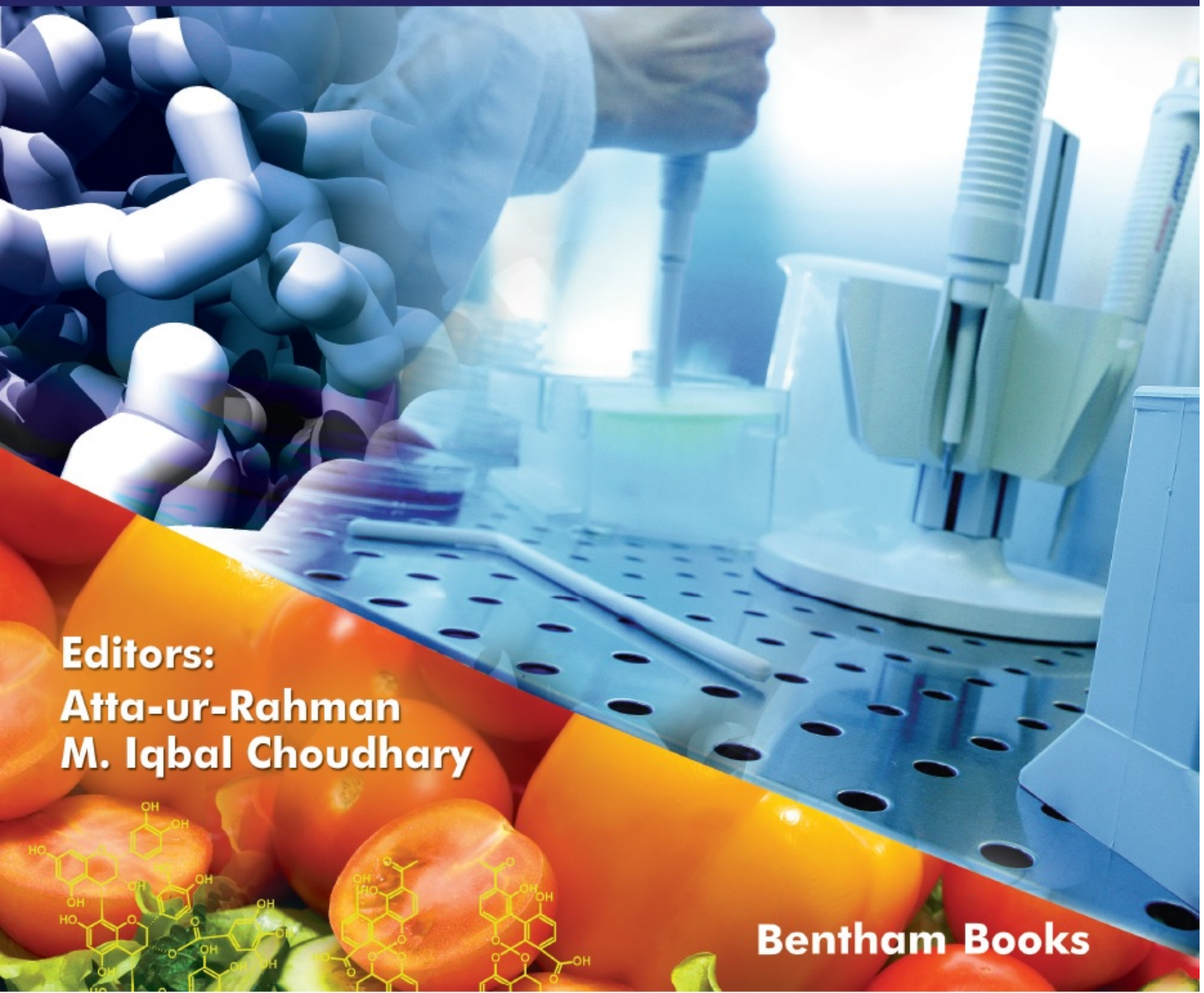
APPLICATIONS OF NMR SPECTROSCOPY



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Applications of NMR Spectroscopy

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CONTENTS

PREFACE

Nuclear Magnetic Resonance (NMR) spectroscopy has emerged as one of the most powerful techniques for the identification of materials, and for the study of their dynamic properties. As a result, the technique has found tremendous uses in almost all fields of physical, natural, and health sciences.

Volume 8 of the book series entitled ***Applications of NMR Spectroscopy*** is mainly focussed on the practical uses of NMR spectroscopy in solving various key problems in biomedical, health, and food sciences. The contents include NMR based analysis of common sugars, plant based constituents, nucleic acids and proteins, as well as NMR- based metabolomics and MRI for the diagnosis of chronic and acute health disorders.

The review contributed by Yang *et al*. provides an overview of the use of quantitative NMR (qNMR) techniques for the analysis of six common sugars in complex food matrices, after derivatization with naphthimidazole (NAIM). Coffee plants contain many constituents which effect cognitive functions. Valderrama *et al*. have analysed the constituents of various coffee types, and correlated them through the use of the psychological attention test. Evran *et al*. have reviewed the recent literature on the NMR-based structures of aptamers (single stranded DNA and RNA molecules) selected via an iterative process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX). The applications of NMR for the identification of the ligand binding mechanisms are discussed. The review contributed by Ibrahim and Gopalsamy describes various NMR techniques used in metabolomics-based diagnosis of common diseases. The next chapter by Asmaa and Mansour provides a critical analysis of various MRI-based diagnostic approaches to study diverse human cancers. Proteins are fascinating molecules, both because of their complex structures and their interactions with other biomolecules. NMR techniques have evolved over the years to determine the structures and functions of protein molecules. This is the key theme of chapter 6 by Bashir and Rashid.

We wish to thank all the eminent scientists for their scholarly contributions. The editorial team of Bentham Science Publishers, particularly Ms. Fariya Zulfiqar (Manager Publications) and team leader Mr. Mahmood Alam (Director Publications), deserves our deepest appreciation for compiling an excellent volume in a time efficient manner. We are confident that like the previous volumes of this book series, the current treatise will also receive wide appreciation for both the readers and practitioners of NMR spectroscopy.

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CHAPTER 1

qNMR as a Tool for Determination of Six Common Sugars in Foods

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Abstract: Nuclear magnetic resonance (NMR) spectroscopy is capable of quantifying molecules. The term so called quantitative NMR (qNMR), has been used for determination of the concentration and purity of small molecules. Carbohydrates are found in various beverages and dietary foods, including crops, milk, fruits, and vegetables. Commercial products frequently use "added sugar" in soft drinks, cookies, candies, and foods. The added sugar in beverages can be sucrose, high-fructose corn syrup (HFCS) and glucose. Here, we report a quantitative method to measure 6 common sugar ingredients in foods from a single one-dimensional ^{1}H-NMR and by using naphthimidazole (NAIM) derived sugars, which are chemically tagging aldoses with 2,3-naphthalenediamine (NADA) at the reducing ends to assist assignment of sugars. The aldoses in native sugars contain α and β anomeric isomers, and may have overlapping signals in ^{1}H-NMR spectra. In contrast, both the anomeric isomers can be converted into a single sugar-NAIM derivative, which resolves the problem of overlapping signals to simplify the NMR quantitative analysis. This NAIM method is especially useful for identification and quantification of multiple kinds of sugars in beverages and foods. This study is to facilitate the quantification of six common sugars in beverages and foods. Our results suggest that a simple treatment of beverage and food with the NAIM labeling method provides a more extensive success rate for the quantification of sugar ingredients.

Keywords: Beverage, Food, Fructose, Galactose, Glucose, Lactose, Maltose, Naphthimidazole (NAIM), q-NMR, Quantitative analysis, Sugar, Sucrose, ^{1}H-NMR spectrometry.

INTRODUCTION

Carbohydrates are found in various beverages and dietary foods, including rice, noodles, bread, meat, milk, fruit, vegetables, and drink [1, 2]. Carbohydrates are

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also used as “added sugar” in soft-drinks, cookies, candies, and many kinds of foods. For example, the added sugar in beverages can be sucrose, fructose, glucose, maltose and other sweeteners. Though carbohydrates are needed for living, an excessive uptake of sugar may induce health problems such as decayed teeth and chronic diseases [3 - 5]. In addition, foods of low glycemic index (GI) are suggested for diabetic patients. It is important to know the content and quantity of sugar in foods. Thus, developing a rapid and convenient qualitative/quantitative method for sugar measurement in foods is needed. Furthermore, many countries have introduced the sugar tax and soft-drink tax in order to reduce sugar consumption [6]. Therefore, a suitable method to verify the sugar content in foods can be provided to the government for policy implementation. The appropriate “fine sugar” or “added sugar” intake is 25 grams per day according to the scientific recommendation by the World Health Organization (WHO) [7]. Since August 2015, Taiwan Food & Drug Administration (TFDA) has proposed to regulate common sugars in foods, including glucose (Glc), galactose (Gal), fructose (Fru), lactose (Lac), maltose (Mal), and sucrose (Suc). The amounts of sugars must be labeled in the “Nutrition Facts Panel” for the products of beverages and foods. Even though the information of sugar content surely benefits consumers, this regulation will impose challenges to the food industry concerning the identification and quantification of the six common sugars in beverages and foods.

At present, high performance liquid chromatography (HPLC) and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) are more common instrumental methods for sugar determination in foods. NMR spectroscopy is also a powerful method for identification and quantification of low molecular weight compounds. Though ^{1}H-NMR spectra are commonly used in the routine quantitative analysis of individual sugars [8, 9], using NMR to identify each sugar in a mixture and simultaneously quantify its content is still challenging because the spectrum is usually complicated by the existence of anomeric isomers and by the similar structures of sugar components. The quantitative NMR (qNMR) technique is designed for determination of the concentration and purity of small molecules [10]. qNMR can be applied for direct quantification of multiple components in a mixture without pretreatment of sample. However, recording a qNMR spectrum would take a much longer acquisition time than a routine ^{1}H-NMR spectrum. In another approach, we performed a simple treatment on beverages and foods with a naphthimidazole (NAIM) labeling kit to provide the sugar-NAIM derivatives for quantification by ^{1}H-NMR spectral analysis. This method combining NAIM derivatization and NMR analysis is successfully applied to the measurement of six common sugars in foods. Our objective is to establish a convenient method for profiling and quantifying sugar ingredients in beverages and foods by using one-dimensional

^{1}H-NMR spectroscopy *via* a simple treatment with NAIM labeling kit.

RESULTS

Workflow 1: Measurement of 6 Common Sugars in Foods

Using ^{1}H-NMR for six common sugars (Glc, Gal, Fru, Suc, Mal, and Lac), the identification process was followed stepwise by sample preparation, NMR processing and statistical analysis. Fig. (**1**) shows the flowchart.

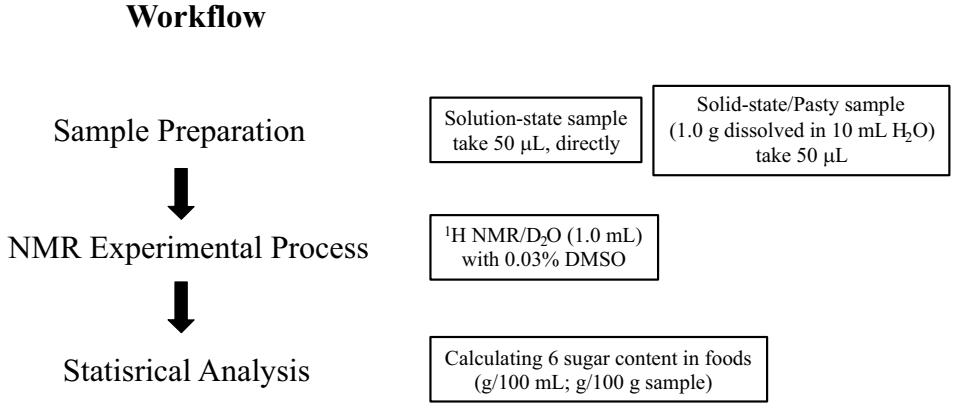

Fig. (1). Workflow of using ^{1}H-NMR for determination of six common sugars.

Sample Preparation

Six standard sugar solutions (Glc, Gal, Fru, Suc, Mal, and Lac) were prepared in varied concentrations using 5.0, 2.5, 1.25 and 0.25 mg, respectively. The samples of beverage and food in solution-state were ready for determination without pretreatment or separation. A less than 50 μL of sample solution was directly taken to reduce the absorption of H_2O (at 4.8 ppm) in ^{1}H-NMR spectra. For solid-state or paste samples, 1.0 gram was dissolved in 10.0 mL of H_2O, and 50 μL of the solution was taken for measurement. Sample solution was concentrated in vacuum (3 min), and then deuterium solution was added for NMR experiment.

NMR Experimental Process

The deuteriated water (D_2O, 99.9%, Sigma Aldrich, USA) 1.0 mL with 0.03 mol% of dimethylsulfoxide (DMSO, 99.9%, extra dry, H_2O < 50 ppm, Acros, New Jersey, USA) as internal standard was added to a dried sample in 5 mm NMR tube for recording the ^{1}H-NMR spectrum. The ^{1}H-NMR spectra were recorded on a Bruker AV600 MHz NMR spectrometer (Bruker BioSpin GmbH,

CHAPTER 2

Correlation Between VIP Scores and ^{1}H NMR to Extract Information of Psychological Attention Tests Applied Before and After Coffee Intake

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Abstract: This chapter presents the correlation between coffee compounds identified by ^{1}H Nuclear Magnetic Resonance (^{1}H NMR) spectroscopy with psychological attention tests in order to verify which compounds are related to the focus and/or diffuse attention. Psychometric tests applied by a clinical psychologist, before and after coffee intake, were the focus attention AC-vector and the diffuse attention TADIM, and the focus attention TACOM-B and the diffuse attention TEDIF, respectively. Different tests to measure the attention before and after coffee intake were used to avoid learning effects. After AC-vector and TADIM tests, each volunteer consumed a total of 40 mL of coffee with different cup qualities (four different coffee blends – 10 mL per beverage) and indicated the order of preference in relation to the smell. This approach was used to create a greater metabolic variation between the samples tested, allowing to build a robust chemometric model. For each preferred coffee, a ^{1}H NMR spectrum was obtained and a chemometric data treatment based on Partial Least Squares (PLS) regression and Variable Importance in Projection scores (VIP scores) was used to correlate the spectra with the psychological test results and to verify which metabolites of the coffee beverage could be related to the focus or diffuse attention. In general, our results showed that coffee intake attenuated diffuse attention and improved focus attention in most volunteers. The major metabolites that contributed to both diffuse and focus attention were caffeine, trigonelline, chlorogenic acids, acetate, lipids, lactate, γ-quinide, and polysaccharides. Among metabolites exclusively important to focus attention, formate, choline, myo-inositol, citrate, and malate were the most important. Therefore, the ^{1}H NMR profile, in combination with chemometric tools, is interesting to assess the correlation between coffee compounds and human attention.

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Keywords: Attention Performance, Chemometrics, Coffee, Coffee compounds, Coffee ingestion, Coffee smell, Cup quality, Metabolomics, NMR spectroscopy, Pilot Study, PLS Regression, Psychometric Tests, VIP scores.

INTRODUCTION

Coffee is globally one of the most widely-consumed beverages that contain over 1000 compounds responsible for its pleasant flavor and aroma [1]. Coffee is known for its stimulant, beneficial and nutritional properties leading researchers to seek to understand how these properties are correlated to their chemical composition. For example, coffee drinking habits have been associated with a decrease in the risk of developing Alzheimer's and Parkinson's disease [2], and a decrease in the incidence of cardiovascular disease and type 2 diabetes mellitus [3 - 5]. Recently, it was shown that the risk of Alzheimer's disease was lower in those who regularly consume coffee than those who do not drink it [6].

The bioactivities related to coffee consumption are related to several compounds. Of these, caffeine (1,3,7-trimethylxanthine) is the most widely studied. Caffeine is a psychoactive and neurostimulator substance that exerts most of its biological effects as an adenosine receptor antagonist, inducing a generally stimulating effect in the central nervous system [7, 8]. Studies have demonstrated the role of caffeine in improving cognitive skills, such as improving attention, increasing alertness rates, reducing tiredness and sleep duration [9, 10]. The amount of caffeine normally found in a cup of coffee can produce psychostimulant effects and increase the performance of individuals in clinical behavioral tasks [11]. However, there are hundreds of compounds in coffee, several of which with potential to contribute to coffee bioactivities directly or indirectly, for instance, some of them by interaction with caffeine [8, 9, 12].

The international coffee trade is concerned with only two coffee species: *Coffea arabica* L. and *Coffea canephora*. Both species proved to be sources of biologically active compounds, such as nicotinic acid, trigonelline, quinolinic acid, tannic acid, pyrogallic acid, chlorogenic acids and especially caffeine [10]. Considering this, and the fact that several coffee metabolites can act on the central nervous system or potentiate the effect of caffeine, it is extremely important to develop tools to gain a complete picture of the metabolites present in the whole biological matrix, and understand which compounds in the beverage are directly related to focus and diffuse attention when performing psychological tests.

Psychological attention tests are a way of examining the level of human attention. They are used in several situations, such as psychological diagnosis assessments, personnel selection, driving permission, and to assess information processing speed. Psychometric tests to check focus and diffuse attention are the most used in

these cases. Focus attention is defined as the ability to select a source of information from all available at a given time and to be able to direct the attention (focus) to stimulus or tasks to be performed over time [13]. Diffuse attention is the mental function that focuses at the same time on various spatially dispersed stimuli, performing a quick collection of information and providing instant knowledge to the individual. Diffuse attention aims to investigate, evaluate and observe how quickly or slowly a person can discriminate against dispersed stimuli, such as a driver who drives on highways [14].

To understand which metabolites of coffee beverage can be related to human attention, a systematic method involving a wide variety of metabolites (metabolomic profiling) could be a very useful contribution. Nuclear Magnetic Resonance (NMR) spectroscopy is the analytical technique that can provide the most complete "metabolome" profile in a single analysis. ^{1}H NMR spectroscopy is rapid, reproducible, and stable over time, requiring a very simple sample preparation, and provides both qualitative and quantitative information about the metabolites present in a sample [15, 16]. Several studies have been carried out showing the application of ^{1}H NMR for the analysis of green and/or roasted coffee beans. For instance, this technique has been used to monitor changes in the composition of coffee during roasting [17, 18], to check adulteration in roasted coffee using corn, coffee husks, barley, and soybean [15], to discriminate coffee beans from different geographical origins [19], to differentiate coffee from different production systems [20], to evaluate the quality of green coffee or coffee beverages [17, 21], and to evaluate the anti-amyloidogenic properties of coffee and its constituents [22].

^{1}H NMR spectroscopy can provide the "metabolome", that is a chemical profile or fingerprint of whole tissues. Today, metabolomics constitutes a potent approach for the investigation and discovery of biomarkers in a large diversity of research domains [16, 22]. In this sense, NMR spectroscopy is a powerful tool capable of detecting a range of different types of metabolites simultaneously, providing valuable structural information with high reproducibility, although with low sensitivity (sub-millimolar concentrations) [23]. The fact that NMR has been routinely used for classical metabolic studies to characterize complex metabolite mixtures, has, in fact, made NMR the preferred technology in the field of metabolomics [24]. Thousands of metabolites in a single analysis are simultaneously monitored. The extraction of meaningful information from these large and complex datasets requires strategies, such as chemometrics tools that become essential for knowledge discovery in metabolomics [22]. In view of the above, in this study, we report the application of ^{1}H NMR coupled to PLS regression and VIP scores to identify the coffee metabolites potentially related to the human focus and diffuse attention. The success of a metabolomics approach

CHAPTER 3

NMR Spectroscopy for Probing the Structural Determinants of Aptamer Optimization and Riboswitch Engineering

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Abstract: Nucleic acid aptamers are single-stranded DNA or RNA molecules that can fold into unique conformations and specifically recognize various targets, such as small molecules, proteins, cells, and tissues. Aptamers are selected *in vitro* through an iterative process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX). As aptamers possess several advantages over antibodies, several diagnostic and therapeutic applications have emerged in recent years. Aptamers also attract interest as they form the receptor domain of RNA-based riboswitches that function as natural modulators of gene expression. Aptamer domain of riboswitch can sense the metabolite and this binding event is transduced into a conformational change, thereby transcriptional or translational control is achieved. Riboswitch engineering has gained importance due to the potential use of artificial riboswitches in biosensors and next-generation therapeutics. Therefore, understanding the structural basis of ligand binding and conformational change is critical for the success of optimization or re-engineering of aptamers. Since crystallization of aptamer-small molecule target complexes is particularly difficult, NMR provides an indispensable tool for structural analysis. In this chapter, we first give a brief information about aptamers and riboswitches. Then, we review the NMR structures of aptamers and riboswitches reported to date. We highlight the importance of NMR for identification of ligand binding mechanism, post-SELEX optimization of aptamers, as well as for the design of artificial riboswitches. In this context, we also give some examples of aptamer studies involving a combination of NMR and other techniques.

Keywords: Aptamer, Aptamer-ligand interaction, NMR-guided design, Riboswitch.

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INTRODUCTION

Selection of Aptamers

Aptamers are single-stranded DNA or RNA oligonucleotides that can bind to their targets with high affinity and specificity due to their ability to form specific three-dimensional structures [1]. Aptamers are selected by using an *in vitro* process called SELEX (Systematic Evolution of Ligands by EXponential Enrichment) [2, 3]. The SELEX process consists of three basic steps: binding, elution and amplification (Fig. **1**). In the first step, the target of interest is incubated with initial DNA or RNA library. Initial library of a typical SELEX process consists of about 10^{13} to 10^{15} different sequences. The library is composed of chemically synthesized oligonucleotides that contain a random region of 20 to 100 nucleotides, which are flanked by specific primer binding sites at the 5' and 3' ends. Following incubation of the target and the library at pre-defined conditions, unbound sequences are removed by washing with buffer. The binding sequences are then amplified by polymerase chain reaction (PCR) *via* the primer binding sites. With repetitive cycles of binding, elution and amplification, the initial random pool of oligonucleotides is reduced to the enriched sequences that show the highest affinity and specificity to the target molecule. Binding properties of the selected aptamers depend on the molecular structure of the target, design of the initial random library, selection conditions, and the ratio of the target to the library. Typically, gradually increasing stringent conditions are applied to obtain aptamers with high affinity and specificity. The stringency can be achieved by reducing target concentration, increasing the number of wash steps, or by decreasing incubation time. Enrichment of high-affinity sequences indicates that the SELEX process can be finalized. The enriched pool is then sequenced and the aptamer candidates are chemically synthesized for further characterization of binding properties.

Aptamers bind to their targets through non-covalent interactions such as van der Waals forces, hydrogen bonding and electrostatic interactions [4]. Binding affinity and specificity of aptamers are comparable to antibodies. Moreover, aptamers are superior to antibodies due to their small size, stability, low cost, ease of chemical synthesis and unlimited target range [5]. Aptamers targeting small molecules, metal ions, peptides, proteins and cells can be developed *in vitro* by excluding the need for living systems. With these properties, aptamers have enormous potential to be used in therapeutics and diagnostics [6, 7]. Aptamers can be designed for many different purposes, such as modulating the immune system, inhibiting enzyme activity, drug transport, and blocking receptor binding [8].

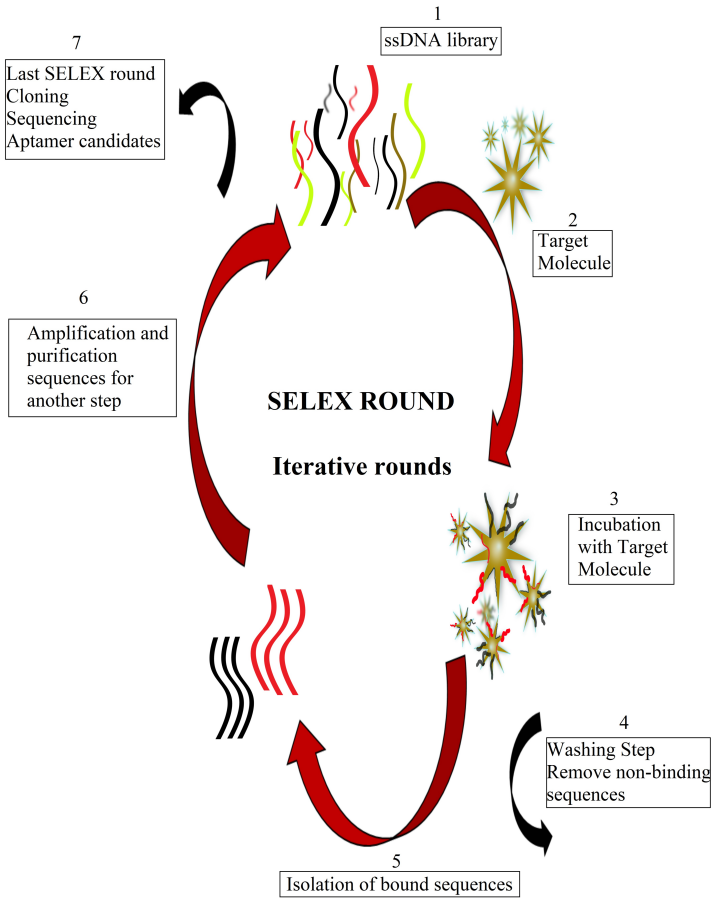

Fig. (1). Schematic representation of SELEX.

Post-SELEX Modifications of Aptamers

Therapeutic application of aptamers is usually limited by short half-lives due to rapid degradation by nucleases. For instance, the half-life of a 16-mer oligonucleotide in rat plasma is less than 1 minute [9]. The oligonucleotide that is promising as an anticoagulant has a limited half-life of 108 seconds [10]. Hence, post-SELEX optimization is a powerful approach to overcome therapeutic limitations since it allows modifications to the aptamer structure to improve stability and binding properties [11 - 16].

Truncation is one of the post-SELEX optimization strategies that relies on shortening the aptamer by removing the nucleotides that are not involved in target binding. The constant primer binding sites of aptamers were shown to contribute

CHAPTER 4

Applications of NMR Spectroscopy in Medical Diagnosis

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Abstract: Nuclear magnetic resonance (NMR) is a special branch of spectroscopy which exploits the magnetic properties of atomic nuclei for molecular elucidation and identification. A technique that was initially developed to analyze chemical and physical molecular structure is now widely used in medical diagnosis. The non-invasiveness, non-destructiveness and simplicity of sample preparation make NMR the preferred technique for metabolomics study. Various body fluids such as urine, saliva, blood, plasma, serum and sweat have been explored to identify potential biomarkers of diseases. Psychiatric disorders, specifically alcohol-use disorder and neurological disorders such as Parkinson's disease, have been investigated with the aid of NMR spectroscopy. Cancer has been one of the most widely studied areas and the research also includes determination of biomarkers which not only could detect the presence of cancer but also potentially predict the various cancer processes in cancer cell lines. Infectious diseases including the compounds produced by the microorganisms such as in tuberculosis and pneumonia have also been explored. Besides, NMR metabolomics has also been used to establish a metabolic fingerprint for risk stratification and early detection of cardiovascular disease (CVD). The samples of subjects with the diseases were collected and the metabolites were compared against controls such as healthy individuals using complex chemometrics and multivariate data analysis such as principal component analysis, partial least square and orthogonal partial least square analyses to distinguish the potential biomarkers. In terms of the various uses of NMR metabolomics in the subject of diagnostic medicine, more improvements to overcome the analytical limitations are expected, making it one of the most notable diagnostic tools of the future. This chapter reviewed some of the published articles in cancer, psychiatric and neurological diseases to provide examples of using NMR spectroscopy in diagnosing human disorders.

Keywords: Cancer, Metabolomics, Neurological disorders, Nuclear Magnetic Resonance Spectroscopy, Psychiatric disorders.

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INTRODUCTION

Since ancient times, humans have utilized urine, saliva and other bodily fluids for the identification of various ailments. The advancement and utilization of analytical techniques for the evaluation of these biofluids have brought about the discovery of various disease biomarkers [1]. The integration of NMR, mass spectrometry (MS) and multivariate statistical techniques became the cornerstone for metabolomics-based disease diagnosis [2]. Metabolomics can be described as an in-depth study of chemical processes involving metabolites in a biological system [3]. Another terminology which is frequently used interchangeably with metabolomics is metabonomics. Metabonomics is defined as the quantitative measurement of metabolic responses of living systems against time to pathophysiological stimuli or genetic modification [4]. The primary objective of NMR-based medical diagnosis is to identify metabolites that precisely correspond to a particular disease for the early detection and treatment of said illness. In this chapter, we will analyze recent publications and highlight the advancements in experimental techniques, sample preparation, discovery and quantification of metabolites using chemometric tools used to identify biomarkers through NMR. One study for each disease will be reviewed in detail to explain this technique.

WORKING PRINCIPLE OF NMR IN MEDICAL DIAGNOSIS

The principle behind NMR is that the nuclei in atoms are charged and hence is detectable by NMR, due to the formation of magnetic dipoles. When an external magnetic field is applied through NMR spectrometer, the base energy is shifted to a higher energy level. The energy transfer produces a wavelength that is measured and processed to produce NMR spectrum for the particular nucleus. With the help of chemometrics software, the area under the curve or peak height/intensity of the spectra can be calculated and used to identify significant/important compounds of diseases. This approach is known as metabolomics. The two main system used in metabolomics study are Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). They both have different advantages and setbacks but both techniques are able to provide complementary information. The MS is usually combined with liquid chromatography (LC) or gas chromatography (GC) and has higher sensitivity compared to NMR. Thus, it is the preferred choice for metabolomics studies where a particular group of compounds are being targeted such as lipid compounds [5]. Nevertheless, MS sample preparation is extensive. It usually involves many steps such as solvent extraction, ultrafiltration, solid-phase extraction and a chemical derivatization. The presence of other chemical species may influence matrix effects, ionization suppression and enhancement and cause inconsistent results. Furthermore, samples are destroyed in the process [6, 7].

Meanwhile, NMR is fast and does not require tedious sample processing [8]. The samples are also preserved thus can be stored and re-run for further analysis. Newer NMR machines allow automation and can run samples in large quantities [9]. It is also non-selective thus is a preferred choice for bulk analysis in metabolomics studies to identify discriminating metabolites without any prior knowledge [10]. In addition to that, NMR provides insight to the molecular dynamics and mobility of a particular metabolite [11]. One major drawback of NMR is its sensitivity and resolution which is lower compared to MS [12]. However, newer NMR is continuously being developed with higher sensitivity and resolution.

Besides *in-vitro* NMR spectroscopy, *in-vivo* magnetic resonance spectroscopy is also a non-invasive method which can complement the magnetic resonance imaging (MRI) in the characterization of tissue and can be used to study metabolic changes in brain related disorders such as stroke, depression, tumors, dementia and seizures. In comparison, traditional investigational techniques such as biopsy is more invasive and has more risks and side effects. In this chapter, selected studies of NMR-based metabolomics applications with reference to specific diseases are discussed.

NMR in the Diagnosis of Lung Cancer

Background of Lung Cancer

Lung cancer poses a serious health burden in most developed nations and it is estimated that there will be roughly 228,150 new cases of lung and bronchus cancer in 2019 [13]. Recent studies suggest that there may be a strong association between inherited genes and development of lung cancer. Nevertheless, there are very few genes that have been associated to lung cancer hitherto [14]. Besides hereditary factor, smoking has been shown to be one of the main risk factors for lung cancer [15]. Similar to other cancers, pathogenesis of lung cancer is induced through carcinogens, followed by a period of promotion and progression in a multistep process [16, 17]. Even though cancer risk may decrease after smoking cessation, another carcinogen may still carry on the process [18]. Table **1** shows the classification of lung cancers and its features.

Early detection is imperative to increase patient survival in lung cancer; nonetheless, available diagnostic techniques are insufficient. Diagnostic work-up for lung cancer still relies heavily on clinical perspectives and no single clinically based algorithm can be applied to all the cases [1, 19]. Definitive diagnosis of lung cancer is primarily based on the histopathological analysis of the lung cells. Due to the lack of screening tests and the onset of tumor growth generally do not display any signs or symptoms, diagnosis is frequently deferred. Therefore, to

CHAPTER 5

Applications of NMR Spectroscopy in Cancer Diagnosis

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Abstract: Cancer is a category of diseases characterized by uncontrolled cell growth and high potential to disseminate to other parts of the body. Cancer diagnosis is challenging due to the high structure similarity between normal and cancerous cells and the aggressive diagnostic procedures. Early diagnosis of cancer is crucial to increase the remission probability and avoid complications. A number of techniques have been involved in cancer diagnosis including biopsy, laboratory tests, computerized tomography (CT) scan, Ultrasonography, X-ray imaging, and nuclear magnetic resonance (NMR) spectroscopy. NMR has been applied both *in vivo* (known as magnetic resonance imaging) and *in vitro* to aid in cancer diagnosis. This chapter discusses the application of *in vitro* NMR in diagnosis and prognosis of different types of cancer with emphasis on the metabolic alterations at early stages of malignancy. The signature metabolites of brain, breast, epithelial ovarian, prostate, lung, colorectal, bladder, and oral cancers have been presented. A perspective overview of the role of NMR spectroscopy in cancer diagnosis has also been presented. This chapter shed the light on the important role of NMR spectroscopy in cancer diagnosis and treatment follow up. The applications introduced are not meant to provide a complete list of existing studies, but to present a wide overview of the current progress in this field. The chapter will cover the following topics:

Keywords: Applications, Bladder cancer, Brain cancer, Breast cancer, Cancer diagnosis, Colorectal cancer, Epithelial ovarian cancer, Lung cancer, Nuclear magnetic resonance (NMR) spectroscopy, Oral cancer, Perspective, Prostate cancer, Technical aspects.

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INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is a powerful analytical technique for both identification and quantification of analytes in solutions as well as in solid states [1]. NMR phenomenon was discovered in 1940s [2] and since then, there has been a rapid progress with regard to both method development and applications, expanding from physics to chemistry, biochemistry, pharmacy, physiology, food science, biology, and medicine [3].

In the field of medical diagnosis, NMR spectroscopy provides a non-invasive metabolic window on the biochemical processes within the body [4]. Its use is no longer restricted to research to investigate pathophysiological processes, but extends to drug assessment, personalized medicine as well as biochemical characterization and diagnosis of diseases [5]. The use of NMR-based metabolomics to aid in human disease diagnosis would give a more complete picture as it reflects the integrated functions of organs [6]. Furthermore, metabolic changes can be detected in biological fluids using NMR spectroscopy before the clinical symptoms develop, generating useful fingerprints for early diagnosis of diseases [7].

NMR spectroscopy would also help in the challenge of cancer diagnosis, especially in brain tumor, by providing another non-invasive approach besides clinical history and radiological examination [8]. The additional metabolic information provided by NMR spectroscopy can help making clinical decisions about cancer patient management without surgical diagnostic procedure [9]. NMR spectroscopy also has a great impact on metabolite-based discovery of diagnostic and prognostic biomarkers of several human diseases [10]. More sensitive boimarkers are urgently needed because traditional biomarkers of diseases are not sensitive enough and only increase after the presence of substantial diseases [6].

The use of NMR spectroscopy for medical diagnosis can be conducted both *in vitro* and *in vivo*. The biomedical applications of i*n vitro* NMR include the analysis of body fluids (such as plasma or urine), extracts of tissue or small biopsy-sized specimens of intact tissues [11]. On the other hand, *in vivo* NMR spectroscopy, commonly known as magnetic resonance spectroscopy (MRS), can be done on the whole-body using a clinical magnetic resonance imaging (MRI) scanner, as an adjunct to standard examination, to obtain metabolic and functional information complementary to anatomical changes [12].

This chapter focuses on the recent applications of NMR spectroscopy in medical diagnosis and how it could offer the potential for a holistic approach to clinical medicine *via* improving disease diagnosis, biomarkers discovery as well as understanding disease mechanisms. The selected applications provide a wide

overview of the current progress in this field, and the future trends.

OVERVIEW OF NMR SPECTROSCOPY

Since NMR was first described in 1938 by Isidor Rabi, the applications and the number of publications are steadily growing [13]. Fig. (**1A**) shows the total number of publications (Journal articles, book chapters, patents, conference abstracts,) with the key word "NMR" using the Semantic Scholar search engine in the last two decades. NMR is one of the most widely used techniques in metabolomic studies (Fig. **1B**). The principle of NMR spectroscopy has been discussed in a number of text books [14, 15]. In this section, the types of NMR spectroscopy used in cancer diagnosis and the pros and cons of the technique will be discussed.

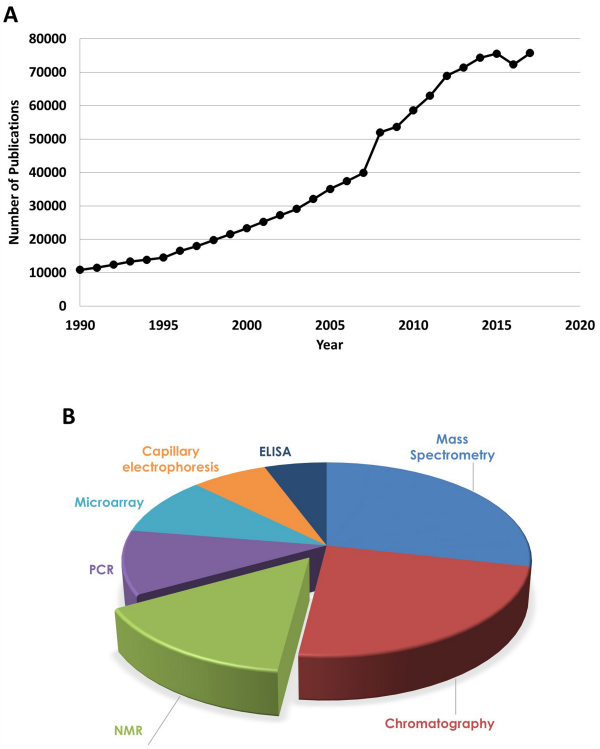

Fig. (1). A) The number of publications with keyword "NMR" in the last 20 years. **B)** A pie chart for the different techniques used in metabolomics.

Types of NMR Spectroscopy Used in Cancer Diagnosis

NMR can be classified according to the number of atoms ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P, *etc*,

CHAPTER 6

NMR as a Tool for Exploring Protein Interactions and Dynamics

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Abstract: Proteins are vital players that mediate a vast majority of cellular functions. NMR spectroscopy originally developed by physicists for investigation of nuclear properties, now represents highest applications in chemistry and biochemistry. NMR has been extensively utilized by structural biologists for exploring protein-ligand interactions and by medicinal chemists for drug discovery. The ligands investigated involved small organic molecules, peptides, proteins and nucleic acids. Recently, there has been increasing interest in the dynamic studies of these protein-ligand interactions. These applications are provided by a multitude of NMR experiments ranging from the simple one-dimensional ^{1}H spectrum to complex multidimensional NMR approaches. Chemical shift perturbation analysis allows for delineation of the binding interface, determination of the dissociation constants and estimation of ligand binding kinetics. Paramagnetic Relaxation Enhancement NMR spectroscopy has been widely used to visualize the weakly populated states and describes the process of protein complex formation. These approaches have been demonstrated for substrate binding, allostery, state equilibria and macromolecular self-association. NMR spectroscopy allows for characterization of minor conformational dynamic differences in structurally similar proteins. Target Immobilized NMR screening represents another approach to drug discovery that allows ligand screening for challenging targets. NMR spectroscopy can also be applied in combination with other techniques including X-ray crystallography and various computational methods to achieve greater coverage than any of the individual methods. This chapter is focused on the applications of NMR in exploring protein-ligand interactions and dynamics.

Keywords: Chemical shift perturbation, Encounter complex, Ligand binding, NMR, Paramagnetic relaxation enhancement, Protein dynamics, Protein-ligand interaction, Spectroscopy, Specific complex, Target immobilized NMR screening.

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INTRODUCTION

Protein-ligand interactions are vital for the maintenance and proper functioning of biological systems. A variety of cellular processes are carried out by proteins through interactions with multiple ligands including other proteins, small molecules and nucleic acids. These processes include signal transduction [1, 2], electron transport [3, 4], cellular metabolism [5, 6], muscle contraction [7, 8], membrane transport [9, 10], gene expression by transcription factors [11, 12], regulation of cytoskeleton [13, 14], enzymatic reactions and enzyme inhibition by intracellular inhibitors [15 - 18]. Abnormality in these interactions can lead to diseases like cancer, Alzheimer's and Creutzfeldt-Jakob disease [19, 20]. Protein-ligand interactions are the physical events, directed by the biochemical events of electrostatic forces, hydrophobic effect, hydrogen bonding, van der Waals and pi interactions. The affinity of protein-ligand interaction is a thermodynamic property described by the dissociation constant (K_d), which is ratio of the individual rate constants of dissociation (k_{off}) and association (k_{on}). The K_d values can range from 10^{-2} M to 10^{-16} M [21, 22]. Depending on the function performed, the protein-ligand interactions are tuned in terms of the strength, specificity and life time of the final complex. On one hand are the specific and static complexes of antigens and antibodies as well as enzymes and their inhibitors. Such proteins have single partners and avoid interactions with other cellular components. In such cases, strong binding is essential to lock the complexes in a single, well-defined orientation. These complexes are characterized by their low dissociation constant (10^{-15} M to 10^{-16} M), high binding energy (up to −21 kcal/mol) and long life-times even up to several days [23]. On the other hand are the transient, weak complexes involved in signal transduction and electron transport. Such events require a high turnover and fast association/dissociation of the partners. Proteins involved in these interactions may recognize multiple ligands, and a high specificity in these complexes is avoided to gain a rapid dissociation. Such complexes have high dissociation constants (μM to mM), low binding energies and short life times of millisecond time scale [24].

The study of protein-ligand interactions is important for the understanding of mechanisms underlying the cellular processes and for drug development. Protein-ligand interactions have been studied at increasing pace by a wide range of experimental techniques [25] including UV-Visible spectroscopy [26], analytical ultracentrifugation [27], microscale thermophoresis [28], surface plasmon resonance [29], isothermal titration calorimetry [30], circular dichroism [31], dynamic light scattering [32], atomic force microscopy [33], mass spectrometry [34], differential scanning fluorimetry [35], small angle X-ray scattering [36], fluorescence microscopy [37], quartz crystal microbalance [38] and NMR [39]. These methods provide information on multiple aspects of protein-ligand

interactions including association, dissociation, conformational changes on binding, kinetic and thermodynamics parameters, with some limitations associated with each technique. It has become increasingly recognized that proteins and ligands do not behave as static objects in solution, rather they are dynamic bodies [40, 41]. There are different kinds of fluctuations, transitions, conformational changes, movements, bond vibrations and rotations going on. They correspond to the local fluctuations of chemical bonds, regional flexibility of residues relative to each other and global movements of protein domains. It also encompasses relative movements of proteins or ligands on or around the binding interface. In terms of structure, kinetics and thermodynamics, NMR is a versatile and powerful technique that presents site-specific information of protein-interactions. It comprises a number of experiments that allow for description of the binding interface, derivation of thermodynamic parameters and characterization of protein dynamics. This chapter highlights the subset of NMR experiments that can be utilized to explore protein-ligand interactions and dynamics.

Protein Dynamics and the Encounter Complex

Protein dynamics cover a broad range of movements within or across the protein surface. Within living organisms, proteins are in constant motion and are interacting with other biomolecules to convey biological messages. A protein must physically interact with its ligand and form a productive complex for successful execution of the assigned task. This interaction has to be very specific to allow for binding with a specific molecule, thereby avoiding interactions with other cellular components. This is achieved by the presence of a specific binding interface that allows for selective recognition of the desired ligand. This binding interface is composed of small surface patches on the protein and ligand and is small as compared to the whole protein surface. If the ligand has to find and bind to the specific interaction interface through mere diffusion-driven collisions in solution, most of the collisions will be nonproductive due to the small chance of directly hitting the small interface. However, in most biological processes such as signal transduction cascades and electron transfer reactions, a fast association of the interacting molecules is crucial. This is achieved by the formation of a dynamic encounter complex [42] that accelerates the formation of the final specific complex by increasing the number of successful collisions, even if they are not directly on target (Fig. **1**).

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ATTA-UR-RAHMAN, *FRS*

Prof. Atta-ur-Rahman, Ph.D. in Organic Chemistry from Cambridge University (1968) has 1,232 international publications (45 international patents and 341 books). He received the following awards: Fellow Royal Society (FRS) London (2006), UNESCO Science Prize (1999), Honorary Life Fellow Kings College, Cambridge University (2007), Academician (Foreign Member) Chinese Academy of Sciences (2015), Highest Civil Award for Foreigners of China (Friendship Award, 2014), High Civil Award Austria ("Grosse Goldene Ehrenzeischen am Bande") (2007), Foreign Fellow Chinese Chemical Society (2013), Sc.D. Cambridge University (UK) (1987), TWAS (Italy) Prize (2009). He was the President of Network of Academies of Sciences of Islamic Countries (NASIC), Vice President TWAS (Italy), Foreign Fellow Korean Academy of Science & Technology, President Pakistan Academy of Sciences (2003-2006) and (2011 – 2014). He was the Federal Minister for Science and Technology of Pakistan (2000 – 2002), Federal Minister of Education (2002) and Chairman Higher Education Commission/Federal Minister (2002-2008), Coordinator General of COMSTECH (OIC Ministerial Committee) (1996-2012), and the Editor-in-Chief of Current Medicinal Chemistry.

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