Design and Synthesis of Fluorescent Peptide Sensors for Monitoring Transition Metal Ions

Department of Chemistry, Graduate School
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Design and Synthesis of Fluorescent Peptide Sensors for Monitoring Transition Metal Ions

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List of Abbreviations

Abbreviations
A= Acceptor
Å = Angstrom
Boc= tert-butoxy carbonyl
CD = Circular Dichroism
CHEF =Chelation Enhanced Fluorescence
CHEQ = Chelation Enhanced Quenching
CHES = N-Cyclohexyl-2-aminoethanesulfonic acid
D= Donor
d-Pro = d-proline
DCC = Dicyclohexyl Carbodiimide
DCM = Dichloromethane
DIPC = di-isopropyl carbodiimide
DIPEA = di-isopropyl ethyl amine
DMF = N, N-dimethyl formamide
DMSO = diemethyl sulfoxide
DNA = de-oxy ribonucleic acid
DTT = dithiothreitol
DTNB = di-thiobis-2-nitro benzoic acid
DMAP = di-methyl amino pyridine
E = Efficiency of the process
EDTA = Ethylene Diamine Tetraacetic Acid
ESI = Electro Spray Ionization
EPA = Environmental Protection Agency
Fmoc = 9-fluorenylmethoxy carbonyl
FRET = Fluorescence Resonance Energy Transfer
GSH/GSSG = redox pair of glutathione
HOMO = Highest Occupied Molecular Orbital
H₂O₂ = Hydrogen peroxide
Hepes = N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid
HoBt = 1-hydroxybenzo triazole
H₂SO₄ = Sulfuric acid
HTM = Heavy and Transition Metal ions
hυ = Photon
J = Spectral Overlap
Kₐ = Association Constant
LMCT = Ligand to Metal Charge Transfer
LUMO = Lowest Occupied Molecular Orbital
MALDI-TOF = Matrix Assisted Laser Desorption Ionization-Time of Flight
MBHA resin = Methyl Benzhyridil Amino Resin
MCL = Maximum Contaminant Level
MT = Metallothioneins
MES = 2-(N-morpholino) ethanesulfonic acid
MerP = Mercury Binding Protein
MLCT = Metal to Ligand Charge Transfer
MW = Microwave
mM = milimole
NMR Spectroscopy = Nuclear Magnetic Resonance Spectroscopy
NEM = N-ethyl morpholine
NaCl = Sodium Chloride
NaNO₂ = Sodium Nitrite
OH = Hydroxyl Radicals
PEG-PS-Resin = Polyethylene Glycol-Polystyrene Resin
PET = Photo Induced Electron Transfer
PCT = Photo Induced Charge Transfer
PyBop = benzotriazol-1-yloxytris (pyrrolidino) phosphonium hexafluorophosphate
ROS = Reactive Oxygen Species
RNS = Reactive Nitrogen Species
RP- HPLC = Reverse Phase High Pressure Liquid Chromatography
RNA = Ribonucleic acid
SAM = Self Assembled Mono Layer
SOD = Superoxide Dismutase
SPPS = Solid Phase Peptide Synthesis
Single letter amino acid code
A = Alanine, R = Arginine, K = Lysine, H = Histidine, D = Aspartic acid, E = Glutamic acid, F = Phenyl alanine, M = Methionine, W = Tryptophan, V = Valine, I = Isoleucine, L = Leucine, P = Proline, C = Cysteine, G = Glycine, Q = Glutamine, N = Asparagine, S = Serine, Y = Tyrosine and T = Thrreonine
TCEP = tri-carboxy ethyl phosphin
Tris = 2-Amino-2-hydroxymethyl-propane-1, 3-diol
TFA = trifluoro acetic acid
TFFH = Tetramethylfluoroformamidinium hexafluorophophosphate
TIS = Tri-isopropyl silane
UV-vis = Ultra Violet-visible

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ABSTRACT

Selective fluorescent sensors for detection and quantification of various types of molecular targets are very important in many fields including environmental chemistry, medicine, and biology. This dissertation describes the efforts for designing fluorescent peptide sensor for various transition metal ions and reactive oxygen species. Chapter 1 describes general approach for the principle of signaling mechanism and the design of various kinds of fluorescent sensors. In the second chapter, we described about the FRET and CHEF/CHEQ based ratiometric peptide sensor for the recognition of various heavy and transition metal ions like Hg\textsuperscript{2+}, Cd\textsuperscript{2+}, Ag\textsuperscript{+}, Pb\textsuperscript{2+}, Zn\textsuperscript{2+} and Cu\textsuperscript{2+}. The design consists a synthetic peptide template (-CXXXC-), a common motif found in many metalloproteins, enzymes and heavy metal binding proteins flanked by two fluorophores which act as FRET pair. Most of the peptides exhibited a turn-on response for Hg\textsuperscript{2+}, Cd\textsuperscript{2+}, Ag\textsuperscript{+}, Pb\textsuperscript{2+}, Zn\textsuperscript{2+} and “turn-off” response for Cu\textsuperscript{2+}. Various fluorescent peptide probes for the detection of Zn\textsuperscript{2+} were synthesized on the basis of zinc binding ligands in zinc enzymes. The peptides displayed a great selectivity for Zn\textsuperscript{2+} in the presence of several transition metal ions in aqueous solution. The reversibility, binding stoichiometry, binding affinity, and pH sensitivity of the sensor were studied. Further, on-bead application of the peptide as chemosensors was demonstrated. Substituting the zinc binding amino acids by other amino acids allows us to finely tune the selectivity for other metal ions. Utilizing amino acid substitution in zinc binding peptides, we developed a sensor for monitoring Pb\textsuperscript{2+}, which can monitor Pb\textsuperscript{2+} selectively. To develop selective peptide probes, we further illustrated the control of selectivity between Zn\textsuperscript{2+} and Cu\textsuperscript{2+} with different secondary structure. Metal
selective fluorescent peptide probes (dansyl-Cys-X-Gly-His-X-Gly-Glu-NH₂, X= Pro or Gly) were developed by synthesizing peptides containing His, Cys, and Glu residues with Pro-Gly sequence to stabilize a turn structure and Gly-Gly sequence to adopt a random coil. The probe containing two Gly-Gly sequences exhibited marked selectivity only for Cu²⁺ over 13 metal ions under physiological buffer condition. The probe containing double Pro-Gly sequences showed high selectivity for Zn²⁺. The peptide probe containing one Pro-Gly sequence exhibited selectivity for Zn²⁺ and Cu²⁺.
We further conjugated these peptides on resin and investigated the property of resin bound peptides for monitoring and extracting metal ions. The resin conjugate peptides exhibited similar property to that of soluble form in terms of selectivity. The reversibility and resin recycle studies were performed using aqueous EDTA solution. The resin conjugate peptides exhibited reversibility with EDTA and fluorescence can be regenerated.
Recently, ratiometric sensors which work in aqueous solution have increased attention of the scientific community. The peptide based ratiometric sensor for selective monitoring of Hg²⁺ and Ag⁺ were reported and discussed in chapter 6.
In chapter 7, the fluorescent peptide was synthesized for monitoring reactive oxygen species by the different distance between quencher and fluorophore in the oxidized and reduced form. In further applications, this peptide sensor was used to investigate the UV mediated reduction of disulfide bond.
In a separate study, an optimized procedure was developed for reducing the reaction time and improving the yield of esterification reaction in solid phase synthesis of pseudopeptides containing an ester bond by utilizing microwave
irradiation. We selected a pseudodipeptide (Fmoc-Lysψ [COO] Leu-NH₂) as model system and optimized the microwave-assisted esterification reaction in solid phase synthesis using Fmoc chemistry. The optimized procedure was further applied towards the synthesis of various pseudopeptides and compared yield and purity to the method without microwave. The structure activity relationship was studied for pseudopeptides and suggested that the main chain hydrogen bonding played an important role for maintaining α-helical structure. The antimicrobial activity was retained for all pseudopeptides whereas the hemolytic activity was lost with the decrease in alpha helicity. The stability of pseudopeptides against trypsin enzyme suggested the rapid degradation as compare to control peptide.
다양한 종류의 분석물질을 탐색하고 그 양을 측정하는 선택적 형광 센서는 환경화학, 의학, 생물학 분야에서 매우 중요하다. 본 논문은 다양한 전이 금속과 과산화 수소수를 모니터링하는 형광 펩타이드 센서의 설계에 대한 내용을 다루었다. 1장은 시그널 기작에 대한 원리 및 다양한 종류의 형광센서 설계에 대한 일반적인 연구 결과에 대해서 다루었다.

2장에서는 수온(II), 카드뮴(II), 은(I), 납(II), 아연 (II), 구리 (II), 등 다양한 전이금속이온을 인식하는 FRET 및 CHEF/CHEQ 기반으로 하는 ratiometric 센서에 대해서 논하였다. -CXXXC-을 가지고 Trp과 dansyl을 가진 형광 펩타이드를 합성하여 다양한 금속이온에 대한 선택성을 조사하였다. 이 형광 펩타이드는 Hg$^{2+}$, Cd$^{2+}$, Ag$^{+}$, Pb$^{2+}$, Zn$^{2+}$에 대하여 형광이 증가하는 반응을 보였으며 에 Cu$^{2+}$에 대해서는 형광이 감소하였다.

효소의 아연 결합리간드를 바탕으로 아연이온에 대한 형광 펩타이드 센서를 개발하였다. 이 펩타이드는 수용액상에서 다양한 전이금속중에서 아연에 대한 높은 선택성을 보인다. 이 펩타이드 센서의 가역성, 결합 정도, pH 에 따른 감도등에 대해서 조사하였고, 펩타이드의 레진 공액을 통한 응용에 대해서 연구하였다. 아미노산 치환을 이용하여 아연 결합 형광 펩타이드로부터 납이온을 선택적으로 모니터링하는 형광센서를 개발하였다. 선택적 펩타이드 센서를 개발하기 위하여, 우리는 다른 이차원 구조를 통한 아연이온과 구리 이온의 선택성 조절에 대한 연구를 수행하였다.

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His, Cys, Glu 잔기와 갯임 구조를 안정화 시켜주는 Pro-Gly, 코일형 구조를 가지는 Gly-Gly 기를 가지는 펩타이드 합성을 통하여 특정 금속에 선택적인 형광 펩타이드 센서를 개발하였다. 두개의 Gly-Gly 잔기를 가진 센서는 생체 완충 용액조건에서 13개의 테스트 금속이온중 구리이온에 대해서만 선택적으로 작용한다. 두개의 Pro-Gly 잔기를 가진 센서는 아연이온에 대해서만 선택성을 가지며 하나의 Pro-Gly 잔기를 가진 센서는 구리이온과 아연에 대해서 선택성을 가진다.

이 펩타이드를 레진에 공액하여 금속이온의 모니터링과 추출 능력에 대해서 조사하였다. 레진에 공액된 형광 펩타이드는 용액상태의 형광 펩타이드와 동일한 선택성을 보인다. EDTA 수용액을 이용한 레진 펩타이드의 가역성, 레진 재활용에 대해서 연구하였다. 레진 형광 펩타이드는 성공적으로 재활용이 가능하였다.

최근에 수용액에서 작용하는 ratiometric 형광 센서에 대한 연구가 중요한 이슈로 떠오르고 있다. 6장에서는 수은과 은이온에 대해서 수용액상에서 ratiometric 형광 변화를 보여주는 형광 센서에 대하여 논의하였다.

7장에서는 산화 환원시 quencher 와 acceptor 의 거리가 변화됨을 이용하여 활성 산소종을 모니터링 하는 형광 펩타이드 센서를 개발하였다. 이 형광 펩타이드를 이용하여 UV 에 의한 이황화 본드의 환원에 대하여 연구하였다.

이외로 에스터 본드를 가진 유사 펩타이드의 마이크로파를 이용한 고체 합성에 대한 연구를 진행하였다. Fmoc-Lysψ[COO]Leu-NH2 유사 펩타이드를 모델로 선택하여 마이크로파를 이용한 고체상,
Fmoc chemistry을 이용한 에스터합성 방법을 최적화 하였다. 그리고 이를 이용하여 다양한 위치에 에스터 본드를 가진 유사펩타이드를 합성하고 그 구조와 항균 활성을 조사하였다. 구조연구를 통하여 수소결합이 나선구조에 중요한 역할을 할 수 있었고, 나선구조가 줄어든 유사펩타이드는 펩타이드에 비하여, 균에 대한 선택성이 높아졌지만, 용혈 활성도는 낮은것으로 밝혀졌다. 흥미롭게도 유사펩타이드는 펩타이드보다 혈액내 효소에도 더 빨리 분해됨을 발견하였다.
CHAPTER I

1. General Introduction

1.1 Heavy Metals in the Environment and Biology
The use of heavy metal ions have increased in several industries. The combustion of fossil fuels introduces a large amount of heavy metals into the atmosphere and the aquatic environment. Heavy metals are released to the ecosystem with the exponential growth of metal mining, the following processes and their industrial use. Heavy metals show a large tendency to form complexes, especially with nitrogen, sulphur and oxygen containing ligands of biological matter. Among heavy metal ions, mercury, cadmium, and arsenic are highly toxic and some heavy metals such as iron, copper, zinc, manganese, cobalt, nickel, tin, and selenium are essential to many organisms. These heavy metal ions along with amino acid, fatty acids, and vitamins are required for various biochemical processes such as respiration, biosynthesis and metabolism. Nature has evolved a number of tight metal regularly proteins for transition metal homeostasis and several artificial metal ion receptors were developed by protein engineering method. An undersupply of these so called trace metals leads to deficiency, while oversupply results in toxic effects.

1.2 Conventional Methods for the Determination of Heavy Metals
A variety of analytical methods for monitoring heavy metal ions are available. However, only some of them have been found in the application for routine analysis. Conventionally several methods including the use of photometric methods, flame or atomic absorption spectroscopy (AAS), ion exchange chromatography have been used. However, these methods offer very sensitive detection but require high cost of analytical instruments. The
necessary of collection and transportation and pretreatment of a sample are time consuming and provides a potential source of error. These methods do not make it possible for continuous monitoring so these techniques cannot be applied for directly investigating of metal ions in biological samples and other biochemical reactions. The toxicity of heavy metals makes a continuous supervision of drinking or ground water and enable on-line and field monitoring and therefore can be a useful alternative tool.

Thus, development of chemical sensors for a variety of application has been growing. Similarly, chemical sensors provide the valuable information for understanding the biochemical reactions and cellular concentration of heavy metal ions.

1.3 Chemosensors

Significant effort has recently been devoted to the design and construction of molecular sensory systems for a broad range of environmental and biological analyses. Chemical sensors, peptide sensors (usually called biosensors), solid surface conjugated sensors and optical sensors among the different types of sensors, have grown fast. Fluorescence appears one of the most powerful transduction mechanisms due to its high sensitivity and continuous monitoring of samples. In the last two decades, a large number of new fluorescent sensing probes have been synthesized. Till now, the general trend adopted for designing a sensor device contains three elements: a receptor, a signal transducer and a read-out (Fig. 1.1).

The receptors have ability to discriminate and bind a specific target substance known as the analyte. Selective receptor–analyte complex formation depends on the size, shape and binding energy of the receptor and analyte molecules. Signal transduction is the process through which an
interaction of receptor with analyte yields a measurable form of energy change and is converted to a signal change that can be read and quantified. The read-out domain is the part responsible for reporting the binding event. The chemosensors described in this dissertation are designed to monitor various transition metal ions such as Zn$^{2+}$, Cu$^{2+}$, Hg$^{2+}$, Pb$^{2+}$, Cd$^{2+}$, Ag$^{+}$ and reactive oxygen species (hydrogen peroxide). The individual chapters will provide detailed descriptions of work in these fields while this introductory chapter will provide a general background to chemosensing and the general strategy utilized herein.

Figure 1.1 Schematic illustration of chemosensor design

1.4 Properties of an Ideal Chemosensors
The ultimate goal for the development of chemosensor is to study physiological processes in their native environment and the presence of target analyte in environment. Hence the first and foremost property of ideal chemosensor should be good solubility in aqueous system. Fluorescence detection is ideal since it is noninvasive and exhibits a low background signal. The properties of an ideal fluorescent chemosensor have been
discussed in depth elsewhere. Briefly, chemosensors should rapidly bind the species that is relevant for signaling with good selectivity and signal sensitivity. Additionally, the binding affinity for the target analyte should approximate the expected concentration; this condition prevents disruption of normal cellular signaling processes and allows detection of increases and decreases of the analyte. The chemosensor should be stable to all aspects of the sensing environment varying in different pH and have appropriate fluorescence properties. Finally, the chemosensor should be easily introduced, distributed uniformly within a cell and should be reversible. Ratiometric property is also important. Ratiometric sensors are desirable because they make it possible to measure the analytes more accurately with minimization of background signal.

1.5 Fluorescent Sensors for Heavy and Transition Metal ions

To date, various chemosensors were reported for monitoring of heavy and transition metal ions exploiting the various kinds of molecular frameworks as analyte binding site varying from simple chemical to large biomolecules. The recent examples for various kinds of sensors used for monitoring heavy and transition metal ions have been nicely reviewed and discussed in depth elsewhere. Some representative examples for different metal ions are presented in the following sections.

1.5.1 Sensors for Zn$^{2+}$
Figure 1.2 Some representative examples of Zinc selective sensors.$^{10a-e}$
1.5.2 Sensors for Cu\textsuperscript{2+}

![Chemical structures for Cu\textsuperscript{2+} sensors]

Figure 1.3 Some representative examples of Cu\textsuperscript{2+} selective sensors.\textsuperscript{11a-d}

1.5.3 Sensors for Hg\textsuperscript{2+}, Pb\textsuperscript{2+} and Ag\textsuperscript{+}
Figure 1.4 Some representative examples of Hg$^{2+}$ (1-11, 1-12), Pb$^{2+}$ (1-13, 1-14) and Ag$^+$ (1-15, 1-16) sensors. 

$^{12a-e}$
Considering these various examples for different molecular targets, we focused our study on peptide receptors which will be discussed in the consecutive sections.

1.6 Ratiometric Sensors
Ratiometric sensors were first invented by Tsien and coworkers in the late 80’s for the sensing of calcium.\textsuperscript{13} Ratiometric behavior can be anticipated when analyte binding changes the electronic properties of a chromophore, resulting in absorption or emission at a different wavelength. Thus, a fluorescent ratiometric sensor responds to an analyte by a shift in its emission maximum, which may or may not be concomitant with the variation in intensity. This shift in the emission wavelength should be enough to distinguish the emission maximum of the coexisting free and bound analyte species, thus allowing the determination of the ratio of emission maxima of the two species. Together with the known binding constant of the sensor, the unknown analyte concentration can be determined. Several ratiometric sensors were developed for various analytes. The forthcoming chapters will elaborate clear insight on peptide receptors used for ratiometric sensing towards many analytes.

1.7 Surface Conjugated Fluorescent Sensors
Many artificial receptors and chemosensors have been developed in recent years, and their sensing abilities were extensively evaluated in homogeneous solution for various metal ions.\textsuperscript{14} Generally, any chemical or peptide sensor when attached to solid supports such as self assembled mono layer, film, glass surface or an electrode, frequently suffers from the fact that the excellent original function is greatly suppressed by such immobilization.\textsuperscript{15}
Similarly, immobilization of the receptors using monolayer films provides very weak signals in response to guest-binding and requires special equipment in many cases.\textsuperscript{16} Parallel to the production of polymeric materials, new trends in material science for chemical sensing are emerging. A number of materials such as silica particles, glass and gold surfaces, quantum dots, Langmuir–Blodgett films, vesicles, liposomes, and others are used combined with many chemical receptors to create sensitive fluorescent materials.\textsuperscript{17} However, resin immobilized bead sensors were rarely reported. Most of the surface conjugated sensors were suffered due to slow response towards analyte, rarely incorporated in to commercial instruments for evaluation and always not reversible.\textsuperscript{17a,d} To overcome with these problems, we developed resin bead sensors and their sensing abilities were evaluated and discussed individually in chapter 5.

1.8 Molecular Recognition and Common Building Blocks
Molecular recognition implies the (molecular) storage and (supramolecular) read out of molecular information. These terms have become characteristic of the language of supramolecular chemistry. Although the concepts of recognition and information were used in connection with biological systems, they effectively pervaded the realm of chemistry only at the beginning of the 1970’s in connection with, and as generalization of, the studies on selective complexation of metal ions. Since then molecular recognition has become a major area of chemical research and a very frequently used term. Various artificial receptors were designed based on different building blocks such as peptides and proteins, carbohydrates, aza crown ethers, calixarenes, crown ethers, cyclodextrins and cyclophanes.\textsuperscript{18,21} In general, these scaffolds share some common traits such as structural rigidity, preorganization of the
functionality, adoptable modular synthesis and solubility in appropriate solvents. Considering these common traits, various fluorescence sensors were reported for selective recognition of the target by means of fluorescence techniques, in the past 20 years.

1.9 Advantage and Disadvantage of Peptide-Based Sensors
A fluorescent sensor derived from a modular peptide scaffold offers several advantages. The first and foremost application is excellent solubility in aqueous system and make feasible for the monitoring of analyte concentration without any organic solvents. The modular architecture allows incorporation of both natural and un-natural amino acids via solid-phase peptide synthesis. Optimization and tuning can be easily achieved by altering the peptide sequence. A number of fluorescence-based sensing mechanisms can be easily implemented due to easy synthesis. Incorporation of other functionalities is also feasible via amide bond formation, e.g. an additional fluorophore for an internal standard or a cellular internalization sequence to transport the chemosensors into cells. In addition, the chemosensors could be attached to a solid support for further fabrication and could be used as reusable sensor. The peptide sensor also provides excellent reversibility and provide metal free spectrum easily with chelating reagents. Peptide motifs favored natural properties and provides less toxicity.

In addition using peptide probes have some disadvantages such as poor cell penetration efficiency due to their hydrophilic nature and deprived stability against enzymes.

1.10 Fluorescence Signaling Phenomena
Based on the originality of the responsive moieties, fluorescent sensors can be divided into two broad classes i.e. chemosensors and biosensors. These sensors detect analytes via several signaling mechanisms and more general are the PET, CHEF/CHEQ and FRET. The developed sensors which are described in this dissertation also follow these mechanisms for signaling. Thus, the consecutive chapter will allow us to get insight the brief mechanism of signaling.

1.10.1 Photo Induced Electron Transfer (PET)

This type of signaling mechanism has been extensively studied.\textsuperscript{19} Fig. 1.5 illustrates how a cation can prevent the electron charge in a fluoroionophore in which the cation receptor is an electron donor (e.g. amino group) and the fluorophore plays the role of an acceptor. Upon excitation of the fluorophore, an electron of the highest occupied molecular orbital (HOMO) is promoted to the lowest unoccupied molecular orbital (LUMO), which enables PET from the HOMO of the donor (belonging to the free cation receptor) to that of the fluorophore, causing fluorescence quenching of the latter. Upon cation binding, HOMO of the receptor becomes lower in energy than that of the fluorophore; consequently, PET is not possible any more and fluorescence quenching is suppressed. In other words, fluorescence intensity is enhanced upon cation binding. In most of PET sensors, the cation receptor involves aliphatic or aromatic amines acting as quenchers. Usually, some paramagnetic metal ions like Cu\textsuperscript{2+} shows “turn-off” response with fluorescence or more commonly called PET quenching. This can be happened when the energy level of the cation LUMO is between the energy levels of the fluorophore HOMO and LUMO, the binding of the cations by
the receptor provides a non-radiative path to disperse the excitation energy, resulting in a quenching of the fluorescence of the chemosensor.

Figure 1.5 Principle of cation recognition by fluorescent PET sensors. (Adopted from B. Valeur et.al *Coordination Chemistry Reviews*, 2000, 205, 3–40)

1.10.2 Chelation Enhanced Fluorescence/Quenching (CHEF/CHEQ)

CHEQ or CHEF effects on the fluorescence emission resulting from the binding of protons are rather common. Systems of this type have been used to construct ON/OFF switches, which is an important goal in the growing field of molecular recognition. In this dissertation, the fluorescence mechanism followed via CHEF with Zn$^{2+}$ and CHEQ with Cu$^{2+}$. Thus, the following explanation with Zn$^{2+}$ and Cu$^{2+}$ will be helpful to describe these mechanisms.

Principally, the inhibition of PET process after binding cation is called CHEF. The CHEQ mechanism usually occurs with Cu$^{2+}$ ion due to its unfilled d-orbital. The mechanism for this process is depicted in Figure 1.6.
When the fluorophore is excited, the non-radiative energy or electron transfer occurs between fluorophore and unfilled d-orbital of metal ions. Thus, the fluorescence shows quenching rather than enhancing.\textsuperscript{21} On the other hand, especially with Zn\textsuperscript{2+}, the complete filling of the 3d orbitals prevents electron circulation between fluorophore and d-orbital resulting in the enhancement rather than quenching. This is just reverse of the process which occurred with Cu\textsuperscript{2+}.

1.10.3 Fluorescence Resonance Energy Transfer (FRET)

FRET relies on the distance-dependent transfer of energy from a donor molecule to an acceptor molecule. Due to its sensitivity to distance, FRET has been used to investigate molecular interactions. FRET is the radiationless transmission of energy from a donor molecule to an acceptor molecule. The transfer of energy leads to a reduction in the donor’s fluorescence intensity.
and excited state lifetime, and an increase in the acceptor’s emission intensity. A pair of molecules that interact in such a manner that FRET occurs is often referred to as a donor/acceptor pair.

The primary conditions to occur are; the donor and acceptor molecules must be in close proximity to one another (typically 10-100 Å) and the absorption or excitation spectrum of the acceptor must overlap the fluorescence emission spectrum of the donor.

The degree to which they overlap is referred to as the spectral overlap integral (J). Forster demonstrated that the efficiency of the process (E) depends on the inverse sixth-distance between donor and acceptor as shown in equation 1.1.

\[
E = \frac{R_0^6}{R_0^6 + r^6} \quad (1.1)
\]

Where \( R_0 \) is the Forster distance at which half of the energy is transferred and \( r \) is the actual distance between donor and acceptor. This system generally provides a ratiometric probe, which makes it possible to measure the analytes more accurately and with minimization of background signal. The schematic representation of the FRET mechanism is shown in Figure 1.7.

![Figure 1.7 Schematic representation of FRET mode of signaling, where D and A represents donor and acceptor respectively.](image)
1.11 General Synthesis

All peptides considered in this dissertation were synthesized following the Fmoc-protocol using solid phase synthesis as conceived by Merrifield (1959) followed by Sheppard (1975). Briefly, Small porous solid beads are treated with functional units (‘linkers’) on which peptide chains can be built. The peptide will remain covalently attached to the bead until cleaved from it by a reagent such as trifluoroacetic acid. The general principle of SPPS is one of repeated cycles of coupling and deprotection steps. The free N-terminal amine of a solid-phase attached peptide is coupled (Scheme 1.1) to a single N-protected amino acid unit. This unit is then deprotected, revealing a new N-terminal amine to which further amino acid will be attached. After complete synthesis, the peptides were achieved by cleaving it from solid support and removal of protection groups. The crude peptides were characterized using analytical HPLC and mass spectrometry. Purification of peptides was performed with semi-prep column using reverse phase HPLC. The final products were stored in -20°C.
Scheme 1.1 General scheme for solid phase peptide synthesis
1.12 Aim of the Research

The purpose of this dissertation is to develop optimized sensor based on peptide scaffold for detecting specific heavy metal ions such as lead, silver, mercury, cadmium, zinc and copper. The sensing strategy uses molecular recognition of the analyte by peptides that change their fluorescence and structure upon analyte binding. Research problems that are being addressed by this dissertation include:

1) Sensor design strategy based on peptide scaffold and fluorescent mechanism.

2) Creation of metal binding sites and labelled them with fluorophores in existing proteins with characteristic scaffold for monitoring heavy metal ions.

3) These studies also contribute to the development of improved metal selective peptides. The knowledge of peptide secondary structure was applied for tuning the metal selectivity.

4) The resin conjugation of metal selective peptides is performed to explore their metal monitoring process via regeneration of the fluorescence of the peptide immobilized resin beads.

5) Design and synthesis of ratiometric probe for monitoring of Ag\(^+\), Hg\(^{2+}\) and for hydrogen peroxide.

6) In a separate part, the reaction condition is optimized for reducing the reaction time, to increase the yield and purity using microwave assisted irradiation method. The structure activity relationship of pseudopeptides was studied.
1.13 Literature Cited


CHAPTER II

2. Ratiometric and Turn-on Monitoring for Heavy and Transition Metal Ions in Aqueous Solution with a Fluorescent Peptide Sensor

2.1 Introduction

Chemical sensors that monitored metal ions are of current topical interest because a variety of natural and environmental contaminations of heavy and transition metal (HTM) ions causes serious problems for human health and ecology.1-3 As monitoring of a low level contamination of HTM ions such as Hg^{2+}, Cd^{2+} and Pb^{2+} has become significant, fluorescent chemical sensors that provide immediate and sensitive response to HTM ions are particularly valuable. Many kinds of fluorescent chemical sensors for heavy metal ions such as Hg^{2+}, Cd^{2+}, and Pb^{2+}, have been reported.4-15 Most of the known fluorescent chemical sensors monitored these cations by fluorescence quenching mechanism via enhanced spin-orbital coupling (e.g. Hg^{2+}) or energy or electron transfer and suffered limitations due to poor solubility in aqueous solution. Thus, the sensors that exhibit turn on response for heavy metal ions in mixed organic-aqueous or aqueous solution are required. In addition, ratiometric sensors for heavy metal ions are desirable because they make it possible to measure the analytes more accurately with minimization of background signal.16-17 However, ratiometric fluorescent probes useful for heavy metal ions in aqueous solution were rarely reported.18-20

Various proteins including enzymes interact with small molecules such as metal ions and this event induces conformational change of the proteins, resulting in the alternation of binding affinity and catalytic activity of the enzymes. Several metal binding motifs have been identified in various metallo-proteins.21-25 Cysteine rich metal ion binding motifs that commonly shared Cys-X_{2,4}-Cys sequence have been identified in metallo-proteins such
as metallothioneins, Menkes, MerR, and CueR protein. Furthermore, the small peptide fragments based on the metal binding motifs showed nanomolar and micromolar affinity for several HTM ions (Group 11 and 12) including Hg$^{2+}$, Cd$^{2+}$ and Zn$^{2+}$. Thus, the peptides containing metal binding motifs have potential applications as chemical sensors. In the present study, we focused on the design of a ratiometric fluorescent sensors for monitoring HTM ions on the basis of metal binding motif because peptide sensors have several advantages for further development after conjugation in a solid device. Peptides consisting of natural amino acids can be easily synthesized by solid phase synthesis with 9-fluorenylmethoxycarbonyl (Fmoc) or tert-butyloxycarbonyl (Boc) chemistry. Peptide sensors are generally working well in aqueous solution or mixed organic-aqueous solution due to their good solubility, and their sensitivity and selectivity can be optimized by further tuning of the amino acid sequences. Peptides can be facilely conjugated into solid support in the device for further applications. Peptides and proteins do not have fluorescent properties to be essential for sensing. Conjugation of fluorophore group (a signal transduction site) into the peptide based receptor is a critical step for developing peptide sensors. If the fluorophore site may directly contact the bound metal ions, the metal recognition event will be fully monitored. We and several other research groups independently reported the synthesis of fluorescent peptide probes containing dansyl fluorophore. The emission spectrum of dansyl group is sensitive to its microenvironments. The dansyl group of the peptide probes was supposed to be directly participated in the metal binding and some of the peptide probes responded with metal ions by enhancement of emission intensity via chelation enhanced fluorescence (CHEF). Thus, a short fluorescent peptide sensor consisting of less than eleven amino acids was
designed and dansyl group was chosen as a fluorophore group. To design a ratiometric peptide sensor, Trp residue as a donor and dansyl group as an acceptor were introduced into the peptide (Scheme 2.1).}

\[ \text{Scheme 2.1 Structure of 3G and possible binding mode} \]

We synthesized a series of fluorescent peptide probe as shown in table 2.1 on the basis of the amino acid sequence of the metal binding motifs. As His residue like Cys residue was known as a heavy metal binding ligand, the peptide probe contained Cys-X-X-His-Cys as a metal ion binding site. A Glu residue in the peptide probe was introduced for improving solubility.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Peptide Sequence</th>
<th>HPLC (Acn. %)</th>
<th>Calcd./Obs. Mass (M+H(^+))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1G</td>
<td>Dans-CAAHCWAE-NH$_2$</td>
<td>37.86</td>
<td>36.96</td>
</tr>
<tr>
<td>2G</td>
<td>Dans-ACAAHCWAE-NH$_2$</td>
<td>40.72</td>
<td>39.01</td>
</tr>
<tr>
<td>3G</td>
<td>Dans-AACAAHCWAE-NH$_2$</td>
<td>39.76</td>
<td>35.65</td>
</tr>
<tr>
<td>1A</td>
<td>Dans-CAAHCA-NH$_2$</td>
<td>53.41</td>
<td>57.59</td>
</tr>
<tr>
<td>2A</td>
<td>Dans-ACAAHCWA-NH$_2$</td>
<td>51.73</td>
<td>56.28</td>
</tr>
<tr>
<td>3A</td>
<td>Dans-AACAAHCWA-NH$_2$</td>
<td>58.77</td>
<td>60.95</td>
</tr>
<tr>
<td>Ac-1G</td>
<td>Ac-CAAHCWAE-NH$_2$</td>
<td>31.76</td>
<td>29.95</td>
</tr>
</tbody>
</table>

\[ \text{Table 2.1 Characterization data for peptide probes using HPLC and mass} \]
As shown in scheme 2.1, we assumed that when the peptide probe interact with metal ions, it might fold and brought the Trp and dansyl closer to each other, resulting in the increase of emission intensity via a fluorescence resonance energy transfer (FRET) mechanism. In addition, if direct interactions between the dansyl fluorophore and metal ions might occur, enhanced emission intensity will be observed via a CHEF mechanism. The entire peptide probe depicted in Table 2.1 successfully exhibited turn-on fluorescent response for several heavy metal ions in aqueous solution. Among them, peptide 3G provided the significant shift and large enhancement after binding with metal ions, thus considered for further study. The peptide probe showed a ratiometric response with heavy metal ions such as Hg$^{2+}$, Cd$^{2+}$, Zn$^{2+}$, and Ag$^{+}$ via a FRET as well as a CHEF mechanism. We described the fluorescent enhancement, binding affinity, detection limit and pH sensitivity of the peptide probes for HTM ions.

2.2 Results and Discussion

2.2.1 Fluorescence Response of 3G to Heavy Metal Ions

The peptide sensor is fully water soluble and we investigated the fluorescence response of the probe to HTM ions in 10 mM HEPES buffer solution (pH 7.4). As shown in scheme 2.1, fluorescence response of the probe to metal ions might occur via two ways. If metal ions directly interact with dansyl fluorophore, the emission intensity of dansyl group will change via a CHEF mechanism. If the peptides would fold in the presence of metal ions, the distance between Trp (donor) and dansyl (acceptor) may decrease, resulting in the increase of emission intensity via a FRET mechanism. Thus, fluorescent spectra of 3G to HTM metal ions was measured by excitation at
a wavelength of 295 nm for the Trp residue or 330 nm for the dansyl fluorophore, respectively.

The fluorescence response of 3G toward Hg$^{2+}$ was measured with a wavelength of 295 nm in HEPES buffer solution (pH=7.4). Figure 2.1 showed that 3G exhibited a fluorescence turn-on response with Hg$^{2+}$.

![Figure 2.1](image)

**Figure 2.1** Fluorescence response of 3G to the addition of Hg$^{2+}$ at 0, 2, 4, 6, 8, 9, 10, 11 µM with an excitation at (A) 295 nm and (B) 330 nm in 10mM HEPES buffer (pH 7.4). Inset A; ratiometric curve I_{495}/I_{340}.

When increasing concentration of Hg$^{2+}$ was added, a significant increase of the emission intensity around 495 nm and decrease at 340 nm were observed with an isosbestic point at 407 nm. The intensity ratio at 495 nm and 340 nm increased from 0.9 to 8.9. Upon the addition of 1 equiv Hg$^{2+}$, about 4-fold increase of the intensity at 495 nm and 30 nm blue-shift from 525 nm to 495 nm of the maximum emission intensity were observed. This result indicated that the sensor 3G showed ratiometric, turn-on fluorescence response with Hg$^{2+}$ via a FRET mechanism.
When the probe was excited with a wavelength of 330 nm for dansyl group, 3G exhibited a fluorescence turn on response with Hg$^{2+}$. Upon the addition of 1 equiv Hg$^{2+}$, about 3-fold intensity increase and 30 nm blue-shift from 525 nm to 495 nm of the maximum emission intensity were observed. This result indicated that direct interactions between dansyl fluorophore with Hg$^{2+}$ resulted in the increase of emission intensity by a CHEF mechanism. The blue shift may be due to the change of circumstances of dansyl fluorophore because the emission intensity of dansyl fluorophore is sensitive to its local environment. The blue shift indicated that when the peptide probe fold in the presence of Hg$^{2+}$, the hydrophilic environment of dansyl group of the free peptide probe was changed into hydrophobic environment.

Figure 2.2 indicated that 3G showed ratiometric, turn-on response with Cd$^{2+}$. Upon the addition of 1 equiv Cd$^{2+}$, 7.5-fold intensity increase and 30 nm blue shift of the maximum emission intensity were observed with a wavelength of 295 nm.

![Figure 2.2](image)

**Figure 2.2** Fluorescence response of 3G to the addition of Cd$^{2+}$ at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 µM with an excitation at (A) 295 nm and (B) 330 nm in 10mM HEPES buffer (pH 7.4).
When the probe was excited with a wavelength of 330 nm, 4.3-fold increase and 30 nm blue shift of the maximum emission intensity were observed. In the presence of \( \text{Hg}^{2+} \) and \( \text{Cd}^{2+} \), the enhancement of emission spectrum measured by excitation with 295 nm was greater than those measured by excitation with 330 nm. Overall results indicated that \( 3G \) exhibited a fluorescence response with \( \text{Hg}^{2+} \) and \( \text{Cd}^{2+} \) via a FRET as well as a CHEF mechanism.

2.2.2 Binding Stoichiometry and Binding Affinity

To investigate the response of the peptide to various metal ions, we measured the fluorescence change of the peptide probe in the presence of several metal cations (\( \text{Ca}^{2+}, \text{Cd}^{2+}, \text{Co}^{2+}, \text{Pb}^{2+}, \text{Cu}^{2+}, \text{Ag}^{+}, \text{Mg}^{2+}, \text{Mn}^{2+}, \text{Ni}^{2+}, \) and \( \text{Zn}^{2+} \) as perchlorate anion and \( \text{Na}^{+}, \text{Al}^{3+}, \text{K}^{+} \), as chloride anion). When \( 3G \) was excited with a wavelength of 330 nm or 295 nm respectively, \( 3G \) exhibited a fluorescence turn on response with HTM ions including \( \text{Hg}^{2+}, \text{Ag}^{+}, \text{Cd}^{2+}, \text{Pb}^{2+} \) and \( \text{Zn}^{2+} \) whereas \( 3G \) exhibited a turn off response with \( \text{Cu}^{2+} \) (Figure 2.3). However, the probe exhibited no response with a representative selection of alkali and alkaline earth metal ions. This selectivity of the peptide sensor for HTM ions would be anticipated because the metal binding motif (CXXXC) of the peptide was reported to bind with several HTM ions including \( \text{Hg}^{2+}, \text{Cd}^{2+}, \text{Pb}^{2+} \), and \( \text{Zn}^{2+} \).25

We investigated the binding stoichiometry and affinities of the peptide sensor for \( \text{Hg}^{2+}, \text{Ag}^{+}, \text{Cd}^{2+}, \text{Pb}^{2+} \), and \( \text{Zn}^{2+} \). In the titration curve, about 10 \( \mu \text{M} \) of \( \text{Hg}^{2+} \) was required for the saturation of the emission intensity of \( 3G \) (20 \( \mu \text{M} \)), indicating that the binding ratio between the peptide with \( \text{Hg}^{2+} \) is 2:1 (Figure 2.1). Moreover, Job’s plot analysis was conducted to determine the binding stoichiometry.17, 46, 47 Job’s plot of \( 3G \) exhibited a maximum at
0.3~0.4 mole fraction of for all heavy metal ions (Figure 2.4). Generally, a Job’s plot with a maximum at any other value besides 0.5 reveals that the peptide did not form 1:1 complex. In the titration curve for Cd$^{2+}$, Zn$^{2+}$, Pb$^{2+}$ and Ag$^+$, 0.5 equiv of metal ion was required for the saturation of the emission intensity of 3G.
Figure 2.3 Fluorescence response of 3G (20 µM) in the presence of various metal ions (1equiv.) with an excitation at (A) 295 nm and (B) 330 nm in 10 mM HEPES buffer solution (pH=7.4).

Job’s plot exhibited a maximum at 0.3~0.4 mole fraction of Cd$^{2+}$, Zn$^{2+}$, Pb$^{2+}$ and Ag$^+$ respectively (Figure 2.4). The titration data and a Job’s plot analysis indicated that a 2:1 complex might be predominant. It is not surprising that two peptides that contained two Cys residues was required to bind one heavy metal ion because metal binding sites in metalloproteins often include four Cys residues (i.e. CXXXXC—CXXXC) and tetrahedral coordination geometries between a protein and a metal ion were observed.\textsuperscript{30}
The job plots for 3G with HTMs. The total concentration of 3G and HTM ions was 2.5 µM.

The association constants of 3G for Hg$^{2+}$, Cd$^{2+}$, Zn$^{2+}$, Pb$^{2+}$ and Ag$^{+}$ were calculated by 2:1 complex model. Table 2.2 summarized the association constants and enhancement factors for several heavy metal ions. The fitting of titration curve provided less than 15% errors. Even though this error was acceptable, this was larger than those of the reported small chemical sensors because the emission intensity increased via FRET as well as CHEF and some heavy metal ions showed quenching effect on the emission of Trp. It is observed that the association constants did not correlate well with the enhancement factors measured by excitation with 295 nm or measured by excitation with 330 nm. The enhancement factors depend on the electron configuration of metal species and the distance between the dansyl fluorophore and the metal ion in the peptide-metal complex. Although $K_a$ for Zn$^{2+}$ is similar to that for Pb$^{2+}$, the enhancement factors measured by excitation with 295 nm are considerably different.

The large difference between the enhancement factors (295 nm) can be explained by several factors such as the secondary structure of peptide-metal complex, and the quenching effect of metal ions. When peptides fold in the
presence of metal ions, the secondary structure would be different depending on the metal species. However, the main reason may be the quenching effect of some metal ions.

<table>
<thead>
<tr>
<th>Metal</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>Enhancement factor (Abs=295 nm)</th>
<th>Enhancement factor (Abs= 330 nm)</th>
<th>Detection limit (µg/L)</th>
<th>Ka (M&lt;sup&gt;-2&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>495</td>
<td>4.0</td>
<td>2.6</td>
<td>5.2</td>
<td>5.5±0.6×10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cd&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>495</td>
<td>7.5</td>
<td>4.3</td>
<td>4.6</td>
<td>2.6±0.3×10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pb&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>495</td>
<td>3.6</td>
<td>2.2</td>
<td>11.0</td>
<td>4.5±0.6×10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ag&lt;sup&gt;+&lt;/sup&gt;</td>
<td>495</td>
<td>4.5</td>
<td>3.5</td>
<td>4.3</td>
<td>3.3±0.3×10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>495</td>
<td>9.0</td>
<td>4.8</td>
<td>2.8</td>
<td>4.0±0.4×10&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 2.2 Enhancement factors, detection limits, and association constants of 3G

It was reported that if heavy metal ions might be close to Trp residue, the emission spectrum of Trp residue was often quenched by some heavy metal ions (Pb<sup>2+</sup> and Hg<sup>2+</sup>). To confirm this explanation, we synthesized the peptide containing a Trp residue (Acetyl-Cys-Ala-Ala-His-Cys-Trp-Ala-Glu-CONH<sub>2</sub>) and measured emission intensity in the presence of various metal ions. (Figure 2.5) Noticeably, Pb<sup>2+</sup>, Hg<sup>2+</sup>, and Ag<sup>+</sup> showed some quenching effects on the emission spectrum of Trp of this peptide. The quenching effect of Pb<sup>2+</sup> was the greatest and 1 equiv of Pb<sup>2+</sup> almost completely quenched the emission intensity of the Trp residue. As some of heavy metal ions (Pb<sup>2+</sup>, Hg<sup>2+</sup>, and Ag<sup>+</sup>) might have a quenching ability for the emission intensity of the Trp residue of 3G, we made a titration curve by excitation with 330 nm
and calculated $K_a$ values for Pb$^{2+}$, Hg$^{2+}$, and Ag$^+$. Except Pb$^{2+}$, $K_a$ values on the basis of the titration curve measured by excitation with 330 nm was similar to those for HTM ions on the basis of the titration curve measured with 295 nm. As Pb$^{2+}$ showed a strong quenching effect on the emission spectrum of the Trp residue, fluorescence titration curve for Pb$^{2+}$ was obtained by excitation with 330 nm and the association constant was calculated.

![Figure 2.5](image.png)

**Figure 2.5** Fluorescence emission change of peptide Ac-1G (10μM) with HTMs.

### 2.2.3 Detection Limit

The binding process of HTM ions to the peptide probe in buffer solution was found to be fast. After adding the metal ions, the emission intensity instantly increased and reached the plateau region less than 2-3 seconds, suggesting that equilibrium might reach instantly and the probe has rapid detection ability for HTM ions. The fluorescence response of the probe for HTM ions
was reversible because the addition of excess EDTA to the probe-metal complex resulted in the return of the original metal free emission spectrum. The detection limits of the probes for important heavy metal ions were measured. The detection limits of 3G for \( \text{Hg}^{2+}, \text{Ag}^+, \text{Cd}^{2+}, \text{Pb}^{2+}, \) and \( \text{Zn}^{2+} \) were calculated and summarized in Table 2.2. (Figure 2.6)

![Figure 2.6](image.png)

**Figure 2.6** The linear relationships between the emission intensity change and concentration of HTMs for probe 3G

The detection limits for \( \text{Zn}^{2+} \) and \( \text{Ag}^+ \) were much lower than the EPA’s drinking water maximum contaminant level (MCL)\(^{50}\) and the detection limits
for Cd\(^{2+}\), Pb\(^{2+}\) were also below the EPA’s drinking water MCL of 5 and 15 
\(\mu g/mL\), respectively. Even though the detection limits for Hg\(^{2+}\) was slightly 
higher than the EPA’s drinking water MCL of 5.2 \(\mu g/mL\), this value is much 
lower than the detection limits of the previously reported Hg\(^{2+}\) chemical and 
peptide sensors\(^{39, 44, 51, 52}\). We expect that this detection limits can be 
optimized by further tuning of the amino acid sequences and can be 
改进ed by several optimization techniques such as a more intense light 
source, a longer integration time, slit size, and tuning of amino acid. 
Holcombe et al. reported a turn-on fluorescent peptide sensor based on the 
mercury binding protein MerP.\(^{44}\) The peptide that has a long size (23mer) 
showed turn-on response with several heavy metal ions in aqueous phase by 
a FRET mechanism. However, the peptide probe has more than two binding 
sites for heavy metal ions and the detection limit was much higher than that 
of 3G. For example, the detection limit (103\(\mu g/ml\)) for Cd\(^{2+}\) was at least 20 
times higher than that of 3G, whereas the detection limit (496\(\mu g/ml\)) for Ag\(^+\) 
was almost 100 times higher.

2.2.4 Fluorescence Study in the Presence of Alkali and Alkaline Earth 
Metal Ions and Different pH

We investigated the fluorescence response of 3G-Hg\(^{2+}\), 3G-Cd\(^{2+}\), and 3G-
Zn\(^{2+}\) in the presence of group I, II metal ions, and Al\(^{3+}\). The metal-dependent 
emission intensities of the 3G-Hg\(^{2+}\), 3G-Cd\(^{2+}\), and 3G-Zn\(^{2+}\) complex were 
not affected by the presence of 5 mM alkali and alkaline earth metal ions 
(Figure 2.7). We investigated the pH influence on the fluorescence intensity 
of the peptide probes in the absence and presence of heavy metal ions. Figure 
6 indicated that the probe in the presence or absence of HTM ions exhibited 
little fluorescence intensity at pH lower than 5.5. (Figure 2.8) This might be
due to the protonation of the dimethylamino group (pK_a~4) of the dansyl fluorophore. At pH > 6.5, the intensity of 3G increased by increasing the pH and the enhancement factors also increased by increasing the pH. Overall results indicated that 3G is useful for monitoring HTM’s in neutral and basic pH.
**Figure 2.7** Emission intensity of 3G in the presence HTM ions (a; Zn$^{2+}$, b; Cd$^{2+}$, and c; Hg$^{2+}$) and additional various metal ions at pH 7.4 (10 mM HEPES buffer). HTM ions (Zn$^{2+}$, Cd$^{2+}$, and Hg$^{2+}$) are one equivalent to 3G (20 µM) and Na$^{+}$, K$^{+}$, Ca$^{2+}$, and Mg$^{2+}$ are used at 250 equivalent.

**Figure 2.8** pH dependence of fluorescence response of 3G (5 µM) and 3G-HTM ions (Hg$^{2+}$, Cd$^{2+}$, Pb$^{2+}$, Ag$^{+}$, and Zn$^{2+}$)

2.3 Conclusion
The fluorescent peptide probe containing Trp as a donor and dansyl as an acceptor was synthesized on the basis the amino acid sequences of the metal binding motifs. This peptide probe showed fluorescent “turn-on” response for several heavy metal ions via FRET mechanism as well as a CHEF mechanism. The peptide probe successfully exhibited ratiometric response for several heavy metal ions in aqueous solution with significantly low detection limit. The peptide sensor is useful for monitoring HTM’s in neutral and basic pH.

2.4 Materials and Methods

2.4.1 Reagents

Fmoc protected amino acids, N, N’-diisopropylcarbodiimide, 1-hydroxybenzotriazole, and Rink Amide MBHA resin were from Novabiochem. Other reagents for peptide synthesis including trifluoroacetic acid (TFA), tri-isopropylsilane (TIS), dansyl chloride, triethylamine, diethyl ether, dimethyl sulfoxide (DMSO), N, N-dimethylformamide (DMF) and piperidine were purchased from Aldrich.

2.4.2 Apparatus

Peptides were synthesized on Biotage Microwave Initiator. All fluorescence and UV-measurements were performed on Perkin-Elmer LS-55 Perkin-Elmer Lambda-40 (United Kingdom) instruments respectively. The synthetic peptides were characterized by prep HPLC (Gilson, France) using waters C18 column and purified by Vydac semi prep column. The mass spectra measured by using ESI mass spectrometer (Micromass, Platform II) or a MALDI TOF mass spectrometer (Voyager-DE STR, Applied Biosystem). The dual wavelength UV light (ENF-260C) was from Spectroline (USA).
2.4.3 Peptide Synthesis
The peptide was synthesized using Fmoc-chemistry by solid phase peptide synthesis with Fmoc chemistry.\textsuperscript{31} The coupling of dansyl chloride was performed by applying the following procedure. To the resin bound peptide (65 mg, 0.05 mmol), dansyl chloride (40 mg, 0.15 mmol, 3 equiv) in DMF (3 ml) containing triethylamine (20 \( \mu l \), 0.15 mmol, 3 equiv) were added. Deprotection and cleavage was achieved by treatment with a mixture of trifluoroacetic acid (TFA)/TIS/H\(_2\)O (9.5:0.25:0.25, v/v/v) at room temperature for 3-4 h. After cleavage of the product from resin, the peptides were purified by preparative-HPLC using a water (0.1% TFA)/acetonitrile (0.1% TFA) gradient (5-50% acetonitrile over 45 min). The peptide mass were characterized by ESI mass spectrometer (Platform II, Micromass, Manchester, UK). The homogeneity (>95\%) of the compound was confirmed by analytical HPLC on a C\(_{18}\) column.

2.4.4 General Fluorescence Measurements
Fluorescence emission spectrum of a peptide probe in a 10 mm path length quartz cuvette was measured in 10 mM HEPES buffer solution (pH =7.4) using a Perkin-Elmer luminescence spectrophotometer (model LS 55). Emission spectra (300-650 nm) of the peptide probes (20 \( \mu M \)) in the presence of various metal ions (Ca\(^{2+}\), Cd\(^{2+}\), Co\(^{2+}\), Pb\(^{2+}\), Ag\(^+\), Mg\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\), and Zn\(^{2+}\) as perchlorate anion; and Na\(^+\), Al\(^{3+}\), and K\(^+\), as chloride anion) were measured by excitation with 295 nm for FRET measurement or by excitation with 330 nm for chelation enhanced fluorescence measurement. The slit size for excitation and emission was 6 nm, respectively. The concentration of peptide probe was confirmed by UV absorbance at 330 nm for dansyl group.
2.4.5 Determination of Binding Stoichiometry and Binding Constant

The binding stoichiometry of peptides with metal ions was determined by using Job’s plot. A series of solutions with varying mole fraction of metal ions were prepared by maintaining the total peptide and metal ion concentration constant (2.5 μM). The fluorescence emission was measured for each sample by exciting at 330 nm or 295 nm and spectra were measured from 300 to 650 nm. The fitting data were acquired by plotting a straight line through the maximum or minimum emission intensity in the titration curve and were plotted against the mole fraction of the metal ion versus emission intensity.

The association constants were calculated based on the titration curve of the probes with metal ions. Association constants were determined by a nonlinear least squares fit of the data with the following equation.

\[
F(x) = \frac{a + b* c^x}{1 + c^x}
\]

Equation 2.1

Where \(x\) is the concentration of metal ions, \(F(x)\) is the intensity, \(a\) is the intensity of probe without metal ions, \(b\) is the intensity at the saturation, \(n\) is the binding stoichiometry, and \(c\) is the association constant.

2.4.6 Calculation of Detection Limit (MDL)

The detection limit is the lowest concentration level that can be determined to be statistically different from a blank (99% confidence). To determine the S/N ratio, only peptide was scanned using fluorescence spectroscopy without addition of analyte. The scan was performed 7 times to get the lower standard deviation. The calibration curve (Intensity vs. analyte
concentration (µM)) was plotted based on the fluorescence titrations. The detection limit is then calculated using the formula
\[ C_m = \frac{(S_m - S_{bi})}{m} \] …equation (2.2)
Where, \( S_m = K \sigma_{bi} \) and \( m \) = slope value.

\( K \) = constant value from appendix (3.143 and is constant), \( \sigma_{bi} \) = standard deviation of baseline. To evaluate the system, three independent experiments were performed and based on the standard deviation of the slope, final detection limits were reported.

Note: The apparatus and common reagents used for peptide synthesis, synthesis method for peptides and general fluorescence measurements are common throughout the experiment unless otherwise indicated and will not be mentioned in consecutive chapters.

2.5 Literature Cited
1) Harris, H. H.; Pickering, I.; George, G. N. Science 2003, 301, 1203.


CHAPTER III

3. Fluorescence Based Approach for Designing Selective Sensors for Metal Cations: Tuning Selectivity with Ligand Variation

3.1 Introduction

Detecting cations is of great interest to many scientists, including chemists, biologists, clinical biochemists and environmentalists. Various metal ions belong to metalloenzymes like copper and zinc. In chemical oceanography, it has been demonstrated that some nutrients required for the survival of microorganisms in sea water contain zinc, iron, and manganese as enzyme cofactors. As earlier discussed in chapter 1, the methods based on fluorescent sensors offer distinct advantages in terms of sensitivity, selectivity, response time, local observation (e.g. by fluorescence imaging spectroscopy). Therefore, considerable efforts are being made to develop selective fluorescent sensors for cation detection. This chapter will focus on the design of fluorescent peptide sensors for cations with a classification according to the nature of the Chelation Enhanced Fluorescence (CHEF or CHEQ) which is responsible for photophysical changes upon cation binding.

3.1.1 Development of Fluorescent Peptides for Monitoring Zn$^{2+}$

Zn$^{2+}$ has significant role in various biological phenomenon and second abundant transition metal followed by iron. Metallothioneins, zinc finger proteins and a large number of zinc containing metalloenzymes are the best studied among them and are frequently reviewed in various peer reviews and textbooks of bioorganic chemistry.

The total concentration of $\text{Zn}^{2+}$ in different cells varies from the nanomolar range up to about 0.3mM.\textsuperscript{10} Thus, optimized chemical probes are required to monitor zinc concentration ranging from nanomolar to micromolar. Although several fluorescence-based chemical and peptide probes for $\text{Zn}^{2+}$ have been developed, most of them suffered limitations due to tight binding affinity (subnanomolar affinity), interference of other metal ions, susceptibility to pH, difficult synthesis, and poor solubility in physiological buffer solution.\textsuperscript{11} It is therefore necessary to develop new probes that have micromolar affinity for $\text{Zn}^{2+}$ with a high selectivity. In the present study, we focused on the development of short fluorescence peptide sensors for $\text{Zn}^{2+}$ for the following reasons. As it is difficult for peptide sensors to penetrate into cells due to their hydrophilic property, they can be used for monitoring extracellular or environmental metal ions. However, as incorporation of additional functionality is also feasible via amide bond formation, a cellular internalization sequence can be appended to transport the probes into cells in future applications.\textsuperscript{12} Several fluorescence-based peptide sensors have been developed for $\text{Zn}^{2+}$. Zinc peptide sensors were synthesized based on zinc finger domains.\textsuperscript{11i} These peptides, consisting of 24 amino acids, suffered limitations with subnanomolar affinity, difficult synthesis due to their size, and the susceptibility of the Cys residues to air oxidation. Peptide sensors containing an unnatural amino acid and $\beta$-turn sequence have been synthesized.\textsuperscript{11j} These peptides form 1:1 or 1:2 complexation with $\text{Zn}^{2+}$ and their affinities ranged from nanomolar to 1 µM. However, the peptide probes with micromolar binding affinity for $\text{Zn}^{2+}$ bind tightly with $\text{Mg}^{2+}$ and $\text{Cd}^{2+}$ and require the difficult synthesis. Thus, the design and synthesis of selective peptide probes with micromolar binding affinity for $\text{Zn}^{2+}$ remains a significant challenge. Considering the drawbacks of the previous peptide
sensors, we synthesized a series of new type of peptide probe for zinc ion and studied their sensing ability towards various metal cations, binding affinity, binding stoichiometry, pH sensitivity and selectivity.

### 3.1.2 Development of Fluorescent Peptides for Monitoring Pb$^{2+}$

Among heavy metal ions, lead is the most abundant and certainly the most toxic causing health problem such as digestive, neurologic, cardiac and mental troubles. Lead is particularly dangerous for children causing mental retardation. Similarly, the possible molecular targets of lead include calcium- and zinc-binding proteins that control cell signaling and gene expression, respectively.\(^{13}\)

Thus selective detection of lead using fluorescence sensors is highly desirable. In the recent reports, there are only few examples of fluoroionophore developed for heavy metal ions particularly for lead which can work in aqueous solution. In order to obtain a selective fluorescence probe for Pb$^{2+}$, we now report the synthesis and the fluorescence properties of peptide based sensors designed after changing the ligand sites from the zinc binding peptides.\(^{14}\) The reported sensor exhibited the selective signaling with Pb$^{2+}$ in presence of many other transition metal ions.

### 3.2 Design and Strategy

The strategy employed in the design of peptide probes for zinc is to link a fluorophore unit with a zinc binding motif. The two units are linked to each other in such a way that the binding of zinc ion to the binding motif causes considerable changes in the fluorescence. For easy synthesis, we designed a small peptide probe (5-7 mer) consisting of natural amino acids using dansyl group as a fluorophore. We designed zinc-binding motif of the peptide
probes for the following reasons. As His, Glu, and Cys residues are frequently found as the ligands of zinc enzymes,\textsuperscript{15} we designed peptide probes containing Cys, His, and Glu residues as a zinc binding amino acid. As a pre-organization of the peptide ligand site may result in the increase of binding of metal ions\textsuperscript{11j} we designed the peptides containing Pro-Gly amino acids to have a turn structure.\textsuperscript{16} We developed a CP1 containing Cys, His and Glu residues in combination with Pro-Gly sequence as a model system. To understanding the role of individual ligand on Zn\textsuperscript{2+} binding, we synthesized various analogs by rotation of the ligand or appending additional binding site. The peptides named from \textbf{cp1-a} to \textbf{Cp1-c} were synthesized which would be expected to possess no distinct secondary structure. Furthermore, in peptide Cp1-d, the additional His residue was incorporated instead of Cys to investigate the effect of Cys in zinc binding and structural change caused by it. Additional amino acid sequences were appended in \textbf{Cp1-e} to investigate either rotation of Pro-Gly has any effect on sensing ability for zinc or not. Similarly, in \textbf{Cp1-f}, Glu residue was eliminated to investigate its role in zinc binding.

As thiol and carboxylate groups of Cys and Glu residue are considered to coordinate with lead under physiological condition.\textsuperscript{17} Keeping this in mind, we further designed peptide \textbf{Cp1-g} and \textbf{h} to alter the selectivity from zinc to lead ions by incorporating the additional Glu residue instead of His. To investigate the role of individual ligand for selective monitoring of these metal ions, the control peptide \textbf{P1} was synthesized that has no functional groups in the amino acid side chains.

\textbf{3.3 Results and Discussion}

\textbf{3.3.1 Peptide Synthesis and Characterization}
All the peptide probes were synthesized using Fmoc-chemistry\textsuperscript{18} in solid phase peptide synthesis according to the literature procedure as discussed earlier and characterized. The HPLC and mass data for all peptides are shown in Table 3.1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Retention time (min.)</th>
<th>Calculated/Observed mass (M+H\textsuperscript{+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP1</td>
<td>Dan-CPGHE-NH\textsubscript{2}</td>
<td>26.08</td>
<td>773.20/774.90</td>
</tr>
<tr>
<td>Cp1-a</td>
<td>Dan-HPGCE-NH\textsubscript{2}</td>
<td>23.85</td>
<td>773.20/773.97</td>
</tr>
<tr>
<td>Cp1-b</td>
<td>Dan-EPGCH-NH\textsubscript{2}</td>
<td>23.08</td>
<td>773.20/773.84</td>
</tr>
<tr>
<td>Cp1-c</td>
<td>Dan-CHPGE-NH\textsubscript{2}</td>
<td>24.05</td>
<td>773.20/773.95</td>
</tr>
<tr>
<td>Cp1-d</td>
<td>Dan-HHPGE-NH\textsubscript{2}</td>
<td>14.57</td>
<td>807.25/807.95</td>
</tr>
<tr>
<td>Cp1-e</td>
<td>Dan-CCHPGE-NH\textsubscript{2}</td>
<td>18.55</td>
<td>876.20/876.91</td>
</tr>
<tr>
<td>Cp1-f</td>
<td>Dan-CPGH-NH\textsubscript{2}</td>
<td>24.17</td>
<td>644.15/645.09</td>
</tr>
<tr>
<td>Cp1-g</td>
<td>Dan-CPGEE-NH\textsubscript{2}</td>
<td>26.05</td>
<td>765.18/765.90</td>
</tr>
<tr>
<td>Cp1-h</td>
<td>Dan-ECPGEE-NH\textsubscript{2}</td>
<td>17.90</td>
<td>894.20/894.72</td>
</tr>
<tr>
<td>P1</td>
<td>Dan-GGGGGG-NH\textsubscript{2}</td>
<td>17.50</td>
<td>535.12/535.98</td>
</tr>
</tbody>
</table>

Table 3.1 HPLC and mass data of the peptides.

### 3.3.2 Fluorescence Response of Probes with Various Metal Ions

We investigated the fluorescence change in physiological buffer solution without co-solvent due to the excellent solubility of peptides. We measured the fluorescence emission spectra of all peptides in the presence of 1 equivalent of various metal ions (Cd\textsuperscript{2+}, Co\textsuperscript{2+}, Pb\textsuperscript{2+}, Cu\textsuperscript{2+}, Ag\textsuperscript{+}, Mn\textsuperscript{2+}, Ni\textsuperscript{2+},...
Zn$^{2+}$ and Al$^{3+}$) except biologically important metal ions like Ca$^{2+}$, Mg$^{2+}$, Na$^+$ and K$^+$, which were used in 5 mM. The peptides from CP1 to Cp1-f shows similar trend with Zn$^{2+}$ and enhances the fluorescence. Similarly, these peptides bind with Cd$^{2+}$ and Cu$^{2+}$. It is very difficult to discriminate between Zn$^{2+}$ and Cd$^{2+}$ due to their similar electronic configuration and ionic radius. Interestingly, the peptide Cp1-d did not bind with Cd$^{2+}$ and showed only fluorescence enhancement with Zn$^{2+}$. This might be due to the absence of free thiol group which has great affinity to bind with Cd$^{2+}$. Generally, the peptides without thiol moiety did not bind with Cd$^{2+}$ and CP1-d contain two imidazole group instead of thiol group. This peptide analog has successfully discriminated its sensing ability between zinc and cadmium due to the poor binding affinity of cadmium towards imidazole ring. The fluorescence quenching of these probes with Cu$^{2+}$ is not unusual because imidazole ring of His and sulfonamide group of dansyl have binding affinity with Cu$^{2+}$ ions. As a representative example of the series from CP1-CP1-f, we only present the fluorescence response of peptide CP1 with various metal ions in Figure 3.1.
Figure 3.1. Fluorescent response of peptide CP1 (10 µM) with 1 equivalent of various metal ions except Mg$^{2+}$, Ca$^{2+}$, Na$^+$, and K$^+$ which were used at 5 mM. The emission spectra were obtained using 330 nm as excitation wavelength in 10 mM Hepes buffer, pH 7.4.

The fluorescence response of peptide Cp1-g with metal ions; where the His residue of peptide CP1 is substituted by Glu residue, is shown in Figure 3.2. As a representative example for both peptides, we showed here peptide CP1-g and its fluorescence response with various metal ions. The fluorescence data indicated that both peptides showed the fluorescence enhancement only with Pb$^{2+}$ via shift in emission intensity from 550 to 505 nm. Other tested metal ions have no effect in the emission intensity except the minimal change with Zn$^{2+}$ and Cu$^{2+}$. In all cases from peptide Cp1-Cp1-i the blue shift was observed after the binding event occurred with metal ions. Among them, Pb$^{2+}$ with the target peptide exhibited the large shift ~ 45 nm, where as with Zn$^{2+}$ a nominal shift was occurred. This blue shift in the emission
spectrum most likely resulted from the fluorophore moving to a less polar environment upon metal binding.  

![Figure 3.2](image.png)

**Figure 3.2.** Fluorescent response of peptide CP1-g (10 µM) with 10 equivalent of various metal ions except Mg²⁺, Ca²⁺, Na⁺ and K⁺ which were used at 5 mM. The emission spectra were obtained using 330 nm as excitation wavelength in 10 mM Hepes buffer, pH 7.4.

### 3.3.3 Fluorescence Titrations with Metal Ions

To investigate the binding affinity, the fluorescence titrations were investigated in 10 mM Hepes Buffer, pH 7.4 by adding different concentrations of representative metal ions. As a representative example of the series from CP1-CP1-f, the titration data for CP1 is shown in **Figure 3.3**. In other peptides from CP1-a to CP1-f, approximately 1 equivalent of Zn²⁺ was required for the complete saturation of the peptides emission intensity as compare to CP1.
Figure 3.3 Fluorescent titration of peptide CP1 (10 µM) with various concentration of Zn(II) obtained by excitation at 330 nm in 10 mM Hepes buffer, pH 7.4.

As these peptides have exhibited the fluorescence change with Cd$^{2+}$ and Cu$^{2+}$, individual titrations were plotted to investigate the binding affinity of these metal ions. Addition of Zn$^{2+}$ to the control peptide P1, (Figure 3.4) which is incapable of binding metal, resulted in no fluorescence change, which supports the individual contribution of ligands in their own perspectives for metal binding.

The fluorescence titrations were plotted for peptide CP1-g and CP1-h with Pb$^{2+}$. Only titration data for peptide Cp1-g is shown in Figure 3.5 as representative example for lead selective peptides. As the Pb$^{2+}$ binds with peptide, a “turn–on” fluorescence was observed with concomitant shift from 550 nm to 505 nm.
**Figure 3.4** Fluorescent titration of peptide P1 (10 µM) with various concentration of Zn(II) obtained by excitation at 330 nm in 10 mM Hepes buffer, pH 7.4.

**Figure 3.5** Fluorescent titration of peptide CP1-g (10 µM) with various concentration of Pb(II) obtained by excitation at 330 nm in 10 mM Hepes buffer, pH 7.4 and inset Sigmoidal curve. Sigmoidal curve suggested the EC50 for this probe with Pb$^{2+}$ is 41.5µM.

### 3.3.4 Determination of Binding Affinity

Peptide binding characteristics were determined via titrations with target metal ions and Job’s plot analysis to determine the affinity and stoichiometry of complex formation. Titration datas were fitted with the programme Enzfitter supplied by Elsvier biosystem using non-linear curve fitting equation as earlier discussed in chapter 2.
The association constant (Ka), are shown in Table 3.2. The binding affinity as shown in table 3.2 indicated that peptides from Cp1 to CP1-f can detect Zn\(^{2+}\) and CP1-g to CP1-h for Pb\(^{2+}\) in micromolar range. In addition, the EC50 value was calculated for peptide Cp1-g with Pb\(^{2+}\) using sigmoidal fit and the obtained value is 41.5 µM.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Metal ion</th>
<th>Ka Value (M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP1</td>
<td>Zn(^{2+})</td>
<td>3.1 ± 0.4 × 10(^6)</td>
</tr>
<tr>
<td>Cp1-a</td>
<td>Zn(^{2+})</td>
<td>4.0 ± 0.4 × 10(^6)</td>
</tr>
<tr>
<td>Cp1-b</td>
<td>Zn(^{2+})</td>
<td>2.2 ± 0.2 × 10(^6)</td>
</tr>
<tr>
<td>Cp1-c</td>
<td>Zn(^{2+})</td>
<td>3.0 ± 0.4 × 10(^6)</td>
</tr>
<tr>
<td>Cp1-d</td>
<td>Zn(^{2+})</td>
<td>1.0 ± 0.3 × 10(^6)</td>
</tr>
<tr>
<td>Cp1-e</td>
<td>Zn(^{2+})</td>
<td>2.6 ± 0.6 × 10(^6)</td>
</tr>
<tr>
<td>Cp1-f</td>
<td>Zn(^{2+})</td>
<td>3.0 ± 0.5 × 10(^6)</td>
</tr>
<tr>
<td>Cp1-g</td>
<td>Pb(^{2+})</td>
<td>3.4 ± 1.2 × 10(^4)</td>
</tr>
<tr>
<td>Cp1-h</td>
<td>Pb(^{2+})</td>
<td>2.1 ± 1.1 × 10(^4)</td>
</tr>
</tbody>
</table>

Table 3.2 Calculation of association constant for peptides applying 1:1 binding stoichiometry. (Note, the fitting error in zinc binding peptides is less than 10\%, where as with Pb\(^{2+}\) binding peptides are slightly higher than zinc binding peptides; might be due to large shift in emission intensity)
3.3.5 Reversibility of the Peptide-Metal Complex with EDTA

Reversibility is an important property of a fluorescent sensor. After the analysis process is performed, the ligand-analyte complexation should be readily reversed. In this process, the analyte (Zn$^{2+}$ and Pb$^{2+}$, herein) was released from the peptide-metal complex, so the peptide was recovered to its free state. We illustrate the reversibility of these peptides with indicated metal ions in Figure 3.6. As a representative example, CP1-Zn$^{2+}$ and CP1-g-Pb$^{2+}$ complexes and their reversibility are discussed here.

![Figure 3.6](image)

Figure 3.6 Fluorescence emission spectra of (A) CP1 with Zn$^{2+}$ and EDTA, (B) CP1-g with Pb$^{2+}$ and EDTA. The emission spectra ($\lambda_{ex}=330$ nm) of all peptides (10 µM) were measured at 25°C in 10 mM HEPES buffer, pH 7.4.

Figure 3.6 indicated that addition of 10 µM of EDTA to the CP1–Zn complex instantly resulted in the return of the original, zinc-free spectrum, which demonstrates the readily reversibility of the signaling mechanism of this peptide. The similar trend was found with all zinc binding peptides. Similarly, about 8 eq. of EDTA was required to get the metal free spectrum of peptide Cp1-g- Pb$^{2+}$ complex. About one equivalent of EDTA to that of metal ion concentration required to obtain the metal free spectrum from both
metal complexes. Overall, an excellent reversibility was observed for all peptide-metal complexes.

3.3.6 Fluorescence Response of Peptides with and without Zn$^{2+}$ at Different pH

We investigated the pH influence on the fluorescence intensity of the peptide in the absence and presence of Zn$^{2+}$ for only zinc binding peptides (Figure 3.7). As a representative example for all the series, we illustrated here peptide CP1 with and without Zn$^{2+}$. At pH lower than 5.5, the peptide exhibited very weak fluorescence intensity, regardless of the presence or absence of Zn$^{2+}$. Previously synthesized probes containing dansyl moiety also showed a very weak emission intensity in acidic pH, which was explained by protonation of the dimethylamino group (pKa ~ 4) of the dansyl fluorophore. At pH > 6.5, the intensity difference between CP1 and CP1–Zn$^{2+}$ complex increased with increasing pH, however the emission intensity of the peptide probe in the absence of Zn$^{2+}$ was not considerably changed. Considering pKa values of His and Cys residues, the negative charge of side groups of His and Cys residues must increase with increasing pH, which might enhance the interactions between the peptide and Zn$^{2+}$, resulting in the fluorescence increase of the probe in the presence of Zn$^{2+}$ in this condition. At pH > 9.5, the intensity of free CP1 slightly increased with increasing pH. This might be due to the deprotonation of the nitrogen atom of the sulfonamide group (pKa ~ 10), which increased the electron density on the naphthyl ring. In addition, the increase of negative charge of the nitrogen atom might promote complexation of CP1–Zn$^{2+}$ complex, resulting in the slight increase of intensity at pH > 9.5. Overall, the titled peptide CP1 can be utilized for monitoring Zn$^{2+}$ at varying pH from 6.5 to 11.5. In addition, the
pH response of the peptide CP1-g with Pb\(^{2+}\) was measured at varying pH as we did in peptide CP1. This peptide only shows the fluorescence enhancement in physiological pH.

![Graph showing fluorescence intensity of CP1 at different pH values.](image)

**Figure 3.7** Fluorescence intensity of CP1 at different pH values. The emission intensity at 520 nm was measured by excitation at a wavelength of 330 nm.

### 3.3.7 Investigation of Secondary Structure with and without Metal Ions

To investigate the structural implications of ligand replacement within the zinc binding, peptide CD spectra were obtained with and without metal ions. Among the series, we selected three peptides CP1, CP1-e and CP1-g for further structural investigation. The apo CD spectra indicated that there is no big structural change among the series (Figure 3.8).

The peak around 223 and 232 nm indicated that the peptides have turn structure which is common among these peptides. In peptide CP1, the zinc induced structural change is minimal which indicates that apo-peptide itself has pre-organized structure and zinc induced secondary structure is not much influenced. Specifically the band observed between 250 and 270 nm is a feature not commonly observed for peptides which do not contain Trp, Tyr
and Phe, and may result from the dansyl chromophore. As Cu$^{2+}$ also exhibited the fluorescence response with these peptides and structural study was investigated in the presence of this metal ion. The CD spectra of CP1 with Cu$^{2+}$ also exhibited the similar structural change as compare to zinc. This result clearly indicates that metal induced secondary structure is not pre-dominant with both metal ions. The structural change induced by metal ion can be further recovered to its original zinc free spectrum with 1 equivalent of EDTA. (Figure 3.9)

This result is in best agreement with the reversibility shown by fluorescence study. Unlike peptide CP1, peptide CP1-e illustrates the zinc induced structural change with deep negative band at 227 nm, which is hallmark of turn structure. The Cu$^{2+}$ induced structural change is minimal with this peptide, which is consistent with the small change in emission intensity exhibited by this peptide with Cu$^{2+}$. Interestingly, the zinc dependent structural change was not observed with peptide CP1-g which is well consistent with the fluorescence data. Overall, structural study concluded that the different binding affinity observed with metal ions is due to different feature of secondary structure induced by metal ions.
**Figure 3.8** Near and Far UV CD spectrum of (A) CP1, (B) CP1-e and (C) CP1-g with and without metal ions in 10 mM HEPES buffer solution (pH = 7.4). The peptide concentration was 200µg/mL.

**Figure 3.9** Near and Far UV CD spectrum of peptide CP1 (200µg/ml) with 1 equivalent of Zn\(^{2+}\) and CP1-Zn\(^{2+}\) complex with 1 equivalent of EDTA in 10 mM HEPES buffer solution (pH = 7.4).
3.3.8 Competition Fluorescence Study

The fluorescence response of the peptide sensor in the presence of each metal ion (Ca\(^{2+}\), Cd\(^{2+}\), Co\(^{2+}\), Pb\(^{2+}\), Cu\(^{2+}\), Ag\(^{+}\), Mg\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\), Hg\(^{2+}\), Zn\(^{2+}\) as per chlorate anion and Na\(^{+}\), Al\(^{3+}\), K\(^{+}\) as chloride anion) was investigated. CP1 was selected as a representative example among the zinc binding series from CP1-CP1-f and investigated the selectivity over various competitive metal ions. Figure 3.10 shows the fluorescence emission change of the probe CP1 upon the addition of each metal ion at pH 7.4. The probe did not exhibit a fluorescence response with almost any of the metal ions except Cd\(^{2+}\) and Cu\(^{2+}\). Specially, the zinc-dependent fluorescence was not affected by the presence of a 5 mM concentration of biologically important metal ions such as Na\(^{+}\), K\(^{+}\), Ca\(^{2+}\), and Mg\(^{2+}\). Generally, chemical or peptide probes are rarely able to differentiate between Zn\(^{2+}\) and Cd\(^{2+}\).\(^{11g,i}\) As the concentration of Cd\(^{2+}\) in biological systems is generally low compared to Zn\(^{2+}\) and the probe had a more potent binding affinity for Zn\(^{2+}\) (Kd = 1.4×10\(^{-6}\) M) than Cd\(^{2+}\) (Kd = 2.3×10\(^{-6}\) M) at 10 µM of peptide concentration, CP1 can be applied for monitoring Zn\(^{2+}\) in biological system with further modification. Interestingly, the fluorescence intensity of CP1 vanished in presence of Cu\(^{2+}\). Generally, other chemical and peptide probes containing dansyl moiety have strong binding affinities for Cu\(^{2+}\), resulting in the decrease of emission intensity.\(^{22}\) Our control peptide, P1, also showed the considerable decrease of emission intensity in the presence of 2–10 µM concentration of Cu\(^{2+}\). (data not shown) The similar trend was observed along the series for zinc binding peptides from Cp1-a to CP1-f.
Figure 3.10 Fluorescence response of peptide CP1 (10 µM) in the presence of Zn^{2+} (10 µM) and/or various metal ions at pH 7.4 (10 mM HEPES buffer). All metal ions were evaluated at one equivalent to Zn^{2+} except Na^+, K^+, Ca^{2+}, and Mg^{2+}, which were used at 5 mM.

Similarly, the peptide CP1-g has no significant variation in the emission intensity in presence of 10 equivalent of various metal ions except biologically important metal ions (Mg^{2+}, Ca^{2+}, Na^+, K^+) which were used in 5 mM (Figure 3.11). The selectivity of Pb^{2+} over Ca^{2+} and Zn^{2+} is highly desirable because Pb^{2+} targets both Ca^{2+} and Zn^{2+} binding sites in vivo suggesting that these ions would be likely competitors for Pb^{2+} detection in cells. The selectivity result of probe Cp1-g indicates that the probe is highly selective for Pb^{2+} and that neither Zn^{2+} nor Ca^{2+} interferes with the response of the probe.
Figure 3.11 Fluorescence response of peptide CP1-g (10 µM) in the presence of Pb\(^{2+}\) (100 µM) and/or various metal ions at pH 7.4 (10 mM HEPES buffer). All metal ions were evaluated at one equivalent to lead ion except Na\(^+\), K\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\), which were used at 5 mM.

3.3.9 Immobilization of Peptide CP1 on resin

To test the utility of the peptides in further application, zinc binding peptide CP1 was immobilized on the solid support and the binding affinities for various metal ions were investigated. For this, we synthesized a CP1–resin conjugate by synthesizing the peptide probe on PEG–PS–resin in consideration of the water swelling characteristics of PEG–PS–resin. The fluorescence emission of the conjugate with various metal ions was measured in 10 mM HEPES buffer (pH 8.4).
Figure 3.12 Fluorescence response of CP1–resin conjugate (300 µg/mL) in the presence of various metal ions at pH 8.4 (10 mM HEPES buffer). All metal ion concentration was 100 µM except for Na⁺, K⁺, Ca²⁺, and Mg²⁺, which were used at 5 mM. The concentration of EDTA was 100 µM. Fluorescence excitation was provided with a handheld UV lamp (ENF 260) set on long wavelength (365 nm). The image was acquired after 30 min of incubation.

Figure 3.12 indicates that CP1–resin conjugate showed a considerable emission intensity increase only in the presence of Zn²⁺, whereas it lost its original emission intensity in the presence of Cu²⁺. The addition of EDTA to the resin-Zn²⁺ complex that exhibited strong emission intensity resulted in the return of the original zinc-free intensity. This result confirmed that immobilized peptide similar to CP1 displayed a great selectivity for Zn²⁺ and Cu²⁺ in the presence of several transition metal ions and this conjugate was easily regenerated through simple washing procedures with or without EDTA solution.

3.4 Conclusion
In the present study, we successfully synthesized a novel peptide sensor for monitoring Zn²⁺ and Pb²⁺ and demonstrated the strategy for developing selective sensor by tuning ligand. The binding affinity, reversibility and
selectivity of these probes for monitoring these ions were studied. The 6 peptides named from \textbf{CP1} to \textbf{CP1-f} were found as selective probe to monitor \(\text{Zn}^{2+}\) in micromolar concentration. Substitution of Cys residue by His discriminates the fluorescence signaling between zinc and cadmium. The structural information provided by CD spectroscopy revealed that pre-organization structure of the probes is not much affected by addition of \(\text{Zn}^{2+}\). Rotation of the ligand along the peptide chain has no significant change in binding affinity as well as in structure. The pH titration data for zinc binding peptide indicated that these peptides can monitor the zinc ion at varying pH from 6.5 to 11.5. Further on-bead application of peptide CP1 as a representative of zinc binding peptides was demonstrated by immobilizing it into Tentagel resin. The fluorescence response with \(\text{Zn}^{2+}\) in resin conjugate peptide was essentially maintained similar to its soluble analog. Substituting the His residue to Glu, allows us to monitor \(\text{Pb}^{2+}\) selectively and reversibly. The peptides developed in this study have several advantages like good solubility in buffer system, micromolar affinity, and easy conjugation with solid support. However, all the peptides are too hydrophilic to penetrate into cells and are useful for monitoring extracellular or environmental monitoring of these metal ions.

3.5 Materials and Methods

3.5.1 Buffer Solutions

All buffers were prepared by using high-purity water obtained. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was obtained from Sigma and used without further purification. All buffers were degassed prior to study. To study the pH influence on the sensitivity of both peptides, the
buffers were selected based on the pKa Value of buffer. The buffers used for fluorescence study is shown in Table 3.3.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Buffer</th>
<th>Effective pH Range</th>
<th>pKa</th>
<th>Measured pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acetate Buffer</td>
<td>3.56 - 5.6</td>
<td>4.76</td>
<td>3.5, 4.5</td>
</tr>
<tr>
<td>2</td>
<td>MES Buffer</td>
<td>5.5 - 6.7</td>
<td>6.10</td>
<td>5.5</td>
</tr>
<tr>
<td>3</td>
<td>HEPES Buffer</td>
<td>7.6 - 8.6</td>
<td>8.00</td>
<td>7.4</td>
</tr>
<tr>
<td>4</td>
<td>Tris Buffer</td>
<td>7.5 - 9.0</td>
<td>8.06</td>
<td>8.5</td>
</tr>
<tr>
<td>5</td>
<td>CHES Buffer</td>
<td>8.6 - 10.0</td>
<td>9.50</td>
<td>9.5, 10.5, 11.5</td>
</tr>
</tbody>
</table>

Table 3.3 Buffers used for measuring the fluorescence response of the probe with and without metal ions.

3.5.2 Circular Dichroism Studies

The secondary structure of the peptides was studied in 10 mM Hepes buffer in the presence and absence of metal ions using Jasco J-815 Spectropolarimeter. The peptide concentration 200 µg/mL was kept constant throughout the experiment. The mean residual ellipticity was calculated and the data were plotted between mean residual ellipticity vs. wavelength.
3.6 Literature Cited


4. Synthesis of Highly Selective Fluorescent Peptide Probes for Metal Ions: Tuning Selective Metal Monitoring with Secondary Structure

4.1 Introduction

Transition and heavy metal ions play an important role in many biological and environmental processes. Much research effort has focused on developing various fluorescent probes for the detection of metal ions. Development of metal monitoring peptide probes has received attention for the following reasons. Peptide probes can be facilely synthesized by solid phase synthesis and the selectivity and sensitivity for specific metal ions can be optimized by further amino acid replacement. In addition, peptide probes can be used in physiological buffer solution due to their good solubility and can be easily conjugated to solid support for further applications.

Several fluorescence peptide probes for monitoring various metal ions have been developed. Most of these consisted of a metal-binding site (receptor) and a signal transduction site (fluorophore). Binding of the receptor with metal ions resulted in a fluorescent spectrum change of fluorophore as a result of electron or energy transfer. Much effort in the field of metallo-protein research have resulted in the identification of various small peptide motifs (< 15 amino acids) that bind specifically to metal ions. Several peptide sensors for metal ions have been synthesized on the basis of the primary structures of various metal-binding motifs. Generally, short peptides containing a metal-binding motif tend to adopt a random coil structure due to the removal of their protein environments for the binding motif. Thus, the binding affinity and selectivity of short peptide probes are

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2) Major part of this chapter was published as Joshi, B. P. and Lee, K. H. *Bioorg. Med. Chem.* **2008**, *16*, 8501.
much lower than those of proteins. It is generally accepted that pre-organized structures of the short peptide probes are necessary for metal binding. Thus, specific amino acid sequences to stabilize specific secondary structures were included in the primary structures of the probes to improve metal-binding affinity and selectivity. However; almost all short peptide probes suffered limitations with binding affinity or selectivity.

In the present study, we investigated whether or not the selective monitoring of a peptide probe for metal ions was tuned by modulating the secondary structure.

4.2 Design and Strategy
We designed heptapeptide probes consisting of fluorophore and a metal-binding site due to the easy synthesis and the convenience of the secondary structure characterization by using circular dichroism. 1,5-dimethylaminonaphthalene sulfonamide (dansyl) group as a fluorophore was introduced to the N-terminal of the peptides because the dansyl group can display a large Stoke shift along with varying quantum yield by changing its local environment. His, Glu, and Cys residues were frequently found in several metal-binding motifs in various metallo proteins and previously, we developed a metal binding peptide probe containing these amino acids. As shown in Table 4.1, we synthesized peptide probes containing these amino acids to afford ligands for metal ions. To change the secondary structure of the peptide probes, a Pro-Gly sequence to stabilize a type II β-turn structure and a Gly-Gly sequence to adopt a random coil structure were included in the peptides. First, we synthesized three peptide probes (GG2, dansyl-Cys-Gly-Gly-His-Gly-Gly-Glu-NH₂; PG2, dansyl-Cys-Pro-Gly-His-Pro-Gly-Glu-NH₂; PG1, dansyl-Cys-Gly-Gly-His-Pro-Pro-
Gly-Glu-NH₂) by solid phase peptide synthesis. Among these, GG2 exhibited great selectivity only for Cu²⁺ among 13 metal ions including competitive transition metal ions, PG2 containing two Pro-Gly sequences showed great selectivity for Zn²⁺. PG1 containing one Pro-Gly sequence exhibited selectivity for Cu²⁺ and Zn²⁺. We investigated the binding stoichiometry, binding affinity, reversibility and pH sensitivity of each peptide probe. CD spectra indicated that the secondary structures caused by the Pro-Gly and Gly-Gly sequences changed the selectivity for detecting metal ions. Further to confirm the role of Cys residue in zinc binding, same analogs were synthesized using Acm-protected Cys as a mimic of Cys residue. To evaluate the turn structure and its role in selective monitoring of metal ions especially for zinc, we further synthesized the different analog containing d-pro which provides the different turn propensity as compare to Pro-Gly.

4.3 Results and Discussion

4.3.1 Synthesis of Peptide Probes and their Characterization

Peptide probes were synthesized by Fmoc-chemistry by solid phase peptide synthesis as discussed earlier. After cleavage of the crude product from resin; the peptides were purified using semi-preparative HPLC with a C18 column. The successful synthesis and purity (>95%) were confirmed by analytical HPLC with a C18 column and a MALDI TOF mass spectrometer. The HPLC and mass data for peptides are shown in Table 4.1.
### Table 4.1 HPLC and Mass Data of the Peptides

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name</th>
<th>Sequence</th>
<th>Ret. Time (min.)</th>
<th>Calcd./Obs. Mass (M+H⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PG2</td>
<td>Dan-CPGHPGE-NH₂</td>
<td>24.77</td>
<td>928.26/928.79</td>
</tr>
<tr>
<td>2</td>
<td>PG1</td>
<td>Dan-CGGHPGE-NH₂</td>
<td>22.36</td>
<td>888.24/888.47</td>
</tr>
<tr>
<td>3</td>
<td>GG2</td>
<td>Dan-CGGHGGE-NH₂</td>
<td>27.5</td>
<td>848.48/848.60</td>
</tr>
<tr>
<td>4</td>
<td>PG2-acm</td>
<td>Dan-C- (acm)- PGHPGE-NH₂</td>
<td>26.88</td>
<td>998.37/999.34</td>
</tr>
<tr>
<td>5</td>
<td>PG1-acm</td>
<td>Dan-C- (acm) -GGHPGE-NH₂</td>
<td>24.34</td>
<td>958.35/959.25</td>
</tr>
<tr>
<td>6</td>
<td>GG2-acm</td>
<td>Dan-C- (acm)- GGHGGE-NH₂</td>
<td>22.97</td>
<td>918.59/919.31</td>
</tr>
<tr>
<td>7</td>
<td>PG2-d</td>
<td>Dan-CPGHPGE-NH₂</td>
<td>28.67</td>
<td>928.26/928.39</td>
</tr>
<tr>
<td>8</td>
<td>PG1-d</td>
<td>Dan-CGGHPGE-NH₂</td>
<td>24.21</td>
<td>888.24/888.64</td>
</tr>
</tbody>
</table>

#### 4.3.2 Investigation of Secondary Structure

To investigate the secondary structures of the peptide probes, we measured the CD spectrum of the peptide probes in HEPES buffer solution (pH=7.4) without any co-solvent. CD spectra (Figure 4.1) ranged from 190 nm to 250 nm revealed that three peptides in the absence of metal ions might adopt different secondary structures. The strong negative bands at 203 nm and near 215 nm indicated that **GG2** containing two Gly-Gly sequences might adopt random coil and β-sheet structures, whereas the negative band at 227 nm indicated that **PG1** containing one Pro-Gly sequence might have a turn structure. A negative band at 205 nm and a strong positive band at 225 nm in the CD spectrum of **PG2** were characteristic bands of β-hairpin structure. However, CD spectrum with a maximum at 228 nm and a minimum at 205 nm are hallmarks of the polyproline II structure and the maximum in CD spectrum of a polyproline II can shift to higher or lower
wavelengths because of contributions from other secondary structures at nearby wavelengths. Thus, we assume that PG2 containing two Pro-Gly sequences might have turn structure or polyproline II structure.

![Graph showing UV CD spectrum of peptide probes](image)

**Figure 4.1** Far UV CD spectrum of peptide probes in 10 mM HEPES buffer solution (pH=7.4).

### 4.3.3 Fluorescence Spectra of Probes in the Presence of Various Metal Ions

As the peptide probes are fully water soluble, we measured the fluorescence spectrum of the probes in 10 mM HEPES buffer solution (pH 7.4) without any co-solvent. Fluorescence spectra ranging from 350 to 650 nm were measured by excitation at a wavelength of 330 nm. To investigate metal selectivity of the probes, we measured the fluorescence change of the peptide probes in the presence of various metal cations (Ca^{2+}, Cd^{2+}, Co^{2+}, Pb^{2+}, Cu^{2+}, Ag^{+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} as perchlorate anion and Na^{+}, Al^{3+}, K^{+}, as chloride anion). **Figure 4.2** shows the fluorescent emission spectrum of GG2,
PG1, and PG2 (10 µM) in HEPES buffer solution (10 mM, pH 7.4) containing each metal ion (1 eq.). GG2 did not exhibit a fluorescence response with any of the metal ions except Cu²⁺. Upon addition of Cu²⁺, the emission intensity at 510 nm vanished. PG1 did not have fluorescence response with all test metal ions except Zn²⁺ and Cu²⁺; the emission intensity increased in the presence of Zn²⁺, whereas fluorescence intensity vanished in the presence of Cu²⁺. Fig 4.2C showed that PG2 did not exhibit a fluorescence response with almost any of the metal ions including Cu²⁺, except Zn²⁺ and Cd²⁺. Upon Zn²⁺ addition, the emission intensity of PG2 at 510 nm increased. Even though Cu²⁺ and Zn²⁺ have similar size and electronic configuration, the peptide probe exhibited great selectivity for Zn²⁺ over Cu²⁺. The dansyl fluorophore of these short peptide probes is supposed to be directly participated in the metal binding. The peptide probes responded with Zn²⁺ by enhancement of emission intensity via chelation enhanced fluorescence mechanism. However, the probes monitored Cu²⁺ by fluorescence quenching mechanism via energy or electron transfer of Cu²⁺. Generally, the chemical or peptide sensors containing dansyl moiety and His (imidazole) residue were reported to show a fluorescence response with Cu²⁺ due to the high affinity of the imidazole moiety for Cu²⁺. However, PG2 even containing dansyl and imidazole moiety did not show a fluorescence response with Cu²⁺. Interestingly, in the absence of metal ions, GG2 showed higher emission intensity than did PG1. PG2 showed the lowest emission intensity at the same concentration. This must be due to the different environment for dansyl group caused by different secondary structures of the peptide probes. Furthermore, we investigated the fluorescence response of the peptide probes to various metal cations in the
presence of 140 mM NaCl. The peptide probes showed the same response as measured in HEPES buffer solution without 140 mM NaCl [data not shown]. To evaluate this system, we further investigated the fluorescence response of different analogs of these peptides containing \textit{d-Pro} which provides the different turn propensity as compare to Pro-Gly. The fluorescence spectra \textbf{(Figure 4.3)} with various metal ions followed the similar trend as the previously studied peptides.
Figure 4.2 Fluorescence response of (A) GG2 (10 µM), (B) PG1 (10 µM), and (C) PG2 (10 µM) in the presence of various metal ions (1eq.) in 10 mM Hepes buffer solution (pH = 7.4)
Figure 4.3 Fluorescence response of (A) PG2-d (10 µM) and (B) PG1-d (10 µM in the presence of various metal ions (1eq.) except Mg²⁺, Ca²⁺, Na⁺ and K⁺, which were used 5mM in 10 mM HEPES buffer solution (pH=7.4).
4.3.4 Reversibility Study of Peptide-metal Complex with EDTA

The fluorescence response of the probes for Zn\(^{2+}\) and Cu\(^{2+}\) upon the addition of excess EDTA (1~10 eq.) to the probe-metal complex instantly return to the original metal free emission spectrum demonstrates that the signaling mechanism of this peptide is readily reversible. As a representative example, we presented the reversibility of the peptide PG2 –zinc complex as shown in Figure 4.4.

![Figure 4.4 Fluorescence emission spectra of PG2 with Zn\(^{2+}\) and EDTA. The emission spectra (\(\lambda_{ex} = 330\) nm) of peptide (10 µM) was measured at 25°C in 10 mM HEPES buffer, pH 7.4.](image)

4.3.5 Binding Affinity Study

We investigated the binding stoichiometry and affinities of the peptides for Zn\(^{2+}\) and/or Cu\(^{2+}\) because both metal ions play a vital role in various biological functions such as gene expression, apoptosis, enzyme regulation, and amyloid fibril formation.\(^1\)
We have used the criteria to determine binding stoichiometry: a Job’s plot analysis and the fitting model used for the association constant calculation. Job’s plot analysis was conducted to determine the binding stoichiometry. Job plot analysis of PG2 and GG2 exhibited a maximum at 0.5 mole fractions with Zn$^{2+}$ and Cu$^{2+}$, respectively (Figure 4.5). Similarly, PG1 also exhibited the maximum at 0.5 mole fraction with both metal ions. (Figure 4.6) As suggested by Job’s plot, it is likely that PG2 forms a 1:1 complex with Zn$^{2+}$ in micromolar range and GG2 also forms a 1:1 complex with Cu$^{2+}$.

Figure 4.5 A Job plot for (A) PG2 and (B) GG2. The total [PG2] + [Zn$^{2+}$] = 2.5 µM and the total [GG2] + [Cu$^{2+}$] = 10 µM.

However, Job’s plot that was unsymmetrical indicated more complex equilibrium might be possible, and the peptides did not form exclusively a 1:1 complex. Imperial et al. reported that the fluorescent peptide probes forming 1:1 and 1:2 complex exhibited a large shift in the maximum emission wavelength, whereas the peptide forming only a 1:1 complex did not show a shift. As shown in Figure 4.7 and 4.8, the peptide probes (GG2 and PG1) showed a large shift in the maximum emission intensity.
when the probes interacted with Cu$^{2+}$, whereas the probes (PG2 and PG1) showed a little shift in the maximum emission wavelength when interacted with Zn$^{2+}$.

**Figure 4.6** A Job plot for (A) PG1 with Zn$^{2+}$ and Cu$^{2+}$. The total [PG1] + [Zn$^{2+}$] = 5 µM and the total [PG1] + [Cu$^{2+}$] = 10 µM.
Figure 4.7 Fluorescence emission spectra of GG2 upon addition of Cu$^{2+}$ and fit obtained from titration data using 1:2 complexations. Fluorescence emission spectra of GG2 (2.5 µM) upon addition of Cu$^{2+}$ (0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 µM) were measured in 10mM HEPES buffer (pH 7.4).
When we calculated the association constants for Zn$^{2+}$ and Cu$^{2+}$, we assumed that the peptide probes form 1:1 complex or 1:2 complexes, respectively. When the probes interacted with Zn$^{2+}$, 1:1 complexation model provides a better fitting and low error in the titration (*Figure 4.7 to 4.9*). However, when the probes interacted with Cu$^{2+}$, 1:2 complexation models provides a better fitting and low error in the fitting of titration data. (*Table 4.2*) Thus, we concluded that even though the peptide probes might have mixed...
complex, when the probes interacted with Zn\(^{2+}\), 1:1 complex might be predominant, whereas when the probes interacted with Cu\(^{2+}\), 1:2 complex might be predominant.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>(K_a) value for Zn(^{2+}) (M(^{-1}))</th>
<th>(K_a) value for Cu(^{2+}) (M(^{-2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:1 complex</td>
<td>1:2 complex</td>
</tr>
<tr>
<td>PG2</td>
<td>Dan-CPGHPGE-NH(_2)</td>
<td>3.0±0.2×10(^6)</td>
<td>NR</td>
</tr>
<tr>
<td>PG1</td>
<td>Dan-CGGHPGE-NH(_2)</td>
<td>2.1±0.1×10(^6)</td>
<td>3.0±0.4×10(^{12})</td>
</tr>
<tr>
<td>GG2</td>
<td>Dan-CGGHGGE-NH(_2)</td>
<td>NR</td>
<td>3.3±0.4×10(^{12})</td>
</tr>
</tbody>
</table>

**Table 4.2** The sequence and association constants of the peptide probes for metal ions at pH 7.4 (10 mM HEPES buffer solution).

As the probes contained a Cys residue, it can be expected that inter-disulfide bond formation in the presence of metal ions would occur. Thus, we measured the binding affinities of the probes for Zn\(^{2+}\) and Cu\(^{2+}\), in the presence of DTT (1 eq.) as a reducing reagent. This concentration of DTT has no interference on the interaction between peptides with metal ions.\(^{19}\)

The titrations were also performed with DTT containing buffer and binding constants were measured. The titration and fitting data for all peptides are shown in **Figure 4.10 and Table 4.3**.

Furthermore, the peptide probes (10 \(\mu M\)) were incubated in the presence of Zn\(^{2+}\) (1 eq.) or Cu\(^{2+}\) (1eq.), respectively and the peptide-metal complex was analyzed by HPLC. HPLC of GG2 and PG2 in the presence of Cu\(^{2+}\) and Zn\(^{2+}\) are illustrated as a representative of all cases as shown in (**Figure 4.11**). The HPLC spectra revealed that dimerization of the peptide probes in the presence of metal ions was not observed. **Table 4.2 and 4.3** showed the association constants of the peptide probes for Zn\(^{2+}\) by assuming 1:1 complex and the association constants for Cu\(^{2+}\) by assuming 1:2 complexes. The binding affinities of all probes for Zn\(^{2+}\) and Cu\(^{2+}\) were not changed in
the presence of DTT, suggesting that the disulfide bond formation might not formed in the metal monitoring process.

![Graphs showing fluorescence change](image)

**Figure 4.10** Comparative study of fluorescence change (a) PG2 with Zn$^{2+}$; (b) GG2 with Cu$^{2+}$; (c) PG1 with Zn$^{2+}$ and (d) PG1 with Cu$^{2+}$ with and without DTT containing 10 mM Hepes buffer. The peptide concentration was 2.5 µM in all cases.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>$K_a$ value for Zn$^{2+}$ (M$^{-1}$)</th>
<th>$K_a$ value for Cu$^{2+}$ (M$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:1 complex</td>
<td>1:2 complex</td>
</tr>
<tr>
<td>PG2</td>
<td>Dan-CPGHPGE-NH$_2$</td>
<td>2.9±0.2×10$^6$</td>
<td>NR</td>
</tr>
<tr>
<td>PG1</td>
<td>Dan-CPGHGGE-NH$_2$</td>
<td>2.5±0.2×10$^6$</td>
<td>2.0±0.1×10$^{12}$</td>
</tr>
<tr>
<td>GG2</td>
<td>Dan-CGHHGGE-NH$_2$</td>
<td>NR</td>
<td>2.2±0.2×10$^{12}$</td>
</tr>
</tbody>
</table>

**Table 4.3** The sequence and association constants of the peptide probes for metal ions at pH 7.4 (10 mM HEPES buffer solution) containing 1 equivalent DTT.

**Figure 4.11** HPLC of (a) PG2 in the absence of Zn$^{2+}$ ions; (b) with 1 eq. Zn$^{2+}$; (c) GG2 in the absence of metal ions and (d) with 1 equivalent Cu$^{2+}$ after 10 min. incubation at room temperature.
4.3.6 Fluorescence Spectra Change of Probes in Various pH

We investigated the pH influence on the fluorescence intensity of the peptide probes in the absence and presence of Cu$^{2+}$ or Zn$^{2+}$. As shown in Figure 4.12, all peptide probes in the presence or absence of the metal ions exhibited little fluorescence intensity at pH lower than 5.5. This might be due to the protonation of the dimethylamino group ($pK_a$~4) of the dansyl fluorophore.$^{20}$ At pH > 6.5, the intensity of GG2 itself increased with increasing pH, whereas GG2-Cu$^{2+}$ complex exhibited little emission intensity in these pH range (3.5~10.5). The emission intensity difference between PG1 and PG1-Zn$^{2+}$ complex maintained at pH > 6.5, because the intensity of PG1 and PG1-Zn$^{2+}$ complex similarly increased with increasing pH. However, PG1-Cu$^{2+}$ complex exhibited little emission in the whole pH range (3.5~11.5), and the emission intensity difference between PG1 and PG1-Cu$^{2+}$ complex increased with increasing pH. The emission intensity difference between PG2 and PG2-Zn$^{2+}$ complex was maximum at pH = 9.5; the emission intensity of PG2-Zn$^{2+}$ complex increased more than 4.5 times than that of PG2 at this pH. Overall results indicated that the peptide probes are useful for monitoring Zn$^{2+}$ or Cu$^{2+}$ in the neutral and basic pH.
Figure 4.12 Fluorescence responses of (A) GG2, (B) PG1, and (C) PG2 in the presence and absence of metal ions at different pH values.

To investigate binding affinity change of the probes in different pH, the association constants of the peptide probes for Zn\textsuperscript{2+} and Cu\textsuperscript{2+} were measured at pH 11.5 and summarized in Table 4.4. According to association constants measured at pH 7.4 and 11.5, the binding affinities of PG1 and PG2 for Zn\textsuperscript{2+} were increased with increasing pH, whereas the association constants of PG1 and GG2 for Cu\textsuperscript{2+} were not significantly changed at higher pH. Considering
the pK$_{a}$ values of Cys, a Cys residue in PG1 and PG2 played an important role in the increase of binding affinity for Zn$^{2+}$ at higher pH.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>$K_a$ value for Zn$^{2+}$ (M$^{-1}$)</th>
<th>$K_a$ value for Cu$^{2+}$ (M$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:1 complex</td>
<td>1:2 complex</td>
</tr>
<tr>
<td>PG2</td>
<td>Dan-CPGHPGE-NH$_2$</td>
<td>6.8±0.7×10$^6$</td>
<td>NR</td>
</tr>
<tr>
<td>PG1</td>
<td>Dan-CGGHPGE-NH$_2$</td>
<td>4.2±0.5×10$^6$</td>
<td>2.2±0.2×10$^{12}$</td>
</tr>
<tr>
<td>GG2</td>
<td>Dan-CGGHGGE-NH$_2$</td>
<td>NR</td>
<td>4.9±0.1×10$^{12}$</td>
</tr>
</tbody>
</table>

Table 4.4 The sequence and association constants of the peptide probes for metal ions at pH 11.5 (10 mM CHES buffer solution).

To confirm the role of a Cys residue, Cys-protected analogs with acm (acetamidomethyl) groups (GG2-acm, dansyl-Cys(Acm)-Gly-Gly-His-Gly-Gly-Glu-NH$_2$; PG2-acm, dansyl-Cys(Acm)-Pro-Gly-His-Pro-Gly-Gly-Glu-NH$_2$; PG1-acm, dansyl-Cys(Acm)-Gly-Gly-His-Pro-Gly-Gly-Glu-NH$_2$) were synthesized and fluorescence response were measured at pH=7.4. All Cys-protected analogs did not give response with Zn$^{2+}$. However, the binding affinities ($K_a = 1.6 \times 10^{12}$ M$^{-2}$ GG2-acm, $K_a = 2.4 \times 10^{12}$ PG1-acm M$^{-2}$) with Cu$^{2+}$ were not significantly different from those of GG2 and PG1. Overall results indicate that the Cys residue of PG1 and PG2 is essential for zinc ion binding, whereas the Cys residue of GG2 and PG1 might not play an important role in Cu$^{2+}$ binding.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>$K_a$ value for Zn$^{2+}$ (M$^{-1}$)</th>
<th>$K_a$ value for Cu$^{2+}$ (M$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1:1 complex</td>
<td>1:2 complex</td>
</tr>
<tr>
<td>PG2-d</td>
<td>Dan-CpGHPGE-NH$_2$</td>
<td>2.0±0.1×10$^6$</td>
<td>NR</td>
</tr>
<tr>
<td>PG1-d</td>
<td>Dan-CpGHGGE-NH$_2$</td>
<td>1.3±0.3×10$^6$</td>
<td>1.3±0.1×10$^{12}$</td>
</tr>
</tbody>
</table>

Table 4.5 The sequence and association constants of the peptide probes containing $d$-Pro for metal ions at pH 7.4 (10 mM HEPES buffer solution) containing 1 equivalent DTT.
Generally, heavy metal binding motif contained a Cys residue and several potent peptide probes for Zn$^{2+}$ contained Cys residues because the soft ligands, such as thiol group are preferred in the binding of Zn$^{2+}$. Similarly, the binding affinity of PG1-d and PG2-d were investigated in 10 mM Hepes buffer, pH 7.4. The binding affinity for PG1-d and PG2-d are slightly in lesser extent as compare to PG1 and PG2. (Table 4.5)

4.3.7 Competition Fluorescence Study
We investigated the fluorescence response of the GG2-Cu$^{2+}$, PG1-Zn$^{2+}$, and PG2-Zn$^{2+}$ complexes in the presence of one equivalent of transition metal ions or hundred equivalent of group I and II metal ions because the concentration of group I and II metal ions in mammalian cells are in mM range, whereas the transition metal ions in the cell or environment were relatively low. As shown in Figure 4.13, the addition of any other metal ion did not alter the emission intensity of the GG2-Cu$^{2+}$ complex. The zinc-dependent emission intensity of the PG1-Zn$^{2+}$ complex was not affected by the presence of any other metal ion except Cu$^{2+}$ in Figure 4.14. The binding affinity in Table 1 indicated that PG1 showed more potent binding affinity for Cu$^{2+}$ than Zn$^{2+}$. Even though Cu$^{2+}$ was reported to cause significant quenching for fluorescent chemical and peptide sensors with imidazole and dansyl moiety, interestingly, the zinc-dependent fluorescence of PG2 was not affected by the presence of any other metal ions such as competitive transition metal ions including Cu$^{2+}$ in Figure 4.15. PG2 showed slightly fluorescence response to Cd$^{2+}$. When the concentration of PG2 was 2.5 µM, the association constant for Cd$^{2+}$ ($K_a = 1.5 \times 10^6$ M$^{-1}$) indicated that PG2 ($K_a = 3.0 \times 10^6$ M$^{-1}$ with Zn$^{2+}$) had a more potent binding affinity for Zn$^{2+}$ than
Cd\(^{2+}\). It was concluded that PG2 developed in this study had a great selectivity for Zn\(^{2+}\).

**Figure 4.13** Emission intensity of GG2 (10 µM) in the presence Cu\(^{2+}\) (10 µM) and additional various metal ions at pH 7.4 (10 mM HEPES buffer). All metal ions are evaluated at one equivalent to Cu\(^{2+}\) except Na\(^{+}\), K\(^{+}\), Ca\(^{2+}\), and Mg\(^{2+}\), which are used at 500 equivalent. The emission intensities are calculated from three independent experiments performed in duplicate.

**Figure 4.14** Emission intensity of PG1 (10 µM) in the presence Zn\(^{2+}\) (10 µM) and additional various metal ions at pH 7.4 (10 mM HEPES buffer). All metal ions are evaluated at one equivalent to Zn\(^{2+}\) except Na\(^{+}\), K\(^{+}\), Ca\(^{2+}\), and Mg\(^{2+}\), which are used at 500 equivalents. The emission intensities are calculated from three independent experiments performed in duplicate.
Figure 4.15 Emission intensity of PG2 (10 µM) in the presence Zn$^{2+}$ (10 µM) and additional various metal ions at pH 7.4 (10 mM HEPES buffer). All metal ions are evaluated at one equivalent to Zn$^{2+}$ except Na$^+$, K$^+$, Ca$^{2+}$, and Mg$^{2+}$, which are used at 500 equivalent. The emission intensities are calculated from three independent experiments performed in duplicate.

4.3.8 Secondary Structure of Peptides in the Presence of Metal Ions

To investigate the secondary structure change of the peptide probes in the presence of metal ions, we measured the CD spectrum of the peptide probes in the presence of Zn$^{2+}$ or Cu$^{2+}$. CD spectra (Figure 4.16) suggests that GG2 in the absence of metal ions may adopt a random coil and β-sheet structures, however the secondary structure of GG2 was changed in the presence of Cu$^{2+}$. GG2 might adopt a random structure and a turn structure in the presence of Cu$^{2+}$ on the basis of the weak negative band near 225 nm. Near-UV CD spectrum ranged from 250-300 nm was measured because near-UV CD bands are used as an indication of the environment of aromatic residue such as Phe, Tyr, and Trp. Generally, near UV CD bands are weaker than those in the far UV.\textsuperscript{15(b)} Interestingly, the large positive band at 265 nm corresponding to the absorbance of dansyl group was observed in the CD
spectrum measured in the presence of Cu$^{2+}$. The CD spectrum indicated that the environment of dansyl group was totally changed when GG2-Cu$^{2+}$ was formed. The CD spectra and the large blue shift of the emission intensity in the presence of Cu$^{2+}$ indicates that when GG2 folds in the presence of Cu$^{2+}$, the hydrophilic environment around dansyl fluorophore may be changed into hydrophobic environment.

(A)

(B)
Figure 4.16 Near and Far UV CD spectrum of (A) GG2, (B) PG1, and (C) PG2 in the presence of metal ions (1 equiv.) in 10 mM HEPES buffer solution (pH = 7.4).
The far UV CD spectrum of \textbf{PG1} in the presence of Zn$^{2+}$ or Cu$^{2+}$ indicated that \textbf{PG1} might adopt different secondary structures depending on the presence of Zn$^{2+}$ or Cu$^{2+}$. Far UV CD spectrum including the negative band at 230 nm indicated that \textbf{PG1} adopted more turn structures in the presence of Zn$^{2+}$ than \textbf{PG1} did in the absence of metal ions, whereas \textbf{PG1} might adopt less turn structures in the presence of Cu$^{2+}$ than \textbf{PG1} did in the absence of metal ions. The near UV CD spectrum indicated that the environment of dansyl group was not greatly changed when \textbf{PG1} interacted with Zn$^{2+}$, whereas the environment of dansyl group was greatly changed when \textbf{PG1} interacted with Cu$^{2+}$. Far UV CD spectrum (200-230nm) revealed that the secondary structure of \textbf{PG2} was not greatly changed when the peptide interacted with Zn$^{2+}$ and \textbf{PG2} seemed to have a pre-organized structure for Zn$^{2+}$ binding. However, a change of negative band at 240 nm indicated that the environment of dansyl was changed when the peptide-Zn$^{2+}$ complex was formed. CD spectrum indicated that \textbf{PG1} and \textbf{PG2} may adopt different turn structures in the absence of metal ions and the secondary structure of the peptide probes play an important role for detecting the specific metal ions.

To validate the secondary structures of the peptide probes and to elucidate the metal-binding mode, we are currently investigating the secondary structure and folding of the peptide probes in the presence of metal ions by using NMR measurements.

Several research groups including us have synthesized various fluorescent probes for monitoring Zn$^{2+}$ \cite{4, 13, 19, 21} because Zn$^{2+}$ plays an important role in many biological and environmental processes.\cite{1, 2} The total concentration of Zn$^{2+}$ in different cells varies from the nanomolar range up to about hundreds micromolar.\cite{1} Thus, optimized chemical probes are required to monitor zinc concentration ranging from nanomolar to micromolar. Even
though several fluorescence-based chemical and peptide probes for Zn$^{2+}$ have been reported, most of them suffered limitations due to tight binding affinity (subnanomolar affinity) and interference of other metal ions. Specially, the probes with micromolar binding affinity for Zn$^{2+}$ interacted with other transition metal ions. 4(b), 13, 19

Almost all chemical or peptide probes containing dansyl moiety and His (imidazole) residue, were reported to show a large decrease of fluorescence emission intensity in the presence of Cu$^{2+}$ due to the high affinity of the imidazole moiety for Cu$^{2+}$ and quenching effect of Cu$^{2+}$. 4b, 8b, 10b Thus, the development of selective peptide probes with micromolar binding affinity for Zn$^{2+}$ remains a significant challenge. The peptide probe, PG2 developed in this study exhibited several advantages because of micromolar affinity for Zn$^{2+}$ in physiological buffer solution, no interference of other metal ions, easy synthesis, and good solubility in physiological buffer solution. Further information obtained from peptide PG2-d and GG2-NG also confirmed that turn structure for selective monitoring of Zn$^{2+}$ was required rather than the random structure. Thus, we expect that the peptide probe has a great possibility to be used for monitoring extracellular and environmental Zn$^{2+}$ concentration.

4.4 Conclusion

The overall study results confirmed the successful synthesis of selective fluorescent peptide probes for detecting Cu$^{2+}$ or Zn$^{2+}$, respectively and demonstrated that the metal detecting selectivity was tuned by modulating the secondary structure. We also demonstrated that while a pre-organized secondary structure is not required for the selective detection of Cu$^{2+}$ ion, it is for the detection of Zn$^{2+}$. 
4.5 Materials and Methods

4.5.1 Determination of Binding Stoichiometry and Binding Constant

The binding stoichiometry of peptides with metal ions was determined by using Job’s plot.\textsuperscript{4a, 22} A series of solutions with varying mole fraction of metal ions were prepared by maintaining the total peptide and metal ion concentration constant (For example, \([\text{GG2}]+[\text{Cu}^{2+}] = 10 \ \mu\text{M}, \ [\text{PG2}] + [\text{Zn}^{2+}] = 2.5 \ \mu\text{M}, \ [\text{PG1}]+[\text{Cu}^{2+}] = 10 \ \mu\text{M} \ \text{and} \ [\text{PG1}]+[\text{Zn}^{2+}] = 5 \ \mu\text{M}\)). The fluorescence emission was measured for each sample by exciting at 330 nm and spectra were measured from 350 to 650 nm. The fitting data were acquired by plotting a straight line through the maximum or minimum emission intensity in the titration curve and were plotted against the mole fraction of the metal ion versus emission intensity. The association constants were calculated based on the titration curve of the probes with metal ions.

4.5.2 Circular Dichroism Studies

CD spectra were recorded on a Jasco J-715 spectropolarimeter (Tokyo, Japan) using a quartz cell of 1 mm path length between 190 and 300 nm at room temperature. The concentration of peptides was 150µg/mL in 10 mM HEPES buffer (pH 7.4). Two scans with a scan speed of 10nm/min. were averaged for each peptide. CD spectra were expressed as the mean residue ellipticity.
4.6 Literature Cited


18) Association constants were obtained using the computer program ENZFITTER, available from Elsevier-BIOSOFT, 68 Hills Road, Cambridge CB2, 1LA, United Kingdom.


CHAPTER V
5. Design, Synthesis and Evaluation of Peptide-Resin Conjugates for Selective Monitoring of Metal Ions

5.1 Introduction
Several efforts were made in the past decades on the design and preparation of fluorescent chemosensors for the purpose of monitoring transition metal ions in solutions.\(^1\) Recently, studies of the design and preparation of fluorescent sensors for the monitoring of Cu\(^{2+}\) and Zn\(^{2+}\) have attracted increasing attention.\(^2\) This is because Cu\(^{2+}\) is a significant environmental pollutant and an essential trace element in biological system.\(^3\) Similarly, Zn\(^{2+}\) is associated with several biological phenomena including gene expression, apoptosis and protein synthesis as we discussed earlier in chapter 3 and 4.\(^4\) Several fluorescence peptide and chemical sensors were reported for the selective monitoring of these metal ions in solution phase.\(^1,2,5\) However, fluorescence sensing of these metal ions using bead sensor are very rare. In terms of practical application, the bead sensor possesses more favorable properties than other soluble chemosensors. For example, they can be easily made in to devices; shows high binding affinity towards analyte, easily regain the fluorescence by simple washing and can be restored without any loss in fluorescence property.

Chemical immobilization of small molecules onto surface is one of the important approaches and provides a convenient way to produce surfaces with specific chemical functionalities that permit the precise tuning of surface properties.\(^6\) For example, through surface modification, both Cu\(^{2+}\) binding ligands and fluorophores were immobilized on organic polymer nanoparticles\(^7\) and on inorganic silica nanoparticles to realize sensing copper
ions. Similarly, copper-binding ligands were immobilized in tentagel resin and CPG resin beads for sensing copper ions. However, conjugation of small chemicals into the surface always poses a synthetic challenge. In terms of synthetic feasibility, immobilization of proper binding ligand for specific metal ions on resin is much easier than other surface modifications. Additionally, compared to other surface modified sensors like nanoparticle-conjugation, film sensors, self assembled monolayer sensors; bead sensors are much easier to handle in the process of utilization. More likely the selectivity can be easily tuned by altering the amino acids, which endow resin-bead based fluorescent sensors to become increasingly prevalent.

In comparison with soluble chemosensors, the report on the peptide immobilized resin bead sensors for Zn$^{2+}$ and Cu$^{2+}$ are very rare where the fluorescence techniques was applied for monitoring. Our group has been especially interested in developing such kinds of resin immobilized sensors due to following reasons: a) control of selectivity mainly due to ligand choice for metal ions; b) simplicity and ease of synthesis; c) modular nature and easily optimization of the system for monitoring using fluorescence.

In the present study we investigated whether the selective monitoring of the peptide probe for metal ions was tuned by modulating the amino acid sequence or not in the peptide resin-conjugate. Thus, we designed heptapeptide probes consisting of fluorophore and metal binding site. 1, 5-Dimethylaminonaphthalene sulfonamide (dansyl) group as a fluorophore was introduced to the N-terminal of the peptides because the dansyl group can display a large stock shift along with varying quantum yield by changing its local environment. His, Glu and Cys residues are frequently found in several metal binding motifs in various metalloproteins and previously we
also developed the selective probe for monitoring Cu\(^{2+}\) and Zn\(^{2+}\) ions containing these amino acids.\(^{12k,l}\) Keeping this in mind, we immobilized these sequences and synthesized GG2-C, Dansyl-CGGHGGE-resin; PG2-C, Dansyl-CPGHPGE-resin and PG1-C, Dansyl-CGGHPGE-resin (Scheme 5.1) using solid phase Fmoc-chemistry.

![Scheme 5.1 Structures of Peptide-resin conjugates](image)

The peptides were immobilized on Tentagel resin due to its well known water swelling property mixed with 2 % mole ratio of Rink amide MBHA resin to confirm the successful synthesis using HPLC and Mass spectrometry. Similarly, as a negative control same sequences were immobilized on MBHA resin containing 2 % mole ratio of Rink amide MBHA resin. The GG2-C exhibited the great selectivity for monitoring Cu\(^{2+}\) ions in presence of several other transition metals. Similarly PG2-C showed only selectivity with Zn\(^{2+}\) whereas the PG1-C exhibited fluorescence response for both metal ions in aqueous buffer solutions. Interestingly, the peptides immobilized on
MBHA resin did not show fluorescence change with any of the metal ions tested in aqueous buffer solutions. The swelling effect in different solvent for the change in fluorescence emission intensity was also studied for Tentagel-resin conjugate peptides. Furthermore, the inter-disulfide bridge of Tentagel resin conjugate peptides was facilitated between cysteine residues using oxidized DTT and their fluorescence response was monitored with various metal ions. The inter-disulfide bridge containing peptide PG1-C and GG2-C exhibited the fluorescence response with Cu$^{2+}$ with lesser extent in sensitivity, whereas PG1 did not show any response with Zn$^{2+}$. Similarly, PG2 also did not exhibit the fluorescence change with Zn$^{2+}$ whereas exhibited slight response with Cu$^{2+}$. We investigated the reversibility, selectivity and resin recovery of these peptide probes. Confocal microscopy was also applied for studying the fluorescence change and reversibility of conjugate peptides with metal ions. In spite of the several reports of surface-conjugate fluorescent sensors for selective monitoring of metal ions, to the best our knowledge, this is the first example of the peptide probes’ metal selectivity being tuned by modulating the change in amino acid sequence and resin can be reused nondestructively for further sensing application.

5.2 Results and Discussion

5.2.1 Peptide Synthesis and Characterization

Peptide probes were synthesized by Fmoc-chemistry in solid phase peptide synthesis in Tentagel resin mixed with 2% mole ratio of Rink amide MBHA resin. The peptide PG1 and PG2 were further synthesized on MBHA resin mixed with 2% mole ratio of Rink amide MBHA resin to look at metal binding characteristics with different properties from Tentagel resin.
After cleavage of the crude product from resin, the peptides were characterized by HPLC with Waters C18 column and further analysed by ESI and MALDI-TOF mass. The HPLC retention time, and observed mass of each peptide were depicted in Table 5.1. The HPLC retention time and observed mass as shown in Table 5.1 indicated that the desired product obtained from both resin is same with >95% purity.

(Scheme 5.1) After cleavage of the crude product from resin, the peptides were characterized by HPLC with Waters C18 column and further analysed by ESI and MALDI-TOF mass. The HPLC retention time, and observed mass of each peptide were depicted in Table 5.1. The HPLC retention time and observed mass as shown in Table 5.1 indicated that the desired product obtained from both resin is same with >95% purity.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>HPLC ret. time (min)</th>
<th>Calcd. mass (M+H)+</th>
<th>Obs. mass (M+H)+</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG1-C</td>
<td>24.22</td>
<td>888.24</td>
<td>887.92</td>
</tr>
<tr>
<td>PG2-C</td>
<td>28.07</td>
<td>928.26</td>
<td>927.90</td>
</tr>
<tr>
<td>GG2-C</td>
<td>20.97</td>
<td>848.48</td>
<td>848.60</td>
</tr>
</tbody>
</table>

Table 5.1 HPLC and mass data of peptide resin conjugates.

5.2.2 Fluorescence Studies

The resin-immobilized peptides (50μM resin bound peptide) were taken in cuvette cell for fluorescence measurement using Luminescence spectrophotometer with continuous stirring. Before observing the fluorescence response with metal ions, the optimum swelling time was checked by swelling the resin in 10 mM Hepes buffer, pH 7.4. The fluorescence spectra was measured at different time intervals and found that 30 min swelling time is enough to diffuse the polystyrene bead and to access the analyte into the inner core of the bead. The optimum swelling time (30 min) was kept constant throughout the entire fluorescence measurement. This swelling time is in best agreement with the swelling time reported by-
Holcombe et.al.\textsuperscript{10(b)} for monitoring the metal binding affinity of the Tentagel resin beads.

To know the effect of solvent in the swelling property of Tentagel resin beads, several solvents were considered from non-polar to polar. The fluorescence of resin beads \textbf{Pg1-C} with Cu\textsuperscript{2+} in aqueous buffer solution exhibited the perfect quenching and resin beads were no longer able to show the fluorescence. This is due to the well-known water swelling property of Tentagel resin beads and the analyte can easily diffuse the resin beads in this condition.\textsuperscript{16} Thus, for further experiment, 10 mM Hepes buffer was considered for monitoring the fluorescence with each metal cation.

The evaluation of the resin immobilized peptides as fluorescence signaling for metal cations was achieved with perchlorate salts of individual metal ions via fluorescence spectrophotometer. As shown in \textbf{Figure 5.1}, the fluorescence change of peptide \textbf{PG1-C} exhibits the fluorescence enhancement with Zn\textsuperscript{2+} and fluorescence quenching with Cu\textsuperscript{2+} whereas, other tested metals have no fluorescence response. Similarly, \textbf{GG2-C} containing two Gly-Gly units exhibited the fluorescence quenching with Cu\textsuperscript{2+} and \textbf{PG2-C} containing two Pro-Gly units only shows the fluorescence enhancement with Zn\textsuperscript{2+} with negligible change for Cu\textsuperscript{2+} and Cd\textsuperscript{2+}.

Even though Cu\textsuperscript{2+} and Zn\textsuperscript{2+} have similar size and electronic configuration, the peptide probe exhibited great selectivity for Zn\textsuperscript{2+} over Cu\textsuperscript{2+}. Generally, the chemical or peptide sensors containing dansyl moiety and His (imidazole) residue were reported to show a fluorescence response with Cu\textsuperscript{2+} due to the high affinity of the imidazole moiety for Cu\textsuperscript{2+}.\textsuperscript{17,11 (d)} However, \textbf{PG2-C} even containing dansyl and imidazole moiety only shows the fluorescence response to Zn\textsuperscript{2+} with slight interference with Cu\textsuperscript{2+}. Interestingly, each peptide immobilized resin bead showed different
emission intensity at the same concentration of peptide probe with out metal ion. To investigate this, we observed the secondary structure of the soluble peptide and found that each peptide showed the different secondary structure as earlier discussed in chapter 4. Thus, we assumed that the difference in emission intensity and varying selectivity of each immobilized peptide towards Cu$^{2+}$ and Zn$^{2+}$ might be due to the different environment for dansyl group caused by different secondary structures of the peptide probes.

![Figure 5.1](image.png)

**Figure 5.1** Fluorescence response of PG1-C (50 μM), GG2-C (50 μM), and PG2-C (50 μM) in the presence of various metal ions (1eq.) in 10 mM HEPES buffer solution (pH=7.4; excited at 330 nm). The points were recorded from their maximum emission intensity with and without metal ions.

Then, the fluorescence titration was performed for each resin-immobilized peptide with different concentration of Cu$^{2+}$ and Zn$^{2+}$. The fluorescence titration of PG1-C with Cu$^{2+}$ (Fig 5.2) indicates that with the increasing concentration of Cu$^{2+}$, the fluorescence intensity of the resin bead decreases
dramatically and became saturated with 1 equivalent of Cu$^{2+}$ with respect to resin bound peptide concentration. The figure as shown in inset of Fig. 5.2 represents the fluorescence change with Cu$^{2+}$ versus time measured at 510 nm using 330 nm as excitation wavelength and indicates that the complete saturation of the resin conjugate peptide with 1 equivalent Cu$^{2+}$ occurs with in 20 min. This observation also supports that the optimum swelling time for Tentagel resin is below 30 min. The titration and time dependent curve both indicated that the initial fluorescence response with Cu$^{2+}$ occurs very fast. It is due to the binding ligands located towards the outer core of the resin bead and can be easily approachable to the Cu$^{2+}$ ions rather then the binding site located in the inner core of the beads. Most of the ligands (40~60%) in Tentagel resin are located towards outer core rather than the inner core of the bead.\textsuperscript{18}

![Graph showing fluorescence titration](image)

**Figure 5.2** Fluorescent titration of PG1-C (50 μM) upon addition of Cu$^{2+}$ (10, 20, 30, 40, 50 and 60 μM) excitation and emission slit were used as 10 and 10 nm respectively), Inset:
Fluorescence change of PG1 versus time (50 µM peptide with 1 equivalent of Cu\(^{2+}\)) in 10mM HEPES buffer, pH 7.4.

The fluorescence titration for PG1-C with Zn\(^{2+}\) and PG2-C with Zn\(^{2+}\) (Figure 5.3) were conducted and results indicated that both peptides with 50µM of Zn\(^{2+}\) completely saturate the fluorescence emission. 

![Figure 5.3](image)

**Figure 5.3** Fluorescent titration of (a) PG1-C (50 µM) upon addition of Zn\(^{2+}\) and (b) PG2-C (50 µM) upon addition of Zn\(^{2+}\) excited at 330 nm in 10mM HEPES buffer, pH 7.4

Similarly, resin conjugate GG2-C with Cu\(^{2+}\) (Fig. 5.4) exhibited the similar fluorescence response with Cu\(^{2+}\) as did PG1-C. Overall, either the fluorescence enhancement with Zn\(^{2+}\) or quenching with Cu\(^{2+}\) is due to the chelating effect of the ligand-binding site with metal ions around the resin bead. The fluorescence sensitivity and response time shown by the peptide resin conjugates with these metal ions were much faster than other surface modified sensors.\(^{19}\)
Figure 5.4 Fluorescent titration of GG2-C (50 μM) upon addition of Cu$^{2+}$ (10, 15, 20, 25, 30, 35, 40, 50 and 60 μM) excitation and emission slit were used as 6 and 6 nm respectively), Inset: Fluorescence change versus time (50 μM peptide with 1 equivalent of Cu$^{2+}$) in 10mM HEPES buffer, pH 7.4.

To investigate the function of PEG units of Tentagel resin-conjugate in the fluorescence change, same sequences were synthesized on MBHA resin and studied the efficiency of the sensor beads for monitoring these metal ions. Peptide PG1-MBHA and PG2-MBHA (scheme 5.1) did not show any kinds of fluorescence change with Cu$^{2+}$ and Zn$^{2+}$ in 10 mM Hepes buffer solution at pH 7.4. As a representative example for both peptides the fluorescence response of peptide PG1-MBHA is shown in Figure 5.5.

This indicated that, no change in fluorescence emission intensity is due to poor swelling property of MBHA resin in aqueous buffer system. Similarly, there is no linker like PEG units as in TG resin; which supports to make good transport between polystyrene bead and ligand binding site.
Since our design strategy is modular, by altering only the ligand site, one can design the sensors for the selective recognition of other metals. Thus the receptor units of all immobilized peptides conjugated with Tentagel resin were modified via on-bead inter-disulfide bond formation between cysteine residues. The successful dimerization between cysteine residues was confirmed by Ellman’s assay. The fluorescence response of each peptide containing inter-disulfide bridge was measured with all 13 metal ions, as in non-dimerized peptides (Figure 5.6).

The cysteine residue have critical role for monitoring of Zn\(^{2+}\); thus the ligand-binding site for monitoring with Zn\(^{2+}\) has been changed. Interestingly, none of the inter-disulfide bridge containing peptide showed the fluorescence response with Zn\(^{2+}\). However; PG1-C and GG2-C even after dimerization can monitor the Cu\(^{2+}\) with slightly lesser extent in emission intensity in the same condition to that of the free cysteine containing peptide.
Similarly, both dimerized peptides showed some fluorescence quenching with Co\(^{2+}\). The change in fluorescence with Co\(^{2+}\) ions for dimerized peptides might be due to the change in conformation of peptides. Generally, peptides containing Cys and His residues have binding preferences with Co\(^{2+}\) based on the position of amino acid and conformation of peptides.\(^{21}\) The inter-disulfide bond containing peptide PG2 also showed the minimal change in fluorescence with Cu\(^{2+}\) via quenching.

![Figure 5.6](image)

**Figure 5.6** Fluorescence response of inter-disulfide bridge containing peptide PG1, GG2 and PG2, 50\(\mu\)M each in the presence of various metal ions (1eq.) in 10 mM HEPES buffer solution (pH=7.4)

In order to further explore the utility of non-dimerized peptide resin conjugates as selective fluorescent bead sensor for Zn\(^{2+}\) and Cu\(^{2+}\), we investigated the fluorescence response of the GG2-C, PG1-C, and PG2-C in the presence of one equivalent of other metal ions because the transition metal ion concentration in the cell or environment are relatively low. As shown in Fig. 5.7, the addition of any other metal ion did not change the
emission intensity of the GG2-C except Cu$^{2+}$. The zinc-dependent emission intensity of the PG1-C was not affected by the presence of any other metal ions except Cu$^{2+}$ in Fig. 5.8. The Cu$^{2+}$ was reported to cause significant quenching for fluorescent chemical and peptide sensors with imidazole and dansyl moiety,\textsuperscript{17} interestingly; the zinc-dependent fluorescence of PG2-C was not affected by the presence of any other metal ions such as competitive transition metal ions including Cu$^{2+}$ in Fig. 5.9. From the selectivity data studied for all resin-immobilized peptides, it was concluded that PG2-C developed in this study had a great selectivity for Zn$^{2+}$. PG1-C can selectively monitor the Zn$^{2+}$ in presence of all the metal ions tested except Cu$^{2+}$, whereas GG2-C can selectively monitor only Cu$^{2+}$. The response time for selective monitoring of Cu$^{2+}$ and Zn$^{2+}$ exhibited by peptide GG2-C, PG1-C and PG2-C was very rapid in presence of several other metal ions.

![Graph showing emission intensity of GG2-C with Cu(II) and other metal ions](image)

**Figure 5.7** Emission intensity of GG2-C (50µM) in the presence Cu$^{2+}$ (50 µM) and additional various metal ions at pH 7.4 (10 mM HEPES buffer). All metal ions were evaluated at one equivalent to Cu$^{2+}$. The emission intensity was calculated based on the maximum emission intensity.
Figure 5.8 Emission intensity of PG1-C (50 µM) in the presence Zn$^{2+}$ (50 µM) and additional various metal ions at pH 7.4 (10 mM HEPES buffer). All metal ions were evaluated at one equivalent to Zn$^{2+}$. The emission intensity was calculated based on the maximum emission intensity.

Figure 5.9 Emission intensity of PG2-C (50 µM) in the presence Zn$^{2+}$ (50 µM) and additional various metal ions at pH 7.4 (10 mM HEPES buffer). All metal ions were evaluated at one equivalent to Zn$^{2+}$. The emission intensity was calculated based on the maximum emission intensity.
The fluorescent selectivity of PG1-C resin beads for Cu\textsuperscript{2+} and Zn\textsuperscript{2+} is also visually evidenced by naked eye monitoring using hand held UV-lamp excited at 365 nm. The resin beads in aqueous buffer solution exhibited the green color. However, in the presence of Cu\textsuperscript{2+} the color of the resin beads disappeared, indicating that Cu\textsuperscript{2+} quenched the fluorescence whereas with Zn\textsuperscript{2+}, the fluorescence of resin beads became strongly fluorescent. Other metal ions did not show any kinds of color changes with the resin beads and the image is essentially the same as that of the control beads suggesting that other metal ions have no effect in the fluorescence of resin beads. The images acquired after treatment of various metal ions for peptide PG1-C is shown in Figure 5.10. Similarly, peptide GG2-C and PG2-C only shows the fluorescence change with Cu\textsuperscript{2+} and Zn\textsuperscript{2+}, respectively.

**Figure 5.10** Fluorescence response of PG1-C (300μg/ml) in the presence of various metal ions at pH 8.4 (10 mM HEPES buffer). The numbers indicated as 1= PG1, 2= Zn\textsuperscript{2+}, 3 = Ca\textsuperscript{2+}, 4 = Mn\textsuperscript{2+}, 5 = Ag\textsuperscript{+}, 6 = Ni\textsuperscript{2+}, 7 = Cu\textsuperscript{2+}, 8 = Co\textsuperscript{2+}, 9 = Mg\textsuperscript{2+}, 10 = Al\textsuperscript{3+} All metal ion concentration was 100 μM. Fluorescence excitation was provided with a handheld UV lamp (ENF 260) set on long wavelength (365 nm). The image was acquired after 30 min of incubation.

### 5.2.3 Regeneration of the Fluorescence in Resin Beads

The fluorescence response of the immobilized peptide to Cu\textsuperscript{2+} and Zn\textsuperscript{2+} is fully reversible, and can be regenerated by treatment with EDTA solution. The fluorescence of peptide-Cu\textsuperscript{2+} complex or Peptide-Zn\textsuperscript{2+} complex with
mM concentration of EDTA demonstrated the reversible phenomena of the resin beads. As a representative example, the peptide PG1-C-Cu$^{2+}$ and PG1-C-Zn$^{2+}$ complex and their reversibility with EDTA are shown in Fig. 5.11.

![Fluorescence emission spectra of PG1-C in the presence of (a) Cu$^{2+}$ and EDTA and (b) Zn$^{2+}$ and EDTA. The emission spectra ($\lambda_{ex}=330$ nm) were measured in 10 mM HEPES buffer (pH 7.4).](image)

**Figure 5.11** Fluorescence emission spectra of PG1-C in the presence of (a) Cu$^{2+}$ and EDTA and (b) Zn$^{2+}$ and EDTA. The emission spectra ($\lambda_{ex}=330$ nm) were measured in 10 mM HEPES buffer (pH 7.4).

Similarly, we studied the reversibility of the GG2-C-Cu$^{2+}$ and PG2-C-Zn$^{2+}$ complex and indicated similar trend to that of the peptide PG1-C. Previously, we studied the reversibility of the peptide-metal complexes in soluble peptides and the required concentration of EDTA is 1-10 equivalent to regain the fluorescence.

We expect that the higher concentration of EDTA, which was required to fully regain the fluorescence of resin-conjugate peptide, might be due to the higher binding affinity of the immobilized peptides with metal ions. Usually, the surface immobilized sensor, film sensor or nanoparticle conjugated sensor showed the higher binding affinity as compare to their soluble counterpart.

Additionally we confirmed the non-specific binding affinity of the PEG units with EDTA. As explained in experimental section, soluble peptide PG1-C was mixed with 0.50 mg of Tentagel resin beads without any immobilized
peptides in 10 mM HEPES buffer, pH 7.4. The fluorescence enhancement of soluble peptide with 1 equivalent of Zn$^{2+}$ became saturated, which is fully recovered by adding 1 equivalent EDTA (Fig. 5.12).

![Graph showing fluorescence response of PG1 with Zn$^{2+}$ and EDTA](image)

**Figure 5.12** Fluorescence response of PG1 (10μM, soluble peptide mixed with 0.50 mg of Tentagel resin) with Zn$^{2+}$ and EDTA. Inset: Fluorescence response of PG1 (10μM, soluble peptide mixed with 0.50 mg of Tentagel resin) with Zn$^{2+}$ and EDTA as a function of time. The PG1-Zn$^{2+}$ complex was recovered with 1 equivalent (10μM) EDTA.

This also implies that PEG units have no non-specific binding with EDTA and the need of higher concentration of EDTA to regain the fluorescence of peptide-metal complex in its original form is due to tight binding affinity of conjugated peptides with metal ions. Due to the lack of proper techniques and enough references in this field for calculating the binding affinity of such kinds of resin immobilized sensors, the exact binding affinity with metal ions is not quite clear at this moment and will be reported in due course.
5.2.4 Resin Recycle Study
Reversibility and reusable approach has wide application in terms of fluorescent chemosensor. To test whether the immobilized resin beads can act as reusable sensor or not, the resin recycling studies was performed applying the method as discussed in experimental section. As a representative example, first we optimized the method for peptide PG1-C. The peptide PG1-C exhibited the fluorescence with out any loss even after three consecutive cycles. (Fig. 5.13)

After, optimizing the method for peptide PG1-C, we studied the recycling study for peptide GG2-C using the same method. The peptide GG2-C with Cu$^{2+}$ could also be fully regenerated like PG1. (Fig 5.14).

![Graph showing reversibility of Cu$^{2+}$ binding to PG1-C resin conjugates upon addition of EDTA with excitation at 330 nm. Inset: emission intensity versus cycle number showing the decrease and restoration of fluorescence upon addition of 1 equiv Cu$^{2+}$ and 2 mM EDTA respectively, over the course of three cycles.](image)

Figure 5.13 Reversibility of Cu$^{2+}$ binding to PG1-C resin conjugates upon addition of EDTA with excitation at 330 nm. Inset: emission intensity versus cycle number showing the decrease and restoration of fluorescence upon addition of 1 equiv Cu$^{2+}$ and 2 mM EDTA respectively, over the course of three cycles.
Figure 5.14 Reversibility of Cu$^{2+}$ binding to GG2-C resin conjugates upon addition of EDTA with excitation at 330 nm. Inset: emission intensity versus cycle number showing the decrease and restoration of fluorescence upon addition of 1 equiv Cu$^{2+}$ and 2 mM EDTA respectively, over the course of three cycles.

From these results, it was found that the fluorescence response of the resin conjugate peptide could be easily restored by treatment with EDTA in very short time. Usually, film sensors and SAM sensors\textsuperscript{19} also exhibited the reversibility after treatment with aqueous EDTA solution or treatment with acid, however the response time was very long (72 hours). On the other hand, handling of film sensors during utilization is also challenging task as compare to resin beads. Compared with soluble fluorescent chemosensors, the reversibility and reusability of the resin-conjugate peptide opens up the possibility for making reversible sensor with proper surface modification for specific metal ions based on this approach.
5.2.5 Fluorescence Quenching and Regeneration Study via Confocal Microscopy

Confocal microscopy has been used as useful technique to investigate the functional group location within a bead and their access to analyte concentration.\textsuperscript{18, 23} Thus, we considered the monitoring of resin bead with metal ions and its reversibility with EDTA. As representative example, we monitored the fluorescence change of peptide PG1-C with Cu\textsuperscript{2+} and its reversibility with EDTA using 405 nm as excitation wavelength. The swelling time and concentration of Cu\textsuperscript{2+} and EDTA was same as used in fluorescence monitoring. First we analyzed the dispersion of fluorescence throughout the resin bead using optical slicing applying the technique called Z-stack. The consistent dispersion of fluorescence throughout the slices of resin beads suggests that the peptide is consistently localized in all parts of the resin bead. (Fig 5.15, A)

Later, the resin beads were treated with Cu\textsuperscript{2+} and images were obtained. It was found that the fluorescence of PG1-C conjugate was completely quenched with Cu\textsuperscript{2+} (Fig. B). Optical slicing of the resin beads treated with Cu\textsuperscript{2+} was performed and indicated that the Cu\textsuperscript{2+} ions diffused the polystyrene beads and fully quenched the fluorescence. The Peptide-Cu\textsuperscript{2+} complex beads were further treated with EDTA and fluorescence regeneration was studied at different time intervals. (Fig. C–F). The resin beads becomes slightly fluorescent after treating them with EDTA and then intensity increases in time dependent manner and fully recovered after 30 min. This result supports that the Cu\textsuperscript{2+} and EDTA can easily diffuse the resin bead and exhibited the fluorescence change. The optical analysis results obtained by confocal microscopy are consistent with the results observed by fluorescence spectrophotometer.
Several research groups including us have synthesized various fluorescent probes for monitoring $\text{Zn}^{2+}$ and $\text{Cu}^{2+}$ because these metals play an important role in many biological and environmental processes. Thus, optimized chemical probes are required to monitor these metal ions. Even though several fluorescence-based chemical and peptide probes for these metal ions have been reported in solution phase however reversible monitoring is rare in solution phase and difficult to reuse. The surface oriented sensors for monitoring $\text{Cu}^{2+}$ ions were developed and most of them were possess synthetic challenge, slow response with analyte, interference with other metals and tuning selectivity is beyond the way.
Thus, the development of selective peptide probes with synthetic feasibility, fast response, high selectivity and tunable nature for these metal ions remains a significant challenge. The peptide resin conjugate developed in this study exhibited several advantages like easy synthesis, high selectivity without interference of other metals, easily tuned the selectivity, fast response, reversibility and reusability. Thus, we expect that the resin conjugate peptides studied here have great possibility to be used for monitoring extracellular and environmental concentration of $Zn^{2+}$ and $Cu^{2+}$ and can be reused and stored for future analysis.

### 5.3 Conclusion

In this study, we developed the fluorescent resin conjugate peptides for the selective monitoring of $Cu^{2+}$ and $Zn^{2+}$ in aqueous solution. The slight alteration in the amino acid sequence has found a significant change for the selective monitoring of these metal ions. The peptide resin conjugates exhibited a fluorescence emission, which is selectively enhanced by $Zn^{2+}$ in **PG2-C** and selectively quenched by $Cu^{2+}$ in **GG2-C**. Similarly, **PG1-C** can
selectively enhance the fluorescence with Zn\textsuperscript{2+} except in presence of Cu\textsuperscript{2+}. The fluorescence response of inter-disulfide bridge containing peptides has confirmed that selectivity can be tuned easily even in resin bound peptides. The fluorescence of peptide-metal complex from resin-conjugate can be regenerated with EDTA solution and reused even after using three consecutive cycles.

5.4 Material and Methods

5.4.1 on-bead Disulfide Bond Formation

The peptides PG2-C, GG2-C and PG1-C immobilized on Tentagel resin were dimerized through inter-disulfide bond formation between cysteine residues to monitor their sensing ability for metal ions. To accomplish the inter-disulfide bond formation between cysteine residues, 150 mM oxidised DTT was added to the resin beads (50 mg/mL) for 12 hours. The resin was subsequently washed with DMF and methanol repeatedly and dried under high vacuum. The completion of disulfide bond formation was confirmed by using Ellman’s assay on the basis of absorbance at 412 nm\textsuperscript{15}.

5.4.2 Fluorescence Measurements

Fluorescence emission spectrum of a peptide probe in a 10 mm path length quartz cuvette was measured in 10 mM HEPES buffer solution (pH =7.4) using a Perkin Elmer luminescence spectrophotometer LS 55 model. Sample concentration of peptide probe was calculated by loading yield of last amino acid as compare to the first step loading yield using Fmoc quantitation by UV-vis absorbance at 301 nm for Fmoc-piperidine adduct. Fluorescence spectra ranging from 350 to 650 nm was measured in the presence and absence of metal ions by excitation with 330 nm with continuous stirring.
The resin was treated with DTT (200 mmol) for 6 hour to ensure the reduction of disulfide groups and washed with DMF/ methanol thrice and dried under high vacuum. The resin was pre-swollen for about 30 min before taking the fluorescence spectra.

5.4.3 Non-specific Binding Study of PEG units with EDTA
The non-specific binding of EDTA with PEG units of the Tentagel resin was confirmed by mixing soluble peptide with Tentagel resin beads and the fluorescence was monitored with Zn$^{2+}$ and EDTA. The fluorescence was recovered with 1 equivalent of EDTA in the similar way as we reported earlier.\textsuperscript{12k}

5.4.4 Resin Recycle Study
Each resin-conjugate peptide containing 50 $\mu$M of bound peptide was taken in cuvette cell and swollen for 30 min and the fluorescence was measured. To the cell 1 eq. of Cu$^{2+}$ was added and further incubated for 30 min. The fluorescence was checked and complete quenching occurs with Cu$^{2+}$ ions. Subsequently, 2 mM EDTA was added to the cuvette cell, incubated for 30 min and monitored the fluorescence until it was fully regained. The EDTA-Cu$^{2+}$ complex from resin was washed with buffer (3 times) to make it free from metal ions and EDTA. The first cycle was completed and recovered resin was further used. Three consecutive cycles were repeated using the same method as in first cycle and found that there is no loss of fluorescence intensity of resin beads. As a representative example, resin recycle study was performed for \textbf{PG1} with Cu$^{2+}$ ions. After optimization the method for peptide \textbf{PG1, GG2} resin beads were recycled applying the same method as
we did in PG1. The peptide-Zn\(^{2+}\) complex also exhibited the similar reversible phenomena like peptide-Cu\(^{2+}\) complex.

### 5.4.5 Fluorescence Quenching and Regeneration Study via Confocal Microscopy

The confocal microscopy (Carl Zeiss) was used to monitor the fluorescence quenching and regeneration for peptide-resin conjugates with Cu\(^{2+}\) and EDTA. Based on the information accumulated from fluorescence experiment, the pre-swollen resin for 30 min with and without Cu\(^{2+}\) was detected through confocal microscopy and images were acquired. The swelled Tentagel resin beads were placed on a microscope slide with a cover slip and analyzed under a confocal microscope. Later, the reversibility with EDTA was checked at different time intervals until the fluorescence was fully regained and fluorescence images were acquired.

### 5.4.6 Fluorescence of Resin Conjugate Peptides with UV–lamp Excitation at 365 nm

Fluorescence response of peptide-resin conjugate (300 µg/mL) in the presence of various metal ions at pH 8.4 (10 mM HEPES buffer) was monitored using 100µM metal ion concentration except Na\(^{+}\), K\(^{+}\), Ca\(^{2+}\), and Mg\(^{2+}\), which were used at 5 mM. The reversible monitoring of peptide resin beads was accomplished with 2 mM EDTA. Fluorescence excitation was provided with a handheld UV lamp (ENF 260) set on long wavelength (365 nm) and the images were acquired after 30 min of incubation at room temperature. As a representative example, the PG1 peptide was considered
which showed the fluorescence change with Cu\(^{2+}\) and Zn\(^{2+}\) among other metal ions tested.

5.5 Literature Cited


2003, 100, 3617.


CHAPTER VI

Part 1

6-1. A Highly Selective and Reversible Peptide Sensor for Monitoring Ag⁺ ions in Physiological Buffer

6-1.1 Introduction

Recently, fluorescence sensors have created insurgency for the process of measuring metal cations with biological and environmental interests such as Na⁺, Ca²⁺, Cu²⁺, Zn²⁺, Ag⁺, Pb²⁺ and Hg²⁺. The development of fluorescent molecular sensors, which can detect these metal ions in aqueous media and shows reversibility have always been of particular interest.¹ Considerable efforts have been devoted to the elaboration of fluorescence sensors for other heavy and transition metal ions² in the last two decades, there are only a rare reports on Ag⁺ ion sensing in aqueous solution monitored by emission enhancement.³ Because of the enormous demand of silver compounds in various commercial sectors and their increased industrial sludge, severe contamination of the environment by Ag⁺ is increasing.⁴ Similarly, the interaction of Ag⁺ ions with essential nutrients has further elevated the consciousness of its potential toxicity.⁴ Regardless of the few sensors that work in organic or mixed organic solutions for Ag⁺, devoted fluorescence sensors for Ag⁺ are still required that show emission enhancement with concomitant shift and selective in presence of other frequently found metal ions.

In this regard, we consider the small peptide motifs containing Cys and His having enough solubility in aqueous buffer system as shown in scheme 6-1.1. For the ease of monitoring via fluorescence the N-terminus was labelled with dansyl chloride. Considering the previous silver binding motif,³ we assumed that soft ions like sulphur and nitrogen interacts well with silver ion. To get
the more pre-organized structure Pro-Gly sequence was appended. Cys residue facilitates the formation of inter-disulfide bond and provides more ligand for metal binding. We assumed that inter-disulfide bond formation may bring two His residue closer to provide the effective binding site for Ag⁺. To investigate the role of Pro-Gly sequence another peptide named as P8-dimer containing Gly-Gly was synthesized. To get insight the individual role of His residue in Ag⁺ binding P17- dimer was synthesized which do not contain His residue. Previously, individual research groups have reported Ag⁺ binding chemosensors; most of them suffered limitations like (1) poor solubility in aqueous buffer system and only worked in mixed organic solvents (2) irreversibility and (3) possess synthetic challenge for tuning selectivity. To overcome with these demerits, the ideal sensor is still required. In addition, sensors which provide large spectral shifts either in absorption or emission spectra would be highly desirable, because they allow the ratiometric monitoring of the analyte. However, ratiometric fluorescent probes useful for selective detection of silver ions in aqueous solution were not reported. To the best of our knowledge, small peptide motifs which bind specifically to silver are currently unknown and the presented sensory device in this chapter is the first example which can selectively recognize silver ions and acts as a redox switch.

6-1.2 Results and Discussion
The peptides were synthesized by using Fmoc-SPPS method. All dimer peptides were obtained from their linear counter part via inter-disulfide bond formation between Cys residue using oxidized DTT. The successful synthesis and the homogeneity (>95%) of the probes was confirmed by
analytical HPLC and ESI mass spectrometry. The HPLC retention time and observed mass are depicted in Table 6-1.1.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence</th>
<th>Ret. Time (min.)</th>
<th>Calcd./Obs. Mass (M+H⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp1-i</td>
<td>(Dan-CPGH-NH₂)₂</td>
<td>31.28</td>
<td>1286.39/1287.47</td>
</tr>
<tr>
<td>P8-dimer</td>
<td>(Dan-CGGH-NH₂)₂</td>
<td>44.25</td>
<td>1206.38/1207.33</td>
</tr>
<tr>
<td>P17-dimer</td>
<td>(Dan-CGGG-NH₂)₂</td>
<td>43.89</td>
<td>1046.3/1047.28</td>
</tr>
</tbody>
</table>

Table 6-1.1 HPLC and mass data of the peptides

All synthesized peptides have good solubility in aqueous buffer system; thus we investigated the fluorescence change in physiological buffer solution without any co-solvent. To investigate the binding properties of peptides toward metal ions, the fluorescence spectrum changes were investigated upon addition of various metal ions as perchlorate and chloride salts (Ca²⁺, Cd²⁺, Co²⁺, Pb²⁺, Cu²⁺, Ag⁺, Mg²⁺, Mn²⁺, Ni²⁺, Hg²⁺ and Zn²⁺ as perchlorate anion and Na⁺, Al³⁺, K⁺, as chloride anion). Figure 6-1.1 shows the fluorescent emission spectrum of Cp1-i (10 µM) in HEPES buffer solution (10 mM, pH 7.4) containing each metal ion (1 eq. except biologically important metal ions such as Mg²⁺, Ca²⁺, Na⁺ and K⁺ which were used in 5 mM). Sensor Cp1-i did not have fluorescence response with all test metal ions except Ag⁺, Hg²⁺ and Cu²⁺; the emission intensity increased in the presence of Ag⁺, whereas fluorescence intensity vanished in the presence of Cu²⁺ and Hg²⁺.
Similarly, peptide P8-dimer did not exhibit any significant change with Ag⁺ as compare to Cp1-i but showed fluorescence quenching with Cu²⁺. Peptide P17-dimer which does not contain His residue did not exhibit any kinds of fluorescence response with Ag⁺. (Figure 6-1.2) This result indicated that His residue played an important role for Ag⁺ binding.

The binding properties of Cp1-i with silver ions were investigated by carrying out the fluorescence titrations. (Figure 6-1.3). A gradual emission enhancement with Ag⁺ (~4 fold) was observed by a concomitant shift in emission intensity from 545 to 513 nm. This blue shift probably resulted from the dansyl fluorophore moving to a less polar environment upon metal binding. Furthermore; the ability of sensing for sensor Cp1-i with Ag⁺ was investigated by UV-vis spectroscopy study in aqueous media. (Figure 6-1.4) The UV-vis spectra indicated that when silver ion binds to Cp1-i the shift in wavelength from 333 to 325 nm was observed with increase in absorbance. This result is in well agreement with the fluorescence titration as shown in Figure 6-1.3, where silver ions shows enhancement with significant shift. Individually, the fluorescence titration experiments were performed for Cp1-i with Cu²⁺ and Hg²⁺ as these metal ions also illustrated the fluorescence response. (Figure 6-1.5)
Furthermore, to investigate the binding stoichiometry, Job’s Plot analysis and mass spectra were conducted. (Figure 6-1.6) The mole fraction at 0.5 in Job’s plot indicates the 1:1 binding between Ag$^+$ and Cp1-i. Similarly, sharp and single mass peaks at m/z 1287.86 and 1393.86 corresponding to free one and Cp1-i-Ag$^+$ respectively are clearly observed, suggesting the solid evidence for predominant 1:1 complex. (Figure 6-1.7) Thus, assuming 1:1 complex formation between host and guest we calculated the dissociation constant using non-linear least square equation. The dissociation constant ($5.7 \times 10^{-7}$ M$^{-1}$) indicated that the probe Cp1-i can be a potential candidate to detect Ag$^+$ in the micromolar concentration to sub nanomolar concentration.

Figure 6-1.1 Fluorescence response of Cp1-i (10 µM) in the presence of various metal ions (1eq.) except biologically important metal ions Mg$^{2+}$, Ca$^{2+}$, Na$^+$ and K$^+$ which were used 5 mM in 10 mM HEPES buffer solution (pH=7.4) (excited at 330 nm, excitation slit size 7.0 and emission slit 7.0 nm)
Figure 6-1.2 Fluorescence response of peptide P8-dimer and P17-dimer (10µM each) with 1 equivalent of metal ions in 10 mM Hepes buffer, pH 7.4.

Figure 6-1.3 Fluorescence titration of Cp1-i (2.5µM) with 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0µM of Ag⁺ excited at 330 nm, inset: Sigmoidal fit allows the determination of EC50 with a value of 1.3 µM.
Figure 6-1.4 Uv-Vis spectra of probe cp1-i (130 µM) upon addition of Ag⁺ in 10mM HEPES buffer (pH 7.4).

Figure 6-1.5 Fluorescence titration of Cp1-i (10µM) with (a) Cu²⁺ and (b) Hg²⁺. The concentration of Cu²⁺ and Hg²⁺ varying from 0-10 µM with 1 µM interval
Figure 6-1.6 Determination of binding stoichiometry using Job's Plot analysis; the total concentration of host and guest = 2.5µM

Figure 6-1.7 MALDI-TOF mass spectra of Cp1-i:Ag⁺ complex. The peak corresponding to m/z at 1288.0 and 1393.9 refers to the free and 1:1 Cp1-i:Ag complex.

The property of reversible sensing exhibited by sensors is of utmost importance. To test the reversibility of **Cp1-i-Ag⁺ complex**, the titrations
with EDTA was conducted as shown in Figure 6-1.8. About 1.5 mM of EDTA was required to get the metal free spectrum. The time course plot indicated that the response time of sensor with Ag⁺ and Ag⁺ complex with EDTA was rapid and exhibited 100% sensitivity with in 5 second.

![Graph showing reversibility of Cp1-i: Ag⁺ complex (2.5:5µM) with EDTA.](image)

**Figure 6-1.8** Reversibility of Cp1-i: Ag⁺ complex (2.5:5µM) with EDTA. The concentration of EDTA varies from 0.125mM to 2 mM. Inset: Time course of the peptide-silver complex and reversibility with EDTA.

The fluorescence response of Cp1-i with Ag⁺ in the presence of each metal ion was investigated. (Figure 6-1.9) Biologically important metal ions Ca²⁺, Mg²⁺, Na⁺ and K⁺ which exist in high concentrations in cells, did not show any spectral change even at 5mM concentration and 1 equivalent of other transition metals including Mn²⁺, Pb²⁺, Cd²⁺, Co²⁺, Zn²⁺, Ni²⁺ did not interfere silver binding. The silver fluorescence for Cp1-i is quenched only with Cu²⁺ and Hg²⁺ which are known to be effective fluorescence quenchers.⁷
Interestingly, the silver dependent fluorescence can be achieved with Hg\(^{2+}\) and Cu\(^{2+}\) in slightly lesser extent to that of free form even though these metal ions showed quenching. This clearly indicates that the titled sensor Cp1-i has great selectivity for Ag\(^{+}\) ion and silver dependent emission was not interfered with any tested metal ions. The fluorescence reappearance of probe-Cu\(^{2+}\) complex with Ag\(^{+}\) is quite interesting and it was further confirmed by MALDI-TOF mass spectroscopy. (Figure 6-1.10)

![Figure 6-1.9](image_url)  
**Figure 6-1.9** Fluorescence response of Cp1-i (10 µM) in the presence of Ag\(^{+}\) (20 µM) and/or various metal ions at pH 7.4 (10 mM HEPES buffer). All metal ions were evaluated at one equivalent to silver ion except Na\(^{+}\), K\(^{+}\), Ca\(^{2+}\), and Mg\(^{2+}\), which were used at 5 mM.

The addition of 2 equivalent Cu\(^{2+}\) to the probe provides exclusively the free and Cu\(^{2+}\)-probe complex at m/z 1288.03 and 1349.92 respectively. Addition of 2 equivalents Ag\(^{+}\) to the probe-Cu\(^{2+}\) complex regenerated new peak at 1393.68 which is corresponding to probe-Ag\(^{+}\) complex. This result is in best agreement with the selectivity data depicted in Figure 6-1.9.
Figure 6-1.10 Mass spectra of Cp1-i with 2 eq. Cu²⁺ and Cp1-i: Cu²⁺ complex with 2 eq. Ag⁺

The fluorescence titration was conducted to investigate the interference of these metal ions for the selective recognition of silver in presence of 1 equivalent of Hg²⁺ and Cu²⁺. The silver ion exhibited the turn-on fluorescence (~450 fold in presence of Cu²⁺, and ~5 times in presence of Hg²⁺) with concomitant shift in emission intensity (Figure 6-1.11). This result can be rationalized by the displacement approach where Hg²⁺ and Cu²⁺ were replaced by Ag⁺ and occurred enhancement in fluorescence. As Hg²⁺ and Cu²⁺ are well known environmental pollutant and monitoring silver in presence of these metal ions is still challenging job. The large change in emission intensity of probe Cp1-i with Ag⁺ in presence of either Cu²⁺ or Hg²⁺ would make it more advantageous for measuring silver ion concentration. Further to get insight the role of individual ligand in Ag⁺ binding ¹H NMR titration was conducted for peptide Cp1-i and P8-dimer in dmso-d6. As shown in Figure 6-1.12, the sharp singlet peak at δ 8.9 and δ7.2
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Similarly, the peak around $\delta$ 7.3 to 7.4 can be attributed to sulfonamide proton.$^{10}$ When Ag$^+$ binds with probe Cp1-i imidazolium C-H proton displayed a large up-field shift ($\delta$ 8.9-8.6) and peak becomes broader in the spectrum of Cp1-i. Similarly, the sulfonamide protons at $\delta$7.3 were disappeared and other aromatic protons were significantly shifted towards up-field. Interestingly, the imidazolium C-H protons were not much influenced with Ag$^+$ in peptide P8-dimer. This excludes the possibility of interaction of Ag$^+$ ion with imidazole group of His residue. The proton shift of imidazole and sulfonamide group is similar with previously reported sensor where similar binding ligands were used for monitoring different target molecules.$^9$-$^{10}$ The similar binding mechanism of Ag$^+$ with His residue was previously suggested by Verma et.al.$^{11}$

Recently, the remarkable progress has been accomplished towards the synthesis of various fluorescent chemosensors for monitoring Ag$^+$ ion.$^3$ The poor solubility in aqueous buffer system, irreversibility of the probes and interference by competitive metal ions may impede them from their proper application. In several respects, the probe Cp1-i developed by us has many advantages like good solubility, turn-on fluorescence with large emission shift, easy synthesis, highly selective even in presence of other heavy and transition metal ions, tunable nature and can be simply conjugated with resin or other devices.

6-1.3 Conclusion

In conclusion, we designed and synthesized a dual labelled fluorophore based on peptide motif which was able to selective monitor of Ag$^+$ with reversible phenomena. The large emission shift would make it possible for
ratiometric analysis of free silver ions. To the best of our knowledge, this is the first example of peptide based silver sensor which works in aqueous solution.

Figure 6-1.11 Fluorescence titration of (a) Cpl1-i:Cu$^{2+}$ complex with Ag$^+$ and (b) Cpl1-i: Hg$^{2+}$ complex with Ag$^+$. The concentration of Cpl1-i, Cu$^{2+}$ and Hg$^{2+}$, each were used 10 µM and Ag$^+$ concentration varies from 1-12µM

Figure 6-1.12 Partial $^1$H NMR spectra (400 MHZ) of peptides (6.0 mM) with 2 equivalents of Ag$^+$; (a) Apopeptide P8-dimer, (b) with Ag$^+$, (c) apopeptide Cpl1-i and (d) with Ag$^+$.
6-1.4 Literature Cited


CHAPTER 6-2
6-2. A Highly Sensitive and Selective Ratiometric Fluorescent Sensor for Monitoring Hg$^{2+}$ in Aqueous Solution
6-2.1 Introduction
Among heavy and transition metal ions, mercury is considered as a highly toxic element, and its contamination is prevalent which occurs from various sources such as ocean and volcanic emission, gold mining, combustion of fossil fuels and solid waste incineration.$^{1}$ Due to these potential problems caused by mercury contamination, much attention has been devoted in the past decades to the development of new fluorescent chemosensors for the detection of mercury.$^{2}$ Thus, designing fluorescent sensors for detection of mercury has drawn attention of the scientific community.$^{2}$ Recently, various fluorescent chemosensors based on the calix[4]arene scaffold, rhodamine derivatives, peptide scaffold and other chemical modification has been synthesized for the detection of various metal ions including mercury.$^{2,3,4}$ Most of the known fluorescent chemical sensors monitored these cations by fluorescence quenching mechanism via enhanced spin-orbital coupling (e.g. Hg$^{2+}$) or energy or electron transfer, suffered limitations due to poor solubility in aqueous solution and interfere by other competitive metal ions. Thus, the sensors that exhibit turn-on response for selective detection of mercury in aqueous solution are highly desirable. In addition, ratiometric sensors for monitoring mercury ions are of current topical interest because they make it possible to measure the analytes more accurately with minimization of background signal.$^{5}$ However, ratiometric fluorescent probes useful for selective detection of mercury ions in aqueous solution were rarely reported.$^{6}$
Amino acids and peptides are known to bind metal ions and in nature, metal binding is achieved with a high degree of selectivity using peptide motifs rather than macrocyclic ligands. Our research involves the design, synthesis, and evaluation of peptide-based receptors selective for heavy and transition metal ions designed after various active parts of the metal binding domains. Very recently, we reported a selective fluorescent sensor for copper and zinc based on zinc binding amino acid sequences. It is well-known in coordination chemistry that the complexation of mercury with a ligand containing nitrogen, sulfur and sulfide is favored. Considering these ligands, herein, we wish to report a selective fluorescence sensor (Dcys) for \( \text{Hg}^{2+} \) containing two dansyl groups obtained via simple conjugation of Cys residue with fluorophore. In the presence of \( \text{Hg}^{2+} \) ions, the receptor Dcys undergoes ratiometric fluorescence enhancement at 505 nm with significant shift and single isosbestic point at 553 nm. To the best of our knowledge, this is the first report where a conjugation of single amino acid with two dansyl moieties has been used as a receptor for monitoring of \( \text{Hg}^{2+} \) via turn-on ratiometric and reversible signaling in physiological pH.

6-2.2 Results and Discussion

The probe Dcys was obtained via simple coupling of Cys residue to fluorophore as shown in scheme 6-2.1. The merits of multichromophoric sensors have already been mentioned in terms of sensitivity of detection and improved photochemical stability, in particular for sensors based on dansyl dendrimers. As shown in scheme 6-2.1, the probe Dcys, consisting of single amino acid was synthesized in SPPS using Fmoc-chemistry. After cleavage of the compound Mcys from the solid support the inter-disulfide bond was made to
provide the binding site for the detection of mercury. The product was purified by semi-prep HPLC with a Vydac C-18 column using a water (0.1% TFA)-acetonitrile (0.1% TFA) gradient (5-40% acetonitrile for 35 min). The successful synthesis and the homogeneity (>95%) of the peptide was confirmed by HPLC and ESI mass (Calculated/observed mass (M+H)$^+$ = 704.16/705.11) spectrometry.

Scheme 6-2.1 Scheme for the synthesis of Dcys

To obtain insight into the binding properties of Dcys toward metal ions, the fluorescent spectrum changes were investigated upon addition of various
metal perchlorate and chloride salts (Ag\(^+\), Ca\(^{2+}\), Cd\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), Na\(^+\), Cu\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\), Mg\(^{2+}\), Hg\(^{2+}\), Pb\(^{2+}\), K\(^+\) and Al\(^{3+}\)) to Hepes buffer solution of Dcys.

The experimental results suggest that Dcys shows an outstanding selectivity to Hg\(^{2+}\) ion with turn-on response and concomitant shift ~ 33 nm in emission intensity. As depicted in Fig. 6-2.1, Dcys shows scarcely any response with other metal ions including Pb\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\) and Cu\(^{2+}\). The highly selective recognition of Hg\(^{2+}\) with Dcys can be attributed via its binding affinity with sulfide group and sulfonamide group. Usually, when sulfonamide group participates directly in binding event, it shows signaling via large shift in emission intensity.\(^{11}\)

\[\text{Figure 6-2.1} \text{ Fluorescence response of Dcys (10 µM), in the presence of various metal ions (1eq.) except Mg\(^{2+}\), Ca\(^{2+}\), Na\(^+\) and K\(^+\) which were used 5 mM in 10 mM HEPES buffer solution (pH=7.4) (excited at 330 nm, excitation slit size 10.0 and emission slit 10.0 nm)\]
The UV-vis absorbance data with Hg\textsuperscript{2+} also supports the role of sulfonamide group in metal binding event. (Figure 6-2.2) The change in absorbance at 330 nm with three isosbestic points at 350, 301 and 264 nm indicated the direct interaction of fluorophore with Hg\textsuperscript{2+} and change in local environment of fluorophore which causes the significant shift.

Addition of Hg\textsuperscript{2+} to Dcys causes a 33 nm blue shift of the fluorescence spectra and \~3.0-fold enhancement of the emission intensity with 1 equivalent to that of probe concentration (Figure 6-2.3). When increasing concentration of Hg\textsuperscript{2+} was added, a significant increase of the emission intensity around 505 nm and decrease at 565 nm were observed with an isosbestic point at 553 nm. The blue shift is rationalized by the deprotonation of the sulfonamide group upon mercury binding, which is in best agreement with the previously reported dansyl containing probe.\textsuperscript{12} Overall; results indicated that the observed shift of the emission spectra with
an isosbestic point at 553 nm allows us a ratiometric measurement for mercury. The ratiometric calibration curve was shown in the inset of Figure 6-2.3 with Hg$^{2+}$, provides the EC50 of 3.5 µM.$^{13}$

**Figure 6-2.3** Fluorescence emission spectra of Dcys upon addition of Hg$^{2+}$ and inset ratiometric curve. Fluorescence emission spectra of Dcys (10 µM) upon addition of Hg$^{2+}$ (0, 1, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 µM) were measured in 10mM HEPES buffer (pH 7.4). Addition of ~2 mM of EDTA to the Dcys-Hg$^{2+}$ complex results in an immediate fluorescence decrease to its original metal free spectrum indicates reversible behavior of Dcys with Hg$^{2+}$. (Figure 6-2.4)

Furthermore, to investigate the binding stoichiometry, Job’s Plot analysis and mass spectra were conducted. (Figure 6-2.5) The mole fraction at 0.5 in Job’s plot indicates the 1:1 binding between Hg$^{2+}$ and Dcys. Similarly, mass peaks at m/z 903.1 and 705.51 corresponding to Dcys-Hg$^{2+}$ and free one respectively are clearly observed, confirming a 1:1 complex. Thus, assuming
1:1 complex formation between host and guest we calculated the association constant using non-linear least square equation and the observed association constant was $4.0 \times 10^6$ M$^{-1}$.

![Figure 6-2.4](image)

**Figure 6-2.4** Reversibility of Dcys-Hg (II) complex with 0, 125, 250, 375, 500, 750, 1000, 1125µM EDTA in 10 mM Hepes buffer, pH 7.4. The peptide and Hg (II) concentration, each were used 10 µM.

To explore the utility of Dcys as an ion-selective fluorescence chemosensor for Hg$^{2+}$, competition experiments were further carried out. Consequently, compound Dcys (10µM) was treated with 1 equiv. Hg$^{2+}$ in the presence of different competitive metal ions (1 equiv. or 500 equiv.). As shown in **Figure 6-2.6**, the tested competitive metal ions exhibited no interference with the detection of Hg$^{2+}$ ion, indicating that Dcys could be used as a potential practical Hg$^{2+}$-selective fluorescent sensor.
To get insight the possible role of ligands in Hg$^{2+}$ binding, $^1$HNMR experiment was conducted. As shown in the $^1$H NMR spectra of Figure 6-2.6, emission intensity of Dcys (10 µM) in the presence Hg$^{2+}$ (10 µM) and additional various metal ions at pH 7.4 (10 mM HEPES buffer). All metal ions are evaluated at one equivalent to Hg$^{2+}$ except Na$^+$, K$^+$, Ca$^{2+}$, and Mg$^{2+}$, which are used at 500 equivalents.
2.7, treatment of Hg$^{2+}$ ions resulted in significant shifts in the aromatic proton towards up field indicated that the metal induced environmental change of dansyl fluorophore. The disappearance of the sulfonamide proton peak at $\delta$ 7.11 and 7.2 pointed out that the active role of sulfonamide group during mercury binding. The aliphatic $-$CH$_2$ protons adjacent to S-S bond were also shifted from $\delta$ 2.7 to $\delta$ 2.9 after Hg$^{2+}$ binding.

**Figure 6-2.7** Partial $^1$H NMR spectra for Dcys (a) 5.7 mM of Dcys and (b) Hg$^{2+}$-Dcys complex obtained by the reaction of 1 eq. Hg$^{2+}$ measured in dmso-d$_6$.

For rationalizing the observed fluorescence enhancement with significant shift, two factors may be considered. First, the low fluorescence intensity of Dcys in buffer without Hg$^{2+}$ ion may be attributed to PET quenching from the lone pair of sulfonamide group to dansyl fluorophore.\textsuperscript{14} When Hg$^{2+}$ ion coordinates with the lone pair of the sulfonamide group, the PET quenching might be retarded, leading to a substantial increase in the fluorescence intensity.\textsuperscript{12,15} Secondary, the significant shift caused by metal induced local environment change which brought the fluorophore group from hydrophilic environment to hydrophobic environment.

**6-2.3 Conclusion**

In conclusion, we have presented a new easily available turn-on fluorescent chemosensor Dcys based on simple conjugation of Cys residue bearing two dansyl subunits, which showed a remarkable ratiometric enhanced
fluorescent intensity (about 3-fold) with significant shift in the presence of Hg\(^{2+}\) ion and a high selectivity toward Hg\(^{2+}\) ion over a wide range of metal ions in physiological buffer. Moreover, background metal ions did not show any kinds of interference with the detection of Hg\(^{2+}\), indicating that sensor Dcys could be used as an efficient Hg\(^{2+}\) selective turn-on fluorescent chemosensor, and might have potential practical applications for monitoring of environmental and biological samples.

6-2.4 Literature Cited


13) The concentration of Hg\(^{2+}\) required to achieve 50% of the total increase is ~ 3.5µM, which is usually called effective concentration.


CHAPTER VII

7. Design and Synthesis of a New Type of Peptide Sensor Bearing Dansyl and Quenching System and its Application

7.1 Introduction

The development of optimized sensor for measuring hydrogen peroxide or other active ROS has been studied extensively since last decade due to their formation in cells as an unavoidable consequence of aerobic life. ROS such as hydrogen peroxide (H$_2$O$_2$), hydroxyl radicals (OH$^-$), superoxide anions (O$_2^-$) and nitric oxide (NO) are known to cause damage to proteins, nucleic acids, lipids, and other macromolecules. Among these, hydrogen peroxide can mediate signal transduction through reversible oxidation and reduction of cysteine thiols and other redox active groups. The complex, reversible oxidation biology of the cell and its broad implications in human health and disease provide inspiration for developing new ways to study dynamic redox chemistry in living beings. It is, therefore, essential to develop methodologies for the study of such kinds of redox biology. The fluorescence methodology, associated with the use of suitable probes, is an excellent approach to measure ROS because of its high sensitivity, simplicity in data collection, and high spatial resolution in microscopic imaging techniques. Recently, several probes were synthesized for measuring ROS or redox potential within cells. However, most of them were chemodosimisters, which could not reversibly monitor ROS. In addition, a few precedent examples suffered from interference of background fluorescence, side reactions by thiol moieties present within cells, or solubility problem in buffer solution. Thus, here we synthesized the peptide probe having enough solubility in biological buffer system and shows the reversible phenomenon.
Furthermore, we demonstrated the application of this probe for understanding the mechanism of protein denaturation study. As the intrinsic fluorescence method is one of the popular approaches that have been routinely used in numerous studies of proteins for decades.\textsuperscript{7} The method is connected with the exposure of proteins to ultraviolet irradiation, which is absorbed by aromatic chromophore like Trp, Tyr or Phe residues. Irradiation with UV light (254 nm) to the oxidized peptide containing Trp residue resulted in the large increase of emission intensity, indicating that this peptide probe is useful as UV-irradiated denaturation protein model. We successfully synthesized fluorometric redox and UV active peptide probe bearing dansyl and quenching system. This new type of the peptide probe will be applicable in various fields such as for monitoring reaction catalyzed by protein-disulfide isomerase or oxidase\textsuperscript{8-10} and for monitoring reactive oxygen species.\textsuperscript{11,12}

\textbf{7.2 Design and Strategy}

The disulfide bond is the only readily reversible bond present in native proteins. Thus, we considered our design for the monitoring of reversible oxidation-reduction by the extensive use of disulfides as redox switch. Our strategy for developing peptide probes for reversibly monitoring reactive oxygen species and UV-mediated denaturation is based on modulating fluorescence resonance energy transfer (FRET) by the different distance between quencher and acceptor in the oxidized and reduced form as shown in Scheme 7.1.
Scheme 7.1 Strategy for the design of peptide probes

Even though the synthesis of short polypeptide is easy and the bioavailability is greater, it is difficult to apply FRET pairs into short polypeptide with less than 20 amino acids since the Forster radius values of most FRET pairs of organic compounds are longer than 20 Å. This indicates that FRET occurs similarly in both reduced and oxidized form of short peptides. Herein, we synthesized a short peptide probe (1G) containing FRET pairs (Trp as a donor, dansyl group as an acceptor) and Glu residue as a quencher. When oxidized, the peptide probe may adopt a conformation in which the quencher is in close proximity to a dansyl group, resulting in the decrease of emission intensity. When reduced, the peptide probe may adopt random coil conformation that spatially separates the dansyl group and quencher, allowing emission to occur. We chose the middle α-helical region (CAAHCLFR) of Tenecin 1, an insect defensin protein as a starting point for the development of the peptide probe. Like other insect defensin proteins, Tenecin 1 shares a common structural feature of short amphipathic α helix followed by C-terminal antiparallel β sheet structure, which is stabilized by two disulfide bridges (named cysteine stabilized α/β motif). Previously reported CD analysis and α helical wheel diagram has indicated that the
peptide corresponding to α helix region of the protein adopted a random coil structure in the reduced state but amphipathic turn structure in the oxidation state.\textsuperscript{14}

Thus, we supposed that when oxidized, N-terminal and C-terminal ends of the peptide would be close to each other. To provide a FRET pair in the peptide, Phe residue was replaced by Trp which acts as a donor, and dansyl group (dans) was introduced to the N-terminal as an acceptor. Dansyl group has been frequently used in chemical sensors because this group can display a large Stoke shift along with varying quantum yield by changing its local environment.\textsuperscript{15} Glu-residue was introduced at the C-terminal of the peptide as a quencher of dansyl group and also to provide the better solubility in 100% aqueous solution. To confirm the role of Glu residue the peptide (1A) without Glu residue was also synthesized. As disulfide bond is effective quencher for Trp emission, we synthesized the peptide Ac-1G without dansyl group to confirm the role of disulfide bond in Trp emission intensity.

### 7.3 Results and Discussion

#### 7.3.1 Synthesis of Peptides and Characterization

The peptides were synthesized following the Fmoc-protocol on solid phase synthesis as mentioned everywhere.\textsuperscript{16} The HPLC retention time and observed mass of the probes are shown in Table 7.1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences</th>
<th>Reduced (Calc./Obs. Mass)</th>
<th>Oxidised (Calc./Obs. Mass)</th>
<th>Reduced (Ret. Time, Min)</th>
<th>Oxidised (Ret. Time Min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1G</td>
<td>Dans-CAAHCWA</td>
<td>1121.57/1120.7</td>
<td>1119.5/1118.91</td>
<td>42.86</td>
<td>41.96</td>
</tr>
<tr>
<td>Ac1G</td>
<td>Ac-CAAHCWA</td>
<td>930.3/930.18</td>
<td>928.3/927.24</td>
<td>36.76</td>
<td>34.95</td>
</tr>
<tr>
<td>1A</td>
<td>Dans-CAAHCWA</td>
<td>992.73/994.13</td>
<td>990.73/989.14</td>
<td>58.41</td>
<td>62.59</td>
</tr>
</tbody>
</table>

Table 7.1 Peptide sequence, HPLC retention time, calculated and observed masses.
7.3.2 Spectroscopic Properties of Peptide Probes

The fluorescence change between the oxidized and reduced forms was measured in 100% aqueous solution (10 mM Hepes Buffer, 150 mM NaCl, pH 7.4). The peptides have FRET pair in which Trp ($\lambda_{\text{ex}}$: 295 nm, $\lambda_{\text{em}}$: 340 nm) acts as a donor, and dansyl group ($\lambda_{\text{ex}}$: 330 nm, $\lambda_{\text{em}}$: 510 nm) at the N-terminal acts as an acceptor.\textsuperscript{17} We measured emission intensity by two excitation wavelengths $\lambda_{\text{ex}}$: 295 nm and 330 nm respectively. As shown in Fig. 7.1, the reduced form (1G) showed about 2.5 fold increase in emission intensity at 515 nm compared to that of the oxidized form (1Gox) when the excitation wavelength was either 295 nm or 330 nm.

A slight shift in emission maximum between oxidized and reduced form was observed because a local environment change of dansyl group is known to change its emission spectrum.\textsuperscript{15,18} The decrease of emission fluorescence in the oxidized form must be due to the close proximity of the quencher group of the Glu residue to a dansyl group. To confirm this result, we investigated emission spectrum change between the oxidized and reduced form of 1A, which does not contain a Glu residue (Figure 7.2). The oxidized and reduced forms provided similar fluorescence emission spectra and the emission maximum of the oxidized form is slightly shifted. Furthermore, we measured the emission spectrum of the reduced form of 1A in the presence of TCEP. The addition of TCEP into the reduced form of 1A resulted in the decrease of emission intensity at 515 nm (Figure 7.3), which is consistent with the result shown by peptide 1G.
The reduced form of the peptide probe (1G) showed the similar emission intensity at 515 nm by excitation whether 295 nm or 330 nm were used as the excitation wavelength. This result indicates that when the peptide is in the reduced form, Trp and dansyl group are close enough for FRET because Forster radius value for this pair is approximate 20 Å. However, in comparison to emission spectrum of the oxidized form, the excitation at 295 nm produced a slightly lower emission intensity than that from excitation at 330 nm. This may be due to the quenching effect of the disulfide bridge to emission spectrum of Trp residue. To confirm the role of disulfide bridge to the emission spectra of Trp residue, the reduced and oxidized form of Ac-1G (Ac-CAAHCWAE-NH₂) was synthesized and investigated fluorescence emission spectrum of Trp in both forms (Figure 7.4). The decreased emission intensity of the oxidized form in comparison to that of reduced form also supports our assumption.
Figure 7.2 Fluorescence emission spectra of peptide 1A in 10 mM HEPES buffer solution (pH = 7.4) containing 150 mM NaCl (A) excitation at 295nm (B) excitation at 330nm. Bold line indicates the reduced form and dotted line indicates the oxidized form.

Figure 7.3 Peptide 1A reduced (10 μM) treated with different concentration of TCEP (tri-carboxy-ethyl phosphin) excited at 330 nm in 10 mM HEPES buffer with 150 mM NaCl, pH 7.4. (excitation slit 2.5 and emission slit 4.5 nm.)
7.3.3 Secondary Structure of the Probes

We investigated the secondary structures of reduced and oxidized form of the peptides 1G, and Ac-1G in 100% aqueous buffer solution without any co-solvent using a CD spectroscopy (Figure 7.5). The CD spectrum indicated that the reduced form did not have a complete random structure in buffer solution, whereas the oxidized form adopted a turn structure on the basis of the negative ellipticity at 210 nm. This result supports that the oxidized form has a turn structure in which the quencher (Glu residue) might be close to the dansyl group, resulting in the decrease of the emission intensity. Unexpectedly, the reduced form might not have a random conformation, which can be explained by interaction between Trp and dansyl group. To support our assumption, we investigated the secondary structure of the reduced and oxidized form of the peptide Ac1G that does not have a dansyl group. The CD spectrum pointed out that Ac1G reduced form had a
random structure, which revealed the reason why the reduced form of 1G does not have a random structure in buffer solution.

**Figure 7.5** Circular Dichroism spectra of peptide (A) 1G reduced and oxidized and (B) Ac-1G reduced and oxidized in 10 mM phosphate buffer solution (pH = 7.4).

### 7.3.4 Fluorescence Response with Hydrogen Peroxide and Reversible Monitoring with DTT

The peptide 1G was considered for further study with hydrogen peroxide due to its large difference in emission intensity between its reduced and oxidized
form. Figure 7.6 shows the rapid response and high sensitivity of the peptide probe for monitoring H$_2$O$_2$. Peptide 1G (2µM) exhibits a fluorescence change when incubated with only 75 equiv. of H$_2$O$_2$ (150 µM). Excitation either at 295 nm or at 330 nm produced a large fluorescence change at 510 nm when the probe was exposed to H$_2$O$_2$. Fluorescence change of this condition was completed within 30 min.

To investigate the reversibility of the probe, DTT was added to the resulting solution after the treatment of H$_2$O$_2$ and fluorescence was monitored. As shown in Figure 7.7, fluorescence enhancement of solution triggered by 100 µM DTT (50 equiv.) was completed within 12 min. The change in fluorescence is almost similar to that of the original reduced form. This result reveals that the probe (1G) displays a quite rapid response and reversibility for monitoring H$_2$O$_2$.

![Figure 7.6](image)  
**Figure 7.6** Fluorescence emission changes of 1G (2.0 µM) in the presence of H$_2$O$_2$ (75 equiv.) (A) excitation at 295 nm and (B) excitation at 330 nm at different time interval measured in 10 mM HEPES buffer solution (pH = 7.4) containing 150 mM NaCl.
Figure 7.7 Fluorescence emission changes of 1G (2.0 µM) in the presence of DTT (50 equiv.) at different time intervals (excitation at 330 nm) measured in 10mM HEPES buffer solution (pH = 7.4) containing 150 mM NaCl.

We also confirmed the conversion of 1G into 1Gox by H₂O₂ using HPLC and MALDI-TOF mass (Figure 7.8). HPLC spectra and mass data indicated that the 1Gred is fully oxidized with hydrogen peroxide.
The peptide probe (1G) synthesized in this study has several advantages for future optimization. First, the response time and sensitivity of the probes for ROS can be optimized by tuning amino acid sequence. Second, incorporation of the additional functionality is also feasible via amide bond formation. For example, cellular internalization sequence could be appended to transport the probe into cells.¹⁹

### 7.3.5 Illumination of the Peptide Probe with UV Light and Characterization

To evaluate whether the peptide probe can be a proper model for UV mediated protein denaturation or not, we monitored fluorescence change of oxidized form of 1Gox by irradiation with UV-light at 254 nm and 350 nm for 6 hr, respectively. Irradiation with UV light (254 nm) resulted in the change of fluorescence spectrum, while fluorescence spectrum was not changed by irradiation with 350 nm UV light (Figure 7.9, 7.11 A). Figure
7.9 showed that the fluorescence intensity of the probe at 510 nm increased as a function of time of UV light irradiation (254 nm). HPLC and MALDI TOF mass spectrum of the UV irradiated product revealed that the oxidized form of peptide 1G was converted to its reduced form by UV irradiation. Furthermore, the conversion after 6 hr was confirmed by Ellman’s reagent and data is shown in Figure 7.10. The UV-absorbance observed at 412 nm further indicated that the probe was reduced after illumination at 254 nm. According to our control experiment, the oxidation state of 1Gox has not been changed even after incubation for 24 hrs in the absence of UV light (Figure 7.11, b).

Figure 7.9 Fluorescence emission spectra of 1Gox (2 μM) (A) excitation at 295 nm and inset conversion % as a function of time (B) excitation at 330 nm as a function of time of UV irradiation (254 nm). Fluorescence emission spectrum excited with 330 nm was measured in 10 mM HEPES buffer solution (pH = 7.4) containing 150 mM NaCl.

Also, the reduced state of 1G is not auto-oxidized in buffer solution (pH = 7.4) without oxidizing reagent such as H₂O₂ and oxidized DTT. This result showed that the UV absorption of Trp residue played an important role in the
reduction of disulfide-bridge, which is in consistent with the result that UV irradiation resulted in Trp-mediated reduction of disulfide bonds of Cutinase.\textsuperscript{20} This indicated that peptide 1G can be a useful model for UV-denatured protein and biomarker for UV denaturation protein.

**Figure 7.10** UV-Vis absorbance of 1Gox before and after UV-illumination. The peak at 412 nm indicates the formation free thiol after illumination.

**Figure 7.11** Fluorescence change of (A) 1Gox (2µM) before and after illumination at 350 nm, (B) time drive course of 1G and 1Gox (each 2µM) excited at 330 nm and emission was observed at 515 nm. The samples were measured in 10 mM Hepes buffer, pH 7.4.
7.4 Conclusion

In the present work, we first introduced a novel FRET-quenching system into the peptide probes for monitoring $\text{H}_2\text{O}_2$. The peptide probe consisting of entire natural amino acids is easily synthesized by solid phase synthesis method which exhibits a marked difference in fluorescence change between oxidized and reduced state. We have successfully synthesized the first example of peptide sensor with a rapid response, great sensitivity and reversibility for monitoring $\text{H}_2\text{O}_2$. Further application of the probe for understanding the model to elucidate UV irradiated protein denaturation reaction and to be a biomarker for UV denaturation protein was demonstrated.

7.5 Materials and Methods

7.5.1 Synthesis of Intra-disulfide Bridged Peptides

The linear form of peptide was diluted in 10mM phosphate buffer, pH 7.4 in the presence of 10% (v/v) DMSO. After being stirred at room temperature, the oxidation reaction was monitored according to the method of Ellman et al.\textsuperscript{21} After completion of the oxidation, the peptide was purified by semi prep HPLC. ESI mass spectrometer (Micromass, Platform II) or a MALDI TOF mass spectrometer (Voyager-DE STR, Applied Biosystem) were used to characterize the successful synthesis and purity (>95%) of oxidized peptides.

7.5.2 DTNB Assay for Free Thiol Detection

The detection of free thiols was monitored using Ellman’s reagent during the formation of intra-disulfide bond in peptide as mentioned in.\textsuperscript{23} 20µM of peptides in 10 mM Hepes buffer at pH 7.4 was incubated with Ellman’s reagent for 30 min. The yellow color, due to the formation of TNB\textsuperscript{2-}
indicates the presence of free thiol which shows the absorbance at 412 nm. If no absorbance at 412 nm indicates the completion of the disulfide bond.

### 7.5.3 Circular Dichroism

CD spectra were recorded on a Jasco J-715 spectropolarimeter (Tokyo, Japan) using a quartz cell of 1 mm path length between 190 and 250 nm at room temperature. The concentration of peptides was 200 μg/mL in 10 mM Phosphate buffer (pH 7.4). Two scans with a scan speed of 10 nm/min were averaged for each peptide. CD spectra were expressed as the mean residue ellipticity.

### 7.5.4 UV-irradiation of Peptide

Oxidised peptide (2 μM) was placed in a 1-cm path length quartz cell containing 10 mM HEPES buffer solution (pH = 7.4) with 150 mM NaCl inside UV box. The cell was exposed to UV light from Hg-lamp ENF-260C. The UV illumination set up ensured the luminous flux (350 μW/cm²). Then, fluorescence was measured at every 1 hr. The temperature of the sample was kept at 20°C. Finally, after complete saturation, the peptide was characterized by HPLC, mass spectroscopy and Ellman’s assay.
7.6 Literature Cited

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Chapter VIII
Part 1

8-1. Application of Rapid Microwave Method to the Solid Phase Synthesis of Pseudopeptides Containing Ester Bond

8-1.1 Introduction

8-1.1.1 A Brief Summary of Microwave Theory

The use of microwave irradiation in organic synthesis has become increasingly popular within the pharmaceutical and academic fields, because it is a new facilitating technology for drug discovery and development.\(^1\) By taking advantage of this efficient source of energy, compound libraries for lead generation and optimization can be assembled in a fraction of the time required by classical thermal methods. Recently, thermally driven organic transformations take place by either of two methods: conventional heating or microwave accelerated heating. In the first method, reactants are slowly activated by a conventional external heat source. Heat is driven into the substance, passing first through the walls of the vessel in order to reach the solvent and reactants. This is a slow and inefficient method for transferring energy into the reacting system. In the second way, microwaves couple directly with the molecules of the entire reaction mixture, leading to a rapid rise in temperature. Since the process is not limited by the thermal conductivity of the vessel, the result is an instantaneous localized superheating of any substance that will respond to either dipole rotation or ionic conduction: the two fundamental mechanisms for transferring energy from microwaves to the substances being heated. Based on experimental data from numerous studies that have been performed over the past ten years,

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3) Part of this chapter was published as Joshi, B.P.; Park, J.W.; Kim, J. M.; Lohani, C.R.; Cho, H.; Lee, K. H. *Tetrahedron Lett.* **2008**, *49*, 98-101
chemists have found that microwave-enhanced chemical reaction rates can be faster than those of conventional heating methods by as much as 1,000-fold.¹

8-1.1.2 Application of Microwave Method to Solid Phase Chemistry
Solid phase synthesis has been extensively used for the preparation of peptides, pseudopeptides and organic compounds.² This technique offers many advantages over conventional synthesis in terms of efficiency as well as convenient work up and purification procedures. However, the main problems with this method are the difficulties for the reagents to reach the active sites located in the solid phase, resulting in slow reaction and degradation of the resin under long reaction conditions, and requirements of excess reagents. Recently, significant increase in reaction rate, improving yield and high purity of the product have frequently been observed in solid phase synthesis with microwave irradiation.³ Several successful attempts for the application of microwave irradiation for the solid phase synthesis of peptides, peptoid and beta polymer have been published.⁴ However, the microwave irradiation method has not extensively been applied for the solid phase synthesis of pseudopeptides.

8-1.2 Design and Strategy for the Synthesis of Pseudopeptides Utilizing Microwave Method
Pseudopeptides have received much attention due to their better bioavailability than peptides and the easy development of non-peptide drugs. Thus, recently many classes of pseudopeptides have been reported.⁵,⁶ We are interested in the solid phase synthesis of pseudopeptides containing ester
bond as following reasons. The amide and ester bonds are very much similar in terms of structural and conformational preferences.\(^6\) (**Scheme 8-1.1**)

Thus, replacement of amide bond with ester bond is well-known strategy for investigating the role of hydrogen bonding of amide bond in proteins and peptides for their structures and biochemical interactions.\(^7\) In addition, pseudopeptides containing ester bond have better bioavailability, and increasing hydrophobicity. Even though pseudopeptides containing ester bond exhibited unique properties, pseudopeptides containing ester bond are rarely synthesized because the synthesis of the pseudopeptides is not straightforward and very often possess a synthetic challenge due to the low nucleophilicity of hydroxyl group compared to amino group. Pseudopeptides containing ester bond was synthesized by using solid phase synthesis with α-hydroxy acids or unnatural α-hydroxy acids.\(^6b, 7a, 8\) Compared to the solid phase peptide synthesis, the reaction time was relatively long (4 hr~16 hr) and even double coupling reaction provided low yield (70 %).\(^6b, 7a, 8\) In the era of the proteomics, the time required for the synthesis of the target pseudopeptides became an important issue and it would be beneficial to increase the yield and reaction rate for the synthesis of the pseudopeptides. Considering this, we developed a microwave irradiation procedure for the purpose of reducing the reaction time and increasing the reaction yield for
the synthesis of pseudopeptides containing ester bond in solid phase synthesis.

8-1.3 Results and Discussion

We chose the dipeptide containing ester bond (Fmoc-Lysψ [COO] Leu-NH₂) as a model system and optimized microwave irradiation procedures for solid phase synthesis (Scheme 8-1.2).

![Scheme 8-1.2](image)

**Scheme 8-1.2** Structure of model dipeptide and its derived pseudopeptide

The (S)-2-Hydroxy-4- OH) and (S)-6-[(Phenylmethoxy) carbonyl] amino]-2-hydroxy hexanoic acid (α-hydroxy-Lys (z)-OH) were synthesized by diazotization method in acidic condition with sodium nitrite from the respective amino acid.⁹ (Scheme 8-1.3)

![Scheme 8-1.3](image)

**Scheme 8-1.3** Synthesis scheme for the compound a and b; where a represents α-hydroxy Lys (Z)-OH and b = α-hydroxy-Leu.
DIPC among various coupling reagents provided the best yield in the previous esterification reaction for pseudopeptides in solid phase synthesis.\textsuperscript{3b, 7, 10} We chose diisopropylcarbodiimide (DIPC) as a coupling reagent and DMAP/NEM as base and investigated yields of the esterification reaction with microwave irradiation in various temperature and solvents for the optimization of this model reaction. In this model reaction, the pseudopeptide was synthesized by using Rink-amide MBHA resin as shown in scheme 7-4.

The α-hydroxy acid was coupled to the resin by activation of 3 eq. of alpha hydroxy acid with the same equivalent of DIPC and HOBt in the presence of DMF with microwave irradiation. The coupling reaction was repeated until no color was observed in ninhydrin test.\textsuperscript{11} Then, the esterification reaction was carried out as will be discussed in experimental section. As hydroxyl group is known to be insensitive with ninhydrin color test; therefore the coupling yield for esterification was measured by Fmoc quantitation assay based on the absorbance observed at 301 nm for Fmoc-piperidine adduct.

In conventional solid phase synthesis, different ratio of DCM/DMF was employed as solvent for the coupling reaction.\textsuperscript{12} The esterification reaction was attempted for model pseudodipetide using DMF/DCM (1:1) and the obtained yield was not significant (~56%). Also, some amino acids have not
good solubility only in DCM. As shown in Table 8-1.1, the esterification reaction with microwave irradiation was performed in NMP as solvent due to its high boiling point. However the yield was not significantly improved\textsuperscript{16} and Fmoc-deprotection of the conjugated Fmoc amino acid to the resin was observed in this condition, perhaps due to high temperature in the presence of base.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Power (W)</th>
<th>Temp (°C)</th>
<th>Time (min)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMF/DCM (1:1)</td>
<td>150</td>
<td>90</td>
<td>12</td>
<td>56</td>
</tr>
<tr>
<td>NMP</td>
<td>200</td>
<td>150</td>
<td>12</td>
<td>58</td>
</tr>
<tr>
<td>DMF</td>
<td>150</td>
<td>90</td>
<td>7</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>78</td>
</tr>
</tbody>
</table>

*Table 8-1.1* Coupling yield of the model pseudo-dipeptide at different time and different solvent under microwave irradiation performed under closed vessel

Then, we selected DMF as solvent and investigated esterification reaction with microwave irradiation. *Table 8-1.1* indicates that the reaction reached a level of 80% at 12 min. To improve coupling yield, we increased reaction time but the yield was not improved. We performed the esterification reaction in DMF solvent with microwave irradiation (150W) at high
temperature (120 °C) however the yield (80%) was not improved. Thus, the reaction condition with microwave irradiation (Temp. 90°C, power 150 watt, time 12 min.) was kept constant for further synthesis of pseudopeptides in this study.

Additionally, several coupling reagents were reconsidered for the esterification reaction by applying this microwave irradiation condition (Temp. 90°C, power 150 watt, time 12 min.). The coupling yield with various coupling reagents was summarized in Table 8-1.2.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Coupling Reagent</th>
<th>Base</th>
<th>Coupling Yield a %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DIPC/ HoBt</td>
<td>DMAP/NEM</td>
<td>72</td>
</tr>
<tr>
<td>2</td>
<td>DIPC</td>
<td>DMAP/NEM</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>TFFH</td>
<td>DIEA</td>
<td>61</td>
</tr>
<tr>
<td>4</td>
<td>PyBop/HoBt</td>
<td>DIEA</td>
<td>49</td>
</tr>
<tr>
<td>5</td>
<td>DCC /HoBt</td>
<td>DMAP/DIEA</td>
<td>53</td>
</tr>
</tbody>
</table>

Table 8-1.2 Microwave-assisted yields of Fmoc-Lysψ [COO] Leu-NH₂ with various coupling reagents. (The resin (0.025 mmol) containing α-hydroxy-Leu was mixed with Fmoc-Lys (Boc)-OH (3 equiv) in the presence of the coupling reagent and the reaction was performed under microwave irradiation (temp 90°C, power 150 W, time 12 min).

As shown in Table 8-1.2, the DIPC mediated coupling reaction provided the higher yield. The activation of an Fmoc-amino acid with coupling reagent 1, 4, and 5 resulted in the formation of the corresponding OBT ester that was
known to provide low racemic product. However, the OBt ester provided low yield in this condition. Thus, this coupling reagent and microwave irradiation condition was applied for the synthesis of various pseudodipeptides containing ester bond (Table 8-1.3).

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequence</th>
<th>Yield (%)</th>
<th>Microwave (12 min)</th>
<th>Without microwave (480 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fmoc-Kψ[COO]L-NH₂</td>
<td>80</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Fmoc-Eψ[COO]L-NH₂</td>
<td>83</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Fmoc-Sψ[COO]L-NH₂</td>
<td>78</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Fmoc-Fψ[COO]L-NH₂</td>
<td>78</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Fmoc-Aψ[COO]L-NH₂</td>
<td>78</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Fmoc-Lψ[COO]K-NH₂</td>
<td>82</td>
<td>65</td>
<td></td>
</tr>
</tbody>
</table>

Table 8-1.3 Coupling yield of various pseudo-dipeptides containing an ester bond

To compare the yield of pseudodipeptides synthesized by microwave method, same sequences were synthesized by using without microwave irradiation method and their yield and purity were compared. The coupling yield of each reaction was measured by Fmoc quantitation assay. After completion of the reaction, the pseudopeptides were cleaved and deprotected by treatment of
TFA: H₂O (95:5) solution. The resulting product was analyzed using C₁₈ reverse phase HPLC and an ESI mass spectrometer. HPLC and ESI mass analysis of the final product mixtures indicated that both methods provided the target pseudopeptide as a major product. (Fig 8-1.1 and Table 8-1.4)

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Sequence</th>
<th>Calcd./Obs. Mass</th>
<th>HPLC Ret. Time (Min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Without</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Microwave</td>
</tr>
<tr>
<td>1</td>
<td>Fmoc-Kψ[COO]L- NH₂</td>
<td>481.58/482.0</td>
<td>42.81</td>
</tr>
<tr>
<td>2</td>
<td>Fmoc-Eψ[COO]L- NH₂</td>
<td>482.23/482.95</td>
<td>48.08</td>
</tr>
<tr>
<td>3</td>
<td>Fmoc-Sψ[COO]L- NH₂</td>
<td>440.13/439.93</td>
<td>44.26</td>
</tr>
<tr>
<td>4</td>
<td>Fmoc-Fψ[COO]L- NH₂</td>
<td>500.23/500.03</td>
<td>53.94</td>
</tr>
<tr>
<td>5</td>
<td>Fmoc-Aψ[COO]L- NH₂</td>
<td>424.22/424.99</td>
<td>53.93</td>
</tr>
<tr>
<td>6</td>
<td>Fmoc-Lψ[COO]K- NH₂</td>
<td>481.58/482.0</td>
<td>44.46</td>
</tr>
</tbody>
</table>

Table 8-1.4 HPLC retention time and observed mass of synthesized pseudo-dipeptides peptides by normal and microwave method

Table 8-1.3 illustrated that the coupling yields for the various pseudodipeptides containing ester bond synthesized in microwave irradiation method are better than those achieved using the coupling reaction without
microwave irradiation. By applying the microwave irradiation method, the reaction time was significantly reduced from 480 min to 12 min. After testing the microwave-assisted procedure for the synthesis of various pseudodipeptides containing ester bond, we chose α-helical peptide, which are known as a difficult sequence\textsuperscript{13} to synthesize in solid phase synthesis as model peptide and synthesized the corresponding pseudopeptides by using the microwave irradiation procedure. The 5-mer and 11-mer pseudopeptides containing hydroxy group at their N-terminus were synthesized on Rink-amide MBHA resin in solid phase synthesis. Then esterification reaction was performed by utilizing microwave assisted esterification method. The same pseudopeptides were synthesized by using without microwave irradiation and compared the yield with that obtained by microwave methods.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Sequence</th>
<th>Coupling Yield %</th>
<th>Normal</th>
<th>Microwave</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fmoc-L\textsubscript{ψ}[COO]K-KLLK-NH\textsubscript{2}</td>
<td>45</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>K\textsubscript{ψ}[COO]LLL-KWLK-KLLK-NH\textsubscript{2}</td>
<td>44</td>
<td>79</td>
<td></td>
</tr>
</tbody>
</table>

\textbf{Table 8-1.5} Coupling yield of 6- and 12-mer pseudopeptide containing an ester bond by using microwave irradiation method

As shown in \textbf{Table 8-1.5}, the coupling yield achieved with the microwave-assisted method was about two times higher than that obtained by the esterification reaction without microwave irradiation. The HPLC and ESI mass analysis of 6-mer pseudopeptide indicated that both methods provided
the target pseudopeptide as a major product whereas, the purity obtained by using microwave irradiation was better than that obtained by the method without microwave irradiation.

Even though we did not use HOBt in this reaction, no racemic product was observed in the microwave assisted reaction. To investigate the racemization, we separately synthesized the 6-mer pseudopeptide (Fmoc-.D-LeuΨ[COO]Lys-Lys-Leu-Leu-Lys-NH₂) using D-Leu at N-terminus. Both pseudopeptides (D and L form) were characterized by HPLC. Furthermore, both peaks were co-injected and analysed by HPLC. Two distinct peaks were observed with different retention time. This clearly indicates that, no racemic product formation occurred during microwave reaction. The 12 mer pseudopeptide was also successfully synthesized using microwave assisted procedure. (Fig. 8-1.2)

The HPLC and ESI mass spectrum pointed out that microwave method provided target molecule as only sole product, whereas the normal method provided two major products in HPLC chromatogram (Fig.8-1.2b). MALDI TOF mass spectrum (Fig 8-1.3) showed that the product corresponding to peak 1 in Fig 8-1.2b was the unreacted product (HO-LLL-KWLK-KLLK-NH₂), while the product corresponding to peak 2 was the target pseudopeptide (Fig. 8-1.4b). HPLC and mass spectrum of the crude product synthesized by microwave-assisted reaction indicated that no racemic product was observed in this microwave assisted reaction (Fig 8-1.4a).

8-1.4 Conclusion

In conclusion, the use of microwave irradiation allowed a rapid and high yield preparation of pseudopeptides containing ester bond for Fmoc based solid phase peptide synthesis. In a direct comparision of our optimized
protocol with normal methods, the pseudodipeptides, 6-mer pseudopeptide and 12-mer pseudopeptide synthesized by microwave irradiation method were achieved in high yield and significant purity. This study has demonstrated that controlled microwave heating would be advantageous for the synthesis of pseudopeptides containing ester bond.

**Figures from 8-1.1 to 8-1.4**
Figure 8-1.1 HPLC and ESI-mass spectrum of [Fmoc-Kψ (COO)-L-NH₂]. (A) microwave (B) without microwave and (C) ESI Mass spectrum. (calculated mass [M+H⁺] = 481.58, measured mass [M+H⁺] = 482.0, M+Na⁺ = 503.9)

Figure 8-1.2 HPLC of 12-mer pseudopeptide Kψ [COO]-LLL-KWKLLK-NH₂: (A) with microwave irradiation, (B) without microwave irradiation
Figure 8-1.3 The mass spectra corresponding to peak 1 in the synthesis of 12-mer pseudopeptide ψ[COO]-LLL-KWLK-KLLK-NH$_2$ without microwave irradiation. The mass is equivalent to the un-reacted product, HO-LLL-KWLK-KLLK-NH$_2$ (Calculated mass [M+H$^+$] = 1396.25, measured mass m/z [M+H$^+$] = 1396.76, M+Na$^+$ = 1418.77)
Figure 8-1.4 The mass spectra of 12 -mer pseudopeptide Kψ [COO]-LLL-KWLK-KLLK-NH₂ (A) with microwave irradiation (B) without microwave irradiation (mass corresponding to peak 2 in Fig 8-1.2B)

8-1.5 Materials and Methods
8-1.5.1 Synthesis of α-hydroxy acid
A solution of NaNo2 (0.4 gm, 5.8 mMol) in water was added drop wise to an ice cooled and stirred solution of α-amino acid (0.4 gm, 1.42 mMol) in 10 ml 2N H2So4/Acetonitrile or water. The mixture was stirred for an additional 3 hour after the addition of NaNo2 at 0-5 dc and left to stand 24 hours at room temperature. The completion of reaction was monitored with ninhydrin as visualizing reagent. After completion of the reaction, the reaction was quenched by methyl amine (1 mL) and dried the solvent using rotavapour. Water was added to the dry suspension and washed the aqueous part with diethyl ether (3 times). Then the organic solvent was evaporated again by rota vapour and high vacuum.

Characterization data of Compound a
Yield = 60 %, TLC: Eluent 20:1:1 (DCM, MeOH: AcOH), Rf = 0.3, Melting point obs. = 76-78°C, literature 79- 81°C.

1H NMR, (200 MHz, CDCl3) δ 1.20 (m, 4H, CH₂ and γ CH₂), 1.40-1.50 (m, 2H, β CH₂), 3.2 (t, 2H, ε CH₂), 4.25 (m, 1H, α CH), 5.1 (d, 2H, CH2Ph), 5.45 (s, 1H, NH), 7.2-7.3 (m, 5H, Ph H), 9.0 (s, OH)

Characterization data of Compound b
Yield = 75%, TLC: Eluent condition, EA: MeOH: AcOH (2:1:1), Rf = 0.357 Melting Point obs. = 77-78 °C, literature: 78 –80 dc.
$^1$H NMR, (200 MHz, CDCl₃) δ 1.0 (q, 6H, CH₃ proton), 1.60 (t, 2H, β CH₂), 1.9 (m, 1H, γ CH), 4.15 (t, 1H, α CH), 7.1 (s, OH)

8-1.5.2 Coupling of α-hydroxy acid and Fmoc-amino acid to α-hydroxy acid in Resin

The coupling of α-hydroxy acid to the resin was accomplished using the similar method to amide bond coupling. α-hydroxy acid is activated with 3 equivalent of DIPC/HoBt in DMF and reaction was either kept in room temperature for four hour or coupled by microwave reaction method. The formation of ester bond between the growing α-hydroxy acid part in resin and Fmoc-amino acid as follows. Activated Fmoc-amino acid with DIC/DMAP in DMF (3 mL) was mixed with the hydroxy Leu attached resin followed by NEM. The reaction mixture was placed in a microwave vial and irradiated at 90°C, supplying 150-W power for 12 min in the Biotage Microwave Initiator System. The reaction was cooled with N₂ gas and the resin was washed with DMF and MeOH, respectively (3 mL, three times each). Similarly, normal reaction procedure was conducted to compare the yield with microwave method. The reaction was kept for 8 hr in room temperature except the microwave heating. The cleavage and characterization methods of pseudopeptides were performed using the similar method as earlier discussed in chapter 2.

8-1.5.3 Determination of Coupling yield by Fmoc-quantitation Method

The dry resin was weighed (3–5 mg) in an eppendorf tube and added to a 50% piperidine/DMF solution. The mixture was incubated for 30 min at room temperature with continuous stirring. Then, the absorbance of the Fmoc-piperidine adduct was measured at 301 nm by using a UV–vis
spectrophotometer (Lambada 40, Perkin Elmer, UK). The blank titrations were performed without resin containing supernatant. The loading yield of the esterification reaction was compared to that of the loading yield of Fmoc-protected α-hydroxy leucine. The degree of substitution was calculated by using the following equation;

\[
(m\text{Mol/g}) = \frac{\text{absorbance at } 301\text{ nm} \times \text{volume (mL)} / 7800 \times \text{wt. (mg)}}{(8-1.1)}
\]

8-1.6 Literature Cited


195
8-2. Structure-Activity Relationship of Pseudopeptide Analogs Corresponding to the Cationic α-Helical Peptides

8-2.1 General Introduction

Antimicrobial peptides have been found in almost all higher eukaryotic organisms.\(^1\) However; they are also expressed in eubacteria, protists, archeae, invertebrates and plants revealing that these peptides were present early in evolution.\(^2\) These are small polypeptides less than 50 amino acid sequences having broad range of activities from bacteria, fungi and viruses. The activity spectrum is unique for every peptides and single amino acid substitution can affect the activity.\(^3\) The net charge of nearly all antimicrobial peptide is positive, and therefore they are designated as cationic antimicrobial peptides (CAMPs). Most of the antimicrobial peptides are membrane active and lyse the target cell by disrupting the integraty of the membrane. Many models regarding their mode of action have been proposed of which the Barrel Steve, Torroidal and Carpet models are well established.\(^4\) Apart from directly kill or inhibit growth of microorganisms, antimicrobial peptides also have other functions in immunity. Many other activities have been attributed to peptides such as neutralising LPS, increasing phagocytosis, induction of mast cell degranulation, and participation in the regulation of the compliment system.\(^5\)

8-2.2 Strategy for the Design of Pseudopeptides

Positively charged peptides have been used extensively as vehicles to deliver functional DNA molecules into cultured cells.\(^6\) Cationic Polypeptides such as polylysine and polyarginine have been shown to efficiently complex negatively charged DNA molecules and to introduce the complexed DNA into the nuclei of recipient cultured cells.\(^7\) However, being composed of
amino acids, these peptidic carriers should be digested by proteolytic enzymes present within the circulation and within cells, and hence their action in vivo is limited. It is well known that replacement of backbone amide bonds by isosteric units stabilizes biologically active peptides towards enzymatic degradation. In addition, incorporation of amide bond isosteres is a convenient way to elucidate the role of selected amide bonds in receptor binding, biological activity and their influence on the secondary structure of peptides. In this regard, replacement of backbone amide bond to corresponding ester bond is well known strategy to investigate the main chain hydrogen bonding and its effect on secondary structure change. We choose the alpha-helical Lysine and Leucine rich cationic antibacterial peptide as a model system. To investigate the role of backbone amide bond and its effect on structure activity, 1 and 7 position amide bond substituted analog by ester bond were synthesized.

8-2.3 Results and Discussion

8-2.3.1 Design and Synthesis

A series of pseudopeptides containing ester bonds were synthesized using solid-phase Fmoc chemistry as discussed earlier. The overall synthetic procedure selected and the experimental conditions developed in this study resulted in a high yield of pseudopeptides. The successful synthesis of pseudopeptides was characterized by analytical HPLC and MALDI-TOF spectroscopy. To investigate the function of ester bond in the structure activity relationship, we chose a cytolytic peptide, Ac-Kc (Ac-KLLL KWLK KLLK-NH2); as a model peptide and synthesized the pseudopeptides containing ester bond in different positions. A cytolytic peptide was selected because it was reported to be active against both bacteria and mammalian
cells and to have high hydrophobicity and high α-helicity in membrane-mimicking condition.\textsuperscript{10}

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Rt. time</th>
<th>Calculated/ Observed mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-Kc</td>
<td>Ac-KLLL-KWLK-KLLK-NH2</td>
<td>53.25</td>
<td>1565.07 / 1566.63</td>
</tr>
<tr>
<td>Ac-Ke2</td>
<td>Ac-Kψ[COO]LLL-KWLK-KLLK-NH2</td>
<td>52.52</td>
<td>1566.06 / 1566.79</td>
</tr>
<tr>
<td>Ac-Ke3</td>
<td>Ac-KLLL-KWL ψ[COO]K-KLLK-NH2</td>
<td>44.05</td>
<td>1566.06 / 1566.75</td>
</tr>
</tbody>
</table>

**Table 8-2.1** Peptide sequence, retention time and observed mass for the peptide and pseudopeptides.

As shown in Table 8-2.1, ester bond was incorporated into the N-terminus of Ac-Kc to form Ac-Ke1 maintaining the same net positive charge without changing the secondary structure. Similarly, 7 position amide bond was substituted by ester bond having decreased α-helicity and hydrophobicity. The N-terminus for all peptides was acetylated to prevent the ionization at neutral pH, increasing hydrophobicity and decreasing the net charge.

**8-2.3.2 Investigation of Secondary Structure in Different Condition**

Usually, antimicrobial peptides exhibit their membrane permeability after adopting the secondary structure such as α-helices or β-sheet. Thus the secondary structure in lipid membrane rather than in aqueous buffer should be well correlated with activity against bacterial and mammalian cells. The secondary structure of peptide and pseudopeptides was investigated in various membrane-mimicking conditions.\textsuperscript{11} Figure 8-2.1(A) shows CD spectra measured in TFE-water (50:50, v/v).
Figure 8-2.1 Circular Dichroism spectra of model peptide and its derived pseudopeptides (a) 50 % TFE (b) 25 mM SDS (c) DPC and (d) buffer. Peptide Concentration = 200 µg/mL.

The CD spectrum of Ac-Kc exhibited double minimum bands at 208 and 222 nm, which indicated that peptide must adopt a well-defined α-helical structure in this condition. The CD spectrum of each pseudopeptide was also measured in the presence of TFE-water (50: 50, v/v) and the α-helicity was calculated. As shown in Table 8-2.2, ester bond containing peptide exhibited α-helicity in lesser extent to that of control peptide. Peptide Ac-Ke1 has almost similar helicity to that of Ac-Kc, whereas the peptide Ac-Ke3 has found drastically decrease α-helicity in all conditions. This result indicated
that the perturbatory effect of this bond on α-helical structure depended on its position in the primary structure of the peptide. Similarly, as shown in Figure 7-2.1(B), we also measured CD spectra in Tris- buffer containing 25% SDS micelles. SDS, which consists of an aliphatic tail and a negatively charged head group, mimicked the lipid membranes of bacteria.\textsuperscript{11b} The measured α-helicity in this condition also followed the similar trend as we observed in 50% TFE condition. The CD spectra measured in DPC micelle achieved slightly higher α-helicity for Ac-Kc1 whereas reduced in case of Ac-Ke1 and Ac-Ke3. The secondary structural behavior of this peptide in buffer remains unstructured; showing predominant random structure which is in best agreement with previous results.\textsuperscript{12}

\begin{table}
\begin{tabular}{llll}
\hline
Name & Helicity (%) TFE / SDS / DPC & Net charge \\
\hline
Ac-Kc & 23.15 / 24.26 / 27.41 & +5 \\
Ac-Ke2 & 21.26 / 24.16 / 10.66 & +5 \\
Ac-Ke3 & 8.14 / 1.92 / 1.3 & +5 \\
\hline
\end{tabular}
\caption{Calculation of alpha helicity and net charge of model peptide and its derived pseudopeptides}
\end{table}

Overall, structural studies revealed us that modification of N-terminus amide bond with ester bond did not change the structure much effectively, whereas replacement of middle position amide bond drastically reduced the α-helicity. This can be explained by the role of main chain hydrogen bonding in maintaining the secondary structure. In pseudopeptides containing ester bond, the carbonyl oxygen has less tendency for hydrogen bonding which is critical for maintaining the secondary structure.\textsuperscript{8}
8-2.3.3 Haemolytic Activity
To investigate the hemolysis on mammalian erythrocytes, the hemolytic activity of the pseudopeptides was examined on human RBCs at various peptide concentration levels (Figure 8-2.2). As envisaged, melittin, a potent lytic peptide used as a positive control, exhibited very strong hemolytic activity for most tested concentrations, and Ac-Kc and Ac-Ke1 showed a strong hemolysis with ~100 % lysis at 25µg/mL peptide concentration. However, peptide Ac-Ke3 did not show complete hemolytic activity at 100 % level in any concentration of peptide up to 200µg/mL. The trend in hemolytic activity is in best agreement with the result of structural determination measured in different lipid mimic conditions.

![Figure 8-2.2](image)

**Figure 8-2.2** Dose response curve of the hemolytic activity of the peptide and its derived pseudopeptides towards human RBCs

8-2.3.4 Antimicrobial Activities
The antimicrobial activities of the designed peptides against bacteria and fungi were determined by measuring their MIC (Table 7-2.3). Ac-Kc and Ac-Ke1 exhibited good antibacterial activity against both Gram-negative and
Gram-positive bacteria tested with MIC ranging between 3.12 and 25 μg/ml. Ac-Ke3 also exhibited activity against all the bacteria tested, but less active towards gram negative bacteria like *E. coli* and *P. aeruginosa*. All the pseudopeptides clearly possessed an excellent and broad spectrum of activities against bacteria as the naturally occurring peptides.

<table>
<thead>
<tr>
<th>Name</th>
<th>Minimum inhibitory concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>M. luteus</em> ATCC9341</td>
</tr>
<tr>
<td>Ac-Kc</td>
<td>3.12</td>
</tr>
<tr>
<td>Ac-Ke2</td>
<td>3.12</td>
</tr>
<tr>
<td>Ac-Ke3</td>
<td>12.5</td>
</tr>
<tr>
<td>melittin</td>
<td>3.12</td>
</tr>
</tbody>
</table>

Table 8-2.3 Minimal Inhibitory concentrations of the model peptide and its derived pseudopeptides; Melittin was used as a positive control.

The broad spectrum of antimicrobial activities with different bacteria can be explained by the differences in the composition of bacterial lipid membranes varying from negatively charged to neutral charge.

When we correlate the structure activity relationship between peptide and pseudopeptides, it revealed us that the maintained structure is important for maintaining the activity. Peptide Ac-Kc and Ac-Ke1 both have similar α-helicity and hydrophobicity provided similar hemolytic activity and
antibacterial activity whereas Ac-ke3 with less helicity afforded decreased hemolytic and antibacterial activities. The CD spectra of Ac-Kc and Ac-Ke1 in the presence of 50% (v/v) TFE and 25 mM SDS have similar \( \alpha \)-helicity to that of the model peptide. Several studies on peptides, where N-terminal amino acid has been substituted with d-amino acid also indicated that the N-terminal amino acid was less important in maintaining \( \alpha \)-helical structure.\textsuperscript{11c,13} Interestingly; the CD spectra of Ac-Ke3 resulted in less \( \alpha \)-helicity under these conditions. Overall, study indicated that the main chain hydrogen bonding is important for maintaining the secondary structure which is requirement for sustaining the antibacterial and hemolytic activities.

8-2.4 Conclusion
In conclusion, we have demonstrated that the main chain hydrogen bonding is important for maintaining the \( \alpha \)-helical structure. Incorporation of ester bond perturbed \( \alpha \)-helical structure and also suggest that the backbone of membrane active peptides are important in the maintenance of secondary structure for interactions with lipid membranes. \( \alpha \)-helical propensity has a substantial effect on antimicrobial activity and hemolytic activity.

8-2.5 Materials and Methods
8-2.5.1 Materials
The common reagents were already discussed. DPC, SDS micelle, Tx-100 and melittin were purchased from Antrace and Sigma Aldrich respectively.

8-2.5.2 Determination of Secondary Structure
CD spectra were recorded on a Jasco J-815 spectropolarimeter (Tokyo, Japan) using a quartz cell of 1-mm path length between 190 and 250 nm at room temperature. The concentration of peptides was 200µg/mL in 10 mM Tris-buffer (pH 7.4) containing 50% trifluoroethanol (TFE, v/v) or 25 mM sodium dodecyl sulfate (SDS) or 50 mM DPC. Two scans with a scan speed of 20nm/min were averaged for each peptide. CD spectral results were expressed as the mean residue ellipticity and the α-helix content was calculated from the mean residue ellipticity [θ] at 222 nm.

The helicity was calculated based on the equation

\[
\text{Helicity} \% = \{-([\theta]_{222} +2340)/30300\} \times 100
\]

8-2.5.3 Hemolytic Assay
The method is described in detail in the literature.\textsuperscript{14} Packed human erythrocytes were washed three times with buffer (150 mM KCl, 5 mM Tris-HCl, pH 7.4) and were then suspended in 10 volumes of the same buffer. The cell stock suspension was diluted 25-fold with the same buffer to a final erythrocyte concentration of 0.4% (v/v). Test samples were added into the erythrocyte solution. After incubation for 1 hour at 37°C, samples were centrifuged at 4,000×g for 5 min and the supernatant absorbance was determined at 540 nm. Hemolysis affected by 0.1% Triton X-100 was considered as 100%.

8-2.5.4 Antibacterial Assay
A description of an in vitro antimicrobial assay is readily available in the literature.\textsuperscript{15} An antibiotic medium 3 (M3; pH 7.0 at 25°C, Difco) was used as the antibacterial assay media. Cells freshly grown on an antibiotic medium 3 agar plate were suspended in physiological saline at 10^5 cells per 1 ml of a
2×-concentrated medium and were used as the inoculum. Peptide was added to 96-well microplates (100 μl/well) and serially diluted by twofold. Antifungal assay was done in Sabraud-2% dextrose broth (SB; pH 5.6 at 25°C, Merck) and the plates were incubated at 30°C for 24 hrs. All minimum inhibitory concentrations (MICs) were determined from two independent experiments performed in duplicate.

8.2.6 Literature Cited


FUTURE OUTLOOK

Peptides are very efficient and versatile ligands for various molecular targets with an outstanding affinity. The great structural change of the complex formation processes of peptides comes from the different arrangements of donor atoms both in the backbone and the side chains of the molecules. As a consequence, small modifications of the amino acid sequences can result in significant changes for the recognition of target. These studies we discussed in this dissertation can help to understand the metal ion-peptide interaction under biological conditions, and contribute to the design of new complexing agents with high metal ion selectivity. Furthermore, amino acid sequences can be optimized for particular target metal ion to improve selectivity and binding affinity.

From the examples published in literature, immobilization of fluorescent probes to several materials has been proven very useful in terms of device implementation because it allows the production of stable and reusable materials. Additionally, combinatorial methods to select the best system or to enhance the performance for particular target are paving the way towards efficient sensors. Among the possible substrates, immobilization of the sensing probes on surfaces will produce efficient fluorescent chemosensors because of their simplicity, efficiency, and high stability. The study conducted by us regarding the grafting of the receptor units on resin beads leads to the realization of sensing devices that can be recovered and recycled after using.

This study will open the way for further designing different peptide receptors for various molecular targets. One can design cyclic peptide to improve the binding affinity and to make it more robust chemosensor after looking the systems we studied. In addition, further modification either by appending
cell penetrating peptide sequence or by fluorophore replacement, one can design novel optimized probe for real time monitoring in cells. The separate study conducted on optimization method for synthesizing pseudopeptides will help to chemist to generate novel peptidomimetics in short time with significant yield. The structure activity relationship study for various pseudopeptides containing ester bond will permit the new design of peptidomimetic to investigate the more robust system having broad range of activities.