the one probe will anneal to the complementary bases of the other probe. After the incubation period, DNA polymerase is added to extend the hybridization oligonucleotide over the oligonucleotide of the other probe to create a DNA template that can be detected by real-time PCR. The use of DNA polymerase improved upon PLA analysis because the DNA polymerase was less susceptible to enzymatic inhibition by plasma components, thereby improving recovery and eliminating the need for data normalization with an internal standard [119]. The use of a polymerase with $3' - 5'$ exonuclease activity reduced background signals by degrading the remaining free DNA ends, preventing random proximity events and extension products from forming during the extension phase. Demonstration of the assay's utility using interleukin-8 (IL-8) and glial cell-line derived

neurotrophic factor (GDNF) resulted in a limit of quantification of 0.1 pM with a detection range of at least four orders of magnitude. The creators of the PEA also demonstrated that the sensitivity of assays using low affinity antibodies could be improved by including sephadex G100 beads in the incubation of probes and sample. The beads take up water, artificially increasing the concentration of proximity probes and antigen to improve target binding. The assay was amenable to multiplex format, and was used to analyze 23 analytes including CA 19-9, by using unique probe oligonucleotides, corresponding extension primers, and different primer pairs for quantitative PCR [119].

1.6.3 Binding-induced DNA annealing

The research group of Tomasz Heyduk developed a family of sensors based on DNA annealing and subsequent fluorescence generation that is induced upon the binding of a pair of target recognition elements to the biomolecule of interest [120]. As shown in Figure 1.8, the assay uses two probes each with an affinity ligand that recognizes a different epitope of the target. Nanoscale flexible linkers are used to conjugate the affinity ligands to complementary signal oligonucleotides. Both oligonucleotides are labelled with a fluorophore, or one is labelled with a fluorophore and the other is labelled with a quencher. The complementary regions are short, for example seven nucleotide bases [121], and present at low concentrations (nanomolar) in solution, so that very little association of the probes will occur in the absence of target. If the target is present in solution, the affinity ligands will bind to the target, increasing the local

concentration of oligonucleotides and promoting efficient annealing of the strands. The proximity of the probes allows for signal generation via fluorescence resonance energy transfer (FRET). The target can be quantified by measuring the quenching of the donor signal, the intensity of the acceptor signal, or the ratio of acceptor to donor signal [121, 122].

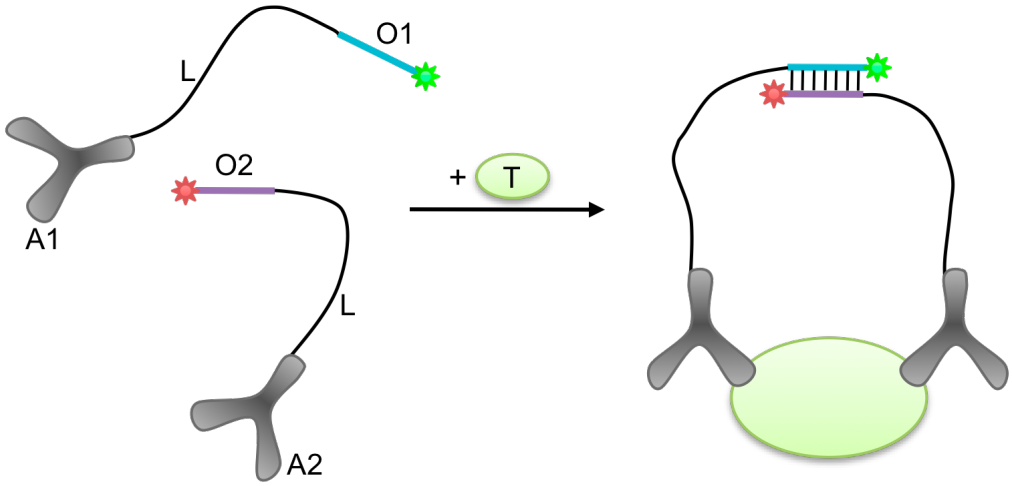

Figure 1.8 Schematic of the molecular pincer assay based on binding-induced DNA annealing. Complementary oligonucleotides (O1 and O2) are linked to affinity ligands (A1 and A2) via flexible linkers (L). When the probes bind the target (T), the probes hybridize and produce a fluorescent signal via FRET. Adapted from reference [120].

The first assay of this type recognized DNA binding proteins using DNA oligonucleotides each containing half of the sequence for the DNA binding site [122, 123]. To apply the sensor to other biomolecules, it was necessary to create sensor pairs with different affinity ligands. Thrombin was detected using aptamers that recognize non-overlapping epitopes of the target [121]. Protein sensors called

molecular pincers used antibodies as the affinity ligand and were demonstrated to detect cardiac troponin I, C-reactive protein [124], insulin, C-peptide [125], and bacteria cells [126]. Mixed sensors, whereby one affinity ligand is an antibody and the other is double-stranded DNA specific for a DNA binding protein, were used to detect GST tagged transcription factor NF-κB and the tumor suppressor protein p53 [124]. Peptide-based sensors could detect antibodies in a direct assay format, or could be used in an indirect competitive assay format to detect proteins [127]. The peptide-based sensors could also be utilized for the detection of enzymatic activity, as phosphorylation of the peptide by protein kinase A prevents recognition of the peptide by the antibody with a resultant decrease in signal [127].

The probe concentration can be manipulated to achieve certain detection limits and assay sensitivity [127]. For certain targets the limit of detection was similar to ELISA assays, and the researchers noted that it would be difficult to achieve sensitivities in the picogram per millilitre range due to the fact that the molecular pincer assays do not involve a signal amplification step [124]. The assays were compatible with diluted plasma or serum [121, 124, 127], with the exception of five times diluted serum due to large background serum autofluorescence [127]. For the analysis of insulin and C-peptide in serum, the detection limit was near the concentrations typically observed in serum, and the assay sensitivity would have to be improved and the background of the serum reduced before the sensor would be appropriate for serum analysis [125].

With the aim of point-of-care analysis combining the sensitivity of PLA with the simple and rapid format of molecular pincer assays, Easley and co-workers developed the homogeneous electrochemical proximity assay (ECPA) [128], shown in Figure 1.9. The assay involves a pair of probes that can be aptamers or antibody-oligonucleotide conjugates. One of the probes hybridizes to a DNA oligonucleotide that is modified with methylene blue (MB) at the other end. A strand of DNA that is complementary to the other probe is attached to the gold electrode via a thiolated bond. The simultaneous binding of the probes to the same target molecule allows for proximity-dependent hybridization of the thiolated DNA and MB-DNA, facilitating electron transfer from MB to the gold electrode. This results in an increase in current that is proportional to the target concentration. Background is minimized by the use of a competitor DNA strand that hybridizes to the thiolated DNA to block hybridization of MB-DNA in the absence of target. The detection limit for aptamer-based detection of thrombin was 50 pM. Using antibody-oligonucleotide probes, the detection limit of insulin was 128 fM with a four orders of magnitude dynamic range [128].

Our research group was involved in the creation of a binding-induced fluorescence turn-on assay using a silver nanocluster beacon to report target detection [129]. The concept was demonstrated using human α-thrombin as the target, with two aptamers recognizing different binding sites. A poly(T) spacer was used to link each aptamer to a 12 base stem sequence that is complementary to the stem sequence of the other probe. The stem of one probe is linked to a silver nanocluster nucleation sequence at the 5′ end while the other probe has a

guanine-rich DNA sequence attached to the stem at the 3′ end. Binding of the aptamers to the target promotes hybridization of the complementary regions, allowing the guanine-rich overhang to come into close proximity with the silver nanoclusters and enhance their fluorescence [129, 130]. After optimizing assay conditions to obtain the best signal to noise ratio, the dynamic range for α-thrombin analysis was 5 nM to 4 μM and the detection limit was 1 nM [129].

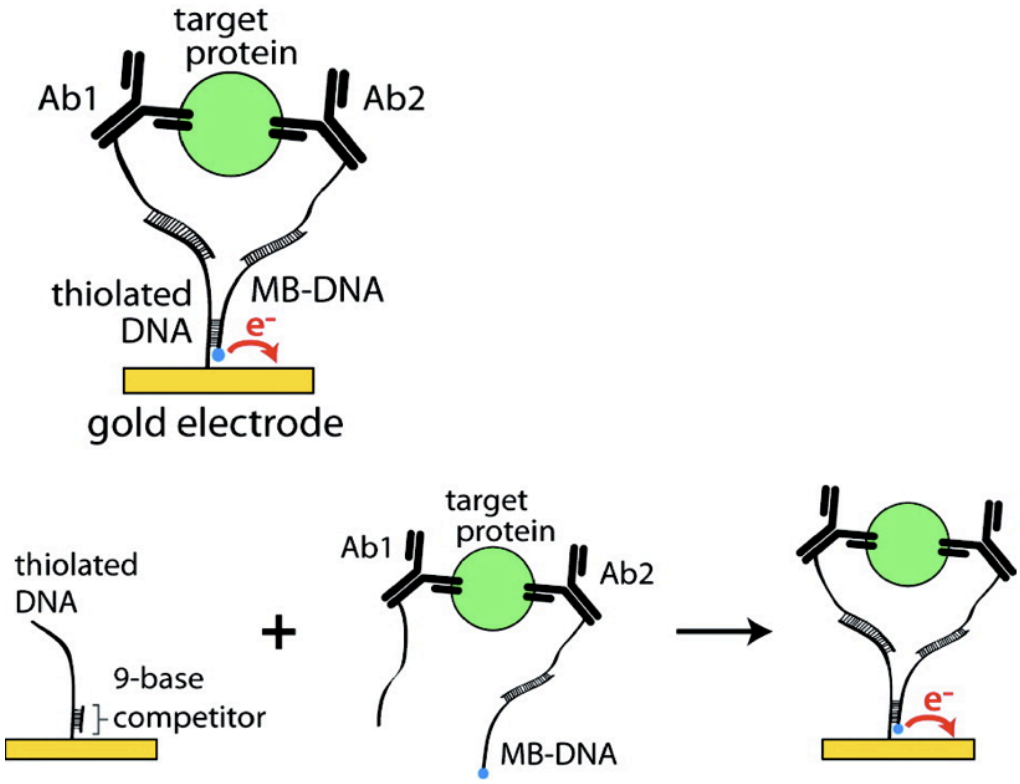

Figure 1.9 Schematic of the electrochemical proximity assay. Target binding and hybridization of probes to the DNA immobilized on the gold electrode results in electron transfer. Reprinted with permission from reference [128]. Copyright 2012 American Chemical Society.

1.6.4 Binding-induced DNA assembly

Our research group has also been involved in the development of other protein assays using nucleic acid assembly. Building off of previous homogeneous immunoassays involving the use of DNA to detect proteins, Hongquan Zhang developed a novel immunoassay based on the principle of binding-induced DNA assembly (BINDA) [131]. The assay involves two probe constructs of an oligonucleotide linked to an affinity ligand. Block oligonucleotides that anneal to the probes are also present in solution, and function to reduce probe hybridization in the absence of target. Binding of the target molecule by the affinity ligands increases the local concentration of the probes. The probe oligonucleotides preferentially hybridize to each other rather than the blocks due to the increased stability of the closed-loop structure that is formed between the probes and target. Ligation of the two probe oligonucleotides and amplification of the combined DNA sequence with real-time PCR is used to determine the target concentration. Affinity ligands can be aptamers or antibodies, as was demonstrated by the analysis of PDGF and PSA. PDGF-BB was analyzed in buffer and cell lysate with a linear dynamic range of 1×10^{-15} M to 1×10^{-11} M and a detection limit of 1×10^{-15} M (2 zeptomole). PSA was analyzed in buffer and diluted goat serum, and as low as 1×10^{-16} M (200 yoctomole) could be detected using only 2 μL of sample [131].

The increase in local concentration of oligonucleotides induced by binding to the target molecule is also used as the basis for a molecular translator that acts by binding-induced DNA strand displacement (Figure 1.10) [132].

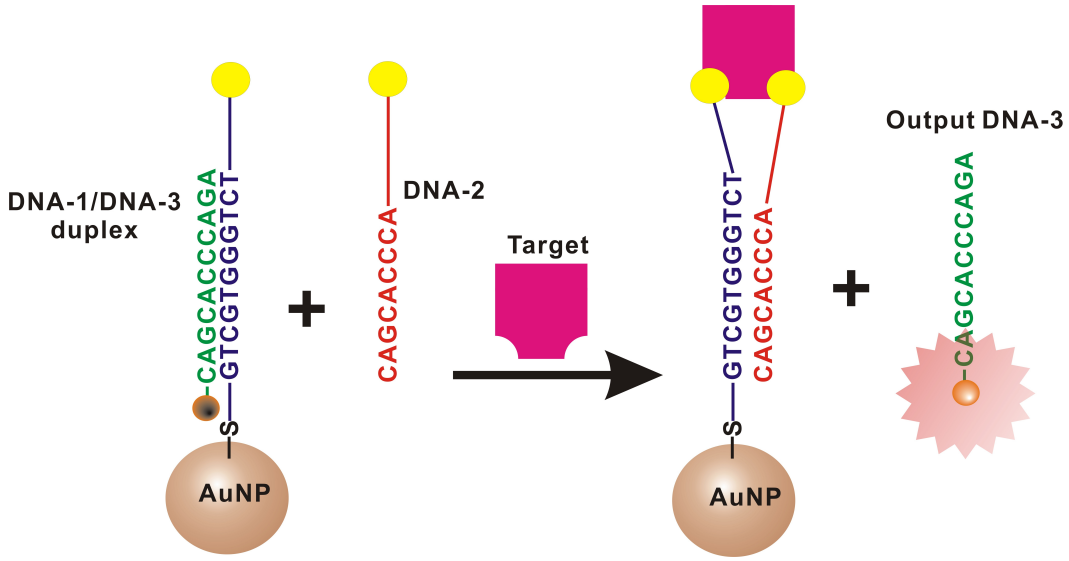

Figure 1.10 Schematic of the binding-induced molecular translator. Reprinted with permission from reference [132] and John Wiley and Sons. Copyright © 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Gold nanoparticles (AuNPs) serve as scaffolds for DNA assembly and the strand displacement reaction. The AuNPs are functionalized with DNA oligonucleotides (DNA1) that are conjugated to a ligand capable of target recognition. The other affinity ligand is conjugated to the competing DNA strand (DNA2) that has 9 complementary bases to DNA1. The output DNA strand (DNA3) has 11 complementary bases to DNA1 and is labelled with a fluorescent dye. In the absence of target, DNA1 and DNA3 form a stable duplex and the AuNP quenches fluorescence. Binding of the affinity ligands to the target brings DNA1 and DNA2 into close proximity, allowing competing DNA2 to displace DNA3 and hybridize with DNA1. A fluorescent signal is produced, as DNA3 and the linked fluorophore are no longer in the vicinity of the AuNP scaffold. The molecular

translator was first demonstrated using biotin as the affinity ligand for streptavidin detection, and the fluorescence intensity was proportional to the concentration between 0.1 and 25 nM streptavidin. The assay was applied to the detection of PDGF-BB using an aptamer as the affinity ligand, and the fluorescence intensity could be improved by conjugating both DNA1 and the aptamer to the AuNP. Background fluorescence could be reduced by the use of a blocking oligonucleotide that partially hybridizes to the competing DNA2 [132].

1.7 Research Objectives

The BINDA assay was initially developed for detection of minute quantities of the proteins PSA and PDGF. The aim of this thesis project is to develop a BINDA assay for the detection of CA 19-9 and demonstrate for the first time the use of BINDA for carbohydrate analysis. The research objectives are to: (1) apply the principle of BINDA to the detection of streptavidin in buffer as a proof of concept; (2) optimize the BINDA assay for the detection of CA 19-9; and (3) test the ability of the assay to detect CA 19-9 in human serum.

Chapter 2 describes the concept and design of the assay, and demonstration of the assay's potential for biomolecule analysis by using streptavidin as a model target. Optimization of the method for the detection of CA 19-9 is described in Chapter 3, with an evaluation of assay performance using a biological matrix. Chapter 4 provides a summary of the research and comparison to other DNA-assisted homogeneous assays. The limitations of this study and possible trajectories for further research are also discussed.

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CHAPTER 2

Binding-Induced DNA Assembly using Streptavidin as a Model Target

2.1 Introduction

Before developing an assay for carbohydrate antigen 19-9 (CA 19-9), the principle of binding-induced DNA assembly (BINDA) is first demonstrated by creating an assay for streptavidin with biotin as the affinity ligand. This study is essentially a proof of concept to demonstrate that the assembly of probes upon target binding and the resulting formation of a new DNA oligonucleotide can be used to quantitate the target without washing or immobilization steps.

Streptavidin, from the bacterium *Streptomyces avidinii*, is a 60 kDa protein composed of four identical subunits that can each bind one molecule of the vitamin biotin within its β-barrel structure [1]. The equilibrium dissociation constant (K_d) of streptavidin and biotin is 4×10^{-14} M, indicating that this noncovalent interaction is remarkably strong [2]. In the current work, streptavidin serves as a useful model because the strong binding affinity for biotin allows other assay parameters to be optimized without the influence of potentially weak binding affinities between the target and target specific ligand. The choice of model in the preliminary investigation is relevant because the strong interaction between streptavidin and biotin will later be exploited to link oligonucleotides to antibodies to create probes for the detection of CA 19-9.

In this chapter, the principle of BINDA and the design of the oligonucleotides will be described. The important parameters affecting performance of BINDA are demonstrated using the detection of streptavidin as a model.

2.2 Experimental

2.2.1 Reagents

Streptavidin, biotin, bovine serum albumin (BSA), ATP disodium salt, and Tris-HCl solution (1 M, pH 7.4) were purchased from Sigma-Aldrich (Oakville, ON). UltraPure™ distilled water, $MgCl_2$, SYBR® GreenER™ qPCR Supermix Universal, and T4 DNA Ligase (1 U/μL) were obtained from Invitrogen Canada (Burlington, ON). NaOH and 10× phosphate buffered saline (PBS) were obtained from Fisher Scientific (Ottawa, ON). To prepare 1× PBS buffer, 10× PBS was diluted with distilled water, resulting in 137 mM NaCl, 11.9 mM phosphate, and 2.7 mM KCl, at pH 7.4.

The DNA oligonucleotides, listed in Table 2.1, were synthesized, labelled, and purified by Integrated DNA Technologies (Coralville, IA). Biotin was attached to the 5′ end of oligonucleotide probe-F via tetra-ethyleneglycol (TEG), a 15-atom spacer arm. Probe-R was phosphorylated at the 5′ end, and labelled with biotin-TEG at the 3′ end. Both oligonucleotide probes were purified by reverse-phase HPLC. Since the block oligonucleotides and primers are shorter they were purified by a standard desalting method. The underlined segments of probe-F and probe-R are the six complementary bases that hybridize to form the stem of the

hairpin structure. The bolded nucleotide bases are the complementary regions between probe-F and the corresponding block-F, and between probe-R and the corresponding block-R.

Table 2.1 Oligonucleotide sequences for analysis of streptavidin

Oligonucleotide	**Sequence**
Probe-F	5′-Biotin-ACTGTGTCTCGTCGTTGGTGTTTTGT**TTTG TTTTAGGC**TGGTCGCTTTGTTTTGCGAC-3′
Probe-R	5′-**CAGCCTTTTTG**TTTGTTTTGTTTTTTTTGATGGA GCAGGTGTCAGATC-Biotin-3′
Block-F8	5′-TTT**GCCTAAAA**TTT-3′
Block-F9	5′-TTT**GCCTAAAAC**TTT-3′
Block-F10	5′-TTT**GCCTAAAACA**TTT-3′
Block-F11	5′-TTT**GCCTAAAACAA**TTT-3′
Block-F12	5′-TTT**GCCTAAAACAAA**TTT-3′
Block-R8	5′-TTT**AAAGGCTG**TTT-3′
Block-R9	5′-TTT**AAAAGGCTG**TTT-3′
Block-R10	5′-TTT**AAAAAGGCTG**TTT-3′
Block-R11	5′-TTT**CAAAAAGGCTG**TTT-3′
Forward primer	5′-ACTGTGTCTCGTCGTTGGTG-3′
Reverse primer	5′-GATCTGACACCTGCTCCATC-3′

2.2.2 Preparation of oligonucleotide solutions

The tubes containing the lyophilized oligonucleotides were centrifuged before opening. Probe and block oligonucleotides were reconstituted in 1× PBS and the primers were reconstituted in distilled water. Stock solutions were prepared of 50 μM probes, 100 μM blocks, and 100 μM primers. Working solutions were prepared by further dilution of the probes to 1 nM in 1× PBS, the blocks to 2 μM in 1× PBS, and the primers to 2 μM in 50 mM Tris-HCl. Aliquots of the working solutions were stored at -20°C.

2.2.3 Preparation of ATP solution

A 5 mM ATP solution with 5 mM $MgCl_2$ is required for the ligation reaction. Tris-HCl (1 M) and $MgCl_2$ (50 mM) were diluted in distilled water to concentrations of 50 mM and 5 mM, respectively. ATP was dissolved in the solution to obtain a concentration of 5 mM. Drops of 1 M NaOH were added to adjust the pH to 7 – 7.5, as determined by adding the ATP solution to a pH-indicator strip (EMD Millipore, Billerica, MA). Aliquots of the ATP solution were stored at -20°C.

2.2.4 Preparation of streptavidin solutions

Lyophilized streptavidin from Sigma-Aldrich (Oakville, ON) was dissolved in 1× PBS with 0.1% BSA to prepare a 25 μM solution. This solution was further diluted to 100 nM streptavidin using 1× PBS with 0.2% BSA, and aliquots were stored at -20°C. For each experiment, a new aliquot was used to

prepare sample streptavidin solutions by serial dilution using 1× PBS with 0.1% BSA.

2.2.5 Analysis of streptavidin using binding-induced DNA assembly

Incubation solutions were prepared in 0.2 mL PCR tubes at a volume of 20 μL, and consisted of 1× PBS with 0.2% BSA solution containing 100 pM of probe-F and probe-R and 100 nM of block-F and block-R, plus the desired concentration of streptavidin or buffer for blanks. The solutions were incubated at 37°C for 30 minutes. A master mix of the reagents required for PCR was prepared in distilled water, and 18 μL of the mix was added into the wells of a MicroAmp® Fast Optical 96-well Reaction Plate (Applied Biosystems, Foster City, CA). An aliquot (2 μL) of each incubation solution, or distilled water for the no template controls, was then added in triplicate to the wells of the reaction plate. The final 20 μL solutions contained 10 μL SYBR® GreenER™ qPCR Supermix Universal, 0.1 μL (0.125 μM) ROX reference dye, 0.1 μM forward and reverse primers, 0.4 units T4 DNA ligase, and 100 μM ATP. The plate was sealed with MicroAmp® Optical Adhesive Film (Applied Biosystems), and then was gently vortexed and centrifuged at 3000 *g* for 5 minutes. The plate remained at room temperature for a total of 10 minutes before being loaded into a 7500 Fast Real-Time PCR System (Applied Biosystems). The PCR run method consisted of 50°C for 2 minutes, 95°C for 10 minutes, and 55 cycles of 95°C for 15 seconds and 60°C for 1 minute, with subsequent melting curve analysis to verify that the correct product was amplified.

2.2.6 Data analysis

Experiments were analyzed using the 7500 software and Microsoft Excel. At the end of each run, an amplification plot of ΔRn (logarithmic scale) as a function of cycle number was produced. ΔRn is obtained by normalization of SYBR Green fluorescence to the fluorescence of ROX reference dye, and subtraction of the baseline (background fluorescence of initial PCR cycles). The threshold, a level of ΔRn, is set in the exponential phase of amplification at a level that minimizes the standard deviation of replicate groups. The cycle number at which the fluorescence crosses the threshold is the threshold cycle (Ct). The mean and standard deviation was calculated for the Ct values from triplicate analysis of each incubation solution. If a value was suspected to be an outlier, the Q-test was applied and the data point was rejected if the calculated Q value was greater than the reference Q value at 90% confidence.

2.3 Results and Discussion

2.3.1 Principle of binding-induced DNA assembly

BINDA requires the use of two probes, designated probe-F and probe-R, that are each composed of a single-stranded DNA oligonucleotide attached to an affinity ligand. In the case of streptavidin detection, the affinity ligand is biotin that has been attached at the 5′ end of probe-F and 3′ end of probe-R via a TEG spacer. For the detection of other targets, the interaction between biotin and streptavidin could be used to attach the DNA to a biotin labelled antibody or aptamer.

A major difference between BINDA and other homogeneous assay formats (described in Chapter 1) is the use of a hairpin, or stem-loop structure, in one of the probes. Hairpin structures result from intramolecular base pairing that forms a duplex ("stem") with unpaired bases forming a loop. The stability of hairpin structures depends on the length of the loop, the length of the base pairing region, and the number of guanine (G) and cytosine (C) bases in the stem, as G and C form three hydrogen bonds as opposed to the two hydrogen bonds formed by the pairing of adenine (A) and thymine (T). Oligonucleotide probe-F was designed to have a stable hairpin structure that allows the oligonucleotide to loop around and provide a free 3′ end to connect with probe-R by enzymatic ligation. A phosphate group was attached to the 5′ end of probe-R to facilitate the ligation reaction.

The principle of BINDA is illustrated in Figure 2.1, with the corresponding oligonucleotide sequences listed in Table 2.1. When the target, streptavidin, is present in the sample, biotin will bind to the target, bringing the probes into close proximity. The increase in the local concentration of the probes facilitates hybridization of the complementary probe regions, forming a closed-loop structure consisting of the target and two probes. Enzymatic ligation of the probe oligonucleotides forms a DNA template that can be amplified and detected by real-time PCR. Self-assembly can also occur in the absence of target binding, though to a lesser extent. Amplification of the self-assembly product can interfere with target detection, so it is important to determine the background by including a blank solution in each experiment.

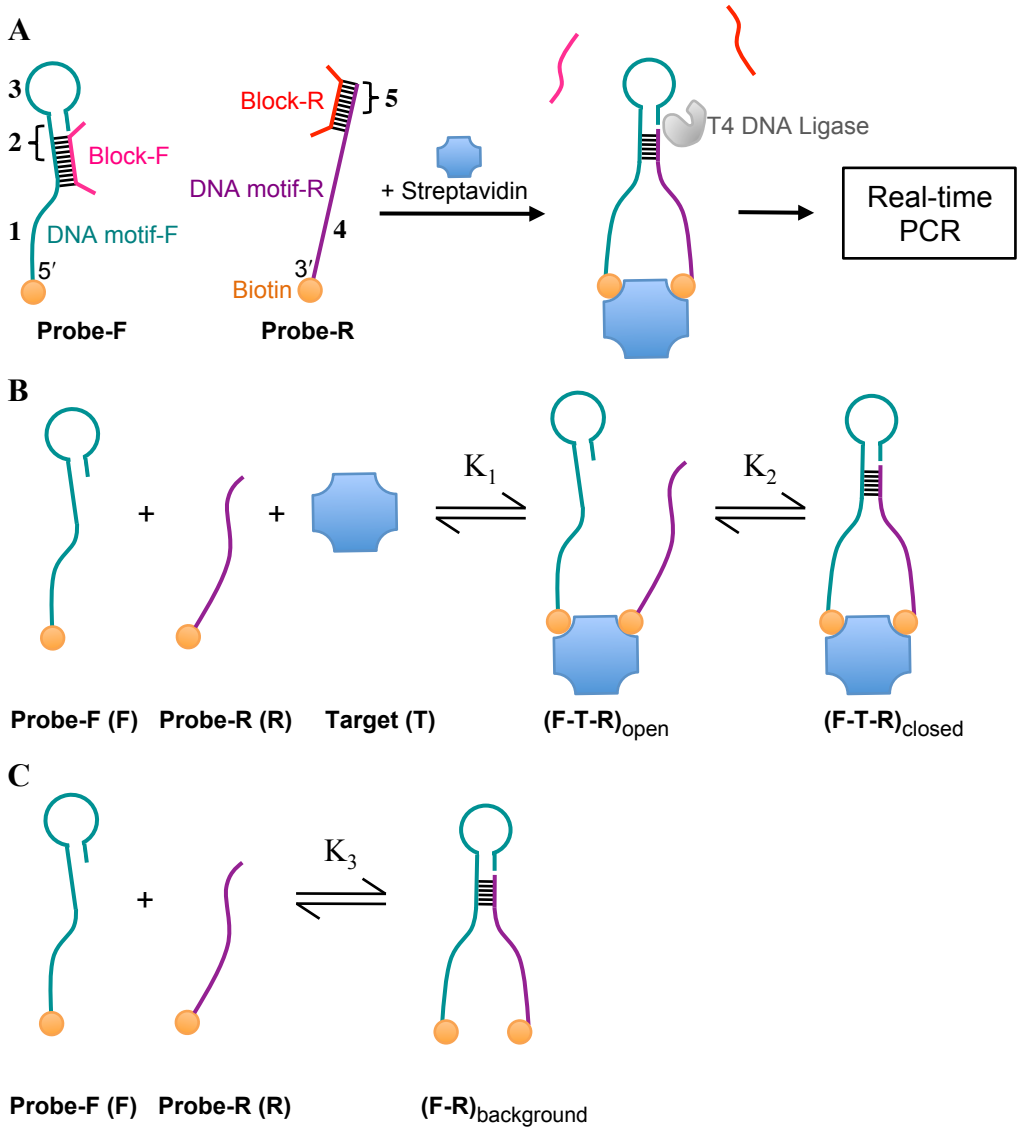

Figure 2.1 Schematic representing the principle of binding-induced DNA assembly using streptavidin as a model target. (A) The probe and blocks are incubated with streptavidin, and upon target binding the probes hybridize and are joined by T4 DNA ligase. The new DNA strand is then amplified and detected by real-time PCR. (B) Equilibria related to target binding and hybridization of the probes to form the closed-loop structure. (C) Equilibrium relating to target-independent assembly of probes that is responsible for background. Adapted from reference [3].

The assembly of probes in the presence (signal) and absence (background) of target can be represented by the following equation, where F represents probe-F, R represents probe-R, $[T]$ represents the target concentration, and K represents equilibrium constants. As illustrated in Figure 2.1, K_1 is the equilibrium constant for the formation of the open structure of probe-F and probe-R bound to the target, and is mainly influenced by the binding affinity of the ligand to the target. K_2 represents the formation of the closed-loop structure, and K_3 represents the assembly of probes in the absence of target; as assembly is due to probe hybridization, K_2 and K_3 are mainly determined by the length and GC content of the complementary stem sequences.

$$\frac{Signal}{Background} = \frac{\left[F \bullet T \bullet R_{closed}\right]}{\left[F \bullet R\right]} = \frac{K_1 K_2 [T]}{K_3}$$

To reduce background due to target-independent hybridization, block oligonucleotides are also incubated with the probes and sample. Block-F and block-R are complementary to probe-F and probe-R, respectively, and hybridize to a segment of the stem sequence of the probes, reducing probe hybridization. If the target is present in the sample, the probes will preferentially hybridize to each other rather than the corresponding block oligonucleotides due to the formation of the stable closed-loop structure.

2.3.2 Design of oligonucleotide sequences

Two DNA motifs were required to create the probes, and were designed to have a region of six complementary bases to allow for hybridization (stem sequences 2 and 5 in Figure 2.1; underlined bases in Table 2.1). These sequences

were designed so the closed-loop structure produced by BINDA would have a higher melting temperature (T_m) than the hybrid structure formed from target independent assembly, such that the complex $(F\text{-}T\text{-}R)_{closed}$ would be stable at room temperature and the hybrid $(F\text{-}R)_{background}$ would be unstable. OligoAnalyzer 3.1 from Integrated DNA Technologies, available online, was used to estimate the T_m using 5 nM DNA, 50 mM Na^+, and 3 mM Mg^{2+} as the input parameters. For $(F\text{-}T\text{-}R)_{closed}$, 100 to 130 thymines were used to represent the closed-loop, and the difference in T_m between $(F\text{-}T\text{-}R)_{closed}$ (T_m 35.4 – 37.4°C) and $(F\text{-}R)_{background}$ (T_m < 10°C) was approximately 30°C [3]. The instability of the six complementary bases would aid in reducing background.

After selection of suitable complementary sequences (sequence 2 and 5), the oligonucleotides were expanded and modified. A spacer sequence of 32 bases (sequence 1), labelled with biotin at the 5′ end, was placed before sequence 2. Hairpin sequence 3, composed of 18 nucleotides, was added to the other end of sequence 2 to complete DNA motif-F. Sequence 3 was designed to form a short, stable hairpin due to base pairing between the first and last five bases, leaving eight unpaired bases in the loop. DNA motif-R consists of a spacer sequence of 42 nucleotides (sequence 4) with a biotin moiety attached to the 3′ end and stem sequence 5 that is phosphorylated at the 5′ end. The spacer sequences were evaluated to ensure they would not form secondary structures or hybridize to form a heterodimer that would contribute to background.

The block oligonucleotides were designed to hybridize to the stem sequence of the corresponding probe. The different block-F oligonucleotides each

hybridize to a 4 base portion (AGGC) of probe-F sequence 2, plus 4 to 8 bases of sequence 1. The different block-R oligonucleotides hybridize to all 6 bases of stem sequence 5 (CAGCCT) of probe-R, plus 2 to 5 bases of sequence 4. Block-F is only complementary to 4 of the 6 sequence 2 bases of probe-F so that block-F and block-R only have a 4 base complementary region. This should prevent the blocks from hybridizing to each other, allowing the blocks to be present in the incubation solution at a concentration 1000-fold greater than the concentration of the probes. The addition of thymine repeats at the 5′ and 3′ end of each block also helps prevent the block oligonucleotides from hybridizing and forming PCR byproducts.

The design of appropriate primers is an important consideration for PCR. Primers are typically 16 to 28 nucleotides long, and should not contain stretches of the same nucleotide, form secondary structures, or have complementary sequences, as this would result in the formation of primer dimers. In addition, the pair of primers should be similar in length, have a GC content between 40 – 60%, and their T_m should only differ by 5°C or less. Primers were designed with the aid of Primer Express 3.0 Software (Applied Biosystems) to ensure they would not form secondary structures or heterodimers [3]. Both the forward and reverse primers were 20 nucleotides in length with a GC content of 55% and similar T_m of 57.2°C and 55.1°C, as calculated using OligoAnalyzer 3.1 using a Na^+ concentration of 50 mM. The reverse primer is complementary to a portion of sequence 4 of probe-R, and the forward primer shares the same sequence as a portion of sequence 1 of probe-F.

2.3.3 Analysis of streptavidin

Before developing an assay for carbohydrate analysis based on BINDA, the detection of streptavidin is used as a model system to investigate the effect of experimental parameters. Previous research tested probe-F with different probe-R oligonucleotides having complementary sequences of five to eight bases [3]. Probe-R with six complementary bases to probe-F resulted in the largest difference between signal and background, and was selected for the current study. The DNA strand that results from the ligation of probe-F and probe-R is 106 bases in length. Since the amplification products are detected using SYBR Green, a dye that intercalates into double-stranded DNA, melting curve analysis is conducted at the end of the PCR run to verify the correct DNA product has been amplified. The temperature is slowly increased and when the T_m is reached the duplex of the 106 base template and its complement denature, resulting in a rapid decrease in fluorescence. The specific PCR product that forms as a result of BINDA has a T_m of approximately 76°C using the experimental conditions applied for streptavidin analysis.

As previously mentioned, block oligonucleotides are included in the incubation solution to reduce the background by preventing target-independent hybridization of the probes. Block oligonucleotides have been used in other assay formats, such as the molecular translator based on binding-induced DNA strand displacement [4], and the electrochemical proximity assay [5]. In the triple-binder proximity ligation assay (3PLA) and 4PLA, hairpin-loop structured blocks prevent hybridization of the probes in the absence of target, and ligation of the

blocks to the probes further prevents target-independent generation of the amplifiable DNA strand [6, 7].

Prior BINDA studies showed that the use of blocks with 11 complementary bases to the probes produced the largest difference between background and signal [3], so these blocks were the focus of the current investigation. As shown in Figure 2.2, the inclusion of block-R11 with various lengths of block-F (8 to 12 complementary bases) in the incubation solution resulted in similar threshold cycle (Ct) values for detection of 1 pM streptavidin. The exception was block-F12 with 12 complementary bases to probe-F, as the Ct for the signal was 6 cycles greater than that produced by sample solutions containing the other block combinations. The longest block-F formed a more stable interaction with probe-F, and better prevented hybridization to probe-R. Background signals were negligible for all combinations of blocks, as background was only detected in one out of three wells for each combination, or in no wells at all. In a similar experiment, block-F11 was tested with various lengths of block-R (8 to 11 complementary bases) and similar Ct values were obtained for the detection of 1 pM streptavidin (Figure 2.3). In both instances, the Ct values were likely similar because the length of only one block was altered. If the length of both blocks was altered, as in block-F8 tested in combination with block-R8, block-F9 with block-R9, and so on, a difference in Ct values may have been apparent [3].

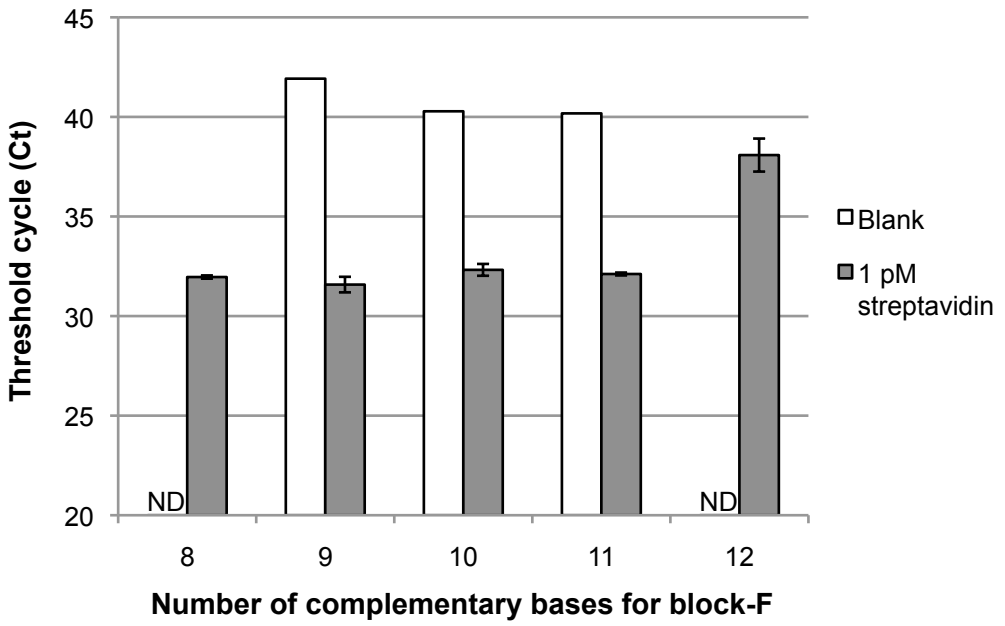

Figure 2.2 Effect of the length of block-F in combination with block-R11 on signal and background. Background was not detected (ND) with block-F8 or F12 and was only detected in 1 out of 3 wells containing block-F9, F10, or F11.

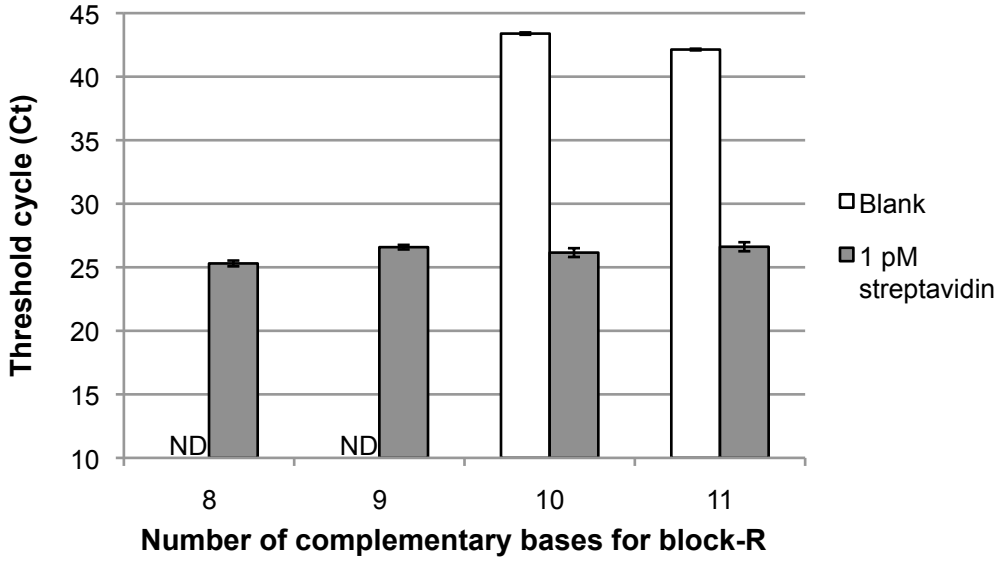

Figure 2.3 Effect of the length of block-R in combination with block-F11 on signal and background. Background was not detection (ND) with block-R8 or block-R9 and was detected in 2 out of 3 wells containing block-R10 or R11.

After the probes and blocks are incubated with streptavidin, or buffer for the blanks, at 37°C for 30 minutes, the solutions are transferred to wells containing the components necessary for ligation and PCR. Before the amplification reaction commences, the solutions remain at room temperature to allow the T4 DNA ligase to catalyze the reaction that joins the oligonucleotides of probe-F and probe-R. Ligation periods ranging from 5 to 30 minutes were tested to investigate the effect on the signal produced from analysis of 1 pM streptavidin and the background produced by blank solutions. As shown in Figure 2.4, the signals produced by ligation times of 5, 10, and 20 minutes were similar. For the 30-minute ligation period, background was observed, and the increased Ct value from 1 pM streptavidin indicates that longer ligation times may not be suitable for detection of low concentrations of the target. A ligation time of 10 minutes was selected as it resulted in a good signal with undetectable background, and compromised between having enough time to seal the wells and centrifuge the plate without adding too much additional time to the procedure.

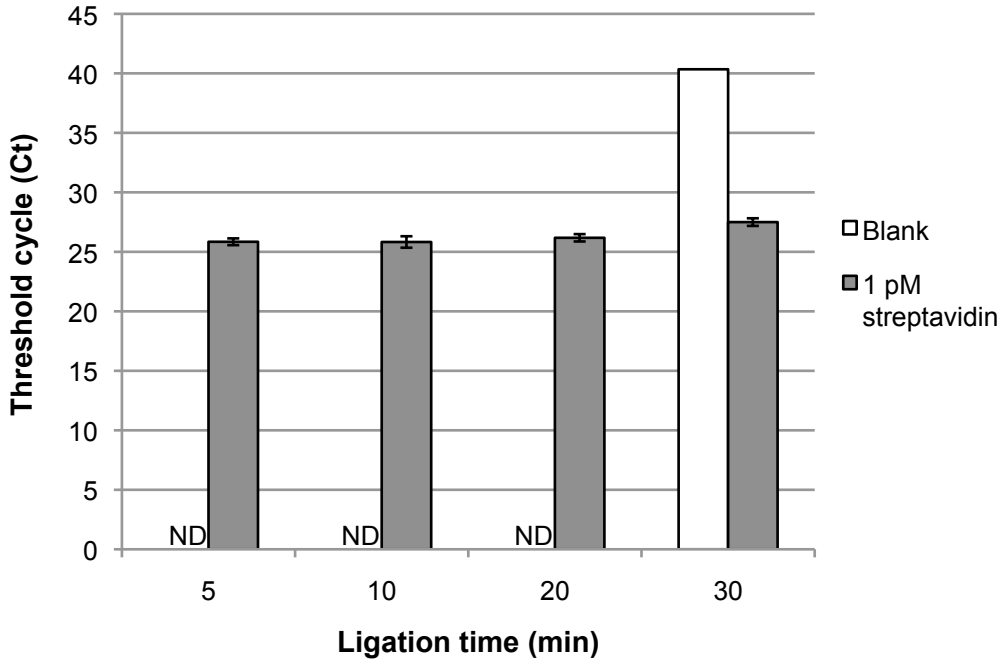

Figure 2.4 Effect of ligation time on the background and signal from 1 pM streptavidin in the incubation solution. The solutions contained block-F11 and block-R10, and were added at different time points to the PCR solution containing T4 DNA ligase. Background was observed in 1 out of 3 wells with the solution that had undergone a 30-minute ligation period, and was not detected (ND) with the other ligation times. The Ct values for the different ligation times were similar, with the exception of 30 minutes where the Ct was over 1 cycle greater.

Based on the principle of BINDA, as the target concentration increases, more probes should bind to the target and hybridize, resulting in a greater amount of DNA template. The greater the amount of starting DNA, the fewer amplification cycles before the threshold of detection is reached, and the lower the threshold cycle (Ct) value. This was experimentally demonstrated by incubating the probes and different block combinations with streptavidin concentrations from

1×10^{-15} M (1 fM) to 1×10^{-12} M (1 pM) (Figure 2.5 and Figure 2.6). There is an inverse linear relationship between the threshold cycle and streptavidin concentration when the concentration is plotted on a logarithmic scale. As shown in Figure 2.5, using block-R10 with block-F10 or block-F11 produced similar Ct values for the streptavidin concentrations tested. Block-F11 in combination with block-R11 resulted in Ct values approximately one cycle less than block-F10 in combination with block-R11 at each streptavidin concentration analyzed (Figure 2.6).

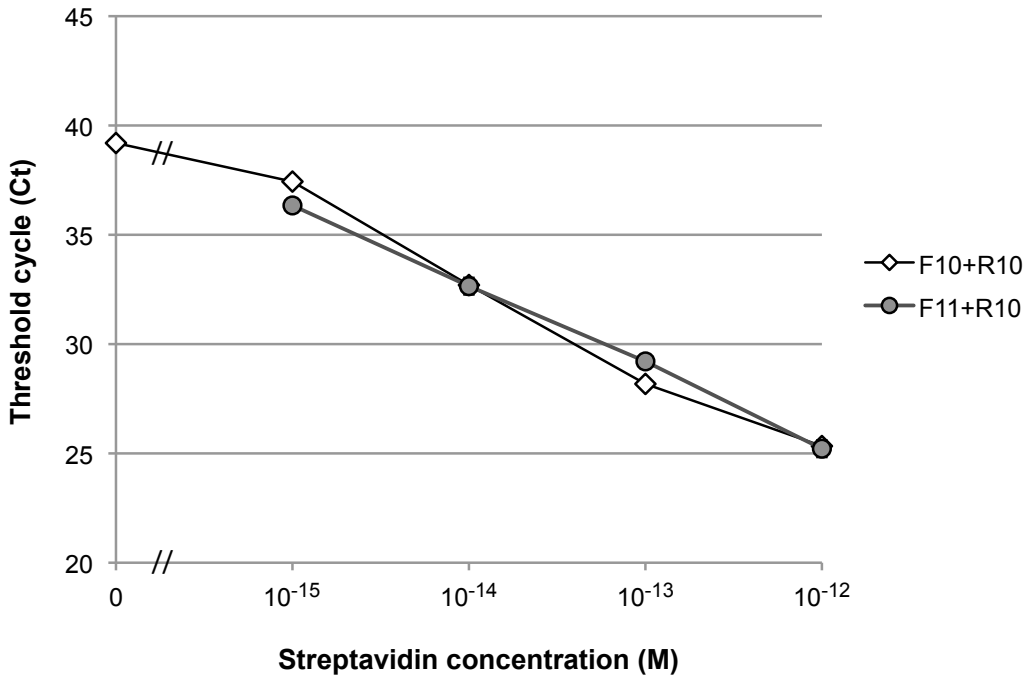

Figure 2.5 Analysis of streptavidin with block-F10 or block-F11 in combination with block-R10. Background was not detected using block-F11 with block-R10.

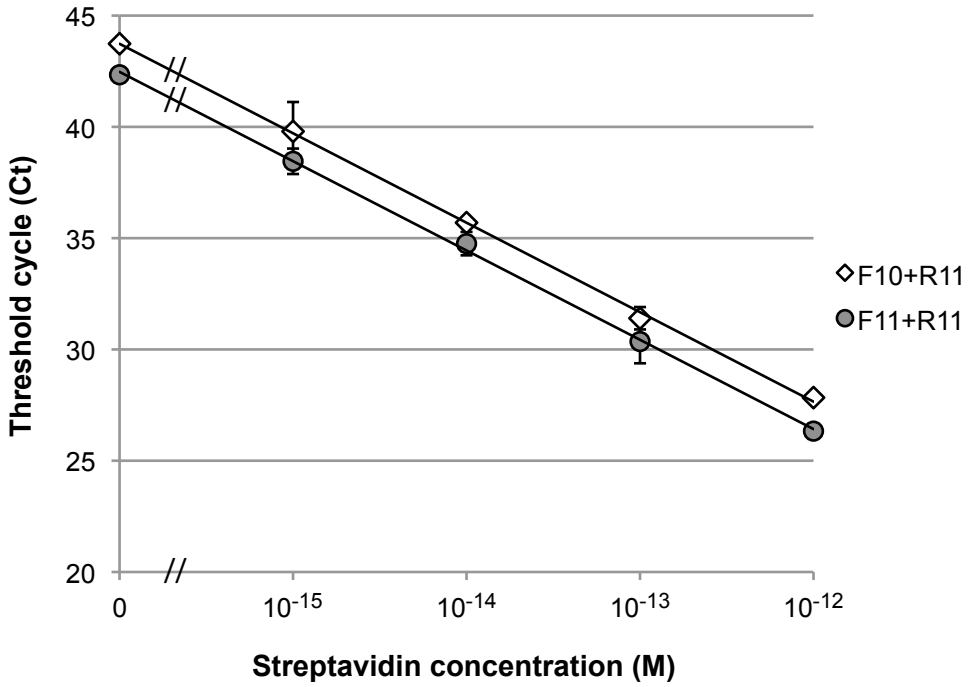

Figure 2.6 Analysis of streptavidin with block-F10 or block-F11 in combination with block-R11. Background was observed in only 1 out of 3 wells for both combinations.

It is difficult to determine the limit of detection for the analysis of streptavidin using three times the standard deviation of the background Ct, as the background was often not detectable. A background Ct value was determined for a blank solution analyzed using block-F10 and block-R10, and the standard deviation was 0.1 cycles (Figure 2.5). Because streptavidin concentrations lower than 1×10^{-15} M were not analyzed, the detection limit was estimated as 4×10^{-16} M (0.4 fM) using three times the standard deviation and the equation of the linear regression line.

2.4 Conclusions

The successful detection of streptavidin in buffer demonstrates the potential of the BINDA strategy for biomolecule analysis. BINDA requires dual probe recognition of the target, and due to the presence of complementary sequences in the probe oligonucleotides, the probes hybridize to form a closed-loop structure. As the background due to the self-assembly of probes in the absence of target is low, or even undetected, the unbound probes do not have to be separated from bound probes. Therefore, the assay is homogeneous, and eliminates the requirement for time consuming and potentially erroneous wash and separation steps. Conventional methods for protein analysis are often based upon fluorescent or chemiluminescent detection. In the BINDA assay, assembly and ligation of DNA converts the protein concentration into a DNA template that can be detected by real-time PCR. Using this method, concentrations of streptavidin from 1×10^{-15} M to 1×10^{-12} M were analyzed. As the concentration increased, there was a corresponding decrease in the threshold cycle value, as a greater target concentration allows for a greater amount of template DNA to form. The estimated detection limit of 4×10^{-16} M indicates that assays based on BINDA have the potential to detect small amounts of target in 2 μL samples.

2.5 References

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CHAPTER 3

Development of a Binding-Induced DNA Assembly Assay for CA 19-9

3.1 Introduction

Our research group has developed the principle of binding-induced DNA assembly (BINDA) with PCR amplification for ultrasensitive detection of proteins. We have demonstrated its use for the analysis of proteins such as streptavidin (Chapter 2), platelet-derived growth factor-BB (PDGF-BB), and prostate specific antigen (PSA) [1]. The BINDA assay utilizes a pair of probes that consist of an antibody linked to a DNA oligonucleotide containing a sequence that is complementary to the other probe. Nonspecific DNA assembly in the absence of target is reduced by block oligonucleotides that hybridize to the oligonucleotide-antibody probes. Upon target recognition by the probe pair, specific DNA assembly occurs: the DNA strands hybridize to each other rather than the block oligonucleotides due to the formation of a stable closed-loop structure. Enzymatic ligation of the hairpin structure of one probe to the other DNA strand creates a template that can be amplified and detected by real-time PCR. DNA assembly is initiated by affinity recognition of the target using an aptamer or antibody; therefore, we reason it is conceivable that the same assay format could be applied to any target provided an affinity ligand for the target of interest is available.

The aim of the thesis research was to demonstrate a novel assay format for carbohydrate analysis using the BINDA principle. Carbohydrates play important functional and structural cellular roles, and aberrant expression of a particular carbohydrate can be indicative of a disease state. Carbohydrate antigen 19-9 (CA 19-9) was chosen as a model target because of its clinical applications. CA 19-9 is a serological marker for patients with pancreatic cancer, and serial measurements are used to monitor a patient's response to treatment [2-4]. Many of the current CA 19-9 analysis methods, whether they are automated methods or ELISA kits, are sandwich immunoassays that require a series of washing or separation steps [5, 6]. Capture antibodies immobilized on a solid phase (plate or paramagnetic microparticle) capture CA 19-9. The reporter antibodies that bind CA 19-9 are then added to produce a fluorescent or chemiluminescent signal that is proportional to the antigen concentration. In contrast, the assay based on BINDA is homogeneous, and quantitation of the target antigen is based on amplification and detection of a DNA template by real-time PCR.

In this chapter, the design and development of a BINDA assay for CA 19-9 is described. We optimized assay parameters that affect target recognition and assembly of the probes to produce the best signal to background ratio, and then demonstrated the detection of CA 19-9 in phosphate buffered saline (PBS) and in human serum.

3.2 Experimental

3.2.1 Safety considerations

The experiments were performed in a level 2 certified biosafety laboratory following the appropriate procedures for working with biohazards. The antigen preparation contains a small amount of sodium azide as a preservative, and should be handled with care.

3.2.2 Reagents

Purified native human CA 19-9 antigen (purity greater than 95% by SDS-PAGE) was purchased from Fitzgerald Industries International (Acton, MA). It was provided at a concentration of 59 147 U/mL in PBS buffer, pH 7.4, with 0.1% sodium azide (NaN_3) added to prevent microbial contamination. CA 125 is present as a contaminant at 1128 U/mL (2%), and CA 15-3 is present at 19.1 U/mL (<1%). Monoclonal antibody to CA 19-9, clone M101221, was supplied by Fitzgerald Industries International at a concentration of 3.45 mg/mL (by OD_{280nm}), in PBS buffer, pH 7.4, with 0.1% sodium azide. This antibody of the IgG_1 subclass had been protein A purified from ascites of a mouse host and 0.2 μm filtered. Fujirebio Diagnostics, Inc. (Malvern, PA) supplied the monoclonal antibody clone C192, also of the IgG_1 subclass, at a concentration of 2.5 mg/mL in a 0.15 mol NaCl solution.

Integrated DNA Technologies (Coralville, IA) synthesized, labelled, and purified the DNA oligonucleotides listed in Table 3.1. The underlined bases are the complementary probe sequences, and the bolded sequences of the blocks are

complementary to the bolded sequence of the corresponding probe. Streptavidin, biotin, bovine serum albumin (BSA), ATP disodium salt, Tris-HCl (1 M, pH 7.4), fetal bovine serum (FBS), human serum, and polyethylene glycol (PEG) 10000 were from Sigma-Aldrich (Oakville, ON). UltraPure™ distilled water, $MgCl_2$, SYBR® GreenER™ qPCR Supermix Universal, and T4 DNA Ligase were from Invitrogen Canada (Burlington, ON). NaOH was from Fisher Scientific (Ottawa, ON), as was the phosphate buffered saline (10× PBS) that was diluted with distilled water to prepare 1× PBS (137 mM NaCl, 11.9 mM phosphate, 2.7 mM KCl, pH 7.4).

Table 3.1 Oligonucleotide sequences used in BINDA assay for CA 19-9

Oligonucleotide	**Sequence**
DNA motif-F	5′-Biotin-ACTGTGTCTCGTCGTTGGTGTTTTGT**TTTG TTTTAGGC**TGGTCGCTTTGTTTTGCGAC-3′
DNA motif-R	5′-**CAGCCTTTTTG**TTTGTTTTGTTTTTTTTGATGGA GCAGGTGTCAGATC-Biotin-3′
Block-F10	5′-TTT**GCCTAAAACA**TTT-3′
Block-F11	5′-TTT**GCCTAAAACAA**TTT-3′
Block-R10	5′-TTT**AAAAAGGCTG**TTT-3′
Block-R11	5′-TTT**CAAAAAGGCTG**TTT-3′
Block-R12	5′-TTT**ACAAAAAGGCTG**TTT-3′
Forward primer	5′-ACTGTGTCTCGTCGTTGGTG-3′
Reverse primer	5′-GATCTGACACCTGCTCCATC-3′

3.2.3 Biotinylation of CA 19-9 antibody

Antibodies against CA 19-9 were biotinylated and purified using the Biotin-XX Microscale Protein Labelling Kit from Invitrogen (Burlington, ON) as per the manufacturer's instructions. Briefly, 100 μg of the CA 19-9 antibody was transferred to a reaction tube, and 1 M sodium bicarbonate solution was added to adjust the pH. The 14.93 nmol/μL reactive biotin stock solution was prepared immediately prior to use by the addition of 10 μL of distilled water to a vial of biotin-XX sulfosuccinimidyl ester (biotin-XX, SSE) sodium salt. The amount of biotin-XX added to the reaction tube was based on the suggested molar ratio of 18 for optimal degree of labelling of IgG [7]. After thoroughly mixing the reaction solution, it was incubated at room temperature for 15 minutes. Biotinylated antibody was separated from unreacted biotin by pipetting the reaction mixture into a Nanosep® MF 0.2 μm centrifugation device containing Bio-Gel P-6® fine resin and centrifuging the spin filter at 16 000 *g* for 1 minute. The purified conjugate in PBS at pH 7.2 was collected in the tube under the spin filter. Aliquots of the conjugate were either used immediately to synthesize probes, or stored at -20°C. The concentration of purified conjugate was determined using a NanoVue Plus™ Spectrophotometer from General Electric Healthcare (Piscataway, NJ) by pipetting 2 μL of the antibody onto the sampling plate and measuring the absorbance at 280 nm (A_{280}) in IgG mode.

3.2.4 Preparation of probes

The DNA oligonucleotides of probe-F and probe-R had been labelled with biotin at the 5′ end and 3′ end, respectively, in order for streptavidin to link the oligonucleotides to biotin conjugated antibodies. DNA motif-F and DNA motif-R were diluted to 400 nM in 1× PBS containing 0.1% BSA, and each oligonucleotide was mixed with an equivalent volume of 400 nM streptavidin in 1× PBS with 0.1% BSA. The mixtures were incubated for 30 minutes at 37°C followed by 30 minutes at room temperature. Afterwards, 100 μL of 200 nM biotinylated anti-CA 19-9 antibody in 1× PBS containing 0.1% BSA was added to 100 μL of each mixture. The two solutions were incubated at room temperature for 1 hour and then at 4°C for 2 hours to allow for probe formation. The probe solutions were then diluted to 50 nM in 1× PBS supplemented with 0.1% BSA and 1 mM biotin, and incubated at 4°C for 1 hour. Aliquots of probe-F and probe-R were stored at 4°C for use within 2 weeks, or stored at -20°C.

3.2.5 BINDA assay and detection by real-time PCR

The desired concentration of CA 19-9, or buffer for blanks, was incubated in a 20 μL solution containing 100 pM of probe-F and probe-R and 500 nM of block-F and block-R in 1× PBS with 0.1% BSA at 37°C for 60 minutes. The reagents required for PCR amplification were mixed in distilled water, and from this master mix, 18 μL was added into the wells of a MicroAmp® Fast Optical 96-well Reaction Plate (Applied Biosystems, Foster City, CA). After the addition of 2 μL of each incubation solution in triplicate, or distilled water for the no template

controls, the reaction solution contained 10 μL SYBR® GreenER™ qPCR Supermix Universal (Invitrogen, Burlington, ON), 0.1 μL ROX reference dye, 0.1 μM forward and reverse primers, 0.4 units T4 DNA ligase (Invitrogen), and 100 μM ATP. The plate was sealed with MicroAmp® Optical Adhesive Film (Applied Biosystems) and remained at room temperature for 10 minutes. For serum analysis, 0.8 units of ligase and a 20-minute ligation period were used. During the ligation period the plate was gently vortexed and centrifuged at 3000 *g* for 5 minutes. The plate was loaded into a 7500 Fast Real-Time PCR System (Applied Biosystems) and PCR was performed using a temperature program of 50°C for 2 minutes, 95°C for 10 minutes, and 55 cycles of 95°C for 15 seconds and 60°C for 1 minute, with subsequent melting curve analysis. Changes to this procedure, such as when investigating the effects of various parameters, will be indicated in the results section to follow.

3.2.6 Preparation of serum

Aliquots of human serum from a male of AB blood type (Sigma-Aldrich, Oakville, ON) were stored at -20°C. Prior to analysis, the serum was thawed and mixed with an equivalent volume of 10% PEG 10000 in 1× PBS. The mixture was incubated at 4°C for 30 minutes, centrifuged at 6000 rcf at 4°C for 20 minutes, and then the supernatant was removed for the experiment.

3.3 Results and Discussion

3.3.1 Principle of binding-induced DNA assembly assay for CA 19-9

The general principle of the BINDA assay for CA 19-9 is illustrated in Figure 3.1, with the DNA sequences listed in Table 3.1. The assay involves recognition of CA 19-9 by a pair of probes, designated probe-F and probe-R, that consist of an antibody attached to a DNA oligonucleotide. DNA motif-F and DNA motif-R are labelled with a biotin moiety at the 5′ end and 3′ end, respectively, to allow streptavidin to link the oligonucleotide to a biotin labelled antibody. DNA motif-F is 58 nucleotide bases in length and consists of three sequence regions with different functions. Sequence 1 includes the 32 bases starting from the biotinylated 5′ end and serves as a spacer to link the antibody to sequence 2. Complementary sequence 2 is 6 nucleotides in length and is linked to sequence 3 (18 nucleotides) that forms a hairpin structure and provides a 3′ end for ligation to DNA motif-R. DNA motif-R is 48 nucleotides in length with spacing sequence 4 (42 nucleotides) at the 3′ end to link the antibody to complementary sequence 5 (6 nucleotides). A free phosphate at the 5′ end of DNA motif-R facilitates ligation to DNA motif-F. Block-F and block-R partially hybridize to complementary sequences 2 and 5, respectively, in order to minimize probe hybridization in the absence of target. When a pair of probes binds CA 19-9, the local concentration of probes increases, allowing the oligonucleotide sequences 2 and 5 to hybridize and assemble a stable closed-loop structure involving the probes and target. The addition of T4 DNA ligase joins the 3′ end of DNA motif-F to the 5′ end of DNA motif-R, creating a template that is amplified

and detected by real-time PCR. Thus, determination of the concentration of CA 19-9 in solution is achieved through the detection of the amplifiable DNA template. As shown in Figure 3.2, the higher the concentration of CA 19-9, the greater the formation of the closed-loop structure. This results in a greater amount of initial template DNA, so when amplified the threshold will be reached sooner and the threshold cycle (Ct) value will be lower.

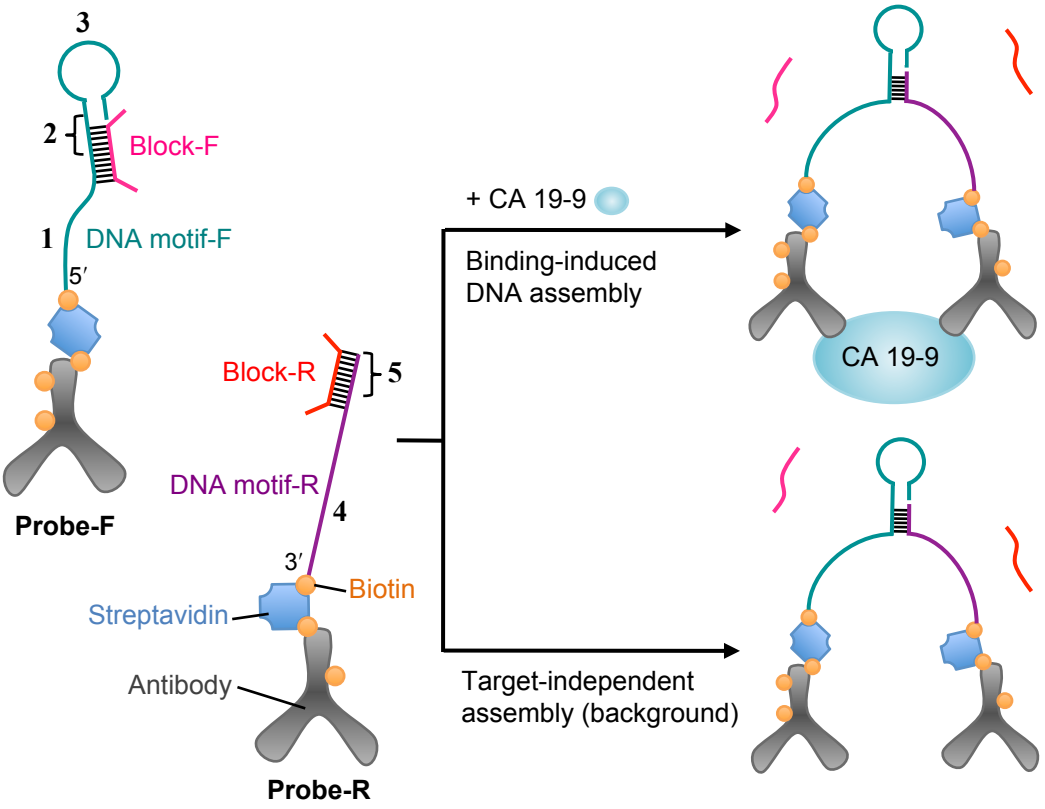

Figure 3.1 Schematic of binding-induced DNA assembly for CA 19-9 detection. Incubation of the probes with a sample containing CA 19-9 results in the formation of the closed-loop structure via BINDA (top). Assembly of the DNA motifs in the absence of CA 19-9 produces background (bottom). Real-time PCR is used to amplify and detect the DNA template that forms as a result of enzymatic ligation of the DNA motifs.

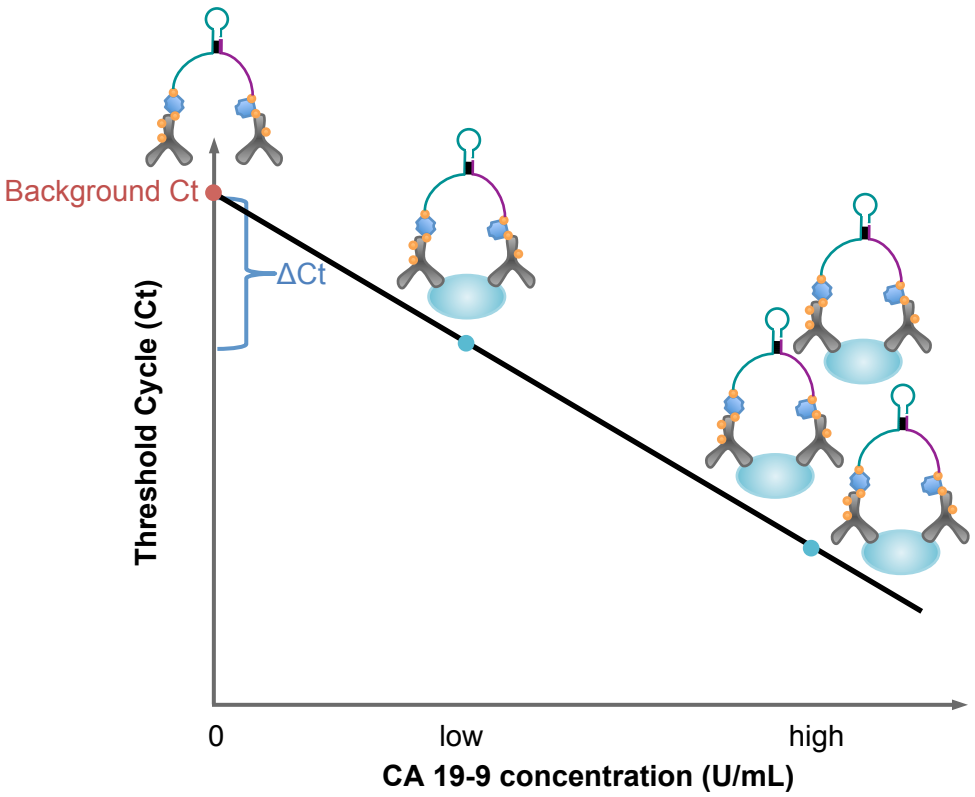

Figure 3.2 Representation of the threshold cycle (Ct) values obtained from background signal (no CA 19-9) and low and high concentrations of CA 19-9. Optimal experimental parameters are those that produce low background (high Ct) and a large difference in Ct values (ΔCt) between sample and blank.

Several factors are important to the successful development of the BINDA assay for CA 19-9, including the type of antibody, the design of probe-F and probe-R, the concentration of probes and blocks, the length of the block sequence, the incubation time and temperature after mixing the probes with sample, and the ligation time prior to real-time PCR. The effect of these parameters was evaluated by performing the reaction in 1× PBS supplemented with 0.1% BSA. Optimal conditions were those that satisfied the following criteria: (1) a low target-

independent background signal, observed as a high Ct value; and (2) a large difference between signal and background, determined by the difference in threshold cycle (ΔCt) between the sample and blank solution. The meaning of the Ct and ΔCt values is indicated in the hypothetical graph of Figure 3.2.

3.3.2 Antibody selection and preparation of probes for CA 19-9

Since mucins carry many carbohydrate chains [8, 9], it was thought that multiple CA 19-9 epitopes would be attached, and the same antibody could be used for both probes. The anti-CA 19-9 antibody clone 1116-NS-19-9 is used in several automated clinical assays for CA 19-9 [5, 6]. However, many commercially available 1116-NS-19-9 antibody preparations are supplied at low concentrations with BSA as a carrier protein. Prior to conjugation of the antibody with biotin, the antibody would have to be purified to remove BSA and preconcentrated to the optimal concentration for the biotinylation reaction. This adds laborious steps to the procedure for preparing the probes, and the addition of steps increases the chance of probe contamination. An antibody preparation supplied at a concentration of at least 1 mg/mL, without the addition of stabilizer proteins, was required.

To create the probes, we conjugated DNA motif-F and DNA motif-R to separate aliquots of biotinylated antibodies via a streptavidin linkage, such that the ratio of DNA to antibody was 1:1. A 1:1 ratio was used because probes with a 2:1 ratio of DNA to antibody could not distinguish the signal from CA 19-9 concentrations of 10 to 1000 U/mL from the background signal (data not shown).

Two antibodies of the IgG_1 subclass, clones C192 and M101221, were examined for their effects on signal generation, as different antibodies have different binding affinities for CA 19-9 [10]. Probes prepared with antibody C192 produced the same background Ct value as probes of antibody M101221 (Figure 3.3), a finding that is consistent with the background signal being due to random hybridization of probe pairs. Probes with C192 were selected for the assay as they produced a larger ΔCt between the blank and 10 and 100 U/mL CA 19-9, providing a better detection limit. In addition, C192 has been used as a capture and tracer antibody in both the CanAg® CA 19-9 EIA kit from Fujirebio Diagnostics, Inc. [11] and the Access® GI Monitor assay on the UniCel® Dxl 800 Immunoassay System from Beckman Coulter [12], as well as in the proximity ligation assay [13]. The association constant (K_a) of C192 is 9.0 x 10^8 M^{-1} (K_d = 1.1 x 10^{-9} M), similar to the K_a of 9.6 x 10^8 M^{-1} (K_d = 1.0 x 10^{-9} M) for the commonly used antibody 1116-NS-19-9, as determined in HEPES-buffered saline at pH 7.4 [10].

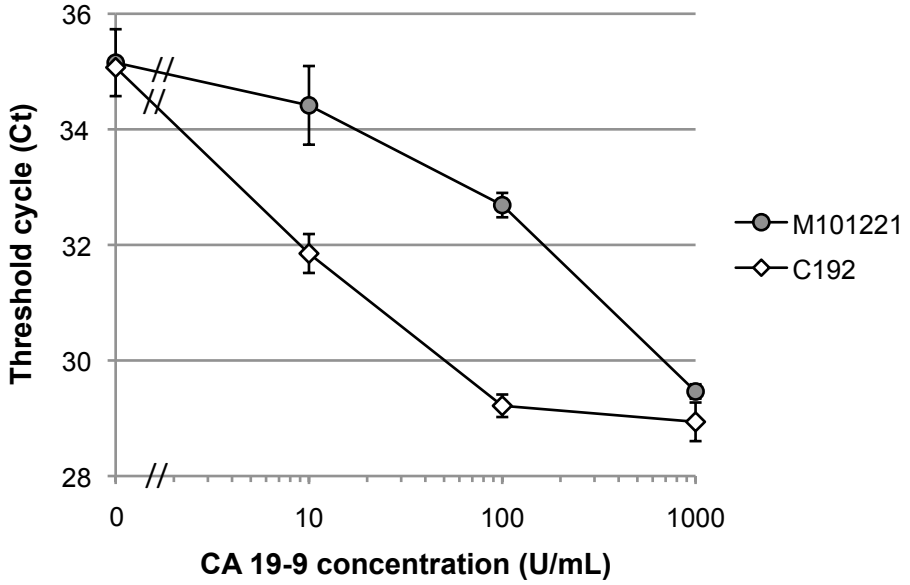

Figure 3.3 Comparison of probes prepared with different antibodies for the detection of various concentrations of CA 19-9. Probes prepared with antibody C192 produced a larger difference between the blank and 10 and 100 U/mL CA 19-9 (ΔCt of 3.2 and 5.9), than probes with antibody M101221 (ΔCt of 0.7 and 2.5). Probe-F and probe-R (each 100 pM) were incubated with block-F11 and block-R11 (each 100 nM) for 30 minutes at room temperature.

3.3.3 Optimization of experimental parameters

Two DNA motifs have been developed for BINDA protein assays, and applied to the detection of streptavidin (Chapter 2), PDGF-BB, and PSA [1]. We proposed that these oligonucleotides, listed in Table 3.1, are useful for developing a BINDA assay for carbohydrates because these assays are based on the common principle of target-mediated DNA assembly. However, the assay parameters must be optimized specifically for CA 19-9 detection.

Hybridization of block-F and block-R to their corresponding probes competes with probe hybridization, reducing the background signal. Without the addition of blocks to the incubation solution, the background signal is large, and the Ct value from 10 U/mL CA 19-9 cannot be differentiated from the blank Ct (Figure 3.4). There is a reduction in signal as the length of the blocking oligonucleotides increases from 10 to 12 nucleotides, because longer blocks enhance the stability of the probe-block duplex, and are more effective at preventing hybridization of the probes. For the longest block combination there was also a reduction in ΔCt, affecting the detection limit of the assay. Block-F11 and block-R11, each with an 11-nucleotide sequence that is complementary to the corresponding probe, were selected for further experiments.

The impact of probe and block concentration on CA 19-9 analysis was first investigated by incubating 10 U/mL CA 19-9 with 100 nM of each block and increasing concentrations (20, 50, 100, 250, and 500 pM) of each probe (data not shown). Probe concentrations of 100 pM and 250 pM were selected for further analysis. Figure 3.5 shows the results of testing different combinations of probe and block concentrations. An increase in the concentration of probes from 100 pM to 250 pM enhances the signal from CA 19-9 detection as more hairpin structures can form, but there is a concomitant increase in the background signal. Increasing the concentration of block oligonucleotides better hinders undesirable hybridization, thereby reducing the background signal. For the low background signal and large ΔCt of 6.4, a probe concentration of 100 pM and block concentration of 500 nM was selected.

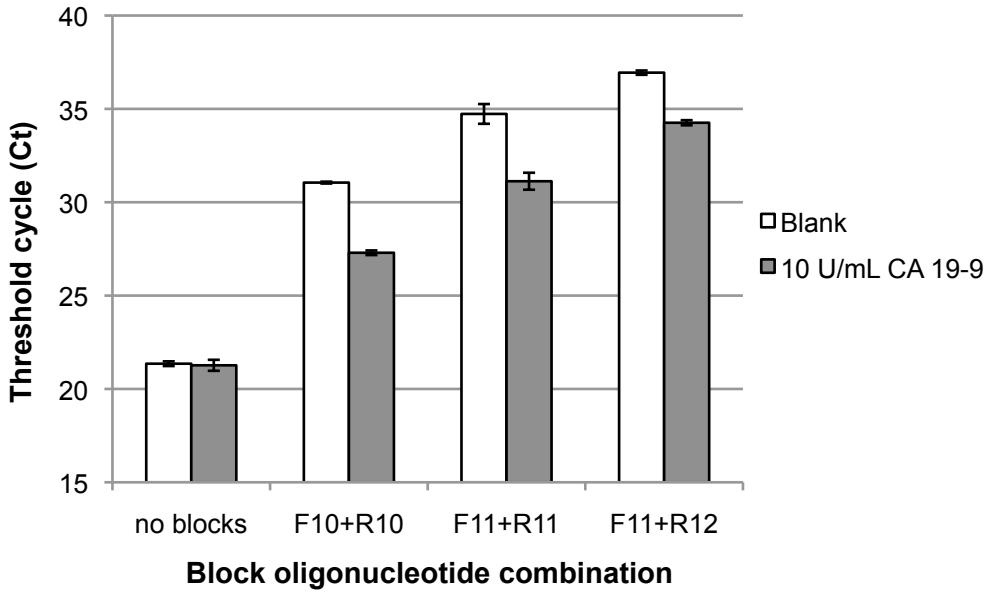

Figure 3.4 Effect of block oligonucleotide length on background and signal from 10 U/mL CA 19-9 in the incubation solution. Probe-F and probe-R (each 100 pM) were incubated with blocks (500 nM) at 37°C for 30 minutes. Increasing the length of block oligonucleotides reduces the background signal. The ΔCt was 3.8 for block-F10 and block-R10, 3.6 for block-F11 and block-R11, and 2.7 for block-F11 and block-R12.

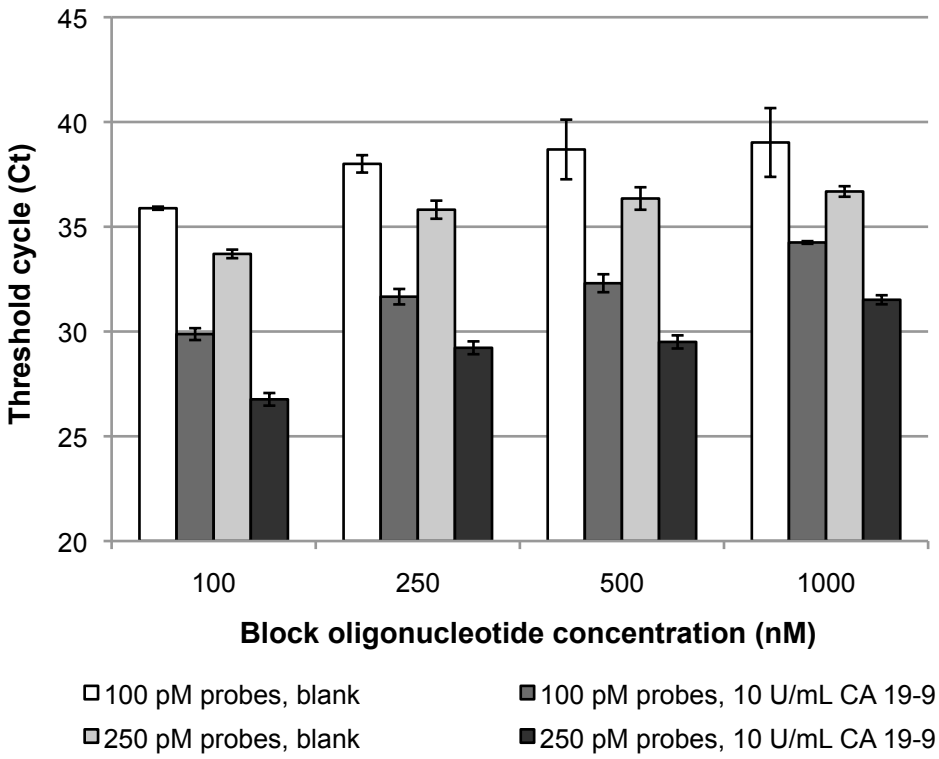

Figure 3.5 Effect of probe concentration (100 and 250 pM) and block concentration (100 to 1000 nM) on background and signal from 10 U/mL CA 19-9. The samples were incubated at room temperature for 30 minutes.

After the probes and blocks are mixed with a test solution (blank or sample), the solution is incubated to allow for target recognition and DNA assembly. Figure 3.6 presents the results of incubating the solutions at 4°C, room temperature, or 37°C; an incubation temperature of 37°C was selected for further experiments. Investigation of incubation periods from 30 to 120 minutes revealed that 90 and 120-minute incubations produce greater background (Figure 3.7). An incubation time of 60 minutes was selected for further experiments as it resulted in low background and large ΔCt between blank and sample. Enzymatic ligation

joins the DNA strands of the probes, and the time allowed for ligation to occur prior to amplification was examined to determine the effect on signal and background. Comparison of 5 to 30 minute ligation periods shows there is a slight increase in background and sample signals (decrease Ct) with longer ligation times (Figure 3.8). Ligation periods of 5, 10, and 20 minutes produced similar ΔCt values, indicating that slight variations in this parameter are tolerable. A ligation time of 10 minutes was chosen for the assay based on analysis of CA 19-9 in 1× PBS with 0.1% BSA.

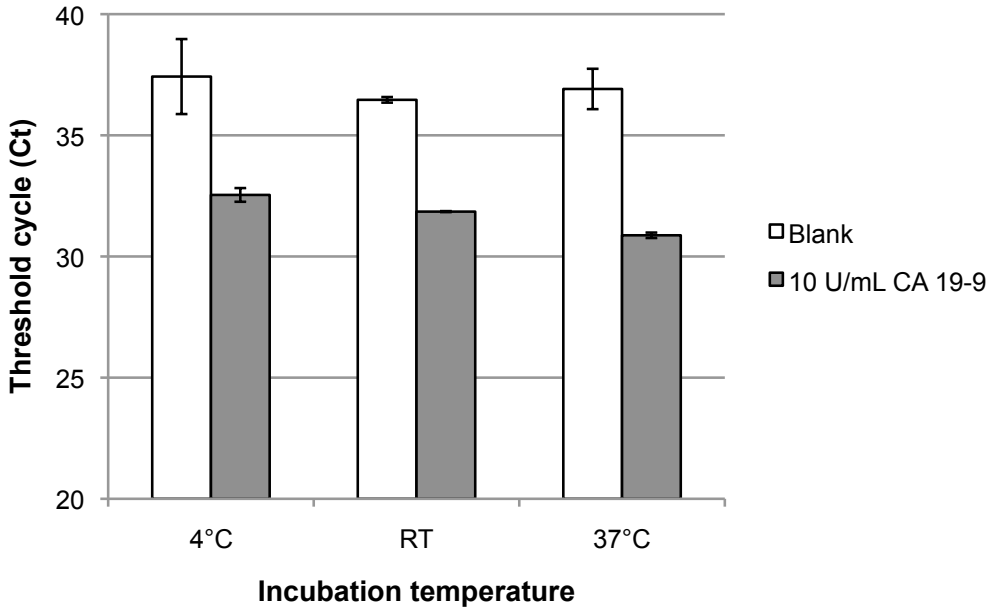

Figure 3.6 Effect of incubation temperature on the background and signal from 10 U/mL CA 19-9 in the incubation solution. Probe-F and probe-R (each 100 pM) were incubated with block-F11 and block-R11 (each 500 nM) for 30 minutes. RT designates room temperature. Incubation at 37°C produced a ΔCt of 6.0, at least one Ct greater than the ΔCt from incubation at 4°C (4.9) or room temperature (4.6).

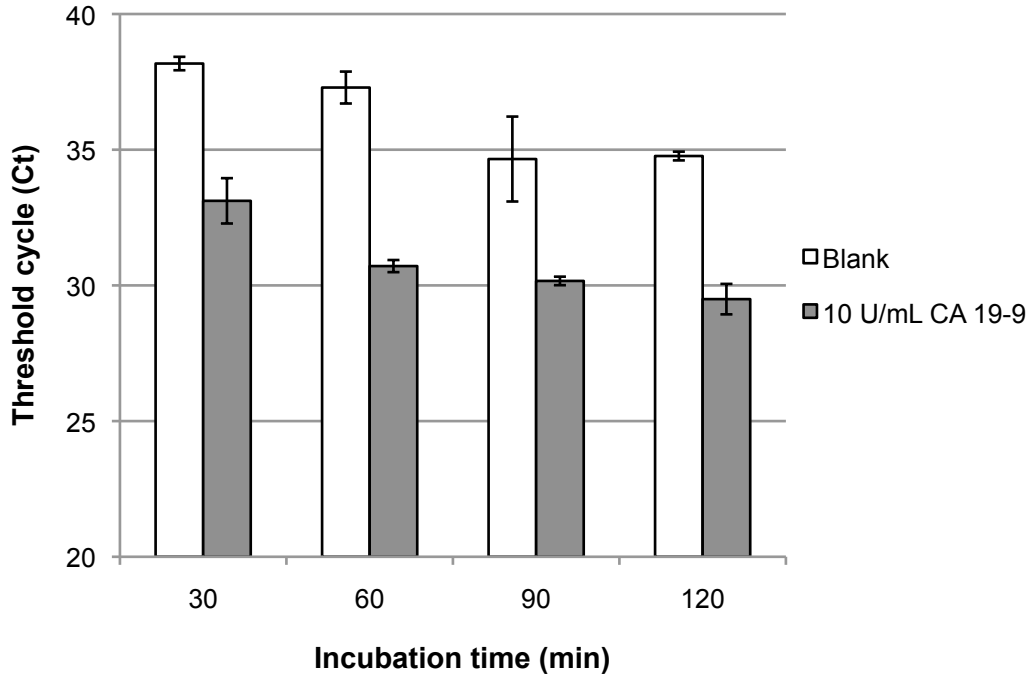

Figure 3.7 Effect of incubation time on the background and signal from 10 U/mL CA 19-9 in the incubation solution. Probe-F and probe-R (each 100 pM) were incubated with block-F11 and block-R11 (each 500 nM) at 37°C for periods ranging from 30 to 120 minutes. Incubation times of 30, 60, 90, and 120 minutes produced ΔCt of 5.1, 6.6, 4.5, and 5.3 cycles, respectively.

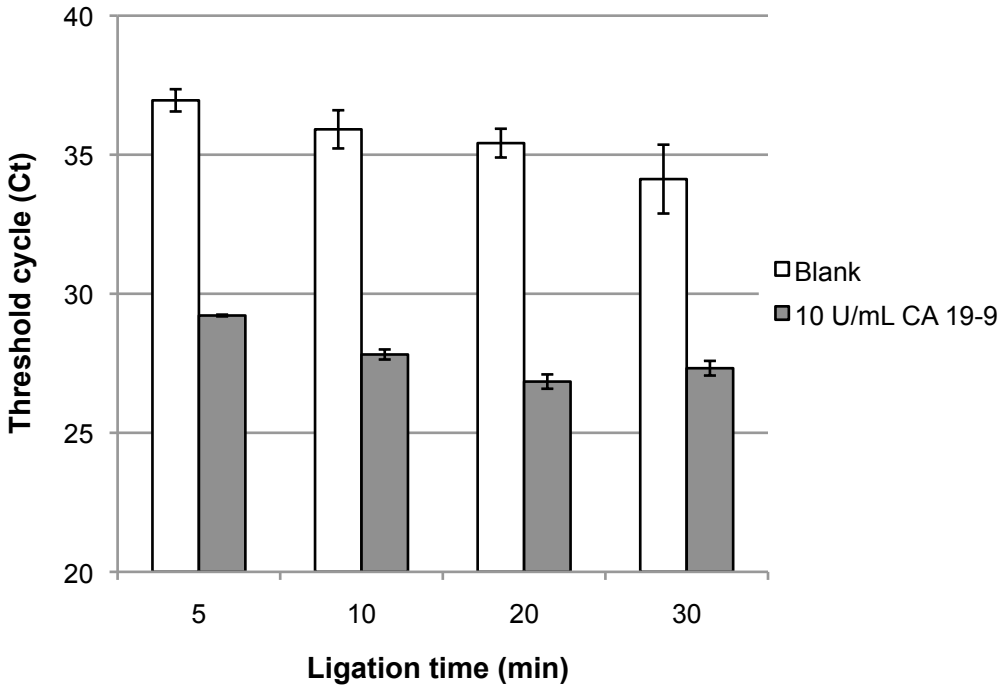

Figure 3.8 Effect of ligation time on the background and signal from 10 U/mL CA 19-9 in the incubation solution. Test solutions were incubated with probe-F and probe-R (each 100 pM) and with block-F11 and block-R11 (each 500 nM) at 37°C for 1 hour, and then added to the PCR solution containing T4 DNA ligase. Ligation times of 5, 10, 20, and 30 minutes resulted in ΔCt of 7.7, 8.1, 8.6, and 6.8 cycles, respectively. A ligation time of 10 minutes was used for analysis of CA 19-9 in 1× PBS with 0.1% BSA, but was increased to 20 minutes for the analysis of CA 19-9 in diluted serum.

3.3.4 Analysis of CA 19-9 in buffer

To test the performance of the assay, we applied the optimized conditions to the detection of CA 19-9 (incubation solution concentrations of 0.1 to 1000 U/mL) in 1× PBS buffer with 0.1% BSA. Based on the curve shown in Figure 3.9,

the limit of detection was 0.2 U/mL for CA 19-9 in buffer. The ΔCt values increased with increasing CA 19-9 concentrations until 100 U/mL, defining the dynamic range as 0.2 to 100 U/mL. There is a reduction in signal at 1000 U/mL because the probability that two probes will bind the target is reduced [1, 14]. This is essentially the hook effect that can occur in immunoassays when excess antigen saturates the binding sites of antibodies without the formation of signal producing complexes, resulting in falsely low values [15, 16].

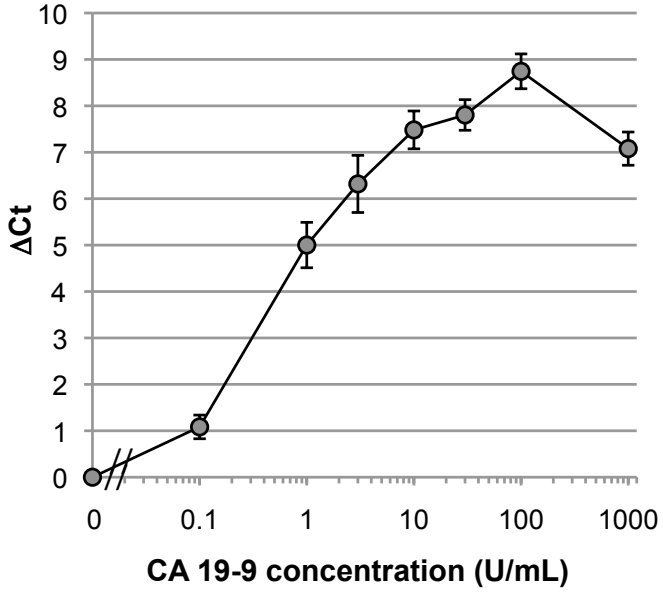

Figure 3.9 Analysis of CA 19-9 in 1× PBS buffer supplemented with 0.1% BSA. Probe-F and probe-R (each 100 pM) were incubated with block-F11 and block-R11 (500 nM) and 0.1 to 1000 U/mL CA 19-9 at 37°C for 1 hour. ΔCt is the Ct value of the CA 19-9 sample subtracted from the Ct of the blank (background signal).

3.3.5 Analysis of CA 19-9 in serum

The performance of the assay was further investigated using a serum matrix. CA 19-9 at concentrations of 5 and 50 U/mL in 20 times diluted human serum or fetal bovine serum (FBS), and in buffer, were tested; CA 19-9 could be detected in the buffer solutions, but it could not be detected in the serum samples. This is likely due to interference from components in the serum samples. Initially we tried to remove large particles in the samples using centrifugation at 13 000 rpm for 5 minutes and then filtering the supernatant using a 0.45 μm PVDF syringe filter. Both human serum and FBS were centrifuged and filtered; however, this did not lead to successful detection of the spiked CA 19-9, as indicated by the high Ct values and melting curve analysis that revealed the amplification product was not the DNA template corresponding to BINDA.

We then considered removing some major components (e.g. immunoglobulins) in the serum using polyethylene glycol (PEG) precipitation [17]. The CA 19-9 target was successfully detected in human serum after PEG treatment. The ligation time and amount of ligase required for the BINDA assay were doubled, as the inhibition of ligase was observed in another homogeneous assay [13]. Using the previously optimized conditions with the altered ligation parameters, CA 19-9 (incubation solution concentrations of 0.1 to 1000 U/mL) was analyzed in 20 times diluted serum (Figure 3.10). The detection limit was estimated as 0.3 U/mL, and the linear range was 0.3 to 30 U/mL, corresponding to 6 to 600 U/mL in undiluted serum. As seen with the analysis in buffer, there was also a reduction in signal at CA 19-9 concentrations greater than 100 U/mL. For

analysis of high concentrations of CA 19-9, the sample could be diluted [16]. It could also be possible to extend the dynamic range by increasing the concentration of probes, but this would also increase the background.

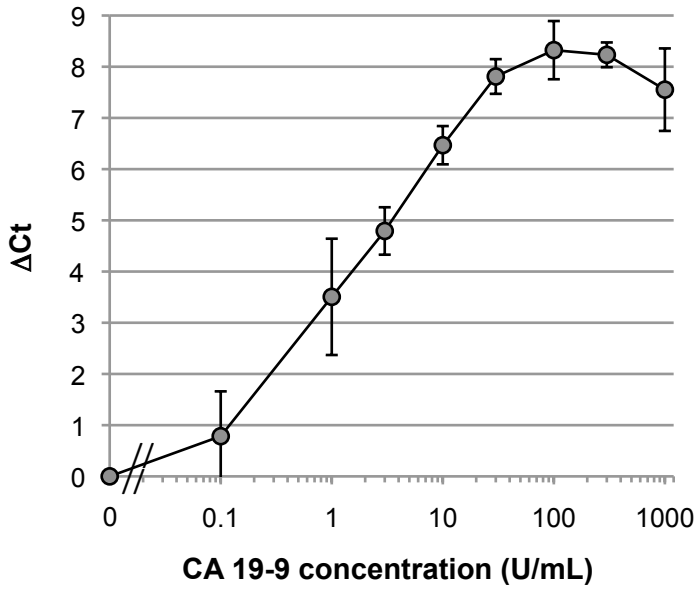

Figure 3.10 Analysis of CA 19-9 in human serum. Probe-F and probe-R (each 100 pM), block-F11 and block-R11 (500 nM), and 0.1 to 1000 U/mL CA 19-9 were incubated in 20 times diluted serum at 37°C for 1 hour. The ligase concentration and ligation time were double that of the buffer analysis conditions.

The basic premise of PEG precipitation is that PEG reduces the availability of solvent, causing an increase in the protein concentration [17, 18]. Precipitation occurs once the proteins exceed the concentration at which they are soluble. The human serum was turbid and yellow, and after PEG precipitation and subsequent centrifugation, a white pellet was at the bottom of the tube and the serum was yellow and clear. Comparison of serum diluted with solutions of 2.5, 5, 7.5, and 10% PEG in PBS determined that 10% PEG (5% PEG when mixed with

serum) was most suitable for treatment of the serum prior to the BINDA assay (data not shown). Despite the technique being relatively specific for immunoglobulins in serum, one concern is the possible precipitation of CA 19-9, thus depleting the sample of the target analyte. To address this concern, CA 19-9 was spiked into serum before and after treatment with PEG. As shown in Figure 3.11, the Ct values for the pretreatment and post-treatment spikes are similar in two of the experiments. In the third experiment (Exp 3 in Figure 3.11), the Ct values for CA 19-9 spiked before or after treatment differ by a little less than one cycle; the higher Ct values obtained from spiking the serum before treatment with PEG suggests the possibility of a loss of some CA 19-9 during the treatment step. However, it should be noted that this was only the result of one experiment, and further experiments should be conducted to rule out the possibility of experimental error and to determine the recovery of CA 19-9 spiked into serum.

For the analysis of serum, matrix components interfered with the detection of CA 19-9. While PEG precipitation was useful, the ligation time and ligase concentration had to be doubled to achieve suitable ΔCt values. It is possible that components in the serum could interfere with the ligation reaction. In a proximity ligation assay for multiple biomarkers in serum, inhibition of DNA ligase was noted, and detection of green fluorescent protein was used as an internal reference to normalize the biomarker data [13]. However, the use of an internal reference protein would require the design of additional probes and the multiplexing capabilities of the BINDA assay are not known.

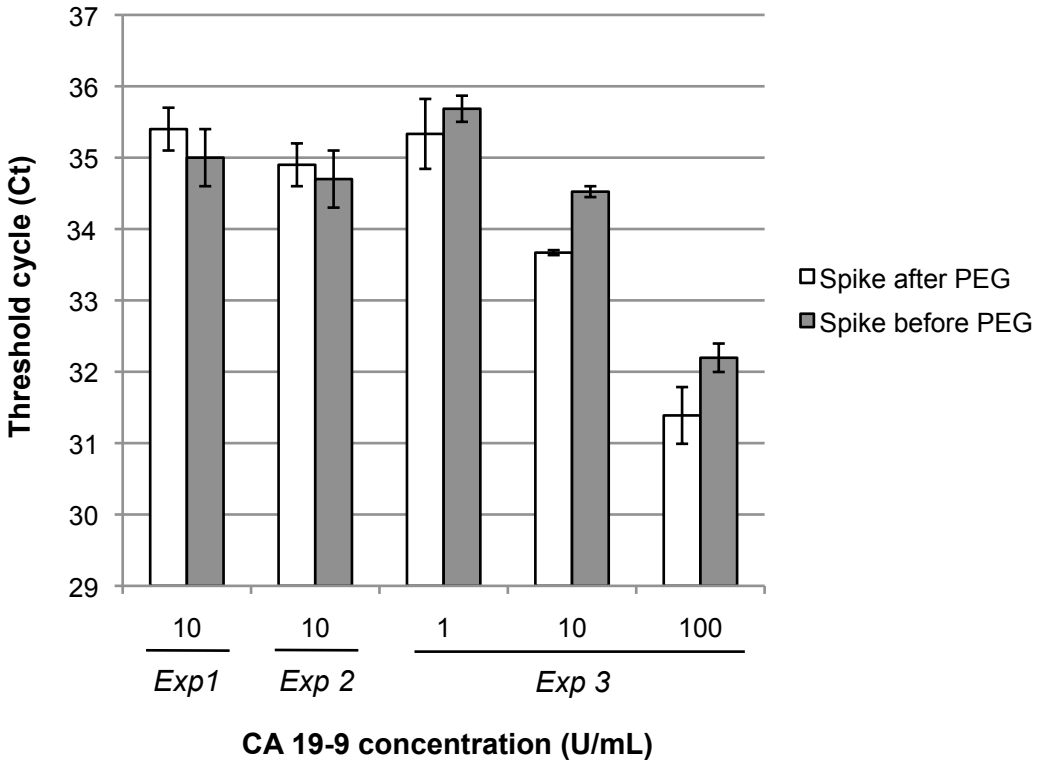

Figure 3.11 Comparison of the analysis of human serum spiked with CA 19-9 before or after treatment with 10% PEG solution in three different experiments (Exp). The incubation solution concentration of CA 19-9 was 10 U/mL in experiments 1 and 2, and 1, 10, or 100 U/mL in experiment 3.

3.4 Conclusions

We have developed a homogeneous assay based on the principle of BINDA for the analysis of CA 19-9. This demonstrates that BINDA can be applied to the detection of carbohydrates in addition to proteins. The detection limit of 0.3 U/mL in diluted serum corresponds to 6 U/mL in undiluted serum. While below the commonly used cut-off values of 35 and 37 U/mL to distinguish normal CA 19-9 levels from elevated levels, the detection limit of the BINDA

assay is higher than that of automated clinical methods [3, 6, 19]. The BINDA assay for CA 19-9 has several potential advantages relative to conventional immunoassays such as ELISA. In sandwich immunoassays, separation steps are necessary to remove unbound reporter antibodies, but these steps are a potential source of error. The BINDA method is simple to perform, does not involve immobilization, separation, or washing steps, and requires 80 minutes for incubation and ligation. The assay format also eliminates problems that can arise when covalent modifications necessary for antibody immobilization to a solid phase cause steric hindrance of the ligand-binding site [20]. Commercially available ELISA assays for CA 19-9 state the detection limit as 1 or 10 U/mL CA 19-9 [21, 22], and the BINDA assay was able to achieve a similar limit of detection. The BINDA assay could potentially be an alternative to ELISA kits, after further optimization and validation.

3.5 References

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CHAPTER 4

General Discussion and Future Research

4.1 Summary

The current work addressed the question of whether the principle of binding-induced DNA assembly (BINDA) could be applied to the analysis of carbohydrates. First, as a proof of concept, the protein streptavidin was detected using BINDA in order to understand the parameters influencing the performance of the assay. Afterwards, probes for carbohydrate antigen 19-9 (CA 19-9) were prepared and a series of experiments were conducted to optimize the experimental parameters. Detection of CA 19-9 was achieved in buffer and in diluted human serum, with the dynamic range of the assay spanning concentrations of CA 19-9 that would be seen in clinical samples. This thesis research was important because it demonstrated that an assay format developed by our research group could be used for the detection of carbohydrates in addition to proteins. The extension of the BINDA assay to the analysis of CA 19-9 supports the idea that the assay format can be applied to various types of biomolecules provided a suitable affinity ligand is available.

BINDA and several of the homogeneous assays based on DNA assembly discussed in Chapter 1 operate based on the following characteristics: (1) the use of probes constructed by attachment of an affinity ligand (aptamer or antibody) to a DNA oligonucleotide; (2) each oligonucleotide has a short sequence that is complementary to the other oligonucleotide of the probe pair; (3) binding of the

probe pair to the target increases the local concentration of the oligonucleotides, favouring hybridization of the complementary sequences; (4) assembly of the DNA produces a signal that is used as an indicator of the target concentration. In both BINDA and the proximity ligation assay (PLA), ligation of the probe oligonucleotides creates a DNA strand that is amplified by real-time PCR to determine the concentration of the target analyte. However, PLA requires the use of a connector oligonucleotide to hybridize to each probe oligonucleotide and bring the free ends together in order to facilitate ligation [1-5]. In the BINDA assay there is no need for a connector oligonucleotide, as the hairpin structure of probe-F aligns the free ends of the oligonucleotides.

4.2 Limitations of Research

The probe preparation procedure was simple in that it only required the addition of reagents and incubation steps, without the requirement of purification steps. One concern is that in addition to creating DNA-antibody conjugates, the procedure also allows for the formation of streptavidin saturated with oligonucleotides or for unbound components to remain in solution. Gullberg et al. [2] noted that such impurities could affect assay performance. Free antibodies could block probe binding, thereby reducing the signal and the ability to detect CA 19-9. Free oligonucleotides and oligonucleotides attached to streptavidin without a conjugated antibody could hybridize and increase the background. Niemeyer et al. [6] used a self-assembly protocol to create conjugates of biotinylated antibody, streptavidin, and DNA, and the conjugates were purified on

a fast protein liquid chromatography (FPLC) gel filtration column and analyzed by non-denaturing gel electrophoresis. An alternative would be to use covalent attachment of the oligonucleotides to the antibody followed by purification to remove the components that did not react. Methods to purify DNA-antibody conjugates include size exclusion FPLC [7], native polyacrylamide gel electrophoresis (PAGE) [8], protein precipitation and filtration [2], and high performance liquid chromatography (HPLC) [9].

Several experimental parameters that could have potentially enhanced the performance of the assay were not investigated. For labelling of the antibodies with biotin to allow for linkage to the oligonucleotides via streptavidin, the amount of biotin reacted with antibody was based on the suggested molar ratio for optimal degree of labelling of IgG antibodies [10]. It is not known whether adjusting the amount of biotin labelling of antibodies could create more effective probes. The probe-F and probe-R oligonucleotides were designed to have a region of six complementary bases. CA 19-9 detection may or may not be improved by the use of probes with a longer hybridization region to enhance the stability of the hairpin structure, or with a shorter hybridization region that reduces target-independent hybridization. It would also be important to determine the stability of the probes after being stored for various lengths of time at -20°C.

For the analysis of CA 19-9 in serum, different concentrations of CA 19-9 were spiked into a commercial human serum, and the dynamic range and limit of detection were determined. Human serum was used as the biological matrix since it is relevant if the BINDA assay was to ever be used to analyze clinical samples.

However, it is not known whether the commercial serum contains low concentrations of CA 19-9, which would affect the determination of the dynamic range and limit of detection.

4.3 Questions and Future Work

Over the course of the current study, a number of questions arose, including: (1) whether or not the BINDA assay for CA 19-9 is suitable for analysis of clinical samples; (2) whether it would be possible to use aptamers as the affinity ligand; (3) whether the probes could be prepared by a different conjugation method; and (4) whether it would be possible to develop a multiplex format of BINDA. The following section discusses these questions and possible avenues for future research.

4.3.1 Demonstration of assay for clinical application: patient samples

Due to difficulty obtaining discarded patient samples, the assay was only tested using a commercially available human serum. To demonstrate the clinical application of the BINDA assay for analysis of CA 19-9, patient serum samples should be tested. Future research could involve obtaining serum samples that are no longer required for patient management from a medical diagnostic laboratory. The discarded samples would be anonymized to remove patient identifiers, and the only information provided would be the clinically determined CA 19-9 concentration. Experiments to investigate assay performance would be designed with the aid of recommendations for feasibility and validation studies [11, 12] and

evaluation protocol documents from the Clinical and Laboratory Standards Institute (CLSI) [13].

Standards with different concentrations of CA 19-9 would be used to construct a calibration curve from which the CA 19-9 concentration of the samples would be determined. The concentration determined by the BINDA assay could be compared to the concentration determined by the standard clinical method to assess the accuracy of BINDA analysis; this can be accomplished by using linear regression analysis to determine the correlation, or by preparing bias plots [14, 15]. Within-laboratory precision would be assessed by repeated measurement of low, medium, and high CA 19-9 concentration serum over multiple days [14, 16]. If the BINDA assay is found to be accurate and precise, then its potential use for analysis of patient samples is better supported.

Another possibility for future work is to investigate possible sources of interference [17]. Endogenous and exogenous substances may alter the determination of the CA 19-9 concentration by cross-reacting to the antibody, or interfering with antibody binding or signal production [18, 19]. La'ulu and Roberts [20] evaluated the performance of five automated assays for CA 19-9 and conducted interference studies by supplementing serum samples with red blood cell hemolysate, bilirubin, or intralipid, to mimic conditions of hemolysis, icterus, and lipemia, respectively. Other potential substances to test include rheumatoid factor [21], chemotherapeutic agents, and human anti-mouse antibodies (HAMA) [22], as well as other cancer biomarkers.

4.3.2 Aptamers as affinity ligands for CA 19-9

For assays that are based on biomolecule recognition, aptamers may be an alternative to the use of antibodies. Nucleic acid aptamers are single-stranded DNA or RNA that have been selected from a library of oligonucleotides for their high binding affinity to the target using the process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [23-25]. The oligonucleotide sequence determines the secondary structure composed of stems and loops, which fold to produce the tertiary structure that binds to the target molecule via hydrogen bonding, van der Waals forces, and electrostatic interactions. The use of aptamers in assays confers several advantages over the use of antibodies. Aptamers are much smaller; for example, an aptamer might be 10 000 Da, while an IgG antibody is 150 000 Da. Unlike antibody generation, once an aptamer has been sequenced it can be synthesized by chemical means with no batch variation. The aptamer nucleotide sequence can be shared among researchers, aiding in the standardization of laboratory assays [1]. While antibodies are sensitive to heat and repeated freeze-thaw cycles, aptamers can undergo repeated denaturation, and the stability of aptamers can further be enhanced by chemical modification of the bases.

Aptamers have been used in a number of assays, including the PLA [1] and BINDA [26] assays for platelet-derived growth factor-BB (PDGF-BB). With all the advantages that aptamers offer, we wondered whether construction of probe-F and probe-R using an aptamer for CA 19-9 would be possible. Currently there is no known aptamer for CA 19-9, so we would have to identify candidates

using SELEX. The research group of Yu et al. [27] selected RNA aptamers for sialyl Le^x, a cell surface glycan and a ligand for selectin proteins that is a positional isomer of sialyl Le^a (CA 19-9) [28]. While the binding affinity of the aptamers (K_d approximately 10^{-9} M to 10^{-11} M) was comparable or better than that of a commercially available antibody, the binding affinity was only 5 to 10 times stronger for sialyl Le^x than the Le^a, sialyl Le^a, and Le^x oligosaccharides [27]. In contrast, antibodies for one isomer generally do not react with the other isomer [28]; for example, commonly used anti-CA 19-9 antibody clones C192 and 1116-NS-19-9 do not cross-react with sialyl Le^x [29]. Therefore, the use of aptamers for carbohydrate analysis may be problematic due to the possibility of cross-reactivity. The challenge of selecting aptamers for carbohydrates is partly due to their structural variability relative to proteins [30]. It is possible that similar difficulties would arise with the selection of an aptamer for CA 19-9, as sialyl Le^a exhibits conformational flexibility in aqueous solutions [31].

4.3.3 Alternative conjugation strategies

The conjugation strategy employed to prepare probes for the BINDA assay was a self-assembly protocol based on the binding of streptavidin to biotinylated antibodies and biotin labelled DNA [26, 32]. In a comparison of triple-binder proximity ligation assays with probes prepared with a noncovalent biotin-streptavidin-biotin linkage versus probes prepared by covalent attachment of DNA to streptavidin, probes with a noncovalent DNA-antibody linkage extended the upper end of the dynamic range with the added advantage of having

an inexpensive, simple, and rapid preparation procedure [32]. While the biotin-streptavidin linkage has been used in several assays, it is not without drawbacks. Subsequent incubation steps with incomplete coupling of reagents results in a complex mixture that may contain free components and streptavidin saturated with oligonucleotides, in addition to the conjugates that can function as probes [33]. As Niemeyer et al. [6] noted, the conjugate preparation method lacks reproducibility, and therefore "seems unsuitable for a highly standardized routine method."

Alternative means of conjugating oligonucleotides to the affinity ligands include covalent attachment of streptavidin to DNA strands via maleimide or hydrazine based chemistry for linkage to biotin labelled antibodies [2, 34], by direct covalent linkage of DNA strands to antibodies using bifunctional cross-linking agents [2, 7, 35], or by the formation of a hydrazone bond between hydrazine-modified antibodies and aldehyde-modified oligonucleotides [9]. The chemical reactions and purification steps for covalent conjugation can be tedious and inefficient, making the use of a kit appealing. The Solulink™ "Antibody-Oligonucleotide All-in-One Conjugation Kit" claims to generate high-purity conjugates, without the need for chromatographic purification, by the formation of a hydrazone linkage between the oligonucleotide and antibody [36]. The Solulink™ technology has been used to prepare the probes for an *in situ* PLA for phosphorylated PDGF receptor β [37]. Experiments to enhance the performance of the BINDA assay for CA 19-9 could include testing probes prepared using the Solulink™ conjugation kit.

4.3.4 Multiplex BINDA assay

A major goal for assay development is the ability to simultaneously measure multiple biomarkers in a single sample. Multiplexed PLA has been created for the analysis of cancer biomarkers [4, 5, 38, 39]. The BINDA assay has the potential to be adapted for multiplex analysis by the development of additional probe pairs that form a unique DNA sequence upon binding to their target. The probe oligonucleotides would be designed so that hybridization is only possible between probes for the same target. Real-time PCR could be performed with the use of unique primers and TaqMan probes with different dyes to monitor the amplification of the DNA strands that each correspond to a particular target.

4.4 Conclusion

The objective of this thesis work was to develop an assay for carbohydrate analysis based on the principle of BINDA. Herein we described the successful application of the concept to the analysis of CA 19-9, the first demonstration of BINDA for the detection of a carbohydrate target. However, the limitations of the described research should be addressed, and a comparison of the assay to a standard clinical method should be conducted before any conclusions are to be made regarding the potential utility of the BINDA assay for analysis of patient samples. The current project could also lead to future research involving alternative methods for probe preparation and the multiplexing capabilities of BINDA.

4.5 References

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