Development of programmable biosensor by on-chip peptide probe synthesis and in situ label free detection

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Japan Advanced Institute of Science and Technology

Doctor Dissertation

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Development of programmable biosensor by on-chip peptide probe synthesis and in situ label free detection

by

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Thesis Title	DEVELOPMENT OF PROGRAMMABLE BIOSENSOR BY ON-CHIP PEPTIDE PROBE SYNTHESIS AND IN SITU LABEL FREE DETECTION.
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Abstract

Preparing and combating against for the worst case scenario, much research has been exploited to develop an effective yet simple biosensing system. Even so, the threats to the human race and the existence of others have been increasingly dangerous and endangered. Because of these potential threats, there is a great need for a system that can quickly, reliably, and accurately detect any contaminations in the environment or in the atmosphere. Biosensors have developed in the recent few decades, but these sensors are specific only to specific target molecule. Thus, programmable biosensor is proposed to flexibly use and detect various other target molecules during on-demand situation at any place.

To realize the proposed novel idea of programmable biosensor, simple and effective microchip system to be used for synthesizing peptide is initially developed. Very simple inlet and outlet microchannel with reaction chamber on the microchip is designed. Pillar-like structures at the reaction chamber is incorporated to be more effective while synthesizing peptides on microfluidic chip. The optimal conditions for on-chip peptide synthesis is studied here and successfully estimated. The optimum coupling reaction time for the developed system is 15 min and 5µl/min of flow rate is the optimum flow rate. Conventionally, coupling time of peptide synthesis is usually 2-3 hours.

Through vigorous consideration, 'programmable biosensors' is proposed here to flexibly change the target analyte by changing the probe molecule based on on-site and on-demand situation. This may be achieved by synthesizing short peptide as probe molecules on-chip following traditional Fmoc-SPPS strategy. Linear hepta-peptide probe (-NH-PPGQPHH-NH_2) to be synthesised on-chip (optimized coupling time of 15min at 5µl/min flow rate) is proposed here in this research and successfully performed in situ detection of the target biomolecule on the same microchip. Highly specific peptide probe containing HPQ sequence is synthesised on-chip and study the interaction of probe with streptavidin biomolecule.

A novel approach of quick and rapid synthesis of peptides on SPR gold surface is introduced in this research. In this, synthesising a short peptide on SPR sensor chip through surface modification chemically is focused. Short peptide, EYYY, a tetramer is synthesised on the modified CM5 sensor chip directly and study the interaction with an atrazine, a herbicide. Development of very sensitive and massive sample analytical device from around the world has been realised in many different fields.

Keywords: Programmable biosensor, microchip, on-chip SPPS, label-free sensor, Surface plasmon resonance

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LIST OF ABBREVIATION

Fmoc-SPPS	9-fluorenylmethyloxycarbonyl-solid phase peptide synthesis
SPR	Surface Plasmon Resonance
VLSI	Very Large Scale Integration
DNA	Deoxyribonucleic acid
CDR	Complimentary determining region
Gly	Glycine (G)
His	Histidine (H)
Ala	Alanine (A)
Leu	Leucine (L)
Met	Methionine (M)
Phe	Phenylalanine (
Ille	Isoleucine (I)
Gln	Glutamine (Q)
Cys	Cystine (C)
Tyr	Tyrosine (Y)
Glu	Glutamic acid (E)
BSA	Bovine serum albumin
PyBOP	(Benzotriazol-1-yl-oxy)tripyrrolidinophosphonium hexafluorophosphate
HOBt.H_2O	Benzotriazol-1-ol hydrate
DMF	N,N-Dimethylformamide
PDMS	Poly(dimethyl)siloxane
FET	Field Effect Transistor

ISFET	Ion-sensitive FET
SPW	Surface plasma wave
SPP	Surface plasmon polariton
μTAS	Micro-total analysis system
POC	Point-of-care
POCT	Point-of-care technique
MEMS	Micro elecctromechanical system
SPRi	Surface plasmon resonance imaging
SERS	Surface-enhanced resonance spectroscopy
LSPR	Localize surface plasmon resonance
QCM	Quartz
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
EDTA	N,N,N,N-tetraacetic acid
DMSO	Dimethylsiloxane
EDC	1-ethyl-3-(-3-dimethylaminopropyl)-carbodiimide
NHS	N-hydroxysuccinimide
DPTU	Diphenylthiourea
HPLC	High-performance liquid chromatography
PBS	Phosphate buffered saline
EM-CCD	Electron multiplying charge coupled device
KH_2PO_4	Potassium dihydrogen phosphate
$Na_2HPO4\text{-}7H_2O$	Disodium hydrogen phosphate
NaCl	Sodium Chloride

UNIT	
mM	milli molar
pmol	picomol
µmol	micromol
µM	micro molar
µg/ml	microgram per milliliter
µl/min	microlitre per minute
kDa	kilo dalton
v/v	volume per volume
mg/ml	milligram per milliliter

CHAPTER 1

GENERAL INTRODUCTION

Abstract

The brief overview of the rapid advancement of biosensors and the application of the biosensor in various spheres of our life is mentioned here in this very chapter. The motivation behind to study and to develop a microchip sensing system as programmable biosensor is also discussed here concisely. In the world of danger and uncertainty, miniaturized high throughput diagnostic device is required and biosensor has been very promising because of high sensitivity and specificity. And recently, peptide based biosensor has received considerable interest because of the inherent property of peptide which can act as bio-recognition element. And because of the advancement in microtechnology, biotechnology, biochemistry etc., biosensors have been used in various fields to detect and to analyse. The general information about the detection method by labeling and non-labeling methods in biosensors is also mentioned. And in order to implement the idea of programmable biosensor, microchip sensing system has been improved during this research.

1.1 INTRODUCTION TO BIOSENSOR

Recently there are numerous cases of terrorists' attacks using dangerous biological weapons and various other methods in and around the world. Deliberate release by using biological agents like bacteria, virus, toxins or other harmful agents causing illness or death in people, animals, or plants is termed as bioterrorism. An example of this is the sarin nerve gas incident on the 21st of August 2013 where 1429 people were killed and 2200 people were injured in the Damascus suburbs in Syria using [1]. Not only this, but there is also outbreaks of diseases like Ebola which threaten the world. The current outbreak of the Ebola disease in west Africa (first cases notified in March 2014) is considered one of the most complex and largest Ebola outbreaks since the Ebola virus was first discovered in 1976 [2]. Threats to the existence and well-being of human beings are happening very frequently and also very unexpectedly. Because of these potential threats, there is a great need for a tool that can rapidly detect any contaminations in the environment or in the atmosphere with accuracy and reliability [3, 4]. Biosensor can serve essentially as low-cost, highly specific and highly efficient devices for this purpose in addition to being used in other day-to-day applications. A biosensor may be defined as an analytical device which is used for the detection of an analyte. This device comprises of a specific bio-element and a transducer, whereby specific bio-element recognizes a particular analyte and the interaction of bio-element with analyte usually gives to electrical readable signals by the transducer [5]. Much advancement has been made so far in the area related to biosensors and is still going on around the world. One of the issues related with biosensors is that they are specific only for particular target molecules [6-9]. For example, a glucose biosensor is specific only for glucose analysis [10].

The dissertation focuses on developing a programmable biosensor which would flexibly change the target molecules according to on-site and on-demand situation. This could

be achieved by on-chip peptide probe synthesis using *Fmoc*-solid phase peptide synthesis (*Fmoc*-SPPS) and in situ label free detection. The idea of a programmable biosensor has been proposed in which target molecules are flexibly changed by changing the probe molecules. In this case, probe molecules are the short peptides which can be easily synthesised by following the protocol of *Fmoc*-SPPS on a microchip system. After synthesising a certain probe molecules, they are allowed to interact with target molecules and thus it is possible to study the binding affinity and interaction. Another different probe molecule can be synthesised following the same protocol to detect and analyse different target molecules in the same micro-system. In this way, flexibility of detecting various target molecules could be possible in the programmable biosensor.

In this chapter, we briefly introduce the background knowledge to understand the principle with the discussion on label and label-free detection biosensors. The current fascinating and highly sensitive sensors, which focus mainly on fluorescent microscopy as a label detecting system and Surface Plasmon Resonance (SPR) as a label-free detecting system, are mentioned. At the end of the chapter, we stated the limitation of the existing biosensor as well as the requirement for improvement of better sensitive and flexible designs. These problems will be improved by proposing a novel idea called programmable biosensor based on short peptide probes synthesised on a microchip system which can flexibly change the target molecules by changing the peptide probes in the system. The application of the biosensor is also demonstrated in the following chapters of the dissertation.

1.1.1 General history and development of biosensors

In the year 1962, Leland C. Clark developed an enzyme electrode in which the concept of biosensors was demonstrated by trapping glucose oxidase at a Clark oxygen electrode using a dialysis membrane [11]. Therefore, the glucose concentration could be measured because it

was proportional to the decreasing concentration of measured oxygen. This is considered the primary enzyme electrode. In 1975, Clark's idea was commercialized in the product of glucose analyzer which was based on amperometric analysis of hydrogen peroxide [12, 13]. Ever since the introduction of glucose analyzer, research communities (VLSI, Physics, Chemistry, and Materials Science) from various fields across the world have come together to devise better and higher reliable and more accountable biosensing devices for various applications. Biosensors have been applied in the fields of medicine, agriculture, and environment, as well as in military and bioterrorism detection and prevention [14-18]. This may be possible because of the integration of specialized fields like biotechnology, biochemistry, nanotechnology, and microtechnology.

Biosensors: Different terminologies are being used depending on the field of application and areas. They are known as: immunosensors, optrodes, chemical canaries, resonant mirrors, glucometers, biochips, biocomputers, and so on [19, 20]. Various definitions are also used to define what biosensors are. But in our research fields, we may define biosensors as: "biosensor is a diagnostic device that combines a biological component with a physicochemical detector which is used for detecting an analyte". Schematic representation of biosensor is shown in Fig. 1.1.

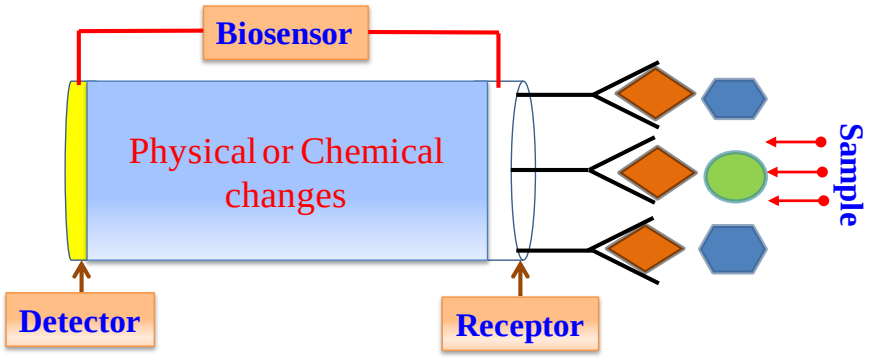

Fig.1.1 *Schematic representation of Biosensor*

The device is a combination of mainly two parts [21, 22]:

(1) A bio-element or biological probe, and

(2) A sensor element.

The sensitive biological component/probe or bio-element includes biomolecules such as tissues, microorganisms, enzymes, antibodies, antigens, hormone receptors, DNA, hybrid bilayer membranes, organelles, cell receptors, nucleic acids, etc., as the core components of biosensor. Fig. 1.2 provides the details of the bio-element and sensing element. The critical function of these biomolecules is that they bind to or recognize specifically the complimentary target or analyte in a complex environment. A specific "bio" element (e.g., enzyme) recognizes a specific analyte and the "sensor" element transduces the change in the biomolecule into an electrical signal. The bio element is very specific and sensitive to the analyte, but it doesn't recognize other analytes. Fig. 1.3 shows how the biosensor is working as a sensor for a specific bio-element or probe.

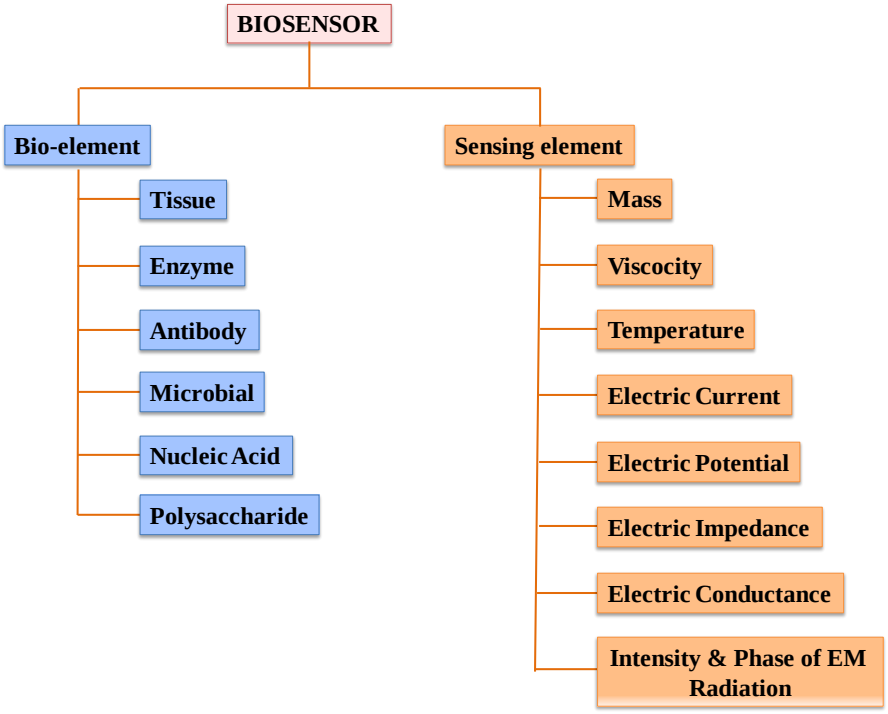

Fig. 1.2 *Elements of Biosensor*

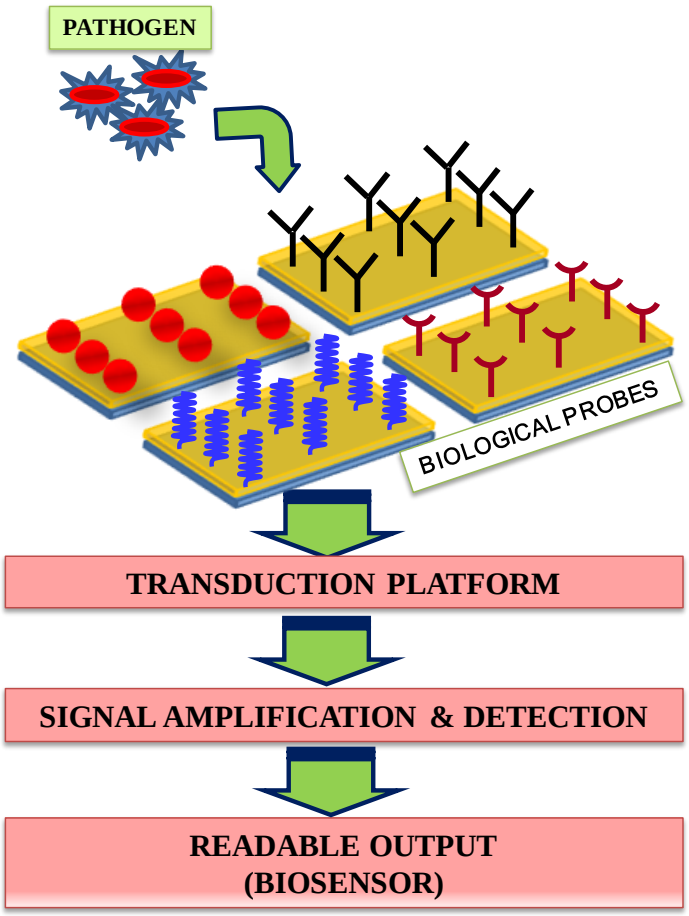

Fig. 1.3 *Fundamental principle of Biosensor*

1.1.2 Bio-element or Bio-probe

As mentioned in Fig. 1.2, the bio-elements of a biosensor may be different kinds of biomolecules, like enzymes, antibodies, proteins or nucleic acids, etc., that play as one of the most important roles in developing the biosensors [23-29]. As explained earlier, a bio-probe recognizes the complimentary analyte and binds specifically only to the analyte in the complex solution. Once a probe molecule interacts and binds with the analyte, the change in the biomolecule transduces with the help of a sensing element to a readable electrical signal. Biosensors take advantage of the affinity between probe and analyte molecules. Hence specificity of probe:analyte interaction is very critical for the development of an efficient

biosensor. In this section, we would like to discuss in more detail about the peptides as bio-elements or –probes, and the significance of peptide probes in developing programmable biosensors on microchips.

The peptide remains one of the biomolecules which has the potential to interact with various molecules [31, 32]. This may be because of the fact that peptides can form various tertiary structures and possess highly Complimentary Determining Regions (CDR). Recently there has been report of using short peptides for the design of artificial receptors [33, 34] because of the following reasons:

(i) Stability in harsh environments,

(ii) Different molecules can be obtained by combining 21 natural amino acids,

(iii) The peptide can be synthesised using an automated peptide synthesizer,

(iv) Relatively low cost to prepare a highly purified product (i.e., in comparison with monoclonal antibody technologies),

(v) Modification to enhance binding affinity is relatively eased, and

(vi) Fast screening of peptide libraries is available both for molecular biology and chemical techniques.

So, it is our interest and wish to mimic the molecular recognition mechanism of peptides which is occurring in biomolecules such as enzymes, antibodies, transmembrane proteins etc. and thus to be able to use as probe molecules in biosensing applications.

1.1.3 On-chip peptide probe designed

Chow and Gooding reported that amino acids and peptides can be used as the bio-elements of electrochemical sensors for metal ion detection [35]. They discussed the interesting phenomena in which very simple short peptides like tripeptide could interact with

metal ions like copper, nickel and zinc. Gly-Gly-His peptide sequence is selective for copper [36], whereas its isomer Gly-His-Gly cross-reacts with copper and zinc. As shown in Table 1.1, many short peptides have been used in various sensors in recent years as bio-elements. They are powerful tools to developing sensing systems or devices for metal ions, small molecules, and proteins. So in our approach, we synthesised short peptide sequence following *Fmoc*-SPPS protocol and study the binding affinity with the target molecules in the microchip system. The interaction between the peptide bioprobe with target biomolecule is detected and analysed by optical detection method. The important advantage of the research is that on-chip peptide probe synthesis can be achieved within a very short time period and detection could be done in the same micro-system. So in this case, one microchip can be used both for chemical peptide synthesis and detection of target analytes.

Table 1.1 *Sensors based on synthetic short peptides*

Ref	Target	Sensing peptide	Sensor	Sensitivity
37	Cu^{2+}	3-mer GGH	Silicon nanowire FET	1nM to 1 mM
38	Cu^{2+}	3-mer GGH	Polypyrrole nanowire electrode	20-300 nM
39	Cu^{2+}	3-mer GGH	Microcantilever	1 μM to 1 mM
40	Atrazine	EYYY and other 4-mer	SPR	200 μM to 1 mM
41	Methotrexate	11-mer SIFPLCNSGAL	QCM and SPR	Range 2-50 μM, K_d 22.3 μM
42	Porphyrin	5-mer HASYS	QCM	0.01-100 μg/ml, LOD 0.01 μg/ml
43	Dioxins	FLDQPhenylglycine	On-bead fluorescence microscopy	LOD 0.05 ng/ml

1.2 Types of biosensors

Based on the transducing mechanism used [44, 45], biosensors can be of many types such as:

(i) Resonant biosensors,

(ii) Optical-detection biosensors,

(iii) Thermal-detection biosensors,

(iv) Ion-sensitive FET (ISFET) biosensors, and

(v) Electrochemical biosensors.

Fig. 1.4 shows the types of biosensor based on Label-free and Label-based techniques. In our research we focused on using an optical-detection system, so we would like to discuss in more details about the optical-detection biosensor. An optical-detection biosensor may be categorized again into two sections:

(a) Label-based biosensor (Fluorescence), and

(b) Label-free biosensor (SPR).

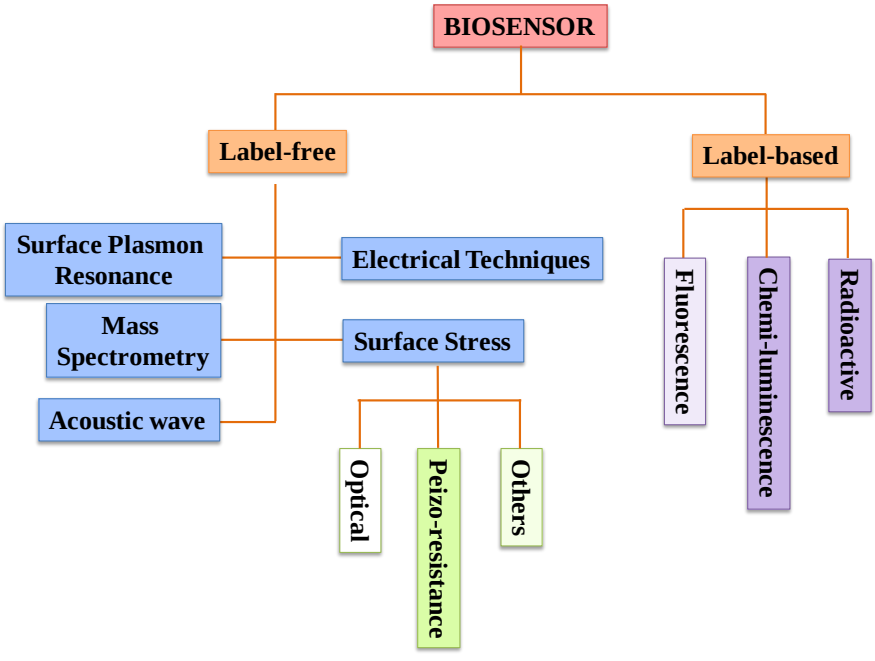

Fig. 1.4 *Types of Biosensor*

1.2.1 Fluorescence as label-based optical biosensor

Fluorescence spectroscopy is a technique suitable for detecting very small concentrations of analytes. Even at low concentration, signal can be very strong because fluorescence provides signal amplification in which a single fluorophore absorbs and emits many photons. In addition, real-time monitoring of concentration fluctuation is possible. Since fluorescent properties only respond to changes related to the fluorophore, fluorescence spectroscopy techniques can be highly selective. For cases in which the intrinsic fluorescence of biomaterials is of limited use, smart synthetic fluorophores have designed, which are used as probes and labels as well, to study and analyze under a fluorescent microscope. Using these properties, research has been carried across the world to develop fluorescent detection system. In 2001, Didenko developed bioaffinity sensors, labeled with fluorophores, and used to detect DNA hybridization and single nucleotide polymorphisms [46]. In contrast to bioaffinity sensors, biosensors being devices capable of detecting target ions using biological reactions can be modified to utilize fluorescence for detecting, identifying or quantifying target ions. For example, many fluorescent chemosensors, including fluorophore-labeled organic chelators [47, 48] and peptides [49] have been developed for metal ion detection.

In our research, we synthesize peptides on a solid resin bead as a probe molecule on a microchip. After that, a biomolecule labeled with a fluorescent molecule (streptavidin biomolecule as an analyte) is allowed to interact with synthesised peptide probe molecules. The binding affinity between the probe and the analyte molecule is monitored and studied under a fluorescent microscope.

1.2.2 SPR as label-free sensor

A label free sensor may consist of a sensing element or probe/receptor biomolecule which is tethered to a stable sensing surface. A sensing transducer detects the probe:analyte interaction and provides a measureable signal for the binding reaction. Label free do not require secondary or tertiary reactions to generate measureable signals and, thus, are very suitable for continuous and real time monitoring of detection or interaction between the probe:analyte [51]. There are many label free techniques which are used for the biosensing application, but in our research we would like to focus on surface plasmon resonance and its application on a biosensing system for developing the proposed target programmable biosensor because of its sensitivity.

The Surface Plasmon Resonance affinity biosensor is an optical sensor which exploits special electromagnetic waves – surface plasmon polariton (SPP) – to probe interaction between an analyte in solution and a biomolecular recognition element immobilized on the sensing surface as shown in Fig. 5. The main fundamental is that the surface plasma wave (SPW) or SPP propagates along the boundary between the dielectric and metal, which is behaving like quasi-free electron plasma. Various biomolecular recognition elements have been studied by incorporating with sensors such as proteins, antibody-antigen, nucleic acids and enzymes [52-54].

Because of the very sensitive nature of the SPR, biosensing application using SPR provides higher throughput, but also give highly selective and accurate analysis. Real-time monitoring with SPR may provide information regarding the kinetics of the binding affinity with analytes, concentration, detection limit etc. An important feature for SPR biosensing is that it is label-free sensing method without radioactive and fluorescence. In addition, without exhibiting any special properties of fluorescence or characteristic absorption and scattering

bands, SPR transduction can be used to study various interactions [55, 56]. SPR biosensor can detect effectively the binding of molecules as small as 2 kDa, but smaller molecules generate insufficient changes in bound mass and so cannot be directly measured adequately [57]. Recently, signal to noise ratio has been tried to be improved in order to measure smaller analytes [58].

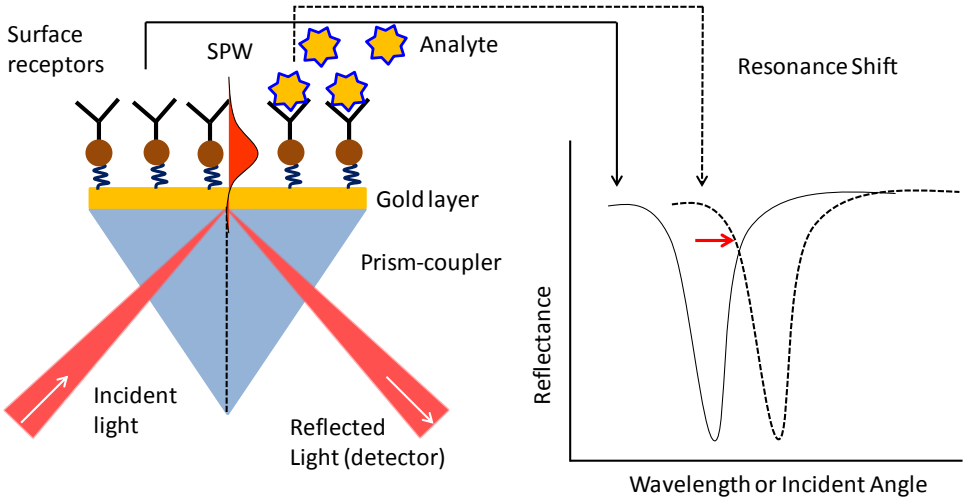

Fig. 1.5 *Principle of surface plasmon resonance biosensor*

Biosensors have received significant attention in recent times from various experts and thus improved in many areas and factors. Such advanced development in detection or analysis and wide ranges of applications are possible due to the advancement and introduction of microfluidic technology and micro total analysis systems (μTAS) in the past few decades to provide the best results in terms of selectivity and lifetime [59, 60].

1.3 Miniaturised microfluidic diagnostic device

Application of engineering in developing diagnostic devices has been successful and excellent, not only in terms of sensitivity, specificity and accuracy, but in terms of size and overall operation, it can be smaller and simplified respectively to be more user friendly. Technology and principles or techniques related to analytical systems have been independently improved from large sizes to handy devices, complicated operations to user friendliness, single analysis to massive arrays, expensive to cheaper devices etc. One of the most significant factors that made these improvements possible is the miniaturization of the system. For example, miniaturized design of clinical systems leads to medical results at point-of-care (POC), i.e., bedside, ambulance, or other remote location [61]. This could be achieved due to improvements and innovation in microtechnology which provides tons of applications of micro total analysis system (μTAS) in basic science research, clinical medicine, and field work [62].

The μTAS, also called "lab on a chip" or miniaturized analysis system, have grown rapidly most likely from the inception of the novel idea in microtechnology and microfluidics. The advancement in this field allows many analytical systems to be miniature in size, high-throughput results, have increase productivity and efficiency, microarray analysis and be less expensive, be portable, environmentally safer, etc. [63, 64]. Thus, because of the numerous advantages which are offered by miniaturization techniques, many fields of science have incorporated the technology of microfluidics and microtechnology. From pure science to the applied science and technology, this novel technique has integrated to develop better, wider and more advanced applications. One of the prominent integration of micro total analysis system is the linking of biotechnology and nanotechnology in recent decades of development. This may be illustrated in the following Fig. 1.6.

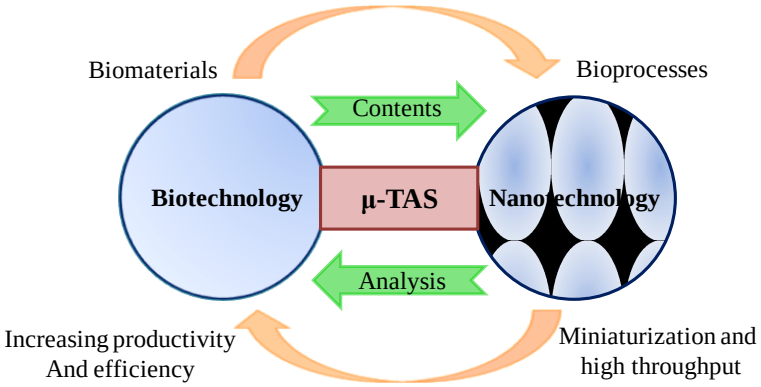

Fig. 1.6 *The micro total analysis systems linking biotechnology and nanotechnology*

In our research, we incorporate the techniques of microtechnology and other fields of science to develop a high throughput analysis system. The analytical system, a micro-reaction chamber, is fabricated following the microfabrication techniques and photolithography. Since microfluidics is capable of analyzing a small sample volume (10^{-9} – 10^{-18}) and minimizing costly reagent consumption as well as automating sample preparation and reducing processing time, it is expected that by sandwiching microtechnology with the principles including biochemistry, chemistry and physics, more advanced technology and many applications could be devised [65-70].

1.4 Programmable biosensor

As we have discussed about the biosensor and the development of the analysis system so far, it has gained lots of attention because of widespread application and its potentiality. Due to the advancement of microtechnology and integration of the micro total analysis system, biosensors have developed in various aspects. Biosensors have gained popularity because of the high specificity, high accuracy and sensitivity to the specific target molecules. They can analyse and detect target molecules, for example, glucose sensor is very specific to glucose

concentration. And any monitoring or analyzing system is specific for its particular target molecule. There may be other limitation of conventional biosensors as well, but mainly we would like to focus on flexibility of the current biosensors of target molecules. Because of this issue, target molecules cannot be detected with the biosensor on-site and on-demand situation.

So we proposed a sensor called "programmable biosensor" in which the target analyte molecules can be detected by flexibly changing the probe molecule in the analytical system. As illustrated in Fig. 1.3, each biosensor has a very specific analyte to be detected due to probe:analyte binding specificity or complimentary with each other. However, if the probe molecules or bio-element can be changed flexibly from one probe molecule to another on the sensing surface, multiple analytes could be detected with one analytical system (i.e., biosensing system). In the mean time, on-site and on-demand situation could be met too because by changing, the probe molecule would be changed according to the target analyte as shown in Fig. 1.7.

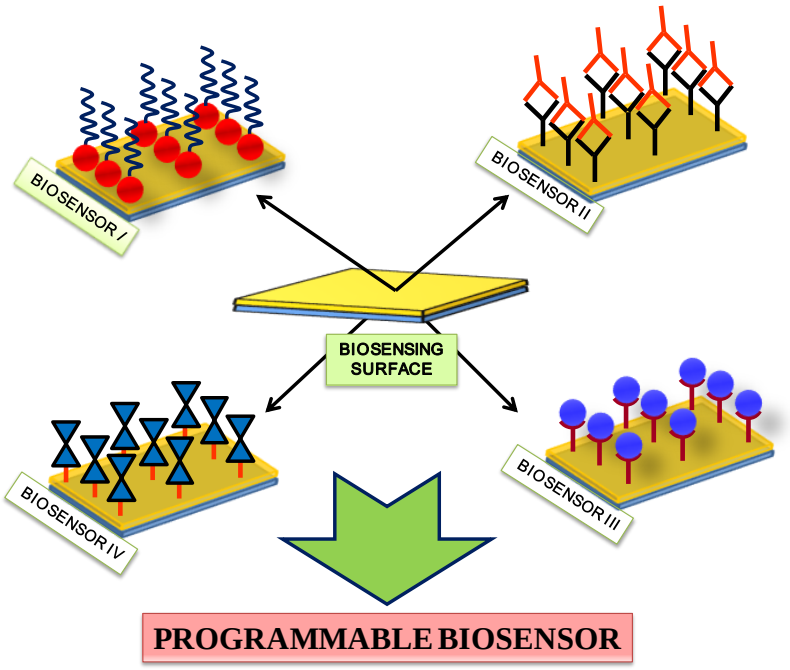

Fig. 1.7 *Illustrating the principle of programmable biosensor*

So, programmable biosensor is the high-throughput diagnostic device which can flexibly change the probe molecule on the sensing surface according to the target analyte. The advantages of developing such advanced biosensor are as follow:

(i) Flexible detection of target analytes by changing probe molecules on sensing surface.

(ii) Detection of multiple analytes in parallel.

(iii) Capable of meeting on-site and on-demand situation.

(iv) Point-of-care techniques which meet the demanding situation at bedside, ambulance or remote areas.

(v) Massive detection of analytes is possible.

(vi) Integration of many biosensors into one biosensor.

1.5 Research Objective

Biosensors as diagnostic device have gained much popularity recently because of their selectivity, sensitivity and specificity even though the technology is a few decades. We proposed a programmable biosensor by exploiting the property of biologically active peptides as probe molecules. The proposed biosensor is to meet on-site and on-demand situation.

- ✧ The concept of programmable biosensor has been proved by previous researcher in our lab. But there are few weak points like using pump to stabilize the resin bead at the reaction chamber and introducing resin bead manually.

  Research Objective I:

  To improve further for more practical purpose, it is our aim to develop a simple biosensing system for on-chip peptide probe synthesis by incorporating pillar-like structures at the center of the reaction chamber for an effective on-chip peptide synthesis.

- ✧ Programmable biosensing system has been developed to detect and analysed with specific target molecule.

  Research ObjectiveII:

  To evaluate the biosensing system for programmable biosensor, binding affinity of synthesised peptide probe molecule with one of the biomolecules is studied. This study of interaction with known biologically active molecule would enhance the ability to use the developed system for the detection of various other biomolecules and thus to realise programmable biosensor.

- ✧ Labeling technique has inherent limitations by interacting with non-target molecule and high background and signal/noise ratio. Label free techniques can be generalized for biosensing elements and detection and are very sensitive and specific.

Research Objective III:

To evaluate programmable biosensor using surface plasmon resonances (SPR) sensor by direct on-chip peptide probe synthesis and study the detection with biomolecules in situ. By studying the interaction of biomolecules with the synthesised peptide probe by label free detection method, the programmable biosensor can be generalized for biosensing to be realised.

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CHAPTER 2

DEVELOPMENT OF BIOSENSING SYSTEM FOR RAPID AND QUICK ON-CHIP PEPTIDE PROBE SYNTHESIS

Abstract

To combat against and to prepare for the worst case scenario, much research has been done to develop effective yet simple biosensing system. However the threats to the human race and the existence of others have been increasingly dangerous and endangered. Because of these potential threats, there is a great need for a system that can quickly, reliably, and accurately detect any contaminations in the environment or in the atmosphere. Biosensors have developed in the recent few decades, but these sensors are specific only to specific target molecule. Thus, programmable biosensor is proposed to flexibly use and detect various other target molecules during on-demand situation at any place. To realize the proposed novel idea of programmable biosensor, simple and effective microchip system to be used for synthesizing peptide is initially developed. Very simple inlet and outlet microchannel with reaction chamber on the microchip is designed. Pillar-like structures at the reaction chamber is incorporated to be more effective while synthesizing peptides on microfluidic chip. The optimal conditions for on-chip peptide synthesis is studied here and successfully estimated. The optimum coupling reaction time for the developed system is 15 min and 5µl/min of flow rate is the optimum flow rate. Linear peptides are synthesised after optimization of the reaction condition of on-chip peptide synthesis.

2.2 Introduction *(partially published in JJAP by the author [1])*

Peptides are known for the ability to form various structures that contain highly specific complimentary determining regions (CDRs) which are recognition sites for many antigens. And they not only compose proteins but are also endogenous compounds which play an extremely vital role in living system [2-5]. There are many who study the property of proteins and peptide and their interaction to understand the reaction mechanism better. There are many applications because of their characteristic and dynamic in nature. Peptides can be represented a good candidate if one wishes to study and mimic the molecular recognition mechanism which are happening in biomolecules such as enzyme, antibodies, drug receptors, and transmembrane proteins [6-7]. The binding affinity of proteins with biomolecules may be because of the presence of very specific biding site in which multiple interactions can be formed. Mostly, peptides are obtained from solid phase peptide synthesis (SPPS) which was developed by R.B. Merrifield in 1984 [8]. Ever since the development of solid phase peptide synthesis (SPPS), SPPS has been rapidly improved and this method has been applied for various applications mainly because of good yields of highly purified products and easier processes. However, the SPPS has certain limitations like:

1. Long reaction periods,
2. Excessive reagents consumption, and
3. Expensive apparatus.

Thus, it is required to further improve the methods to:

1. High-efficient synthesis,
2. Miniaturized system,
3. Environment-friendly, and
4. Low cost synthesis.

Because of the rapid development of microtechnology and integration of biotechnology and nanotechnology in recent years, much progress has been made even in the field of peptide chemistry. In recent years, microarrays of highly throughput is possible due to advancement in microtechnology, which are based on SPPS, were experimented and integrated by means of micro electromechanical systems (MEMS), but these are often carried with the help of light, electricity or, in some cases, they need special reactants [9-12]. However, on-chip SPPS may possess more advantages when compared with the microarray based peptide synthesis. The points may be mentioned below:

1 Continuous flow of fresh reagents,

2 Use of traditional reagents,

3 Simple reaction chamber/chip,

4 Low-cost and

5 Disposability.

2.2 Research Objective

The research objective is to develop a microchip reaction system whereby peptide can be synthesised following the protocol of Fmoc-solid phase peptide synthesis. This may be achieved by following sub-objectives:

(i) Design of microchip reaction system,

(ii) Microfabrication of the designed microchip,

(iii) Optimization of the reaction/experimental condition,

(iv) Short peptide synthesis and characterization.

2.3 Experimental Approach

The information regarding the instruments and equipments, the chemicals, the microchip designed, Fmoc-solid phase peptide synthesis (Fmoc-SPPS) protocols and peptide synthesis cycle are explained as followed.

2.3.1 The materials and equipments

The materials and equipments for the designing and fabrication of the microchip are listed in Table 2.1

Table 2.1 *List of materials and equipments used for designing and fabrication of the microchip*

Materials and equipments	**Suppliers**
Adobe Illustrator CS5 software	Adobe System Inc.
Laptop Computer	Toshiba, Japan
Spin Coater 1H-DX2	Mikasa, Japan
Double-View Mask Aligner PEM-800	Union, Japan
Plasma Cleaner PDC 210	Yamato, Japan
Glass slides	Matsunami Glass Ind. Ltd
Sonicator	As One, Japan
Conditioning Mixer	Thinky, Japan
Constant Temperature Oven DKN 301	Yamato, Japan
Silicone	Laboran, Japan

2.3.2 Chemicals used

The chemicals which are used during the designing and microfabrication are listed here in Table 2.2

Table 2.2 *Chemicals used during microfabrication of microchip*

Chemicals	**Suppliers**
SU 8 3050	Micro-Chem, Japan
Silpot 184	Dow Corning Toray, Japan
Silpot 184 Cat	Dow Corning Toray, Japan
Ethanol	Cica, Japan
Acetone	Wako, Japan
2-propanol	Wako, Japan
SU-8 developer	Micro-Chem, Japan

2.3.3 Design of pattern using adobe illustrator

A very simple design of the microchip is proposed. Adobe illustrator software is used to design. And after designing, the patterns of the design would be bought as photomask from company. In our design, both the designs have only one outlet and inlet microchannel passing through the main reaction chamber is designed. In the second design, pillar like structures are added to trap the single resin bead. The dimension of the microchip designs are shown in Fig. 2.1

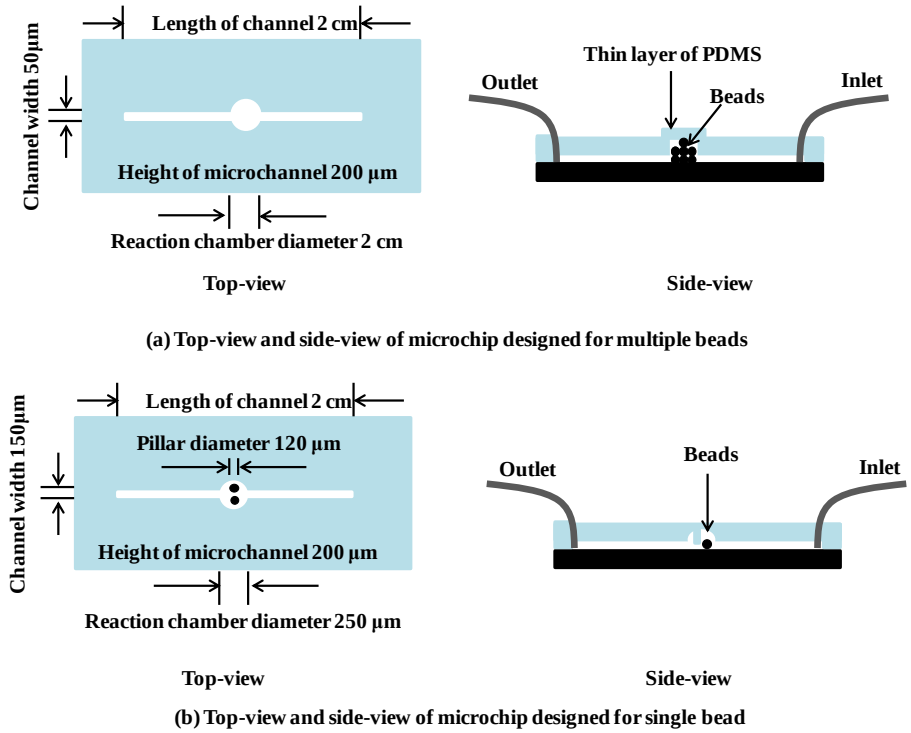


Fig. 2.1 *Dimension of the microchip designed for peptide synthesis. (a) Top-view (left) and side-view (right) of the microchip designed for multiple beads. (b) Top-view (left) and side-view (right) of the microchip designed for single bead with pillar like structure at the center.*

2.3.4 Microfabrication of designed chip

Many technological achievements have been made the convergence of microfabrication, automation sciences, and biological analysis possible. The microelectronics industry developed photolithography and associated techniques to fabricate integrated circuits. The most common used soft photolithography technique for fabrication PDMS microfluidic devices is replica molding.

Fabrication of the microchips usually involves the following steps:

(I) Washing or Cleaning the photomask and Si wafer with ethanol,

(II) Coating with SU-8 by Spin Coater,

(III) Pre-baking at high temperature,

(IV) UV Irradiation,

(V) Post-baking at high temperature,

(VI) Developing with SU-8 developing,

(VII) Washing or rinsing with 2-propanol, and

(VIII) Drying the under N_2 gas.

Each fabrication steps are explained in more detail as follows:

(a) Cleaning of silicon wafer and treatment of glass substrate:

1. Silicon (Si) wafer was cleaned with ethanol under sonication for 5 min.
2. The wafer was dried by blowing N_2 gas.
3. Glass substrate was sonicated for 10 min in acetone first. Then washed with distilled water.
4. The substrate was dried with N_2.

(b) Spin-coating:

1. SU-8 3050 is used as coating material on the Si wafer.
2. The thickness of the coated material is around ≥ 100 μm.
3. The condition of the spinning of the device is as follow:

Table 2.3 *Condition for spin-coating*

Sl. No.	Slope/time	rpm
1	5	
2	10	500
3	3	
4	30	1000
5	10	

(c) Treatment of the coated substrate on hot plate:

Pre-treatment of substrate on hot plate is required to obtain a uniform and proper exposure later. This is to activate the surface and following pre-treatment baking is given in the table.

Table 2.4 *Pre-treatment or Soft-baking*

Thickness (in µm)	**Temperature (in °C)**	**Time (in min)**
100	65	
	95	45

(d) Photo-lithography or exposure to UV light:

Mask aligner is used for exposing UV light to prepared mask. 40 sec of UV light is exposed on the mask.

(e) Post-treatment baking:

After the exposure of UV-light to the mask, the mask is further treated by baking on hot plate. This treatment will harden and

Table 2.5 *Post-treatment baking*

Thickness (in µm)	**Temperature (in °C)**	**Time (in min)**
100	65	1
	95	5

(f) Developing and washing:

1. A SU-8 developer is used to develop the coated SU-8 surface in SU-8 developer solution for 15 min. The Si wafer is sonicated in SU-8 developer solution.
2. After 15 min, the substrate is taken out from the solution and washed with IPA

(Iso-propyl alcohol). This is treated for 10 min again. (note: if there are white patches on the Si wafer, the developing hasn't done well, so, the wafer could be treated under developer again for few more minutes).

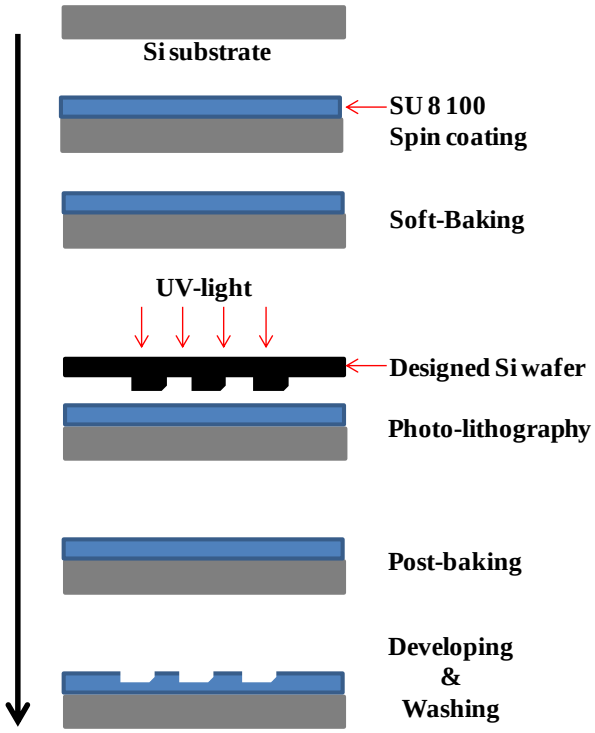
3. The substrate is taken out from IPA solution and rinse with ultra pure water.
4. Then the substrate is dried with N_2 gas.

(g) PDMS preparation:

1. 20g of Silpot 184 and 2g of Silpot 184 Cat are taken in the ratio of 10:1 (w/w). the solution is mixed well under the mixer.
2. The prepared solution is then poured on to the surface of the substrate where there is a replica of the desired mask pattern. The design is cornered and surrounded using polymer of certain thickness to obtain the desired thickness of the PDMS pattern/mold.

(h) Curing of PDMS mold in oven:

1. The PDMS solution on the mask substrate is cured in the oven at 65 °C for about 90 min. (Note: the physical properties of PDMS mold would depend on the temperature and time of the curing).
2. After curing, the substrate and mold is cooled down.
3. Then, PDMS mold is peel-off from the substrate very slowly. (Note: the Si wafer substrate is retained and is kept properly because it can be re-used for further molding to a number of extent)



Scheme 2.1 *Micro-fabrication steps and procedure of the designed microchip with the patterns on it. All the steps and procedures of lithography, and fabrication steps (baking, coating, exposure, developing and washing the substrate) were carried out using the clean room facility.*

(i) Punching holes on PDMS mold:

Inlet-holes and out-let holes are punched with the help of puncher. In the first design, reaction chamber is punched and thin layer of PDMS is used to seal the chamber whereas in second design, reaction chamber is closed and intact.

(j) Bonding of glass substrate and PDMS mold by oxygen plasma:

1. Clean the substrate and PDMS mold properly with ethanol.
2. Dried them properly by heating in oven or hot plate.
3. After that, the PDMS molds and glass substrate were placed in the plasma.
4. Then oxygen plasma is exposed on the surface of the substance which will help them to have an irreversible bonding.

2.3.5 On-chip peptide synthesis

Peptides are usually prepared and synthesised by following the protocol of Fmoc-SPPS, developed by RB. Merrifield. Lesser reagents and continuous introduction of fresh solution makes this microfluidic very attractive for chemical synthesis. Materials and equipment while synthesising peptide on-chip and the chemicals used are also mentioned below.

2.3.5.1 Materials and equipments used

Table 2.6 *List of equipments and materials that are used during on-chip peptide synthesis.*

Materials and equipments	**Suppliers**
Microscope (SZ×12)	Olympus
Micro glass slides	Matsunami Glass Ind. Ltd
Cover glass slides	Matsunami Glass Ind. Ltd
Peptide sequencer, 492HT	Applied Biosystem,
Syringe needle	Terumo, Japan
Micro Syringe Pump ESP-64	Elcom Corp, Japan
Syringe	Terumo, Japan
Silicone tube	Laboran, Japan
Syringe	Hamilton, Japan
Eppendorf	PhysioCare Concept

2.3.5.2 Chemical used:

The following chemicals are used while synthesising peptides.

Table 2.7 *List of chemical used for Fmoc-SPPS*

Chemicals	**Suppliers**
NovaSyn® TG amino resin	NovaBiochem
Fmoc-Ala-OH	NovaBiochem
Fmoc-Gly-OH	NovaBiochem
PyBOP	NovaBiochem
HOBt.H_2O	NovaBiochem
Piperidine	Wako pure chemical industries, Ltd.
N,N'-Dimethylformamide	Nacalai Tesque, Inc, Japan

2.3.5.3 Preparation of reagents and amino acid solution

N,N-Dimethylformamide (DMF) is used as solvent while preparing amino acid solution. The following are the basic steps we followed:

(i) Amino resins were soaked in DMF solution before doing any synthesis for at least 30 min, to swell the polymer uniformly for better reaction.

(ii) All the required chemicals were taken out from the freezer to equilibrate to room temperature before using any of them.

(iii) 20μmol of amino acids solution (Fmoc-Ala-OH, Fmoc-Gly-OH) were prepared in DMF in bottle.

(iv) 20μmol of PyBOP, 30μmol of HOBt.H_2O of solution were prepared in DMF.

(v) 30% piperidine was prepared in DMF.

2.3.5.4 Procedure for peptide synthesis

Conventional Fmoc-SPPS strategy was followed to synthesis peptide on microchip.

(a) Amino resin beads were introduced to the reaction chamber of the microchip designed for multiple beads.

(b) Coupling step:

The activated Fmoc-protected amino acid (with coupling reagents) was introduced to the reaction chamber in order to form an amide bond between free amine terminal group of resin beads and the free carboxyl terminal group of amino acid.

(c) Washing step:

The microchip was washed with DMF two to three times.

(d) De-protection step:

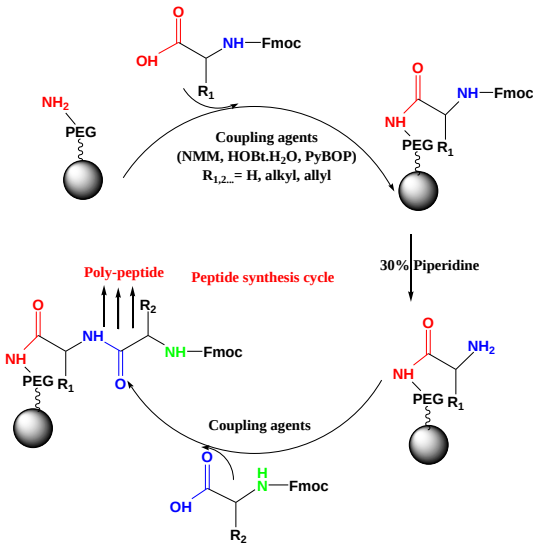
30% Piperidine was introduced to the reaction chamber to cleave Fmoc- group which is meant to protect amine group of the amino acids in order to elongate the chain length.

(e) Washing:

The reaction chamber was washed again to remove any unreacted piperidine and other waste from the chamber.

(f) In this way, step (b), (c), (d) and (e) repeat the cycle to synthesis the target or desired peptide sequence.

From Scheme 2.2, it's known that peptide chain could be elongated following the cyclical procedure of deprotection, activation, coupling and washing in each cycle of peptide synthesis.



Scheme 2.2 *Cycle of traditional peptide synthesis including introduction of amino acids along with coupling agents, deprotection of Fmoc-group by 30% piperidine and then introduction of another amino acids along with coupling agents.*

2.3.5.4.1 Optimization of coupling time

Since on-chip peptide synthesis is realised first time in the system, reaction conditions were optimized. For coupling the free $–NH_2$ and free –COOH group to form an amide bond (-CO-NH-), the following condition were applied. A very short peptide, a dimer (Gly-Ala), was synthesised and checked the flow rate and coupling time.

Table 2.8 *Condition for optimizing of coupling time to synthesis peptide on-chip*

Peptide	Flow rate	Coupling time
Dimer (Gly-Ala)	5µl/min	3 min
	5µl/min	5 min
	5µl/min	10 min
	5µl/min	15 min
	5µl/min	20 min

2.3.5.4.2 Optimization of flow rate

After optimizing the coupling time, optimization of flow rate was checked and evaluated. The following conditions were checked and confirmed the optimum condition to synthesis peptide.

Table 2.9 *Condition for optimizing flow rate to synthesis peptide on-chip*

Peptide	Flow rate	Coupling time
Dimer (Gly-Ala)	3 µl/min	15 min
	5 µl/min	15 min
	10 µl/min	15 in

2.3.6 Synthesis longer peptide with optimized condition

Nona-peptide (9-mer peptide) chain was synthesised with the following peptide sequence Gly-Ala-Ala-Leu-Val-Met-Ille-Met-Phe. The peptide sequence was confirmed by peptide sequencer.

2.3.7 Characterisation of the peptide by peptide sequencer

After peptides were synthesised, the sequence of the peptide could be confirmed and characterized by protein sequencer. The following steps were performed:

(i) The cartridge A or B was removed from the system and placed a cartridge seal to the lower part of the glass. The membrane was then smeared with 15µl of BioBrene Plus solution, and then this was dried under Ar gas.

(ii) The cartridge was put back to the system and run the pre-sequence cycle.

(iii) After pre-cycle run was finished, the cartridge was taken out and a single bead was placed on the membrane (cartridge seal).

(iv) Then the required number cycle was input to get the sequence.

2.4 Results and discussions

2.4.1 Microfabrication of microchip:

Microchip for peptide syntheses on both multiple beads and single bead has been fabricated. Microchip for multiple beads has inlet and outlet holes for the tube to be connected to reagent bottles and syringe pump respectively. It has reaction chamber at the center of the microchannel where many beads around 20-30 beads were put with the help of pipette. But due to clogging of beads towards the outlet channel, homogeneity of the reaction could not be met. In another microchip design for single bead, pillar-like structures at the reaction chamber was also introduced successfully as observed under scanning electron microscopy (SEM). The design of the microchip was in such a way to trap or block the amino resin bead in the reaction chamber. The bead was introduced to reaction chamber by applying pressure. To avoid clogging towards the outlet channel, creating a pillar-like structures or barrier at the reaction chamber was necessary to give proper fluidic movement. This has been incorporated in such a way to give proper space for the peptide reaction on the bead. Homogeneity of the reaction around the bead may enhance due to this approach by avoiding clogging towards the outlet. This would also likely give good flow across the microchannel since organic solvents and reagents have to continuously introduce.

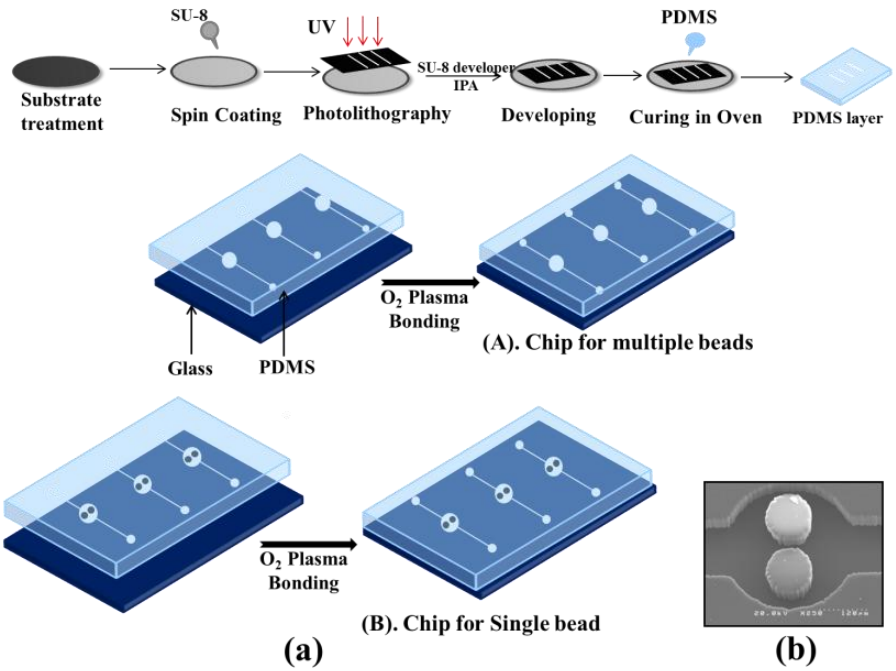


Fig.2.2 ***(a)*** *The fabrication process of the microchip following substrate treatment, SU 8 coating, UV irradiation or photolithography, developing and curing of PDMS. (A). Microchip designed for synthesis of peptides on multiple beads. (B). Microchip designed to synthesize on single bead with pillar-like structure.* ***(b)*** *Scanning electron microscopy (SEM) image of pillar like structure in the reaction chamber.*

2.4.2 Optimization of on-chip peptide synthesis

On-chip peptide synthesis experimental overview may be shown in the following Fig. 2.3. The silicone tubing was used for inlet and outlet tubing connecting the microchip to sample bottles and syringe pump respectively. Around 20-30 amino resin beads were put into the reaction chamber with the help of pipette and the reaction chamber was sealed-off with thin layer of PDMS. The beads were observed and confirmed by the microscope.

For microchip with pillar like structure, an amino resin bead was introduced from inlet channel. The amino resin bead would be trapped at the reaction chamber when pressure was

applied. The other steps of peptide synthesis on the resin bead remained the same as that on the multiple beads. After each synthesised, peptide sequence was confirmed by protein sequencer.

2.4.2.1 Optimization of coupling time

Short di-peptide (Gly-Ala) was synthesised following the protocol of Fmoc-SPPS initially on the developed microchip system for peptide synthesis. 5μl/min of flow rate was kept constant while synthesising peptide (di-mer) for 3 min, 5 min, 10 min, 15 min and 20 min coupling time.

First of all, the peptide was synthesised on multiple bead. After that, single bead was taken out and confirmed the peptide sequence with Protein Sequencer. Sequencer sequenced the peptide from terminal end which means the last amino acid that was synthesised would be degraded and gave the chromatogram first with Edman degradation process. Retention of Alanine (Ala) or Glycine (Gly) of the synthesised was compared with the standard Ala or Gly peak and thus confirmed the success of peptide synthesis on the microchip. The chromatogram of the dipeptide is shown in Fig. 2.3. So, the peptide was synthesised for the above mentioned coupling time. Fig. 2.4 shows the graph of coupling time and from this, it is known and proved that within 10 min of coupling time, peptide could be synthesised on the microchip.

There was some clogging of resin beads towards the outlet channel due to which the reaction couldn't be performed well as illustrated in Fig. 2.5. And it seemed that the reaction of peptide synthesis may not be uniformly reacted or synthesised due to this problem. So, we designed another microchip for single bead. After single bead was introduced to the reaction chamber, the bead was trapped in between the pillar-like structure and dimer (Gly-Ala) were synthesised following the same reaction condition as before. The chromatogram of di-peptide

for single bead is shown in Fig. 2.6, whereas, the optimization of coupling time for single bead is shown in Fig. 2.4. Since there is better and uniform interaction of bead with the solution, formation of peptide or amide bond is higher as we can see from the amount of detection of the amino acid forming amide bond.

2.4.2.2 Optimization of flow rate

After optimization of coupling time of reaction, same dimer was synthesised to optimized flow rate using 15 min as time of coupling for both the microchip systems. 5µl/min of flow rate gave the highest detection of synthesised peptide by protein sequencer for both the cases, multiple and single as shown in Fig. 2.7. With this flow rate and coupling time, peptides could be synthesised successfully in the designed microchip. This may be explained by the fact that, the fresh reagents are continuously introduced to the reaction chamber and with this flow rate, amino acid coupling can be done at the highest maximum rate.

2.4.3 Longer peptide synthesis on microchip system

After optimization of coupling time and flow rate, nona-mer (9-mer peptide sequence of Gly-Ala-Ala-Leu-Val-Met-Ille-Met-Phe) was synthesised on microchip designed for single bead. The chromatogram of nona-mer peptide is shown in Fig. 2.8. This experiment is performed to ascertain the fact that peptide could be synthesised in microchip quicker and faster than the conventional Fmoc-SPPS method. Traditionally, coupling reaction time is 2-3 hours [15], but in microchip we could synthesise peptide with coupling time of just 15 min.

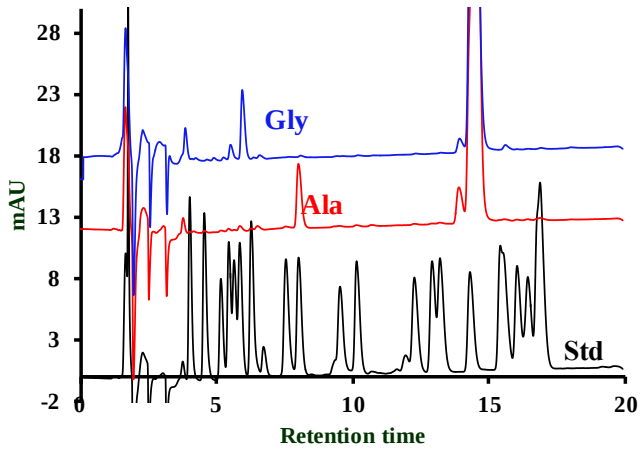


Fig. 2.3 *Chromatogram of dipeptide (Gly-Ala) comparing with chromatogram of standard amino acids.*

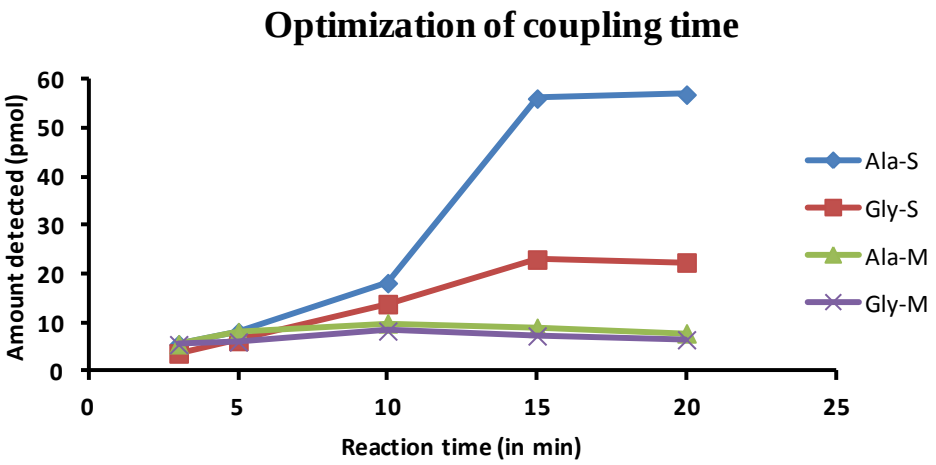


Fig. 2.4 *Optimization of coupling time of on-chip peptide synthesis. Ala-M, Gly-M represents the individual amino acids synthesised on multiple beads whereas Ala-S, and Gly-S represents the individual amino acid synthesised in microchip designed for single bead. 3 min, 5 min, 10 min, 15 min and 20 min of coupling time were analysed while synthesising di-mer peptide (Gly-Ala).*

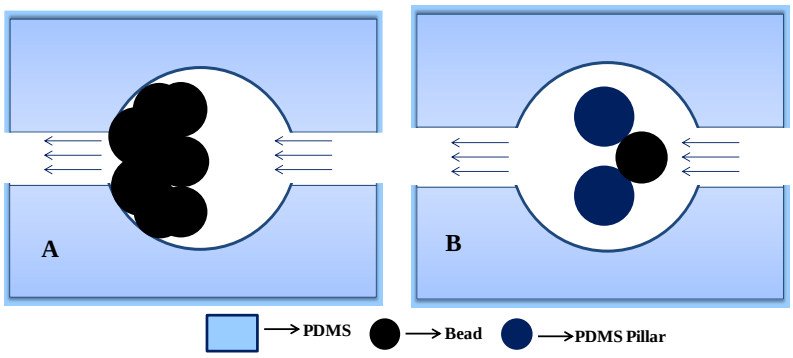


Fig. 2.5 *Illustrating the clogging of multiple beads towards the outlet as shown in (A, left image) and trapping of single bead by pillar-like structure at the center of the chamber (B, rght image).*

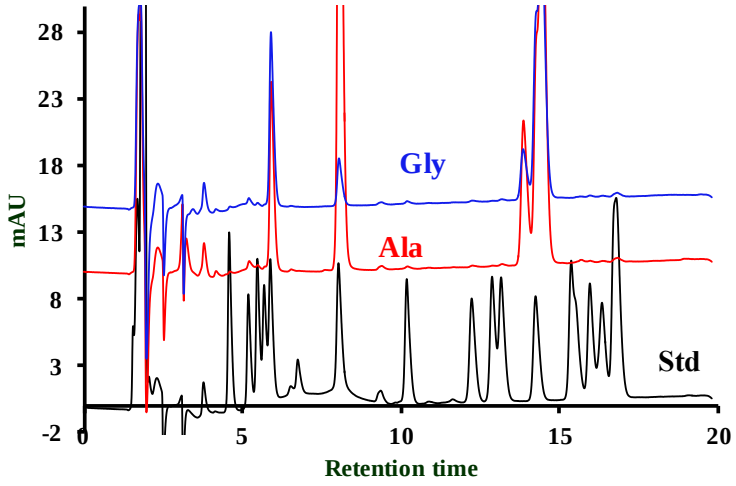


Fig. 2.6 *Chromatogram of dimer (Gly-Ala) on microchip system designed for single bead. The peak for Gly, and Ala are compared with the standard chromatogram peak.*

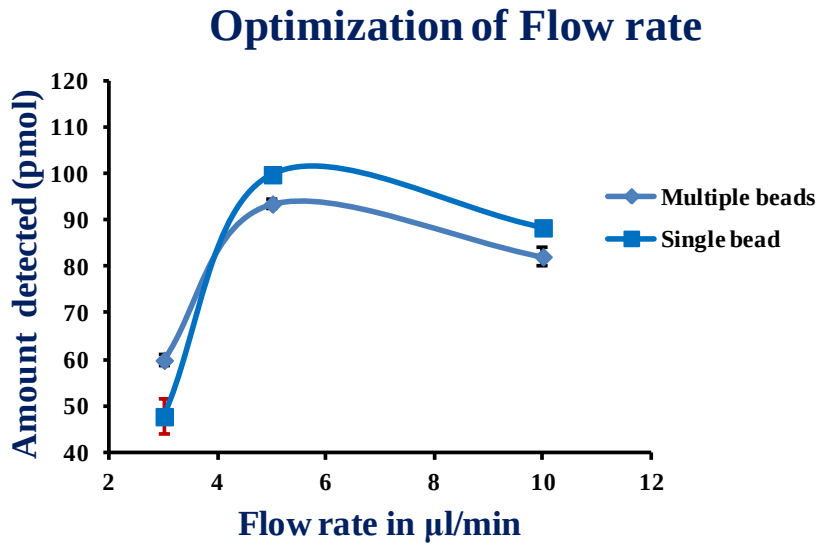


Fig. 2.7 *Optimization of flow rate by synthesising dimer on the microchip designed for both multiple beads and single bead. 3µl/min, 5µl/min and 10µl/min flow rates were analysed.*

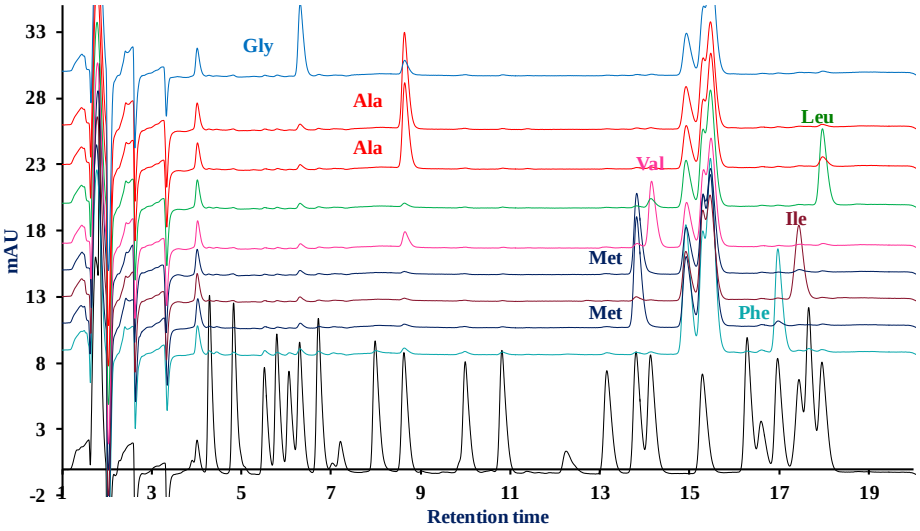


Fig. 2.8 *Chromatogram of nona-mer peptide sequence synthesised on the microchip designed for single bead. The peptide sequence of Gly-Ala-Ala-Leu-Val-Met-Ille-Met-Phe is successfully synthesised on the microchip.*

2.5 Summary

We have successfully developed a microchip system to synthesize peptide using conventional peptide synthesis method. After designed microchips were fabricated using all the resources and facility in clean room, Fmoc-SPPS methods is utilize to synthesis peptide.

Initially, on-chip peptide synthesis coupling time was optimized for the two systems that developed. For the microchip system designed for multiple beads, coupling 10 min gave the highest detection amount of around 10 pmol. Whereas with the microchip designed for single bead, coupling time of 15 min gave the highest detection amount twice than the microchip designed for multiple beads. Traditionally, coupling time is around 2-3 hours, but we could achieve the coupling time to 10-15 min.

Finally, nona-mer peptide sequence was synthesised on the microchip system designed for single bead to confirm the system possibility of peptide synthesis. So, in this way, it is our desire to synthesize peptide probe quickly or rapidly to develop a programmable biosensor.

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CHAPTER 3

DETECTION OF STREPTAVIDIN BIOMOLECULE USING ON-CHIP SYNTHESISED PEPTIDE PROBE MOLECULE

Abstract

Development of high throughput diagnostic devices is highly demanded due to constant outbreak of unknown yet deadly microbial pathogens and continuous threats of bio-warfare or-terrorism to the mankind. The next generation, novel point-of-care devices have thus been trying to devise to combat against all odds. Due to advancement and integration with various fields and technologies, miniaturized diagnostic and analytical device called 'biosensors' has been developed lately. Yet, in spite of the selectivity, accuracy and sensitivity of traditional biosensors as diagnostic devices, they cannot meet of all the demanding situations. This problem arose because the sensing element or probe molecules cannot change flexibly within the system easily. Through vigorous consideration, 'programmable biosensors' is proposed here to flexibly change the target analyte by changing the probe molecule based on on-site and on-demand situation. This may be achieved by synthesizing short peptide as probe molecules on-chip following traditional Fmoc-SPPS strategy. Linear hepta-peptide probe (-NH-PPGQPHH-NH_2) to be synthesised on-chip (optimized coupling time of 15min at 5µl/min flow rate) is proposed here in this research and successfully performed in situ detection of the target biomolecule on the same microchip.

3.1 General Introduction *(Partially published in JJAP by the author [1])*

Development of biosensors, as a high throughput device, is an enormous step forward to combat against all these odds in the present generation. They are very successful in dealing with the current clinical or non-clinical analysis because of their high selectivity, sensitivity and accuracy. The specific interaction between sensing element and target analyte (ligand:analyte interaction) makes this biosensors very efficient in detection resulting in a highly sensitive biosensor. But, recent biological warfare and outbreaks of unknown microbial pathogens around the in and around the world, emphasize clearly the need of a new generation and rapid diagnostic device, which can take information generated from the study of genomics, proteomics, metabolomics and glycomics [2, 3].

Due to advancement of μ-total analysis system (μ-TAS), innovative incorporation of lab-on-chip [4] and the prospects of microfluidics [5], diagnosing in these modern days uses miniaturized devices like biosensors and point-of-care techniques (POCT). With such development, human life becomes better and more secure from various biothreats, and also solving the problems associating with traditional clinical related issues [6]. Biosensors are used in a wide array and microarray for forensic sciences [7], food science [8], protein [9], clinical and non-clinical applications which is including rapid clinical diagnostics [10, 11, 12], DNA analysis [13, 14], etc.

The conventional biosensors are strictly restricted themselves to a particular target analyte yet they are very sensitive even in the low concentration (e.g. glucose sensor is specific or sensitive to glucose level in the body only). The main issue of how to analyse any incoming target analytes or multiple target molecules within the same system is still remained unsolved. In simpler words, target molecules cannot detect flexibly according to the demanding situation. Besides the sensing molecules or probe molecules cannot quickly prepare or synthesize to

analyse. Traditional biosensors use natural probe molecules (like antibody-antigen, proteins etc) which are obtained from a market or other sources.

A novel next generation diagnostic device called 'programmable biosensor' is proposed here in this research. This biosensor may flexibly change the target molecule by changing the probe molecules in order to be used in on-demand and on-site situations. In order to evaluate the proposed solution, short peptides as probe molecules may be synthesised on the microchip following the strategy of 9-fluorenylmethyloxycarbonyl-solid phase peptide synthesis (*Fmoc*-SPPS) [15]. Streptavidin is a tetrameric protein produced by *Streptomyces avidinii* which binds to the vitamin H (biotin) ($K_d \sim 10^{-15}$) with affinity higher than any other known biological binding sites [16]. HPQ sequence has great affinity with streptavidin molecule [17]. Therefore linear heptapeptide -NH-PPGQPHH-NH_2 containing HPQ sequence is proposed as peptide ligand/probe.

3.2 Research Objective:

The main objective of the research is to detect streptavidin biomolecule by on-chip peptide probe molecules using previously developed microchip reaction chamber. The authors may proceed by categorizing into sub-objectives as follow:

(I) Design and fabricate the microchip for peptide synthesis,

(II) Propose and synthesis peptides as probe molecule,

(III) Study the interaction of streptavidin with synthesised peptide through fluorescent microscope

3.3 Experimental methods

Since optimization of peptide on microchip has been successfully done, the optimized conditions are utilized for the synthesis of proposed target peptide sequence. In this experimental part, we would like to discuss:

(I) The microfabrication of the microchip,

(II) The synthesis of the target peptide sequence on microchip,

(III) Detection of the interaction between the ligand and analyte under microscope

3.3.1 Materials and equipment used

Since, materials and equipments for microfabrication steps used the same with the previous chapter 2. The list can be obtained from Table 2.1 But for detection, the following list of equipment and materials have been utilized as shown in Table 3.1

Table 3.1 *List of materials and equipment for detection of ligand and analyte interaction*

Materials and Equipments	**Suppliers**
Microscope (1×71)	Olympus, Japan
Microscope (SZ×12)	Olympus, Japan
Glass slides	Matsunami Glass Ind. Ltd
EM-CCD camera C9100	Hamamatsu, Japan
Adobe Photoshop 7 software	Adobe System Inc.

3.3.2 Chemicals used

Some of the chemicals used in this research have been already listed in Table 2.2, and Table 2.3. But there are few chemicals that have added to the list during the experimentation of detection of streptavidin biomolecule and peptide probe as shown in Table 3.2

Table 3.2 *Chemicals used for peptide synthesis and to study the interaction of peptide probe with target streptavidin biomolecule*

Chemicals	**Suppliers**
Streptavidin-fluorescent polymer	Sigma Aldrich
Sodium Chloride	Nacalai Tesque, Japan
Disodium hydrogen phosphate	Wako, Japan
Potassium dihydrogen phosphate	Wako, Japan
Fmoc-Gly-OH	NovaBiochem, Japan
Fmoc-Ala-OH	NovaBiochem, Japan
Fmoc-Gln-OH	NovaBiochem, Japan
Fmoc-Pro-OH	NovaBiochem, Japan
Fmoc-His-OH	NovaBiochem, Japan
Fmoc-Cys-OH	NovaBiochem, Japan
BSA	SigmaAldrich

3.3.3 Microfabrication of microchip

Microchip designed for single bead was fabricated using the clean room facility following the same procedure as explained in chapter of this thesis. The microchip for single bead was used as system because this gave a better result and higher amount of detection.

3.3.4 Peptide probe synthesis

Peptide probe containing HPQ sequence was proposed to be synthesised. Linear hepta-peptide probe (Pro-Pro-Gly-Gln-Pro-His-His) was synthesised in fabricated microchip by following Fmoc-SPPS conventional protocol. Resin bead was soaked in DMF solvent for at least 30 min before introducing to the center of the microchip. After that, Pro was introduced with the activating reagents for 15 min at the flow rate of 5μl/min.

Another peptide sequence was also synthesised which doesn't contain HPQ sequence in it. Linear peptide (Gly-Gln-His-Pro-Ala) was synthesised following the same procedure in the microchip system. After synthesising the peptide probes were confirmed their sequence through protein sequencer because specific sequence of peptide interact with specific biomolecule. The experimental set-up is shown in Fig. 3.1(A). The inlet and outlet channel were connected by silicone tube to the reagent bottle and syringe pump respectively.

3.3.5 Detection using peptide probes

3.3.5.1 Preparation of buffer

PBS (10 mM, pH 7.4 with BSA) buffer solution was prepared initially. To prepare this, 0.26 g of KH_2PO4, 2.17 g $Na_2HPO4\text{-}7H_2O$, 8.71 g of NaCl were dissolved in 800 mL of milli-q water. The pH of the solution was adjusted to 7.4 and made up the volume to 1L with milli-q water.

3.3.5.2 Detection of interaction between the peptide probes and streptavidin biomolecule

The resin bead with the synthesized peptide was first washed with (40×3) µl of the buffer solution (PBS (10mM, pH 7.4 with bovine serum albumin) from the inlet holes with the help of pipette. This would be followed by the introduction of 40µL (6µg/ml and so on) of fluorescent-labeled streptavidin biomolecules (prepared by diluting 100µl of the 100x concentrated (product code S3441) with 10 ml of the PBS and BSA). (40×3)µl of buffer solution was used to rinse the residual again. The fluorescent image was captured during every steps of procedure by EM-CCD camera attached to the inverted microscope. The experimental set-up of the fluorescent imaging could be shown as in the following Fig. 3.1(B). The intensity of the captured image was calculated using adobe adobe photoshop 7.0. The interaction of fluorescently labeled streptavidin and resin bead without any peptide was also measured using the same analysis procedure.

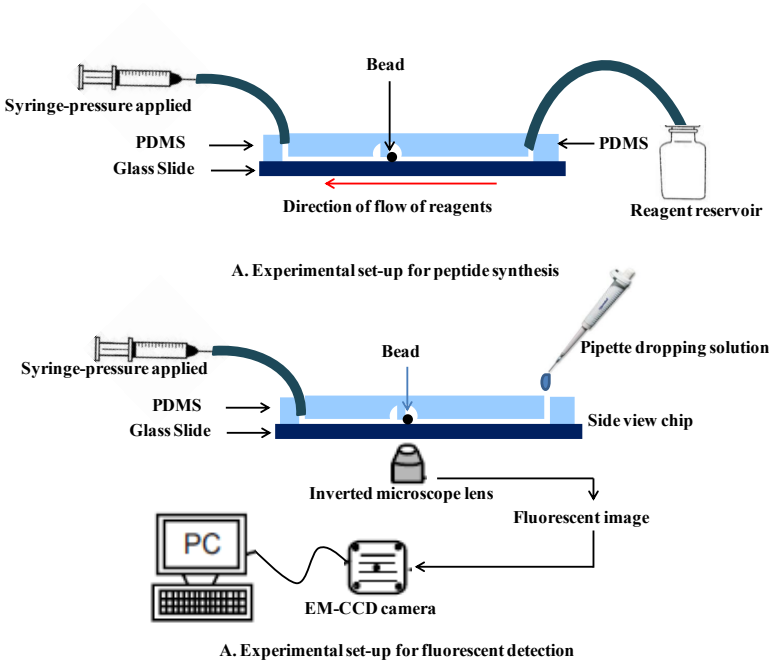

Fig. 3.1 *Experimental set-up of peptide synthesis on microchip designed for single bead (A), and experimental set-up for fluorescent detection (B).*

3.4 Results and discussion

3.4.1 Peptide probe synthesis

Fmoc-SPPS protocol is used to synthesize peptide and the mechanism behind the amide bond formation may be illustrated in Fig. 3.2. The free amine group and activated carboxylic group are reacted and form an active ester, during which these two functional groups can be interacted. But in order to free the amine group from the peptide, Fmoc-group has to be cleaved. 30% piperidine is used to cleave and the mechanism may be demonstrated in Fig. 3.3.

The proposed peptide ligand containing HPQ sequence was synthesized on single bead. The synthesized peptide was then characterized by the protein sequencer. The chromatogram obtained is shown in the Fig. 3.4. The retention time of the individual amino acid was compared with that of the standard amino acids confirming the sequential degradation by the Edman

degradation chemistry. The degraded amino acid then undergoes analysis by high-performance liquid chromatography (HPLC) technique and thus gives the chromatogram.

Histidine (His) was last introduced to the proposed peptide sequence which was degraded first. The intense peak at 8.1 min is His peak confirming by comparing with the standard amino acids. The peak for glycine (Gly) in the chromatogram also the same with retention time of synthesised and standard glycine peak which is at 6.4 min. The retention for glutamine (Gln) is at ~5.9 min both for standard amino acid solution and the synthesised peptide. The retention time for proline (Pro) amino acid of the standard amino acid is at 13.2 min and that of the synthesised proline peak comes out to be at the same retention time.

The strong peak coming out at 14 min in the case of the synthesised peptide is for the peak for diphenylthiourea (DPTU), a byproduct after Edman degradation chemistry. After confirming the sequence of the successful synthesis of peptide on-chip, the synthesised peptide would like to be used as probe molecule for the detection.

3.4.2 Detection using peptide probes

Streptavidin (fluorescently labeled) was used as a target analyte in this measurement using fluorescent microscope. The intensity of the fluorescent peak of bead having no peptide probe has slight increment during the course of detection due to the interaction of bead polymer and the fluorescent dye though it's negligible compare with the peptide probe. As shown in Fig.3.5, the proposed peptide ligand with streptavidin has strong interaction with the target known molecule which is fluorescently labeled streptavidin biomolecule.

The interaction of peptide probe as we increase the concentration of streptavidin biomolecules, the intensity also increased very much which showed that HPQ containing sequence of peptide has good affinity with streptavidin as shown in Fig. 3.6. The peptide probe

can be removed from the bead via chemical removal process and can synthesize another peptide probe molecules on the same microchip for another target molecules. This method may be used for the detecting many target molecules flexibly by changing probe peptides. So, this may suggest that probe peptide can be synthesised on-chip and in-situ analysis or detection could also be done simultaneously accordingly during on-site and on-demand situations.

The overall reaction steps from fabrication of microchip to peptide synthesis and to study the detection with the microchip is illustrated properly in Fig. 3.7.

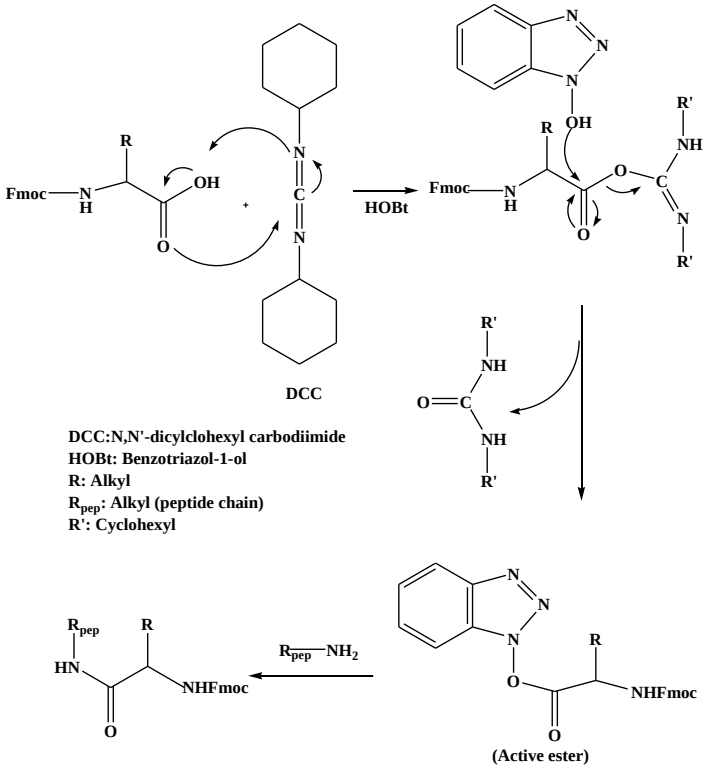

Fig. 3.2 *Mechanism of amide bond formation and peptide synthesis. Activating reagents activate the carboxylic group andupon interacting with free amine group, amide bonding is possible.*

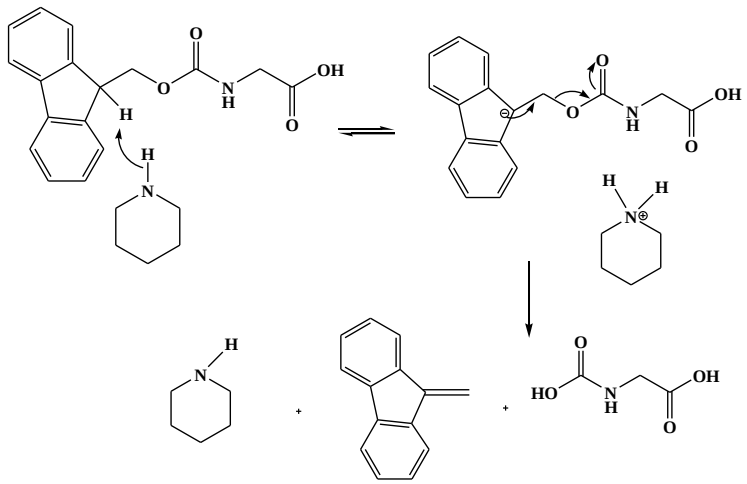

Fig. 3.3 *Mechanism of cleavage of Fmoc-group from amino acid. Piperidine is used for cleaving the Fmoc-group to free amine functional group.*

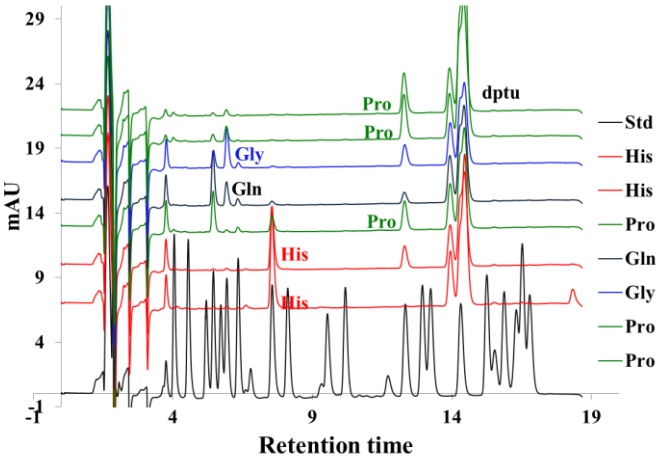

Fig. 3.4 *Chromatogram of propose peptide probe synthesised on microchip designed for single amino resin bead. Pro-Pro-Gly-Gln-Pro-His-His is synthesised successfully on bead in microchip.*

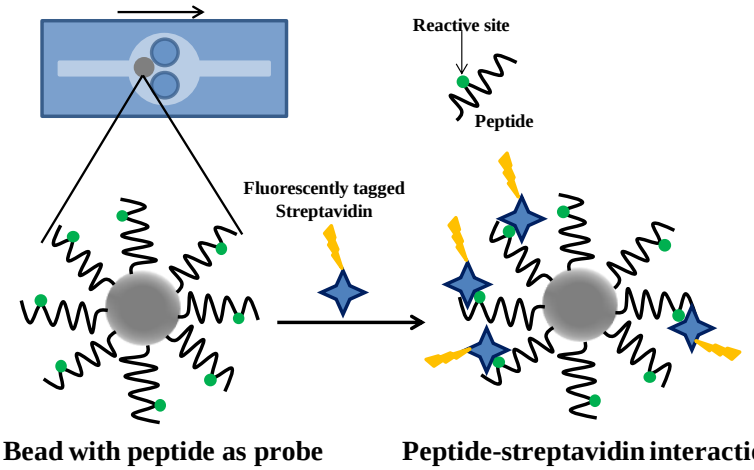

Fig. 3.5 *Illustrate the interaction of streptavidin biomolecule with synthesised peptide probe. Streptavidin with fluorescent tagged is interacting with peptide probe synthesised on bead using the following Fmoc-SPPS in the developed microchip system.*

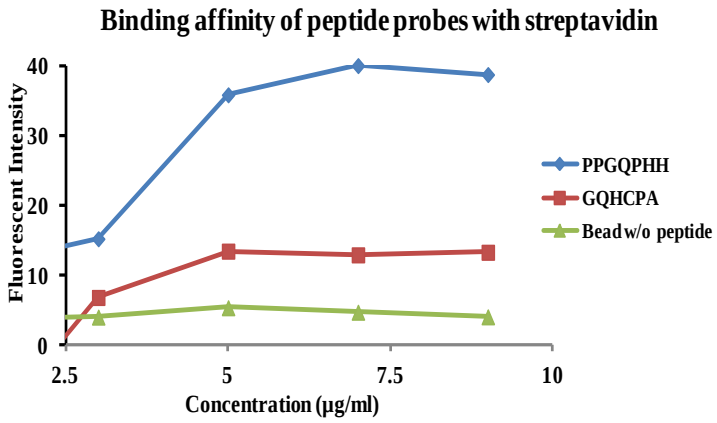

Fig. 3.6 *Detection of streptavidin with synthesised peptides in the microchip system designed for the single resin bead.*

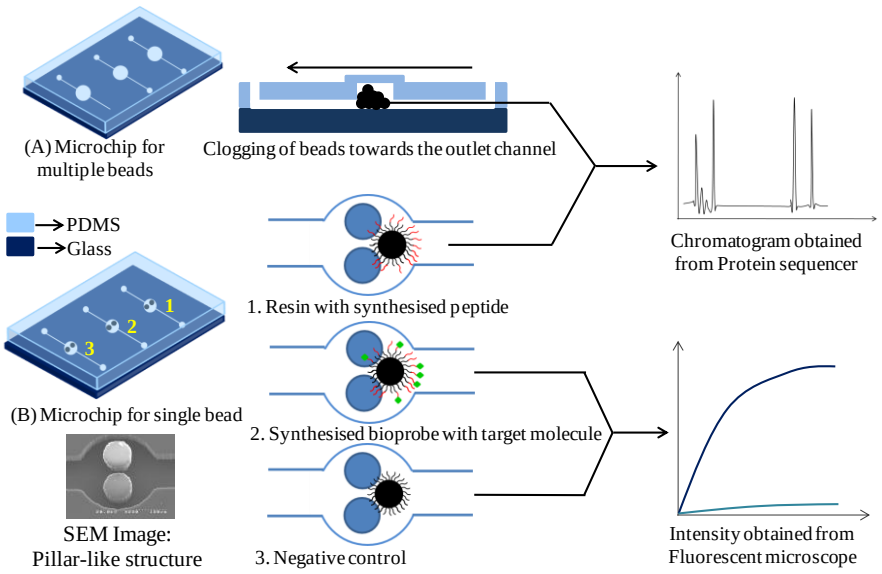

Fig. 3.7 *Illustrates the overall steps of detection of target sample is done in the microchip system after synthesising probe peptide.*

3.5 Summary

The detection of streptavidin by two probe molecules were performed in the microchip system. There are few things we may be concluded:

(i) Peptide probe synthesis and detection using peptide probe to target molecules can be performed within the same chip itself without any difficulties. This allows the potential possibility to reduce the sample and handling problem.

(ii) Peptide probes were successfully synthesised and analysed with peptide sequencer in the microchip system with optimized coupling time and flow rate.

(iii) From the literature, HPQ sequence is highly interactive with streptavidin and the system we developed can be used as detecting system as well. Two peptide probes were synthesised and with the binding affinity, streptavidin could differentiate the probe molecules very effectively.

The research may be summarized as the highly innovative resource in which the development of next generation programmable biosensor could be possible in the near future. The probe molecules on the sensing surface could flexibly change according to the target analytes by synthesizing probe peptides. Since, the coupling time of the on-chip peptide synthesis is within 15 min in microscale synthesis as compare to conventional SPPS of 1-2 h coupling time [14], quicker way of synthesizing probe molecule may solve the hurdle while developing novel sensors that able to prepare various probes on-site, on-demand. And the assaying of peptide probe with target molecule could be analysed showing high affinity with each other. It’s the desire of the authors to furthermore improve the system and method for better application and to verify the significant of this approach in the near future.

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CHAPTER 4

DIRECT OBSERVATION OF THE QUICK SYNTHESIS OF PEPTIDES ON GOLD PLANAR SURFACE BY FMOC-SOLID PHASE PEPTIDE SYNTHESIS ON SURFACE PLASMON RESONANCE CHIP

Abstract

A novel approach of quick and rapid synthesis of peptides on SPR gold surface is introduced in this research. There is always an issue of long sample preparation and difficulty in the synthesis of natural probe molecules. But in this case, we focus on synthesising a short peptide on SPR sensor chip through surface modification chemically. Short peptide, EYYY, a tetramer is synthesised on the modified CM5 sensor chip directly and study the interaction with an atrazine, a herbicide. The new finding opens up a different dimension with great potentiality to explore because SPR is very sensitive and specific. Peptide has been used as probe in biosensing and it is our wish to synthesis peptide for the programmable biosensor in which flexibility to change the probe molecule is the main criteria. Surface plasmon resonance has been used in many analytical applications due to its sensitivity, so it is our desire to utilize in the research. Development of very sensitive and massive sample analytical device from around the world has been realised in many different fields.

4.1 General introduction

Surface plasmon resonance (SPR) principle has been incorporated to develop an analytical tool for studying biomolecular recognition at the surfaces because it can be used to monitor the interaction of proteins and immobilized ligands in real time [1-5]. And optical sensors based on the excitation of the surface plasmons are referred to as surface plasmon resonance sensors (SPR sensors). In principle, SPR sensors are refractometers that measure changes in the refractive index happening at the interface of metal film support of the substrate. The plasmon excited by light waves propagates along the metal film and any changes on the surface can be easily monitored because even small changes could be detected as illustrated in Fig. 4.1

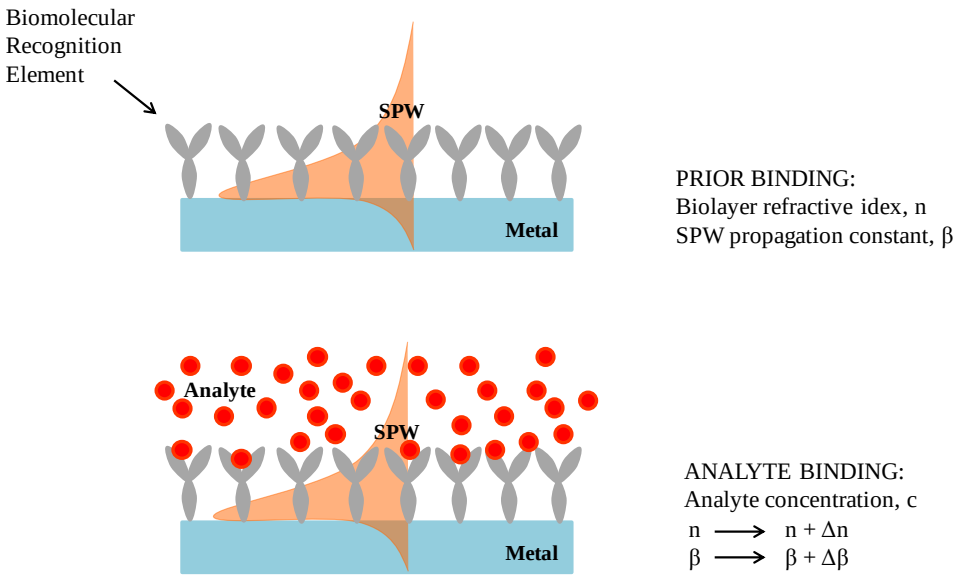


Fig. 4.1 *Illustrate the mechanism of surface plasmon under which change in refractive index corresponds with the change in propagation constant of surface plasmon wave (SPW).*

In the meanwhile, there are various application of the plasmonic technologies [6] may be suggested as shown in Fig. 4.2. SPR, LSPR, SPRi and SERS exploit and use plasmonic property of the metal to apply in:

(i) Point of care,

(ii) Biomolecular Imaging,

(iii) Biosensors

(iv) Medical diagnosis

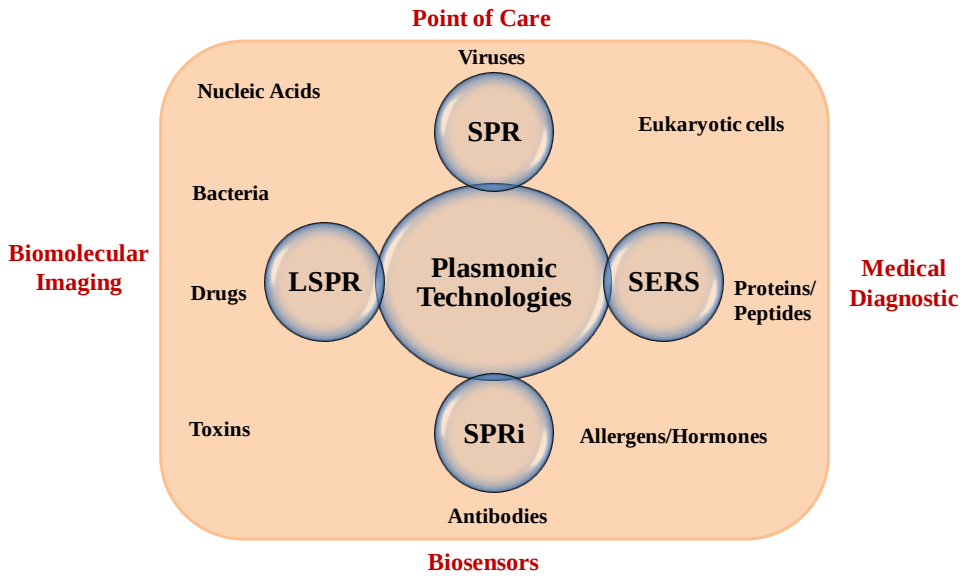


Fig. 4.2 *Illustrate the various applications of plasmon waves called plasmonic technologies.*

Recently, surface plasmon resonance has become an important sensing technology in the fields of biology, biochemistry and medical science because of its real-time, label free, and non-invasive nature. This may be because of the following characters and potentiality of the technology [7-10]:

(a) SPR can measure the physical quantities,

(b) SPR can be used as chemical sensing, and

(c) SPR as biosensing

The peptides as probe molecules have been used in biosensor. And peptides can be easily synthesised in laboratory scale using conventional peptide synthesis protocol. And in modern research, there have been development of peptide synthesis on microchip [11, 12] which can provide the following advantages:

(i) Coupling time of peptide synthesis is faster than conventional method,

(ii) Lesser reagent used,

(iii) Higher yields of peptide, and

(iv) Lesser side reaction or contamination.

But in these papers, the biosensing is done with the help of labeling technique which have again inherent limitation regarding the signal/noise ratio, background noise and other disadvantages. The other limitation is that beads are used in which peptide may not be able to synthesised uniformly in a short span of time giving less reactive and sensitive.

4.2 Research Objective

Biosensors using peptides as ligand has been motivated due to the various complimentary recognition sites specific of peptides and proteins. Recently, short peptides have been used as probe molecules for developing various sensors or detectors. And in this research, it our objectives to directly synthesis of peptide probe molecules on SPR sensor chip and study the interaction with target molecules.

(i) Optimization of coupling reaction time of amide formation on SPR chip

(ii) Synthesis of peptide tetramer (EYYY) as ligand on the planar surface of SPR chip

(iii) Study the interaction of ligand and analyte (analyte being atrazine).

4.2 Experimental Approach

4.2.1 Materials and equipment used

During this experiment, the materials and equipment that has been utilized are listed in the Table 4.1

Table 4.1 *Lists of materials and equipment used*

Materials and equipment	**Suppliers**
CM5	GE healthcare, Sweden
Biamaintenance kit	GE healthcare, Sweden
HBS-EP Buffer, BR-1001088	GE healthcare, Sweden
Biacore J	Biaocore

4.2.2 Chemical used

The chemicals used during the synthesis of peptide on Biacore J, an SPR machine, are listed here in Table 4.2

Table 4.2 *Lists of chemicals used*

Chemicals	**Suppliers**
HEPES	Dojindo, Japan
EDTA	Dojindo, Japan
Tween 20	Wako, Japan
Sodium chloride (NaCl)	Nacalai Tesque, Japan
Fmoc-Tyr-OH	NovaBiochem
Fmoc-Glu-OH	NovaBiochem

DMSO	Wako, Japan
Ethanol amine	SigmaAldrich
Atrazine	Wako, Japan
Carbohydrazide	TCI, Japan

4.2.3 Sample preparation

HBS-EP Buffer preparation:

Chemical used:

a) HEPES

b) NaOH

c) EDTA

d) Tween 20

e) NaCl

(I) Preparation of 0.5 M HEPES buffer (pH 7.4) (500 mL)

0.5 M or 500 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer is prepared by mixing 59.6 g of HEPES powder with 400 mL of milli-q water. pH of 7.4 is adjusted with NaOH.

(II) Preparation of 0.5 M EDTA (pH 8.0) (500 mL)

93.1 g of ethylenediamine-N.N,N',N'-tetraacetic acid disodium salt (EDTA.2Na) is mixed with 400 mL of milli-q water. The pH is adjusted to 8.0 with NaOH and after which milli-q water is added to 500 mL.

Note: EDTA.2Na will not be completely dissolved until the pH reaches 8.0.

(III) Preparation of 20 % (v/v) polysorbate 20 (500 mL)

400 mL milli-q water is added to 100 mL of polyoxyethylene (20) sorbitan monolaurate (Tween 20).

(IV) 10 × HBS-E (500 mL)

100 mL of 0.5 M HEPES buffer, pH 7.4, is mixed with 30 mL of 0.5 M EDTA, pH 8.0 and 44 g of sodium chloride (NaCl). Milli-q water is added to 500 mL.

(V) Preparation of 1 × HBS-EP (2 L)

400 mL of 10 × HBS-E buffer is taken and milli-q water is added to make it 2 L. After that 0.5 mL of 20% (v/v) polysorbate (20) is added.

(1) Preparation of EDC/NHS:

Chemical used:

a) 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)

b) N-hydroxysuccinimide (NHS)

0.4 M (400 mM) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) is prepared in milli-q water in a vial. In the other vial, 0.1 M (100 mM) of N-hydroxysuccinimide (NHS) is prepared with milli-q water as solvent

(2) Preparation of 50% ethanol amine

50% ethanol amine is prepared in DMSO.

10 mL of ethanol amine is taken and dissolve in 10 mL of DMSO in vial.

4.2.4 Peptide synthesis on SPR chip

1 1:1 ratio of NHS and EDC was introduced for 6 min.

2 Carbohydrazide was injected for 6 min.

3 Ethanol amine HCl was injected to remove the un-reacted and residual from the sensor chip.

4 Amino acid with activating group (as NHS/EDC) with the ratio of 1:2:2 was injected and allowed to interact with the activated CM5 surface.

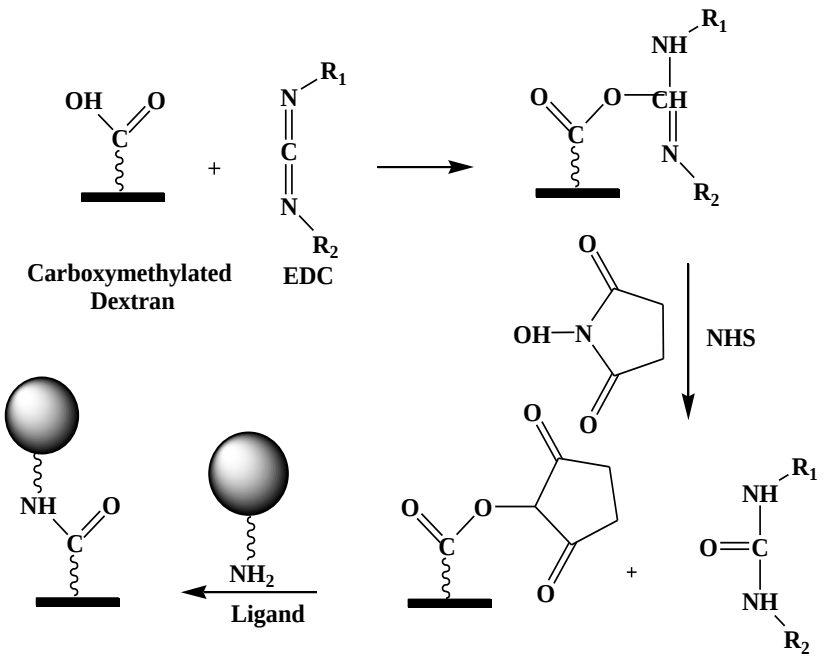
5 Regeneration buffer was injected for 6 min to regenerate the surface.

Buffer solution was kept running in order to avoid the contamination of the CM5 sensor surface. New buffer solution to be used while synthesising peptide was also prepared [15].

4.3 Results and discussion

CM5 is known for its many good qualities that impart on the sensor surface. One of the main important advantages of using CM5 is that it increases the surface capacity for ligand immobilisation [16, 17]. And another one is CM5 surface can be chemically modified and one such example of chemical modification is shown in Fig. 4.3 and 4.4 whereby illustrating amine coupling and aldehyde coupling respectively.

The normal representation of Sensorgram of SPR signal is illustrated in Fig. 4.5 in which association steps, dissociation steps and regeneration steps are depicted.



R_1 & R_2 : alkyl group
NHS: N-hdroxy succinimide
EDC: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide

Fig. 4.3 *Amine coupling of ligands to the sensor surface*

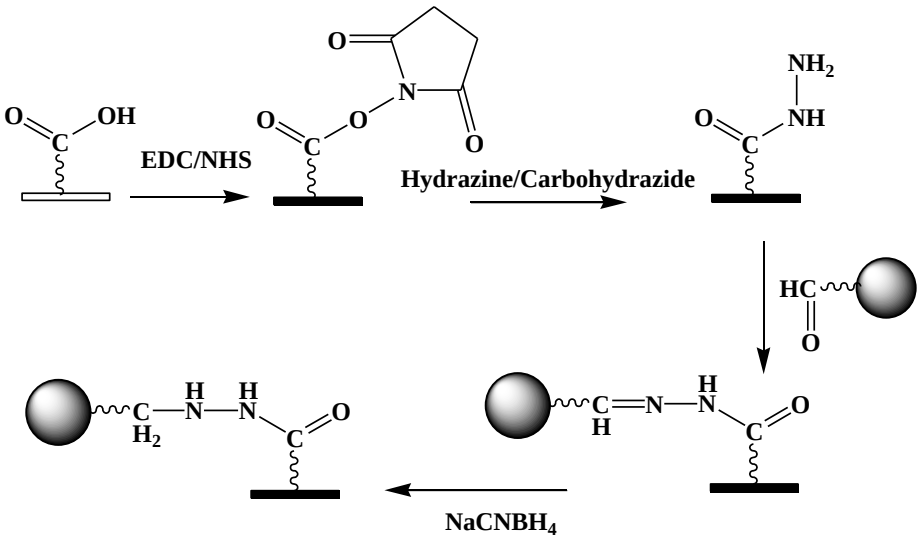


Fig. 4.4 *Aldehyde/carboxy coupling of ligand to the sensing surface*

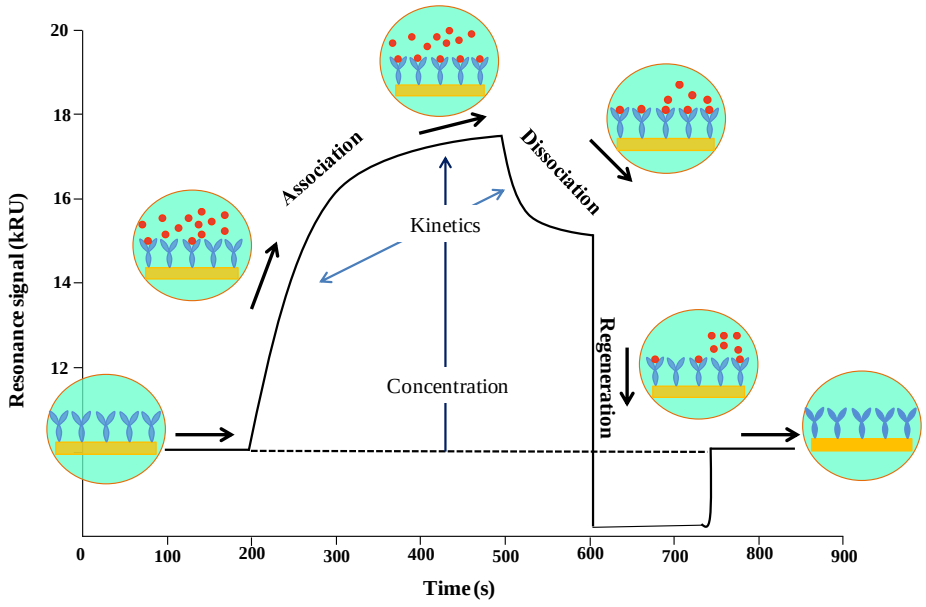


Fig 4.5 *Illustration of SPR signal during the interaction or modification of sensor surface due to binding affinity and because of the change in refractive index after every modification, the change in response against time is plotted.*

4.3.1 Effect of concentration of amino acid on sensor chip

In the research, we were interested mainly on peptide synthesis on the sensor surface. So, first of all, an amino acid (Fmoc-Tyr-OH) was introduced and allowed to interact with the already activated CM5 surface. Different concentration of 0.5 mM and 0.05 mM were tested and checked. However the Sensorgram for both the concentration gave almost same results as shown in Fig. 4.6. Thus in the later parts of the experiment, 0,05 mM concentration of amino acids were used. The difference between the response unit (RU) from the base line before introduction or injecting the sample and after injecting the sample gave the information how much refractive index has changed.

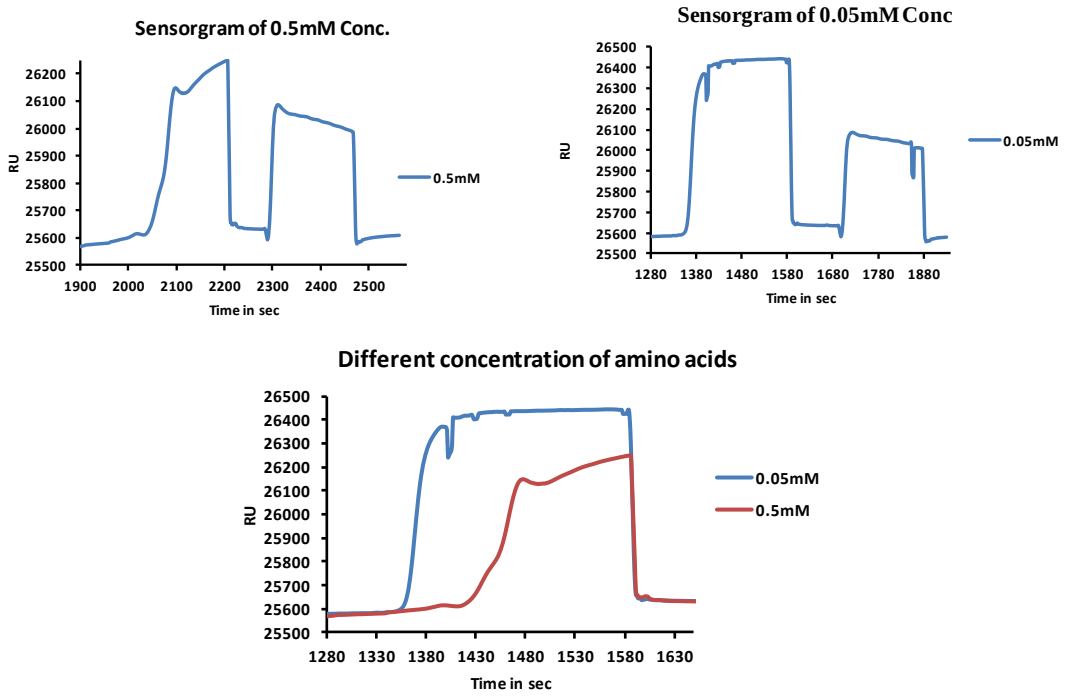


Fig.4.6 *Sensorgram of different concentration of amino acid. 0.05 mM concentration is taken as the concentration in later part of the experiment.*

4.3.2 Deprotection of Fmoc-group

To elongate the peptide sequence from the base, Fmoc-group has to be removed. Normally, 20-30% of Piperidine is used for cleaving the Fmoc-group from the amino acids. But in the Biacore system, not more than 50% DMSO can be used. However, 50% ethanolamine can be also used for deprotecting the Fmoc-group [13]. So, in our research, 50% of ethanolamine was used for the cleavage step of Fmoc-group. The cleaving time was monitored by injecting the solution for 1 min, 2 min and 3 min. The Sensorgram of the cleavage step could be analysed as shown in Fig. 4.7. In Fig. 4.8, the change in response unit against time of deprotection was plotted. Since the graph is exponential, we could conclude that 4-5 min of deprotection is enough for the cleaving step. Though the mass of Fmoc-group is small, the removal of Fmoc-group on the Sensorgram can be studied and in the later experiment, 5 min of deprotection was used.

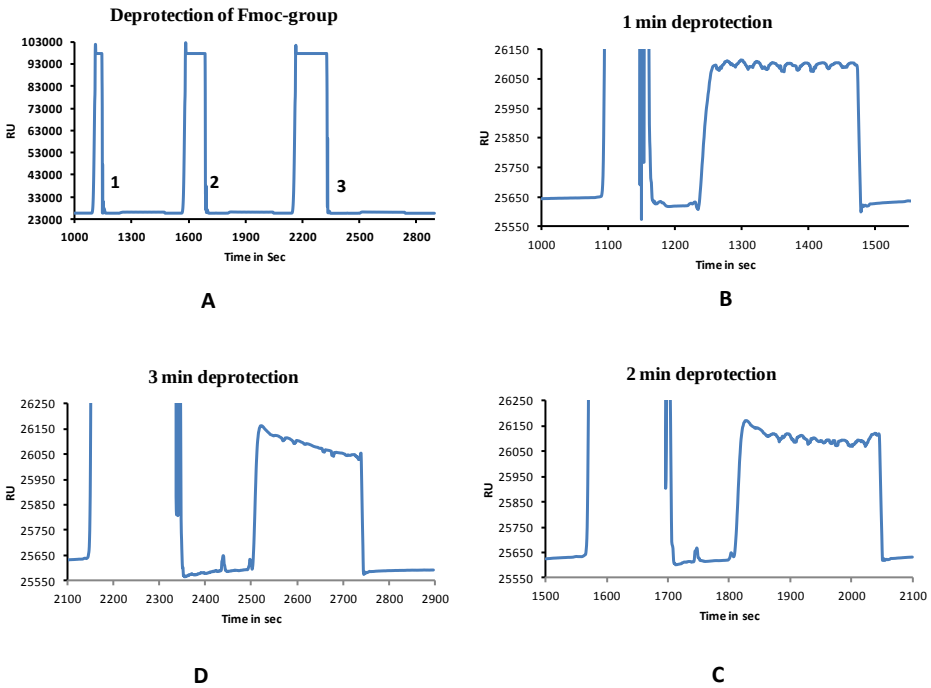


Fig.4.7 *Deprotection of Fmoc-group to free the –NH_2 functional group to elongate the peptide chain. B, C, and D are the zoomed image of A of 1, 2 and 3 min respectively.*

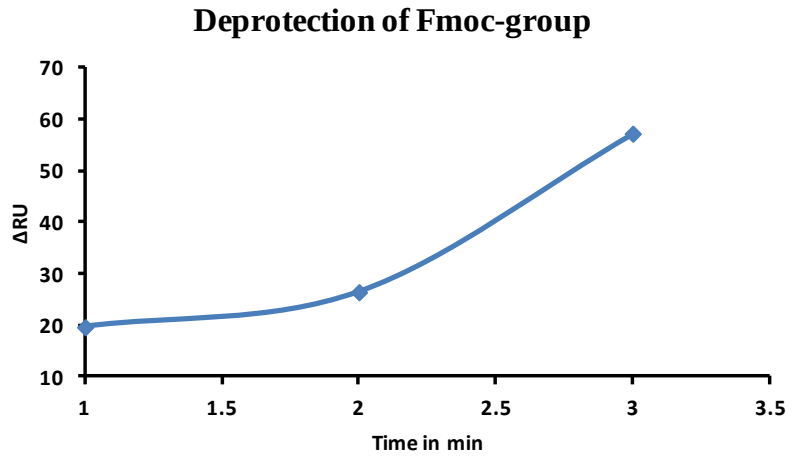


Fig.4.8 *Graph between the change in response unit (RU) and deprotection time.*

4.3.4 Coupling time on CM5 sensor chip

Coupling reaction time is very important step in peptide synthesis, so it is required to be optimized and studied for this system. As far as my knowledge serve, peptide synthesis in the SPR machine seem to be first time. After activating the surface of CM5 with NHS/EDC and carbohydrazide, an active ester intermediate is formed from –COOH group. But since, the incoming solution or amino acid is also containing –COOH group, it has to be activated. Normally, PyBOP and HOBt.H_2O are used for activating the Fmoc-amino group for Fmoc-SPPS. But instead, we used NHS/EDC for activating the –COOH group of the amino acids just like the first step. The activated amino acid solution in 5% DMSO buffer solution was introduced or injected for 1-7 min as shown in Fig. 4.9. The Sensorgram is shown in Fig. 4.10 as well. The data was obtained by subtraction of the signal change from before and after of injecting the reagent. This coupling rate is faster which may be because of the nature of the reaction area. In this case, the area of the sensor chip is planar, so interaction with each active functional group is easier and faster with less hindrance.

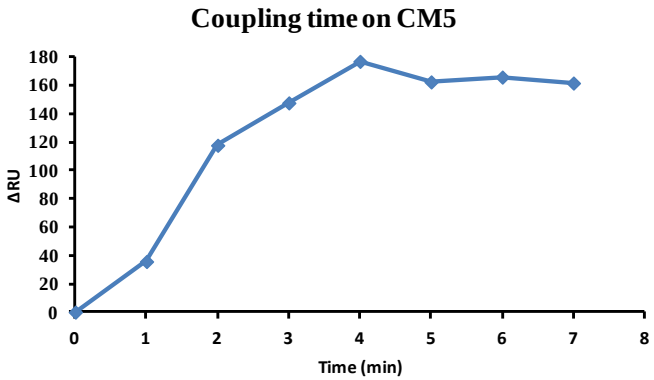


Fig. 4.9 *Coupling reaction time of amino acid on the surface of CM5. 4 min is sufficient enough to form an amide bond.*

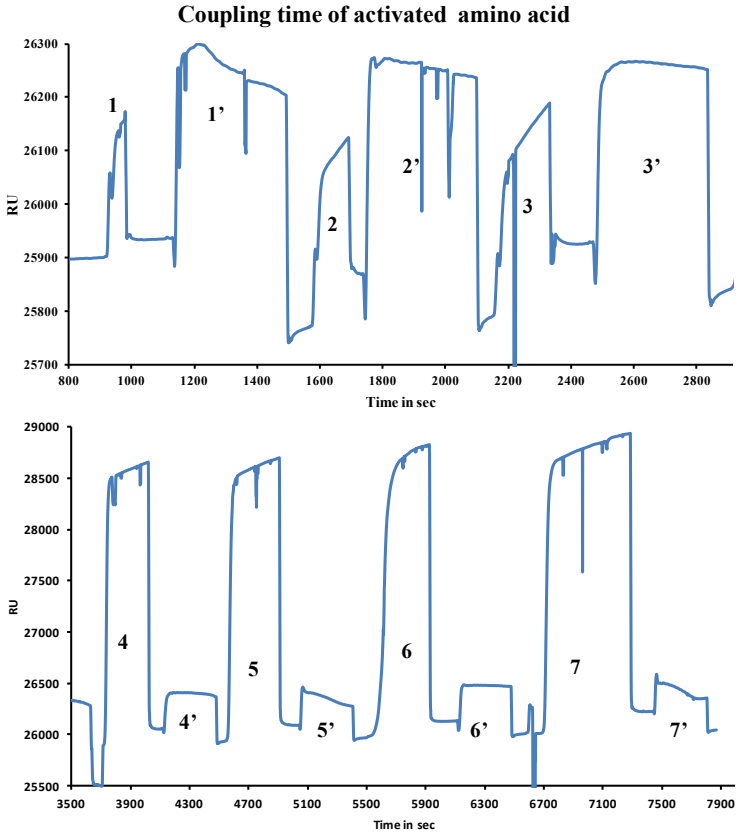


Fig. 4.10 *Coupling time of activated amino acid on CM5 surface. 1-7 numbers indicate the time in minutes and 1'-7' indicates the deprotection of Fmoc-group from the amino acid after every synthesis.*

4.3.5 Peptide probe synthesis on CM5 sensor chip

After confirming the possible reaction of peptide synthesis in the system, peptide probe was synthesised and monitored the synthesis on SPR sensor. First of all, CM5 surface was modified as explained before with NHS/EDC, and then to make the surface reactive to –CHO and –COOH group, carbohydrazide was introduced and injected for 6 min (normally hydrazine is used for activation, but carbohydrazide is less toxic and less dangerous). After that, the following amino acids were introduced. Firstly, Glutamic acid (E or Glu) was injected for 6 min into the sensor chip. After that, the chip was wash with buffer solution and then 50% of ethanolamine was injected for 4 min. Then activated Tyrosine (Y or Tyr) was injected for 6 min to form second amide linkage. Fmoc-group was cleaved to introduce another amino acid to get the target peptide chain of EYYY. The baseline of Sensorgram of the amino acid before introduction and after introduction was analysed because the difference of these two baseline would give how much has the surface been changed.

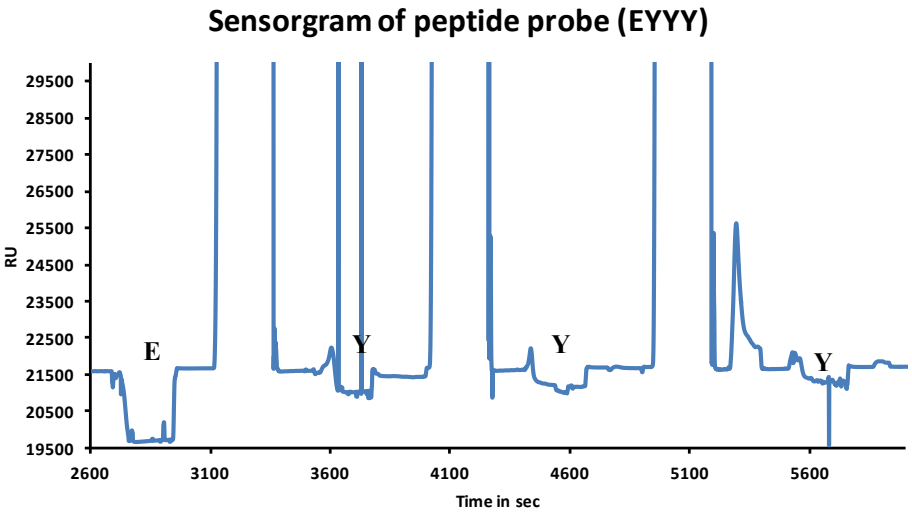


Fig. 4.11 *Sensorgram of peptide probe synthesised on activated CM5 sensor chip in Biacore J instrument.*

4.3.6 Detection of atrazine with peptide probe

After synthesising peptide probe on the sensor chip, atrazine as analyte was introduced. Different concentration of atrazine ranging from 1 mM, 5 mM and 10 mM were analysed as shown in Fig. 4.12. It is a known interaction between the atrazine and the peptide sequence [14]. But the sensitivity was from 200 mM to 1 mM in SPR sensors. The graph of the interaction between the atrazine and peptide ligand was plot as shown in Fig. 4.13. In the research, the interaction may be studied more thoroughly taking into account of other factors which may affect to the sensitivity of the interaction.

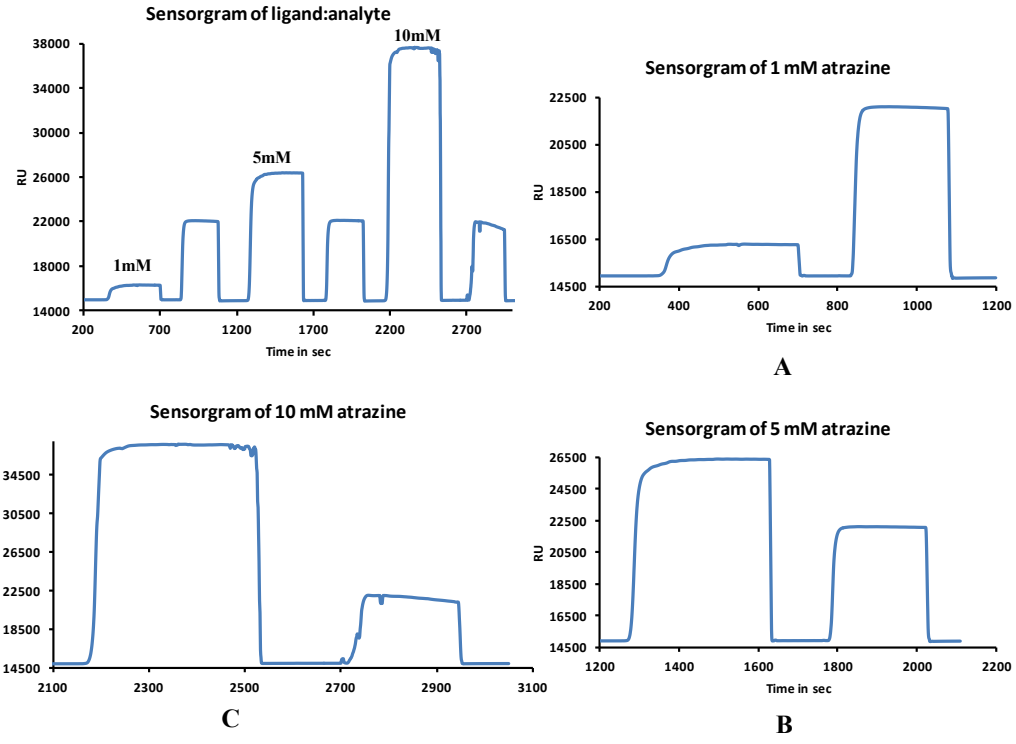


Fig. 4.12 *Sensorgram of atrazine and EYYY interaction on CM5 sensor as solid support. 1 mM, 5 mM and 10 mM of atrazine were analysed with the synthesised peptide probe [EYYY]. (A), (B) and (C) Sensorgram are the image being zoomed out of the interaction.*

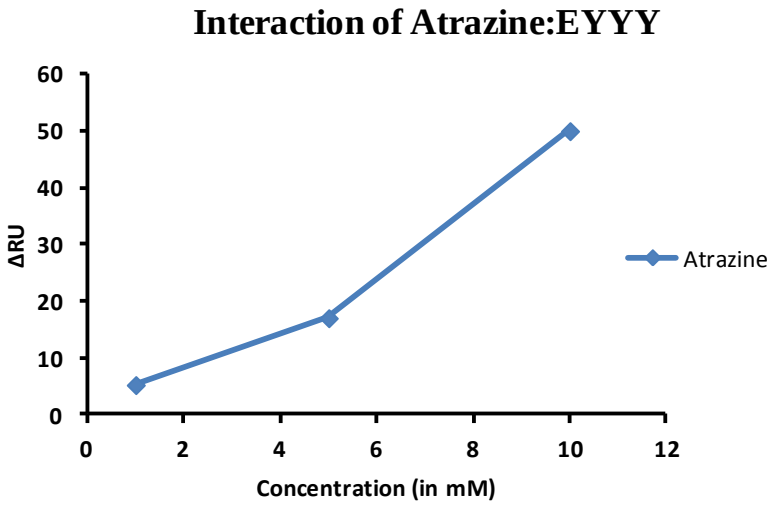


Fig.4.13 *Interaction of atrazine as analyte and EYYY as peptide probe detected by SPR sensors.*

4.4 Summary

The label-free detection of atrazine with the synthesised peptide probe was successfully demonstrated in Biacore J instrument. There may be some inherent limitation of the current commercialized SPR instrument, but in spite of that, direct and rapid synthesis of peptide was possible. The following points could be worth mentioning.

(i) Coupling time is faster (within 6 min) than that of the previous chapter.

(ii) The deprotection step and concentration of amino acid solution to be reacted were also optimized somehow. 4 min of deprotection time and 0.05 mM concentration of amino acids were used.

(iii) After confirming with the Sensorgram, EYYY, a tetramer peptide as probe molecule was synthesised on the CM5 surface.

(iv) Atrazine as analyte was used to detect and thus study their interaction through SPR.

It is our honest desire to extend the research further to check for more sensitivity. And to develop a programmable biosensor, probe molecules have to be flexibly changed and rapidly synthesised another target probe molecules on the same sensor surface without much problem.

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CHAPTER 5

CONCLUSION

A simple biosensing microchip system was developed in which peptide probe molecules can be synthesised using Fmoc-solid phase peptide synthesis protocol. Few points may be accounted while developing programmable biosensor in this research:

(i) Pillar-like structures were introduced at the center of reaction chamber of microchip to trap the single resin bead for peptide synthesis. Detection amount of peptide was 2 times increment from peptides synthesised on multiple beads without pillar-like structure. Coupling reaction time in the developed microchip system was 15 min compare to 2-3 hours conventionally. 5µl/min flow rate was used for synthesising peptides in microchip. Short peptide probe sequence can be synthesised effectively within few hours.

(ii) Binding affinity of streptavidin biomolecule with synthesised probe peptide was demonstrated successfully within the microchip system. Since streptavidin biomolecule is well-known biomolecule having high affinity with biotin naturally, study the binding affinity of streptavidin with synthesised peptide probe can be generalized. The potentiality to recognize such biomolecule with the developed system enhances the programmability of the sensor to detect possibly with large selectivity.

(iii) Peptide probe (EYYY) was synthesised on the modified CM5 and real-time in situ interaction with the target analyte (Atrazine) was demonstrated by SPR method. Since SPR method is a general and known label-free technique and can be used for the peptide probe synthesis and detection with biomolecules, thus, we could conclude that the developed programmable biosensor has the potential to realise even in general sensing method.

ACHIEVEMENTS

Paper Published

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2. Rahul Bhardwaj, Ngashangva Lightson, Yoshiaki Ukita and Yuzuru Takamura, Development of oligopeptide-based novel biosensor by solid-phase peptide synthesis on microchip, *Sensors and Actuators B: Chemical*, 192, **2014**, 192, 818-825.

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2. Lightson Ngashangva, Rahul Bhardwaj, Tue Trong Phan, Manish Biyani, Yuzuru Takamura, Highly sensitive label-free detection of biomolecules by in situ on-chip synthesised peptide probe molecules.

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