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Development of an Optical Method for the Detection of Homocysteine as a Disease
Biomarker Using Fluorescein-Aldehydes

by

Aabha Barve

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
in
Chemistry

Dissertation Committee:
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Portland State University
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ABSTRACT

Homocysteine is a natural occurring aminothiol. It is an intermediate product in the metabolism of methionine. Methionine is an essential amino acid required for protein synthesis. Metabolic irregularities disrupt homocysteine levels in plasma. Elevated homocysteine levels are directly linked to folate and cobalamin (vitamin B12) deficiencies, and are an independent risk factor for cardiovascular diseases. High homocysteine levels have also been associated with Alzheimer's, osteoporosis, renal failure, cancer, birth defects and pregnancy complications. The association of elevated homocysteine levels with cardiovascular disease and other diseases has generated great interest in the detection of homocysteine.

An optical method for the detection of homocysteine has been developed using fluorescein mono- and dialdehydes. Selectivity for homocysteine was achieved based on the characteristic differences between 5- and 6-membered ring heterocyclic amines formed upon the reaction with fluorescein mono- and dialdehydes. 6-membered ring homocysteine-derived thiazinane-4-carboxylic acids were found to be more basic than 5-membered cysteine-derived thiazolidine-4-carboxylic acids. Fluorescence enhancement in response to homocysteine was thus attained by tuning pH and excitation wavelengths. Furthermore, the design and synthesis of a more sensitive fluorophore, fluorescein tri aldehyde has been accomplished based on the aforementioned findings to enable the detection of homocysteine at physiological levels. Calculations of Mulliken charges revealed that the formation of thiazinanes results in modulation of the electron density on the fluorophore leading to higher fluorescence.

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TABLE OF CONTENTS

ABSTRACT.....	i
ACKNOWLEDGEMENTS.....	ii
LIST OF TABLES.....	vii
LIST OF FIGURES	viii
LIST OF ABBREVIATIONS.....	xi
CHAPTER 1 INTRODUCTION	1
1.1 Hcy, Cys and GSH: structural features and functions.	1
1.2 Metabolism of Hcy	3
1.3. Hcy and human health	8
1.3.1 Hcy: A biomarker or a risk factor!.....	13
CHAPTER 2 AN OVERVIEW – TECHNIQUES INVOLVED IN HCY DETECTION	17
2.1 Introduction.....	17
2.2 Existing methods for the detection of Hcy	17
2.3 Methods based on electrochemistry and nano materials.....	19
2.4 Optical methods.	22
2.4.1 Optical detection based on the redox chemistry of Hcy	22
2.4.2 Methods for detection of Cys and Hcy based on Schiff base formation and subsequent cyclization to form a heterocycle.	24

CHAPTER 3 SELECTIVE DETECTION OF HCY USING FLUORESCINE ALDEHYDES.....	27
3.1 Background.....	27
3.2 Mechanism of signal transaction in aldehyde bearing fluorophore probes upon reaction with aminothiols.....	30
3.3 Previous work- response of fluorescein aldehydes to Cys and Hcy	31
3.4 Present work – results and discussion.....	34
3.5 Conclusion	45
3.6 Experimental section.....	46
3.6.1 Materials	46
3.6.2 Methods.....	46
3.6.3 Spectrophotometric measurement.....	47
3.6.4 Deproteinization of plasma	47
CHAPTER 4 MECHANISTIC STUDIES.....	48
4.1 Introduction.....	48
4.2. Result and discussion.....	48
4.2.1 Synthesis of Cys- and Hcy-derived analogs 4.1, 4.3, 4.5, 4.2, 4.4 and 4.6.....	49
4.2.1 Estimated pK_a values of Cys- and Hcy-derived analogs 4.1, 4.3, 4.5, 4.2, 4.4 and 4.6.....	49
4.3 Conclusion	61

4.4 Experimental section.....	61
4.4.1 Materials	61
4.4.2 Methods.....	61
4.4.3 Spectrophotometric measurement.....	62
4.4.4 pK_a measurements.....	62
4.4.5 Synthesis of compounds 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6.....	63
CHAPTER 5 SYNTHESIS OF FLUORESCEIN TRI ALDEHDYE AND ITS APPLICATION IN THE DETECTION OF HCY	67
5.1 Introduction.....	67
5.2 Duff reaction and its application.....	67
5.3 Result and discussion.....	69
5.5 Conclusion	80
5.4 Experimental section.....	81
5.4.1 Materials	81
5.4.2 Methods.....	81
5.4.3 Spectrophotometric measurement.....	82
5.4.3 Deproteinization of plasma	82
5.4.4 Mulliken charges calculation	82
5.4.5 Synthesis of 5.1	82

REFERENCES	84
APPENDIX A : CHARACTERIZATION OF COMPOUNDS 4.1 AND 4.2	95
APPENDIX B : CHARACTERIZATION OF COMPOUND 4.3 AND 4.4.....	98
APPENDIX C : CHARACTERIZATION OF COMPOUND 4.5 AND 4.6.....	101
APPENDIX D : CHARACTERIZATION OF COMPOUNDS 5.1	104
APPENDIX E : COLORIMETRIC RESPONSE OF THIOLS TOWARDS 5.1	106
APPENDIX F : ENERY MINIMIZED SIMULATED 3D STRUCTURE OF 5.1, 5.1A AND 5.1B'	107

LIST OF TABLES

Table 4.1. Estimated pK_a values of the protonated amine moiety of 2-substituted thiazolidine/thiazinane-4-carboxylic acids.	51
Table 5.1 Comparison Mulliken charges on the 'N' and 'S' of Cys and Hcy derived heterocycles formed upon reaction with 5.1	79
Table 5.2 Comparison Mulliken charges on the phenolate 'O' and ether 'O' of 5.1 and Cys and Hcy derived heterocycles formed upon reaction with 5.1	80

LIST OF FIGURES

Figure 1-1. Structures of selected biological thiols	1
Figure 1-2. Homocysteine-methionine metabolism.....	5
Figure 1-3. Redox and methylation reaction mechanism of methionine synthase:	7
Figure 2-1. Structure of desyl derivative probe DN-2	24
Figure 2-2. Structure of phosphorescence sensor for Hcy based on iridium (III) complex, Ir(pba) ₂ (acac) (Hpba = 4-(2-pyridyl)-benzaldehyde; acac = acetylacetone)	25
Figure 2-3. Structures of P-Hcy-1 and P-Hcy-2.	25
Figure 2-4. Structure of aldehyde bearing bodipy based sensor for Hcy detection	26
Figure 3-1. General reaction scheme for reaction of carbonyl with Cys and Hcy.....	27
Figure 3-2. Mechanism of signal transduction in generic aldehyde-bearing aminothiols probes.	30
Figure 3-3. Change in the absorbance spectra of 3.3 upon addition of Cys in buffer, pH 9.5 at room temperature.	33
Figure 3-4. Quenching of the fluorescence emission of 3.3 upon addition of Cys, excited at 460 nm, pH 9.5 at room temperature	33
Figure 3-5. Quenching of the fluorescence emission of 3.3 upon addition of Hcy , excited at 460 nm , pH 9.5 at room temperature	34
Figure 3-6. Optical sensing behavior of 3.1 towards Hcy and Cys at pH 9.5.....	36
Figure 3-7. Optical sensing behavior of 3.1 towards Hcy and Cys at pH 5.5.....	36
Figure 3-8. Optical sensing behavior of 3.1 towards Hcy and Cys at pH 6.0.....	37
Figure 3-9. Optical sensing behavior of 3.1 towards Hcy and Cys at pH 9.5.....	38

Figure 3-10. Optical sensing behavior of 3.1 towards Hcy and Cys at pH 5.5.....	39
Figure 3-11. Optical sensing behavior of 3.1 towards Hcy and Cys at pH 6.0.....	39
Figure 3-12. Optical sensing behavior of 3.1 towards Cys and Hcy as compared to other biologically relevant species.	40
Figure 3-13. ^1H NMR of 3.1, 3.1 in the presence of Hcy (3 equiv) and 3.1 in presence of Cys (3 equiv) at pH 6.0.	41
Figure 3-14. ^1H NMR of 3.1 and 3.1 in the presence of Gly (3 equiv) at pH 6.....	42
Figure 3-15. Optical sensing behavior of 3.3 towards Hcy and Cys at pH 6.0.....	43
Figure 3-16. Optical sensing behavior of 3.3 towards Hcy and Cys at pH 6.0.....	43
Figure 3-17. Optical sensing behavior of 3.3 towards Cys and Hcy at their biological concentrations at pH 6.0.	44
Figure 3-18. Spectral response of 3.3 towards increasing levels of Hcy in phosphate buffer (100 mM, pH 6.0).....	44
Figure 3-19. Optical sensing behavior of 3.3 towards Cys and Hcy in deproteinized bovine plasma at pH 6.0.....	45
Figure 4-1. Average of triplicates of (a) pH titration curve and (b) derivatives of 4.1 and 4.2.....	52
Figure 4-2. Average of triplicates of (a) pH titration curve and (b) derivatives of 4.3 and 4.4.....	53
Figure 4-3. Average of triplicates of (a) pH titration curve and (b) derivatives of 4.5 and 4.6.....	54
Figure 4-4. ^1H NMR of salicylaldehyde-Cys derived thiazolidines 4.1 and	

salicylaldehyde-Hcy derived thiazinanes 4.2 in D ₂ O:H ₂ O	55
Figure 4-5. Time dependent fluorescence spectral changes and enhancement factor (inset) of fluorescence of 3.1 with Cys and Hcy,(a) pH 5.5, (b) pH 6.0, (c) pH 6.5, (d) pH 7.0, (e) pH 7.4. (f) comparison of fluorescence response of 3.1 towards Cys and Hcy at different pH.....	60
Figure 5-1. Optical sensing behavior of 5.1 towards Hcy and Cys at pH 6.0.....	71
Figure 5-2. Comparison of magnitude of response of Hcy towards (a) 5.1, (b) 3.3, (c) 3.1.	72
Figure 5-3. Optical sensing behavior of 5.1 towards Cys and Hcy at their biological concentrations at pH 6.0.	73
Figure 5-4. Optical sensing behavior of 5.1 towards Hcy and Cys with and without amino acids and GSH at pH 6.0.....	74
Figure 5-5. (a) and (b)- Spectral response of 5.1 towards increasing levels of Hcy in phosphate buffer (100 mM, pH 6.0 at 2h).	75
Figure 5-6. Optical sensing behavior of 5.1 towards Cys and Hcy in deproteinized plasma at pH 6.0.after 2h.	76
Figure 5-7. Optical sensing behavior of 5.1 towards Cys and Hcy in deproteinized plasma at pH 6.0.after 2h.	77
Figure 5-8. Optical sensing behavior of 5.1 towards Cys and Hcy in buffer at different pH, after 2h.	78
Figure 5-9. Predominant species of compounds 5.1, 5.1a and 5.1b at pH 6.0. Calculated Mulliken charges are shown for the oxygen atoms in the fused ring system.	80

LIST OF ABBREVIATIONS

Hcy	Homocysteine
Cys	Cysteine
GSH	Glutathione
Met	Methionine
SAHH	S-adenosylhomocysteine hydrolase
SHA	S-adenosyl-Hcy
MAT	Methionine adenosyltransferase
MTHFR	Methylenetetrahydrofolate reductase
CBS	Cystathionine β - synthase
S-AdoMet	S-adenosylmethione
BMT	Betaine methyl transferase
S-AdoHcy	S-adenosylhomocysteine
SAC	S-adenosyl-cysteine
CVD	Cardiovascular diseases
AD	Alzheimer's disease
NTD	Neural tube defects

TG	Thyroglobulin
SAC	S-adenosyl-cysteine
LDA	Lactate dehydrogenase
MSN	Mesoporus silica nanoparticle
PEI	Polyethyleneimine
RSL	Resonance light scattering technique
MWCNT	Multiwalled carbon nanotubes
CPE	Carbon paste electrode
HBA	Homocysteine biosensor array
PET	Photoinduced electron transfer
ICT	Intramolecular charge transfer
AAOx	D-amino acid oxidase
SAH	S-adenosylhomocysteine
FMA	Fluorescein mono aldehyde
FDA	Fluorescein di aldehyde
FTA	Fluorescein tri aldehyde
HMTA	Hexamethylenetetraamine

CHAPTER 1 INTRODUCTION

The low molecular weight thiols homocysteine (Hcy), cysteine (Cys) and glutathione (GSH) play a crucial role in metabolism and homeostasis. They are vital cell components that serve as antioxidants to maintain the redox balance in the human body. Aberrant levels in their concentrations results in metabolic disturbances that are linked to several diseases [1].

1.1 Hcy, Cys and GSH: structural features and functions.

Hcy is a sulfur containing non-essential amino acid. It is a metabolic intermediate derived from the essential sulfur containing amino acid methionine (Met). Hcy shares structure resemblance with another low molecular weight thiol, Cys, with the exception that it contains one more methylene group in its side chain, as shown in Figure 1-1. Thiol-like properties of Hcy are common to GSH and Cys.

Figure 1-1. Structures of selected biological thiols

Hcy is present in various forms in human plasma. Total Hcy is the sum of all different species of Hcy i.e. protein bound, oxidized and reduced or free forms and it is

referred as **tHcy** [2]. The major form is disulfide bound to proteins, mainly albumin. The protein bound form of Hcy constitutes 70-80 % of total Hcy. Other oxidized forms such as Hcy-Hcy (Hcy disulfide) accounts for 5-10%, Hcy-Cys disulfide accounts for 5-10% of the tHcy, and reduced Hcy sulfhydryl accounts for less than 1% in human plasma. In general, the levels of tHcy in a healthy humans is approximately 5-15 μM , and increases with ageing. The condition in which the level of Hcy in human plasma rises above 15 μM is called 'hyperhomocysteinemia'. There are three stages of hyperhomocysteinemia; mild, in which the Hcy level is 15-30 μM ; moderate, in which the level is 30-100 μM and for the level above >100 μM of Hcy, it is termed as severe hyperhomocysteinemia [3].

Cys is the lowest molecular weight biological thiol. Among the three major biological thiols, cysteine is the most abundant extracellular thiol. It varies from 150-300 μM [4, 5]. Cys is produced during Hcy metabolism. Therefore, Hcy and Cys levels are highly related to each other. Cys plays an essential role in protein synthesis, redox chemistry and peptide cross-linking, detoxification and other metabolic processes [6, 7].

GSH is a tripeptide wherein glycine and Cys are linked by normal peptide linkage and amine group of Cys is attached to the carbonyl group of glutamate side chain *via* gamma peptide linkage. Thus, GSH is indirectly linked to homocysteine metabolism. It is known for its antioxidant properties. It is also involved in gene expression, cell proliferation and apoptosis [8]. GSH concentrations are very high in cells. They range from 1 to 5 mM, whereas its concentration in plasma is 1 to 6 μM [5, 8].

1.2 Metabolism of Hcy

The metabolism of Hcy is intricately linked to the metabolic pathway of methionine, one of the essential amino acids [9]. Hcy is thus a vital part of cellular homeostasis in human body. Methionine is essential for protein synthesis; it acts as a methyl donor for the synthesis of a wide range of vital methylated compounds such as DNA, RNA etc. [10]. Hcy is the key intermediate of the transsulfuration pathway to produce several other sulfur-bearing amino acids such as, cystathionine, Cys, taurine, GSH, etc. [11]. Hcy is the foremost source of sulfur for inorganic sulfate. It also is linked to polyamine biosynthesis. Furthermore, Hcy plays a crucial part in folate metabolism, as well as in the catabolism of choline and betaine [10].

Hcy metabolism is shown in Figure 1-2. Hcy levels depend on the proper functioning of the enzymes involved in its metabolism. These include enzymes involved in the remethylation reaction pathway to form methionine (Met), such as betaine methyltransferase (BMT), *S*-adenosylhomocysteine hydrolase (SAHH), methionine synthase (MS), methionine adenosyltransferase (MAT) and *S*-adenosylmethione (*S*-AdoMet)-dependent methyl transferases e.g., glycine methyltransferase. Methionine synthase depends on methionine synthase reductase, vitamin B12 and 5-methyltetrahydrofolate obtained from methylenetetrahydrofolate reductase (MTHFR). A second pathway in Hcy metabolism is transsulphuration to produce cystathione, Cys and inorganic sulphates. This latter pathway mainly involves the enzyme cystathionine β -synthase (CBS) [12].

In the remethylation step, Hcy is converted to Met by a complex 5-methyltetrahydrofolate- dependent reaction [13]. 5-methyl tetrahydrofolate is produced by 5,10-methylenetetrahydrofolate in the presence of MTHFR. The remethylation reaction is catalyzed by MS. This enzyme gets reductively methylated by methionine synthase reductase. The synthesis of methionine synthase reductase requires cobalamine (vitamin B12) and *S*-AdoMet. Additionally, Hcy can be remethylated to Met in a process promoted by betaine *via* betaine methyl transferase (BMT). Remethylating enzyme BMT is only found in liver, kidney and brain. MS, however, it has a wide tissue distribution [14].

Met is transformed to its active form *S*-AdoMet. This reaction is catalyzed by methionine adenosyl transferase (MAT) [13]. *S*-AdoMet acts as a methyl donor for the production of a large number methylated biomolecules such as methylated DNA, RNA, lipids and proteins [14]. To date, 39 *S*-AdoMet dependent methyl transferases have been discovered in mammals [1]. After donating a methyl group, *S*-AdoMet becomes *S*-adenosylhomocysteine (*S*-AdoHcy). To complete the Hcy metabolism cycle, *S*-AdoHcy undergoes hydrolysis to produce Hcy and adenosine. The enzyme Ado-Hcy hydrolase catalyzes this hydrolysis [14]. *S*-AdoMet also undergoes decarboxylation to provide a propyl amine moiety for the synthesis of spermine and spermidine[13]. However, this reaction consumes less than 10% of the total *S*-AdoMet[15]. (Figure 1-3)

Figure 1-2. Homocysteine-methionine metabolism.

Enzymes involved in Hcy metabolism : MAT-methionine adenosyltransferase, X-MT-various *S*-AdoMet dependent methyltransferases, SAHH-S-adenosylhomocysteine hydrolase, MS-methionine synthase, BMT-betaine methyltransferase, MTHFR methylenetetrahydrofolate reductase, CBS cystathionine β-synthase, γCL, γ-cystathionase, SHM serine hydroxymethylase. This figure was adopted from [14] and redrawn with some modifications.

In the alternate Hcy transsulfuration [11] pathway, the first step involves condensation of Hcy with serine to produce cystathionine. This condensation takes place in the presence of cystathionine β-synthase (CBS). Pyridoxal 5'phosphate (vitamin B₆) is a cofactor for CBS. In the second step, cystathionine converts to Cys and a by-product α-oxo butyrate. The enzyme β- cystathionase catalyzes this step and, like CBS, requires pyridoxal 5'phosphate [14]. Cys is essential for the synthesis of the antioxidant glutathione. By a variety of enzymatic reactions and oxidation, cysteine produces

inorganic sulfates [16].

1.2.1 Summary of enzymes involved in the regulation of Met and Hcy

(1). ATP: L-Methionine *S*-Adenosyltransferase: This enzyme facilitates the formation of vitally important *S*-AdoMet by catalyzing the transfer of adenosyl moiety from ATP to Met [14]. This reaction takes place in the presence of Mg^{2+} and K^{+} [17]. Two of the forms of *S*-AdoMet are specific to liver i.e. a tetramer MAT(I), which is an α form and a dimer MAT(III), which is a β form. The gene *MAT1A* encodes both forms in the liver. This gene is capable of reacting with high Met immediately.

(2). *S*-AdoMet-dependent methyltransferases (glycine N-methyltransferase GNMT): GNMT is one of the *S*-AdoMet-dependent methyltransferases that converts glycine to sarcosine by the transfer of a methyl group from *S*-AdoMet [14]. By donating a methyl group, *S*-AdoMet transforms to *S*-AdoHcy. *S*-AdoHcy is an inhibitor of the enzyme MS. Any disturbance in the ratio of *S*-AdoMet to *S*-AdoHcy affects the activity of MS [14].

(3). *S*-AdoHcy Hydrolase: This enzyme has a binding site for adenosine. Therefore, it facilitates *S*-AdoHcy hydrolysis to form Hcy and release adenosine [9].

(4). Methionine Synthase (5-Methyltetrahydrofolate: Homocysteine Methyltransferase): This enzyme catalyzes the remethylation reaction to form Met [9]. MS assists the transfer of the methyl group from 5-methyl THF to Hcy to form Met. MS maintains the folate and Met cycle to endow enough Met for *S*-AdoMet-dependent transmethylation reactions [14]. This enzyme encompasses zinc and four domains to bind Hcy, *S*-AdoMet, cobalmine cofactor and methylene tetrahydrofolate [18].

(5). Methionine Synthase Reductase (Methionine Synthase Cob(II)alamine Reductase): MS would be inactive if the cobalamine factor exists as cob(II)alamine [14]. To activate methionine synthase, cob(II)amine needs to be reduced to cob(I)alamine. Reduction requires conversion of *S*-AdoMet to *S*-AdoHcy. This overall process is expedited by the enzyme methionine synthase reductase [14]. A complete reaction mechanism is described in Figure1-4.

Figure 1-3. Redox and methylation reaction mechanism of methionine synthase: methionine synthase (MS) reductase; and tetrahydrofolate (THF). This figure was adopted from [14] and redrawn with some modifications.

(6). Betaine Homocysteine Methyltransferase (BMT): This enzyme also transforms Hcy to Met [14]. Betaine acts as a methyl donor in the process [13]. Betaine is the product of the catabolism of choline [13]. Betaine methyltransferase is mainly present in the liver and the kidney.

(7) 5,10 Methylene tetrahydrofolate Reductase (MTHFR): 5-methyltetrahydrofolate is synthesized from 5,10-methylene tetrahydrofolate with the help of MTHFR [9]. This NADPH-associated reduction process provides a methyl group for vital compounds such as DNA and RNA and controls the folate coenzyme entry in the remethylation pathway for Hcy metabolism [14].

(8). Cystathionine β -synthase (CBS): L-cystathionine, the precursor for the synthesis of Cys, is produced by the condensation of Hcy and serine [14]. This condensation is expedited by CBS which is a cytosolic pyridoxal 5' phosphate-requiring enzyme. This enzyme mainly occurs in human liver [14].

1.3. Hcy and human health

As described in the previous section, Hcy is the key intermediate in the Met and Cys synthesis. Any alternation in these two metabolic pathways i.e. remethylation and transsulfuration, may result in abnormal levels of Hcy. Elevated levels of Hcy are directly linked to a number of pathologies mainly cardiovascular diseases [19], Alzheimer's [20], birth defects and pregnancy complications [21], osteoporosis [22], renal failure [23], cancer [24], inflammatory bowel disease [25], and cancer [24]. Normal Hcy levels can be disturbed by several factors such as a deficiency of enzymes involved in Hcy metabolism (for example cystathionine β -synthase (CBS), MTHFR, MS) [11], inadequacy of vitamin B₆, B₁₂ and/or folic acid [2], overburdening of dietary methionine and buildup of excessive reactive oxygen species (ROS) [3]. Other factors that affect Hcy homeostasis include heavy smoking, caffeine and alcohol consumption, specific drugs, renal dysfunction, life style, age and gender [26]. A deficiency of folate or defects in MTHFR

alter the formation of 5-methyltetrahydrofolate that results in lower concentrations of *S*-AdoMet and overloading of Hcy for the transsulfuration pathway. Furthermore, insufficient levels of *S*-AdoMet also suppress the transsulfuration pathway, and this overall process leads to the accumulation of excessive Hcy in the cells, followed by its transfer to the blood stream producing hyperhomocysteinemia [11]. It is also caused by defects in CBS, responsible for transsulfuration pathway, which forces Hcy to proceed *via* remethylation to produce excess *S*-AdoMet. This eventually leads to feedback inhibition of MTHFR impeding the remethylation process. This causes severe hyperhomocysteinemia wherein the entire Hcy metabolism is impaired [11]. A number of diseases associated with hyperhomocysteinemia are listed below.

Cardiovascular diseases (CVD)

Hcy has been established as an independent risk factor for cardiovascular diseases. A number of published studies indicate the direct link between CVD and elevated levels of Hcy [27-30]. According to an early study by WHO, CVD mortality rate and Hcy levels are closely related to each other.

In 1969, McCully published a report about two patients, 2 months and 8 years old, with homocystinuria. Homocystinuria is a form of very severe hyperhomocysteinemia wherein homocysteine overload leads to its excretion in urine. McCully's young patients suffered from premature atherosclerosis due to a deficiency of CBS [31]. Since that time, a wide variety of studies have been published suggesting modestly higher level of Hcy are a risk factor for atherosclerosis [32]. An association

between plasma Hcy and mortality rate was published by Refsum *et al.* Their study revealed that the mortality rate was 24.7 % in CVDs patient with plasma Hcy >15 μ M [19]. Recently, Tentolouris *et al.* indicated that acute Met-induced hyperhomocysteinemia affects myocardial performance and impairs aortic distensibility [27].

The occurrence of hyperhomocysteinemia is assessed to be 5% in the general population and 13% to 47% among patients suffering from symptomatic atherosclerotic vascular disease [26]. A meta-analysis of cross-sectional epidemiologic studies revealed that a 60% higher occurrence of ischemic heart disease was noticed with increase in each 5 μ M concentration level of plasma Hcy [33]. Cross-sectional studies and prospective studies both confirm that the incidence of myocardial infarction was increased by 3.4 fold when the concentration of Hcy was more than 16 μ M. The mortality rates of CVD patients was 4.5 fold higher at a Hcy concentration higher than 20 μ M [34].

A multivariate analysis conducted by Boutouyrie *et al.* indicated that out of Hcy, Cys and GSH, only Hcy was directly linked with carotid artery remodeling, suggesting that Hcy is the only plasma thiols that acts as an independent risk factor for CVD [35]. Meta-analysis studies presented a correlation of plasma Hcy concentration with ischaemic heart disease, deep vein thrombosis, pulmonary embolism and stroke [32]. Both genetic and prospective studies revealed lowering Hcy concentration by 3 μ M reduced the risk of ischaemic heart diseases by 16%, deep vein thrombosis by 25 % and stroke by 24 % [32]. Also, a prospective study conducted on women with a risk of myocardial infarction found that fasting Hcy levels are positively related with myocardial infarction risk in women [36].

Alzheimer's disease (AD)

Hyperhomocysteinemia has been also considered to be an independent risk factor for cognitive impairment, dementia and Alzheimer's disease (AD) [20]. According to the National Health and Nutrition Examination Survey (NHANES III), hyperhomocysteinemia is directly linked to weak memory in elderly people. A survey was conducted on participants over 60 years of age. Participants with greater Hcy levels and lower folate levels received a lower score on short delayed recall tests [37].

AD has been associated with an increased level of Hcy, along with a deficiency of vitamin B₆, B₁₂ and folic acid [38]. Elevated levels of Hcy and S-AdoHcy deter the remethylation processes of Met and S-AdoMet. This affects the methylation of various vital components such as DNA and protein, eventually damaging the vascular structure, neurotoxicity and causing apoptosis.

According to Lehman *et al.* unusual high levels of Hcy were found in patients suffering from AD as compared to a control group [39]. Ueland *et al.* published a study that presented considerably higher levels of Hcy along with a deficiency of folate and vitamin B12 in the AD patients. It also revealed that slight increases in Hcy levels have a strong effect on the risk of AD, and are 4.5 times greater in patients with Hcy levels $\geq 14\mu\text{M}$ compared to patients with $\leq 11\mu\text{M}$ [40]. Another case study conducted at the interval of 8 years on 1092 elderly patient stated that risk of AD was increased by 40% with 5 μM increases in Hcy concentrations, whereas risk was doubled at Hcy concentrations $\geq 14\mu\text{M}$ [20].

Neural tube defects (NTD)

It is well known that the intake of folic acid during pregnancy can reduce the risk of neural tube birth defects. Levels of folate and vitamin B₁₂ were found to be lower in the women carrying fetuses with NTD than the women with normal fetuses [41]. However, despite folate and vitamin B12 supplements, the risk of NTD remains elevated indicating that other metabolic factors are also responsible for this defect [42]. A case study reported by Scott *et al.* showed abnormal levels of Hcy present in the women with NTD fetuses. According to the authors, the abnormal levels were related to the defects in the enzyme methionine synthase [43]. This enzyme is necessary for methylation reaction such as DNA and myelin a basic protein, which may be a possible mechanism for NTD. Another factor responsible for NTD is the C677T and A1298C mutations in the (MTHFR) gene [44]. This can be regulated by folate supplement. However MTHFR polymorphism is a moderate risk factor for NDT in comparison to the methionine synthase.

Osteoporosis

In a 2005 review Herrmann *et al.* described a direct link between osteoporosis and Hcy levels. They explained that Hcy is not merely a risk indicator, it is also involved in bone metabolism [22]. A study conducted by Mudd *et al.* shows that ‘bone mineral density’ (BMD) was found to be lower in patients suffering from homocystinuria. Moreover, a risk of osteoporosis was found to be 50% by the age of 16 [45]. Studies conducted on large populations revealed that increases in Hcy levels elevate the risk of fragile bone

fractures [46, 47] Abnormal skeletal growth, skeletal deformities, an elongated appendicular skeleton and flattening of the vertebral bodies have been observed in homocystinuric patients [48-50]. According to a double-blind placebo-controlled intervention trial conducted by Sato et al. "Hcy lowering therapy treatment (5 mg of folate and 1500 µg of vitamin B12) was found to be effective in reducing the hip fracture rate in hemiplegic stroke patients [51]. The role of Hcy in osteoporosis is not clearly understood. It is believed that Hcy can affect bone metabolism in different ways; for instance, it can inhibit osteoblast formation and accelerate the osteoclast formation thereby affecting bone resorption. It can also lead to decreased bone blood flow. It inhibits collagen cross-linking, thereby reducing bone extracellular matrix (ECM) quality [22].

In addition to the aforementioned disorders, Hcy has been linked to inflammatory bowel disease [25], diabetes [52] and cancer [24].

1.3.1 Hcy: A biomarker or a risk factor!

Several clinical studies have successfully demonstrated a strong link between Hcy with CVD [19]. However, it has been a subject of debate from decades, whether Hcy is merely a biomarker or in fact a risk factor [53]. A proper mechanism for the role of Hcy in CVD has not been clearly understood. The efficiency of folic acid supplementation in stroke prevention was examined by a meta-analysis study [54]. The results indicated that folic acid supplementation may effectively reduce the risk of stroke for the patient [54]. A randomized controlled trial was conducted by Hess *et al.* on 553 patients to study the effect of Hcy lowering therapy by a combined supplement of folic acid, vitamin B6 and

B12. The results revealed that after percutaneous coronary intervention, the frequency of major adverse events was substantially reduced [55]. Improvement in the stroke mortality was also reported in a government study after folic acid fortification in United States and Canada [56].

Nevertheless, another meta-analysis of randomized and controlled trial conducted by Saver *et al.* showed that folic acid and vitamin B12 combined together provided a very mild benefit to prevent primary stroke in male patient [57]. Similarly, McQueen *et al.* investigated the efficacy of Hcy-lowering therapy on stroke risk, severity and disability. Their 5 year study on 5522 adults with CVD demonstrated that a combined supplement of folate and vitamin B decreased the frequency of overall stroke but stroke severity and disability remained unaffected [58]. On the other hand, supplementation of vitamin B12 along with folate as Hcy-lowering therapy for secondary prevention in patients that suffered from acute myocardial infarction was published by Rasmussen *et al.* Their investigation revealed Hcy-lowering therapy had no impact on the risk of recurring CVD after myocardial infarction, in fact vitamin B12 had an adverse effect [59]. Similar cases were reported by Ebbing *et al.* where secondary prevention by folic acid and vitamin B supplementation in patients with coronary artery disease or aortic valve stenosis was found to be ineffective on CVD and total mortality [60]. Possible reasons for the failure of Hcy lowering therapy were explained by Blom *et al.* These include (1) the duration of these trials and interventions were not long enough to decide whether Hcy lowering therapy is helpful to prevent the risk of CVD. Most of these trials were conducted for less than 5 years. Since atherosclerotic plaque develops 30-40 years from initial growth to

clinical trials, Hcy lowering therapy may require a much longer period of trial than a relatively few years of study[61]. Reason two is that the majority of interventions understudied patients with greater levels of homocysteinemia ($> 20\mu\text{M}$), as mostly patients with just slightly higher levels of Hcy were included in the trials. Reason 3 is that the adverse effects of folate and vitamin B supplements were not taken into consideration in aggravating CVD. Fourth, a better understanding of the role of Hcy in damaging the vascular system is needed so that a proper study and treatment can be designed, apart from folate and Vitamin B supplementation. A fifth reason given is that Hcy lowering therapy was found to be ineffective because patients already had advanced CVD with a vascular system so impaired that folate and vitamin B supplementation was insufficient or too late to control the disease [61].

Regardless of whether Hcy is a biomarker or risk factor, a strong correlation between Hcy and CVD as well as other diseases generates a great demand to determine Hcy levels. Therefore, in this study, a method to detect Hcy has been developed.

The main objectives of this project are:

1. Development of an optical method and design of a sensor for selective detection of Hcy.
2. To detect Hcy at its physiological levels in addition to the elevated levels.
3. To achieve a selective response of Hcy in presence of other related potentially interfering analytes, especially Cys, as well as in biological media.

This thesis has been divided into five chapters. After an introduction in the first chapter, an overview of the current state of the art of techniques for Hcy detection is described in the second chapter. The third chapter explains the development of a novel optical method for the selective detection of Hcy using aldehyde bearing fluorophores as sensors. In the fourth chapter, the mechanism behind the selectivity for Hcy is described. The fifth chapter includes the design and synthesis of a novel aldehyde bearing fluorophore to achieve a sensitive and selective fluorescent signal in response to Hcy at biologically-relevant concentrations.

CHAPTER 2 AN OVERVIEW – TECHNIQUES INVOLVED IN HCY DETECTION

2.1 Introduction

As describes in chapter one, elevated levels of Hcy are linked to a number of diseases, especially cardiovascular diseases. The association of Hcy levels with such life threatening diseases has generated a great interest in the scientific world to develop different methodologies for detection of this biomarker[62]. For example, high performance liquid chromatography (HPLC) [63-65], liquid chromatography/mass spectroscopy (LCMS) [66, 67], electrochemical [68-70], enzymatic [71, 72], and immunoassay [73] [72] and optical methods [74-79] have been employed to detect Hcy.

2.2 Existing methods for the detection of Hcy

In current diagnostic laboratories, techniques such as HPLC [80], LCMS [81], enzymatic procedures [82-84], and immunoassays [85-87] are more prevalent for detection of Hcy. Their popularity is due to the sensitive and precise measurements of Hcy. However, these techniques also suffer from some disadvantages. For example, HPLC and UHPLC involve an additional derivatization step with a suitable reagent, and also require thorough sample preparation and labor intensive work with expensive instruments [2, 62, 88-90]. LC/MS involved complicated sample preparation methods and expensive instruments [67, 91].

The first immunoassay method was developed by Shipchandler and Moore in 1995 [92]. tHcy is reduced to free Hcy by a suitable reducing agent. Next, Hcy is

enzymatically transformed to *S*-adenosyl-Hcy (SAH) by the action of *S*-adenosyl-Hcy hydrolase (SAHH). Anti SAH antibodies are used for the immunoassay of SAH *via* different techniques such as fluorescence polarization immunoassay (FPIA), chemiluminescence immunoassay (ICL), enzyme-linked immunoassay (EIA), the immune precipitation assay (IP) [93]. For instance, Zappacosta *et al.* used anti-*S*-AdoHcy antibodies bound to polystyrene particles to bind SAH [73]. SAH had to compete for anti-SAH antibodies with thyroglobulin (TG) conjugated *S*-adenosyl-cysteine (SAC). The presence of SAH caused a weaker aggregation of polystyrene particles. In absence of SAH, TG-conjugated SAC induces a complete aggregation of polystyrene particles. The signal is measured by the scattered light which is inversely associated to SAH content [73]. However, the accuracy and adequate specificity of immunoassay techniques have been questioned in the past [94-96]. These methods suffer from the interferences such as effects of anticoagulants, icterus, hemolysis, lipemia, and sample storage [94, 95, 97]. Apart from that, analyte dependent interferences are produced by the interaction of sample component with one or more antibodies such as human anti-animal antibodies, auto-analyte, heterophilic antibodies, rheumatoid factor and other proteins [94, 95]. These interferences may lead to false results.

In enzymatic procedures, cystathionine - β -synthase (CBS) was used to synthesize cystathionine from Hcy and serine [71]. With the help of γ -cystathionase lyase, cystathionine was converted to Cys, ammonina and pyruvate. Using lactate dehydrogenase (LDA), the reduction of pyruvate was carried out along with the oxidation of NADH to NAD. The oxidation of NADH is measured by the spectroscopic methods

[71]. Immunoassays and enzymatic procedures also require fragile and relatively expensive biological material such as antibodies and other biomolecules. Therefore, their proper washing, handling and storage is necessary [2, 83]. Regardless of these complications, enzymatic procedures are the more commonly used methods on the clinical level compared to immunoassays, HPLC and LCMS. The requirements of small amounts of reagents, less complicated procedures, sensitivity and high output make enzymatic procedure a method of interest for Hcy detection for regular clinical applications [71, 73, 83]. However, a comparative study of all these techniques for tHcy determination shows imprecision and variations in the results [98].

2.3 Methods based on electrochemistry and nano materials

Other techniques for Hcy detection have been reported in the literature that includes, the use of nano particles [99, 100] and electrochemical techniques[69, 88, 100, 101] etc. Recently, a silica nanoparticle based sensor was reported by Wu *et al.* [102]. They developed a mesoporous silica nanoparticle (MSN) based nanostructure as fluorescence sensor for detection of Hcy. They prepared a fluorescence sensor by embedding the anthracene nitroolefin inside the MSNs. In the absence of biothiols, the electron deficient nitroolefin moiety quenches the fluorescence of electron rich anthracene scaffold *via* intramolecular charge transfer (ICT). Later, Michael addition of thiols to the nitroolefin moiety prevents ICT, resulting in fluorescence enhancement. Thus the probe acts as a fluorescence sensor for biological thiols. Selectivity for Hcy was achieved with the help of covalently bound polyethylene glycol (PEG 5000) chains on the surface of the MSNs. Based on the polarity and the size of the molecules, these chains serve as a barrier for the

diffusion of different biological thiols inside the mesoporus nanoparticle. According to them, relative lower content of polar groups and smaller size make Hcy best candidate among all biological thiols, to pass through the barrier fastest and give a fluorescence turn on signal by Michael addition reaction. However size and the polarity of Cys is similar to that of Hcy but no explanation was given why Cys was not able to pass the barrier. The sensor responded well towards Hcy, however equimolar concentrations of other thiols such as Cys and GSH were used in the detection method. Therefore, Cys is expected to cause interference at physiological levels, making the sensor ineffective for specific responses to Hcy in plasma.

Scheme 2-1. Michael addition of Hcy on the nitroloefin moiety of 2.1 to prevent ITC that results in the fluorescence of 2.2.

Yan *et al.* designed polyethyleneimine capped Ag-nanoclusters for Hcy detection by resonance light scattering technique (RSL) [99]. Hcy served as a bridge between the Ag core and polyethyleneimine (PEI) chains. This linking was due to the affinity of SH group of Hcy for the Ag core and the ionic interaction of the carboxylate group of Hcy with hyperbranched PEI chains. This resulted in the formation of a Ag-nanocluster assembly. These nanoclusters interacted with one another to produce oscillation coupling, enhancing the RSL signal. This assembly showed selectivity for Hcy over Cys and

glutathione. However the instability of the Ag-particles makes it difficult to run the Hcy assay in practice [103].

Several electrochemical detection techniques for Hcy detection have also been reported in the literature [69, 88, 100, 101]. For example, Campton *et al.* achieved responses for Hcy by an electrocatalytic reaction and 1,4 Michael addition of Hcy with catechol using a modified carbon nanotube glassy carbon electrode (CNT–GCE) [68, 69]. Though the electrochemical detection of Hcy was carried out in the presence of Cys, GSH and ascorbic acid using carbon electrode, the response for Hcy was not achieved at a physiological level in the presence of other thiols and amino acids as well as in any biological media [68].

Kerman *et al.* developed another electrochemical method wherein electrodeposition of gold on carbon nanotubes was employed for the enhanced electrochemical detection of Hcy. They recorded the response of Hcy at very high concentrations (mM level), and no information was given about other interfering thiols, amino acids and biological media [100].

On the other hand, an amperometric Hcy biosensor array (HBA) was developed by Ching *et al.* for the determination of Hcy. They fabricated amperometric HBA with a D-amino acid oxidase (AAOx) immobilized on a screen printed carbon electrode[104]. Silver-silver chloride (Ag-AgCl) was used as a reference electrode. Detection sensitivity was found to be the best with 8 working electrodes. Though this method was sensitive, it involved several steps and was found to be time consuming. Moreover, they do not report

the response of Cys and GHS, capable of undergoing the same oxidation mechanism as Hcy, eventually causing interference with the response of Hcy. (Scheme 2-2)

Scheme 2-2. The enzymatic oxidation of homocysteine by D-amino acid oxidase to produce H₂O₂

2.4 Optical methods.

Optical methods have received a great deal of attention for the detection of biological thiols [74-79]. The popularity of these methods is due to sensitivity, relative ease, and low cost; however, to date there have been relatively few optical methods, especially fluorescence methods, reported that can reliably distinguish Hcy over Cys, GSH and other amino acids [105-110].

2.4.1 Optical detection based on the redox chemistry of Hcy

Our group has published a photochemical method for the detection of homocysteine in whole plasma using viologens as redox based indicators [106, 107]. This mechanism, as shown in Scheme 2-3, relies on the reduction of viologens by the α -amino carbon centered radical of Hcy generated by intramolecular hydrogen atom transfer (HAT) of its thiyl radical [106, 107]. Addition of Hcy to a solution of viologen resulted in change in the absorbance spectra the viologen.

Scheme 2-3. Mechanism for the detection of Hcy involving by generation of thiyl radical, hydrogen atom transfer and reduction of BV^{2+}

Lately, Wang *et al.* also published a redox based fluorometric detection of homocysteine using a dansyl derivative as fluorescence probe [108] (Figure 2-1). The selectivity for Hcy was proposed due to the oxidation of dansyl derivative probe DN-2 by Hcy. Selectivity of the probe for Hcy over Cys was due to the formation of a favorable five membered intermediate derived from Hcy, as opposed to the strained four membered ring derived from Cys. They claimed the selectivity of Hcy over Cys and GSH, nonetheless the response of Hcy in presence Cys and GSH towards the probe, had a relatively very high fluorescence enhancement than the Hcy itself (almost two folds). Under physiological conditions, where levels of Cys are 15-20 times higher than Hcy, it would be confusing to decide whether the high response is from Hcy or Cys.

Figure 2-1. Structure of desyl derivative probe DN-2

2.4.2 Methods for detection of Cys and Hcy based on Schiff base formation and subsequent cyclization to form a heterocycle.

It is well known that, Cys and Hcy form thiazolidine and thiazinane heterocycles upon reaction with aldehyde moiety (for more details refer chapter 4). Based on this concept, some research groups have reported the detection of Hcy over Cys using aldehyde bearing fluorophores [105, 109, 110]. Huang *et al.* reported a phosphorescence sensor for Hcy detection based on an iridium (III) complex. Figure 2-2). Photoinduced electron transfer (PET) from Cys-derived thiazolidine was believed to be responsible for specificity of iridium (III) complex towards Hcy over Cys. Enhancement of the photoluminescence upon addition of Hcy and quenching of the optical response with respect to Cys was obtained. This complex produced a very good signal for Hcy, nevertheless a response was achieved at a very high concentration (400 μ M), far more than the physiological levels, and the use of excess of DMSO (90%) as a reaction medium makes it inapplicable in the biological sample.[109]

Figure 2-2. Structure of phosphorescence sensor for Hcy based on iridium (III) complex, $\text{Ir}(\text{pba})_2(\text{acac})$ (Hpba = 4-(2-pyridyl)-benzaldehyde; acac = acetylacetonate)

Yoon *et al.* reported aldehyde bearing pyrene probes (P-Hcy-1 and P-Hcy-2) for the selective detection of Hcy [105] (Figure 2-3). Addition of Hcy to a solution of pyrene aldehydes resulted in fluorescence enhancement. They stated that the distinct fluorescence response of the probes towards Hcy and Cys was due to intramolecular charge transfer (ICT) and photoinduced electron transfer (PET). However, the response of Hcy at physiological levels in the presence of other interfering thiols was not reported.

Figure 2-3. Structures of P-Hcy-1 and P-Hcy-2.

Recently, an aldehyde bearing bodipy based turn on NIR fluorescent probe containing a partially exposed aldehyde group at the meso position for the detection of

Hcy over Cys was published [110] (Figure 2-4). At the equimolar concentration of Hcy and Cys, after four hours, a 30 fold increase in the fluorescence was obtained for Hcy and a 9 folds increase in the fluorescence for Cys was observed. Under physiological conditions where Cys levels are 15-20 times higher than Hcy; hence, Cys will interfere with the selectivity for Hcy. This makes the sensor unsuitable for Hcy detection in the biological samples.

Figure 2-4. Structure of aldehyde bearing bodipy based sensor for Hcy detection

Keeping the above information in mind, it is safe to say that it has been very challenging to design a molecule and develop a method which can deliver a distinctive response to Hcy in the presence of other biological thiols and amino acids at physiologically-relevant levels.

CHAPTER 3 SELECTIVE DETECTION OF HCY USING FLUORESCEINE

ALDEHYDES

3.1 Background

It has been known for almost 90 years that Cys reacts with aldehydes and forms thiazolidines [111]. Aldehyde-functionalized probes have received a great deal of attention for the optical detection of amino thiols and amino acids [76-78, 105, 112-114]. First aldehyde-bearing fluorophore for the detection of Cys and Hcy was developed by our group in 2004 [76]. These reactions are based on the well-known cyclization of the aldehyde group with amino thiols to form thiazinane/thiazolidine heterocycles, as shown in the Figure 3-1. The formation of these heterocycles affords a change in the electronic properties of the chromophore and gives a different optical response than the molecule itself.

Figure 3-1. General reaction scheme for reaction of carbonyl with Cys and Hcy

Interestingly, this class of probes has responded to their respective analytes *via* either fluorescence quenching or fluorescence enhancement on a case by case basis. For instance, Glass *et al.* reported the detection of amino acids using aldehyde bearing

coumarin probes, wherein the reaction of the aldehyde with amino acids to form imines produced an enhancement of fluorescence [112]. Concurrently, Strongin and co-workers presented the detection of biological thiols with aldehyde bearing fluorophores [76, 77] based upon the well-known reaction of aldehydes with *N*-terminal cysteine residues to form heterocyclic thiazolidines [115] and *N*-terminal homocysteine residues to form heterocyclic thiazinanes. They reported the fluorescence quenching of fluorescein aldehyde probes due to the formation of thiazolidines/thiazinanes heterocycles, upon reaction of Cys and Hcy [76]. Afterwards, several groups have implemented this concept of aldehyde bearing probes for the detection of Cys and Hcy [75]. For instance, Kim *et al.* observed the fluorescence quenching of an aldehyde bearing amino coumarin based fluorophore in response to Cys/Hcy [113]. Hoang *et al.*, reported an aldehyde bearing hydroxy coumarin [114] in which addition of Cys/Hcy resulted in enhancement of the fluorescence.

Another point of interest is that these aldehyde-based probes [76-78, 105, 112-114, 116] have generally responded to the entire classes of analytes including amino acids[112] or biological thiols [113, 114] without specificity for individual analytes within each class. It is therefore of great interest and utility to develop a better understanding of the mechanisms behind different signal transduction in these related aldehyde-based probes. Such knowledge will lead to improved probes with increased sensitivity for the selected aminothiols of interest, specifically for Hcy over Cys.

Besides understanding the mechanism of signal transduction for respective analytes, it is equally important to focus on the difference in the spectroscopic responses

of the probe itself and the probe-analyte complex with respect to excitation wavelength (λ_{ex}). In general, fluorescence responses of an analyte-indicator complexes are observed at an excitation wavelength that corresponds to the absorption maximum of the dye or indicator. However, the chosen excitation wavelength may not give the optimal response, since it corresponds to the maximum absorption of the dye in the absence of analyte [112]. In 2010, Strongin *et al.* had reported the optimum excitation wavelength for α,β – unsaturated aldehyde-based sensor for the detection of biological thiols, which was different than the maximum absorption of sensor[78].

Usually, selective detection is obtained due to the specific and distinctive interactions of analytes with sensors or receptors. However, different analytes or biomolecules present in biological media may interact with the indicator through similar supramolecular or covalent interactions. Therefore, the presence of these indicator-bound species may interfere with the selective detection of the desired analyte. To overcome this problem, Strongin *et al.* studied a simple approach for the selective detection of a target biomolecule [117]. This new approach is based on the concept that the structural differences among various complexes which were formed by the interaction of various analytes with the same sensor may produce distinctive absorption and emission responses. This potentially allows a multianalyte detection *via* a single optical indicator. Based on this concept, a tunable detection of specific saccharides was achieved with the help of the single optical indicator by the judicious selection of excitation and emission wavelengths[117]. For instance, selective detection of fructose and ribose derivatives was attained at different excitation and emission wavelengths as a function of time. A

rhodamine boronic acid was used as an indicator in that study.

3.2 Mechanism of signal transaction in aldehyde bearing fluorophore probes upon reaction with aminothiols

It is important to note the differences in the reaction of aldehyde-bearing probes with aminothiols compared to amino acids. Amino acids react to form Schiff bases [112] whereas, aminothiols such as Cys and Hcy react to form heterocyclic thiazolidines and thiazinanes respectively [76]. These differences result in unique optical responses towards aminothiols. The mechanism of signal transduction in fluorescein aldehyde probes upon their reaction with aminothiols was investigated in this work.

Figure 3-2. Mechanism of signal transduction in generic aldehyde-bearing aminothiol probes.

(a) PET-based fluorescence quenching following reaction of Cys/Hcy with the aldehyde group. (b) PET inhibition through hydrogen bonding between the amine-containing heterocycle and adjacent groups. (c) PET inhibition through protonation of the amine-containing heterocycle.

There are several phenomena that impact the fluorescence response of aldehyde-

bearing probes. Figure 3-2 depicts the reaction of Cys/Hcy with the aldehyde group of a generic aldehyde-bearing probe. The mechanistic understanding described herein, has led to control over signal transduction in such a way that the probe can be tuned to respond to Cys/Hcy through either quenching or enhancement. Importantly, the response can be controlled in such a manner that Hcy is detected selectively over Cys.

It is well established that the free electrons of the amino group in the heterocycle can quench the fluorescence of the probe (Figure 3-2a) through photo induced electron transfer (PET) [113]. PET-induced responses have been modulated through tuning the functionality of the fluorescent probes [118],[114]. For example, hydrogen bonding in aqueous media between the amine-containing heterocycles and adjacent groups (Figure 3-2b) has been reported to inhibit PET leading to fluorescence enhancement upon reaction with both Cys and Hcy with a coumarin aldehyde [114]. Conversely, PET inhibition leading to selective fluorescence enhancement for Hcy/Cys can be achieved *via* a mechanism involving protonation of the amino group of the heterocycle. (Figure 3-2c)

In this work, the above mentioned concepts were implemented based on the structural differences between the complexes of fluorescein dialdehyde (FDA) or /fluorescein monoaldehyde (FMA) with Cys and Hcy. *Via* different signal transduction mechanisms, a specific response for Hcy was achieved by varying the excitation and emission wavelengths as a function of time at specific pH values.

3.3 Previous work- response of fluorescein aldehydes to Cys and Hcy

Previously, in 2004, Strongin *et al.* investigated the reaction of **3.1**(FMA), and **3.3** (FDA)

with Cys and Hcy at pH 9.5 [76, 77]. As described below in Scheme 3-1, this is a very simple method for the detection of Cys or Hcy and doesn't involve any complicated biochemical techniques or preparative separation [76, 77]. This protocol was developed on the same aforementioned concept i.e. the reaction of aldehyde with N- terminal Cys residue to form thiazolidines [115, 119].

Scheme 3-1. Reaction of FMA (3.1) and FDA (3.3) with Cys and Hcy to generate thiazolidines/ thiazinanes heterocycles respectively.

In Scheme 3-1, as shown above, the addition of Cys and Hcy to a solution of 3.3 results in a change in solution color from yellow to orange. Optical studies have shown that the

addition of Cys to the solution of **3.3** produces a red shift in the absorption spectrum from 480 nm to 495 nm which is shown in the Figure 3-3. Further, addition of Cys and Hcy to a solution of **3.3** has also been shown to result in a monitorable fluorescence quenching (excited at 460 nm) due to the formation of thiazolidines/thiazinanes as shown in Figure 3-4 and Figure 3-5. Moreover, NMR spectra proved the formation of thiazolidines/thiazinanes in this reaction [76]. "Figure 3-3, Figure 3-4 and Figure 3-5 were adapted with permission from [76] Copyright (2004) American Chemical Society."

Figure 3-3. Change in the absorbance spectra of **3.3** upon addition of Cys in buffer, pH 9.5 at room temperature. Each spectrum was acquired after 5 min. As the concentration of Cys increases, a red shift from 480 nm to 495 nm was observed.

Figure 3-4. Quenching of the fluorescence emission of **3.3** upon addition of Cys, excited at 460 nm, pH 9.5 at room temperature

Figure 3-5. Quenching of the fluorescence emission of **3.3** upon addition of Hcy , excited at 460 nm , pH 9.5 at room temperature

In these optical studies, under basic conditions (at pH 9.5) both Cys and Hcy displayed the same behavior towards **3.1** and **3.3** i.e. quenching of the fluorescence emission of fluorescein aldehydes. This was attributed to the photoinduced electron transfer (PET) of the lone pairs present on the nitrogen atom of heterocycles derived from Cys and Hcy.

3.4 Present work – results and discussion

In the current study, the objective was to optimize the system to develop a simple, convenient and inexpensive fluorescence method to serve as a sensitive analytical tool for the detection of Hcy. It was hypothesized that at sufficiently low pH, the amine of the heterocycles, which formed upon reaction with the probe, would be protonated, thereby inhibiting PET and leading to fluorescence enhancement. Moreover, we anticipated that the protonated amino groups in the thiazinane and thiazolidine would have different pKa values due to hybridization differences as a function of ring sizes. Hence, it was decided to study of response Hcy and Cys towards **3.1** and **3.3** at different pH values.

The behavior of the reaction products was found to vary at different pH, as shown in Figure 3-6, Figure 3-7 and Figure 3-8. At higher pH (9.5), Hcy and Cys both exhibited expected quenching of the fluorescence of **3.1** as both retain their amine lone pairs. At lower pH (5.5 and 6.0), Hcy enhanced the fluorescence of the fluorophore. However Cys displayed fluorescence quenching (Figure 3-8) at pH 6.0 (at pH = 5.5 there was no signal change). These results suggest that at pH = 6 the amine of the Hcy thiazinane was protonated (PET quenching off) and that of the cysteine thiazolidine was neutral (lone pairs retained and PET quenching on).

In the case of a 5-membered Cys-derived thiazolidine ring, the C-N-C bond angle is smaller than that in a 6-membered Hcy-derived thiazinane ring. This results in greater % *S*-character of the N-H bond in the thiazolidines, rendering them less basic than the thiazinanes[120-122]

Figure 3-6. Optical sensing behavior of **3.1** towards Hcy and Cys at pH 9.5

(a) fluorescence spectral changes of **3.1** with Hcy and Cys after 30 min. (b) Time dependent fluorescence spectral changes of **3.1** with Hcy and Cys. Solutions are composed of 4 μM of **3.1** with 1 mM of analyte in carbonate buffer (100 mM, pH 9.5) DMSO 99:1 at 20 $^{\circ}\text{C}$, $\lambda_{\text{ex}} = 480 \text{ nm}$ and $\lambda_{\text{em}} = 515 \text{ nm}$.

Figure 3-7. Optical sensing behavior of **3.1** towards Hcy and Cys at pH 5.5.

(a) fluorescence spectral changes of **3.1** with Hcy and Cys after 2h. (b) Time dependent fluorescence spectral changes of **3.1** with Hcy and Cys. Solutions are composed of 4 μM of **3.1** with 1 mM of analyte in phosphate buffer (100 mM, pH 5.5) DMSO 99:1 at 20 $^{\circ}\text{C}$, $\lambda_{\text{ex}} = 480 \text{ nm}$ and $\lambda_{\text{em}} = 515 \text{ nm}$.

Figure 3-8. Optical sensing behavior of **3.1** towards Hcy and Cys at pH 6.0.

(a) Fluorescence spectral changes of **3.1** with Hcy and Cys after 2h. (b) Time dependent fluorescence spectral changes of **3.1** with Hcy and Cys. Solutions are composed of 4 μ M of **3.1** with 1 mM of analyte in phosphate buffer (100 mM, pH 6.0) DMSO 99:1 at 20 $^{\circ}$ C, λ_{ex} = 480 nm and λ_{em} = 515nm.

The hypothesis and results showed that lowering the pH of the reaction medium results in the enhancement of the fluorescence of **3.1** in the presence of Hcy. After studying the responses to thiols as a function of pH, a study was conducted to explore the response of **3.1** towards Hcy and Cys at a different excitation wavelengths. It was again hypothesize that the fluorescence response would be maximum when excited at wavelength which is near the absorption maxima of the reaction product, instead of the absorption maxima of the probe itself. Based on this concept, maximum response for the detection of amino acids and Cys as well as tunable detection of saccharides[117] have been reported in the literature [78, 112, 117]. Absorbance spectra of **3.1**, show that the addition of Hcy or Cys to a solution of **3.1**, produces a red shift from 480 nm to 495 as a result of the formation of thiazinane/thiazolidine heterocycles (

Figure 3-9) [76].

To prove this second hypothesis, the optical response of Cys and Hcy towards **3.1** was monitored at higher excitation wavelengths, i.e. λ_{ex} at 495 nm, near the absorption maxima of the heterocyclic reaction product. On exciting at 495 nm, enhancement in the fluorescence of **3.1** in response to Hcy was found to be 2 to 3 fold higher than the excitation at lower wavelength (at 480 nm, lambda max of the probe itself) as shown in Figure 3-10. Another interesting observation was that, on adjusting the pH from 5.5 to 6.0, the signal is modulated such that Hcy enhances the fluorescence of **1** while there is minimal change upon reaction with Cys, as shown in Figure 3-11. These observations led to a new method to achieve a maximum response of Hcy over Cys *via* adjusting the pH of the reaction medium and the excitation wavelength. In the present case, the best parameters are pH 6.0 and excitation wavelength, λ_{ex} of 495nm.

Figure 3-9. Optical sensing behavior of **3.1** towards Hcy and Cys at pH 9.5.

(a) Time dependent absorption spectral changes of **3.1** with Hcy over 30 min. (b) Time dependent absorption spectral changes of **3.1** with Cys over 30 min. Solutions are composed of 4 μM of **3.1** with 1 mM of analyte in carbonate buffer (100 mM, pH 9.5) DMSO 99:1 at 20 $^{\circ}\text{C}$

Figure 3-10. Optical sensing behavior of **3.1** towards Hcy and Cys at pH 5.5.

(a) Fluorescence spectral changes of **3.1** with Hcy and Cys after 2h. (b) Time dependent fluorescence spectral changes of **3.1** with Hcy and Cys. Solutions are composed of 4 μ M of **3.1** with 1 mM of analyte in phosphate buffer (100 mM, pH 5.5) DMSO 99:1 at 20 $^{\circ}$ C, λ_{ex} = 495 nm and λ_{em} = 515nm.

Figure 3-11. Optical sensing behavior of **3.1** towards Hcy and Cys at pH 6.0.

(a) Fluorescence spectral changes of **3.1** with Hcy and Cys after 2h. (b) Time dependent fluorescence spectral changes of **3.1** with Hcy and Cys. Solutions are composed of 4 μ M of **3.1** with 1 mM of analyte in phosphate buffer (100 mM, pH 6.0) DMSO 99:1 at 20 $^{\circ}$ C, λ_{ex} = 495 nm and λ_{em} = 515nm.

The response of **3.1** towards GSH and various amino acids at pH 6.0 was also investigated. As shown in the Figure 3-12, it was found that only Hcy promotes fluorescence enhancement.

Figure 3-12. Optical sensing behavior of **3.1** towards Cys and Hcy as compared to other biologically relevant species.

Fluorescence response of **3.1** upon addition of different analytes (Cys, Hcy, GSH, Asn, Ala, Gln, Thr, Asp, Arg, His, Glu, 1 mM). All Solutions are composed of 4 μ M of **3.1** with 1 mM analyte in phosphate buffer (100 mM, pH 6.0):DMSO 99:1 at 20 $^{\circ}$ C, λ_{ex} = 495nm λ_{em} = 515 nm

^1H NMR study of **3.1** in the presence of Hcy and Cys under these conditions also confirms the formation of thiazinane and thiazolidine, upon reaction of Cys and Hcy with **3.1**. Figure 3-13 shows disappearance of aldehyde resonance at 10.39 ppm and appearance of methine resonance at 6.81 ppm substantiate formation of these heterocycles. Whereas, ^1H NMR experiment of **3.1** with glycine shows the formation of a Schiff base (an imine), in the Figure 3-14, the Schiff base resonance at 9.11 ppm along with CHO resonance at 10.38 ppm shows the Schiff base formation was in equilibrium with the **3.1**.

Figure 3-13. ^1H NMR of **3.1**, **3.1** in the presence of Hcy (3 equiv) and **3.1** in presence of Cys (3 equiv) at pH 6.0.

Figure 3-14. ^1H NMR of **3.1** and **3.1** in the presence of Gly (3 equiv) at pH 6.

Although highly selective for Hcy over Cys, **3.1** was not sensitive enough to produce an appreciable response of Hcy at the lower levels. Therefore, it was decided to study the response of Hcy and Cys towards **3.3**, i.e. fluorescein dialdehyde.

The absorption spectra of **3.3** in the presence of Hcy and Cys are analogous to those using **3.1**, including a red shift in the absorption maxima upon addition of Hcy and Cys from 480nm to 495 nm (Figure 3-15). Further, on exciting at a higher wavelength ($\lambda_{\text{ex}} = 495 \text{ nm}$), **3.3** produces a >5 fold enhancement in the fluorescence upon addition of Hcy (Figure 3-16). The signals using **3.3** were approximately twice as intense as from **3.1**. The response of **3.3** towards Hcy was easily observed in as little as 15 min and

plateaus near 45 min.

Figure 3-15. Optical sensing behavior of **3.3** towards Hcy and Cys at pH 6.0.

(a) Time dependent absorption spectral changes of **3.3** with Hcy over 2h. (b) Time dependent absorption spectral changes of **3.3** with Cys over 2h. Solutions are composed of 4 μM of **3.3** with 1 mM of analyte in Phosphate buffer (100 mM, pH 9.5) DMSO 99:1 at 20 $^{\circ}\text{C}$

Figure 3-16. Optical sensing behavior of **3.3** towards Hcy and Cys at pH 6.0.

(a) Fluorescence spectral changes of **3.3** with Hcy and Cys after 2h. (b) Time dependent fluorescence spectral changes of **3.3** with Hcy and Cys. Solutions are composed of 4 μM of **3.3** with 1 mM of analyte in phosphate buffer (100 mM, pH 6.0) DMSO 99:1 at 20 $^{\circ}\text{C}$, $\lambda_{\text{ex}} = 495 \text{ nm}$ and $\lambda_{\text{em}} = 515 \text{ nm}$.

Having identified the optimal conditions for the discrimination of Hcy over Cys, the response of the more sensitive dialdehyde **3.3** towards normal healthy concentrations of aminothiols in pH 6.0 buffer was investigated. As shown in the Figure 3-17, No response

was observed toward 250 μM Cys, while 15 μM Hcy resulted in a $\sim 10\%$ increase in the fluorescence. The fluorescence response of **3.3** towards Hcy increased linearly with the increase in the Hcy concentration, and the limit of detection was found to be as low as 2.5 μM as shown in the Figure 3-18.

Figure 3-17. Optical sensing behavior of **3.3** towards Cys and Hcy at their biological concentrations at pH 6.0.

Fluorescence spectra of **3.3** with Cys and Hcy after two hours. Solutions are composed of 30 μM of **3.3** with 250 μM of Cys and 15 μM of Hcy in phosphate buffer (100 mM, pH 6.0):DMSO 99:1 at 20 $^{\circ}\text{C}$, $\lambda_{\text{ex}} = 494 \text{ nm}$.

Figure 3-18. Spectral response of **3.3** towards increasing levels of Hcy in phosphate buffer (100 mM, pH 6.0).

Solutions are composed of 4 μM of **3.3** with 0-80 μM of Hcy in phosphate buffer (100 mM, pH 6.0):DMSO 99:1 at 20 $^{\circ}\text{C}$, $\lambda_{\text{ex}} = 494 \text{ nm}$ and $\lambda_{\text{em}} = 515 \text{ nm}$.

The response of **3.3** in deproteinized plasma at elevated levels of Hcy was also investigated. To measure a known amount of Hcy deproteinized plasma was spiked with Hcy (100 μ M). Under this condition, **3.3** functioned well in the presence of spiked Hcy. The response of physiological levels of Cys (i.e. 250 μ M) towards **3.3** was insignificant as shown in the Figure 3-19.

Figure 3-19. Optical sensing behavior of **3.3** towards Cys and Hcy in deproteinized bovine plasma at pH 6.0.

Solutions are composed of 25 μ M of **3.3** with 250 μ M of Cys and 100 μ M of Hcy in phosphate buffer (100 mM, pH 6.0):DMSO 99:1 at 20 oC after 2 hr, λ_{ex} = 495 nm.

3.5 Conclusion

In conclusion, the optimum conditions to attain the specific detection of Hcy *via* a characteristic fluorescence increase with **3.1** and **3.3** were identified. Under basic conditions, addition of Hcy and Cys to a solution of fluorescein aldehyde (**3.1** or **3.3**), resulted in fluorescence quenching due to the PET from the lone pair of electron, present on the nitrogen atom of heterocycles derived from Hcy and Cys. Whereas under acidic conditions, protonation of amine of Hcy/Cys derived heterocycles inhibits PET and produces fluorescence enhancement. pH 6.0 was found to be the optimal pH for the

detection of Hcy, at this pH the response to Hcy of **3.1** and **3.3** led to fluorescence enhancement, whereas the response to Cys was minimal.

Apart from the pH dependence, the detection of Hcy was also found to be wavelength dependent. The magnitude of the response was at the maximum when excited in the region near the absorption maxima of the analyte-probe complex ($\lambda_{\text{ex}} = 495\text{nm}$) rather than the absorption maxima of probe itself ($\lambda_{\text{ex}} = 480\text{nm}$). Fluorescein dialdehyde (**3.3**) was found to be more sensitive than fluorescein mono aldehyde (**3.1**). The reaction of **3.3** with Hcy resulted in more than a fivefold enhancement in fluorescence. **3.3** was also found to function in deproteinized plasma, detecting relatively high levels of Hcy (100 μM).

3.6 Experimental section

3.6.1 Materials

All chemicals were purchased from Sigma Aldrich and used as received. Ultra-pure water (18.2 $\text{M}\Omega\cdot\text{cm}$ at 25 $^{\circ}\text{C}$) obtained from a Milli-Q direct water purification system was used to prepare all aqueous solutions. All spectroscopic measurements were carried out in DMSO:buffer (1:99) solutions. **3.1** and **3.3** were synthesized from the procedure reported in the literature based on the Reimer Tiemann reaction for formylation [77].

3.6.2 Methods

^1H -NMR spectra were recorded on an ARX-400 Advance Bruker spectrometer performing at 400.13 MHz (^1H). $\text{D}_2\text{O}:\text{H}_2\text{O}$ (1:9) solvent system was used as NMR solvent. All chemical shifts (δ) were reported in ppm using DSS (sodium 2,2-dimethyl-2-

silapentane-5-sulfonate) as an internal standard ($\delta=0.000$) for ^1H -NMR studies.

3.6.3 Spectrophotometric measurement

UV-visible spectra were recorded on a Cary 50 UV-Vis spectrophotometer (Agilent Technologies). Fluorescence spectra were obtained on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) with slit widths set at 5 nm for both excitation and emission, respectively. Fluorescence spectra were corrected for the wavelength dependent response of the R928 photomultiplier tube with the help of a manufacturer generated correction file. All spectrophotometric measurements were conducted at room temperature.

3.6.4 Deproteinization of plasma

Deproteinization of plasma was carried out by reconstituting the lyophilized bovine plasma with ultrapure water to 1/3 of the reconstitution volume. Two equivalents of acetonitrile (2/3 of the reconstitution volume) were added. This solution was vortexed for 10 min. after letting the solution stand for 10 minutes, it was centrifuged at 4000 RPM for 30 minutes to pellet the proteins. The decanted supernatant was aliquoted into sample vials and re-lyophilized. The vials were then sealed and stored in a refrigerator (4 °C).

CHAPTER 4 MECHANISTIC STUDIES

4.1 Introduction

Fluorescence enhancement due to PET Inhibition is a well-known phenomenon. [123] PET based fluorescence sensors have been widely used to detect an individual or a class of analytes [109, 113, 114, 123-125]. This effect has been observed in the case of fluorescein aldehydes. As seen in previous chapter, under acidic conditions, amine of Hcy-Cys derived heterocycles is protonated causing inhibition of PET and fluorescence enhancement. However, at an intermediate pH, the response of Hcy was observed but Cys seems to give minimal or insignificant response.

4.2. Result and discussion

Here the objective was to understand the difference in the fluorescence response of Hcy-derived thiazinane and Cys-derived thiazolidine which are formed upon reaction of Hcy and Cys with **3.1** and **3.3** respectively. The difference in the response of Hcy than Cys towards **3.1/3.3** was possibly due to the difference in the pK_a value of protonated amine of Hcy-derived thiazinane and the Cys-derived thiazolidine. Since, the C-N-C bond angle for 5-membered Cys-derived thiazolidine ring is smaller than the 6-membered Hcy-derived thiazinane ring, it generates a greater *S*-character of the N-H bond in the thiazolidines, making them less basic than the thiazinanes [120-122]. To prove this hypothesis, it was necessary to understand the difference in the basicity of amine of Hcy/Cys derived heterocycles. Previously, the basicity of the amine of thiazolidine-4-carboxylic acids has been studied [126-129] but the basicity of the amine of the Hcy-derived thiazinane-4-carboxylic acids had not been reported. Therefore, a series of 5-

and 6-membered 2-substituted thiazolidine/thiazinaneS-4-carboxylic acids were synthesized as model compounds of **3.1** and **3.3**.

4.2.1 Synthesis of Cys- and Hcy-derived analogs 4.1, 4.3, 4.5, 4.2, 4.4 and 4.6

To understand the difference in the basicity of 5- and 6-membered heterocyclic amines, the Cys- and Hcy-derived analogs **4.1**, **4.3**, **4.5**, **4.2**, **4.4** and **4.6** were synthesized. **4.1**, **4.3** and **4.5** were prepared by the reaction of Cys with salicylaldehyde, benzaldehyde and formaldehyde respectively in aqueous medium at room temperature. Several attempts were made for the synthesis of Hcy derived heterocycles analogs **4.2**, **4.4** and **4.6**. Reaction of Hcy was carried out with salicylaldehyde, benzaldehyde and formaldehyde respectively in aqueous and/or ethanol reaction medium by changing the number of equivalents, water/EtOH ratio and time over a period of few hours to 3 days. **4.2** and **4.4** were obtained as colorless crystals from the reaction medium. However **4.6** remained soluble in the aqueous medium therefore ethanol was added to the reaction mixture and after 24 hours shiny colorless crystals of **4.6** were obtained.

4.2.1 Estimated pK_a values of Cys- and Hcy-derived analogs 4.1, 4.3, 4.5, 4.2, 4.4 and 4.6

To confirm the hypothesis that the pH dependent Hcy enhancement was related to the difference in the basicity of 5- and 6-membered heterocyclic amines, the estimated pK_a values of the protonated amine of the Cys- and Hcy-derived analogs were measured. The values are reported in the Table 4.1. estimated pK_a values were calculated from the pH titration curves which are shown in Figure 4-1, Figure 4-2 and Figure 4-3. Cys-derived **4.1**, **4.3** and **4.5** are known [130, 131] and their previously published pK_a values

are in general agreement with those in Table 4.1. As seen in Table 4.1, the protonated amine pK_a of 6-membered thiazinanes is higher than that of 5-membered thiazolidines by at least one unit. Therefore, based on the pK_a of these analogs, we expect the protonated amine pK_a of Hcy-derived **3.2b** to be approximately 1 unit greater (~6.7) than Cys-derived **3.2a** (~5.7). One unit difference in the pK_a of amine of Hcy and Cys derived heterocycles will affect the ionization state of amine at a given pH. For instance, at pH 6.0 major species for the amine of Hcy-derived thiazinane would exist mainly as the ammonium ion whereas the amine of the Cys-derived thiazolidine would be at a nearly equal concentration to the ammonium form.

Table 4.1. Estimated pK_a values of the protonated amine moiety of 2-substituted thiazolidine/thiazinane-4-carboxylic acids.

Compound	$pK_{a \text{ exp}}$, ($pK_{a \text{ lit}}$)	Compound	$pK_{a \text{ exp}}$
<p>4.1</p>	NA, ^a (5.67 [130])	<p>4.2</p>	6.67 ^b
<p>4.3</p>	5.50, ^a (5.31[130])	<p>4.4</p>	6.85 ^b
<p>4.5</p>	6.44, (6.19)	<p>4.6</p>	7.76

^a**4.1** and **4.3** were found to be relatively unstable showing signs of decomposition in aqueous solution during the course of the titration (Figure 4-1, Figure 4-2 and Figure 4-4).

^b**4.2** and **4.4** showed no indication of decomposition (Figure 4-1, Figure 4-2 and Figure 4-4)

Figure 4-1. Average of triplicates of (a) pH titration curve and (b) derivatives of **4.1** and **4.2**

Figure 4-2. Average of triplicates of (a) pH titration curve and (b) derivatives of **4.3** and **4.4**

Figure 4-3. Average of triplicates of (a) pH titration curve and (b) derivatives of **4.5** and **4.6**

From the pH titration of thiazolidine-4-carboxylic acids, derivatives **4.1** and **4.3** were found to be unstable in the aqueous solutions; however, thiazinane-4-carboxylic acids **4.2** and **4.4** appeared stable as shown in Figure 4-1 and Figure 4-2. Further, the stability of thiazolidines/thiazinane-4-carboxylic acids **4.1** and **4.2** were studied by ^1H NMR experiments. As shown in the , ^1H NMR spectra of **4.1** and **4.2** were carried out in a mixture of $\text{D}_2\text{O}:\text{H}_2\text{O}$ (9:1). In the ^1H NMR of **4.1**, the appearance of an aldehyde resonance at 9.88 ppm shows that **4.1** decomposes and goes back to starting material i.e

salicylaldehyde. On the other hand ^1H NMR of **4.2** doesn't show any aldehyde proton peak and the presence of a methine resonance at 5.7 ppm demonstrates the stability of this compound in aqueous medium, as shown in Figure 4.4. The instability of thiazolidine-4-carboxylic acids **4.1** and **4.3** in aqueous solutions has also been mentioned previously[130] (particularly in strongly basic and acidic medium [132]).

Figure 4-4. ^1H NMR of salicylaldehyde-Cys derived thiazolidines **4.1** and salicylaldehyde-Hcy derived thiazinanes **4.2** in $\text{D}_2\text{O}:\text{H}_2\text{O}$

Table 1 and pH titration curves (Figure 4-1, Figure 4-2 and Figure 4-3) show the higher basicity of the amine of the thiazinane-4-carboxylic acids than the amine of thiazolidine-4-carboxylic acids at least by one unit. This difference in basicity results in the selectivity

for Hcy over Cys. For the purpose of investigating the mechanism responsible for this unique selectivity, first the simpler monoaldehyde **3.1** was studied. The response of **3.1** towards Hcy and Cys over a range of pH values was investigated (Figure 4-5). From the pH dependent experiment, it is evident that, with a change in the pH values, the ratio of amine to ammonium ion in the Hcy/Cys derived heterocycles changes. That eventually affects the fluorescence response of **3.1** i.e. FMA (Figure 4-5). As the pH is lowered, the percent of ammonium ion species increases, consequently the fluorescence enhancement increases linearly as a result of PET inhibition by the ammonium ion. Due to the higher pK_a value of Hcy-derived thiazinane, there is a greater percent of ammonium ion species in the solution than the Cys-derived thiazolidines.

At the appropriate pH, the ratio of ionization states of the amine moiety in thiazolidine **3.2a** would be such that fluorescence quenching by the lone pair electrons is cancelled by PET inhibition from the ammonium species **3.2a'** (Scheme 4-1). At this pH, the amine group in thiazinane **3.2b** exists predominately as the more fluorescent ammonium **3.2b'** (Scheme 4-1) because the pK_a of **3.2b'** is greater than that of **3.2a'**. At pH 6.0, the reaction between **3.1** and Cys does not result in a change in fluorescence while reaction with Hcy results in fluorescence enhancement.

Scheme 4-1. Different ionization states of the the amine moieties in Cys- and Hcy-derived heterocycles at pH 6.0.

Thus pH 6.0 is optimal for the detection of Hcy. Raising the pH increases the ratio of amine to ammonium which leads to quenching, while decreasing the pH leads to enhancement as clear from Figure 4-5.

Figure 4-5. Time dependent fluorescence spectral changes and enhancement factor (inset) of fluorescence of **3.1** with Cys and Hcy, (a) pH 5.5, (b) pH 6.0, (c) pH 6.5, (d) pH 7.0, (e) pH 7.4. (f) comparison of fluorescence response of **3.1** towards Cys and Hcy at different pH.

Solutions are composed of 4 μ M of **3.1** with 1 mM analyte in phosphate buffer (100 mM, pH 6.0):DMSO 99:1 at 20 $^{\circ}$ C, λ_{ex} = 495 nm and λ_{em} = 515 nm. For Time dependent fluorescence spectral changes, λ_{ex} = 495 and λ_{em} = 515. Enhancement factor (EF) is the response of **3.1**+ analyte divided by 1. When EF < **3.1** quenching is observed, while when EF > **3.1** enhancement is observed. The vertical line in the enhancement factor plots is the experimentally determined optimal excitation wavelength of 495 nm. At pH 6.0 (b inset), excitation at 495 results in enhancement for Hcy but no enhancement for Cys in response to **3.1**.

4.3 Conclusion

In conclusion, a new approach for the selective detection of Hcy using fluorescein aldehyde-based probes has been developed. This method is based on intrinsic differences between the pK_a of 5- and 6-membered thiazolidines and thiazinanes formed upon reaction with the aldehyde-bearing probes. At an optimum pH (6.0), amine of Hcy-derived thiazinane is protonated to a greater extent ($pK_a \sim 6.7$) causing PET inhibition and fluorescence enhancement. Due to lower pK_a value (~ 5.7) of the protonated amine of the Cys-derived thiazolidine, at pH 6.0, amine and ammonium ion species were seen in equal quantity and therefore, no optical response was observed.

4.4 Experimental section

4.4.1 Materials

All chemicals were purchased from Sigma Aldrich and used as received. Ultrapure water obtained from a Milli-Q direct water purification system was used to prepare all aqueous solutions. All spectroscopic measurements were carried out in DMSO:buffer (1:99) solutions. All experiments were performed at room temperature.

4.4.2 Methods

^1H NMR and ^{13}C NMR were recorded on an ARX-400 Advance Bruker spectrometer performing at 400.13 MHz (^1H) and 100.61 MHz (^{13}C). All chemical shifts (δ) were reported in ppm relative to DMSO- d_6 (2.50 ppm, ^1H ; 39.52 ppm, ^{13}C) unless otherwise indicated. For the study of stability of **4.1** and **4.3** in aqueous media, ^1H NMR experiment was conducted in $\text{H}_2\text{O}:\text{D}_2\text{O}$ mixture (9:1) using DSS (sodium 2, 2-dimethyl-2-

silapentane-5-sulfonate) as an internal standard ($\delta=0.000$). ESI-HRMS (high resolution mass spectrometry) were recorded at the PSU Bioanalytical Mass Spectrometry Facility on a ThermoElectron LTQ-Orbitrap high resolution mass spectrometer. IR spectrum was obtained by Fourier transform infrared (FT-IR) spectroscopy operated on a Thermo Scientific Nicolet iS10 spectrophotometer equipped with a single-bounce diamond ATR attachment.

4.4.3 Spectrophotometric measurement

Fluorescence spectra were obtained on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) with slit widths set at 5 nm for both excitation and emission, respectively. Fluorescence spectra were corrected for the wavelength dependent response of the R928 photomultiplier tube with the help of a manufacturer generated correction file.

4.4.4 pK_a measurements

pK_a values of the amine of 2-substituted thiazolidines/thiazinane-5-carboxylic acids, **4.1**, **4.3**, **4.5**, **4.2**, **4.4**, and **4.6** were obtained by the titration of their aqueous solutions with 0.01M NaOH by following the procedure reported in the literature [126]. In the case of **4.1** and **4.2**, 0.1 M NaOH was used. The pH measurements were conducted using an Orion 410A pH meter, which was standardized with Scientific Products potassium hydrogen phthalate pH 4.00 buffer, BDH pH 7.00 and Scientific Products boric acid/potassium hydroxide pH 10.00 buffer. All pH titrations were conducted in triplicates and their average was reported.

4.4.5 Synthesis of compounds 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6

Scheme 4-1. Synthetic route of 4.1, 4.3 and 4.5

Scheme 4-2. Synthetic route of 4.2, 4.4 and 4.6

(2*S*,4*R*) and (2*R*,4*R*)-2-(2-hydroxy phenyl) thiazolidine-4-carboxylic acid (4.1)

L-Cys (1.67 mmol) and salicylaldehyde (1.67 mmol) were added in 5 mL of DI H₂O. Reaction mixture was stirred at RT for 2 h. The precipitates from the reaction mixture were filtered, washed with ice cold H₂O and dried under vacuum to yield **4.1** as a mixture of distereoisomers, colourless solid (307 mg, 83%); $\nu_{\max}$ (film)/cm⁻¹ 1738 (C=O); M.P.180-181 °C; ¹H NMR (400 MHz, DMSO) δ 7.49 (m, 1H, Ar), 7. (m, 1H, Ar), 6.91 (m, 2H, Ar), 5.84 (s, 1H, Ar-CH), 5.65 (s, 1H, Ar-CH), 4.26 (m, 1H, NH-CH), 3.82 (m,1H, S-CH₂), 3.11 (m, 1H, S- CH₂); ¹³C NMR (101 MHz, DMSO) 172.9-172.4 (C=O), 155.1, 154.5, 129.00, 128.2, 126.00, 124.2, 119.00, 118.7, 115.6, 115.00, 67.6, 66.6, 63.2, 38.00, 37.00; Electrospray ionization (ESI) mass spectrometry (positive electron ionization) m/z :226.05602 ([M+H]⁺,100%), calculated for C₁₀H₁₁NO₃S, m/z :225.04596.

(2*S*,4*S*) and (2*R*,4*S*)-2-(2-hydroxyphenyl)-1,3-thiazinane-4-carboxylic acid (4.2)

L-Hcy (1.88 mmol) and salicylaldehyde (1.88 mmol) were added in a mixture of 6 mL of distilled water and 0.25 mL of ethanol. Reaction mixture was stirred at RT for 48 hrs. The resultant precipitates were filtered, washed with ice cold H₂O and dried under vacuum to yield **4.2** as a mixture of distereoisomers, colourless solid.

(322 mg, 82%); $\nu_{\max}$ (film)/cm⁻¹ 1738 (C=O); M.P.131-132 °C; ¹H-NMR (400 MHz, DMSO-*d*₆); δ (ppm): 7.35 (m, 1H, Ar), 7.15 (m, 1H, Ar), 6.87 (m, 2H, Ar), 5.43 (s, 1H, Ar-CH), 3.59 (m, 1H, COCH), 3.22 (m, Hz, 1H, S-CH₂), 2.89 (m, 1H, S-CH₂), 2.08 (m, 1H, CH-CH₂), 1.50 (m,1H, CH-CH₂); ¹³C-NMR (101 MHz, DMSO) 173.49(C=O), 155.2, 154.1, 128.7, 127.8, 126.4, 125.8, 125.8, 118.9, 115.6, 59.3, 56.1, 54.8, 54.50, 28.7, 27.9, 26.4, 25.3 ; Electrospray ionization (ESI) mass spectrometry (positive electron ionization) m/z : 240.07 182 ([M+H]⁺,100%), calculated for C₁₁H₁₃NO₃S, m/z 239.06161.

(2*S*,4*R*) and (2*R*,4*R*)-2-phenylthiazolidine-4-carboxylic acid (4.3)

The synthesis of compound **4.3** has been reported in the literature [133]. Here an improved and simpler procedure has been used for its synthesis. Compound **4.3** was prepared according to the procedure described for **4.1**. L-Cys (1.67 mmol) and (1.67 mmol) benzaldehyde were added in 5 mL of DI H₂O. Reaction mixture was stirred at RT for 2 h. The precipitates from the reaction mixture were filtered, washed with ice cold H₂O and dried under vacuum. Compound **4.3** was obtained as a mixture of distereoisomers, colourless solid (488 mg, 99%); $\nu_{\max}$ (film)/cm⁻¹ 1668 (C=O); M.P.175-176 °C; ¹HMNR resonance for the major isomer are shown here, ¹H-NMR (400 MHz, DMSO-

d_6) δ (ppm) 7.4 (m, 5H, Ar) 5.67 (s, 1H, Ar-CH), 4.24 (q, $^3J_{\text{H-H}} = 5$ Hz, 1H, CO-CH), 3.80 (m, 1H, CH-CH₂), 3.15 (m, 1H, CH-CH₂); ^{13}C NMR (101 MHz, DMSO- d_6) (ppm) 172.95, 172.19 (C=O), 141.18, 139.91, 128.31, 127.25, 127.57, 126.91, 71.73, 71.06, 65.40, 64.85, 38.39, 37.94; Electrospray ionization (ESI) mass spectrometry (positive electron ionization) m/z : 210.06017 ($[\text{M}+\text{H}]^+$, 100%), calculated for $\text{C}_{10}\text{H}_{11}\text{NO}_2\text{S}$, m/z 209.05105.

(2*S*,4*S*) and (2*R*,4*S*) -2-phenyl-1,3-thiazinane-4-carboxylic acid (4.4)

L-Hcy (1.88 mmol) and benzaldehyde (1.88 mmol) were added in 6 mL of DI water. Reaction mixture was stirred at RT for 72 hrs. The resultant precipitates were filtered washed with ice cold H₂O and dried under vacuum. Compound **4.4** was obtained as a mixture of distereoisomers, colorless solid (273 mg, 65%); ν_{max} (film)/cm⁻¹ 1738 (C=O); M.P. 177-178°C; ^1H -NMR (400 MHz, DMSO- d_6) δ (ppm) 7.55 (m, 5H, Ar), 5.27 (s, 1H, Ar-CH), 3.60 (dd, $^3J_{\text{H-H}} = 9, 3$ Hz, 1H, CO-CH), 3.28 (td, $^3J_{\text{H-H}} = 11, 3$ Hz, 1H, S-CH₂), 2.91 (dt, $^3J_{\text{H-H}} = 10, 4$ Hz, 1H, S-CH₂), 2.10 (dq, $^3J_{\text{H-H}} = 7.0, 3$ Hz, 1H, CH-CH₂), 1.51 (qd, $^3J_{\text{H-H}} = 9, 4$ Hz, 1H, CH-CH₂); ^{13}C NMR, (101 MHz, DMSO d_6) δ (ppm) 173.5 (C=O), 140.8, 128.3 (overlapping), 127.8, 126.5, 64.1, 59.2, 28.9, 27.7; Electrospray ionization (ESI) mass spectrometry (positive electron ionization) m/z : 224.07707 ($[\text{M}+\text{H}]^+$, 100%) calculated for $\text{C}_{11}\text{H}_{13}\text{NO}_2\text{S}$, m/z 223.06670.

(*R*)-thiazolidine-4-carboxylic acid (4.5)

Synthesis of compound **4.5** has been reported in the literature, but for this work a modified and simpler procedure was used [126, 127]. Compound **4.5** was prepared by

following the procedure described for **4.1**. Formaldehyde was used as the starting material instead of salicylaldehyde. Compound **4.5** was obtained as a mixture of distereoisomers, colourless solid. (152 mg, 68.5%). $\nu_{\max}$ (film)/cm⁻¹ 1617 (C=O); M.P.181-182 °C; ¹H NMR (400 MHz, D₂O) δ 4.52 (m, 1H CO CH and 2H, NH₂CH₂), 3.47 (m, 2H, CH-CH₂). ¹³CNMR (101 MHz, D₂O) δ (ppm) 171.9 (C =O), 63.9, 48.6, 32.9. Electrospray ionization (ESI) mass spectrometry (positive electron ionization) m/z : 134.0252 ([M+H]⁺, 100%), calculated for C₄H₇NO₂S, m/z 133.01975.

(S)-1,3-thiazinane-4-carboxylic acid (4.6)

The synthesis of compound **4.6** has been reported in the literature [134]. Herein, a modified procedure to synthesize **4.6** has been used for the synthesis. L-Hcy (1.67 mmol) and formaldehyde (1.67 mmol) were added in 6 mL of DI water. The reaction mixture was stirred at RT for 48 h. The precipitates from the reaction mixture were filtered. Ethanol was added to the filtrate. The solution was kept at RT for 24 hrs. Transparent shiny crystal formation in the filtrate was observed. Crystals were filtered, washed with ice cold ethanol and dried under vacuum to yield **4.6** as colorless shiny crystals (135mg, 55%) ¹H NMR (400 MHz, D₂O) δ 4.29 (m, 2H, NH-CH₂-S), 3.68 (dd, ³J_{H-H} = 10, 3 Hz, 1H, CO-CH), 3.05 (m, 1H, S-CH₂-CH₂), 2.89 (m, 1H, S-CH₂-CH₂), 2.56 (m, 1H, CH-CH₂), 2.04 (qd, ³J_{H-H} = 10, 3 Hz, 1H, CHCH₂); ¹³CNMR (101 MHz, D₂O) 173.3 (C=O), 59.4, 45.1, 28.1, 26.0; Electrospray ionization (ESI) mass spectrometry (positive electron ionization) m/z : 148.04422 ([M+H]⁺, 100%), calculated for C₅H₉NO₂S, m/z 147.03540.

CHAPTER 5 SYNTHESIS OF FLUORESCEIN TRI ALDEHDYE AND ITS APPLICATION IN THE DETECTION OF HCY

5.1 Introduction

In previous chapters, optimum conditions for the detection of Hcy using **3.1** (FMA) and **3.3** (FDA) have been discussed. The mechanism behind this selectivity is based on differences in the basicity of Hcy and Cys derived heterocycles was also described. Based on this mechanism, the next goal was to design a more sensitive aldehyde-bearing fluorophore that can detect Hcy at its physiological levels in biological media. Compounds **3.1** and **3.3** didn't exhibit an sufficiently low detection limit for an appreciable response from Hcy at concentrations below ~50 μM in plasma (Figure 3-17). To achieve this goal fluorophore reactivity was increased by adding another reactive moiety and rendering the fluorophore ring more electrophilic. To implement this plan, it was decided to increase the number of aldehyde moieties on the fluorescein scaffold. As noticed before, the sensitivity of **3.3** was found to be twice of the sensitivity of **3.1**. Therefore it seemed reasonable that to improve the sensitivity of fluorophore, the number of aldehyde moieties should be increased. Based on this concept, fluorophore, fluorescein trialdehyde was designed.

5.2 Duff reaction and its application

Several methods have been reported in the literature for the formylation of aromatic compounds [135, 136]. For example, the Riemer Tiemann reaction [137], Gatterman reaction, Gattermann-Koch reaction [138], Vilsmeier-Haack reaction [139], and Duff

reaction [140] are well known name reactions for the formylation of aromatic compounds. The Riemer Tiemann reaction was applied towards the synthesis of **3.1** and **3.2**. Both were obtained from the same reaction *via* a separation, unlike the long synthesis reported by Lippard *et al.* [141] where at first, 4,5-dimethyl fluorescein was synthesized from the reaction of 2-methyl resorcinol and phthalic anhydride, later hydroxyl groups of 4,5-dimethyl fluorescein were protected with the help of benzoic anhydride. In the next step, Bromination of 4,5-dimethyl groups was carried out over a period of more than 7 days. Finally, bromo-methyl groups were converted to aldehyde groups by Swern oxidation to yield **3.2** with 36% yield [141].

To synthesize fluorescein trialdehyde, a new synthetic strategy was used for the diformylation of the phenolic ring. Previously, *ortho* (to a hydroxyl) diformylation using The Duff reaction had been published [142] by Grylo *et al.* using a diverse range of phenols as shown in Scheme 5-1. The Duff reaction also has some advantages including relative simplicity and inexpensive, stable and relatively nontoxic reagents.

Scheme 5-1. Duff formylation of phenol

In 1934, An English scientist James Cooper Duff found that the reaction of hexamethylenetetramine with phenolic compound in acetic acid yielded a Schiff base, which upon hydrolysis in acidic medium, resulted in the *ortho* formyl product [143, 144].

Since then The Duff reaction has been utilized for the direct diformylation of aromatic compounds in the synthesis of various drugs [145, 146], sensors [147, 148], and receptors [149] with some modifications. In the present work, the main focus was on the first application of The Duff reaction for the formylation of fluorescein to produce fluorescein trialdehyde. Attempts for the Synthesis of fluorescein tetra aldehyde were also made.

5.3 Result and discussion

Under The Duff reaction conditions, formylation of commercially available fluorescein was carried out by the reaction of fluorescein with hexamethylenetetramine in trifluoroacetic acid under an inert atmosphere followed by acid hydrolysis to afford **5.1** i.e. fluorescein tri aldehyde (FTA) in a single step (Scheme 5.2)

Scheme 5-2. Synthesis of fluorescein tri aldehyde (**5.1**)

Fluorescein tri aldehyde was obtained in 2% yield. The reason for the small yield was that, with the insertion of electron withdrawing aldehyde group in the fluorescein scaffold the nucleophilicity of the fluorophore decreases. Therefore the reactivity of the molecule towards electrophilic aromatic substitution for further formylation also reduces. As a result small yield of **5.1** was obtained and fluorescein tetra aldehyde could not be synthesized. Synthesis of **5.1**, under The Duff reaction was attempted several times by

changing the number of equivalents of reactants and duration of the reaction. It was found that the ratio of fluorescein to HMTA (1:10 eqv) and duration of reaction *i.e.* 72 hrs were the best parameters to synthesize **5.1**.

Before the application of The Duff reaction, for the synthesis of fluorescein tri and tetra aldehyde, different reaction routes were also implemented. Formylation of tetra bromo fluorescein in THF with butyl lithium and DMF under various reaction conditions did not yield the desired product. formylation of tetra bromo fluorescein by isopropyl bromide and DMF under different reaction conditions did not produce **5.1** either. Later, hydroxyl groups of tetra bromofluorescein were protected by benzoyl chloride . OH-protected tetra bromofluorescein was subjected to formylation by isopropyl bromide and DMF. However this reaction did not succeed to give desired product. Finally, phosphoryl chloride and DMF were used for the formylation of fluorescein under a number of different reaction conditions but none of them could produce formylated product.

Study of response of Hcy and Cys towards 5.1

To examine the sensitivity of this newly synthesized fluorophore **5.1**, the reaction of Hcy and Cys with **5.1** was studied at pH 6.0 (Figure 5-1). As expected, in response to Hcy, a high fluorescence enhancement (~7 fold) was observed, whereas Cys didn't show any appreciable change in the fluorescence. Later, the magnitude of the response towards Hcy with **3.1** and **3.3** (i.e. FMA and FDA) was also compared. The magnitude of fluorescence response of **5.1** towards Hcy was found to be more than higher than that of **3.1** and **3.3** (Figure 5-2).

Figure 5-1. Optical sensing behavior of **5.1** towards Hcy and Cys at pH 6.0. (a) Fluorescence spectral changes of **5.1** with Hcy and Cys after 2h. (b) Time dependent fluorescence spectral changes of **5.1** with Hcy and Cys. Solutions are composed of 4 μM of **5.1** with 1 mM of analyte in phosphate buffer (100 mM, pH 6.0) DMSO 99:1 at 20 $^{\circ}\text{C}$, $\lambda_{\text{ex}} = 495 \text{ nm}$ and $\lambda_{\text{em}} = 595 \text{ nm}$.

Figure 5-2. Comparison of magnitude of response of Hcy towards (a) **5.1**, (b) **3.3**, (c) **3.1**. Solutions are composed of 4 μM of probe with 1 mM of analyte in phosphate buffer (100 mM, pH 6.0) DMSO 99:1 at 20 $^{\circ}\text{C}$, $\lambda_{\text{ex}} = 495 \text{ nm}$ and $\lambda_{\text{em}} = 515 \text{ nm}$

After investigating the relative magnitude of the signal in the detection of Hcy with **5.1**, its behavior towards Hcy and Cys was evaluated at physiologically relevant levels. On adding Hcy (15 μM) in the presence of Cys (250 μM) to a solution of **5.1**, an

enhancement in the fluorescence (~ 1.5 fold) was obtained. Cys at 250 μM does not produce a change in the optical response of **5.1**, as shown in the Figure 5-3.

Figure 5-3. Optical sensing behavior of **5.1** towards Cys and Hcy at their biological concentrations at pH 6.0.

(a) Fluorescence spectra of **5.1** with Cys and Hcy after two hours. Solutions are composed of 30 μM of **3.3** with 250 μM of Cys and 15 μM of Hcy in phosphate buffer (100 mM, pH 6.0):DMSO 99:1 at 20 $^{\circ}\text{C}$, λ_{ex} = 494 nm and λ_{em} = 525 nm.

The response to Hcy was also investigated in the presence of various amino acids (Gly, Ala, Met, Gln, Glu, and Asp) along with Cys. Figure 5-4 shows that Hcy produces a distinct response in the presence of these related amino acid analytes. Other amino acids

and Cys produced a very small quenching in the fluorescence of **5.1**. Whereas, Hcy produced almost 3 folds enhancement in the fluorescence. That shows the specificity of Hcy over other amino acids and Cys towards **5.1**. Glutathione (GSH) produced no significant change in the fluorescence signal (Figure 5-4). Overall, these results demonstrated that **5.1** exhibited a distinct selectivity for Hcy.

Figure 5-4. Optical sensing behavior of **5.1** towards Hcy and Cys with and without amino acids and GSH at pH 6.0

fluorescence emission of **1** with Cys and Hcy in presence and absence of amino acids and GSH after 2h. Solutions are composed of 4 μM of **5.1** with 50 μM of each analyte in phosphate buffer (100 mM, pH 6.0):DMSO 99:1 at 20 $^{\circ}\text{C}$, $\lambda_{\text{ex}} = 495 \text{ nm}$

Figure 5-5. (a) and (b)- Spectral response of **5.1** towards increasing levels of Hcy in phosphate buffer (100 mM, pH 6.0 at 2h). Solutions are composed of 4 μM of **5.1** with 0-25 μM of Hcy in phosphate buffer (100 mM, pH 6.0):DMSO 99:1 at 20 $^{\circ}\text{C}$, $\lambda_{\text{ex}} = 495 \text{ nm}$ and $\lambda_{\text{ex}} = 525 \text{ nm}$

A concentration dependent experiment was also performed which indicated that the fluorescence response of **5.1** increased linearly with a limit of detection at 1.88 μM as shown in Figure 5-5. LOD was calculated with the help of standard error, slope and intercept from the calibration curve graph. Standard error was obtained from the regression analysis in Microsoft Excel 2010.

Further, at the physiological levels of thiols, the response of **5.1** towards Hcy and Cys was investigated in deproteinized plasma. Results show that probe **5.1** was also capable of detecting Hcy in biological media. To measure the known amount of Hcy, deproteinized plasma was spiked with the known concentration of Hcy. In deproteinized plasma, addition of Hcy to a solution of **5.1** enhances the fluorescence. In contrast, no significant change in the emission intensity was observed upon addition of Cys. (Figure 5-6) With an increase in the concentration of Hcy, increase in the fluorescence emission was also observed in deproteinized plasma, as shown in Figure 5-7.

Figure 5-6. Optical sensing behavior of **5.1** towards Cys and Hcy in deproteinized plasma at pH 6.0. after 2h.

Solutions are composed of 20 μM of **5.1** with 250 μM of Cys and 15 μM of Hcy in phosphate buffer (100 mM, pH 6.0):DMSO 99:1 at 20 $^{\circ}\text{C}$, $\lambda_{\text{ex}} = 495 \text{ nm}$

Figure 5-7. Optical sensing behavior of **5.1** towards Cys and Hcy in deproteinized plasma at pH 6.0. after 2h.

Solutions are composed of 20 μM of **5.1** with 5, 10, 15, 20 and 25 μM of Hcy in phosphate buffer (100 mM, pH 6.0):DMSO 99:1 at 20 $^{\circ}\text{C}$, λ_{ex} = 495 nm

As previously explained fluorescence enhancement of **5.1** by the addition of Hcy was due to the formation of thiazinane-4-carboxylic acid, and subsequent protonation of the amine, producing an ammonium species at pH 6.0 to inhibit PET. To confirm the PET inhibition based response of Hcy to **5.1**, the reaction of Hcy and Cys was studied at lower pH (5.0), intermediate pH (6.0) and higher pH (9.5). In Figure 5-8, it is evident that at lower pH (5.0), the thiazinane and thiazolidines are in the ammonium form, since fluorescent enhancement occurs. Conversely, at pH 6.0, the ammonium of the thiazinane is the predominant species in contrast to Cys-derived thiazolidine, wherein the ammonium and amine species exist in nearly equal amounts. At higher pH (9.5), the amine of both heterocycles have their lone pairs available for PET quenching.

Figure 5-8. Optical sensing behavior of **5.1** towards Cys and Hcy in buffer at different pH, after 2h. Fluorescence spectra of **5.1** with Cys and Hcy (a) at pH 5.5 in phosphate buffer, (b) at pH 6.0 in phosphate buffer, (c) at pH 9.5 in carbonate buffer. Solutions are composed of 4 μ M of **5.1** with 1 mM of analyte in 100 mM buffer solution: DMSO(99:1) at 20 $^{\circ}$ C, λ_{ex} = 495 nm

Apart from being selective, the enhanced sensitivity of the probe **5.1** is due to the presence of three electron withdrawing aldehyde groups that makes the fluorophore scaffold more electrophilic and reactive. Previously, Huang *et al.* calculated the surface charges based on density functional theory (DFT) for an aldehyde-bearing iridium (III) complex. They found that charges on the N and S atoms of thiazolidine are more negative than the charges on the N and S atoms of thiazinane. According to them, higher negative charges on the thiazolidine can result in an intramolecular electron-transfer process from the thiazolidine group to the excited complex, causing a weak photoluminescence of the probe-Cys complex [109].

In the current case, as shown in Table 5.1, the Mulliken charges on the N and S

atoms for Cys-derived thiazolidine are more negative (N,-0.650223 and S,-0.384138) when compared to the N and S atoms of Hcy-derived thiazinane (N,-0.399059 and S,-0.078137). This is in agreement with the results reported by Huang *et al.* for the intramolecular electron transfer process from the thiazolidine group resulting in fluorescence quenching or weak enhancement [109].

Table 5.1 Comparison Mulliken charges on the ‘N’ and ‘S’ of Cys and Hcy derived heterocycles formed upon reaction with **5.1**

	Mulliken charges	Mulliken charges
5.1 +Hcy ‘N’	-0.202843, -0.194407, -0.209806	‘S’ -0.020108, - 0.043911, -0.059903
5.1 +Cys ‘N’	-0.542689, -0.544021, -0.529787	‘S’ -0.136779, -0.218184, -0.358607

Further, the selective signaling for Hcy in terms of ionic charges can also be explained. From the Mulliken charge calculations, it was found that the phenolate anion and the ether oxygen of the thiazinane (Hcy)-dye adduct bear higher negative charges (-0.729, -0.686 and -0.326, respectively) than of the Cys-derived thiazolidine (-0.508, -0.621 and -0.280, respectively) and **5.1** (-0.659, -0.576 and -0.261, respectively, Table 5.2 and Figure 5-9). The development of enhanced negative charge density on the fluorophore phenolate oxygen upon formation of the Hcy-derived thiazinane results in an increase in fluorescence emission.

Figure 5-9. Predominant species of compounds **5.1**, **5.1a** and **5.1b** at pH 6.0. Calculated Mulliken charges are shown for the oxygen atoms in the fused ring system.

Table 5.2 Comparison Mulliken charges on the phenolate 'O' and ether 'O' of **5.1** and Cys and Hcy derived heterocycles formed upon reaction with **5.1**

Mulliken charges		
5.1	Phenolate oxygen, ether oxygen	-0.65882, -0.575738, -0.261133
5.1+Hcy	Phenolate oxygen, ether oxygen	-0.729433, -0.686371, -0.325709
5.1+Cys	Phenolate oxygen, ether oxygen	-0.508089, -0.620732, -0.280251

5.5 Conclusion

In conclusion, a highly selective fluorescein trialdehyde for the detection of Hcy at its

physiological level in biological media has been developed. At pH 6.0, protonation of the amine of the Hcy-derived thiazinane takes place due to its higher basicity compared to the amine of the Cys-derived thiazolidine. The protonation results in inhibition of PET quenching and selective signaling for Hcy. The presence of three electron-withdrawing aldehyde groups makes the fluorophore highly reactive and enhances the Hcy detection limit. Mulliken charge calculations reveal that the Hcy-derived thiazinane renders the fluorophore phenoxide oxygen more electron-rich leading to fluorescence enhancement as compared to the Cys-derived thiazolidine.

5.4 Experimental section

5.4.1 Materials

All chemicals were purchased from Sigma Aldrich and used as received. Ultra-pure water (18.2 M Ω ·cm at 25 °C) obtained from a Milli-Q direct water purification system was utilized to prepare all aqueous solutions. All spectroscopic measurements were conducted in DMSO:buffer (1:99) solutions.

5.4.2 Methods

^1H NMR and ^{13}C -NMR were recorded on a ARX-400 Advance Bruker spectrometer performing at 400.13 MHz (^1H) and 100.61 MHz (^{13}C). All chemical shifts chemical shifts (δ) were reported in ppm using DSS (sodium 2, 2-dimethyl-2-silapentane-5-sulfonate) as an internal standard ($\delta=0.000$) for ^1H NMR studies. CF_3COOD was used as a NMR solvent to record ^1H NMR spectra. ^{13}C NMR of **5.1** was obtained in CDCl_3 and CF_3COOD (1:8) solvent system. ESI-HRMS (high resolution mass spectrometry) spectra

was recorded at the PSU Bioanalytical Mass Spectrometry Facility on a ThermoElectron LTQ-Orbitrap high resolution mass spectrometer. IR spectrum was obtained by Fourier transform infrared (FT-IR) spectroscopy operated on a Thermo Scientific Nicolet iS10 spectrophotometer equipped with a single-bounce diamond ATR attachment.

5.4.3 Spectrophotometric measurement

UV-visible spectra were recorded on a Cary 50 UV-Vis spectrophotometer (Agilent Technologies). Fluorescence spectra were recorded on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) with slit widths set at 5 nm for both excitation and emission, respectively. Fluorescence spectra were corrected for the wavelength dependent response of the R928 photomultiplier tube with the help of a manufacturer generated correction file. All spectrophotometric measurements were conducted at room temperature.

5.4.3 Deproteinization of plasma

Deproteinization of plasma was carried out by following the procedure described in the section 3.6.4.

5.4.4 Mulliken charges calculation

Molecular models of compounds **5.1**, **5.1a** and **5.1b** were built and subjected to geometry optimization and Mulliken population analysis using the PM7 hamiltonian in MOPAC2012 [150, 151].

5.4.5 Synthesis of 5.1

Fluorescein (1 eqv, 7.52 mmol) was dissolved in a minimum amount of trifluoroacetic

acid. Hexamethylene tetraamine (10 eqv, 75.23 mmol) was added slowly to the fluorescein solution under Ar atmosphere at RT. Reaction mixture was stirred at 70°C for 72 hrs under Ar and then at 100°C for extra 4 hrs. Afterward, 30 mL of 1N HCl was added to the reaction mixture and stirred at 100 °C for additional 1hr. This resulted in the formation of orange precipitates. The reaction mixture was left at RT for 15 hrs. Orange precipitates were filtered, washed with water and dried under vacuum. Later, probe **5.1** was separated by column chromatography (CHCl₃: MeOH, 98:2 and DCM: EtOAc 97:3). Product was obtained as colorless solid with 2 % yield (62 mg). R_f = 0.3 (3% Ethyl acetate in dichloromethane, SiO₂); M.P. > 200 °C; ¹H NMR (400 MHz, CDCl₃) δ 10.74 (s, 1H, CHO), 10.62 (s, 1H, CHO), 9.93 (s, 1H, CHO), 8.17 (m, 1H), 7.84 (s, 1H), 7.74 (m, 2H), 7.25 (m, 2H), 6.94 (m, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 189.7 (CHO), 187.6(overlapping, CHO), 166.3 (COOH), 153.7, 152.8 , 137.9 , 133.6 , 131.9, 127.8, 125.1, 122.4, 122, 120.8 , 117.9 , 115.5, 111.9, 110.8, 109.6 -108 (overlapping), 104.4; $\nu_{\max}$ (film)/cm⁻¹ 1738 (C=O);. Electrospray ionization (ESI) mass spectrometry (negative electron ionization) m/z : 415.04610 (100% (M-H)⁻, calculated for C₂₃H₁₂O₈ m/z : 416.05322 (100.0%).

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APPENDIX A : CHARACTERIZATION OF COMPOUNDS 4.1 AND 4.2

Figure A-1. ¹H NMR of **4.1** and **4.2**

Figure A-2 ^{13}C NMR of **4.1** and **4.2**

SAL CYS F1 #1 RT: 0.00 AV: 1 NL: 7.54E5
T: FTMS + pESI/Full ms [100.00-260.00]

SAL HC1 #1 RT: 0.00 AV: 1 NL: 9.08E5
T: FTMS + pESI/Full ms [100.00-260.00]

Figure A-3. Mass spectrum of 4.1 and 4.2

APPENDIX B : CHARACTERIZATION OF COMPOUND 4.3 AND 4.4

Figure B-2. ¹³CNMR of **4.3** and **4.4**

BANCYS #1 RT: 0.00 AV: 1 NL: 1.79E5
T: FTMS + pESIFull.ms [100.00-250.00]

BAN HCl #1 RT: 0.01 AV: 1 NL: 3.79E5
T: FTMS + pESIFull.ms [100.00-250.00]

Figure B-3. Mass spectrum of 4.3 and 4.4

¹³C NMR (CDCl₃) spectrum of compound **4.5**. The chemical structure of **4.5** is shown above the spectrum. The spectrum displays peaks at 1774.8965, 1749.8484, 1739.6051, 1384.8898, 1377.4874, 1372.8059, 1365.3635, 1344.3967, 1338.6348, 1332.3128, 1326.5509, 1170.8603, 1163.0178, 1155.0152, 703.0284, 259.5243, 251.0015, 242.5588, 3.1210, -0.1200, and -3.3611 ppm.

¹H NMR (CDCl₃) spectrum of compound **4.5**. The chemical structure of **4.5** is shown above the spectrum. The spectrum displays peaks at 1727.0410, 1708.1949, 1707.8347, 1695.1106, 1481.2811, 1478.8404, 1469.3973, 1466.9965, 1196.7887, 1185.1450, 1183.2243, 1141.7709, 1019.2111, 1016.0100, 822.9073, 811.1835, 0.8003, and -0.1200 ppm.

¹³C NMR (CDCl₃) spectrum of compound **4.6**. The chemical structure of **4.6** is shown above the spectrum. The spectrum displays peaks at 1774.8965, 1749.8484, 1739.6051, 1384.8898, 1377.4874, 1372.8059, 1365.3635, 1344.3967, 1338.6348, 1332.3128, 1326.5509, 1170.8603, 1163.0178, 1155.0152, 703.0284, 259.5243, 251.0015, 242.5588, 3.1210, -0.1200, and -3.3611 ppm.

¹H NMR (CDCl₃) spectrum of compound **4.6**. The chemical structure of **4.6** is shown above the spectrum. The spectrum displays peaks at 1727.0410, 1708.1949, 1707.8347, 1695.1106, 1481.2811, 1478.8404, 1469.3973, 1466.9965, 1196.7887, 1185.1450, 1183.2243, 1141.7709, 1019.2111, 1016.0100, 822.9073, 811.1835, 0.8003, and -0.1200 ppm.

101

Figure C-2. ^{13}C NMR of **4.5** and **4.6**

Figure C-3. Mass spectrum of 4.5 and 4.6

APPENDIX D : CHARACTERIZATION OF COMPOUNDS 5.1

Figure D-1. ^1H NMR of 5.1

Figure D-2. ^{13}C NMR of 5.1

APPENDIX E : COLORIMETRIC RESPONSE OF THIOLS TOWARDS 5.1

Figure E-1. Optical sensing behavior of **5.1** towards Hcy at pH 6.0, Time course absorbance spectral changes of **5.1** with Hcy, Solutions are composed of 4 μM of **5.1** with 50 μM of analyte in phosphate buffer (100 mM, pH 6.0):DMSO 99:1 at 20 $^{\circ}\text{C}$

Figure E-2. Optical sensing behavior of **5.1** towards Cys at pH 6.0, Time course absorbance spectral changes of **5.1** with Cys, Solutions are composed of 4 μM of **5.1** with 50 μM of analyte in phosphate buffer (100 mM, pH 6.0):DMSO 99:1 at 20 $^{\circ}\text{C}$

**APPENDIX F : ENERY MINIMIZED SIMULATED 3D STRUCTURE OF 5.1,
5.1A AND 5.1B'**

Figure F-1. Energy minimized 3D structure of **5.1**

Figure F-2. Energy minimized 3D structure of **5.1b** (Hcy-**5.1**)

Figure F-3. Energy minimized 3D structure of **5.1a** (Cys-**5.1**).