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BIOSENSOR DEVELOPMENT FOR THE ANALYSIS OF FOOD QUALITY

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ABSTRACT

This thesis describes the development and evaluation of a number of biosensors for food applications.

The first part of this thesis deals with the development of Surface Plasmon Resonance (SPR) biosensor systems, coupled with Polymerase Chain Reaction (PCR) for the detection of GMO related amplified nucleic acids in foodstuffs.

The first SPR biosensor described, used streptavidin-biotin linkage chemistry to attach a P35S nucleic acid probe on dextran-coated SPR transducer chips. Methodologies were developed for both the PCR stage and post-PCR sample preparation for the sensitive, rapid and cost-effective detection of GMO-specific amplified DNA sequences. The final embodiment of the method was an asymmetric PCR amplification system with a simple sample processing step (0.3 M NaOH for 30 min in 20 % v/v formamide). The developed PCR-SPR system was successfully applied to the screening of samples of GMO origin.

The second SPR biosensor reported herein, is based on a SPR chip immobilised single-stranded thiolated DNA. The thiolated probe exhibited a hybridisation capacity of 95 RU (Resonance Units) for 100 nM of complementary DNA target and a detection limit of 5 nM. The potential of the current probe system for the detection of symmetrically amplified DNA sequences of short length was subsequently confirmed.

The second part of this thesis involved preliminary studies into the development of simple, disposable screen–printed electrodes for the electrochemical determination of glucose and L-amino acids in horticultural products. The dynamic range of the developed biosensors was up to 10 mM for glucose and up to 1 mM for L-leucine determination. The developed glucose biosensor exhibited encouraging analytical performance in fresh fruit samples. However, the L-amino acid oxidase electrodes consistently underestimated the amino acid content of the fruit samples. The latter observation was found to be primarily due to inhibitory components in the matrix.

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COMMONLY USED ABBREVIATIONS

AAO	Amino Acid Oxidase
Ag/AgCl	Silver/Silver Chloride
CV	Coefficient of Variation
DNA	Deoxyribonucleic Acid
GOD	Glucose Oxidase
H_2O_2	Hydrogen peroxide
OD	Optical Density
P35S	Promoter region cauliflower mosaic virus ribosomal RNA
SD	Standard Deviation
SPR	Surface Plasmon Resonance
TNOS	Terminator of the nopalin synthase gene originating from the soil bacterium *Agrobacterium tumefasciens*

CHAPTER 1

BIOSENSORS AS AN ANALYTICAL TOOL FOR FOOD CONTROL

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A biosensor (Figure 1.1) has been defined as an analytical device that associates a biological sensing element and a transducer (Coulet, 1991). Examples of biological elements specifically coupled to transducers are enzymes, antibodies, cell receptors, nucleic acids, and microbes. Transduction elements can be optical, electrochemical, calorimetric, magnetic, or mass sensitive (Tothill and Turner, 2003).

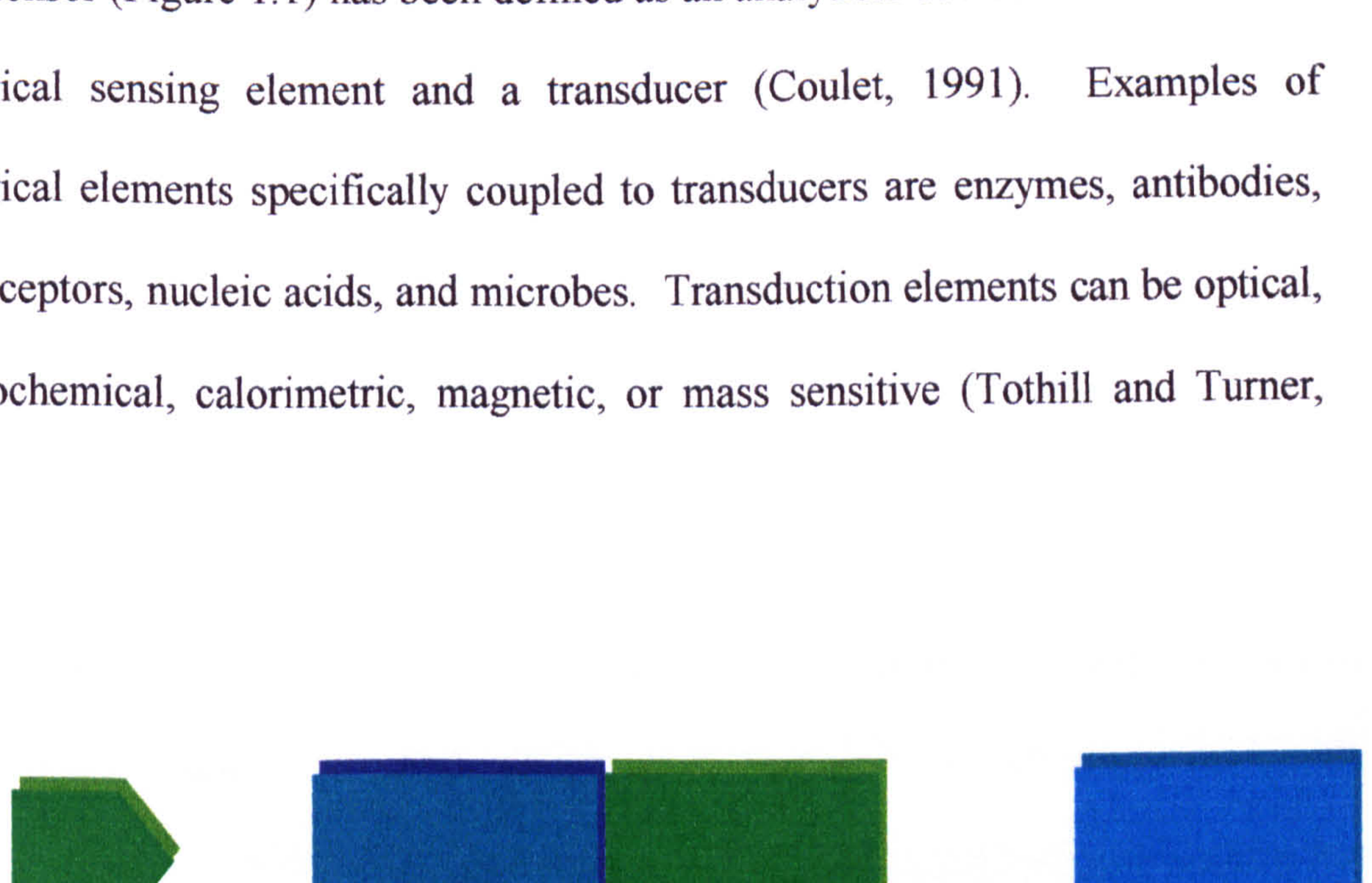

Figure 1.1: Schematic representation of the biosensor. The biological recognition element is combined with a transducer and produces a signal (Tothill and Turner, 2003).

The analyte reacts with the biological component and the transducer transforms the specific recognition signal of the analyte into an electronic, optical or similar signal, which may be readily quantified (Scott, 1998). Thus, both specificity and rapidity are achieved.

Since Clark and Lyons first created a biosensor for glucose monitoring in 1962, biosensors have shown great potential in many areas, such as medicine, food analysis, biotechnology, and environmental monitoring.

Food safety has a major effect in consumer health and well-being. Foodborne illnesses associated with microbial pathogens (e.g. Listeria, Salmonella), biotoxins (e.g. domoic acid in mussles), viruses (e.g. hepatitis A) and chemical contaminants could put at risk public health and considerably damage the profile of a food company. Although associated to some extent with consumer health, food quality has mainly an economical impact, since a food of poor quality has limited consumer acceptability. Food quality encompasses sensory properties (skin appearance, taste, flavour), nutritive values, mechanical and functional properties and defects (Abbott, 1999). Quality and safety assurance concerns a number of organisations, including the food industry and governmental agencies. The food industry needs to ensure the quality and safety of food products to comply with the legislation in force (additives, allergens, genetically modified organisms). On the other hand, governments need to use surveillance to ensure legislative requirements are met and that consumer safety is assured. Therefore,

food analysis is of primary importance as there is the need for accurate, sensitive and validated analytical methods.

Food analysis is not an easy task, considering the complexity of the food matrices and the sensitivity requirements. Food analysis is also challenged by factors such as the form of the analyte, access to the analyte and the stability of the analyte during and after isolation (Scott, 1998). To have commercial interest, the biosensor must prove to be analogous to the standard analytical techniques offering, additionally, rapidity and cost-efficiency of measurement, without the need for time-consuming or expensive sample preparation.

Developed biosensors implicated in food quality analysis have been mainly concerned with the enzyme catalysed detection of glucose, using glucose oxidase (Centonze *et al.*, 1997; Mannino *et al.*, 1997; Gavalas *et al.*, 2000; Palmisano *et al.*, 2000). In a lesser extent, detection of other carbohydrates, such as fructose (Kinnear and Monbouquette, 1997; Boujtita and Murr, 2000) and lactose (Amárita *et al.*, 1997) has also been reported. From the naturally occurring L-amino acids, L-malic acid (Gajovic *et al.*, 1997; Arif *et al.*, 2002), L-glutamic acid (Matsumoto *et al.*, 1998; Kwog *et al.*, 2000) and L-lysine (Curulli, *et al.*, 1998) have predominantly been studied with a few studies looking at the total L-amino acid content (Lee and Huh, 1999; Sarkar *et al.*, 1999).

Biosensor measurements of analytes associated with food safety include the detection of bacterial pathogens (Che *et al.*, 2000; Hamid *et al.*, 1998) and

chemical contaminants, like herbicides (Harris *et al.*, 1999) and insecticides (Nunes *et al.*, 1998). Biosensor-based methods for the detection of genetically modified organisms (Minunni *et al.*, 2001, Mannelli *et al.*, 2003; Mariotti *et al.*, 2002; Feriotto *et al.* 2002) and food allergens (Mohammed *et al.*, 2001) could facilitate compliance with current legislations in force.

To be regarded as a valid analytical method, the developed biosensor must face the challenge of real food matrices. In other words, the biosensor performance must be assessed in real food samples against standard analytical techniques. As this is not always demonstrated, there is a gap between the academic developments and potential of commercialisation.

The current thesis attempts to develop biosensors for food applications. The developed biosensors presented in this study, are optimised in standard conditions and their performance is subsequently challenged in real food samples of raw materials and /or finished products.

CHAPTER 2

GENERAL INTRODUCTION-PART 1: SPR BIOSENSOR DEVELOPMENT FOR THE DETECTION OF GMOS

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2.1. Genetically modified organisms

Genetic change occurs continuously in nature. Micro-organisms have evolved adaptations of high diversity that represent an invaluable genetic resource. These changes may arise by mutations or other combination of genes created by fertilisation of eggs, pollen transfer or other naturally occurring DNA exchange. However, during the past decades, molecular techniques have been developed that allow direct modification of the genetic material of living organisms, such as micro-organisms and plants. All organisms that have a DNA sequence from another organism, are known as 'genetically modified'. Introduction of nucleic acid of a DNA segment into a plant gene is called transformation. Such genetically modified organisms (GMOs) may contain genetic information and exhibit characteristics that have evolved in the context of completely unrelated species.

2.1.1 Insect-pathogen, and herbicide-resistant plants

The main goal of plant biotechnology is to develop new varieties of cultivated plants that exhibit improved propagation or nutritional/end product characteristics. The development of varieties that exhibit pest and disease resistance, increase yields and reduce the cost associated with the use of potentially hazardous pesticides. Other transgenic plants are resistant to herbicides, thus reducing the loss of crop production due to weed infestations (Glick and Pasternak, 2003).

High levels of viral resistance can be achieved via expression of a viral coat protein, linked to a specific virus. Although the mechanism by which the plant acquires resistance is not understood, the ability of the specific virus to infect the plant and initiate a propagation cycle is greatly reduced. In this way, transgenic tobacco, potato and tomato plants have been 'engineered'. In an alternative embodiment transgenic plants synthesise antisense RNA copies of virus coat protein genes. Transgenic plants of this type develop enhanced resistance against viral infections.

For resistance against a broader range of viruses, other approaches have been sought. These include the expression of the ribonuclease (RNAase) III (*E. coli*) in wheat that act by cleaving double-stranded RNA, the genetic material of most viruses (Glick and Pasternak, 2003).

Other approaches have been investigated with respect to the development of transgenic plants conferring fungal and bacterial resistance. Many plants naturally respond to fungal or bacterial pathogens by the production of salicylic acid. Salicylic acid induces the synthesis of a group of proteins, known as pathogenesis-related (PR) proteins that include protease inhibitors and chitinases. Plants (e.g. tobacco) engineered to overproduce salicylic acid or constitutively express high levels of one or more PR proteins should prove more resistant against fungal pathogens. Approaches to the development of pathogenic bacteria resistant plants, include the expression of bacteriophage T4 lysozyme and antimicrobial peptides (Glick and Pasternak, 2003).

Strategies to confer resistance against insect predators have involved a gene of an insecticidal protoxin, produced by one of the several subspecies of *Bacillus thuringiensis*. For example, *B. thuringiensis* subsp. *kurstaki* is toxic to lepidopteran larva, including those of moths and butterflies. *B. thuringiensis* subsp. *israelensis* kills diptera, such as mosquitoes and butterflies. Transgenic plants that express and synthesise a functional form of a Bt-protoxin at sufficient levels, are able to prevent damage from insect predators. Insecticidal proteins expressed in transgenic tobacco and cotton plants are the Cry1Aa, Cry1Ab, Cry1Ac. Each Bt protoxin is effective only to a narrow range of insect predators (Glick and Pasternak, 2003).

Strategies for resistance over a broader range of potential predators include the over-expression of protease inhibitors. Protease inhibitors are naturally produced by some plants, such as cowpea. When digested, the protease inhibitors prevent the insect

from hydrolysing plant proteins and the insect starves. Transgenic crop plants in which increased expression of these compounds has been achieved include rice and tobacco (Glick and Pasternak, 2003).

The herbicide glyphosate acts as an inhibitor for the 5-enolpuryruvylshikimate-3-phosphate synthase (EPSPS), an enzyme involved in the synthesis of aromatic amino acids. Herbicide-resistant plants have been engineered to overproduce the herbicide-sensitive target enzyme, so that adequate amounts remain available for cellular functions, despite the action of the herbicide. Transgenic tomato, potato and cotton plants that produce resistant *E. coli* EPSPS are resistant to the effects of glyphosate (Glick and Pasternak, 2003).

Another mode of herbicide resistance is due to herbicide inactivation. Resistant plants (e.g. tobacco, cotton) express the nitrilase gene from *Klebsiella ozaenae* that inactivates bromoxynil (3, 5-dibromo-4-hybenzonitrile), a herbicide that acts by inhibiting photosynthesis.

2.1.2 Stress- and senescence–tolerant plants

Transgenic plants tolerant to adverse environmental conditions appear to offer the benefits of high production yield. On the other hand, reduction in premature fruit ripening and softening is beneficial to the fruit market.

High salt levels, freezing and drought all stimulate the formation of reactive oxygen species in plant cells. Membranes, proteins and nucleic acids, especially in mitochondria and chloroplasts can be damaged by oxygen radicals, such as the superoxide anion (O_2^-), which results in oxidative stress. One approach for enhancing oxidative stress tolerance is the modification of the enzyme superoxide dismutase. In normal plants, superoxide dismutase detoxifies the superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2). Hydrogen peroxide is subsequently converted to H_2O by various peroxidase or catalase reactions. Tobacco plants have been transformed to express a superoxide dismutase enzyme offering reduced oxygen radical damage under conditions of oxidative stress (Glick and Pasternak, 2003).

The cellulase and polygalacturonase enzymes are induced during the senescence process in relation to fruit ripening. By inhibiting the expression of these enzymes, it has been possible to delay the fruit ripening process. For example, the expression of an anti-sense RNA gene introduced into tomatoes, has led to the reduction of polygalacturonase mRNA and enzymatic activity by 90 %. This genetically engineered tomato is known as 'FLAVR SAVR' and has been commercialised.

More recently, genetic engineering has moved towards the development of transgenic plants with improved nutritional content. Modification of the amino acid content, fatty acid composition and other taste compounds have already been investigated in the laboratory.

As more genetically modified foods are commercialised, public concern upon the safety of GMOs has intensified. Toxicity and allergenicity, caused by the foreign

protein expression, has recently been evidenced in some oil-seed rape crops that use *Bacillus amyloliquifaciens* RNAase as part of a terminator approach (Hodgson, 2001).

Various methods have been employed to determine GMO markers in food products for consumer peace of mind. Immunological detection of the proteins derived from genetic engineering is usually based on the Enzyme-Linked ImmunoSorbent Assay (ELISA), a highly quantitative analytical method. A drawback of the system however, is that the modified protein may not be expressed or is only expressed in very low levels in a part of the plant that it is not used in food production. For example, the immunochemical detection of the Bt-toxin protein in the kernels of Bt - 176 maize, is not immunologically possible, as the protein is expressed in very low amounts in the maize kernels compared to other parts of the plant (Pöpping, 2001). In addition, some introduced DNA sequences are not expressed as proteins, as in the case of transgenic tomato where only RNA is produced to inhibit the translation of a protein that influences the ripening process (Pöpping, 2001). Another disadvantage of the ELISA method is the possibility of denaturation of the trangenic protein as a result of processing (Hübner *et al.*, 1999a). On the other hand, chemical detection of the transgenic protein using gas chromatography/mass spectrometry (GC-MS), fast protein liquid chromatography (FLPC), or capillary electrophoreis (CE) is reliable in certain cases (e.g the detection of chymosin by FLPC), but nevertheless, highly dependent on the protein expression levels. This eliminates its usefuleness, as many modified proteins in GM plants are present in very low concentrations (Pöpping, 2001). DNA-based methods offer the advantage of high specificity and selectivity.

Studies using DNA to detect GMOs are mainly based on PCR amplification techniques. A suitable DNA-based screening method able to detect 26/28 GMOs plants has been proposed by Pietsch *et al.* (1997) and is officially established on a European scale (Lipp *et al.*, 1999). The method detects two regulatory sequences commonly found in transgenic plants. These sequences are contained in the promoter region (35s) of the CAMV (cauliflower mosaic virus) ribosomal RNA and the NOS terminator of the nopalin synthase gene originating from the soil bacterium *Agrobacterium tumefasciens.*

Real-time monitoring of DNA hydridisation is also feasible by the use of technologically advanced PCR instruments, with signal generations based on a variety of fluorescence techniques. An example of real-time quantitative PCR detection of GMOs was provided by Hoehne *et al.* (2002). The method was able to detect and quantify the P35S content of GMO food materials by the use of the ABI prism 7700 sequence detection system. Competitive PCR is another detection method for GMOs that also provides a quantitative analysis of the target material (Hübner *et al.*, 1999 a, b).

In this thesis, the use of DNA biosensors is suggested as an alternative to the usual DNA-based methods. Biosensors offer great sensitivity, easiness, specificity, low cost and amenability to field based usage. These characteristics make biosensors an attractive analytical method applied to various fields, from environmental monitoring to clinical analysis.

2.2 Objective of part 1: SPR biosensor development for the detection of GMOs

In response to the increasing debate surrounding GMOs and their usage, the European Commission has adopted several proposals for the regulation on Novel Foods (1139/98, 258/97). These mainly govern rules for authorisation and labelling of GMO derived products, as consumers wish to obtain complete information about what they consume. Another regulation (1139/98) covers the labelling of foodstuff derived from the GMO derived 'Round Up Ready' and 'Novartis Bt-176' corn, as these products were commercialised before the Novel Foods Law went into effect. This law sets a one percent threshold for adventitious (accidental) contamination such as during cultivation, harvest, storage and processing (www.useu.be/agri/GMOs.html, 2002).

An essential step in the labelling procedure of the GMO products is the availability of analytical methods, capable of reliably identifying evidence of GMO derived products in foodstuffs.

This thesis focuses on the development of Surface Plasmon Resonance (SPR) DNA biosensor-based analytical methods for the detection of DNA-DNA hybridisation with respect to detection of GMO derived ingredients.

The study focused on two key areas: i) the optimised coupling of an SPR DNA biosensor and Polymerase Chain Reaction (PCR) for the detection of PCR amplified nucleic acids (Chapter 3) and ii) the realisation of a functionalised thiolated DNA probe as a part of a mixed Self-Assembled Monolayer (SAM) system (Chapter 4).

In both developed biosensors, the hybridisation reaction was allowed to proceed between the target sequence (P35S) and a complementary sequence ('probe') immobilised on the sensor surface.

2.3 Principles of DNA biosensors

DNA is composed by four repeating nucleotide bases: adenine, thymine, cytocine and guanine. DNA is usually in the form of a double helix that consists of two strands held together by hydrogen bonds. To fulfil its biological role (replication or expression), the DNA double helix has the ability to separate the two strands without disrupting the covalent bonds that hold each strand intact. This temporary separation of the DNA strands and the subsequent reformation occurs under physiological conditions at (rapid) rates needed for the maintenance of these genetic functions. The same features that allow DNA to perform its biological role in a cell make it possible to manipulate the nucleic acids *in vitro*. The noncovalent forces that stabilise the double-stranded DNA (dsDNA) structure may be disrupted by heat or exposure to low concentrations of salts. Dissociation of the hydrogen bonds between the bases results in the formation of entirely separated strands. The process of strand separation is called denaturation. The single-stranded DNA (ssDNA) product is relatively stable, but removal of the denaturing conditions allows the DNA to re-form (re-anneal) the double-stranded helix. Hybridisation is an extension of the re-annealing process, in which the annealing of complementary DNA strands from different sources is performed.

Complementarity is governed by the rules for base pairing between adenine-thymine and cytocine-guanine bases. The stability of hybridisation in a double-stranded DNA molecule depends on the percentage of complementarity.

This basic property of DNA to hybridise has laid the foundation for DNA-based detection systems. Hybridisation can be performed in two ways: solution (liquid) hybridisation and filter (solid phase) hybridisation (Lewin, 1997). Hybridisation in solution involves the mixing of two solution preparations of single-stranded DNA. The drawback of solution hybridisation is the possibility of increased non-specific hybridisation when one or both of the two preparations used contain duplex DNA. In that case, the original complementary single strands can re-nature. This drawback is usually overcome by the solid phase hybridisation process, where one of the two complementary single strands is immobilised onto a solid support. DNA sensors also incorporate a suitable transducer, such as an electrode, crystal, or chips and thus are usually based on solid-phase hybridisation.

The creation of biosensors based on single-stranded nucleic acids, involves several steps. First, a single-stranded DNA fragment containing the sequence of interest must be isolated from cells or synthesised. Single-stranded DNA molecules bearing complementarity to the 'target' sequence, and hence known as the 'probe' are then immobilised onto a solid support of a sensor. Immobilisation should be performed using an appropriate immobilisation chemistry that allows stability and high concentration of immobilised material. Recognition between

the 'probe' and the 'target' sequence is achieved under specific conditions (such as appropriate ionic strength, pH, length of nucleic acid fragments), resulting in formation of the complementary complex (Yevdokimov *et al.*, 1995). The complex formation between the 'target' and the 'probe' leads to a change in physicochemical parameters, such as mass, absorption or diffusion coefficients, and capacity of the layer formed on the transducer surface (Yevdokimov *et al.*, 1995). The most well-known example of ssDNA-based biosensors, in which mass changes resulting from complementary complex formation is detected is the piezoelectric biosensor (Thompson *et al.*, 1991; Caruso *et al.*, 1997; Storri *et al.*, 1998b). Another transduction methodology utilised for DNA-based biosensors is based on electrochemical signal generation. In this case, the hybridisation product can be detected by using redox-active metal/polypuridine complexes that associate selectively and reversibly with dsDNA. The presence of the immobilised double strand results in a higher DNA concentration near the transducer surface and thus higher voltammetric peaks are observed with respect to the single-stranded immobilised material alone (Millan *et al.*, 1993 and 1994). Another type of DNA biosensor is that based on optical transduction, which will be discussed in the following section.

2.4 Affinity biosensors

Optical techniques for chemical analysis are well established. Optical sensors detect changes in the properties of light, which are caused by interaction between electromagnetic waves and matter. Useful optical parameters includes amongst others: absorbance, fluorescence, phosphorescence, refractive index, evanescence wave, total internal reflection and surface plasmon resonance. It is in the scope of this thesis to deal with the optical affinity biosensing based on surface plasmon resonance (SPR) transduction and its application in DNA hybridisation studies. Other evanescent wave optical biosensors will be briefly described.

According to Rogers, 1998:

"Affinity-based biosensors are analytical devices that use an antibody, sequence of DNA, or receptor protein interfaced to a signal transducer to measure a binding event".

Specific binding interactions between molecules form the basis of many biologically controlled processes. These molecular binding reactions are addressed in various biological systems with differing molecular complexity: from low molecular weight species like hormones, amino acids and sugars, to more complex structures such as peptides, proteins, cells and whole organisms. The chemical/biological implications of these reactions can be found in drug-receptor, enzyme-substrate and DNA-protein technology. The role of an affinity-

based biosensor is to probe these reactions, the kinetics governing the binding mechanisms and the concentration of the reactants.

The first affinity-based bio-analytical assay, using antibodies as recognition elements, can be dated back in 1950s, when Yalow and Berson developed an radio-immunoassay for insulin measurement (Yalow and Berson, 1959). Since then, a wide variety of affinity-based biosensor configurations has been developed.

For affinity biosensor devices to be realised, three important steps need to be performed: the fabrication of an appropriate transducer, the selection of a biomaterial able to probe the analyte of interest and the immobilisation of a suitable probe onto the surface of the transducer. Transducer mechanisms with bioaffinity recognition elements include optical, acoustic, electrochemical and thermal. Bioaffinity elements that have been used in the development of an affinity biosensor include antibodies, receptors, and nucleic acids.

Due to their high affinity and versatility, antibodies are the most widely used in the development of affinity-based biosensors. The use of monoclonal antibodies is generally the most favoured due to the high density of binding sites that can be immobilised onto the sensor's surface (Rogers, 1998). Affinity biosensors based on monoclonal antibodies have been well reported in the literature (Quinn *et al.*, 1997; Shimomura *et al.*, 2001).

More limited with respect to antibodies is the use of receptors. Biologically derived receptors are generally more difficult to isolate and reconstruct in vitro,

requiring specialised techniques. As many receptors originate from biological membranes, they often require reconstitution into a lipid environment in order to express their physiological action (Rogers, 1998). One of the most studied receptor proteins is the nicotinic acetylcholine receptor (nAChR) (Changeux *et al.*, 1984).

The use of nucleic acids has shown considerable potential in hybridisation studies for the detection of complementary oligonucleotides (Watts *et al.*, 1995; Peterlinz *et al.*, 1997), and intercalation of compounds that may be detected optically (Pandey and Weetall, 1995). Peptide nucleic acid (PNA) has demonstrated remarkable hybridisation properties towards complementary oligonucleotides and thus its use within affinity biosensor probes is rapidly developing (Wang, 1998; Sawata *et al.*, 1999).

Most affinity biosensors rely on competitive formats for analyte detection. These formats are considered to be either direct or indirect. For the direct assay format the antibody or receptor needs to be immobilised onto the transducer surface, while detection follows the binding of a labelled traced analyte. Indirect assay format uses enzyme-labelled analyte wherein catalytic activity provides the analytical signal after removal of the unbound to the probe analyte. Another assay format in antibody-based biosensors relies on the use a labelled analyte tracer that competes with an unlabeled analyte for the antibody binding sites on the transducer surface (Rogers, 1998).

2.5 Evanescent wave optical DNA biosensors

Optical sensors detect changes in the properties of light caused by an interaction between electromagnetic waves and matter. In the intrinsic mode, the electromagnetic wave propagates in a waveguide and interacts with the sample at a surface within the evanescent field.

Waveguides are physical media through which light can be directed. The propagation wave (light ray) may be viewed as being quantized into discrete propagation states called modes. In optical waveguides, light propagation occurs through total internal reflection (TIR). To obtain this phenomenon in a waveguiding medium and thus a minimal leakage of the light to the surroundings, the refractive index of the guiding medium (n_{medium}) has to be higher with respect to the refractive index of the surroundings ($n_{surroun}$). TIR can occur at a boundary interface between two media having different refractive indices, provided that a critical angle of reflection is met, as defined by Snell's law (Earp and Dessy, 1998).

$$\sin\theta = n_{medium} / n_{surroun}$$

Snell's law defines a minimum angle of incidence for a particular waveguide interface. In case that the angle of the incident light is less than the critical angle of reflection, the light ray undergoes refraction. Snell's law also suggests that the critical angle for a particular waveguiding system depends on the ratio of the refractive indices of the media involved.

There is another criterion for the light to be propagated and this refers to the phase shift that occurs when a wave is reflected at a dielectric boundary. To provide a constructive interference for the wave to travel through the waveguide, the phase shift must be a multiple of 2π for every two wave reflections. If this criterion is not satisfied the wave will quickly be attenuated (Earp and Dessy, 1998).

When a beam of light is in the interface between two transparent media under conditions of total internal reflection, an electromagnetic field is produced and it propagates along the surface decaying exponentially perpendicular to it. The electromagnetic field generated is called evanescent energy wave and penetrates into the medium with low refractive index ($n_{surroun}$) (Earp and Dessy, 1998).

Emission and absorption of electromagnetic radiation are very useful phenomena for chemical analysis. Optical sensors have been used for this purpose for many years and they are among the oldest and best established techniques for direct sensing of biomolecular interactions. Several kinds of optical transducers may be exploited to form the basis of a DNA biosensor. These include resonance mirrors (RM), total internal reflection fluorescence (TIRF) and surface plasmon resonance (SPR).

2.5.1 Optical DNA biosensors based on fluorescence

Total internal reflection fluorescence is the process whereby fluorophores that are either attached to, or in close proximity to, the surface of a waveguide are selectively excited via an evanescent wave (Sapsford *et al.*, 2002).

TIRF systems usually involve competitive assay formats where the unlabelled analyte competes with a fluorescently labelled analyte-analogue for binding to a immobilised material at the sensor surface (Earp and Dessy, 1998). The binding of the analyte to the immobilised substrate is measured via interaction of the fluorophore with the evanescent wave arising from TIR of a light beam (Earp and Dessy, 1998). In TIRF sensors the waveguide may be an optical fiber, planar or a prism. The optical fiber has a cylindrical geometry, while planar and prism formats use TIR at their planar surfaces (Earp and Dessy, 1998).

In fiber optic evanescent-based biosensors, fluorescent DNA-intercalating dyes, fluorophore-labelled target DNA or probe DNA (molecular beacon) have been reported. A commonly used fluorescent DNA stain is ethidium bromide. The fluorescent ethidium cation strongly associates with the dsDNA in the base stacking region and, in some cases the major groove of the double helix structure (Monaco and Hausheer, 1993). One of the first biosensors for direct analysis of DNA hybridisation by use of an optical fiber was developed by Piunno (Piunno *et al.*, 1994). Hybridisation between the covalently immobilised oligomers with the available complementary ssDNA was detected by the use of ethidium bromide.

The first integrated fiber-optic DNA sensor array able to monitor multiple hybridisation events simultaneously was proposed by Healey (Healey *et al.*, 1997). The DNA sensor array involved the immobilisation of different oligonucleotide probes that hybridised with 5'-fluorescein isothiocyanate labelled target oligonucleotides. The system was able to detect point mutations in DNA. Another example of fluorophore-conjugated target DNA was provided by Lü (Lü *et al.*, 2000). Hybridisation of the immobilised probe sequence was achieved with fluorescein conjugated target sequence and studied in situ by the use of an optical fiber. An optical fiber-based Single Molecule Detection (SMD) method was used for the imaging of single molecular beacon DNA molecules (Fang and Tan, 1999).

Molecular beacons comprise a new class of single-stranded oligonucleotide probe, bearing a stem and loop configuration (Tyagi and Kramer, 1996). The loop region is complementary to the target sequence and the two ends are labelled with either a fluorophore (5' prime end) or a quencher (3' prime end). When the stem and loop structure is conserved, fluorescence emission is quenched. In the presence of a target sequence however, hybridisation reaction with the loop sequence, causes the structure to open up. The increased distance between the two ends prevents the efficient quenching of the fluorophore, and thus a fluorescent emission is observed. The study suggested the feasibility of detecting single molecules in solution.

Planar waveguide TIRF has also been used for the study of DNA hybridisation. Peter *et al.*, (2001) used the system to measure hybridisation events using Cy5-labeled target DNA. In this case, the oligonucleotides complementary to the target were immobilised onto the surface of a disposable sensor chip.

2.5.2 Resonant mirror

The resonant mirror comprises a glass prism, the top surface of which is coated with a low refractive index silica spacer layer, which in turn is coated with a thinner high refractive index waveguide of metal oxide. This film is then coated with the desired bioselective layer. A laser light (λ=670nm) is directed at the prism and polarised to produce equal intensities of components of light. At a certain angle of incidence, termed the resonant angle, light passes from the prism through the low refractive index coupling layer and propagates in the high refractive index waveguide layer at the same angle. The bioselective film overlaying this arrangement is a low refractive index layer. Thus, propagating light in the high refractive index waveguide layer will generate an evanescent field extending into the bioselective layer. Refractive index changes within this field cause changes in the resonant angle at which light couples into the resonant structure. Only the light that has travelled within the resonant structure passes through a phase polariser and is eventually collected by the detector. Light not undergoing a phase change is coupled via the low refractive index layer into the

high refractive index layer, where it undergoes multiple total internal reflections at the top interface (Kinning and Edwards, 2002).

This technology has been applied to the direct and rapid detection of ssDNA-ssDNA hybridisation. Oligonucleotide probes have been immobilised onto the surface of the sensor and hybridisation of a target label free oligonucleotide (40-mer) was monitored in real time (Watts *et al.*, 1995).

2.5.3 Surface plasmon resonance

Surface Plasmon Resonance (SPR) is a surface sensing technique that can be used to probe refractive index changes occurring within the vicinity of a sensor surface (Earp and Dessy, 1998).

A surface plasma wave (SPW) or a surface plasmon-polariton is an electromagnetic wave which propagates along the boundary between a dielectric and a metal (Boardman, 1982). Optical excitation of a surface plasmon can be accomplished if a light beam is totally internally reflected at the surface of a glass substrate onto which a thin metal is deposited (Nylander, 1987).

Wavevectors are mathematical expressions that describe the propagation of light and other electromagnetic phenomena (Earp and Dessy, 1998). The wavevector of the evanescent field (Kev) is given by:

$$K_{ev}= \frac{w_o}{c} \eta_g \sin\theta$$

where w_o is the frequency of the incident light , η_g the refractive index of the dense medium (glass), θ the angle of incidence of the light and c the speed of light in a vacuum. The wavevector of a surface plasmon (SP) can be approximated to:

$$K_{sp}=\frac{w_o}{c}\sqrt{\frac{\varepsilon_m \eta_s^2}{\varepsilon_m+\eta_s^2}}$$

where ε_m is the dielectric constant of the metal film and η_s the refractive index of the dielectric medium (Matsubara *et al.*, 1988).

When the wavevector of the light is made to match the wavevector of the surface plasmon, the surface plasmon is resonantly excited, resulting in energy loss from the reflected intensity. Excitation of the surface plasmon occurs under a combination of a specific wavelength, the resonance wavelength of light, and a specific angle of incidence, the surface plasmon resonance angle (SPR angle) (Meléndez *et al.*, 1997).

The surface plasmon resonance angle (SPR angle) is very sensitive to variations of the refractive index of the medium extending about 100nm outside the surface

of the metal (Nylander, 1987). Any changes occurring within the plasmon's electric field can therefore be detected as change of the SPR angle.

The most common method for setting up a surface plasmon is the Kretschmann configuration (Figure 2.1) (Kretschmann and Raether, 1968). The Kretschmann prism arrangement is a structure where a light beam is coupled into a surface plasmon mode that can exist at the surface of a solid metal film (Figure 2.1).

Light which is p-polarised with respect to the metal surface is launched onto the metal's surface and coupled into the surface plasmons (Earp and Dessy, 1998). Metallic films are typically fabricated from gold and silver. Gold is used due to its stability and silver because it provides a sharp SPR peak (De Bruijn *et al.*, 1992). The Otto configuration used in SPR devices has a high degree of similarity with that of the Kretschmann configuration, the difference being that excitation of light occurs in the outer metal boundary through a dielectric layer (Homola *et al.*, 2002).

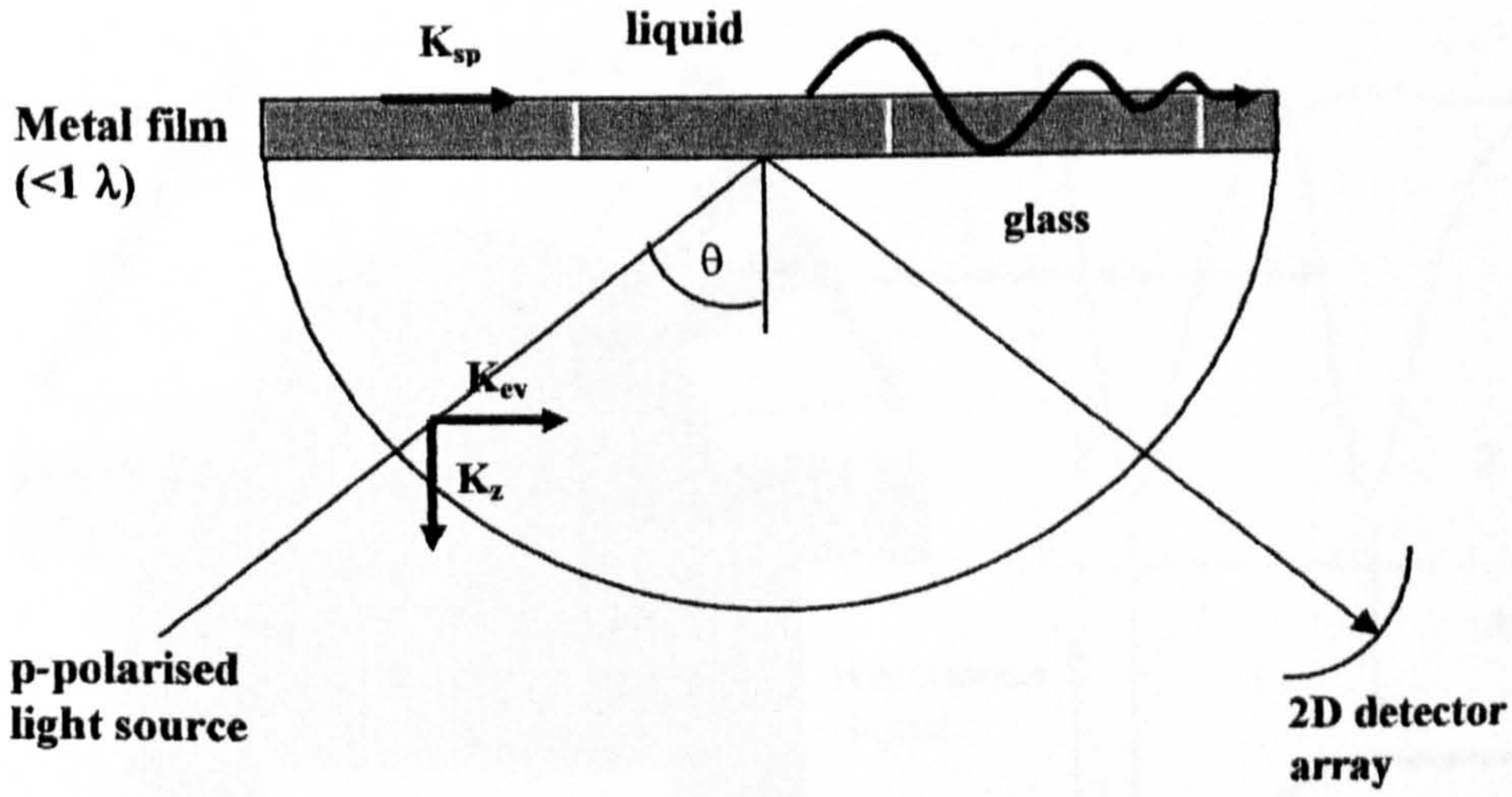

Figure 2.1: The Kretschmann configuration of SPR. Electromagnetic radiation is introduced into the prism and coupled into the SP mode on the metal film. Resonance of the SP mode occurs when the angle of incidence of the incident light is such that the evanescent component of its wave vector (Kev) is equal to the wave vector of the propagating light (Ksp).

To obtain wavevector matching, the wavevector of the light, which is normally smaller than that of the surface plasmons, requires magnification by the use of prisms or gratings.

The first commercial SPR biosensor was launched by BIAcore International AB in 1990 (Pharmacia, Uppsala, Sweden). BIAcore SPR sensors based on the Kretschmann geometry and angular modulation have found many applications, such as biomolecular interaction kinetic analysis, concentration assays and

affinity measurements. Figure 2.2, provides an illustration of the SPR principle used in BIAcore AB sensors.

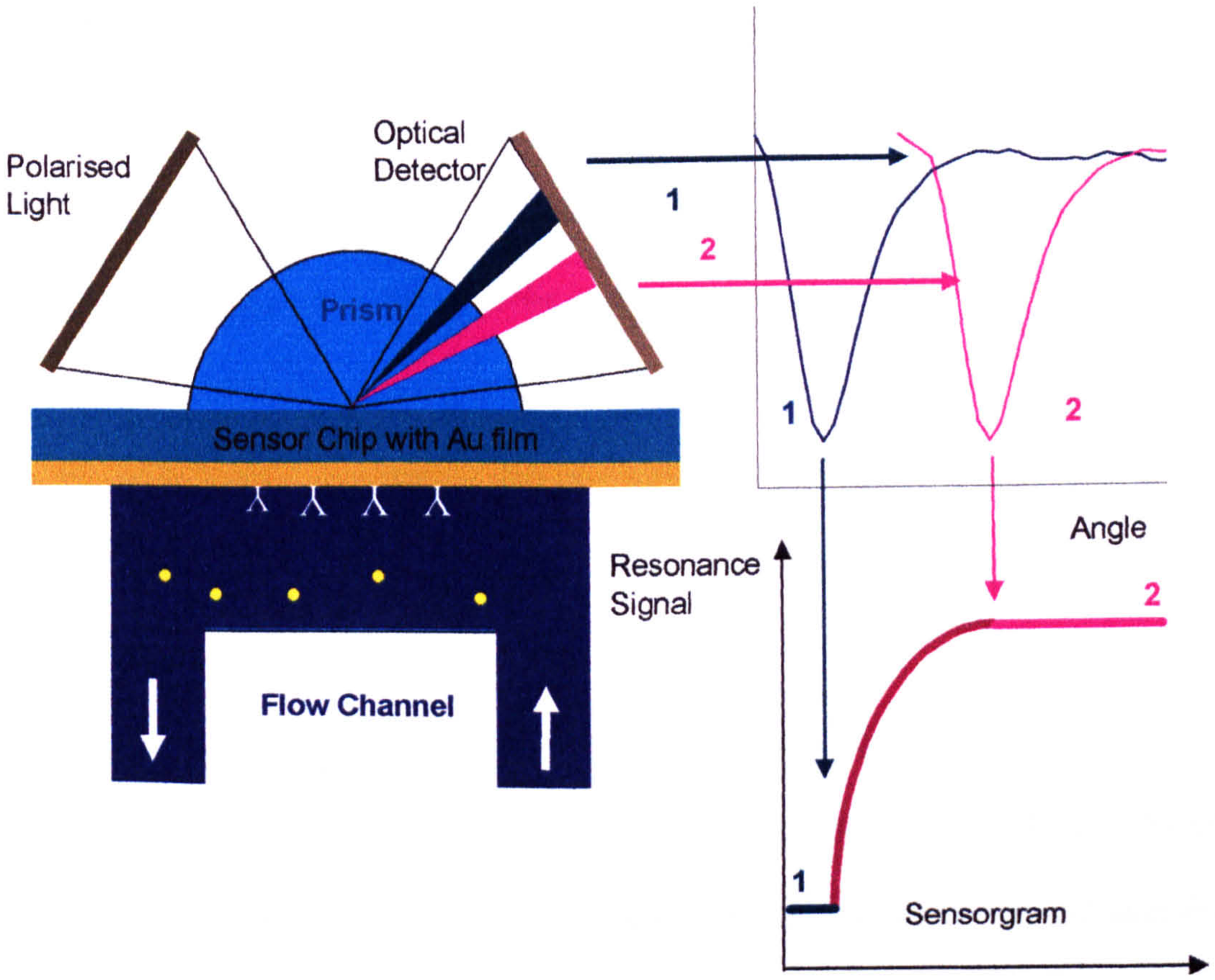

Figure 2.2: SPR principle used in BIAcore™ sensors (Pharmacia, Uppsala, Sweden). Binding of the interactants change the refractive index of the environment close to the SP and alter SPR angle, where maximum absorbance of light occurs. SPR changes are continuously measured and reported in the sensorgram.

To perform an analysis, a biological component is immobilised at the sensor surface that also comprises the wall of the flow cell (Figure 2.2). Antibodies, DNA probes or enzymes are usually chosen as they offer high specificity and selectivity. Fixed wavelength light is then directed over the gold film. The

analyte solution flows over the sensor surface at a specifically controlled flow rate and hence interacts with the immobilised substrate. This interaction leads to molecular binding between the 'receptor' and the 'target' components, thus changing the mass concentration of the material attached to the sensor. This change in the refractive index of the environment close to the surface plasmon causes a shift in the SPR angle, where maximum absorbance of light occurs. A linear relationship exists between the magnitude of this shift and the magnitude of the chemical change. During a binding process, SPR changes are continuously measured to form a sensorgram that provides a complete record of the association or dissociation events between the interacting species (Figure 2.2).

The SPR assay provides a convenient and effective method for the investigation of ssDNA-ssDNA interactions. DNA hybridisation studies based on SPR are relatively few and have been mainly applied in the analysis of clinical samples. Differences in the hybridisation levels between PCR amplified fragments carrying mismatches and fully matched sequences, allow the screening of mutations and pathologically significant DNA (Nilsson *et al.*, 1997; Sawata *et al.*, 1999; Kai *et al.*, 2000; Feriotto *et al.*, 2001). In 2001, Nakatani *et al.*, developed a novel SPR-based system to detect guanine-guanine mismatches, one of the four types of single–nucleotide polymorphism. The novelty of their work, lays within the synthesis and use of ligands able to bind specifically and with high affinity to the G-G mismatches. Experiments with PCR amplified fragments containing a G-G mismatch gave a marked SPR response in comparison to perfectly matched sequences (Nakatani *et al.*, 2001).

2.6 Immobilisation of biomolecules on gold surfaces for SPR sensing

An essential requirement for SPR biosensors is that one of the interacting species must be immobilised onto the sensor surface. Many types of nucleic acid immobilisation on solid support may be applied to the development of an SPR biosensor. Particular aspects of immobilisation that have received significant attention include the efficiency of DNA coupling, the stability of attachment, the accessibility of oligonucleotides for hybridisation and the minimisation of non-specific binding. The focus of this chapter will be the examination of alternative techniques for the immobilisation of DNA molecules onto gold surfaces for SPR sensing.

2.6.1 Physical adsorption of biomolecules

Immobilisation of self–assembled monolayers through physical adsorption is an attractive method, owing to its versatility and simplicity. Physical adsorption of proteins onto unmodified solid surfaces may be accomplished through hydrophobic or electrostatic interactions. However, the technique suffers from serious disadvantages. The method does not ensure the appropriate orientation of material coating the sensor. Poorly defined strand orientation, low packing density and limited mobility often arise due to the passive adsorption process. In certain cases, the subject protein can undergo conformational changes, which affect its functional activity. Protein denaturation and subsequent inactivation

upon direct contact with the metal surface has also been noted (Butler *et al.*, 1992).

2.6.2 Covalent immobilisation based on Self-Assembled Monolayers

Self-Assembled Monolayers (SAMs) are monomolecular layers that exhibit high organisation and are spontaneously formed as a consequence of immersing a solid surface into a solution consisting of amphifunctional molecules. The defining feature of the SAM is the "pair" of the chemisorbing headgroup of the molecule and the substrate (Schreiber, 2000). Many systems are able to undergo self assembly: long chain carboxylic acids at metal oxide substrates, organosilane species ($RSiX_3$, $R2SiX_2$, R_3SiX, where R is an alkyl chain and X a chloro or alkoxy group) at hydroxylated substrates, exemplified by silicon and organosulphur-based species at noble metal surfaces. The latter system has been the most widely studied to date and thus is the best characterised in terms of stability and physicochemical properties. Alkanethiols (R-SH), disulphides (R-S-S-R) and sulphides (R-S-R) have the ability to strongly absorb on a metal surface (Nuzzo and Allara, 1983). Sulphur compounds co-ordinate very strongly to gold, silver, platinum and copper. The alkyl chains are found to be orientated away from the perpendicular plane with respect to the gold surface and the angle (α) is typically between 26° and 28° (Figure 2.3) (Bain *et al.*, 1989; Ferretti *et al.*, 2000).

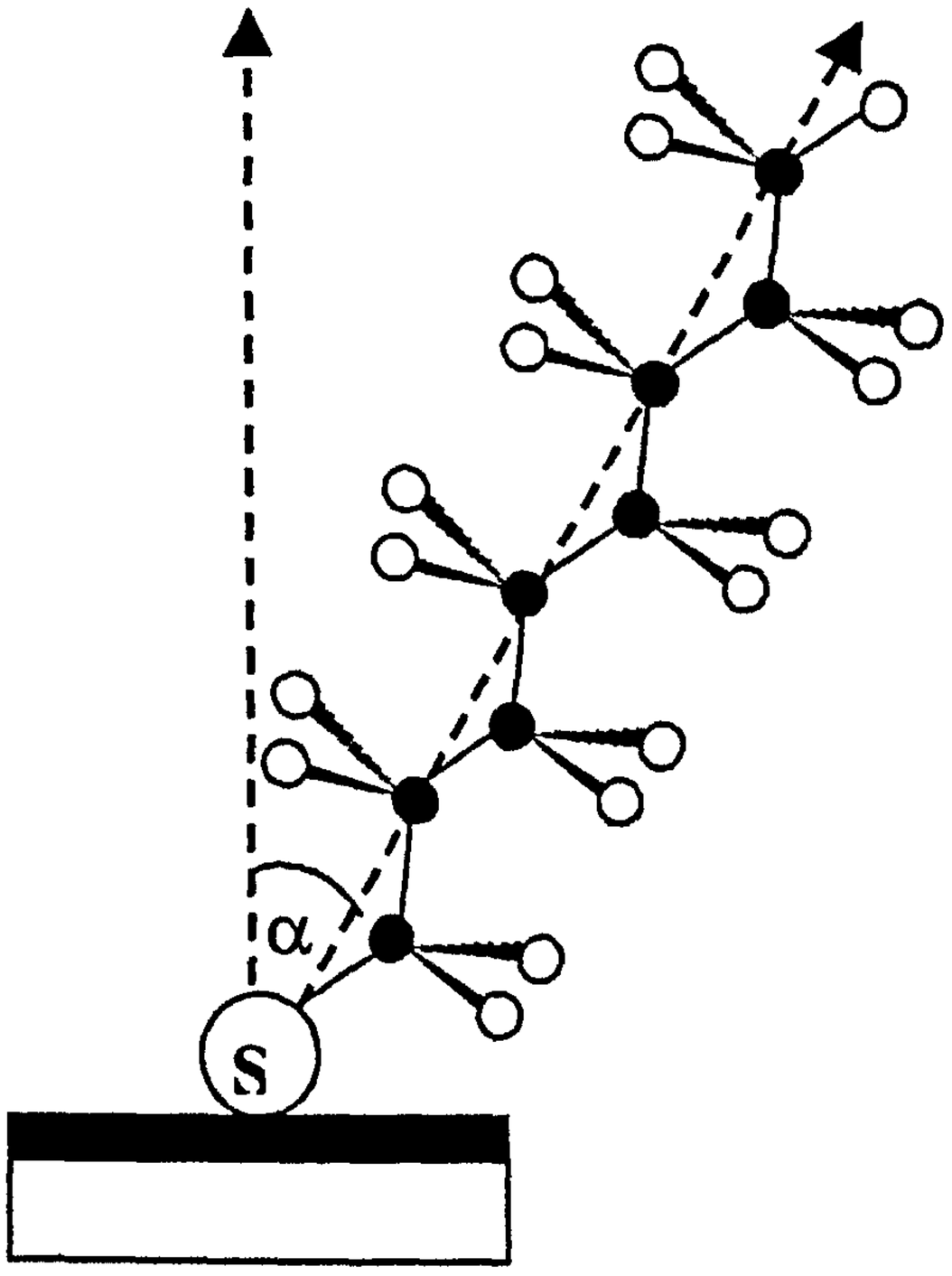

Figure 2.3: Schematic representation of a self-assembled alkanethiol (R-HS) molecule on gold. The tilt of the alkyl chain is at a well defined angle α from the perpendicular with respect to the gold surface (α is approximately 26-28°).

While adsorption is due to the affinity of the head group to the surface, the driving force for organisation originates from the hydrophobic Van der Waals interactions of the long alkyl chains attached to the head group (Mandler and Turyan, 1996). Van der Waals forces orient and stabilise the monolayer. It has been observed that long chain alkanethiols (number of methylene groups n>10) assemble in a crystalline-like way. The key reasons that contribute to the success of the sulphur-containing compounds include amongst others: simplicity and reliability of the method of preparation; flexibility of generating a wide range of surfaces via the incorporation of different groups into the alkyl chain and chain termini of a

SAM; and possibility of applying a wide variety of techniques to the characterisation of the SAM surface (Ferretti *et al.*, 2000).

Sulphur atoms coordinate strongly on a gold substrate. Immobilisation of the sensing layer on the gold chip surface may be accomplished by a number of different approaches. A straightforward method for immobilising SAMs onto solid supports may be provided by physical adsorption (Löfås *et al.*, 1995). However, the limitations of this method make it a non-favourable immobilisation technique. On the other hand, membrane entrapment may preserve the protein structure to a greater extent than the experienced in simple adsorption process, but protein detachment is feasible and probable (Löfås *et al.*, 1995).

An interesting feature of SAM, is the capability of covalent bonding between the molecule to be immobilised and the substrate surface. Various chemical modification procedures permit such coupling by the use of specific molecular tags, containing functional moieties with high affinity for the chosen substrate. Self–assembly of molecules by chemical modification offers a number of advantages, such as reproducibility and stability of the monolayer, controlled density and environment of the immobilised species and reduction of possible random orientations (Ferretti *et al.*, 2000). The strong affinity of sulphur–containing compounds for gold and other noble metals make them an attractive linking structure between the molecule of interest and the substrate surface. Linking of molecules, such as proteins, with sulphur-containing compounds is achieved via functional groups present within the tagging molecule structure able

to react with exposed groups at the surface of the molecule thus providing the immobilisation mechanism.

The formation of SAMs by sulphur-containing compounds is performed either by chemical modification of the molecule to be immobilised or by chemical modification of the substrate where this molecule will be attached. In the first case, the molecule is tagged with the sulphur–containing compound via reaction with specific reactive groups present on the biomolecule surface (in the case of proteins, these could be the ε amino group of exposed lysine residues). The modified molecule is then attached to the metal surface by the sulphur moiety. The substrate modification involves the self assembling of the sulphur-containing compound onto the substrate surface. The chemically modified surface is subsequently linked with the protein biomolecule again via specific moieties present within the biomolecular surface (Ferretti *et al.*, 2000).

Bilayers composed of two separate monolayers have also been documented in the literature. Supported hybrid bilayer membranes composed of a monolayer of alkanethiol and a monolayer of phospholipid attached onto the gold surface, provide a typical example of such structures applied in SPR studies. Plant *et al.*, (1995) demonstrated the development of a lipid hybrid bilayer membrane (HBM) model by firstly coating the gold plasmon resonance surface with a hydrophobic SAM of octadecanethiol, to which phospholipid vesicles containing lipophilic agents of interest were allowed to unfold and fuse. Hydrophobic interactions between the alkyl chains of the alkanethiols and the phospholipid molecules

provide a strong stabilising force in the HBM. The system proved to be reproducible with high specific binding, whereas the presence of the phospholipid vesicles effectively eliminated the non-specific adsorption events, normally observed on the gold surface or the sulphur-containing SAM alone.

Another model of bilayer formation is established by the adsorption of a monolayer of the polypeprtide poly(L-lysine) (PL) onto gold surfaces via formation of multiple ammonium –carboxylate ion pairs via a self-assembled monolayer of the alkanethiol 11-mercaptoundecanoic acid (MUA) (Jordan *et al.*, 1994, Frey *et al.*, 1995). Using chemical modification, a variety of specific adsorption sites (e.g. biotin moieties) can be incorporated into the PL chain. The bilayer was proving to be beneficial in controlling the surface coverage of the specifically adsorbed proteins. The latter was demonstrated by modification of the PL monolayer with varying amounts of biotin that subsequently provided varying numbers of specific adsorption sites for avidin. The PL monolayer was successful in preventing the non-specific adsorption of proteins with relatively high isoelectric points (Frey *et al.*, 1995).

A general method for the immobilisation of proteins for applications on SPR biosensor studies, relies on the use of a carboxy-methyldextran matrix bound to gold surfaces to which recognition bioelements can be attached, thus enabling receptor-ligand or antibody-antigen or DNA-DNA interactions (Figure 2.4). Dextrans are hydrophilic and non-charged natural polymeric carbohydrates which are highly water soluble and form highly hydrated hydrogel compounds. Due to the high concentration of hydroxyl groups in the dextran molecule, chemical

modification is feasible without significantly altering their hydrophilicity. Dextrans possess non-branched polymer chains, which are highly flexible and ligands immobilised in dextran matrices are well accessible (Frazier *et al.*, 2000). The hydrophilic layer of dextran increases the SPR sensitivity and protects the gold surface from non-specific adsorption of proteins, while the reversible binding chemistry of the recognition element to the dextran matrix allows the surface to be regenerated and reused. (Kretschmann, 1968; Liedberg *et al.*, 1993).

A range of synthetic thiolated dextrans of varying molecular weights and degrees of thiol substitution have gained acceptability as well-defined monolayers. For affinity detection, covalently attached dextran layers have become a standard modification for various transducer systems (Löfås *et al.*, 1995; Storri *et al.*, 1998a).

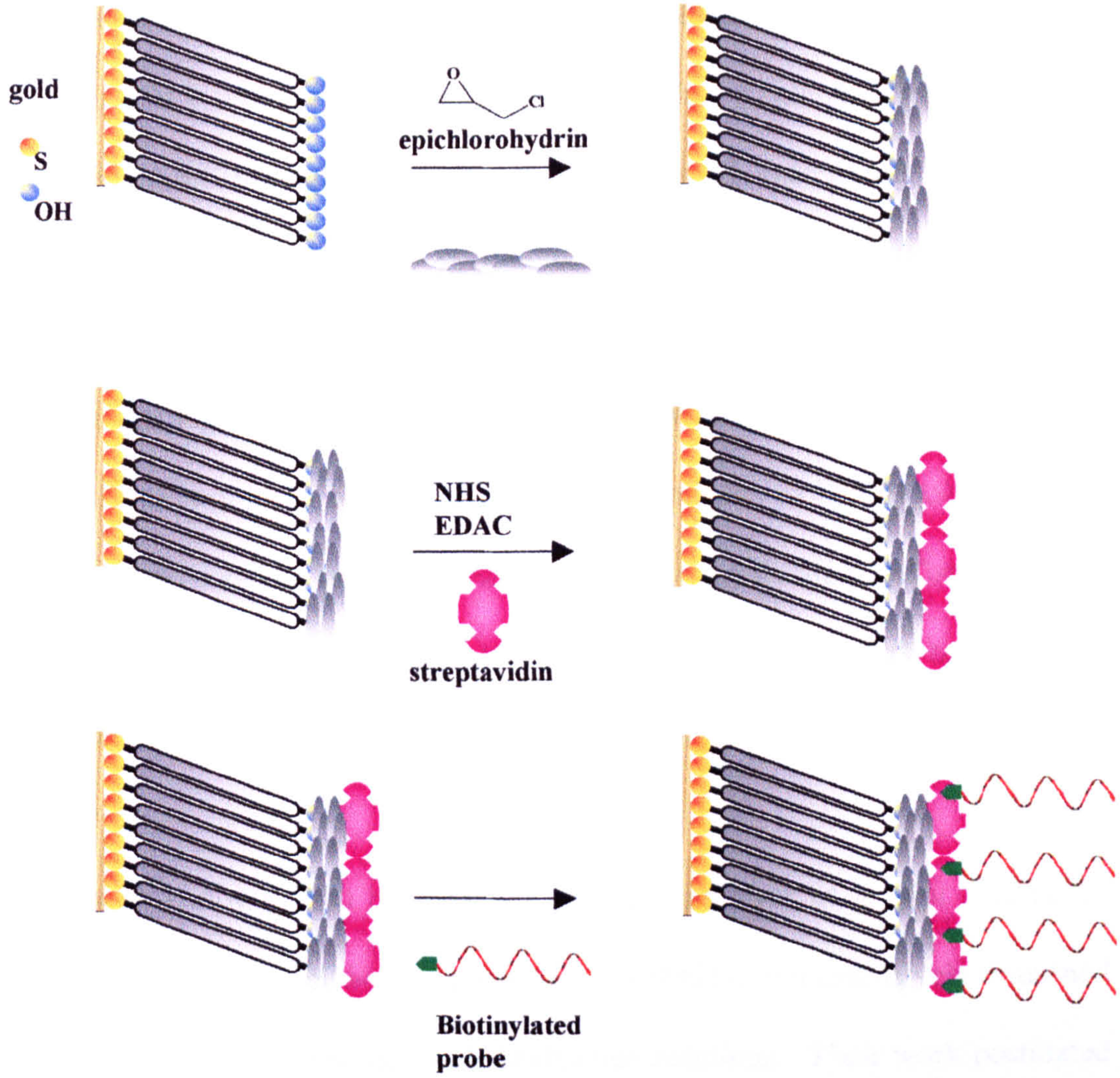

Figure 2.4: Immobilisation of a biotinylated probe on a gold surface modified with thiols and carboxylated dextran. A fraction of the carboxyl groups on the matrix is transformed into reactive N-hydroxysuccinimide (NHS) esters by NHS and N-ethyl-N'-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) treatment. These esters react with streptavidin to form covalent links. Immobilisation of the biotinylated probe is eventually achieved via biotin-streptavidin bonding (Johnsson *et al.*, 1991).

2.6.3 Immobilisation of DNA molecules via self-assembly mechanisms

Self–assembled monolayers of functionalised alkanethiols on gold surfaces have also been applied in the development of DNA sensors. The self–assembly is

governed by the strong interaction and bond formation between the gold and the thiolated DNA molecule. It should be noted that single-stranded DNA will naturally adsorb strongly on a gold surface through interaction with the N-containing bases (purines and pyrimidines). The addition of a thiol moiety however, leads in the re-organisation of the DNA molecule on the gold surface, with the thiol group becoming the primary point of attachment. The covalent immobilisation of DNA onto self-assembled monolayers has been mainly studied and characterised by systems other than SPR transduction. These include between others quartz crystal microbalance and X-ray photoelectron spectroscopy systems.

Herne and Tarlov (1997) developed a mixed monolayer of a thiol derivatised probe and a spacer thiol 6-mercapto-1-hexanol (MCH) and extensively examined the effect of surface coverage on hybridisation reactions. Their work postulated that the thiol-gold interaction results in the adsorption of HS-ssDNA to higher coverages, compared to non-thiolated DNA. The HS-ssDNA monolayer was found not to be a tightly packed monolayer and thus the DNA chains are not mainly oriented perpendicular to the surface. Buffer concentration, in which the probe was suspended, played a critical role in the adsorption of DNA. To minimise intermolecular electrostatic repulsion between neighbouring DNA strands, conditions of high ionic strength were required. The data suggested that maximum surface coverage is obtained in KH_2PO_4 buffer concentrations greater than 0.45 M. It was also proved that dense packing of HS-ssDNA strands inhibits hybridisation with complementary molecules primary due to inaccessibility of

binding sites, but also due to inherent electrostatic inhibition and binding of the similarly charged complementing compounds.

The use of mercaptohexanol (MCH) has proved to be particularly advantageous as it resulted in the removal of the non-specifically adsorbed ssDNA and in the verification of the probe surface to allow less dense packing between the HS-ssDNA molecules (Herne and Tarlov, 1997). The latter inhibited the interaction of nitrogen containing bases with the surface and contributed to a favourable conformation of the HS-ssDNA, thus facilitating successful hybridisation. The length of the MCH molecules (identical to the methylene group spacer in HS-ssDNA) was not of sufficient chain length to interfere with the hybridisation reactions of surface bound DNA (Herne and Tarlov, 1997).

The model of mixed SAMs has been further developed to allow attachment of unmodified double or single-stranded DNA onto gold chips. Two species of thiol, either DNA-terminated or triethylene glycol-terminated, were incorporated into the SAM (Bamdad, 1998). The system was able to detect dsDNA having a single–stranded tail, complementary to the DNA incorporated into the SAM. Further modification involved the enzymatic production of a covalent bond between the 5' end of the incoming dsDNA with the 3' end of the ssDNA immobilised on the SAM. The non covalently bound anti-sense strand can be easily removed by thermal or chemical means, leaving ssDNA presented on the surface for subsequent hybridisation studies.

Covalent immobilisation of double-stranded DNA onto gold surfaces through layer-by-layer self-assembly has been studied with X-ray spectroscopy and by electrochemical methods (Zhao *et al.*, 1999). These methods have involved the formation of the SAMs onto gold electrodes and the subsequent condensation reaction occuring between the 5'-phosphate end or 3'-hydroxy end of the DNA to be immobilised and the terminal group of the SAMs. The influence of different terminal groups of SAMs on the covalent immobilisation of DNA onto the gold surface was investigated, with the candidate terminals of SAM being the hydroxyl, the amino and the carboxyl groups. The hydroxyl terminated SAM, yielded better DNA attachment on the gold surface, through the formation of phosphate ester linkages, with the best being the hydroxyl-terminated SAM.

2.6.4 The biotin–avidin complex

The molecular recognition of biotin by the bacterial protein streptavidin is a well documented example of a high-affinity, protein-ligand interaction. Streptavidin, is a tetrameric protein isolated from the actinobacterium *Streptomyces avidinii*. Streptavidin and the homologous protein avidin are remarkable for their ability to form very strong, non-covalent bonds with biotin. The biotin-streptavidin bond is amongst the strongest noncovalent biosepecific interactions known, and it is kinetically irreversible (Asanov *et al.*, 1998). The high affinity of biotin-streptavidin bonding, results from several factors, including the formation of multiple hydrogen bonds and Van der Waals interactions between the biotin and

the streptavidin entities, and the ordering of surface polypeptide loops that bury the protein interior. Strong binding and high activation energy of dissociation are also produced as a result of the quaternary structural changes of the streptavidin tetramer that take place at the biotin binding site (Weber *et al.*, 1989). Being tetrameric, avidin and steptavidin possess four binding sites per molecule. This is a very useful property that makes possible the formation of mixtures consisting of avidin/streptavidin and polymers bearing several biotinylated moieties (polybiotinylated enzymes). These polymers may still possess some free binding sites for biotin, thus becoming more sensitive detection reagents (Diamandis *et al.*, 1991). Steptavidin–biotin bonding has been extensively used for the immobilisation of DNA molecules on SPR sensors (Bianchi *et al.*, 1997; Sawata *et al.*, 1999; Kai *et al.*, 2000; Mariotti *et al.*, 2002).

A different appoarch on the use of streptavidin on SPR sensors has been reported by Jordan *et al.* (1994). Initially, thiol-terminated ssDNA was attached on a MUA monolayer via a linker molecule (sulfosuccinimidy-l-4-(N-maleimidomethyl) cyclohexane–1-carboxylate) and electrostatically bound poly(L-lysine). Then, biotinylated oligonucleotides were hybridised to the complementary thiol-terminated DNA. This allowed the attachment of streptavidin, which enhanced the SPR signal up to 4-fold. The signal was further amplified by the formation of streptavidin/DNA multilayers. The latter were formed by the subsequent binding of two strands of biotinylated linker oligonucleotides on the streptavidin coated surface followed by a second layer of streptavidin. In this way, up to six layers of streptavidin were formed.

CHAPTER 3

ANALYSIS OF AMPLIFIED NUCLEIC ACIDS BY SURFACE PLASMON RESONANCE: APPLICATION TO GENETICALLY MODIFIED ORGANISM DETECTION

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3.1 Introduction

Since its first publication in 1985 (Saiki *et al.*, 1985) the Polymerase Chain Reaction (PCR) has opened new bio-analytical avenues in forensic, medical, environmental and food sciences. Post-PCR nucleic acid analysis is routinely performed by gel electrophoresis. This method however, fails to provide any sequence information of the amplified DNA. Problems may arise from the amplification of a non-specific sequence having nearly the same length with the desired fragment. Southern blotting satisfies the requirement for sequence information, but it is not recommended for routine analysis, as it involves several steps.

Surface Plasmon Resonance (SPR) detection of PCR products offers a rapid and sequence specific alternative to the usual analytical methods (Bianchi *et al.*, 1997; Nilsson *et al.*, 1997; Kai *et al*, 1999; Sawata *et al.*, 1999; Kai *et al.*, 2000). In the ordinary PCR amplification, the amplified fragments are double-stranded and require denaturation steps of long duration which lack practicality (Sawata *et al.*,

1999). This sample pre-treatment is necessary to obtain single-stranded DNA fragment for the hybridisation with the probe immobilised on the sensor surface.

A previous work (Mariotti *et al.*, 2002) on SPR-based DNA-sensing, revealed that heat denaturation alone (95°C for 5 min and 1 min in ice) of the PCR products is not efficient for strand separation, as it leads to partial re-annealing of the denatured strands. On this basis, more complex sample treatments had to be employed. Magnetic particle separation solved the problem, but it was considered to be an expensive and time-consuming method, unsuitable for routine analysis of a large number of samples.

Several research groups have applied the SPR technology to detect DNA hybridisation with peptide nucleic acid (PNA) (Sawata *et al.*, 1999; Kai *et al.*, 2000; Burgener *et al.*, 2000; Feriotto *et al.*, 2001). Although successful, the system was not cost-effective due to the use of the expensive PNA. Moreover, a heat denaturation step was usually required prior to SPR testing (Sawata *et al.*, 1999; Kai *et al.*, 2000,).

Further development of the detection system employed an optimised PCR procedure that predominantly produces single-stranded DNA fragments (Eggerding *et al.*, 1991; Bianchi *et al.*, 1997, Innis *et al.*, 1998). In the case of Kai *et al.* (1999), two different asymmetrically amplified fragments were produced, differing in length. Following a heat denaturation step, the asymmetrically amplified products were hybridised to produce a unilateral protruding DNA

(UPD) fragment. Despite the sensitivity and reproducibility that the system provides, generation of UPD fragments makes the detection system more complicated. Feriotto *et al.* (2002) applied asymmetric PCR and SPR technology, in particular to the detection of Genetically Modified Organisms (GMOs). The best biosensor performance was achieved upon immobilisation of a PCR biotinylated product on the sensor chip surface and the subsequent hybridisation with its complementary asymmetrically amplified counterpart present in solution.

Moreover, detection of asymmetrically amplified products without a pre-treatment step, has been reported for the diagnosis of human immunodeficiency virus type I (HIV-1) (Bianchi *et al.*, 1997).

Biotechnology is rapidly developing to deliver highly specific, reproducible and cost–effective screening methods for the detection of desired analytes. Moving towards a more straight forward and rapid detection of PCR products, we investigated some amplification and post-amplification strategies for the detection of PCR products based on SPR transduction (BIAcore X™).

The system relies on DNA sensing, based on hybridisation of a DNA 'probe' immobilised on the sensor surface and the complementary DNA sequence ('target') present in solution. A simple DNA sample processing step makes feasible the direct detection of asymmetric PCR products. The objective and novelty of the presented work, lies on the validation of different strategies to obtain sufficient and specific DNA-DNA hybridisation shifts for the detection of specific amplified DNA sequences. Strategies on DNA treatment prior to SPR

testing, included lambda exonuclease digestion of PCR products and optimisation of the denaturation procedure. The different approaches followed, were applied to the analysis of specific DNA sequences characteristic of GMO. These sequences are contained in the promoter region (P35S) of the CAMV (cauliflower mosaic virus) ribosomal RNA.

3.2 Materials and Methods

3.2.1 Apparatus and reagents

For all the experiments the SPR device Biacore X™ and a dextran modified sensor chip (CM5) were used (Biacore AB Uppsala, Sweden). All experiments were conducted at a flow rate of 5 µl min^{-1} and 25 °C.

N-hydroxysuccinimide (NHS), 1-Ethyl-3-(Dimethylaminopropyl) carbodiimide (EDAC) and streptavidin were all purchased from Sigma Aldrich (Milan, Italy). Synthetic oligonucleotides were purchased from Sigma Genosys (Cambridge, UK).

List of buffers and composition:

(TE) Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 8.0);

Immobilization buffer (NaCl 300 mM, Na_2HPO_4 20 mM, EDTA 0.1 mM, pH 7.4);

Acetate buffer (50 mM) for streptavidin suspension;

Hybridisation buffer: (NaCl 150 mM, Na_2HPO_4 20 mM, EDTA 0.1 mM, pH 7.4).

The buffer reagents and ethanol were all of analytical grade and purchased from Merck (Rome, Italy). All the other reagents were purchased from Sigma Aldrich (Milan, Italy).

The sensor chip has two independent flow cells, thus providing the opportunity to immobilise separately two different probe sequences. Probe P35S is complementary to the selected target sequence. A second probe (TNOS) of the same length, non-complementary to the P35S target was used as a control surface, to monitor any possible non-specific binding.

The base sequences of the 5'-biotinylated probes (25-mer) are described below:

Target specific probe (P35S):

5'-biotin-GCCATCGTTGAAGATGCCTCTGCC-3'.

Non-specific probe (TNOS):

5'-biotin-AATGATTAATTGCGGGACTCTAATC-3'.

3.2.2 DNA testing material

25-mer Synthetic Oligonucleotides:

Target sequence (P35S):

5'GGCAGAGGCATCTTCAACGATGGCC 3'

(sequence of interest, complementary to the immobilised probe P35S);

Sequence TNOS specific:

5'TTACTAATTAACGCCCTGAGATTAG 3'

(sequence for negative control on the P35S probe)

Non- specific sequence for P35S and TNOS:

5'GATTAGAGTCCCGCAATTAATCATT 3'

(sequence for negative control on both cells).

25-mer Double-stranded oligonucleotide DNA

A dsDNA helix was constructed by co-incubating equimolar amounts (0.1 μM) of probe and target P35S sequences. Incubation involved 1 hour at 25 °C. The resulted dsDNA oligonucleotide helices were treated as a model type of symmetrically amplified samples (double-stranded PCR products).

DNA samples of higher complexity

A. Commercially available Certified Reference Material (CRM) from soybean powder (2% Roundup Ready™, Fluka, Italy);

B. PBI121 plasmid (13kbp) was purchased from BD Biosciences Clontech (Oxford, UK) and it contains a 814bp fragment of promoter P35S. The plasmid was extracted from genetically modified *E.coli*, previously transformed with the plasmid;

C. P35S containing maize samples from animal feed were provided by an authorised organisation (243 bp and 498 bp).

D. Non- transgenic genomic DNA of *Nicotiana glauca* (0.02 μg μl^{-1}, 0.5 μg per injection) was digested using the enzymes BamHI and Hind III.

E. Non-P35S containing GMO maize event, provided by an authorised organisation.

Following extraction, all the samples A, B, C, and E were PCR amplified and readily provided by the department of Animal Biology and Genetics, University of Florence (Italy). The amplicon of sample B was 243 bp. The amplified sample C was amplified to produce fragments of 243 bp and 498 bp. Sample E was amplified to produce fragments of 540 bp.

The control solution (PCR blank) consisted of all the PCR reagents except the DNA template.

3.2.2.1 DNA extraction and isolation

PBI121 DNA was isolated from *E.coli* cells, using the QIAGEN Plasmid Mini Kit (Qiagen, Milan Italy). Following extraction, the PBI121 DNA samples were suspended in double distilled (dd) H_2O.

Total DNA extraction from axenic plants followed the protocol of Doyle (Doyle *et al.*, 1989), with slight modifications as described in Bogani *et al* (1995).

DNA extraction from the sources A, C, and E followed the instructions of the Nucleospin Plant kit (Macherey-Nagel, Duren, Germany) and the Wizard® Magnetic DNA Purification System for Food (Promega, Milan, Italy). The extracted and purified DNA samples were suspended in the Elution-Buffer provided in the DNA extraction kit.

The concentration of the extracted DNA material was determined by measuring the fluorescence of the Hoechst 33258-DNA complex, according to the method of Labarca and Paigen (1980). Fluorescence was measured by the use of a Hoefer TKO-100 minifluorometer (Amersham- Biotech, Milan, Italy).

3.2.2.2 Symmetric and asymmetric PCR amplification

The functionalised sense (forward) (5'GCTCCTACAAATGCCATCATT 3') and antisense (reverse) (5' CTCCAAATGAAATGAAC 3') primers (MWG-BIOTECH, Florence, Italy) amplified a 243 bp DNA fragment, containing the target sequence P35S (25-mer). Both primers were designed using the OLIGO® Primer Analysis software.

The PCR reaction mixture contained 100-300 ng of isolated DNA from sources A, C and E or 10 ng from B, 2 units of Taq polymerase (Amersham–Biotech, Upsala, Sweden) and 100 mM of each desoxy-ribonucleotide-triphosphate (dNTP) (Amersham–Biotech, Upsala, Sweden).

Symmetric PCR: The 50 µl PCR mixture for symmetric PCR contained equal

amounts of sense and antisense primer solutions (200 nmol). The PCR conditions were: 94 °C for 4 min, 50 °C for 1 min and 72 °C for 2 min (35 cycles).

Asymmetric PCR: The 100 µl PCR mixture contained a sense/antisense ratio of either 1/50 pmol or 2/200 nM. This type of PCR is necessarily performed using one primer in excess. When the limiting primer is depleted, PCR starts to amplify one of the two strands linearly. The PCR conditions were: 94 °C for 4 min, 50 °C for 1 min and 72 °C for 2 min (50 cycles).

All PCR experiments were conducted by a Perkin Elmer Thermal cycler (model 9600) (Perkin Elmer, Shelton, USA).

Following amplification, the PCR products were ethanol precipitated (1 volume of sample per 2.5 volumes of ethanol) and dissolved in sterile ddH_2O or TE buffer. The DNA concentration of the amplified products was determined spectrophotometrically at 260nm. Screening of the PCR products was performed by gel electrophoresis (Campbell, 1996) and visualised through a U.V. transilluminator.

3.2.2.3 Lambda (λ) exonuclease digestion of PCR products

Lambda exonuclease digestion employed a specific set of PCR primers, the one of which was selectively phosphorylated. The Strandase™ kit (Novagen, Madison, USA) is designed for the production of single-stranded PCR products. Lambda exonuclease is an enzyme that preferentially degrades one strand of duplex DNA from a 5' phosphorylated end, releasing 5' phosphomononucleotides (Little *et al.*, 1967).

Only templates that contain the phosphorylated primer are selectively degraded by the enzyme.

3.2.3 Immobilisation of oligonucleotide probes

The presence of a second flow cell on the sensor chip, allows the immobilisation of two different probe sequences. The P35S probe provides the complementary counterpart for the target of interest. The non-specific probe was only used as a control surface. Both probe sequences were immobilised using the same protocol. The sensor chip was docked in the BIAcore instrument and immobilisation buffer was used as the running buffer. The sensor chip is readily modified by the supplier (BIAcore, Uppsala) with carboxylated dextran. For the activation of the carboxylic groups, the sensor surface was treated with a solution of NHS (35 µl of 5.8 mg ml^{-1}) and EDAC (35 µl of 38.5 mg ml^{-1}) in double distilled water (dd)H2O. The dextran sensor chip was further modified with streptavidin (200 µg ml^{-1} in acetate buffer 10 mM, pH: 5.0) (Löfas and Johnsson, 1990). Then, the biotinylated oligonucleotide probe (1 µM in immobilisation buffer) was immobilised, as previously reported (Tombelli *et al.* 2000 a, b).

Considering that 1000 RU correspond to approximately 1ng/mm^2 (BIAcore Manual), the immobilisation of the probe P35S led to approximately 0.63 ng/mm^2 surface coverage. Other authors (Nilsson *et al.*, 1997) also use this estimation for

oligonucleotides, but it should be noted that it has been originally used for proteins.

3.2.4 Denaturation of DNA samples

Prior to SPR testing, the PCR samples were subject to a denaturation step to obtain ssDNA fragments. As a part of our system optimisation strategies, different denaturation methods were employed and their efficacy to obtain adequate amounts of ssDNA for hybridisation was examined.

3.2.4.1 High Temperature denaturation

High temperature denaturation (95°C) was conducted as a reference procedure with or without the addition of formamide (20% v/v). The followed protocol is well documented in many previous studies on DNA-based biosensors (Tombelli *et al.*, 2000a, b; Marrazza *et al.*, 2001; Minunni *et al.*, 2001; Mariotti *et al.*, 2002). It involved a 5 min incubation step at 95°C followed by 1 min in ice.

3.2.4.2 Denaturation in alkaline conditions

The second method employed a combination of alkaline and thermal conditions along with a formamide treatment (Harwood, 1996). Formamide was used in

different concentrations and all in accordance with BIAcore operating instructions (only pulse formamide treatment is allowed by the BIAcore instruction manual). The DNA containing denaturation mixtures were consisted of 0.3 M NaOH, formamide (0%, 10% or 20% v/v) and hybridisation buffer to adjust close to the final volume. The mixture tubes were then incubated at 42 °C for 30 min. Following the incubation step, all reaction tubes were inoculated with highly concentrated HCl solution to obtain a final concentration of 0.3 M HCl and a volume of 60 μl. 25 μl of denaturation mix was then immediately injected in the BIAcore flow system.

3.2.5 Hybridisation with synthetic oligonucleotides

P35S oligonucleotide sequences (25-mer) fully complementary to the immobilised probe, were initially used to characterise the developed biosensor. The effect of denaturation on the hybridisation behaviour of the 25-mer P35S sequences, was investigated by the analysis of denatured (alkaline conditions and 20% formamide v/v) and non -denatured DNA sequences.

Hybridisation reactions of the immobilised P35S probe with the complementary sequence were allowed to proceed by injecting the testing solution in the SPR flow cell. The reaction was monitored for 5 min and the sensor chip was subsequently washed with hybridisation buffer to remove the unbound DNA material. The analytical signal, reported as Resonance Units (RU), was derived

by the difference between the value after the hybridization value and the value recorded before the hybridisation (baseline). Both values were taken when the sensor chip was in contact with the same buffer solution (hybridisation buffer) so that the shift was related only to compounds fixed on the sensor chip during the reaction. Following the completion of hybridisation reaction, any positive RU signal obtained from the control cell was subtracted from the RU shift of the P35S cell.

3.2.6 Regeneration of the probe surface

In all experiments, the single stranded probe was regenerated by an one-minute treatment with 1 mM HCl, which allows the multiuse of the sensor. After each regeneration cycle a successive hybridisation reaction could be monitored. Such treatment could be performed up to 100 times without affecting the hybridisation efficiency of the immobilised probe (Mariotti *et al.*, 2002).

3.3 Results

3.3.1 Baseline stability

The effect of continuous buffer flow conditions on the probe surface may be viewed from the stability of the baseline signal over different days. A stable baseline signal throughout a day of measurements is also indicative of a

successful regeneration procedure, which is applied to the probe surface after a hybridisation reaction.

The stability of the baseline signal recorded at a sensor chip throughout different measurement days and over a number of overnight flow conditions is shown in Figure 3.1.

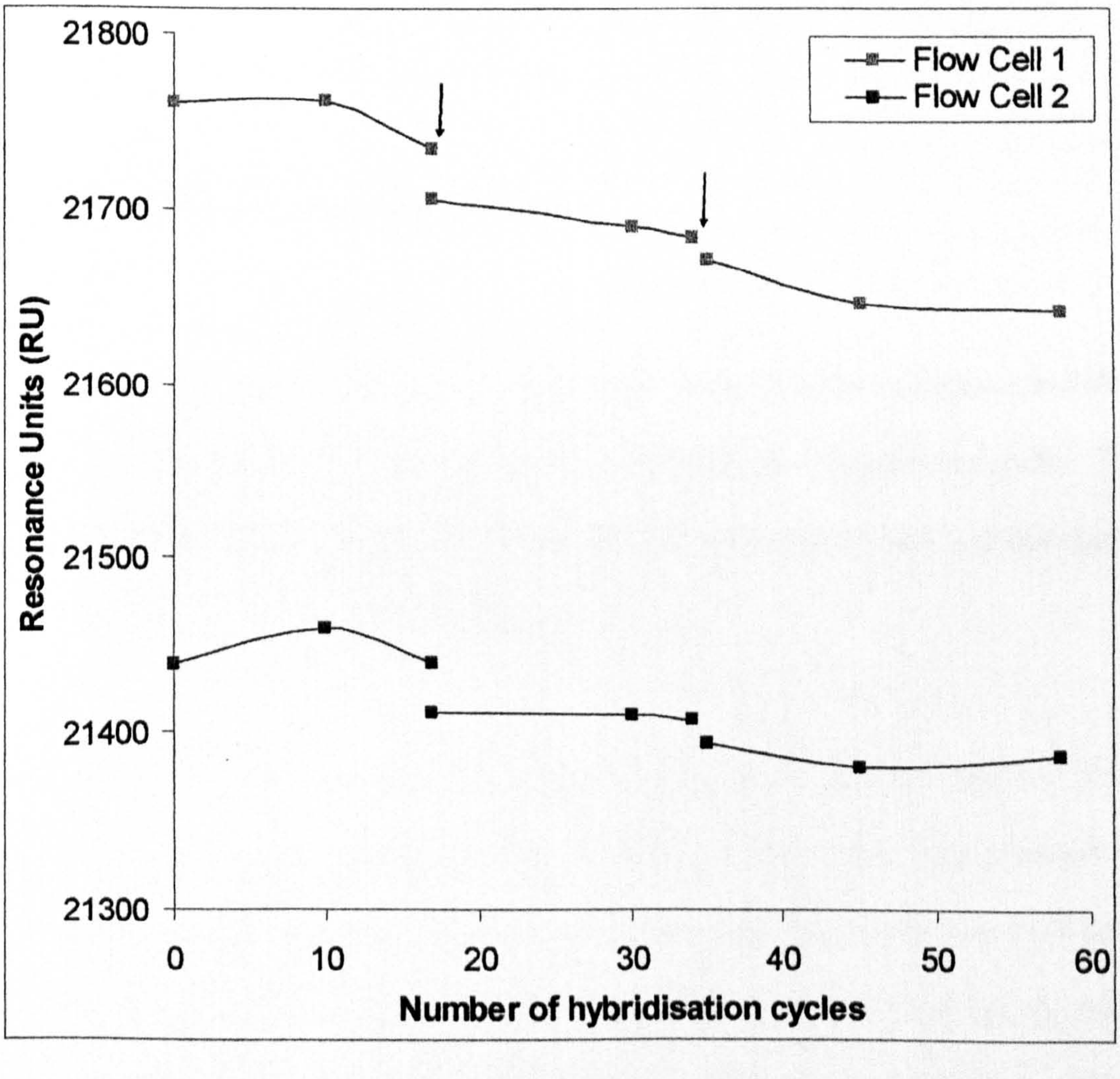

Figure 3.1: The effect of continuous buffer flow conditions and regeneration procedures on the stability of the SPR baseline signal. Baseline signal was recorded after regeneration of the probe surface, when the sensor chip was in contact with the same buffer solution (hybridisation buffer). Data were collected from both flow cells, over a number of overnight flows (indicated by the vertical arrows) and hybridisation cycles.

3.3.2 SPR analysis of DNA samples

The immobilised (25-mer) P35S probe was initially characterised by the use of complementary synthetic oligonucleotides in solution. Following characterisation with the complementary oligonucleotides, DNA samples of higher complexity were analysed.

3.3.2.1 P35S synthetic oligonucleotides

The DNA detection system was optimised using synthetic oligonucleotides, complementary and of the same length (25-mer) with the immobilised probe. The concentration range was between 0 and 150 nM. The curve shows a linear region (0-50nM) followed by a plateau (Figure 3.2).

To check the specificity of the immobilised probes P35S and TNOS, a DNA sequence non–complementary to the immobilised fragments was analysed in concentrations 10 and 100 nM. Traces of hybridisation shifts (<6 RU) were produced in both flow cells (FCs), thus confirming the specificity of the P35S detection system. A target TNOS sequence generated <6 RU in flow cell 2 (FC2) (P35S specific), while an average shift of 452RU (CV= 11 %) was produced in flow cell 1 (FC1) (TNOS specific). Any positive shift produced by P35S target sequence on the control cell (FC1), was subtracted from the shift of FC2. The

analytical signal for the P35S target sequences was produced by the subtraction of the shifts generated in FC2 and FC1, in a manner that $\Delta RU = RU_{FC2} - RU_{FC1}$. Table 3.1 summarises the hybridisation shifts (ΔRU) corresponding to the standard curve, produced by different P35S target concentrations.

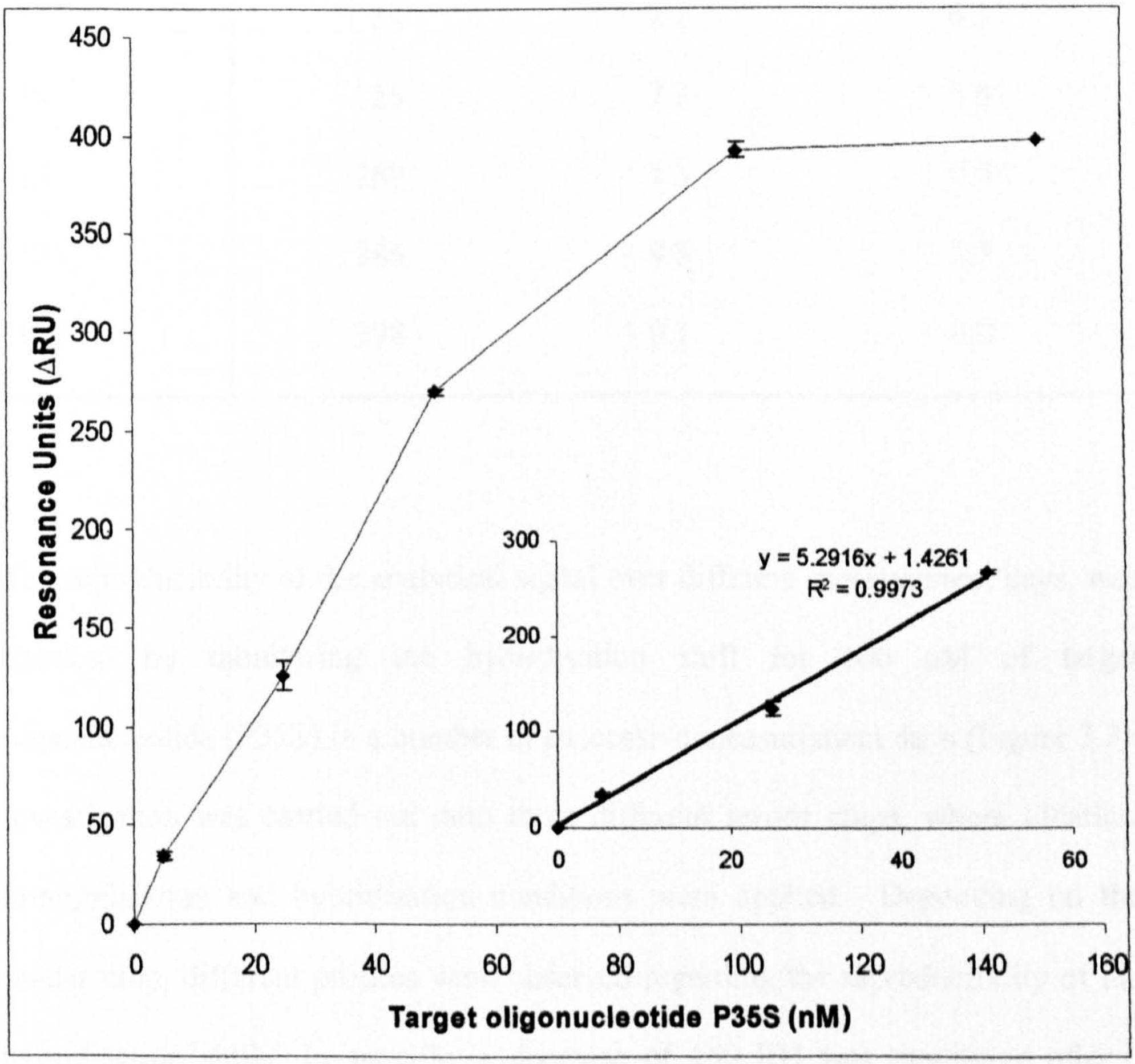

Figure 3.2: Calibration curve for the optical SPR biosensor by the use of synthetic oligonucleotides, complementary to the immobilised P35S sequence. The analytical signal (ΔRU) was produced after subtraction of the observed shifts in flow cell 1 (FC1) and flow cell 2 (FC2), in a way that $\Delta RU = RU_{FC2} - RU_{FC1}$.

Table 3.1: Hybridisation shifts of target P35S oligonucleotides complementary to the immobilised probe. The analytical signal (ΔRU) was produced after subtraction of the observed shifts in flow cell 1 (FC1) and flow cell 2 (FC2), in a way that $\Delta RU = RU_{FC2} - RU_{FC1}$.

P35S target (25-mer) oligonucleotides

P35S (nM)	$Mean\Delta RU_{(FC2-FC1)}$	$Stdev\Delta RU_{(FC2-FC1)}$	CV%
5	34	2.1	6.3
25	125	7.3	5.8
50	269	1.3	0.5
100	386	9.8	2.5
150	398	0.1	0.0

The reproducibility of the analytical signal over different measurement days, was checked by monitoring the hybridisation shift for 100 nM of target oligonucleotide (P35S) in a number of successive measurement days (Figure 3.3). Investigation was carried out onto three different sensor chips, where identical immobilisation and hybridisation conditions were applied. Depending on the sensor chip, different profiles were observed regarding the reproducibility of the hybridisation shift. In specific, a decrease of 150 RU was monitored after 7 overnight flow treatments and 91 measurement cycles in sensor chip 1. Sensor chip 2, encountered a decrease of ~140 RU after 9 overnight flows and 98 measurement cycles. On the other hand, a lower decrease in the hybridisation efficiency was observed in sensor chip 3. After 5 overnight flows and 84

measurement cycles, a maximum decrease of 40 RU was observed in the hybridisation shift for the same target concentration.

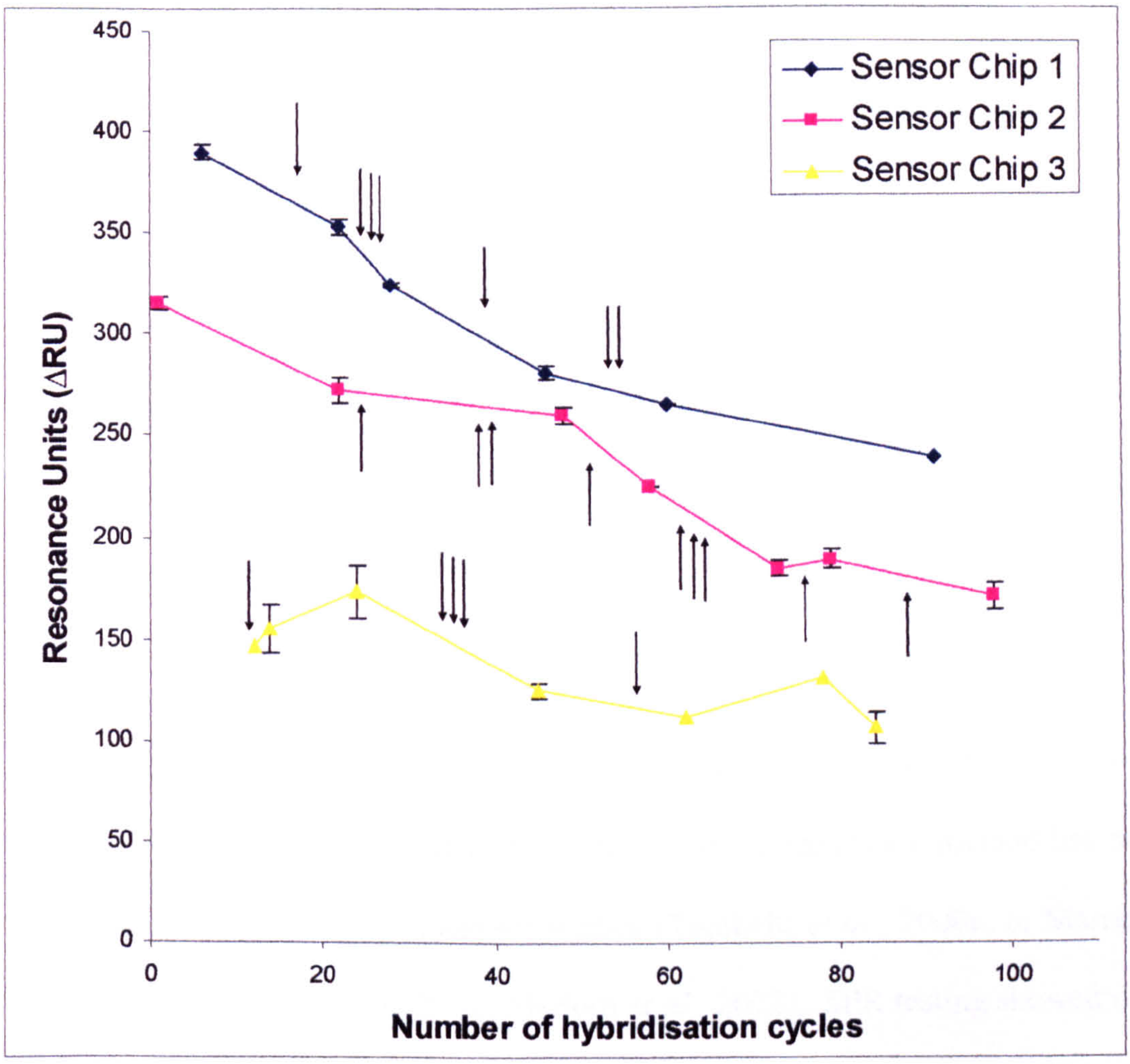

Figure 3.3: The graph shows the reproducibility of the generated analytical signal ($\Delta RU = RU_{FC2}-RU_{FC1}$) for 100 nM of oligonucleotide target P35S, over a number of hybridisation cycles. Investigation was carried out using three different sensor chips (marked by the different colours). Vertical arrows correspond to overnight continuous flow conditions.

Worthnoting, is the inconsistent hybridisation performance between different sensor chips for the same target concentration (Figure 3.3). The average (n= 3) hybridisation shift for 100 nM of target exhibited a CV equal to 38.1 % over the three sensor chips. Considering that the comparative experiment was carried out under identical immobilisation and hybridisation conditions, this difference may

suggest a non–reproducible dextran modification by the supplier (BIAcore, Uppsala).

3.3.3.2 DNA samples of higher complexity

Following sensor characterisation with synthetic 25-mer oligonucleotides, the DNA samples of higher complexity were analysed, in this case PCR amplified samples of 243 bp and 498 bp in length.

PCR symmetrically amplified products were denatured using the high temperature treatment (95°C for 5 min and 1 min in ice). This denaturation method has been well documented in DNA biosensor studies (Tombelli *et al.*, 2000a, b; Marrazza *et al.*, 2001; Minunni *et al.*, 2001; Mariotti *et al.*, 2002). SPR testing showed non-detectable or questionable traces of hybridisation shifts (<10 RU). This was attributed to the re-annealing of the denatured DNA strands before coming in contact with the sensor surface, as also demonstrated by Mariotti *et al.* (2002). Different strategies were then employed to overcome this problem and to obtain greater amounts of the ssDNA target. These are classified in approaches directly made during the amplification step (PCR level) and in improvements of the denaturing conditions prior to SPR testing.

3.3.4 Modifications at the PCR level

These aimed to eliminate one strand of the dsDNA helix prior to SPR testing, so that only the single-stranded DNA that contains our P35S target is available for hybridisation.

3.3.4.1 Lambda (λ) exonuclease digestion

Five different amplified samples were subject to lambda exonuclease digestion and subsequently denatured using high temperature (95 °C) and 20% v/v of formamide. The DNA concentration of the amplified samples varied between 180 nM and 230 nM. SPR testing showed irreproducible and often non-detectable hybridisation shifts (data not shown).

3.3.4.2 Asymmetric PCR amplification

Asymmetrically amplified samples were denatured using high temperature (95 °C) and 20 % of formamide. Subsequent SPR testing monitored non-detectable hybridisation shifts when 2.5 and 5 μM of asymmetrically amplified DNA was tested. The low analytical signals were attributed to the H-bonding in

the intra- and possibly in the inter- strand region of the amplified fragments, due to the lack of adequate and prolonged denaturation. Secondary structures within a ssDNA fragment can be formed in the presence of repetitive sequences.

Figure 3.4, shows a potential configuration of secondary structures in the asymmetrically amplified 243 bp sequence.

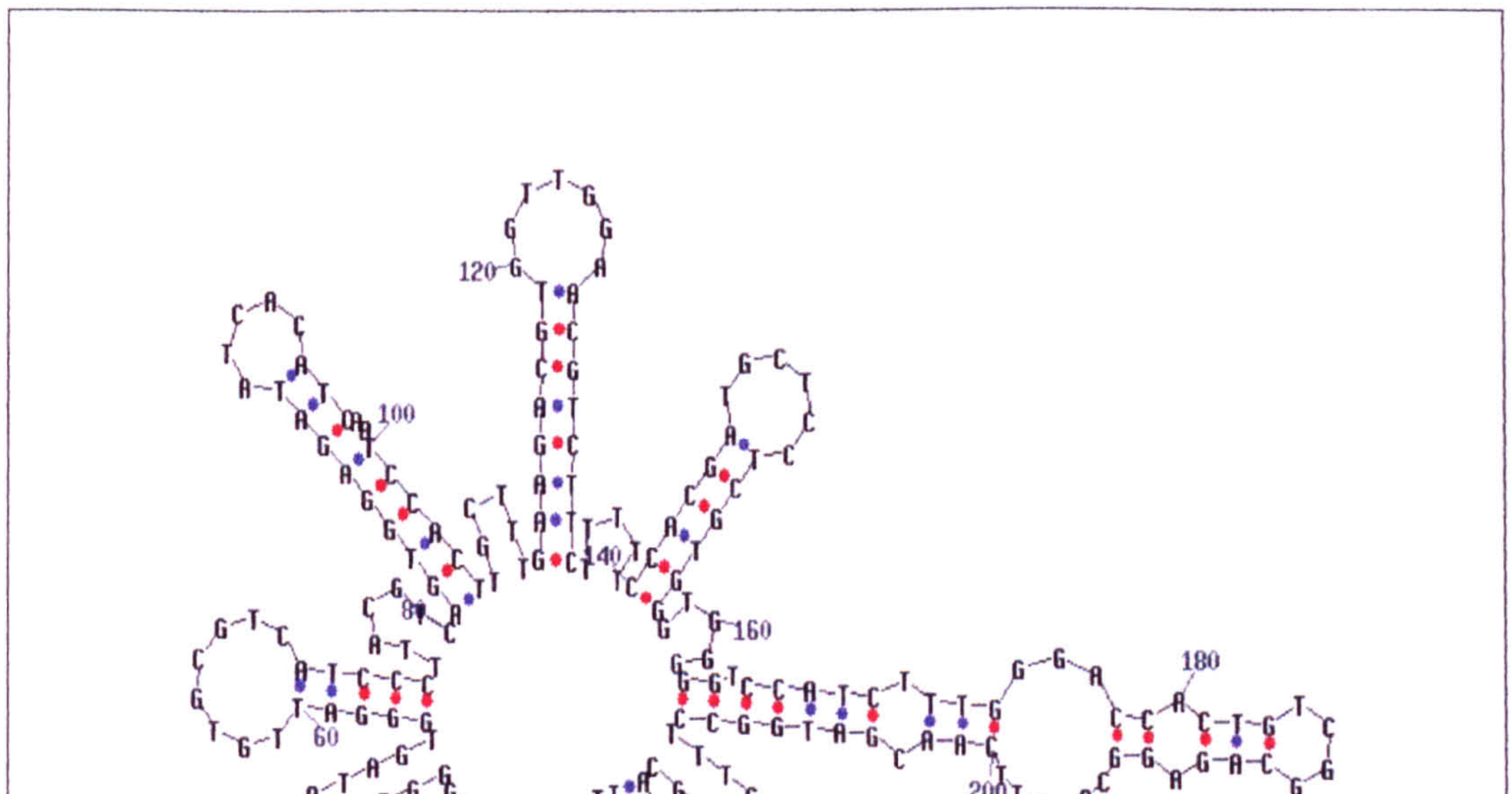

Figure 3.4: One of the potential secondary structures formed by the amplified 243bp PCR product. The 25-mer P35S target is located within the 5' end of the amplified fragment between base numbers 186 and 210. A number of intra-strand hydrogen bonds is evident within the target 25-mer sequence.

Denaturing conditions were optimised by the use of alkaline conditions and an extended duration of formamide treatment prior to SPR testing.

3.3.5 Improvement of denaturing conditions

The denaturation step prior to SPR testing was modified to establish a strong alkaline environment as described in the experimental section. Investigation was first carried out with synthetic oligonucleotide hybrids, which represent a model type of dsDNA.

3.3.5.1 Synthetic oligonucleotide hybrids

The constructed dsDNA hybrids were considered to be a model type of symmetrically amplified DNA and were treated as real samples. Denaturation involved alkaline conditions and different amounts of formamide (0 %, 10 %, and 20 % v/v). In the presence of 20 % v/v formamide, the hybridisation shifts were approximately two times greater with respect to those obtained in the absence of formamide (Table 3.2). Reproducibility was also improved with a CV= 4 %. This confirms the importance of formamide in the denaturing conditions. The optimised alkaline conditions (0.3 M NaOH, 20 % v/v formamide) were compared with the high temperature denaturation method. In an attempt to enhance the denaturing effect of the high temperature method, experiments were performed in the presence and absence of 20 % v/v of formamide. The results clearly suggest the superior efficiency of the optimised alkaline conditions over the high temperature denaturation treatment (Figure 3.5). The addition of

formamide in the high temperature denaturation protocol resulted in insignificant improvement.

Table 3.2: The effect of formamide in the hybridisation process, when alkaline conditions are employed. The dsDNA oligonucleotide molecules were tested in 100nM of concentration.

Ds DNA oligonucleotide hybrid molecules (25-mer)			
Formamide % v/v	Mean$\Delta RU_{(FC2-FC1)}$	Stdev$\Delta RU_{(FC2-FC1)}$	CV%
0	61	23.0	37.9
10	113	7.9	7.0
20	138	6.0	4.3

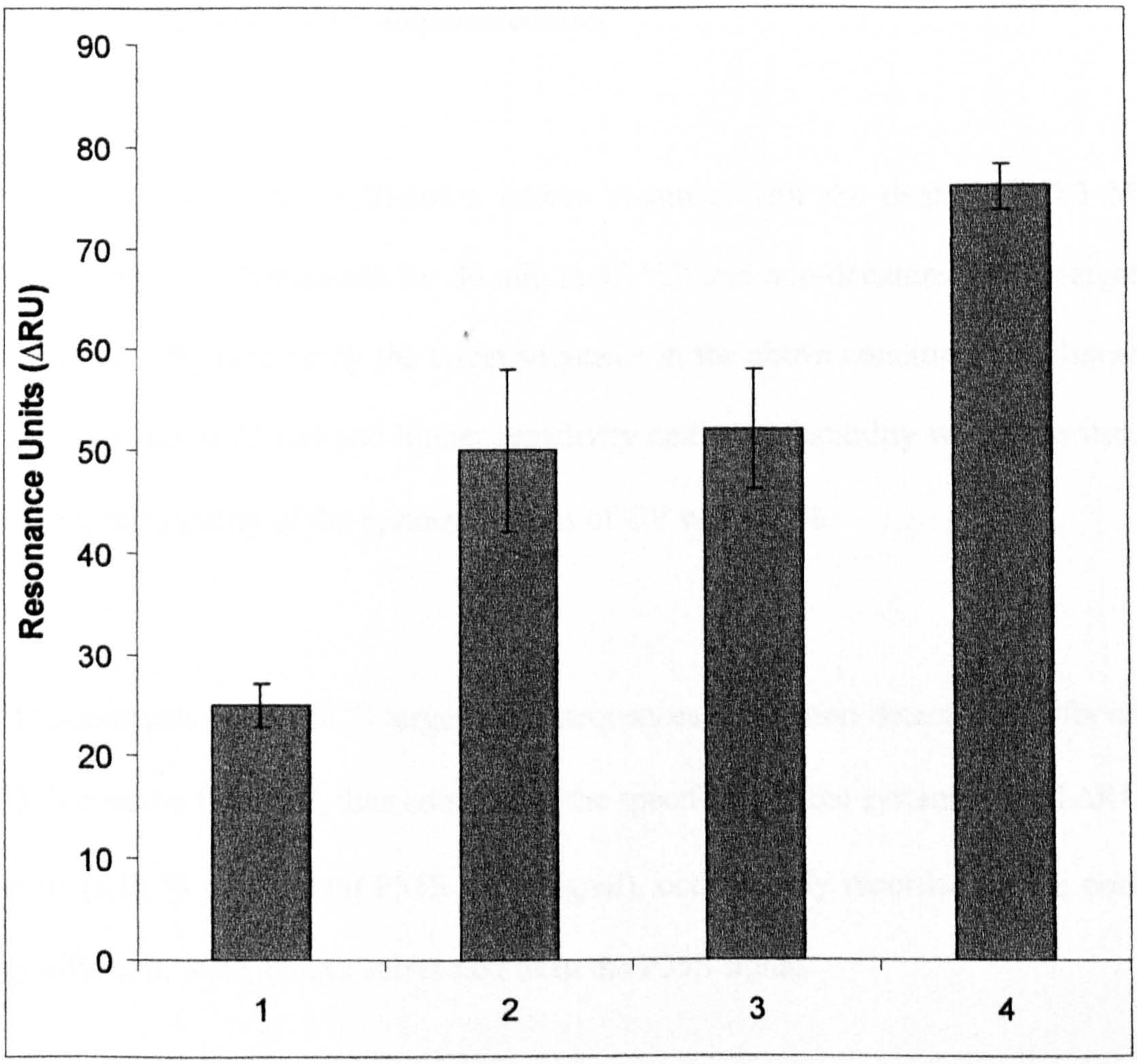

Figure 3.5: Comparison of two denaturation protocols on double-stranded oligonucleotide hybrid molecules (100 nM). *Column* 1: No denaturation CV= 8.6 %; *Column 2*: High temperature denaturation (95 °C for 5 min, cooling in ice) CV= 15.9 %; *Column 3*: High temperature denaturation and 20 % v/v formamide (95°C for 5 min, cooling in ice) CV= 11.5 %; *Column 4*: Alkaline denaturation and 20 % v/v formamide (0.3 M NaOH for 30 min, neutralisation with HCl) CV = 3.1 %;

3.3.5.2 P35S synthetic oligonucleotides

Figure 3.6, shows the calibration curves obtained with the denatured (0.3 M NaOH, 20% v/v formamide for 30 min at 42 °C) and non-denatured P35S target sequences. By denaturing the target sequence in the above conditions, the linear range was up to 25 nM and higher sensitivity and reproducibility were observed. The reproducibility of the system in terms of CV was ≤ 5 %.

Measurements with TNOS target DNA sequences led to non detectable shifts on P35S specific flow cell, thus confirming the specificity of the system. Small ΔRU shifts (≤10 % of the total P35S ΔRU signal), occasionally recorded on the non-specific cell, were always subtracted from the P35S signal.

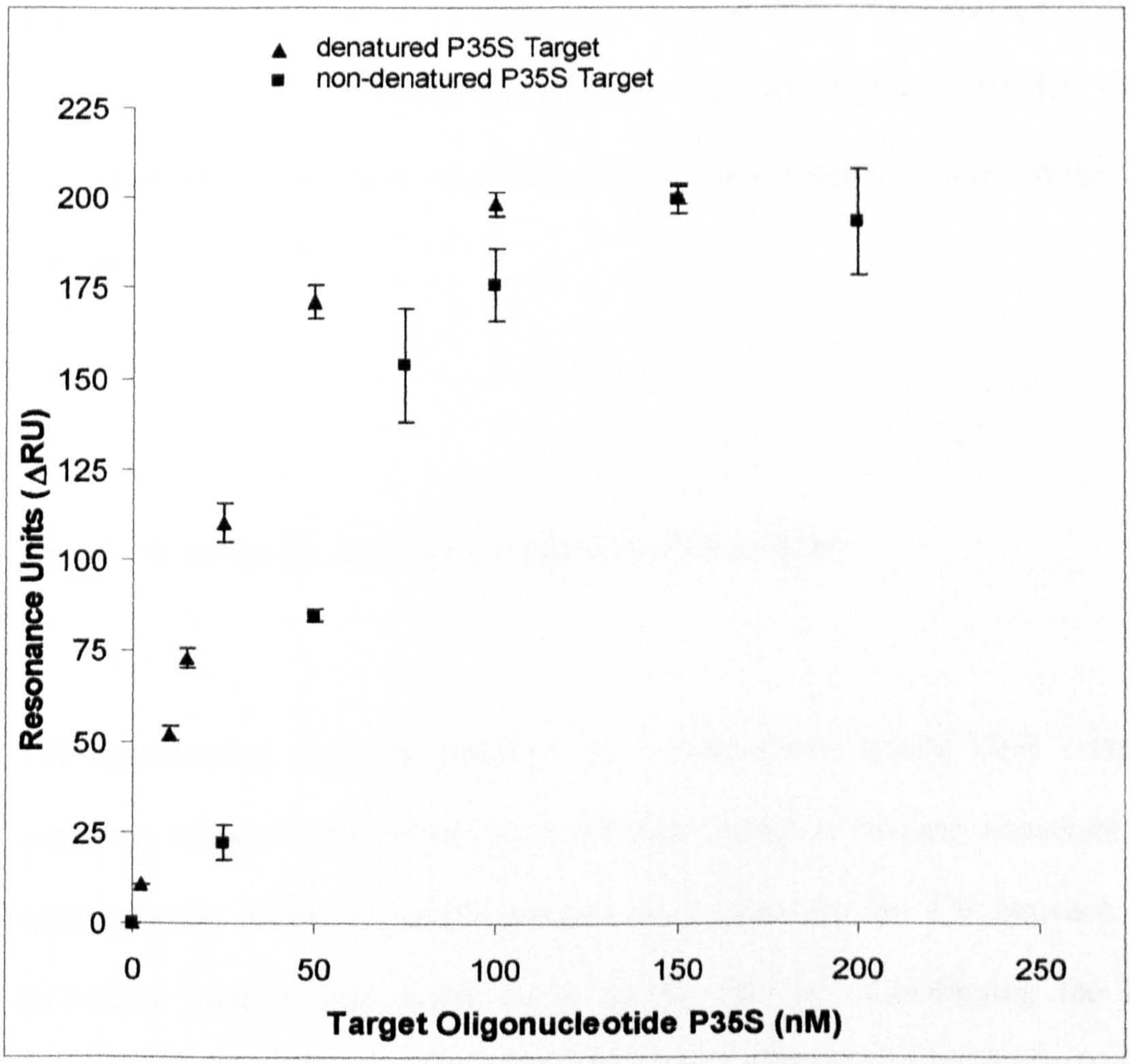

Figure 3.6: Calibration curves for the optical SPR sensor with the immobilised P35S probe sequence. The analytical signal (ΔRU) was produced after subtraction of the observed shifts in flow cell 1 (FC1) and flow cell 2 (FC2), in a way that $\Delta RU = RU_{FC2} - RU_{FC1}$. Denatured (0.3 M NaOH, 20% v/v formamide at 42°C for 30 min) and non-denatured complementary oligonucleotides were tested. Denaturation improved the system sensitivity, reaching a LOD of 2.5 nM.

3.3.5.3 Symmetrically PCR amplified samples

Table 3.3, shows the hybridisation shifts produced from the testing of symmetrically amplified samples using 0, 10 and 20% v/v of formamide. In the

absence of formamide, non-detectable hybridisation shifts were obtained. Using 20 % v/v of formamide positive hybridisation shifts were measured but they were considered to be low and unreliable due to irreproducibility (e.g. sample C (243 bp): CV= 33 %).

3.3.5.4 Lambda (λ) exonuclease digested DNA samples

The hybridisation shifts generated by the λ exonuclease treated DNA samples were very irreproducible. When three different samples of the same concentration were alkaline denatured in the absence of formamide, the CV between the individual samples was found to be up to 132 %. Considering the low reproducibility of the system, no further tests were performed using this particular treatment.

3.3.5.5 Asymmetric PCR amplified samples

The hybridisation shifts obtained from the testing of asymmetrically amplified products are shown in Table 3.3. When 20 % v/v of formamide was used, high reproducibility was detected in both sample B (CV= 5 %, n= 3) and C (CV= 4 %, n= 3), while the high hybridisation shifts ($Mean\Delta RU_B$= 112, $Mean\Delta RU_C$= 66)

indicate the suitability of the technique to identify the presence of GM content. In this case, the system identified the real maize sample (C) as transgenic.

The blank PCR solution was treated under the same conditions as the real samples. No response was obtained in all cases. The cell containing the non-complementary probe, generated a low non-specific response (<10 % of the total P35S specific signal), which was subsequently subtracted from the ΔRU value recorded on P35S cell.

Table 3.3: Hybridisation shifts ($\Delta RU = RU_{FC2} - RU_{FC1}$) obtained from symmetrically and asymmetrically amplified samples. Sample A: CRM 2 %; Sample B: PBI121; Sample C: P35S containing GM Maize. SPR testing was performed following the alkaline-thermal denaturation method combined with different percentages of formamide (0%, 10% and 20% v/v). The samples were diluted in hybridisation buffer up to a final concentration of 0.2 µM (the exception being sample E (0.02µM) and the symmetrically amplified sample A, denatured in 10% v/v formamide, whose concentration was not known)

Sample	% Formamide	ΔRUav	SD (n=3)	CV%
SYMMETRICALLY AMPLIFIED SAMPLES				
PCR blank	0	0		
B	0	0		
C (243bp)	0	0		
PCR blank	10	0		
A	10	27		
PCR blank	20	0		
B	20	14	1	5
C(243bp)	20	11	4	33
ASYMMETRICALLY AMPLIFIED SAMPLES				
PCR blank	0	0		
B	0	0		
C (243bp)	0	0		
PCR blank	10	0		
B	10	20		
PCR blank	20	0		
B	20	112	5	5
C (243bp)	20	66	3	4
E	20	0	0	0

3.3.6 Recovery experiments on the matrix effect

Non-transgenic genomic DNA of *Nicotiana glauca* (0.02 µg μl^{-1}, 0.5 µg per injection) was digested using the enzymes BamHI and Hind III. Different amounts (50 nM and 2nM) of 25-mer ssDNA P35S target were then inoculated into the digested solution of genomic DNA. The hybridisation shifts shown in Figure 3.7 and 3.8 were obtained after denaturing the samples using the optimised technique (alkaline conditions and 20 % v/v formamide), as well as in the absence of denaturation. The hybridisation shifts between the denatured spiked standard solution (i) and the spiked sample of genomic DNA (ii) were comparable, even in low concentrations of P35S 25-mer target. The accuracy between the hybridisation shifts obtained by (i) and (ii) was 0.45 % and –5.7 % in the case of 50 nM and 2 nM spiking, respectively. Non-denatured samples spiked with 2 nM generated very low or not detectable hybridisation shifts and high irreproducibility. This is due to the fact that the concentration of 2 nM P35S target is close to the detection limit of the system.

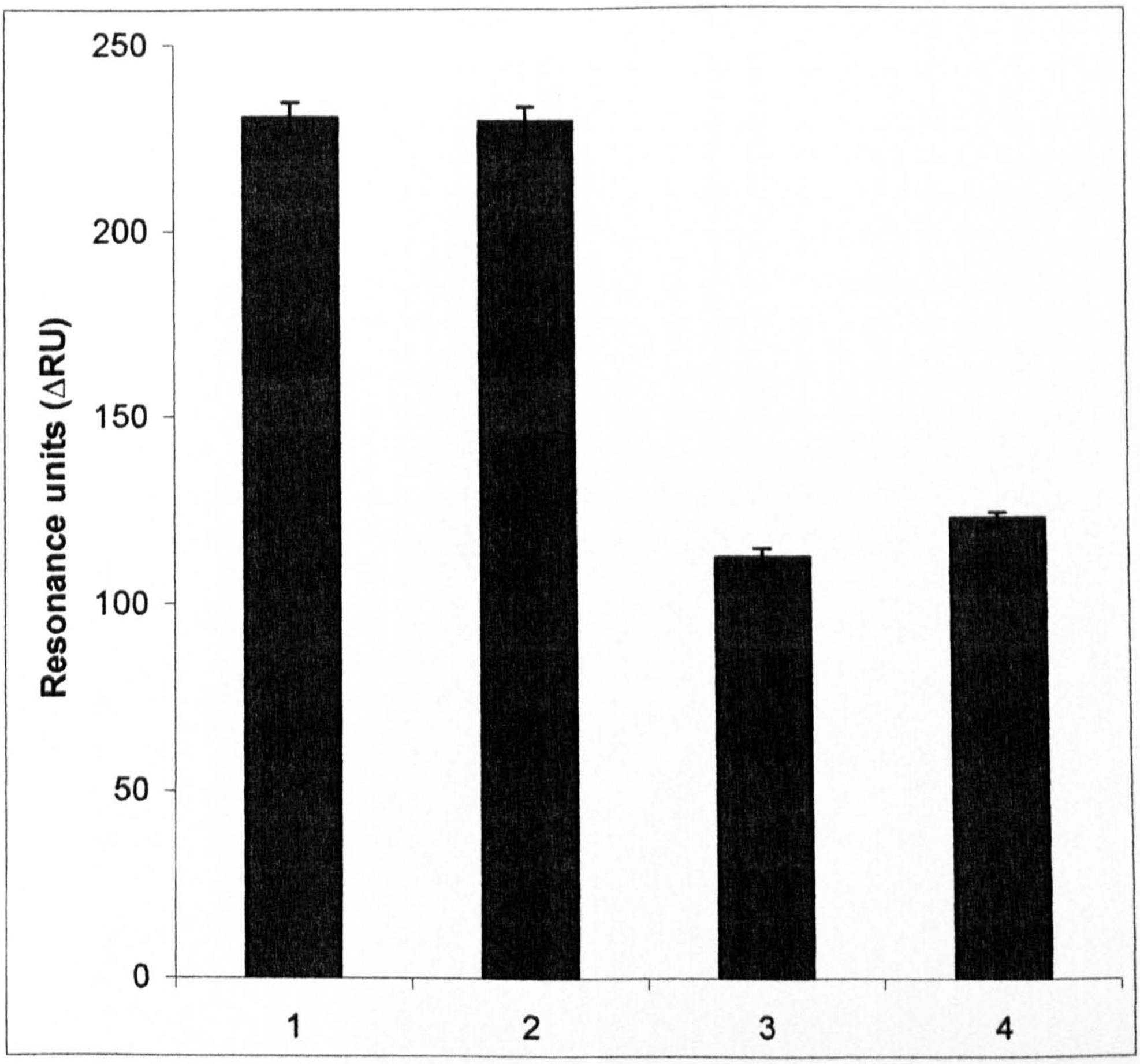

Figure 3.7: The matrix effect of genomic DNA (0.5 µg per injection) on the hybridisation of the P35S target with the immobilised probe.

Column 1: Genomic DNA spiked with 50 nM P35S target, denatured. (n= 3, CV= 1.7 %)

Column 2: 50 nM of P35S target suspended in hybridisation buffer and denatured as real samples (n= 3, CV= 1.7 %)

Column 3: Genomic DNA spiked with 50nM P35S target, non-denatured. (n= 3, CV= 2.0 %)

Column 4: 50nM of P35S target suspended in hybridisation buffer, non-denatured (n= 3, CV= 1.3 %)

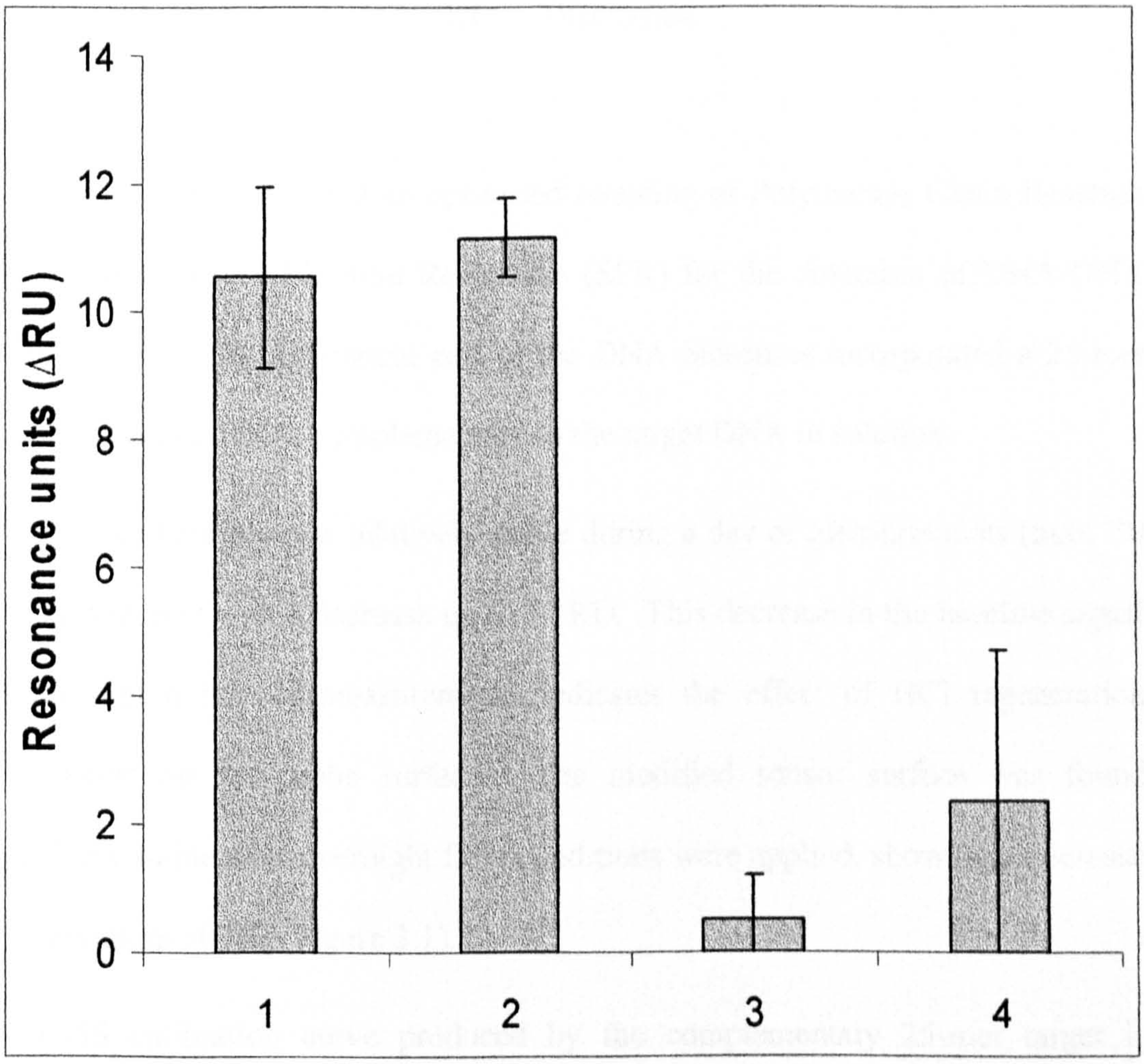

Figure 3.8: The matrix effect of genomic DNA (0.5 µg per injection) on the hybridisation of the P35S target with the immobilised probe.

Column 1: Genomic DNA spiked with 2 nM P35S target, denatured. (n= 3, CV= 13.6 %)

Column 2: 2 nM of P35S target suspended in hybridisation buffer and denatured as real samples (n= 3, CV= 5.4 %)

Column 3: Genomic DNA spiked with 2 nM P35S target, non-denatured. (n= 3, CV= 141 %)

Column 4: 2nM of P35S target suspended in hybridisation buffer, non-denatured (n= 3, CV= 103 %)

3.4 Discussion

This chapter demonstrated an optimised coupling of Polymerase Chain Reaction (PCR) and Surface Plasmon Resonance (SPR) for the detection of DNA-DNA hybridisation. The biological part of the DNA biosensor incorporated a 25-mer oligonucleotide DNA, complementary to the target DNA in solution.

The sensor baseline was relatively stable during a day of measurements (max. 30 measurements) with a decrease up to 30 RU. This decrease in the baseline signal throughout a day of measurements indicates the effect of HCl regeneration treatments on the probe surface. The modified sensor surface was found relatively stable after overnight flow conditions were applied, showing a decrease of maximum 30 RU (Figure 3.1).

A P35S calibration curve produced by the complementary 25-mer target in solution, showed linearity up to 50 nM (Figure 3.2). The empirically established detection limit was 5 nM.

The hybridisation efficiency of the probe surface decreased after successive days of measurement and overnight flows (Figure 3.3). A CV up to 42 % was observed in the hybridisation shift for 100 nM of target after 7 overnight flow treatments and 91 measurement cycles.

Despite the good performance in the detection of single-stranded oligonucleotide target, the biosensor failed to detect PCR amplified samples when heat denaturation (95 °C for 5 min) was applied prior to SPR testing. This was attributed to the re-annealing of the complementary strands before reaching the

sensor surface (Mariotti *et al.*, 2002). The present work evaluated a series of approaches to optimise detection of PCR amplified DNA samples, both at the PCR level and the sample denaturation stage.

At the PCR level, one approach concerned the lambda exonuclease digestion of PCR products by the use of a commercially available kit (Strandase™ -Novagen, Madison, USA). Lambda exonuclease digestion employed a specific set of PCR primers, the one of which was selectively phosphorylated. After the completion of the PCR reaction, the enzyme was used to preferentially degrade one strand of the duplex DNA from a 5' phosphorylated end, releasing 5' phosphomononucleotides (Little *et al.*, 1967). SPR testing of thermally denatured exonuclease-treated PCR products, showed irreproducible and often non-detectable hybridisation shifts.

Another approach at the PCR level involved an asymmetric PCR scheme for the selective amplification of one DNA strand, the one containing the target P35S capable of hybridisation with the immobilised complementary probe. Denatured at high temperature, the asymmetrically amplified PCR products produced non-detectable hybridisation shifts. This was attributed to H-bonding in the intra- and possibly in the inter-strand region of the amplified fragments, due to the lack of adequate and prolonged denaturation. Secondary structures within a ssDNA fragment can be formed in the presence of repetitive sequences, especially in the case of polynucleotide sequences, as the ones analysed in the current experiment (Figure 3.4).

The other strategy focused on the post-PCR amplification stage. The adopted denaturation protocol consisted of strong alkaline conditions (0.3 M NaOH) and 20 % v/v formamide. The effect of formamide was substantial for the efficiency

of the specific denaturation protocol. Experiments with double-stranded oligonucleotides showed that in the presence of 20 % formamide, the hybridisation shifts were more than two times greater with respect to those obtained in the absence of formamide (Table 3.2). Alkaline conditions coupled with 20 % formamide, obtained 1.5 times greater hybridisation shifts with respect to the high temperature denaturation (Figure 3.5). The high temperature treatment was also found inefficient when 20 % formamide was used, resulting again in non–detectable hybridisation shifts. This could be due to the short duration of the formamide treatment. Studies on the thermal denaturation profile of DNA, showed that the denaturation behaviour depends on the duration of formamide treatment (Rauch *et al.*, 2000). Nevertheless, in our case, a longer exposure of the DNA samples to 95 °C would increase the probability of DNA conformational changes.

The improved biosensor performance in the case of denatured ssDNA (25-mer) target (Figure 3.6), was attributed to the elimination of intra-strand H-bonding possible even within the 25-mer target sequence.

The optimised denaturing conditions were applied in both symmetrically and asymmetrically amplified fragments. In the case of symmetrically amplified samples, low analytical signals were detected. When 0.2 μM of symmetrically amplified sample B and C were analysed (n= 3), the generated hybridisation shifts were 14 RU and 11 RU, respectively (Table 3.3). By using the new denaturation protocol, a partial inhibition of the re-annealing process could be observed.

However, the low levels of hybridisation combined with the low reproducibility, put considerable constraints in the potential use of the system as an analytical tool.

Using the alkaline denaturation protocol, λ exonuclease digested PCR products generated irreproducible hybridisation shifts. More specific, when three different samples of the same concentration were alkaline-denatured in the absence of formamide, the CV between the individual samples was found to be up to 132 %. The incapability of the method to produce consistent amounts of ssDNA fragments was attributed to the quality of the kit reagents as provided by the supplier, considering that storage and handling requirements were met in the laboratory.

Asymmetrically amplified PCR products denatured in the optimised alkaline conditions generated reliable and reproducible hybridisation shifts. Asymmetrically amplified sample B generated an average hybridisation shift (n= 3) of 112 RU with a CV= 5%. SPR analysis of 0.2 μM of sample C (243 bp) produced an average (n= 3) hybridisation shift of 66 RU and CV= 4 % (Table 3.3). When a longer amplicon of sample C (498 bp) was analysed, the system was found able to detect the target sequence even at a low PCR product concentration (25 nM). The results suggest the use of a longer amplicon target, when a limited amount of DNA starting material or DNA of poor quality is available. The target of higher molecular weight has a greater effect on the refractive index of the system, thus improving the detection limit.

Recovery experiments with non-transgenic DNA (0.5 μg per injection) of *Nicotiana glauca* clearly suggested the absence of matrix effect with an accuracy of +0.45 % and -5.7 % in the case of 50 nM and 2 nM of P35S spiking (Figures 3.7 and 3.8). Worthnoting is also the absence of diffusion constraints expected to arise from the presence of genomic DNA.

3.5 Conclusions

To obtain single-stranded target DNA available for hybridisation in SPR biosensors, is a difficult task. Even in the case of asymmetrically amplified fragments, the presence of secondary structures puts considerable constrains in hybridisation reactions between the immobilised probe and the target DNA in solution. The presented work evaluated different approaches to obtain single-stranded DNA target sequences prior to SPR testing. Eventually, the system combined an asymmetric PCR amplification system with a simple DNA denaturation step for rapid and efficient detection of DNA hybridisation. Asymmetric PCR amplification led to the selective amplification of the desired DNA strand. The optimised denaturing conditions, eliminated intra-strand complexes within the asymmetrically amplified DNA fragments. When applied to genetically modified organism detection, the system proved to be sensitive, reproducible and rapid, with a strong potential in routine analysis. The system could be used to detect a wide variety of GM crops.

CHAPTER 4

DEVELOPMENT OF AN SPR AFFINITY BIOSENSOR BASED ON A THIOL-FUNCTIONALISED PROBE: APPLICATION TO GENETICALLY MODIFIED ORGANISM ANALYSIS

CHAPTER 4

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4.1 Introduction

The biological component of a biosensor can be immobilised on a solid support in various ways, including adsorption, entrapment, covalent bonding and cross-linking. Desirable characteristics of an immobilisation method are retainmnent of stability and maximum activity of the biological component on the surface of the transducer.

DNA nucleotides can adsorb to gold via multiple moieties, as amines are known to chemisorb weakly to gold surfaces (Xu *et al.*, 1993). At an adsorption state, the hybridisation efficiency of immobilised ssDNA can only reach 36-50% for the first hybridisation cycle (Peterlinz *et al.*, 1997). A model system to study interaction between DNA-functionalised surfaces and free oligonucleotides, consists of thiol-containing probes that are immobilised through self-assembly to gold surfaces. Typically, ssDNA probes are attached to the gold surface through sulphur-gold linkages. Neutron reflectivity studies have indicated that adsorbed layers of HS-ssDNA form compact films, suggesting the presence of multiple contacts between each DNA strand with the surface (Levicky *et al.*, 1998).

The accessibility of the immobilised probe to complementary target in solution and thus the hybridisation efficiency of the probe, can be enhanced by treating the surface with small molecules, acting as blocking agents. MCH (6-mercapto-1-hexanol) effectively displaces adsorbed ssDNA from the gold surface, leaving ssDNA primarily tethered through the thiol end group (Herne and Tarlov, 1997; Levicky *et al.*, 1998). This end-tethered geometry of DNA exhibits nearly 100% hybridisation efficiency (Levicky *et al.*, 1998).

Advantages of the above model include cost efficiency and less time consuming steps for sensor fabrication. Using the above model, preliminary hybridisation studies have been conducted by means of SPR, neutron reflectivity, cyclic voltammetry and chronocoulometry (Levicky *et al.*, 1998; Georgiadis *et al.*, 2000; Steel *et al.*, 2000). Application of the mixed Self-Assembled Monolayers (SAMs) model for diagnostic purposes has been recently reported by Mannelli and co-workers (2002). The QCM study compared the hybridisation efficiency of ssDNA probes immobilised on gold by biotin-streptavidin linkage with the ones immobilised by the HS-ssDNA and blocking thiol procedure. The linear profile in the concentration/frequency curve was found comparable (0 to ~0.1 μM) in both cases. The probe immobilised with the HS-ssDNA and blocking thiol model, successfully detected PCR amplified DNA sequences and it was applied for the detection of GMO food material.

The aim of the current study is to investigate the hybridisation efficiency of the HS-ssDNA (25-mer) and blocking thiol model, by means of BIAcore technology.

The stability of the thiolated probe has been studied under conditions of continuous buffer flow. The hybridisation efficiency has been examined by the use of 25-mer target oligonucleotides, complementary to the immobilised probe. Moreover, the analytical performance of the HS-ssDNA and blocking thiol model has been compared to the thiol-dextran scheme. Finally, the applicability of the specific immobilisation model for the detection of more complex DNA target has been preliminary examined.

4.2 Materials and Methods

4.2.1 Apparatus and reagents

For all the experiments the SPR device BiacoreX™ and a dextran modified sensor chip (CM5) were used (Biacore AB Uppsala, Sweden). All experiments were conducted at a flow rate of 5 µl min^{-1} and 25 °C.

List of buffers and composition:

(TE) Tris-EDTA buffer: 10 mM Tris, 1 mM EDTA, pH 8.0;

Immobilisation buffer : NaCl 300 mM, Na_2HPO_4 20 mM, EDTA 0.1 mM, pH 7.4;

Hybridisation buffer: NaCl 150 mM, Na_2HPO_4 20 mM, EDTA 0.1 mM, pH 7.4, Tween 0.005% v/v (polyoxyethylenesorbitan monolaurate);

Immobilisation solution: KH_2PO_4 1 M, pH 3.8;

The buffer reagents and ethanol were all of analytical grade and purchased from Merck (Rome, Italy). 6-mercapto-1-hexanol (MCH) was purchased from Sigma-Aldrich. All the other reagents were purchased from Sigma Aldrich (Milan, Italy).

Synthetic oligonucleotides (25-mer) functionalised with a group of C_6-SH at the 5'-end were purchased from Sigma Genosys (Cambridge, UK). The base sequences of the 5'-biotinylated probe (25-mer) is described below:

Thiolated Probe (35S):

5' –C_6-SH- GGCCATCGTTGAAGATGCCTCTGCC 3'.

4.2.2 DNA testing material

The DNA testing material consisted of synthetic oligonucleotides 25-mer in length and PCR amplified DNA sequences of GMO origin.

25mer- Synthetic Oligonucleotides:

Target (35S):

5' GGCAGAGGCATCTTCAACGATGGCC 3'

(sequence of interest, complementary to the immobilised 35S probe);

Non-complementary strand:

5' GATTAGAGTCCCGCAATTAATCATT 3'

(sequence for negative control on the 35S- thiolated probe).

A dsDNA helix was also constructed by co-incubating equimolar amounts (0.1 μM) of probe and target 35S sequences (Mariotti *et al.*, 2002). Incubation was conducted for 1 h at 25 °C. The resulted dsDNA oligonucleotide helices were treated as a model type of symmetrically amplified samples.

DNA samples of higher complexity:

- PCR amplified polynucleotide DNA target (243 bp) containing DNA fragment complementary to the immobilised probe. The starting PCR material was maize sample from animal feed. Amplification of the DNA starting material followed an asymmetric scheme, which selectively amplified one DNA strand. That was accomplished by the use of one primer in excess. Once the limiting primer is depleted, the other DNA strand is amplified linearly (Eggerding *et al.*, 1991). Details on the PCR amplification, are described in chapter 3 (paragraph 3.2.2.2).

- Restriction digested DNA material was extracted from a non-transgenic *Nicotiana glauca* plant (0.02 μg μl^{-1}, 0.5 μg per injection).

In addition, a control solution (PCR blank), consisting of all the PCR reagents except the DNA template, was tested.

The samples of higher complexity, along with the PCR blank were kindly provided from Prof. Buiatti (Laboratory of Genetics, University of Florence, Italy).

4.2.3 Denaturation of DNA samples

To facilitate hybridisation with the immobilised probe, the PCR amplified DNA sequences were exposed to a treatment step to obtain ssDNA fragments. The treatment step consisted of denaturing conditions, as detailed in section 3.2.4.2 of Chapter 3. The denaturation step employed a combination of alkaline and thermal conditions along with a formamide treatment (Harwood, 1996). Formamide was used in different concentrations and all in accordance with BIAcore operating instructions. The DNA containing denaturation mixtures, were all of 60 µl final volume and consisted of: 0.3 M NaOH, 10 % or 20 % v/v formamide and hybridisation buffer to adjust close to the final volume. The mixture tubes were then incubated at 42 °C for 30 min. Following the incubation step, all reaction tubes were inoculated with highly concentrated HCl solution to obtain a final concentration of 0.3 M HCl. A 25 µl volume of denaturation mix was then injected in the BIAcore flow system.

4.2.4 Immobilisation of oligonucleotide probes

The immobilisation procedure was performed outside of BIAcore instrument, according to the protocol described in Herne and Tarlov (1997). Initially, the gold sensor surface was removed from the plastic cover of the chip and was cleaned in a boiling solution, consisting of H_2O_2 (30 % v/v), NH_3 (30 % v/v) and milliQ H_2O (mQH_2O) in a 1:1:5 ratios. The gold sensor surface was dipped in the above

solution for 10 min. Thereafter, the sensor surface was thoroughly washed with mQH_2O.

The cleansed gold surface was then modified by the C_6-SH–oligonucleotide probe. To achieve this, the sensor surface was treated with the thiolated probe (1 μM) in immobilisation solution for 2 h. After washing with immobilisation solution, the modified sensor surface was treated, for a further hour, with 1 mM of MCH (blocking thiol) suspended in immobilisation solution. Following a final milliQH_2O washing-step, the gold sensor was mounted in the plastic cover of the chip and docked into the BIAcore instrument, readily available for hybridisation reactions.

4.2.5 Hybridisation with synthetic oligonucleotides/PCR amplified DNA sequences

Hybridisation reactions of the immobilised 35S probe with the complementary sequence were achieved by injecting the testing solution into the SPR flow cell. An injection volume of 25 μl was selected, due to the restricted use of formamide in the BIAcore system. The reaction was monitored for 5 min and subsequently the sensor chip was washed with hybridisation buffer to remove the unbound DNA material. Due to the restricted use of formamide in the BIAcore system, no higher injection volume was permitted (a pulse formamide treatment is allowed, as instructed by the BIAcore user manual). The analytical signal, reported as Resonance Units (RU), is derived by the difference between the value before

(baseline) and after the hybridisation. Both values are taken when the sensor chip is in contact with the same buffer solution (hybridisation buffer), so that the shift is related only to compounds bound to the sensor chip. In all experiments, the single-stranded probe was regenerated by a one-minute treatment with 1 mM HCl, which allows the multi-use of the sensor. After each regeneration cycle a successive hybridisation reaction could be monitored.

4.3 Results

4.3.1 Probe characterisation with 25-mer target DNA

The probe surface was characterised by the use of synthetic oligonucleotides (25-mer) of the same length and complementarity to that of the immobilised DNA molecules.

4.3.1.1 Baseline stability

It is important to examine the stability of the baseline signal, as it is directly related to the stability of the probe surface.

Following the immobilisation procedure, the sensor chips were transferred in the BIAcore instrument, where hybridisation buffer flow conditions (5 $\mu l\ min^{-1}$) were

applied. Running buffer flow over freshly immobilised sensor surface led to a decrease in the observed baseline signal as shown in Figure 4.1.

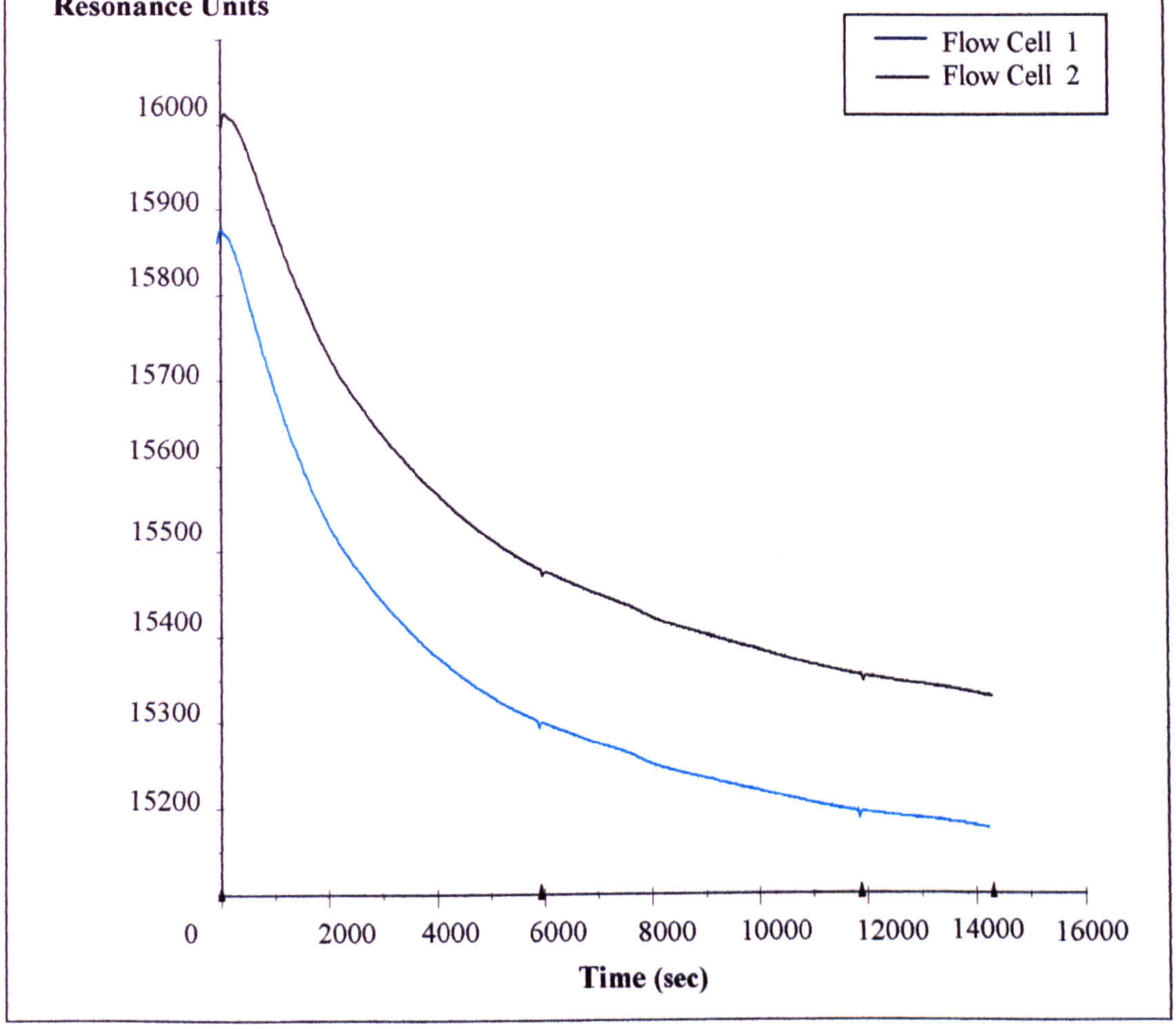

Figure 4.1: Observed decrease in the baseline signal of the freshly immobilised SPR sensor surface, after rinsing with hybridisation buffer. The curves correspond to the baseline signal recorded in flowcells 1 and 2, both immobilised with the thiol-functionalised probe P35S.

Upon achieving an initial stable baseline, the effect of successive hybridisation and regeneration cycles on the probe surface was investigated. It is clear that failure to regenerate the probe surface adequately, would lead to an increase of the baseline signal. This would be due to the retention of target ssDNA molecules in

the form of a double helix with the immobilised complementary strands, which increases the amount of molecules bound to the sensor surface. Moreover, the partial preservation of the double-stranded form makes some of the probe unavailable for subsequent hybridisation reactions.

For the purpose of this investigation, the baseline signal was monitored after hybridisation with 25-mer untreated DNA target. It was observed that 24 successive hybridisation cycles under 5 μl min^{-1} conditions of running buffer flow resulted in an increase of the baseline signal equal to 32 RU in flow cell 1 (FC1), while a decrease of 5 RU was recorded in flow cell 2 (FC2) (Figure 4.2). The effect of overnight continuous flows (5 μl min^{-1}) on the probe surface was also examined by monitoring the difference of the baseline signal before and after overnight flows. After each overnight flow the baseline signal was decreased <90 RU for both flow cells (Figure 4.2).

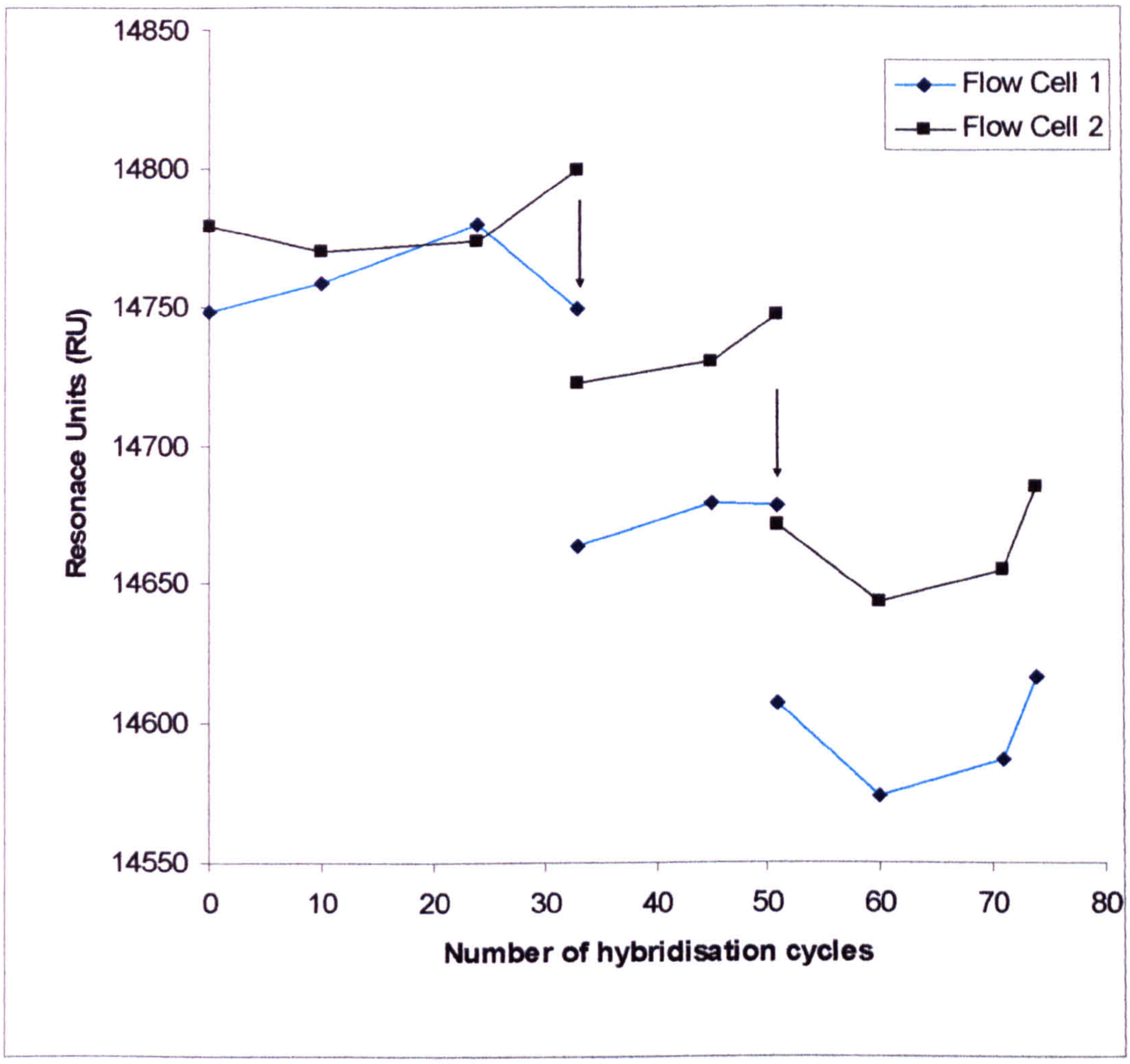

Figure 4.2: The effect of continuous buffer flow conditions and regeneration treatments on the stability of the SPR baseline signal. Baseline signal was recorded on regenerated probe surface, when the sensor chip was in contact with the same buffer solution (hybridisation buffer). Data were collected from flow cells 1 and 2, both immobilised with the thiol-functionalised probe P35S. Overnight flows are symbolised with vertical arrows.

The above results indicate that the stability of the baseline was more vulnerable to overnight flow conditions in comparison to the large number of successive hybridisation/regeneration cycles.

4.3.1.2 Hybridisation capacity for 25-mer target oligonucleotides

Initial hybridisation experiments were conducted without the addition of Tween 20. In the absence of Tween 20, some of the produced hybridisation shift remained on the probe surface after 1 or 2 regeneration cycles. More specifically, hybridisation with 50 nM of oligonucleotide target and subsequent regeneration, increased the baseline signal up to <15 RU (12.35 % of the observed hybridisation shift). With 100 nM of target oligonucleotide, the effect was more pronounced leading to a baseline increase up to 83 RU (35.5 % of the observed hybridisation shift). This is an indication of non-specific binding events, difficult to recover with one regeneration cycle. Accumulation of material bound on the probe surface increases the baseline signal and eventually reduces the hybridisation efficiency of the probe with respect to repeatability. The probe surface recovers after successive regeneration cycles and continuous flow of running buffer.

Improvement of the hybridisation conditions was accomplished by the addition of Tween 20. Tween 20, is a non-ionic surfactant used in hybridisation studies to reduce non-specificity. Spiking of the hybridisation buffer with 0.005 % v/v of Tween 20, effectively eliminated non-specificity, but also reduced the observed hybridisation shift by 23 % in FC1 and 42 % in FC2. Nevertheless, it led to well regenerated surfaces, thus indicating the specificity of the observed hybridisation shift.

Reduction of non-specific binding effects is probably due to the blocking effect of Tween 20. By being non-ionic in nature, no interaction with the charged DNA

molecules is expected. Both running buffer and hybridisation solutions were spiked with 0.005 % v/v of Tween 20. All the presented data was produced using these conditions.

Figure 4.3, shows the calibration curve of P35S target oligonucleotides (25-mer), complementary to the thiolated immobilised probe, using 0.005 % v/v of Tween 20.

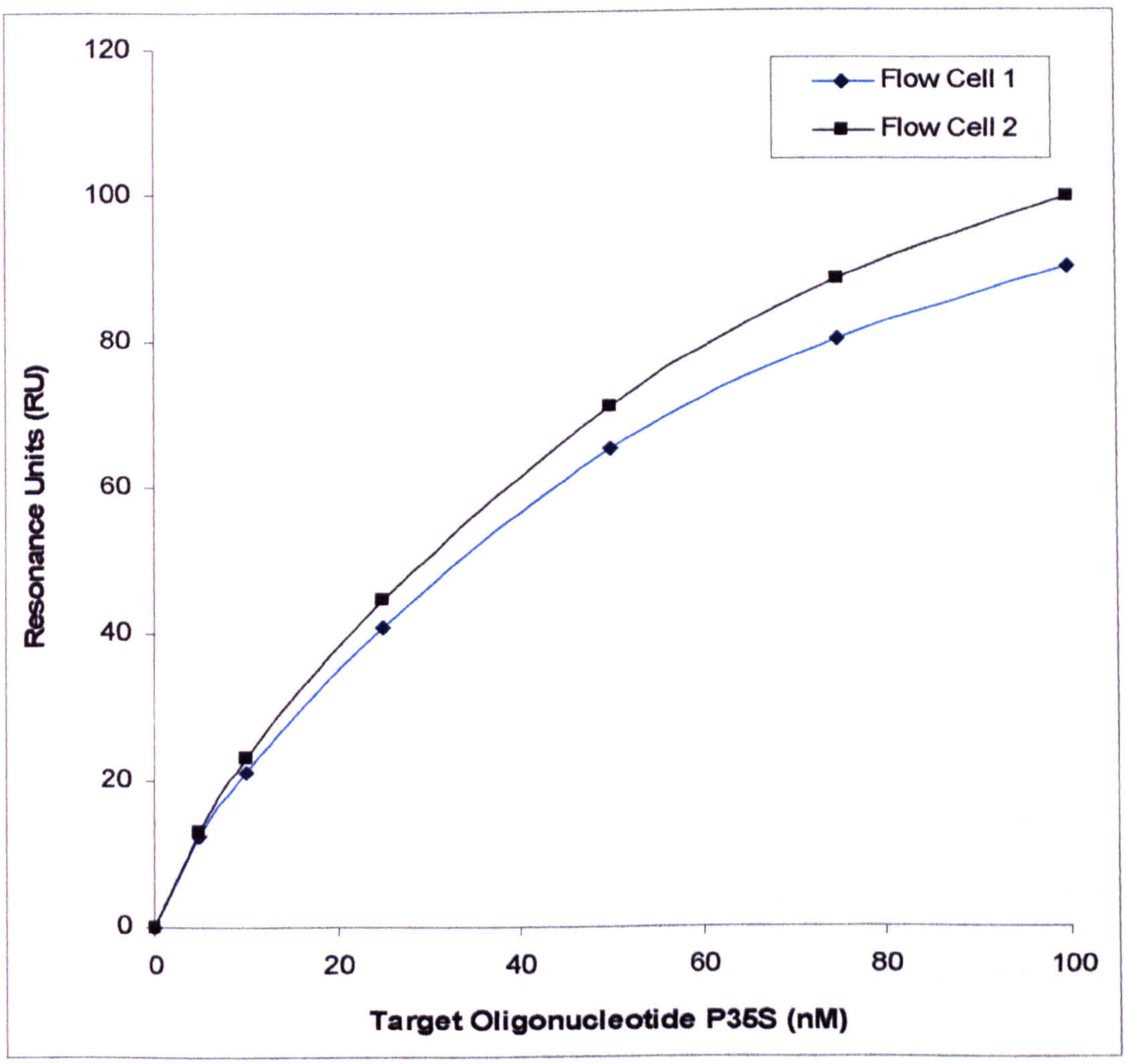

Figure 4.3: Calibration curve for P35S synthetic oligonucleotides (25-mer), using a complementary thiol-functionalised sequence immobilised onto the gold surface of the sensor chip, as a part of a thiolated mixed SAM. Flow cells 1 and 2, are replicates of the same immobilisation treatment.

The precision in terms of CV was lower in concentrations at the lower end of the calibration curve (<11 %). Higher concentrations of target analyte (>25 nM) resulted in improved precision that did not exceed the 2.2 %. Non-target (TNOS) oligonucleotides produced <8 RU, thus confirming the specificity of the probe. Considering the RU shifts generated by non-target sequences, only shifts >10 RU in the case of target P35S were considered as reliable.

Using this criterion, the experimental limit of detection for the 25-mer P35S target sequence, empirically established, was found to be 5 nM (RU_{FC1}=12, RU_{FC2}=13). Comparison of the hybridisation shifts obtained from FC1 and FC2, showed a variation less than 10 %, which suggests a fairly homogenous active probe along the sensor surface (Table 4.1).

To check the reproducibility of the generated response, the hybridisation shifts for 100 nM of target oligonucleotide (P35S) were monitored on each day of measurement over the course of three successive days. The average hybridisation shift (n=3) for 100 nM over 2 overnight continuous flows and 28 hybridisation cycles showed a CV<9 % for both flow cells. This suggests that the hybridisation capacity of the thiolated probe is quite stable and stress-resistant, with regard to the number of hybridisation reactions and continuous flow treatments. The repeatability of the hybridisation shifts (n=3) for 100 nM of untreated target DNA, recorded over a day of measurements did not exceed 5 % in terms of CV.

Table 4.1: Hybridisation shifts obtained from flowcells (FC) 1 and 2. The concentrations in nM refer to target P35S oligonucleotides (25mer), complementary to the immobilised probe sequence. A 25-mer oligonucleotides sequence, non-complementary to the immobilised probe (TNOS) was used as a negative control.

P35S target (25mer) oligonucleotides						
nM	Mean RU_{FC1}	Mean RU_{FC2}	Stdev $_{FC1}$	Stdev $_{FC2}$	$CV\%_{FC1}$	$CV\%_{FC2}$
5	12	13	1.3	0.9	10.6	7.0
10	21	23	2.2	2.5	10.3	10.8
25	41	45	0.9	0.8	2.2	1.7
50	65	71	0.5	0.2	0.8	0.3
75	80	88	0.8	0.1	1.1	0.1
100	90	99	1.0	1.1	1.1	1.1
TNOS (25mer) oligonucleotides (Negative Control)						
100	2	3	2.6	3.9	131	124

Keeping the inlet velocity (5 µl min^{-1}) and concentration constant, the effect of injection volume on assay performance was studied. Variation in the injection volume provides information regarding the diffusivity of the mobile species under the specific conditions of flow. Table 4.2, summarises the hybridisation shifts obtained in FC1 and FC2, using different injection volumes of the target oligonucleotide solution (100 nM P35S).

Table 4.2: Hybridisation shifts for 100nM of oligonucleotide target P35S, using different injection volumes of target solution.

Injection volume (µl)	$MeanRU_{FC1}$	$MeanRU_{FC2}$	$CV\%_{FC1}$	$CV\%_{FC2}$
50	108	118	2.0	1.6
25	93	100	5.2	4.3
15	70	75	1.4	0.9

4.3.1.3 Regeneration of the probe

Each hybridisation cycle was followed by a 1 min regeneration step, which was aimed at removing the H-bonded target molecules and leave the probe free for new hybridisation cycles. The regeneration agents used, were NaOH and HCl in different concentrations. 1 mM HCl or 7.5 mM NaOH were sufficient to regenerate the probe when low concentrations of P35S target (<25 nM) were tested. For higher concentrations of target, 5 mM HCl or 10 mM NaOH were successfully applied.

After 24 hybridisation cycles, the baseline signal was increased by 32RU in FC1 and decreased only by 5 RU in FC2. This decrease can be regarded as relatively small, if the number of hybridisation cycles is considered.

4.3.1.4 Reproducibility of the ΔRU signal over different chips

The hybridisation performance between two sensor chips was compared using 100 nM of target P35S oligonucleotide. Measurements were taken from fresh and stabilised probe surfaces. The average (n=3) hybridisation shift for 100 nM of target, was found to be in the range of 100-140 RU over two individually immobilised sensor chips. Considering that the immobilisation conditions (flow rate, concentration of probe sequences, probe aliquots, temperature, time allowance for immobilisation) were kept constant in both cases, this small difference indicates that the hybridisation capacity of the thiolated mixed SAM varied only slightly in each case.

4.3.1.5 Probe characterisation with pre-treated 25-mer target DNA

To examine if a sample pre-treatment step could increase the hybridisation efficiency of the probe for the target DNA, hybridisation experiments were performed using the sample pre-treatment step optimised with the biotinylated probe (chapter 3, section 3.3.5). The use of a sample pre-treatment step was aimed at the denaturation of polynucleotide amplified sequences prior to SPR testing, in order to facilitate hybridisation with the immobilised probe. The optimised denaturation step employed in chapter 3, consisted of 0.3 M NaOH and 20 % v/v formamide and it was found to be able to eliminate the secondary structures within the target DNA sequence.

To test the hybridisation efficiency of the probe surface with denatured DNA samples and the effect of these conditions on the probe surface, initial experiments were carried out using P35S oligonucleotides of 25-mer. Table 4.3, provides data regarding the hybridisation shifts for 5 nM and 100 nM P35S in denaturing conditions. A comparison with the hybridisation shifts obtained in the absence of denaturing conditions is provided at the same table. Application of the optimised denaturation method improves the detection limit of the system, leading to approximately a four-fold greater hybridisation shift at the 5 nM level.

The results below suggest the importance of a sample pre-treatment step to improve the detection limit. The pre-treated 100 nM target DNA produced an average hybridisation shift of 161 RU in FC1 and 173 RU in FC2, exhibiting a 1.7-fold higher analytical signal to that of untreated target. A 5 nM concentration of non target solution (TNOS) was tested under the same conditions, to provide information regarding the specificity of the RU shifts, generated by target concentrations on the detection limit, as shown in Table 4.3.

Table 4.3: Hybridisation shifts (RU) for P35S oligonucleotides (25-mer), produced in the presence and absence of a sample pre-treatment step. Hybridisation shifts recorded from flowcells 1 and 2. Specificity of the generated hybridisation shifts was confirmed by a non-target 25-mer sequence (TNOS). Mean values (n=3).

Denatured (25mer) P35S oligonucleotides

	$MeanRU_{FC1}$	$MeanRU_{FC2}$	$StevRU_{FC1}$	$StevRU_{FC2}$	$CV\%_{FC1}$	$CV\%_{FC2}$
5 nM P35S	42	49	5.8	0.9	13.7	1.9
100 nM P35S	161	173	22.9	18.6	14.2	10.8
5 nM TNOS	6	11	5.6	5.8	87.4	50.4

Non-denatured (25mer) P35S oligonucleotides

5nM P35S	12	13	1.3	0.9	10.6	7.0
100nM P35S	90	99	1.0	1.1	1.1	1.1
5nM TNOS	1	1	2.3	2.1	>100	>100

When the denaturing treatment was employed, the generated hybridisation shifts were described as less repeatable in comparison to the ones produced by untreated

samples (Table 4.3). In addition, difficulties were encountered in regenerating the probe surface after hybridisation with pre-treated samples. Up to 10 % of the observed hybridisation shift for a 100 nM concentration of target sequence remained on the probe surface after a regeneration cycle. For 5 nM of target, <34 % of the observed hybridisation shift remained on the probe surface after a regeneration cycle. Complete regeneration of the probe was usually accomplished by successive washing steps (1000 µl min^{-1}) or overnight buffer flow treatment.

Injection of 5, 10 and 20 % v/v of formamide alone in hybridisation solution did not produce any RU shift. However, injection of the denaturing reaction mixture in the absence of target sequence led to a 39 RU shift, which proved resistant to removal by regeneration cycles.

Further investigations on the effect of formamide were carried out on a newly immobilised probe surface. Smaller amounts of formamide (5 and 10% v/v) in the reaction mixture, generally led to more easily regenerated surfaces, while repeatability of the observed hybridisation shift was not improved.

4.3.1.6 Sensor lifetime

One criterion for defining sensor lifetime was the number of hybridisation cycles and overnight washes that led to specific hybridisation events with untreated oligonucleotide solutions. Detachment of the mixed SAM from the sensor surface, as a result of the continuous flow conditions, would be expected to result

in patches of bare gold. These sites could be vulnerable to adsorption of DNA that would be more difficult to recover under the regenerating conditions used. Moreover, probe detachment could eventually decrease the hybridisation capacity of the probe. Considering the non-specific events associated with the use of formamide, the regenerating behaviour of the probe surface was monitored after hybridisation with untreated oligonucleotide solutions. After a period of 4 overnight washes and 74 hybridisation cycles, at least 8 % of the generated hybridisation shift for 100 nM of target was not removed from the probe surface by the use of a 5 mM HCl regenerating solution. It is postulated that the 8 % of RU shift corresponds to DNA material adsorbed on the gold surface.

The hybridisation capacity of the probe is also indicative of sensor lifetime. The study was conducted on a single sensor chip, by monitoring the sensor response to 100 nM of target DNA, over 3 overnight washes and 50 hybridisation cycles. The hybridisation shift for 100 nM of target was reduced from 90 RU to 74 RU in FC1 (18% decrease) and from 99 RU to 77 RU (22 % decrease) in FC2.

4.3.2 Matrix effect of genomic DNA

To investigate whether the presence of genomic DNA could have an effect on the hybridisation efficiency of the probe for a known target concentration, non-transgenic genomic DNA was inoculated with 50 nM of target DNA. The non-transgenic genomic DNA was extracted from *Nicotiana glauca* (0.02 $\mu g\ \mu l^{-1}$, 0.5

μg per injection) and was digested using the enzymes BamHI and Hind III. A final concentration of 50 nM of P35S target was then inoculated into a digested solution. Due to the non-specific events generated by the use of 20 % v/v formamide, the use of formamide was reduced to 10 %. The hybridisation shifts shown in Figure 4.4, were obtained after denaturing the samples using 0.3 M NaOH and 10 % v/v formamide, as well as in the absence of denaturation. In the absence of denaturing conditions, comparable hybridisation shifts were obtained using 25-mer target in hybridisation solution and in genomic DNA. Intra-strand interactions between the 25-mer target and the digested genomic DNA were, in principle, not expected due to the double stranded nature of the genomic DNA. Interestingly however, the reaction under the current conditions (hybridisation time, flow rate etc) was found to be independent on the presence of genomic DNA, that could cause diffusion constrains.

When denaturing conditions were applied (10% v/v formamide, 0.3 M NaOH), the system generated slightly higher hybridisation response in the presence of the genomic matrix (<17 RU). The small RU difference observed between the spiked genomic sample and the standard target solution was attributed to sample preparation.

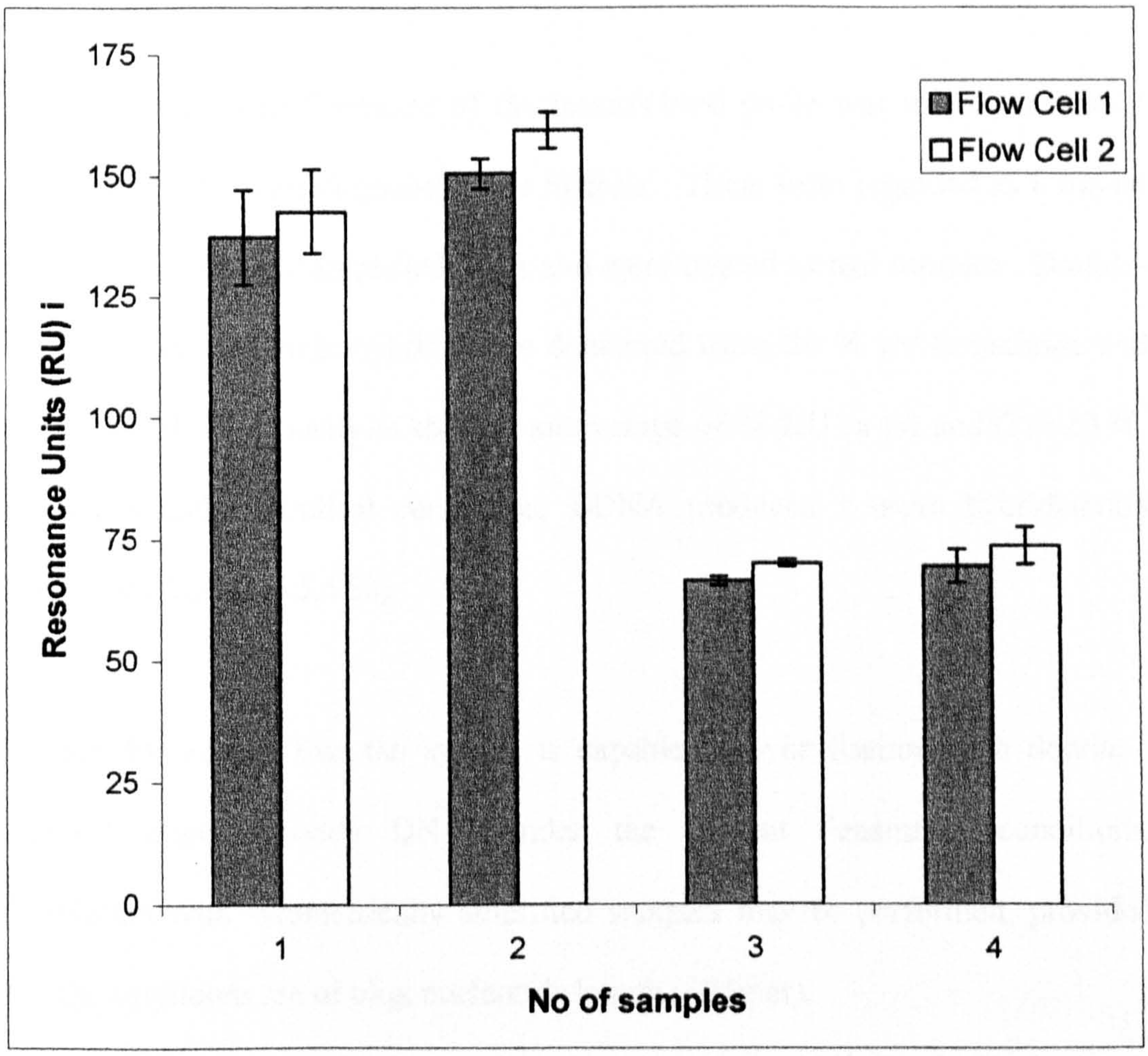

Figure 4.4: The matrix effect of genomic DNA (0.02 μg μl^{-1}, 0.5 μg per injection) on the hybridisation of the P35S target with the immobilised probe.
Sample 1: 50 nM target P35S in hybridisation buffer (NaCl 300 mM, Na_2PO_4 20 mM, EDTA 0.1 M, pH:7.4). Denatured in 0.3 M NaOH and 10 % v/v formamide
Sample 2: 50 nM target P35S in genomic DNA of *Nicotiana glauca*. Denatured in 0.3 M NaOH and 10 % v/v formamide.
Sample 3: 50 nM target P35S in hybridisation buffer (NaCl 300 mM, Na_2PO_4 20 mM, EDTA 0.1 M, pH: 7.4). No denaturation was applied.
Sample 4: 50 nM target P35S in genomic DNA of *Nicotiana glauca*. No denaturation was applied.

4.3.3 Double-stranded oligonucleotide DNA

The hybridisation performance of the immobilised probe was initially assessed using double stranded oligonucleotide hybrids. These were regarded as a model type of symmetrically amplified DNA and were treated as real samples. Double-stranded oligonucleotide hybrids were denatured using 20 % v/v formamide and 0.3 M NaOH. SPR analysis showed an average of 58 RU (n=3) and CV<20 %. Denatured under identical conditions, ssDNA produced a mean hybridisation shifts of 96 RU (CV<5.5 %).

The results suggest that the system is capable of hybridisation with double - stranded oligonucleotide DNA, under the present denaturing conditions. Correlation with symmetrically amplified samples may be performed, provided that the amplicons are of oligonucleotide length (~25mer).

4.3.4 SPR analysis of asymmetrically PCR amplified DNA

Asymmetrically amplified SF210 maize (200 nM, 243bp) was denatured using 10 % v/v formamide and 0.3 M NaOH. SPR testing showed an average hybridisation shift of 24 RU (n=3) in both flow cells. Reproducibility in terms of CV was found to be <10 %. Considering that the present denaturing conditions could give rise to non-specific binding effects, this hybridisation shift was considered too

small to support the argument that the system is able to specifically hybridise with PCR amplified sequences (243 bp).

Higher amount of formamide (20 % v/v) resulted in slightly higher resonance shifts (31 RU), thus indicating the low capacity of the system to generate high hybridisation signals regardless of the formamide concentration. SPR testing with non-denatured asymmetrically amplified samples resulted in non-detectable hybridisation shifts.

4.3.5 Comparison of the thiol-functionalised DNA and blocking thiol model with the scheme based on biotin-streptavidin linkage on dextran matrix.

The HS –ssDNA and blocking thiol model was compared with the well optimised on biotin-streptavidin based immobilisation scheme. The immobilisation procedure of the biotin-streptavidin scheme was based on the protocol of Löfas and Johnsson, (1990), as previously reported (Tombelli *et al.*, 2000 a, b; Mariotti *et al.*, 2002).

Calibration curves (RU response *vs* target concentration) produced by both immobilisation schemes are shown in Figure 4.5.

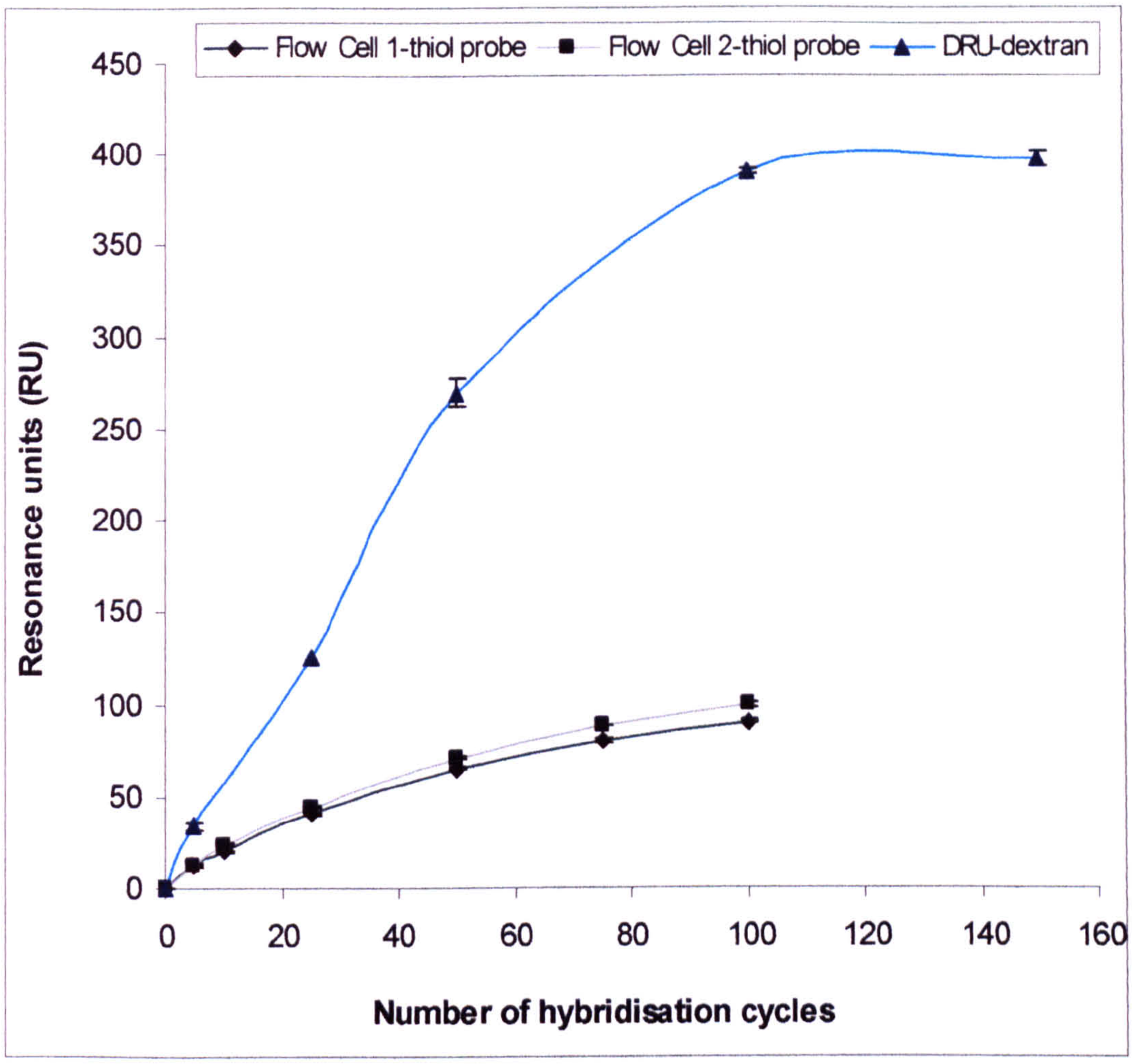

Figure 4.5: Comparison of response calibration curves generated by identical probe molecules, attached on the gold SPR sensor by two alternative immobilisation schemes. The triangular data points correspond to the calibration curve produced by the scheme based on biotin-streptavidin linkage on dextran matrix ($\Delta RU_{(FC2\text{-}FC1)}$). The square and diamond data points relate to the calibration curves produced by the HS–ssDNA and blocking thiol model (flowcells 1 and 2).

The reproducibility of the generated resonance shifts between the different sensor chips was studied by both immobilisation schemes. Investigations were performed on two sensor chips for each immobilisation method studied. As previously described in section 4.3.1.4, hybridisation reactions with the HS–ssDNA probe system in two sensor chips, generated shifts in the range of 100-140 RU for 100 nM of target P35S. It is worth noting, that two different experiments

with the dextran-biotin-streptavidin method, when conducted under the same immobilisation and hybridisation conditions, produced hybridisation shifts in the range of 150-390RU for 100nM of target P35S.

Comparison of the generated shifts obtained for the reference sample analysis (100 nM of untreated target P35S) on different days, provided an indication of probe stability and sensor lifetime. Figure 4.6, shows a comparison of the two immobilisation schemes in terms of reproducibility of hybridisation shifts between different days of measurement. After 28 hybridisation cycles and 1 overnight wash, the hybridisation shift produced by the biotinylated probe was reduced by 17 %. Nevertheless, using the thiolated probe, the resonance unit response for the same concentration of target remained relatively constant (FC1= 95±5 RU, FC2= 103± 4 RU) over 30 cycles and 2 overnight washes. A 27% loss of hybridisation response was eventually observed after 82 hybridisation cycles and 3 overnight flow treatments (5 $\mu l\ min^{-1}$).

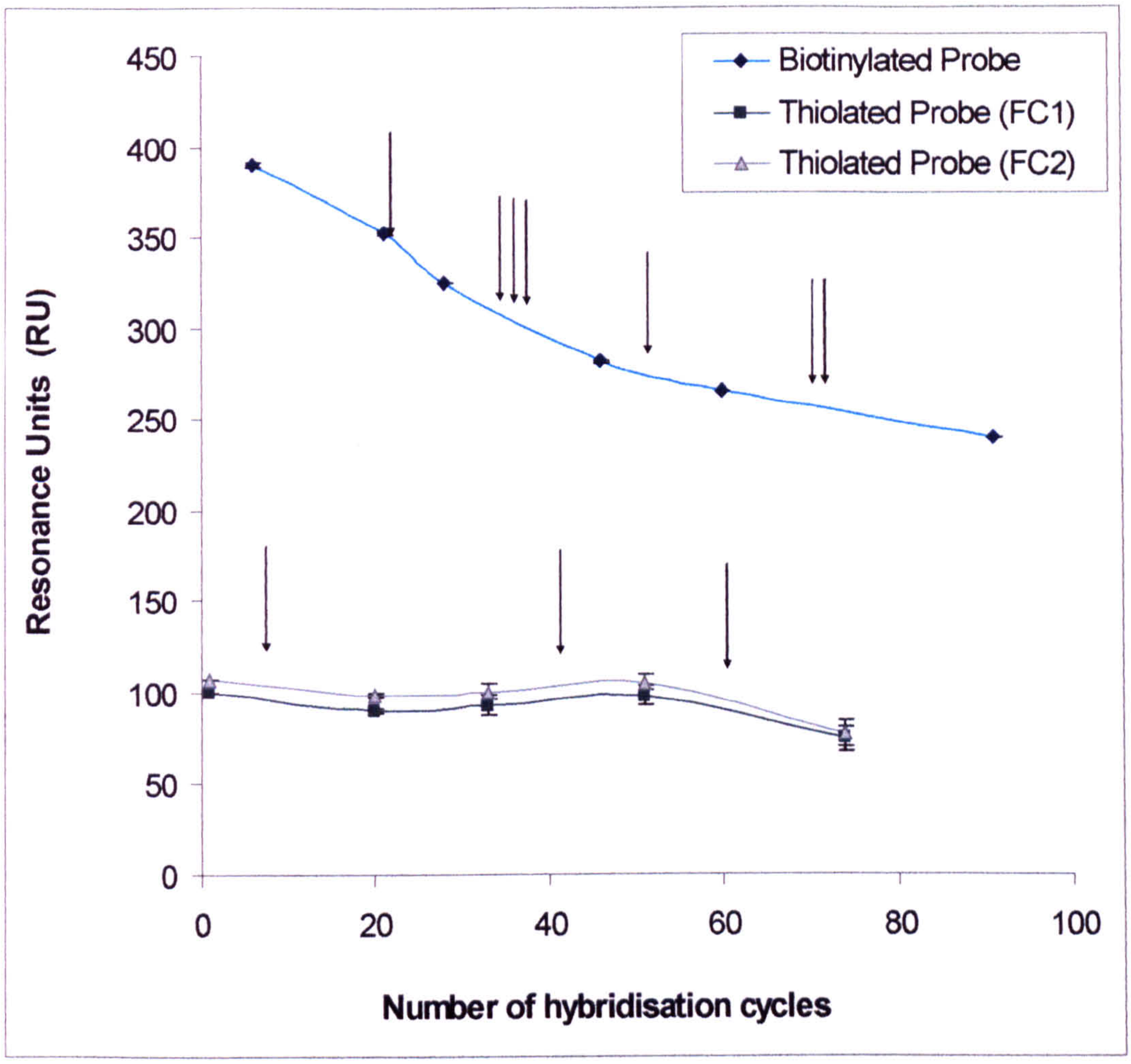

Figure 4.6: Comparison between two alternative immobilisation schemes, in terms of reproducibility of SPR analytical signal for 100 nM of 25-mer P35S target, in a course of different days of measurement. Indicative arrows correspond to overnight flow treatments.
Series 1(diamonds): Hybridisation shifts produced by the biotinylated probe attached to a dextran matrix by biotin-streptavidin linkage. Error bars correspond to CV% for the mean DRU response (n=3).
Series 2 and 3 (squares and triangles): Hybridisation shifts produced by the HS – ssDNA probe in flow cells 1 and 2, respectively. Error bars correspond to CV% for the mean RU response (n=3).

4.4. Discussion

The biosensor developed herein, is based on a thiolated probe, part of a mixed SAM immobilised on a gold SPR sensor surface. The current study investigated the stability and hybridisation capacity of the thiolated probe by means of BIAcore SPR technology.

Following immobilisation of the thiolated mixed SAM, the sensor surface was exposed to running buffer flow conditions (5 µl min^{-1}). This initial exposure of the immobilised probe surface to running buffer flow conditions, led to a decrease in the baseline signal. This decrease is considered to be as a result of detachment of material from the sensor surface. With regard to this issue, immobilisation studies on cantilever sensors, using thiol-modified DNA oligonucleotide sequences, suggested a three phase immobilisation process (Marie *et al.*, 2002). During a first phase, the thiol-modified DNA oligonucleotides self-assemble on the gold-coated cantilever via thiol-gold binding. Once this layer is completed, or nearly completed, DNA oligonucleotides continue to adsorb, forming a multilayer structure held together by hydrogen bonding, essentially a low-energy interaction. When the system is rinsed with milliQ water those oligonucleotides, which are not strongly bound to the surface by thiol-gold bond are desorbed and the cantilever signal decreases. The cantilever stabilisation value was interpreted as the surface stress in a single layer of strongly bound DNA oligonucleotides.

Even in the case of mixed SAM, loss of material is attributed to the displacement of the thiolated probe rather than the loss of the mercaptohexanol. Control SPR experiments (Georgiadis *et al.*, 2000) showed that the maximum coverage of mercaptohexanol onto bare gold (1.8×10^{14} molecules/cm^2) is much greater than the HS-C6-ssDNA (7.6×10^{12} molecules/cm^2). Therefore, loss of large amounts of mercaptohexanol would leave much of the gold surface bare. Such a surface is vulnerable to non-specific binding of DNA. In the case of non-specific binding events, the target DNA molecules are considered to be at an adsorption state through substrate/backbone contacts. In control experiments, no non-specific adsorption of ssDNA was observed on this surface (Peterlinz *et al.*, 1997).

The current study also confirms the specificity of the generated hybridisation shifts on freshly immobilised sensor surfaces. SPR testing of DNA sequences non-complementary to the immobilised probe yielded very low (<8 RU) or non-detectable shifts. Therefore, adsorption related events onto freshly immobilised sensor surface, is an argument not supported. Further decrease of the baseline signal was found to be dependent on the number of hybridisation/regeneration cycles and overnight washes. Overnight flow conditions appeared to have a greater effect on the baseline, by decreasing the baseline signal up to 90 RU per operation.

Characterisation of the probe surface was conducted by the use of oligonucleotides (25-mer) bearing the same length and complementarity to the immobilised probe. The experimental limit of detection for the 25-mer

oligonucleotide was found to be 5 nM ($FC1_{RU}$= 12, $FC2_{RU}$= 13). Non-target sequence (TNOS) produced a <8 RU shift, thus confirming the specificity of the probe.

Sikavitsas *et al.* 2002, described the parameters governing transport processes in the BIAcore biosensor. Evolution of the concentration profile of the mobile species in the flow channel is controlled by conditions, such as the inlet velocity of the buffer, the inlet concentration and also the diffusivity of the mobile species in the buffer. In the current work, the diffusivity of the DNA oligonucleotides in solution (100 nM) was investigated, by keeping the other parameters (inlet concentration, velocity) constant. An injection volume of 15 µl produced a decrease of <25 % in hybridisation shifts, in comparison to the 25 µl injection volume. The use of 50 µl sample volume, increased the resultant hybridisation shift by <15 %, leading to a less pronounced difference in response. These results are indicative of the reaction kinetics, under the present conditions of buffer flow and DNA target concentration.

Comparison of the thiolated probe-based immobilisation method with the alternative of biotinylated probe to dextran by a streptavidin linkage revealed the superiority of the latter in terms of hybridisation shifts. Working in the plateau region of the P35S standard curve in both immobilisation schemes, showed that the thiolated probe had the hybridisation capacity to produce average shift of around 120 RU, whereas the biotinylated probe generated shifts values of at least 200 RU. Although the method failed to generate high hybridisation shifts with

respect to the optimised immobilisation scheme, the thiolated probe yielded an encouraging good detection limit of 5 nM for target DNA.

The superiority of the dextran-based immobilisation protocol in terms of hybridisation shifts is not surprising considering that the method has formed the immobilisation platform for various transducer systems (Löfås *et al.*, 1995; Storri *et al.*, 1998a) and has been used as a standard method for SPR BIAcore applications (Uppsala, Sweden). On the contrary, the thiolated DNA-based immobilisation protocol was only preliminary investigated under continuous buffer flow conditions in the present study.

Despite the loss of probe material from the sensor surface as a result of the continuous flow conditions (Figure 4.1), the hybridisation capacity of the HS – ssDNA probe was highly reproducible over different days of measurement. The thiolated probe proved to be more stable in terms of hybridisation capacity than the biotinylated probe, generating relatively constant hybridisation shifts for 100 nM of target ($FC1_{RU}$= 95± 5, $FC2_{RU}$= 103± 4) over 30 cycles and 2 overnight flows.

Examination of the variation in the hybridisation efficiency between different sensor chips was found to be more pronounced in the case of the biotinylated probe attached to a dextran matrix by streptavidin linkage. Two different sensor chips treated with identical immobilisation and hybridisation conditions, produced hybridisation shifts in the range of 150-390 RU for 100nM of target P35S using the streptavidin/biotin immobilisation approach. Correspondingly, the HS–

ssDNA generated shifts in the range of 100-140 RU for 100 nM of target P35S. This considerable variation in the case of the dextran modified surface, observed under reproducible conditions, may be due to the several steps involved in the immobilisation procedure. It may be suggested that, in the case of the mixed SAM, the limited numbers of steps for sensor-surface modification reduce the introduction of variability into the procedure, hence producing more reproducible results.

To extend the analytical applications of the system for PCR amplified DNA target, a pre-treatment step was employed. The sample pre-treatment step was aimed at the denaturation of the amplified DNA target, under strong alkaline conditions (0.3 M NaOH) and formamide (10 or 20 % v/v), in order to facilitate hybridisation with the immobilised probe. The specific pre-treatment step has been successfully applied in hybridisation studies of biotinylated probe immobilised on dextran modified gold surface (chapter 3, section 3.3.5). Upon implementation of the selected sample pre-treatment conditions, the probe exhibited poor repeatability and regeneration problems. Specifically, the hybridisation shifts generated when using a target concentration of 100 nM, produced a CV value of at least 10 %, in both flow cells. Moreover, upon regeneration of the probe surface, up to 10 % of the observed hybridisation shift was still apparent on the probe surface.

Formamide is a highly polar solvent, capable of hydrogen bonding. Sur and Lakshminarayanan (2001), reported the effect of formamide to SAMs based on the barrier properties of the latter to formamide. The study investigated whether

ferrocene electron transfer through the monolayer was facilitated by the ability of the solvent molecules to interact with the thiol molecules and hence disorganise the monolayer. The study revealed excellent monolayer blocking properties to ferrocene, which was attributed to the two dimensional (layered) hydrogen bonded structure of the formamide molecules. Due to the association of formamide molecules by strong hydrogen bonding, formamide did not solvate the alkyl tails of alkanethiol molecules and therefore, no disorganisation took place which would permit passage of the ferrocene molecules to the sensor surface. In the current study, injection of 5, 10, and 20 % v/v of formamide alone in the reaction mix did not produce any change in the refractive index, thus confirming the absence of interaction between the formamide molecules and the mixed SAM.

The presence of strong alkaline conditions in the formamide reaction mixture gave rise to non-specific binding effects, recognised by regeneration problems and reduced repeatability in the generated hybridisation shifts. When oligonucleotide and polynucleotide target DNA molecules were analysed, regeneration problems were encountered. Identical sample treatment was successfully applied in the case of dextran modified sensor surface, where no refractive index artefacts were observed on the negative control probe surface. It is postulated that this phenomenon may result from interaction of the OH-terminated thiol molecules with the amide salt, produced by the following reaction (Rodriguez *et al.*, 2000):

$$NaOH + HCONH_2 \longrightarrow CHCONH(Na)H + H_2O$$

SPR analysis of denatured dsDNA of oligonucleotide length (25-mer), showed an average hybridisation shift of 58 RU (n=3). This result suggests the capability of the probe system to detect dsDNA, in the presence of adequate denaturing conditions. Correlation to symmetrically PCR amplified target is possible, as far as the amplicons are of short length. Nevertheless, hybridisation studies with polynucleotide target were less promising. SPR testing of PCR amplified polynucleotide target, produced an average hybridisation shift of 24 RU (n=3). Considering the non-specific events observed with the use of denaturing mix, this hybridisation shift is insignificant to support the argument that the probe system is able to hybridise with PCR polynucleotide target. Although the poor quality of the DNA starting material may have contributed to this low hybridisation shift, it should be considered that a probe able to generate an average (n=3) of 120 RU for 25-mer target sequences is difficult to generate high hybridisation shifts with DNA sequences of 243 bp. The current results come into agreement with the work of Huang *et al* who studied the hybridisation efficiency of mixed SAMs for polynucleotide target, using QCM crystals (Huang *et al.*, 2001). His study suggested that even if there is adequate space for the PCR amplified sequences to approach the immobilised probe, the flexibility of such long DNA sequences in the vicinity of the surface might not be as high as that of DNA in the bulk solution. As a result, the short portion in the amplified sequence which is complementary to the immobilised target might not have the suitable alignment for hybridisation.

4.5 Conclusions

The current work characterised a thiol-derivatised DNA (HS-C6-ssDNA) probe immobilised on gold, by means of BIAcore technology. The thiolated probe was found capable of hybridisation with complementary oligonucleotide molecules of the same length, with a detection limit of 5 nM. A stable hybridisation capacity was observed over a course of 50 hybridisation cycles and a period of two overnight continuous flow treatments. The hybridisation efficiency of the probe was not affected by the presence of a genomic matrix. The potential of the current probe system to detect symmetrically PCR amplified DNA of short length, was confirmed by the use ds-hybrids (25-mer). However, insignificant hybridisation efficiency was observed towards polynucleotide target molecules. This is not unexpected, when the low hybridisation capacity (~95 RU for 100 nM of target) of the probe and the disorganisation and steric hindrance of the bulky polynucleotide is considered.

CHAPTER 5

GENERAL INTRODUCTION-PART 2: BIOSENSOR DEVELOPMENT FOR THE MEASUREMENT OF GLUCOSE AND L-AMINO ACIDS IN HORTICULTURAL PRODUCE

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5.1. Horticultural produce quality in terms of sugars and amino acids

Fructose, glucose and sucrose are the predominant sugars found in fresh fruits and are responsible for the sweetness. Acidity and flavour rely upon the total concentration of organic acids and amino acids. Distinct flavours may be due to single amino acids or the combination of these, but the role of amino acids in contributing to flavour is not clear. It has been suggested that certain amino acids are precursors of volatile aroma constituents (Hobson and Davies, 1971).

In apples, as in any other fruit, amino acid levels usually decline during fruit maturation. This is probably related to the processes of protein synthesis and degradation during maturation (Ackermann *et al.*, 1992). Principal amino acids in apples are asparagine and aspartic acid. Sucrose is the major sugar in apples, and it is steadily increased during development. Fructose and glucose fluctuate during development around the same values until just before harvest when they suddenly increase in concentration (Ackermann *et al.*, 1992).

The sugar content of tomatoes increases rapidly during ripening, reaching a level with little subsequent changes (Davies *et al.*, 1969). Experiments showed that acidity also

tends to increase around mid-July and decreases rapidly afterwards (Davies *et al.*, 1969). Premature picking of the fruit seems to decrease the total sugar content, whereas no significant variation in the fruits' content of reducing sugars is observed during the postharvest up to seven days (Auerswald *et al.*, 1999). The principal amino acid in tomatoes is glutamic acid, followed by aspartic acid (Kader *et al.*, 1978).

The excessive darkening of potatoes after frying is an important quality defect, unacceptable in the potato industry. The colour development after frying is primarily due to the high reducing sugar content (Pritchard and Adam, 1994). Consequently, industry uses the levels of reducing sugars (glucose, fructose), as an indicator for colour development (Roe *et al.*, 1990). However, the content of reducing sugars fails to explain colour development in cultivars with reducing sugar content <60 mg 100 g^{-1} (Rodriguez-Saona and Wrolstad, 1997a). A minor effect to chroma development has been attributed to increased levels of the amino acid glutamine, which appears to be the major amino acid in the development of colour intensity (Rodriguez-Saona *et al.*, 1997b). Levels of reducing sugars and amino acids explained most of the variations (75-88 %) in the chip colour (Rodriguez-Saona *et al.*, 1997b). In the Russet Burbank potatoes, fry colour was most closely associated with glucose during 8°C storage (Pritchard *et al.*, 1994). For example, glucose concentration >1.6 mg g^{-1} results in undesirably dark fries (Pritchard *et al.*, 1994).

Sugars, carboxylic and amino acids are naturally abundant in numerous crude foods such as fruit, vegetable, milk and honey. The determination of amino acids and sugars is thus of great significance in nutritional and biotechnological fields and many

efforts have been devoted to analytical chemical methods. Chromatography is the most widely used analytical chemical procedure for amino acid and sugar measurement in food.

The chromatographic methods currently in use to determine sugars and amino acids are high-performance liquid chromatography (HPLC), gas chromatography (GC) and capillary electrophoresis (CE). Nevertheless, chromatography of sugars often suffers from low sensitivity and time–consuming processes. The necessity of other detection methods, such as mass spectrometry is necessary especially in cases where natural matrices are present (Molnár-Perl, 2000). HPLC is a successful approach for amino acid detection. Amino acid detection by chromatographic techniques is however, hindered by the absence of a strong chromophore, and formation of derivatives is often required to enhance the absorbivity (Casella *et al.*, 2000). Photometric detection of amino acids by the ninhydrin assay (Hamilton, 1963) is a widely used method, but lacks practicality.

Electrochemical detection has proved to be superior to chromatography in terms of selectivity, sensitivity and rapidity. The challenge of providing a rapid, sensitive and cost–effective measurement of commercially important analytes has been met by the development of biosensors. Enzyme electrodes, in particular amperometric sensors, have increasingly been used in food analysis.

5.2 Objective of part 2: Biosensor development for the measurement of glucose and L-amino acids in horticultural produce

Glucose and free L-amino acids play a critical role in the maturation stages of the fruit, thus affecting shelf life, ripeness and quality. Specific varieties of tomatoes, potatoes and apples were tested. Comparison of the biosensor method to standard spectophotometric assay methods (test kit, ninhydrin) was one of the major concerns of this part of the project. In this manner, the quality of the electrochemical measurements and thus the applicability of the biosensor for measurement in real fruit samples was assessed against commercialised and commonly accepted monitoring methods.

5.3 Principles of electrochemical biosensors

Electrochemistry is the science of the interaction of phases containing electrons and phases containing ions (Hibbert, 1993). Electron transfer occurs at interfaces (or phase boundaries) between a metallic conductor (an electrode) and an ionic conductor (an electrolyte).

Several parameters are known to influence the rate of electron transfer reactions (Fisher, 1996):

- The electrode potential;
- The reactivity of electroactive species;
- The nature of the electrode surface;
- The structure of the metal electrode-electrolyte solution interface.

5.3.1 The electrode potential

The difference in potential $\Delta\phi$ between two phases in contact, determines the electrochemical processes occurring at the interface (Bard and Faulkner, 1980). Indeed, $\Delta\phi$ can control the direction of the chemical reaction. The quantity representing the half-cell potential relative to the potential value at equilibrium, is known as overpotential or activation overpotential (η) (Hibbert, 1993):

$$\eta = \Delta\phi - \Delta\phi_e$$

where $\Delta\phi_e$ is the potential of the half-cell at equilibrium.

The direction of the electrochemical reaction is determined by η in the context that oxidation occurs under a positive η and reduction under a negative η.

5.3.2 Reactivity of electroactive species

For the general reaction of charge transfer:

$$O + ne^- \longleftrightarrow R$$

the balance of reactants and product concentration at a potential is at equilibrium with their formal potential, according to the Nernst equation.

$$E_e = E^\circ + (R\,T / n\,F)\ln[O]/[R]$$

The equilibrium potential (E_e) depends on the standard electrode potential (E) of the reaction and the concentrations of O and R at the electrode surface. Also, R= gas constant, F= faraday constant, *n*= total number of charges on an ion and T= temperature in Kelvins.

5.3.3 Nature of the electrode/solution interface

The currently accepted model of the electrical double layer is shown in Figure 5.1.

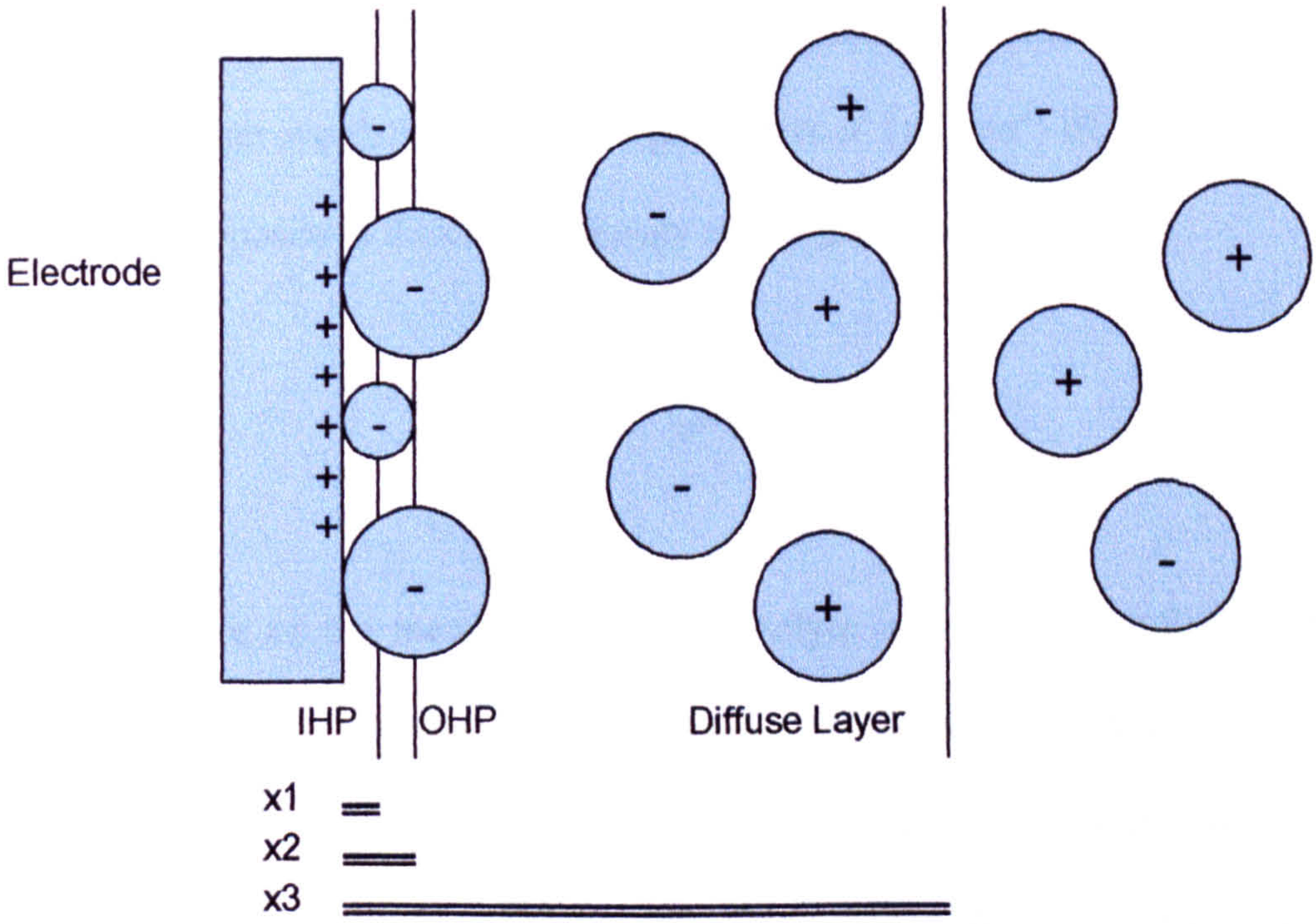

Figure 5.1: Grahame's model of the electrode-solution interface, showing the Inner Helmhotz Plane (IHP) at a distance x1, the Outer Helmhotz Plane (OHP) at a distance x2 and the diffuse layer that extends in a distance x3, from the OHP into the bulk of the solution.

At a given potential the electrode-solution interface is characterised by an array of charged species and oriented dipoles, known as electrical double layer (Bard and Faulkner, 1980). This consists of different layers that spread out in the solution phase. At a minimum distance from the metal electrode (x_1), extends the inner Helmholtz plane (IHP), which is occupied by specifically adsorbed species. Solvated ions are electrostatically attracted to the metal electrode and approach the metal to a minimum distance x_2, a function of the solvation shell dimensions. This layer corresponds to the outer Helmholtz plane (OHP). These solvated ions are known to be non-specifically adsorbed and are distributed in the diffuse layer, the thickness of which depends on the total ionic strength of the solution. The metal electrode-electrolyte interface acts like a capacitor. A charging current equalises the charge between the metal electrode surface and the diffuse layer, resulting in an equilibrium state. In other words, the total charge density σ^s ($\mu C/cm^2$) of the solution becomes equal and opposite to the charge density of the metal electrode σ^m:

$$\sigma^m = - \sigma^s$$

The structure of the metal electrode-electrolyte interface can influence the rate of electron transfer. The structure of the double layer is dependent on the applied potential and the ionic strength of the solution. Therefore, non-specifically adsorbed species approaching the metal electrode experience different potentials. On the other hand, in electrochemical reactions that involve electroactive species of low concentration, the charging current may be higher than the Faradaic one (Bard and Faulkner, 1980). Therefore, the effects of the double layer should be taken into account in electrochemical experiments.

5.4.4 Transport processes at an electrode

In a chemical reaction, the reactant species need to be transported to the metal surface and the products transported back into the bulk solution. Therefore, the rate of transport contributes to the overall kinetics of a chemical reaction. The mechanisms that govern the movement of ions through a solution are: diffusion, migration and convection.

Diffusion arises from uneven concentration distribution and acts to maximise entropy (Fisher, 1996). The diffusion process has been described by Fick's law, in which the quantity of a species diffusing through a unit area per unit time, the 'diffusional flux J' (mol sec^{-1} cm^{-2}), is equal to the concentration gradient of the species (B) at that location. In one dimension x,

$$J = -D\partial[B] / \partial x$$

where $\partial[B]/\partial x$ is the concentration gradient in direction x and the D is the proportionality coefficient known as the diffusion coefficient (cm^2/s). In an electrode process, the formation of a concentration gradient may be due to the consumption of an electroactive species at the electrode surface.

Migration, by definition, is the movement of a charged body under the influence of an electric field (Bard and Faulkner, 1980). The electrostatically driven movement of charged species at the electrode/solution interface, as a result of an applied potential, is a migration effect.

Finally, convection is the movement that occurs when mechanical force acts on a solution (Fisher, 1996). Apart from the natural convection, which is due to thermal or density gradients within a solution, another type of convection is identified. The forced convection is due to external forces, such as heat application and/or stirring. The height of the diffusion layer at the electrode surface can be modified by the rate of the forced convection at the electrode surface. As the rate of the convection increases, the diffusion layer becomes smaller.

5.4.5 The electrochemical cell

An electrochemical cell may contain three electrodes. The electrode where the electrochemistry of interest takes place, is known as the working or indicator electrode. The potential of this electrode is controlled with respect to the reference electrode, which operates in a potentiometric mode. The current in the electrochemical cell passes between the working electrode and a third electrode, termed the auxilliary electrode.

The working electrode is where the electrochemical reaction of interest takes place. Electrode materials must be good electron conductors. The range of electrode materials includes metals and other solids with metallic conductivity and good semiconductors. Carbon is a widely used chemically inert electrode. The presence of π-electron clouds in the graphite atoms, increases its absorbance affinity for a variety

of atoms (Hibbert, 1993). Carbon is used in the form of glassy carbon, graphite and carbon paste.

The role of a reference electrode is to provide an accurate and stable potential value as a reference potential.

The silver/silver chloride electrode often consists of a silver wire, coated with a film of insoluble silver chloride as a result of an oxidation process. When immersed in a solution of chloride ions, a potential is established with the half-cell reaction:

$$AgCl + e^{(-)} \longleftrightarrow Ag + Cl^{-} \qquad E^{\circ} = +0.2224V$$

The concentration of free silver ions is determined by the solubility of the silver electrode. If the Cl^{-} activity is kept constant, the electrode potential is steady. The Ag/AgCl electrode is often used as a reference electrode or "inner electrode" in membrane electrodes.

The calomel electrode is similar, with mercury (I) replacing silver. The half-cell reaction is:

$$Hg_2Cl_2(s) + 2e^{(-)} \longleftrightarrow 2Hg(I) + 2Cl^{-} \qquad E^{\circ} = +0.2681\ V$$

By keeping the activity of chloride constant the potential of the half-cell is fixed.

Finally, the auxillary or counter electrode is used for the completion of the electrical circuit. The counter electrode may be a separate third electrode, but when the current flow is minimal it may also be substituted by the reference electrode.

5.5 Electroanalytical methods

Conductimetry, potentiometry and voltammetry (including amperometry) are amongst the three types of electroanalytical methods.

A very large number of electrochemical devices based on these methods have been developed to determine the concentration of electroactive species involved in biological systems.

In brief, Conductimetry measures solution resistance as a means of determining charge concentration. It is not ion-selective, but it can determine the total ion concentration against a maximum or minimum value.

Potentiometry is the measurement of the electrical potential difference between two electrodes in the electrochemical cell. An electrochemical cell consists of two electrodes that are connected by an electrolyte solution (ion conductor). The maximum difference in potential obtained when no current flow exists in the cell, is called an electromotive force. The absence of a current flow in the cell is a necessary requirement for an accurate potential measurement.

Voltammetry measures the current as a function of the applied potential and comprises a group of electroanalytical techniques, related to different types of potential waveforms. In potential sweep techniques, the potential of the working electrode is swept along a chosen region of potential at a fixed rate of potential change with time. The current response produced from the electrochemical reaction is measured.

Amperometry is a specific kind of voltammetry, where the potential remains constant with time. Thus, amperometry is the measurement of the current passing through the working electrode, when a constant potential is applied between the working and counter electrodes.

The oxidation or reduction of an electroactive species takes place at the working electrode and gives rise to an electrical current. The steady-state electrical current (I) may be related to the concentration of an electroactive analyte (B) in solution, which undergoes chemical transformation due to the applied potential, such that:

$$\mathbf{I} = nFAD[\mathbf{B}] / \delta$$

where n the number of electrons transferred per mole, and F the Faraday's constant: 9.64846 $*10^4$ Coulombs, A the area of the electrode surface, D the diffusion coefficient and δ is the diffusion layer thickness.

5.6 Amperometric enzyme biosensors

According to Mulchandani (1998):

"*An enzyme amperometric biosensor is an analytical device that combines an enzyme with an amperometric transducer to produce a signal proportional to target analyte concentration*".

The enzymatic reaction generates a current, resulting from oxidation/reduction of electroactive species at the surface of the working electrode. The amperometric transducer measures the current flow across the working electrode at a steady potential, controlled with respect to a reference electrode.

To improve biosensor response, the charge transfer between the working electrode and the molecules in solution must be facilitated. Some important biosensor elements that influence the redox response are briefly described in the following paragraphs.

5.6.1 Oxidoreductases

Oxidoreductases have been widely used in the development of biosensors. They form a large group of enzymes that catalyze a wide variety of oxidation/reduction reactions. In this study, glucose oxidase and L-amino acid oxidase were used for the construction of the biosensors under development.

Glucose 1-oxidase (GOD, EC: 1.1.3.4) oxidises glucose as a main substrate to produce gluconic acid, according to the reaction:

$$C_6H_{12}O_6 + O_2 \xrightarrow{\text{GOD}} C_6H_{10}O_6 + H_2O_2$$

A schematic presentation of glucose oxidation is provided in Figure 5.2. Other types of enzymes that oxidise glucose as their main substrate are the glucose dehydrogenases, quinoprotein glucose dehydrogenases and glucose 2-oxidases

(Wilson and Turner, 1992). Although the former two enzymes are both specific for β-D glucose, glucose dehydrogenase requires a soluble cofactor whilst the quinoprotein dehydrogenase is relatively unstable. On the other hand, glucose 2-oxidase suffers from low substrate specificity, as it may also oxidise other carbohydrates such as xylose and gluconolactone (Wilson and Turner, 1992). These drawbacks have eliminated their widespread use.

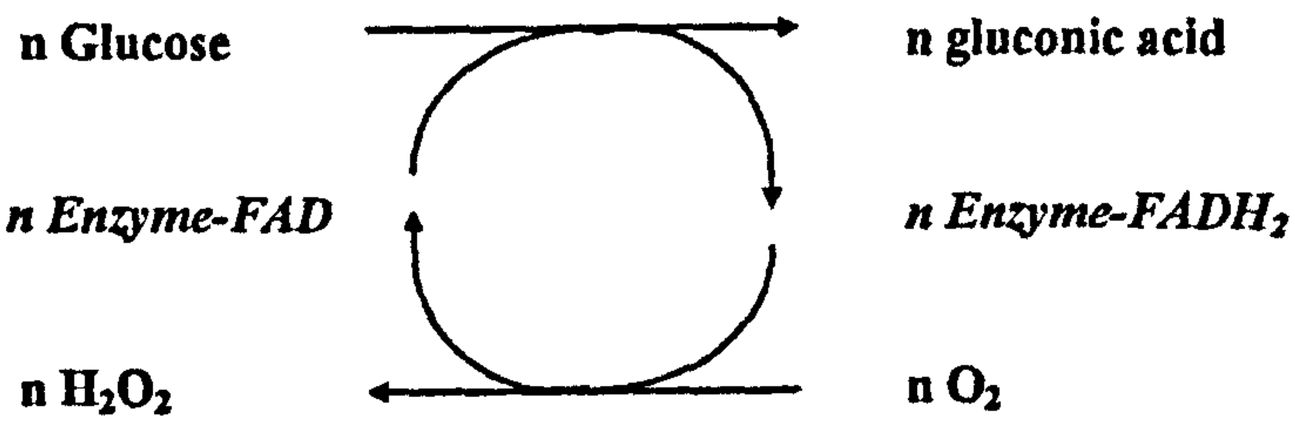

Figure 5.2: Enzymatic reaction process for the electrochemical reaction of glucose oxidation at the sensor surface. The hydrogen peroxide product is subsequently oxidised and quantified amperometrically at a +350 mV poised working electrode.

Glucose 1-oxidase (GOD) was discovered by Muller in 1928 (Bentley, 1963). It has been isolated from various sources, including bacteria (Dowling and Levine, 1956) and insects (Schepartz and Subers, 1964). GOD has been described as a branched glycoprotein (O'Malley and Weaver, 1972), whose carbohydrate component partly surrounds a protein core (Degani and Heller, 1988). This shell appears to act as a barrier for electron transfer between GOD and the working electrode in amperometric biosensors (Degani and Heller, 1988).

The amino acid oxidase group of enzymes comprises two different types of enzymes: L- and D- amino acid oxidases. Both enzymes catalyse the oxidation of amino acids to α-keto acids and ammonia, according to the general scheme:

$$RCHNH_2COOH + O_2 + H_2O \xrightarrow{AAO} RCOCOOH + NH_3 + H_2O_2$$

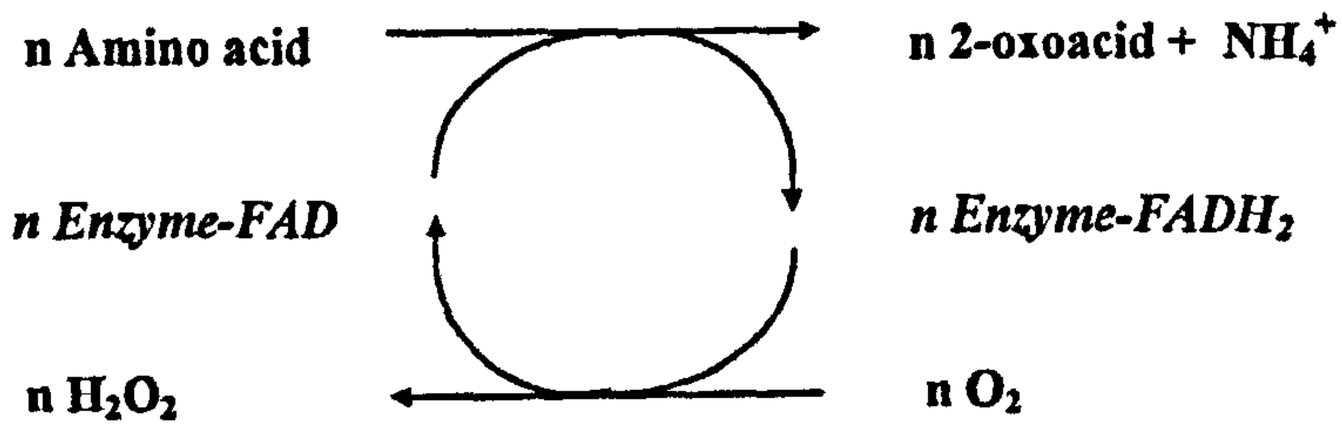

Figure 5.3: Enzymatic reaction process for the electrochemical reaction of amino acid oxidation at the sensor surface. The hydrogen peroxide product is subsequently oxidised and quantified amperometrically at a +350 mV poised working electrode.

Immobilised L-amino acid oxidase (L-AAO) degrades L-amino acids to 2-oxo-acid, ammonia and hydrogen peroxide. The enzyme L-amino acid oxidase (L-AAO) (EC: 1.4.3.2) may be purified from the venom of several kinds of snakes. These include the species *Agkistrodon blomhoffii* (Sun *et al.*, 1999), *Agkistrodon acutus* (Xu *et al.*, 2000), *Crotalus atrox* (Torii *et al.*, 1997) and *Crotalus adamanteus* (Varadi *et al.*, 1998). L-AAO is a cytotoxic glycoprotein, acting primarily on the membrane lipid bilayer (Dufton and Hider, 1988). The enzyme also showed cytotoxic activity against cancer cells, probably due to inhibition of thymidine incorporation and interaction with DNA (Ahn *et al.*, 1997). Characterisation of L-AAO from the venom of king cobra, *Ophiophagus Hannah*, revealed a dimer composition (Ahn *et al.*, 1997).

Effective enzyme immobilisation is a crucial part for the construction of a biosensor, as it is directly correlated with the preservation of enzyme activity and stability.

5.6.2 Enzyme immobilisation procedures

Adsorption of enzymes on insoluble supports is a result of polar, ionic, hydrogen bonding or hydrophobic interactions (Bardeletti *et al.*, 1991). Simplicity and appropriate enzyme treatment, in order to minimise denaturation effects, are the main advantages of the method. The weak nature of the enzyme linkages makes them susceptible to pH changes, temperature and ionic strength (Mulchandani *et al.*, 1998).

Entrapment or retainmnent of an enzyme within a gel matrix dates back in 1967, when Updike and Hicks (1967) immobilised glucose oxidase in a polyacrylamide gel at the surface of an oxygen electrode. Apart from polyacrylamide, enzymes can be entrapped in agar, agarose and chitosan polymer (Mulchandani *et al.*, 1998). The method involves a non-denaturing procedure, but suffers mainly from diffusional limitations for the substrates and products as well as loss of enzyme by leakage. A good alternative is the negatively charged perfluorinated sulphonate polymer Nafion™ (DuPont-NEN, Boston, MA, USA). The polymer combines enzyme preservation and suppression of anionic-interferences (such as ascorbic acid) due to charge repulsion. Using the latter polymer, membrane casting can be done directly at the surface and the thickness of the formed layer can be smaller (Gorton *et al.*, 1991). More recently, pectin has been used as a novel immobilisation matrix for biosensor construction (Jawaheer *et al.*, 2003). Pectin can be reconstituted into a thin gel, while

its high sugar content stabilises the enzyme in an environment compatible to a prolonged operating lifetime. Moreover, pectin can form a paste with the metalised carbon of screen-printable consistency, which could find potential in mass production of readily immobilised screen-printed electrodes.

Another immobilisation method for biosensor applications involves the use of electrochemically deposited matrices. The method involves enzyme entrapment during electrogeneration of the organic polymer at the electrode surface (Cosnier, 1999).

Polypyrrole and polyaniline are amongst the conducting electrochemically deposited polymers (Foulds and Lowe, 1986; Shinohara *et al.*, 1988). The main advantages of the method are the one-step procedure and the exact control of the polymer thickness (Cosnier, 1999). Entrapment of GOD into conducting polypyrroles has been documented (Foulds and Lowe, 1986; Barlett and Whitaker, 1987). Non-conductive polymers, like polyphenols, (Centonze *et al.*, 1992) are used to prevent interferences from fouling the biosensor signal.

Immobilisation via covalent bonding provides strong binding links between an enzyme and a support. Polytyramine bears free amine groups on the polymer backbone, where enzymes can attach via peptide bonds (Situmorang *et al.*, 1999). Covalent attachment of GOD at the surface of Polytyramine involves a sequential procedure, due to the harsh conditions under which polymerisation is conducted, that is 0.3M NaOH in methanol solution (Situmorang *et al.*, 1998). Covalent attachment of the enzyme is then accomplished by carbodiimide coupling. Dissolvement of tyramine with a mixed methanol-water solvent, allowed polymerisation under conditions that retained enzyme activity, thus making electropolymerisation and

enzyme immobilisation a one step procedure (Situmorang *et al.*, 1999). A drawback of covalent bonding is that it may involve toxic chemicals, although the strong binding forces make it a popular option.

Coupling of enzymes to amino-containing supports can be achieved by crosslinking agents, like glutaraldehyde. L-AAO has been immobilised to polytyramine, with glutaraldehyde offering a crosslinkage between the enzyme and the polymer (Cooper and Schubert, 1994).

Simple immobilisation is achieved by the use of commercially available pre-activated membranes, where the enzyme can be covalently attached or crosslinked. An example of commercially available membrane is the Immobilon (Millipore, Bedford, MA). Following enzyme immobilisation, the membrane is placed on the surface of the working electrode.

5.6.3 Biosensor stability

A desirable characteristic of biosensors is to maintain activity for an extended period of time. Many studies have focused on the elevation of the operational stability and the shelf-stability of biosensors. The production of reproducible, stable biosensors mainly relies on enzyme stability. A few methods employed to enhance enzyme stability are described in this section.

Naturally thermostable enzymes, isolated from archaebacteria, appear to have enhanced stability characteristics and provide an alternative method in the construction of stable biosensors (Raia, 1995).

The use of dilute solutions of polyelectrolytes in buffer has proved to partially protect the enzyme against thermal inactivation, when stressed at a temperature of 75 °C in aqueous environment (Gibson *et al.*, 1996). The formation of protein-polyelectrolyte complexes has improved the stability characteristics of biosensors. The use of a combination of polyelectrolytes, such as diethylaminoethyl (DEA) dextran and dextran sulphate has shown that a high level of stability may be conferred to enzymes in the dehydrated state (Gibson and Woodward, 1992). Enzymically active enzyme–polyelectrolyte complexes were first reported by Gibson (1996) and provided another mechanism of stabilisation. The immobilisation of malate dehydrogenase and lactate oxidase led to the production of shelf-stable biosensors for malate and lactate (Gibson *et al.*, 1996).

Transglutaminase from a variant of *Streptoverticillium mobaraense* (MTGase), was applied for the immobilization of biomolecules on electrode surfaces. This enzyme catalyses the acyl-transfer between gamma-carboxyamide groups and various primary amines. Suitable polypeptides (poly-L-lysine, poly-L-glutamine) or other proteins, such as casein, which served as substrates for MTGase, were added to generate networks by the entrapped and/or covalently attached sensing enzymes. It was demonstrated that MTGase catalyzed enzyme layers on disposable screen printed lactate enzyme electrodes exhibited high operational and storage stability, combined with high sensitivity (Josten *et al.*, 1999).

Another approach to increase stability is the use of biosensors with solid binding matrix (SBM)-based composite transducers. SBM-based composite transducers have been used for development of series of multibiosensor systems applicable in various fields, including food processing and safety (Miertus *et al.*, 1998). Katrlik *et al.*, recently reported a biosensor for the selective determination of L-lactate and L-malate in wine, based on robust solid composite transducers. Transducers comprised a solid binding matrix having hydrophobic skeleton, e.g. 2-hexadecanone, graphite and $NAD^{(+)}$. The biosensor showed an excellent long-term stability, after five months storage at room temperature. The L-malate sensor exhibited almost 100 % and L-lactate 90 % of the initial sensitivity (Katrlik *et al.*, 1999).

Application of polyturamine as an immobilisation matrix for GOD, resulted in a stable device that showed no loss in electrode response after four months of storage, while exhibited minor loss after 20 days of repeated use (Situmorang *et al.*, 1998).

5.6.4 Increasing sensitivity against interferences

Hydrogen peroxide is present in many biological reactions. It is a substrate for peroxidases and a product of several oxidation reactions, such as the oxidation of glucose that yields gluconic acid and hydrogen peroxide. Its detection forms the basis for the operation of many oxidoreductase based biosensors.

The amperometric detection of hydrogen peroxide may suffer from the presence of other electroactive species, which act as interferents. Due to the positive applied

potential required for the oxidation of hydrogen peroxide, other substance present capable of being oxidised at these potentials may cause interference. The interference species are oxidised at the electrode to give an additive positive current response. Common interference species in biological and food samples, include ascorbic acid, uric acid, cysteine and acetaminophen (Higson *et al.*, 1994; Garjonyte and Malinauskas, 1999).

A variety of polymer membranes has been employed to discriminate between hydrogen peroxide and other oxidisable compounds. The first polymer perm-selective membrane for hydrogen peroxide was reported by Malitesta *et al.*, in 1990. That was a poly(o-phenylenediamine) membrane capable of immobilising glucose oxidase during electropolymerisation and of rejecting ascorbic acid.

Centenze and Palmisano (Centonze *et al.*, 1992; Palmisano *et al.*, 1993; Palmisano *et al.*, 1995) reported that overoxidised polypyrrole exhibits high selectivity for hydrogen peroxide. Both the poly(o-phenylene diamine) and the overoxidised polypyrrole polymers show perm-selective properties based on hydrophobic-hydrophilic and/or charge interaction between solutes and the membrane (Palmisano *et al.*, 1995). The poly(o-phenylene diamine)-based biosensor was modified by the addition of another electroactive layer, that was placed between the substrate electrode and the poly(o-phenylene diamine) film. In a lactate sensitive biosensor, described by Palmisano *et al.* (1995), the enzyme lactate oxidase was immobilised in a poly(o-phenylene diamine) film grown on polypyrrole-modified platinum electrode.

Size–exclusion coatings have been employed for the construction of hydrogen peroxide transducers. Membranes evaluated for use in the biosensors include the PVC, polysulphone (PS), sulphonated polyether-ether sulphone-polyether sulphone (SPEES-PES) (Benmakroha *et al.*, 1995). In cellulose acetate membranes, molecular size exclusion and anion rejection are effectively combined to minimise the interference from ascorbate and urate and to a lesser extent from acetaminophen (Benmakroha *et al.*, 1995).

Another approach to avoid interference is the use of selective electrocatalysts to allow the electrochemical oxidation and detection of hydrogen peroxide at reduced operating potentials. Examples of electrocatalysts are mainly metal hexacyanoferrates, such as Prussian blue. Garjonyte and Malinauskas, recently reported hydrogen peroxide sensing biosensors based on Cu_2O or CuO modified carbon paste electrodes, as well as carbon paste electrodes, modified by some metal hexacyanoferrates, such as ferrous (Garjonyte and Malinauskas, 1998 a, b). The electrocatalytic rhodinised carbon (MCA 4a) has proved to have a good response to hydrogen peroxide, with reduced interference effects (White *et al.*, 1994). Although bare carbon failed to generate any measurable signal for 0.1 mM H_2O_2 at potentials $\leq$+600 mV, a response of 3 μA was recorded at the HEC / MCA 4a working electrode at +300 mV (Kröger *et al.*, 1998). This indicates the ability of MCA 4a catalyst to amplify the signal and offers a degree of specificity in the system, due to the selection of lower working potentials.

Diffusional electron mediators are often used to establish adequate electrical communication between the redox enzymes and the electrode interfaces as well as to

minimise interferences. The presence of mediators facilitates the electron transfer between the redox-enzymes and the electrode surface, at lower voltages, thus reducing the possibility of other interfering species being oxidised. Ferrocenes are the most extensively studied of all mediators. The use of ferrocene as a mediator dates from 1984, when Cass *et al.* described a mediated glucose enzyme electrode, using electrochemically regenerated ferrocene to re-oxidise the reduced flavine adenine dinucleotide prosthetic group of reduced glucose oxidase (Cass *et al.*, 1984). Ferrocene typically allows electron transfer from glucose oxidase at +240 mV.vs Ag/AgCl (Maines *et al.*, 1996). Other mediators include the ferricyanide and Meldola's Blue (Maines *et al.*, 1996). The ExacTech™ disposable glucose sensor, used by diabetics, uses a mediated glucose enzyme electrode.

Another practical method, applied to minimise interference from ascorbate, is the use of the enzyme ascorbate oxidase. However, this method is only effective against ascorbate and does not affect other interfering species. Currently, another method is under investigation. Signal subtraction refers to the use of a second working electrode ('compensator' electrode) with no enzyme to register the sample background signal. In this way, the total interfering signal can be determined and removed.

CHAPTER 6

DEVELOPMENT OF AN ELECTROCHEMICAL BIOSENSOR FOR THE DETECTION OF GLUCOSE AND L-AMINO ACIDS IN HORTICULTURAL PRODUCE

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6.1 Introduction

The development of simple, field-based devices for rapid quality control is of growing interest for both consumer and food industries. Glucose and free L-amino acids play a critical role in fruit maturation thus affecting shelf-life and quality. A simple biosensor device for glucose and L-amino acids, could provide a useful, rapid and field-based tool for the analysis of the mayor fruit maturity and potato colour development indicators.

Simplicity, selectivity and cost efficiency were all considered important assay criteria in this project, due to the intended field-based usage of the developed biosensors. Rhodinised carbon has proved to be more sensitive and selective than the platinised carbon (White *et al.*, 1994) for the direct detection of hydrogen peroxide at reduced operating potentials. The latter benefit reduces the likelihood of high levels of oxidation of other potential interfering species present in the fruit matrix, such as ascorbic acid and malic acid. Moreover, the previously reported non–mediated oxidation of peroxide at rhodinised carbon electrodes (Kröger *et al.*, 1998; Sarkar *et al.*, 1999; Arif *et al.*, 2002; Jawaheer *et al.*, 2003) suggests that such sensors may be simply manufactured at low cost. Consequently, the

current study used the approach of electrocatalytic rhodinised carbon for the non-mediated oxidation of peroxide in both biosensors under development.

6.1.1 Latest advances on glucose biosensor

The electrochemical detection of glucose has been mainly based on immobilised glucose oxidase (GOD). GOD is the most widely used oxidoreductase for the construction of enzyme electrodes and is based on either O_2 depletion or H_2O_2 detection. Desirable features of the enzyme include high specificity, high stability and high turnover. There are numerous applications regarding the use of GOD in the development of biosensors used in food analysis (Maines *et al.*, 1996; Mannino *et al.*, 1997; Loechel *et al.*, 1998; Volotovsky and Kim, 1998). Recently, a fabrication approach for individual enzyme biosensors has been demonstrated for β-D-glucose, sucrose, total D-glucose and ascorbic acid, aiming at the development of an integrated single-device biosensor (Jawaheer *et al.*, 2003). The work showed that the use of a cellulose acetate membrane (1 %-2 % w/v) was able to extend the dynamic range of the sensor about five-fold compared to sensor array lacking membranes.

6.1.2 Latest advances on L-amino acid biosensor

Immobilised L-amino acid oxidase (L-AAO) degrades L-amino acids to 2-oxo-acid, ammonia and hydrogen peroxide. Enzyme electrodes for amino acids have been developed by coupling L-AAO to a membrane (Simonian *et al.*, 1991). The levels of amino acids are determined by potentiometric measurement of ammonia or hydrogen peroxide (Puchades *et al.*, 1989; Guibault *et al.*, 1987). An L-amino acid biosensor has been developed to detect ammonia, by immobilising L-AAO to nylon membrane (Lee and Huh, 1998). L-amino acids were degraded and a potentiometric electrode measured the ammonia produced. The sensor showed high relative activities for L-amino acids, with a linear response to phenylalanine and isoleucine up to 10 mM (Lee and Huh, 1998). A biosensor specific for L-lysine was developed with immobilised L-lysine α-oxidase on a rhuthenium and rhodium modified surface. The metallised electrode allowed peroxide detection at +100 mV *vs* Ag/AgCl. The selectivity of the enzyme against interferences (ascorbic acid and L-cysteine) was optimised through the selection of the temperature (30 °C) and flow rate (0.5 ml min^{-1}). The dynamic range of the sensor was determined to be from 2 μM to 125 μM (Kelly *et al.*, 2000).

Although both GOD and L-AAO-based screen-printed electrodes have been documented, data upon the analytical performance of the developed biosensors in real horticultural samples has rarely been reported.

This preliminary work aims at the development of cheap, disposable screen–printed electrodes for the electrochemical measurement of glucose and L-amino

acids in horticultural products. The screen-printing fabrication offers simplicity and mass-reproducibility, thus making sensor manufacture easy and of low cost. The analytical performance of the GOD and L-AAO-based biosensors in real fresh fruit samples is examined against standard photometric assays.

6.2 Materials and Methods

6.2.1 Apparatus and reagents

The electrochemical measurements were performed at room temperature using an Autolab Electrochemical Analyser with the General Purpose Electrochemical Software (GPES 3) operating system (Ecochemie, Ultrecht, Netherlands).

All chemicals and reagents were supplied by Sigma (Poole, Dorset, UK), unless otherwise stated.

Hydroxyethylcellulose (HEC) was purchased from Fluka (Buchs, Switzerland). The screen-printer meshes were cleaned using 2-butoxyethyl acetate (BEA 99 %, Aldrich, Gillingham, Dorset, UK).

Glucose oxidase (GOD, EC 1.1.3.4, from *Aspergillus niger*, specific activity 8156 U ml^{-1}) and L-amino acid oxidase (L-AAO, from *Crotalus adamanteus* type VI, specific activity 380 U ml^{-1}) were purchased from Biozyme (Gwent, UK) and Sigma (Gillingham, Dorset, UK), respectively.

All solutions were prepared in deionised reverse osmosis water (The Elgastat System, Elga, High Wycombe, UK).

Phosphate Buffer (PB): pH: 7.4, NaH_2PO_4-Na_2HPO_4 (1 M, 0.1 M).

Phosphate Buffer (PB)-electrolyte: pH: 7.4, NaH_2PO_4-Na_2HPO_4 0.1 M, 100 mM (KCl).

6.2.2 Fabrication of screen-printed sensors

Screen–printing is a form of graphic reproduction where a quantity of ink or other viscous compound is forced through a mesh incorporating a stencil design and hence is deposited as a film in a controlled manner to form a repeatable pattern of certain thickness (Goldberg *et al.*, 1994).

Three-electrode devices (Figure 6.1) were manufactured in-house by a multi-stage screen-printing process using a DEK 248 machine (DEK™, Weymouth, UK) and screens with appropriate stencil design (60 per screen) were fabricated by DEK Presicion Screen Division. The stainless-steel screen mesh was mounted at 45° to the print stroke with 77 wires^{cm-1} and emulsion thickness of 13 and 18 μm for the solvent and water-resistant screens, respectively.

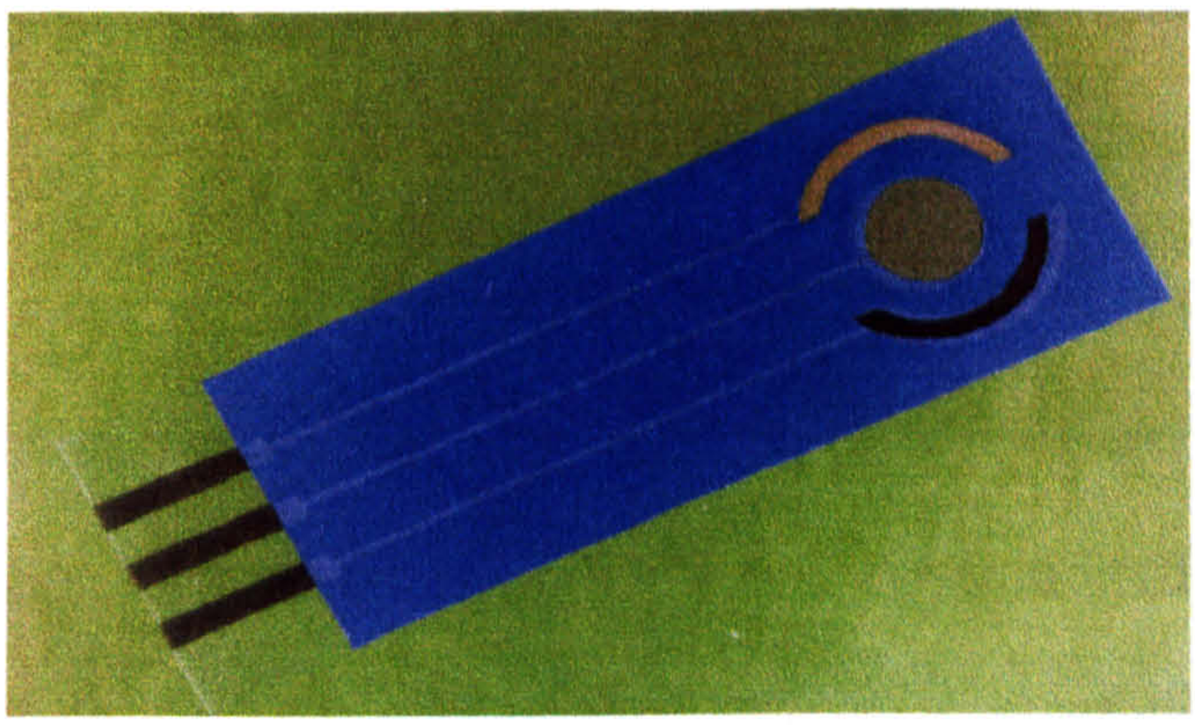

Figure 6.1: Biosensor comprised by a three-electrode system. From the left: Counter electrode, Working electrode, Reference electrode (Tothill and Turner, 2003).

Devices were printed onto 250 μm thick polyester sheet (Cadillac Plastic, Swindon, UK). The circular electrocatalytic working electrode (planar area 0.16 cm^2) was fabricated from MC4 4a electrocatalyst (MCA Services Ltd., Cambridge, UK), a commercially available carbon powder containing 5 % rhodium plus promoters, made into a screen-printable paste by mixing 1:4 in 2.5 % w/v HEC in buffer-electrolyte. The reference electrode ink contained 15 % silver chloride in silver-paste (MCA Services Ltd.). The counter electrode and basal tracks were fabricated from 145R carbon ink (MCA Services Ltd.). The basal tracks were insulated from the measurement solution using 242-SB epoxy-based protective coating ink (Agmet ESL, Reading, UK). The electrodes were then heated at 125 °C for 2 h, for the epoxy resin to be cured and the electrocatalytic pad to be stabilised, thus allowing prolonged use of the device in aqueous solutions. The screens were cleaned using of 2-butoxyethyl acetate.

6.2.3 Enzyme immobilisation

The enzymes were immobilised on the surface of the working electrode, via physical adsorption, for it shows simplicity and rapidity of preparation. Aliquots (10 µl) containing various amounts of GOD or L-AAO in buffer-electrolyte (pH: 7.4, 10 mM PB, 0.1 M KCl) were pipetted onto the working electrode and left to dry for >90 min at 25 °C. The dried electrodes were stored wrapped in foil at 4 °C until required.

6.2.4 Electrochemical test procedure

A 1.1 cm diameter Whatman 114 filter disc (Whatman, Maidstone, UK) was placed over the three-electrode assembly which completed the electrochemical circuit, when wetted with the sample.

Glucose, amino acids and fruit sample solutions were all prepared in buffer-electrolyte PB (phosphate buffer). The working electrode was poised at a potential of +350 mV *vs* the Ag/AgCl reference electrode. Following sample addition to the filter disc, amperometric measurement was initiated and allowed to proceed for 240 sec.

The 'background' electrochemical response of the buffer-electrolyte PB on the enzyme electrodes, was subtracted from the analyte specific response in a way such that:

$$\Delta Rs = \text{Analyte specific Response}_{(amps)} - \text{Buffer-electrolyte Response (PB)}_{(amps)}$$

where ΔRs, the analytical signal. When fruit samples were tested, the equation was modified to include the non-specific response produced by readily electroactive interfering species (e.g. ascorbic acid, malic acid etc). To do so, the non-specific response of each sample was determined using enzyme-free electrodes and was subtracted from the equivalent enzyme electrode response, i.e.:

$$\Delta Rs = \text{Analyte specific Response}_{(amps)} - \text{Buffer-electrolyte Response}_{(amps)} - \text{Non-specific Response}_{(amps)}$$

where ΔRs, the analytical signal of the electrochemical measurement.

6.2.5 Test kit method

Sensor performance has been validated against a commercially available method for the detection of glucose. This method used the protocol and reagents included in the D-glucose Test Kit (Cat. No. 716 251, Boehringer Mannheim, GmbH, Mannheim, Germany). The experiment was carried out at room temperature and the absorbance was read at 340 nm.

The principle of D-Glucose measurement is as follows: D-Glucose is phosphorylated to D-glucose-6-phospate (G-6-P) in the presence of the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) with the simultaneous formation of adenosine-5'-diphosphate (ADP):

$$\text{D-GLUCOSE} + \text{ATP} \xrightarrow{\text{HK}} \text{G-6-P} + \text{ADP}$$

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidised by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH):

$$\text{G-6-P} + \text{NADP}^{+} \xrightarrow{\text{G6P-DH}} \text{D-gluconate-6-phosphate} + \text{NADPH} + \text{H}^{+}$$

Therefore, the amount of NADH formed in this reaction is stoichiometrically related to the amount of D-glucose consumed. The increase in NADH is measured by means of its light absorbance at 340 nm.

6.2.6 Ninhydrin test method

Determination of free amino acids was also carried out, using the ninhydrin assay (Figure 6.2) (Moore and Stein, 1948). The test reagent was prepared using 150 ml

of glycerol, 0.625 g of ninhydrin, 18.387 g of citric acid and 133.3 µl of 150 mM $MnSO^4$, made up to 250 ml in water. The test reagent (2 ml) was added to 0.1 ml of sample or standard solution in a test tube. After vortexing, the mixture was heated at 100°C for 12 min, and diluted two-fold in water. The absorbance was recorded at 750nm. The ninhydrin method works for all amino acids, except proline.

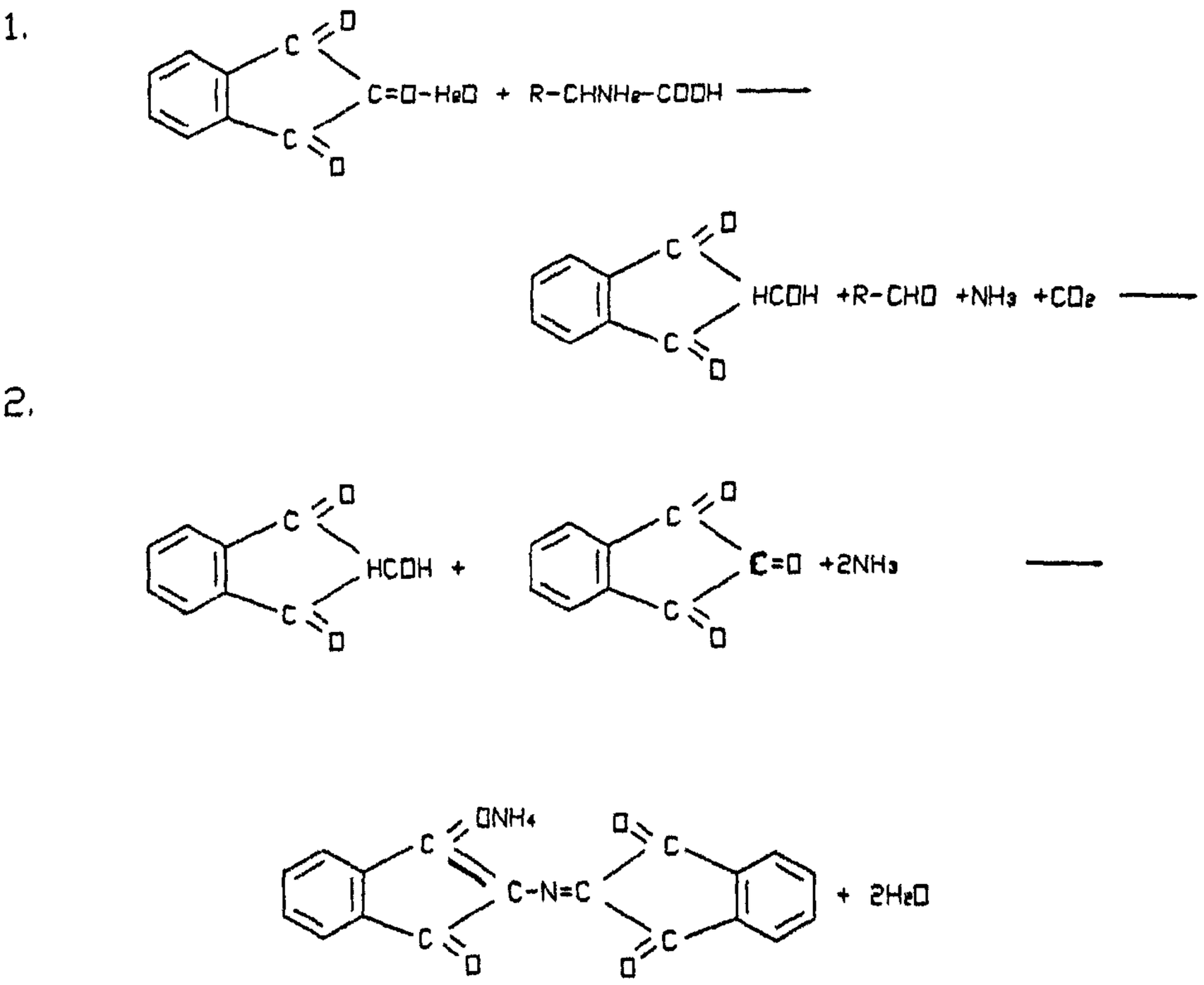

Figure 6.2: Ninhydrin reaction with amino groups proceeds in two steps and eventually produces an intensely coloured product, which can be measured spectophotometrically.

6.2.7 Real fruit samples

The fruit samples were prepared by grating the fruit and pressing the resultant material through a fine food–grade cotton filter (Boots company, Nottingham, UK). The use of cotton filters eliminated the presence of large solid particles in the extracted juice. For glucose testing, samples were diluted in buffer-electrolyte as follows: Jonagold apples x40, Mini-plum tomatoes x40, Russel Burbank potatoes x10. For L-amino acid determination, a different dilution pattern was employed due to concentration dissimilarities and the lower linear range of the corresponding L-AAO biosensor: Bramley apples x10, Mini-plum tomatoes x100 and King Edward potatoes x100. The extracted fruit juices were kept at 4 °C until required. Prior testing, the samples were suitably diluted in buffer-electrolyte PB, containing 100 mM of KCl and tested for glucose and L-amino acid content within 48 h.

6.3 Results

6.3.1 Optimisation of sensor analytical performance

L-leucine was chosen as the standard amino acid substrate for L-AAO, since *Crotalus adamanteus* L-AAO exhibits high K_m value for this substance (Albery *et al.*, 1996). L-Phenylalaline was selected as an additional standard substrate for L-AAO, as previous L-AAO biosensor studies showed a high current response

(0.272 μA) towards for the specific analyte compared to L-leucine (Sarkar *et al.*, 1999).

6.3.1.1 Optimisation of enzyme loading activity

The enzyme loading activity was optimised in both detection systems, by the determination of the minimum enzyme loading capable of generating the highest current response from the system. Experiments were carried out on screen-printed electrodes, containing a rhodinised carbon working electrode with glucose oxidase (GOD) or L-amino acid oxidase (L-AAO) immobilised via direct physical adsorption. The amperometric response generated at different enzyme loadings was monitored and a calibration curve was constructed, as shown in Figures 6.3 and 6.4. GOD loadings were tested in amounts from 5 to 250 mU, using substrate of glucose (10 mM) in PB (Figure 6.3).

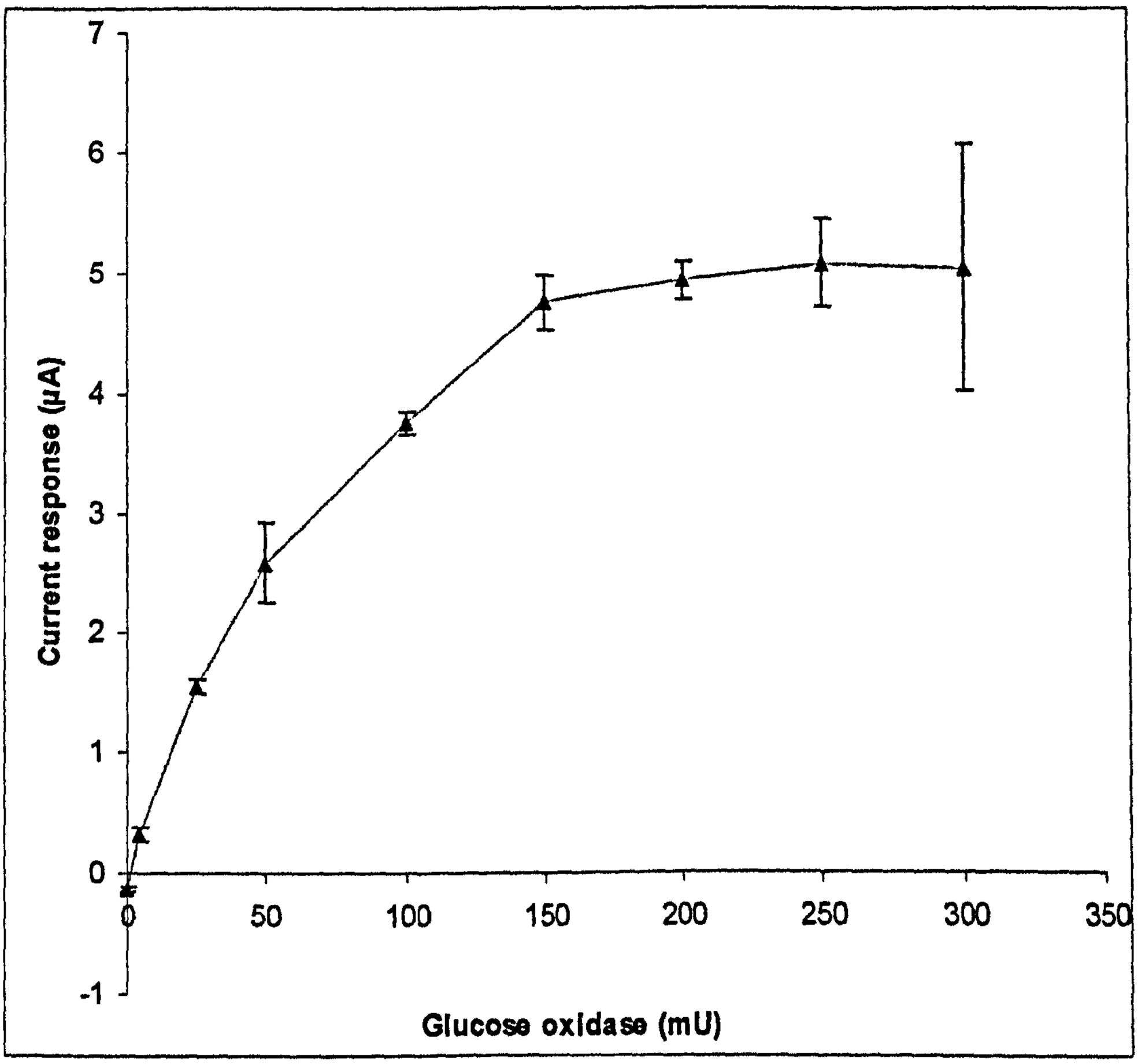

Figure 6.3: Current (μA) versus glucose oxidase (GOD) loading on rhodinised carbon screen-printed electrodes. Experiments were carried out using 10 mM of glucose in phosphate buffer (pH: 7.4, 100 mM NaH_2PO_4-Na_2HPO_4, 100 mM KCl). The selected potential was +350 mV against the Ag/AgCl reference.

Figure 6.4, shows the response of L-AAO, tested across the range from 5 to 70 mU, using L-leucine (1 mM) as substrate.

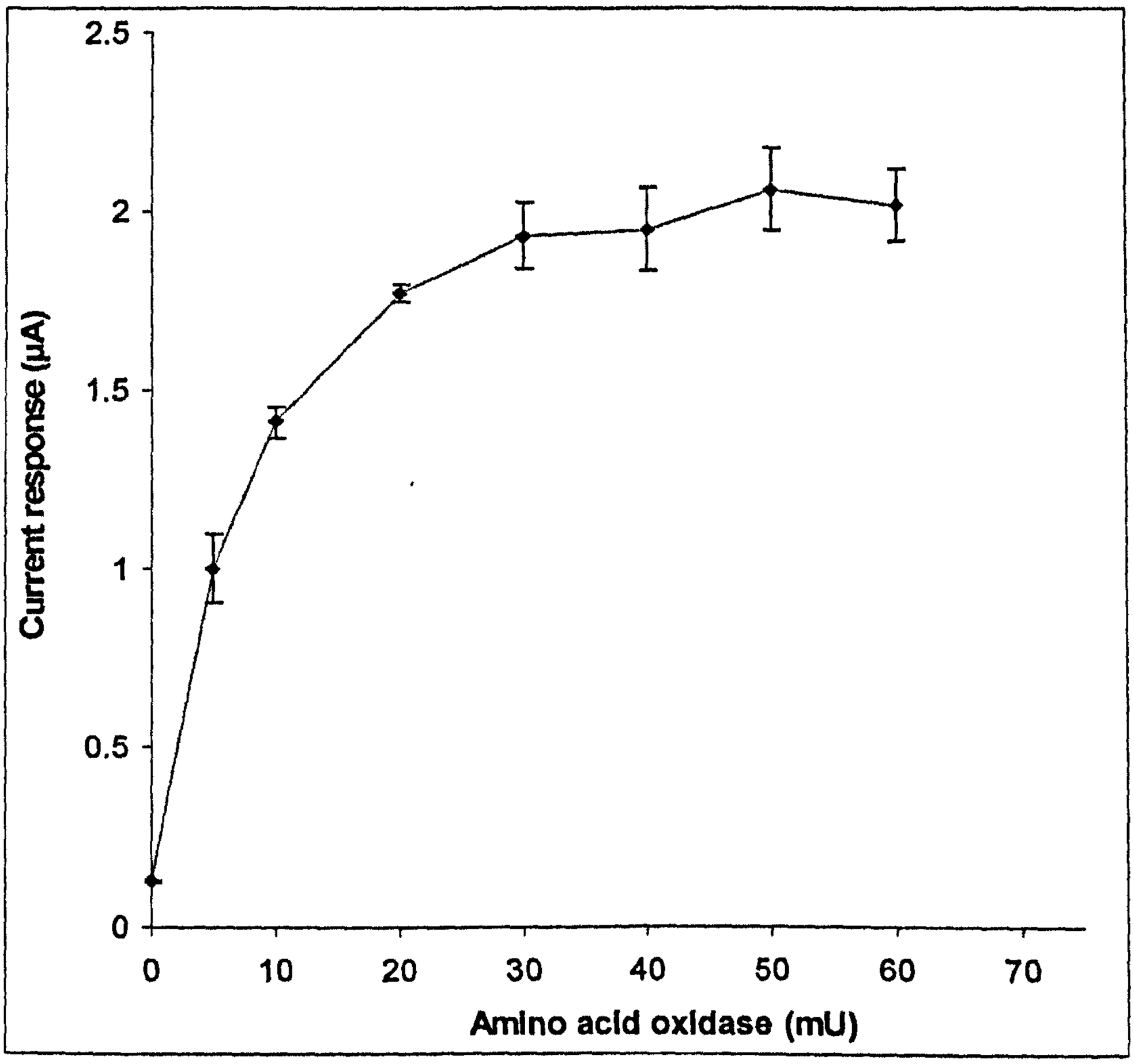

Figure 6.4: Current (µA) *versus* amino acid oxidase (AAO) loading amount on MCA 4a rhodinised carbon screen-printed electrodes. Experiments were carried out using 1 mM of leucine in phosphate buffer (pH: 7.4, 100 mM NaH_2PO_4-Na_2HPO_4, 100 mM KCl. The selected potential was 400 mV against the Ag/AgCl reference.

The optimum enzyme loading activity was observed at ≥150 mU of glucose oxidase and ≥30 mU of amino acid oxidase per electrode. Accordingly, these optimised enzyme loadings, (150 mU and 30 mU for GOD and L-AAO, respectively) were used for subsequent experiments.

6.3.1.2 Optimisation of operational detection potential

Step-amperometry was used to define the optimised working detection potential of the electrochemical systems under investigation. The experiments were carried out under stirred conditions and changing potential status (from +150 to +450 mV in 25 or 50 mV steps). The choice for the working detection potential was based on the signal to noise ratio (S/N) of H_2O_2 on bare MCA 4a carbon electrodes (Table 6.1). The 'noise', corresponds to the background charging effects inherent in the PB buffer.

Table 6.1: Signal to noise ratio (S/N) *versus* potential (mV) profile for 100 µM of H_2O_2 in PB (pH: 7.4, 100 mM NaH_2PO_4-Na_2HPO_4). MCA 4a rhodinised carbon screen-printed electrodes. Tests carried out in triplicate (n=3).

H_2O_2 (100 µM) in PB *Vs* PB	
Potential (mVolts)	Signal /noice (s/n)
200	79.22
250	85.57
300	100.01
350	109.52
375	109.25
400	86.55
450	58.11

The higher S/N value was recorded at +350 mV. This detection potential was therefore used in subsequent experiments.

6.3.2 Analytical performance of the amperometric GOD- and L-AAO-based biosensors in standard solutions

The analytical performance of the developed amperometric biosensors was examined, using GOD or L-AAO modified screen-printed electrodes, according to the method described in 6.2.4. The corresponding substrate solutions were prepared using buffer-electrolyte PB (100 mM NaH_2PO_4-Na_2HPO_4, 100 mM KCl).

6.3.2.1 Glucose oxidase–based amperometric biosensor

The linear range of the glucose detection system was determined by the use of glucose solutions (0-18 mM) in PB buffer. A linear relationship between glucose concentration and current response is observed up to 10 mM of glucose (Figure 6.5). Higher glucose concentrations (>10 mM) produced current responses that correspond to a plateau, an enzyme saturation status.

The limit of detection (LOD) for glucose, calculated as 3x the standard deviation of the zero analyte response, was 0.162 mM. The experimentally established linear range of the glucose biosensor was 3-10 mM.

Table 6.2, summarises the current responses corresponding to the glucose calibration curve (Figure 6.5), produced by different glucose concentrations in phosphate buffer.

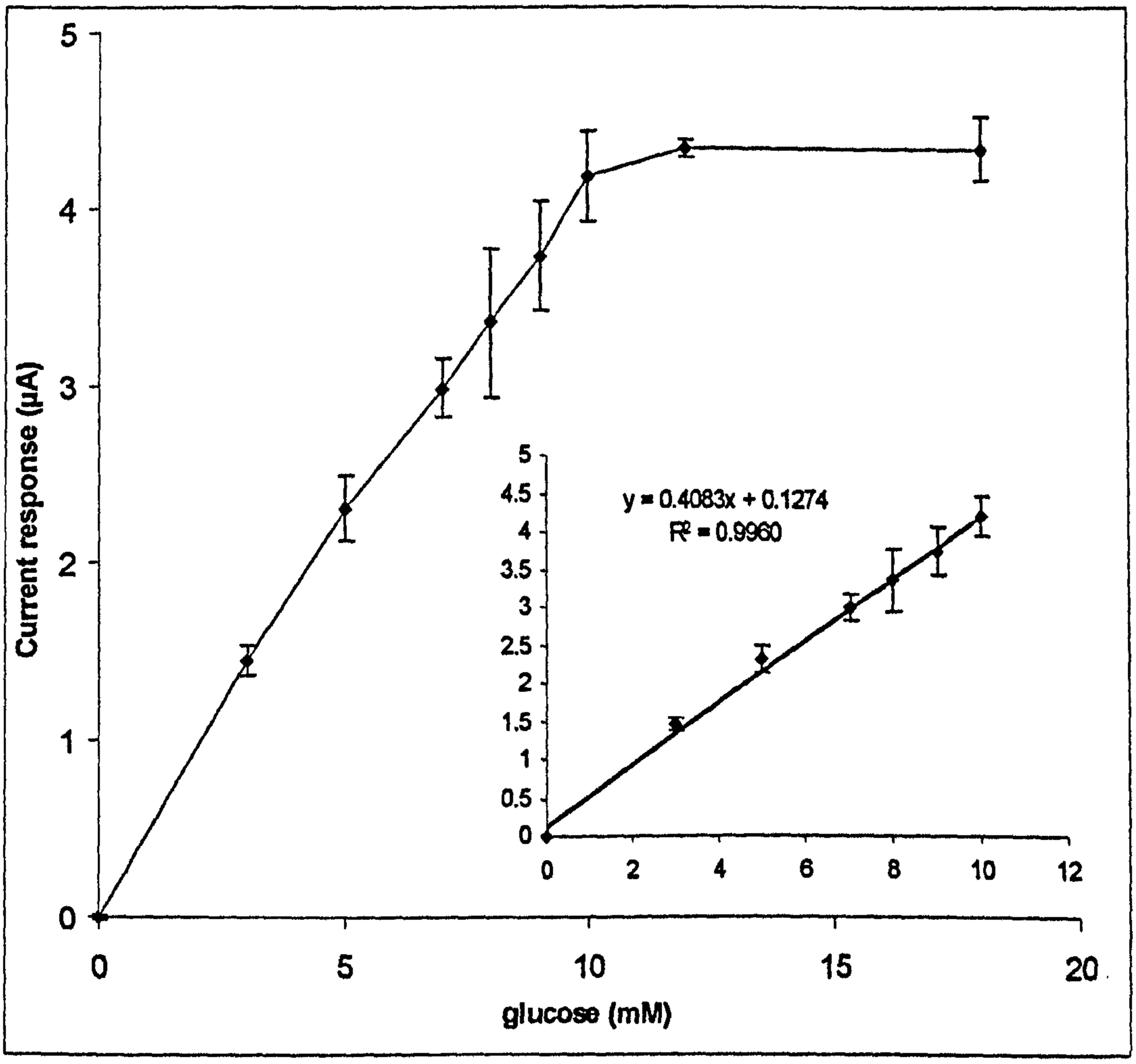

Figure 6.5: Calibration curve for the amperometric glucose oxidase (GOD) modified screen-printed electrodes. The substrate glucose solution (0-18 mM) was prepared in phosphate buffer PB. Background response values have been subtracted. Tests were carried out in triplicate (n=3).

Table 6.2: Summary of current responses (μA) of GOD modified screen-printed electrodes to glucose substrate solutions of different concentrations. Background (PB) response values have been subtracted.

Glucose in phosphate buffer *(*100 mM NaH_2PO_4-Na_2HPO_4, 100 mM KCl*)*			
Concentration (mM)	Mean $_{(n=3)}$ response in μA	Stdev	CV%
0	0.00	0.007	12.96
3	1.45	0.090	6.21
5	2.31	0.181	7.84
7	2.99	0.170	5.69
8	3.36	0.419	12.47
9	3.74	0.310	8.29
10	4.19	0.260	6.21
12	4.35	0.045	1.04
18	4.34	0.179	4.12

The glucose sensor was compared against a commercially available D-glucose photometric test-kit, the results being in Figure 6.6.

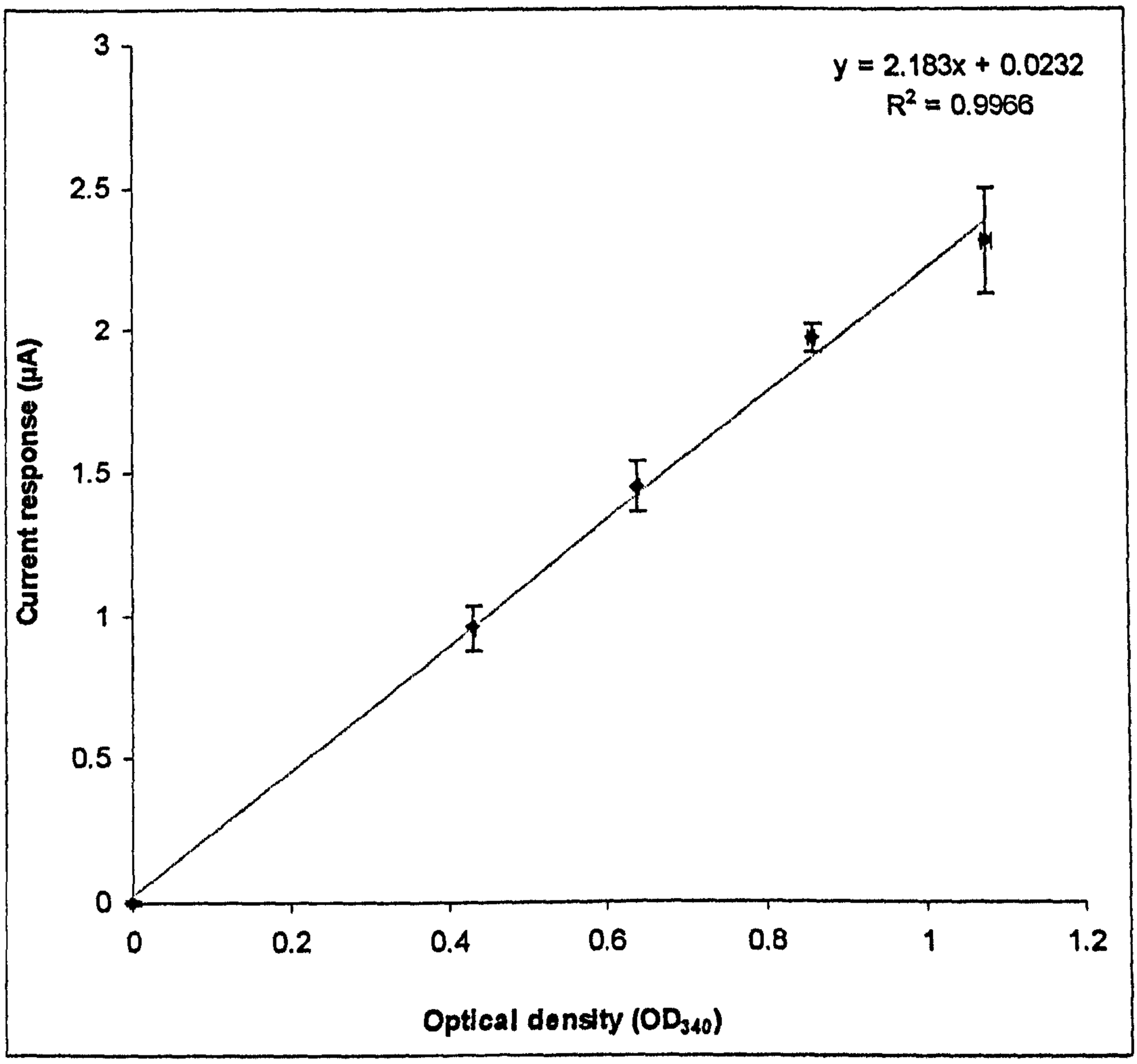

Figure 6.6: Correlation between amperometrical and photometric response for glucose concentrations (2-5 mM). Background response values have been subtracted. Tests were carried out in triplicate (n=3).

The amperometrically and photometrically determined measurements for glucose, showed a very good correlation, with a linear relationship of $R^2 = 0.9966$. The concentration range over which both methods can be directly compared is up to 1 mM.

6.3.2.2 L-amino acid oxidase –based amperometric biosensor

The L-amino acid biosensor was realised by the direct physical immobilisation of L-amino acid oxidase (L-AAO) onto screen-printed electrodes, modified with rhodinised carbon. For both L-amino acid substrates tested, the current versus concentration profile shows a linear region, followed by a tendency towards a plataeu.

A linear relationship between L-leucine concentration and current response is observed up to 1.2 mM, with a correlation coefficient value (r^2) of 0.9989 (Figure 6.7).

The limit of detection (LOD) for leucine, calculated as 3x the standard deviation of the zero analyte response, was 0.07 mM. The experiments showed a linear range for leucine between 0.2-1.2 mM. Analytical data of current (μA) versus concentration (mM) for leucine is given in Table 6.3.

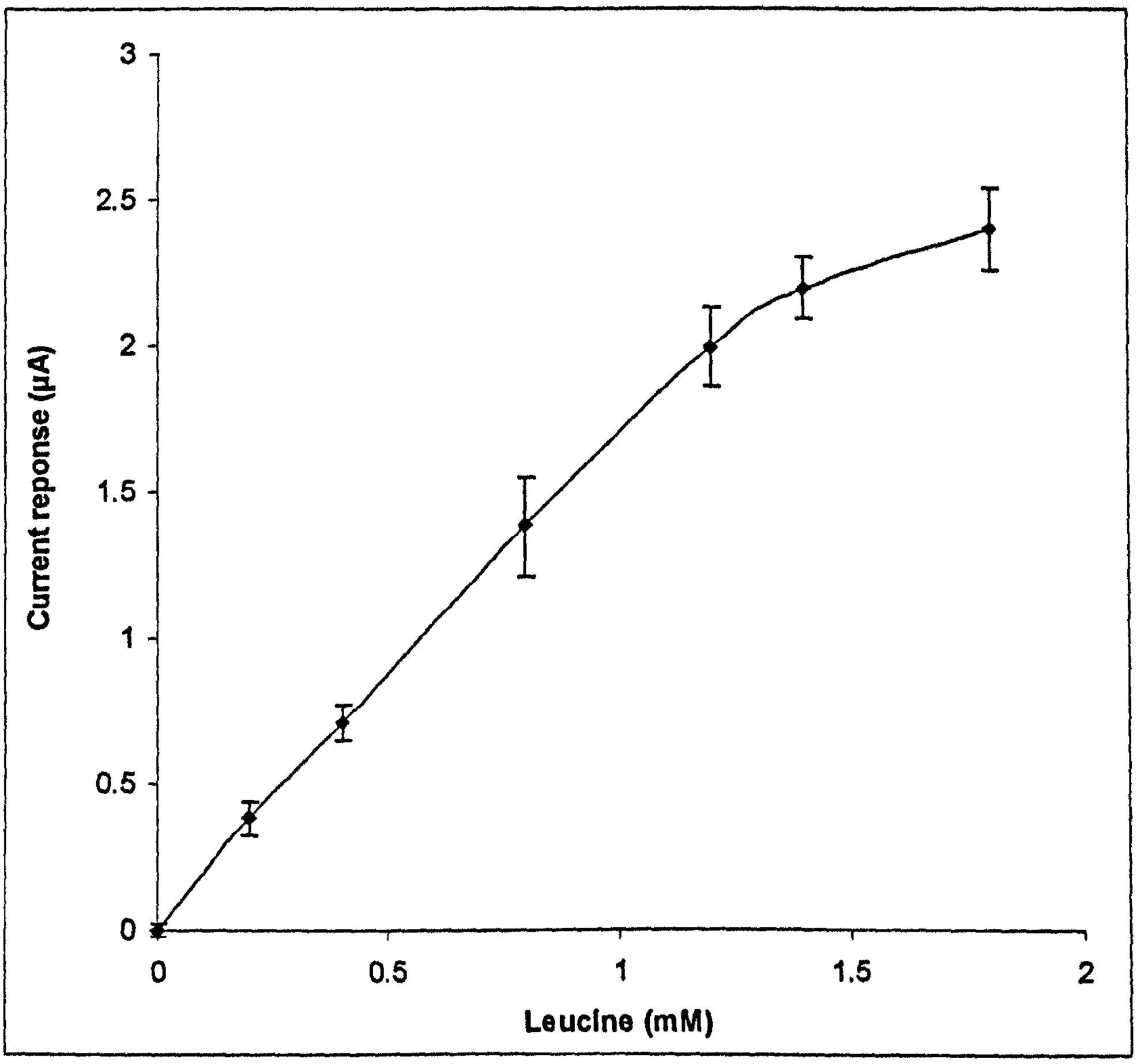

Figure 6.7: Calibration curve for L-leucine with rhodinised carbon screen-printed electrodes, containing immobilised L-AAO. Background response values have been subtracted. Error bars= SD; (n=3).

Table 6.3: Summary of current responses (μA) of L-AAO modified screen-printed electrodes to L-leucine solution of different concentrations. Background response values have been subtracted.

L-leucine in PB(100 mM NaH_2PO_4-Na_2HPO_4, 100 mM KCl)			
Concentration (mM)	Mean $_{(n=3)}$ response in μA	Stdev	CV%
0	0	0.02	-
0.2	0.38	0.06	14.81
0.4	0.71	0.06	8.52
0.8	1.38	0.17	12.31
1.2	2.00	0.14	6.97
1.4	2.20	0.11	4.87
1.8	2.40	0.14	5.79

The limit of detection for L-phenylalanine was calculated 0.08 mM (3x the standard deviation of the zero analyte response). The linear range of detection was from 0.2 mM to 0.8 mM, with a correlation coefficient value (r^2) of 0.9973 % (Figure 6.8). Analytical data of current (μA) versus concentration (mM) is given in Table 6.4.

Table 6.4: Summary of current responses (μA) of L-AAO modified screen-printed electrodes to L-phenylalanine solution of different concentrations. Background (PB) response values have been subtracted.

L-phenylalanine in PB *(*100 mM NaH_2PO_4-Na_2HPO_4, 100 mM KCl*)*			
Concentration (mM)	Mean $_{(n=3)}$ response in μA	Stdev	CV%
0	0	0.03	-
0.2	0.37	0.03	9.18
0.4	0.67	0.12	17.39
0.6	1.11	0.11	9.55
0.8	1.43	0.13	9.32
1	1.45	0.13	9.27

The L-amino acid biosensor was compared against the standard ninhydrin photometric test (Figure 6.9 and 6.10).

A linear relationship was observed for both L-amino acid substrates, indicative of a good correlation between the standard photometric assay and the biosensor measurements. The concentration range over which both methods were directly comparable was 0.2-1.0 mM for leucine and 0.2-0.8 mM for phenylalanine, as dictated by the linear range of the biosensor method.

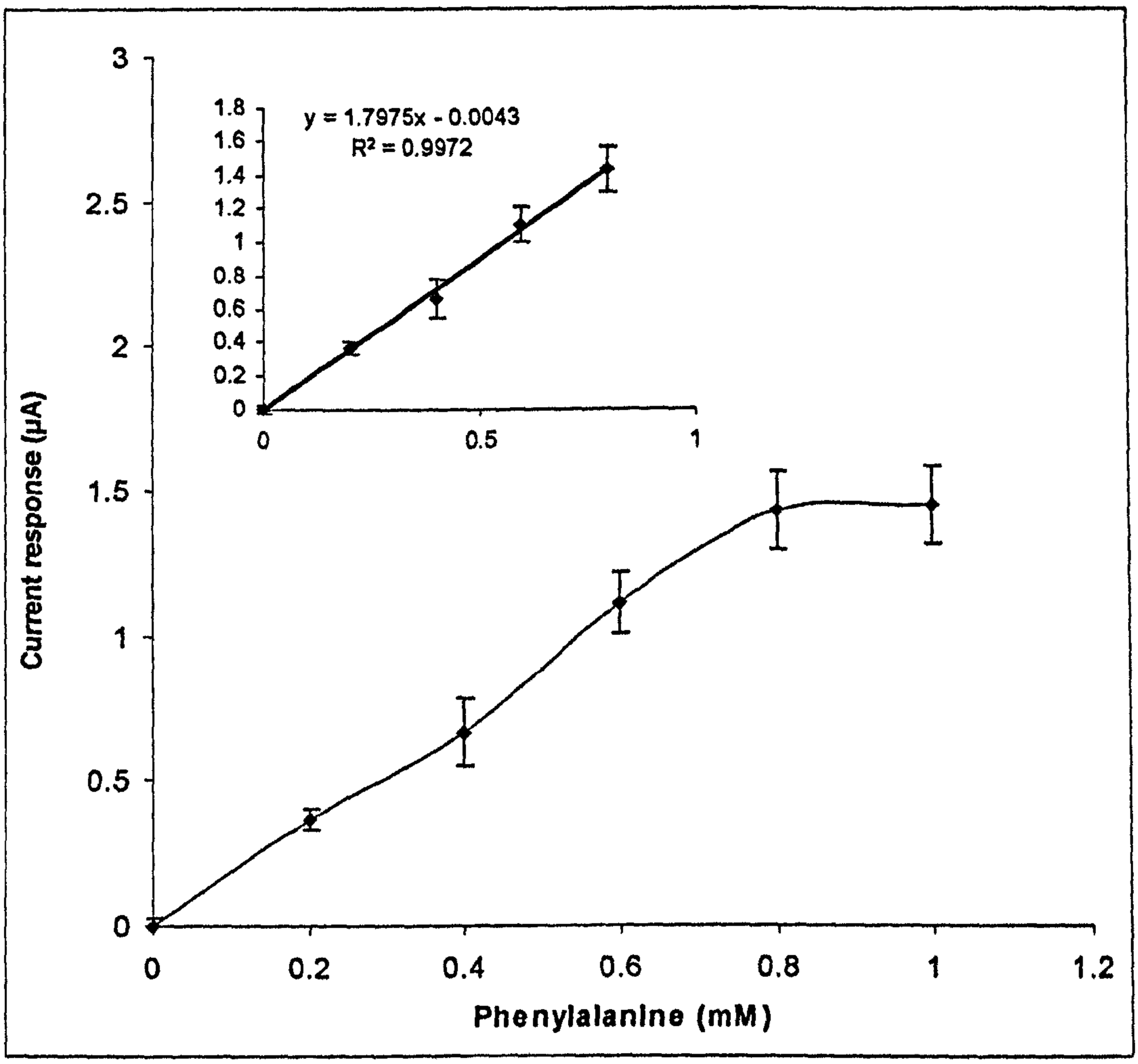

Figure 6.8: Calibration curve for L-phenylalanine with rhodinised carbon screen-printed electrodes, containing immobilised L-AAO. Background response values have been subtracted. Error bars= SD; (n=3).

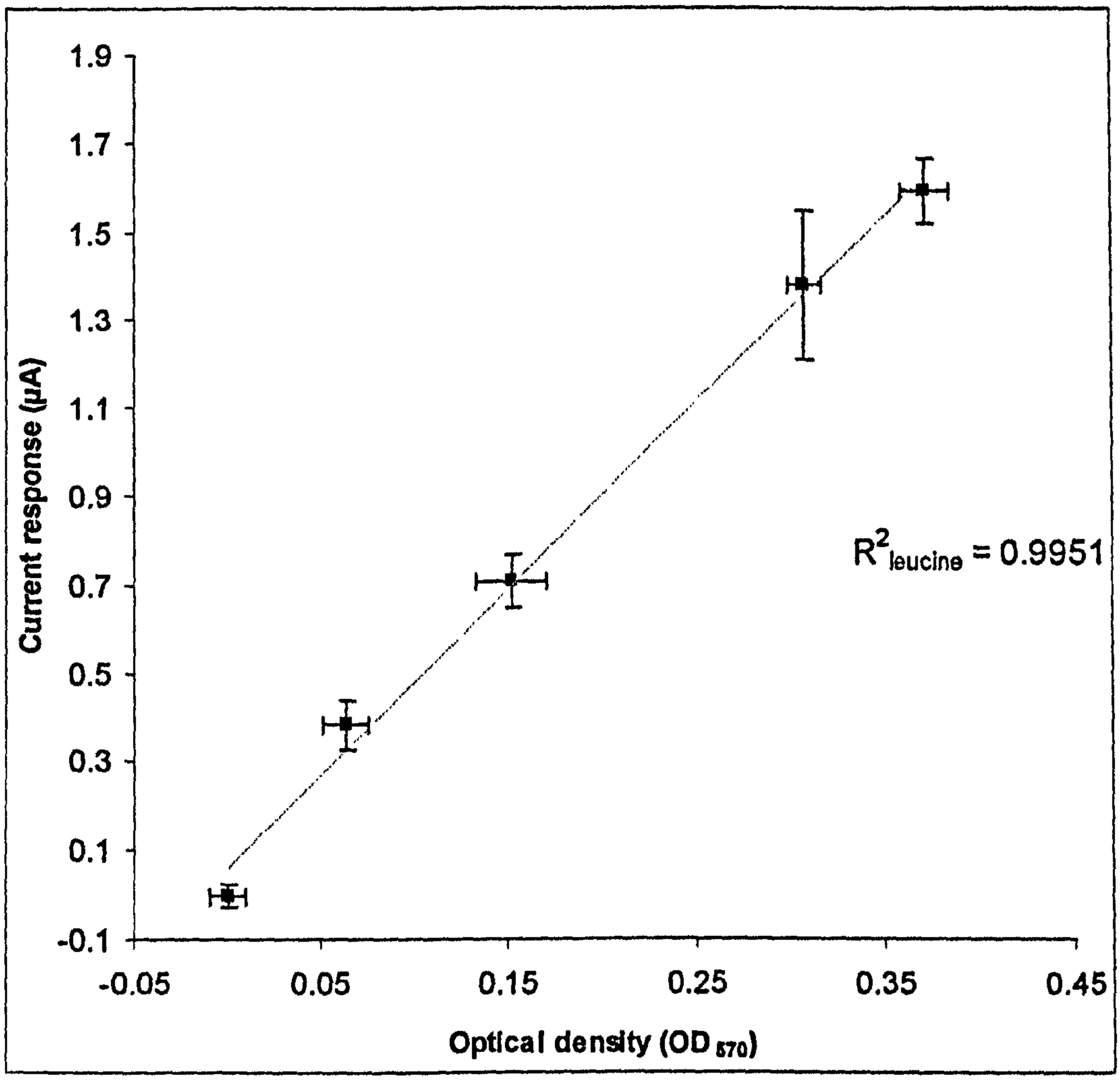

Figure 6.9: Correlation between L-AAO electrode and standard photometric assay response for the measurement of 0-1 mM of leucine. The background responses from both analytical methods have been subtracted. Error bars= SD; (n=3).

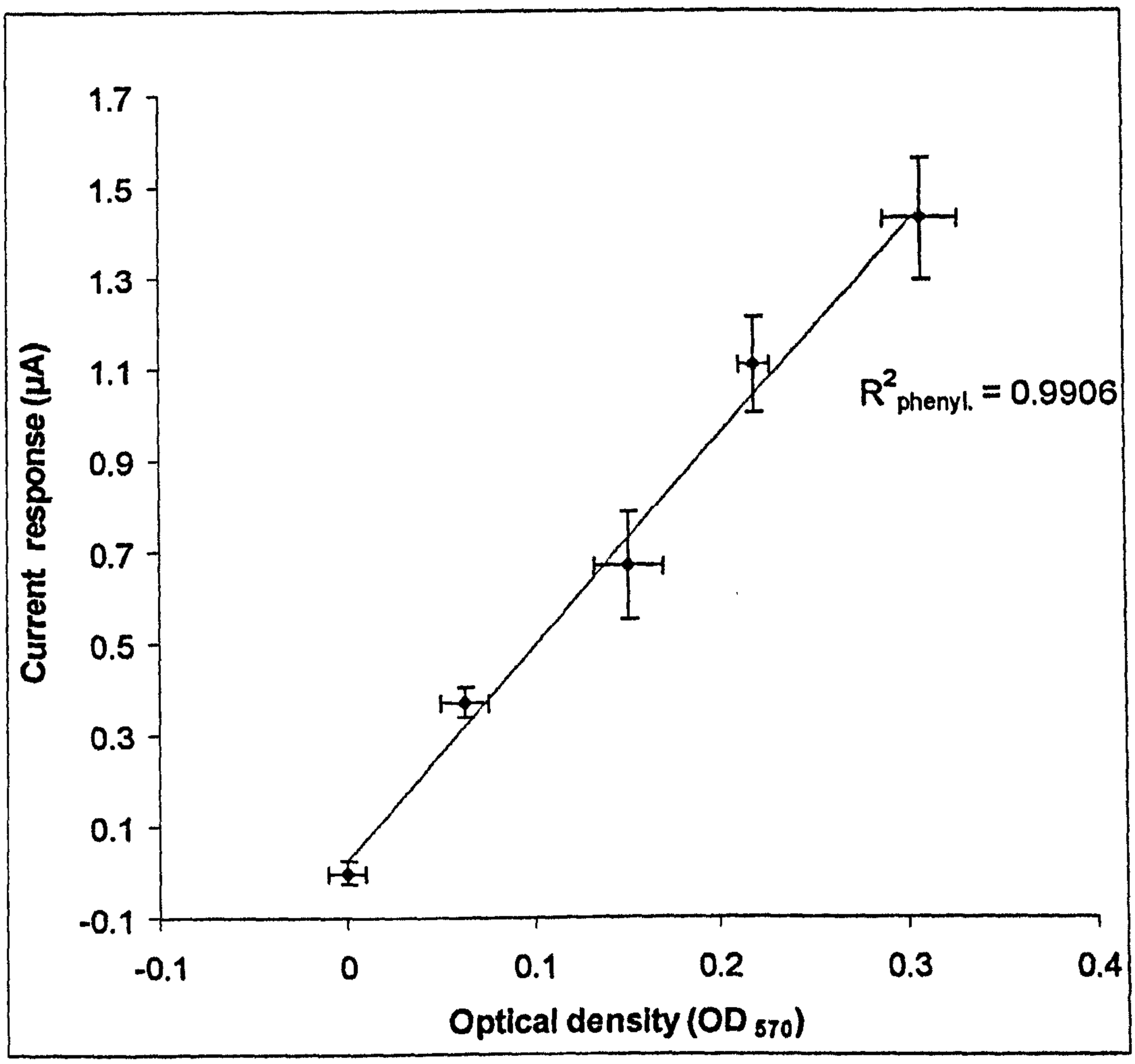

Figure 6.10: Correlation between L-AAO electrode and standard photometric assay response for the measurement of 0-0.8 mM of phenylalanine samples. The background responses from both analytical methods have been subtracted. Error bars= SD; (n=3).

6.3.3 Selection of sample processing step

The effect of sample preparation on the electrochemical and spectophotometric glucose measurement has been investigated on tomato samples (T_1 and T_2). The effect of filtration (section 6.2.7), centrifugation (13000 rpm for 15 min) and lack of sample processing on the electrochemical and spectophotometric detection of glucose levels is shown in Figure 6.11.

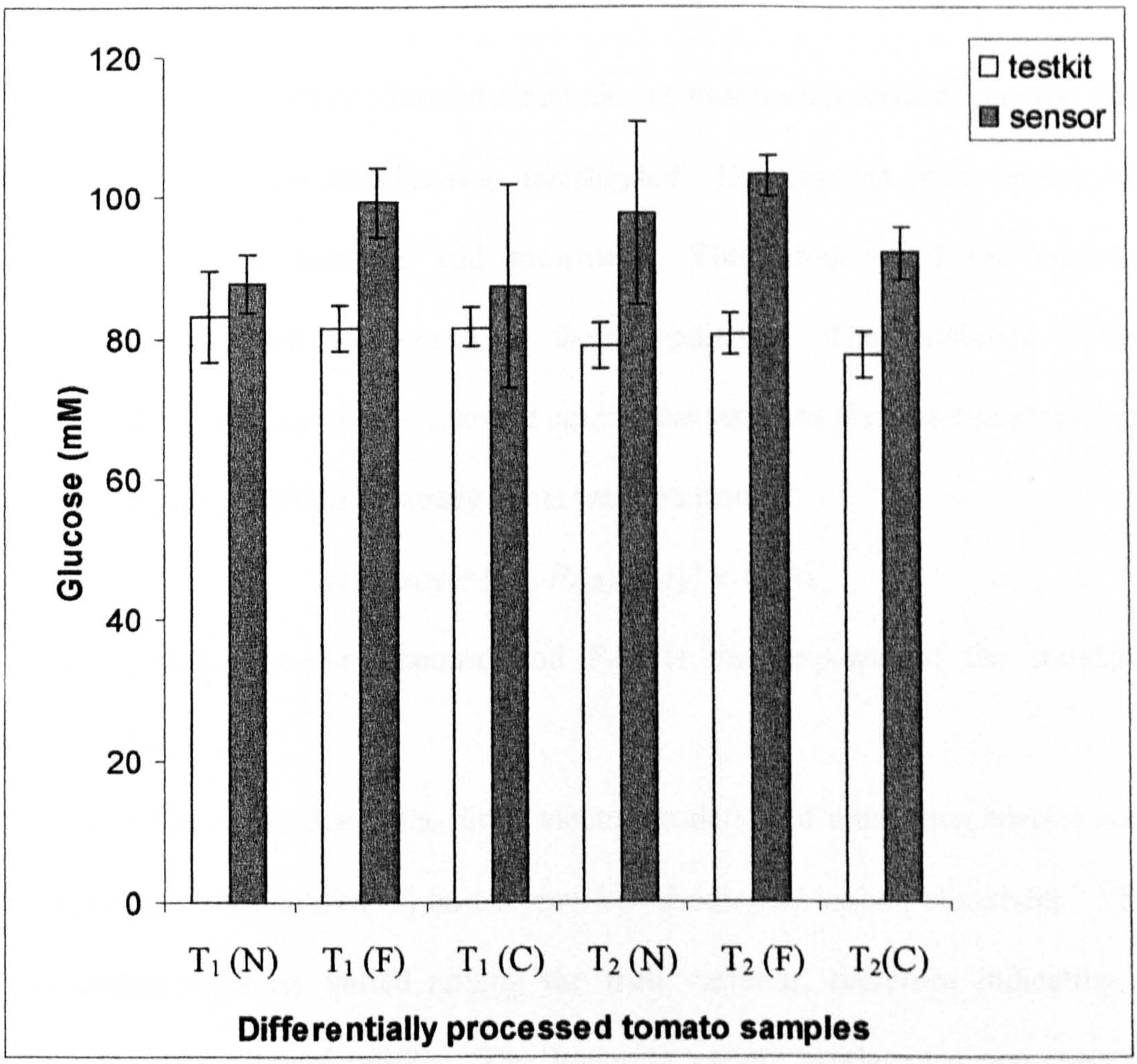

Figure 6.11: Chart shows sensor and test kit measurements of glucose in two tomato samples (T_1 and T_2), differentially processed prior testing [no processing (N), filtrated (F) and centrifugated (C)]. Error bars= SD; (n=3)

The centrifugated samples exhibited slightly greater comparability between the sensor and the test kit response. However, centrifugation may be considered as less–convenient and inappropriate due to the intended field-based usage of the device. Therefore, filtration was proposed as an alternative sample preparation method to minimise the presence of large particulates in the juice extracts.

6.3.4 Sensor performance in real fruit samples

The potential of the screen-printed electrodes as monitoring tools of glucose and L-amino acids in real samples was investigated. Experiments were carried out with fresh apples, potatoes and tomatoes. The selection of the specific horticultural products was based on their popularity. The reliability of the enzyme-based biosensors was assessed against the standard photometric assays, as already described and the accuracy value was determined:

$$\text{Accuracy} = [(S_R\text{-}PA_R)/\ PA_R] \text{ x } 100\%,$$

where S_R is the sensor response and PA_R is the response of the standard photometric assay).

The current response due to the direct electro-oxidation of interfering species was determined in triplicate (n=3) on enzyme-free rhodinised carbon electrodes. The interference response varied among the fruit varieties, therefore indicating a different compositional profile. The Jonagold variety produced a non-specific response of <6.25 μA, more than 2 times higher to the one generated by Bramley apples (<2.56 μA). In potatoes, the non-specific response was <3.45 μA in Russel Burbank and <1.49 μA in King Edwards samples. Mini-plum tomatoes produced a significantly higher interference response, that was determined to be <25.80 μA. The background (non-specific) response of each fruit sample using enzyme-free (or blank) electrodes was subtracted from the equivalent enzyme electrode response, in such a way that:

$$\Delta Rs = \text{Enzyme electrode}_{(amps)} - \text{Blank electrode}_{(amps)},$$

where ΔRs, the electrochemical analytical signal.

6.3.4.1 Glucose measurement in real samples

The performance of the GOD-based biosensor in measuring glucose levels in fresh fruits was determined and the results were compared against the test-kit response (Table 6.5). Jonagold apples, Russel Burbank potatoes and Mini–plum tomatoes were tested, following the preparation step detailed in 6.2.7.

Table 6.5: Comparison of GOD-based amperometric glucose biosensor with the standard photometric test kit assay for analysis of glucose in real fruit samples. Values shown are in mM with n=3.

GOD-based Sensor *versus* Test-Kit results for Glucose							
	Test Kit (mM)	$Stdev_{TK}$	$CV\%_{TK}$	Sensor (mM)	$Stdev_{SR}$	$CV\%_{SR}$	Accuracy %
Apples -Jonagold							
1	100.10	0.35	0.39	101.96	6.58	5.60	**+1.8**
2	118.34	1.25	1.09	125.19	6.83	4.94	**+5.8**
3	116.60	0.60	0.54	117.21	5.70	4.60	**-0.5**
4	117.89	0.43	0.39	108.16	3.07	2.33	**+9.0**
5	140.94	0.62	0.46	163.56	14.82	8.62	**-13.8**
Potatoes-Russel Burbank							
1	9.62	0.66	3.83	8.91	2.46	17.96	**-7.4**
2	6.99	0.35	0.73	5.28	0.11	6.22	**-24.5**
3	8.09	0.35	0.63	4.13	0.97	12.77	**-48.9**
4	10.96	0.32	0.23	8.82	0.78	8.91	**-19.5**
5	8.03	0.38	1.10	7.65	0.59	8.57	**-4.7**
Tomatoes-Mini Plum							
1	88.54	0.08	0.12	61.16	3.83	4.39	**-30.9**
2	91.55	0.62	0.71	50.07	13.00	12.96	**-45.3**
3	89.74	0.33	0.40	74.28	2.83	3.59	**-17.2**
4	79.08	0.83	0.97	99.26	3.92	4.34	**+25.5**
5	78.14	0.42	0.46	103.06	12.96	13.06	**+31.9**

6.3.4.2 L-amino acid measurement in real samples

Samples of extracted juice were prepared from Bramley apples, King Edward potatoes and Mini–plum tomatoes, as described in 6.2.7. The analysed were analysed using the L-AAO-based biosensor and the results were compared with the response of the ninhydrin assay (Table 6.6).

Table 6.6: Comparison of L-AAO-based amperometric amino acid biosensor with the standard photometric ninhydrin assay for analysis of amino acids in real fruit samples. Values shown are in mM with n=3.

L-AAO-based Sensor *versus* Ninhydrin results for L-amino acids							
	Ninhydrin (mM)	$Stdev_{NH}$	$CV\%_{NH}$	Sensor (mM)	$Stdev_{SR}$	$CV\%_{SR}$	Accuracy %
Apples –Bramleys							
1	1.20	0.24	4.25	1.32	0.30	12.14	**10.0**
2	0.87	0.40	6.00	0.82	0.09	4.81	**-5.7**
3	1.01	0.11	1.55	0.58	0.15	7.85	**-42.6**
4	1.51	0.21	3.15	1.01	0.05	2.59	**-33.1**
5	1.65	0.46	6.58	0.47	0.00	0.12	**-71.5**
Potatoes-King Edwards							
1	109.25	0.32	0.28	55.24	1.90	3.41	**-49.4**
2	98.04	2.30	2.34	49.76	3.19	6.26	**-49.2**
3	101.63	10.56	10.38	64.25	5.15	7.80	**-36.8**
4	79.91	1.48	1.83	62.63	2.12	3.35	**-21.6**
5	110.31	15.40	13.95	63.96	4.85	7.46	**-42.0**
Tomatoes-Mini Plum							
1	51.82	2.09	4.02	13.23	0.45	3.03	**-74.5**
2	23.82	0.55	2.24	9.28	0.65	5.76	**-61.0**
3	54.15	0.36	0.64	12.50	1.13	7.68	**-76.9**
4	25.28	0.83	3.24	9.90	0.13	1.18	**-60.8**
5	49.91	2.48	4.94	14.24	0.13	0.89	**-71.5**

6.3.5 Inhibition effects on the screen-printed electrodes

A number of electroactive species found in horticultural samples can produce an electrochemical response and therefore interfere with the enzyme specific signal. In addition, the viscocity and proteinaceous nature of the fruit extracts may cause diffusion constraints for the analyte of interest, thereby interfering with its ability to diffuse through the electrode surface. Inhibitory effects were determined by preparing solutions containing tomato sample extracts containing different concentrations of the analytes of interest and monitoring the consequent biosensor response. Tomato samples were selected as they exhibited the lowest accuracy values between the biosensor and the standard photometric assay response. The amperometric response of the spiked samples was compared with the unspiked counterparts and with solutions in buffer-electrolyte PB (Figure 6.12 and 6.13).

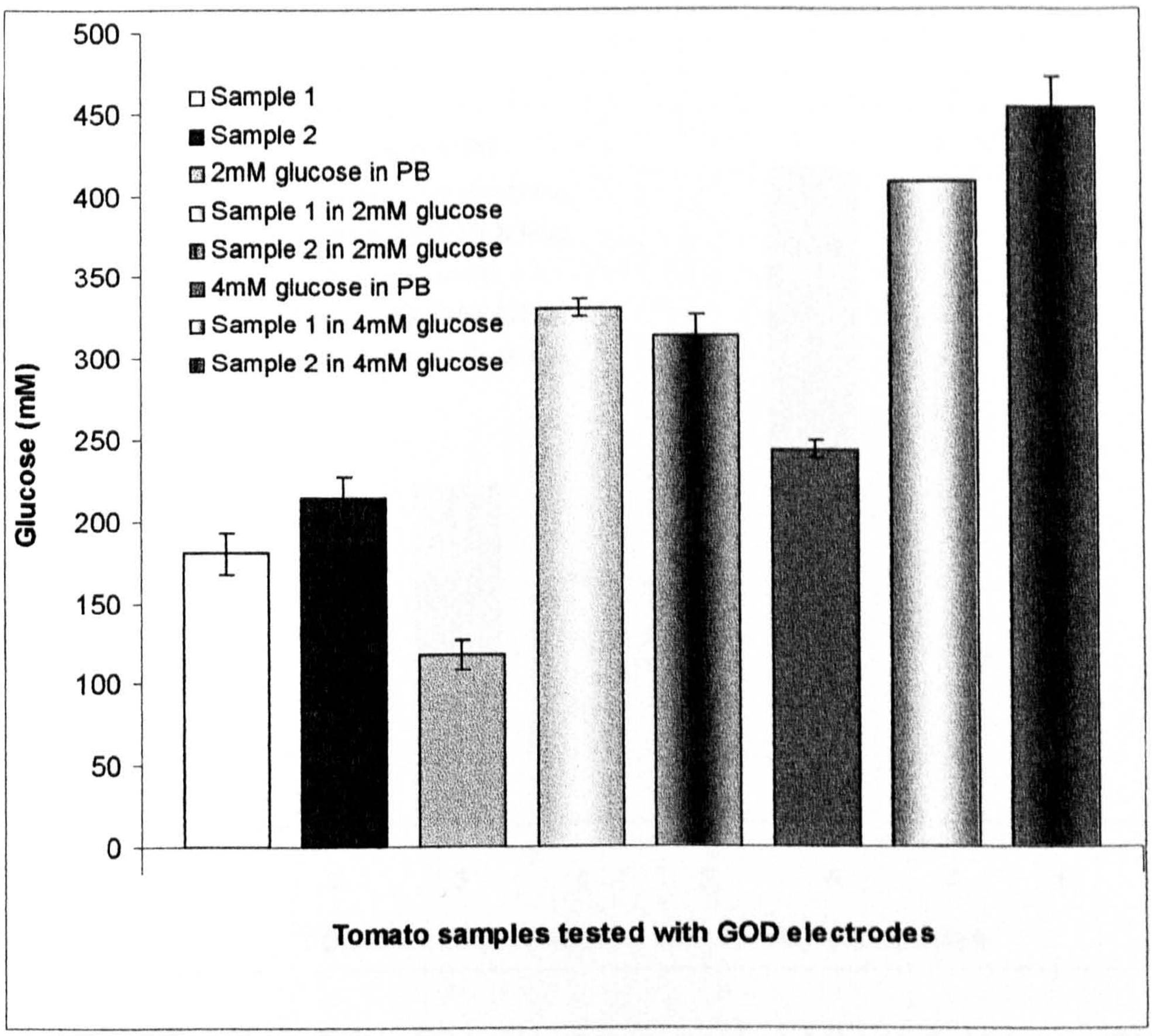

Figure 6.12: The effect of tomato matrix on GOD-based screen-printed electrodes.
Concentration (mM) of glucose measured in two tomato samples. Samples were tested: Unspiked (*columns* 1 and 2); Spiked in 2 mM of glucose (*columns* 4 and 5); and Spiked with 4 mM of glucose (*columns* 7 and 8).
Glucose in PB in concentrations of 2 mM and 4 mM is shown in *columns* 3 and 6, respectively. All samples were diluted accordingly to contain 100 mM NaH_2PO_4-Na_2HPO_4, 100 mM KCl. Background values from electrodes lacking GOD were subtracted. Error bars= SD; (n=3).

Accordingly, inhibitory effects on the L-AAO were determined with solutions containing 0.3 mM or 0.6 mM of leucine in tomato samples.

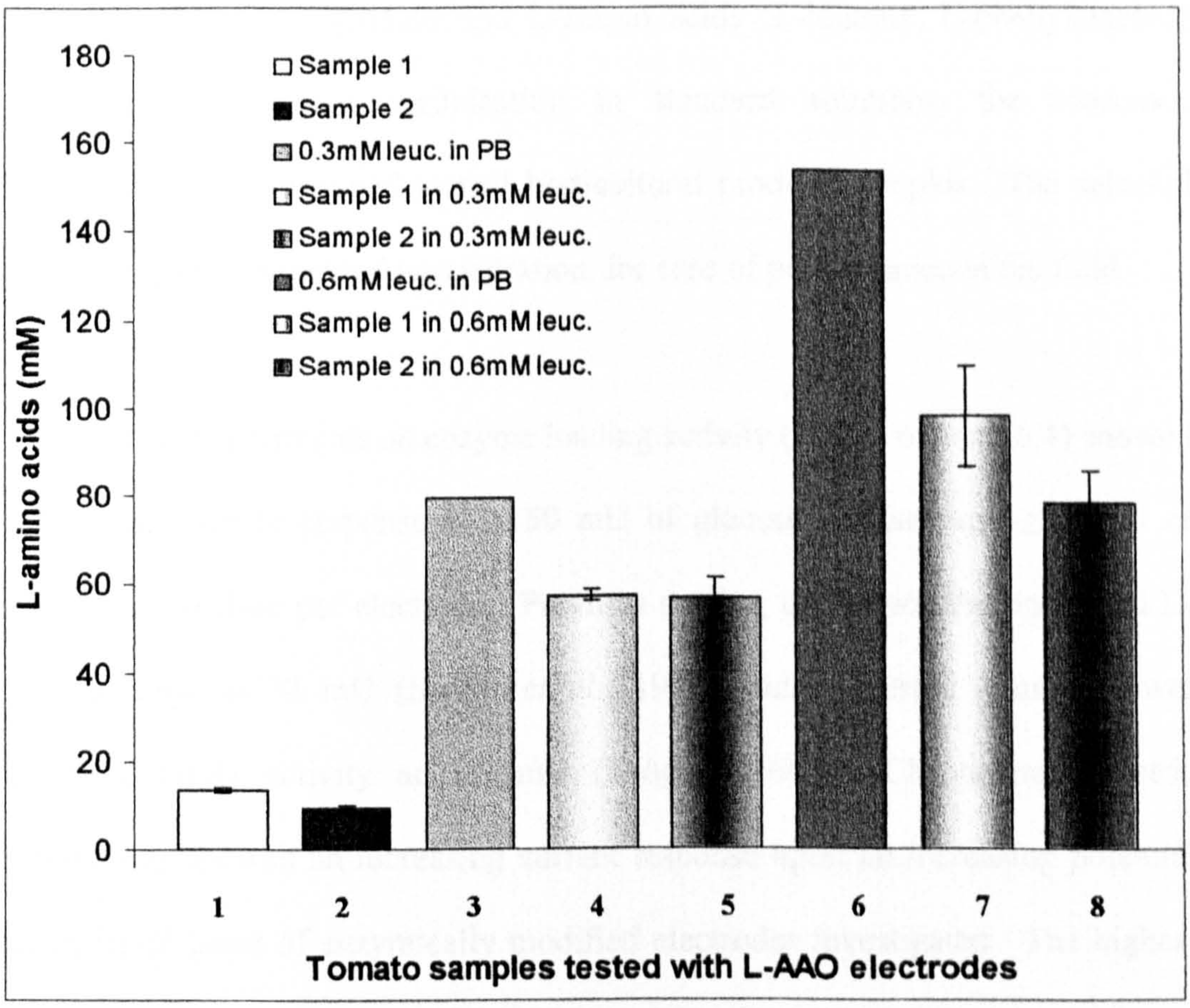

Figure 6.13: The effect of tomato matrix on L-AAO-based screen-printed electrodes.
Concentration (mM) of L-amino acids measured in two tomato samples. Samples were tested: Unspiked (*columns* 1 and 2); Spiked in 0.3 mM of leucine (*columns* 4 and 5); and Spiked with 0.6 mM of leucine (*columns* 7 and 8).
Leucine in PB in concentrations of 0.3 mM and 0.6 mM is shown in *columns* 3 and 6, respectively. All samples were diluted accordingly to contain 100 mM NaH_2PO_4-Na_2HPO_4, 100 mM KCl. Background values from electrodes lacking L-AAO were subtracted. Error bars= SD; (n=3).

6.4 Discussion

The present study involves a preliminary investigation on the biosensor performance in glucose and L-amino acid detection in real horticultural produce. Initially, the glucose and L-amino acid biosensors were characterised using

standard solutions of glucose and L-amino acids (L-leucine, L-phenylalanine) substrates. Following oprimisation in standard solutions, the biosensor performance was examined in real horticultural produce samples. The selected sample preparation method was filtration, for ease of performance in the field.

Optimisation experiments on enzyme loading activity (Figure 6.3 and 6.4) showed a maximum sensor response at ≥150 mU of glucose oxidase and ≥30 mU of amino acid oxidase per electrode. Previous studies, confirmed the optimised L-AAO activity at 30 mU (Sarkar *et al.*, 1999), but suggested a much lower optimised GOD activity at 7.8 mU (Kröger, 1998). Chronoamperometric experiments showed an increasing current response upon an increasing potential status, in all cases of enzymically modified electrodes investigated. The highest signal-to-noise (S/N) ratio (~109:1) obtained for 100 μM of H_2O_2 on bare MCA 4a carbon electrodes was at +350 mV *vs* Ag/AgCl reference electrode (Table 6.1). This potential value was selected as the working detection potential for subsequent experiments. Higher potential values would increase the likelihood of the oxidation of other interfering electroactive species. Previous studies have selected +400 mV *vs* Ag/AgCl as the operational potential for an amino acid biosensor (Sarkar, 1998) and 300 mV for a glucose biosensor (Kröger *et al.*, 1998).

The analytical range of the developed glucose biosensor extended to 10 mM with a correlation factor (r^2) of 0.9960 (Figure 6.5). Comparison of the biosensor to the test kit showed a correlation factor (r^2) 0.9966 (Figure 6.6). The L-amino acid

biosensor was tested on leucine and phenylalanine standard solutions. Both amino acids were shown to exhibit linearity up to 1 mM with correlation factors (r^2) 0.9989 and 0.9972, respectively (Figures 6.7 and 6.8). A correlation of (r^2) 0.9951 and 0.9906 was calculated by comparing the ninhydrin method to L-amino acid sensor in standard leucine and phenylalanine solutions, respectively (Figure 6.9 and 6.10). Despite the excellent biosensor performance in standard solutions, testing in real fruit samples yielded poorer accuracy values.

No published Km data was found for L-AAO (*Crotalus adamanteus*), however data was found for L-AAO *Agkistrodon piscivorus piscivorus*, isolated from the snake venom from cottonmouth moccasin, in which a Km value of 1 mM for L-leucine has been recorded (Tris buffer, pH 7.2, 38°C). Barman (1969) stated that enzyme snake venom from either *A. p. piscivorus* or *C. adamanteus* attacks L-monoamino monocarboxylic acids with the greatest facility, the most rapidly attacked being: leucine, methionine, phenylalanine, norvaline, norleucine, cysteine, tyrosine and tryptophan. Histidine, arginine and ornithine are slowly oxidised and glycine, alanine, serine, threonine and valine exhibit very little or no activities. Given that both types of L-AAO are isolated from snake venom and exhibit a similar amino acid reactivity series, a Km value of around 1 mM would be realistic for L-AAO isolated from *C. adamanteus*, with a slightly lower value for L-phenylalanine.

Test kit and biosensor measurements of glucose in Jonagold apples yielded an accuracy of -13.8 to +9.0 %, indicating a good agreement between the two

methods (Table 6.5). A poorer correlation between the standard method and the sensor measurement was observed in L-amino acid detection (Table 6.6). The accuracy values for L-amino acid detection in Bramleys apples were in the range of -71.5 to +10.0 %. Repeatability in both developed sensors was evident with a precision (n=3) of 2.33 to 8.62 % for glucose and 0.12 to 12.4 % for L-amino acids. The direct electroactivity of sample elements on enzyme-free electrodes generated a non-specific response <6.25 μA in Jonagold and <2.56 μA in Bramley fruits.

Glucose data for Russel Burbank potatoes showed an acceptable accuracy of -48.9 to -4.7 % (Table 6.5). The accuracy values for L-amino acids in King Edwards potatoes, were between -49.4 and -21.6 % (Table 6.6). Biosensor precision (n=3) for glucose was between 6.22 to 17.96 % and for L-amino acids between 3.35 to 7.80 %. The interference response on enzyme-free electrodes was <3.45 μA in Russel Burbank and <1.49 μA in King Edwards potatoes.

Glucose biosensor data for the Mini-plum tomatoes yielded acceptable accuracy (-45.3 to +31.9 %) and a precision (n=3) of 3.59 to 13.06 % (Table 6.5). Accuracy for L-amino acid measurement was low and of the range of -76.9 to -60.8 %, thus indicating a particular poor biosensor performance (Table 6.6). Precision (n=3) values for L-amino acid measurement was between 0.89 to 7.68 %. The presence of readily oxidisable species generated interference response <5.80 μA. This particularly high interference response indicates the presence of fruit elements in high amounts, readily oxidised at the poised potential. Davies

and Winsor (1969) found that smaller tomato fruited varieties, such is Mini-plum, had higher acidity. Acidity in tomatoes is mainly attributed to citric acid and malic acid, which could interfere with the electrochemical detection of the desired analyte at the selected working potential (Arif *et al.*, 2002).

In overall, the performance of the GOD-based biosensor was good, indicating a satisfactory degree of correlation with the standard test-kit method. No matrix component was observed to inhibit the function of the GOD enzyme, as shown in Figure 6.12. The best agreement between the two methods was observed in the case of apples, where the potential of the electrochemical sensor as a reliable rapid (<6 min) analytical method for glucose measurement is evident.

The poorer biosensor performance in the case of potatoes may be due to the fouling on the surface of the working electrode, caused by proteinaceous or polysaccharide materials present in the potato matrix. Presence of solid particles, adsorpted in the MCA 4a layer would dramatically decrease the diffusion of the desired analyte and its subsequent electrochemical detection.

In tomatoes, the high non-specific response from readily electroactive interfering species may be the reason for the low accuracy values. The reduction of the operational potential may be a suggestion in certain cases, although good responses to glucose at potentials lower than +300 mV is normally obtained by the use of more complicated methods than the simple adsorption of GOD onto the working electrode. High sensitivity to H_2O_2 and glucose at low potentials

(+200 mV) was obtained by the immobilisation of GOD on platinum covered by a polymerised heteropolypyrrole film (Li *et al.*, 1999).

Interaction and possible inhibition of the L-AAO enzyme by fruit components was observed in Figure 6.13, thus explaining the broad agreement between the biosensor and the ninhydrin measurement. Some variation between the sensor and the ninhydrin test is anyway expected, as these methods measure different analytes. The quantitative method of ninhydrin test permits the determination of all free amino acids, the exception being proline, and possibly other compounds bearing amino groups. Nevertheless, L-amino acid biosensor detects exclusively free L-amino acids, due to the nature of the recognition element. Additionally, it should be remembered that L-AAO has a wide number of L-amino acid substrates, each of which exhibits different affinity for the enzyme. The sensor sensitivity varies according to the amino acid type, the differences being a function of the amino acid side chain and the Km of the enzyme for each amino acid. An excess of low affinity amino acids would result in a low amperometric response. The L-AAO sensor was calibrated against L-leucine for which the enzyme has a moderate affinity. Asparagine and aspartic acid seem to be the principal amino acids in apples, arginine in potatoes, glutamic acid and aspartic acid in tomatoes (Kader *et al.*, 1978). Despite the fact that the latter amino acids exhibit higher or same affinity to L-AAO, according to Sarkar *et al.* (1999), underestimation of L-amino acid levels was observed in all the samples tested.

6.5 Conclusions

This preliminary study demonstrated the potential of biosensors for horticultural produce quality monitoring. The developed glucose biosensor exhibited encouraging analytical performance in the real fruit samples tested (apples, tomatoes, potatoes). The L-AAO electrodes consistently underestimated the amino acid content of the fruit samples. The latter was found to be primarily due to inhibition effect of the matrix components, but the different affinity of the amino acid substrates to the enzyme may have contributed as well.

CHAPTER 7

SUMMARY OF CONCLUSIONS AND FUTURE CONSIDERATIONS

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SUMMARY OF CONCLUSIONS AND FUTURE CONSIDERATIONS

This chapter outlines the main conclusions of the present work and states some future considerations for each study.

PART 1

GMOs are subject to legislative regulations, rendering the need for their screening particular important. Based on SPR technology, the proposed optical biosensor allows monitoring of DNA hybridisation reactions between DNA molecules in solution ('target') and immobilised counterparts ('probe') on the surface of a gold chip.

Study 1:

Analysis of amplified nucleic acids by surface plasmon resonance (SPR): application to genetically modified organism detection.

The surface of the gold chip is readily modified by a thiol-dextran matrix. The probe, a complementary fragment to the target P35S, was immobilised via a biotin-streptavidin linkage.

Motivation for the study:

The use of complicated and expensive sample post-PCR treatment for the detection of DNA-DNA hybridization using SPR technology.

Aim of the study:

Optimised coupling of Polymerase Chain Reaction (PCR) and Surface Plasmon Resonance (SPR) for rapid and cost-effective DNA hybridization monitoring with application to GMO analysis. The performance of different strategies was assessed.

Main outcomes and future considerations:

- Both P35S and TNOS probes were highly target specific, generating maximum interference response of <6 RU.
- The sensor baseline showed acceptable stability over a day of measurements (12 h period-30 measurements maximum) with a decrease of up to 30 RU, as a result of the effect of HCl and the running buffer flow conditions on the probe surface.
- Hybridisation efficiency decreased over successive days of measurement, due to the overnight flows and the hybridization reactions. A CV of 42 % was observed in the hybridization shift of target DNA (100 nM) after 7 overnight flow treatments and 91 hybridisation cycles.
- The detection limit, experimentally established was <5 nM. Therefore, the dynamic range of the sensor for the target P35S (25-mer) was 5-50 nM.

- Optimisation at the PCR amplification stage was achieved by the selective amplification of the target strand, using an asymmetric amplification scheme. The target strand contained the 25-mer P35S sequence of interest, complementary to the immobilised probe.
- Optimisation of the post-PCR amplification stage was accomplished by the use of strong denaturing conditions, consisting of 0.3 M NaOH and 20 % v/v formamide. The adopted denaturing conditions eliminated the possible intra-strand secondary structures within the asymmetrically amplified target strand (243 bp).
- Using the optimised conditions, the SPR system successfully detected PCR amplified samples of GMO origin. Screening of PCR amplified samples is simple and rapid, with 30 min of sample treatment and 5 min of measurement.

This work has demonstrated the potential use of the optimised SPR sensing for routine GMO screening analysis. Most laboratories in the food industry use conventional PCR to which SPR technology can be successfully coupled for GMO analysis. The usefulness of SPR technology in routine analysis is the elimination of the gel electrophoresis post-amplification control, which is time consuming and does not provide any sequence information for the amplified samples. Based on DNA-DNA hybridisation reaction monitoring, the proposed optimised system offers sequence specificity and rapidity of measurement. Improvements on the PCR-SPR coupling can include further optimisation of the PCR conditions. Modification of the PCR conditions may include fewer cycles

that could still produce amplified products beyond the detection limit of the SPR system. This would further increase the rapidity of sample screening. To provide estimation for the GMO content, further optimisation of the PCR cycles may be useful. Within the linear range of the amplification curve, the PCR products may be directly correlated with the quantity of the DNA starting material. Therefore, choosing the cycle number that corresponds to the linear range of amplification and leads to PCR products beyond the SPR detection limit would provide an approximation of the GMO content, if an appropriate calibration curve is prepared and the DNA starting material is not denatured.

Study 2:

Analysis of amplified nucleic acids by surface plasmon resonance (SPR): application to genetically modified organism detection.

A thiol-functionalised probe was immobilised on the surface of a gold chip, as described by Herne *et al.*, 1997. Immobilisation was carried out outside the BIAcore instument. The DNA part of the thiolated probe (25-mer) was complementary to the target P35S DNA in solution.

Motivation of the study:

To investigate an alternative to the thiol-dextran based immobilisation method for cost-effectiveness and simplicity.

Aim of the study:

Preliminary investigation on the characterisation and application of a thiol-functionalised probe for SPR BIAcore applications.

Main outcomes and future considerations:

- Optimisation of the hybridisation conditions was accomplished by the addition of Tween 20 (0.005 % v/v), which minimised non-specific events.
- The probe system based on the thiol-derivatised probe was highly specific for the target sequence, generating minimum interference shifts for non-complementary sequences (<8 RU).
- The experimentally established detection limit was 5 nM of untreated target P35S (25-mer).
- The thiol-functionalised probe showed significant stability and stress-resistance against the overnight flow treatments and the successive hybridisation reactions. A CV<9 % in the average (n=3) shift for 100 nM of target P35S was produced, over 2 overnight flows and 28 hybridisation cycles. The repeatability of the generated signal in one day of measurement expressed in CV, was 5 % for 100 nM of untreated target P35S.
- Reproducibility of hybridisation efficiency between different sensor chips (100-140 RU hybridisation shifts for 100nM of P35S target over two different chips).

- The mixed SAM-modified gold chip showed good lifetime. Reduction of 18-22 % of the hybridisation shift of untreated P35S (100 nM) was recorded after 3 overnight flows and 50 hybridisation cycles.
- In comparison to the thiol-dextran immobilisation method, the HS-functionalised probe exhibited higher reproducibility and repeatability of the generated signal, but lower hybridisation capacity.
- No matrix effects were observed in the presence of large amounts of genomic DNA.
- The denaturing conditions consisted of 0.3M NaOH and 20 % v/v formamide led to regeneration problems indicating non-specific events. Up to 10 % of the generated hybridisation shift for 100 nM of treated P35S target, was found to remain on the probe surface after one regeneration cycle. It was postulated that this phenomenon was due to the interaction of the OH-terminated blocking thiol molecules with the amide salt.
- SPR analysis of denatured oligo-dsDNA using 0.3M NaOH and 20 % formamide (v/v) led to an average (n=3) hybridisation shift of 58 RU and a CV< 20 %, suggesting the capability of the system to detect dsDNA of short length (25-mer).
- Less promising were the results with polynucleotide DNA sequences, indicating the need of optimised denaturing conditions and improvements in the immobilisation protocol.

Optimisation of the SPR system should be considered at the probe level, PCR level and the sample pre-treatment stage. At the probe level, optimisation is

needed for the system to be capable of hybridisation with polynucleotide target, such as PCR amplified DNA fragments. This may be achieved by a controlled spatial distribution of the thiolated probe molecules on the gold surface. Optimisation at the PCR level may include the amplification of shorter amplicons to aid hybridisation with the oligonucleotide probe. Optimisation at the level of sample treatment is also essential. Strategies at the sample pre-treatment level may include more sophisticated procedures, such as the use of paramagnetic particles, since the low cost of the sensor can compromise the cost of a more expensive sample treatment.

PART 2

Glucose and L-amino acids may be used as indicators for fruit maturation and colour development in potatoes, and therefore their determination is important in food analysis. Based on electrochemical detection of H_2O_2, the screen-printed electrodes are of particular interest due to their potential field–based use.

Study 3:
Development of an electrochemical biosensor for the detection of glucose and L-amino acids in horticultural produce.

Amperometric detection of glucose and L-amino acids was based on immobilised glucose oxidase and L-amino acid oxidase on the surface of rhodinised carbon electrodes. The analytical performance of the developed biosensors was assessed against standard methods of detection.

Motivation for the study:

The lack of validated biosensors for field-based use for glucose and L-amino acid measurement in horticultural produce.

Aim of the study:

To produce and optimise screen-printed glucose oxidase (GOD) and L-amino acid oxidase (L-AAO) electrodes. The analytical performance of the developed screen-printed electrodes was investigated in fresh fruit sample testing.

Main outcomes and future considerations:

- Comparison of the screen-printed electrodes to standard or commercially avalaible techniques, revealed a correlation factor of 0.9966 (R^2) for glucose and 0.9951/0.9906 for L-leucine/L-phenylalanine.
- The experimentally established analytical range of the developed biosensors was 3-10 mM for glucose detection, 0.2-1.0 mM for L-leucine and 0.2-0.8 mM for L-phenylalanine.
- Filtration was selected as the sample preparation method, prior to testing, for ease of performance in the field.
- The glucose oxidase-based biosensor showed encouraging performance in real horticultural sample analysis. No enzyme-inhibition effects were observed. In Russel Burbank potatoes an acceptable accuracy of -48.9 to -4.7 % was observed. Similar accuracy values were yielded in Mini-plum tomatoes analysis with an accuracy of -45.3 to +31.9 %. The best

biosensor performance was monitored in Jonagold apples with an accuracy of +1.8 to -13.8 %.

- The L-amino acid oxidase-based biosensor showed poorer analytical performance in real horticultural sample analysis. The accuracy values in Bramleys apples were in the range of -71.5 to 10.0 %. Analysis of Mini-plum tomatoes led to accuracy values of -76.9 to -60.8 %, whereas the best biosensor performance was observed in King Edwards potatoes with accuracy values between -49.4 and -21.6 %. This broad agreement between the sensor and the ninhydrin measurements was attributed to enzyme inhibition effects.

Further optimisation is needed to improve sensor selectivity, compatibility and to eliminate interference response of the developed L-AAO and GOD biosensors. The use of polymeric membranes is an option that could be taken into consideration. Enhanced sensitivity and interference free response in glucose detection was achieved by the covalent immobilisation of GOD and further covering with a non-conductive heteropolypyrrole film (Li *et al.*, 1998).

CHAPTER 8

REFERENCES

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CHAPTER 9

PRESENTATIONS AND PUBLICATIONS

CHAPTER 9

PRESENTATIONS AND PUBLICATIONS

Giakoumaki, E., Setford, S.J and Tothill.

Measurement of horticultural produce quality, using a developed glucose and an L-amino acid biosensor.

2nd Postgraduate Conference, Cranfield University at Silsoe, 2001

Giakoumaki, E., Tombelli, S., Mannelli, I., Minunni, M., Mascini, M.

Optimisation of a DNA biosensor coupled with PCR for GMOs detection.

Attualità ed Interdisciplinarità della Chimica Analytica

Conference in memory of Prof. Liberti, 2002

Giakoumaki, E., Tombelli, S., Minunni, M., Tothill, I.E., Mascini, M.

Improved DNA sensing based on SPR transduction coupled with PCR for GMOs detection.

World Congress in Biosensors: 'Biosensors 2002', Kyoto, Japan, 2002.

Giakoumaki, E., Minunni, M., Tombelli, S., Tothill, I.E., Mascini, M., Bogani, P., Buiatti, M. (2003). Combination of amplification and post-ampification strategies to improve oprical DNA sensing. *Biosensors and Bioelectonics*, **19**, 337-344.

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CHAPTER 9

PRESENTATIONS AND PUBLICATIONS

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PRESENTATIONS AND PUBLICATIONS

Giakoumaki, E., Setford, S.J and Tothill.

Measurement of horticultural produce quality, using a developed glucose and an L-amino acid biosensor.

2nd Postgraduate Conference, Cranfield University at Silsoe, 2001

Giakoumaki, E., Tombelli, S., Mannelli, I., Minunni, M., Mascini, M.

Optimisation of a DNA biosensor coupled with PCR for GMOs detection.

Attualità ed Interdisciplinarità della Chimica Analytica

Conference in memory of Prof. Liberti, 2002

Giakoumaki, E., Tombelli, S., Minunni, M., Tothill, I.E., Mascini, M.

Improved DNA sensing based on SPR transduction coupled with PCR for GMOs detection.

World Congress in Biosensors: 'Biosensors 2002', Kyoto, Japan, 2002.

Giakoumaki, E., Minunni, M., Tombelli, S., Tothill, I.E., Mascini, M., Bogani, P., Buiatti, M. (2003). Combination of amplification and post-ampification strategies to improve oprical DNA sensing. *Biosensors and Bioelectonics*, **19**, 337-344.

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Combination of amplification and post-amplification strategies to improve optical DNA sensing

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Abstract

The work evaluated a series of approaches to optimise detection of polymerase chain reaction (PCR) amplified DNA samples by an optical sensor based on surface plasmon resonance (SPR) (BiacoreX™). The optimised procedure was based on an asymmetric PCR amplification system to amplify predominantly one DNA strand, containing the sequence complementary to a specific probe. The study moved into two directions, aiming to improve the analytical performance of SPR detection in PCR amplified products. One approach concerned the application of new strategies at the level of PCR, i.e. asymmetric PCR to obtain ssDNA amplified fragments containing the target capable of hybridisation with the immobilised complementary probe. The other strategy focused on the post-PCR amplification stage. Optimised denaturing conditions were applied to both symmetrically and asymmetrically amplified fragments. The effective combination of the two strategies allowed a rapid and specific hybridisation reaction. The developed method was successfully applied in the detection of genetically modified organisms.

Keywords: Surface plasmon resonance; DNA sensing; PCR; Denaturation; GMO

1. Introduction

Since its first publication in 1985 (Saiki et al., 1985) polymerase chain reaction (PCR) has opened new bio-analytical avenues in forensic, medical, environmental and food sciences. Post-PCR nucleic acid analysis is routinely performed by gel electrophoresis. This method however, fails to provide any sequence information of the amplified DNA. Problems may arise from the amplification of a non-specific sequence having nearly the same length with the desired fragment. Southern blotting satisfies the requirement for sequence information, but it is not recommended for routine analysis, as it involves several steps. Surface plasmon resonance (SPR) detection of PCR products offers a rapid and sequence specific alternative to the usual analytical methods (Kai et al., 1999, 2000; Sawata et al., 1999; Bianchi et al., 1997; Nilsson et al., 1997). Prior to SPR testing, DNA fragments which, amplified by ordinary PCR, are double-stranded, require denaturation steps of long duration which lack practicality (Sawata et al., 1999). This sample pre-treatment is necessary to obtain single stranded DNA fragments capable of hybridisation with the probe immobilised on the sensor surface. A previous work from our group (Mariotti et al., 2002) on SPR based DNA sensing, revealed that heat denaturation alone (95 °C for 5 and 1 min in ice) of the PCR products is not efficient for strand separation, as it leads to re-annealing of the denatured strands. On this basis, more complex sample treatments had to be employed. Magnetic particle separation solved the problem but it was considered to be an expensive and time-consuming method, unsuitable for routine analysis of a large number of samples.

Several research groups have applied the SPR technology to detect DNA hybridisation with peptide

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nucleic acids (PNA) (Kai et al., 2000; Sawata et al., 1999; Burgener et al., 2000; Feriotto et al., 2001). Although successful, the system was not cost-effective due to the use of expensive PNA. Moreover, a heat denaturation step was usually required prior to SPR testing (Kai et al., 2000; Sawata et al., 1999).

Further development of the detection system employed an optimised PCR procedure that predominantly produces single-stranded DNA fragments (Bianchi et al., 1997; Innis et al., 1998; Eggerding et al., 1991). In the case of Kai et al. (1999), two different asymmetrically amplified fragments were produced, differing in length. Following a heat denaturation step, the asymmetrically amplified products were hybridised to produce a unilateral protruding DNA (UPD) fragment. Despite the sensitivity and reproducibility that the system provides, generation of UPD fragments makes the detection system more complicated.

Feriotto et al. (2002) also applied asymmetric PCR and SPR technology, in the detection of genetically modified organisms (GMOs). The best biosensor performance was achieved upon immobilisation of a PCR biotinylated product on the sensor chip surface and the subsequent hybridisation with its complementary asymmetrically amplified counterpart present in solution.

Moreover, detection of asymmetrically amplified products without a pre-treatment step has been reported for the diagnosis of human immunodeficiency virus type I (HIV-1) (Bianchi et al., 1997).

Biotechnology is rapidly developing to deliver highly specific, reproducible and cost-effective screening methods for the detection of desired analytes. Moving towards a more straight forward and rapid detection of PCR products, we investigated some amplification and post-amplification strategies for the detection of PCR products based on SPR transduction (BiacoreX™).

In this work, several approaches were examined to obtain significant and specific DNA–DNA hybridisation. The different approaches were applied in the analysis of specific DNA sequences present in various GMOs. These sequences are contained in the promoter region (35S) of the cauliflower mosaic virus (CAMV) ribosomal RNA.

To improve the analytical performance of SPR detection in PCR amplified products, different strategies both at the PCR and post-PCR level were evaluated. At the level of PCR, an optimised scheme was applied to obtain ssDNA amplified fragments containing the target 35S capable of hybridisation with the immobilised complementary probe. At the post-PCR amplification stage, optimised denaturing conditions were applied to both symmetrically and asymmetrically amplified fragments.

Improvement of the denaturing conditions was performed by the combination of a strong alkaline environment and a formamide treatment at 42 °C. Denaturation of dsDNA in strong alkaline conditions is well established. At a pH $\geq$ 13 the charge of the DNA bases changes, thus preventing H-bond formation (Alberts et al., 1994). On the other hand, the influence of organic compounds such as formamide, urea and formaldehyde on the thermal DNA denaturation process has been well documented (Sambrook et al., 1989). Formamide is a helix destabiliser that replaces the native DNA bases for inter-strand hydrogen bonds, thus inducing the denaturation of dsDNA (Bhattacharyya and Feingold, 2001). High temperature denaturation (95 °C for 5 min followed by 1 min in ice) was here used as a reference method.

2. Materials and methods

2.1. Apparatus and reagents

For all the experiments the SPR device BiacoreX™ and a dextran modified sensor chip (CM5) were used (Biacore AB Uppsala, Sweden). All experiments were conducted at a flow rate of 5 μl/min and 25 °C.

N-hydroxysuccinimide, 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDAC) and streptavidin were all purchased from Sigma Aldrich (Milan, Italy). Synthetic oligonucleotides were purchased from Sigma Genosys (Cambridge, UK). The buffer reagents and ethanol were purchased from Merck (Rome, Italy). All the other reagents were purchased from Sigma Aldrich (Milan, Italy).

The base sequences of the 5′-biotinylated probes (25-mer) were: probe 35S (5′ biotin-GGCCATCGTTGAAGATGCCTCTGCC 3′) and non-specific probe (5′ biotin-AATGATTAATTGCGGGACTCTAATC 3′).

2.2. DNA samples

DNA samples consisted in synthetic oligonucleotides and PCR amplified products of DNA extracted from different sources.

2.2.1. 25mer-Synthetic oligonucleotides

The sequence of interest, complementary to the immobilised 35S probe was: target 35S (GGCAGAGGCATCTTCAACGATGGCC). A sequence for negative control was also used (non-complementary strand: GATTAGAGTCCCGCAATTAATCATT).

A dsDNA helix was also constructed by co-incubation of equimolar amounts (0.1 μM) of probe and target 35S. Incubation was conducted for 1 h at 25 °C. The resulted dsDNA oligonucleotide helices were treated as a model type of symmetrically amplified samples (see below).

2.2.2. DNA samples of higher complexity

A) Commercially available Certified Reference Material (CRM) from soybean powder (2% Roundup Ready™, Fluka, Italy);
B) PBI121 plasmid (13 kbp) was purchased from BD Biosciences Clontech (Oxford, UK) and it contains a 814 bp fragment of promoter 35S. The plasmid was extracted from genetically modified *E. coli*, previously transformed with the plasmid;
C) Maize samples from animal feed.

Following extraction, all the samples A, B, C were PCR amplified.

The control solution (PCR blank) consisted of all the PCR reagents except the DNA template.

2.3. DNA extraction and isolation

PBI121 DNA was isolated from *E. coli* cells, using the QIAGEN Plasmid Mini Kit (Qiagen, Milan Italy). Following extraction, the PBI121 DNA samples were suspended in ddH_2O.

DNA extraction from the sources A and C, followed the instructions of the Nucleospin Plant kit (Macherey-Nagel, Duren, Germany) and the Wizard® Magnetic DNA Purification System for Food (Promega, Milan, Italy). The extracted and purified DNA samples were suspended in the elution buffer provided in the DNA extraction kit.

The concentration of the extracted DNA material was determined by measuring the fluorescence of the Hoechst 33258-DNA complex, by the use of a Hoefer TKO-100 minifluorometer (Amersham-Biotech, Milan, Italy).

2.4. Symmetric and asymmetric PCR amplification

The sense (5′ GCTCCTACAAATGCCATCATT 3′) and antisense (5′ CTCCAAATGAAATGAAC 3′) primers (MWG-BIOTECH, Florence, Italy) amplified a 243 bp DNA fragment, containing the target sequence 35S (25-mer). Both primers were designed using the OLIGO® Primer Analysis software.

The PCR reaction mixture contained 100–300 ng of isolated DNA from sources A and C or 10 ng from B, 2 units of Taq polymerase (Amersham-Biotech, Upsala, Sweden) and 100 mM of each desoxy-ribonucleotide-triphosphate (dNTP) (Amersham-Biotech, Uppsala, Sweden).

The 50 μl PCR mixture for symmetric PCR contained equal amounts of sense and antisense primer solutions (200 nmol). The PCR conditions were: 94 °C for 4 min, 50 °C for 1 min and 72 °C for 2 min (35 cycles). The asymmetric PCR was performed in the same conditions for 50 cycles but with a sense/antisense primer ratio of 1/50 pmol. All PCR experiments were conducted by a Perkin Elmer Thermal cycler (model 9600) (Perkin Elmer, Shelton, USA).

Following amplification, the PCR products were ethanol precipitated (1 volume of sample per 2.5 volumes of ethanol) and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA concentration of the amplified products was determined spectrophotometrically at 260 nm. Screening of the PCR products was performed by gel electrophoresis (Sambrook et al., 1989) and visualised through a UV transilluminator.

2.5. Denaturation of DNA samples

Prior to SPR testing, the PCR samples were subjected to a denaturation step in order to obtain ssDNA fragments. As a part of our optimisation strategies, different denaturation methods were employed and their efficiency to obtain adequate amounts of ssDNA for hybridisation was examined.

2.5.1. High temperature denaturation

High temperature denaturation (95 °C) was conducted as a reference procedure with or without the addition of formamide (20%). The followed protocol is well documented in many previous studies on DNA-based biosensors (Mariotti et al., 2002; Tombelli et al., 2000a,b; Minunni et al., 2001). It involves a 5 min incubation at 95 °C followed by 1 min in ice.

2.5.2. Denaturation in alkaline conditions

The second method employed a combination of alkaline and thermal conditions along with a formamide treatment (Harwood, 1996). Formamide was used in different concentrations and all in accordance with Biacore operating instructions. The DNA containing denaturation mixtures, were all of 60 μl final volume and consisted of: 0.3 M NaOH, 0 or 10 or 20% formamide and hybridisation buffer (NaCl 150 mM, Na_2HPO_4 20 mM, EDTA 0.1 mM, pH 7.4) to adjust close to the final volume. The mixture tubes were then incubated at 42 °C for 30 min. Following the incubation step, all reaction tubes were inoculated with highly concentrated HCl solution to obtain a final concentration of 0.3 M HCl. Twenty-five microlitre of denaturation mix was then injected in the Biacore flow system.

2.6. Immobilisation of oligonucleotide probes

The presence of a second flow-cell on the sensor chip, allows the immobilisation of two different probe sequences. The 35S probe provides the complementary counterpart for the target of interest. The non-specific probe was only used as a control surface. Both probe sequences were immobilised using the same protocol.

The dextran sensor chip was further modified with streptavidin (200 μg/ml in acetate buffer 100 mM, pH 5.0) (Löfås and Johnsson, 1990). Then, the biotinylated oligonucleotide probe (1 μM in immobilisation buffer (NaCl 150 mM, Na_2HPO_4 20 mM, EDTA 0.1 mM, pH 7.4)) was immobilised, as previously reported (Tombelli et al., 2000a,b).

2.7. Hybridisation with synthetic oligonucleotides

Hybridisation reactions of the immobilised 35S probe with the complementary sequence, were achieved by injecting the testing solution in the SPR flow-cell. The reaction was monitored for 5 min and subsequently the sensor chip was washed with hybridisation buffer to remove the unbound DNA material. The analytical signal, reported as resonance units (RU), derives from the difference between the value before (baseline) and after the hybridisation.

In all the experiments, the single stranded probe was regenerated by an 1-min treatment with 1 mM HCl, which allows the multi-use of the sensor.

3. Results and discussion

3.1. Synthetic oligonucleotides

25-mer 35S oligonucleotide sequences fully complementary to the immobilised probe, were initially used to characterise the developed biosensor. The calibration curve is reported in Fig. 1 (curve a). Measurements with non-complementary DNA sequences led to non detectable results, thus confirming the specificity of the system (data not shown).

3.2. DNA samples of higher complexity

Following sensor optimisation using synthetic 25-mer oligonucleotides, the sensor performance was examined by testing DNA samples of higher complexity. These samples were obtained by symmetric and asymmetric PCR amplification. To aid hybridisation with the immobilised probe, the samples were denatured in order to obtain a single stranded form of the target fragment. The denaturing treatment was performed at high temperature with or without formamide and in alkaline conditions (low temperature) with different percentages of formamide.

3.2.1. High temperature denaturation

3.2.1.1. Symmetric PCR amplified samples. DNA PCR symmetrically amplified products were denatured using the high temperature treatment (95 °C for 5 min and 1 min in ice). SPR testing showed non-detectable or questionable traces of hybridisation shifts. This was attributed to the re-annealing of the denatured DNA strands before coming in contact with the sensor surface, as also demonstrated by Mariotti et al. (2002). The high temperature denaturation was also found inefficient when 20% formamide was used, resulting in non-detectable hybridisation shifts. This demonstrated that high temperature denaturation was not sufficient to keep the two strands separated in solution, even after the addition of formamide. A new PCR amplification scheme was then approached to minimise the chances of

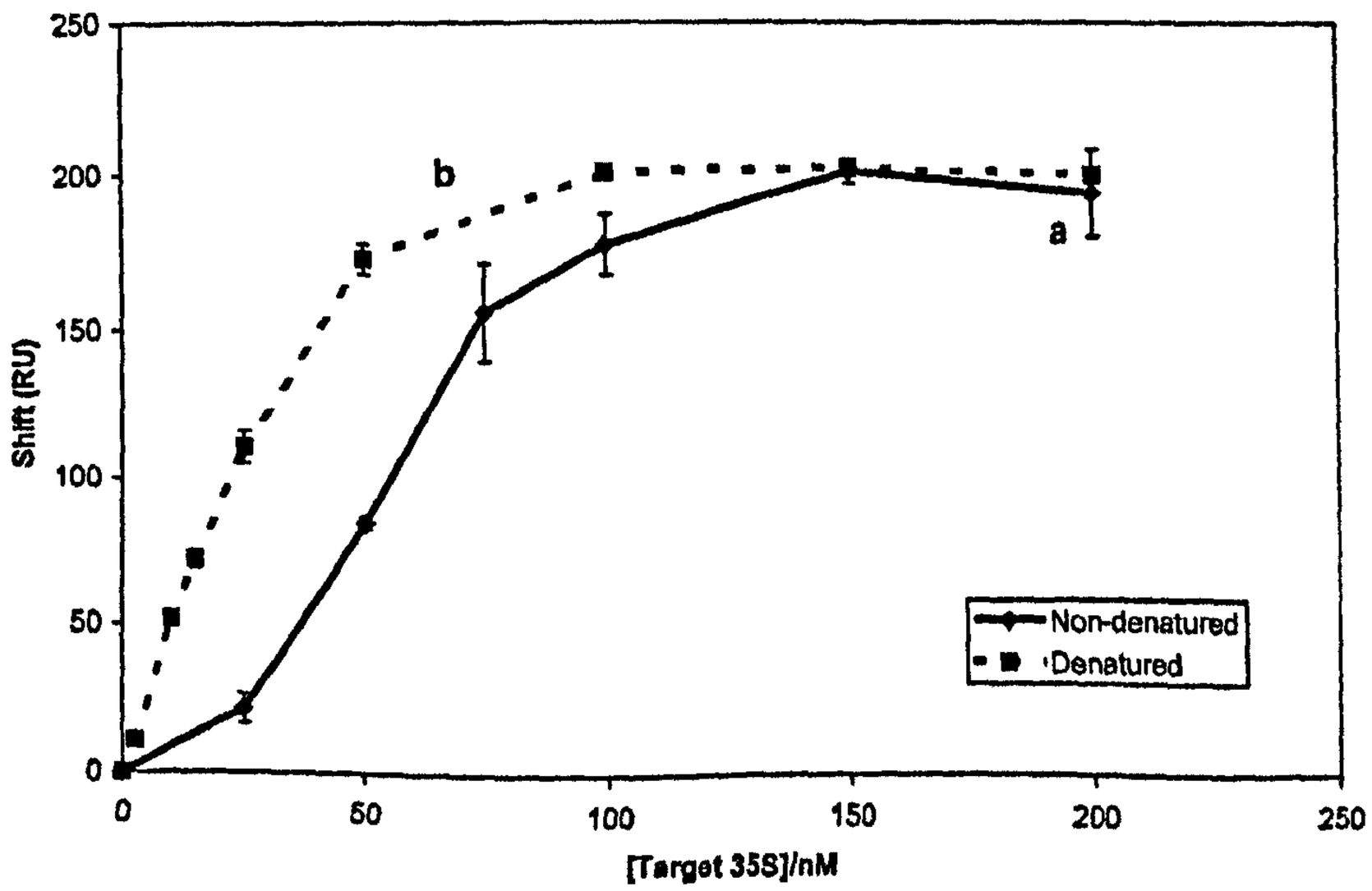

Fig. 1. Calibration curves with the immobilised 35S probe sequence. (a) Curve obtained with synthetic 35S target; (b) curve obtained with denatured synthetic 35S target; denatured with 0.3 M NaOH, 20% formamide at 42 °C for 30 min.

strand re-annealing by the synthesis of single-stranded PCR fragments.

3.2.1.2. Asymmetric PCR amplified samples. Asymmetrically amplified samples were denatured using high temperature (95 °C) and 20% of formamide. Subsequent SPR testing monitored non-detectable hybridisation shifts when 2.5 and 5 μM of asymmetrically amplified DNA was tested. The low analytical signals were attributed to the H-bonding in the intra- and possibly in the inter-strand region of the amplified fragments, due to the lack of adequate and prolonged denaturation. A contributing factor to the insufficient denaturing conditions could also be the short duration of the formamide treatment. Nevertheless, a longer exposure of the DNA samples to 95 °C would increase the probability of DNA conformational changes.

Secondary structures within a ssDNA fragment can be formed in the presence of repetitive sequences.

Fig. 2 shows a potential configuration of secondary structure in the asymmetrically amplified 243 bp sequence (Zuker, 2002).

To overcome this additional problem in the hybridisation reaction, denaturing conditions were optimised by the use of alkaline conditions and an extended duration of formamide treatment prior to SPR testing.

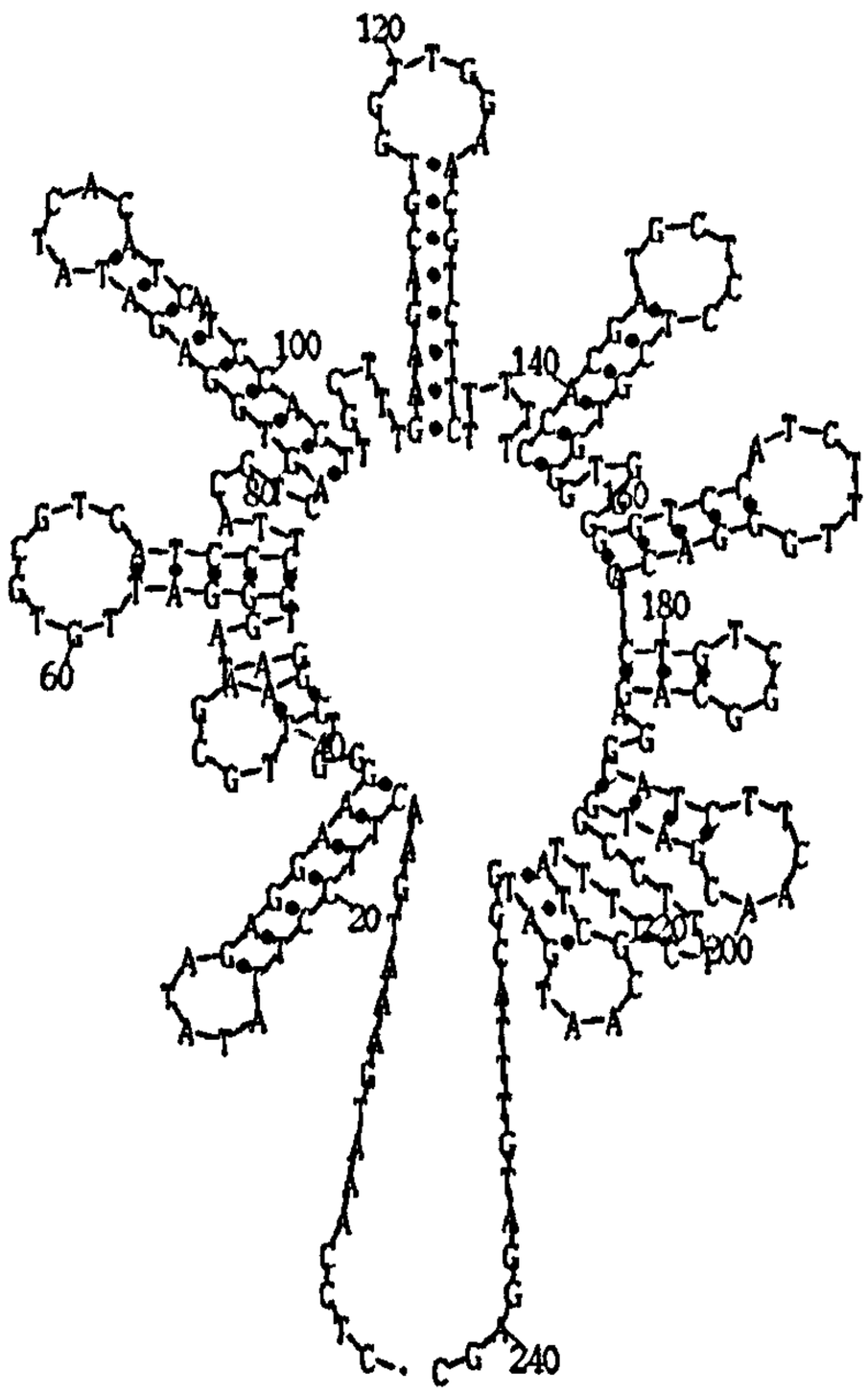

Fig. 2. One of the potential secondary structures formed by the 243 bases asymmetric PCR product. The 25-mer 35S target is located within the 5′ end of the amplified fragment between base numbers 186 and 210. A number of intra-strand hydrogen bonds are evident within the target 25-mer sequence.

3.2.2. Improvement of denaturing conditions

The denaturation step prior to SPR testing was modified to establish a strong alkaline environment as described in the experimental section.

3.2.2.1. Synthetic oligonucleotides. To study the effect of denaturing conditions on the hybridisation behaviour of the synthetic 25-mer 35S sequences, denatured (alkaline conditions and 20% formamide) and non-denatured synthetic DNA sequences were tested.

Fig. 1 shows the calibration curves obtained with the denatured (curve b) (0.3 M NaOH, 20% formamide for 30 min at 42 °C) and non-denatured 35S target sequences (curve a). By denaturing the target sequence in the above conditions, the linear range was up to 25 nM and higher sensitivity and reproducibility were observed. More specifically, the detection limit was improved, reaching a value of 2.5 nM. The reproducibility of the system in terms of CV% was $\leq 5\%$.

The improved biosensor performance in the case of the denatured 35S oligonucleotide target, was attributed to the elimination of intra-strand H-bonding possible even within the 25-mer target sequence.

dsDNA hybrids, were constructed to mimic symmetrically amplified DNA and were treated as real samples. Denaturation involved alkaline conditions and different amounts of formamide (0, 10, 20%). In the presence of 20% formamide, the hybridisation shifts ($\Delta RU = 138 \pm 6RU$) were approximately two times greater with respect to those obtained in the absence of formamide ($\Delta RU = 61 \pm 23RU$). Reproducibility was also improved with a CV% = 4%. This confirms the importance of formamide in the denaturing conditions.

Based on the findings obtained with "synthetic" dsDNA, the optimised denaturing treatment was applied to symmetrically and asymmetrically amplified samples.

3.2.2.2. Symmetric PCR amplified samples. Fig. 3(a) shows the hybridisation shifts produced with symmetrically amplified samples using 0, 10 and 20% of formamide. In the absence of formamide, non-detectable hybridisation shifts were obtained. Using 20% of formamide positive hybridisation shifts were measured but they were considered to be low and unreliable due to irreproducibility (e.g. sample C: CV% = 33%). By using the new denaturation protocol, a partial inhibition of the re-annealing process could be observed.

3.2.2.3. Asymmetric PCR amplified samples. The hybridisation shifts obtained with asymmetrically amplified products are shown in Fig. 3(b). When 20% of formamide was used, high reproducibility was obtained in

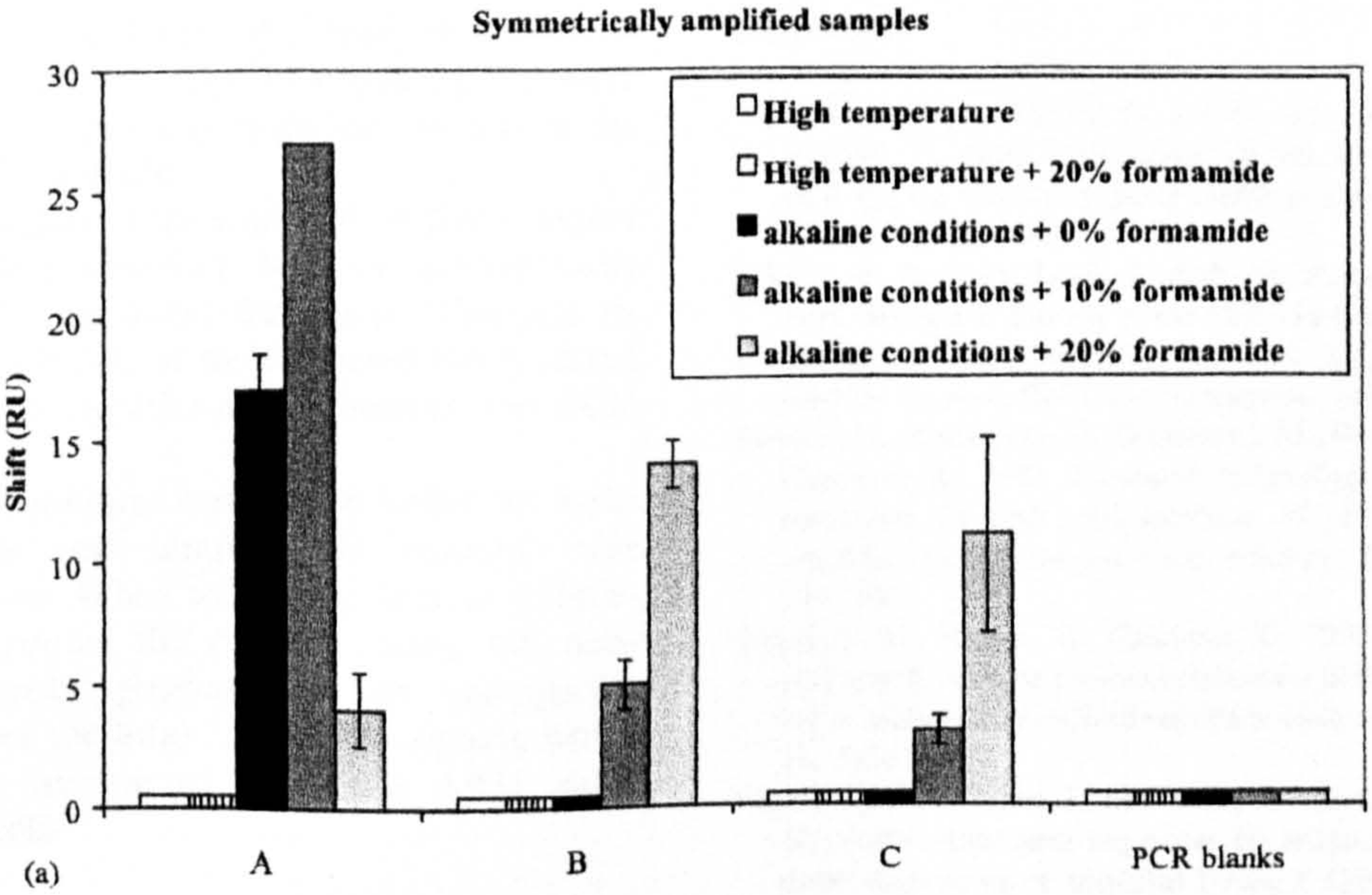

Fig. 3. Hybridisation shifts obtained from symmetrically (a) and asymmetrically (b) amplified samples. Sample A: CRM 2%; Sample B: PBI121; Sample C: GM Maize. SPR testing was performed following different denaturing conditions: high thermal denaturation, high thermal denaturation with 20% formamide, alkaline-thermal denaturation combined with different percentages of formamide (0, 10 and 20%). The samples were diluted in hybridisation buffer up to a final concentration of 0.2 μM. The reported shifts provide the mean value calculated over 3 measurements for each sample ($n = 3$).

sample B (CV% = 5%, $n = 3$) and C (CV% = 4%, $n = 3$), while the high hybridisation shifts ($\Delta RU_B = 112RU$, $\Delta RU_C = 66RU$) indicate the suitability of the technique to identify the target sequence.

All the reported results are summarised and compared in Fig. 3. High temperature denaturation led to encouraging results only when synthetic oligonucleotides were tested. However, thermal treatment with or without formamide, was found to be insufficient to generate reproducible and significant SPR signals when PCR fragments of hundreds of base pairs were tested. In the latter case, the formation of secondary structures needs to be eliminated for the hybridisation reaction to be favoured. The strategies used to obtain single-stranded DNA in solution, focused on the improvement of the denaturing conditions by the use of a strong alkaline environment (in the presence of formamide) and by an asymmetric PCR amplification scheme.

The optimised alkaline denaturing conditions resulted in low and irreproducible signals when applied to

symmetrically amplified fragments, while better results in terms of reproducibility and recorded signals were achieved using asymmetrically amplified samples in the presence of 20% formamide.

When using samples from source A (CRM), higher irreproducibility was observed, both in symmetrically and asymmetrically amplified fragments. This can be attributed to degradation of the extracted DNA (Alari et al., 2002) which significantly influences the PCR amplification.

The blank PCR solution was treated under the same conditions as the real samples. No response was obtained in all cases. When testing the sample complementary to 35S probe, the cell containing the non-complementary probe generated a low non-specific response ($<10\%$ of the total 35S specific signal), which was subsequently subtracted from the ΔRU value recorded on 35S cell.

4. Conclusions

To obtain single-stranded target DNA available for hybridisation in SPR biosensors, is a difficult task. Even in the case of asymmetrically amplified fragments, the presence of secondary structures puts considerable constrains in hybridisation reactions between the immobilised probe and the target DNA in solution. The presented work evaluated different approaches to obtain single stranded DNA target sequences prior to SPR testing. Eventually, the system combined an asymmetric PCR amplification system with a simple DNA denaturation step for rapid and specific detection of DNA hybridisation. Asymmetric PCR amplification led to the selective amplification of the desired DNA strand. The optimised denaturing conditions, eliminated intra-strand complexes within the asymmetrically amplified DNA fragments. The resulting system provides an alternative method for DNA detection. In our case, the potential use of this system in the detection of various GMOs, is demonstrated.

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