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Enrichment of Lipids and Their Complexes from Biological Samples by Affinity Chromatography and Analysis by Different Analytical Techniques

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Chemistry

at the Institute of Chemical Sciences,

Bahauddin Zakariya University, Multan, Pakistan

By

Rabia Javeed

Roll No. PHD-C-14-04 Registration No. 2011-bzch-13

Supervisor

Prof. Dr. M. Najam-ul-Haq

Institute of Chemical Sciences

Bahauddin Zakariya University

Multan, Pakistan

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It is hereby certified that the research work presented in this thesis entitled **"Enrichment of Lipids and their Complexes from Biological Samples by Affinity Chromatography and Analysis by Different Analytical Techniques"** is submitted in its present form by **Miss Rabia Javeed** to the Institute of Chemical Sciences, Bahauddin Zakariya University, Multan, Pakistan for the partial fulfillment of the degree of **Doctorate of Philosophy in Chemistry**.

Prof. Dr. M. Najam-ul-Haq
PhD Supervisor
Institute of Chemical Sciences
Bahauddin Zakariya University
Multan, Pakistan

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The plasma samples (Visucon-F frozen normal control plasma, citrated and 0.02 M HEPES buffered) were obtained from Affinity Biologicals (Ancaster, Canada). Furthermore, it is confirmed that the experimental protocols to execute the described experiments were approved by the Ethical Committee. It is clarified that the reported study in this work does not directly involve any *in vivo* animal studies.

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DEDICATION

This study is dedicated to my beloved parents ***Mr. and Mrs. Javeed Riaz*** and brothers ***Ali Javeed, Ahsan Javeed*** *and* ***Asad Javeed***; who gave me strength when I thought of giving up, who continually provide their moral, emotional, spiritual and financial support.

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ABSTRACT

Lipids are simply classified into simple, compound and derived lipids. Compound lipids are phospholipids, glycolipids, sulfolipids and lipoproteins. Lipoproteins are macromolecular complexes of lipids with proteins. Lipoprotein's surface is covered by phospholipids, having phosphate groups. Lipoproteins are separated by the interaction of anion exchanger with the phosphate groups and elution takes place at specific pH. HDL is isolated by precipitating the very low density lipoproteins (VLDL) and low density lipoproteins (LDL) with standard precipitating reagent, followed by delipidation by liquid/liquid extraction. Isolated Apolipoproteins are analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). Polymeric separation media are popular in separation science due to its versatility and chemical stability. Poly(GMA/DVB), poly(GMA/EGMA) and poly(GPE/DVB) are derivatized as strong anion exchangers and used for lipoproteins separation. These materials prove their worth by the selective enrichment of lipoproteins.

High-density lipoproteins (HDLs) have anti-inflammatory and antioxidant properties and are potentially cardio-protective. Defective HDL function is caused by the alterations in both proteome and the lipidome of HDL particles. As potential biomarker, analytical methods are required to be developed for the enrichment of HDLs. Therefore, a method for the selective enrichment of HDLs is developed. Immobilized metal ion affinity chromatography (IMAC) and metal oxide affinity chromatography (MOAC) solid phase extraction (SPE) based isolation of HDLs from whole serum is adopted as an alternate to the traditional ultracentrifugation method followed by SDS-PAGE. The enrichment mechanism relies on the isoelectric points of lipoproteins and metal oxide. Negatively charged lipoprotein particles interact with positively charged metal oxides and IMAC affinity which acts as a cation. Identified proteins from HDL through MALDI-MS analysis are apo A-I, A-II, A-IV, C-I, C-II, C-III, E, J, M, H, serum amyloid A, and other non-apoproteins that are part of HDL particles and perform cellular functions. This serum based proteomics approach gives insight into the functional role of HDL. HDL associated phospholipids have also been analyzed by LDI-MS (without traditional matrix). The results suggest that the adopted analytical strategy is useful to extract lipoproteins from serum. A comparative study of healthy and diseased samples by using this approach can provide valuable information in future.

Apolipoproteins are the structural and functional protein constituents of lipoproteins. Apolipoproteins are reported to be differentially regulated, in various types of cancers, for example profile changes in 8.9 kDa isoform of Apo A-II in serum from individuals with prostate cancer, ApoA-I for ovarian cancer, colorectal cancer and pancreatic cancer.

Apo-H is a plasma glycoprotein and has high content of carbohydrates relative to other proteins. Up and down regulation and structural changes of protein glycans are hallmark of disease states and provide diagnostic value for disease detection. An efficient, sensitive and optimized method is developed for Apo-H N-glycans analysis by MALDI-TOF MS in positive mode. This bioanalytical method development includes two main steps, the sample preparation and sample purification followed by detection. Several branched; bi- and tri- antennary glycans with fucosylated and sialylation structure are identified. Reproducibility of developed method is tested by multiple replicates of human plasma where same glycans are consistently identified. The applicability of this method is expected for the Apo-H glycans profiling of large clinical cohorts and hold future for therapeutic purposes.

Synthesis of Fe_3O_4/ZIF-8/APBA offers a simple and effective approach, in which the boronic acid ligand is easily and well functionalized on to the composite substrate materials. Boronic acid affinities usually show specific and reproducible enrichment. In basic conditions, boronic acid binds through cis-1,2-diols and in acidic conditions, the target molecules are released. Boronic acid affinity has enriched the world of glycoproteins and glycopeptides. Here this affinity is being used for the glycolipids i.e. gangliosides (sialic acid containing biomolecules) enrichment. Mixture of gangliosides is poured on the material and incubation, washing and elution steps are followed. Eluents are analyzed by MALDI-MS. Analysis conditions are optimized and analyzed in positive and negative modes.

Gangliosides with different number of sialic acids are identified in eluent spectra. Supernatant and wash spectra are free of any particular peaks. Gangliosides are altered during the pathological conditions and particularly in cancers. This strategy will be helpful to analyze gangliosides in biological samples.

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CHAPTER # 1
INTRODUCTION

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INTRODUCTION

1.1 Lipids

Unlike proteins, polysaccharides, nucleic acids lipids are not polymers, they are small molecules. Lipids have diversity in their structures and functions and are involved in constructing membranes and energy storage in physiological systems. In the last decade, lipids have expanded and considered as main factor responsible for apoptosis, inflammation, cell signaling, immunity and metabolic errors.

Lipids molecules are water insoluble however they are soluble in organic based solvent systems. "International Lipid Classification and Nomenclature Committee" defined lipids as “hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion-based condensation of thioester (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids and polyketides) and/or by carbocation-based condensations of isoprene units (prenol lipids and sterol lipids)”.

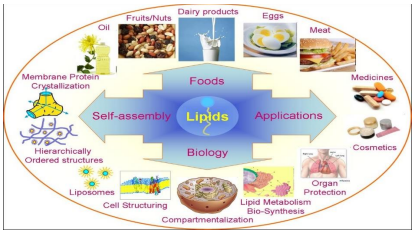

Figure 1.1: Lipids in human life

1.2 Lipidomics

The recent developments in modern mass spectrometry instrumentation, especially in soft ionization techniques (MALDI and ESI) in terms of resolution and mass accuracy, has increased the success of lipidomics to answer lipid metabolic questions. Lipidomics is the latest area of Omics which is applicable to all therapeutic areas and diagnosis of several diseases including cardiovascular diseases (CVD), neurological diseases, diabetes, autoimmune, inflammatory diseases, and cancer.

Lipidomic technology is a valuable tool in disease evaluation and in understanding mechanisms of action of new therapeutic drugs [1]. Lipid profiles can be linked with data about genome, transcriptome, proteome, and other metabolites of a system [2]. Presently used technologies facilitate studies in all biological materials, with cultured cells, biofluids (serum, urine, cerebrospinal fluid, blood, plasma, etc.) and tissue from clinical samples. Good sample preparation methodology, efficient sample handling and short storage times are pre-requisites in accurate lipidomics analysis.

1.3 Traditional Methods of Lipid Analysis

Traditionally, lipid analysis is done by chromatographic methods, mainly by TLC and LC. Both techniques are used however these techniques are limited by low resolution (TLC) or need long-standing experience (LC). Another modern technique of phospholipids (PLs) analysis is high resolution ^{31}P-NMR spectroscopy which is not suitable for general lipids. This technique requires expensive equipment [3].

1.4 Lipids Classification

Lipids are categorized as (Figure 1.2).

- Simple lipids
- Derived lipids
- Compound lipids

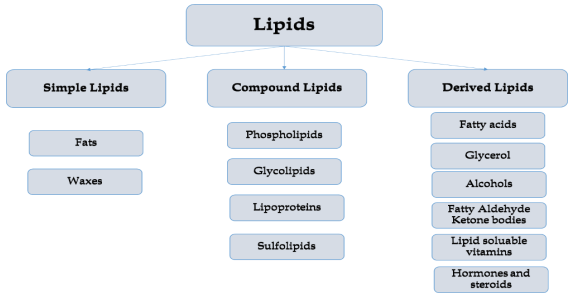

Figure 1.2: Classification of lipids

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1.4.1 Simple Lipids

They are the fatty acids esters having various alcohols. Fats are the esters of fatty acids having glycerol. They are found in nature in large quantities. They preserve body heat by acting as an insulator. They protect internal organs by providing cushion effect. They are tasteless, odorless and neutral in reaction. Their melting points are low. Waxes are fatty acids esters having alcohols and glycerol. They are important for making ointment and cosmetics.

1.4.2 Derived Lipids

They are produced by the hydrolysis of complex and simple lipids. Derived lipids include ketone bodies, hydrocarbons, glycerol, lipid soluble vitamins, and steroid hormones. They are fatty acids, steroids, prostaglandins, cholesterol, vitamin A and D.

1.4.3 Complex or Compound Lipids

These lipids contain additional substances e.g. sulfur, phosphorous, amino group, carbohydrate or proteins besides fatty acid and alcohol. Complex or conjugated lipids are classified according to the nature of additional group:

- Phospholipid
- Glycolipid
- Lipoproteins
- Sulfolipid

1.4.3.1 Phospholipids

Phospholipids have both hydrophilic as well as lipophilic characteristics. Phospholipids have role in many biological processes and are the main components of cell membrane (Figure 1.3).They are important to maintain physical structure and function, such as selective permeability, active transportation and electrical conductivity.

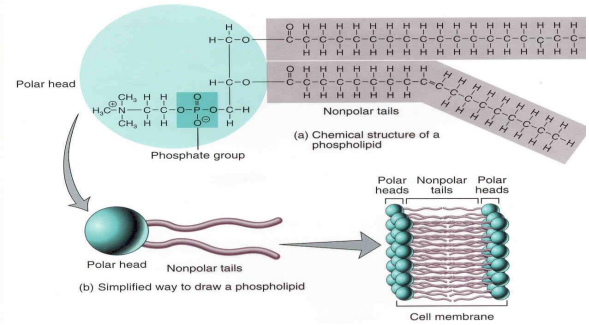

Figure 1.3: Phospholipids structure and its role in cell membrane

Phospholipids (PLs) are further classified into two major groups; sphingolipids and glycerophospholipids. Phosphatidyl inositol (PI), Phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and phosphatidyl choline (PC) are the examples of glycerophospholipids. On the other hand, sphingomyelin (SM), a famous sphingolipids, originated from sphingosine [4].

PLs are helpful to human health. They decrease the risk of cardiovascular diseases (CVD) [5]. Phospholipids play role in a variety of pathologies [6] as shown in Table 1.1.

Table 1.1: Phospholipids related disorders

Phospholipids	Related Disorders
PC	Distress syndrome Respiratory disease Acute respiratory Cystic fibrosis (CF) Asthma
PC, PE	Neurological dysfunction
PE, PC	Alzheimer’s disease
PI	Down’s syndrome,

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PE, PI	Diabetes
Cardiolipin (CL)	Heart failure
Oxidized phospholipids	Atherosclerosis

Phospholipids are also extensively used as emulsion stabilizers by the food industry. Best sources of PLs are dairy products: PC, PI, PE, PS, and SM are found in milk fat. Lipoproteins are fenced with these amphipathic phospholipids (PL) monolayer. PLs polar head groups are pointed toward the aqueous plasma matrix. Lipoproteins PL structural study is important for better understanding of disease states. Main PLs on the monolayer surface of lipoproteins are PE, PC, lysophosphatidylcholines LPC, PI and SM. Each PL species have specific molecular shape because of differences in their head group and acyl chains which vary by carbon chain length and number of double bond [7].

Firstly lipids were extracted by Folch and Bligh-Dyer method [8,9] and then PLs were isolated from total polar lipids. For the separation of PL classes, the common method is thin-layer chromatography (TLC) [10] or liquid chromatography (LC) normal-phase, [11] and separation of molecular species by gas chromatography (GC) [12] or by reversed-phase HPLC [13]. The analyte is lost during the conversion of PL species to GC suitable compounds which is difficult and time consuming process and LC sensitivity and resolution is not fully satisfactory [14].

MS can also be employed for analyzing PLs. Softer ionization techniques like MALDI [15,16-17], ESI and FAB [16] are recommended for recording intact PLs [18].

1.4.3.2 Gangliosides

Gangliosides are present in the nervous system in large abundance. They are also found in all body fluids and vertebrate cells. Structures of ganglioside and sialic acid are given in Figure 1.4. Gangliosides belong to GSLs (glycosphingolipids) categorized having sialic acid groups attached to the galactose part of the oligosaccharide chain connected to ceramide tail. They are sub-categorized on the basis of number of sialic group, where mono-sialogangliosides (GMs) have one, di-sialogangliosides (GDs) have two, tri-sialogangliosides (GTs) have three, tetra-sialogangliosides (GQs) have four, and penta-sialogangliosides (GPs) have five sialic acid groups as depicted in Figure 1.5.

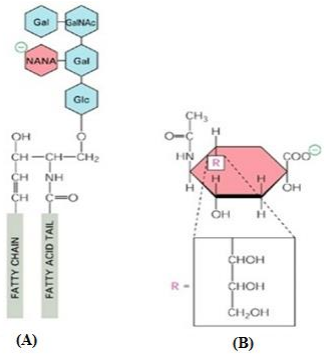

Figure 1.4: A) Ganglioside structure **B)** and Sialic acid

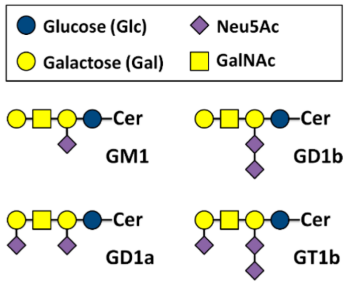

Figure 1.5: Ganglioside classification on the basis of number of sialic acid groups

GSLs serve as receptor molecules and antigens and as functional membrane components. Their dis-regulation and over-accumulation cause numerous lysosomal storage disorders including Gaucher's, Hunter's, and Tay Sachs, neurodegenerative diseases including Parkinson's,

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Alzheimer's, and the Guillain-Barré syndrome, and cancers including melanoma, neuroblastoma and even breast cancer stem cells [19].

1.4.3.2.1 Separation, Identification and Quantification

Gangliosides analysis is a big challenge due to its structural complexity. The developments of reliable bioanalytical methods are of great significance to measure gangliosides.

1.4.3.2.1.1 TLC

For many decades, TLC has been the conventional method of ganglioside profiling. TLC is the simplest method for the analysis of glycosphingolipid (GSL) mixture. Various types of solvents have been used for the separation of complex mixtures. In addition, enzymatic, colorimetric, radiochemical and immunological detection procedures are also available for their recognition. TLC has no ability to determine the exact chemical structure of separated molecules, but generally gives fast, economical, and easily accessible preliminary data.

1.4.3.2.1.2 GC-MS

GC-MS is a valuable technique for gangliosides analysis. This technique is capable of analyzing the sphingosine base and fatty acid chains; specifically, the cis/trans and Regioisomers.

1.4.3.2.1.3 LC-MS

Most lipids are not volatile inherently; and typically require time consuming derivatization protocols for efficient analysis. Consequently, GC-MS is not always the best method for analysis of intact gangliosides components. Today, LC interfaced to electrospray MS is becoming the standard of analysis due to its improved sensitivity and selectivity while also permitting the potential for high through-put strategies.

1.4.3.2.1.4 Solid Phase Extraction (SPE)

SPE offers a selective and flexible method for the separation, concentration and purification of lipids. SPE gives better separation and recoveries and is a cost-effective method. SPE is valuable with lesser sample preparation times. For the fractionation and isolation of lipids, SPE methods mainly utilize silica, aminopropyl and C18 phases; however, the developments of exemplary

phases offer lipid chemists with opportunities for development of better separation strategies [20].

1.4.3.3 Lipoproteins

Macromolecular Complexes of Lipids with Proteins

Species from insects to mammals, lipoproteins perform important role in extracellular transport of lipids [21]. The shape of plasma lipoproteins is spherical, these are macromolecules soluble in water having phospholipids and neutral lipids that are non-covalently bound to apolipoproteins. Cholesterol esters and triglycerides (TG) are non-polar lipids. They are present in core and phospholipids, free cholesterol, and apolipoproteins are on the surface of lipoprotein particle. Lipoproteins help in the mobility of cholesterol and triglycerides and from the place it manufactured and absorbs to the place of consumption [22]. In humans, along with other mammals, two lipoproteins are those having ApoA-I (HDL) and those having ApoB (chylomicrons, LDL, VLDL).

1.4.3.3.1 Lipoproteomics

"Proteomics" represents proteins study [23,24]. For the earlier analysis of disease, proteins best indicate the pathological or physiological states [25].

Figure 1.6: Proteins

Different fields are glyco-proteomics, lipo-proteomics and phospho-proteomics [26]. Nearly 50% of proteins are phosphorylated [27]. In glycoprotein, the glyco (sugar) group is attached to the protein as a result of PTM [28]. Glycoproteomics is the study of glycoproteins. The investigations related to multiple aspects of lipoproteins is termed as "lipoproteomics".

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1.4.3.3.2 Classes of Lipoproteins

Lipoproteins are sub-divided according to the composition of apolipoprotein, and particle density. Five classes of lipoproteins include HDL, LDL, IDL, VLDL, and chylomicrons (Figure 1.7).

Ratio of protein and lipid content and diameters of lipoproteins particles are given in Table 1.2.

Table 1.2: Content of lipoprotein particles

Lipoprotein Particle	Diameter (nm)	Protein %	Phospholipid %	Triglycerides %	Cholesterol %
Chylomicrons	100-1000	<2	7	84	8
VLDL	30-80	10	18	50	22
IDL	25-50	18	22	31	29
LDL	18-28	25	21	4	50
HDL	5-15	33	29	8	30

1.4.3.3.2.1 Chylomicrons

Chylomicrons (CM) are the largest lipoproteins in humans. CM main protein is Apolipoprotein B-48 and these are triglyceride-rich lipoproteins [29].

1.4.3.3.2.2 VLDL (Very Low-Density Lipoprotein)

Apo B-100 is important for the preliminary VLDL assembly, moreover the particle also contains apolipoproteins C and E. VLDL is harmful because it carries more TG and increase the heart disease risk. By hydrolyzation process, VLDL is converted to IDL.

1.4.3.3.2.3 IDL (Intermediate-Density Lipoprotein)

They come from the degradation of VLDL. IDLs are made up of triacyl glycerols and cholesterol esters and carry fatty acids. These IDL particles are removed in liver cells by receptor-mediated endocytosis (RME).IDL further hydrolyzes and the particle density reaches the LDL. When IDLs cholesterol content become higher than triglyceride, it becomes LDL.

1.4.3.3.2.4 LDL (Low-Density Lipoprotein)

It is made up of cholesterol, proteins, phospholipids and triacyl glycerol (50%, 25%, 20% and 5% respectively). Apo B-100 binds lipoproteins to the surface of cells through LDL-specific receptors [30]. LDL is the product of hydrolysis of intermediate density lipoprotein. Its main function is to carry cholesterol around the body. LDL is notorious as “bad cholesterol”. It is the main lipoprotein particle that is involved in atherogenesis.

1.4.3.3.2.5 HDL (High-Density Lipoprotein)

HDLs are the densest and the smallest particles. HDL is composed of mainly phospholipids and lipoproteins. Cholesterol is picked by HDLs from around body and take it to liver for recycling or for its release as bile acid from the body [31]. Other than density, HDLs exist in different forms like HDL 3 and HDL 2. Many apolipoproteins are part of HDL and ApoA-I is the dominating protein [32]. HDLs play role in compromised/diseased body conditions and decrease atherosclerosis and defend against CAD [33].

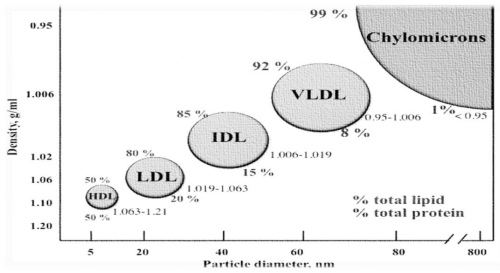

Figure 1.7: Classes of lipoproteins

1.4.3.3.3 Lipoproteins Associated Diseases

Clinically lipoproteins are important because of putting potential threat for many abnormal body functions.

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1.4.3.3.3.1 Hypertension

It is a persistent high BP and barely going away without medication. Hypertension is a condition caused by disturbed levels of lipids and lipid tagged proteins in blood and changes in lipid metabolism are observed. Many studies show that TC, TG and nearly all lipoprotein portions show abnormal quantities in high blood pressure conditions when related to normal. TC are significantly up-regulated in the blood of high blood pressure individuals [34].

1.4.3.3.3.2 Coronary Artery Disease (CAD)

CAD is the major reason of deaths in advanced world. Changed cholesterol levels in plasma are the major risk of atherosclerosis and lead to many cardio vascular diseases [35,36]. Genetic factors or environmental conditions may alter the production, catabolism and removal of lipoproteins from circulation. 60 mg/dL is the protective HDL-C levels.

1.4.3.3.3.3 Lipoproteins and Diabetes

Changes in plasma lipoproteins are also associated with diabetes mellitus [37]. Diabetic individuals of Type 1 show lipid disorders and have higher content of TG, LDL and decreased concentration of HDL. Furthermore in LDL, the patterns of apolipoproteins PTMs are increased i.e. LDL oxidation, and glycation. Type 2 diabetes patients show qualitative and quantitative changes of lipoproteins [38].

1.4.3.3.3.4 Hypothyroidism and Lipoproteins

Biological condition where thyroid gland reduces the production of T3, T4 and thyroid hormones is termed as hypothyroidism. These thyroid hormones alter lipid metabolism, thyroid and control cholesterol formation/degradation and enzymes activity of biological pathways. Thyroid hormones lower cholesterol levels by increasing the LDL-C receptors in liver and peripheral organs. Hypothyroidism is the abnormal levels of lipids. Hypothyroidism shows higher concentrations of LDL and TC altered levels of apolipoproteins and other lipoproteins in serum [39].

1.4.3.3.3.5 Alzheimer’s Disease (AD) and Lipoproteins

AD shows a relation between cholesterol metabolism in the brain and the formation of amyloid plaques. Amyloid-β (AB) production and deposition is caused by the elevated brain cholesterol

[40]. AD Sera are analyzed for all the lipids and lipid related content in cerebral cortex. AD patients have elevated levels of LDL, lower level of HDL cholesterol and Apo-B. This indicates that higher serum cholesterol, mainly LDL-C effects the AD associated pathology [41].

1.4.3.3.3.6 Lipoproteins and Cancer

Around the globe, cancer is fatal and effecting public life quality. It's premature tracing is beneficial for timely control and stopping the propagation [42]. Lipoproteins are potential biomarkers of several cancers. LDL-C tracing in blood are associated to higher cancer threat [43]. In blood, HDL-C can be a clinical prognostic factor for gastric cancer [44] and breast cancer [45].

1.4.3.3.4 Lipoproteins Separation Techniques

Characterization of very low analyte concentrations is always difficult. Sample complexity reduction step using selective enrichment method is necessary [48]. For detecting and characterizing the lipoproteins, many techniques have been established;

- Affinity chromatography (anion exchange)
- Cholesterol and cholic acid affinity
- Density-gradient ultracentrifugation (UC)
- Immuno-affinity techniques
- HPLC/FPLC
- Size exclusion chromatography

All these methods have their gains and losses, yet no method has been developed as a perfect method for lipoproteins isolation.

1.4.3.3.4.1 Ultracentrifugation (UC)

Originally lipoproteins are distinct by their densities. For analytical analysis of lipoproteins, first reliable technique for their separation is UC. The UC separation is achieved with very less sample (half milliliters serum). Serum sampling is usually performed with automatic dilutor [49]. DGUC is used to separate plasma lipoproteins. NaBr or iodixanol are used as medium. The purity of individual lipoprotein class is enhanced by running two UC runs successively [50].

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Centrifugation is also performed by sucrose solution which is usually used as UC density gradient medium [13].

For the quantitative analysis of plasma lipoproteins, ultracentrifugation is preferred technique. But this method is clumsy and time-taking and this technique requires large volumes (0.5–2.0 mL)of sample as compared to other techniques [50].

1.4.3.3.4.2 SEC (Size Exclusion Chromatography)

It is another strategy for lipoproteins separation. SEC methods can separate lipoproteins on the basis of specific sizes, but there is a drawback of proteins contamination when plasma or serum is used [51].

1.4.3.3.4.3 HPLC & FPLC

CAD patients show irregular lipoproteins profile. The analysis of major plasma lipoproteins, cholesterol has been performed by HPLC gel permeation chromatography (GPC). The results indicate that HPLC is an effective analysis method. HPLC have been employed for isolating and analyzing lipoproteins and the related content to CAD. HPLC through GPC columns is a better way in the quantification and classification of lipo- content [52].

FPLC works on principle of gel filtration. For isolation of lipoproteins, this method is fast and gentle where the content keeps its integrity without losing the structural activity. FPLC is a validated technique for the isolation of blood lipo- content. The sample volume is required in very less quantity. Few features make this technique unfavorable as pumps are needed for the mobility of sample content and mobile phases through the analytical columns.

1.4.3.3.4.4 Immunoaffinity

One way for lipoproteins separation is immuno-sorption. Each lipoprotein class (HDL, VLDL, LDL, Chylomicrons) has a dominant protein. According to this protein, a specific antibody is used to separate lipoprotein i.e. anti-apoA-I antibodies can be employed to separate HDL whereas anti-apoB-100 separates LDL [53]. If immunocapturing is done on a protein chip, this method is called SELDI® (surface enhanced LDI) method for the determination of HDL bound proteins by mass spectrometry. ApoA-I or apoA-II antibodies bind to HDL from blood [54]. For lipoproteins separation, this technique is good but expensive.

1.4.3.3.4.5 Cholesterol and Cholic Acid Affinity

Lipoprotein particles have lipids such as PLs, triacyl glycerols and cholesterol which are selected from number of alternative ligands to capture the lipoproteins. Cholic acid and cholesterol are also tried. Cholic acid and cholesterol affinities does not interact with other plasma proteins. For LDL and HDL lipoproteins, adsorbents with these ligands show strong affinity. Small quantity of albumin is the only contamination that is identified. From protein solutions, these adsorbents show high selectivity and adsorbent capacity towards lipoproteins. In an affinity-chromatography it would be a favorable ligand system for the lipoproteins capturing from biological sample [55].

1.4.3.3.4.10 Anion Exchange Chromatography

Lipoproteins from blood are mostly separated through UC however this method takes long time. Alternate methods are being developed to make lipoproteins analysis simple and easy. Anion-exchange chromatography is one of the alternate method [56]. Complexity of sample can be reduced by specific cum selective targeting strategies which play part in enriching the very low concentrations of analyte present.

1.4.3.3.4.10.1 Hydroxyapatite

Hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ usually serves as stationary phase. Nucleic acids and proteins have been separated by hydroxyapatite LC column. Lipoproteins can be isolated from human serum by hydroxyapatite media without any ultracentrifugation (UC) step. Potassium phosphate buffer and its pH value affect protein retention on hydroxyapatite column. The separations of human serum lipoproteins from blood like albumin (ALB) and globulins are usually performed on hydroxyapatite columns. Elution can be stepwise or gradient by PBS buffer system pH 7.4. Eluent from column is monitored by UV/Vis. spectrophotometer at the wavelength of 280 nm which matches to the maximum lipoproteins absorption [57]. The calcium on hydroxyapatite interact with the acidic groups on the proteins. Phosphate buffer as eluting buffer compete with the protein phosphate groups to approach the hydroxyapatite calcium sites. At both 6.8 and 7.4 pH, the lipoproteins have negative charge and bind with the calcium sites of$Ca_{10}(PO_4)_6(OH)_2$ and in elution step, eluting (phosphate) buffer competes for the calcium sites to elute lipoproteins [58].

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1.4.3.3.4.10.2 Diethylaminoethyl Anion-Exchange

Here lipoprotein classes are separated by commercially available ProtEx-DEAE anion-exchange column [59]. This type of column has polymeric diethylaminoethyl- ligand gel that is non-porous and used to separate lipoproteins by using sodium perchlorate as eluting buffer by step gradient elution from serum.

Structure of DEAE Anion Exchange polymer is as follows.

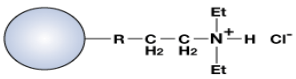

Figure 1.8: DEAE Anion Exchange polymeric structure

With the help of anion exchanger, five major classes of serum lipoproteins have been separated within 20 minutes [50].

1.4.3.3.4.10.3 AEX-HPLC

AEX-HPLC is a method for lipoproteins isolation and analyses. Method is reproducible. Five major lipoproteins classes have been separated by AEX-HPLC from rabbit and human blood aliquots. With small modifications, a similar HPLC equipment has been used previously for lipoproteins isolation. An AEX column contains non-porous polymeric gel with diethylaminoethyl ligand. Elution with high ionic strength solvent system is required to disrupt lipoproteins from AEX. In type II diabetes, lipoprotein cholesterol levels are also measured using AEX-HPLC [60].

1.4.3.3.5Apolipoproteins (APOS)

Lipoproteins are made of lipids and proteins, proteins only are recognized as “apolipoproteins” (Apolipo/Apos). Outer core of lipoproteins is made-up of cholesterol and triglycerides esters, a hydrophobic core stabilized via protein part; apolipoproteins. They have role of transporting lipids and regulate its metabolism by the activation/inhibition of enzymes. Quantitative changes of Apos. and variations in their structure and their link with disease condition is a current research topic [61]. LDL binds to ApoB-100 as a major apolipoprotein through receptor and are important for the uptake of LDL from circulatory system. The key apolipoproteins are ApoA-I

and A-II, other apos. are ApolipoA-IV, ApolipoA-V, ApolipoC-I, ApolipoC-II, ApolipoC-III, Apolipo-D, Apolipo-E, Apolipo-F, Apolipo-H, Apolipo-J, Apolipo-L, and Apolipo-M which are also attached to lipoproteins particles. Major Apos. of HDL is ApoA-I and 70% of HDL content is apolipoprotein. ApoA-I is a serum protein with mass of 28 kDa. ApoC-I is also an HDL apolipoprotein and it is a 6.6 kDa protein.

In LDL proteome study, ApolipoB-100, ApolipoC-II, ApolipoC-III (3 isoforms), ApolipoA-I (2 isoforms), ApolipoA-IV, Apolipo-E (4 isoforms), Apolipo-J (clusterin), etc. are detected [62]. So, the apolipoproteins (Apos.) are major part of lipoproteins and regulate its metabolism. The up and down regulation of these apolipoproteins are markers of various diseases. The apolipoproteins biological functions are affected by post translational modifications (PTMs) [63]. Some main apolipoproteins and their distribution in different lipoprotein particles are given in Table 1.3.

Table 1.3: Lipoproteins and its Apolipoproteins

Apolipoproteins	Lipoproteins Distribution
ApolipoA-I	HDL
ApolipoA-II	HDL
ApolipoB-48	Chylomicrons
ApolipoB-100	LDL, VLDL
ApolipoC-I	HDL, VLDL
ApolipoC-II	HDL, VLDL, Chylomicrons
ApolipoC-III	HDL, VLDL, Chylomicrons
Apolipo-D	HDL
Apolipo-E	HDL, VLDL, Chylomicrons
Apolipo-H	HDL, LDL, VLDL, Chylomicrons

1.4.3.3.5.1 Apolipoproteins as Cancer Biomarker

Apolipoproteins are important part of lipoprotein particles and are also the cancer biomarkers. Up and down regulations of Apos. predict the cancer and its stage. Structural changes of Apos., their link with cancer and change in their levels are active field of research. Analytical

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techniques are being developed to investigate Apos. MALDI-MS and ESI-MS are developed to identify the Apos. For given samples, they provide structural information [64].

In various types of cancers, for example advanced liver cancer, colorectal and ovarian, ApoA-I is differentially regulated and shows its abnormal levels in blood [65]. ApoA-I is a prognostic biomarker. Some recent studies show that ApoA-I is signaling molecule for prognosis of pancreatic and ovarian cancer. Low levels of ApoA-I and HDL are linked to prostate cancer [66]. ApoA-II, the major HDL apolipoprotein is present as homodimer form in human blood. The appearance of mass-spectral peak intensity of ApoA-II is high in the serum taken from prostate cancer patients and ApoA-II dimer mass-spectral peak intensity is low in the serum of pancreatic cancer patient [42]. Apolipoproteins and their association with possible cancer biomarkers are summed up in Table 1.4.

Table 1.4: Apolipoproteins as cancer biomarkers

APOLIPOPROTEIN	CANCER
Apolipoprotein A-I	Colorectal (CRC) Ovarian (OC) Gastric Pancreatic
Apolipoprotein A-II	Prostate Pancreatic
Apolipoprotein A-IV	Bladder
Apolipoprotein C-I	Hepatocellular (HCC) CRC
Apolipoprotein E	Bladder Cancer
Apolipoprotein H	HCC

Other aspects of lifestyle such as body mass index (BMI), cigarette smoking, alcohol intake, hypertension, diabetes and dyslipidemia also contribute to change the levels of circulating ApoA-I and HDL [35].

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1.4.3.3.5.2 Apo-H (Beta-2-Glycoprotein)

Apolipoprotein H (Apo-H), also well-known as beta 2-glycoprotein I (ß2-GPI) [67] is 48-50 kDa plasma glycoprotein which is predominantly synthesized in hepatocytes. It is a single chain (Figure 1.9) highly glycosylated, 326 amino acids polypeptide. Apo-H circulates in blood in free form or bound to lipoproteins; LDL, VLDL, HDL and chylomicrons [36]. Apo-H contains multiple N-glycosylation sites [68]. One site of O-glycosylation is linked to domain III and IV of the protein. Hence, there is a growing interest in studying this protein, its PTMs and its glycans in clinical context.

1.4.3.3.5.2.1 Biological Significance

Apo-H is an acute phase protein. Apolipoprotein-H is associated to physiological pathways including coagulation. It acts as an activator of lipoprotein lipase and is clinically known as the essential antigen of antiphospholipid syndrome (APS) [69].Apo-H is present in atheroscloretic plaque and may work as diagnostic molecule. Levels of apo-H are positively correlated with increasing age, smoking and hyperlipidemia.

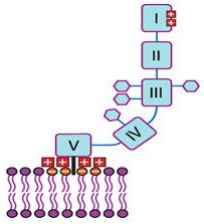

Figure 1.9: Structure of Apo-H

Apo-H glycans structure changes are observed in hepatocellular carcinoma (HCC) and APS patients. Apo-H relates to number of diseases. High levels of this protein are considered as clinical marker of CVD, type 2 diabetics, and metabolic diseases.

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1.4.3.3.5.3 Non-Apolipoproteins

Other than apolipoproteins, non-apolipoproteins are found in lipoprotein particles. They are ALB, CETP, serum amyloid A, PON1, DBP, component C3, A1AT, AGP, AHSA, α-1B-glycoprotein (A1BG), Filamin-A (FLNA) phospholipid transfer protein (PTP), Serotransferrin (ST), transthyretin, component C4A, C4B, VN, bikunin, Alpha-2-AP, ITIH3, AGT, Complement C4 beta chain (C4B), serotransferrin (TF) Kininogen-1 (KNG1), Prenylcysteine oxidase and Hemopexin (HPX) [70]. SAA is a putative biomarker for lung cancer. Transthyretin (TTR) is down-regulated in ovarian cancer and C3 is putative biomarker of liver cancer [70].

1.4.3.3.6 Post-Translational Modifications (PTMs)

PTM is the modification of a group on amino acid (Figure 1.10). PTMs are result of attachment/addition of biochemical functional groups; phosphates, carbohydrates, acetates and lipids to amino acids. These PTMs cause structural changes. It is an event of covalent processing based on proteolytic cleavage. Changes of all types that occur in a protein before during and after the translation process are counted as PTMs. Protein folding is also considered as PTM. Analytical chemists are exploring PTMs and about ≥200 PTMs have been analyzed. Some of the PTMs are phosphorylation, sulfation and glycosylation. These PTMS are essential for proper functioning of proteins based on their structural conformations. Modifications assist protein stability, its solubility, attachment in cell and work as molecular switches that regulate the proteins biological functions. The proper analysis of protein's PTMs is important in context to its importance in disease conditions like heart diseases, diabetes, cancers, and neurodegenerative diseases.

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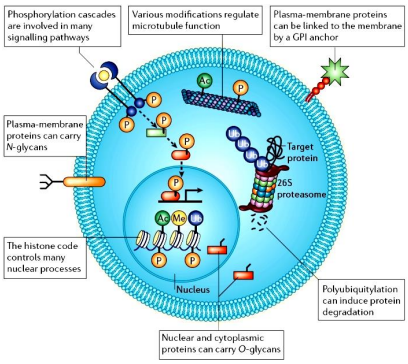

Figure 1.10: Cellular post-translational modifications

These changes can be tracked by decrease and increase in their molecular weights. Some modifications can be easily identified e.g. shift of mass with +16 Da is the addition of oxygen.

Apolipoprotein modifications exist in different forms such as phosphorylation, glycosylation, oxidation [13]. Glycosylation through O-linking occurs in Apolipoproteins PTM [71].

ApolipoA-I PTM due to reactive carbonyls hinder the cholesterol transport. Different isoforms of Apolipo-C have been analyzed from biological samples in its de-glycosylation and glycosylation forms [72].

1.4.3.3.6 .1 Glycosylation

Glycosylation process is induced by enzyme that establishes glycosidic bonds between saccharides and other saccharides, lipids, proteins and metabolites [73,74]. One of the most observed PTM of protein is glycosylation which contributes in approximately all biological pathways [75]. Defects in protein glycosylation is linked to several diseases which indicate that the glycome carries significant biological information [76]. Proteins have their proper glycosylation sites however heterogeneity exists for their glycans structure. Human plasma N-glycome (PNG) is stable within the individuals but pathophysiological processes cause changes

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in PNG and reflect the cell state. Changes in the glycosylation of proteins and elevated level of glycosylated metabolites profile have been correlated with diseases; cancers [77,78], type-2 diabetes mellitus, hyperlipidemia, kidney disease [78], Alzheimer [79] and cardiovascular diseases (CVD) [80,81]. Studies investigate N-glycan patterns in the serum of rheumatoid arthritis (RA) patients [82]. The glycans are studied as prognostic markers and the glycans study of biological fluids and tissues has medical importance [83]. The progress in analytical strategies has impact on glycomics research for sensitive and high-throughput analysis of glycan structures. Glycosylation of proteins is shown in Figure 1.11.

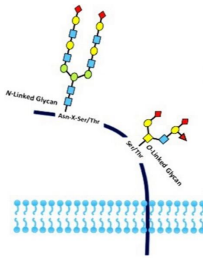

Figure 1.11: Glycosylation of proteins

Apo-H N-glycans are sialylated [84] and fucosylated [85]. Glycan part is composed of hexose; galactose, mannose, N-acetylglucosamine, fucose, and N-acetyl neuraminic acid (NeuAc) which is also known as sialic acid. Decrease in sialylated tri-antennary glycans structure and an increase of sialylated bi-antennary Apo-H glycan in APS patient samples is observed as compared to controls [86]. MALDI-TOF-MS has been used for proteins analysis as it generates simple charged ions and offers high tolerance against buffers and salts. Its high throughput protein profiling from complex biological samples is another advantage [87]. This technique is limited for protein glycans unbiased analysis because of the ability of sialic acid glycosidic bonds in MALDI source.

To overcome this difficulty, sialic acids can be stabilized by methods including permethylation, dimethyl amidation and ethyl esterification which enhance the sensitivity and ease the mass spectrometry interpretations. This has become favorable for glycans profiling with glycans

derivatization prior to MS analysis. Linkage-specific derivatization allows accurate MALDI-TOFMS analysis of isomeric sialylated glycans. Ethylation is used to selectively derivatize α(2,6) sialic acids, while α(2,3) sialic acids are lactonized [88]. Hydrophilic interaction liquid chromatography (HILIC) solid phase extraction prior to MS analysis is ideal for rapid cleanup, desalting and concentration of glycans. N-glycans are detected in m/z 1500–4000 in reflector positive mode with super-DHB as matrix. 1 M NaOH is added to the matrix solution to suppress K-adduct formation while dominant specie is $[M + Na]^+$ [89].

Identification of low microgram levels of Apo-H glycoprotein is still a challenge. An optimized and sensitive protocol for Apo-H glycans identification and structure characterization is important. Apo-H captured by Anti Apo-H beads is verified by SDS-PAGE and LC-MS/MS analysis. Both in-solution and in-gel Apo-H N-linked glycans release is optimized and compared. Characterization of unstable Apo-H glycans in MALDI-MS is carried out by controlled sialic acid derivatization followed by HILIC SPE to enrich glycans.

1.7 Serum/Plasma as Diagnostic Fluid

In comparison to plasma, serum is preferred because serum is anticoagulants interference free. Stability wise plasma is better than serum. Some studies show comparable results from serum and plasma[90] and for some studies a changed profile is observed. For biomarker discovery, the choice of plasma or serum for proteomics study is still a question. Most of the research uses serum for proteomics study. For better results serum and plasma both should run but it will take longer time and complicate the data analysis. These fluids carry small biomolecules and huge amount of information. For prognosis of several diseases, the analysis of serum or plasma proteome by different analytical technique work perfectly [3].

1.8 Mass Spectrometry (MS)

Thousands of biomolecules of varying nature can be identified by MS. For the finding of biomarkers, MS analyses are important. Mass spectrometry is capable for distinguishing peptides with amino acid sequence. Therefore MS is the best approach for biomarker identification [16].

1.8.1 MALDI/TOF-MS

It is employed for proteins analysis. Variety of matrices have been used in MALDI. Sinapinic acid is used for the detection of high molecular weight molecules like proteins that are larger

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than 5000 Daltons and recorded in MALDI positive mode. HCCA is taken as matrix for medium ranged molecular weight content like peptides that are smaller than 5000 Daltons and work best in MALDI positive mode.1,5-Diaminonaphthalene (1,5 DAN) is used for lipid imaging in both positive and negative modes and 2,5-dihydroxybenzoic acid (DHB) is used for small molecules like peptides, lipids, nucleotides, oligosaccharides in LDI positive mode. Matrix is dried on a MALDI plate with analyte. Matrix assists the ionization process by proton transfer and proteins that are present in solid phase are evaporated. A laser flash (usually nitrogen; N_2) transfers the ions that are in a gas phase. A high voltage create an electric field where ions get speeded up and move to TOF analyzer in vacuum. TOF analyzer works on the principle of mass to charge ratio. Ions will be isolated on m/z ratios. MALDI/TOF-MS has reflector for the reflection of ions that improves resolution. Each peptide is assigned +1 charge and the time of flight (TOF) separates them with monoisotopic masses. Calibration step improves the mass accuracy and database searches for obtained mass list [91]. The working principle of MALDI-TOF MS is shown in Figure 1.12.

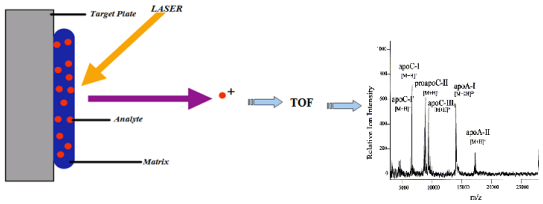

Figure 1.12: Working principle of MALDI-TOF MS

MS is of great significance; high throughout molecule-specific, high sensitivity, no labeling and the ability of *in situ* localizing of number of biomolecules in single run from a tissue sample. There are different ionization methods. FAB is relatively harsh and insensitive, which causes fragmentation of biomolecules. On the other hand, ESI and MALDI have been utilized in the analysis of gangliosides with considerable efficiency.

1.8.1.1 MALDI-MS for Protein Analysis

MALDI-TOFMS is a widespread and powerful analytical tool in biological analysis due to its sensitivity, wide mass range (1-300 kDa) and high accuracy. It is commonly used for the analysis of all types of biomolecules like proteins, peptides, metabolites, nucleic acids and carbohydrates. MALDI-TOF-MS with other analytical techniques like liquid chromatography, solid phase extraction, gel electrophoresis, is suitable for the identification of proteins. MALDI-TOFMS method allows a detailed analysis of PTMs.

1.8.1.2 MALDI-MS for Lipids Analysis

MALDI-TOF MS is a well-known analytical tool especially in proteins and peptides research. Its special gains are the fast and simple performance, robustness against sample impurities, its high sensitivity in the femtomolar range, and low tendency to give fragmentation. MS is also handy in lipid analysis. For investigation of lipid molecular species from mixtures, mass spectrometry based techniques are the superior choice. MS-based approaches are thus commonly used for lipids analyses [92]. Lot of work has been done with "lipidomics" by using MALDI-TOF MS and still further work is necessary to evaluate the abilities of the technique in this field [3].

1.8.1.2.1 MALDI-MS for Gangliosides Analysis

MALDI and ESI possess capacity for the analysis of gangliosides and their structural characterization. However, the ionization process is a reason for glycosidic bond breakages, either by dehydration, and/or decarboxylation reactions.

LC/ESI-MS/MS is currently being employed for the separation and identification of Regio-isomeric gangliosides, including GD1a, -1b, and -1c, GM1a and -1b, and GT1a, -1b, and -1c.

1.8.2 Peptide Mass Fingerprinting (PMF)

It is a term used for the identification of proteins by unique amino acids (AA) sequence. Proteins are tryptically digested that cleave proteins. α-Chymotrypsin breaks the bond from C-terminal side of tyrosine (Y), phenylalanine (F), tryptophan (W) and leucine (L). Chemical cleavage is performed by cyano-bromide that cleaves proteins from ethionine C-terminal. Typically, in-solution digestions are performed at 37 °C for 12 hours. To reduce this long digestion time, different techniques have been employed. Microwaves assisted proteolysis reduces the digestion time from 5 to 20 min, ultrasonic waves further reduce to 1 min. Recently alternating current

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(AC) technique minimizes the time of in-solution tryptic proteolysis to 5min. Each protein has profile of its distinctive peptides and be easily traced. After MS analysis, the obtained masses are compared with different database masses [93].

1.8.3 MELDI®

MELDI-MS is a profiling technique. MELDI is a suitable approach for pattern analysis and gives reproducible and accurate results and is helpful in various fields of life. All carrier materials have specific spectra. Robotic MELDI is a developed MELDI technique that has automatic devices for liquid handling and are used for the analysis of clinical related samples mainly for the profiling of multiplexed proteins [46,47].

1.9 Affinities used in Separation Science

Proteins and lipids play part in biological processes. Chemical methods and variety of affinities are used for their enrichments. Chemical methods are hydrazide and boronic acid chemistry, [47,76] and affinity enrichment through lectin [94], boronate [95], IMAC, metal oxides [96], hydrophilic interaction chromatography (HILIC), and ion exchanger chromatography.

1.9.1 IMAC and MOX

Different strategies have been explored and studied for proteins enrichment. Two main methodologies that are commercially available for research applications are immobilized metal ion affinity chromatography (IMAC)with Fe^{3+} [97], Ti^{4+} [98], Zr^{4+} [99], and Sm^{3+} [100], etc. and metal oxide affinity chromatography (MOAC)like Fe_3O_4 [101], TiO_2 [102], ZrO2 [103], CeO_2 [104], and La_2O_3 [105], etc. Metal oxides can be synthesized directly or maintained on other materials to control the binding capacity and surface area. IMAC materials need support for the immobilization of metal ions by chelation. Ti^{4+} based IMAC has high acceptance for the acidic buffers [106].

1.9.2 Boronic Acid Affinity

Chromatography where boronic acid works as ligand is denoted as boronate affinity chromatography. The interaction between boronic acid and cis-diol compounds holds the analytes. In aqueous solution, the boronate is hydroxylated and changes to boronate anion under basic conditions which form esters with cis-diols with five- or six-membered rings. Geometrical

changes occur from trigonal coplanar to a tetrahedral. This process is exemplified in Figure 1.13. Under acidic conditions, the resulting cyclic diester is hydrolyzed and the reaction is reversed [107,108]. Two covalent bonds are formed and this is reversible process in aqueous solution [109].

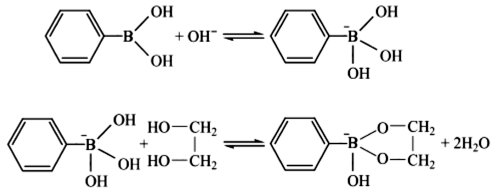

Figure 1.13: Boronic acid showing binding with cis-diol

Boronate affinity chromatography is simple, rapid and accurate for the analysis of glycated proteins [110]. For the evaluation of diabetic states, boronate chromatography for glycosylated proteins study is a method of choice [111]. Similar to other affinity chromatography [112], in boronate-based separations, the support material is important. Materials that have been considered to carry boronate affinity are dextran [113], silica [112], agarose [114], cellulose [115], polyacrylamide [116], polystyrene [117] and polymethacrylate [116].

1.9.3 Ion Exchange Chromatography

The physical or chemical properties of specific protein are used to separate a specific protein from a crude mixture. Proteins are adsorbed to various solid phases in a very selective way under appropriate buffer and pH conditions. Proteins have charged groups that are positioned on protein surface. Analyte is separated by creating opposite charges to the stationary phase. The introduction of positive charge on stationary phase is known as an anion exchanger. Most proteins carry negatively charge at pH 6-8. Therefore, anion exchanger is the first choice to isolate lipoproteins because these proteins have negative charge molecules on their surface. For ideal separation, the choice of buffer and pH is made to isolate the target protein from contaminants. Generally anion exchanger is used to bind the target molecules and non-bound contaminants are washed off.

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In ion exchange method, the proteins enrichment is based on the ionic interactions. In the separation process, attraction between proteins with surface charge of ion exchange adsorbent occurs. The charged groups on the surface of material are surrounded by oppositely charged ions in an aqueous condition. Ions are exchanged reversibly without any alteration in the nature and properties of analyte. On the basis of structure of material onto which ion exchange affinity is present, the support is hydrophilic or hydrophobic which also contributes in isolation process. Mostly highly substituted polystyrene introduce hydrophobic character to the affinity material. First ion exchangers synthesized had hydrophilic base that contained modified cellulose with highly substituted ionic groups. Cellulose has challenging mechanical properties: cellulose fibers are fragile, making it anion exchange-cellulose material debatable for enrichment of analytes in buffer and pH conditions. There are various types of anion exchangers (Table 1.5).

Table 1.5: Anion Exchangers

Diethyl-aminoethyl (DEAE)	$(\text{-}OCH_2CH_2N^+H(CH_2CH_3)_2)$
Quaternary aminoethyl (QAE)	$(OCH_2CH_2N^+(C_2H_5)_2CH_2CH(OH)CH_3)$
Quaternary ammonium (Q)	$(\text{-}CH_2N^+(CH_3)_3)$

Ion exchangers containing quaternary ammonium groups are strong ion exchangers.

The enrichment process has few steps: equilibration of material (ion exchanger) with an equilibration buffer where ionic strength and pH are adjusted. Loading of sample reversibly binds analyte to the material. Un-wanted proteins do not bind to the material under the optimized conditions. Non-specifically bound molecules are eliminated by washing the material with washing buffer and with deionized water. Last step is the elution of analyte of interest. By changing eluting buffer composition, the bound molecules are desorbed. Elution is also affected by increasing the concentration of counter ions and by pH changes.

Anion exchange affinity is mostly used for the separation of biomolecules (proteins, polypeptides, nucleic acids, polynucleotides, etc.). Separation principle of ion exchange is simple and easy. One of the advantages of anion exchange affinity method is the elution of analyte under soft conditions. The ion exchange materials are stable and can be regenerated and used for several times.

1.10 Types of Materials used in Separation Science

Irrespective to the enrichment approach, the nature of support material is important. The structural design of support material is not inert; hydrophilic/hydrophobic supports can enhance the enrichment. In case of IMAC [118,119] and MOAC [120,121], the hydrophobic interactions may cause non-specific binding of hydrophobic peptides. The affinities are commonly immobilized on various support materials (cellulose, carbon based [122], polymeric [123], silica [124] and magnetic [125]) for the enrichment of proteins/peptides, lipids, other metabolites and nucleic acids. The nature of support material affects separation process [126]. They possess inherent hydrophilic and hydrophobic characteristics [127]. Cellulose is a hydrophilic natural polymer. Cellulose is non-toxic and biocompatible and works best as stationary phase [128].

1.10.1 Composite Materials

A composite material is the combination of two or more materials. Both the materials have different properties. The two materials work together and give unique properties to the composite. In the composite, materials do not dissolve or blend into each other. Natural composites are present in both plants and animals. Wood is a composite and it contains long cellulose fibers that are composed of lignin which is a weaker substance. Animal bone is another example of composite. Carbon nanotubes (CNTs) is one of the materials used to create new composites.

1.10.2 Core-shell

Core encapsulated by another shell material is called core-shell material (Figure 1.14). Core-shell materials have different types of cores and shell materials with different morphologies and properties. They also vary by synthetic techniques which include solvothermal synthesis, emulsion polymerization, sol-gel method, microemulsion polymerization, and many more [129].

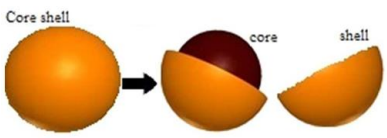

Figure 1.14: Core-Shell particles

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1.10.3 Magnetic Materials

For the analysis of sample, pretreatment step is important such as liquid-liquid extraction (LLE), solid-phase extraction (SPE) and solid-phase microextraction (SPME). Recently, magnetic solid-phase extraction (MSPE) has drawn analyst's attention. Target analytes are adsorbed by dispersing magnetic adsorbent into liquid sample, and magnetically separate with the help of magnet. This technique is rapid and simple. To obtain the best extraction efficiency, nano-size magnetic materials are synthesized and different functionalities are introduced onto them [130]. MSPE process is explained in Figure 1.15.

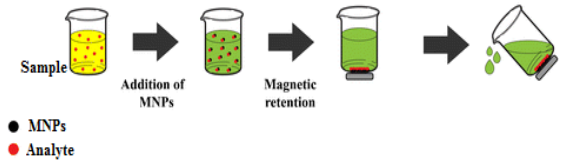

Figure 1.15: Magnetic Solid-phase Extraction (MSPE) process

1.10.4 Polymeric Materials

Natural organic polymers, synthetic polymers and inorganic materials have their applications as stationary phases. Highlighted feature of organic polymers depict stability under wider pH series. Media is chemically derivatized for specific binding of analyte. For example, epoxy group on glycidyl methacrylate is modified with different functionalities and work as affinity materials. In this work, strong anion exchange functionality is introduced onto poly(GMA/DVB)[132]. The optimum pH promotes stronger interactions between biomolecules and quaternary ammonium functionality.

Poly(N,N,N-triethylammonium-2-hydroxypropyl-methacrylate-co-divinylbenzene) monolith is synthesized having required physical properties for separation of biomolecules (oligonucleotides and nucleotides). Separation of nucleotides with mono-, di- and triphosphates have been achieved by using this monolith [130-131].

1.10.5 Monoliths

Monoliths journey started from methacrylate based hydrogel, followed by polymeric foams and compressed beds. They further developed into rigid macro-porous organic polymers, silica rods [133] and polymeric disks (Figure 1.16) [134].

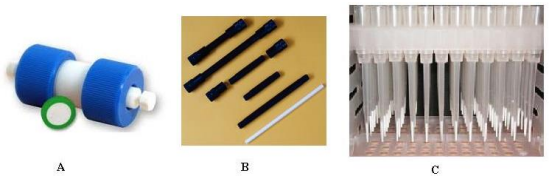

Figure 1.16: Monolithic disc and column (A), columns (B), and pipette tips (C).

For monoliths preparation, the polymerization mixtures are the combination of monomers, cross-linker, initiator, and porogen. Monoliths porosity can be controlled by the concentration of cross-linker and more importantly the porogen. Currently polymeric monoliths that are organic in nature are used in separation science can operate at high pH and are suitable with variety of solvents. Some of these polymer substrates are sensitive to organic solvents and suffer some shrinkage and swelling in spite of high cross-linking, and sensitive to temperatures above 200 °C. Polymer based monoliths have low surface area and are suitable for large molecules separation than small molecules [135]. Silica monoliths show relatively high surface area (about 200gm^{-2} up to 648 gm^{-2}).Variety of inorganic monolithic materials have been synthesized based on ceria, iron, nickel and zirconia. There are also metal/metal-oxide monoliths and composite monoliths. Composite monoliths are formed from two or more materials; a supporting silica or organic monolithic base with metallic layer [136].

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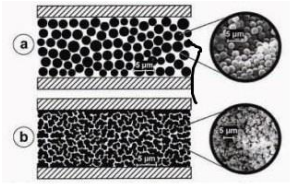

Figure 1.17: Comparison of traditional packed and monolith columns

The main benefits of monolithic stationary phase are their easier synthesis, no packing difficulties, zero void volume, no backpressure (Figure 1.17), speedy mass transfer, fast separation speed and the best efficiency [137].

1.10.6 Carbon based Materials

Carbon based materials have their applications in different fields (optics, electronics, medicine, nanotechnology, etc.).Their application in analytical chemistry is very important. Carbon-based materials are versatile, chemically interact with different elements and with other carbon-based materials and form strong covalent bonds. They show properties such as high strength, hardness and density [138]. Carbon nanotubes (CNTs) have unique properties and are used in different fields. CNTs also serve as separation materials including adsorption separation and membrane-based separation [139].

Recent development in the area of separation science has provoked the use of various diamond-related materials as solid media in different separation techniques. Diamond-related materials are capable to be used as chromatographic stationary phase having chemical, thermal and mechanical stability, low reactivity, and biocompatibility [140].

1.10.7 Metal Organic Frameworks (MOFs)

Specific symmetry, high surface area, easier modification, and availability of various ligands are the reasons of MOFs usage in various fields. MOFs have higher selectivity, sensitivity, and reproducibility. Glycopeptides, phosphopeptides, and low mass peptides have been enriched by

MOF materials. They have also been used as laser desorption/ionization (LDI) matrices. Their properties can be controlled like porosity, surface area and nature of functional groups [141]. They consist of organic ligands and metal ions [142]. MOFs have applications in different fields; gas adsorption [143], drug delivery [144], enzyme immobilization [145], adsorbents [146], separations [147], and enrichment of glycopeptides [148], phosphopeptides, endogenous peptides [149], and nucleosides [74].

MOF is a highly ordered microporous material (Figure 1.18). MOFs are hybrid structures having metal ions and organic ligands. They are porous materials with proper symmetry through inter-molecular forces or coordination bonding. MOFs have further applications in biomedical sciences, energy, environmental science, agriculture, food, medicine, health, and natural sciences. Simple MOFs have only one ligand and metal ion. Hybrid and composite MOFs are synthesized in various forms such as cubes, particles, membranes, films and many other configurations. MOFs can be modified with desirable characteristics. Their hydrophilicity and surface functionalities can be controlled and their working efficiency can be improved [126].

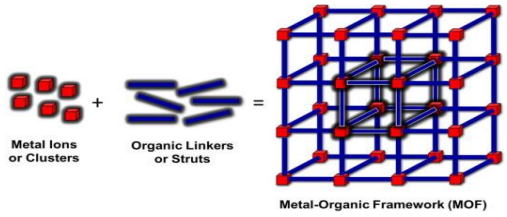

Figure 1.18: Metal Organic Framework (MOF)

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1.11 Aims and Objectives

- Serum lipoproteins isolation from other proteins in selective manner without prior step of ultracentrifugation achieved through synthetic materials having affinity for lipoproteins
- Positive charges (N^+) generated on organic polymers to enrich lipoproteins from blood under optimized pH conditions
- The synthesis of organic polymeric solid media with free epoxy groups on polymer for further derivatization
- Polymeric materials as anion exchangers with triethylamine having affinity for lipoproteins
- Anion exchanger binding between positively charged groups of anion exchanger and negatively charged phosphate groups in lipoproteins as per the following mechanism of interaction (Figure 1.15)

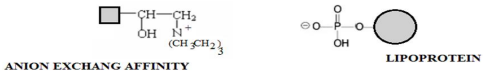

Figure 1.15: Interaction site of lipoprotein for anion exchanger

- Characterization by FT-IR, Scanning electron microscope (SEM) imaging and Energy-dispersive X-ray (EDX) spectroscopy.
- Synthesis of IMAC and metal oxides (MOXs); Cellulose-SiO_2-IMAC-Sm^{3+}, SiO_2-La_2O_3 composite and Fe_3O_4@Sm_2O_3 core-shell nanocomposites
- Tested for lipoproteins enrichment from serum samples on the principle of isoelectric point of affinity materials and lipoproteins at binding and eluting pH
- Detection of enriched content by MALDI-MS
- Lipoproteins phospholipids analyzed with Au-nanoparticles LDI-MS technique
- Development of an optimized and sensitive protocol for Apo-H glycans identification
- Apo-H captured by Anti Apo-H beads, verified by SDS-PAGE and LC-MS/MS analyses
- Both in-solution and in-gel Apo-H N-linked glycans compared
- Characterization of unstable Apo-H glycans in MALDI-MS by controlled sialic acid derivatization followed by HILIC SPE to enrich glycans

- Development of complete work strategy to analyze Apo-H glycans (Figure 1.16)

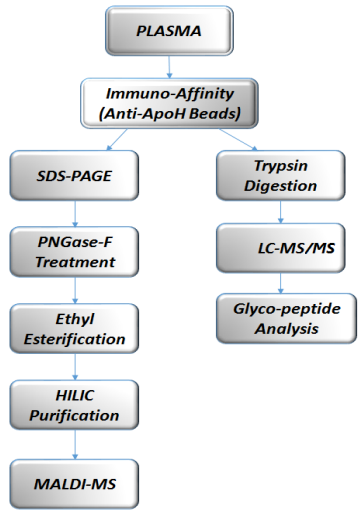

Figure 1.16: A complete work strategy to analyze Apo-H glycans

- Synthesis of magnetic ZIF-8 functionalized with boronic acid (Fe_3O_4/ZIF-8/APBA) to enrich gangliosides
- MALDI-MS protocol optimized to analyze gangliosides

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CHAPTER # 2

EXPERIMENTAL

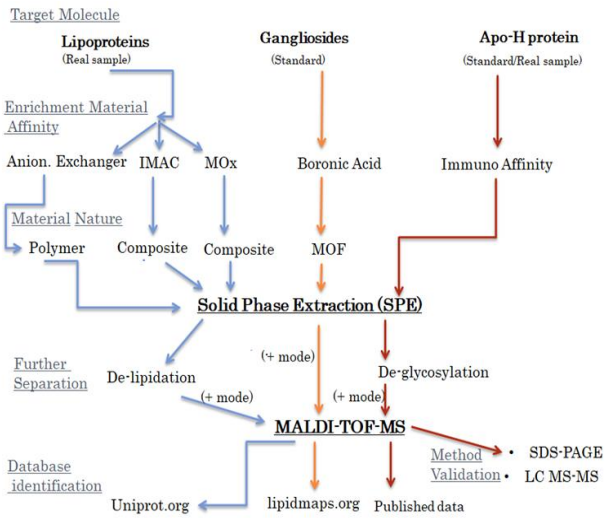

Figure 2.1: Systematic Design of Experiments

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EXPERIMENTAL

2.1 Instruments

Electronic equilibrium (Shimadzu Corporation, AW No. D432300456, type 220), electronic stove from Technico Supply based in Lahore, Pakistan, magnetic stirrer hot plate (Stuart Technical Attractive Stirrer Hot plate, Serial R000102033), FTIR-spectrophotometer i.e. Perkin Elmer 1760, MALDI-MS (Ultraflex I TOF MS, Bruker, Germany), Gel imager, LC-MS/MS (Orbitrap Mixture Lumos Figure spectrometer, Thermo-Scientific).

2.2 Chemicals

B2-Glycoprotein (Apo-H) was purchased from Invitrogen (USA), apo-H affinity matrix from Thermo-Scientific and the Visucon F standard control was from Affinity Biological Inc. Ammonium salt of total Ganglioside extract was from Avanti Polar lipids. Divinyl benzene (80%, DVB, Technical grade), sinapinic acid (99%), glycidyl methacrylate (97%, GMA), α,α-azoisobutyronitrile (AIBN), ethylene glycol dimethacrylate (EGDMA), ethanol (99.9% for HPLC), dithiothreitol (DTT, 99.0%), glycidyl propargyl ether (GPE, ≥90%), trifluoroacetic acid (TFA, for protein sequence), acetonitrile, 1,4-butane diol (≥99.9%), acetonitrile (ACN), ammonium bicarbonate (NH_4HCO_3), and Trypsin were purchased from Promega, USA. Sodium perchlorate, EDTA and HCl were purchased from local dealers. Trizma base was from Sigma Aldrich, St. Louis, MO, USA. Phosphotungstic acid, polyethylene glycol (PEG), dihydroxy benzoic acid (DHB), iodoacetamide (IAA), gold nanoparticles (Au-NPs) were acquired from Sigma Aldrich, St. Louis, USA. Deionized water was from Milli Q, Millipore. Formic acid (98%), and Super-DHB for MALDI-MS (≥99.0%) were bought from Fluorochem, Hadfield, UK. PNGase-F was from Roche Analytical, Mannheim, Germany. Peptide calibration standard was purchased from Bruker, Bremen, Germany. Trifluoroacetic acid (TFA), sodium chloride (NaCl), ethanol, di-sodium biphosphate dehydrate i.e. $Na_2HPO_4.2H_2O$, glacial acetic acid, SDS (sodium dodecyl sulphate), chloroform, and ammonium hydroxide were acquired from Merck, Darmstadt, Germany. 2-Mercaptoethanol and acetonitrile (ACN) were purchased from Biosolve (Valkenswaard, Netherlands). Protein Ladder 10-250 kDa, Laemmli buffer Standard were acquired from Bio-Rad, Bis-Tris GEL Nu–PAGE (4-12%), NuPAGE MES SDS buffer and Blue SafeStain were bought from Invitrogen, USA.

2.3 Buffers Preparation

Filling Buffer: Produced of Trizma base and it was melted in water to prepare Tris HCl. HCl was used to adjust the pH.

Elution: Tris HCl (50 mM) was produced by dissolving Trizma in water. HCl was used to adjust the pH. 1 mM EDTA and 500 mM sodium perchlorate ($NaClO_4$) were prepared in water. HCl was used for pH adjustment.

Washing Buffer I: 25 mM sodium bicarbonate of pH 8

Reduction Buffer: 10 mM DTT

Alkylation Buffer: 55 mM IAA

2.4 Synthesis of Affinity Materials

2.4.1 Polymeric Anion Exchanger

Batch extraction technique was used for lipoproteins selective enrichment. Organic polymer supports including poly(EGDMA/GMA), poly(GPE/DVB) and poly(GMA/DVB) were synthesized and modified using triethyl amine to form anion exchange affinity.

2.4.1.1 Preparation of Poly(Glycidyl Methacrylate/Divinyl Benzene)

A mixture of DVB (2.5 mL), azobisisobutyronitrile (62.5 mg) and acetonitrile (120 mL) was prepared. The mixture was heated at 60 °C for 4 hours under nitrogen. Then 5 mg GMA was added followed by the addition of AIBN and heated at 70 °C for another 16 hours. Reaction mixture was cooled to room temperature, filtered and washed with ACN and methanol. Finally, the obtained product was placed in vacuum oven for drying and stored in desiccator [150].

2.4.1.2 Preparation of Poly(Glycidyl Methacrylate/Ethylene Glycol Dimethacrylate)

Synthesis of poly(Glycidyl Methacrylate/Ethylene Glycol Dimethacrylate) was done by thermal polymerization. A mixture of 3.5 mL 1-propanol, 1 mL EDMA, 4 mL butanediol, 3 mL GMA and initiator, AIBN (62.5 mg) was polymerized for 24 h at 60 °C. Obtained product was filtered, washed and dried for further use [151].

2.4.1.3 Synthesis of Poly(GPE/DVB)

Monomers, GPE and DVB were polymerized by a reported method with slight variation. Briefly, 3 mL divinyl benzene, initiator AIBN (0.120 g) and acetonitrile (60 mL) were heated for 4 h at

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60 °C. Afterwards, 0.10 g further AIBN was added along with GPE (4 mL) and the mixture was stirred at 60 °C for another 16 hours. The mixture was cooled to room temperature. Solvent was removed by filtration and un-reacted species were washed with acetonitrile and methanol. Obtained product placed in desiccator for drying [123].

2.4.1.4 Derivatization of Synthesized Polymers

Poly(GMA/EGDMA), poly(Glycidyl Methacrylate/Divinyl Benzene) and poly(Glycidyl Propargyl Ether/Divinyl Benzene) were derivatized to introduce affinity. 150 mL triethylamine was added to 4 g polymer and mildly stirred for 16 h by heating at 90 °C. Resultant anion exchange affinity was washed by methanol, deionized water, and dried in vacuum oven [131].

2.4.2 Preparation of Immobilized Metal Ion Affinity Chromatography (IMAC) and MO_X Affinity Materials

Cellulose-SiO_2-IMAC-Sm^{3+} [100] as Immobilized Metal Ion Affinity Chromatographic material and Metal Oxide Affinity Materials, i.e. SiO_2-La_2O_3 [105] and Fe_3O_4@SiO_2@Sm_2O_3 [125] were synthesized using the reported protocols.

2.4.3 Synthesis of Boronic Acid Functionalized Metal Organic Frameworks

2.4.3.1Synthesis of ZIF-8

0.492 g of 2-methylimidazolate and 0.297 g of $Zn(NO_3)_2.6H_2O$ were dissolved in 50 mL deionized water. The mixture was transferred to Teflon lined stainless steel autoclave and placed in electrical oven for 3 hours at 50 ºC to carry out the hydrothermal reaction. The obtained product was cooled to room temperature, washed with ethanol and overnight dried at 50 ºC.

2.4.3.2Synthesis of ZIF-8/APBA

Attachment of APBA (aminophenyl boronic acid mono hydrate)with ZIF-8 was done via the sol-gel method. 80 mg APBA was dissolved in deionized water. 120 mL of 3-isocyanatopropyltriethoxysilane was added and stirred for 24 hours at room temperature. The mixture containing APBA and triethoxysilane reagent was mixed with 0.1 M acetate buffer solution and pH was maintained at 5.2 and reaction was continued for 12 hours at room temperature. Finally, ZIF-8-APBA was filtered, washed and overnight dried at 50 ºC [152].

2.5 Characterization of Synthesized Materials

Synthesized combinations of affinity materials were characterized by ATR-FTIR. SEM, TEM and EDX.

2.6 Analysis of Lipoproteins Captured by Affinities Materials

Following protocols were used to enrich lipoproteins and peptides by the affinity materials including polymeric anion exchangers of polymers, i.e. poly(Glycidyl Propargyl Ether/Divinyl Benzene), poly(Glycidyl Methacrylate/Ethylene Glycol Dimethacrylate), poly(Glycidyl Methacrylate/Divinyl Benzene), IMAC/MOx materials (above mentioned) and boronic acid functionalized metal organic framework.

2.6.1 Proteins Enrichment Protocol

2.6.1.1 Precipitation of Dominating Proteins

Serum was diluted by one volume of serum and twice the volume of acetonitrile and stirred at room temperature for 30 minutes, followed by centrifugation at 12000 rpm for 15 minutes. Highly abundant proteins including Albumin were precipitated and obtained pellets were separated. Supernatant was collected and saved for further experiments [153].

2.6.1.2 Lipoprotein Enrichment Protocol

200 µL equilibration buffer (1 mM EDTA + Tris HCl) of pH 7.5 was added to the supernatant obtained from above step and vortexed for 2 min. Content was centrifuged for 5 min and supernatant was discarded. 200 µL human plasma (10 µL plasma diluted with 180 µL Milli-Q water) was added and mixed for 2 minutes. After incubation for 1 hour at 28 ºC, the mixture was centrifuged. Washing was carried out with 200 µL water to remove the salts and non-specific species.

At the end, 200 µL elution buffer (1 mM EDTA, 50 mM Tris HCl and 500 mM sodium perchlorate) of pH 7.5 was used for the elution of bound lipoproteins. Elution buffer was added to above mixture, vortexed for 2 minutes followed by keeping at 28 ºC for 10 min. Eluted lipoproteins in the supernatant were collected and saved for MS analysis. Figure 2.2 represents the batch extraction method for the enrichment of lipoproteins.

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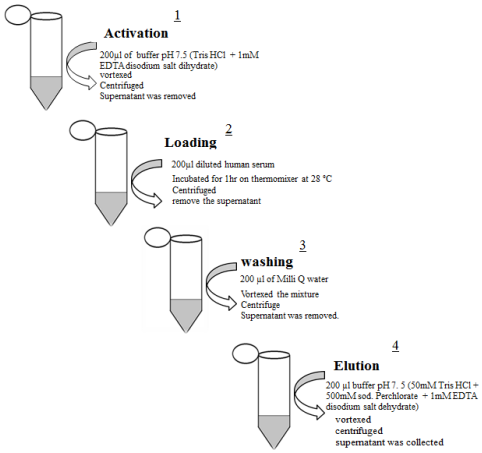

Figure 2.2: Steps for lipoproteins enrichment by batch extraction method

2.6.1.3 Separation of HDL from other Lipoproteins

LDL were precipitated by using standard precipitating reagent [154]. Eluted lipoproteins (100 µL) were taken in a vial and an organic buffer, i.e. 0.2 M 2(N-morpholino) gasoline sulfonic acid (MES) was added for lowering the pH to 4.8-5.8 which is the isoelectric point of LDL. 0.4% phosphotungstic acid (precipitating agent) was added which formed in soluble complex with LDL particles. To improve the precipitation, 2.5% PEG was also added. 10 mL EDTA was added to form complex with ions and precipitate formed were centrifuged and separated.

2.6.1.4 Delipidation of Lipoproteins

Eluted HDL (100 µL) was added drop wise to vortexing methanol (1 mL) and ethyl ether (4 mL) was added to the mixture. After cooling for 10 minutes, mixture was centrifuged at 10000 rpm for 4 minutes and supernatant (fats) was removed. Obtained precipitate (pellets of apoprotein)

were washed with methanol. After excessive washing, the pellets were dissolved in 250 mM ammonium bicarbonate solution.

2.6.1.5 MALDI-MS Analysis of Apoproteins

20 mL matrix solution was prepared by dissolving sinapinic acid (Bruker Daltonics) into 0.1% TFA in 50% ACN, vortexed for 2 minutes, sonicated for 5 minutes and centrifuged at 15000 rpm for 4 minutes. 0.5 µL eluted protein samples were spotted on stainless steel MALDI target plate followed by 0.5 µL matrix solution and mixed thoroughly by aspirating and de-aspirating by micropipette tip. The spots were dried in air at room temperature before MALDI-MS analysis [33]. MALDI-MS analysis of apolipoprotein is summarized in Figure 2.2.

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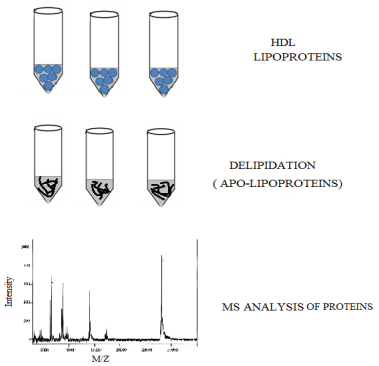

Figure 2.3: Enrichment and MS analysis of HDL Apolipoproteins

2.6.2 Peptides Enrichment Protocol

2.6.2.1 Protein Digestion

Abundant proteins were removed by the same procedure as described above. Reduction of proteins was done by 10 mM dithiothreitol (DTT) at 37 ºC for 45 min followed by alkylation in 55 mM iodoacetamide in dark. Proteins were fragmented by trypsin (proportion of 50:1 protein to trypsin) at 37 ºC for 12 hours. Quenching of digestion was performed by 10% FA (formic acid). Zip-Tips C18 were used for desalting prior to the sample loading, enrichment and MS analysis [155].

2.6.2.2 Enrichment of Peptides and MALDI-MS Analysis

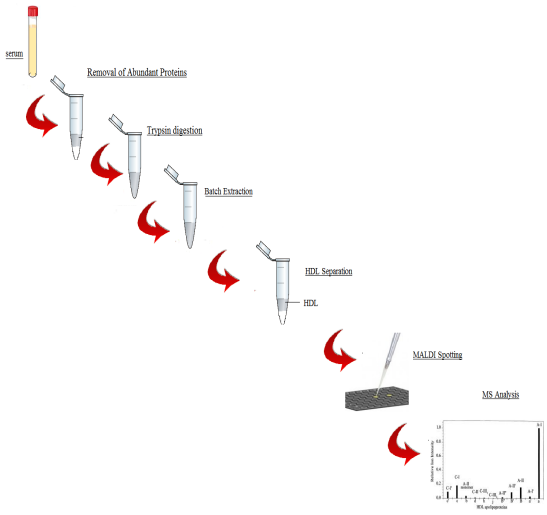

Figure 2.4: Lipoproteins enrichment, HDL separation and MS analysis from digested serum

Enrichment conditions for peptides, HDL separation from other lipoproteins and MALDI-MS analysis conditions were the same as described above with the exception of DHB matrix used for peptides instead of sinapinic acid. Equilibration, loading and elution was completed as stated above (2.6.1.2).

Gold nanoparticles were used for the LDI-MS analysis of lipids. SwissProt database (http://www.uniprot.org/) and (www.phosphosite.org/) were used for proteins identification and Lipidmaps database (www.lipidmaps.org/) (http://www.hmdb.ca/) for the lipid fractions.

2.7 Apolipoprotein-H (APO-H) Glycans Analysis

2.7.1 Sample Preparation

B2-Glycoprotein (Apo-H) was diluted with Milli-Q water with the concentration as 10 µg/50 µL.

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2.7.2 Enrichment Protocol

Affinity Capturing

Apo-H protein was captured by Anti Apo-H affinity beads diluted with PBS. 15 µL beads were applied to 96-well plate. Beads were washed three times with PBS by vacuum filtration. 10 µg standard and 50 µL (10 µg) Visucon-F sample was applied with 50 µL PBS and put the plate on plate shaker for 60 min at 1000 rpm to assist the binding. Washing of plate was done with PBS and Milli-Q water thrice to remove the unbound proteins. Wash fractions were collected to check the loss of required protein. Elution of bound proteins was done by 100 µL of 100 mM formic acid (FA). 6 mM FA was also tried to find out the right eluting condition (Figure 2.5). Both fractions were incubated for 5 min on a shaker at 1000 rpm, followed by centrifugation for 2 min at 500 rpm. Collected proteins were dried in Speed Vac for 1 hour at 60 °C. The eluted proteins were analyzed by SDS-PAGE. An appropriate quantity of the samples was saved for in-solution and in-gel glycans release comparison.

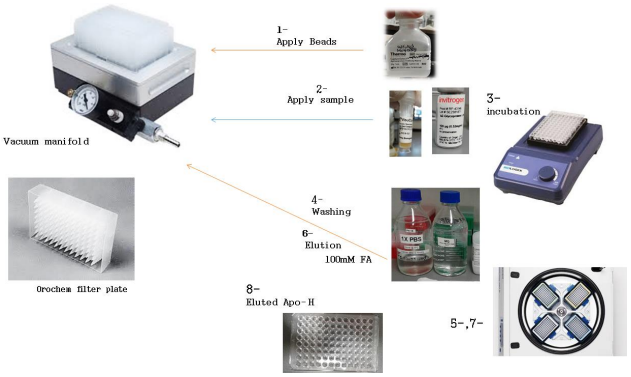

Figure 2.5: Apo-H protein affinity capturing procedure

2.7.3 SDS PAGE

Dried proteins were dissolved in reducing Laemmli buffer. The solution was vortexed and incubated at 93 ºC for 10 min to reduce, denature and charge the proteins. 20 µL sample and 3 µL protein ladder were loaded on Nu-PAGE gel and run at 200 mV for 45 min in Nu-PAGE MES SDS running buffer (Figure 2.4). Staining with blue stain and de-staining with Milli-Q water was completed. Gel picture was taken by gel imaging system (Hoefer Ultima).

Figure 2.6: SDS PAGE Setup

2.7.4 In-Gel Glycans Release

Gel bands were cut, chopped and washed with 25 mM sodium bicarbonate and acetonitrile. Reduction was performed by 10 mM DTT at 56 °C for 30 min and alkylation with 55 mM IAA for 20 min in dark. For glycans release, 2U PNGase-F/sample prepared in 4% NP-40/acidic 5 x PBS was used and put in the oven overnight at 37 °C for complete digestion. Next day, 20 µL MQ was added to gel pieces and sonicated for 30 min. Supernatant was collected for further analysis.

2.7.5 In-Solution Glycans Release

For in-solution glycan analysis, dried proteins from step 1 were dissolved in 10 µL PBS. 20 µL 2% SDS was added and shaken for 5 min. The incubation of mixture was carried out at 60 °C for 10 min. After cooling to room temperature, 20 µL release mixture (1 µL PNGase F, 10 µL acidic 5 x PBS and 10 µL 4% NP-40) was added, shaken for 15 minutes and incubated at 37 °C for 14 hours. It was used immediately for ethyl esterification [88].

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2.7.6 Ethyl Esterification

2 µL released glycans (in-gel/in-solution) were added to 20 µL ethylating reagent containing 0.5 M HOBt and 0.5 M EDC in ethanol in each PCR well-plate and incubated for 60 min at 37 °C. After cooling to room temperature, protein precipitation was carried out by the addition of 20 µL ACN and incubation for 15 min [88]. 2,6-linked, 2,3-linked sialic acid structural changes after ethyl esterification are shown in Figure 2.7.

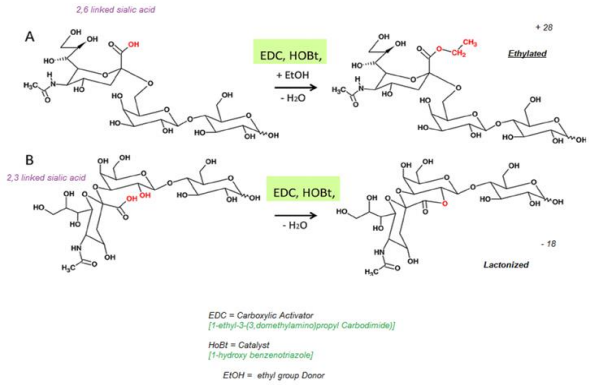

Figure 2.7: Ethyl Esterification of Sialic acid groups (2,6-linked and 2,3-linked)

2.7.7 Cotton HILIC Purification

To suppress the excessive salt effect, glycans were purified by HILIC SPE. 3 mm cotton rope was inserted in 20 µL tip and 50 kPa pressure was applied to fix inside the tip. Cotton was equilibrated by thrice pipetting up and down 20 µL of 85% ACN and 20 µL of Milli-Q. Loading of sample was carried out by pipetting up-down the ethyl esterified released glycans about 20 times. The sample was washed thrice with 1% TFA in 85% ACN and then with 85% ACN. Elution was done with 10 µL Milli-Q water and the whole experiment was repeated five times. A

multichannel pipette with Cotton HILIC tips and collected purified glycans are shown in Figure 2.8.

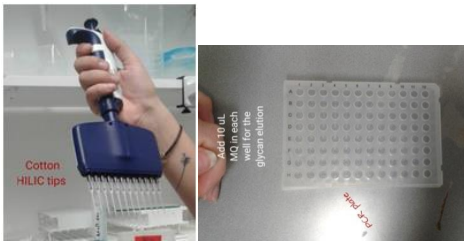

Figure 2.8: Cotton HILIC purification

2.7.8 MALDI-TOF-MS Analysis

Super-DHB was prepared by dissolving 20 mg in 5mL of 50% ACN. 1 µl of 1 M NaOH was added to highlight the Na^+ adduct. 1 µL matrix and 2 µL HILIC purified glycans were spotted onto MTP AnchorChip MALDI target plate and air dried at room temperature. FlexControl 3.4 software Build 119 was used for MALDI-TOF-MS (Bruker Daltonics)operation. Before sample analysis, manual calibration of the instrument was carried out using a peptide calibration standard provided by Bruker Daltonics. 1000-4500 m/z mass range was selected for glycans. To avoid the matrix ions, mass up to 950 Da was suppressed. 1000 Hz frequency, 10,000 shots and 200 shots per spot was selected for the analysis.

2.7.9 LC-MS/MS Verification

ApoH after elution and drying were re-dissolved in NH_4HCO_3. ACN was added and shaken for 5 min, followed by reduction with DTT at 95 °C for 5 min. Sample was brought to room temperature and alkylated with 125 mM IAA, incubated in dark for 30 min at room temperature. 1 µg TPCK trypsin was added per sample and incubated overnight at 37 °C.

The sample was injected to Orbitrap Fusion Lumos Tribrid LC-MS/MS (ThermoScientific) and the generated data was analyzed by Byonic software for protein identification and glycans investigation. Orbitrap Fusion Lumos Mass-spectrometer is shown in Figure 2.9.

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Figure 2.9: Orbitrap Fusion Lumos Mass-spectrometer (ThermoScientific)

2.8 Ganglioside Analysis

2.8.1 Ganglioside Standard Preparation

1mg/mL ganglioside standard stock solution was prepared in 50% methanol. Standard dilutions were also carried out with 50% methanol.

2.8.2 Enrichment of Standard Gangliosides with Boronic Acid Affinity

2 mg MOF-boronic acid material was taken in a vial. 1mL standard gangliosides (diluted 5 times) was taken as sample, pH was adjusted with ammonium hydroxide. 500 µL sample was loaded onto the material. Material was vortexed and incubated at 37 ºC for 1 hour on incubating shaker at 1000 rpm. After centrifugation for 10 minutes, supernatant was collected and saved for MS analysis.

Washing step was carried out with 500 µL distilled water by vortexing followed by centrifugation. Supernatant from wash fraction was also collected for MS analysis. Finally, 2% formic Acid (1mL) was added for elution, vortexed for 3 minutes, incubated for 30 min followed by centrifugation. Supernatant was collected, dried in speed-Vac for 2 hours and stored for MS analysis.

2.8.3 Alkaline Treatment of Eluted Gangliosides

To fly the lipids in MALDI-MS, gangliosides were treated with 500 µL ammonium hydroxide, vortexed for 5 minutes and gangliosides mixture was lyophilized. Mixture was re-dissolved in MeOH:H_2O (5: 1) for MALDI-MS analysis.

2.8.4 MALDI Matrix Preparation

50mg/mL DHB matrix was prepared in 50% ACN. 1 µL of 1M NaCl was added as cation dopant and other with 50mg DHB in 1mL MeOH.

2.8.5 MALDI Spotting

2 µL sample was taken in 500 µL sample vial. 8 µL matrix was added, mixed with pipette tip and spotted on MALDI plate, MTP target frame III (Bruker).

2.8.6 MALDI-MS Analysis

Mass spectrometric analysis was performed using an Autoflex MALDI-MS (Bruker Daltonics) with N_2 Laser of 337 nm. Acquired data was recorded in the range of 1000–3000 m/z. MS spectra was calibrated externally using a peptide calibration standard.

Gangliosides identification was done via LIPID MAPS (http://www.lipidmaps.org/tools/index.html). Obtained data was compared with accurate mass measurements from published literature as well.

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CHAPTER # 3

RESULTS & DISCUSSION

RESULTS AND DISCUSSION

3.1 Solid Phase Extraction (SPE)

In analytical chemistry, solid phase extraction is used to concentrate and purify samples before their analysis. SPE is used to separate analytes of interest from complex mixtures (urine, blood, water, beverages, animal tissue and soil samples). In comparison to traditional methods (ultracentrifugation, immuno-affinity and liquid chromatography), SPE is simple, cost effective with fewer steps, less sample handling, less solvent consumption, less exposure to toxic agents and minimal sample transfers. Keeping in view the benefits, SPE based separation strategy is adopted in this study. A comparison of designed SPE based strategy with traditional methods for the separation of lipoprotein is shown in Figure 3.1.

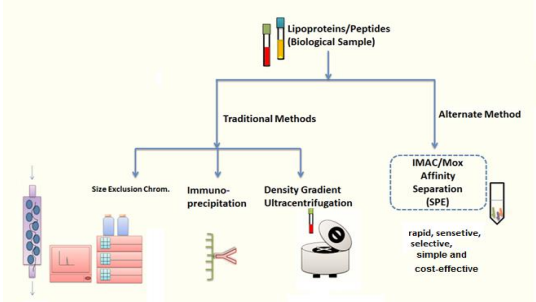

Figure 3.1: Comparison of designed SPE based simple and cost-effective strategy for lipoproteins separation with traditional methods.

3.2 Selection of Affinity Materials

Varity of separation media have been studied for the isolation of proteins and lipids from their matrices. Affinities with different supports are tried to achieve the best separation/isolation results. In current study, variety of affinities for lipoproteins and glycolipids isolations from biological sample are synthesized and applied before their mass spectrometric analysis.

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3.2.1 Selection of Affinity Materials for Lipoproteins

- Anion Exchangers
- IMAC and MOXs

3.2.1.1 Choice of Anion Exchanger for Lipoproteins Enrichment

3.2.1.1.1 Phospholipids

There is an outer layer of apolipoproteins (Apos.) and phospholipids (PL) on lipoproteins [157]. The main part of HDL lipidome is made-up of phospholipids [158]. Phospholipids are of different types like PG, PS and PI. These phospholipids are acidic in nature and produce negatively charged ions. Phospholipids like PA and PPI are categorized as acidic and show greater than 1 negative charge at physiological pH of 7.4. An inositol ring have various phosphate groups. At physiological pH, PI possesses 1 negative charge, PA two, PIP three, PIP_2 five and PIP_3 greater than seven [159].

Phospholipids layer in lipoprotein and structure of phospholipid are shown in Figures 3.2 and 3.3 respectively.

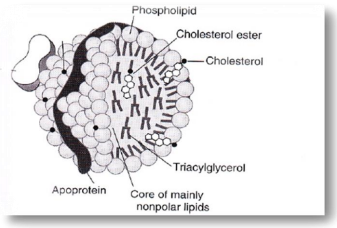

Figure 3.2: Phospholipids in lipoproteins

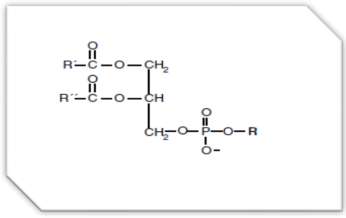

Figure 3.3: Structure of phospholipid

At surface of lipoprotein particles, hydrophilic phospholipids are organized. To enrich the lipoproteins, anion exchange affinity works the best towards phosphate group of phospholipids.

3.2.1.1.2 Selection of Strong Anion Exchanger (SAX)

Physical or chemical properties of proteins are considered to isolate them from crude mixture. Proteins can bind to variety of materials in optimized conditions. Proteins are charged species that is why it is a reasonable approach to separate proteins of interest by creating opposite charge on the stationary phase; by creating positive charge. Maximum of proteins are negatively charged at pH 6-8 and anion exchanger is the best choice to enrich proteins. Lipoproteins may also be isolated from other proteins (contaminants) by the same principle and for the optimal separation, proper selection of pH is made. By adjusting the buffer composition and pH, the target molecules (lipoproteins) bind to anion exchanger and non-bound proteins are washed away. Lipoproteins have a monolayer of apolipoproteins (Apos.). Phospholipid carries charges from -1 to -7 at pH 7.4 [159]. The affinity is provided to negatively charged phosphate group of phospholipids to enrich the lipoproteins through anion exchanger. Strong anion exchangers remain positively charged even at high pH which enhances its sample loading (binding) capacity whereas weak anion exchanger becomes neutral at high pH.

3.2.2 Selection of IMAC and MOXs for Lipoproteins Enrichment

Lipoproteins (HDLs) can be separated by IMAC and MOx based SPE isolation strategy. Lipoproteins possess anionic charge because of both the apoprotein and lipid parts of the particles. The lipid ingredients of lipoproteins affect charge density directly by being charged

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components and indirectly by modifying the protein conformation. Phospholipid has small influence on HDL charge however triglyceride and cholesteryl ester increase the total negative charge on HDL by changing the conformation of apoprotein. ApoA has net negative charge. An increase in ApoA1 negative charge increases the net negative charge on HDL particles.

3.2.2.1 IMAC/MOX and Lipoproteins Interaction Mechanism

Different proteins show different pKa values. At a specific pH, the proteins show specific net charge. This characteristic helps in separating the proteins in this study. Additionally, buffers improve lipoproteins separation by decreasing the non-specific interactions. A comparison of binding and eluting conditions of phosphopeptides, phospholipids and lipoproteins by IMAC affinity is given in Table 3.1.

Table 3.1. Comparison of binding and eluting conditions for phosphopeptides, phospholipids and lipoproteins from IMAC affinity

Analyte	Phosphopeptides	Phospholipids	Lipoproteins
Affinity	IMAC	IMAC	IMAC
Binding Condition	50 µL of 0.1% (v/v) TFA; pH 2-3	0.1% acetic acid in 1:1 MeOH-ACN	200 µL buffer of Tris HCl with1 mM EDTA disodium salt dihydrate of pH 7.5
Eluting condition	10 µL of 1.5% NH_4OH solution; pH 11-12	0.3 N NH_4OH in 1:1 MeOH-ACN	500 mM sodium perchlorate + 50 mM Tris HCl + 1 mM EDTA disodium salt dehydrate of pH 7.5
Reference	[100]	[160]	Present Study

Normally lanthanum oxides form bi-dentate complex with phosphopeptides. However, in case of lipoproteins, the enrichment mechanism is different. La_2O_3 achieves the isoelectric point at pH 10. Below this pH, it has positive charge. At pH 7.5 (binding pH), positively charged metal oxide

shows electrostatic interaction with anionic lipoproteins (Figure 3.4). La_2O_3 thus behaves as cation and works as an anion exchanger.

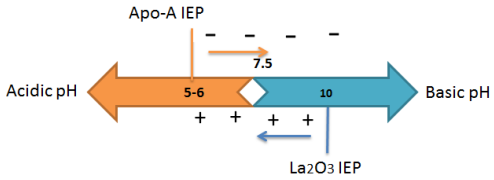

Figure 3.4: Interaction mechanism of anionic lipoproteins with La_2O_3 based on their iso-electric points (IEP)

Binding conditions are optimized by varying pH from 3 to 7.5 and then 8. At 3 and 8 pH, phosphopeptides are also observed, however at 7.5 only lipo-peptides are obtained [161].

3.2.3 Selection of Boronic Acid Affinity for Gangliosides

Gangliosides are a family of glycosphingolipids (GSLs), ceramide lipid chain attached to an anionic oligosaccharide moiety that varies in complexity based on the level of sialylation (N-acetyl neuraminic acid or NeuAc). Gangliosides are generally separated by DEAE. In this study, SPE method is presented for the enrichment of gangliosides using metal organic frameworks (MOFs) based boronic acid affinity.

In recent era, boronic acid (BA) has attracted attention and has been studied widely in the context of glycoproteins enrichment. Boronic acids form cyclic esters with cis-diol-containing compounds such as sugars, glycoproteins, and nucleosides at relatively high pH. The resulting complexes dissociate reversibly at low pH values. Here, this interaction is employed for the selective capturing and elution of gangliosides.

MOFs are well-known in materials science due to their versatile properties and applications in proteomics. For example, for the glycans analysis from biological samples, MOF boronic acid affinity captures them through electrostatic forces existing between amino groups of MOF affinity and negatively charged carboxylic groups of sialic acid that is part of glycans. In the

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binding process, hydrogen bonding also exists between -OH groups of glycans and amino groups of MOF affinity.

3.2.3.1 Gangliosides Enrichment by SPE based ZIF-8/APBA NPs

An analytical method of SPE followed by MALDI-MS analysis is established. SPE based on ZIF-8/APBA offers the selective enrichment of gangliosides. Zeolitic imidazolate framework-8 (ZIF-8) is functionalized with phenyl boronic acid through the sol-gel technique to produce ZIF-8/APBA. Synthesized materials have high capacity to adsorb target molecules. MS analyses conditions are optimized. The results of this study provide an efficient enrichment and determination method for gangliosides.

3.3 Choice of Support Media

3.3.1 Choice of Polymer as Support Material

Inorganic or organic, natural or synthetic polymers are used in isolation techniques. Polymers have been used in various fields. To stand with higher pressures, agarose beads stability can be improved by chemically hardening and then cross linking. Major drawback of agarose is its low binding capacity.

The efficiency and selectivity of polymeric materials can be improved by introducing different functionalities. These polymers are easy to derivatize and are pH stable. After derivatization into different functional groups, these polymers can interact with variety of analytes. These polymers are selective and sensitive towards analytes. In this work, GPE is polymerized with divinyl benzene (DVB) to produce poly(GPE/DVB) which is further derivatized.

3.3.1.1 Epoxy Group

They have epoxy group that can be converted into different functional groups. The epoxy group is also called oxirane where two carbon atoms are linked to one oxygen atom and make a crown. Epoxy group is the highlighted feature of polymers for the introduction of functionalities. Through basic chemical reactions, the epoxy groups can be derivatized. For example, GPE is one of the monomers used in this study. The terminal epoxy groups in GPE are modified and acrylic and allyl functionality copolymerizes with several of other vinyl monomers. Biomolecules (more specifically proteins and peptides)have different polarities and analytical chemists look for affinity materials, achieved by modification of polymers [66].

Polymers with epoxy groups are converted to anion-exchanger by triethylamine [162].

Polymers have properties like surface polarity, porosity, wide pH stability and reactivity. These organic polymers are thus popular separation media. Synthetic polymers have been presented in chromatography in 1964. The best commercially available polymers are the copolymers of divinyl benzene (DVB) with styrene and are available in various particles sizes. After treating with acid or base groups, these materials are converted to ion exchanger for proteins separation [163]. GMA epoxy part can bechemically modified into various functionalities. Here, we have introduced strong anion exchange affinity on poly(GMA/DVB), poly(GMA/EGMA) and poly(GPE/DVB). These polymers can be synthesized easily.

3.4 Eluting Buffer Conditions for Lipoproteins

The pH range in which ion exchange affinity chromatography works is limited because of proteins stability. Buffer pH should be higher (at least by 1 pH unit) than the protein pI. At this pH, proteins show negative charge and result in good interaction. The choice of buffer ions is a major factor for isolating proteins. The proteins bind with anion exchanger and proteins are selectively release from the material [58].

The lipoproteins and anion exchanger binding is interrupted by the buffer counter ion of eluent [13]. Elution is attained with counter ion. The counter ions compete with the phosphate groups of lipoproteins. Lipoprotein particles interact mainly with N^+ sites present on anion exchanger and a suitable buffer is selected for their elution [54].

3.5 Lipoproteomics Workflow

3.5.1 Lipoproteomics Workflow Using Anion Exchanger as SPE Affinity Material

Numbers of peptides are generated by the enzymatic digestion of proteome increasing the complexity which is not directly compatible with MS analysis. Because of closely related physico-chemical properties and complex structures, the separation and identification of proteins is challenging. Bringing together experimental components into one platform can increase time efficiency and data management. Figure 3.5 displays a workflow for lipoproteomics using experimental setup together with separation/fractionation of sample. Initially SPE is conducted for the separation of lipoproteins and then analyzed by mass spectrometry. After elution and washing, the material is regenerated. This workflow is suitable for both intact proteins and

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peptides enrichment. All the steps in workflow are simple and easy to perform. Lipoproteins are enriched by SPE method and analyzed; further HDL is separated from all other lipoproteins by using precipitating agent (phospho tungstic acid) and analyzed. In the next step HDL fraction is delipidated by LLE (liquid/liquid extraction) to obtain lipids and apoproteins. Lipoproteins lipid content is clinically important for its association to various diseases whereas apoproteins are considered as potential biomarkers for several diseases. Thus all fractions obtained using this workflow are of clinical relevance and complete information can be obtained for serum sample. All protein fractions are analyzed by MALDI-MS at each step. All the steps are reproducible and suitable for both lipoproteins and peptides. Small serum volume (10 µL) is good enough for the whole analysis.

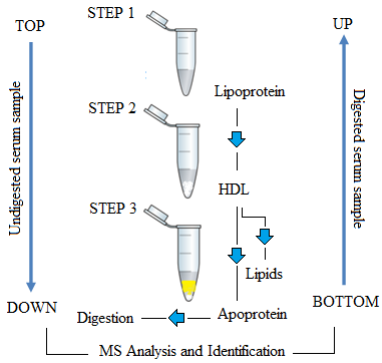

Figure 3.5: Lipoproteomics workflow for the analysis of intact and digested lipoproteins, HDL, apoproteins and lipids from serum

3.5.2 Lipoproteomics Workflow Using IMAC and MOX as SPE Affinity Material

In this experiment, a similar workflow is adopted. The designed HDL-omics workflow includes SPE based enrichment strategy from human serum. In the first step, immobilized metal ion affinity and metal oxides are used as SPE materials. Workflow includes analysis of intact

apolipoproteins, apopeptides and lipids from a single fraction of 200 µL diluted serum sample (Figure 3.6).

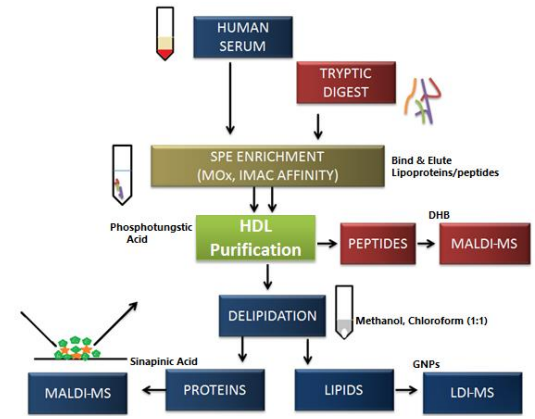

Figure 3.6: HDL-Omics workflow: Serum lipoproteins/lipo-peptides enrichment by IMAC/MOx affinities, HDL purification, delipidation and MS analysis of HDL proteome and phospholipidome

3.6 Characterization

3.6.1 Characterization of Anion Exchanger

3.6.1.1 FT-IR Spectra for Anion Exchanger

Solid state IR spectra are taken which avoid KBr disc preparations. The ring opening reaction of epoxy group is justified by the presence of C-N stretch peak at 1080-1360 cm^{-1}. C-O-H peak at 1415 cm^{-1} confirms the formation of anion exchanger (Table 3.2). FT-IR spectra of poly(GMA/DVB) anion exchanger, poly(GMA/EGMA) anion exchanger and poly(GPE/DVB) anion exchanger are shown in Figure 3.7, 3.8 and 3.9 respectively.

Table 3.2: IR functional groups bands

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Functional Group	Wavenumber cm^{-1}
C-N	1080-1360
C-O-H	1415
O-H	3500-3000
Ester	1750-1735
Unsaturation	1680-1620

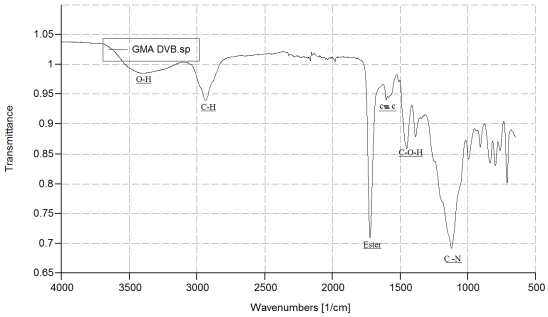

Figure 3.7: FT-IR spectrum of poly(GMA/DVB) anion exchanger

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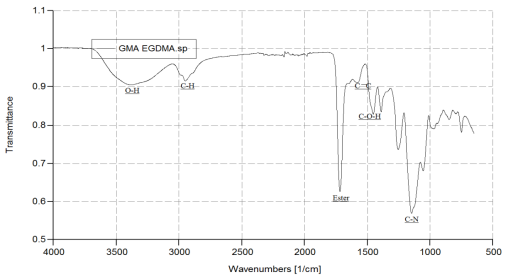

Figure 3.8: FT-IR spectrum of poly(GMA/EGMA) anion exchanger

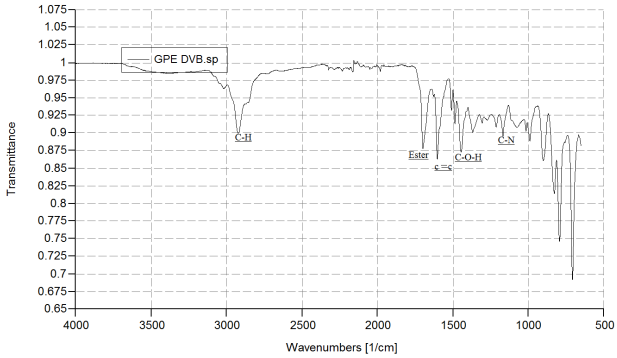

Figure 3.9: FT-IR spectrum of poly(GPE/DVB) anion exchanger

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3.6.1.2 Scanning Electron Microscopy (SEM), Energy Dispersive X-ray Spectroscopy (EDX)

The characterization of polymers is further carried out using SEM and EDX. EDX analytics provide the purity of polymers whereas SEM micrographs reveal the surface morphology (Figure 3.10, Figure 3.11 and Figure 3.12).

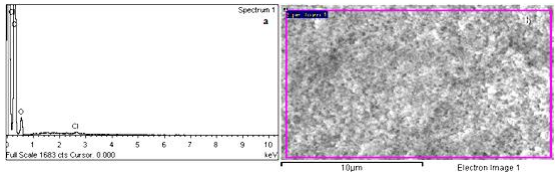

Figure 3.10: EDX spectrum (a) and SEM image (b) of poly(GMA/DVB)

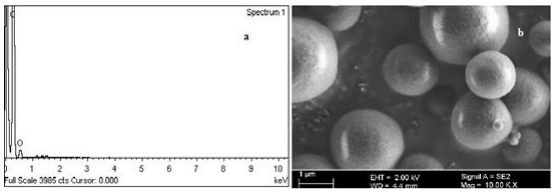

Figure 3.11: Dispersive X-Ray (a) and SEM image (b) of poly(GPE/DVB)

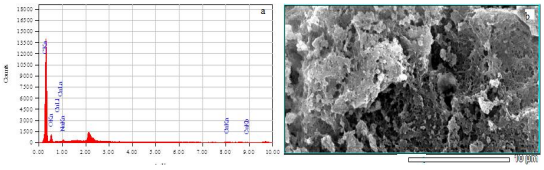

Figure 3.12: Dispersive X-Ray (a) and SEM image (b) of poly(GMA/EGDMA)

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Three polymeric materials are used as support materials for anion exchange affinity i.e. poly(GMA/DVB), poly(GMA/EGDMA) and poly(GPE/DVB). All three materials have different hydrophilic and hydrophobic strength which may affect morphology, porosity and on the whole enrichment efficiency. Poly(GMA/EGDMA) is more hydrophilic than poly(GMA/DVB) and poly(GPE/DVB) due to the presence of DVB as cross linker. The purpose of using three polymers in present workflow is to observe change in enrichment by varying polymeric material of the sorbent. Derivatization protocol for three polymers to anion exchange is similar. The buffer conditions of enrichment are also same. All three polymers show comparable results and thus any anion exchange material can be selected for the developed workflow. Hydrophilicity of poly(GMA/EGDMA) strengthens the anion exchange ability to bind lipoproteins and results in good S/N ratio which is favorable in case of complex samples.

3.6.2 Characterization of ZIF-8/APBA NPs

Transmission electron microscopy (TEM), SEM imaging and EDX analysis of synthesized ZIF-8/APBA NPs is performed. Images are shown in Figures 3.13, 3.14 and 3.15.

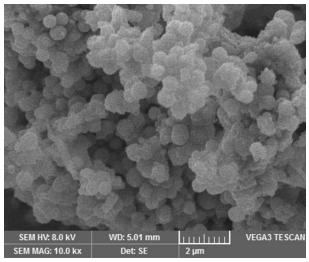

Figure 3.13: Scanning electron microscope (SEM) image of ZIF-8/APBA NPs

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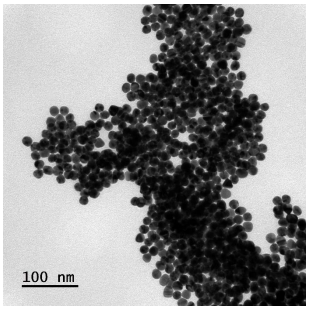

Figure 3.14: Transmission electron microscopy (TEM) image of ZIF-8/APBA NPs

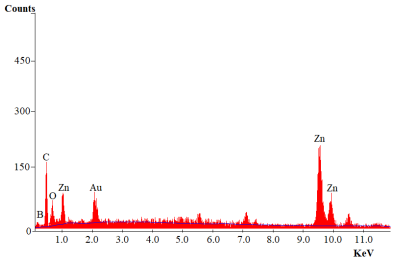

Figure 3.15: Energy-dispersive X-ray (EDX) spectrum of ZIF-8/APBA NPs

3.7 MALDI-MS Analysis

3.7.1MALDI-MS Analysis of Lipoproteins Captured by SAX

HDL particle carries number of non-apolipoproteins that are less abundant. Standard precipitation method is used for HDL isolation, followed by the delipidation and MS analysis is performed at each step. Various apoproteins are identified which include ApoA-II, ApoC-I, ApoC-III and ApoC-IV, in addition to Apo-M and serum amyloid A as shown in Table 3.3. More proteins are detected prior to delipidation as compared to the fraction collected after because the

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fraction is collected using SPE material through selective and optimized enrichment. Delipidation on the other hand depends on the choice of solvents in liquid/liquid extraction.

Table 3.3: Apoproteins, their masses and SwissProt accession number derived from HDL fraction before and after delipidation from serum sample

No.	Apoprotein	Mass (Da)	Accession No.	Prior to Delipidation	After Delipidation
1	ApoC-I	6,998	P02654	+	+
2	ApoC-III	9,470	P02656	+	+
3	ApoC-III	10,005	P02656	+	-
4	ApoA-II	11,877	P02652	+	-
5	SAA4	12,972	P35542	+	+
6	Apo-D	19,050	P05090	+	-
7	Apo-M	21,517	O95445	+	+
8	ApoA-I	25,232	P02647	+	-
9	ApoA-I	28,127	P02647	+	-
10	Apo-E	30,912	P02649	+	-
11	ApC-IV	15,839	P55056	-	+
12	ApoA-II	18,873	P02652	-	+

Intact apolipoproteins in undigested serum are identified from eluted lipoproteins (Table 3.4) which include various isoforms of Apo A-I, Apo A-II, Apo M, Apo-E and Apo B-100. Analysis of intact lipoproteins is rare in literature. In this study, intact lipoproteins having mass between 6 kDa to 36 kDa are analyzed as shown in Figure 3.16.

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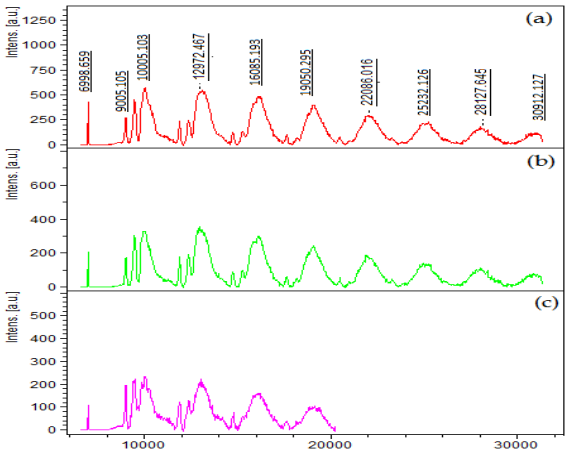

Figure 3.16: MS spectra for HDL intact proteins obtained after anion exchange enrichment using (a) poly(GMA/DVB) (b) poly(GMA/EGMA) and (c) poly(GPE/DVB) anion exchangers

Apo C-I from HDL is identified in MS spectra of poly(GMA/DVB), poly(GMA/EGMA) and poly(GPE/DVB) anion exchangers (Figure 3.17a-c). Apo-E, Apo B-100, Apo A-I, Apo A-II, Apo-M, Apo-J, SAA4 and many other proteins are present in LDL. The binding of SAA to HDL may change the HDL metabolism [33]. HDL proteins are detected by MS in the first step of workflow and further explored for other content related to lipoproteins.

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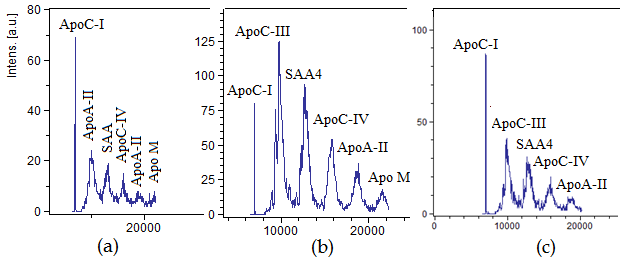

Figure 3.17: MS spectra of HDL intact apolipoproteins using (a) poly(GMA/DVB) (b) poly(GMA/EGMA)and (c) poly(GPE/DVB) anion exchangers

Table 3.4: Detail of apoproteins, their masses and accession number for intact lipoprotein fraction obtained in step-1 from undigested serum

No	Protein	Mass (kDa)	Accession No.
1	ApoC-I	6	P02654
2	ApoC-II	8	P02655
4	ApoC-III	10	P02656
5	ApoA-II	11	P02652
		9	P02652
6	ApoC-IV	15	P55056
7	SAA4	12	P35542
		14	
		17	
8	Apo-D	19	P05090
9	Apo-M	21	O95445
		13	O95445
		26	O95445
10	α-1-acid glycoprotein 2	23	P19652
11	ApoA-I	25	P02647

		28	P02647
		31	P02647
12	ApoB-100	29	P04114
		30	P04114
13	Apo-E	34	P02649
		36	P02649
14	Apo-F	35	Q13790

3.7.1.1 Bottom up Approach for HDL Apoproteins

Serum digest is subjected to SPE using the three polymeric anion exchangers. Figure 3.18a for poly(GMA/DVB); 3.18b for poly(GMA/EGMA) and 3.18c for poly(GPE/DVB) which include Apo A-I, Apo A-II, Apo C-I, Apo CIII, Apo-M, C3, C9, hemopexin, Apo-D, Apo-J, Apo-L, Kininogen-1, SAA and Apo-E. All identified content is shown in Table 3.5. More than one peptide is found for some apoproteins [35]. ApoB-100 is the LDL apoprotein identified by its characteristic peptides in the first fraction. The results confirm that ApoA-I, ApoA-II, ApoCII, ApoE, clusterin (Apo-J), and Apo-M are present in the lipoproteins. The results also reveal the association of HDL with complement factors C3, C9 in addition to the clusterin. The digested sample in lipoproteomics workflow has increased number of detected apoproteins as more sites are exposed for interaction.

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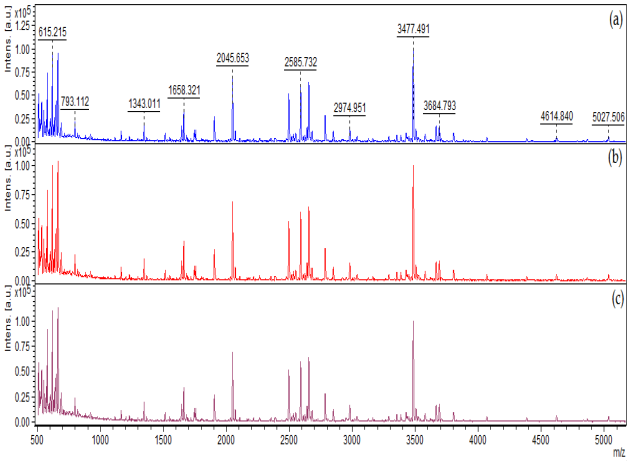

Figure 3.18: HDL peptides enriched by (a) poly(GMA/DVB), (b) poly(GMA/EGMA)and (C) poly(GPE/DVB) anion exchangers

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Table 3.5: Apoproteins, their masses and SwissProt accession No's enriched from digested serum using three anion exchangers

No.	Protein Name	Mass (Da)	Accession No.
1	Apo-D	505.037	P05090
2	TF	529.947	P02787
3	Apo-M	547.121	O95445
4	ApoA-IV	575.082	P06727
5	Kininogen-1	591.098	P01042
6	ApoA-I	615.127	P02647
7	Complement component C9	631.175	P02748
8	TF	663.518	P02787
9	SAA4	685.489	O95445
10	Hemopexin	795.336	P02790
11	Apo-E	817.351	P02649
12	ApoA-V	1160,756	Q6Q788
13	Albumin	1343,011	P02768
14	ApoB-100	1658,321	P04114
15	Apo(a)	1743,863	P08519
16	Alpha-1-antitrypsin	1753,799	P01009
17	Apo(a)	1900,790	P08519
18	Albumin	2045,653	P02768
19	C3	2491,522	P01024

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20	C4B	2780,044	P0C0L5
21	C3	2846,488	P01024
22	Apo-F	3478,908	Q13790
23	Apo-F	3688,389	Q13790

This confirms that serum sample contains LDL along with HDL lipoproteins. The digestion opens lipoproteins bulky molecule and generate peptides with attached PLs for better binding due to exposed interaction sites and reduce steric hinderance.

3.7.1.2 pH Optimization

The lipoproteins interact with N^+ sites of anion exchanger through negatively charged phosphate group of phospholipids. Weak hydrophobic/hydrophilic interactions can also develop between polymeric support material and lipoproteins. Such interactions can be reduced through binding and washing conditions. Phosphopeptides/proteins offer maximum interference due to similarity of affinity site and interaction chemistry via phosphate group. The background of phosphopeptide/protein is much lighter as compared to bulky lipoproteins which results that the former offer more strong binding and a shift to high pH is used for elution whereas in later case interactions between lipoproteins and anion exchanger are disrupted by eluent containing perchlorate as counter ion; a chaotropic ion [56]. It is reported that the binding of phosphopeptides/proteins is favorable at low pH whereas phospholipids have more than one negative charge at physiological pH 7.4. At pH 7.5, a single phosphopeptide is eluted along with lipoproteins while at pH 3 and 8, more non-specific peaks are observed (Figure 3.19).

It is customary to mention that at pH 3, the ionic strength of eluting buffer decreases due to release of protons (Tris HCl → H^+Tris and Cl^-) which neutralize the negative functional groups of proteins/peptides and thus the interaction is reduced causing elution. When pH is increased to 8, all the phosphate interaction with material decreases. Thus phosphopeptides get co-eluted with lipopeptides at pH 3 and 8 as shown in Figure 3.19 which is not the case at pH7.5.

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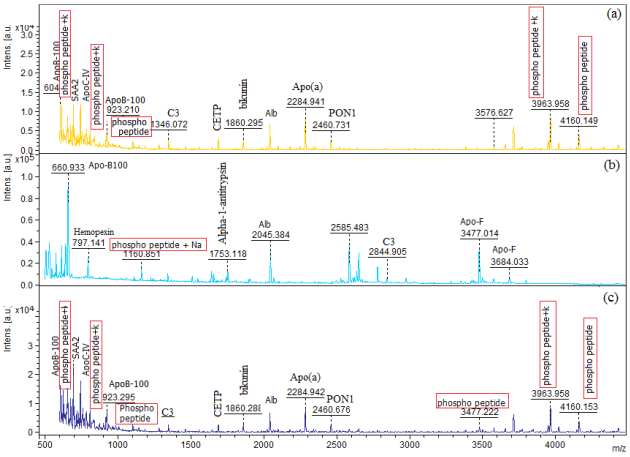

Figure 3.19: pH optimization for lipoproteins enrichment and elution using poly(GMA/DVB)anion exchanger at (a) pH 3, (b) pH 7.5 and (c) pH 8

3.7.1.3 Reproducibility

The developed workflow is repeated step by step using all three polymeric materials and reproducible results are obtained. Figure 3.20 (a1 and a2) depicts the batch-to-batch reproducibility for poly(GMA/EGDMA) used for serum samples following the similar protocol for both batches. Similar results are obtained for other two materials (data not shown) which confirm that the materials provide reproducible results. The significance of any workflow is enhanced if all the conducted steps show reproducibility too. Therefore the extracted lipoprotein fractions from batch 1 and 2 of poly(GMA/EGDMA) are further delipidated for apoproteins (Figure 3.20 b1and b2), apopeptides (Figure 3.20 c1 and c2) and lipids (Figure 3.20 d1 and d2). MS analysis of these fractions confirm the reproducibility of presented workflow.

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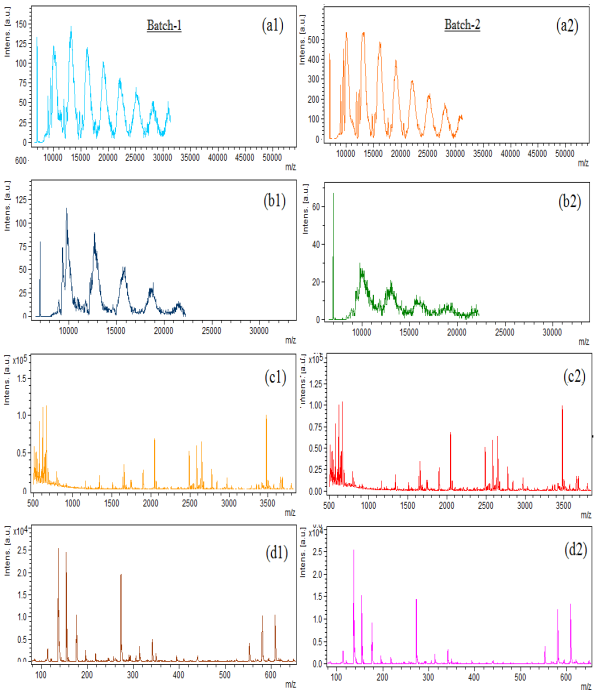

Figure 3.20:MS analysis for two batches (1 and 2) of poly(GMA/EGDMA) for lipoprotein fraction (a1 and a2) showing batch to batch reproducibility; HDL's apoproteins (b1 and b2), (c1 and c2), HDL fraction from digested serum (c1 and c2) and lipid fraction (d1 and d2) showing reproducibility of workflow.

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3.7.1.4 Lipids Profiling

Lipid classes of lipoproteins are key structural components. Phosphatidylcholine, cholesterol, lysophosphatidylcholine, sphingomyelin, cholesteryl ester, ceramide and phosphatidyl ethanolamine (PE) content has been analyzed in lipoproteins by the workflow. Sphingolipid (SP) and glycerol phospholipid (GP) species regulate the lipoprotein and cell function. Ischemic heart disease is inversely correlated with the abundance of ceramide [158]. Cardiolipin (CL), phosphatidylglycerol (PG), phosphatidylserine (PS) are difficult to detect because of their low abundance. Lipoproteins lipid fraction carries various fatty acid chains. Different lipid classes are identified from the digested and undigested serum samples at the stage of delipidation (Figure 3.21 and Figure 3.22). As digestion has no effect on lipid profile, the information is conserved in the designed workflow, either adopting top-down or bottom up approach.

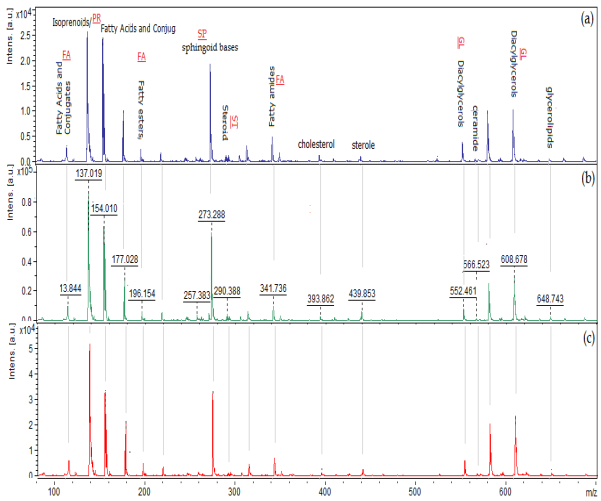

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Figure 3.21: Lipoprotein lipid profile from undigested serum using (a) poly(GMA/DVB), (b) poly(GMA/EGMA), and (c) poly(GPE/DVB) anion exchangers

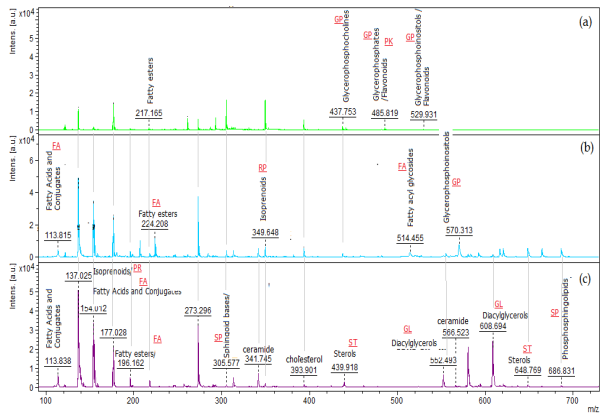

Figure 3.22: Lipoprotein lipid profile from digested serum using (a) poly(GMA/DVB), (b) poly(GMA/EGMA), and (c) poly(GPE/DVB) anion exchangers

3.7.2 MALDI-MS Analysis of Enriched Lipoprotein by IMAC/MOX Affinities

It is difficult to enrich intact proteins due to their large size. IMAC affinity shows high interaction for lipoproteins/peptides through metal cations at set pH. A rapid, sensitive, and selective method for isolating lipoproteins from biological sample is thus carried out (Figure 2.23).