

**Proteomic Studies of Erythrocytes
In Sickle Cell Disease, Erythrocyte
Membrane Skeleton And Post
Translational Modifications**

**Thesis Submitted for the Degree of
Doctor of Philosophy (Science)
In**

Biotechnology

By

Avik Basu

**University of Calcutta, India
2014**

Contents

Abbreviations	1
Synopsis	3
Chapter 1: General Introduction	
1. Human Erythrocyte	6
1.1 Brief Description	6
1.2 Erythrocyte Membrane	8
1.3 Erythrocyte Membrane Proteins	8
1.3. A. Integral Membrane Proteins	9
1.3. B Membrane Skeleton Proteins	10
1.3. C. Lipid Bilayer-Membrane Skeleton Interaction	13
1.3. D. Defects in Membrane Skeleton and Related Disorders	14
1.4. Erythrocyte Cytosolic Proteins	15
1.4. A. Hemoglobins	15
1.4. B. Redox Regulators	17
1.4. C. Other Cytosolic Proteins	19
1.5. Abnormal Hemoglobins and Hemoglobinopathies	20
1.5. A. Sickle Cell Disease	21
2. Introduction to Proteomics	23
2.1. Brief Overview	23
2.2. Gel Based Proteomics	23
2.2. A. Two-Dimensional Differential In-Gel Electrophoresis (2D-DIGE)	24
2.3. Liquid Chromatography Based Proteomics	26
2.4. Biological Mass Spectrometry	27
2.4. A. Matrix Assisted Laser Disruption Ionisation (MALDI)	28
2.4. B. Electron Spray Ionisation (ESI)	29
2.4. C. Time of Flight (ToF) and ToF-ToF Analyzers	30
2.4. D. Iontrap and Orbitrap Analyzers	34
2.5. The Data Analysis Software	34
2.5. A. SEQUEST	35
2.5. B. MASCOT	35
2.6. Interactome Study	35
2.7. Post Translational Modification (PTM) Analysis	36
3. Proteomics of Erythrocytes	38
3.1. Gel Based Erythrocyte Proteomics	38
3.1. A. Erythrocyte Membrane Proteomics	39
3.1. B. Erythrocyte Cytosolic Proteomics	39
3.2. Liquid Chromatography (LC) based Proteomics in Erythrocyte	40
3.3. Erythrocyte Interactome	42
3.4. Post translational Modifications of Erythrocyte Proteins	43
3.5. Proteomics of Erythrocytes in Hemoglobinopathies	44
4. Objectives of the Proposed Thesis Work	47
References	

Chapter 2: Proteomics Study of Erythrocyte in Sickle Cell Disease

1. Prologue	57
2. Material and Methods	59
2.1. Materials	59
2.2. Sample Collection:	59
2.3. Erythrocyte Processing and Hemoglobin Depletion	61
2.4. Fluorescence Differential in Gel Electrophoresis (DIGE) Experiment	61
2.5. MALDI ToF/ToF Mass Spectrometry	63
2.6. Immunoblotting (1D and 2D)	64
2.7. Statistical Analysis	65
2.8. Estimation of Reactive Oxygen Species	65
2.9. Confocal Imaging of ROS Generation	65
2.10. Oxyblot™ Analysis for Measuring Oxidative Stress in Erythrocytes	66
3. Results	66
3.1. Differential Protein Profiling Between Normal and SCD Hb Depleted Cytosol	66
3.2. Validation by Immunoblotting	69
3.3. Prdx2 Dimerization	70
3.4. Catalase Phosphorylation	71
3.5. Reactive Oxygen Species Analysis	72
3.6. Oxyblot Analysis	74
4. Discussion and Conclusion	75
References	

Chapter 3: Hemoglobin Interacting Proteins & Implications of Spectrin Hemoglobin Interaction

1. Prologue	82
2. Material and Methods	83
2.1. Materials	83
2.2. Erythrocyte Processing	85
2.3. Hemoglobin Depletion	85
2.4. Hemoglobin Purification	85
2.5. Globin Chain Purification	85
2.6. Two Dimensional Gel Electrophoresis	86
2.7. 1D and 2D Far Western Blotting	86
2.8. The Pull-down Experiment	87
2.9. Immunoprecipitation	87
2.10. MALDI ToF/ToF Mass Spectrometry	88
2.11. Co-immunoprecipitation	88
2.12. Spectrin Purification and FITC Labeling	89
2.13. Globin Chain Peroxidase Activity Measurements	89
2.14. Globin Chain Aggregation Assay	90
2.15. Measurement of Dissociation Constants between F-Spectrin and HbS	90
3. Results	91
3.1. Interacting Partners of Hemoglobin.	91
3.1.A. Identification of Hb Interacting Proteins from Erythrocyte Cytosol	91
3.1.B. Validation of the Identified Cytosolic Hb Interacting Partners	94
3.1.C. Identification of Hb Interacting Proteins from Erythrocyte Membrane	95
3.2. Functional Implication(s) of Spectrin- globin Chain Interactions	97
3.3. Characterization of Spectrin- HbS Interaction by Fluorescence Analysis	100
4. Discussion and Conclusion	101

References

Chapter 4: Proteomic Study of Erythrocyte Membrane Skeleton

1. Prologue	107
2. Material and Methods	109
2.1. Material	109
2.2. Sample Collection	109
2.3. Preparation of Triton Skeletons	110
2.4. Filter Assisted Sample Preparation (FASP)	110
2.5. LC-ESI-Orbitrap and Q Exactive	111
2.6. LC Data Processing	112
3. Results	112
3.1. Triton Skeleton Proteome	112
3.2. Argonaute-2 in Erythrocyte Membranes and Membrane Skeletons	117
4. Discussion and Conclusion	119
References	

Chapter 5: Phosphorylation and Ubiquitination of Erythrocyte Membrane Proteins

1. Prologue	123
2. Material and methods	124
2.1. Material	124
2.2. Sample Collection	125
2.3. Filter Assisted Sample Preparation (FASP)	125
2.4. Phospho-peptide Enrichment	125
2.5. Ubiquitin Remnant Enrichment	126
2.6. LC-ESI-Orbitrap and Q Exactive	126
2.7. MS Data Processing	127
3. Results	128
3.1. Erythrocyte Membrane Phosphoproteomics	128
3.1. A. Phospho-peptide Enrichment (TiO ₂ vs. IMAC)	128
3.1. B. Phosphoproteome Analysis	130
3.2. Ubiquitinome Analysis of Erythrocyte Membrane Proteins	134
3.2. A. Enrichments of Ubiquitinated Peptides	134
3.2. B. Ubiquitinome Analysis	136
4. Discussion and Conclusion	148
References	

Chapter 6: Summary & Conclusions

Publications

Appendix in CD (MS Data for All the Identified proteins and PTM Sites)